frontiers | Research Topics

Insights in veterinary pharmacology and toxicology 2023

Edited by
Arturo Anadón

Published in
Frontiers in Veterinary Science

FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-8325-5732-7
DOI 10.3389/978-2-8325-5732-7

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Insights in veterinary pharmacology and toxicology: 2023

Topic editor
Arturo Anadón — Complutense University of Madrid, Spain

Citation
Anadón, A., ed. (2024). *Insights in veterinary pharmacology and toxicology: 2023*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5732-7

Table of contents

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 07 June 2023
DOI 10.3389/fvets.2023.1208221

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Aneliya Milanova,
Trakia University, Bulgaria
Ayhan Filazi,
Ankara University, Türkiye

*CORRESPONDENCE
Hector Sumano
✉ sumano@unam.mx

RECEIVED 18 April 2023
ACCEPTED 16 May 2023
PUBLISHED 07 June 2023

CITATION
Gutierrez L, Guzman-Flores A, Monroy-Barreto M, Ocampo L and Sumano H (2023) Oral pharmacokinetics of a pharmaceutical preparation of florfenicol in broiler chickens. *Front. Vet. Sci.* 10:1208221. doi: 10.3389/fvets.2023.1208221

COPYRIGHT
© 2023 Gutierrez, Guzman-Flores, Monroy-Barreto, Ocampo and Sumano. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Oral pharmacokinetics of a pharmaceutical preparation of florfenicol in broiler chickens

Lilia Gutierrez[1], Aline Guzman-Flores[1], Minerva Monroy-Barreto[2], Luis Ocampo[1] and Hector Sumano[1]*

[1]Departamento de Fisiología y Farmacologia, Faculta de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad de México, Mexico, [2]Departamento de Química Analítica, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad de México, Mexico

Introduction: The use of florfenicol must follow particular pharmacokinetic/pharmacodynamic (PK/PD) ratios, i.e., it requires achieving serum concentrations at or slightly above the pathogen's minimum inhibitory concentration (MIC) during the dosing interval and that the ratio of area under the concentration vs. time curve (AUC)/MIC should be as high as possible (still undetermined for poultry). As an alternative to the standard soluble florfenicol that is administered to the flock through drinking water, florfenicol premix is often recommended as feed medication in Latin America. However, no particular pharmaceutical design has been proposed.

Methods: This study compared the PK of two preparations of florfenicol in broiler chickens and pondered the possibility of each covering the referred PK-PD ratios as predictors of clinical efficacy. The preparations comprise a pharmaceutical form as FOLA pellets (F = bioavailability; O = optimum; and LA = long-acting) and the premix formulation. The former are small colored pellets with vehicles and absorption enhancers of florfenicol designed for long action, and the latter is the reference premix of the antibiotic. First, these two pharmaceutical forms of florfenicol were administered as oral boluses (30 mg/kg), aided by a probe. In a second trial of the dosing form, both pharmaceutical preparations of florfenicol were administered in feed and ad libitum (110 ppm; ~30 mg/kg).

Results: In both cases, FOLA-florfenicol presented much higher relative bioavailability (3.27 times higher) and mean better residence time than florfenicol premix (two times high when forced as bolus dose). Consequently, FOLA-florfenicol possesses better PK/PD ratios than less sensitive pathogens, i.e., *E. coli*. It is proposed that if a metaphylactic treatment of a bacterial outbreak in poultry is implemented with florfenicol prepared as FOLA, better PK/PD ratios will be obtained than those of standard florfenicol premix.

Discussion: Clinicians must confirm that feed consumption in the flock has not been affected by the particular disease if FOLA pellets of florfenicol are used.

KEYWORDS

florfenicol-premix, florfenicol-FOLA, broiler-chicken, pharmacokinetics, oral dosage

Introduction

Florfenicol is a wide-spectrum, synthetic antibacterial structurally related to chloramphenicol and thiamphenicol. To date, there is no evidence of toxicity or relevant adverse effects in poultry for this derivative. In contrast, chloramphenicol has been banned in most countries due to its involvement in human toxicity, i.e., aplastic anemia that can be developed even with residual amounts (1). The recommended dose in poultry is 30–40 mg/kg bw/day for 3 days *via* drinking water, and its pharmacokinetics has been defined

for this vehicle (2, 3). In contrast, no published data recommend its use as an in-feed medication in poultry species. Nevertheless, several florfenicol premix preparations are available for poultry in Latin American countries. However, in broiler chickens, the oral bioavailability appears to be lower in fed (55%) than in fasted animals (87–96%) (3, 4). This feature may indicate that administering florfenicol in poultry as an in-feed medication may or may not achieve adequate serum and tissue concentrations and consequently may or may not deliver good clinical efficacy. Florfenicol shows an elimination half-life of at least 106.6 min in poultry when administered through their drinking water (5, 6), and it exhibits a reasonably good apparent volume of distribution at a steady state (3.5 ml/kg) (6). When experimentally administered by an oral gavage at a dose of 40 mg/kg bw, the duration of therapeutic plasma concentrations can be stretched up to 8 h (7). If administered *ad libitum* through the drinking water (approximate dose of 26 mg/kg bw) in an 18:6 dark:light cycle for 3 days, the estimated mean serum concentration of florfenicol averaged ∼ 0.7 μg/ml, and no florfenicol was detected in serum 72 h after the terminal dose. Florfenicol is partially metabolized into florfenicol amine, which is still bioactive (3).

Florfenicol is usually prescribed for treating gastrointestinal and respiratory tract infections in poultry. The presence of a bacterial disease must be established to administer florfenicol as a metaphylaxis treatment (8). Based on the above features of florfenicol, it is postulated that it could be administered in poultry as feed medication and achieve good bioavailability in broiler chickens. According to Murugayan et al. (8), it is necessary to seek alternatives in dosing antibacterial drugs to chicken to optimize pharmacokinetic/pharmacodynamic ratios and reduce the emergence of bacterial resistance caused by the misuse of these drugs. This trial was set to test this hypothesis; that is, a comparative pharmacokinetic study was carried out in broiler chickens, evaluating an experimental long-acting dosage form of florfenicol, prepared as small colored pellets, and named FOLA [Patent No. MX/a/2012/013222 and PCT/MX2013/000137, Universidad Nacional Autónoma de México [UNAM]; FOLA stands for bioavailability (F), optimum (O), and long-acting preparation (LA)]. A standard commercial premix of florfenicol for in-feed administration was taken as a reference preparation.

Materials and methods

The study design and animal handling complied with Mexican regulations for experimental animals, as stated by CICUA No. 0673 (Internal Committee for the Care and Use of Animals, UNAM) and Mexican prescripts in NOM-062-ZOO-1999. This trial was carried out in an experimental chicken house in Mexico City. Overall, 360 15-day-old Ross-308 broiler chickens, weighing approximately 450 g with a daily gain of 60 g, were distributed by simple randomization in four groups. They were allocated within a single chicken house. It was divided by wire mesh into eight smaller areas to contain 45 chicken broilers per group, i.e., a group and its replica as follows: for bolus dose, ($F_{REF-bolus}$) florfenicol premix from the product NF-180® 8% (PiSA Agropecuaria S.A. de CV, Mexico; https://www.avicultura.mx/producto/nf-180-8) approved in Mexico and other Latin American countries for poultry.

The bolus administration of the florfenicol premix or the florfenicol FOLA was carried out at 6 a.m. Each pharmaceutical preparation was weighed to meet individual broiler chicken weights. Each preparation was suspended in tap water with 2% gelatin and stirred prior to its dosing. The administration was achieved by employing a plastic probe attached to a 10 ml syringe and introduced into the included samples. In both groups, the established dose was 30 mg/kg (15 mg/chicken broiler). Once ensured that no regurgitation occurred, broiler chickens were allowed to feed and water *ad libitum*. For the *ad libitum* dosing ($F_{REFad-lib}$ and $F_{FOLAad-lib}$), florfenicol, either as premix from NF-180® 8% or FOLAs, was incorporated into their powdered feed at a rate of 110 ppm of florfenicol, considering a feed intake of 140 g/chicken and a final dose of ∼ 30 mg/kg/day (15 mg/chicken, considering a 10% feed-waste). Medicated feeds were prepared daily. In addition, FOLAs were added to the powdered feed as a dressing at the same dose rate, establishing visually that the distribution of pellets was even. In both *ad libitum* groups,

TABLE 1 Diet composition for this trial with 15-day-old Ross-308 broiler chickens, weighing ∼ 450 g.

Ingredients	Amount (kg)
Corn	590.50
Soybean meal	344.80
Soybean oil	23.80
Salt	2.40
Calcite limestone	9.50
Dicalcium phosphate	18.00
DL-methionine	1.75
L-lysine	2.15
Vitamins[a]	2.50
Minerals[b]	1
Sodium bicarbonate	3.60
Total	1,000
Crude protein	207.90
Calcium	8.80
Available phosphorus	4.40
Methionine	4.90
Sulfur amino acids	8.20
Lysine	12.70
Potassium	8.00
Sodium	2.20
Chlorine	1.90
Linoleic acid	26.00
Metabolizable energy, MJ/kg	12.56

[a] Amount/kg: Retinol 0.9 g, cholecalciferol 0.019 g, d-alpha-tocopherol 0.004 g, phylloquinone 1.0 g, riboflavin 4.0 g, cyanocobalamin 0.060 g, pyridoxine 3.0 g, calcium pantothenate 13.0 g, niacin 25 g, biotin 0.063 g, and choline chloride 250 g.

[b] Amount/kg: selenium 0.2 g, cobalt 0.1 g, iodine 0.3g, copper 10 g, zinc 50 g, iron 100 g, and manganese 100 g.

florfenicol was administered for 3 days, making feeders available from 6:00 a.m. to 12:00 p.m. during these days. Diet composition is presented in Table 1.

The pharmacokinetic approach of this study was to obtain PK parameters following a type of naïve pooled sampling since each animal was not sampled more than two times. Hence, for the oral bolus dosing ($F_{REF-bolus}$ and $F_{FOLA-bolus}$) of this trial aided by technical assistance, ∼ 1 ml of blood samples was obtained by jugular or radial wing vein puncture with 3 ml syringes and 25g x 1 in pediatric needles. A total of 10 broiler chickens were bled-sampled each time, i.e., 5 from each group and 5 from each repetition. The set times were as follows: 0.5, 1, 2, 4, 6, 8, 12, and 24 h. No bird was sampled more than twice in a 24-h period.

For the *ad libitum* dosing, blood samples (1 ml per chicken) were obtained from 5 animals in each group and 5 from its repetition during 3 days of medication at fixed times as follows: 2, 4, 8, 14, and 24 h after dosing, i.e., 8 a.m., 10 a.m., 2 p.m., 8 p.m., and 6 a.m. the next morning and on each day. Paint marking of the sampled broiler chickens allowed for an even sampling in each group. Blood samples were centrifuged at 1,000 × *g* for 5 min, and serum was harvested and stored at −20°C until analyzed.

The FOLA pellets of florfenicol were manufactured in our laboratory as described in Patent No.MX/a/2012/013222 and CT/MX2013/000137 (UNAM), observing good laboratory practices. In brief, 1% carpool with butylhydroxytoluene as an antioxidant was mixed in a base of 1:1 parts of wheat and corn flour. Then, a yellow-orange vegetable dye was added. Finally, florfenicol was incorporated at a rate of 10%. The mixture was mixed and then extruded at temperatures not higher than 30°C, using ethylalcohol and cotton-seed oil as lubricants. The final concentration of florfenicol in FOLAS was 97.6%, as determined by high-performance liquid chromatography (HPLC) based on the established technique (10).

Concentrations of florfenicol and its active metabolite florfenicol-amine were determined in plasma samples by HPLC using the method described by Kowalski et al. (11), with thiamphenicol as an internal standard. In brief, the extraction procedure was initiated by thawing the plasma samples at 20–25°C laboratory temperature. Then, 0.5 ml of plasma aliquot thiamphenicol was added as internal standard (0.5 μg in 0.2 ml), along with 0.2 ml of 1.0 M sodium hydroxide and 3 ml of ethyl acetate. Each sample was vortex mixed and centrifuged at 5,000 *g* for 15 min, and the organic layer was carefully transferred to another tube. The supernatant was dissolved in 0.5 ml of the mobile phase. Then, the samples were filtered through a membrane (nylon 0.45 μm) and injected into the HPLC with a 0.6 ml/min flow. Acetonitrile–water (25:75, v/v), adjusted to a pH of 2.7 with 85% orthophosphoric acid, was utilized as the mobile phase. Detection and quantitation were performed at 224 nm for excitation wavelength and 290 nm for emission wavelength. Calibration curves for florfenicol and florfenicol-amine were prepared from 0.05 to 20.48 μg/ml ($n = 5$).

The apparatus used was a Jasco XLC HPLC system (LC-2000Plus; Jasco Benelux, the Netherlands) with a Symmetry-C18 column (4.6 mm × 100 mm, 3.5 μm; Waters, USA) and equipped with a fluorescence detector. Data were analyzed using Empower-3 software from Waters (Mexico). The chromatographic method was validated, and the analytical procedure was demonstrated as specific. The method produced a linear result from 0.05 to 20.48 μg/ml ($r^2 = 0.984$; y = 500030 x−107 046). Recovery of florfenicol and florfenicol-amine was calculated by applying a linear regression analysis. Precision was demonstrated by the inter-day coefficient of variance (3.0) and the inter-assay error value (<3.8). The lower quantification limit for florfenicol in plasma was 0.05 μg/ml, with a detection limit of 0.008 μg/ml, and linearity was established from 0.05 to 20.48 μg/ml.

A pharmacokinetic analysis of plasma concentration–time data for florfenicol was carried out using PKAnalyst® (Micromath, Scientific Software, SLM, USA). The pharmacokinetic model (11) was based on choosing the most similar one having the highest r after examining the concentration–time curves ($r > 0.98$). Then, the number of exponential terms required to describe the plasma concentration–time data for each dosing form was determined by applying Akaike's information criterion (12). The peak concentration in serum (C_{MAX}) and the time to C_{MAX} (T_{MAX}) were estimated by observing data from tabulated plasma concentrations. Relative bioavailability (Fr%) was derived by comparing AUC_{0-24} with florfenicol-FOLA and florfenicol-reference groups.

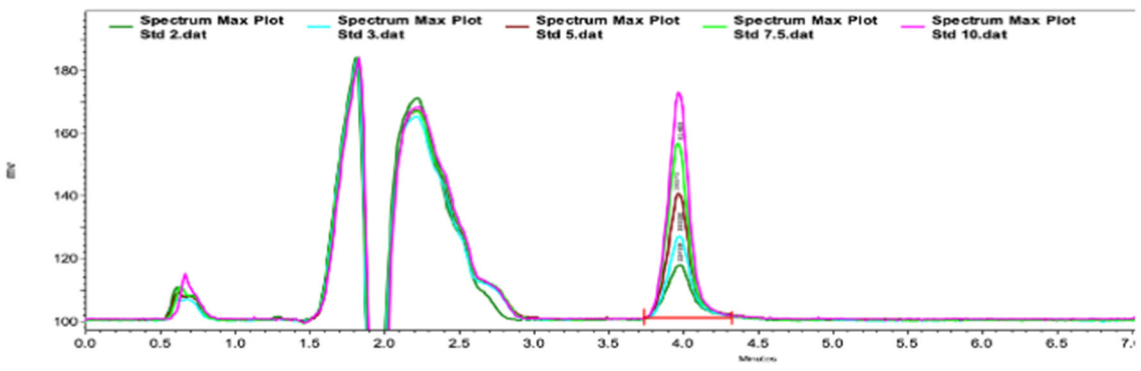


FIGURE 1
Type chromatogram of florfenicol in chicken serum samples. The retention time for florfenicol was 4 min.

The serum concentration *vs.* time data was graphed with Origin Lab-Pro 8C. Areas under the concentration *vs.* time curve (AUC) on days 1 and 3 were calculated through the trapezoidal method and confirmed with PKAnalyst®. Statistical AUC values were compared with ANOVA and successive Dunnet's test.

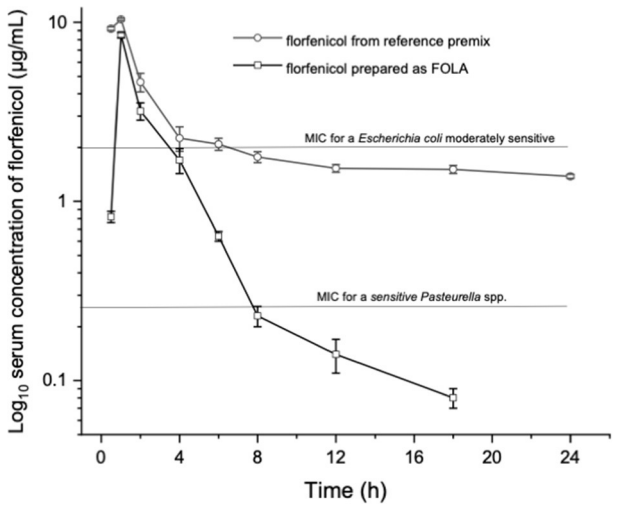


FIGURE 2
Florfenicol serum concentrations in broiler chickens treated with 30 mg/kg florfenicol either as a reference premix ($F_{REFbolus}$) or as FOLA pellets ($F_{FOLAbolus}$), employing a probe and suspending either preparation in water plus 2% gelatin.

Results

Figure 1 shows an example of the chromatograms obtained. The method utilized showed linearity when florfenicol-fortified chicken serum samples were analyzed. Mean ± 1 SD serum concentrations *vs.* time profiles of florfenicol in broiler chickens after forced oral bolus medication with either florfenicol premix from NF-180® or florfenicol prepared as FOLAs are presented in Figure 2. Figure 3 shows the serum concentrations of florfenicol after its administration *ad libitum* (110 ppm) for 3 days and their pharmacokinetic variables are shown in Table 2. Pharmacokinetic data obtained for oral bolus administration are shown in Table 3. In addition, two pharmacokinetic/pharmacodynamic (PK/PD) ratios (AUC_{0-24}/MIC and %T ≥ MIC) are presented for two pathogenic bacteria whose MIC values were taken from the formal literature: one sensitive (0.25 µg/ml) and the other moderately sensitive (2.0 µg/ml) (13, 14). The relative bioavailability value of $F_{FOLA-bolus}$ was 328%. Comparisons for MRT and T½β showed that these variables were statistically longer ($P < 0.05$) in $F_{FOLA-bolus}$ compared with $F_{REF-bolus}$. The time the serum florfenicol levels remain above or equal to MIC values (T≥MIC) and the AUC_{0-24}/MIC ratios for $F_{FOLA-bolus}$ show that the latter had larger ratios than the former. Florfenicol administered as premix remained above MIC values of the theoretically sensitive

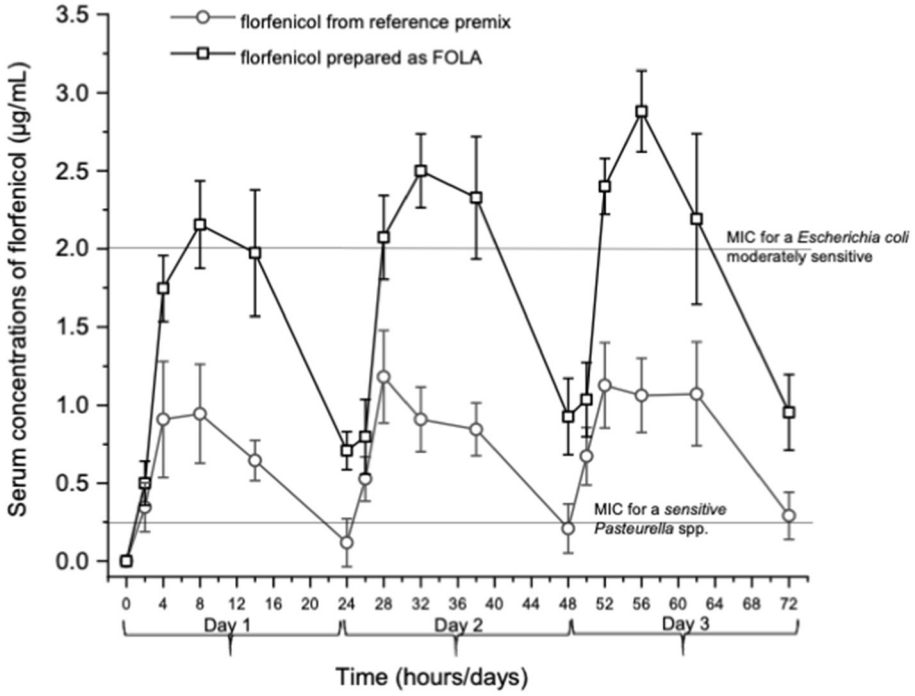


FIGURE 3
Serum concentrations of florfenicol in broiler chickens receiving two pharmaceutical forms of florfenicol. Feed medication was made available to chickens at 6:00 a.m. using powdered premix (NF-180®, PiSA Agropecuaria S.A. de CV, Mexico) or small colored pellets (FOLA-see text). In both instances, 110 ppm of florfenicol was incorporated into the feed. Given a food intake of 140 g/chicken (weighing 450 g), a dose of ~30 mg/kg was administered.

TABLE 2 Mean ± 1 SD pharmacokinetic parameters of florfenicol in broiler chickens receiving two pharmaceutical forms of florfenicol as in-feed medication and *ad libitum*: powdered premix (NF-180®, PiSA Agropecuaria S.A. de CV, Mexico) ($F_{REFad-lib)}$, and as FOLA ($F_{FOLAad-lib)}$.

Parameter	$F_{FOLAad-lib}$		$F_{red-lib}$	
	Mean	±1 SD	Mean	±1 SD
Ka (h^{-1})	0.12[a]	0.6	0.6[a]	0.3
T½β_1 (h)	3.17[a]	0.2	1.8[a]	0.8
T ½ β_2	6.2[a]	0.3	2.3[a]	0.4
T ½ β_3	7.0[a]	0.6	4.2[b]	1.2
AR_{Cmax}	2.33	0.2	1.42	0.2
AR_{AUC}	1.13	0.2	1.09	0.1
Fr (%)	250.6			
AUC_{0-72} (µg/mL·h)	132. 6[a]	6.5	52.9[b]	4.9
AUC_{0-24} (µg/mL·h)	44.0[a]	2.5	17.6[b]	1.8
AUC_T (µg/mL·h)	137.2[a]	8.5	53.7[b]	6.2
MRT_1 (h)	16.9[a]	3.2	14.1[b]	2.1
RT_1 (h)	16.9	3.2	14.1	2.1
RT_{lastT} (h)	16.8	3.4	14.0	2.4
AUC_{24-48} (µg/mL·h)	17.95	2.67	46.24	5.31
AUC_{48-72} (µg/mL·h)	18.92	3.65	46.90	5.28
$AUMC_1$ (µg/mL·h^2)	745.9	13.5	248.0	9.14
$AUMC_{lastT}$ (µg/mL·h^2)	739.5	14.1	247.3	10.1

Ka, absorption rate constant; T½$\beta_{1,2,3}$, elimination half-life on days 1, 2, and 3; Fr, relative bioavailability ($F_{FOLAad-lib}/F_{REF}$ x 100); AUC_{0-72}, area under the curve from 0 to 72 h; stAUC, first-day area under the curve from 0 to 24 h; $1^{st}AUC_T$, first-day area under the curve-trapezoidal method; RT_1, Residence time at first dose; RT_{lastT}, Residence time at last T; AR_{Cmax}, Accumulation ratio using C_{max} as a comparative value; AR_{AUC}, Accumulation ratio using AUC_{0-24} as a comparative value; $AUMC_1$, area under the moment curve at first dose; $AUMC_{last}$ T, area under the moment curve at last T.
In both instances, 110 ppm of florfenicol were incorporated into feed, which, given a food intake of 140 g/chicken (weighing 450 g), a 30 mg/kg dose was calculated.
[a,b]Different letters mean statistical differences between groups ($P < 0.5$).

TABLE 3 Mean ± 1 SD of the oral pharmacokinetic parameters of florfenicol in broiler chickens after a 30 mg/kg administration either as a reference premix ($F_{REFbolus}$) or as FOLA pellets ($F_{FOLAbolus}$), utilizing a probe and suspending either preparation in water plus 2% gelatin.

Parameter	$F_{REFbolus}$		$F_{FOLAbolus}$	
	Mean	±1 SD	Mean	±1 SD
Ka (h^{-1})	0.86[a]	0.04	0.23[b]	0.04
T½β (h)	2.35[a]	0.31	8.72[b]	0.48
T_{MAX} (h)	1.23[a]	0.31	1.07[a]	0.01
C_{MAX} (µg/mL)	7.17[a]	0.52	10.31[b]	1.25
AUC_{0-24} (µg/mL·h)	17.25[a]	2.42	41.18[b]	5.1
$AUC_{0-\infty}$ (µg/mL·h^{-2})	42.32[a]	5.74	162.01[b]	13.68
MRT (h)	3.45[a]	0.71	7.03[b]	0.45
AUC_T (µg/mL·h)	18.14[a]	2.32	59.62[b]	6.61
F %	100%		328.7	
PK/PD ratios				
$AUC_{0-24}/CMI^{*}_{0.25}$	69		165	
$\%T \geq CMI^{*}_{0.25}$	31%		>100%	
$AUC_{0-24}/CMI^{\#}_{2.0}$	8.6		20	
$\%T \geq CMI^{\#}_{2.0}$	16%		30%	

Ka, absorption rate constant; $K½_{ab}$, absorption constant; C_{MAX}, Maximum plasma concentration; T_{MAX}, time at which C_{MAX} is achieved; AUC_{0-24}, area under the curve from 0 to 24 h; MRT, mean residence time; $AUC_{0-\infty}$, area under the curve from zero to infinity; AUC_T, area under the curve-trapezoidal method; Fr, relative bioavailability ($F_{FOLAbolus}/F_{REFbolus}$ x 100); PK/PD ratios: $AUC_{0-24}/CMI_{0.25} > 125$; $\%T \geq CMI_{2.0} > 100\%$.
*_Pasteurella spp._ sensitive 0.25 µg/mL.
#_Escherichia coli_ moderately sensitive 2.0 µg/mL.
Relevant PK/PD ratios are also presented.
[a,b]Different letters mean statistical differences between groups ($P < 0.5$).

bacteria (0.25 µg/ml) 31% of the dosing interval (24 h), while florfenicol prepared as FOLA was capable of covering the whole dosing interval. However, the same T ≥ MIC ratio is reduced to 16% and 30% for $F_{REF-bolus}$ and $F_{FOLA-bolus}$, respectively, if the challenged bacteria are moderately sensitive (2.0 µg/ml). AUC_{0-24}/CMI ratios follow the same pattern favoring the FOLA form of florfenicol.

In the *ad libitum* administration, a decisive difference in the serum profiles of florfenicol for the FOLA form of florfenicol is appreciated. The values of AUC_{0-72} and $1^{st}AUC_{0-24}$ and 1st MRT and T½β were statistically higher than those achieved with the reference florfenicol premix ($P < 0.05$ in all cases). Consequently, this complies well with a lower Ka obtained for $F_{FOLAbolus}$ and $F_{FOLAad-lib}$.

Discussion

Respiratory diseases are the primary problem in poultry production worldwide, and although much can be done with good husbandry, bacterial disease outbreaks occur and must be treated (15, 16). Antimicrobial drugs are then chosen and are critical to solving the problem. However, their utility has been compromised in recent years by the emergence of resistance to antimicrobial drugs. Given the increasingly limited availability of antimicrobial drugs for poultry production, it is reasonable to think that an immediate operational line of research is to optimize the pharmaceutical design of each active principle (9). The study on the absorption and bioavailability processes of antibacterial drugs in poultry is limited (17). Most pharmaceutical forms available for poultry medicine have been the product of trial and error. They were not designed to optimize their PK/PD ratios when included in food or drinking water (18). Thus, when an outbreak of a respiratory disease occurs, such as complicated chronic respiratory disease, infectious coryza (*Haemophilus paragallinarum*), or fowl cholera (*Pasteurella multocida*), soluble florfenicol is often administered as the drug of choice. Early intervention is required (metaphylaxis), and it has been postulated that sick birds tend to reduce feed or water intake, but their consumption patterns before signs of the referred diseases appear have yet to be established; that is, unless a critical part of the flock is affected, water or feed intake variations are rarely detected during the early stages of the disease. This is a task that requires careful research. However, in this context, several preparations

of florfenicol in premix became available in Latin America for metaphylaxis treatment.

Florfenicol is rapidly eliminated from the broiler chickens' plasma. This study conceived and tested an attempt to extend the clearance of florfenicol with its inclusion in FOLA pellets and as an in-feed medication. The results show improved florfenicol PK/PD ratios. It is postulated that the gastrointestinal retentive properties of carbopol in FOLA pellets modify absorption into a type of sustained release, and therefore, elimination is extended.

Prudent use of highly potent antimicrobial drugs in veterinary medicine, such as florfenicol, is required (9, 17). To achieve this goal, it is necessary to design pharmaceutical preparations that better comply with each drug's competent PK/PD ratios (19). In turn, this may contribute to maintaining its efficacy in the future (10, 20, 21). It is in this context that florfenicol in FOLAs was conceived. FOLAs can be described as pharmaceutically designed carriers prepared as small color pellets that are readily consumed by poultry, which allow the inclusion of vehicles and help improve the absorption of the active ingredient by modulating the GI-transit time (10, 21).

The pharmacokinetic parameters obtained for the reference florfenicol as premix were very similar to what was achieved in other studies with soluble florfenicol. Minor differences can be attributed to the use of different bloodlines of chicken, different ages, feeding, and housing peculiarities (22, 23). More efficient absorption of florfenicol, when prepared as FOLA pellets, was anticipated as sustained-release formulations tend to achieve better bioavailability values (20, 24), and such behavior has already been obtained with FOLAs for doxycycline and tylosin in poultry (19, 23). Furthermore, the lower Ka obtained for $F_{FOLAbolus}$ and $F_{FOLAad-lib}$ may suggest that a flip-flop phenomenon is occurring. In this study, the $F_{FOLAbolus}$ preparation achieved better values in C_{MAX} and AUC_{0-24} than the premix formulation. In addition, MRT and T½β were statistically higher than the premix formulation ($P < 0.05$). Consequently, these features generate notable differences in the bioavailability of the preparations, with a notable advantage for the pharmaceutical form of type FOLA, i.e., Fr from 250 to 328%. Having higher plasma concentrations of the antibacterial drug and a total load of florfenicol achieved (AUC/CMI), as well as slower elimination parameters for florfenicol FOLA compared with reference florfenicol premix (T $\geq$ CMI), signify a notable improvement in the pharmacokinetic ratios of this drug. Florfenicol is considered a time-dependent antibacterial, and it would seem that there is no advantage in using FOLAs when treating sensitive bacteria (MIC = 0.25 μg/ml). However, it is evident that if a more resistant pathogen is involved in an outbreak, such as *E. coli* (24), only florfenicol prepared as FOLA will achieve adequate PK/PD ratios. This latter view is even more evident in the *ad libitum* dosing, where the AUC values, T½B, and MRT parameters were substantially higher.

In summary, during the metaphylactic treatment of a bacterial outbreak in poultry, the clinician can safely use florfenicol prepared as FOLA, as better PK/PD ratios than those achieved with florfenicol premix will be obtained. In turn, better clinical results are foreseen. These pharmacokinetic considerations apply if the clinician can confirm that feed consumption in the flock has not been affected by the particular disease. In addition, given the prolonged MRT, AR_{Cmax}, and AR_{AUC} found for FOLA preparations, a new withdrawal time may be necessary to avoid unauthorized drug residues. Nevertheless, according to the standards proposed by the European Medicines Agency for the modified release of pharmaceutical preparations, the obtained value for the FOLA pellets ($AR_{AUC} < 1.25$) can be considered as leading to non-relevant drug accumulation (24).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by CICUA No. 0673 (Internal Committee for the Care and Use of Animals, UNAM) and Mexican prescripts in NOM-062-ZOO-1999.

Author contributions

HS and LG conceived and designed the study and carried out the pharmacokinetic and statistical analyses. AG-F and LO carried out the clinical trial. MM-B and AG-F performed the analytical phase. All authors have read and accepted the manuscript as it is presented to the journal.

Funding

The support from the PAPIIT-UNAM IT200222 project for carrying out this research is acknowledged.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Pooja S, Bansode FW, Singh RK. Chloramphenicol Toxicity: a review. *J Med Medic Sci.* (2011) 13:1313–6.

2. Shen J, Wu X, Wu D, Jiang H. Pharmacokinetics of florfenicol in healthy and *Escherichia coli*-infected broiler chickens. *Res Vet Sci.* (2002) 73:137–40. doi: 10.1016/S0034-5288(02)00033-4

3. Anadón A, Aranzazu M, Martinez M, Rios A, Caballero V, Ares I, Martinez-Larrañaga MR. Plasma and tissue depletion of florfenicol and florfenicol amine in chickens. *J Agric Food Che*. (2008) 56:11049–56. doi: 10.1021/jf802138y

4. Afifi NA, El-Sooud KA. Tissue concentrations and pharmacokinetics of florfenicol in broiler chickens. *British Poultry Sci.* (1997) 38:425–8. doi: 10.1080/00071669708418013

5. Shen J, Hu D, Wu X, Coats JR. Bioavailability and pharmacokinetics of florfenicol in broiler chickens. *J Vet Pharmacol Therapy.* (2003) 26:337–41. doi: 10.1046/j.1365-2885.2003.00495.x

6. Lohani M, Ahmad AH, Singh KP, Verma S. Pharmacokinetics and residual study of florfenicol following multiple-dose oral administration in poultry. *J App Anim Res.* (2010) 38:9–12. doi: 10.1080/09712119.2010.9707145

7. Chang JL, Davis CN, Cheng RH, Shien MK, Koh C, Chou C. Pharmacokinetics and tissue depletion of florfenicol in leghorn and Taiwan native chickens. *J Vet Pharmacol Therap.* (2010) 33:471–9. doi: 10.1111/j.1365-2885.2009.01155.x

8. Muryagan J, Kumar PA, Rao GS, Iskandar K, Hawser S, Hays JP, et al. Progress in alternative strategies to combat antimicrobial resistance: focus on antibiotics. *Antibiotics.* (2022) 11:200. doi: 10.3390/antibiotics11020200

9. Gutierrez L, Marcos X, García-Guzman P, Monroy-Barreto M, Sumano H. Pharmaceutical characterization and pharmacokinetics of florfenicol-loaded alginate dried beads in rabbits. *World Rabbit Science.* (2022) 30:153–62. doi: 10.4995/wrs.2022.16381

10. Kowalski P, Konieczna L, Chmielewska A, Oledzka I, Plenis A, Bieniecki M, et al. Comparative evaluation between capillary electrophoresis and high-performance liquid chromatography for the analysis of florfenicol in plasma. *J Pharm Biomed Anal.* (2005) 39:983–9. doi: 10.1016/j.jpba.2005.05.032

11. Yamaoka K, Nakagawa T, Uno T. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. *J Pharmacokinet Biopharm.* (1978) 6:165–75. doi: 10.1007/BF01117450

12. Shin SJ, Kang SG, Nabin R, Kang ML, Yoo HS. Evaluation of the antimicrobial activity of florfenicol against bacteria isolated from bovine and porcine respiratory disease. *Vet Microbiol.* (2005) 106:73–7. doi: 10.1016/j.vetmic.2004.11.015

13. Li XS, Wang GQ, Du XD, Cui BA, Zhang SM, Shen JZ. Antimicrobial susceptibility and molecular detection of chloramphenicol and florfenicol resistance among *Escherichia coli* isolates from diseased chickens. *J Vet Scie.* (2007) 8:243–7. doi: 10.4142/jvs.2007.8.3.243

14. Glison JR. Bacterial respiratory diseases of poultry. *Poult Sci.* (1998) 77:1139–42. doi: 10.1093/ps/77.8.1139

15. Kursa O, Tomczyk G, Adamska K, Chrzanowska J, Sawicka-Durkalec A. The microbial community of the respiratory tract of commercial chickens and turkeys. *Microorganisms.* (2022) 10:987. doi: 10.3390/microorganisms10050987

16. Hu YS, Prisca XYT, Tao TMN, Hui TC, Shangshe X, A. critical review of the pharmacokinetics, pharmacodynamics, and safety data of antibiotics in avian species. *Antibiotics.* (2022) 11:741. doi: 10.3390/antibiotics11060741

17. Gray P, Jenner R, Norris J, Page S, Browning G. Australian veterinary association Ltd and animal medicines Australia. Antimicrobial prescribing guidelines for poultry. *Aust Vet J.* (2021) 99:181–235. doi: 10.1111/avj.13034

18. Rodriguez-Gascon A, Soliní MA, Isla A. The role of PK/PD analysis in the development and evaluation of antimicrobials. *Pharmaceutics.* (2021) 13:833. doi: 10.3390/pharmaceutics13060833

19. Gutierrez L, Zermeño J, Alcala Y, Sumano H. Higher bioavailability of doxycycline in broiler chickens with novel in-feed pharmaceutical formulations. *Poult Sci.* (2017) 96:2662–9. doi: 10.3382/ps/pex036

20. Gutierrez L, Tapia G, Gutierrez E, Sumano H. Evaluation of a tasteless enrofloxacin pharmaceutical preparations for cats. Naïve pooled-sample approach to study its pharmacokinetics. *Animals.* (2021) 11:2312. doi: 10.3390/ani11082312

21. Ismail M, El-Kattan YA. Comparative pharmacokinetics of florfenicol in chicken, pigeon, and quail. *British Poult Sci.* (2009) 50:144–9. doi: 10.1080/00071660802613286

22. Watteyn A, Russo E, Garmyn A, De Baere S, Pasmans F, Martel A, et al. Clinical efficacy of florfenicol administered in the drinking water against *Ornithobacterium rhinotracheale* in turkeys housed in different environmental conditions: a pharmacokinetics/pharmacodynamics approach. *Avian Pathology.* (2013) 42:47448. doi: 10.1080/03079457.2013.823144

23. Gutierrez L, Alcala Y, Bernad MJ, Sumano H. Increased bioavailability of tylosin phosphate as in-feed medication formulated for long action pellets in broiler chickens. *J Appl Poult Res.* (2018) 27:16–22. doi: 10.3382/japr/pfx035

24. Scheerans C, Heinig R, Mueck W. Proposal for defining the relevance of drug accumulation derived from single dose study for modified release dosage forms. *Biopharm drug Dispos*. (2015) 36:93–103. doi: 10.1002/bdd.1923

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 18 October 2023
DOI 10.3389/fvets.2023.1268658

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Ian Sutherland,
AgResearch Ltd., New Zealand
Guillermo Virkel,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

*CORRESPONDENCE
Gracia Merino
✉ gmerp@unileon.es

RECEIVED 28 July 2023
ACCEPTED 05 October 2023
PUBLISHED 18 October 2023

CITATION
Blanco-Paniagua E, Álvarez-Fernández L, Millán-García A, Rivas G, Álvarez AI and Merino G (2023) Coadministration of ivermectin and abamectin affects milk pharmacokinetics of the antiparasitic clorsulon in Assaf sheep.
Front. Vet. Sci. 10:1268658.
doi: 10.3389/fvets.2023.1268658

COPYRIGHT
© 2023 Blanco-Paniagua, Álvarez-Fernández, Millán-García, Rivas, Álvarez and Merino. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Coadministration of ivermectin and abamectin affects milk pharmacokinetics of the antiparasitic clorsulon in Assaf sheep

Esther Blanco-Paniagua[1,2], Laura Álvarez-Fernández[1,2], Alicia Millán-García[1,2], Guillermo Rivas[2], Ana I. Álvarez[1,2] and Gracia Merino[1,2]*

[1]Department of Biomedical Sciences-Physiology, Faculty of Veterinary, University of León, León, Spain, [2]Instituto de Desarrollo Ganadero y Sanidad Animal (INDEGSAL), University of León, León, Spain

In veterinary field, drug exposure during milk production in dairy cattle is considered a major health problem which concerns dairy consumers. The induced expression of the ABC transporter G2 (ABCG2) in the mammary gland during lactation plays a significant role in the active secretion of many compounds into milk. The main objective of this study was to determine the involvement of ABCG2 in the secretion into milk of the antiparasitic clorsulon in sheep as well as the possible effect of the coadministration of model ABCG2 inhibitors such as macrocyclic lactones on this process. Cells transduced with the ovine variant of ABCG2 were used to carry out *in vitro* transepithelial transport assays in which we showed that clorsulon is a substrate of the ovine transporter. In addition, ivermectin and abamectin significantly inhibited clorsulon transport mediated by ovine ABCG2. *In vivo* interactions were studied in Assaf sheep after coadministration of clorsulon (in DMSO, 2 mg/kg, s.c.) with ivermectin (Ivomec®, 0.2 mg/kg, s.c.) or abamectin (in DMSO, 0.2 mg/kg, s.c.). After ivermectin and abamectin treatment, no relevant statistically significant differences in plasma levels of clorsulon were reported between the experimental groups since there were no differences in the area under the plasma concentration-curve (AUC) between clorsulon treatment alone and coadministration with macrocyclic lactones. With regard to milk, total amount of clorsulon, as percentage of dose excreted, did not show statistically significant differences when macrocyclic lactones were coadministered. However, the AUC for clorsulon significantly decreased ($p < 0.05$) after coadministration with ivermectin (15.15 ± 3.17 μg h/mL) and abamectin (15.30 ± 3.25 μg h/mL) compared to control group (20.73 ± 4.97 μg h/mL). Moreover, milk parameters such as half-life ($T_{1/2}$) and mean residence time (MRT) were significantly lower ($p < 0.05$) after coadministration of macrocyclic lactones. This research shows that the milk pharmacokinetics of clorsulon is affected by the coadministration of ABCG2 inhibitors, reducing drug persistence in milk.

KEYWORDS

ABCG2, ivermectin, clorsulon, milk, sheep

1. Introduction

Helminth infections are responsible for the most relevant diseases of livestock globally due to their negative impact on production efficiency in livestock systems. Consequently, control of helminth infections is essential and is mainly based on the use of anthelmintic drugs (1–3). Fascioliasis, a zoonotic disease, causes high economic loses in meat and milk production in livestock throughout the world (4). A number of existing anthelmintic drugs have been used to treat fascioliasis, among them clorsulon (4, 5).

Clorsulon is an antiparasitic drug that belongs to the benzenesulphonamide antiparasitic family used for treatment against adult and mature liver flukes (6). Blocking energy-producing pathways in the fluke is the main mode of action of clorsulon; specifically, it inhibits two enzymes involved in glycolysis of the parasite: 3-phosphate-glyceratekinase and phospho-glyceromutase (6, 7). Regarding its pharmacokinetics, clorsulon is well absorbed after oral administration and eliminated by renal excretion without being metabolized (7, 8). Several previous studies have reported its plasma pharmacokinetics in sheep, goats, cattle and rats (6, 8–10). Commonly, clorsulon is marketed with the macrocyclic lactone ivermectin, combining nematicide and flukicide effects (11, 12). The effectiveness of this combination has been reported in sheep, rats and cattle (13–17).

In veterinary field, potential mechanisms or factors that could modify drug exposure and impact on efficacy as well as on the appearance of drug residues in milk are vital to study. Drug–drug interactions may be one of these factors and must be taken into consideration. Transporter-based interactions in particular have been reported to affect the pharmacokinetics of drugs (17, 18). These interactions are related to coadministration of a drug that is an inhibitor or an inducer of the transporter which may affect the transport of another drug described as a substrate (19). ATP-binding cassette (ABC) transporters are some of the transporters involved in drug–drug interactions and Breast Cancer Resistance Protein (BCRP/ABCG2) is one of the proteins included in this superfamily of transporters, which is the focus of our study.

The ABCG2 transporter extrudes a wide range of drugs from cells due to its localization in the apical membrane of epithelial cells in several tissues such as intestine, kidney, liver, brain and testicles. The main biological function of ABCG2 is to limit toxin accumulation in cells and to modulate of pharmacokinetic processes such as xenobiotic absorption, distribution and elimination (20, 21). Moreover, ABCG2 is expressed in alveolar epithelial cells of the mammary gland during lactation and is one of the main factors involved in active secretion of many compounds into milk (22, 23). Consequently, drugs described as ABCG2 substrates can accumulate in milk, posing a health risk to dairy consumers. Drug–drug interactions mediated by the ABCG2 transporter that lead to the inhibition of ABCG2 affecting drug secretion into milk or plasma availability have been reported (24–30).

Clorsulon has been characterized as an *in vitro* substrate of murine and human variants of ABCG2, and its involvement in the secretion of clorsulon into milk has been reported using ABCG2-knockout mice; furthermore, a drug–drug interaction with the macrocyclic lactone ivermectin has been shown given that its coadministration decreased secretion of clorsulon into milk by ABCG2 in mice (31). However, interaction with the ovine variant of ABCG2 and its potential role in the secretion of clorsulon into milk in sheep is unknown. The purpose of this study was to determine whether clorsulon is an *in vitro* substrate of ovine ABCG2 and to explore its role in the secretion of this flukicide into milk. The effect of macrocyclic lactones, ivermectin and abamectin, on the pharmacokinetics of clorsulon in sheep was also evaluated.

2. Materials and methods

2.1. Standards and chemicals

Clorsulon and abamectin were purchased from Biosynth Carbosynth (Berkshire, United Kingdom) and albendazol-2 aminosulfone from LGC Standards (Molsheim, France). Ivermectin and Lucifer Yellow were obtained from Sigma-Aldrich (St. Louis, MO, United States). For *in vivo* assays, ivermectin (Ivomec®) was purchased from Boehringer Ingelheim (Barcelona, Spain). All the additional chemicals used were reagent grade and were available from commercial suppliers.

2.2. Cell cultures

This study employed Madin-Darby Canine Kidney (MDCKII) cells that had been previously transduced with ovine ABCG2. The conditions for culturing have been previously described (32). Briefly, cells were grown in DMEM (Dulbecco's modified Eagle's medium) enriched with glutamax (Life Technologies, Inc., Rockville, MD, United States) and supplemented with penicillin (50 units/mL), streptomycin (50 µg/mL), and 10% (v/v) fetal calf serum (MP Biomedicals, Solon, OH, United States) at a temperature of 37°C and 5% of CO_2. Every 3 to 4 days, cells were trypsinized for subculturing.

2.3. Transcellular transport studies

Transcellular transport experiments using ovine ABCG2-transduced cells were conducted following the method previously described (33). Cells were seeded onto microporous polycarbonate membrane filters (3.0 µm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) at a density of 1.0×10^6 per well. The tightness of the monolayer was assessed by measuring its transcellular resistance using Millicell ERS (Millipore Burlington, MA).

Two hours before the beginning, preincubation was carried out replacing medium in both compartments with 2 mL of transport medium [Hanks' balanced salt solution supplemented with HEPES (25 mM)] with or without the macrocyclic lactones (10 µM ivermectin and 2.5 µM abamectin). The experiment began with the replacement

Abbreviations: ABC, ATP-binding cassette; AP-BL, Apical to basal transport; AUC, Area under concentration-time curves; BCRP/ABCG2, Breast cancer resistance protein/ATP-binding cassette transporter G2; BL-AP, Basal to apical transport; C_{max}, Maximum peak concentration; HPLC, High-performance liquid chromatography; LOD, Limit of detection; LOQ, Limit of quantification; MDCKII, Madin-Darby canine kidney; MRT, Mean residence time; s.c., Subcutaneous; T_{max}, Time to maximum concentration; $T_{1/2}$, Half-life.

of the medium in either the apical or basal compartment with transport medium containing clorsulon (10 μM), with or without the same concentration of macrocyclic lactones. Aliquots of 100 μL at 1, 2 and 3 h in the opposite compartment were taken, this volume being replaced with fresh medium. At 4 h, 600 μL aliquots were taken in both compartments. Until analysis by high-performance liquid chromatography (HPLC), samples were stored at −20°C. After concluding the experiment, the confluence of the monolayer was evaluated by means of the Lucifer Yellow permeability assay (34). Transport proficiency of these cells was consistently verified through the evaluation of a typical ABCG2 substrate, danofloxacin (25).

The apparent permeability coefficients (P_{app}) across MDCKII parent and MDCKII oABCG2 cells monolayers in both apical to basal (A-B) (P_{app} A-B) and basal to apical (B-A) (P_{app} B-A) directions were calculated using following equation: $P_{app} = (\Delta Q/\Delta t)^*(1/A^*\text{Co})$, where $\Delta Q/\Delta t$ is the rate of corresponding clorsulon appearing in the receiver chamber, which was obtained as the slope of the regression line on the transport time profile of clorsulon across the cell monolayers (from 0 to 4 h); Co is the initial concentration of drug; A is the cell monolayer surface area (4.67 cm^2). The efflux ratio is the P_{app} B-A/P_{app} A-B quotient.

2.4. Pharmacokinetic experiments with Assaf sheep

Studies with sheep were carried out on the Experimental Farm of the University of León, following institutional guidelines in accordance with European legislation (2010/63/EU). Procedures were also approved by the Animal Care and Use Committee of the University of León ULE-008-2019 (25/09/2019). Eighteen lactating sheep (3–4 months in lactation) of between 77–83 kg were used. Animals were previously dewormed and they had *ad libitum* access to drinking water. Sheep were randomly distributed in three experimental groups. All received a subcutaneous (s.c.) injection of clorsulon at 2 mg/kg (dissolved in DMSO, 80 mg/mL); one group coadministrated with another s.c. injection of ivermectin at 0.2 mg/kg (Ivomec® 1%, Merial, France) and another group with another s.c. injection of abamectin at 0.2 mg/kg (dissolved in DMSO, 8 mg/mL). Blood samples were taken from the jugular vein, while milk samples were obtained after completing milking of the gland before each treatment and at intervals of 1, 2, 4, 6, 8, 10, 12, 24, 30, 48, 72, 96, 120 and 168 h thereafter. Plasma was separated by centrifuging at 1200 g 15 min. Samples were stored at −20°C until analyzed.

2.5. High performance liquid chromatography analysis

Samples were analyzed by HPLC under conditions which have been previously described (31). Briefly, the chromatographic system used included a Waters 2695 separation module and a Waters 2998 UV photodiode array detector. Separation was performed on a reversed-phase column (4 mm particle size, 250 × 341 4.6 mm, Max-RP 80Å, 362 Phenomenex®, Torrance, CA, United States). The mobile phase consisted of potassium phosphate 0.01 M (pH 7): acetonitrile (75:25). The flow rate was set at 1.20 mL/min, with UV absorbance measured at 225 nm, and the column temperature maintained at 35°C.

In vitro samples were injected directly into the HPLC system. *In vivo* samples, milk and plasma, were extracted following a formerly described method (31) with minor modifications. For milk and plasma samples, 10 μL of albendazole-2-aminosulfone (6.25 μg/mL) as internal standard and 200 μL of ethyl acetate were added to each 100 μL. Then, the mixture was vortexed horizontally for 1 min and centrifuged at 8000 g for 10 min at 4°C. The resulting supernatant was collected and subjected to evaporation under N_2 at 30°C until dryness. To the evaporated samples, 500 μL of hexane and 300 μL of acetonitrile was added, the mixture was again vortexed horizontally for 1 min and centrifuged at 3000 g for 10 min at 4°C. Hexane was removed and the rest was evaporated to dryness under nitrogen stream. After being resuspended in 100 μL of cold methanol, samples were injected into the HPLC system.

Standard samples of clorsulon in the appropriate drug-free matrix were prepared at concentrations of 0.078–10 μg/mL for culture samples, 0.078–2.5 μg/mL for plasma samples, and 0.078–5 μg/mL for milk samples. Correlation coefficients for clorsulon ranged between 0.98–0.99. Precision coefficients of variation were ≤20%, and relative standard deviations (accuracy) values were ≤20%. Determination of the limit of detection (LOD) and limit of quantification (LOQ) was carried out in accordance with the procedure explained by Taverniers et al. (35). LOD was 0.01 μg/mL for cell culture samples and sheep plasma and 0.03 μg/mL for sheep milk. LOQ was 0.02 μg/mL for culture samples, 0.04 μg/mL for sheep plasma and 0.09 μg/mL for sheep milk.

2.6. Pharmacokinetic calculations and statistical analyses

Plasma and milk concentrations for each animal were analyzed using PK solution 2.0 computer program (Summit Research Services, Ashland, OH) to calculated pharmacokinetic parameters as previously described (27).

Statistical analysis for significant differences was performed using SPSS Statistics software (v. 26.0; IBM, Armonk, New York, NY, United States). Normal distribution of data was analyzed by the Shapiro–Wilk test. The ANOVA and Kruskal–Wallis tests were applied for normally and not normally distributed data, respectively. A probability of $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. *In vitro* transport of clorsulon: inhibition by ivermectin and abamectin

To determine the *in vitro* role of ovine ABCG2 in transport of clorsulon, MDCKII parental cells and subclone transduced with ovine ABCG2 were used, transepithelial translocation of clorsulon across monolayers on porous membrane filters was measured (Figure 1) and relative efflux ratios were calculated (Table 1).

MDCKII parental cells showed a similar transport in both directions, basolateral and apical (Figure 1A), which is also reflected in the relative efflux ratio, 1.07 ± 0.06. However, translocation in the apical direction was significantly higher ($p < 0.05$) than translocation in the basolateral direction in MDCKII ovine ABCG2 cells

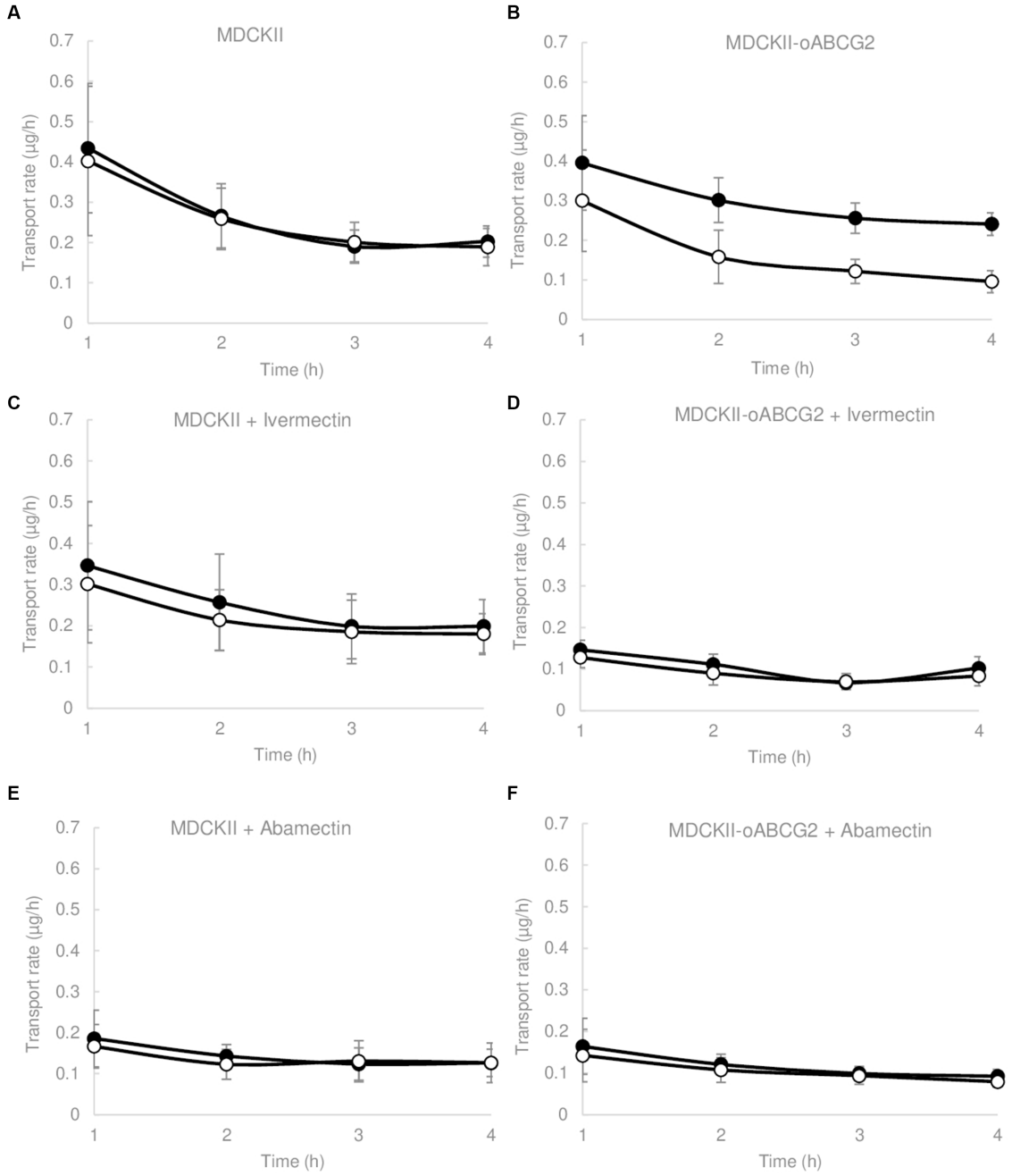


FIGURE 1
Transepithelial transport assays of clorsulon (10 µM) in MDCKII (parental) cells and its subclones transduced with ovine ABCG2 (oABCG2) **(A,B)**, in presence of ivermectin 10 µM **(C,D)** and abamectin 2.5 µM **(E,F)**. The assay was started with the addition of medium in either the apical or basal compartment with fresh transport medium containing clorsulon with or without ivermectin or abamectin. Results are represented as mean ± SD. Closed circles represent basal to apical (B-A) transport rate and open circles represent apical to basal transport rate (A-B) ($n \geq 3$).

(Figure 1B), with a relative efflux ratio significantly higher ($p < 0.05$) in comparison to MDCKII parental cells (Table 1). Specific transport by ABCG2 was checked using a specific inhibitor of ABCG2, Ko143 (36), resulting in a complete inhibition of clorsulon transported by ovine ABCG2-transduced cells. These results reveal that clorsulon is efficiently transported by ovine ABCG2.

We also studied the effect of two macrocyclic lactones, ivermectin and abamectin on the ovine ABCG2-mediated transport of clorsulon and performed transepithelial transport assays in the presence of ivermectin 10 µM (Figures 1C,D) and abamectin 2.5 µM (Figures 1E,F) using MDCKII parental and ovine ABCG2 cells. For ovine ABCG2-transduced cells, clorsulon transport was effectively inhibited in the presence of ivermectin (Figure 1D) and abamectin (Figure 1F), resulting in a similar apical and basolateral translocation, with efflux ratios similar to those in the parental cells (Table 1). These results demonstrate a highly effective *in vitro* inhibitory effect of ivermectin and abamectin on clorsulon transport mediated by ovine ABCG2.

TABLE 1 Apparent permeability (P_{app}) values for transepithelial transport of clorsulon (10 μM) across cells monolayers in MDCKII cells and the ovine-ABCG2 transduced cells (oABCG2) with or without ivermectin (10 μM), abamectin (2.5 μM) and Ko143 (1 μM) ($n \geq 3$).

		BL-AP, x10^{-6} cm/s (P_{app} B-A)	AP-BL, x10^{-6} cm/s (P_{app} A-B)	Efflux ratio P_{app} B-A/P_{app} A-B
MDCKII	Clorsulon	2.41 ± 0.46	2.27 ± 0.46	1.07 ± 0.06
	Clorsulon + Ivermectin	2.83 ± 0.97	2.61 ± 0.71	1.09 ± 0.17
	Clorsulon + Abamectin	2.29 ± 0.60	2.37 ± 0.93	0.99 ± 0.13
	Clorsulon + Ko143	2.21 ± 0.40	2.15 ± 0.54	1.04 ± 0.08
MDCKII oABCG2	Clorsulon	3.23 ± 0.88	1.13 ± 0.16	2.92 ± 0.93*
	Clorsulon + Ivermectin	1.43 ± 0.20	1.22 ± 0.15	1.17 ± 0.11#
	Clorsulon + Abamectin	1.67 ± 0.26	1.48 ± 0.30	1.15 ± 0.17#
	Clorsulon + Ko143	1.59 ± 0.29	1.50 ± 0.21	1.06 ± 0.09#

Results are mean ± SD. *$p \leq 0.05$: significant differences from MDCKII cells. #$p \leq 0.05$: significant differences from MDCKII oABCG2 clorsulon alone treatment.

For MDCKII parental cells with ivermectin (Figure 1C) and abamectin (Figure 1E), we showed that clorsulon apical and basolateral translocation were similar. Moreover, relative efflux ratios were comparable to previous efflux ratios obtained in clorsulon alone treatment in MDCKII parental cells (Table 1), thus indicating no effect of these macrocyclic lactones on the parental cells.

3.2. Plasma and milk clorsulon pharmacokinetics in sheep

To evaluate the potential *in vivo* drug–drug interactions between macrocyclic lactones and secretion into milk of clorsulon, its coadministration with ivermectin and abamectin was carried out in sheep.

Mean plasma concentrations are shown in Figure 2A. Clorsulon plasma concentrations were significantly lower ($p < 0.05$) at 8 h (1.59 ± 0.20 μg/mL) after coadministration with ivermectin compared to clorsulon alone treatment (1.95 ± 0.24 μg/mL). Regarding plasma pharmacokinetics parameters (Table 2), $T_{1/2}$ and MRT values were lower ($p < 0.05$) after coadministration with abamectin compared to clorsulon administration. However, no significant differences were reported on comparing the AUC for clorsulon between clorsulon alone treatment and coadministration with ivermectin and abamectin. These data indicate that plasma availability was not affected by coadministration of macrocyclic lactones.

Regarding milk, mean concentrations (Figure 2B) and pharmacokinetic parameters of clorsulon (Table 3) were calculated. No differences were reported in mean milk concentrations between clorsulon alone administration and coadministration with ivermectin or abamectin (Figure 2B). Nevertheless, the milk AUC for clorsulon was around 25% lower ($p < 0.05$) after coadministration with ivermectin and abamectin compared to clorsulon alone treatment (Table 3). No differences were reported for the AUC milk-to-plasma values or total amount of clorsulon as percentage of dose excreted between clorsulon alone treatment and ivermectin or abamectin coadministration (Table 3). With regard to pharmacokinetics parameters, the $T_{1/2}$ values were lower ($p < 0.05$) after coadministration with ivermectin and abamectin compared to clorsulon administration. Furthermore, the MRT showed a reduction ($p < 0.05$) in milk values after coadministration of ivermectin and abamectin compared to clorsulon alone treatment.

These results indicate that milk pharmacokinetics of clorsulon is modified following its simultaneous administration with the macrocyclic lactones ivermectin and abamectin in sheep. In addition, a reduction in the persistence of clorsulon in milk was reported.

4. Discussion

Animal helminthic infections have an important unwanted impact not only on livestock production, but also on animal welfare, the environment and human health. Prevention or treatment of these diseases using chemotherapeutics is essential for their control. Nevertheless, there are undesired side-effects related to anthelmintic use such as anthelmintic resistance development and anthelmintic residues in the environment and animal-derived products (3). Moreover, antiparasitic combinations are often used to enhance the spectrum of activity. For example, clorsulon with the macrocyclic lactone ivermectin is a marketed combination used in veterinary medicine as a broad-spectrum anthelmintic formulation thanks to the association of a nematicide and a flukicide (11, 12). However, the administration of different anthelmintic drugs in combination can lead to unpredictable drug–drug interactions that must be considered. In particular, transporter-based interactions may affect plasma and milk pharmacokinetics parameters of drugs (19, 23, 37, 38).

Recently, we showed that clorsulon was efficiently transported *in vitro* by murine ABCG2 and human ABCG2, and also that ivermectin inhibited murine and human ABCG2-mediated transport of clorsulon. Moreover, we reported a reduction in milk levels of clorsulon after coadministration of ivermectin in mice (31). In accordance with these outcomes, in the present study we show that clorsulon is an *in vitro* substrate for ovine ABCG2 and that there is an important effect of two macrocyclic lactones, ivermectin and abamectin, as model ABCG2 inhibitors, on the transfer of clorsulon into sheep milk.

In vitro transcellular transport assays using MDCKII cells transduced with ovine variant of ABCG2 showed that clorsulon is efficiently transported by ovine ABGC2 with an efflux transport ratio of 2.92 ± 0.93 (Table 1, Figure 1B). Similar ratios were previously obtained in murine ABCG2 (2.20 ± 0.13) and human ABCG2 (1.63 ± 0.17) transduced cells lines (31). Beforehand, different compounds were described as substrates of ovine ABCG2 such as antibiotics and non-steroidal anti-inflammatories (28, 30, 32, 39).

We also demonstrate an efficient *in vitro* inhibition of ABCG2-mediated transport of clorsulon in ovine variant by the macrocyclic lactones ivermectin and abamectin (Table 1). Ivermectin and abamectin are avermectin compounds known for their anthelmintic and insecticidal effects (40). Both of these have been described as inhibitors of the ABC transporter P-glycoprotein (41). Nevertheless, only ivermectin has been previously identified as an *in vitro* inhibitor of ABCG2, with an inhibition potency value of 45.9% at 25 μM in ovine ABCG2-transduced cells (32, 42). Furthermore, this inhibitory effect was confirmed using transport assays with well-known ABCG2 substrates such as danofloxacin (25), albendazole sulphoxide (43) or

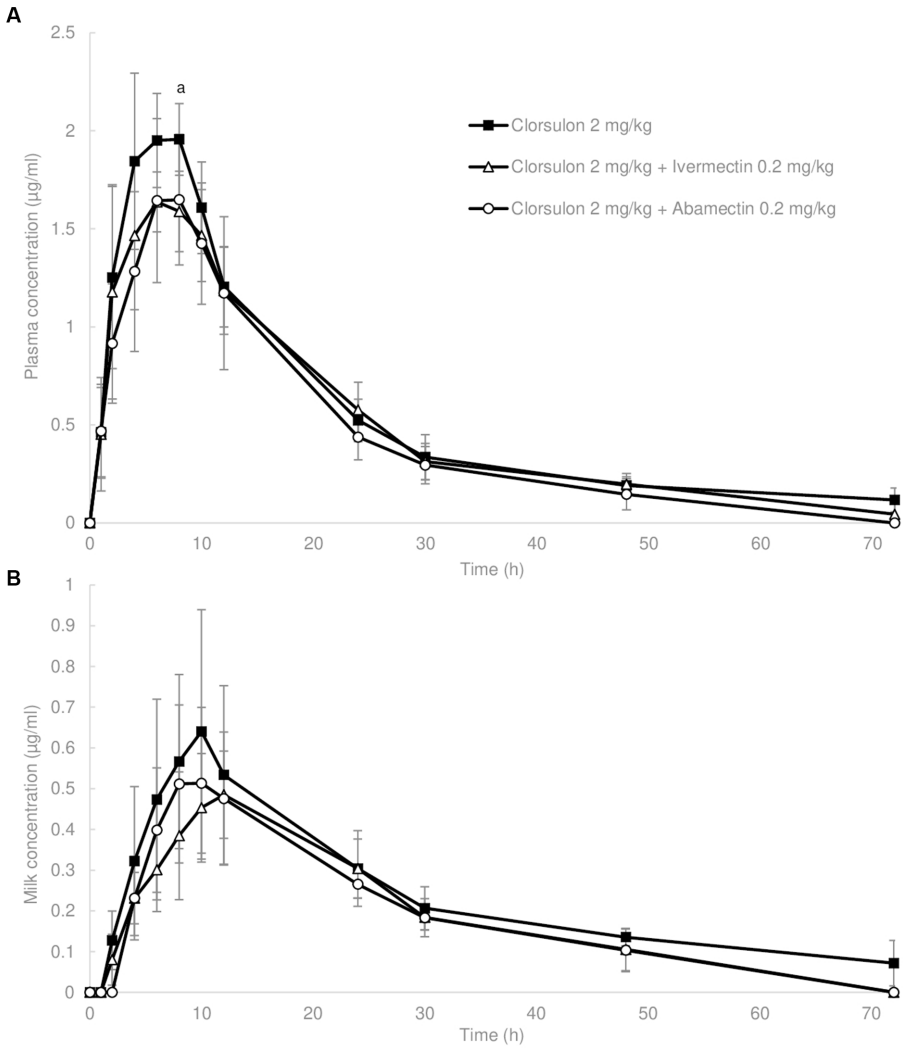


FIGURE 2
Concentration in plasma **(A)** and milk **(B)** vs. time curves for clorsulon obtained from sheep after s.c. administration of clorsulon at dosage of 2 mg/kg and co-administered with ivermectin at 0.2 mg/kg (s.c.) and abamectin at 0.2 mg/kg (s.c.). Results are represented as mean ± SD ($n = 6$). Lowercase letter (a) represents significant differences ($p \leq 0.05$) between clorsulon alone dose and ivermectin coadministration.

TABLE 2 Pharmacokinetic parameters (mean ± SD) for clorsulon in plasma after s.c. administration at 2 mg/kg in sheep co-administrated with ivermectin (0.2 mg/kg, s.c.) and abamectin (0.2 mg/kg, s.c.) ($n = 6$).

Parameters[a]	Clorsulon	Clorsulon + ivermectin	Clorsulon + abamectin
C_{max} (µg/mL)	2.13 ± 0.28	1.72 ± 0.19	1.70 ± 0.40
T_{max} (h)	6.67 ± 1.93	6.00 ± 1.79	7.67 ± 0.82
$T_{1/2}$ (h)	18.97 ± 2.38	15.74 ± 3.83	12.34 ± 2.65*
MRT (h)	25.00 ± 3.97	22.85 ± 5.20	18.82 ± 2.96*
AUC $_{(0-\infty)}$ (µg h/mL)	43.27 ± 6.40	38.77 ± 6.10	33.68 ± 9.07

*$p \leq 0.05$, significant differences from clorsulon alone treatment.
[a]C_{max}, maximum concentration; T_{max}, time to maximum concentration; $T_{1/2}$, half-life; MRT, mean residence time; AUC, area under the curve.

novel substrates like meloxicam (30). Apart from ivermectin, other avermectins have been reported to have inhibitory effects on ABCG2 such as doramectin (32), eprinomectin (29) and selamectin (42). However, the interaction between abamectin and ABCG2 has not yet been described. Due to its similar structure with the rest of the avermectins, its hydrophobic properties, previously related as a common chemical feature of inhibitors of ABCG2 (44), as well as its current importance in the agriculture field (40), it was interesting to address the potential role of abamectin as an inhibitor of ovine ABCG2.

Following the meaningful inhibition obtained in the *in vitro* assays, the extent of *in vivo* ABCG2-mediated drug–drug interaction involving macrocyclic lactones and the ovine ABCG2 substrate

TABLE 3 Pharmacokinetic parameters (mean ± SD) for clorsulon in milk after s.c. administration at 2 mg/kg in sheep co-administered with ivermectin (0.2 mg/kg, s.c.) and abamectin (0.2 mg/kg, s.c.) ($n = 6$).

Parameters[a]	Clorsulon	Clorsulon + ivermectin	Clorsulon + abamectin
C_{max} (μg/mL)	0.65 ± 0.29	0.52 ± 0.12	0.57 ± 0.19
T_{max} (h)	10.00 ± 1.26	11.00 ± 1.67	10.00 ± 1.79
$T_{1/2}$ (h)	25.88 ± 3.72	17.44 ± 5.51	17.90 ± 4.56
MRT (h)	40.77 ± 6.15	30.45 ± 7.83*	30.27 ± 6.91*
AUC $_{(0-\infty)}$ (μg h/mL)	20.73 ± 4.97	15.15 ± 3.17*	15.30 ± 3.25*
AUC milk/AUC plasma	0.49 ± 0.14	0.39 ± 0.03	0.47 ± 0.11
Dose excreted (%) in milk (72 h)	0.86 ± 0.28	0.75 ± 0.40	0.59 ± 0.18

*$p \leq 0.05$, significant differences from clorsulon alone treatment.
[a]C_{max}, maximum concentration; T_{max}, time to maximum concentration; $T_{1/2}$, half-life; MRT, mean residence time; AUC, area under the curve.

clorsulon was determined carrying out the coadministration of clorsulon with ivermectin and abamectin in sheep. Clorsulon was administrated at the recommended dose of 2 mg/kg by the s.c. route, as is commonly used in cattle (6), and ivermectin and abamectin were tested at doses based on the recommended dose rate (45).

Plasma concentrations of clorsulon (Figure 2A) were in the same range as previous pharmacokinetics studies carried out in cattle in which after s.c. administration of 2 mg/kg of clorsulon, the maximum plasma concentration was 2.5 μg/mL at 6 h (6). We showed that plasma concentrations were lower at 8 h after coadministration with ivermectin. Differences in $T_{1/2}$ and MRT after coadministration with abamectin were reported with lower values compared to clorsulon alone administration. Despite these differences in clorsulon plasma concentrations at certain single time points and pharmacokinetics parameters, no significant differences between AUC values in clorsulon alone treatment compared to coadministration of macrocyclic lactones were reported, although these differences tended to be lower after the coadministrations (Table 2). Lack of differences in clorsulon plasma availability in sheep after macrocyclic lactone coadministration is in agreement with the lack of differences in clorsulon plasma levels in mice after coadministration with ivermectin (31). In particular, there were no differences in plasma concentrations of clorsulon between wild-type and Abcg2$^{-/-}$ mice in the treatment either with clorsulon alone or with the combination of clorsulon and ivermectin. Even when comparing treatments with or without ivermectin in both types of mice, no differences in plasma concentrations of clorsulon were reported. Although our *in vitro* results evidently showed that clorsulon is a substrate of ovine ABCG2 and that this transporter can have an influence on the plasma disposition of its substrates (20), it is important to consider that other factors, including the potential involvement of other transporters *in vivo*, might conceal the effect of ABCG2 on the systemic disposition of clorsulon.

Focusing on milk pharmacokinetics of clorsulon, in our study the effect of ivermectin and abamectin on clorsulon pharmacokinetics in milk was also studied in Assaf sheep (Figure 2B and Table 3). Milk AUC values decreased following coadministration of ivermectin and abamectin compared to treatment with clorsulon alone (Table 3). Moreover, the values of the parameters $T_{1/2}$ and MRT in milk were also lower after coadministration of macrocyclic lactones compared to clorsulon alone treatment (Table 3), showing a reduction in the persistence of clorsulon in milk. Previous studies reported a reduction in milk levels of ABCG2 substrates such as danofloxacin (25) or meloxicam (30) after coadministration of ivermectin in sheep, without any effect in plasma levels. In addition, the coadministration of different ABCG2 inhibitors in sheep, such as triclabendazole metabolites (28) or soy isoflavones (26) were also reported to affect milk pharmacokinetics parameters of moxidectin and danofloxacin, respectively.

Our results show no significant differences in AUC milk-to-plasma values of clorsulon between experimental groups. We cannot rule out the possibility that the coadministration of macrocyclic lactones affected systemic exposure of clorsulon by some unknown mechanism since reduced AUC in plasma was also shown although not statistically significant, probably due to high interindividual variability. Consequently, reduction in milk AUC might be a consequence of this potential plasma reduction. In any case, these results show that coadministration of macrocyclic lactones such as model ABCG2 inhibitors causes a reduction in the persistence of clorsulon in milk, limiting potential exposure of the offspring and consumers of dairy products to this xenobiotic and enhancing the understanding of the potential factors that could affect the pharmacokinetics of contaminants in milk.

5. Conclusion

Our study defined clorsulon as an *in vitro* substrate of ovine ABCG2 and showed the *in vitro* inhibitory effect of ivermectin and abamectin on clorsulon transport mediated by ovine ABCG2. In sheep, the coadministration of ABCG2 inhibitors such as macrocyclic lactones affected milk pharmacokinetics of clorsulon.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were approved by Animal Care and Use Committee of the University of León (ULE-008-2019). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EB-P: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. LÁ-F: Data curation, Formal analysis, Methodology, Investigation, Writing – review & editing. AM-G: Investigation, Writing – review & editing. GR: Methodology, Writing – review & editing. AÁ: Methodology, Validation, Conceptualization, Funding acquisition, Supervision, Writing – review & editing. GM: Methodology, Conceptualization, Funding acquisition, Project administration, Resources, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the research projects RTI2018-100903-B-I00 and PID2021-125660OB-I00 (MCIN/AEI/10.13039/501100011033/FEDER "Una manera de hacer Europa"); and by the predoctoral grants (FPU18/01559 grant to EB-P, FPU19/04169 grant to LÁ-F) from Spanish Ministry of Education, Culture and Sport.

Acknowledgments

The authors are grateful to James McCue (Caledonia English Academy) for assistance in language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Duthaler U, Smith TA, Keiser J. *In vivo* and *in vitro* sensitivity of *Fasciola hepatica* to triclabendazole combined with artesunate, artemether, or OZ78. *Antimicrob Agents Chemother*. (2010) 54:4596–604. doi: 10.1128/AAC.00828-10

2. Statham JME. Control of liver fluke: an emerging issue in terms of veterinary residues. *Vet Rec*. (2015) 177:519–21. doi: 10.1136/vr.h6287

3. Vercruysse J, Charlier J, Van Dijk J, Morgan ER, Geary T, von Samson-Himmelstjerna G, et al. Control of helminth ruminant infections by 2030. *Parasitology*. (2018) 145:1655–64. doi: 10.1017/S003118201700227X

4. Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. Fascioliasis and fasciolopsiasis: current knowledge and future trends. *Res Vet Sci*. (2021) 134:27–35. doi: 10.1016/j.rvsc.2020.10.011

5. Martínez-Valladares M, Cordero-Pérez C, Rojo-Vázquez FA. Efficacy of an anthelmintic combination in sheep infected with *Fasciola hepatica* resistant to albendazole and clorsulon. *Exp Parasitol*. (2014) 136:59–62. doi: 10.1016/j.exppara.2013.10.010

6. European Medicines Agency. Committee for medicinal products for veterinary use. European public MRL assessment report (EPMAR). *EMEA/CVMP/358525/2008*. (2008). Available at: https://www.ema.europa.eu/en/documents/mrl-report/clorsulon-cattle-european-public-mrl-assessment-report-epmar-committee-veterinary-medicinal-products_en.pdf

7. Richter D, Richter J, Grüner B, Kranz K, Franz J, Kern P. *In vitro* efficacy of triclabendazole and clorsulon against the larval stage of echinococcus multilocularis. *Parasitol Res*. (2013) 112:1655–60. doi: 10.1007/s00436-013-3321-7

8. Sundlof SF, Whitlock TW. Clorsulon pharmacokinetics in sheep and goats following oral and intravenous administration. *J Vet Pharmacol Ther*. (1992) 15:282–91. doi: 10.1111/j.1365-2885.1992.tb01018.x

9. Schulman MD, Valentino D, Cifelli S, Lang R, Ostlind DA. A pharmacokinetic basis for the efficacy of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide against *Fasciola hepatica* in the rat. *J Parasitol*. (1979) 65:555–61. doi: 10.2307/3280320

10. Schulman MD, Valentino D, Cifelli S, Ostlind DA. Dose-dependent pharmacokinetics and efficacy of MK-401 against old, and young-mature infections of *Fasciola hepatica* in the rat. *J Parasitol*. (1982) 68:603–8. doi: 10.2307/3280917

11. Dupuy J, Alvinerie M, Ménez C, Lespine A. Interaction of anthelmintic drugs with P-glycoprotein in recombinant LLC-PK1-mdr1a cells. *Chem Biol Interact*. (2010) 186:280–6. doi: 10.1016/j.cbi.2010.05.013

12. Meaney M, Fairweather I, Brennan GP, McDowell LSL, Forbes AB. *Fasciola hepatica*: effects of the fasciolicide clorsulon *in vitro* and *in vivo* on the tegumental surface, and a comparison of the effects on young- and old-mature flukes. *Parasitol Res*. (2003) 91:238–50. doi: 10.1007/s00436-003-0863-0

13. Heredia R, Aguilar E, Romero C, Bautista L, Mendoza G. Evaluation of five treatments to control intestinal parasites in sheep in Ayapango, state of Mexico. *Vet World*. (2016) 9:1233–7. doi: 10.14202/vetworld.2016.1233-1237

14. Sibille P, Calléja C, Carreras F, Bigot K, Galtier P, Boulard C. *Fasciola hepatica*: influence of gender and liver biotransformations on flukicide treatment efficacy of rats infested and cured with either clorsulon/ivermectin or triclabendazole. *Exp Parasitol*. (2000) 94:227–37. doi: 10.1006/expr.2000.4501

15. Geurden T, Bartram D, Van Brussel L, Bo L, Scott-Baird E, Rugg D. Evaluation of the comparative efficacy of a moxidectin plus triclabendazole pour-on solution against adult and immature liver fluke, *Fasciola hepatica*, in cattle. *Vet Parasitol*. (2012) 189:227–32. doi: 10.1016/j.vetpar.2012.04.019

16. Ibarra-Velarde F, Vera-Montenegro Y, Nájera-Fuentes R, Sánchez-Albarran A. Efficacy of combined chemotherapy against gastrointestinal nematodes and *Fasciola hepatica* in cattle. *Vet Parasitol*. (2001) 99:199–204. doi: 10.1016/S0304-4017(01)00460-5

17. Marquez B, Van Bambeke F. ABC multidrug transporters: target for modulation of drug pharmacokinetics and drug–drug interactions. *Curr Drug Targets*. (2011) 12:600–20. doi: 10.2174/138945011795378504

18. Hillgren KM, Keppler D, Zur AA, Giacomini KM, Stieger B, Cass CE, et al. Emerging transporters of clinical importance: an update from the International Transporter Consortium. *Clin Pharmacol Ther*. (2013) 94:52–63. doi: 10.1038/clpt.2013.74

19. Zhang L, Reynolds KS, Zhao P, Huang S-M. Drug interactions evaluation: an integrated part of risk assessment of therapeutics. *Toxicol Appl Pharmacol*. (2010) 243:134–45. doi: 10.1016/j.taap.2009.12.016

20. Vlaming MLH, Lagas JS, Schinkel AH. Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice. *Adv Drug Deliv Rev*. (2009) 61:14–25. doi: 10.1016/j.addr.2008.08.007

21. Meyer zu Schwabedissen HE, Kroemer HK. *In vitro* and *in vivo* evidence for the importance of breast cancer resistance protein transporters (BCRP/MXR/ABCP/ABCG2) *Handb Exp Pharmacol*. (2011). 201:325–71. doi: 10.1007/978-3-642-14541-4_9

22. Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, et al. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat Med*. (2005) 11:127–9. doi: 10.1038/nm1186

23. Garcia-Lino AM, Álvarez-Fernández I, Blanco-Paniagua E, Merino G, Álvarez AI. Transporters in the mammary gland—contribution to presence of nutrients and drugs into milk. *Nutrients*. (2019) 11:2372. doi: 10.3390/nu11102372

24. Pulido MM, Molina AJ, Merino G, Mendoza G, Prieto JG, Alvarez AI. Interaction of enrofloxacin with breast cancer resistance protein (BCRP/ABCG2): influence of flavonoids and role in milk secretion in sheep. *J Vet Pharmacol Ther*. (2006) 29:279–87. doi: 10.1111/j.1365-2885.2006.00744.x

25. Real R, Egido E, Pérez M, González-Lobato L, Barrera B, Prieto JG, et al. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the secretion of danofloxacin into milk: interaction with ivermectin. *J Vet Pharmacol Ther*. (2011) 34:313–21. doi: 10.1111/j.1365-2885.2010.01241.x

26. Perez M, Otero JA, Barrera B, Prieto JG, Merino G, Alvarez AI. Inhibition of ABCG2/BCRP transporter by soy isoflavones genistein and daidzein: effect on plasma and milk levels of danofloxacin in sheep. *Vet J*. (2013) 196:203–8. doi: 10.1016/j.tvjl.2012.09.012

27. Otero JA, García-Mateos D, Alvarez-Fernández I, García-Villalba R, Espín JC, Álvarez AI, et al. Flaxseed-enriched diets change milk concentration of the antimicrobial danofloxacin in sheep. *BMC Vet Res*. (2018) 14:14. doi: 10.1186/s12917-018-1341-3

28. Barrera B, González-Lobato L, Otero JA, Real R, Prieto JG, Álvarez AI, et al. Effects of triclabendazole on secretion of danofloxacin and moxidectin into the milk of sheep: role of triclabendazole metabolites as inhibitors of the ruminant ABCG2 transporter. *Vet J*. (2013) 198:429–36. doi: 10.1016/j.tvjl.2013.07.033

29. Garcia-Lino AM, Garcia-Mateos D, Alvarez-Fernandez I, Blanco-Paniagua E, Medina JM, Merino G, et al. Role of eprinomectin as inhibitor of the ruminant ABCG2 transporter: effects on plasma distribution of danofloxacin and meloxicam in sheep. *Res Vet Sci*. (2021) 136:478–83. doi: 10.1016/j.rvsc.2021.03.026

30. Blanco-Paniagua E, Garcia-Lino AM, Alvarez-Fernández L, Alvarez AI, Merino G. Ivermectin inhibits ovine ABCG2-mediated *in vitro* transport of meloxicam and reduces its secretion into milk in sheep. *Res Vet Sci*. (2022) 153:88–91. doi: 10.1016/j.rvsc.2022.10.019

31. Blanco-Paniagua E, Álvarez-Fernández L, Rodríguez-Alonso A, Millán-Garcia A, Álvarez AI, Merino G. Role of the Abcg2 transporter in secretion into milk of the anthelmintic clorsulon: interaction with ivermectin. *Antimicrob Agents Chemother*. (2023) 67:e0009523. doi: 10.1128/aac.00095-23

32. González-Lobato L, Real R, Herrero D, de la Fuente A, Prieto JG, Marqués MM, et al. Novel *in vitro* systems for prediction of veterinary drug residues in ovine milk and dairy products. *Food Addit Contam A*. (2014) 31:1026–37. doi: 10.1080/19440049.2014.908261

33. Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. *Mol Pharmacol*. (2005) 67:1758–64. doi: 10.1124/mol.104.010439

34. Mahnke H, Ballent M, Baumann S, Imperiale F, von Bergen M, Lanusse C, et al. The ABCG2 efflux transporter in the mammary gland mediates veterinary drug secretion across the blood-milk barrier into milk of dairy cows. *Drug Metab Dispos*. (2016) 44:700–8. doi: 10.1124/dmd.115.068940

35. Taverniers I, De Loose M, Van Bockstaele E. Trends in quality in the analytical laboratory: II. Analytical method validation and quality assurance. *Trends Anal Chem*. (2004) 23:535–52. doi: 10.1016/j.trac.2004.04.001

36. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther*. (2002) 1:417–25. Available at: https://aacrjournals.org/mct/article/1/6/417/233728/Potent-and-Specific-Inhibition-of-the-Breast

37. Neodo A, Schulz JD, Huwyler J, Keiser J. *In vitro* and *in vivo* drug-drug interaction study of the effects of ivermectin and oxantel pamoate on tribendimidine. *Antimicrob Agents Chemother*. (2019) 63:e00762–18. doi: 10.1128/AAC.00762-18

38. Ballent M, Canton C, Dominguez P, Bernat G, Lanusse C, Virkel G, et al. Pharmacokinetic-pharmacodynamic assessment of the ivermectin and abamectin nematodicidal interaction in cattle. *Vet Parasitol*. (2020) 279:109010. doi: 10.1016/j.vetpar.2019.109010

39. Wassermann L, Halwachs S, Lindner S, Honscha KU, Honscha W. Determination of functional ABCG2 activity and assessment of drug–ABCG2 interactions in dairy animals using a novel MDCKII *in vitro* model. *J Pharm Sci*. (2013) 102:772–84. doi: 10.1002/jps.23399

40. El-Saber Batiha G, Alqahtani A, Ilesanmi OB, Saati AA, El-Mleeh A, Hetta HF, et al. Avermectin derivatives, pharmacokinetics, therapeutic and toxic dosages, mechanism of action, and their biological effects. *Pharmaceuticals*. (2020) 13:196. doi: 10.3390/ph13080196

41. Lespine A, Martin S, Dupuy J, Roulet A, Pineau T, Orlowski S, et al. Interaction of macrocyclic lactones with P-glycoprotein: structure-affinity relationship. *Eur J Pharm Sci*. (2007) 30:84–94. doi: 10.1016/j.ejps.2006.10.004

42. Merino G, Real R, Baro MF, Gonzalez-Lobato L, Prieto JG, Alvarez AI, et al. Natural allelic variants of bovine ATP-binding cassette transporter ABCG2: increased activity of the Ser581 variant and development of tools for the discovery of new ABCG2 inhibitors. *Drug Metab Dispos*. (2009) 37:5–9. doi: 10.1124/dmd.108.022715

43. Muenster U, Grieshop B, Ickenroth K, Gnoth MJ. Characterization of substrates and inhibitors for the *in vitro* assessment of Bcrp mediated drug–drug interactions. *Pharm Res*. (2008) 25:2320–6. doi: 10.1007/s11095-008-9632-1

44. Egido E, Müller R, Li-Blatter X, Merino G, Seelig A. Predicting activators and inhibitors of the breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1) based on mechanistic considerations. *Mol Pharm*. (2015) 12:4026–37. doi: 10.1021/acs.molpharmaceut.5b00463

45. European Medicines Agency. European public MRL assessment report (EPMAR). clorsulon (bovine milk)—after the provisional maximum residue limit (MRL). *EMA/CVMP/752339/2013* (2014) Available at: https://www.ema.europa.eu/en/documents/mrl-report/clorsulon-bovine-milk-european-public-maximum-residue-limit-assessment-report-epmar-cvmp_en.pdf

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 09 November 2023
DOI 10.3389/fvets.2023.1297221

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Ayhan Filazi,
Ankara University, Türkiye
Abdurrahman Aksoy,
Ondokuz Mayıs University, Türkiye

*CORRESPONDENCE
Thimmasettappa Thippeswamy
✉ tswamy@iastate.edu

†These authors have contributed equally to this work and share first authorship

RECEIVED 19 September 2023
ACCEPTED 24 October 2023
PUBLISHED 09 November 2023

CITATION
Vasanthi SS, Massey N, Nair SN, Mochel JP, Showman L and Thippeswamy T (2023) Exploring the benefits of in-diet versus repeated oral dosing of saracatinib (AZD0530) in chronic studies: insights into pharmacokinetics and animal welfare.
Front. Vet. Sci. 10:1297221.
doi: 10.3389/fvets.2023.1297221

COPYRIGHT
© 2023 Vasanthi, Massey, Nair, Mochel, Showman and Thippeswamy. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Exploring the benefits of in-diet versus repeated oral dosing of saracatinib (AZD0530) in chronic studies: insights into pharmacokinetics and animal welfare

Suraj S. Vasanthi[1†], Nyzil Massey[1†], Suresh N. Nair[2], Jonathan P. Mochel[3], Lucas Showman[4] and Thimmasettappa Thippeswamy[1*]

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA, United States, [2]Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Thrissur, India, [3]Precision One Health, Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA, United States, [4]W.M. Keck Metabolomics Research Laboratory, Iowa State University, Ames, IA, United States

Saracatinib/AZD0530 (SAR), a Src tyrosine kinase inhibitor, mitigates seizure-induced brain pathology in epilepsy models upon repeated oral dosing. However, repeated dosing is stressful and can be challenging in some seizing animals. To overcome this issue, we have incorporated SAR-in-Diet and compared serum pharmacokinetics (PK) and brain concentrations with conventional repeated oral dosing. Saracatinib in solution or in-diet was stable at room temperature for >4 weeks (97 ± 1.56%). Adult Sprague Dawley rats on SAR-in-Diet consumed ~1.7 g/day less compared to regular diet (16.82 ± 0.6 vs. 18.50 ± 0.5 g/day), but the weight gain/day was unaffected (2.63 ± 0.5 g/day vs. 2.83 ± 0.2 g/day). Importantly, we achieved the anticipated SAR dose range from 2.5–18.7 mg/kg of rat in response to varying concentrations of SAR-in-Diet from 54 to 260 ppm of feed, respectively. There was a strong and significant correlation between SAR-in-Diet dose (mg/kg) and serum saracatinib concentrations (ng/ml). Serum concentrations also did not vary significantly between SAR-in-Diet and repeated oral dosing. The hippocampal saracatinib concentrations derived from SAR-in-Diet treatment were higher than those derived after repeated oral dosing (day 3, 546.8 ± 219.7 ng/g vs. 238.6 ± 143 ng/g; day 7, 300.7 ± 43.4 ng/g vs. 271.1 ± 62.33 ng/g). Saracatinib stability at room temperature and high serum and hippocampal concentrations in animals fed on SAR-in-Diet are useful to titer the saracatinib dose for future animal disease models. Overall, test drugs in the diet is an experimental approach that addresses issues related to handling stress-induced variables in animal experiments.

KEYWORDS

saracatinib, Src tyrosine kinases, serum, LC–MS/MS, hippocampus, pharmacokinetics

1. Introduction

Saracatinib, also known as AZD0530, is a selective inhibitor of Fyn or Src family of nonreceptor tyrosine developed by AstraZeneca. Fyn/Src is highly expressed in proliferating cells such as cancerous cells (1, 2) neurons and glial cells of the central nervous system in response to brain insults such as exposure to neurotoxins (3) or seizures (4, 5) or in chronic neurodegenerative diseases such as Alzheimer's disease (6). Considering the role of Fyn/Src kinases in cell proliferation, saracatinib has been in clinical trials for various types of cancers, such as bone, ovarian, and breast cancer (7–9). Excessive production of Fyn/Src and its phosphorylation in reactive glial cells or neurons causes hyperexcitability, neuroinflammation, and neurodegeneration (10, 11). Therefore, saracatinib has been tested as a disease modifier in experimental models of epilepsy and AD (3, 12) and in clinical trials for AD and Parkinson's disease (13, 14).

Saracatinib is orally active, crosses the blood brain barrier, and is a potent modulator of ABCB1-mediated multidrug resistance (9, 15). However, long-term treatment with this drug at an optimal dose is required for debilitating and chronic diseases such as cancer and neurodegenerative diseases to achieve beneficial effects with minimum adverse effects. In humans, saracatinib has been tested in clinical trials for some of these conditions for varying periods (8, 9). In experimental models of epilepsy, we tested repeated oral dosing of saracatinib for about a week and observed some disease-modifying effects depending on the severity of the disease and the models of epilepsy (3, 16). In the kainate (KA) model of temporal lobe epilepsy, treating for a week with saracatinib was sufficient to mitigate long-term effects such as reactive gliosis, neurodegeneration, epileptiform spiking, and spontaneously recurring seizures (3). However, such protective effects of saracatinib treatment for a week were limited in organophosphate (OP) induced epilepsy models, perhaps due to widespread peripheral effects and extended duration of body clearance of OP (16). Furthermore, acute exposure to OP impacts gut dysbiosis (17), in contrast to the more centrally localized effect of KA, therefore, the drugs administered via oral gavage for a shorter duration may have limited effects in OP models. Chronic and debilitating diseases such as cancer, AD, PD, and epilepsy require long-term treatment, and oral medication is ideal for attaining optimum plasma concentration from a translational perspective.

In experimental models, daily administration of test drugs by oral gavage for long term is tedious, impractical, and stressful for animals due to repeated handling, which could confound the real effects of interventional drugs. To mitigate these issues, we incorporated saracatinib in rat chow to achieve the required dose during the 24 h feeding cycle and compared the feed consumption, weight gain, and the pharmacokinetics of saracatinib in serum and brain with repeated daily oral dosing of saracatinib for a week. We also tested the serum saracatinib concentrations when the animals were fed different concentrations of saracatinib in the diet for long term. LC–MS/MS confirmed the saracatinib concentrations in the formulated diet. The serum saracatinib in both groups showed a strong correlation from day 2 through day 7, and there were no significant differences between groups. Overall, saracatinib stability at room temperature and high serum and hippocampal concentrations in animals fed on SAR-in-Diet are useful for titrating the required dosing regimen in chronic disease models. Incorporating test drugs in the diet is a translational approach and abates stress-induced variables in animal experiments.

2. Materials and methods

2.1. Animal studies

Twenty eight adult male Sprague Dawley rats (8 weeks old; 250-300 g; Charles River, United States) were used in the study. Animals were housed in individual cages with a 12-h light–dark cycle in an enriched environment. The experiments were conducted after 2–3 days of acclimatization as per the approved protocols by the Institutional Animal Care and Use Committees (IACUC protocol: 21–109) and complied with the NIH ARRIVE Guidelines for the Care and Use of Laboratory Animals. Blood sampling was done by retro-orbital puncture as per IACUC protocol. Maximal blood collection was limited to less than 7.5% of the animal's body weight in one week. The serum was separated by centrifugation at 1,000–1,500 × g for 10 min and stored at −80°C until further analysis. All animals were euthanized at the end of the study with pentobarbital sodium and phenytoin sodium (100 mg/kg, i.p.) as per the American Veterinary Medical Association Guidelines for euthanasia. Tissue samples were collected and stored at −80°C until further analysis.

The experimental design is illustrated in Figure 1. SAR-in-Diet at 260, 210, 160, and 50 ppm was prepared by LabDiet (Lan O'Lakes, Inc) to achieve the saracatinib dose range of 20-5 mg/kg of rat. The required concentration of saracatinib to be incorporated in the rat chow was estimated based on the average daily food consumption (18-21 g/day) from eight male adult rats of the same age (8 weeks to start with) and a similar weight range (206–230 g). Saracatinib was prepared in 0.5% hydroxypropyl methylcellulose at 5 mg/mL and added to the diet at 260, 210, 160, and 50 ppm. The Saracatinib incorporated diet and the control (regular rat chow) diet had similar composition/ingredients and was purchased from the same source, Purina 5P07 (Prolab RMH 1000). Saracatinib >99% pure by Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) was supplied by AstraZeneca under the Open Innovation Program.

The animals were randomized into four experimental groups ($n = 4$–8). In group 1 (24 h study; Figure 1A), eight animals received a single oral gavage of saracatinib (20 mg/kg), and the blood sampling was done at 0.5, 1, 2, 4, 6, 8, 12, and 24 h post-treatment. Four animals each were euthanized at 4 h and 24 h, respectively. In group 2 (7-day repeated oral dosing study; Figure 1B), eight animals received repeated daily oral dosing of saracatinib (20 mg/kg of rat) at 24 h intervals; the blood sampling was done daily at 24 h intervals before the next dosing. Four animals were euthanized 24 h after the third repeated oral dosing, i.e., day 3 and the other four animals were euthanized at day 7. Group 3 (7-day SAR-in-Diet study; Figure 1C), received a single oral gavage of saracatinib (20 mg/kg of rat) followed by 24 h later SAR-in-Diet for 7 days (260 ppm). The blood sampling was done at 24 h post oral and daily at 24 h intervals for 7 days while the animals were on SAR-in-Diet. Four animals each were euthanized on day 3 and 7. Group 4 (Figure 1D) included four animals that were given 7 repeated doses of oral saracatinib (20 mg/kg/day for a week) followed by *ad-libitum* SAR-in-Diet at varying concentrations fed for up to 18 weeks (260 ppm diet for 2 weeks, 210 ppm diet for a week, 160 ppm for 4 weeks, and 50 ppm diet for 11 weeks) The tapering concentrations of saracatinib in the diet from 260 to 50 ppm was done to target a daily saracatinib dose rate in the range of 20–5 mg/kg respectively, in a 24 h feeding cycle. Blood samples were collected at the end of each diet change.

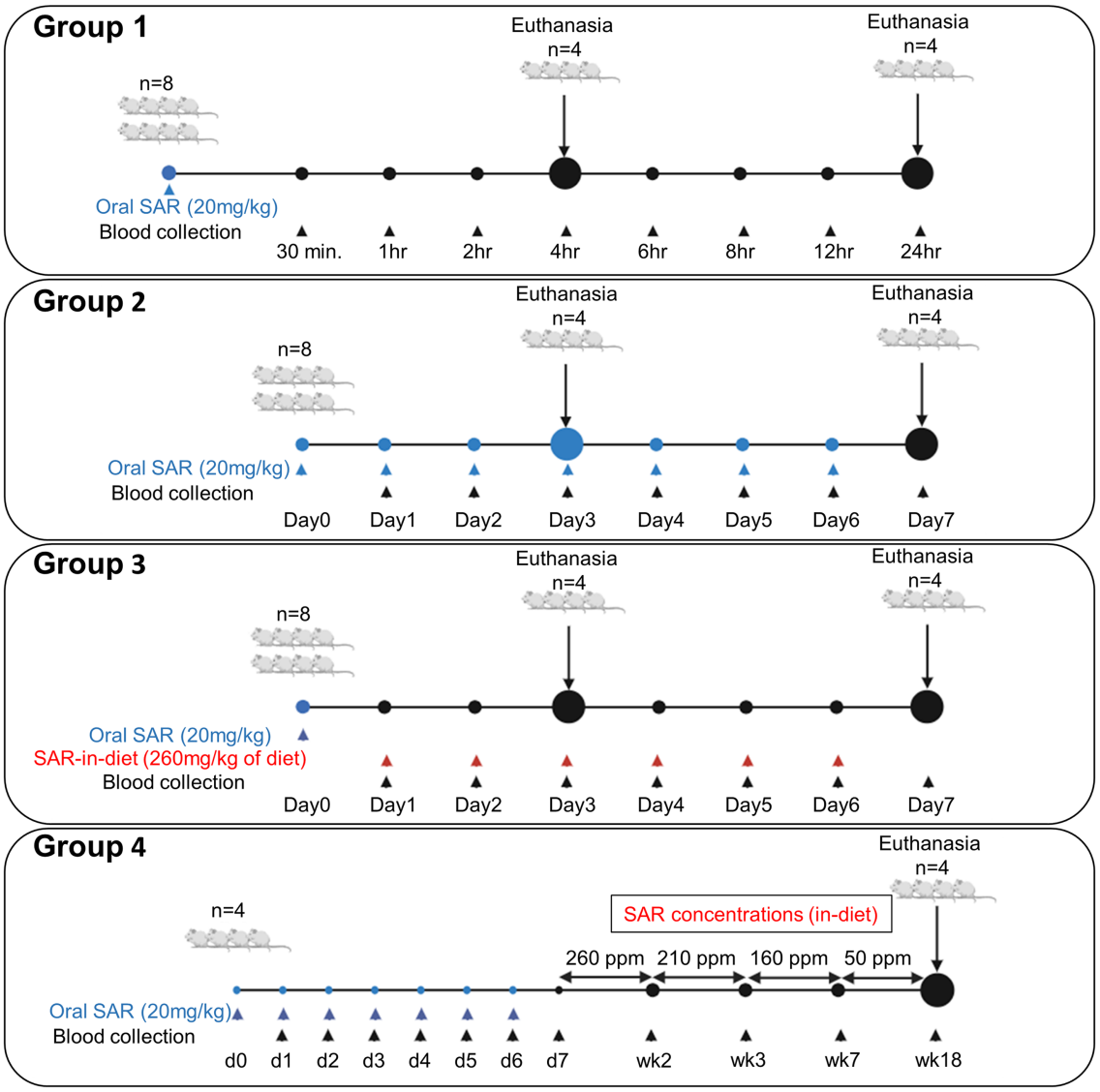


FIGURE 1
Experimental design for a single dose pharmacokinetics of saracatinib [24 h study, **(A)**], repeated daily oral dose [for 7 days, **(B)**], SAR-in-Diet [for 7 days, **(C)**], and for long term studies **(D)**.

2.2. Serum and hippocampal samples preparation for LC–MS/MS

Samples were submitted to the W.M. Keck Metabolomics Research Laboratory (Office of Biotechnology, Iowa State University, Ames, IA) for targeted saracatinib LC–MS/MS quantification. Serum and hippocampus preparations were conducted using a modified version of the previously available extraction and sample preparation methods previously established (18, 19). 50–75 µL or 50–75 mg wet weight of each sample was added to microcentrifuge tubes. A Reagent control sample was generated for each sample batch, 75 µL of 0.9% NaCl which was extracted in the same fashion as the samples. The weighed samples were then spiked with the internal standard, 1.25 µg of S-Hexylglutathione (Sigma-Aldrich CO., St. Louis, MO), added as 10 µL of from a 0.125 mg/mL solution in LC–MS grade 9:1 methanol: acetonitrile (Fisher Scientific, Waltham, MA). The extraction was initiated by the addition of 0.4 mL of 90% ice-cold LC–MS grade methanol with 10% LC–MS grade water (Fisher Scientific, Waltham, MA). For the hippocampus samples, after adding the 90% methanol to the samples, two 2.4 mm metal beads were added to each sample, the tissue was then homogenized using a Bead Mill 24 Homogenizer (Thermo Fisher Scientific, Inc., Waltham, MA, United States). All samples were then vortexed 10 s and placed on ice for 10 min before the addition of 1.0 mL of 3:1 of LC–MS grade acetonitrile: water (Fisher Scientific, Waltham, MA). The samples were again vortexed for 10 s before being placed in an ice-cold sonication water bath (Branson Ultrasonics, Brookfield, CT) for 10 min. Samples were vortexed for 10 min and centrifuged for 7 min at maximum speed (16,000 g). The serum sample supernatant extracts were transferred to LC vials and subjected directly to LC–MS/MS analysis. The hippocampus samples sample extracts were filtered with 0.2-micron centrifugal filters (Cat. No. UFC30LG25, Millipore Sigma, Burlington, MA) prior to LC–MS/MS analysis. A saracatinib

(AstraZeneca, Cambridge, England, United Kingdom) standard curve with a range of 0.49 to 1,000 ng per sample was prepared as 1:1 serial dilutions in 90% methanol before being extracted and subjected to LC–MS/MS analysis in the same manner as the biological samples.

2.3. Food pellet sample preparation for LC–MS/MS

Samples were submitted to the W.M. Keck Metabolomics Research Laboratory (Office of Biotechnology, Iowa State University, Ames, IA) for targeted saracatinib LC–MS/MS quantification. One or two food pellets (1.5–3 g) were homogenized by mortar-and-pestle before being transferred and weighed in glass tubes with Teflon-lined screw caps. A reagent control sample was generated for each sample batch which was prepared in the same fashion as the samples. The weighed samples were then spiked with the internal standard, 250 µg of S-Hexylglutathione (Sigma-Aldrich CO., St. Louis, MO), added as 1.0 mL of from a 0.25 mg/mL solution in LC–MS grade 9:1 methanol: acetonitrile (Fisher Scientific, Waltham, MA). The extraction was initiated by the addition of 8 mL of 90% LC–MS grade methanol with 10% LC–MS grade water (Fisher Scientific, Waltham, MA). The samples were then vortexed 10 s and allowed to sit on ice for 10 min before the addition of 12 mL of 5:1 of LC–MS grade acetonitrile: water (Fisher Scientific, Waltham, MA). The samples were again vortexed for 10 s before being placed in an ice-cold sonication water bath (Branson Ultrasonics, Brookfield, CT) for 10 min. Samples were vortexed for 10 min and centrifuged for 30 min at 900 g). The food pellet sample supernatant extracts were transferred to LC vials and subjected directly to LC–MS/MS analysis. A saracatinib (AstraZeneca, Cambridge, England, UK) standard curve with a range of 75 to 6 00 µg per sample was prepared as 1:1 serial dilutions in 90% methanol before being extracted and subjected to LC–MS/MS analysis in the same manner as the food pellet samples.

2.4. LC–MS/MS

LC separations were performed with an Agilent Technologies 1,290 Infinity Binary Pump UHPLC instrument equipped with an Agilent Technologies Eclipse C18 1.8 µm 2.1 mm × 100 mm analytical column that was coupled to an Agilent Technologies 6,540 UHD Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). A volume of 6 µL of each sample (0.5 µL of food pellet samples) was injected into the LC system. Chromatography was carried out at 40°C with a flow rate of 0.400 mL/min. Running solvents were A: water with 0.1% formic acid and B: acetonitrile with 0.1% formic acid. Initial solvent conditions were 1% B which increased on a linear gradient to 50% B over 7 min before increasing to 100% B over 2 min, 100% B was held for 6 min before returning to 1% B over 2 min. A 5-min post run at 1% B was conducted after each LC–MS/MS acquisition. Saracatinib was detected using electrospray ionization in positive ionization mode. Nitrogen was used as the service gas for the ion source with a drying gas flow rate of 12 L/min at a temperature of 350°C, a nebulizing pressure of 25 psi, and a sheath gas flow of 11 L/min at 400°C. The capillary and nozzle voltages were 4,000 and 1,750 volts, respectively. The mass spectrometer was operated in high resolution (4Gz) mode with a scan range from m/z 100 to m/z 1,700 for MS and m/z 60 to m/z 350 for MS/MS. An acquisition rate of 10 spectra per second was used for MS and 5 spectra per second for MS/MS, respectively. During LC–MS data acquisition, reference masses were monitored for continuous mass calibration: m/z 121.050873 with m/z 922.009698. Two targeted MS/MS selections were acquired: for saracatinib m/z 542.2 with a narrow isolation width and a collision energy of 25 eV; for the internal standard m/z 392.2 with a narrow isolation width and a collision energy of 25 eV. Data evaluation and peak quantitation were performed using Agilent MassHunter Qualitative Analysis (version 10.0) and Agilent MassHunter Quantitative Analysis (version 10.0) software (Agilent Technologies, Santa Clara, CA). Target peaks were quantified using the LC–MS/MS extracted ion chromatographs for saracatinib 542.2- > 127.1218 ± 50 ppm and the internal standard 392.2- > 246.1143 ± 50 ppm. Saracatinib quantification was finally determined by relative abundance to the internal standard and linear saracatinib standard curve before being made relative to the measured sample mass and volumes. The limit of detection (LOD) and quantification (LLOQ) were determined by the signal-to-noise ratio. The LLOQ was determined by the concentrations of spiked calibration standards.

2.5. Pharmacokinetic parameter analysis

A noncompartmental pharmacokinetic (or statistical moments) analysis was performed using PKanalix software ©Lixoft version 2023 to derive the following pharmacokinetic parameters for saracatinib after a single oral dose (20 mg/kg) 24 h study: elimination half-life ($T_{1/2}$), elimination rate constant (K_{el}), maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), apparent volume of distribution ($V_{d/F}$), apparent systemic clearance (CL/F). The first saracatinib concentration post dose below the LLOQ was inferred to be LLOQ/2, and subsequent data points were excluded from the analysis. Area under concentration vs. time curves was determined using the linear/log trapezoidal rule. The slope of the terminal phase for saracatinib (λz) was derived by linear regression between Y (log(concentrations)) and the X (time) using a $1/Y^2$ weighting method.

2.6. Experimental rigor and statistics

The experimental groups and samples were coded/blinded until the raw data were compiled and analyses were completed. We used GraphPad Prism 9.0 for statistical analysis. Normality tests were performed using the Shapiro–Wilk test. Based on the normality of the data, t-test or Mann–Whitney test was applied to compare data between the two groups. A mixed-effects model was performed to determine differences at different time points. Further statistical details are included in the figure legends of the corresponding figures.

3. Results

The experimental design is illustrated in Figure 1.

3.1. Feed consumption and bodyweight comparison between groups

In this study, the daily food intake and body weight during the one week experimental period were compared between the animals fed on a regular diet (Group 2) and SAR-in-Diet (Group 3). When the daily food consumption was compared between the two groups, the

SAR-in-Diet group consumed significantly less food on days 6–8 (Figure 2A); however, there were no significant differences in the average diet consumption before and after the first saracatinib oral dosing (Figure 2B). Interestingly, despite the reduction in daily food consumption in SAR-in-Diet group, there were no significant differences in the weight gain between the groups at any day (Figures 2C,D).

3.2. Authentication of saracatinib and its stability at room temperature in solution or when it was incorporated into the diet: measured by LC–MS/MS

Before the saracatinib was tested in animals, its identity and purity (>99%) was determined by LC–MS/MS. We first determined response linearity for saracatinib (Figure 3A). The retention time for saracatinib and the internal standard S- Hexylglutathione was 4.315 and 4.992 min, respectively (Figures 3B,C). When peak area (y) was plotted against the ascending saracatinib standard, a good correlation coefficient R^2 of 0.9998 was obtained, which was within the accepted range and showed a linear relationship. The slope (m) and intercept (c) of the calibration curve were 0.0214 and 0.0286, respectively. The LOD and LLOQ of the method was 7.35 ng/mL or ng/g and 22.65 ng/mL or ng/g, respectively. The SAR-in-Diet or in solution at room temperature was stable for >4 weeks (97 ± 1.56%) as detected by LC–MS/MS.

3.3. Serum pharmacokinetics and hippocampal concentrations of saracatinib after a single oral dose (20 mg/kg) 24 h study

After a single bolus dose of saracatinib (20 mg/kg, oral gavage), the absorption of the drug seems biphasic. The initial absorption from the GI lumen was slow for up to 2 h, followed by a steep increase in the rate of absorption. The peak serum concentration attained by saracatinib was 954.42 ng/mL at 6 h (Figure 4A). The mean volume of distribution was 15.59 L/kg, and the clearance was 2.83 L/h/kg. Saracatinib was detected in the serum up to 24 h after a single oral dose. The estimated elimination half-life of saracatinib was 4.12 h with a rate constant of 0.18 per hour. The area under the concentration-time curve (AUC) from 0 to 24 h was 6992.15 (ng/mL*h) (Supplementary Table S1). The hippocampal levels were > 500 ng/g at 4 h, and by 24 h, the concentrations dropped to nearly half compared to 4 h. The hippocampal concentrations were higher than the serum concentrations at 4 h and 24 h (Figure 4B).

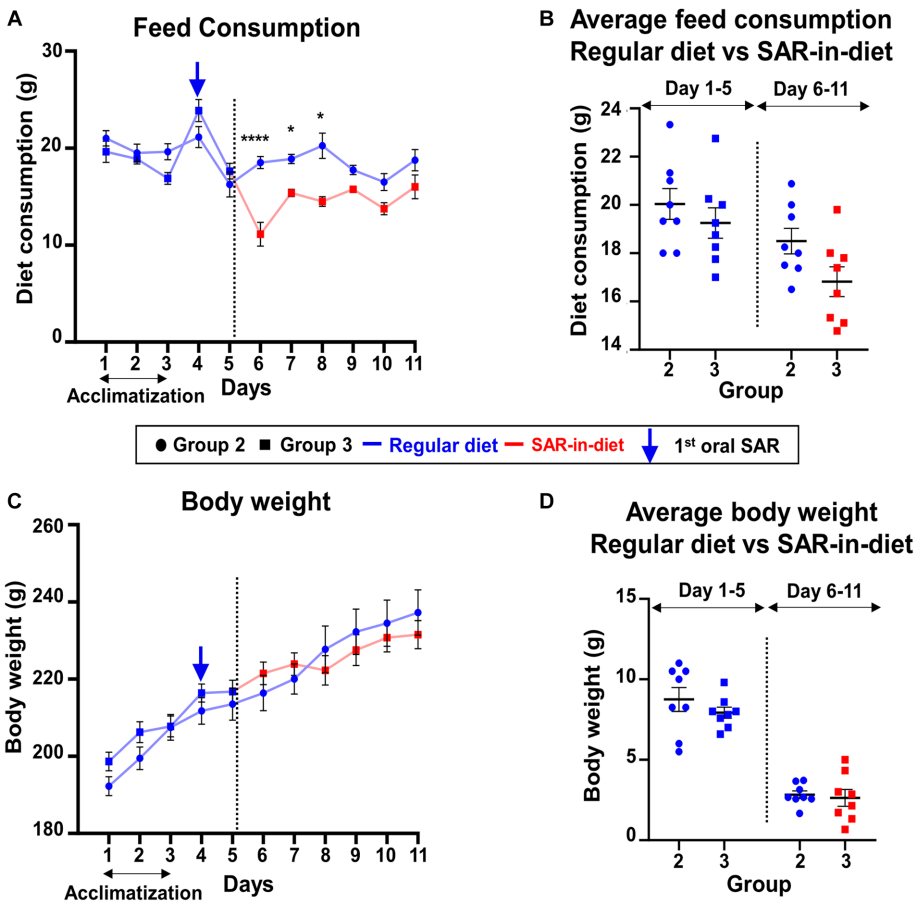


FIGURE 2
Comparison of feed consumption **(A,B)** and weight gain **(C,D)** between the animals that were on regular diet (Group 2) versus SAR-in-Diet (Group 3) for a week. $n = 4–8$ **(A,C)** mixed-effects analysis (Šídák's multiple comparisons test); $n = 8$ **(B,D)** two-way ANOVA (Tukey's multiple comparisons test); *$p < 0.05$, ****$p < 0.0001$.

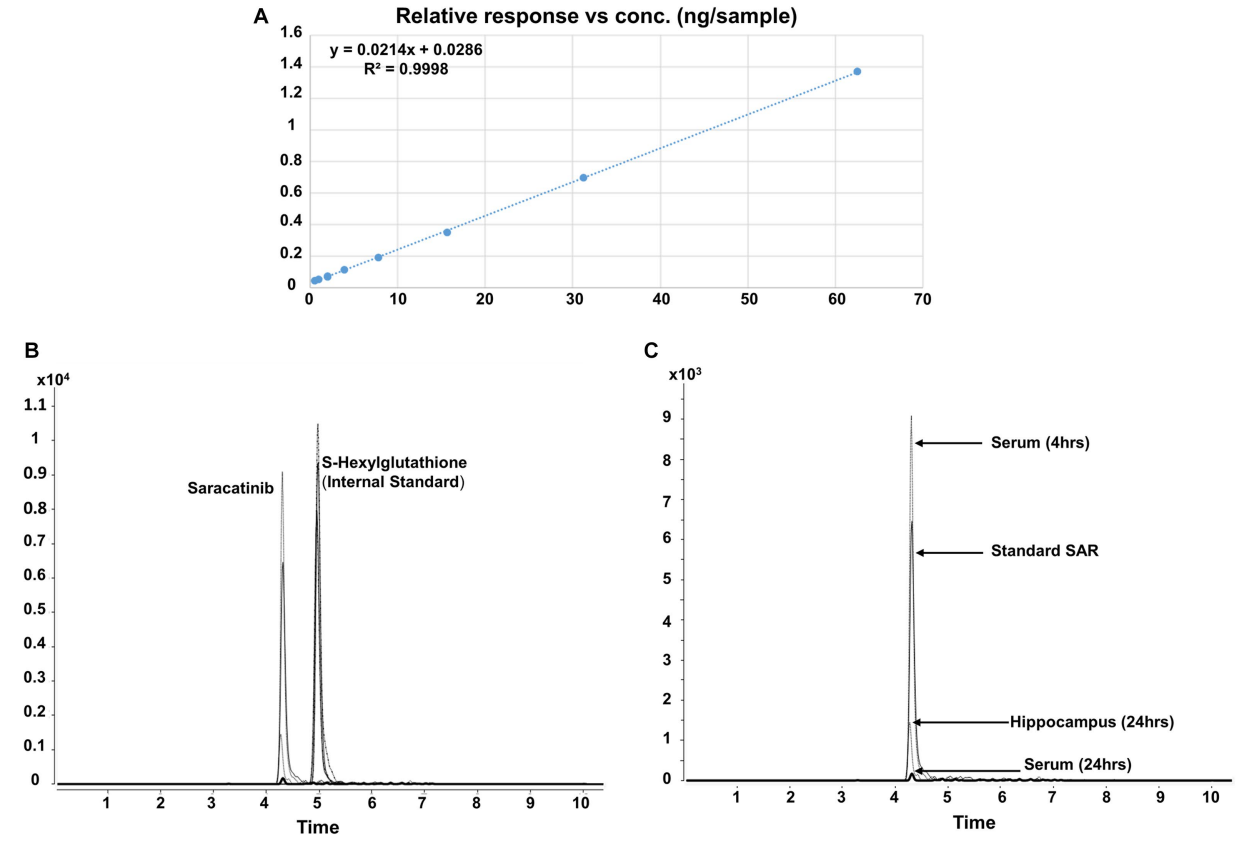


FIGURE 3
Method validation for saracatinib using LC–MS/MS. Response linearity of saracatinib **(A)**, chromatogram of saracatinib and internal standard (S-Hexylglutathione) **(B)**, chromatogram of saracatinib in the serum, the hippocampus and the standard saracatinib **(C)**.

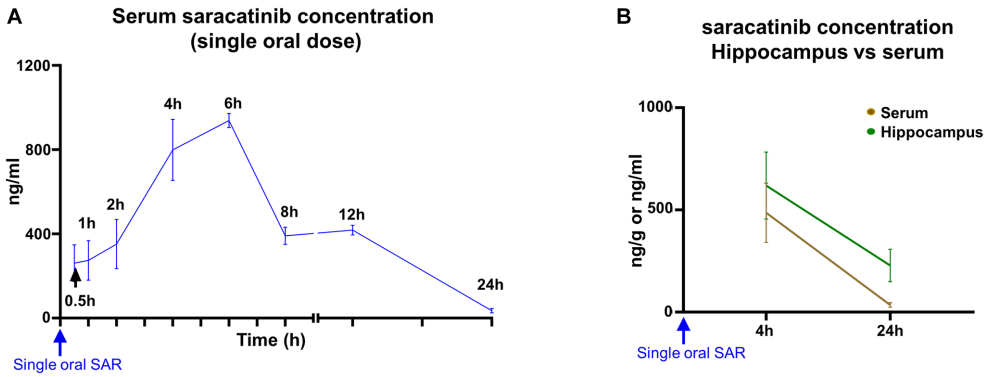


FIGURE 4
Mean (± SEM) serum and hippocampal concentrations of saracatinib after a single oral dose (20 mg/kg). $n = 4–8$ **(A)**; $n = 4$ **(B)** mixed-effects analysis (Šídák's multiple comparisons test).

3.4. Serum and hippocampal concentrations of saracatinib in repeated daily dosing and SAR-in-Diet groups in the 7-day study groups

In the repeated daily dosing of saracatinib (20 mg/kg, oral gavage), blood sampling was done at 24 h intervals before the next oral dosing. The serum saracatinib concentrations were maintained at ~100 ng/mL for the first three days, while trough serum concentration of saracatinib steadily increased during 3–6 days to reach a plateau at day 7 (Figure 5A). In the SAR-in-Diet group, the serum concentration steadily increased during days 1–3, 5, and 6 with a reduction in saracatinib systemic levels at day 4 and day 7 (Figure 5A). However, there were no significant differences in the serum concentrations of saracatinib between the repeated daily oral dosing (group 2) and SAR-in-Diet (group 3) except on day 3

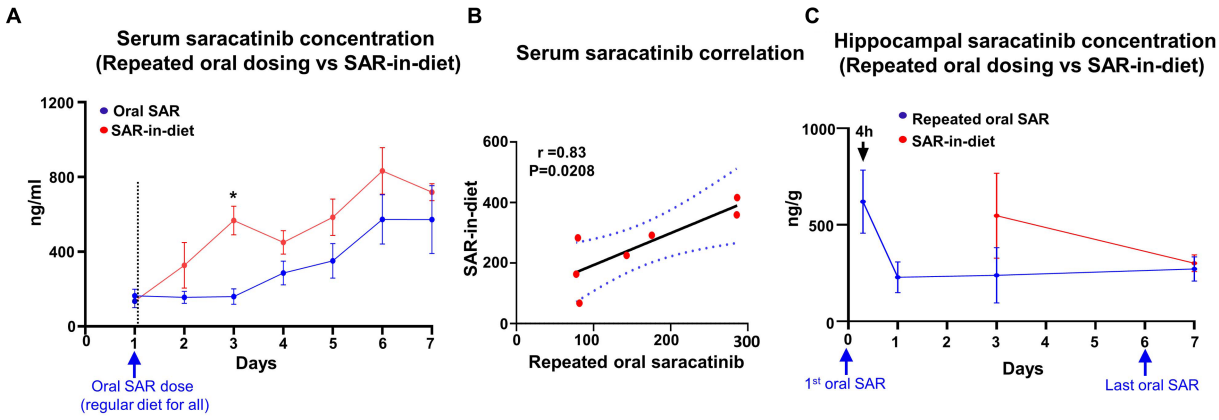


FIGURE 5
Comparison between mean (± SEM) serum concentrations of saracatinib after repeated oral administration (20 mg/kg) and in SAR-in-Diet (20 mg/kg) groups **(A)**. Correlation between the serum concentration of saracatinib between repeated oral administration (20 mg/kg) and in SAR-in-Diet (20 mg/kg) groups **(B)**. Hippocampal SAR concentrations **(C)**. Significant increase in the serum concentrations of SAR at only day 3 in SAR-in-Diet versus repeated oral dosing **(A)** $n = 4$, mixed-effects analysis (Šídák's multiple comparisons test) $^*p < 0.05$.

(Figure 5A), which could be due to reduction in food intake in the SAR-in-Diet group during the previous 24 h (Figure 2A). Overall, there was a strong and significant positive correlation in the serum concentrations of saracatinib between the repeated daily oral dosing and SAR-in-Diet groups (Figure 5B). Hippocampal saracatinib concentrations in the repeated oral dosing maintained nearly constant levels at days 3 and 7 (Figure 5C). There were no significant differences in the hippocampal saracatinib concentrations when compared between the repeated daily oral dosing versus the animals fed on SAR-in-Diet at 3 and 7 days (Figure 5C).

TABLE 1 SAR concentration in diet and SAR dose rate in long term study (18 weeks).

Expected SAR-in-Diet (ppm)	Actual SAR conc. (ppm)	Target dose rate of SAR (mg/kg)	Dose rate of SAR achieved (mg/kg)	No. of weeks on the diet
260	260.15	20	17.47	2
210	219.41	15	13.75	1
160	165.45	10	8.95	4
50	54.43	5	2.75	11

3.5. Serum concentrations of saracatinib when the animals were fed with different concentrations of SAR-in-Diet for long term

SAR-in-Diet of different concentrations were prepared, and the drug concentrations were confirmed in the prepared diet by LC–MS/MS (Table 1). The animals first received daily oral dosing of saracatinib (20 mg/kg of rat) for a week and then fed with SAR-in-Diet of varying/tapering concentrations. The concentrations of saracatinib in the diet were 260, 210, 160, and 50 ppm and were given for 2, 1, 4, and 11 weeks, respectively (group 4). Blood samples were collected at the end of each period before the diet was changed. The experimental design for group 4 is illustrated in Figure 1D. The overview of the serum saracatinib concentrations is represented in Figure 6.

3.6. Correlation analysis of serum SAR levels and SAR-in-Diet consumption at different concentrations

In group 3, animals were given saracatinib with an initial single oral dose followed by 6 days of SAR-in-Diet at 260 ppm. The serum concentration of saracatinib and the rate at which the drug was consumed (mg/kg of rat) via SAR-in-Diet is presented in Figure 7A. A positive correlation was observed between serum concentration and the SAR-in-Diet consumption rate (Figure 7B). In group 4, animals were given repeated oral dosing of saracatinib for 7 days followed by SAR-in-Diet at 260 ppm, 210 ppm, 160 ppm, and 50 ppm until the end of week 2, week 3, week 7 and week 18, respectively. The serum concentration of saracatinib and the rate at which the drug was received through SAR-in-Diet is presented in Figure 7C. Overall, a strong and positive significant correlation was observed between serum saracatinib concentrations and the drug consumption rate via the diet (Figure 7D).

4. Discussion

In this study, we demonstrated the feasibility of incorporating various concentrations of an orally active test drug saracatinib, for about 18 weeks in healthy adult male rats. We also compared the serum disposition kinetics, feed consumption, and weight gain between orally administered saracatinib and diet-incorporated saracatinib in a week-long study. Our descriptive PK analysis confirmed that saracatinib is well absorbed when administered orally, reaches a peak concentration at approximately 6 h, and is

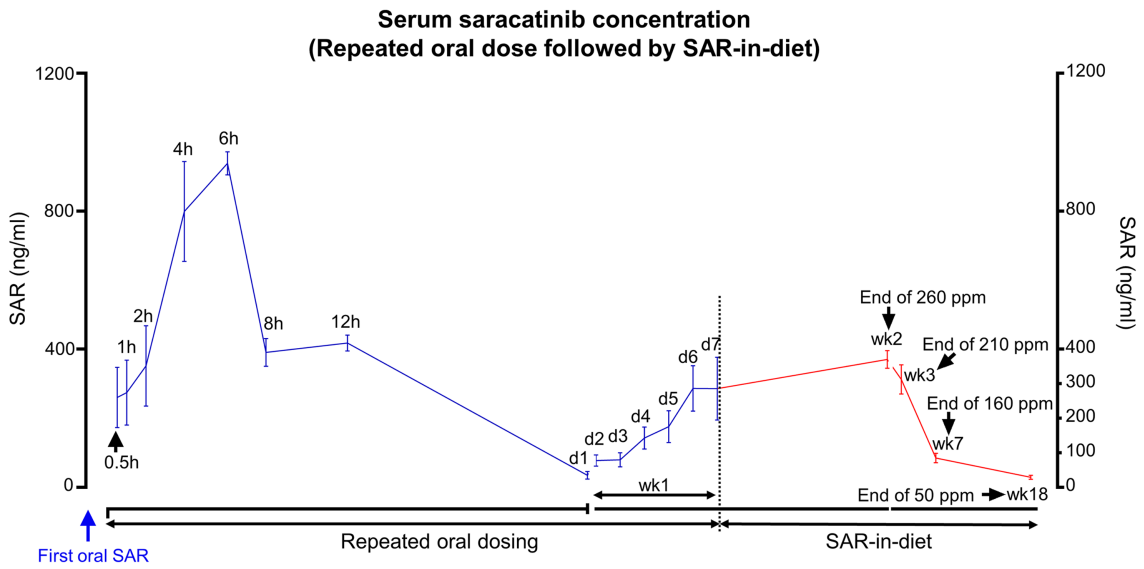


FIGURE 6
Serum SAR concentrations in animals that received repeated daily oral dosing of SAR (20 mg/kg) followed by varying concentrations of SAR-in-Diet for long term. $n = 4–8$.

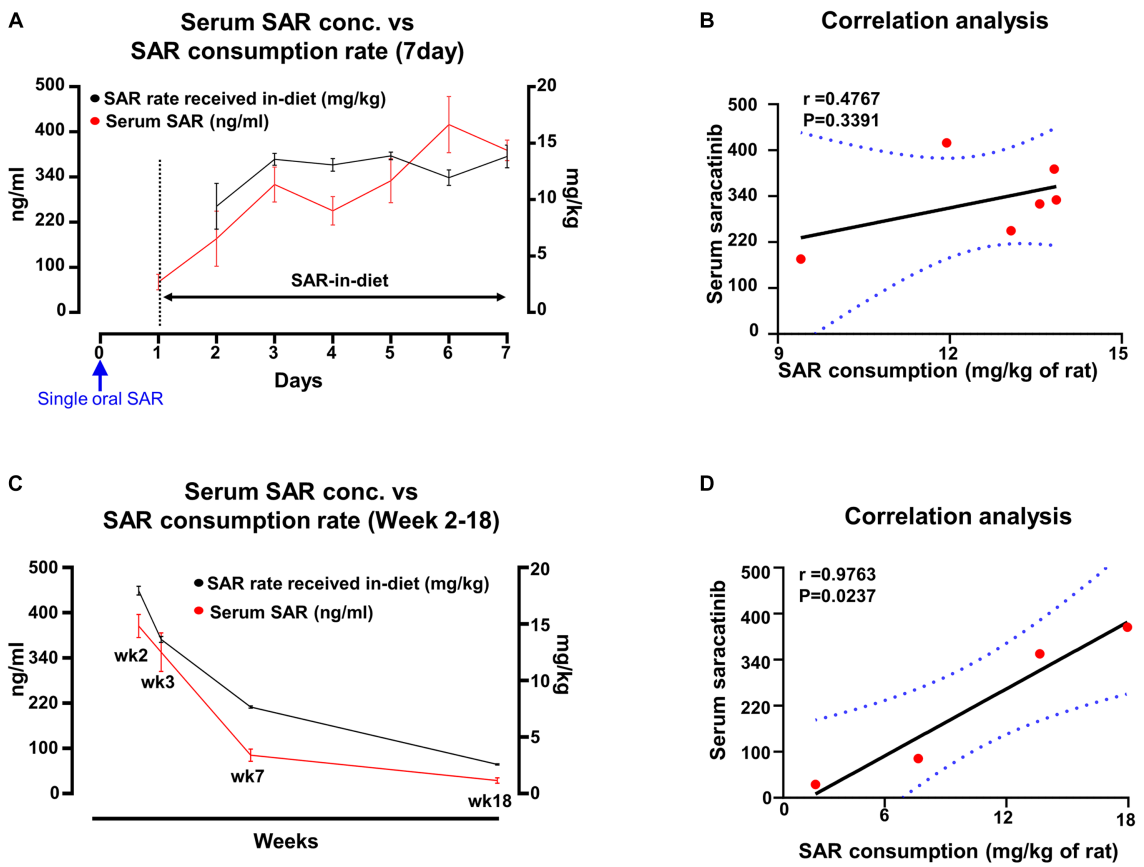


FIGURE 7
Comparison and correlation analysis of serum concentration of saracatinib and its consumption rate when the animals were fed with varying concentrations of SAR-in-Diet. $n = 4–8$. **(A,C)** mixed-effects analysis (Šídák's multiple comparisons test); $n = 4–8$ **(B,D)** Pearson correlation analysis.

well-distributed to the brain. Clinical pharmacokinetics of saracatinib in healthy humans at the dose rate of 250 mg/day once a day daily for 10–14 days has shown excellent pharmacokinetic parameters with the $T_{1/2}$ of 40 h and was well tolerated. However, the drug and its metabolites may accumulate in the tissues over time (20). Therefore, a tapering dosing regimen may mitigate such adverse effects in long-term studies. In contrast to human pharmacokinetics, our study in adult rats shows that the half-life of saracatinib was much lower in rats, perhaps due to a higher metabolic rate.

Exposure to DFP and soman can lead to status epilepticus resulting in spontaneous recurrent seizures (21–24). We have previously demonstrated the disease-modifying effects of saracatinib in the rat kainate and DFP models of epilepsy (3, 16). However, saracatinib efficacy seems to depend on the initial severity of the seizures and subsequent brain pathology (25). Therefore, optimizing the effective dose of saracatinib for chronic diseases such as epilepsy, AD, and certain types of cancers, and a long term treatment of saracatinib at decreasing concentrations may be required to minimize adverse effects. Src/Fyn kinases play a significant role in cell proliferation and migration, an active process in cancers and chronic neurodegenerative diseases (for example, gliosis). Therefore, inhibiting Src/Fyn kinase activity can be beneficial to control or modify the progression of the disease.

Saracatinib has been tested in several clinical trials for various types of cancers, AD and PD. In all these trials, saracatinib was administered as oral tablets or capsules for a very long time (13, 26, 27). Since saracatinib is an orally active drug, it has to pass through the hepatic circulation for its metabolism. A repeated oral gavage in experimental models is not feasible for long term. Furthermore, repeated handling of animals for oral gavage causes stress and confounds the experimental results. Therefore, for long term studies test drugs are normally added to the drinking water or the feed (28). In a mouse model of AD, saracatinib incorporated into the diet was fed for up to 9 months and demonstrated its disease-modifying effects (29). In the mouse AD study, they monitored the body weight to determine the food/drug intake. Our current study demonstrates the feasibility, stability, serum and brain pharmacokinetics of saracatinib when the animals were fed for the long term with the drug incorporated in the rat chow. We also compared the differences in serum and brain saracatinib concentrations between daily oral dosing versus SAR-in-Diet in a 7-day study. The brain concentrations were also decreased as the serum concentrations decreased but took a longer time to clear from the brain, which is an advantage from a therapeutic perspective.

The commonly used routes of drug delivery in laboratory animals require repeated handling and restraint that are aversive to animals. This can be a problem in studies that require chronic administration of drugs daily orally. Furthermore, the timing of administration of the test drug may impact the pharmacokinetics and the drug metabolism, which depends on animal's metabolic state during the day or the night. Since rodents are nocturnal, they are metabolically active at night and eat more than during the day. This may be an advantage for rodents if a test drug mixed in the diet was fed, especially for epileptic conditions to control the incidence of nocturnal seizures.

Several studies from other labs have demonstrated the delivery of drugs such as antibiotics or disease-modifying agents in drinking water or by incorporating them into the diet (29, 30). However, the choice of such a delivery method depends on the stability of the test drug at room temperature and whether the test drug remains in solution without precipitation. Although saracatinib in solution is stable at room temperature for several weeks, it may require stirring to prevent precipitation, therefore, not suitable for delivering it in drinking water for long term treatment. Furthermore, when added to water, saracatinib solution alters its taste and impacts daily water consumption. Diet-incorporated saracatinib has been shown to be effective in a mouse model of AD (29). Repeated oral dosing or chronic diet incorporated delivery is required to achieve a steady state of saracatinib serum concentrations. Although we did not find significant differences between the two approaches, sustained and steady optimum serum concentrations of saracatinib are likely to be maintained through diet rather than acute dosing. Acute dosing rapidly increases serum concentrations of saracatinib within 6 h and decreases after that. Such rapid increases of test drug in serum may have off-target effects in contrast to a gradual buildup of serum concentrations when the test drugs were administered through the diet (31–33).

The choice of administration of test drugs in rodents depends on various factors such as rate of absorption, target retention/engagement, and the rate of metabolism, which is high in small rodents and impacts drug clearance (34, 35). Drugs administered in humans or other species with lower metabolic rates and various disease conditions require dosage adjusting to achieve optimum therapeutic levels in serum and the target organs (36). Our data from repeated daily oral dosing versus SAR-in-Diet suggest that a steady state of therapeutic concentration in serum can be achieved through diet incorporation.

The main objective of the translational studies in preclinical rodent models is to acquire adequate and robust scientific data for future drug development for clinical application. In humans, oral medication takes priority over parenteral routes of administration for obvious reasons. Therefore, characterizing the orally active test drugs in preclinical models has a translational potential. Saracatinib has been tested in various experimental models and its PK and toxicity at high doses are also known from clinical trials (8, 37). Our published work has demonstrated the disease-modifying effects of saracatinib in experimental models of epilepsy (3, 25). The long term treatment of saracatinib in epilepsy and AD models, and in various cancerous studies in clinical trials provide adequate evidence for the translational potential of saracatinib for future drug development. Furthermore, saracatinib reverses ATP-binding cassette (ABC) transporters B1 (ABCB1) mediated multidrug resistance (15). Interestingly, saracatinib only inhibits ABCB1 transport function, without altering ABCB1 expression or AKT phosphorylation. Therefore, incorporating orally active antiepileptic drugs and saracatinib in the diet could offer therapeutic benefits in epilepsy.

5. Conclusion

Repeated oral dosing of saracatinib and SAR-in-Diet produced comparable serum saracatinib concentrations with no significant differences between groups, except on day 3 wherein saracatinib serum concentrations were significantly higher in SAR-in-Diet group. Overall, saracatinib concentrations in the serum and the hippocampus reached therapeutic concentrations in rats fed a SAR-in-Diet, which has obvious potential benefits in the management of chronic diseases such as epilepsy. This approach can be extended to many other orally active therapeutic drugs that are intended for use long term.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Iowa State University Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TT: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. NM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SN: Methodology, Validation, Visualization, Writing – review & editing. JM: Methodology, Validation, Visualization, Writing – review & editing. LS: Data curation, Methodology, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the National Institute of Health/NINDS through the CounterACT program, grant number NS117284, and W.E. Lloyd Endowment fund SG2200008.

Acknowledgments

We thank AstraZeneca for providing saracatinib under the Open Innovation Program for this study.

Conflict of interest

This study received the test drug, saracatinib, under the Open Innovation Program from AstraZeneca. However, AstraZeneca was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1297221/full#supplementary-material

References

1. Elias D, Ditzel HJ. Fyn is an important molecule in cancer pathogenesis and drug resistance. *Pharmacol Res*. (2015) 100:250–4. doi: 10.1016/j.phrs.2015.08.010

2. Wakelee HA, Gubens MA, Burns M, Barbeau SL, Perkins S, Pedo-Salcedo MGS, et al. A phase II study of Saracatinib (AZD0530), a Src inhibitor, administered orally daily to patients with advanced Thymic malignancies. *J Thorac Oncol*. (2010) 5:S528–8.

3. Sharma S, Carlson S, Gregory-Flores A, Hinojo-Perez A, Olson A, Thippeswamy T. Mechanisms of disease-modifying effect of saracatinib (AZD0530), a Src/Fyn tyrosine kinase inhibitor, in the rat kainate model of temporal lobe epilepsy. *Neurobiol Dis*. (2021) 156:105410. doi: 10.1016/j.nbd.2021.105410

4. Luo XM, Zhao J, Wu WY, Fu J, Li ZY, Zhang M, et al. Post-status epilepticus treatment with the Fyn inhibitor, saracatinib, improves cognitive function in mice. *BMC Neurosci*. (2021) 22:2. doi: 10.1186/s12868-020-00606-z

5. Putra M, Puttachary S, Liu GH, Lee G, Thippeswamy T. Fyn-tau ablation modifies PTZ-induced seizures and post-seizure hallmarks of early Epileptogenesis. *Front Cell Neurosci*. (2020) 14:e592374. doi: 10.3389/fncel.2020.592374

6. Nygaard HB, van Dyck CH, Strittmatter SM. Fyn kinase inhibition as a novel therapy for Alzheimer's disease. *Alzheimers Res Ther*. (2014) 6:8. doi: 10.1186/alzrt238

7. Baird K, Glod J, Steinberg SM, Reinke D, Pressey JG, Mascarenhas L, et al. Results of a randomized, double-blinded, placebo-controlled, phase 2.5 study of saracatinib (AZD0530), in patients with recurrent osteosarcoma localized to the lung. Sarcoma, 2020 (2020).

8. Oswald AJ, Symeonides SN, Wheatley D, Chan SP, Brunt AM, McAdam K, et al. Aromatase inhibition plus/minus Src inhibitor saracatinib (AZD0530) in advanced breast cancer therapy (ARISTACAT): a randomised phase II study. *Breast Cancer Res Treat*. (2023) 199:35–46. doi: 10.1007/s10549-023-06873-8

9. Williams E, Bagarova J, Kerr G, Xia DD, Place ES, Dey D, et al. Saracatinib is an efficacious clinical candidate for fibrodysplasia ossificans progressiva. *JCI Insight*. (2021) 6. doi: 10.1172/jci.insight.95042

10. Du F, Tang T, Li QZ, Liu JX. Fyn signaling in ischemia-reperfusion injury: potential and therapeutic implications. *Mediat Inflamm*. (2022) 2022:1–10. doi: 10.1155/2022/9112127

11. Guglietti B, Sivasankar S, Mustafa S, Corrigan F, Collins-Praino LE. Fyn kinase activity and its role in neurodegenerative disease pathology: a potential universal target? *Mol Neurobiol*. (2021) 58:5986–005. doi: 10.1007/s12035-021-02518-3

12. Kaufman AC, Salazar SV, Haas LT, Yang J, Kostylev MA, Jeng AT, et al. Fyn inhibition rescues established memory and synapse loss in Alzheimer mice. *Ann Neurol*. (2015) 77:953–71. doi: 10.1002/ana.24394

13. McFarthing K, Buff S, Rafaloff G, Dominey T, Wyse RK, Stott SRW. Parkinson's disease drug therapies in the clinical trial pipeline: 2020. *J Park Dis*. (2020) 10:757–74. doi: 10.3233/JPD-202128

14. van Dyck CH, Nygaard HB, Chen KW, Donohue MC, Raman R, Rissman RA, et al. Effect of AZD0530 on cerebral metabolic decline in Alzheimer disease a randomized clinical trial. *JAMA Neurol*. (2019) 76:1219–29. doi: 10.1001/jamaneurol.2019.2050

15. Liu KJ, He JH, Su XD, Sim HM, Xie JD, Chen XG, et al. Saracatinib (AZD0530) is a potent modulator of ABCB1-mediated multidrug resistance in vitro and in vivo. *Int J Cancer*. (2013) 132:224–35. doi: 10.1002/ijc.27649

16. Gage M, Putra M, Wachter L, Dishman K, Gard M, Gomez-Estrada C, et al. Saracatinib, a Src tyrosine kinase inhibitor, as a disease modifier in the rat DFP model: sex differences, Neurobehavior, gliosis, neurodegeneration, and nitro-oxidative stress. *Antioxidants*. (2022a) 11:61. doi: 10.3390/antiox11010061

17. Gage M, Vinithakumari AA, Mooyottu S, Thippeswamy T. Gut dysbiosis following organophosphate, diisopropylfluorophosphate (DFP), intoxication and saracatinib oral administration. *Front Microb*. (2022c) 1:1006078. doi: 10.3389/frmbi.2022.1006078

18. Jackson KD, Durandis R, Vergne MJ. Role of cytochrome P450 enzymes in the metabolic activation of tyrosine kinase inhibitors. *Int J Mol Sci*. (2018) 19:2367. doi: 10.3390/ijms19082367

19. Sharma S, Carlson S, Puttachary S, Sarkar S, Showman L, Putra M, et al. Role of the Fyn-PKCδ signaling in SE-induced neuroinflammation and epileptogenesis in experimental models of temporal lobe epilepsy. *Neurobiol Dis*. (2018) 110:102–21. doi: 10.1016/j.nbd.2017.11.008

20. Hannon RA, Clack G, Rimmer M, Swaisland A, Lockton JA, Finkelman RD, et al. Effects of the Src kinase inhibitor saracatinib (AZD0530) on bone turnover in healthy men: a randomized, double-blind, placebo-controlled, multiple-ascending-dose phase I trial. *J Bone Miner Res*. (2010) 27:1435–5. doi: 10.1002/jbmr.1557

21. Gage M, Rao NS, Samidurai M, Putra M, Vasanthi SS, Meyer C, et al. Soman (GD) rat model to mimic civilian exposure to nerve agent: Mortality, video-EEG based status epilepticus severity, sex differences, spontaneously recurring seizures, and brain pathology. *Front Cell Neurosci*. (2022b) 15:15. doi: 10.3389/fncel.2021.798247

22. Massey N, Vasanthi SS, Samidurai M, Gage M, Rao NK, Meyer C, et al. 1400 W, a selective inducible nitric oxide synthase inhibitor, mitigates early neuroinflammation and nitrooxidative stress in diisopropylfluorophosphate-induced short-term neurotoxicity rat model. *Front Mol Neurosci*. (2023) 16:1125934. doi: 10.3389/fnmol.2023.1125934

23. Rao NS, Meyer C, Vasanthi SS, Massey N, Samidurai M, Gage M, et al. DFP-induced status epilepticus severity in mixed-sex cohorts of adult rats housed in the same room: behavioral and EEG comparisons. *Front Cell Dev Biol*. (2022) 10. doi: 10.3389/fcell.2022.895092

24. Vasanthi SS, Rao NS, Samidurai M, Massey N, Meyer C, Gage M, et al. Disease-modifying effects of a glial-targeted inducible nitric oxide synthase inhibitor (1400W) in mixed-sex cohorts of a rat soman (GD) model of epilepsy. *J Neuroinflammation*. (2023) 20:163. doi: 10.1186/s12974-023-02847-1

25. Gage M, Putra M, Gomez-Estrada C, Golden M, Wachter L, Gard M, et al. Differential impact of severity and duration of status epilepticus, medical countermeasures, and a disease-modifier, Saracatinib, on brain regions in the rat Diisopropylfluorophosphate model. *Front Cell Neurosci*. (2021) 15:e772868. doi: 10.3389/fncel.2021.772868

26. Gucalp A, Sparano JA, Caravelli J, Santamauro J, Patil S, Abbruzzi A, et al. Phase II trial of Saracatinib (AZD0530), an Oral SRC-inhibitor for the treatment of patients with hormone receptor-negative metastatic breast Cancer. *Clin Breast Cancer*. (2011) 11:306–11. doi: 10.1016/j.clbc.2011.03.021

27. Nygaard HB, Wagner AF, Bowen GS, Good SP, MacAvoy MG, Strittmatter KA, et al. A phase Ib multiple ascending dose study of the safety, tolerability, and central nervous system availability of AZD0530 (saracatinib) in Alzheimer's disease. *Alzheimers Res Ther*. (2015) 7:35. doi: 10.1186/s13195-015-0119-0

28. Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of Substances to laboratory animals: routes of administration and factors to consider. *J Am Assoc Lab Anim Sci*. (2011) 50:600–13.

29. Tang SJ, Fesharaki-Zadeh A, Takahashi H, Nies SH, Smith LM, Luo A, et al. Fyn kinase inhibition reduces protein aggregation, increases synapse density and improves memory in transgenic and traumatic Tauopathy. *Acta Neuropathol Commun*. (2020) 8:96. doi: 10.1186/s40478-020-00976-9

30. Redelsperger IM, Taldone T, Riedel ER, Lepherd ML, Lipman NS, Wolf FR. Stability of doxycycline in feed and water and minimal effective doses in tetracycline-inducible systems. *J Am Assoc Lab Anim Sci*. (2016) 55:467–74.

31. Lin A, Giuliano CJ, Palladino A, John KM, Abramowicz C, Yuan ML, et al. Off-target toxicity is a common mechanism of action of cancer drugs undergoing clinical trials. *Sci Transl Med*. (2019) 11. doi: 10.1126/scitranslmed.aaw8412

32. Van Harken DR, Hottendorf GH. Comparative absorption following the administration of a drug to rats by oral gavage and incorporation in the diet. *Toxicol Appl Pharmacol*. (1978) 43:407–10. doi: 10.1016/0041-008X(78)90020-0

33. Zhao ZM, Ukidve A, Kim J, Mitragotri S. Targeting strategies for tissue-specific drug delivery. *Cells*. (2020) 181:151–67. doi: 10.1016/j.cell.2020.02.001

34. Agoston DV. How to translate time? The temporal aspect of human and rodent biology. *Front Neurol*. (2017) 8:92. doi: 10.3389/fneur.2017.00092

35. Kim J. *Medication routes of administration*. Treasure Island (FL): StatPearls Publishing (2023).

36. Herbrink M, de Vries N, Rosing H, Huitema ADR, Nuijen B, Schellens JHM, et al. Quantification of 11 therapeutic kinase inhibitors in human plasma for therapeutic drug monitoring using liquid chromatography coupled with tandem mass spectrometry. *Ther Drug Monit*. (2016) 38:649–56. doi: 10.1097/FTD.0000000000000349

37. Baselga J, Cervantes A, Martinelli E, Chirivella I, Hoekman K, Hurwitz HI, et al. Phase I safety, pharmacokinetics, and inhibition of Src activity study of Saracatinib in patients with solid tumors. *Clin Cancer Res*. (2010) 16:4876–83. doi: 10.1158/1078-0432.CCR-10-0748

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 03 January 2024
DOI 10.3389/fvets.2023.1320162

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Ayhan Filazi,
Ankara University, Türkiye
James Edward Brereton,
Sparsholt College, United Kingdom

*CORRESPONDENCE
Wilson K. Rumbeiha
✉ wkrumbeiha@ucdavis.edu

RECEIVED 11 October 2023
ACCEPTED 04 December 2023
PUBLISHED 03 January 2024

CITATION
Kwizera E, Rumbeiha WK, Nishimwe K and Nziza J (2024) A survey to document toxic hazards in the zone surrounding volcanoes national park, a habitat for mountain gorillas, an endangered wildlife species in Rwanda. *Front. Vet. Sci.* 10:1320162. doi: 10.3389/fvets.2023.1320162

COPYRIGHT
© 2024 Kwizera, Rumbeiha, Nishimwe and Nziza. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

A survey to document toxic hazards in the zone surrounding volcanoes national park, a habitat for mountain gorillas, an endangered wildlife species in Rwanda

Enock Kwizera[1], Wilson K. Rumbeiha[2]*, Kizito Nishimwe[3] and Julius Nziza[4]

[1]Department of Veterinary Medicine, University of Rwanda, Nyagatare, Rwanda, [2]Department of Molecular Biosciences, University of California, Davis, Davis, CA, United States, [3]Department of Food Science and Technology, University of Rwanda, Musanze, Rwanda, [4]Gorilla Doctors, Musanze, Rwanda

Introduction: In recent years, Volcanoes National Park has seen a rise in its wildlife population, primarily due to the diligent efforts of the Rwandan government in safeguarding endangered species, notably the mountain gorillas (*Gorilla beringei spp. beringei*). This population growth has led to a pressing need for more expansive habitats, ensuring these creatures have ample space, sustenance, and shelter for their wellbeing. Consequently, there are planned park expansion activities on the horizon. However, before initiating this expansion, a critical prelude involves identifying potential threats, particularly toxic substances stemming from agricultural activities in the surrounding environment of Volcanoes National Park.

Methods: To address this concern, a comprehensive study was conducted, aimed at pinpointing potential toxic hazards and assessing the awareness of the local population regarding the harm these hazards pose to wildlife species. Data was collected from individuals with no prior knowledge of the study using a pre-tested questionnaire. The questionnaire was divided into three sections: socio-demographic issues, potential toxic hazards assessment, and a section to determine awareness and risk of potential toxic hazards to humans, animals, and the environment. Respondents were selected based on specific criteria, which included being 18 years or older and residing within the National Volcano Park (NVP) area.

Results: The study's findings revealed four main categories of potential toxic hazards, which include household chemicals, pharmaceutical products, agricultural pesticides, and poisonous plants. These hazards could jeopardize the health and survival of wildlife species if they consume or come into contact with them. Furthermore, the study exposed an inadequacy in the knowledge and skills of the local community in preventing these toxic hazards, which can result in death of wildlife species and ecosystem contamination and degradation.

Conclusion: Study results also underscored the significance of education and training in enhancing the awareness of local communities concerning these toxic threats. Therefore, it is imperative to implement immediate measures to mitigate the adverse effects of these toxic hazards on wildlife species, especially in light of the planned park expansion.

KEYWORDS

chemicals, wildlife, mountain gorillas, VNP, pesticides, pharmaceuticals, poisonous plants, toxicants

1 Introduction

The Volcanoes National Park (VNP) is situated in Northern Rwanda and is part of the Virunga Massif landscape that also consists of the Mgahinga Gorilla National Park in Southwestern Uganda and the Virunga National Park in the Eastern Democratic Republic of Congo (DRC). The VNP, with an area of 160 km^2, encompasses high-altitude volcanic mountains (ranging from 2, 400 to 4, 500 m above sea level), namely Sabyinyo, Gahinga, Muhabura, Bisoke and Karisimbi, with the highest peak at 4, 507 m above sea level (1). As a part of the Albertine rift (The western branch of the East African Rift, covering parts of Uganda, DRC, Rwanda, Burundi and Tanzania, extending from the northern end of Lake Albert to the southern end of Lake Tanganyika), this unique habitat is one of the most biologically diverse regions in the world, supporting various wildlife species, including the endangered mountain gorillas according to the International Union for Conservation of Nature (IUCN) (2, 3). The VNP is a legally protected area by the Government of Rwanda (GoR) to shield endangered species including mountain gorillas (4). In addition, eco-tourism to the VNP generates foreign income, primarily from mountain gorilla-based tourism (5, 6). Thus, the VNP is a world treasure.

Over the last few years, the wildlife population in VNP has increased. For instance, it was estimated that the wildlife population of the Virunga Mass if would increase by around 3% annually, including the 4.4% growth rate of the mountain gorillas under observation (7), thanks to the GoR's efforts to protect those endangered species. As the population has increased, mountain gorillas tend to move out of VNP to the surrounding agricultural communities, which unfortunately creates conflicts with the local communities. To better protect these endangered species, the GoR has initiated the expansion of VNP to the surrounding agricultural communities (8). These communities traditionally use agricultural and household chemicals, pharmaceuticals, and other hazardous chemicals for different reasons. The surrounding areas also contain natural toxins such as poisonous plants and mushrooms. Like communities anywhere in the world, people living in the area surrounding the VNP plant ornamental and/or herbal plants, some of which have toxic properties (9). Therefore, there are concerns that the expansion of VNP to the surrounding agricultural territory will expose the wildlife in the protected area to toxic hazards that will harm or kill them. Some published studies show that farmers in Musanze District, where VNP is located, heavily rely on pesticides to protect their crops from pests and diseases, with some using up to 12 different types of pesticides (10–12). Such heavy use of pesticides in agriculture, particularly in Musanze District, Rwanda, has been a cause for concern because of the potential of these chemicals to harm human health and the environment if they are not well managed or disposed of.

Therefore, the goal of this study was to identify the toxic hazards to wildlife in the future expansion zone surrounding VNP, and to assess people's awareness of the harm these toxic hazards pose to wildlife. We tested the hypothesis that people living in the area surrounding the VNP were not aware of the potential for these toxicants to harm or kill wildlife. This is important because the VNP harbors endangered wildlife species, such as mountain gorillas which must be conserved. Information obtained from this study is crucial for safe expansion of VNP into contiguous areas. Expanding wildlife habitat will provide wildlife species in VNP with sufficient sources of food and shelter, reduce competition, and allow wildlife will thrive in a conductive safe environment.

2 Materials and methods

2.1 Description of the study area

The study area is located in the northern and western provinces of Rwanda. This area is near the borders with the DRC and Uganda. The study was conducted in four sectors surrounding VNP in Musanze District, namely Nyange, Kinigi, Shingiro, and Gataraga (Figure 1). The primary economic activity in the area is agriculture, with Irish potatoes, pyrethrum, wheat, onions, nuts, and maize as major cash crops cultivated (13, 14). Livestock production is mainly cattle (*Bos taurus*), sheep (*Ovis aries*), and goats (*Capra aegagrus hircus*). Beekeeping is also practiced on a small scale. The study area is characterized by heavy pesticide use to boost crop agricultural production (15, 16).

2.2 Sample size determination

Determination of sample size of study participants for this study was based on the estimated population of 63, 972 individuals living in the mentioned sectors surrounding VNP (16). A confidence level of 95% and a margin of error of 5% were used to determine the sample size. The Survey Monkey formula was employed, with a z-score of 1.96 (17). The calculated minimum sample size was determined to be 380 participants, representative of the entire population.

2.3 Target population

The target population was individuals living in the area surrounding VNP, regardless of their occupation. The only inclusion criteria were that the participants were at least 18 years old. Therefore, the study aimed at capturing a diverse range of individuals living in the vicinity of the park, including farmers and non-farmers.

2.4 Study design and sampling method

A cross-sectional study was conducted for identification of potential toxic hazards through interviews with people who lived in the area surrounding VNP. Non-probability (empirical) sampling using the quota approach was used because all study subjects in the research population were not available at the same time. By skipping 3rd or 4th home, survey was conducted until the required number of study subjects was reached.

2.5 Data collection

Data collection was conducted using a questionnaire from November to mid December 2022. Respondents were not

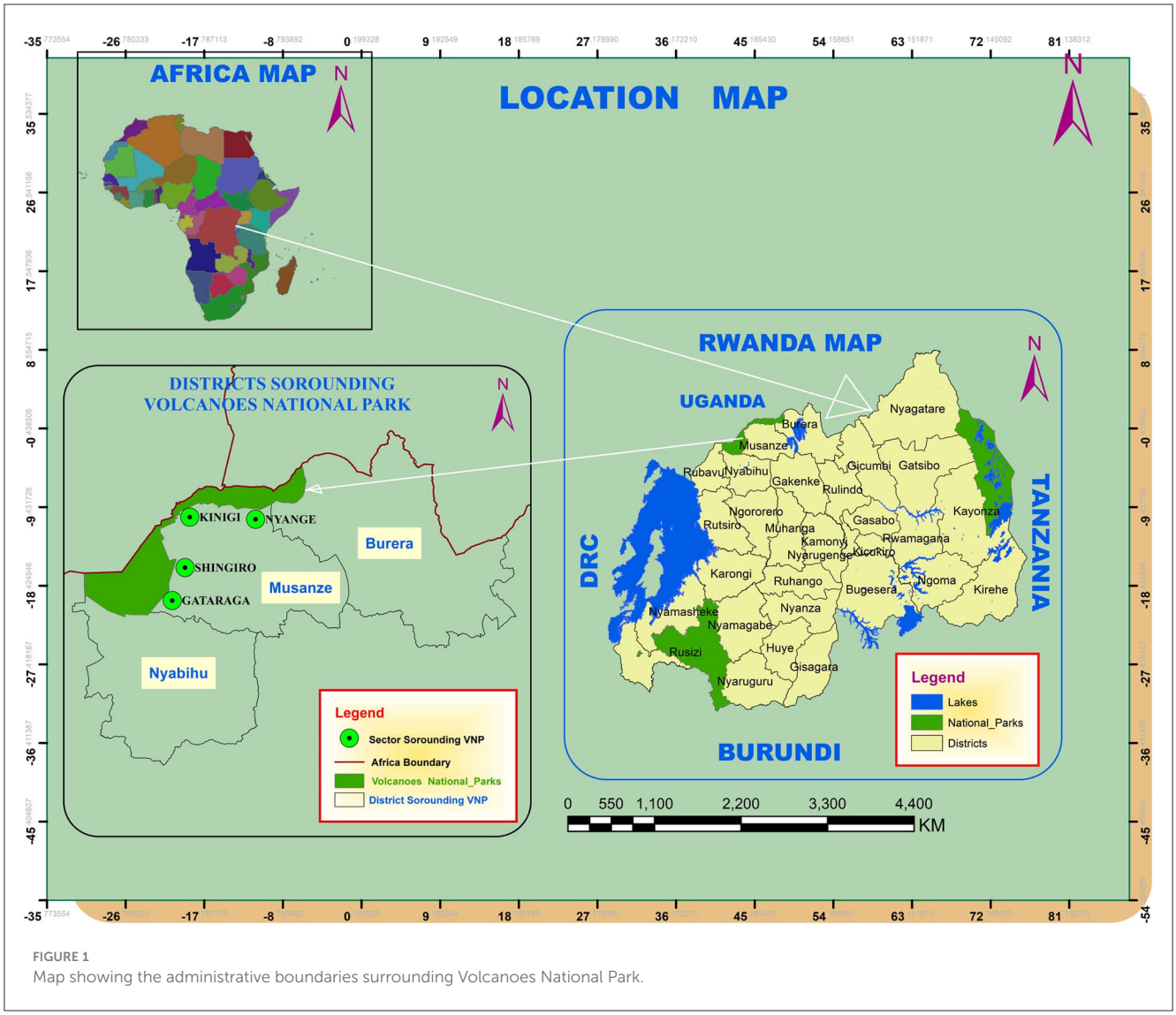


FIGURE 1
Map showing the administrative boundaries surrounding Volcanoes National Park.

pre-informed to avoid biased responses, and the questionnaire was designed based on previously published studies (18–21). Prior to the study, the questionnaire was piloted on a small sample of farmers (22), and questions' clarity and appropriateness were evaluated and edited accordingly. The questionnaire was divided into three main sections: socio-demographic issues, potential toxic hazards assessment, and a section to assess awareness and risk of potential toxic hazards to humans, animals, and the environment. The target respondents were selected based on specific criteria namely being 18 years old or above and living in the area surrounding VNP. The questionnaire consisted of closed questions, and 14 questions were selected to assess the respondents' level of knowledge about potential toxic hazards to wildlife species in the area surrounding VNP (Supplementary material). The questionnaire was inserted into the Kobo Toolbox for quick and reliable data collection (23). The interviews were conducted in the local language (Kinyarwanda), and verbal consent was obtained from all participants involved in the study to keep their anonymity. This study was approved by the University of Rwanda (The office of the director of research & innovation) and Rwanda Development Board (RDB) in charge of wildlife protection. Validation of names of pesticides and pharmaceutical drugs used by the participants in each study area was done by contacting local pesticide retailers and local health centers and pharmacies. The printed photos of listed poisonous plants were shown to respondents during interview in order give accurate data. Furthermore, an android application called LEAF SNAP was used to confirm the identity of plant species by scanning plants' photos (23).

2.6 Data analysis

The raw data collected were first exported into Microsoft Excel (version 2021) and reviewed for the accuracy. The data were then coded, entered, and verified to minimize the risk of errors. The statistical software package, SPSS v.20 (Chicago, IL), was used for data analysis in accordance with the study's objectives. The precision level was set at 5% and the confidence interval at 95%. Descriptive statistics were used to calculate the frequencies and percentages of the responses. Logistic regression was conducted to determine the association between the dependent

TABLE 1 Social demographic characteristics of respondents.

Respondent's characteristics		Frequency (n)	Percentage (%)
Location			
	Kinigi	139	34.4%
	Nyange	72	17.9%
	Shingiro	126	31.3%
	Gataraga	66	16.4%
Gender			
	Female	202	50.1%
	Male	201	49.9%
Age			
	18–25	55	13.6%
	26–30	60	14.9%
	31–35	77	19.1%
	36–40	64	15.9%
	41–45	37	9.18%
	46–50	28	6.95%
	51–55	21	5.21%
	56–60	25	6.21%
	61–65	13	3.24%
	66–70	9	2.23%
	71–75	5	1.25%
	76–80	9	2.23%
Occupation			
	Farmer	400	99.25%
	Pastor	1	0.25%
	Technician	1	0.25%
	Constructor	1	0.25%
Marital Status			
	Divorced	6	1.49%
	Married	354	87.84%
	Single	18	4.47%
	Widower	25	6.2%
Family Size			
	1–5 People	201	49.9%
	6–10 People	75	31.5%
	11–15 People	127	18.6%
Educational Background			
	No formal education	90	22.3%
	Primary	209	51.9%
	Secondary	100	24.8%
	College and University	4	1%

variable, knowledge, and the independent variables, education level, and training status. To evaluate the level of knowledge of the respondents regarding potential toxic hazards around VNP, the 14 questions were scored, with a score of 1 indicating sufficient knowledge and a score of 0 indicating insufficient knowledge. The total number of points was 14. Respondents who scored > 10 points were considered to have sufficient knowledge. Those who scored 7 but <10 points were considered to have moderate knowledge. Those who scored < 7 points were regarded as having insufficient knowledge about potential toxic hazards.

3 Results

3.1 Social demographic characteristics of respondents

The results of this study showed that the majority of the respondents were farmers (99.9%) with a large proportion practicing both crop and livestock farming (43.9%). The respondents were predominantly married (87.8%) and had different family sizes, with almost half of them belonging to households of 1–5 people (49.9%). In terms of educational background, the majority attended primary school (51.9%) and a considerable number had no formal school education at all (22.3%). The age bracket of the majority of respondents was youths, 31–35 years (19.1%). The study had almost an even representation of both genders, with females constituting slightly over half of the respondents (50.1%). The study was conducted in four sectors of the Musanze district, with Kinigi sector having the highest number of respondents (34%) (Table 1).

3.2 Household chemicals

The survey results revealed that households in the vicinity of the VNP had a low usage level of house hold chemicals. Bathroom, toilet cleaners, disinfectants, and surface cleaners were used by only 0.20% of respondents. Deodorants and sprays, bleach, perfumes, nail polish, nail polish remover, hair dyeing and hair styling products, and hair removers were not used at all. In the kitchen, dishwashing detergents and powders were used by 49.0% of respondents. Chemicals used in laundry, such as washing detergents were used by respondents at a high level of 98% (Table 2). Sleeping aids, painkillers, and cough and cold medicines were used only by 29% of respondents, while cosmetics (lotions) were used at a level of 70.5% (Table 3).

3.3 Pharmaceutical drugs used in humans

The findings from the survey revealed the use of various human pharmaceutical drugs. The most commonly used pharmaceutical drugs were antimalarial drugs such as Coartem® (artemether/lumefantrine) which was used by 8.4% of survey participants. The usage of anthelminthic and antiprotozoal drugs was also common. For example, Metronidazole was used by 96.5% of participants, followed by Tinidazole at 96.8% and Albendazole at

TABLE 2 Household chemicals used for laundry in communities around VNP.

Itemized household chemicals	Frequency (n)		Percentage (%)	
	Yes	No	Yes	No
Washing detergents	395	8	98%	2%
Bleach	0	403	0%	100%
Solvents	0	403	0%	100%
Pet flea powders and shampoo	0	403	0%	100%
Metal and wood polish	0	403	0%	100%
Washing soap	1	402	0.20%	99.80%
Antiseptics	1	402	0.20%	99.80%

TABLE 3 Household chemicals used in bedrooms in communities around VNP.

Household chemicals	Frequency (n)		Percentage (%)	
	Yes	No	Yes	No
Usage of chemicals in your bedrooms	286	116	71.1%	28.9%
Sleeping tablets painkillers and cough and cold medicines	2	401	0.0.5%	99.5%
Cosmetics lotions	284	119	70.5%	29.5%
Insect repellents	0	403	0%	100%

97.3%. Antibiotics such as Amoxicillin were also commonly used, with a usage level of 99%, followed by Erythromycin at 26.6% and Cotrimoxazole at 13.3%. Non-steroidal Anti-inflammatory drugs such as Paracetamol and Ibuprofen were used at a very high level of 99.5% and 98.5% of survey participants respectively (Table 4). Results showed that survey participants use pharmaceutical drugs at different frequencies. Those who use pharmaceutical drugs more than once per week were at 4.5%, while those who use pharmaceutical drugs once per week were at 1.2%. The majority of the population surveyed use drugs less frequently, with 40.4% using them once every 3 months, 16.1% using them once every 6 months, and 31.3% using them once a month. The lowest percentage of pharmaceutical drug users was those who use them once per year, at 6.5%.

3.4 Disposal of household chemicals and pharmaceutical drugs

The results showed that people have adopted different methods for disposal of unused household chemicals, pharmaceutical products, and residues. The majority of the respondents (40.1%) reported that they simply throw away unused chemical and packaging materials into dumpsites, while a very small proportion (0.2%) reported wrapping the materials in separate containers before discarding in the open space. Additionally, 15.6% of respondents reported burying the materials, while the majority of respondents (60.8%) reported outdoor burning the materials.

TABLE 4 Itemized household human pharmaceuticals used around VNP.

Medicines category	Medicine	Frequency (n)		Percentage (%)	
		Yes	No	Yes	No
Antimalarial drugs					
	Coartem®	34	369	8.4%	91.6%
Anthelmintic/ antiprotozoal drugs					
	Metronidazole	389	14	96.5%	3.5%
	Tinidazole	390	13	96.8%	3.2%
	Mebendazole	34	369	8.4%	91.6%
	Albendazole	392	11	97.3%	2.7%
	Nystatin	4	399	1%	99%
Antibiotics					
	Amoxicillin	399	4	99%	1%
	Erythromycin	107	296	26.6%	73.4
	Cotrimoxazole	53	350	13.2%	86.8%
NSAID for fever and headache					
	Paracetamol	401	2	99.5%	0.5%
	Ibuprofen	397	6	98.5	1.50%

3.5 Use of veterinary drugs around VNP

The use of Albendazole and Ivermectin at 100% indicates that parasitic infections are the most common diseases affecting livestock in the area. Oxytetracycline, used to treat bacterial infections is an important veterinary drug used by 38% of respondents. Multivitamin supplements used to improve the general health of the livestock was used by all survey participants (100%). Vaccines were used at 55.8% to prevent certain diseases such as Rift Valley Fever, Lumpy Skin diseases, Anthrax, and so on (Table 5). The low usage of disinfectants at 17% suggests a lack of awareness of the importance of hygiene in preventing spread of diseases. The use of Limoxin® 25 (Oxytetracycline 2.5% spray). Spray at 39.2% and Eye Ointments at 4.5% indicates that eye infections are also prevalent in the area. Streptomycin at 70.2% is used to treat bacterial infections, but its frequent use can lead to the development of antibiotic resistance, which is a major concern for public health.

3.6 The fate of veterinary drug residues and their packaging materials

We also studied the fate of unused veterinary drug and their packaging materials to understand the level of environmental contamination from this class of chemicals. The results showed that 46.4% of respondents discard unused veterinary drugs and their packaging materials in the open at the edge of farms 13.2% bury them, 5.5% discard them in public landfills, and 1.7% dispose of them in streams. Interestingly, a significant proportion (62.8%) reported outdoor burning the unused veterinary drugs

10.3389/fvets.2023.1320162

TABLE 5 Veterinary pharmaceuticals commonly used by farmers around VNP.

Veterinary drugs	Frequency (*n*)		Percentage (%)	
	Yes	No	Yes	No
Albendazole	403	0	100%	0%
Oxytetracycline	153	250	38%	62%
Ivermectin	403	0	100%	0%
Multivitamin	403	0	100%	0%
Vaccines	225	178	55.8%	44.2%
Disinfectant	70	333	17%	82.6%
Limoxin® 25 Spray	158	245	39.2%	60.8%
Eye Ointment	18	385	4.50%	95.5%
Streptomycin	141	262	35%	65%
Streptomycin Injection	283	120	70.2%	29.8%

and packaging materials, which could potentially release harmful chemicals into the environment. These findings suggest that there is a need for proper disposal methods for veterinary drug residues and their packaging materials to reduce the potential risks to the environment and wildlife in and around the VNP.

3.7 Agricultural pesticides

Our survey for potential toxic hazards for wildlife species around VNP in Rwanda also involved studying the use of different pesticides in the area. We found that the most commonly used pesticide was SAFARIMAX® (Dinotefuran 20%), which was used at a level of 88.4% in the agricultural sector. THIODAN 4EC® (Endosulfan) was also widely used, with a frequency of 90.3%. DUDU® (Abamectin 20g/L + Acetamiprid 3%) was used by 68.8% of farmers, while DITHANE M.45® (Manconazeb/ dithiocarbametes) had the highest frequency of use at 96.3%. MBOLEA YA MAJIMAJI® (Nitrogen, Potassium and Phosphorous) as Fertilizer was used by 54% of farmers, and ROCKET (Profenofos and Pyrethroid Cypermethrin) had a frequency of use of 60%. Finally, MILLMAX GOLD® (Cymoxanil 6% + Propineb 70%) was used at a level of 0.2% (Table 6). These findings suggest that the use of pesticides is prevalent in the area, particularly for crop farming such as Irish potatoes (*Solanum tuberosum*) and tamarillo /tree tomatoes (*Solanum betaceum*) which rely heavily on pesticide applications to increase yield and prevent agricultural losses.

3.8 The storing, disposing, and factors influencing application of pesticides

Results showed that most of the farmers surveyed have designated storage areas for pesticides (88.80%). However, the study also found that there is a lack of proper disposal practices for unused pesticide and packaging materials. Most of the farmers reported outdoor burning of pesticide containers and residues.

TABLE 6 Itemized insecticides commonly used to increase agricultural production around VNP.

Pesticides	Frequency (*n*)		Percentage (%)	
	Yes	No	Yes	No
SAFARIMAX® (Dinotefuran 20%)	358	47	88.4%	11.6%
THIODAN® (Endosulfan)	365	39	90.3%	9.7%
Dudu® (Abamectin 20g/L + Acetamiprid 3%)	278	121	68.8%	31.2%
DITHANE M-45® (80% Mancozeb)	389	15	96.3%	3.7%
MBOLEA YA MAJIMAJI® (Nitrogen, Potassium and Phosphorous)	218	186	54%	46%
ROCKET® (Profenofos and Pyrethroid Cypermethrin)	242	161	60%	40%
MILLMAX GOLD® (Cymoxanil 6%+Propineb 70%)	1	402	0.2%	99.8%

The findings of the study suggest that there are several factors that could contribute to presence of high quantities of pesticides in the area around VNP. The data indicates that the harmfulness threshold and date fixed in advance were the most influential factors, with 98.8 and 98.3% of respondents respectively. In terms of initiating the treatment, the majority of respondents (98.3%) relied on the harmfulness threshold and date fixed in advance, with regional surveillance playing a slightly larger role at 99.0%. Respondents also took into account the climate when applying pesticides, with 1.5% reporting it as a major contributor to the use of pesticides. In cases where the treatment was ineffective, all respondents (100%) reported increasing the concentration or changing the pesticides used. Only a small percentage (0.2%) of respondents reported consulting a specialist in phytosanitary products.

3.9 Poisonous plants

Our study has also revealed several herbal, ornamental, and poisonous plants in the area. In total, 47.9% of respondents reported having herbal plants around their homes. Those herbal plants that were commonly used include Nasturtium (*Tropaeolum majus*), Soap aloe (*Aloe maculate*), Coleus plant (*Coleus kilimandscharica),* Umutagara *(Crassocephalum multicorymbosum)*, bitter leaf (*Vernonia amygdalina),* ginger bush *(Tetradenia riparia),* African soapberry *(Phytolacca dodecandra), and* holy basil *(Ocimumtenuiflorum)*. In addition to herbal plants, the study also revealed the most common toxic plants in that area and they include pyrethrum, Coral tree (*Erythrina genus*), angel trumpet (*Brugmansia genus*), Bracken *fern (Pteridium aquilinum*) and Castor oil plant (*Ricinus Communis*). Other toxic plants identified include Arum lily, *Arizona cypress, cacti*, and Mushrooms (Table 7).

3.10 Awareness about potential toxic hazards to wildlife species around VNP

The results of our study indicate that the level of awareness about toxic hazards to wildlife around VNP varied among study participants. The majority of the participants had moderate knowledge (57.10%), while 28.8% insufficient knowledge (that chemicals and natural plant toxins can harm wildlife. However, a smaller percentage of participants was aware (14.10%) (Table 8).

3.11 The association between education background of respondents and training on awareness of potential toxic hazards to wildlife in the area around VNP

The findings of our study showed a significant association between the educational background of respondents and their knowledge about potential toxic hazards for wildlife species around VNP. It was observed that the majority of respondents who had attended primary school had moderate knowledge (52.6%) about potential toxic hazards, followed by those who had attended secondary school (76%). On the other hand, respondents without formal education had the lowest proportion (20%). Interestingly, respondents who had attended college or university had sufficient knowledge (50%) compared to those who had attended primary or secondary school. The study also found that respondents who had received training on potential toxic hazards had better knowledge compared to those who had not received any training. Specifically, those who had received training had a higher proportion of respondents with moderate knowledge (60.5%) compared to those who had not received training (43.4%) (Table 9).

4 Discussions

The VNP is home to endangered wildlife species such as the mountain gorillas. The GoR is planning an expansion of the VNP to increase habitat for this endangered species. Mountain gorillas, like other wildlife, are susceptible to poisoning from natural and man-made chemicals. For example, pesticides, including rodenticides, pose significant risks to wildlife (22, 24, 25). Pharmaceutical products and household chemicals released into the environment may also pose threats to wildlife directly or indirectly (26, 27). Herbal or poisonous ornamental plants can also affect wildlife negatively (28). Because people in the potential VNP expansion zone use various chemicals, fertilizers, pharmaceuticals, ornamental plants, etc.; for their livelihoods, it is important to determine potential toxic hazards to wildlife so that the area can be prepared before wildlife are introduced to the area. Furthermore, although the park expansion has not yet commenced, wildlife species in VNP, including buffaloes and mountain gorillas, continue to venture out of the park and into surrounding agricultural fields. This ongoing interaction between wildlife and human settlements increases the risk of these animals being exposed to toxic hazards within the community, posing potential threats to their health and wellbeing. Therefore, this study tackles that too even before park expansion program.

This study has identified four main categories of potential toxic hazards to wildlife. These include household chemicals, pharmaceutical products, pesticides, and poisonous plants. Interestingly, survey results revealed that households in the vicinity of the VNP had a low level of house chemical usage in their homes. This is likely due to a number of factors, including the remote location of the park, the limited availability of household chemicals and the cultural practices of the local people. This is good news as it reduces the chances for environmental contamination. However,

TABLE 7 Poisonous plants found in homesteads around VNP.

Poisonous plant	Frequency (*n*)		Percentage (%)	
	Yes	No	Yes	No
Presence of poisonous plants harmful to people and animals	6	397	1.50%	98.5%
Castor oil (*Ricinus Communis*)	103	300	25.6%	74.4%
Coral tree (*Erythrina genus*)	331	72	82.1	17.9%
Golden dewdrop (*Duranta eracta*)	1	402	0.2%	99.8%
Rhus or Wax tree (*Toxicodendron Succedaneum*)	28	375	6.9%	93.1%
White Cedar tree (*Melia Azedarach*)	1	402	0.2%	99.8%
Angel trumpet (*Brugmansia genus*)	305	98	75.7%	24.3%
Arum lily (*Zantedeschia aethiopica*)	125	278	31%	69%
Belladonna lily and *amalylis belladonna*	1	402	0.2%	99.8%
Cacti and other succulents (Adromischus *spp*)	63	340	15.6%	84.4%
Dumb cane (*dieffendenbachia genus*)	12	391	3%	97%
Euphorbia genus	2	401	0.5%	99.5%
Arizona cypress (*Hesperocyparis arizonica*)	122	281	30.3%	69.7%
Mushrooms and toadstools	110	293	27.3%	72.7%
Bulbs	2	401	0.5%	99.5%
Sticky weed or asthma weed (*Parietaria Judaica*)	1	402	0.2%	99.8%
Bracken fern (*Pteridium aquilinum*)	226	177	56.1%	43.9

TABLE 8 Awareness about toxic hazards to wildlife species around VNP.

Knowledge level	Frequency (*n*)	Percentage (%)
Insufficient	116	28.8%
Moderate	230	57.1%
Sufficient	57	14.1%

TABLE 9 Association between education background of respondents, training on toxic hazards and knowledge toward potential toxic hazards for wildlife species in area around VNP.

Respondent's characteristics	Knowledge			
	Insufficient	Moderate	Sufficient	*P*-value
Educational background				<0.0001
No formal education	30/90 (33.3%)	42/90 (46.7%)	18/90 (20%)	
Primary	73/209 (34.9%)	110/209 (52.7%)	26/209 (12.4)	
Secondary	13/100 (13%)	76/100 (76%)	11/100 (11%)	
College and University	0/4 (0%)	2/4 (50%)	2/4 (50%)	
Specific training on potential toxic hazards of pesticides				0.02
No	33/84 (39.3%)	36/84 (42.9%)	15/84 (17.8.7%)	
Yes	83/319 (26%)	193/319 (60.5%)	43/319 (13.5%)	

use of dishwashing detergents and laundry detergents was observed at varying levels. Some detergents have extreme pH values and exposure to such detergents is corrosive and can cause caustic injury on contact with mouth or skin (29).

Our study also uncovered evidence of significant use of rodenticides in the area. Those include zinc phosphide and sodium monofluoroacetate. However, zinc phosphide was more prevalent. Zinc phosphide, primarily used as a rodenticide, poses a threat to local wildlife because it releases toxic phosphine gas, impacting not only the targeted rodents but also potentially harming non-target species, including humans. All species of animals are susceptible to zinc phosphide poisoning, but avian species, are the most seriously affected and even resulting in death (30). Meanwhile, sodium monofluoroacetate, known as Compound 1080, a potent pesticide used for pest control, can disrupt cellular metabolism and result in organ failure in various animals, making it a substantial risk to both wildlife and humans alike (31, 32).

Rodenticides are widely employed to control rodent populations and to minimize food damage during storage. Rodenticides can affect primary and secondary targets. Some are specific and others are nonspecific. The potential hazards associated with rodenticides can extend beyond their intended targets. Secondary poisoning resulting from the consumption of poisoned rodents by some wildlife (carnivores) and is a well-documented concern. Poisoning on non-target species such mountain gorillas, through ingestion of rodenticides residues or packaging materials is also a serious concern. Additionally, scavengers and other non-target animals may also be exposed to the lethal effects of rodenticides, leading to ecosystem disruptions and unintended consequences for the entire wildlife community.

The high prevalence of the use of human and veterinary pharmaceutical drugs in the area surrounding VNP is a cause for concern. Poorly disposed of pharmaceuticals may be directly accessed and consumed by wildlife, and can easily enter the ecosystem, primarily through wastewater, and reach various surface water bodies such streams, rivers, lakes, wetlands, reservoirs, creeks, and oceans. These drugs may also contaminate ground water sources (33, 34). Primary medicines and their metabolites may also enter the food chain (35). Ultimately, this could harm wildlife including the endangered mountain gorillas. For example, NSAIDs like Ibuprofen are toxic to a wide variety of species (36, 37). Moreover, excessive and inappropriate use of pharmaceuticals, particularly antibiotics in humans and animals, contributes significantly to global antimicrobial resistance (AMR). This misuse leads to antibiotic-resistant bacteria in both groups, endangering public and animal health. In animal agriculture, resistant bacteria can transfer to humans and wildlife through food or the environment, worsening the AMR issue. One of the medications used for treating parasitic infections in livestock was ivermectin. Accidental ingestion of ivermectin by wildlife, whether through contaminated food sources or environmental exposure, can result in a range of toxic effects, including neurological symptoms like tremors and seizures, gastrointestinal disturbances, respiratory distress, ataxia, muscle weakness or paralysis, and lethargy (38–40).

The high frequency of pharmaceutical drug use in the area of the study suggests that there is a potential for mountain gorillas and other wildlife to be exposed to pharmaceutical drugs through a variety of pathways, such as direct ingestion, indirect ingestion through contaminated food or water, or through contact with contaminated surfaces. The results of the survey on the disposal methods of household chemicals and pharmaceutical drugs in the zone surrounding VNP provide crucial insights into potential toxic hazards that may impact the habitat of the endangered mountain gorillas. Alarmingly, results showed a significant proportion of respondents reported poor disposal of pharmaceuticals. They simply throw them away without an adequate disposal. When VNP is expanded it is recommended that proper procedures for disposal of human and animal pharmaceutical drugs is implemented so that wildlife do not get into contact.

Our survey indicates that the use of agricultural insecticides is widespread, with several products being applied at high frequencies. Most of these are potent acetylcholinesterase inhibiting organophosphorus and carbamate pesticides. They are known to be highly toxic to wildlife (41, 42). Ingestion of improperly disposed of these agricultural insecticides not only can kill the primary victim but also causes relay toxicosis which can devastate ecosystems. For example the intentional baiting of carcasses by poachers has killed mammals and vultures simultaneously (43, 44). Therefore, since the use of agricultural insecticides is prevalent, these chemicals must be

collected and properly disposed of by authorities before wildlife is allowed access to the expansion zone.

Natural toxins can poison wildlife. Plant toxins are particularly hazardous to wildlife. Therefore, the discovery of herbal, ornamental, and poisonous plants in the study area raises concerns about the potential risks they pose to the wildlife in VNP, including the mountain gorillas. The prevalence of herbal plants around the homes of local communities is notable, with 47.9% of respondents reporting their presence. While some of these plants are likely beneficial (herbal plant species), toxic plants including the Coral tree, Angel trumpet, Bracken fern, and Castor oil plant (Ricin), and others can be lethal to mammals (45). Pyrethrum, a source of pyrethrin insecticides is commercially grown in the area. Wildlife grazing these plants, which have the potential to grow wild if not controlled, may be hazardous., Ricin (*Ricinus communis*) is a potent toxin which inhibits the synthesis of proteins within cells and can cause severe vomiting, diarrhea, seizures, and death (46). These and other toxic plants identified should be eliminated before this area is accessible to wildlife.

The study also reveals varying levels of awareness by survey participants about natural and chemical hazards to wildlife in areas surrounding VNP. While a significant proportion had moderate knowledge, 28% of the population had insufficient knowledge about the dangers of toxicants they pose to wildlife. This suggests that it is necessary to conduct an education campaign to educate the population about dangers natural and chemical toxicants pose to wildlife. That way the public can assist VNP staff in eliminating toxic hazards identified in this survey before they are relocated. In addition, it is necessary to educate communities surrounding VNP about such dangers to enhance their awareness about these toxic hazards to wildlife. Communities that are aware of toxic hazards can play a proactive role in safeguarding the park's ecosystem and the wellbeing of its precious inhabitants, including the endangered mountain gorillas.

In conclusion, this is the first report of toxic hazards in the zone surrounding the VNP which has been identified for future expansion of the park. This research has revealed the presence of various toxic hazards, including household chemicals, human and veterinary pharmaceutical drugs, agricultural insecticides, and poisonous plants. The VNP is a crucial habitat for the endangered mountain gorillas and other wildlife. More research is recommended to fully document the quantity of toxic hazards identified in this survey. Also, in addition to environmental sampling and laboratory analysis of water bodies, soil, river sediments and poisonous plants, is recommended to fully understand whether use of agricultural chemicals for example have contaminated and negatively impacted the ecosystem. This will require collaborative efforts between health care veterinary professionals, agronomists, local communities, wildlife conservationists, chemists and other professionals. Ultimately, this work will improve our understanding of whether and how these pharmaceuticals, household chemicals, pesticides and poisonous plants impact the delicate ecological balance of VNP. This is a one health issue, crucial for maintaining the health of people, domestic animals and wildlife species such as the endangered mountain gorillas that share this unique and fragile habitat; and this requires a one health approach. It is also important to conduct a targeted education campaign to communities surrounding VNP to increase their awareness of the toxic hazards to wildlife. By increasing their knowledge and awareness we can foster a more sustainable relationship between the communities and the natural environment, contributing to the conservation efforts for the mountain gorillas and their habitat.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the University of Rwanda (The office of the director of research and innovation) and Rwanda Development Board (RDB) in charge of wildlife protection. The interviews were conducted in the local language (Kinyarwanda), and verbal consent was obtained from all participants.

Author contributions

EK: Funding acquisition, Investigation, Writing—original draft, Writing—review & editing. WR: Conceptualization, Supervision, Validation, Visualization, Writing—review & editing. KN: Conceptualization, Funding acquisition, Supervision, Validation, Visualization, Writing—review & editing. JN: Conceptualization, Validation, Visualization, Writing—review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was funded by Morris Animal Foundation (Grant number: MAF Grant ID: D22ZO-603), through Veterinary student scholar program.

Acknowledgments

We acknowledge the joint effort of various stakeholder namely University of Rwanda, University of California Davis, Mountain Gorilla Project (Gorilla Doctors), Rwanda Development Board, the local communities, the authorities of the sectors, districts and province that are adjacent to VNP for their technical support, the facilitation of the process, good will and collaboration during the implementation of this research project.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1320162/full#supplementary-material

References

1. Spinage CA. The ecology and problems of the Volcano National Park, Rwanda. *Biol Conserv.* (1972) 4:194–204. doi: 10.1016/0006-3207(72)90169-3

2. Uwayo P, Nsanzumukiza VM, Maniragaba A, Nsabimana AP, Akimanizanye V. Contribution of former poachers for wildlife conservation in Rwanda volcanoes national park. *J Geosci Environ Prot.* (2020) 8:47–56. doi: 10.4236/gep.2020.84004

3. Rutagarama E, Martin A. Partnerships for protected area conservation in Rwanda. *Geogr J.* (2006) 172:291–305. doi: 10.1111/j.1475-4959.2006.00217.x

4. Musafili I. Willis Oluoch-Kosura, Otieno DJ. An application of choice experiment to assess farmers' willingness to pay for volcanoes national park management attributes in Rwanda. *Br J Environ Stud.* (2022) 2:43–54. doi: 10.32996/bjes.2022.2.1.4

5. Sabuhoro E, Wright B, Munanura IE, Nyakabwa IN, Nibigira C. The potential of ecotourism opportunities to generate support for mountain gorilla conservation among local communities neighboring Volcanoes National Park in Rwanda. *J Ecotourism.* (2021) 20:1–17. doi: 10.1080/14724049.2017.1280043

6. Granjon A-C., Robbins MM, Arinaitwe J, Cranfield MR, Eckardt W, Mburanumwe I, et al. Estimating abundance and growth rates in a wild mountain gorilla population. *Anim Conserv.* (2020) 23:455–65. doi: 10.1111/acv.12559

7. Expanding Volcanoes National Park – Visit Rwanda (2023). Available online at: https://www.visitrwanda.com/gorillas/ (accessed October 2, 2023).

8. Alphonse N, Bigendako M, Fawcett K, Yansheng G. Ethnobotanic study around Volcanoes National Park, Rwanda. *N Y Sci J.* (2010) 3:5. Available online at: https://www.sciencepub.net/newyork/ny0305/07_2374_Ethnobotanic_am_ny0305_37_49.pdf

9. Rutikanga A. *Pesticides Use and Regulations in Rwanda Status and Potential for Promotion of Biological Control Methods*. (2015). Available online at: http://rgdoi.net/10.13140/RG.2.1.2907.8641 (accessed September 6, 2023).

10. *Assessment of Aboveground Insects Associated Within Farm Fields of Musanze District in Rwanda: Abundance and Diversity* (2023). Available online at: https://www.researchgate.net/publication/357877443_457_ (accessed September 6, 2023).

11. Muhamadi S, Boz I. Factors influencing farmers' perception of sustainable agriculture: a case study of Musanze District, Rwanda. *Int J Sustain Agric Manag Inform.* (2022) 8:408. doi: 10.1504/ijsami.2022.126803

12. Ephraim R, Murugesan R. Opportunities for rural development in Musanze district, africa: a rural livelihood analysis. *IJBMER.* (2015) 6:231–48. Available online at: https://www.ijbmer.com/docs/volumes/vol6issue4/ijbmer2015060403.pdf

13. Maniriho A, Bizoza AR. *Determinants of Crop Production in Musanze District, Northern Rwanda* (2018). Available online at: https://edit.ju.se/download/18.2304a3fb164265793121450f/1530153275832/EARP-EF%202018-36%20Maniriho.pdf (accessed September 3, 2023).

14. Uzamukunda S. *Improving the Existing Farming Systems Towards a Climate Smart Agriculture in Musanze District of Rwanda* (2015). Available online at: https://stud.epsilon.slu.se/8578/1/Uzamukunda_S_151022.pdf (accessed September 6, 2023).

15. Maniriho A, Bizoza A. *Financial Benefit-Cost Analysis of Agricultural Production in Musanze District (Rwanda)* (2013). Available online at: https://orbi.uliege.be/handle/2268/264588 (accessed September 6, 2023).

16. *Musanze (District, Rwanda) - Population Statistics, Charts, Map and Location* (2023). Available online at: https://www.citypopulation.de/en/rwanda/sector/admin/43__musanze/ (accessed August 15, 2023).

17. SurveyMonkey. *How to Calculate Sample Size for a Survey* (2023). Available online at: https://www.surveymonkey.com/mp/sample-size/ (accessed May 21, 2023).

18. Fischer BC, Rotter S, Schubert J, Marx-Stoelting P, Solecki R. Recommendations for international harmonisation, implementation and further development of suitable scientific approaches regarding the assessment of mixture effects. *Food Chem Toxicol.* (2020) 141:111388. doi: 10.1016/j.fct.2020.111388

19. Maiga A, Diallo D, Fane S, Sanogo R, Paulsen BS, Cisse B, et al. Survey of toxic plants on the market in the district of Bamako, Mali: traditional knowledge compared with a literature search of modern pharmacology and toxicology. *J Ethnopharmacol.* (2005) 96:183–93. doi: 10.1016/j.jep.2004.09.005

20. Rasool F, Nizamani ZA, Ahmad KS, Parveen F, Khan SA, Sabir N. Phytotoxicological study of selected poisonous plants from Azad Jammu & Kashmir. *Ishtiaq M PLoS ONE.* (2022) 17:e0263605. doi: 10.1371/journal.pone.0263605

21. KoboToolbox (2023). *Kobotoolbox.* Available online at: https://www.kobotoolbox.org/ (accessed May 1, 2023).

22. Capinera LJ. (2021). *Insecticides and Wildlife.* Available online at: https://edis.ifas.ufl.edu/publication/IN881 (accessed January 12, 2023).

23. Dolan C, Mannan B. *Pesticide Use and Wildlife*. (2023). Available online at: https://extension.arizona.edu/sites/extension.arizona.edu/files/pubs/az1481i.pdf (accessed June 23, 2023).

24. Wires. (2023). *Wildlife and Pesticides*. Available online at: https://www.wires.org.au/wildlife-information/wildlife-and-pesticides (accessed June 30, 2023).

25. Daughton CG, Ternes TA. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect.* (1999) 107:907–38. doi: 10.1289/ehp.99107s6907

26. CHEM Trust. (2014). *Medicines in the Environment: A Growing Threat to Wildlife and Drinking Water*. Available online at: https://chemtrust.org/medicines-in-the-environment-a-growing-threat-to-wildlife-and-drinking-water/ (accessed July 17, 2023).

27. Stegelmeier BL, Field R, Panter KE, Hall JO, Welch KD, Pfister JA, et al. *Selected Poisonous Plants Affecting Animal and Human Health. Haschek and Rousseaux's Handbook of Toxicologic Pathology*. London: Academic Press (2013).

28. Krenzelok EP. Liquid automatic dishwashing detergents: a profile of toxicity. *Ann Emerg Med.* (1989) 18:60–3. doi: 10.1016/S0196-0644(89)80315-4

29. DNS. (2023). *Zinc Phosphide*. Available online at: https://www.michigan.gov/dnr/managing-resources/wildlife/wildlife-disease/wdm/zinc-phosphide (accessed September 15, 2023).

30. Moran S. Toxicity of sodium fluoroacetate and zinc phosphide wheat grain baits to *Microtus guentheri and Meriones tristrami* EPPO. *Bull.* (1991) 21:73–80. doi: 10.1111/j.1365-2338.1991.tb00456.x

31. Ward JC, Spencer DA. Notes on the pharmacology of sodium fluoroacetate—compound 1080*†. *J Am Pharm Assoc Am Pharm Assoc.* (1947) 36:59–62. doi: 10.1002/jps.3030360207

32. Daughton CG. Pharmaceuticals and the Environment (PiE): Evolution and impact of the published literature revealed by bibliometric analysis. *Sci Total Environ.* (2016) 562:391–426. doi: 10.1016/j.scitotenv.2016.03.109

33. Holm G, Snape JR, Murray-Smith R, Talbot J, Taylor D, Sörme P. Implementing ecopharmacovigilance in practice: challenges and potential opportunities. *Drug Saf.* (2013) 36:533–46. doi: 10.1007/s40264-013-0049-3

34. Hejna M, Kapuścińska D, Aksmann A. Pharmaceuticals in the aquatic environment: a review on eco-toxicology and the remediation potential of algae. *Int J Environ Res Public Health.* (2022) 19:7717. doi: 10.3390/ijerph19137717

35. Modi CM, Mody SK, Patel HB, Dudhatra GB, Kumar A, Avale M. Toxicopathological overview of analgesic and anti-inflammatory drugs in animals. *J Appl Pharm Sci.* (2012) 30:149–57. Available online at: https://www.japsonline.com/admin/php/uploads/361_pdf.pdf

36. Swiacka K, Michnowska A, Maculewicz J, Caban M, Smolarz K. Toxic effects of NSAIDs in non-target species: a review from the perspective of the aquatic environment. *Environ Pollut.* (2021) 15:115891. doi: 10.1016/j.envpol.2020.115891

37. Caloni F, Cortinovis C, Rivolta M, Davanzo F. Animal poisoning in Italy: 10 years of epidemiological data from the poison control Centre of Milan. *Vet Rec.* (2021). 170:415. doi: 10.1136/vr.100210

38. Trailovic SM, Nedeljkovic H. Central and Peripheral Neurotoxic Effects of Ivermectin in Rats. *J Vet Med Sc.* (2011) 73: 591–9. doi: 10.1292/jvms.10-0424

39. Kumar S. *Environmental Contaminants and Their Impact on Wildlife. In: Toxicology and Human Health : Environmental Exposures and Biomarkers*. Singapore: Springer Nature Singapore (2023).

40. Ogada DL. The power of poison: pesticide poisoning of Africa's wildlife. *Ann N Y Acad Sci.* (2014) 1322:1–20. doi: 10.1111/nyas.12405

41. Ruiz-Suárez N, Boada LD, Henríquez-Hernández LA, González-Moreo F, Suárez-Pérez A, Camacho M, et al. Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain). *Sci Total Environ.* (2015) 505:1093–9. doi: 10.1016/j.scitotenv.2014.10.093

42. Daboné C, Ouéda A, Thompson LJ, Adjakpa JB, Weesie PD. Trade in vulture parts in West Africa: Burkina Faso may be one of the main sources of vulture carcasses. *Bird Conserv Int*. (2023) 33:8. doi: 10.1017/S095927092100054X

43. Odino M, Ogada D. The Intentional Use of Pesticides as Poison in Kenya: Conservation and Ecohealth Impacts. In: SC Underkoffler, HR Adams, editors *Wildlife Biodiversity Conservation*. Cham: Springer (2021).

44. DeFilipps RA, Krupnick GA. The medicinal plants of Myanmar. *PhytoKeys.* (2018) 28:1–341. doi: 10.3897/phytokeys.102.24380

45. *Britannica. 7 of the World's Deadliest Plants | Britannica*. (2023). Available online at: https://www.britannica.com/story/7-of-the-worlds-deadliest-plants (accessed July 23, 2023).

46. Worbs S, Köhler K, Pauly D, Avondet MA, Schaer M, Dorner MB, et al. *Ricinus communis* intoxications in human and veterinary medicine-a summary of real cases. *Toxins.* (2011) 3:1332–72. doi: 10.3390/toxins3101332

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 04 January 2024
DOI 10.3389/fvets.2023.1314029

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Damiano Cavallini,
University of Bologna, Italy
Aneliya Milanova,
Trakia University, Bulgaria

*CORRESPONDENCE
Qiugang Ma
✉ maqiugang@cau.edu.cn

†These authors have contributed equally to this work

RECEIVED 10 October 2023
ACCEPTED 08 December 2023
PUBLISHED 04 January 2024

CITATION
Liu S, Guo Y, Qu H, Dong Y, Zhao S, Fu T, Kang R, Cheng J, Huang S, Zhao L and Ma Q (2024) Pharmacokinetics of florfenicol and its metabolite florfenicol amine in the plasma, urine, and feces of fattening male donkeys following single oral administration. *Front. Vet. Sci.* 10:1314029. doi: 10.3389/fvets.2023.1314029

COPYRIGHT
© 2024 Liu, Guo, Qu, Dong, Zhao, Fu, Kang, Cheng, Huang, Zhao and Ma. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Pharmacokinetics of florfenicol and its metabolite florfenicol amine in the plasma, urine, and feces of fattening male donkeys following single oral administration

Shijie Liu[1†], Yanxin Guo[1†], Honglei Qu[1,2], Yanjie Dong[3], Shancang Zhao[3], Tianze Fu[1], Ruifen Kang[1], Jie Cheng[1,2], Shimeng Huang[1], Lihong Zhao[1] and Qiugang Ma[1*]

[1]State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, China, [2]National Engineering Research Center for Gelatin-Based Traditional Chinese Medicine, Dong-E-E-Jiao Co., Ltd., Liaocheng, China, [3]Shandong Academy of Agricultural Sciences, Jinan, China

Florfenicol (FF) is a commonly used antibacterial agent in animals. We investigated the pharmacokinetics of FF and its metabolite florfenicol amine (FFA) in donkeys. Donkeys were administered FF (30mg/kg bodyweight, p.o.). Pharmacokinetic parameters were calculated using a non-compartmental model. The FF (FFA) pharmacokinetics parameters were characterized by along elimination half-life ($t_{1/2kz}$) of 5.92h (15.95h), plasma peak concentration (C_{max}) of 0.13μg/mL (0.08μg/mL), and the time taken to reach C_{max} (T_{max}) of 0.68h (0.72h). The area under plasma concentration-time curve and mean residence time of FF (FFA) in plasma were 1.31 μg·mL^{-1}·h (0.47 μg·mL^{-1}·h) and 10.37h (18.40h), respectively. The $t_{1/2kz}$ of FF and FFA in urine was 21.93 and 40.26h, and the maximum excretion rate was 10.56 and 4.03μg/h reached at 25.60 and 32.20h, respectively. The respective values in feces were 0.02 and 0.01 μg·h^{-1} reached at 33.40h. The amount of FF and FFA recovered in feces was 0.52 and 0.22μg, respectively. In conclusion, FF (FFA) is rapidly absorbed and slowly eliminated after a single oral administration to donkeys. Compared to FF, FFA was more slowly eliminated. FF (FFA) is mostly excreted through urine.

KEYWORDS

florfenicol, florfenicol amine, pharmacokinetics, donkey, urinary excretion, antibiotic

1 Introduction

Florfenicol (FF) is a fluorinated derivative of thiamphenicol. It is a valuable synthetic reagent used to treat various antibacterial infections in animals, widely used in veterinary clinics. FF exerts its antibacterial effect by inhibiting protein synthesis at the prokaryotic ribosome. However, compared with thiamphenicol and chloramphenicol, FF is much less susceptible to bacterial deactivation as a result of fluorine substituting a hydroxyl group

(1, 2). Considering its safety and high therapeutic potency, FF is used frequently for treating infections induced by *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus suis*, *Pasteurella multocida*, *Manheimia haemolytica* and *Actinobacillus pleuropneumoniae* (3, 4).

FF is mainly used for large ruminants in the treatment of bovine respiratory diseases caused by etiological agents such as *Pasteurella multocida*, *Mannheimia haemolytica*, or *Histophilus somni*. Florfenicol products are also intended for therapeutic use in acute interdigital necrobacillosis, presenting good antimicrobial activity against *Fusobacterium necrophorum* and *B. melaninogenicus* (5), but also in the case of keratoconjunctivitis produced by *Moraxella bovis* (6). Additionally, a study was identified that tested the efficacy of florfenicol in its therapeutic approach to caseous lymphadenitis in sheep and goats caused by *Corynebacterium pseudotuberculosis*. A comparison of the control groups and those who received florfenicol therapy showed an improvement in clinical scores, suggesting the effective treatment and maintenance of remission in caseous abscesses (7). In pigs, FF is used for the treatment of bacterial respiratory diseases caused by agents such as *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, and *Salmonella choleraesuis* (6, 8). A recent study presented florfenicol to be a reliable option for the treatment of arthritis in pigs caused by *Streptococcus suis* (9). The pharmacokinetics of FF has been extensively studied in cattle (10, 11), pigs (12), rabbits (13), dogs (14), llamas (15), sheep (16), chickens (17–19), turkeys (20), ducks (21), and fish (22), but pharmacokinetic studies in donkeys have not been reported.

As known, donkeys are widely used in various aspects of social life, as important tools for transportation and private riding. As livestock, they are also widely used in the food processing industry. Donkey products, such as donkey meat and skin, play an important role in the catering industry and healthcare industry. Donkeys are also frequently infected with diseases, but there are few reports on effective drugs for treating donkey diseases. Based on the effectiveness of FF in other animals, it may be possible to use it as an effective drug for treating donkey diseases, and therefore pharmacokinetics needs to be carried out to further understand the metabolism of FF in donkeys, and provide a theoretical basis for the use of FF in production practice.

However, few studies have been carried out on the pharmacokinetics of FF in equine species. Designing species-specific dosing protocols and identifying the absorption, distribution, metabolism or elimination of FF in such studies is very important. Herein, we studied the pharmacokinetics of FF in the plasma, urine, and feces of donkeys.

2 Materials and methods

2.1 Chemicals and reagents

The concentration of florfenicol standard in methanol is over 99.0% which was purchased from the Research and Monitoring Institute of Environmental Protection (Ministry of Agriculture, China). And florfenicol used in this study was provided by Zhongmu Nanjing Animal Pharmaceutical Co., Ltd. (Nanjing, China).

2.2 Animals and treatment

The study was allowed by the Laboratory Animal Welfare and Animal Experimental Ethical Committee of China Agricultural University (No. AW80803202-1-4). Five 13 months-old male donkeys were selected and housed individually in metabolic cages. The donkeys were allowed to adapt to their surroundings for 7 days and fasting overnight before the experiment. Then, the body weight (BW) of donkeys was measured (173.20 ± 14.39 kg). The samples of blank blood, urine and feces were collected at 6 h prior to the start of the experiment. At the start of the experiment, the FF solution was orally administered through an esophageal tube in a single dose of 30 mg/kg BW. The animals had free access to water during the experimental period. Each donkey received their individual ration of concentrates twice daily (7 am and 5 pm), with weighing 1.5 kg/d per donkey. The composition and nutrient levels of concentrate was reported in Table 1. Meanwhile, each donkey was fed the Leymus chinensis hay four times a day (8 am, 12 am, 4 pm and 8 pm), with averagely 2.1 kg/d per donkey. The analyzed values of dry matter (DM), crude protein (CP), crude fiber (CF), neutral detergent fiber (NDF), acid detergent fiber (ADF), ether extract (EE) and ash were 90.64, 7.78, 35.32, 68.17, 44.19, 1.10 and 5.15%, respectively in the hay.

2.3 Blood, plasma, feces and urine collection

Blood was collected from the jugular vein in heparin anticoagulant tubes before administration (0 h) and 0.08, 0.25, 0.42, 0.58, 0.75, 1.0, 1.5, 2.0, 2.5, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72, 96, and 144 h after administration. The blood samples were centrifuged at 3,000 rpm for 20 min at 4°Cto obtain plasma and were stored at −20°C until analyses. The urine and feces were collected and weighed every 6 h. After each collection, the weight of feces and the volume of urine were recorded and stored into 50 mL centrifuge tubes for subsequent analysis.

TABLE 1 Composition and nutrient levels of concentrate.

Ingredients	Composition (%)	Nutrients	Levels[b]
Corn	45.00	Dry matter (DM)	87.56
Soybean meal	15.00	DE/MJ·kg^{-1}	10.79
Wheat bran	20.00	Crude protein (CP)	22.18
Corn germ meal	10.00	Crude fiber(CF)	6.26
Peanut meal	5.00	Ether extract (EE)	1.38
Premix[a]	5.00	Lysine	1.12
Total	100.00	Calcium	1.07
		Phosphorus	0.65

[a]The premix provided the following per kg of concentrate diets VA 12,000 IU, VD3 1,500 IU, VE 40 mg, VK3 3 mg, VB1 2 mg, VB2 4 mg, VB6 4 mg, VB12 0.01 mg, pantothenic acid 20 mg, nicotinic acid 30 mg, folic acid 1.7 mg, biotin 0.3 mg, Cu 20 mg, Zn 80 mg, Fe 100 mg, Mn 80 mg, I 0.6 mg, Se 0.2 mg.

[b]DE was a calculated value, while the others were measured values; except for DM, the others were air-dry basis.

2.4 Determination of FF and FFA

The concentrations of FF and its metabolite FFA were determined using high performance liquid chromatography (HPLC) tandem mass spectrometry detection. The assay was referenced to previous reports (9).

Briefly, 0.1 mL of FF and FFA standard stock solution, respectively, were placed in a 10 mL volumetric flask. The volume was made up to 10 mL with methanol. A mixed standard working solution of concentration 1 μg/mL was prepared. Then, 0.1 mL of FF-d3 and FFA-d3 isotope internal standard stock solution, respectively, were placed in a 10 mL volumetric flask. The volume was made up to 10 mL with methanol. A mixed internal standard working solution of FF-d3 and FFA-d3 was prepared. An appropriate amount of the standard working solution of FF and FFA was measured accurately. The control solution was diluted with acetonitrile to make concentrations of 5, 10, 20, 50, 100, and 200 ng/mL. The internal standard solution was mixed with a concentration of 50 ng/mL. A series of control solutions from low concentrations to high concentrations was created. A standard curve was created according to the ratio of the obtained peak area to the concentration of the corresponding control solution. We calculated the regression equation and correlation coefficient. The sample solution and control solution of 50 ng/mL (based on the FF concentration) were used for calibration at a single point. The response values of FF and FFA in the control solution and sample solution had to be within the linear range of detection for the instrument. During determination of the sample solution, the reference solution was injected after 10 single batches of samples to facilitate accurate quantification. Subsequently, we weighed 1 g of blood, urine, or feces, respectively, and placed them in 50 mL centrifuge tubes. Then, we added 50 μL of mixed internal standard working solution. Next, we added 5.0 mL of water and 0.5 mL of ammonia solution, and vortex-mixed for 1 min. Then, we added 10.0 mL of acetonitrile and vortex-mixed immediately for 1 min. The next step was ultrasonic agitation for 20 min, followed by addition of NaCl (3 g) and stirring for 1 min. Next, we undertook ultrasonic agitation for 10 min, followed by centrifugation at 8,000 × *g* for 5 min at room temperature. And then, 1 mL of the supernatant was transferred to a test tube and the extraction procedure was repeated, and make the liquid flow out at a constant speed drop by drop through HLB columns (Tianjin Alta Technology, Tianjin, China), lastly put it on the HPLC-MS/MS for determination. The mobile phase was 5% acetonitrile (phase A) and 95% formic acid water (phase B). The flow rate was maintained at 1.0 mL/min.

2.5 HPLC method validation

The method was validation for linearity, sensitivity and recovery in plasma, urine and feces. Standard curves were constructed from the concentrations of 5, 10, 20, 50, 100, and 200 ng/mL FF or FFA in plasma, urine, and feces, respectively, with their peak areas. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated. Recoveries were calculated by the ratio of the peak areas of different concentrations of FF or FFA obtained from spiked samples of plasma, urine, and feces to the peak area of the corresponding FF or FFA standard working solution.

2.6 Pharmacokinetics analyses

We undertook a non-compartmental approach based on the combined linear trapezoidal rule using WinNonlin 8.3.5.0 (Pharsight, Mountain View, CA, United States). The λz was a first-order rate constant associated with the terminal (log linear) segment of the curve. It was estimated by linear regression of terminal data points. The terminal elimination half-life ($t_{1/2kz}$) was calculated as $0.693/\lambda z$. The area under the plasma concentration-time curve (AUC) was calculated using the trapezoid method. The area under the rate curve (AURC) was calculated as the product of time and excretion rate.

The peak plasma concentration (C_{max}) of the drug and the time to reach the peak plasma concentration (T_{max}) were determined from individual plasma concentration-time curves. The ratio of AUCs of FFA and FF was calculated. The concentrations of FF and FFA in plasma, urine, and feces versus time for each donkey were reported as the mean ± SD at each time point.

3 Results

LC/MS for simultaneous determination of FF and FFA was rapid with a high degree of reproducibility. We obtained calibration curves of FF (FFA) for plasma, urine and fecal matrix with R^2 of 0.9988 (0.9992), 0.9971 (0.9980) and 0.9962 (0.9977), respectively. The mean inter-day precision and intra-day precision was <20%. Accuracy ranged from 80.9 to 113.5%. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.5 μg/kg and 1.0 μg/g or μg/mL for FF and FFA that is calculated according to a signal-to-noise ratio >3 and >10. These validation parameters indicate that this assay method used in this study is accurate and precise.

The mean serum concentration of FF and FFA in these five donkeys is shown in Figure 1. The pharmacokinetics parameters for FF after administration (30 mg·kg^{-1} BW, p.o.) to donkeys are shown in Table 2. After administration of FF, the C_{max} was 0.13 ± 0.02 μg·mL^{-1}, T_{max} was 0.68 ± 0.09 h, and elimination half-life was 5.92 ± 3.25 h. The AUC of FF was 1.31 ± 0.46 μg·mL^{-1}·h, and the mean residence time for FF was 10.37 ± 4.80 h. FFA was detected in all donkeys after administration. The C_{max} of FFA was 0.08 ± 0.01 μg·mL^{-1}, T_{max} was 0.72 ± 0.72 h, and elimination half-life was 15.95 ± 14.04 h. The AUC of FFA was 0.47 ± 0.11 μg·mL^{-1}·h, and the mean residence time for FFA was 18.40 ± 13.02 h. Plasma results can show that FF and FFA are absorbed quickly and eliminated slowly in donkeys.

In a semi-logarithmic plot, the relationship between the urinary concentration of FF and FFA and time was evaluated (Figure 2). Table 3 illustrates the individual and mean pharmacokinetics parameters of FF and FFA in urine. FF was excreted mainly unchanged. The urinary concentration of FF and its metabolite FFA was 123.08 ± 51.71 and 63.62 ± 28.76 μg·L^{-1}, respectively. The $t_{1/2kz}$ of FF and FFA in urine was 21.93 ± 3.42 and 40.26 ± 26.00 h, respectively. The maximum excretion rate of FF and FFA was 10.56 ± 6.41 and 4.03 ± 0.59 μg·h^{-1}, and was reached at 25.60 ± 11.70 and 32.20 ± 9.86 h, respectively.

In a semi-logarithmic plot, the relationship between the fecal concentration of FF and FFA and time was evaluated (Figure 3). Table 4 presents the individual and mean pharmacokinetics parameters of FF and FFA in feces. The maximum excretion rate of FF and FFA in feces was 0.02 ± 0.01 and 0.01 μg·h^{-1}, respectively, and was

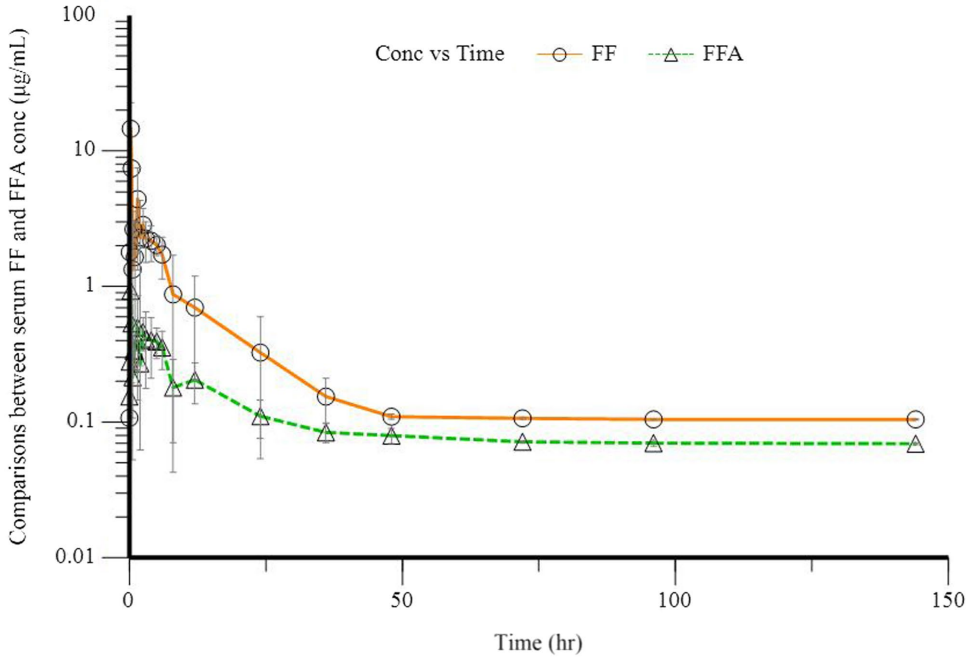


FIGURE 1
Plasma concentration of florfenicol (FF, O) and florfenicol amine (FFA, Δ) in five donkeys after a single oral dose of 30 mg/kg bodyweight.

TABLE 2 Pharmacokinetic parameters of florfenicol in the plasma of five donkeys after single oral administration of 30 mg/kg bodyweight.

Parameter (unit)	Donkey	BW (kg)	λz ($1 \cdot h^{-1}$)	$t_{1/2kz}$ (h)	T_{max} (h)	C_{max} (μg/mL)	$AUC_{0-\infty}$ ($\mu g \cdot mL^{-1} \cdot h$)	MRT (h)
Florfenicol	Boris	156	0.23	3.00	0.75	0.14	0.92	6.22
	John	172	0.22	3.12	0.75	0.14	0.98	7.11
	Jack	196	0.09	7.99	0.75	0.14	1.72	12.66
	Robin	170	0.14	5.00	0.58	0.10	1.01	8.15
	Donald	172	0.07	10.48	0.58	0.11	1.91	17.72
	Mean (SD)	173.20 ± 14.39	0.15 (0.08)	5.92 (3.25)	0.68 (0.09)	0.13 (0.02)	1.31 (0.46)	10.37 (4.80)
Florfenicol amine	Boris	156	0.06	11.07	1.50	0.08	0.62	16.96
	John	172	0.06	12.22	0.25	0.08	0.41	12.44
	Jack	196	0.08	8.64	1.50	0.06	0.53	13.07
	Robin	170	0.10	7.03	0.25	0.07	0.34	8.46
	Donald	172	0.02	40.80	0.08	0.09	0.48	41.06
	Mean (SD)	173.20 ± 14.39	0.06 (0.03)	15.95 (14.04)	0.72 (0.72)	0.08 (0.01)	0.47 (0.11)	18.40 (13.02)

Values are the mean ± SD. BW, bodyweight; λz, first order rate constant associated with the terminal portion of the curve; $t_{1/2\lambda z}$, terminal half-life; AUC, area under the curve; T_{max}, time needed to reach the maximum concentration in plasma; C_{max}, maximum concentration in plasma; MRT, mean residence time.

reached in 33.40 h. The recovery of FF and FFA in feces was 0.52 ± 0.17 and 0.22 ± 0.05 μg, respectively. The area under the feces concentration versus time curve (AURC) of FF and FFA was 0.81 ± 0.36 and 0.34 ± 0.18 μg, respectively. The amount of FF (FFA) excreted through feces is less compared to urine, and it is seen that FF and FFA are mainly excreted through urine.

4 Discussion

Few studies have been carried out on the pharmacokinetics of FF in equine species (23). In the present work, a non-compartmental model best described the time-course of FF in the plasma of donkeys following oral administration. FF was absorbed rapidly through the gastrointestinal tract with a C_{max} of 0.13 μg/mL at 0.68 h. The C_{max} of the present study was much lower than that reported in different animal species. Upon oral administration of FF (22 mg/kg BW), horses had a C_{max} of 13.80 mg/mL and T_{max} of 1.13 h (24). Pigs were orally administered FF at 20 mg/kg BW and 30 mg/kg BW, the C_{max} and T_{max} was 9.9 μg/mL and 1.50 h, 10.84 μg/mL and 1.35 h, respectively (12, 25). The C_{max} and T_{max} of rabbits was 7.96 μg/mL and 0.90 h, 15.14 μg/mL and 0.50 h, respectively, when oral administration of 20 mg/kg body weight and 30 mg/kg body weight FF (13, 26). Also, after administration of FF, the C_{max} (μg/mL) and T_{max} (h) have been

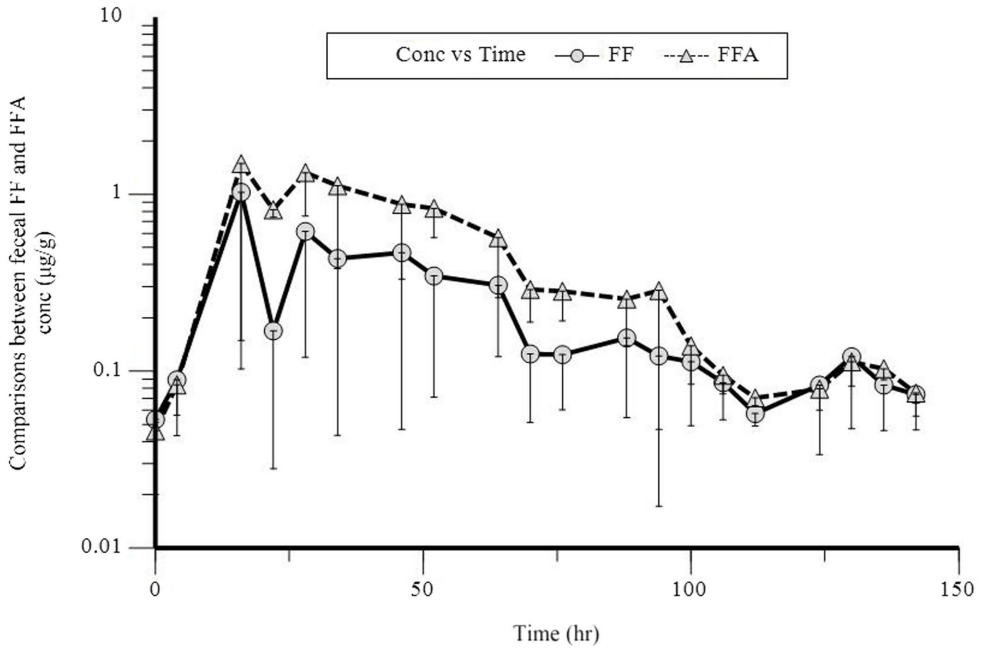


FIGURE 2
Semilogarithmic plot of florfenicol (FF, O) and florfenicol amine (FFA, Δ) urinary concentration vs. time following a single oral dose at 30 mg/kg bodyweight in donkeys.

TABLE 3 Amount of florfenicol and florfenicol amine recovered in urine after single oral administration to donkeys at 30 mg/kg bodyweight.

Parameter (unit)	Donkey	λz ($1 \cdot h^{-1}$)	$t_{1/2kz}$ (h)	$T_{max\ rate}$ (h)	Max rate ($\mu g \cdot h^{-1}$)	Amount recovered (µg)	AURC (µg)
Florfenicol	Boris	0.02	34.59	25.00	7.82	116.66	358.70
	John	0.02	31.71	25.00	12.03	311.62	454.08
	Jack	0.36	1.94	43.00	6.29	87.50	149.54
	Robin	0.02	28.86	49.00	3.04	87.80	147.82
	Donald	0.01	54.16	25.00	5.70	159.94	448.44
	Mean (SD)	0.03 (0.01)	21.93 (3.42)	25.60 (11.70)	10.56 (6.41)	123.08 (51.71)	153.93 (43.59)
Florfenicol amine	Boris	0.02	44.84	37.00	3.05	34.43	96.14
	John	0.02	32.03	19.00	4.02	110.75	151.46
	Jack	0.01	79.44	43.00	4.60	54.61	173.67
	Robin	0.02	37.47	37.00	4.22	51.57	76.16
	Donald	0.09	7.52	25.00	4.28	66.74	142.60
	Mean (SD)	0.03 (0.03)	40.26 (26.00)	32.2 (9.86)	4.03 (0.59)	63.62 (28.76)	128.01 (40.47)

Values are the mean ± SD. λz, first order rate constant associated with the terminal portion of the curve; $t_{1/2kz}$, terminal half-life; AURC, area under the rate curve; $T_{max\ rate}$, time needed to reach the maximum rate; Max rate, maximum excretion rate; amount-recovered, amount of drug excreted in urine.

reported to be 6.18 and 0.94 in dogs (14), 2.41 and 1.16 for chickens (27), 30.47 and 0.50 in geese (28). This difference may have resulted from the formulation type for different species (23) and variation in the FF dose administered (15 versus 20 versus 22 versus 30 mg/kg BW) (29).

λz and $t_{1/2kz}$ are constants that reflect the rate of drug elimination from the body, and there is a reciprocal relationship between them. Oral administration of FF (20 mg/kg BW) led to a $t_{1/2kz}$ (h) of 10.0 in pigs (12), 1.42 in rabbits (13), 1.24 in dogs (14), 2.43 in geese (28), 21.93 in one group of chickens (17), 9.0 in another group of chickens (27). Oral administration of FF (30 mg/kg BW) led to a $t_{1/2kz}$ (h) of 12.39 in pigs (12), 2.57 in rabbits (26), 1.67 in chickens (18), 3.76 in broiler turkeys (20), and 2.77 in ducks (21). In our study, the $t_{1/2kz}$ for FF in the plasma of donkeys was 5.92 h, which differed to the values for the animals mentioned above. Inter-species variability affects pharmacokinetics parameters, and it is a major issue in veterinary pharmacology (23). Age, sex, breed, health status, or gene polymorphisms have also been shown to influence pharmacokinetics indices in different pharmacokinetic studies (30).

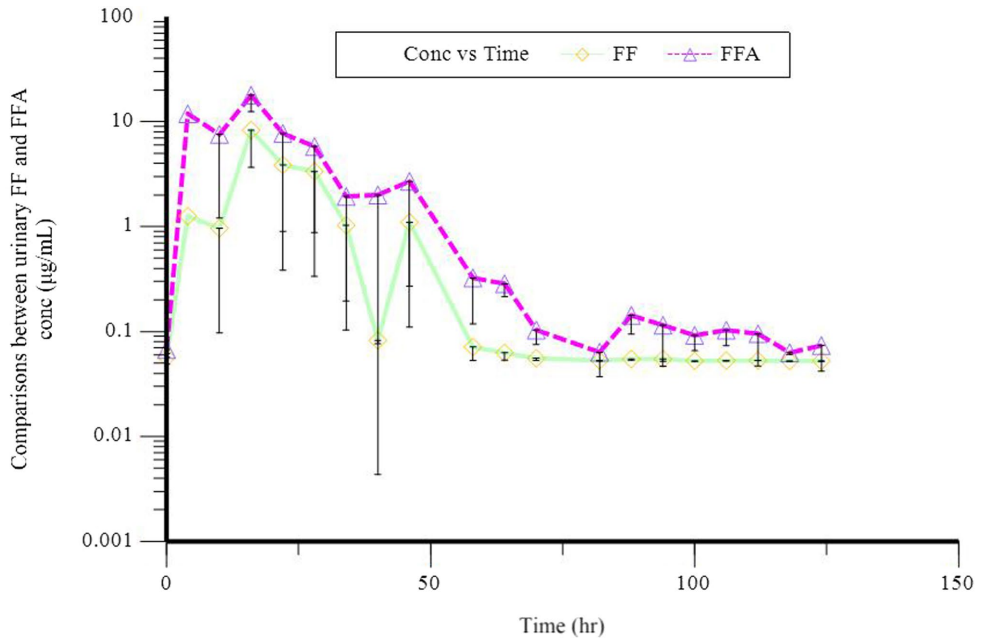


FIGURE 3
Semilogarithmic plot of florfenicol (FF, O) and florfenicol amine (FFA, Δ) fecal concentration vs. time after a single oral dose of 30 mg/kg bodyweight in donkeys.

TABLE 4 Pharmacokinetic parameters calculated from the feces of donkeys administered florfenicol (30 mg/kg bodyweight, p.o.).

Parameter (unit)	Donkey	λz ($1 \cdot h^{-1}$)	$t_{1/2\,kz}$ (h)	$T_{max\ rate}$ (h)	Max rate ($\mu g \cdot h^{-1}$)	Amount-recovered (μg)	AURC (μg)
Florfenicol	Boris	0.017	41.91	25.00	0.012	0.605	0.831
	John	0.009	77.76	43.00	0.012	0.606	1.363
	Jack	0.011	64.83	43.00	0.011	0.222	0.400
	Robin	0.024	29.17	31.00	0.037	0.521	0.624
	Donald	0.011	65.67	25.00	0.012	0.647	0.837
	Mean (SD)	0.01 (0.01)	55.87 (19.77)	33.40 (9.10)	0.02 (0.01)	0.52 (0.17)	0.81 (0.36)
Florfenicol amine	Boris	0.083	8.31	49.00	0.010	0.251	0.248
	John	0.012	59.00	19.00	0.008	0.227	0.293
	Jack	0.004	168.32	43.00	0.008	0.136	0.671
	Robin	0.022	30.83	31.00	0.019	0.249	0.263
	Donald	0.051	13.52	25.00	0.009	0.228	0.242
	Mean (SD)	0.03 (0.03)	56.00 (65.84)	33.40 (12.44)	0.01 (0.00)	0.22 (0.05)	0.34 (0.18)

Values are the mean ± SD. λz, first order rate constant associated with the terminal portion of the curve; $t_{1/2kz}$, terminal half-life; AURC, area under the rate curve; $T_{max\ rate}$, time needed to reach the maximum rate; Max rate, maximum excretion rate; amount-recovered, amount of drug recovered from feces.

The AUC is an important index to evaluate the degree of drug absorption and drug exposure. Upon oral administration of FF at 20 $mg \cdot kg^{-1}$ BW, the AUC has been reported to be 53.45 $mg \cdot h \cdot L^{-1}$ in Equidae (25), 132.1 $\mu g \cdot h \cdot mL^{-1}$ in pigs (12), 23.78 μg·h/mL in rabbits (13), 22.36 mg·h/L in dogs (14), and 37.85 mg·h/L in chickens (27). Upon oral administration of FF at 30 mg/kg BW, the AUC was 65.89 μg/mL/h in pigs (12), 49.02 μg/mL/h in rabbits (13), 27.59 mg·h/L in one type of chicken, 4.15 mg·h/L in another type of chicken (18), 77.62 μg·h/mL in broiler turkeys (20), and 84.00 μg·h/mL in ducks (21). In the present work, when five donkeys were administered FF at 30 mg/kg BW, the AUC in plasma was 1.31 $\mu g \cdot mL^{-1} \cdot h$, which indicated that the degree of drug exposure in the body was very low and very little FF was absorbed. A possible explanation may be associated with the differences in BW and animal species-related cytochrome P450 enzymes. One study revealed that an increase in BW had a significant influence on the variability of pharmacokinetics parameters within the same species (31). These results of plasma pharmacokinetic parameters suggest that donkeys appear to have low bioavailability of FF (FFA) under oral administration, but this requires subsequent in-depth studies.

In the current study, the amount of FF excreted amount in urine was 123.08 μg and the excreted amount of FFA was 63.62 μg. Similarly,

the amount of FF excreted in feces was 0.52 μg and that of FFA was 0.22 μg. These data indicated that FF was excreted mainly in urine. One study found that most of a drug dose was excreted in urine as the parent form, which suggested that major clearance of the drug was in the kidneys (10), which was also evidenced in our work. When FF was administered to calves, FF was excreted in urine as the parent form (10). FF and its metabolite FFA are eliminated primarily by the kidneys through glomerular filtration. Therefore, some portion of the administered drug may be affected by first-pass metabolism or may be excreted by the kidneys without reaching the general systemic circulation (23, 29). However, FF is metabolized by cytochrome P450 3A in the liver to FFA, and the same process is assumed to occur in the kidneys (27, 32). Therefore, when using FF (FFA), in addition to considering the dosage, measures should also be considered to counteract the possible environmental pollution caused by antibiotic urinary excretion. The results of this study provide a reference for the rational use of FF in donkey clinical practice, and selecting the appropriate dosage based on pharmacokinetics will save economic costs.

5 Conclusion

The present study suggests that after a single oral administration of FF (30 mg/kg BW), it was rapidly absorbed and slowly eliminated in male donkeys. Most FF (FFA) was excreted through feces and urine, and the amount excreted in urine was larger than in feces.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved and conducted according to the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, China) and approved by the Laboratory Animal Welfare and Animal Experimental Ethical Committee of China Agricultural University (No. AW80803202-1-4, 8 August 2023). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SL: Investigation, Methodology, Writing – original draft. YG: Investigation, Software, Writing – original draft. HQ: Formal analysis, Investigation, Resources, Writing – review & editing. YD: Methodology, Writing – review & editing. SZ: Methodology, Writing – review & editing. TF: Investigation, Writing – review & editing. RK: Formal analysis, Methodology, Writing – review & editing. JC: Resources, Writing – review & editing. SH: Supervision, Writing – review & editing. LZ: Visualization, Writing – review & editing. QM: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Taishan Leading Industry Talents-Agricultural Science of Shandong Province (grant number LJNY202022).

Acknowledgments

The authors are grateful to the staff of the donkey farm at Shandong Black Donkey Animal Husbandry Technology Co., Ltd. for their help in transferring donkeys and collecting samples.

Conflict of interest

HQ and JC were employed by Dong-E-E-Jiao Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Keyes K, Hudson C, Maurer JJ, Thayer S, White DG, Lee MD. Detection of florfenicol resistance genes in *Escherichia coli* isolated from sick chickens. *Antimicrob Agents Chemother*. (2000) 44:421–4. doi: 10.1128/AAC.44.2.421-424.2000

2. Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev*. (2004) 28:519–42. doi: 10.1016/j.femsre.2004.04.001

3. Farag VM, El-Shafei RA, Elkenany RM, Ali HS, Eladl AH. Antimicrobial, immunological and biochemical effects of florfenicol and garlic (*Allium sativum*) on rabbits infected with *Escherichia coli* serotype O55: H7. *Vet Res Commun*. (2022) 46:363–76. doi: 10.1007/s11259-021-09859-3

4. Zhao X, Cui X, Yang Y, Zhu L, Li L, Kong X. Synergistic effect of quercetin on antibacterial activity of florfenicol against *Aeromonas hydrophila in vitro* and *in vivo*. *Antibiotics*. (2022) 11:929. doi: 10.3390/antibiotics11070929

5. Apley MD. Clinical evidence for individual animal therapy for papillomatous digital dermatitis (hairy heel wart) and infectious bovine pododermatitis (foot rot). *Vet Clin North Am Food Anim Pract*. (2015) 31:81–95. doi: 10.1016/j.cvfa.2014.11.009

6. Papich MG. Florfenicol In:. *Saunders handbook of veterinary drugs*. Philadelphia, PA, USA: WB Saunders (2016). 327–9.

7. Ural K, Kirkan S, Ural DA, Erbas G, Gültekin M, Parın U, et al. Florfenicol therapy during naturally occuring corynebacterium pseudotuberculosis infection in sheep and goats in Aydin, Turkey. *Anim Health Prod Hyg*. (2014) 3:278–83.

8. Wang Y, Zhang L, Ahmed S, Liu Y, Li X. Pharmacokinetic of florfenicol in pulmonary epithelial lining fluid of swine and effects of anesthetic agent on drug plasma disposition kinetics. *Arq Bras Med Vet Zootec*. (2018) 70:1497–504. doi: 10.1590/1678-4162-9770

9. Somogyi Z, Mag P, Kovács D, Kerek Á, Szabó P, Makrai L, et al. Synovial and systemic pharmacokinetics of florfenicol and PK/PD integration against *Streptococcus suis* in pigs. *Pharmaceutics*. (2022) 14:109. doi: 10.3390/pharmaceutics14010109

10. Selimov R, Goncharova E, Koriakovtsev P, Gabidullina D, Karsakova J, Kozlov S, et al. Comparative pharmacokinetics of florfenicol in heifers after intramuscular and subcutaneous administration. *J Vet Pharmacol Ther*. (2023) 46:177–84. doi: 10.1111/jvp.13110

11. Yang F, Lin Z, Riviere JE, Baynes RE. Development and application of a population physiologically based pharmacokinetic model for florfenicol and its metabolite florfenicol amine in cattle. *Food Chem Toxicol*. (2019) 126:285–94. doi: 10.1016/j.fct.2019.02.029

12. Jiang HX, Zeng ZL, Chen ZL, Liu JJ, Fung KF. Pharmacokinetics of florfenicol in pigs following intravenous, intramuscular or oral administration and the effects of feed intake on oral dosing. *J Vet Pharmacol Ther*. (2006) 29:153–6. doi: 10.1111/j.1365-2885.2006.00727.x

13. Park BK, Lim JH, Kim MS, Hwang YH, Yun HI. Pharmacokinetics of florfenicol and its major metabolite, florfenicol amine, in rabbits. *J Vet Pharmacol Ther*. (2007) 30:32–6. doi: 10.1111/j.1365-2885.2007.00809.x

14. Park BK, Lim JH, Kim MS, Hwang YH, Yun HI. Pharmacokinetics of florfenicol and its metabolite, florfenicol amine, in dogs. *Res Vet Sci*. (2008) 84:85–9. doi: 10.1016/j.rvsc.2007.04.001

15. Pentecost RL, Niehaus AJ, Werle NA, Lakritz J. Pharmacokinetics of florfenicol after intravenous and intramuscular dosing in llamas. *Res Vet Sci*. (2013) 95:594–9. doi: 10.1016/j.rvsc.2013.05.009

16. Shen J, Li X, Jiang H, Hsu WH. Bioavailability and pharmacokinetics of florfenicol in healthy sheep. *J Vet Pharmacol Ther*. (2004) 27:163–8. doi: 10.1111/j.1365-2885.2004.00574.x

17. Imran M, Fazal EH, Tawab A, Rauf W, Rahman M, Khan QM, et al. LC-MS/MS based method development for the analysis of florfenicol and its application to estimate relative distribution in various tissues of broiler chicken. *J Chromatogr B*. (2017) 1063:163–73. doi: 10.1016/j.jchromb.2017.08.029

18. Shen J, Hu D, Wu X, Coats JR. Bioavailability and pharmacokinetics of florfenicol in broiler chickens. *J Vet Pharmacol Ther*. (2003) 26:337–41. doi: 10.1046/j.1365-2885.2003.00495.x

19. Chang SK, Davis JL, Cheng CN, Shien RH, Hsieh MK, Koh BW, et al. Pharmacokinetics and tissue depletion of florfenicol in Leghorn and Taiwan native chickens. *J Vet Pharmacol Ther*. (2010) 33:471–9. doi: 10.1111/j.1365-2885.2009.01155.x

20. Bello A, Pozniak B, Smutkiewicz A, Switala M. The influence of the site of drug administration on florfenicol pharmacokinetics in turkeys. *Poult Sci*. (2022) 101:101536. doi: 10.1016/j.psj.2021.101536

21. Tikhomirov M, Pozniak B, Smutkiewicz A, Switala M. Pharmacokinetics of florfenicol and thiamphenicol in ducks. *J Vet Pharmacol Ther*. (2019) 42:116–20. doi: 10.1111/jvp.12714

22. Sumithra TG, Sharma SRK, Prasad V, Gop AP, Gangadharan S, Gayathri S, et al. Pharmacokinetics and tissue distribution of florfenicol and florfenicol amine in snubnose pompano (*Trachinotus blochii*) following oral administration. *Fish Physiol Biochem*. (2023) 49:307–20. doi: 10.1007/s10695-023-01179-4

23. Toutain PL, Ferran A, Bousquet-Mélou A. Species differences in pharmacokinetics and pharmacodynamics In: F Cunningham, J Elliott and P Lees, editors. *Comparative and veterinary pharmacology handbook of experimental pharmacology*. Berlin: Springer (2010). 19–48.

24. McKellar QA, Varma KJ. Pharmacokinetics and tolerance of florfenicol in Equidae. *Equine Vet J*. (1996) 28:209–13. doi: 10.1111/j.2042-3306.1996.tb03774.x

25. Liu J, Fung KF, Chen Z, Zeng Z, Zhang J. Pharmacokinetics of florfenicol in healthy pigs and in pigs experimentally infected with *Actinobacillus pleuropneumoniae*. *Antimicrob Agents Chemother*. (2003) 47:820–3. doi: 10.1128/AAC.47.2.820-823.2003

26. Abd El-Aty AM, Goudah A, Abo El-Sooud K, El-Zorba HY, Shimoda M, Zhou HH. Pharmacokinetics and bioavailability of florfenicol following intravenous, intramuscular and oral administrations in rabbits. *Vet Res Commun*. (2004) 28:515–24. doi: 10.1023/b:verc.0000040241.06642.49

27. Anadon A, Martinez MA, Martinez M, Rios A, Caballero V, Ares I, et al. Plasma and tissue depletion of florfenicol and florfenicol-amine in chickens. *J Agric Food Chem*. (2008) 56:11049–56. doi: 10.1021/jf802138y

28. Tikhomirov M, Pozniak B, Smutkiewicz A, Switala M. Pharmacokinetics of florfenicol and thiamphenicol after single oral and intravenous, as well as multiple oral administrations to geese. *Br Poult Sci*. (2021) 62:25–31. doi: 10.1080/00071668.2020.1824290

29. Houben R, Antonissen G, Croubels S, De Backer P, Devreese M. Pharmacokinetics of drugs in avian species and the applications and limitations of dose extrapolation. *Vlaams Diergeneeskundig Tijdschrift*. (2016) 85:124–32. doi: 10.21825/vdt.v85i3.16338

30. Modric S, Martinez M. Patient variation in veterinary medicine-part II-influence of physiological variables. *J Vet Pharmacol Ther*. (2011) 34:209–23. doi: 10.1111/j.1365-2885.2010.01249.x

31. Switala M, Pozniak B, Paslawska U, Grabowski T, Motykiewicz-Pers K, Bobrek K. Metronidazole pharmacokinetics during rapid growth in turkeys—relation to changes in haemodynamics and drug metabolism. *J Vet Pharmacol Ther*. (2016) 39:373–80. doi: 10.1111/jvp.12283

32. Wang GY, Zheng HH, Zhang KY, Yang F, Kong T, Zhou B, et al. The roles of cytochrome P 450 and P-glycoprotein in the pharmacokinetics of florfenicol in chickens. *Iran J Vet Res*. (2018) 19:9–14. doi: 10.22099/ijvr.2018.4761

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 08 January 2024
DOI 10.3389/fvets.2023.1328698

Check for updates

OPEN ACCESS

EDITED BY
Benito Soto-Blanco,
Federal University of Minas Gerais, Brazil

REVIEWED BY
Clara Marin,
Universidad CEU Cardenal Herrera, Spain
Irene Bueno,
University of Bristol, United Kingdom

*CORRESPONDENCE
Ana M. Sahagún
amsahp@unileon.es
Cristina López
clopcd@unileon.es

RECEIVED 27 October 2023
ACCEPTED 12 December 2023
PUBLISHED 08 January 2024

CITATION
Romero B, Susperregui J, Sahagún AM, Fernández N, López C, de la Puente R, Altónaga JR and Díez R (2024) Drug prescription pattern in exotic pet and wildlife animal practice: a retrospective study in a Spanish veterinary teaching hospital from 2018 to 2022.
Front. Vet. Sci. 10:1328698.
doi: 10.3389/fvets.2023.1328698

COPYRIGHT
© 2024 Romero, Susperregui, Sahagún, Fernández, López, de la Puente, Altónaga and Díez. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Drug prescription pattern in exotic pet and wildlife animal practice: a retrospective study in a Spanish veterinary teaching hospital from 2018 to 2022

Beatriz Romero[1], Julen Susperregui[2], Ana M. Sahagún[1]*, Nélida Fernández[1], Cristina López[1]*, Raúl de la Puente[1], José R. Altónaga[3] and Raquel Díez[1]

[1]Department of Biomedical Sciences, Veterinary Faculty, Institute of Biomedicine (IBIOMED), University of León, León, Spain, [2]Applied Mathematics, Department of Mathematics, University of León, León, Spain, [3]Department of Veterinary Medicine, Surgery and Anatomy, Director of the Veterinary Teaching Hospital of the University of León (HVULE), University of León, León, Spain

Exotic companion animals have had an important role in our society since ancient times. Preserving animal health is necessary to do a responsible use of veterinary medicines. This study aimed to describe the prescription patterns of drugs in exotic pets and wildlife animals attending the Veterinary Teaching Hospital of the University of León (HVULE). A retrospective study was carried out between 2018 and 2022. Birds were the largest group of exotic animals attending the HVULE. Visits were related to emergency reasons and for musculoskeletal disorders. One-third of the animals were eventually euthanised. Regarding pharmacological treatments, the most frequently active ingredients used were pentobarbital, isoflurane, meloxicam, and within antibiotics, marbofloxacin (category B in the classification of European Medicines Agency).

KEYWORDS

antibiotic, prescription pattern, exotic animal, veterinary teaching hospital, wildlife animal

1 Introduction

The adjective *exotic* is a term with several definitions, all equally valid to define an *exotic animal*. However, there are other expressions more commonly used, such as non-traditional or non-domestic animals (1). The American Veterinary Medical Association (AVMA) considers *exotic companion animals* as all species that coexist with humans, both domestic and non-domestic, representing hundreds of animal species of reptiles, amphibians, birds and mammals, the Pisces group, and the phylum *Arthropoda* (2). In Spain, Royal Decree 630/2013, which regulates the Spanish catalog of invasive exotic species, defines an *exotic pet animal* as "an animal of non-native wildlife that is individually dependent on humans, lives with them and has assumed the habit of captivity" (3).

Although according to the AVMA, dogs and cats are by far the largest companion animal population in the United States, between 2017 and 2018, the ownership of other types of pets, such as fish, ferrets, rabbits, hamsters, guinea pigs, turtles, snakes, and lizards, has increased significantly (4). In Europe, the number of ornamental birds is estimated to be approximately

50 million, whereas ornamental fish are approximately 300 million, small mammals 27 million, and reptiles 8 million (5). In Spain, according to data from 2021, there are more than 29 million pets, including nearly 8 million fish, 5 million birds, 1.5 million reptiles, and 1.5 million small mammals (6). However, the recent Law 7/2023 on the Protection of the Rights and Welfare of Animals may change this trend in this country, as it prohibits "the keeping, breeding, trade, sale, offer for sale, exchange or donation and import or export as pets of individuals of species not included in a positive list of pets" (7), which has not yet established.

This growing demand for exotic animals poses new challenges for veterinarians, as they are expected to effectively care for, diagnose, and treat a huge variety of animal species. Specifically, in the case of the different groups of exotic animals, both companion and wild ones, their anatomy and physiology may vary enormously, and also the way in which disorders manifest themselves. This makes the selection and prescription of medicines for these patients a complex process, as pharmacokinetic, efficacy, and safety data are often lacking in exotic animal medicine (8). In many cases, this often leads to the use of drugs in an extralabel manner (9) and, consequently, to an increased risk of adverse effects.

In addition, exotic practitioners are aware that the prevalence of daily emergency events, particularly those resulting in death, is higher among exotic animals than with other species such as dogs or cats (2). Among the reasons that may explain this fact is that most exotic species, as prey animals, tend to mask their diseases. Furthermore, the interaction time of owners with these animals may not be as long as with a dog or cat. Moreover, the behavioral patterns indicative of pain or disease in these animal species are not well known, which may lead to treatment failure to provide help to the animal when it is essential (2).

Among global health problems, zoonosis and antimicrobial resistance best illustrate the *One Health* concept (10). This approach is a global strategy that recognizes the interdependence among human, animal, and environmental health (11, 12), and they should be considered as a whole (13), as animal health is fundamental to ensuring public health, food security and supply, the economy, and preservation of animal species (14). Moreover, these species may be a major source of zoonoses, especially in young children and immunocompromised people (15). As for antimicrobial resistance, several studies have pointed out that antimicrobials are not often used in companion animals according to European recommendations (16–21), thus favoring the development of resistance (22, 23). Recently, increasing attention has been paid to the role of companion animals as potential reservoirs of resistant and multi-resistant pathogens for humans (24–27) as we live in close contact with them (24–26). However, in exotic animal medicine little or no information is available on antimicrobial resistance. A study carried out in wildlife rescue centers revealed that birds may be housed for long periods of time and receive antimicrobial treatments, which could potentially lead to the acquisition and subsequent colonization by antimicrobial-resistant bacteria (28).

In veterinary medicine, drugs are available to preserve animal health provided they are used responsibly (14). Rational use of medicines is based on using the right drug, at the right dose and at the right cost, as described by World Health Organization (WHO) (29). In humans, the assessment of drug utilization patterns with WHO indicators is increasingly necessary to promote rational use of medicines (30, 31). In veterinary practice, these indicators are not still defined, although antimicrobials are the drugs for which most progress has been made. In the European Union (EU), Regulation 2019/6 states that within 8 years from 28 January 2022, data on antimicrobial medicinal products should be collected from animals other than food-producing ones not later than in 2029 (32). More recently, Spanish regulations have had set a deadline to start the collection of pet data on 31 January 2025 (RD 666/2023) (33). As for exotic animals, information is scarce and scattered, often only as guidelines to manage a certain animal species, disorder, or pharmacological group (9, 34, 35), or related to common practice of veterinarians with a certain type of animals (8, 36) but not about the current consumption of drugs. Therefore, this study aimed to describe the prescription patterns of medicines in exotic pets and wildlife animals attending the Veterinary Teaching Hospital of the University of León (HVULE).

2 Materials and methods

2.1 Study site

The HVULE is the Veterinary Teaching Hospital of the Faculty of Veterinary at the University of Leon (Spain). The hospital is organized into two separate clinical departments according to the target species: small animals and large ones, both with their emergency facilities. In 2023, all the teaching staff were qualified veterinarians.

2.2 Study design and data collection

A retrospective study was designed to evaluate the drug prescription patterns in exotic pets and wildlife patients treated at the HVULE between 1 January 2018 to 31 December 2022.

Data of all exotic pets and wildlife (birds, mammals, and reptiles, both wild and kept animals) of the 5-year period were collected through an inductive method. All data used in this study were obtained by reviewing the individual medical records stored in GestorVet (GestorVet, Las Palmas de Gran Canaria, Spain), an online management software, one of the most widely used in veterinary clinics and hospitals in Spain. For each animal, the following data were collected: history number, year of clinical record, medical check-up, species, weight, anatomical system/organ involved, if hospitalization occurred and days of hospitalization, number of treatments and if euthanasia was applied, as well as the different treatments prescribed in the HVULE, route of administration, and dosage regimen. All medications were categorized according to the WHO anatomical-therapeutic-chemical veterinary (ATCvet) classification system (37). Any combination medicine (multicomponent products) was considered as a single medicine. The European Medicines Agency (EMA) Categorization of antibiotics for use in animals was used to classify antibiotics (Category A—Avoid; Category B—Restrict; Category C—Caution; Category D—Prudence) (38). Data were exported to Microsoft Access (Microsoft Office 2019). Patients whose medical records lacked essential information, those who died before or during consultation without having received treatment and those who did not receive any treatment were excluded.

The Strengthening the Reporting of Observational Studies in Epidemiology—Veterinary Extension (STROBE-Vet) Statement was used to report data (39).

2.3 Calculation of the administered daily amount

The administered daily amount (in grams) was also calculated for each given drug administration. For this purpose, the following formula was used:

$$\text{Administered Daily Amount}\left(\text{ADA}\right) = \text{Amount of drug} \times \text{Proportion of active ingredient in this drug}$$

2.4 Calculation of the prescription diversity

Prescription diversity (PD) is defined as "the frequency and variety with which a practice prescribes pharmaceutical classes (PC) within a determined pharmaceutical family (PF)" (40). It is calculated as follows, adjusted to a 0–1 scale where 1 represents maximal diversity:

$$\text{Prescription Diversity}\left(\text{PD}\right) = 1 - \frac{\sum \text{np}\left(\text{np}-1\right)}{\text{NP}\left(\text{NP}-1\right)}$$

np is the number of prescriptions of a particular PC within a PF, and NP is the total number of prescriptions within a PF.

2.5 Data management and statistical analysis

Data analysis was performed with the statistical package IBM SPSS Statistics 26 (IBM Corporation, Armonk, NY, United States). Descriptive statistics (frequencies, mean, standard deviation, ranges, and percentages with 95% confidence intervals) were used to analyse the study population. Odds ratio (OR) was calculated with their respective 95% confidence intervals (95% CI). Multivariable forward-step ordinal logistic regression analysis was conducted to assess the impact of each predictor on euthanasia and antibiotic prescriptions. A p-value of ≤ 0.05 was always considered as significant.

3 Results

Data were available from a total of 17,483 medical records registered in GestorVet for the 5-year period studied. Of those, 1,420 (8.1%) were exotic pets and wildlife animals. After applying exclusion criteria, 503 animals were included in the study, which received 1,081 medical treatments.

Table 1 summarizes the results of some background information about the animals assessed. Birds were the most frequently treated, being 247 (57.8%) birds of prey or raptors (order *Falconiformes, Accipitriformes,* and *Strigiformes*) and 180 (42.2%) synanthropic birds (order *Ciconiiformes*, *Columbiformes,* and *Apodiformes*). As for mammals, 32 were rodents (45.1%), 17 lagomorphs (23.9%), 15 mustelids (21.2%), 4 hedgehogs (5.6%), and 3 other animals (4.2%). Finally, all reptiles were chelonians (5 animals, 1%). Birds were 22 times more likely to have visited the hospital for an emergency than mammals (OR = 22.1; 95% CI: 12.1–40.6). The proportion of cases evaluated increased progressively over the study period approximately 195%, despite the COVID-19 pandemic. In addition, if an animal was admitted for emergency care, it was almost 4 times more probably to be hospitalized (OR = 3.8; 95% CI: 2.3–6.1), and approximately 2 times more likely to be euthanised (OR = 1.9; 95% CI: 1.2–3.2).

TABLE 1 Background information of the exotic pets and wildlife animals visiting the HVULE (Spain) from 2018 to 2022.

Characteristics	Frequency (%) (n = 503)	95% CI
Year of clinical record		
2018	43 (8.5)	0.061–0.110
2019	57 (11.3)	0.086–0.141
2020	131 (26.0)	0.222–0.299
2021	145 (28.8)	0.249–0.328
2022	127 (25.2)	0.215–0.290
Hospital visit		
Emergency	400 (79.5)	0.760–0.830
Medical check-up	97 (19.3)	0.158–0.227
Hospital discharge	3 (0.6)	0.000–0.013
Surgery	2 (0.4)	0.000–0.009
Image diagnosis	1 (0.2)	0.000–0.006
Animal class		
Birds	427 (84.9)	0.818–0.880
Mammals	71 (14.1)	0.111–0.172
Reptiles	5 (1.0)	0.001–0.019

CI, Confidence interval.

Table 2 shows the clinical characteristics of the animals attending the hospital. For those who were hospitalized, the length of stay was 4.56 ± 6.96 days (range 1–71, median 2.0), and the mean administered treatments were 2.15 ± 1.02 (range 1–7, median 2.0). Birds were again 3.4 times more probably to be hospitalized than mammals (OR = 3.4; 95% CI: 1.7–5.9) and 8.4 times more likely to be euthanised (OR = 8.4; 95% CI: 3.3–21.3). However, all animals undergoing surgery were mammals.

Table 3 displays the multivariate analysis carried out to identify those variables associated with euthanasia in exotic animals. Non-hospitalized, birds, emergency visits, and the existence of musculoskeletal disorders had a significant impact on euthanasia. In addition, there was a significant year-on-year increase of 2.1 (CI: 1.1–3.8; p = 0.019).

With respect to the treatments followed (n = 1,081), 1,069 (98.9%) were medications included in the ATCvet classification. Table 4 lists these treatments according to the first level of this classification, showing that nearly half of them belonged to group QN (Nervous system). When the fourth level was considered, the most commonly chemical/therapeutic subgroup was QB05BB (solutions affecting the

TABLE 2 Clinical characteristics of the exotic pets and wildlife animals visiting the HVULE (Spain) from 2018 to 2022.

Clinical characteristics	Frequency (%) (*n* = 503)	95% CI
Anatomical system/organ affected		
Musculoskeletal	297 (59.0)	0.547–0.633
Digestive	112 (22.3)	0.186–0.259
Sense organs	22 (4.4)	0.026–0.062
Respiratory	18 (3.6)	0.020–0.052
Nervous	13 (2.6)	0.012–0.040
Genitourinary	8 (1.6)	0.005–0.027
Infectious	7 (1.4)	0.004–0.024
Cardiovascular	2 (0.4)	0.000–0.009
Others*	24 (4.8)	0.029–0.066
Euthanasia		
No	332 (66.0)	0.619–0.701
Yes	171 (34.0)	0.299–0.381
Hospitalization		
No	247 (49.1)	0.447–0.535
Yes	256 (50.9)	0.465–0.553
Hospitalization days (n = 256)		
1–3	157 (61.3)	0.270–0.351
4–8	70 (27.3)	0.109–0.169
≥9	29 (11.3)	0.037–0.078
Surgery		
No	496 (98.6)	0.976–0.996
Yes	7 (1.4)	0.004–0.024
Number of treatments		
1	120 (23.9)	0.201–0.276
2	251 (49.9)	0.455–0.543
3	101 (20.1)	0.166–0.236
≥4	31 (6.1)	0.041–0.083

CI, Confidence interval. * Others include those animals electrocuted or poisoned.

TABLE 3 Multivariate ordinal logistic regression analysis of factors relevant to being euthanised.

Variables	OR (95% CI)	*p*-value
Hospital visit (emergency)	4.615 (1.005–21.199)	0.049
Animal class (birds)	6.811 (1.108–41.871)	0.038
System affected (musculoskeletal)	31.72 (4.184–240.4)	0.001
Hospitalization	2510.2 (206.4–30529.9)	<0.001
Year	2.078 (1.126–3.838)	0.019

CI, Confidence interval; OR, Odds ratio.

electrolyte balance), which was used in 188 animals, followed by halogenated hydrocarbons (QN01AB, *n* = 186), oxicams (QM01AC, *n* = 173), and barbiturates (QN51AA) for animal euthanasia (*n* = 171). Specifically for each drug (fifth-level code), pentobarbital was administered to 187 animals (171 for animal euthanasia and 16 for sedation) followed by isoflurane (*n* = 184), meloxicam (*n* = 173), and electrolyte solutions (*n* = 153). It should be highlighted that 171 animals were euthanised with drugs from group QN (Nervous system). The HVULE protocol for birds consisted of inhaled isoflurane (5%) and intracardiac pentobarbital (0.1 g/kg). For mammals, intramuscular dexmedetomidine (0.04 mg/kg) or acepromazine (0.1 mg/kg), intravenous propofol (3 mg/kg) and intravenous pentobarbital (2 mg/kg) were used with the same purpose.

In group QN (Nervous system), subgroup analgesics (QN02) were also used (*n* = 111; 22.3%). In this case, all were opioids (QN02A), with a PD of 0.67. Parenteral buprenorphine (45.9%) was the main opioid employed (76.5% in birds and 23.5% in mammals), followed by tramadol (27.9%), butorphanol (22.5%), and methadone (3.6%). Butorphanol and methadone were only administered parenterally to birds and mammals, respectively, whereas tramadol was administered to both birds (90.3%) and mammals (9.7%) by parenteral (77.4%) or oral (22.6%) routes.

All prescriptions of the group QM (Musculoskeletal system) were non-steroidal anti-inflammatory drugs (NSAID), specifically meloxicam (*n* = 173) and robenacoxib (*n* = 3), with a PD for NSAID very small (0.03). Meloxicam was more commonly administered parenterally (79.2%) than orally (20.8%) and more frequently used in birds (85.5%) than mammals (14.5%). As for robenacoxib, this drug was administered only in mammals and by the parenteral route.

Group QJ01 *Antibacterials for systemic use* were used only in 12.5% of treatments, with a PD of 0.58. The most prescribed therapeutic subgroup was QJ01M *Quinolone and quinoxaline antibacterials* (Table 5), with marbofloxacin as the most used compound, followed by metronidazole.

According to the EMA Categorization of antibiotics for prudent and responsible use in animals (38), only 1 (0.7%) of the active ingredients administered were classified as Avoid; 103 (72.0%) as Restrict; 9 (6.3%) as Caution; and 30 (21.0%) as Prudence. Most antibiotics (*n* = 90; 62.9%) were administered as parenteral treatments, followed by oral (*n* = 43; 30.1%) and local treatments (*n* = 10; 7.0%). The PD for all antibiotics was 0.63. Figure 1 shows the antibiotic prescriptions according to the EMA categorization and year of study. Altogether, 2.7 grams of antibacterials for systemic use was documented for exotic animals over the 5-year period (Table 6), with clear differences between birds and mammals. The quinolone and quinoxaline subgroup was the most prescribed one in both animal classes.

Table 7 summarizes the results of the multivariate analysis, identifying those variables associated with antibiotic prescription patterns in exotic animals. The need for hospitalization, being a mammal, non-emergency visits, and any condition different from digestive or musculoskeletal disorders had a significant impact on antibiotic prescription.

Furthermore, when comparing treatments with and without antibiotics, the likelihood of an antibiotic being administered parenterally was 1.8 times higher (OR = 1.8; 95% CI: 0.9–3.7; $p = 0.119$) than topically. The same happened for the oral route, which was 5.2 times higher (OR = 5.2; 95% CI: 2.4–11.5; $p < 0.001$) to be used than the topical one. Finally, if only the two main routes of administration were considered (oral and parenteral), antibiotics were 2.9 times more likely to be administered by the oral route than parenterally (OR = 2.9; 95% CI: 1.9–4.6; $p < 0.001$).

TABLE 4 Anatomical groups (first-level ATCvet) prescribed among exotic animals.

ATCvet anatomical group	Frequency (%) (n = 1,069)	95% CI
Group QA Alimentary tract and metabolism	10 (0.9)	0.004–0.015
Group QB Blood and blood-forming organs	196 (18.4)	0.160–0.207
Group QC Cardiovascular system	2 (0.2)	0.000–0.004
Group QD Dermatologicals	8 (0.7)	0.002–0.013
Group QG Genito urinary system and sex hormones	2 (0.2)	0.000–0.004
Group QH Systemic hormonal preparations, excluding sex hormones and insulins	13 (1.2)	0.006–0.019
Group QJ Antiinfectives for systemic use	133 (12.5)	0.105–0.144
Group QM Musculoskeletal system	176 (16.5)	0.142–0.187
Group QN Nervous system	497 (46.5)	0.435–0.495
Group QP Antiparasitic products, insecticides, and repellents	17 (1.6)	0.008–0.023
Group QS Sensory organs	4 (0.4)	0.000–0.007
ATC anatomical group		
Group S Sensory organs	11 (1.0)	0.004–0.016

CI, Confidence interval.

TABLE 5 J01 therapeutic subgroups (third-level group ATCvet) prescribed among exotic animals.

ATCvet therapeutic subgroup J01	Frequency (%) (n = 133)	95% CI
Subgroup Q01C Beta-lactam antibacterials, penicillins	3 (2.3)	0.000–0.048
Subgroup Q01D Other beta-lactam antibacterials	4 (3.0)	0.001–0.059
Subgroup Q01E Sulfonamides and trimethoprim	13 (9.8)	0.047–0.148
Subgroup Q01F Macrolides, lincosamides and streptogramins	3 (2.3)	0.000–0.048
Subgroup Q01M Quinolone and quinoxaline antibacterials	94 (70.7)	0.638–0.791
Subgroup Q01X Other antibacterials	16 (12.0)	0.065–0.176

CI, Confidence interval.

4 Discussion

To our knowledge, this is the first study which describes the pattern prescription in exotic pets and wildlife animals in a Veterinary Teaching Hospital over a long period of study. Most of the studies found in the literature are surveys (36, 41), and others are focussed on all types of pets (dogs, cats, and exotics) (40, 42).

As shown before, birds were the largest group of exotic animals attending the hospital over the study period. In Europe, 15.7% of pets are birds; 9.5%, small mammals; and 3.7%, reptiles. Italy, Turkey, and Spain have the largest populations of ornamental birds; Russia, Germany, and France of small mammals and France and Spain of reptiles (5). In Spanish households, the dog is the preferred pet (21.9%) followed by the cat (8.2%). However, the presence of other pets, mainly birds and fish, has increased considerably in the last years (43). In addition, according to a survey conducted at the University of Dublin with exotic pet veterinarians, birds occupied the largest number of their visits (96.4%), followed by small mammals (89.3%) and reptiles (78.6%) (44). Dogs and cats visited the veterinary at least once a year (45, 46). In contrast, only 50% of exotic pet owners said that they have visited a veterinary clinic in 2019 (47).

We have observed that birds went to the hospital for an emergency much more than small mammals. According to veterinary professionals, the percentage of emergencies seen daily is much higher in exotic animals than in dogs and cats (2). Several reasons may explain these differences. In this sense, these species may mask their illness until it is well advanced, and probably owners do not spend as much time observing their pets. In addition, they have specific environmental requirements, and their behavioral patterns of pain or illness are not still well understood (2).

Related to hospitalization, it should be taken into account that exotic animals usually visit the veterinarian when pathologies are much more advanced than in other domestic animals. This may be related to the lack of experience with these species, the equipment, or diagnostic tests specifically addressed to other animal species. In addition, the economic constraints of owners (44) for treatment or the lack of authorized medicines for these animals should be considered. In these situations, with very advanced pathological disorders and unfavorable prognosis, euthanasia must be done to reduce the animal's suffering (48). It is important to perform this procedure using techniques that minimize stress (48). For this purpose, in 2013 the AVMA published guidelines for the euthanasia of exotic species (48). Acceptable methods of euthanasia include intravenous administration if performed without causing fear or distress. They recommend intramuscular deep sedation or prior anesthesia in these species, as carried out in the HVULE. Some drugs included in the AVMA guideline are xylazine, opioid analgesics, dexmedetomidine (used for small mammals in the HVULE), alfaxalone, or midazolam, among others (48). Once the animal is unconscious, euthanasia solutions are administered intravenously (route of administration used in small mammals in the HVULE), intraosseous, intracardiac, or intrathoracic (the latter two used in birds in this hospital) (48). Euthanasia rates

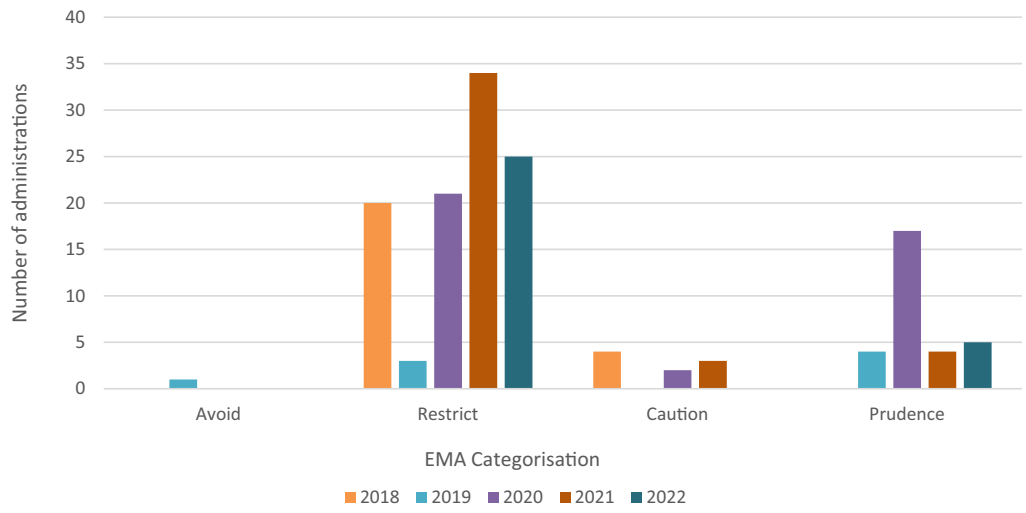


FIGURE 1
Antibiotic prescription according to the European Medicines Agency (EMA) categorization and year of study.

were relatively higher (34.0%) compared to companion animals, such as dogs (1%), cats (2%), or rabbits (4%) (43), but more similar to values observed in backyard poultry (29.9%) (49). These authors related their euthanasia rates to the increased severity of the disorders in these animals (49).

Regarding pharmacological treatments, the most frequent group used was QN, with pentobarbital and isoflurane as the most often prescribed active ingredients, as one-third of the animals assessed in this study were euthanised. Opioids were also used quite frequently. Veterinarians prescribe and administer an important amount of opioid analgesics to treat pain in animals (50). Butorphanol is a synthetic opioid indicated for the treatment of acute pain in birds and small mammals (51, 52), recommended in birds as preoperative and postoperative analgesic medication (51–56), and for mild pain and short-term analgesia in small mammals (57, 58). As for buprenorphine, it is commonly administered in veterinary medicine for analgesia and sedation, although little information is available on its use in exotic animals. It is not recommended for pain control in reptiles, but used in birds, whereas in small mammals there is more research providing evidence of its analgesic and sedative effects (59).

The second most used group was QB (blood and blood-forming organs), in certain solutions affecting the electrolyte balance, which is in accordance with the fact that half of our patients were hospitalized and received fluid therapy. Fluid administration is essential to treat many medical conditions or provide support therapy to patients. Regarding routes of administration, subcutaneous fluids are a valid treatment of choice for hypovolemic avian and reptile patients. In particular, electrolyte solutions are an option for the majority of exotic animal fluid treatment needs (60).

Within the QM group, meloxicam was used quite often as a high number of patients had musculoskeletal disorders. This drug is commonly prescribed off-label for pain and inflammation in many exotic and zoo animals, including reptiles and birds (61). Nowadays, it is the current drug of choice due to its widespread use and low incidence of reported toxicity in exotic animal practices. In a study in which this drug was administered to over 700 captive birds (60 different species) in zoos, no mortality was observed (62). In addition, several studies reported no significant renal, gastrointestinal, or haemostatic adverse events at the doses evaluated (63–66). Vultures are more sensitive to the renal adverse effects of several NSAIDs, except for meloxicam (67). The preference for this NSAID is also reflected in the low PD of our veterinary hospital for the population assessed.

Inappropriate use of antimicrobials is a potential threat to public health (34). Multidrug-resistant bacteria have been reported in several exotic animal species, such as methicillin-resistant staphylococci in rabbits and birds, extended-spectrum β-lactamase-producing enterobacteria in turtles and wild birds, or resistant *Escherichia coli* in wild birds (28, 68–72). Specifically in the Iberian Peninsula, *Pseudomonas* spp. showed the highest levels of resistance among birds, mammals, and reptiles, and multidrug resistance was also significant in *Enterobacterales* (73). In 2015, WHO adopted a global action plan to address antimicrobial resistance and proposed to ban the prophylactic use of antimicrobials in healthy animals (74). To this end, the EU has significantly restricted the prophylactic use of antimicrobials (32). Moreover, since 2014, the EMA has categorized antibiotics for veterinary use taking into account the public health risk of their use in animals due to the potential development of resistance, as well as the need for their use (38).

For those antibiotics belonging to category B (Restrict), we found that marbofloxacin was the most widely used in the HVULE. Fluoroquinolones are frequently used in birds, mainly due to their wide safety margin. However, injectable solutions are highly irritant and may cause tissue necrosis (75). In addition, some studies have recognized that fluoroquinolones have detrimental effects on reproduction in scavenging birds (76). In general, these antibiotics that reduce hatchability and total egg hatch, and cause joint deformities in chicks, increased embryonic heart rate and biochemical signs of stress (77). Since the 1980s, the livestock industry has been using fluoroquinolones as antimicrobials in food-producing animals (78, 79). A 1998–2001 survey of UK veterinarians on dermatological treatments showed that the most commonly used antibiotics in dogs, cats, and exotic animals were cefalexin, amoxicillin, and enrofloxacin (41). In another study carried out in the UK in 2016, fluoroquinolones were the most usually prescribed antibiotic group (49%) in rabbits and third-generation cephalosporins (36%) in cats and amoxicillin with clavulanic acid (29%) in dogs (40). In Germany, amoxicillin with

TABLE 6 Amount of QJ01 Antibacterials for systemic use; QM01A Antiinflammatory and antirheumatic products, non-steroids, and QN02A Opioids prescribed in exotic animals.

	Birds amount in grams	Mammals amount in grams	Total amounts in grams (%)
QJ01 Antibacterials for systemic use			
Subgroup Q01C Beta-lactam antibacterials, penicillins			0.0936 (3.5)
Amoxicillin	–	0.0089	0.0089 (0.3)
Amoxicillin and beta-lactamase inhibitor	–	0.0029	0.0029 (0.1)
Piperacillin and beta-lactamase inhibitor	–	–	0.0820 (3.0)*
Subgroup Q01D Other beta-lactam antibacterials			0.1368 (5.0)
Cefovecin	–	0.1398	0.1368 (5.0)
Subgroup Q01E Sulfonamides and trimethoprim			0.3505 (12.9)
Trimethoprim	–	0.1054	0.1054 (3.9)
Sulfamethoxazole and trimethoprim	0.1980	0.0472	0.2452 (9.0)
Subgroup Q01F Macrolides, lincosamides, and streptogramins			0.0606 (2.2)
Tilmicosin	–	0.0145	0.0145 (0.5)
Clindamycin	0.0461	–	0.0461 (1.7)
Subgroup Q01M Quinolone and quinoxaline antibacterials			1.4679 (54.2)
Enrofloxacin	–	0.1169	0.1169 (4.3)
Marbofloxacin	1.2361	0.1147	1.3509 (49.8)
Subgroup Q01X Other antibacterials			0.6007 (22.2)
Metronidazole	0.4296	0.1711	0.6007 (22.2)
Total	1.9098	0.7181	2.7100 (100)
QM01A Antiinflammatory and antirheumatic products, non-steroids			
Meloxicam	0.1092	0.0081	0.1173 (96.6)
Celecoxib	–	0.0041	0.0041 (3.4)
Total	0.1092	0.0122	0.1214 (100)
QN02A Opioids			
Buprenorphine	0.4298	0.0188	0.4486 (61.5)
Tramadol	0.2535	0.0206	0.2741 (37.5)
Methadone	–	0.0052	0.0052 (0.7)
Butorphanol	0.0021	–	0.0021 (0.3)
Total	0.6854	0.0446	0.7300 (100)

* Prescribed to a chelonian.

TABLE 7 Multivariate ordinal logistic regression analysis of factors relevant to the antibiotic prescription pattern.

Variables	OR (95% CI)	*p*-value
Hospital visit (no emergency)	2.190 (1.469–3.261)	<0.001
Animal class (mammals)	2.075 (1.385–3.109)	<0.001
Organ affected (Others vs. musculoskeletal)	2.081 (1.379–3.135)	<0.001
Organ affected (Others vs. digestive)	2.452 (1.462–4.115)	0.001
Hospitalization	2.477 (1.642–3.732)	<0.001
Year	1.121 (0.969–1.310)	0.121

CI, Confidence interval; OR, Odds ratio.

clavulanic acid was the most prescribed drug in dogs (47.89%) and cats (48.15%) in 2017–18 (42). In contrast, in backyard poultry, the most prescribed antibiotic was enrofloxacin (40.6%), followed by tylosin (19.5%) and amoxicillin with clavulanic acid (12.1%). Furthermore, 92.4% of those antibiotics prescribed for chickens were for systemic administration (oral or injectable) and only 5.2% for topical administration (49). Another survey among Swiss veterinarians on antibiotics usage in exotic pets showed that fluoroquinolones were the most commonly prescribed drugs in rabbits (82%), rodents (86%), birds (83%), and reptiles (97%) (36).

We have observed that antibiotics from category A (Avoid), which should be given to companion animals under exceptional circumstances, were used only once at the HVULE, whereas those belonging to category B (Restrict), whose use should be restricted in animals due to their critical importance in human medicine, were prescribed in the hospital quite frequently. However, antibiotics belonging to category C (Caution), which should be used only when there are no effective category D (Prudence) antimicrobials, are minimal. Finally, although category D is the first group of choice, their use is not very high in our study. The prescription of antimicrobials classified in EMA categories A and B should be based on antimicrobial susceptibility testing whenever possible

(38). Some countries, such as the Netherlands (80), Belgium (81), and Denmark (82), have national legislation that requires susceptibility testing to verify that no other antibiotics in categories C or D are clinically effective before an antimicrobial belonging to fluoroquinolones or third−/fourth-generation cephalosporins can be prescribed, and it is also the case in Spain (33).

As for the PD of antibiotics, our value was 0.63, which is much lower than those reported in 2016 for dogs (0.83) and cats (0.75), and very similar to that determined for rabbits (0.64) in the UK (40). In 2018, the prevalence of PD data increased in Germany for dogs and cats to 0.93 and 0.88, respectively (42). Our data may reflect that evidence-based guidelines for the treatment of infections, including antimicrobial stewardship, are not yet available for exotic animals. In the absence of appropriate veterinary medicines, common practice is based on personal experience of successful strategies implemented by individual veterinarians. As a result, off-label use of critical antimicrobials such as fluoroquinolones, aminopenicillins, third-generation cephalosporins, or macrolides is frequent in exotic pets, which underscores the need for guidance for prudent antibiotic use. The positive influence of these recommendations has already been demonstrated in dogs and cats (34).

Potential limitations of this study include the representativeness of the sample, and results may not be generalisable to other clinics or veterinary hospitals. Although significant efforts were made to identify animal species, demographic characteristics, clinical signs, and pharmaceutical prescriptions using manual and semi-automated methodologies, unclear or missing descriptions may have been overlooked by the authors. Additionally, the COVID-19 pandemic occurred during the period of study and may have had an impact on hospital visits. However, despite these limitations, our findings may help raise further discussions on the prudent use of several pharmacological groups in these types of animals, which are becoming more and more popular among the population.

This is the first study that describes the actual consumption of drugs in exotic pets and wild animals at a Veterinary Teaching Hospital. We have observed that the number of exotic animals increased over time, and they were mainly birds who went to the hospital for an emergency related to the QM group, and one-third were euthanised. As for medicines, almost half belonged to the QN group, with pentobarbital as the most used drug, followed by isoflurane and meloxicam. Regarding antibiotics, marbofloxacin (Category B—Restrict) was the most prescribed one.

Our study provides an insight into the prescription patterns in exotic animals. The findings of the study provide sufficient data to veterinary policymakers and education aimed at improving drug use practices in general and antimicrobial use, in particular in the profession.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Data used in this study are based on these generated for accounting and documentation purposes. Our research does not involve any regulated animals, and no scientific procedures of any kind were performed on animals. For this reason, formal approval by an ethics committee was not necessary under the provisions of the Spanish regulations.

Author contributions

BR: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. JS: Formal Analysis, Writing – review & editing. AS: Data curation, Writing – review & editing. NF: Data curation, Writing – review & editing. CL: Project administration, Writing – review & editing. RP: Resources, Writing – review & editing. JA: Writing – review & editing. RD: Conceptualization, Methodology, Project administration, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Mitchell M, Tully T. History of exotic pets In: *Manual of exotic Pet practice*. St Louis: Elsevier Inc. (2009). 1–3.

2. Brown SA, Nye RR. Essentials of the exotic Pet practice. *J Exot Pet Med*. (2006) 15:225–33. doi: 10.1053/j.jepm.2006.06.009

3. Boletín Oficial del Estado. Real Decreto 630/2013, de 2 de agosto, por el que se regula el Catálogo español de especies exóticas invasoras. *España* (2013). Available at: https://www.boe.es/buscar/act.php?id=BOE-A-2013-8565 (Accessed October 2, 2023).

4. American Veterinary Medical Association. U.S. pet ownership statistics. (2019) Available at: https://www.avma.org/resources-tools/reports-statistics/us-pet-ownership-statistics (Accessed October 12, 2023).

5. FEDIAF European Pet Food. Report the European Pet Food industry 2019. (2020). Available at: https://www.fecava.org/news-and-events/news/fediaf-european-facts-figures-2019/ (Accessed October 12, 2023).

6. Asociación Nacional de Fabricantes de Alimentos para Animales de Compañía. (2021). Available at: https://www.anfaac.org/inicio/ (Accessed October 12, 2023).

7. Boletín Oficial del Estado. Ley 7/2023, de 28 de marzo, de protección de los derechos y el bienestar de los animales. *España* (2023). Available at: https://www.boe.es/buscar/doc.php?id=BOE-A-2023-7936 (Accessed October 6, 2023).

8. Di Girolamo N, Caron M, Brandao J, Reynders RM. Most veterinarians treating exotic animals use formularies to select drug dosages without consistently checking their sources. *J Am Vet Med Assoc*. (2022) 260:1–11. doi: 10.2460/javma.21.03.0116

9. Powers LV, Davidson G. Compounding and Extralabel use of drugs in exotic animal medicine. *Vet Clin North Am Exot Anim Pract*. (2018) 21:195–214. doi: 10.1016/j.cvex.2018.01.005

10. Velazquez-Meza ME, Galarde-López M, Carrillo-Quiróz B, Alpuche-Aranda CM. Antimicrobial resistance: one health approach. *Vet World*. (2022) 15:743–9. doi: 10.14202/vetworld.2022.743-749

11. Kaplan B, Kahn LH, Monath TP. The brewing storm. *Vet Ital*. (2009) 45:9–18.

12. One Health Initiative. One health initiative will unite human and veterinary medicine. (2023). Available at: https://onehealthinitiative.com/ (Accessed October 12, 2023).

13. Souza MJ. One health: Zoonoses in the exotic animal practice. *Vet Clin North Am Exot Anim Pract*. (2011) 14:421–6. doi: 10.1016/j.cvex.2011.05.007

14. Consejo General de Colegios Farmacéuticos. One Health: los medicamentos veterinarios. (2022). Available at: https://www.farmaceuticos.com/wp-content/uploads/2022/07/Punto-Farmacologico-162-Medicamentos-veterinarios.pdf (Accessed September 26, 2023).

15. Chomel BB. Diseases transmitted by less common house pets. *Microbiol Spectr*. (2015) 3:IOL5-0012-2015. doi: 10.1128/microbiolspec.iol5-0012-2015

16. Escher M, Vanni M, Intorre L, Caprioli A, Tognetti R, Scavia G. Use of antimicrobials in companion animal practice: a retrospective study in a veterinary teaching hospital in Italy. *J Antimicrob Chemother*. (2011) 66:920–7. doi: 10.1093/jac/dkq543

17. Sarrazin S, Vandael F, Van Cleven A, De Graef E, de Rooster H, Dewulf J. The impact of antimicrobial use guidelines on prescription habits in fourteen Flemish small animal practices. *Vlaams Diergeneeskd Tijdschr*. (2017) 86:173–82. doi: 10.21825/vdt.v86i3.16287

18. Mateus A, Brodbelt DC, Barber N, Stärk KDC. Antimicrobial usage in dogs and cats in first opinion veterinary practices in the UK. *J Small Anim Pract*. (2011) 52:515–21. doi: 10.1111/j.1748-5827.2011.01098.x

19. Singleton DA, Sánchez-Vizcaíno F, Dawson S, Jones PH, Noble PJM, Pinchbeck GL, et al. Patterns of antimicrobial agent prescription in a sentinel population of canine and feline veterinary practices in the United Kingdom. *Vet J*. (2017) 224:18–24. doi: 10.1016/j.tvjl.2017.03.010

20. Hopman NEM, Van Dijk MAM, Broens EM, Wagenaar JA, Heederik DJJ, Van Geijlswijk IM. Quantifying antimicrobial use in Dutch companion animals. *Front Vet Sci*. (2019) 6:158. doi: 10.3389/fvets.2019.00158

21. De Graef E, Decostere A, Devriese L, Haesebrouck F. Antibiotic resistance among fecal Indicator Bacteria from healthy individually owned and kennel dogs. *Microb Drug Resist*. (2004) 10:65–9. doi: 10.1089/107662904323047826

22. Rhys-Davies L, Ogden J. Vets' and Pet owners' views about antibiotics for companion animals and the use of phages as an alternative. *Front Vet Sci*. (2020) 7:513770. doi: 10.3389/fvets.2020.513770

23. Tompson AC, Mateus ALP, Brodbelt DC, Chandler CIR. Understanding antibiotic use in companion animals: a literature review identifying avenues for future efforts. *Front Vet Sci*. (2021) 8:719547. doi: 10.3389/fvets.2021.719547

24. Lloyd DH. Reservoirs of antimicrobial resistance in pet animals. *Clin Infect Dis*. (2007) 45:S148–52. doi: 10.1086/519254

25. Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. *J Antimicrob Chemother*. (2004) 54:321–32. doi: 10.1093/jac/dkh332

26. Scott WJ. Antimicrobial resistance in companion animals. *Anim Health Res Rev*. (2008) 9:169–76. doi: 10.1017/S1466252308001485

27. Prescott J. Antimicrobial use in food and companion animals. *Anim Health Res Rev*. (2008) 9:127–33. doi: 10.1017/S1466252308001473

28. Prandi I, Bellato A, Nebbia P, Stella MC, Ala U, von Degerfeld MM, et al. Antibiotic resistant *Escherichia coli* in wild birds hospitalised in a wildlife rescue Centre. *Comp Immunol Microbiol Infect Dis*. (2023) 93:101945. doi: 10.1016/j.cimid.2023.101945

29. World Health Organization. *The rational use of drugs and WHO*. Nairobi: Dev Dialogue (1985). p. 1–4. Available at: https://pubmed.ncbi.nlm.nih.gov/12341043/ (Accessed November 28, 2023).

30. World Health Organization. How to investigate drug use in health facilities: selected drug use indicators. (1993). Available at: https://iris.who.int/bitstream/handle/10665/60519/WHO_DAP_93.1.pdf?sequence=1 (Accessed September 1, 2023).

31. Laing RO, Hogerzeil HV, Ross-Degnan D. Ten recommendations to improve use of medicines in developing countries. *Health Policy Plan*. (2001) 16:13–20. doi: 10.1093/heapol/16.1.13

32. Europea Unión. Reglamento (UE) 2019/6 del Parlamento Europeo y del Consejo, de 11 de diciembre de 2018, sobre medicamentos veterinarios y por el que se deroga la Directiva 2001/82/CE. (2019). Available at: https://www.boe.es/buscar/doc.php?id=DOUE-L-2019-80010 (Accessed September 7, 2023).

33. Boletín Oficial del Estado. Real Decreto 666/2023, de 18 de julio, por el que se regula la distribución, prescripción, dispensación y uso de medicamentos veterinarios. *España* (2023). Available at: https://www.boe.es/buscar/doc.php?id=BOE-A-2023-16727 (Accessed July 14, 2023).

34. Broens EM, Van Geijlswijk IM. Prudent use of antimicrobials in exotic animal medicine. *Vet Clin North Am Exot Anim Pract*. (2018) 21:341–53. doi: 10.1016/j.cvex.2018.01.014

35. Zehnder A, Graham J, Antonissen G. Update on Cancer treatment in exotics. *Vet Clin North Am Exot Anim Pract*. (2018) 21:465–509. doi: 10.1016/j.cvex.2018.01.012

36. Hösli M, Overesch G, Willi B, Heim D, Hatt J-M. Survey on the use of antibiotics in exotic pets among Swiss veterinarians [article in German]. *Schweiz Arch Tierheilkd*. (2021) 163:227–37. doi: 10.48350/163644

37. World Health Organization Collaborating Centre for Drug Statistics Methodology. Guidelines for ATCvet classification 2023. (2023). Available at: https://www.whocc.no/filearchive/publications/2023_atcvet_guidelines_web.pdf (Accessed November 27, 2023).

38. European Medicines Agency. Categorisation of antibiotics in the European Union. (2019). Available at: https://www.ema.europa.eu/en/news/categorisation-antibiotics-used-animals-promotes-responsible-use-protect-public-animal-health (Accessed July 6, 2023).

39. O'Connor AM, Sargeant JM, Dohoo IR, Erb HN, Cevallos M, Egger M, et al. Explanation and elaboration document for the STROBE-vet statement: strengthening the reporting of observational studies in epidemiology-veterinary extension. *J Vet Intern Med*. (2016) 30:1896–928. doi: 10.1111/jvim.14592

40. Singleton DA, Sánchez-Vizcaíno F, Arsevska E, Dawson S, Jones PH, Noble PJM, et al. New approaches to pharmacosurveillance for monitoring prescription frequency, diversity, and co-prescription in a large sentinel network of companion animal veterinary practices in the United Kingdom, 2014–2016. *Prev Vet Med*. (2018) 159:153–61. doi: 10.1016/j.prevetmed.2018.09.004

41. Hill PB, Lo A, Eden CAN, Huntley S, Morey V, Ramsey S, et al. Survey of the prevalence, diagnosis and treatment of dermatological conditions in small animals in general practice. *Vet Record*. (2006) 158:533–9. doi: 10.1136/vr.158.16.533

42. Schnepf A, Kramer S, Wagels R, Volk HA, Kreienbrock L. Evaluation of antimicrobial usage in dogs and cats at a veterinary teaching Hospital in Germany in 2017 and 2018. *Front Vet Sci*. (2021) 8:689018. doi: 10.3389/fvets.2021.689018

43. de Agricultura Ministerio, Pesca, y Alimentación. Análisis y caracterización del sector de los animales de compañía-Informe de resultados. (2015). Available at: https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/20160222_informeestudioparapublicar_tcm30-104720.pdf (Accessed July 24, 2023).

44. Goins M, Hanlon AJ. Exotic pets in Ireland: 2. Provision of veterinary services and perspectives of veterinary professionals' on responsible ownership. *Ir Vet J*. (2021) 74:13. doi: 10.1186/s13620-021-00191-5

45. Day MJ, Horzinek MC, Schultz RD, Squires RA. WSAVA guidelines for the vaccination of dogs and cats. *J Small Anim Pract*. (2016) 57:E1–E45. doi: 10.1111/jsap.2_12431

46. Anvik JO. Acetaminophen Toxicosis in a cat. *Can Vet J*. (1984) 25:445–7.

47. Goins M, Hanlon AJ. Exotic pets in Ireland: 1. Prevalence of ownership and access to veterinary services. *Ir Vet J*. (2021) 74:14. doi: 10.1186/s13620-021-00190-6

48. American Veterinary Medical Association. AVMA guidelines for the euthanasia of animals. (2020). p. 1–121. Available at: https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf (Accessed July 18, 2023).

49. Singleton DA, Ball C, Rennie C, Coxon C, Ganapathy K, Jones PH, et al. Backyard poultry cases in UK small animal practices: demographics, health conditions and pharmaceutical prescriptions. *Vet Record*. (2021) 188:e71–e282. doi: 10.1002/vetr.71

50. Clarke DL, Drobatz KJ, Korzekwa C, Nelson LS, Perrone J. Trends in opioid prescribing and dispensing by veterinarians in Pennsylvania. *JAMA Netw Open*. (2019) 2:e186950. doi: 10.1001/jamanetworkopen.2018.6950

51. Pollock C. Postoperative management of the exotic animal patient. *Vet Clin North Am Exot Anim Pract*. (2002) 5:183–212. doi: 10.1016/S1094-9194(03)00053-7

52. Hawkins MG. The use of analgesics in birds, reptiles, and small exotic mammals. *J Exot Pet Med*. (2006) 15:177–92. doi: 10.1053/j.jepm.2006.06.004

53. Sladky K, Krugner-Higby L, Meek-Walker E, Heath T. Serum concentrations and analgesic effects of liposome-encapsulated and standard butorphanol tartrate in parrots. *Am J Vet Res*. (2006) 67:775–81. doi: 10.2460/ajvr.67.5.775

54. Paul-Murphy JR, Krugner-Higby LA, Tourdot RL, Sladky KK, Klauer JM, Keuler NS, et al. Evaluation of liposome-encapsulated butorphanol tartrate for alleviation of experimentally induced arthritic pain in green-cheeked conures (*Pyrrhura molinae*). *Am J Vet Res*. (2009) 70:1211–9. doi: 10.2460/ajvr.70.10.1211

55. Riggs S, Hawkins M, Graigmill A, Kass P. Pharmacokinetics of butorphanol tartrate in red-tailed hawks (*Buteo jamaicensis*) and great horned owls (*Bubo virginianus*). *Am J Vet Res*. (2008) 69:596–603. doi: 10.2460/ajvr.69.5.596

56. Hawkins M, Sanchez-Migallon D, Beaufrère H, Lennox A, Carpenter J. Chapter 5:birds In: JW Carpenter, editor. *Exotic Anima Formulary* (2018). 167–375.

57. Sharp J, Zammit T, Azar T, Lawson D. Recovery of male rats from major abdominal surgery after treatment with various analgesics. *Contemp Top Lab Anim Sci*. (2003) 42:22–7.

58. Garner H, Burke T, Lawhotn D, Storner J, Wssinger W. Butorphanol-mediated antinociception in mice: partial agonist effects and mu receptor involvement. *J Pharmacol Exp Ther*. (1997) 282:1253–61.

59. Rockwell K. Buprenorphine. *J Exot Pet Med*. (2019) 30:12–6. doi: 10.1053/j.jepm.2018.10.002

60. Parkinson L. Fluid therapy in exotic animal emergency and critical care. *Vet Clin North Am Exot Anim Pract*. (2023) 26:623–45. doi: 10.1016/j.cvex.2023.05.004

61. Papich MG. Papich handbook of veterinary drugs. 5 (2020). Available at: https://www.sciencedirect.com/book/9780323709576/papich-handbook-of-veterinary-drugs (Accessed July 13, 2023).

62. Cuthbert R, Parry-Jones J, Green RE, Pain DJ. NSAIDs and scavenging birds: potential impacts beyond Asia's critically endangered vultures. *Biol Lett*. (2006) 3:90–3. doi: 10.1098/rsbl.2006.0554

63. Desmarchelier M, Troncy E, Fitzgerald G, Lair S. Analgesic effects of meloxicam administration on postoperative orthopedic pain in domestic pigeons (*Columba livia*). *Am J Vet Res*. (2012) 73:361–7. doi: 10.2460/ajvr.73.3.361

64. Pereira M, Werther K. Evaluation of the renal effects of flunixin meglumine, ketoprofen and meloxicam in budgerigars (*Melopsittacus undulatus*). *Vet Rec*. (2007) 160:844–6. doi: 10.1136/vr.160.24.844

65. Montesinos A, Ardiaca M, Juan-Sallés C, Tesouro MA. Effects of meloxicam on hematologic and plasma biochemical analyte values and results of histologic examination of kidney biopsy specimens of African grey parrots (*Psittacus erithacus*). *J Avian Med Surg*. (2015) 29:1–8. doi: 10.1647/2013-056

66. Sinclair KM, Church ME, Farver TB, Lowenstine LJ, Owens SD, Paul-Murphy J. Effects of meloxicam on hematologic and plasma biochemical analysis variables and results of histologic examination of tissue specimens of Japanese quail (*Coturnix japonica*). *Am J Vet Res*. (2012) 73:1720–7. doi: 10.2460/ajvr.73.11.1720

67. Swarup D, Patra RC, Prakash V, Cuthbert R, Das D, Avari P, et al. Safety of meloxicam to critically endangered Gyps vultures and other scavenging birds in India. *Anim Conserv*. (2007) 10:192–8. doi: 10.1111/j.1469-1795.2006.00086.x

68. Briscoe JA, Morris DO, Rankin SC, Hendrick MJ, Rosenthal KL. Methicillin-resistant *Staphylococcus aureus*–associated dermatitis in a Congo African grey parrot (*Psittacus erithacus erithacus*). *J Avian Med Surg*. (2008) 22:336–43. doi: 10.1647/2008-014.1

69. Loncaric I, Künzel F. Sequence type 398 meticillin-resistant *Staphylococcus aureus* infection in a pet rabbit. *Vet Dermatol*. (2013) 24:370–2. doi: 10.1111/vde.12023

70. Sousa M, Silva N, Igrejas G, Sargo R, Benito D, Gómez P, et al. Genetic diversity and antibiotic resistance among coagulase-negative staphylococci recovered from birds of prey in Portugal. *Microb Drug Resist*. (2016) 22:727–30. doi: 10.1089/mdr.2015.0266

71. Yilmaz EŞ, Dolar A. Detection of extended-spectrum β-lactamases in *Escherichia coli* from cage birds. *J Exot Pet Med*. (2017) 26:13–8. doi: 10.1053/j.jepm.2016.10.008

72. Cortés-Cortés G, Lozano-Zarain P, Torres C, Castañeda M, Moreno Sánchez G, Alonso CA, et al. Detection and molecular characterization of *Escherichia coli* strains producers of extended-spectrum and CMY-2 type beta-lactamases, isolated from turtles in Mexico. *Vector Borne Zoonotic Dis*. (2016) 16:595–603. doi: 10.1089/vbz.2014.1725

73. Muñoz-Ibarra E, Molina-López RA, Durán I, Garcias B, Martín M, Darwich L. Antimicrobial resistance in bacteria isolated from exotic pets: the situation in the Iberian Peninsula. *Animals*. (2022) 12:1912. doi: 10.3390/ani12151912

74. World Health Organization. Global action plan on antimicrobial resistance. *Sixty-Eight World Health Assembly*. (2015). Available at: https://www.emro.who.int/health-topics/drug-resistance/global-action-plan.html (Accessed September 9, 2023).

75. Yarto E. Farmacología en animales exóticos: ¿qué podemos usar y qué no? Congreso LAVECCS. *Punta del Este* (2017). Available at: https://docplayer.es/54106620-Farmacologia-en-animales-exoticos-que-podemos-usar-y-que-no-10mo-congreso-laveccs-punta-del-este-uruguay-2017.html (Accessed September 21, 2023).

76. Lemus JÁ, Blanco G, Arroyo B, Martínez F, Grande J. Fatal embryo chondral damage associated with fluoroquinolones in eggs of threatened avian scavengers. *Environ Pollut*. (2009) 157:2421–7. doi: 10.1016/j.envpol.2009.03.013

77. Hruba H, Abdelsalam EEE, Anisimov N, Bandouchova H, Havelkova B, Heger T, et al. Reproductive toxicity of fluoroquinolones in birds. *Vet Res*. (2019) 15:209. doi: 10.1186/s12917-019-1957-y

78. Brown SA. Fluoroquinolones in animal health. *J Vet Pharmacol Ther*. (1996) 19:1–14. doi: 10.1111/j.1365-2885.1996.tb00001.x

79. Martinez M, McDermott P, Walker R. Pharmacology of the fluoroquinolones: a perspective for the use in domestic animals. *Vet J*. (2006) 172:10–28. doi: 10.1016/j.tvjl.2005.07.010

80. Ministry of Health, Welfare, and Sport. Animal health register. The Netherlands (2023). Available at: https://wetten.overheid.nl/BWBR0035238/2023-01-01/#Hoofdstuk5_Paragraaf2_Artikel5.8 (Accessed October 18, 2023).

81. Staatsblad Belgisch. Koninklijk besluit betreffende de voorwaarden voor het gebruik van geneesmiddelen door de dierenartsen en door de verantwoordelijken van de dieren. (2016). Available at: https://etaamb.openjustice.be/nl/koninklijk-besluit-van-21-juli-2016_n2016024152.html (Accessed October 18, 2023).

82. Ministry of Food, Agriculture and, Fisheries of Denmark., Danish Veterinary and, Food Administration. Distribution and use of veterinary medicinal products. (2019). Available at: https://en.foedevarestyrelsen.dk/animals/animal-health-/veterinary-medicine/distribution-and-use-of-veterinary-medicinal-products-(Accessed October 11, 2023).

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 January 2024
DOI 10.3389/fvets.2024.1332974

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Raquel Díez Laiz,
University of León, Spain
Muammer Elmas,
Selcuk University, Türkiye

*CORRESPONDENCE
Richard Munn
✉ rmunn@anexa.co.nz

RECEIVED 04 November 2023
ACCEPTED 04 January 2024
PUBLISHED 16 January 2024

CITATION
Munn R and Whittem T (2024) Moxidectin is a candidate for use as an *in vivo* internal standard in pharmacokinetic studies, as demonstrated with use in simultaneous tissue cage and ultrafiltration fluid collection. *Front. Vet. Sci.* 11:1332974. doi: 10.3389/fvets.2024.1332974

COPYRIGHT
© 2024 Munn and Whittem. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Moxidectin is a candidate for use as an *in vivo* internal standard in pharmacokinetic studies, as demonstrated with use in simultaneous tissue cage and ultrafiltration fluid collection

Richard Munn[1,2]* and Ted Whittem[1,3]

[1]Melbourne Veterinary School, Faculty of Science, University of Melbourne, Werribee, VIC, Australia, [2]Cognosco, Anexa Veterinary Services, Morrinsville, New Zealand, [3]College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia

In vivo ultrafiltration has been used in veterinary pharmacokinetics since the early 2000's as an improvement on the tissue cage model which enables sampling of fluids from extra-circulatory compartments. Variability in analyte recovery from ultrafiltration samples, due to membrane fouling or tissue inflammation, has been a concern for this technique. Internal standards may be used to scale or verify the unknown result, such as is common in analytical extractions and *in vivo* microdialysis. Eight merino sheep were implanted with subcutaneous tissue cages and 2 weeks prior to the initiation of the study the sheep were injected with 0.2 mg/kg moxidectin subcutaneously. On the day of the study ultrafiltration probes were inserted subcutaneously. At time zero 4 mg/kg of carprofen was injected intravenously. Plasma, tissue cage, and ultrafiltration samples were taken 30 min before and 0.5, 1, 2, 3, 4, 5, 7, 24, 36, 48, 72 h after dosing. Carprofen and moxidectin concentrations were measured by LC–MS/MS. Pharmacokinetic parameters were estimated using Monolix for both the carprofen concentrations and the moxidectin corrected carprofen concentrations. The ultrafiltration probes failed to consistently produce enough sample volume to analyse. Moxidectin concentrations in the plasma and tissue cage fluid were stable throughout the 72 h sampling window. Moxidectin proved to be suitable as an *in vivo* internal standard for pharmacokinetic research using, tissue cages, plasma sampling and ultrafiltration probes, but the application of ultrafiltration techniques requires refinement.

KEYWORDS

ultrafiltration, internal standard, pharmacokinetics, moxidectin, carprofen, sheep

1 Introduction

In vivo ultrafiltration has been used in veterinary pharmacological research since at least the early 2000's (1) although adoption of the method has been slow since its early description for drug distribution studies in 1992 (2). *In vivo* ultrafiltration is viewed as an improved model for tissue fluid sampling when compared to tissue cage models (3). An ultrafiltration

probe, consisting of multiple loops of a membrane that is porous to molecules less than 30,000 Da, is inserted subcutaneously and connected to a collection vial under negative pressure (vacutainer). The collection vial is replaced at nominated timepoints to give multiple samples. The concentration of the analyte is determined and is regarded to be the average concentration of the sampling period, usually plotted as the mid-point of the sample period. This methodology was developed for glucose monitoring by Janle et al. and reported in 1987 (4).

Temperature, pressure differential, the chosen membrane material and the surface area of membrane affect the flux of analyte across the membrane (2). In addition to these factors fouling has been acknowledged to change the flux across the membrane (5). Imsilp et al. (6) reported inflammatory responses to intramuscular implantation of polyacrylonitrile ultrafiltration probes in sheep, the concern being that inflammation may change the recovery of target analytes. Bungay et al. (7), developed a model to account for the inflammation induced by the implantation of microdialysis probes, which are constructed of the same material as ultrafiltration probes, in neural tissue. The model predicted that the inflammation would lead to an underestimation of analyte concentrations. Previous uses of ultrafiltration probes for pharmacokinetic sampling have not described methods for incorporating an internal control to compensate for these changes in analyte recovery.

In contrast to ultrafiltration, microdialysis techniques use a pumped perfusate that contains a known concentration of a compound as an internal standard. This compound along with the target analyte is measured, and the change in the internal standard is used to calculate the relative recovery of target analyte. Linhares and Kissinger (8) compared ultrafiltration and microdialysis simultaneously for pharmacokinetic measurements of theophylline in a small number ($n = 3$) of rats, the concentrations obtained between the two techniques differed but the slope and shape of the concentration time graph was similar.

Ideal internal standards for microdialysis, as for other analytical techniques such as liquid chromatography, have similar physicochemical properties to the compound being studied. For microdialysis, the internal standard should diffuse through the membrane in a similar way to the compound of interest and be metabolised by similar pathways in the tissue (9). Some authors have employed radioisotopes to achieve this (10). As the internal standard is pumped into the membrane and tissue at a constant rate, in microdialysis the internal standard must have a short elimination half-life so that a steady state can be reached quickly.

A systemically administered drug which achieves steady-state concentration could provide an internal control for ultrafiltration sampling. At steady state concentrations the internal standard would be expected to be at a consistent concentration in any given tissue, thus changes in the concentration particularly decreases would indicate a potential change in the recovery of the analyte of interest in the ultrafiltrate. The relative change in the internal standard could be used to correct for the change in recovery.

Moxidectin is an antiparasitic macrocyclic lactone, commonly used in veterinary medicine for the treatment of intestinal nematodes and external parasites such as lice. In addition, it has activity against heartworm microfiliria (*Dirofilaria immitis*) in dogs. The pharmacokinetics of moxidectin in sheep have been described (11). Due to its long half-life of ~18 and 19 days in sheep and dogs, respectively, it can be dosed monthly for heartworm prevention (12) or provide residual activity against intestinal parasites such as *Haemonchus contortus*. Pseudo-steady state plasma concentrations, relative to short acting drugs, can be achieved after single or infrequent dosing. These characteristics make it a potential candidate for use an *in vivo* internal standard.

Carprofen is a non-steroidal anti-inflammatory drug (NSAID) widely used in veterinary medicine for the treatment of pain and inflammation. It has been widely used in pharmacological studies in multiple species with both tissue cage and ultrafiltration models, including description of its pharmacokinetics in sheep (3, 13–17).

In our previous work in sheep (16) simultaneous samples were collected, from five sizes of tissue cages and from blood, following carprofen dosing. The surface area to volume ratio differed between the tissue cage sizes and a model was constructed to simultaneously fit the pharmacokinetics for plasma and all tissue cages. A linear relationship was used to describe the change in microconstants for flux between the central compartment and the tissue cages. The surface area to volume ratio was demonstrated to affect the pharmacokinetic results in the tissue cages. Ultrafiltration probes have minimal volume and an extremely large diffusable surface area and therefore should represent the gold standard for measuring drugs in tissue fluid as the SA:V is practically infinite.

The aims of this study are two-fold. First, we aim to acquire simultaneous data from a tissue cage model and ultrafiltration probes, allowing contrast or comparison and possible validation of the different experimental models. Second, this study aims to investigate the viability of using moxidectin as an *in vivo* internal standard for tissue fluid (ultrafiltration and/or tissue cage) sampling. Our hypotheses are, ultrafiltration data will resemble plasma pharmacokinetics more than tissue cage data does and moxidectin will provide a pseudo-steady state concentration for the duration of the experiment and changes in moxidectin concentration will reflect changes in carprofen recovery.

2 Materials and methods

Animal work was approved by the University of Melbourne Faculty of Veterinary and Agricultural Sciences Animal Ethics Committee (UoM 2,015,111).

Eight merino wethers, approximately 18 months old and ranging from 42–51.5 kg, were enrolled. Each wether was determined to be healthy by veterinary clinical examination and routine haematological and biochemical testing prior to enrolment. All sheep were housed in a corrugated iron shed on slatted floors with water supplied *ad libitum*. Pellets (Sheep & Cattle Rumevite, Townsville QLD Australia) and lucerne chaff were provided daily. Ventilation was provided by passive air movement through doors and windows, and experiments were conducted between October and November 2020 in Werribee, Victoria, Australia (18).

Animals were anaesthetised and two tissue cages (6 cm and 10 cm length) were inserted under the skin on one side of the neck three weeks prior to the experiment, as previously described (16, 18), with the addition of a local anaesthetic field block being placed around the surgical site with 0.75% ropivacaine. The side of neck into which tissue cages were implanted was alternated so that 4 sheep had the left side implanted and 4 the right side.

Fourteen days prior to the experiment 0.2 mg/kg of moxidectin (Cydectin, Virbac, Milperra NSW Australia) was injected subcutaneously into a hind limb.

On the morning of experiment a cephalic vein was catheterised with an 18 g IV catheter (Jelco Optiva, Smiths Medical Macquarie Park NSW) and an injection port was attached. The port and catheter were secured with tape and flushed with heparinised saline after each sample. The side of the neck without tissue cages was clipped and aseptically prepared with iodine scrub. Two blebs of 2% lignocaine were injected under the skin and a stab incision made through the bleb. A sterile metal introducer trocar was tunnelled subcutaneously from the incision ventrally, the ultrafiltration probe was inserted inside the trocar, the trocar was then removed while maintaining the probe in place. The probe was secured to the sheep by placing butterfly tape wings around the exposed tubing and stapling the wings to the skin with skin staples. The tubing was connected to a double ended needle mounted on a vacutainer sampling bell. A sample vial with negative pressure was placed on the end of the needle.

At time zero, 4 mg/kg of carprofen was injected intravenously into the cephalic vein contralateral to the intravenous catheter. Samples of blood and tissue cage fluid were obtained at timepoints, −0.5, 0.5, 1, 2, 3, 4, 5, 7, 24, 36, 48, 72 h. The sample vial connected to each of the ultrafiltration probes was changed at these same timepoints. Samples of blood were obtained via the cephalic catheter, 1.5 mL of blood was withdrawn and discarded before a 4 mL sample was obtained, the catheter was flushed with heparinised saline. Tissue cage fluid was obtained by percutaneous puncture of the cage with a 20 ga hypodermic needle, analgesia was provided by the Coolsense device (18). All samples were collected into or transferred into sample vials containing lithium heparin.

Samples were stored at 4°C prior to centrifugation, the plasma (blood) or sediment free (tissue cage) fluid was decanted into 1.5 mL microcentrifuge tubes and stored at −80°C until analysis (≤21 days). Carprofen and Moxidectin have been shown to be stable in canine and human plasma, respectively at −80°C (19, 20).

At the end of the in-life phase the sheep were euthanised with pentobarbitone IV through the cephalic catheter. Tissue from around the ultrafiltration probe collected and submitted for histopathological examination.

2.1 Analytical method

Samples were subjected to separate carprofen and moxidectin LCMS analysis. Carprofen analysis was as previously described (16) with the following refinements; sample preparation was simplified to 100 µL of sample in addition to 400 µL of Meclofenamic Acid Internal Standard Working Solution being added to Ostro Pass-through plates (Waters Australia Rydalmere, NSW). Acetonitrile (ACN) was replaced with 50: 50 methanol: ACN as mobile phase B. Mobile phase A was MilliQ water without addition of buffer and the injection volume was reduced to 5 µL. The standard curve was extended to 100 µg/mL. The assay was partially validated for concentrations between 0.25 and 100 µg/mL. Spiked plasma was analysed at each point of the standard curve with 6 samples to calculate inter-sample CV. Additionally intra-assay CV was calculated at 50, 10, 1 and 0.25 µg/mL. All validation was done in a single day, inter-day variability was not assessed. Inter-assay variability was 1.9%–4.7% and intra-assay variability was 0.4%–2.3%.

A moxidectin method was developed and partially validated. Sample preparation for moxidectin analysis was; 400 µL of sample was mixed with 1,000 µL of Abamectin Internal Standard Working Solution (500 ng/mL ACN solvent) in a microcentrifuge tube. This was centrifuged and decanted. The supernatant was evaporated until dry in a Speedvac (Environmental Speedvac Savant, United States) on medium setting. The samples were reconstituted with 200 µL of ACN before transferring to 96-well plates for analysis. A 20 µL injection was made by the autosampler into a Shimadzu LCMS/MS system fitted with a C18 column as previously described (16), the column oven was held at 50°C. The liquid chromatography program began at 30% organic mobile phase (Isopropyl Alcohol:ACN, 75:25) rising to 90% organic at 5 min, the mobile phase was rapidly switched to 95% aqueous (MilliQ water) before returning to the starting conditions for 2 min. The nebulising gas, heating gas and drying gas were set to 2, 10 and 10 L/m, respectively. The interface temperature, DL temperature and heating block were set to 375, 250, and 400°C. Mass spectrometry was carried out in MRM mode with negative electro spray ionisation. Abamectin m/z 871.6 → 229.2 and 565.5 with collision energies of 27 and 28 eV, respectively. Moxidectin m/z was monitored 638.3 → 602.4, 236.2, and 247.1 with CE 20, 27, 26 eV, respectively. The assay was partially validated for concentrations between 10 ng/mL and 0.25 ng/mL. Intra-assay CV was calculated for 10, 5, 2.5, 1, 0.5, 0.25 ng/mL. Inter-assay variability was 5.3%–18.5%, intra-assay variability was 2.1%–5.9%.

Plasma spikes of moxidectin or carprofen were included in each analytical run as quality control. The results of the samples were adjusted based on the ratio of the measured concentration and the known concentration of the spiked plasma.

Since the sample volumes obtained from the ultrafiltration probes were generally low, the LCMS method was modified for small volumes; 60 µL of sample was combined with 150 µL of abamectin ISWS for moxidectin analysis, 100 or 20 µL of sample was combined with 400 or 80 µL of MFA ISWS depending on available sample volume. The rest of the sample preparation followed as above with volumes adjusted to maintain ratios. In most cases the entire sample was used thus preventing repeat analysis of samples.

2.2 Statistical methods

Pharmacokinetic analysis was performed in Monolix (2023R1, SimulationsPlus) using a custom model as previously described (16), the tissue cage concentrations were driven by the central compartment with the length of the cage in centimeters used as the regressor value. The model was run on both raw carprofen concentrations and on the corrected carprofen concentration. Confidence intervals for the parameters estimated by Monolix were generated using the Rsmlx package in R (21).

3 Results

Valid plasma carprofen results were obtained for all timepoints in all sheep except for sheep 6 at 36 h. Plasma moxidectin was not detectable in sheep 1 at −0.5 and sheep 6 at 72 h. In the tissue cages carprofen could not be measured in sheep 2 at 5 h in the 6 cm cage and

sheep 4 at 1 h in the 10 cm cage. Moxidectin could not be measured in 6 samples from the tissues cages in sheep 6 and 8.

Ultrafiltration yielded 74 samples from which carprofen ($n = 71$), moxidectin ($n = 19$) or both ($n = 16$) could be quantified. The carprofen concentrations were generally low with 66 samples having concentrations below 1 ng/mL.

Moxidectin concentrations in plasma had little variation within individual sheep over the sampling time period with mean (CV) plasma moxidectin of 8.55–8.57 (0.05–0.15) ng/mL. Individual cage within subject mean moxidectin concentration in the tissue cages was 8.25–8.58 ng/mL with CV ranging from 0.03%–14%.

Carprofen concentrations were corrected when a valid concurrent moxidectin result was available by multiplying the carprofen result by the mean moxidectin concentration within the respective tissue and sheep divided by the moxidectin result.

3.1 Pharmacokinetics

Pharmacokinetic estimation was performed on the plasma and tissue cage data simultaneously, but insufficient valid results were available from the ultrafiltration probes to perform analysis on these data. A continuous co-variate was included in the model to describe the change in flux between the tissue cage and the central compartment due to the tissue cage size.

The pharmacokinetic parameters estimated for the raw and corrected carprofen concentrations, and the 95% confidence interval are shown in Figure 1. Correction of the carprofen concentrations using moxidectin concentrations did not alter the estimated values of the pharmacokinetic parameters. The degree of uncertainty for these values was unaffected as shown by the 95% confidence intervals. The precise estimates are available in the Supplementary Table S1, but are not reported, as discussed below.

3.2 Histopathology

A single ultrafiltration probe was recovered post-mortem *in situ* and submitted for histology. Six sections of skin to the level of the subcutaneous adipose tissue and skeletal muscles were examined. All sections reveal comparable changes but the changes were more prominent in sections from the distal part of the string implant. Located within the deep subcutis and forming a mantle around cavitated spaces which on occasion contain the cross section of a basophilic tubing was a moderate, mixed inflammatory infiltrate. This infiltrate was dominated by macrophages including multinucleated giant cells, neutrophils and eosinophils along with fewer lymphocytes and rare plasma cells. Also noted within these inflamed areas were free erythrocytes as well as accumulations of amorphous to fibrillar eosinophilic material with the appearance of fibrin and necrosis. There were marginal accumulations of fibroblasts, and small-calibre vessels and collagen fibres (granulation tissue). No bacteria were identified on gram staining. The morphological diagnosis was of Moderate, chronic, multifocal, pyogranulomatous panniculitis with an intralesional foreign body (Figure 2).

4 Discussion

Moxidectin concentrations measured in the plasma and tissue cage fluid showed little variability throughout the sampling period. This demonstrates that moxidectin is suitable as an *in vivo* internal standard for sampling periods of up to 72 h in sheep when given 14 days before the planned first sample. The stability of moxidectin in tissue cage fluid suggests that the removal of sample fluid did not directly influence the pharmacokinetic values obtained. Moxidectin is unlikely to be suitable for studies longer than 72 h as the concentrations would be expected to decrease by >10% with a half-life of 18 days. To

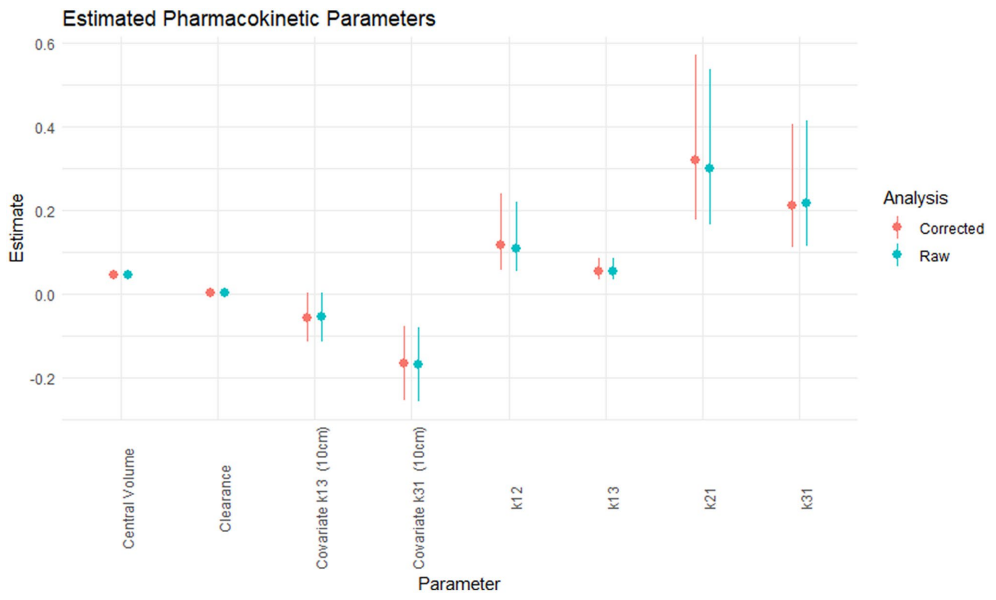


FIGURE 1
The estimated pharmacokinetic parameters; central volume (L/kg), clearance (L/kg.h), covariate for k13 for 10 cm cage (h^{-1}), covariate for k31 for 10 cm cage (h^{-1}), k12 (h^{-1}), k13 (h^{-1}), k21 (h^{-1}), k31 (h^{-1}) for the raw (green) and corrected carprofen.

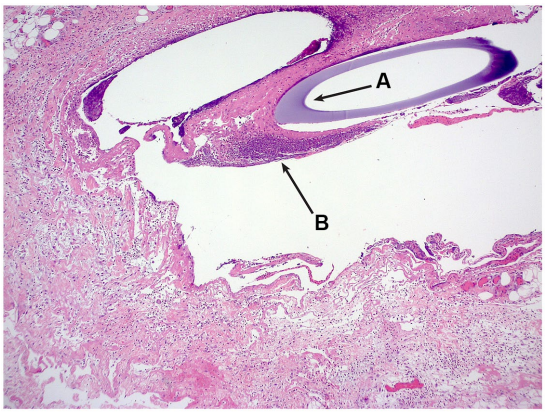


FIGURE 2
H&E photomicrograph, the cross-section of the ultrafiltration probe (A) can be seen, surrounded by inflammatory infiltrate (B).

the authors knowledge this is the first use of an internal standard for the validation of ultrafiltration and/or tissue cages and provides information for the future use of internal standards in these sampling methodologies.

The pharmacokinetic values did not differ in clinically or statistically relevant (22) amounts between the raw carprofen and carprofen adjusted for moxidectin concentrations. The volume of the central compartment calculated on this dataset was approximately 50% smaller than our previous calculations, clearance was reduced (16). This resulted in an estimated half-life of 19.5 h compared to 27.2 h in the previous dataset. The rate constants k21 for distribution into the peripheral compartment was larger while k13, and k31 representing flux of carprofen into and out of the tissue cages were smaller. These differences may be artefactual from the analytical phase as large corrections were required based on the standard curves included in the runs. Additionally the present dataset only contains 2 tissue cage sizes as opposed to 5 in our previous work. The pharmacokinetic parameter estimates in the current study allow comparisons within this study, but are unlikely to be as externally valid as in our previous report (16) and therefore are not relied on; the estimation of pharmacokinetic indices was not an objective of this study.

There are potential implications of using moxidectin as an *in vivo* internal standard when evaluating the pharmacokinetics of other drugs, such as carprofen. Drug–drug interactions between carprofen and moxidectin are possible in sheep, with potential for the pharmacokinetics of one or both drugs to be altered. However, no reports of interactions could be found in the literature for any species. These two drugs are likely to be given coincidentally in dogs, with moxidectin used for heartworm prevention and carprofen a common anti-inflammatory in general practice. No reports of adverse events or interactions were found. To fully validate the use of moxidectin as an *in vivo* internal standard, cross-over studies with the drug of interest could be performed to check for interactions in plasma. Similarly, it would be valuable to evaluate *ex vivo* and analytical interactions that might influence derived sample-drug concentration results.

Recovery sample volume of ultrafiltrate from probes was very poor with only one probe consistently producing sample volumes of 100 µL or more. This necessitated the LCMS methodology to be adapted to small volumes and prevented re-analysis of samples, as no additional sample was available. In the current study the probes were inserted subcutaneously using methodology previously described on the same day as the sampling period began. In contrast, some previous publications have inserted the probes 24 h or more ahead of sampling. Our decision to insert probes on Day One was determined from pragmatic needs, as in both group-housed sheep and for sheep in individual pens, the neighbouring animals attempted to chew the probe tubing. Same-day insertion was also intended to minimise the effect of the inflammatory obstruction of probe membranes previously described (6). Sample volumes from the probes did not increase substantially over the 72 h sampling period, suggesting that the timing of insertion did not influence sample volume recovery.

In contrast to their use by other authors (4) the stab incisions through which the probes were inserted were not closed in this study. This may have led to an air gap between the probe and subcutaneous tissues, which could be maintained by the continued entry of air from the wound. Future work may benefit from sealing the insertion wound. Species differences may contribute to the amount of ultrafiltrate that can obtained. The early work of Janle stated that subcutaneous probes produced 0.5–1 mL of ultrafiltrate per day. However, Plummer et al. (23) also report low sample volumes (maximum of 1 mL per 4–12 h) from ultrafiltration probes in merino sheep. Additionally, Plummer et al. report a high failure and complication rate of UF probes in sheep, similar to our experience in the current study. Similar experiences have been reported in calves. Hauschild et al. (24) reported that drug concentrations of pradofloxacin in ultrafiltrate sample were below the LOD when the sample volume was low, the authors infer there was insufficient vacuum to drive the drug molecules across the membrane, this phenomenon was also observed in our study. Advances in analytical technology allowing smaller samples to be utilised may overcome some of these barriers but will not overcome the poor recovery seen with low flow rates as described by Hauschild and this paper.

In this work 4 mL evacuated tubes were used to apply the vacuum pressure and collect the sample. This similar to other authors (4, 25), although flow rates in those papers were low. The use of larger evacuated tubes, i.e., 10 mL would be expected to apply a greater vacuum pressure and increase the flow rate of the ultrafiltrate.

Cooke et al. (26) overcame some of these difficulties by utilising a hybrid method, by implanting a bundle of microdialysis fibres ($n = 176$) subcutaneously, with both ends accessible. Samples were obtained by flushing the bundle intermittently thus they were able to forgo the perfusate pump system of traditional microdialysis and ensure the sample volume was sufficent for analysis by HPLC. The equilibration of the fibre bundle to the surrounding environment was reported to be 5 min allowing dense pharmacokinetic sampling to occur. Despite the apparent success of this technique, no other reports of its use were found. This technique may warrant further use given the reported difficulties of ultrafiltration and the limitations of microdialysis equipment.

The limited reported use of ultrafiltration probes for pharmacological studies outside laboratory rats gives rise to the possibility of publication bias, the work by Plummer et al. (23), Bidgood and Papich (1), Messenger et al. (3) and this paper were part

of graduate research programs, thus increasing the likelihood of submission of unfavourable results as publication is often a prerequisite for speciality (27, 28). Commercially funded work using ultrafiltration probes that did not produce sufficient volumes for analysis may not have been submitted or accepted for publication (29).

The histopathological findings on the recovered probe were consistent with those reported by Imsilp et al. (6) with granulomatous inflammation without evidence of bacterial infection. This suggests the probes suffered from biofouling and encapsulation as described by Wisniewski et al. (30). There appears to be marked species differences in the reaction to ultrafiltration probes with Underwood reporting mild inflammation in line with suture material reaction when ultrafiltration probes were inserted in equine lamella tissue (25).

Given the consistent plasma and tissue cage fluid concentrations of moxidectin, it would be expected that moxidectin would be recovered in concentrations of a similar order and variability from the ultrafiltration probes if they were functioning correctly. The use of moxidectin as an *in vivo* internal standard for pharmacokinetic studies would allow researchers to detect changes in recovery and correct for them post-hoc. This should lead to more reliable and robust results.

5 Conclusion

Subcutaneously implanted tissue cages are a more reliable model for obtaining non-central compartment samples than commercially available ultrafiltration probes. Unfortunately, tissue cage-derived samples are less likely to represent true physiological spaces than samples obtained by ultrafiltration (16).

Currently reported ultrafiltration probe techniques appear to be unsuitable for pharmacokinetic sampling. In agreement with prior work, we found that the method will require modification or refinement if it is to give consistent samples. Effective modifications and refinements have not been identified.

We were unable to accept or reject our first hypothesis as we could generate sufficient simulataneous data from the two methodologies for comparison.

This work showed that moxidectin is useful as an *in vivo* internal standard for pharmacokinetic studies. In sheep, a single dose proved useful for a duration of approximately 3 days when injected subcutaneously 2 weeks prior to the study commencement. Samples from both tissue cages and ultrafiltration probe studies illustrated this usefulness. Thus we can partially accept the second hypothesis, we did not observe changes in moxidectin concentration of sufficient magnitude to evaluate the second portion of the hypothesis.

Further work to characterize moxidectin as an *in vivo* internal standard in ultrafiltrate samples should await discovery of effective modifications and refinements to ultrafiltration sample techniques which enable reliable acquisition of samples.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by University of Melbourne Faculty of Veterinary and Agricultural Sciences Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RM: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. TW: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors would like to acknowledge Yuchi Chen and Richard Ploeg for the histopathological reporting. The technical assistance of Jess Leung, Sebastien Bauquier, Andrew Woodward, Thierry Beths, Jamie Wearn, Dianne Rees, and Babak Jillian.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1332974/full#supplementary-material

References

1. Bidgood T, Papich MG. Plasma pharmacokinetics and tissue fluid concentrations of meropenem after intravenous and subcutaneous administration in dogs. *Am J Vet Res*. (2002) 63:1622–8. doi: 10.2460/ajvr.2002.63.1622

2. Linhares MC, Kissinger PT. Capillary ultrafiltration: in vivo sampling probes for small molecules. *Anal Chem*. (1992) 64:2831–5. doi: 10.1021/ac00046a029

3. Messenger K, Wofford J, Papich M. Carprofen pharmacokinetics in plasma and in control and inflamed canine tissue fluid using in vivo ultrafiltration. *J Vet Pharmacol Ther*. (2016) 39:32–9. doi: 10.1111/jvp.12233

4. Janle-Swain E, Van Vleet JF, Ash SR. Use of a capillary filtrate collector for monitoring glucose in diabetics. *ASAIO J*. (1987) 33:336–40.

5. Garrison KE, Pasas SA, Cooper JD, Davies MI. A review of membrane sampling from biological tissues with applications in pharmacokinetics, metabolism and pharmacodynamics. *Eur J Pharm Sci*. (2002) 17:1–12. doi: 10.1016/S0928-0987(02)00149-5

6. Imsilp K, Whittem T, Koritz GD, Zachary JF, Schaeffer DJ. Inflammatory response to intramuscular implantation of polyacrylonitrile ultrafiltration probes in sheep. *Vet Res*. (2000) 31:623–34. doi: 10.1051/vetres:2000145

7. Bungay PM, Newton-Vinson P, Isele W, Garris PA, Justice JB Jr. Microdialysis of dopamine interpreted with quantitative model incorporating probe implantation trauma. *J Neurochem*. (2003) 86:932–46. doi: 10.1046/j.1471-4159.2003.01904.x

8. Linhares MC, Kissinger PT. Pharmacokinetic monitoring in subcutaneous tissue using in vivo capillary ultrafiltration probes. *Pharm Res*. (1993) 10:598–602. doi: 10.1023/A:1018914522749

9. de Lange EC. Recovery and calibration techniques: toward quantitative microdialysis In: Müller M *Microdialysis in drug development*. New York: Springer (2012). 13–33.

10. MacLean DA, Ettinger SM, Sinoway LI, Lanoue KF. Determination of muscle-specific glucose flux using radioactive stereoisomers and microdialysis. *Am J Physiol Endocrinol Metab*. (2001) 280:E187–92. doi: 10.1152/ajpendo.2001.280.1.E187

11. Pérez R, Núñez MJ, Palma C, Riquelme J, Arboix M. Plasma disposition kinetics of moxidectin after subcutaneous administration to pregnant sheep. *J Vet Pharmacol Ther*. (2014) 37:550–5. doi: 10.1111/jvp.12127

12. Mealey KL. Canine ABCB1 and macrocyclic lactones: heartworm prevention and pharmacogenetics. *Vet Parasitol*. (2008) 158:215–22. doi: 10.1016/j.vetpar.2008.09.009

13. Brentnall C, Cheng Z, Mckellar QA, Lees P. Influence of oxytetracycline on carprofen pharmacodynamics and pharmacokinetics in calves. *J Vet Pharmacol Ther*. (2013) 36:320–8. doi: 10.1111/jvp.12000

14. Cheng Z, Nolan A, McKellar Q. Anti-inflammatory effects of carprofen, carprofen enantiomers, and NG-nitro-L-arginine methyl ester in sheep. *Am J Vet Res*. (2002) 63:782–8. doi: 10.2460/ajvr.2002.63.782

15. Lees P, Delatour P, Benoit E, Foster AP. Pharmacokinetics of carprofen enantiomers in the horse. *Acta Vet Scand Suppl*. (1991) 87:249–51.

16. Munn R, Whittem T, Woodward AP. The surface area to volume ratio changes the pharmacokinetic and pharmacodynamic parameters in the subcutaneous tissue cage model: as illustrated by carprofen in sheep. *Front Vet Sci*. (2022) 9:801. doi: 10.3389/fvets.2022.905797

17. Welsh EM, Baxter P, Nolan AM. Pharmacokinetics of carprofen administered intravenously to sheep. *Res Vet Sci*. (1992) 53:264–6. doi: 10.1016/0034-5288(92)90123-J

18. Munn R, Woodward A, Beths T, Whittem T. Observations on the use of a pain numbing device for repetitive percutaneous sampling in sheep. *Aust Vet J*. (2021) 99:445–8. doi: 10.1111/avj.13104

19. Shuttleworth JR, Behrens KN, Biggo MR, Horne RL, Cox S, Lakritz J, et al. Effect of storage duration on carprofen concentration measurements in dog plasma. *Vet Med Sci*. (2023) 9:2022–5. doi: 10.1002/vms3.1215

20. Chhonker YS, Sleightholm RL, Murry DJ. Bioanalytical method development and validation of moxidectin in plasma by LC–MS/MS: application to in vitro metabolism. *Biomed Chromatogr*. (2019) 33:e4389. doi: 10.1002/bmc.4389

21. Lavielle M., *Rsmlx: R Speaks 'Monolix'*. Repository CRAN. Eds. Mihaljevic F, Lavielle M, Chauvin J., Pinaud C. (2022). Available at: https://monolix.lixoft.com/rsmlx/

22. Cumming G, Finch S. Inference by eye: confidence intervals and how to read pictures of data. *Am Psychol*. (2005) 60:170–80. doi: 10.1037/0003-066X.60.2.170

23. Plummer C, White PJ, Kimble B, Govendir M, van der Saag D. Preliminary investigation into a novel sustained-release formulation of meloxicam in sheep (*Ovis aries*)—pharmacokinetic profile. *Animals*. (2021) 11:2484. doi: 10.3390/ani11092484

24. Hauschild G, Rohn K, Engelhardt E, Sager M, Hardes J, Gosheger G. Pharmacokinetic study on pradofloxacin in the dog – comparison of serum analysis, ultrafiltration and tissue sampling after oral administration. *BMC Vet Res*. (2013) 9:32. doi: 10.1186/1746-6148-9-32

25. Underwood C, Collins SN, van Eps AW, Allavena RE, Medina-Torres CE, Pollitt CC. Ultrafiltration of equine digital lamellar tissue. *Vet J*. (2014) 202:314–22. doi: 10.1016/j.tvjl.2014.05.007

26. Cooke I, Bevill R, Koritz G. Pharmacokinetics of penicillin G in plasma and interstitial fluid collected with dialysis fiber bundles in sheep. *Vet Res*. (1996) 27:147–59.

27. Wood W, McCollum J, Kukreja P, Vetter IL, Morgan CJ, Hossein Zadeh Maleki A, et al. Graduate medical education scholarly activities initiatives: a systematic review and meta-analysis. *BMC Med Educ*. (2018) 18:318. doi: 10.1186/s12909-018-1407-8

28. *The Australian and New Zealand College of veterinary scientists fellowship candidate handbook*. (2023). Available at: https://ripehosting.blob.core.windows.net/anzcvs-dev-media/42753/20230724-fellowship-candidate-handbook-2023-finalised1.pdf

29. Thornton A, Lee P. Publication bias in meta-analysis: its causes and consequences. *J Clin Epidemiol*. (2000) 53:207–16. doi: 10.1016/S0895-4356(99)00161-4

30. Wisniewski N, Klitzman B, Miller B, Reichert WM. Decreased analyte transport through implanted membranes: differentiation of biofouling from tissue effects. *J Biomed Mater Res*. (2001) 57:513–21. doi: 10.1002/1097-4636(20011215)57:4<513::AID-JBM1197>3.0.CO;2-E

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 12 April 2024
DOI 10.3389/fvets.2024.1351693

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Chandrasekaran Sankaranarayanan,
Annamalai University, India
Şenol Çelik,
Bingöl University, Türkiye

*CORRESPONDENCE
Muhammad Majeed
✉ m.majeed@uog.edu.pk
Hosam O. Elansary
✉ helansary@ksu.edu.sa

†These authors have contributed equally to this work

RECEIVED 06 December 2023
ACCEPTED 18 March 2024
PUBLISHED 12 April 2024

CITATION
Khan AM, Altaf M, Hussain T, Hamed MH, Safdar U, Ayub A, Memon Z-n, Hafiz A, Ashraf S, Amjad MS, Majeed M, Hassan M, Bussmann RW, Abbasi AM, Al-Yafrsi M, Elansary HO and Mahmoud EA (2024) Ethnopharmacological uses of fauna among the people of central Punjab, Pakistan. *Front. Vet. Sci.* 11:1351693. doi: 10.3389/fvets.2024.1351693

COPYRIGHT
© 2024 Khan, Altaf, Hussain, Hamed, Safdar, Ayub, Memon, Hafiz, Ashraf, Amjad, Majeed, Hassan, Bussmann, Abbasi, Al-Yafrsi, Elansary and Mahmoud. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Ethnopharmacological uses of fauna among the people of central Punjab, Pakistan

Abdul Majid Khan[1†], Muhammad Altaf[2†], Tanveer Hussain[2], M. Haroon Hamed[3], Umaira Safdar[1], Amina Ayub[4], Zaibun-nisa Memon[5], Adnan Hafiz[6], Sana Ashraf[7], Muhammad Shoaib Amjad[8], Muhammad Majeed[9]*, Musheerul Hassan[10,11], Rainer W. Bussmann[10,12], Arshad Mahmood Abbasi[13], Mohamed Al-Yafrsi[14], Hosam O. Elansary[14]* and Eman A. Mahmoud[15]

[1]Institute of Zoology, University of the Punjab, Lahore, Pakistan, [2]Institute of Forest Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan, [3]Department of Zoology Wildlife and Fisheries, University of Agriculture Faisalabad, Faisalabad, Pakistan, [4]Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad, Faisalabad, Pakistan, [5]Department of Zoology, Shah Abdul Latif University, Khairpur, Sindh, Pakistan, [6]Department of Zoology, University of Sialkot, Sialkot, Punjab, Pakistan, [7]Department of Zoology, University of Lahore, Sargodha, Pakistan, [8]Department of Botany, Women University of Azad Jammu and Kashmir Bagh, Bagh, Pakistan, [9]Department of Botany, University of Gujrat, Gujrat, Punjab, Pakistan, [10]Department of Ethnobotany, Institute of Botany, Ilia State University, Tbilisi, Georgia, [11]Alpine Institute of Management and Technology, Dehradun, Uttarakhand, India, [12]Staatliches Museum Für Naturkunde, Karlsruhe, Germany, [13]Department of Environment Sciences, COMSATS University Islamabad, Abbottabad, Pakistan, [14]Department of Plant Production, College of Food & Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia, [15]Department of Food Science, Faculty of Agriculture, Damietta University, Damietta, Egypt

Introduction: The utilization of fauna and fauna-based byproducts in ethnomedicinal usages has been a longstanding human activity, practiced across various cultures worldwide. This study focuses on investigating the utilization of animal-based traditional medicine by the people of Pakistan, specifically in the Gujranwala area.

Methods: Data collection took place from January to September 2019 through interviews with local communities. Ethnomedicinal applications of animal products were analyzed using several indices, including Relative Frequency of Citation (RFC), Relative Popularity Level (RPL), Folk Use Value (FL), and Relative Occurrence Percentage (ROP).

Results: The study identified the use of different body parts of 54 species of animals in treating various diseases and health issues. These include but are not limited to skin infections, sexual problems, pain management (e.g., in the backbone and joints), eyesight issues, immunity enhancement, cold, weakness, burns, smallpox, wounds, poisoning, muscular pain, arthritis, diabetes, fever, epilepsy, allergies, asthma, herpes, ear pain, paralysis, cough, swelling, cancer, bronchitis, girls' maturity, and stomach-related problems. Certain species of fauna were noted by informers with high "frequency of citation" (FC), ranging from 1 to 77. For instance, the black cobra was the most frequently cited animal for eyesight issues (FC=77), followed by the domestic rabbit for burn treatment (FC=67), and the Indus Valley spiny-tailed ground lizard for sexual problems (FC=66). Passer domesticus and *Gallus gallus* were noted to have the highest ROP value of 99.

Discussion: The findings of this study provide valuable preliminary insights for the conservation of fauna in the Gujranwala region of Punjab, Pakistan. Additionally, screening these animals for medicinally active compounds could potentially lead to the development of novel animal-based medications, contributing to both traditional medicine preservation and modern pharmaceutical advancements.

KEYWORDS
Gujranwala, ethnozoology, ethnomedicine, zootherapy, animals, communities

1 Introduction

Zootherapy is described as the use of animal or animal-derived products to treat human ailments (1). The utilization of fauna with therapeutic characteristics is still a popular practice across the world (2). Zootherapy methods and materials are used in both folk and modern medicine to treat different kinds of sicknesses (3–7). It has been found that over 13% of the medications used in traditional Chinese medicine are derived from fauna. In Ayurvedic medicine, faunal products make up 15–20% of the medications. More than 111 medications in Tibetan medicine contain fauna-based components (8–10).

Many communities are rapidly losing ethnomedicinal expertise, making it increasingly necessary to capture this information before it is lost (1, 11–17). The utilization of fauna and fauna-based yield in folk therapy has been under-documented, most likely due to the dominance of plants in folk medical systems (18, 19). Pakistan has a rich fauna diversity, including 668 species of birds (20), 195 species of mammals (21), 24 species of amphibians (22), and 195 species of reptiles (23).

Gujranwala has a diverse range of fauna and biodiversity. This region, with its plains and different ecosystems, is home to an amazing variety of wildlife. Dominant avian fauna is documented in Gujranwala, i.e., *Acridothere ginginianus*, *Acridothere tristis*, *Apus affinis*, *Athene brama*, *Bubulcus ibis*, *Cercomela fusca*, *Columba livia*, *Corvus splendens*, *Egretta garzetta*, *Hirundo rustica*, *Hoplopterus indicus*, *Milvus migrans migrans*, *Nectarinia asiatica*, and *Passer domesticus* (24), while important mammalian fauna of the area is reported as, i.e., *Suncus etruscus*, *Funnambulus pennantii*, *Rattus rattus*, *Mus musculus*, *Herpestes javanicus*, and *Herpestes edwardsi* (25–27), and more than 30 species of freshwater are documented along Gujranwala (28), and prominent herptiles are *Saara hardwickii*, Var*anus bengalensis*, *Duttaphrynus stomaticus*, *Hemidactylus flaviviridis*, *Hoplobatrachus tigerinus*, *Aspideretes gangeticus*, *Lissemys punctate*, *Calotes versicolor*, and *Eryx johnii* (29).

Knowing the conservation and management of biocultural systems requires ethnozoological study. Traditional usages of fauna species, e.g., food (30–37), medicine (32, 38–42), trade (43), etc., can endorse attitude that aids in the conservation of these animals; however, if they are practiced in an unsustainable manner or are influenced by economic and political factors, they may have a negative impact on or even endanger these animals. Local populations' usage of animal species in traditional medicine and for cultural purposes must be evaluated in connection to other issues such as climate and habitat changes (44, 45). There is a global need to identify innovative techniques to cope with the current catastrophe of loss of biodiversity (46), and ethnozoology gives crucial insights into local community practices, allowing conservation efforts to successfully collaborate with resource managers to enhance the overall veracity of biological structures (47, 48). A number of studies have been conducted to date that has documented the use of animal parts in traditional medicine in various areas of Pakistan (31, 32, 49–61); however, ethnomedicinal applications of animals in Gujranwala have never been described. This research on the medical applications of fauna by the people of Gujranwala district in Pakistan is part of a larger plan to record the usage of fauna by local populations across Pakistan (18, 29, 62–64).

The research on the ethnopharmacological uses of fauna among the inhabitants of central Punjab, Pakistan, aims to investigate and describe traditional understandings and methods of using local animal resources for medicinal reasons in this area. By conducting an in-depth inquiry into the fauna-based remedies used by communities of indigenous peoples, the study hopes to contribute to the preservation of traditional healing practices, shed light on the possible medicinal qualities of these animals, and provide insights for the conservation of biodiversity.

2 Materials and methods

2.1 Study site and climate

Gujranwala is the city and capital of Gujranwala Division, Punjab, Pakistan. It is also known as the "City of Wrestlers." Gujranwala is Pakistan's fifth-most populous city. Founded in the eighteenth century, Gujranwala is a relatively modern town compared to other nearby old cities in northern Punjab (Figure 1). The people of the area like to eat meat. The coordinates of Gujranwala are 32°9′24″N 74°11′24″E. Gujranwala has a hot, semi-arid climate. During the summer (June to September), the temperature reaches 40 °C. The coldest months are typically November through February, when temperatures can dip to an average of 5°C. During the other months, the average rainfall is approximately 25 mm. There is very little rain from October to May (Figures 2A,B).

2.2 Data collection

Semi-structured interviews and questionnaires were collected from 100 respondents (i.e., traditional health practitioners, farmers, teachers, hunters, and herdsmen). We selected random respondents from the community who have knowledge about traditional therapy. Respondents were chosen based on their ethnomedicinal and ethnocultural recognition of the customary remedial and societal value of herptiles, fish, animals, and fowl. Birds were identified using field guides, "The Birds of Pakistan." Data were statistically analyzed using five indices, including (i) the "fidelity level" (FL), (ii) the "relative popularity level" (RPL), and (iii) "rank order priority."

2.3 The Fidelity level

FL was analyzed with the help of a formula as follows (65):

$$FL(\%) = N_p / FC \times 100.$$

where "N_p" = number of respondents with vital chronic diseases for certain breeds of animals; FC = frequency of citation for ethnocultural utilization of specific creatures.

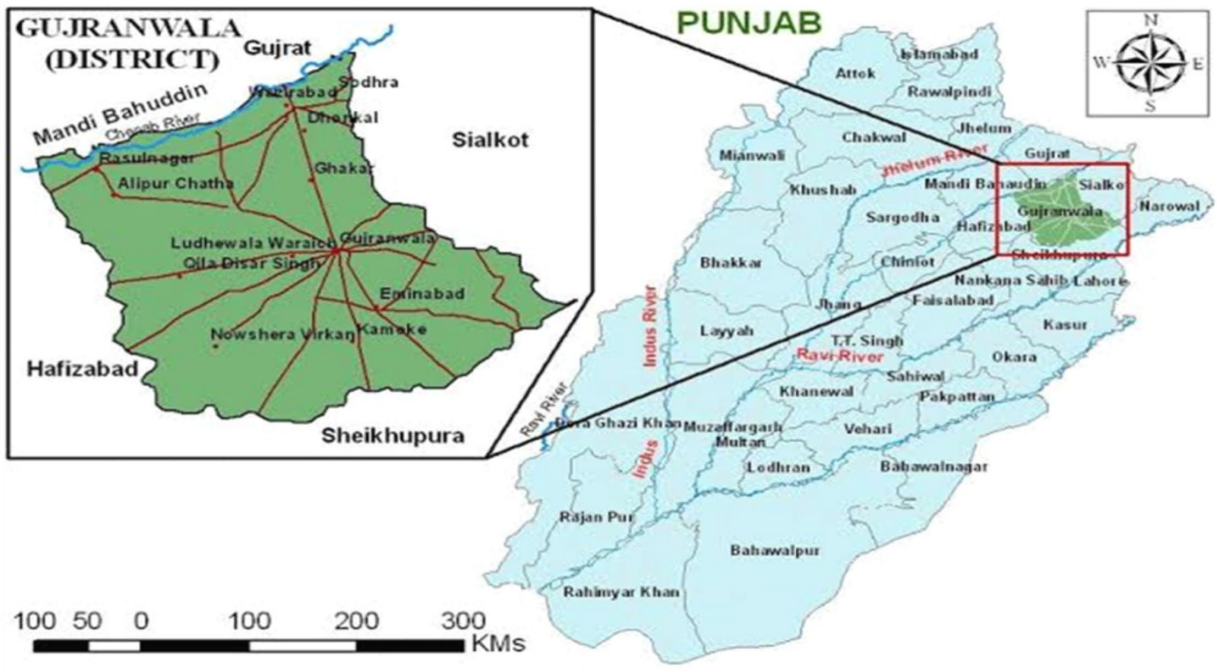


FIGURE 1
Study site map.

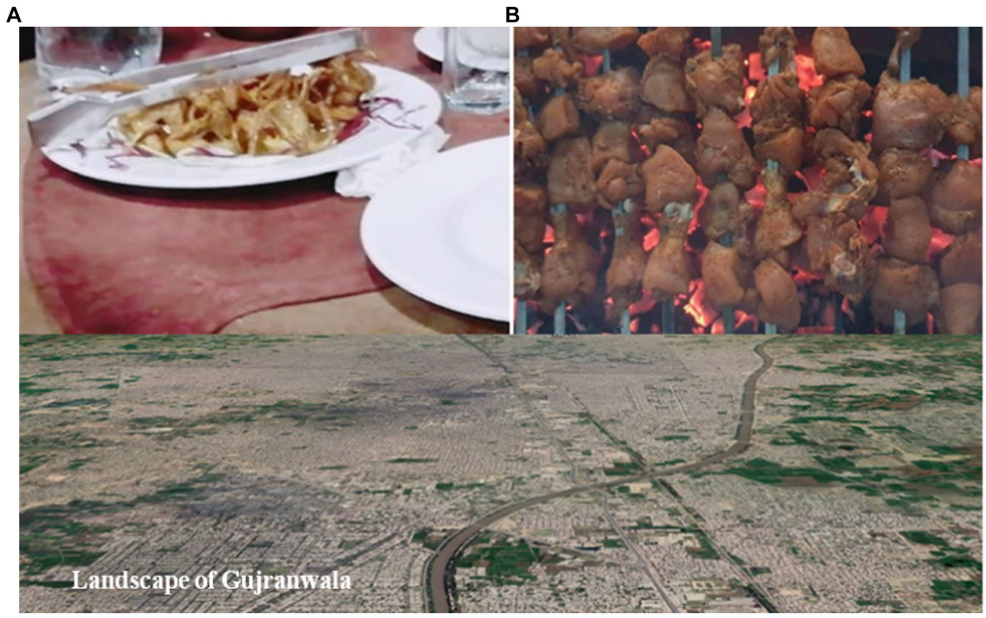


FIGURE 2
(A,B) Famous meat (**A**: quail meat, **B**: chicken) and landscape of Gujranwala.

2.4 The relative popularity level

RPL was described previously by Friedman et al. (66); creatures were separated into two gatherings, for example, (i) "famous" and (ii) "disagreeable." (i) Popular creatures are those species that were expressed for the greater part of the most extreme recurrence of reference (FC). (ii) The left-over creatures were recorded as disagreeable. While for famous (creature species) an even line was non-existent specifically, the normal numeral of employments per creature is free of the recurrence of reference (FC), who perceives the creatures; along these lines, the normal numeral of employments of mainstream creature animal varieties does not improve with the addition to recurrence of reference, who refers to the creature species for any clinical use. For the mainstream creature species, the RPL was set at 1. For creature species in disliked gatherings, the overall notoriety level is under 1.0.

2.5 Rank order priority

ROP is utilized to group the fauna species (66, 67) and was examined using the following formula:

$$ROP = FL \times RPL$$

3 Results and discussion

3.1 Informant selection for ethnozoological data collection

During the years 2018–2020, ethnozoological information was gathered; prior to the data collection, a reconnaissance survey was conducted to ensure accessibility across the study area. Using the snowball technique, we collected the traditional endemic knowledge using semi-structured questionnaires followed by group discussions. The informants selected were mostly from urban (55%). Furthermore, the selected informants were classified into different professions and age groups. The maximum number of informants were educated (92.9%). Upon data collection, men showed ascendency over women due to cultural and religious limitations. Women are not allowed to talk with strangers due to religious and social norms, and they are usually involved in house chores. The complete details can be found in Figure 3. It is documented that older people have more insights about traditional knowledge as compared with younger people, and it is also noted that uneducated and less educated people have more reliance on traditional knowledge as compared with highly educated people.

3.2 Taxonomy and ethnozoological inventory

In the ethno-scientific domain, we documented a total of 54 species used in Gujranwala, Pakistan, classified into different categories, i.e., herptiles ($n = 7$), fish ($n = 16$), mammals ($n = 13$), birds ($n = 15$), and invertebrates ($n = 3$). The complete inventory is provided in Table 1. The maximum usage of the fish species in the region can be attributed to the presence of the two important rivers (Ravi and Chenab) inhabiting varieties of fish fauna; also, the locals have a potential traditional knowledge of fish utilization.

Upon interpreting the results, it was revealed that different body parts of the documented species were employed against a variety of diseases. The most frequently used parts were meat (33%), followed by fat (30%), and brain (16%; Figures 4A–C).

The fat of animals is the second most consumed body part of animals. The fat of 31 species of animals, i.e., Indian Bullfrog, Indus Valley spiny-tail ground lizard, Bengal monitor, rock python, black cobra, Russell's chain viper, grass carp, common carp, silver carp, mrigal carp, Reba carp, raho, Orangefin labeo, catla, spotted snakehead, great snakehead, Nile tilapia, Rita, goonch, Gangetic mystus, zig–zag eel, wallago catfish, cow, buffalo, small Indian mongoose, Indian flying fox bat, bear, tawny eagle, and Bonelli's eagle, is used to treat wounds in feet, small pox, skin diseases, sexual problems, pain in joints, pain in body, pain in backbone, fever, eyesight and eye problems, enhance potential, memory, and immunity, engulf of poisonous things, cold, cancer, burn, and blindness in the night (Figures 4A,B).

Similarly, the brain of animal species, i.e., grass carp, common carp, silver carp, mrigal carp, Reba carp, raho, Orangefin labeo, catla, spotted snakehead, great snakehead, Nile tilapia, Rita, goonch, Gangetic mystus, zig–zag eel, and wallago catfish, is used to treat small pox, sexual problems, pain in joints, pain in the body, eyesight and eye problems, enhance potential, memory, and immunity, cold, burn, and blindness at night (Figures 4A,B).

Similarly, the skin of animal species, i.e., the Indus Valley toad and black cobra catfish, is used to cure skin diseases, sexual problems, and eyesight, while the blood of camel, desert hare, domestic rabbit, and spotted little owlet is used to cure pain in muscles, pain in joints, diabetes, asthma, burns, face paralysis, burn, enhance potential, sexual problems, and skin diseases. However, the milk of camel, goat, and sheep is used to treat pain in muscles, pain in joints, diabetes, sexual problems, cold, fever, burn, and enhance potential (Figures 4A,B).

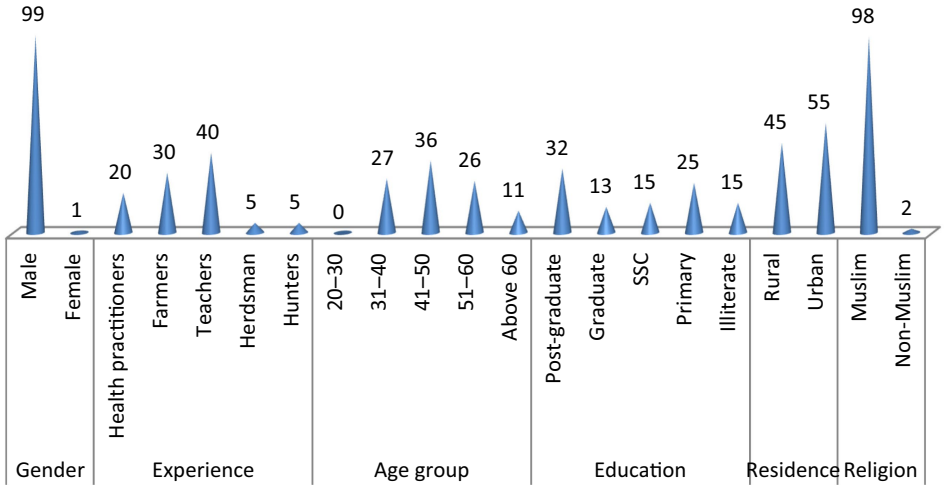


FIGURE 3
Selected informants on the ethnozoological usage of the documented species.

10.3389/fvets.2024.1351693

TABLE 1 A detailed analysis of the ethnozoology of Gujranwala, Punjab.

No.	Common name Scientific name (species authority) Punjabi name	Uses of body parts	Mode of use	Diseases	FC	RFC	Np	RPL	FL	ROP
Herptiles										
1	Indus Valley toad *Bufo stomaticus* (Lutkin, 1862) Ghariallo daddo	S	T	Skin diseases	8.0	0.08	2	0.23	25.00	6
2	Indian Bullfrog *Hoplobatrachus tigerinus* (Daudin, 1802) Wada daddo	F	T	Sexual problems	13.0	0.13	3	0.37	23.08	8
				Pain in backbone	28.0	0.28	6	0.79	21.43	17
				Pain in joints	12.0	0.12	4	0.34	33.33	11
3	Indus Valley spiny-tail ground lizard *Uromastyx hardwickii* (Strauch, 1863) Sanda	F	T	Pain in joints	4.0	0.04	1	0.11	25.00	3
				Pain in backbone	23.0	0.23	8	0.65	34.78	23
				Sexual problems	66.0	0.66	28	1.00	42.42	42
4	Bengal Monitor *Varanus bengalensis* (Daudin, 1802) Goh	F	T	Pain in backbone	2.0	0.02	1	0.06	50.00	3
				Sexual problems	2.0	0.02	1	0.06	50.00	3
5	Rock python *Python molurus* (Linnaeus, 1758) Azdha sap	F	T	Pain in joints	2.0	0.02	1	0.06	50.00	3
				Sexual problems	11.0	0.11	7	0.31	63.64	20
6	Black cobra *Naja naja naja* (Linnaeus, 1768) Kala naag	S, F	T	Sexual problems	19.0	0.19	3	0.54	15.79	8
				Eyesight	77.0	0.77	13	1.00	16.88	17
7	Russell's chain Viper *Daboia russelii russelii* (Shaw and Nodder, 1797) Koriwala	F	T	Sexual problems	9.0	0.09	2	0.25	22.22	6
				Pain in joints	7.0	0.07	2	0.20	28.57	6
Fishes										
8	Grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) Grass carp	B, M, F	T, O	Eye problems	7.0	0.07	2	0.20	28.57	6
				Blindness in night	6.0	0.06	2	0.17	33.33	6
				Enhance memory	10.0	0.1	2	0.28	20.00	6
				Sexual problems	19.0	0.19	3	0.54	15.79	8
9	Common carp *Cyprinus carpio* (Linnaeus, 1758) Gulfam	B, M, F	T, O	Eye problems	8.0	0.08	2	0.23	25.00	6
				Blindness in night	3.0	0.03	2	0.08	66.67	6
				Enhance memory	13.0	0.13	3	0.37	23.08	8
				Sexual problems	10.0	0.1	2	0.28	20.00	6
				Enhance immunity	21.0	0.21	2	0.59	9.52	6
10	Silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844) Silver carp	B, M, F	T, O	Eye problems	8.0	0.08	3	0.23	37.50	8
				Blindness in night	3.0	0.03	1	0.08	33.33	3
				Enhance memory	23.0	0.23	2	0.65	8.70	6
				Sexual problems	28.0	0.28	4	0.79	14.29	11
				Enhance immunity	7.0	0.07	1	0.20	14.29	3
11	Mrigal carp *Cirrhinus mrigala* (Hamilton, 1822) Mori	B, M, F	T, O	Eye problems	5.0	0.05	1	0.14	20.00	3
				Blindness in night	8.0	0.08	1	0.23	12.50	3
				Enhance immunity	12.0	0.12	3	0.34	25.00	8
				Cold	10.0	0.1	3	0.28	30.00	8
				Sexual problems	7.0	0.07	2	0.20	28.57	6
12	Reba carp *Cirrhinus reba* (Hamilton, 1822) Reba Machhali	B, M, F	T, O	Eye problems	1.0	0.01	1	0.03	100.00	3
				Blindness in night	4.0	0.04	1	0.11	25.00	3

(Continued)

TABLE 1 (Continued)

No.	Common name Scientific name (species authority) Punjabi name	Uses of body parts	Mode of use	Diseases	FC	RFC	Np	RPL	FL	ROP
				Enhance memory	5.0	0.05	1	0.14	20.00	3
				Sexual problems	3.0	0.03	1	0.08	33.33	3
				Enhance immunity	5.0	0.05	1	0.14	20.00	3
13	Raho *Labeo rohita* (Hamilton, 1822) Raho	B, M, F	O	Pain in joints	8.0	0.08	2	0.23	25.00	6
				Sexual problems	36.0	0.36	18	1.00	50.00	50
				Eyesight	12.0	0.12	4	0.34	33.33	11
				Enhance memory	14.0	0.14	3	0.39	21.43	8
				Enhance immunity	12.0	0.12	2	0.34	16.67	6
14	Orangefin labeo *Labeo calbasu* (Hamilton, 1822) Kalbans	B, M, F	T, O	Enhance memory	1.0	0.01	1	0.03	100.00	3
				Pain in body	2.0	0.02	1	0.06	50.00	3
				Sexual problems	12.0	0.12	7	0.34	58.33	20
				Enhance immunity	10.0	0.1	3	0.28	30.00	8
15	Catla *Gibelion catla* (Hamilton, 1822) Thaila	B, M, F	O	Enhance memory	4.0	0.04	2	0.11	50.00	6
				Sexual problems	22.0	0.22	3	0.62	13.64	8
16	Spotted snakehead *Channa punctata* (Bloch, 1793) Dola	B, M, F	O	Enhance memory	5.0	0.05	3	0.14	60.00	8
				Enhance immunity	30.0	0.3	20	0.85	66.67	56
17	Great snakehead *Channa marulius* (Hamilton, 1822) Soul	B, M, F	O	Sexual problems	19.0	0.19	11	0.54	57.89	31
				Enhance potential	12.0	0.12	8	0.34	66.67	23
18	Nile tilapia *Oreochromis niloticu* (Linnaeus, 1758) Tilapia	B, M, F	T	Enhance immunity	25.0	0.25	12	0.70	48.00	34
				Burn	4.0	0.04	2	0.11	50.00	6
19	Rita *Rita rita* (Hamilton, 1822) Khaga	B, M, F	O, T	Enhance immunity	13.0	0.13	7	0.37	53.85	20
				Sexual problems	7.0	0.07	4	0.20	57.14	11
20	Goonch *Bagarius bagarius* (Hamilton, 1822) Foji Khaga	B, M, F	O	Enhance immunity	21.0	0.21	9	0.59	42.86	25
				Sexual problems	25.0	0.25	9	0.70	36.00	25
21	Gangetic mystus *Mystus cavasius* (Hamilton, 1822) Tangra Machhali	B, M, F	O	Small pox	2.0	0.02	1	0.06	50.00	3
				Enhance immunity	16.0	0.16	2	0.45	12.50	6
22	Zig-zag eel *Mastacembelus armatus* (Lacepède, 1800) Baam Machhali	B, M, F	O	Sexual problems	7.0	0.07	2	0.20	28.57	6
				Enhance immunity	16.0	0.16	3	0.45	18.75	8
23	Wallago catfish *Wallago attu* (Bloch & Schneider, 1801) Mali	B, M, F	O	Enhance memory	5.0	0.05	2	0.14	40.00	6
				Enhance immunity	11.0	0.11	4	0.31	36.36	11
Mammals										
24	Cow *Bos gaurus* (C. H. Smith, 1827) Gay	B, M, F	O, T	Wounds in feet	12.0	0.12	7	0.34	58.33	20
				Pain in body	21.0	0.21	19	0.59	90.48	54
				Engulf of poisonous things	12.0	0.12	2	0.34	16.67	6
25	Buffalo *Bubalus bubalis* (Linnaeus, 1758) Mujh	B, M, F	O, T	Wounds in feet	12.0	0.12	8	0.34	66.67	23
				Pain in body	37.0	0.37	21	1.00	56.76	57
				Engulf of poisonous things	7.0	0.07	4	0.20	57.14	11

(Continued)

TABLE 1 (Continued)

No.	Common name Scientific name (species authority) Punjabi name	Uses of body parts	Mode of use	Diseases	FC	RFC	Np	RPL	FL	ROP
26	Camel *Camelus dromedaries* (Linnaeus, 1758) Ount	MI, BL, M	Oral Topical	Pain in muscles	2.0	0.02	1	0.06	50.00	3
				Pain in joints	2.0	0.02	1	0.06	50.00	3
				Diabetes	37.0	0.37	22	1.00	59.46	59
27	Goat *Capra aegagrus hircus* (Linnaeus, 1758) Bakri	MI	Oral	Sexual problems	41.0	0.41	22	1.00	53.66	54
				Cold	2.0	0.02	1	0.06	50.00	3
				Fever	2.0	0.02	1	0.06	50.00	3
28	Northern palm squirrel *Funnambulus pennanti* (Wroughton, 1905) Gulahri	M, H	O, T	Epilepsy	7.0	0.07	2	0.20	28.57	6
				Skin diseases	7.0	0.07	2	0.20	28.57	6
				Allergy	7.0	0.07	2	0.20	28.57	6
29	Small Indian mongoose *Herpestes javanicus* (Geoffroy Saint-Hilarie, 1818) Neola	F	T	Sexual problems	6.0	0.06	2	0.17	33.33	6
				Pain in joints	6.0	0.06	2	0.17	33.33	6
				Pain in backbone	6.0	0.06	2	0.17	33.33	6
30	Humans *Homo sapiens* (Linnaeus, 1758) Adam	SA	T	Herpes	27.0	0.27	5	0.76	18.52	14
				Ear pain	27.0	0.27	3	0.76	11.11	8
				Eye problems	27.0	0.27	5	0.76	18.52	14
31	Desert hare *Lepus nigricollis dayanus* (F. Cuvier, 1823) Jungli khargush	M, L, BL	O,T	Asthma	12.0	0.12	4	0.34	33.33	11
				Burn	12.0	0.12	4	0.34	33.33	11
				Face paralysis	12.0	0.12	4	0.34	33.33	11
32	Indian Pangolin *Manis crassicaudata* (É.Geoffroy Saint-Hilaire, 1803) Pangolin, Sipple	SC, M	T	Feet swelling	11.0	0.11	4	0.31	36.36	11
				Sexual problems	2.0	0.02	1	0.06	50.00	3
				Cancer	2.0	0.02	1	0.06	50.00	3
33	Domestic rabbit *Oryctolagus cuniculus* (Linnaeus, 1758) Khargush, Saya	T, BL	T	Burn	67.0	0.67	34	1.00	50.75	51
				Enhance potential	2.0	0.02	1	0.06	50.00	3
				Face paralysis	2.0	0.02	1	0.06	50.00	3
34	Sheep *Ovis aries* (Linnaeus, 1758) Bairh	F, MI, M	O,T	Burn	26.0	0.26	22	0.73	84.62	62
				Enhance potential	26.0	0.26	22	0.73	84.62	62
				Pain in joints	26.0	0.26	22	0.73	84.62	62
35	Indian flying fox bat *Pteropus giganteus* (Brünnich, 1782) Chamga-dar	F	T	Pain in body	2.0	0.02	1	0.06	50.00	3
				Pain in joints	2.0	0.02	1	0.06	50.00	3
				Pain in backbone	2.0	0.02	1	0.06	50.00	3
36	Bear *Ursus thibetanus* (Cuvier, 1823) Richh	F	T	Sexual problems	3.0	0.03	2	0.08	66.67	6
				Pain in joints	3.0	0.03	2	0.08	66.67	6
				Pain in backbone	3.0	0.03	2	0.08	66.67	6
Birds										
37	Common Myna *Acridotheres tristis* (Linnaeus, 1766) Lali	M	O	Cough	9.0	0.09	2	0.25	22.22	6
				Fever	9.0	0.09	2	0.25	22.22	6
38	Domestic Duck *Anas platyrhynchos domesticus* (Linnaeus, 1758) Batakh	E	O	Eye problems	9.0	0.09	2	0.25	22.22	6
				Cold	9.0	0.09	2	0.25	22.22	6
39	Mallard *Anas platyrhynchos* (Linnaeus, 1758) Nilsir	M, E	O	Face paralysis	4.0	0.04	3	0.11	75.00	8
				Cold	4.0	0.04	3	0.11	75.00	8
40	Tawny Eagle *Aquila rapax* (Temminck, 1828) Chhota baaz	F	T	Skin diseases	3.0	0.03	2	0.08	66.67	6
				Cancer	3.0	0.03	2	0.08	66.67	6

(Continued)

TABLE 1 (Continued)

No.	Common name Scientific name (species authority) Punjabi name	Uses of body parts	Mode of use	Diseases	FC	RFC	Np	RPL	FL	ROP
41	Spotted Little Owlet *Athene brama* (Temminck, 1821) Ullo	BL	T	Sexual problems	10.0	0.1	2	0.28	20.00	6
				Skin diseases	10.0	0.1	2	0.28	20.00	6
42	Blue Rock Pigeon *Columba livia* (F.Gmelin, 1789) Jangli kabotar	M, FE	O	Face paralysis	44.0	0.44	22	1.00	50.00	50
				Cold	44.0	0.44	22	1.00	50.00	50
43	Common Quail *Coturnix coturnix* (Linnaeus, 1758) Batera	M	O	Enhance memory	10.0	0.1	7	0.28	70.00	20
				Sexual problems	10.0	0.1	7	0.28	70.00	20
44	Black partridge *Francolinus francolinus* (Linnaeus, 1766) Kala tittar	M	O	Bronchitis	19.0	0.19	11	0.54	57.89	31
				Enhance potential	19.0	0.19	11	0.54	57.89	31
45	Domestic Chicken *Gallus gallus* (Linnaeus, 1758) Murghi	E, M	O	Fever	35.0	0.35	35	0.99	100.00	99
				Cold	35.0	0.35	35	0.99	100.00	99
46	Bonnelli's Eagle *Hieraaetus fasciatus* (Sibley & Monroe, 1990) Baaz	F	T	Cancer	3.0	0.03	1	0.08	33.33	3
				Skin diseases	3.0	0.03	1	0.08	33.33	3
47	House Sparrow *Passer domesticus* (Linnaeus, 1758) Chiri	M	O	Enhance potential	35.0	0.35	35	0.99	100.00	99
				Face paralysis	35.0	0.35	35	0.99	100.00	99
48	Indian Ring Dove *Streptopelia decaocto* (Frivaldszky, 1838) Kogi	M	O	Enhance maturity	5.0	0.05	2	0.14	40.00	6
				Cold	5.0	0.05	2	0.14	40.00	6
49	Oriental turtle Dove *Streptopelia orientalis* (Latham, 1790) Kogi	M	O	Enhance maturity	5.0	0.05	2	0.14	40.00	6
				Cold	5.0	0.05	2	0.14	40.00	6
50	Little Brown Dove *Streptopelia senegalensis* (Linnaeus, 1766) Chhoti kogi	M	O	Enhance maturity	5.0	0.05	2	0.14	40.00	6
				Cold	5.0	0.05	2	0.14	40.00	6
51	Red Turtle Dove *Streptopelia tranquebarica* (Hermann, 1804) Lal kogi	M	O	Enhance maturity	5.0	0.05	2	0.14	40.00	6
				Cold	5.0	0.05	2	0.14	40.00	6
Invertebrates										
52	European honey bee *Apis mellifera* (Linnaeus, 1758) Shahd Makhi	HO	O	Stomach	24.0	0.24	11	0.68	45.83	31
				Eye diseases	24.0	0.24	11	0.68	45.83	31
				Skin diseases	24.0	0.24	11	0.68	45.83	31
				Diabetics	24.0	0.24	11	0.68	45.83	31
53	Earthworm *Pheretima hawayana* (Rosa, 1891) Gandoya	WB	O	Pain in backbone	12.0	0.12	9	0.34	75.00	25
54	Common beak *Libythea lepita* (Moore, 1857) Titli	WB	O	Antibacterial	41.0	0.41	4	1.00	9.76	10

O, oral; T, topical; S, skin; SA, saliva; SC, scale; M, meat; MI, milk; F, fat; E, egg; WB, whole body; HO, honey; H, hair; FE, feather; B, brain; BL, blood; T, tail; L, liver.

3.3 Ethnomedicinal use of herptile and fish

In the present study, we found that the local people use the herptiles and fish for different ailments. Sexual problems were found to be treated by most of the species, followed by "enhanced immunity" (Figure 5).

3.4 Ethnomedicinal use of mammals

The present study revealed that 23 diseases were treated by the documented mammals (Figure 6). The maximum use of the species to treat cold is due to the belief that the meat has the potential to

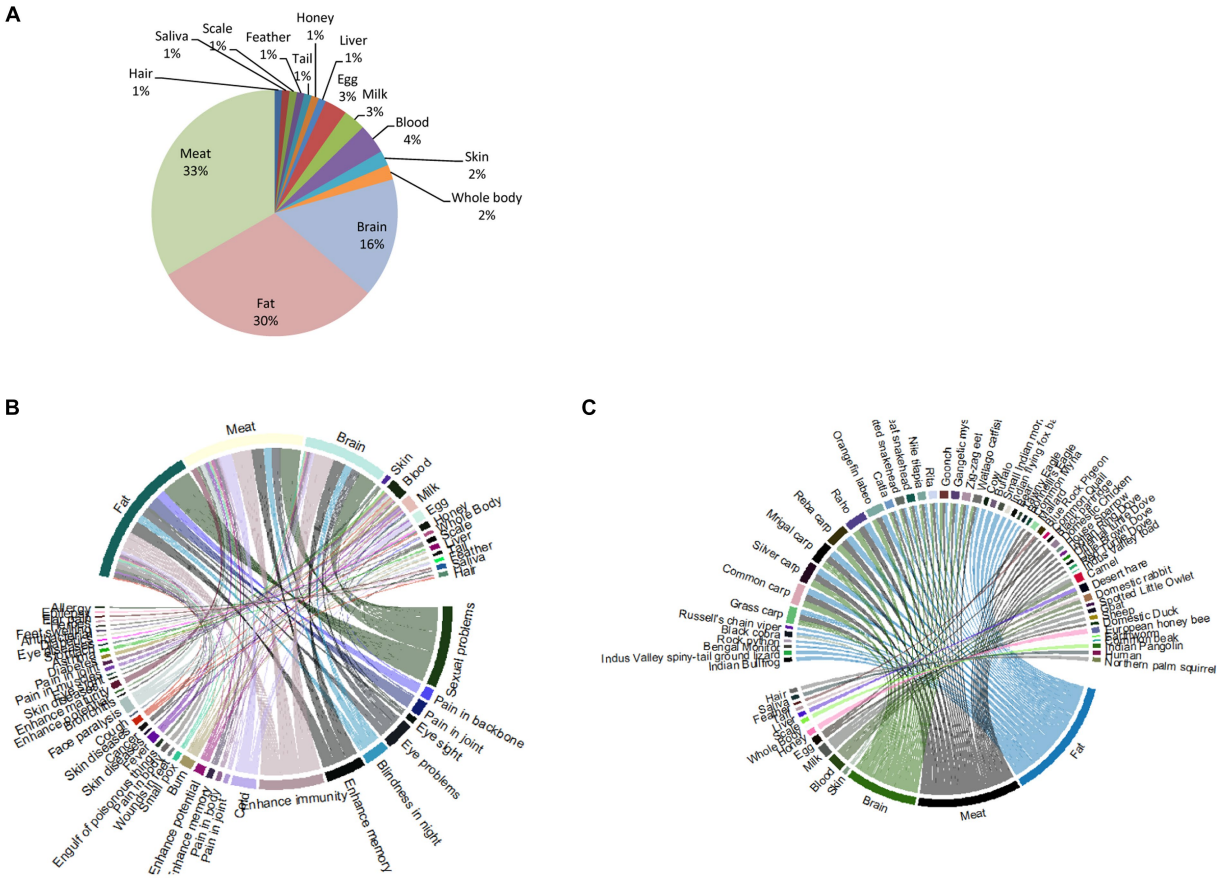


FIGURE 4
(A) Percentage of different body parts of fauna used to treat a variety of diseases in the study area. **(B)** The body parts are used to treat diseases. **(C)** Ethnopharmacological uses of body parts of animal species in the study area.

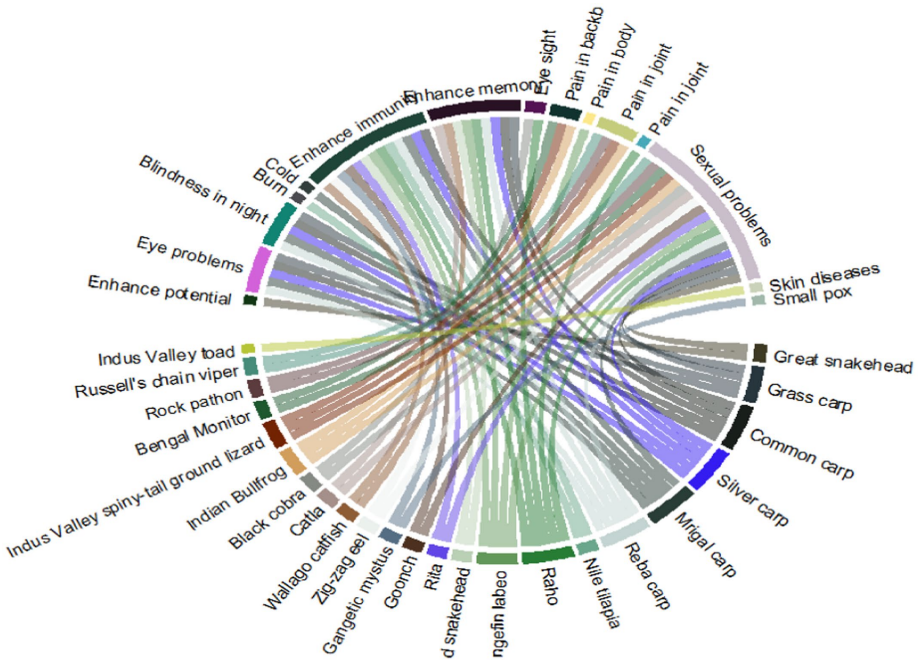


FIGURE 5
Herptiles and fish species used to cure different kinds of sicknesses in the study area.

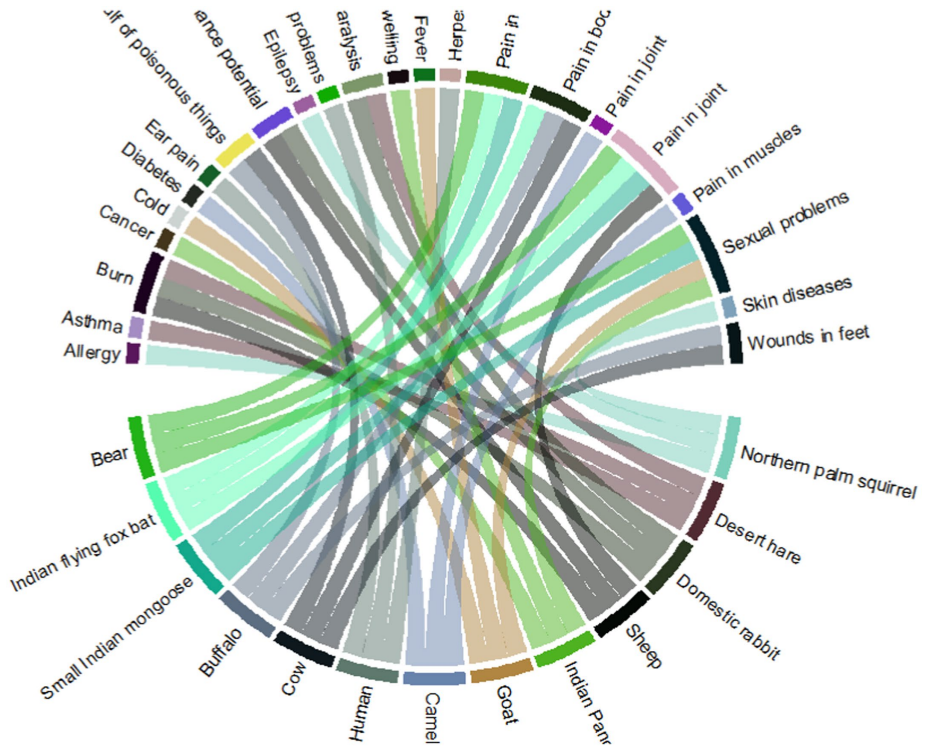


FIGURE 6
Mammalian species used to cure different kinds of sicknesses in the study area.

overcome cough and cold. Also, meat is rich in protein, which in turn provides body strength.

3.5 Ethnomedicinal use of aves and invertebrates

In the present study, 15 birds were reported to treat 13 diseases. The most frequent diseases treated were "cold" followed by "enhanced maturity" and "face paralysis" (Figure 7). Only three invertebrate species were documented, i.e., European honey bee, earthworm, and common beak, to treat different diseases. The European honey bee was recorded for ascendancy as the said species treated a maximum number of diseases (stomach, eye diseases, skin diseases, and diabetics), contrary to the other two invertebrates (Figure 8).

3.6 Frequency of citation

In the present study, different species were reported by a different number of informants. The FC ranged between 1 and 77 (Table 1; Figure 9). The highest value of FC (77) was obtained for Black cobra (for eyesight), followed by domestic rabbit (for burn) with FC = 67, and Indus Valley spiny-tail ground lizard (for sexual problems; FC = 66). The lowest value of FC = 1 was recorded for the Orangefin labeo (to enhance memory) and Reba carp (for eye problems).

3.7 The Fidelity level

According to Altaf et al. (18), FL is used for the identification of the species that are most preferred in a region by the local inhabitants for curing different diseases. Any species with the maximum medicinal uses in a region is known to have the highest fidelity level (54). A complete list of the fidelity levels of the documented species is provided in Table 1. The highest FL (100; Figure 10) was registered for Orangefin labeo, *Labeo calbus* (for enhanced memory); Reba carp, *Cirrhinus reba* (for eye problems); domestic chicken, *Gallus* (for fever and cold); house sparrow (to enhance potential and face paralysis); followed by cows (90.48) to treat pain in the body and *Ovis aries* (84.62) to treat burns, enhance potential, and to treat pain in joints. The lowest FL (8.70) was recorded for *Hypophthalmichthys molitrix* (to enhance memory).

3.8 The relative popularity level

The relative popularity level (RPL) of the species can be seen in Table 1. We grouped the species into two categories (popular and unpopular; Figure 9). Species with RPL 1 are considered to be popular, and these include species, such as Indus Valley spiny-tail ground lizard, *Uromastyx hardwickii;* Black cobra, *Naja naja naja;* Raho, *Labeo rohita;* Buffalo, *Bubalus bubalis;* Camel, *Camelus dromedaries;* Goat, *Capra aegagrus hircus;* Domestic rabbit, *Oryctolagus cuniculus;* Blue Rock Pigeon, *Columba livia;* and Common beak, *Libythea lepita*.

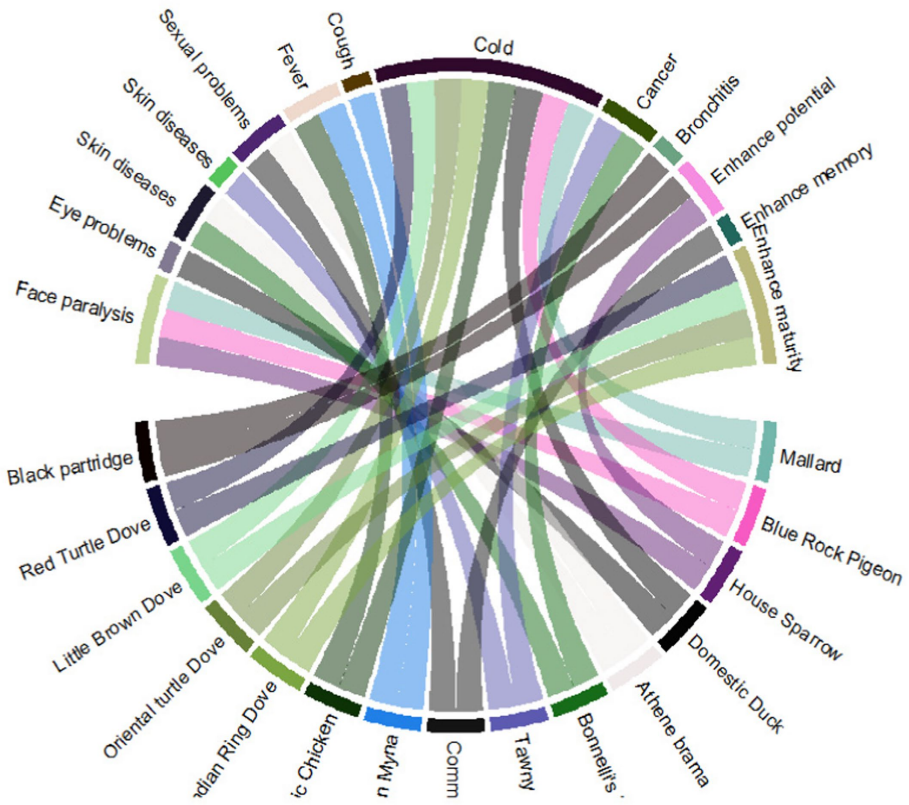


FIGURE 7
Avian species used to cure different kinds of sicknesses in the study area.

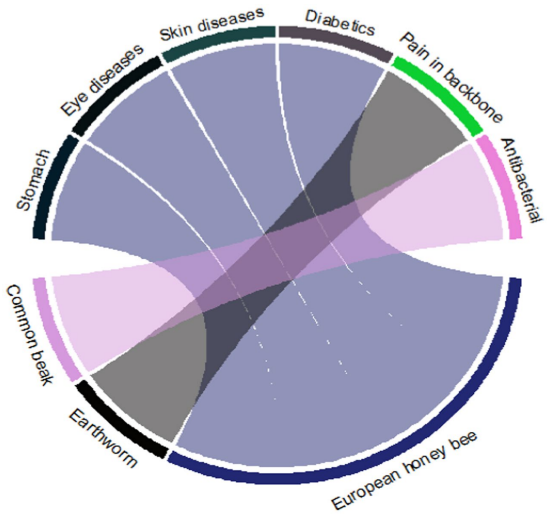


FIGURE 8
Invertebrate species used to cure different kinds of sicknesses in the study area.

3.9 Rank order priority

Rank order priority (ROP) is employed to assign an appropriate grade to the documented species with different FL values. The calculated ROP values for each species are presented in Table 1. *Passer domesticus* and *Gallus gallus* were the species with the highest ROP (99), followed by *Ovis aries* (62), *Camelus dromedaries* (59), *Bubalus bubalis* (57), *Channa punctata* (56), *Labeo rohita* (50), and *Uromastyx hardwickii* (42; Table 1; Figure 7).

4 Discussion

Meat contains nitrogenous and non-nitrogenous substances, water, lipids, sodium, magnesium, glycogen, lactic acid, potassium, iron, calcium, phosphorus, and chlorine (68, 69). Meat composition varies owing to the effects of many environmental elements and internal characteristics such as animal species, diet, muscle, breed, and sex (70). Poultry, cattle, sheep, goat, fish, and pork are the most common meat sources globally. However, in a few nations, particularly in arid and semi-arid regions, camel meat is renowned as the primary source of animal protein that equals, and in some cases exceeds, the commercial importance of other meats (31, 71–74). Bones include up to 95% elastic protein, collagen fibers, and inorganic minerals such as calcium and phosphate, which help to prevent bone fracture. Ethnozoologists discovered that various species of animals, including the Indian gagata, horse, goat, fruit bat, deer, crow, crab-eating macaque, common carp, cinereous vulture, and alpine musk deer, were used to treat a variety of ailments, including wound healing, digestion, heart strength, ear ache, lumbago, skin, chest pain, and urine problems (18, 29, 55, 75–84).

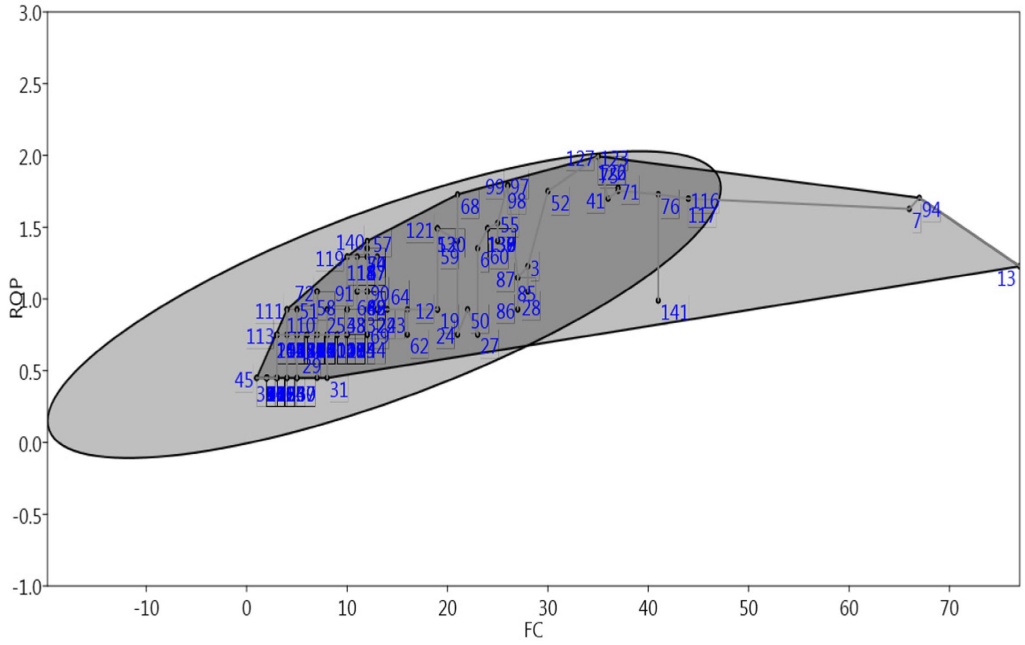


FIGURE 9
A comparison of FC and ROP values.

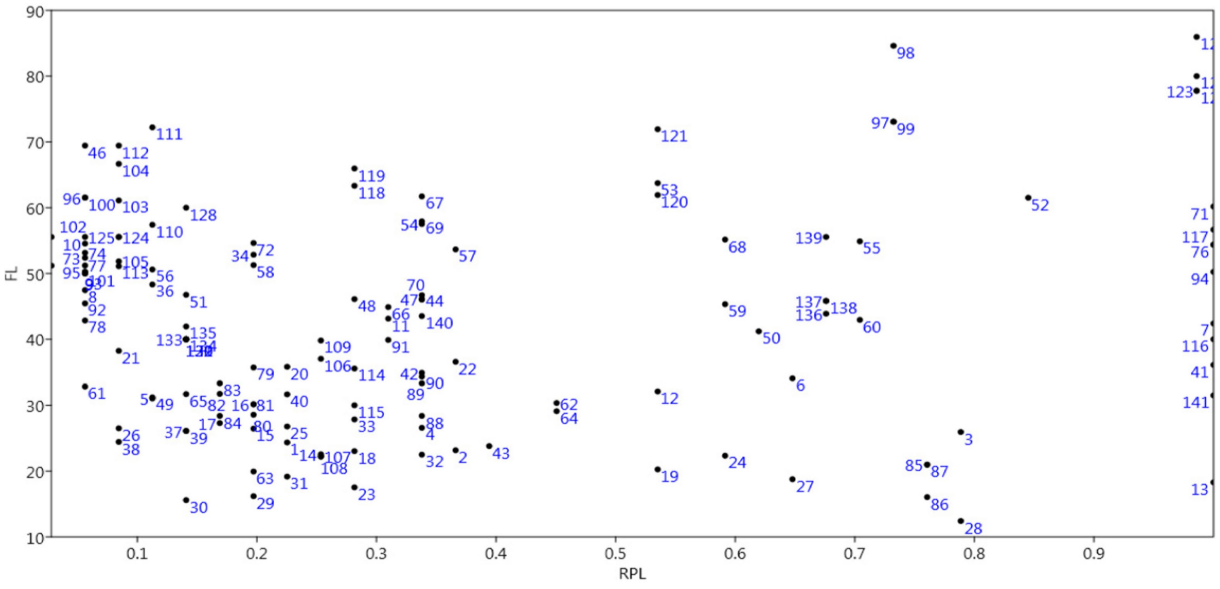


FIGURE 10
A comparison of FL and RPL values.

Nanoparticles can operate as carriers for fish oil ingredients (85), preserving them from degradation in the gastrointestinal system and allowing for regulated release at particular locations throughout the body. Several forms of nanoparticles, including liposomes (86), nanoemulsions (87), and polymeric nanoparticles (88), have been investigated for encapsulating fish oil or its active components, such as eicosapentaenoic acid (89) and docosahexaenoic acid (90). Docosahexaenoic acid has received a lot of interest in the field of nanomedicine because of its potential health advantages and therapeutic qualities. These fatty acids play important roles in a variety of physiological processes, including cancer (91), inflammation (92), and diabetic problems (93). Omega-3 fatty acids in vertebrate fats have been shown to reduce inflammation. Ethnobiologists discovered that lipids are utilized to treat neurological disorders, atherosclerosis, thrombosis, and the effects of aging (32, 94–96). The previous published data showed that fats of various animals species, i.e., wild boar (*Sus scrofa*), turtle

(Aspideretes sp.), streaked prochilod (*Prochilodus platensis*), sheep (*Ovis* sp.), mongoose (*Herpestes* sp.), bat (*Pteropus* sp.), lizard (*Hemidactylus* sp.), Irrawaddy, dolphin (*Orcaella brevirostris*), Indus Valley spiny-tail ground lizard (*Saara hardwickii*), Indian rock python (*Python molurus*), Indian flap-shelled turtle (*Lissemys punctata andersoni*), Indian bullfrog (*Hoplobatrachus tigerinus*), horse (*Equus* sp.), Himalayan Serow (*Capricornis thar*), hen (*Gallus* sp.), jackal (*Canis* sp.), hare (*Lepus* sp.), green pond frog (*Euphlyctis Hexadactylus*), goat (*Capra aegagrus hircus*), deer (Cervidae), cow (Bos sp.), common leopard gecko (*Eublepharis macularius*), cat (*Felis* sp.), buffalo (*Bubalus bubalis*) and Asiatic black bear (*Ursus* sp.), are used to cure different ailments such as wounds and injuries, toothache (97), wound skin burn and crack, pain in back (26, 98), sexual stimulant (18, 26, 29, 82, 97, 99, 100), rheumatism (26, 82), pain in muscles (82, 83), menstruation problem (84), asthma (26, 82, 83), impotency (18, 29), head pain (98), erysipelas (83), ear disease, wounds (84, 97), cancer (29), asthma, wart (26, 97), arthritis (26), anemia, fever, paralysis (26, 83), allergy, wound, skin disease (26, 76, 83), allergy, typhoid, ear infection (82, 101), and pain in joints (100).

Milk is one of the most important and oldest foods. Mammalian species' milk consists of lactose, ash, fats, proteins, solids, and water (102–111). Milk of different mammalian species, i.e., *Panthera tigris*, *Ovis aries*, *Muntiacus muntjak*, *Homo sapiens*, *Equus caballus*, *Equus asinus*, *Equus africanus*, *Capra hircus*, *Capra aegagrus*, *Camelus dromedaries*, *Bubalus bubalis*, and *Bos taurus*, is used to cure different sicknesses, i.e., enhance immunity, wound, whooping cough, skin burn, sexual power, red eyes, pain, muscular pain, jaundice, invigorative, hiccup, hepatitis, headache, gastritis, eye, diabetes, cough, cold, catarrh, and arthritis (7, 18, 26, 82, 84, 112–126).

The egg is a good source of protein and nutrients for people and a source of chemicals and elements such as "phosphorus," "selenium," "amino acid," "iron, vitamins A, B6, B12," and "folic acid." Asthma, high blood pressure, breast cancer, bronchitis, burns, CNS, cold, diabetes, eye diseases, fever, hemorrhoids, indigestion, jaundice, mental disorders, night blindness, nourishment, sinusitis, sprains, tooth, weak eye side, weakness, and weight loss are all treated with eggs (18, 26, 37, 114, 115, 119–121, 124, 127–138). The egg has components that provide the best environment for an embryo's development and growth. Except for vitamin C, it is a major source of important nutrients for humans. Eggs are an incredibly tasty and healthy item that can be utilized in a variety of ways (37).

Feather is utilized as a biomaterial since it is inexpensive and environmentally beneficial. Feathers are made up of "α-helix" and "β-sheet." Bird feathers are utilized for decoration and as toys. Feathers of various species are used in traditional medicine, e.g., *Phalacrocorax brasilianus*, *Nothura boraquira*, *Meleagris gallopayo*, *Coryus splendens*, *Corythaeola cristata*, *Coragyps atratus*, *Columba livia*, and *Ceryle rudis*, to treat alcoholism, asthma, cough, cough, flu, headache, and typhoid (49, 82, 112, 113, 119, 121, 124, 133, 139–142). Feathers are used for a variety of purposes, such as antibacterial activity modification, biosorbents, cell viability enhancement, cosmetic micro- and nanoparticles, and wound dressing in industry. Graphene Oxide is utilized as a bio-composites, bio-fertilizer, biomaterial, feeding supplement, bioplastic, electrode material, fire-resistant substance, leather processing, paper formation, protein for ruminants, regenerated fibers, textile fibers, thermoplastic films, tissue reformation, and wound healing (49, 143–164).

Honey is composed of amino acids (165, 166), minerals (167), organic acids, phenolic compounds (168), solid particles (169), sugars (170), vitamins (171), water, proteins, disaccharides (172–174), and volatile compounds (175). Honey is utilized as a therapy in folk medicine to heal acidity, obesity, allergy, Alzheimer's disease, asthma, atherosclerosis, burn, cancer, cold, cough, diabetes mellitus, diarrhea, expectorant, eye infection, gastritis, hypertension, influenza, migraine, skin, snake-bite, spleen, throat pain, tonsils, toothache, and urinary system (7, 18, 30, 81, 82, 84, 98, 115–117, 119, 120, 123, 124, 126, 137, 176–183). Honey is also used in nanomedicine to heal diverse sicknesses and behaves as oxidative stress, heart, blood pressure, anti-proliferative, antioxidant, anti-inflammatory, anti-fungal endophthalmitis, anti-diabetic, anti-cataract, antibiotic, antibacterial, and anti-apoptosis (184–199). Many fauna species have been shown to be quite adaptable in their applications. The maximum relative significance levels may indicate that animals are easily accessible and affordable (200–202).

5 Conclusion

The folklore animal-based medicinal concept of Gujranwala communities indicates that people have a strong link with ecology. The ethnopharmacological benefits of the fauna in Gujranwala were documented for the first time. In addition, 54 fauna species are employed to treat various human ailments. Different body parts of animals (*viz.* skin, fat, brain, meat, milk, blood, hair, saliva, flesh, liver, scale, tail, egg, feather, honey, and whole body) are used to treat various diseases such as skin infection, sexual problems, pain in the backbone, joint pain, eyesight, night blindness, enhance immunity and memory, cold, weakness, body pain, burn, smallpox, wounds in feet, engulf of poisonous things, pain in muscles, arthritis, diabetes, fever, epilepsy, allergy, asthma, burn, herpes, ear pain, paralysis, cough, swelling, cancer, bronchitis, girls maturity, stomach, and antibacterial infections. The present results provide data that may be constructive for the conservation of fauna in the district of Gujranwala, Punjab, Pakistan. Screening of bioactive materials and "*in vivo*" and/or "*in vitro*" studies of the zoological activities of the species with the highest FC, FL, RPL, and ROP, may be significant for wild animal-based novel medications. Due to financial constraints, the local population lives in isolated, hilly villages that are far from urban areas, primarily involved in agricultural labor, home-based businesses, and livestock rearing. The locals heavily rely on medicinal flora to meet their basic medical needs. While nearby regions such as Malakand, Dir Lower, Chitral, and Swat have been thoroughly investigated for their medicinal plants, this study focuses specifically on Dir Upper. This investigation supported the theory that the local indigenous knowledge would differ significantly from its surroundings. Notably, 80% of people in developing nations rely on herbal health remedies. The primary healthcare needs are met in large part by local healers. Medicinal plants with significant UV protection were found to protect the studied area's biodiversity. Unfortunately, anthropogenic practices, such as overharvesting, overpopulation, and grazing, endanger regional biodiversity. To stop the impending extinction of medicinal plants in the study area, initiatives for cultivating these species must be implemented immediately. This strategy will lessen the risks brought on by human activity and maintain the availability of vital plant resources for future generations.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

AK: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. MA: Conceptualization, Investigation, Methodology, Project administration, Writing – original draft. TH: Project administration, Supervision, Validation, Writing – review & editing. AA: Resources, Validation, Writing – review & editing. Z-nM: Resources, Validation, Writing – review & editing. AH: Data curation, Validation, Writing – review & editing. SA: Data curation, Writing – original draft. US: Data curation, Investigation, Writing – review & editing. MSA: Supervision, Validation, Visualization, Writing – review & editing. MM: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. MH: Validation, Methodology, Writing – review & editing. RB: Supervision, Validation, Visualization, Writing – review & editing. AMA: Conceptualization, Project administration, Supervision, Validation, Visualization, Writing – review & editing. MA-Y: Validation, Visualization, Writing – review & editing. HE: Validation, Visualization, Writing – review & editing. EM: Validation, Visualization, Writing – review & editing. MHH: Methodology, Data curation, Writing – original draft preparation, Writing – review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Research and Innovations "Ministry of Education" in Saudi Arabia (IFKSUOR3-095-6).

Acknowledgments

The authors extend their appreciation to the Deputyship for Research and Innovations "Ministry of Education" in Saudi Arabia for funding this research (IFKSUOR3-095-6).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Faiz M, Adil S, Nawaz A. Chemical composition, ethnopharmacological applications of animal biles-mini review. *J Wildlife Ecol*. (2022) 6:172–7.

2. Altaf M. *Assessment of avian and mammalian diversity at selected sites along river Chenab*. Lahore, Pakistan: University of Veterinary and Animal Sciences (2016).

3. Costa-Neto EM. Animal-based medicines: biological prospection and the sustainable use of zootherapeutic resources. *An Acad Bras Cienc*. (2005) 77:33–43. doi: 10.1590/S0001-37652005000100004

4. Holennavar P. Use of animal and animal derived products as medicines by the inhabitants of villages in Athani taluka of Belagavi District. *Karnataka Int J App Res*. (2015) 1:437–40. doi: 10.1007/s10668-019-00404-6

5. Kassam A. *World resources 2000–2001: people and ecosystems: the fraying web of life*. Washington, DC: World resources institute (2000) (2002). 389 p.

6. Lawal O, Banjo A. Survey for the usage of arthropods in traditional medicine in southwestern Nigeria. *J Entomol*. (2007) 4:104–12. doi: 10.3923/je.2007.104.112

7. Lev E. Traditional healing with animals (zootherapy): medieval to present-day Levantine practice. *J Ethnopharmacol*. (2003) 85:107–18. doi: 10.1016/S0378-8741(02)00377-X

8. Singh V. *A note on the use of wild animal organs in Tibetan medicine. Traffic India*. Lodhi Estate, New Delhi: WWF (2000).

9. Still J. Use of animal products in traditional Chinese medicine: environmental impact and health hazards. *Complement Ther Med*. (2003) 11:118–22. doi: 10.1016/S0965-2299(03)00055-4

10. Unnikrishnan PM. Animals in ayurveda. *Amruth*. (1998):1–15.

11. Arif U, Bhatti KH, Ajaib M, Wagay NA, Majeed M, Zeb J, et al. Ethnobotanical indigenous knowledge of tehsil Charhoi, district Kotli, Azad Jammu and Kashmir, Pakistan. *Ethnobot Res Appl*. (2021) 22:1–24. doi: 10.32859/era.22.50.1-24

12. Arshad F, Waheed M, Harun N, Fatima K, Khan BA, Fatima K, et al. Indigenous farmer's perception about fodder and foraging species of semi-arid lowlands of Pakistan: A case study of district Kasur Pakistan. *Taiwania*. (2022) 67:510–23. doi: 10.6165/tai.2022.67.510

13. Bashir SM, Altaf M, Hussain T, Umair M, Majeed M, Mangrio WM, et al. Vernacular taxonomy, cultural and ethnopharmacological applications of avian and mammalian species in the vicinity of Ayubia National Park. *Himalayan Region Biol*. (2023) 12:609. doi: 10.3390/biology12040609

14. Haq SM, Yaqoob U, Majeed M, Amjad MS, Hassan M, Ahmad R, et al. Quantitative ethnoveterinary study on plant resource utilization by indigenous communities in high-altitude regions. *Front Vet Sci*. (2022) 9:944046. doi: 10.3389/fvets.2022.944046

15. Majeed M, Lu L, Anwar MM, Tariq A, Qin S, El-Hefnawy ME, et al. Prediction of flash flood susceptibility using integrating analytic hierarchy process (AHP) and frequency ratio (FR) algorithms. *Front Environ Sci*. (2023) 10:1037547. doi: 10.3389/fenvs.2022.1037547

16. Majeed M, Tariq A, Haq SM, Waheed M, Anwar MM, Li Q, et al. A detailed ecological exploration of the distribution patterns of wild Poaceae from the Jhelum District (Punjab). *Pakistan Sustainability*. (2022) 14:3786. doi: 10.3390/su14073786

17. Saeed A, Umair M, Altaf M, Hussain T, Abbasi AM. Ethno-veterinary medicines of South Punjab. *Pakistan J Wildlife Ecol*. (2022) 6:64–81.

18. Altaf M, Umair M, Abbasi AR, Muhammad N, Abbasi AM. Ethnomedicinal applications of animal species by the local communities of Punjab Pakistan. *J Ethnobiol Ethnomed*. (2018) 14:55. doi: 10.1186/s13002-018-0253-4

19. Alves RR, Rosa IL. Zootherapy goes to town: the use of animal-based remedies in urban areas of NE and N Brazil. *J Ethnopharmacol*. (2007) 113:541–55. doi: 10.1016/j.jep.2007.07.015

20. Mirza ZB, Wasiq H. *A field guide to birds of Pakistan Bookland*. Lahore: (2007).

21. Roberts TJ. *The mammals of Pakistan*. New Yark: Oxford University Press (1997).

22. Khan MS. Checklist of amphibians of Pakistan. *Pakistan J Wildlife*. (2010) 1

23. Khan MS. *Amphibian and reptiles of Pakistan*. Pakistan: Krieger Publisher Company (2006).

24. Altaf M. Study of avian diversity in urban areas of Gujranwala, Pakistan. *Int J of*. (2021)

25. Altaf M. *Assessment of avian and mammalian diversity at selected sites along river Chenab*. Lahore-Pakistan: University of Veterinary and Animal Sciences (2016).

26. Altaf M, Javid A, Umair M, Iqbal KJ, Rasheed Z, Abbasi AM. Ethnomedicinal and cultural practices of mammals and birds in the vicinity of river Chenab Punjab-Pakistan. *J Ethnobiol Ethnomed*. (2017) 13:1–24.

27. Altaf M, Khan AM, Umair M, Irfan MMA. Status of wild birds and mammals in urban habitats of Gujranwala, Punjab Pakistan. *Punjab Univ J Zool*. (2012) 27:9–12.

28. Altaf M, Javid A, Khan AM, Hussain A, Umair M, Ali Z. The status of fish diversity of river Chenab, Pakistan. *J Animal & Plant Sci*. (2015) 25:564–9.doi: 10.1186/s13002-017-0168-5

29. Altaf M, Abbasi AM, Umair M, Amjad MS, Irshad K, Khan AM. The use of fish and herptiles in traditional folk therapies in three districts of Chenab riverine area in Punjab. *Pakistan J Ethnobiol Ethnomed*. (2020) 16:1–21. doi: 10.1186/s13002-020-00379-z

30. Altaf M, Umair M. Diversity, distribution and medicinal importance of honeybees in the world-A review. *J Wildlife Ecol*. (2020) 4:130–41.

31. Haidar R, Bashir SM. Chemical composition, traditional and modern uses of meat of animals-a review. *J Wildlife Ecol*. (2021) 5:47–55.

32. Ijaz S, Faiz M. Chemical composition, folk and modern uses of fats and oil-a review. *J Wildlife Ecol*. (2021) 5:104–10.

33. Khoja AA, Haq SM, Majeed M, Hassan M, Waheed M, Yaqoob U, et al. Diversity, ecological and traditional knowledge of pteridophytes in the western Himalayas. *Diversity*. (2022) 14:628. doi: 10.3390/d14080628

34. Majeed M, Bhatti KH, Amjad MS. Impact of climatic variations on the flowering phenology of plant species in JHELUM district, Punjab Pakistan. *Appl Ecol Environ Res*. (2021) 19:3343–76. doi: 10.15666/aeer/1905_33433376

35. Majeed M, Bhatti KH, Pieroni A, Sõukand R, Bussmann RW, Khan AM, et al. Gathered wild food plants among diverse religious groups in Jhelum District, Punjab, Pakistan. *Food Secur*. (2021) 10:594. doi: 10.3390/foods10030594

36. Majeed M, Tariq A, Anwar MM, Khan AM, Arshad F, Mumtaz F, et al. Monitoring of land use–land cover change and potential causal factors of climate change in Jhelum district, Punjab, Pakistan, through GIS and multi-temporal satellite data. *Landscape*. (2021) 10:1026. doi: 10.3390/land10101026

37. Tariq S. Chemical composition and traditional uses of eggs of different avian species-A review. *J Wildlife Ecol*. (2020) 4:45–50.

38. Akhtar S. Antimicrobial effect of silver nanoparticles synthesized from skin of *Laudakia agrorensis*. *J Wildlife Ecol*. (2021) 5:176–85.

39. Altaf M, Faiz M. Snake venom-a review. *J Wildlife Ecol*. (2021) 5:146–58.

40. Habib S. Antibacterial activity of biogenic synthesized silver nanoparticles using skin of Kashmir Nadi frog *Paa barmoachensis*. *J Wildlife Ecol*. (2022) 6:07–12.

41. Nazir N. Biosynthesis of silver nanoparticles from skin of Hazara toad (*Bufo melanostictus*) and assessment of antibacterial activity. *J Wildlife Ecol*. (2021) 5:111–9.

42. Zainab S. Antibacterial and antibiofilm activity of bull frog *Hoplobatrachus tigerinus* skin extract. *J Wildlife Ecol*. (2021) 5:32–7.

43. Khan MSH, Ullah S, Hamed MH, Altaf M. A study of illegal wildlife trade and seizures in Pakistan. *J Wildlife Ecol*. (2020) 4:193–210.

44. Alves RR. Relationships between fauna and people and the role of ethnozoology in animal conservation. *Ethnobiol Conserv*. (2012) 1:1–69. doi: 10.15451/ec2012-8-1.2-1-69

45. Alves RRN, Silva JS, da Silva CL, Albuquerque UP. Ethnozoology and animal conservation. *In: Ethnozoology Elsevier*. (2018):481–96. doi: 10.1016/B978-0-12-809913-1.00025-9

46. Boivin NL, Zeder MA, Fuller DQ, Crowther A, Larson G, Erlandson JM, et al. Ecological consequences of human niche construction: examining long-term anthropogenic shaping of global species distributions. *Proc Natl Acad Sci*. (2016) 113:6388–96. doi: 10.1073/pnas.1525200113

47. Dickman AJ. Complexities of conflict: the importance of considering social factors for effectively resolving human–wildlife conflict. *Animal*. (2010) 13:458–66. doi: 10.1111/j.1469-1795.2010.00368.x

48. Saunders CD. The emerging field of conservation psychology. *Hum Ecol Rev*. (2003):137–49.

49. Adil S, Tariq S. Study of traditional and modern applications of feathers-a review. *J Wildlife Ecol*. (2020) 4:141–50.

50. Ali A, Khan MSH, Altaf M. Winter survey of birds at district of the Badin, Pakistan. *J Wildlife Ecol*. (2018) 2:11–22.

51. Altaf M, Abbasi AM, Umair M, Amjad MS, Muhammad N, Iqbal KJ, et al. The usage of freshwater fishes in cultural and folklore therapies among the people along river Jhelum, Punjab Pakistan. *J Wildlife Ecol*. (2021) 5:79–99.

52. Aslam H, Faiz M. Ethnopharmacological and modern applications of milk of various mammalian species-a review. *J Wildlife Ecol*. (2020) 4:211–26.

53. Hassan M, Haq SM, Ahmad R, Majeed M, Sahito HA, Shirani M, et al. Traditional use of wild and domestic Fauna among different ethnic groups in the Western Himalayas—A cross cultural analysis. *Animals*. (2022) 12:2276. doi: 10.3390/ani12172276

54. Hassan M, Haq SM, Majeed M, Umair M, Sahito HA, Shirani M, et al. Traditional food and medicine: Ethno-traditional usage of fish Fauna across the valley of Kashmir: A western Himalayan region. *Diversity*. (2022) 14:455. doi: 10.3390/d14060455

55. Ijaz S, Iftikhar A. Chemical composition, ethnomedicinal and industrial uses of bones-a review. *J Wildlife Ecol*. (2021) 5:56–9.

56. Khan AM, Li Q, Saqib Z, Khan N, Habib T, Khalid N, et al. MaxEnt modelling and impact of climate change on habitat suitability variations of economically important chilgoza pine (*Pinus gerardiana* wall.) in South Asia. *Forests*. (2022) 13:715. doi: 10.3390/f13050715

57. Majeed M, Khan AM, Habib T, Anwar MM, Sahito HA, Khan N, et al. Vegetation analysis and environmental indicators of an arid tropical forest ecosystem of Pakistan. *Ecol Indic*. (2022) 142:109291. doi: 10.1016/j.ecolind.2022.109291

58. Majeed M, Lu L, Haq SM, Waheed M, Sahito HA, Fatima S, et al. Spatiotemporal distribution patterns of climbers along an abiotic gradient in Jhelum district, Punjab. *Pakistan Forests*. (2022) 13:1244. doi: 10.3390/f13081244

59. Mughal S, Pervaz M, Bashir SM, Shamashad SS. Assessment of diversity and ethnopharmacological uses of birds in chakar, Hattian Bala district, Azad Jammu and Kashmir-Pakistan. *J Wildlife Ecol*. (2020) 4:35–44.

60. Noor U, Haider R. Assessment of herpetofauna diversity and human-herpetofauna-interaction in district Sudhnoti, Azad Jammu and Kashmir Pakistan. *J Wildlife Ecol*. (2020) 4:156–63.

61. Saleem R, Altaf M, Umair M, Amjad MS, Abbasi AM. Ethnopharmacological applications of the amphibians and reptiles among the people in the vicinity of Margalla Hill National Park, Islamabad Pakistan. *J Wildlife Ecol*. (2021) 5:13–25.

62. Chattha SA, Malik MF, Altaf M, Mahmood S, Khan J, Ali A, et al. Human pursuits cause of road killing of wild and domestic animals by accident on National Highway of Punjab Pakistan. *J Wildlife Ecol*. (2017) 1:8–16.

63. Hakeem F, Altaf M, Manzoor S, Rauf K, Mumtaz B, Bashir M, et al. Assessment of behavioral study, human activities impacts and interaction with streak laughingthrush (*Trochalopteron lineatum*) in district Bagh, Azad Jammu and Kashmir-Pakistan. *J Wildlife Ecol*. (2017) 1:1–7.

64. Muhammad N, Khan AM, Umair M, Qazi A, Yaqoob M, Ashraf S, et al. Assessment of distribution and ethnocultural uses of the sol (*Channa marulius*) in Punjab. *Pakistan J Wildlife Ecol*. (2017) 1:35–41.

65. Alexiades MN, Sheldon JW. *Selected guidelines for ethnobotanical research: A field manual*. New York: Botanical Garden (1996).

66. Friedman J, Yaniv Z, Dafni A, Palewitch D. A preliminary classification of the healing potential of medicinal plants, based on a rational analysis of an ethnopharmacological field survey among Bedouins in the Negev Desert, Israel. *J Ethnopharmacol*. (1986) 16:275–87. doi: 10.1016/0378-8741(86)90094-2

67. Ali-Shtayeh MS, Yaniv Z, Mahajna J. Ethnobotanical survey in the Palestinian area: a classification of the healing potential of medicinal plants. *J Ethnopharmacol*. (2000) 73:221–32. doi: 10.1016/S0378-8741(00)00316-0

68. Hui YH. *Handbook of meat and meat processing*. US: CRC press (2012).

69. Keeton JT, Eddy S. Chemical composition In: *Yclopedia of meat sciences: Enc*. Oxford: Elsevier Academic Press (2004)

70. Cheung PCK, Mehta BM. *Handbook of food chemistry*. Berlin Heidelberg: Springer (2015).

71. Abrhaley A, Leta S. Medicinal value of camel milk and meat. *J Appl Anim Res*. (2018) 46:552–8. doi: 10.1080/09712119.2017.1357562

72. Faiz M. Folklore or facts: investigating the nutritional and ethnopharmacological roles of animal parts. *J Wildlife Ecol*. (2023) 7:211–6.

73. Schönfeldt HC, Gibson N. Changes in the nutrient quality of meat in an obesity context. *Meat Sci*. (2008) 80:20–7. doi: 10.1016/j.meatsci.2008.05.025

74. Williams P. Nutritional composition of red meat. *Nutr Diet*. (2007) 64:S113–9. doi: 10.1111/j.1747-0080.2007.00197.x

75. Altaf M, Altaf M, Khichi TAA. Cultural significance and medicinal applications of herpetofauna in Bagh, Azad Jammu and Kashmir, Pakistan. *J WIldlife Ecol*. (2023) 7

76. Bullitta S, Re GA, Manunta MDI, Piluzza G. Traditional knowledge about plant, animal, and mineral-based remedies to treat cattle, pigs, horses, and other domestic animals in the Mediterranean island of Sardinia. *J Ethnobiol Ethnomed*. (2018) 14:50. doi: 10.1186/s13002-018-0250-7

77. Ghosh T, Singhamahapatra R, Mandal F. Traditional use of animals among Santhals of Bankura district. *Int J Latest Res Sci Technol*. (2013) 2:95–6.

78. Hall J. *Textbook of medical physiology*. Philadelphia: Elsevier (2011).

79. Hussain T, Altaf M. *Ethnobiology of Cholistan*. Pakistan: PEACE International Publisher (2023).

80. Muhammad N, Altaf M, Abbasi AM, Khan AM, Iqbal KJ. From water to remedy: fishes as ethnopharmacological resources along the river Ravi, Punjab, Pakistan. *J WIldlife Ecol*. (2023) 7

81. Vallejo JR, González JA. Fish-based remedies in Spanish ethnomedicine: a review from a historical perspective. *J Ethnobiol Ethnomed*. (2014) 10. doi: 10.1186/1746-4269-10-37

82. Vijayakumar S, Yabesh JM, Prabhu S, Ayyanar M, Damodaran R. Ethnozoological study of animals used by traditional healers in Silent Valley of Kerala, India. *J Ethnopharmacol*. (2015) 162:296–305. doi: 10.1016/j.jep.2014.12.055

83. Vijayakumar S, Prabhu S, Yabesh JM, Prakashraj R. A quantitative ethnozoological study of traditionally used animals in Pachamalai hills of Tamil Nadu, India. *J Ethnopharmacol*. (2015) 171:51–63. doi: 10.1016/j.jep.2015.05.023

84. Yeshi K, Morisco P, Wangchuk P. Animal-derived natural products of Sowa Rigpa medicine: their pharmacopoeial description, current utilization and zoological identification. *J Ethnopharmacol*. (2017) 207:192–202. doi: 10.1016/j.jep.2017.06.009

85. Kadakia E, Harpude P, Parayath N, Bottino D, Amiji M. Challenging the CNS targeting potential of systemically administered nanoemulsion delivery systems: a case study with rapamycin-containing fish oil nanoemulsions in mice. *Pharm Res*. (2019) 36:1–12. doi: 10.1007/s11095-019-2667-7

86. Hadian Z. A review of nanoliposomal delivery system for stabilization of bioactive omega-3 fatty acids. *Electron Physician*. (2016) 8:1776–85. doi: 10.19082/1776

87. Khoshbou Lahijani L, Ahari H, Sharifan A. Enhancement of food safety using nanoemulsion with emphasize on fish food: A review. *Sustain Aquacul Health Manag J*. (2019) 5:26–44.

88. Miyazawa T, Itaya M, Burdeos GC, Nakagawa K, Miyazawa T. A critical review of the use of surfactant-coated nanoparticles in nanomedicine and food nanotechnology. *Int J Nanomedicine*. (2021) 16:3937–99. doi: 10.2147/IJN.S298606

89. Escobar-García JD, Prieto C, Pardo-Figuerez M, Lagaron JM. Room temperature nanoencapsulation of bioactive eicosapentaenoic acid rich oil within whey protein microparticles. *Nano*. (2021) 11:575. doi: 10.3390/nano11030575

90. Guo P, Si M, Wu D, Xue HY, Hu W, Wong HL. Incorporation of docosahexaenoic acid (DHA) enhances nanodelivery of antiretroviral across the blood-brain barrier for treatment of HIV reservoir in brain. *J Control Release*. (2020) 328:696–709. doi: 10.1016/j.jconrel.2020.09.050

91. Wen X, Reynolds L, Mulik RS, Kim SY, Van Treuren T, Nguyen LH, et al. Hepatic arterial infusion of low-density lipoprotein docosahexaenoic acid nanoparticles selectively disrupts redox balance in hepatoma cells and reduces growth of orthotopic liver tumors in rats. *Gastroenterology*. (2016) 150:488–98. doi: 10.1053/j.gastro.2015.10.008

92. Alaarg A, Jordan NY, Verhoef JJ, Metselaar JM, Storm G, Kok RJ. Docosahexaenoic acid liposomes for targeting chronic inflammatory diseases and cancer: an in vitro assessment. *Int J Nanomedicine*. (2016) 11:5027–40. doi: 10.2147/IJN.S115995

93. Hussein J, Rasheed W, Ramzy T, Nabeeh M, Harvy M, El-Toukhy S, et al. Synthesis of docosahexaenoic acid–loaded silver nanoparticles for improving endothelial dysfunctions in experimental diabetes. *Hum Exp Toxicol*. (2019) 38:962–73. doi: 10.1177/0960327119843586

94. Breteler MM. Vascular risk factors for Alzheimer's disease: an epidemiologic perspective. *Neurobiol Aging*. (2000) 21:153–60. doi: 10.1016/S0197-4580(99)00110-4

95. Haag M. Essential fatty acids and the brain. *Can J Psychiatr*. (2003) 48:195–203. doi: 10.1177/070674370304800308

96. Wilson L. *Fats and oils for optimum health* The Center for Development (2015).

97. Kendie FA, Mekuriaw SA, Dagnew MA. Ethnozoological study of traditional medicinal appreciation of animals and their products among the indigenous people of Metema woreda, North-Western Ethiopia. *J Ethnobiol Ethnomed*. (2018) 14:37. doi: 10.1186/s13002-018-0234-7

98. Sreekeesoon DP, Mahomoodally MF. Ethnopharmacological analysis of medicinal plants and animals used in the treatment and management of pain in Mauritius. *J Ethnopharmacol*. (2014) 157:181–200. doi: 10.1016/j.jep.2014.09.030

99. Haq SM, Calixto ES, Yaqoob U, Ahmed R, Mahmoud AH, Bussmann RW, et al. Traditional usage of wild Fauna among the local inhabitants of Ladakh. *Trans-Himalayan Region Animals*. (2020) 10:2317.

100. Nijman V, Shepherd CR. Ethnozoological assessment of animals used by Mon traditional medicine vendors at Kyaiktiyo, Myanmar. *J Ethnopharmacol*. (2017) 206:101–6. doi: 10.1016/j.jep.2017.05.010

101. Kim H, Song M-J. Analysis of ethnomedicinal practices for treating skin diseases in communities on Jeju Island (Korea). *Indian J Tradit Knowl*. (2014) 13:673–80.

102. Abdullahi A. Camel Milk-A Review. *J Animal Sci Livestock Production*. (2019) 3:13–8.

103. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. *Pediatr Clin*. (2013) 60:49–74.

104. Getaneh G, Mebrat A, Wubie A, Kendie H. Review on goat milk composition and its nutritive value. *J Nutrs Health Sci*. (2016) 3:401–9.

105. Grădinaru AC, Creangă Ş, Solcan G. Milk–a review on its synthesis, composition, and quality assurance in dairy industry. *Human Vet Med*. (2015) 7:173–7.

106. Guo H, Pang K, Zhang X, Zhao L, Chen S, Dong M, et al. Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Joint annual meeting abstracts*. (2007) 90:1635–43. doi: 10.3168/jds.2006-600

107. Hamad M, Baiomy A. Physical properties and chemical composition of cow's and buffalo's milk in Qena governorate. *J food and Dairy Sci Mansoura Uni*. (2010) 1:397–403. doi: 10.21608/jfds.2010.82466

108. Kula JT, Tegegne D. Chemical composition and medicinal values of camel milk. *Int J Res Stud Biosci*. (2016) 4:13–25.

109. Merlin Junior IA, Sifuentes dos Santos J, Grecco Costa L, Grecco Costa R, Ludovico A, de Almeida Rego FC, et al. Sheep milk: physical-chemical characteristics and microbiological quality. *Arch Latinoam Nutr*. (2015) 65:193–8.

110. Spreer E. *Milk and dairy product technology*. US: CRC Press (1998).

111. Wiley AS. *Re-imagining milk: Cultural and biological perspectives*. UK: Routledge (2015).

112. Alonso-Castro AJ. Use of medicinal fauna in Mexican traditional medicine. *J Ethnopharmacol*. (2014) 152:53–70. doi: 10.1016/j.jep.2014.01.005

113. Alves RR, Neto NAL, Brooks SE, Albuquerque UP. Commercialization of animal-derived remedies as complementary medicine in the semi-arid region of Northeastern Brazil. *J Ethnopharmacol*. (2009) 124:600–8. doi: 10.1016/j.jep.2009.04.049

114. Alves RRN, Neta ROS, Trovão D, Barbosa J, Barros AT, Dias TLP. Traditional uses of medicinal animals in the semi-arid region of northeastern Brazil. *J Ethnobiol Ethnomed*. (2012) 8:4269–8. doi: 10.1186/1746-4269-8-41

115. Barros FB, Varela SA, Pereira HM, Vicente L. Medicinal use of fauna by a traditional community in the Brazilian Amazonia. *J Ethnobiol Ethnomed*. (2012) 8:37. doi: 10.1186/1746-4269-8-37

116. Benítez G. Animals used for medicinal and magico-religious purposes in western Granada Province, Andalusia (Spain). *J Ethnopharmacol*. (2011) 137:1113–23. doi: 10.1016/j.jep.2011.07.036

117. Betlloch Mas I, Chiner E, Chiner Betlloch J, Llorca FX, Martín Pascual L. (2014). *The use of animals in medicine of Latin tradition: Study of the Tresor de Beutat, a medieval treatise devoted to female cosmetics: Photon*.

118. Borah MP, Prasad SB. Ethnozoological study of animals based medicine used by traditional healers and indigenous inhabitants in the adjoining areas of gibbon wildlife sanctuary, Assam. *India J ethnobiol ethnome*. (2017) 13:39. doi: 10.1186/s13002-017-0167-6

119. Haileselasie TH. Traditional zootherapeutic studies in Degu'a Tembien, northern Ethiopia. *Curr Res J Biol Sci*. (2012) 4:563–9.

120. Lohani U. Traditional uses of animals among jirels of Central Nepal. *Ethno Med*. (2011) 5:115–24. doi: 10.1080/09735070.2011.11886398

121. Martínez GJ. Use of fauna in the traditional medicine of native Toba (Qom) from the Argentine Gran Chaco region: an ethnozoological and conservationist approach. *Ethnobiol Conserv*. (2013) 2:1–43. doi: 10.15451/ec2013-8-2.2-1-43

122. Mishra N, Rout S, Panda T. Ethno-zoological studies and medicinal values of Similipal biosphere reserve, Orissa, India. *Afr J Pharm Pharmacol*. (2011) 5:6–11.

123. Mootoosamy A, Mahomoodally MF. A quantitative ethnozoological assessment of traditionally used animal-based therapies in the tropical island of Mauritius. *J Ethnopharmacol*. (2014) 154:847–57. doi: 10.1016/j.jep.2014.05.001

124. Padmanabhan P, Sujana K. Animal products in traditional medicine from Attappady hills of Western Ghats. *Indian J Tradit Knowl*. (2008) 7:326–9.

125. WHO. *Technical updates of the guidelines on integrated Management of Childhood Illness (IMCI)*. Geneva: Evidence and recommendations for further adaptations (2005).

126. Yirga G, Teferi M, Gebreslassea Y. Ethnozoological study of traditional medicinal animals used by the people of Kafta-Humera District, northern Ethiopia. *Int J Med Medical Sci*. (2011) 3:316–20.

127. Alonso-Castro AJ, Carranza-Álvarez C, Maldonado-Miranda JJ, del Rosario Jacobo-Salcedo M, Quezada-Rivera DA, Lorenzo-Márquez H, et al. Zootherapeutic practices in Aquismón, San Luis Potosí, México. *J Ethnopharmacol*. (2011) 138:233–7. doi: 10.1016/j.jep.2011.09.020

128. Alves RRN, Oliveira MGG, Barboza RRD, Lopez LCS, Oliveira MGG. An ethnozoological survey of medicinal animals commercialized in the markets of Campina GrandeNE Brazil. *Hum Ecol Rev*. (2010) 17:11–7.

129. Bagde N, Jain S. An ethnozoological studies and medicinal values of vertebrate origin in the adjoining areas of Pench National Park of Chhindwara District of Madhya Pradesh, India. *Int J lifesci*. (2013) 1:278–83.

130. Bagde N, Jain S. Study of traditional man-animal relationship in CHHINDWARA district of Madhya Pradesh India. *J Global Biosci*. (2015) 4:1456–63.

131. Betlu ALS. Indigenous knowledge of zootherapeutic use among the Biate tribe of Dima Hasao District, Assam, Northeastern India. *J Ethnobiol Ethnomed*. (2013) 9:1–16.

132. Chellappandian M, Pandikumar P, Mutheeswaran S, Paulraj MG, Prabakaran S, Duraipandiyan V, et al. Documentation and quantitative analysis of local ethnozoological knowledge among traditional healers of Theni district, Tamil Nadu, India. *J Ethnopharmacol*. (2014) 154:116–30. doi: 10.1016/j.jep.2014.03.028

133. Jacobo-Salcedo M d R, Alonso-Castro AJ, Zarate-Martinez A. Folk medicinal use of fauna in Mapimi, Durango, México. *J Ethnopharmacol*. (2011) 133:902–6. doi: 10.1016/j.jep.2010.10.005

134. Dey A, Gorai P, Mukherjee A, Dhan R, Modak BK. Ethnobiological treatments of neurological conditions in the Chota Nagpur plateau, India. *J Ethnopharmacol*. (2017) 198:33–44. doi: 10.1016/j.jep.2016.12.040

135. Kim H, Song M-J. Ethnozoological study of medicinal animals on Jeju Island, Korea. *J Ethnopharmacol*. (2013) 146:75–82. doi: 10.1016/j.jep.2012.11.011

136. Lohani U. Man-animal relationships in Central Nepal. *J Ethnobiol Ethnomed*. (2010) 6. doi: 10.1186/1746-4269-6-31

137. Oliveira ES, Torres DF, Brooks SE, Alves RR. The medicinal animal markets in the metropolitan region of Natal City, Northeastern Brazil. *J Ethnopharmacol*. (2010) 130:54–60. doi: 10.1016/j.jep.2010.04.010

138. Souto WMS, Barboza RRD, da Silva MJ, Alves RRN. Traditional knowledge of sertanejos about zootherapeutic practices used in ethnoveterinary medicine of NE Brazil. *Indian J Tradit Knowl*. (2012) 11:259–65.

139. Bezerra DMM, de Araujo HFP, Alves A, Alves RRN. Birds and people in semiarid northeastern Brazil: symbolic and medicinal relationships. *J Ethnobiol Ethnomed*. (2013) 9. doi: 10.1186/1746-4269-9-3

140. Bobo KS, Aghomo FFM, Ntumwel BC. Wildlife use and the role of taboos in the conservation of wildlife around the Nkwende Hills Forest reserve; south-West Cameroon. *J Ethnobiol Ethnomed*. (2015) 11:2. doi: 10.1186/1746-4269-11-2

141. dos Santos Soares VM, de Lucena Soares HK, da Silva SS, de Lucena RFP. Local knowledge, use, and conservation of wild birds in the semi-arid region of Paraíba state, northeastern Brazil. *J Ethnobiol Ethnomed*. (2018) 14:77. doi: 10.1186/s13002-018-0276-x

142. Lohani U. Eroding ethnozoological knowledge among Magars in Central Nepal. *Indian J Tradit Knowl*. (2011) 10:466–73.

143. Amieva EJ-C, Fuentes-Ramirez R, Martinez-Hernandez A, Millan-Chiu B, Lopez-Marin LM, Castaño V, et al. Graphene oxide and reduced graphene oxide modification with polypeptide chains from chicken feather keratin. *J Alloys Compd*. (2015) 643:S137–43. doi: 10.1016/j.jallcom.2014.12.062

144. Coward-Kelly G, Chang VS, Agbogbo FK, Holtzapple MT. Lime treatment of keratinous materials for the generation of highly digestible animal feed: 1. Chicken feathers. *Bioresources*. (2006) 97:1337–43. doi: 10.1016/j.biortech.2005.05.021

145. Flores-Hernández CG, Colín-Cruz A, Velasco-Santos C, Castaño VM, Rivera-Armenta JL, Almendarez-Camarillo A, et al. All green composites from fully renewable biopolymers: chitosan-starch reinforced with keratin from feathers. *Polymers*. (2014) 6:686–705. doi: 10.3390/polym6030686

146. Gurav RG, Jadhav JP. A novel source of biofertilizer from feather biomass for banana cultivation. *Environ Sci Pollut Res*. (2013) 20:4532–9. doi: 10.1007/s11356-012-1405-z

147. Karthikeyan R, Balaji S, Sehgal P. (2007). *Industrial applications of keratins–A review*.

148. Khajavi R, Rahimi MK, Abbasipour M, Brendjchi AH. Antibacterial nanofibrous scaffolds with lowered cytotoxicity using keratin extracted from quail feathers. *J Bioact Compat Polym*. (2016) 31:60–71. doi: 10.1177/0883911515598793

149. Kumar SL, Anandhavelu S, Sivaraman J, Swathy M. Modified extraction and characterization of keratin from Indian goat hoof: A biocompatible biomaterial for tissue regenerative applications. *Integr Ferroelectr*. (2017) 184:41–9. doi: 10.1080/10584587.2017.1368642

150. Manivasagan P, Sivakumar K, Gnanam S, Venkatesan J, Kim S-K. Production, biochemical characterization and detergents application of keratinase from the marine actinobacterium actinoalloteichus sp. MA-32. *J Surfactant Deterg*. (2014) 17:669–82. doi: 10.1007/s11743-013-1519-4

151. Nanthavanan P, Arungandhi K, Sunmathi D, Niranjana J. Biological synthesis of keratin nanoparticles from dove feather (*Columba livia*) and its applications. *Asian J Pharm Clin Res*. (2019) 12:142–6. doi: 10.22159/ajpcr.2019.v12i10.34572

152. Poole AJ, Church JS, Huson MG. Environmentally sustainable fibers from regenerated protein. *Biomacromolecules*. (2009) 10:1–8. doi: 10.1021/bm8010648

153. Ramakrishnan N, Sharma S, Gupta A, Alashwal BY. Keratin based bioplastic film from chicken feathers and its characterization. *Int J Biol Macromol*. (2018) 111:352–8. doi: 10.1016/j.ijbiomac.2018.01.037

154. Reddy N, Chen L, Zhang Y, Yang Y. Reducing environmental pollution of the textile industry using keratin as alternative sizing agent to poly (vinyl alcohol). *J Clean Prod*. (2014) 65:561–7. doi: 10.1016/j.jclepro.2013.09.046

155. Reddy N, Shi Z, Temme L, Xu H, Xu L, Hou X, et al. Development and characterization of thermoplastic films from sorghum distillers dried grains grafted with various methacrylates. *J Agric Food Chem*. (2014) 62:2406–11. doi: 10.1021/jf405499t

156. Reddy N, Yang Y. Structure and properties of chicken feather barbs as natural protein fibers. *J Polym Environ*. (2007) 15:81–7. doi: 10.1007/s10924-007-0054-7

157. Rouse JG, Van Dyke ME. A review of keratin-based biomaterials for biomedical applications. *Dent Mater*. (2010) 3:999–1014. doi: 10.3390/ma3020999

158. Sharma S, Gupta A, Chik SMS, Kee CG, Mistry BM, Kim DH, et al. Characterization of keratin microparticles from feather biomass with potent antioxidant and anticancer activities. *Int J Biol Macromol*. (2017) 104:189–96. doi: 10.1016/j.ijbiomac.2017.06.015

159. Sharma S, Gupta A, SMSBT C, CYG K, Poddar PK. Dissolution and characterization of biofunctional keratin particles extracted from chicken feathers In: *Proceedings of the IOP conference series: Materials science and engineering*: IOP Publishing (2017)

160. Tesfaye T, Sithole B, Ramjugernath D, Chunilall V. Valorisation of chicken feathers: application in paper production. *J Clean Prod*. (2017) 164:1324–31. doi: 10.1016/j.jclepro.2017.07.034

161. Tsuda Y, Nomura Y. Properties of alkaline-hydrolyzed waterfowl feather keratin. *Anim Sci J*. (2014) 85:180–5. doi: 10.1111/asj.12093

162. Wang J, Hao S, Luo T, Cheng Z, Li W, Gao F, et al. Feather keratin hydrogel for wound repair: preparation, healing effect and biocompatibility evaluation. *Colloids Surf B: Biointerfaces*. (2017) 149:341–50. doi: 10.1016/j.colsurfb.2016.10.038

163. Xu H, Cai S, Xu L, Yang Y. Water-stable three-dimensional ultrafine fibrous scaffolds from keratin for cartilage tissue engineering. *Langmuir*. (2014) 30:8461–70. doi: 10.1021/la500768b

164. Zhan M, Wool RP. Mechanical properties of chicken feather fibers. *Polymer Comp*. (2011) 32:937–44. doi: 10.1002/pc.21112

165. Hermosín I, Chicón RM, Dolores Cabezudo M. Free amino acid composition and botanical origin of honey. *Food Chem*. (2003) 83:263–8. doi: 10.1016/S0308-8146(03)00089-X

166. Iglesias MT, Martín-Álvarez PJ, Polo MC, de Lorenzo C, González M, Pueyo E. Changes in the free amino acid contents of honeys during storage at ambient temperature. *J Agric Food Chem*. (2006) 54:9099–104. doi: 10.1021/jf061712x

167. Alqarni AS, Owayss AA, Mahmoud AA, Hannan MA. Mineral content and physical properties of local and imported honeys in Saudi Arabia. *J Saudi Chem Soc*. (2014) 18:618–25. doi: 10.1016/j.jscs.2012.11.009

168. Andersen OM, Markham KR. *Flavonoids: Chemistry, biochemistry and applications*. US: CRC press (2005).

169. Castro-Vázquez L, Díaz-Maroto MC, Pérez-Coello MS. Aroma composition and new chemical markers of Spanish citrus honeys. *Food Chem*. (2007) 103:601–6. doi: 10.1016/j.foodchem.2006.08.031

170. Kamal MA, Klein P. Determination of sugars in honey by liquid chromatography. *Saudi J biol sci*. (2011) 18:17–21. doi: 10.1016/j.sjbs.2010.09.003

171. Bonté F, Desmoulière A. Le miel: origine et composition. *Actual Pharmacol*. (2013) 52:18–21. doi: 10.1016/j.actpha.2013.10.004

172. Moreira RFA, Maria CAB, Pietroluongo M, Trugo LC. Chemical changes in the non-volatile fraction of Brazilian honeys during storage under tropical conditions. *Food Chem*. (2007) 104:1236–41. doi: 10.1016/j.foodchem.2007.01.055

173. Sak-Bosnar M, Sakač N. Direct potentiometric determination of diastase activity in honey. *Food Chem*. (2012) 135:827–31. doi: 10.1016/j.foodchem.2012.05.006

174. Won S-R, Li C-Y, Kim J-W, Rhee H-I. Immunological characterization of honey major protein and its application. *Food Chem*. (2009) 113:1334–8. doi: 10.1016/j.foodchem.2008.08.082

175. da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R. Honey: chemical composition, stability and authenticity. *Food Chem*. (2016) 196:309–23. doi: 10.1016/j.foodchem.2015.09.051

176. Abbasi AM, Khan MA, Ahmad M, Zafar M. *Medicinal plant biodiversity of lesser*. Himalayas-Pakistan: Springer Science & Business Media (2011).

177. Chinlampianga M, Singh RK, Shukla AC. Ethnozoological diversity of Northeast India: empirical learning with traditional knowledge holders of Mizoram and Arunachal Pradesh. *Indian J Tradit Knowl*. (2013) 12:18–30.

178. Deb AK, Haque CE. 'Every mother is a mini-doctor': ethnomedicinal uses of fish, shellfish and some other aquatic animals in Bangladesh. *J Ethnopharmacol*. (2011) 134:259–67. doi: 10.1016/j.jep.2010.12.001

179. Dixit A, Kadavul K, Rajalakshmi S, Shekhawat M. Ethno-medico-biological studies of South India. *Indian J Tradit Knowl*. (2010) 9:116–8.

180. Erejuwa OO, Sulaiman SA, Ab Wahab MS. Honey-a novel antidiabetic agent. *Int J Biol Sci*. (2012) 8:913–34. doi: 10.7150/ijbs.3697

181. Jaroli D, Mahawar MM, Vyas N. An ethnozoological study in the adjoining areas of Mount Abu wildlife sanctuary. *J Ethnobiol Ethnomed*. (2010) 6:6. doi: 10.1186/1746-4269-6-6

182. Mahawar MM, Jaroli D. Animals and their products utilized as medicines by the inhabitants surrounding the Ranthambhore National Park. *India J ethnobiol ethnomed*. (2006) 2:46. doi: 10.1186/1746-4269-2-46

183. Waykar B, Alqadhi YA. Biological properties and uses of honey: A concise scientific review. *Indian J Pharmaceutical Biolog Res*. (2016) 4:20–7. doi: 10.30750/ijpbr.4.3.4

184. Al-Waili N, Salom K, Al-Ghamdi A, Ansari MJ, Al-Waili A, Al-Waili T. Honey and cardiovascular risk factors, in normal individuals and in patients with diabetes mellitus or dyslipidemia. *J Med Food*. (2013) 16:1063–78. doi: 10.1089/jmf.2012.0285

185. Aluko EO, Olubobokun TH, Atang DE, Nna VU. Honey's ability to reduce blood pressure and heart rate in healthy male subjects. *Front Sci*. (2014) 4:8–11.

186. Bobiş O, Dezmirean DS, Moise AR. Honey and diabetes: the importance of natural simple sugars in diet for preventing and treating different type of diabetes. *Oxidative Med Cell Longev*. (2018) 2018:1–12. doi: 10.1155/2018/4757893

187. Cernak M, Majtanova N, Cernak A, Majtan J. Honey prophylaxis reduces the risk of endophthalmitis during perioperative period of eye surgery. *Phytother Res*. (2012) 26:613–6. doi: 10.1002/ptr.3606

188. El-haskoury R, Al-Waili N, Kamoun Z, Makni M, Al-Waili H, Lyoussi B. Antioxidant activity and protective effect of carob honey in CCl4-induced kidney and liver injury. *Arch Invest Med*. (2018) 49:306–13. doi: 10.1016/j.arcmed.2018.09.011

189. Erejuwa OO. Effect of honey in diabetes mellitus: matters arising. *J Diabetes Metab Disord*. (2014) 13:23. doi: 10.1186/2251-6581-13-23

190. Francis A, Cho Y, Johnson DW. Honey in the prevention and treatment of infection in the CKD population: a narrative review. *Evid Based Complement Alternat Med*. (2015) 2015:1–8. doi: 10.1155/2015/261425

191. Li X, Huang Q, Ong C-N, Yang X-F, Shen H-M. Chrysin sensitizes tumor necrosis factor-α-induced apoptosis in human tumor cells via suppression of nuclear factor-kappaB. *Cancer*. (2010) 293:109–16. doi: 10.1016/j.canlet.2010.01.002

192. Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. *Asian Pac J Trop Biomed*. (2011) 1:154–60. doi: 10.1016/S2221-1691(11)60016-6

193. Omotayo EO, Gurtu S, Sulaiman SA, Wahab MSA, Sirajudeen K, Salleh MSM. Hypoglycemic and antioxidant effects of honey supplementation in streptozotocin-induced diabetic rats. *Int J Vitam Nutr Res*. (2010) 80:74–82. doi: 10.1024/0300-9831/a000008

194. Oršolić N. Bee honey and cancer. *J apiproduct and apimedical Sci*. (2009) 1:93–103. doi: 10.3896/IBRA.4.01.4.01

195. Rhone M, Basu A. Phytochemicals and age-related eye diseases. *Nutrition* reviews.66:465-472. (2008) 66:465–72. doi: 10.1111/j.1753-4887.2008.00078.x

196. Salehi A, Jabarzare S, Neurmohamadi M, Kheiri S, Rafieian-Kopaei M. A double blind clinical trial on the efficacy of honey drop in vernal keratoconjunctivitis. *Evid Based Complement Alternat Med*. (2014) 2014:1–4. doi: 10.1155/2014/287540

197. Vallianou NG, Evangelopoulos A, Skourtis A, Kazazis C. Honey and cancer--A review. *Current Topics in Nutraceutical Res*. (2014) 12

198. Vit P, Jacob TJ. Putative anticataract properties of honey studied by the action of flavonoids on a lens culture model. *J Health Sci*. (2008) 54:196–202. doi: 10.1248/jhs.54.196

199. Zhao H, Cheng N, He L, Peng G, Liu Q, Ma T, et al. Hepatoprotective effects of the honey of *Apis cerana* Fabricius on bromobenzene-induced liver damage in mice. *J Food Sci*. (2018) 83:509–16. doi: 10.1111/1750-3841.14021

200. Umair M, Altaf M, Bussmann RW, Abbasi AM. Ethnomedicinal uses of the local flora in Chenab riverine area, Punjab province Pakistan. *J Ethnobiol Ethnomed*. (2019) 15:7. doi: 10.1186/s13002-019-0285-4

201. Faiz M, Altaf M, Umair M, Almarry KS, Elbadawi YB, Abbasi AM. Traditional uses of animals in the Himalayan region of Azad Jammu and Kashmir. *Front Pharmacol*. (1951) 13. doi: 10.3389/fphar.2022.807831

202. Majeed M, Bhatti KH, Amjad MS, Abbasi AM, Bussmann RW, Nawaz F, et al. Ethno-veterinary uses of Poaceae in Punjab. *Pakistan PloS one*. (2020) 15:e0241705. doi: 10.1371/journal.pone.0241705

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 02 May 2024
DOI 10.3389/fvets.2024.1362292

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Cengiz Gokbulut,
Balıkesir University, Türkiye
Zigang Qu,
Lanzhou Veterinary Research Institute (CAAS), China
Nora Mestorino,
National University of La Plata, Argentina

*CORRESPONDENCE
Zhenling Zeng
✉ zlzeng@scau.edu.cn
Dongping Zeng
✉ donytsang@scau.edu.cn

†These authors have contributed equally to this work

RECEIVED 28 December 2023
ACCEPTED 12 April 2024
PUBLISHED 02 May 2024

CITATION
Zhang W, Lu Y, Ma M, Yang J, Huang H, Peng X, Zeng Z and Zeng D (2024) *Ex vivo* pharmacokinetic/pharmacodynamic of hexahydrocolupulone against *Clostridium perfringens* in broiler chickens. *Front. Vet. Sci.* 11:1362292. doi: 10.3389/fvets.2024.1362292

COPYRIGHT
© 2024 Zhang, Lu, Ma, Yang, Huang, Peng, Zeng and Zeng. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Ex vivo pharmacokinetic/pharmacodynamic of hexahydrocolupulone against *Clostridium perfringens* in broiler chickens

Wanying Zhang[1,2†], Yixing Lu[1,2†], Minglang Ma[1,2], Jinyu Yang[1,2], Huiguo Huang[1,2], Xianfeng Peng[3], Zhenling Zeng[1,2*] and Dongping Zeng[1,2*]

[1]Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [2]National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, Guangzhou, China, [3]Guangzhou Insighter Biotechnology Co., Ltd., Guangzhou, China

The economic impact of necrotizing enteritis (NE) resulting from *Clostridium perfringens* infection has been significant within the broiler industry. This study primarily investigated the antibacterial efficacy of hexahydrocolupulone against *C. perfringens*, and its pharmacokinetics within the ileal contents of broiler chickens. Additionally, a dosing regimen was developed based on the pharmacokinetic/pharmacodynamic (PK/PD) model specific to broiler chickens. Results of the study indicated that the minimum inhibitory concentration (MIC) of hexahydrocolupulone against *C. perfringens* ranged from 2mg/L to 16mg/L in MH broth. However, in ileal content, the MIC ranged from 8mg/L to 64mg/L. The mutation prevention concentration (MPC) in the culture medium was found to be 128mg/L. After oral administration of hexahydrocolupulone at a single dosage of 10–40mg/kg bodyweight, the peak concentration (C_{max}), maximum concentration time (T_{max}), and area under the concentration-time curve (AUC) in ileal content of broiler chickens were 291.42–3519.50μg/g, 1–1.5h, and 478.99–3121.41μgh/g, respectively. By integrating the *in vivo* PK and *ex vivo* PD data, the $AUC_{0\text{-}24h}$/MIC values required for achieving bacteriostatic, bactericidal, and bacterial eradication effects were determined to be 36.79, 52.67, and 62.71h, respectively. A dosage regimen of 32.9mg/kg at 24h intervals for a duration of 3days would yield therapeutic efficacy in broiler chickens against *C. perfringens*, provided that the MIC below 4mg/L.

KEYWORDS

hexahydrocolupulone, *Clostridium perfringens*, pharmacokinetic/pharmacodynamic (PK/PD), broiler, *ex vivo*

1 Introduction

The incidence of *Clostridium perfringens*-induced necrotic enteritis (NE) in the broiler industry, as well as other subclinical diseases associated with the bacteria, has increased (1, 2). Due to the significant costs associated with disease in broiler production (3), the annual cost of NE to the global poultry industry is estimated to be approximately $6 billion, including production losses and the cost of management measures (4). Antibiotic growth promoters (AGPs) can sustain intestinal well-being and modify the composition of resident

microorganisms, consequently enhancing production efficiency and ameliorating intestinal health in broiler chickens (5). In recent years, numerous countries worldwide, including the European Union and the United States, have ceased the utilization of antibiotics as growth promoters in poultry feed due to the escalating resistance of *C. perfringens* to these medications (6, 7). When high concentrations of antibiotics were used, their residues can be found in the blood, the other tissues of the poultry and feces (8). The drug excreted in the feces of treated animals, and can contaminate the feed of other untreated animals (9). Vegetables may also be contaminated from feces especially in countries in where feces are generally used as a fertilizer (10). This presents a significant risk to public health, underscoring the urgency to find herbal or natural products to replace antibiotics.

As research progresses, certain natural antimicrobial agents utilized in food preservation have demonstrated exceptional efficacy in suppressing microbial proliferation (11). Some of the active ingredients extracted from the plant are considered safe and reliable (12). Hop *(Humulus lupulus* L.) is a dioecious vine belonging to the genus *Humulus* of the Cannabis family and widely cultivated around the world (13). Hop-derived bitter acids and their oxidation products not only give the unique bitter taste and aroma of beer but also exert a wide range of biological effects, including antibacterial (14), anti-inflammation (15), antifibrogenesis (16), and they have been considered as chemopreventive agents. β-acid, a member of bitter acids (17), contains a blend of lupulone homologs such as lupulone, colupulone, and adlupulone (13). Although β-acids have antimicrobial effects, they are unstable and are easily oxidized (18). However, as hydrogenated derivatives of β-acids, hexahydro-β-acids (HBA) are mixtures of analogues such as hexahydrolupulone, hexahydrocolupulone, and hexahydroadlupulone (19). The stability, antibacterial and antioxidant activity of HBA are better than those of β-acids (19, 20). HBA can inhibit the expression of proteins related to DNA replication, transcription, translation, and proteins related to ribosome synthesis of *Listeria monocytogenes*, resulting in a decrease in protein content in cells, thereby hindering normal life activities and physiological metabolism (21). Based on the Federal Regulations of Food and Drug Administration (FDA), hops and their extracts are widely acknowledged as safe (22). The structural formula of hexahydrocolupulone is shown in Figure 1.

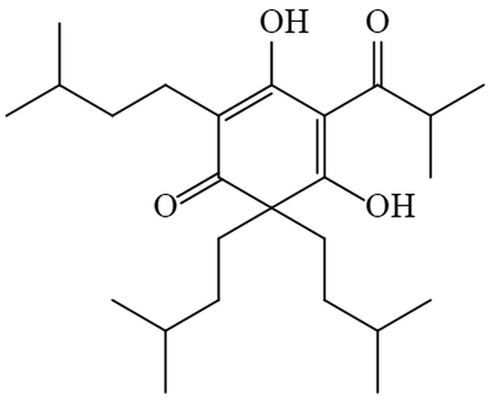


FIGURE 1
Hexahydrocolupulone structural formula.

The objective of this study was to investigate the pharmacokinetic (PK) and pharmacodynamic (PD) properties hexahydrocolupulone in the ileal content. The inhibitory I_{max} model was employed to compute the PK/PD indices necessary for varying levels of antibacterial efficacy. Moreover, the dosage regimen of hexahydrocolupulone in broiler chickens facilitated the determination of an efficacious dose for NE.

2 Materials and methods

2.1 Chemicals

Hexahydrocolupulone (98.6%) was provided by Guangzhou Insighter Biotechnology (Guangzhou, China). Mueller–Hinton (MH) broth and MH agar were obtained from Qingdao Hope Bio-Technology Co., Ltd (Qingdao, China). Tryptone–sulfite–cycloserine (TSC) agar was obtained from Guangdong Huankai Microbial Technology (Guangdong, China).

2.2 Bacteria

A total of 26 isolates of *C. perfringens* were employed in this study, comprising one standard strain (ATCC 13124) procured from the Chinese Veterinary Culture Collection Center and 25 strains derived from broiler chickens in Guangdong Province from 2021 to 2023. All strains were stored at −80°C until use. Before each experiment, these bacteria cultures were subcultured on TSC agar and incubated at 37°C for 18–24 h.

2.3 Animals

This study utilized two-week-old Sanhuang broiler chickens with a weight of 50 ± 5 g, which were in a healthy condition. Prior to conducting the experiments, the broiler chickens underwent a 7 days acclimation period. Throughout the study, the broiler chickens were provided with unrestricted access to antibiotic-free food and water. Food, but not water, was withheld for 12 h before dosing and until 4 h after drug administration. All procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of South China Agricultural University, with the assigned approval number of 2022A016.

2.4 Determination of MIC, MBC, and MPC

The sensitivity of hexahydrocolupulone selected in MH broth was evaluated using the microdilution method recommended by CLSI (23). Following a 24 h incubation period, the MIC was established as the lowest concentration of hexahydrocolupulone that effectively hindered observable bacterial growth. Additionally, the ileal contents were assessed for MIC through the microdilution technique. To determine the minimal bactericidal concentration (MBC), 100 μL suspension from the MIC determination wells was consecutively diluted 10 fold in broth. The colony-forming unit of each dilution was counted by spreading 20 μL onto TSC agar plates after 24 h incubation

at 37°C in anaerobic condition. The MBC was determined as the concentration at which a 99.9% reduction in the bacterial counts was achieved. The agar method was employed to determine the MPC of hexahydrocolupulone (24). The *C. perfringens* strains with a concentration of 10^{10} CFU/mL were inoculated to agar plates that contained varying concentrations of hexahydrocolupulone (1 MIC, 2 MIC, 4 MIC, 8 MIC, 16 MIC, and 32 MIC). These plates were then incubated at 37°C for 72 h. The MPC was defined as the concentration of hexahydrocolupulone that did not facilitate the growth of bacteria on the agar plates.

2.5 *In vitro* and *ex vivo* time-killing curves

Different concentrations of hexahydrocolupulone (1/4 MIC, 1/2 MIC, 1 MIC, 2 MIC, and 4 MIC) were prepared in MH broth. Test tubes were inoculated with 10^6 CFU/mL *C. perfringens* and incubated at 37°C. Bacterial counts (CFU/mL) were determined at 0, 1, 2, 4, 8, 12, and 24 h of incubation. Specifically, 100 μL of culture was collected at each time point and serially diluted. Colony counts were performed the following morning with a limit of detection (LOD) of 50 CFU/mL. All experiments were conducted in triplicate.

The ileal contents were collected at different intervals after oral administration of 20 mg/kg hexahydrocolupulone in a PK test for high-speed centrifugation and sterile filter treatment. Subsequently, an *ex vivo* time-kill curve was established. Viable bacteria were enumerated at specific time points (0, 1, 2, 4, 8, 12, and 24 h) by incubating tubes containing the bacterial culture and intestinal contents at 37°C. The LOD for viable bacteria was set at 50 CFU/mL.

2.6 Pharmacokinetic of hexahydrocolupulone in broiler chickens ileum content

Following a period of 7 days of acclimatization, the 144 chickens were subjected to random allocation into three distinct groups, each comprising 48 chickens. The administered doses were 10, 20, and 40 mg/kg. Subsequently, at specific time intervals after the oral administration of hexahydrocolupulone (0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, and 24 h), four broiler chickens were euthanized in each group to collect ileum contents.

The ileum content was accurately weighed to 0.2 ± 0.02 g and then added with 600 μL of 1% formic acid methanol. The mixture was vortexed for 1 min and centrifuged at 13,000 rpm for 10 min. About 200 μL of supernatant was added with 400 μL of 1% formic acid methanol. The mixture was vortexed for 1 min at 13,000 rpm, centrifuged for 10 min, passed through a 0.22 μm filter membrane, and analyzed by high-performance liquid chromatography (HPLC).

The determination of hexahydrocolupulone concentrations in intestinal contents by using HPLC and UV detectors involved the following conditions: UV detection at 341 nm, a column temperature of 30°C, a mobile phase consisting of water with 0.05% phosphoric acid and methanol, and a sample size of 20 μL injected into the HPLC system (Shimazu LC-20A) with a flow rate of 1 mL/min. Separation was achieved using an A R D-C_{18} (250 mm × 4.6 mm, 5 μm) column. The calibration range for this analysis was 0.25–20 μg/g. The precision levels for intraday and interday measurements ranged from 1.54 to 6.74% and from 4.82 to 7.43%, respectively. The LOD and limit of quantification (LOQ) were determined to be 0.10 and 0.25 μg/g, respectively. A non-compartmental analysis of hexahydrocolupulone concentrations in the intestinal content was conducted using Phoenix WinNonlin® 8.4 (Certara, L.P., Princeton, NJ, United States).

2.7 Analysis of the PK/PD relationship

The *ex vivo* PK/PD relationships of hexahydrocolupulone in the intestine were simulated using the I_{max} model in WinNonlin® 8.4 (Certara, L.P., Princeton, NJ, United States) with the following equation (25):

$$E = \mathrm{E}_0 - \frac{I_{\max} \cdot \mathrm{X}}{\mathrm{IC}_{50} + \mathrm{X}}$$

In this study, E_0 denotes the difference in bacterial count expressed as $\log_{10}$CFU/mL in control samples. I_{max} is the maximum inhibition of antimicrobial growth, determined by the alteration in $\log_{10}$CFU/mL subsequent to hexahydrocolupulone treatment. *X* represents the predictive variable, specifically the ratio of area under the concentration-time curve from 0 h to 24 h to MIC ($AUC_{0\text{-}24h}$/MIC). IC_{50} denotes the *X* value that elicits 50% of the maximum antibacterial effect.

The potential optimal dosage can be calculated using the following equation (26, 27):

$$\mathrm{Dose} = \frac{\left(\mathrm{AUC} / \mathrm{MIC}\right) \cdot \mathrm{MIC} \cdot \mathrm{Cl}}{\mathrm{fu} \cdot \mathrm{F}}$$

where dose (per day) is at a steady state; CL is the clearance per day; AUC/MIC is the targeted endpoint for optimal efficacy in hours; MIC is the target pathogen; F is the bioavailability factor, and *fu* is the free fraction of the drug.

3 Results

3.1 MIC, MBC, and MPC of hexahydrocolupulone against *C. perfringens*

A range of 2–16 mg/L was observed in the MIC of hexahydrocolupulone against 26 strains of *C. perfringens*. The percentages of each MIC (2, 4, 8, and 16 mg/L) were 26.92, 42.31, 26.92, and 3.85%, respectively. The distribution of MICs is depicted in Figure 2. In MH broth, the MIC and MBC of hexahydrocolupulone against *C. perfringens* ATCC13124 were 4 and 16 mg/L, respectively; however, in ileal content, these concentrations were sixteen times higher at 16 and 64 mg/L, respectively. The MPC in the culture medium was found to be 128 mg/L, which was 32 times the MIC. The MIC of hexahydrocolupulone against *C. perfringens* ATCC13124 in the ileum content was determined to be 16 μg/mL, as shown in Table 1.

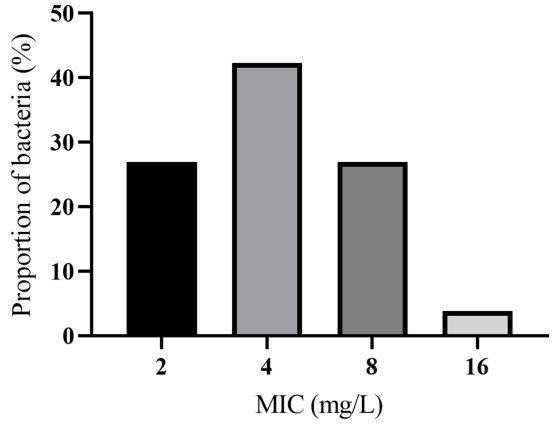


FIGURE 2
MIC distributions of hexahydrocolupulone against 26 *C. perfringens*.

TABLE 1 Antibacterial activity of hexahydrocolupulone against *C. perfringens* ATCC 13124.

	MIC (mg/L)	MBC (mg/L)	MPC (mg/L)
Artificial medium	4	16	128
Ileal content	16	64	–

TABLE 2 Attenuative effect of matrix on *in vitro* susceptibility of hexahydrocolupulone against *C. perfringens* ($n = 26$).

Test matrix[a]	MIC (Mean ± SD, mg/L)
Ileal content	26.46 ± 16.27
MH	4.75 ± 2.63
Ileal content/MH[b]	5.57

[a]MIC represent geometric means (SD) using 26 *C. perfringens* isolates.
[b]Comparison of *C. perfringens* serum/test medium (MH)/ratio differences: $p < 0.01$.

TABLE 3 Pharmacokinetic parameters of hexahydrocolupulone in ileal content following single gavage in healthy broiler chickens (n=4, mean±SD).

Dose (mg/kg)	Ileal content			
	T_{max} (h)	C_{max} (μg/g)	AUC_{last} (μg h/g)	$T_{1/2}$ (h)
10	1.38	291.42	478.99	0.97
20	1.50	440.88	779.48	1.57
40	1	3519.50	3121.41	3.45

T_{max}, time of maximum observed concentration; C_{max}, maximum concentration; AUC_{last}, the area under the concentration-time curve from 0 h to the last sample time point; $T_{1/2}$, half-life.

The calculated MIC of hexahydrocolupulone for *C. perfringens* ATCC13124 in the ileum content (16 mg/L) was found to be four times higher than that in MH broth (4 mg/L), indicating a strong reinforcing effect of MH broth. To further validate the effects of MH broth, we determined the MICs of hexahydrocolupulone in MH broth and ileum content against 26 selected *C. perfringens* isolates. Interestingly, a significant difference in the geometric mean MIC values was observed between MH broth and ileal content, resulting in an ileal content/MH ratio of 5.57 for MICs ($p < 0.01$; Table 2).

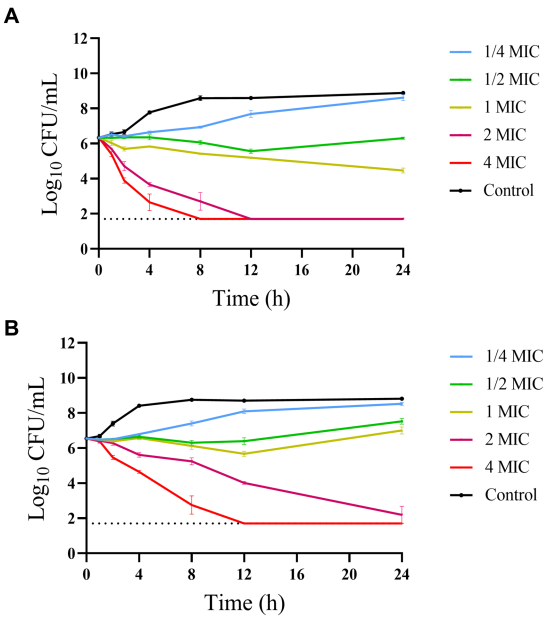


FIGURE 3
In vitro time-kill curve of hexahydrocolupulone against *C. perfringens* ATCC13124 **(A)** and GDZ21C61W **(B)**.

3.2 *In vitro* and *ex vivo* antimicrobial activity

The time-kill curves of hexahydrocolupulone against *C. perfringens* ATCC13124 and GDZ21C61W in MH broth are depicted in Figure 3. The curves revealed that hexahydrocolupulone exhibited a concentration-dependent bactericidal effect. Notably, a sustained inhibitory impact on bacterial growth was observed when *C. perfringens* was exposed to hexahydrocolupulone concentrations exceeding 8 mg/L.

The *ex vivo* time-kill curves were used to assess the effects of hexahydrocolupulone on samples collected at various time points. The *ex vivo* time-kill curves of hexahydrocolupulone against *C. perfringens* ATCC13124, GDZ21C61W, and GDZ21C222W are presented in Figure 4. The results indicated that hexahydrocolupulone exhibited a concentration-dependent effect in *ex vivo*, which was consistent with the observed time-kill curves *in vitro*. Notably, a significant reduction in bacterial count was observed in the high-concentration group (at 0.75–2 h), with no detectable bacteria at 24 h.

3.3 Pharmacokinetics analysis

The concentration-time curve of intestinal contents in broiler chickens after a single oral gavage at a dose of 10, 20, and 40 mg/kg was shown in Figure 5. Table 3 presents the PK parameters of hexahydrocolupulone in intestinal content. The T_{max} in 10 mg/kg, 20 mg/kg, and 40 mg/kg were 1.38, 1.50, and 1 h, respectively. The C_{max} were 291.42 ± 90.01, 440.88 ± 181.09, and 3519.50 ± 752.01 μg/g,

TABLE 4 PK/PD parameter of *ex vivo* data after oral administration hexahydrocolupulone in broiler chickens.

Parameter	Unit	PK/PD fitting parameters
E_0	(log_{10}CFU/mL)	2.10
I_{max}	(log_{10}CFU/mL)	6.97
IC_{50}	h	43.54
AUC_{0-24h}/MIC for bacteriostatic action	h	36.79
AUC_{0-24h}/MIC for bactericidal action	h	52.67
AUC_{0-24h}/MIC for bacterial elimination	h	62.71

E_0, difference in number of bacteria counts (log_{10} CFU/mL) in a drug-free sample between 0 and 24 h; I_{max}, difference in greatest amount of antibacterial reduction (log_{10} CFU/mL); IC_{50} is the AUC_{0-24h}/MIC value producing 50% of the maximal antibacterial effect.

respectively. And the AUC_{last} were 478.99 ± 149.92, 779.48 ± 210.59, and 3121.41 ± 895.08 μg h/g, respectively.

3.4 PK/PD analysis

In ileum content, the I_{max} model effectively elucidated the correlation between the antimicrobial effectiveness of hexahydrocolupulone and the PK/PD parameter represented by the AUC_{0-24h}/MIC ratio in the ileum. The correlation between the efficacy of hexahydrocolupulone against *C. perfringens* and each of the PK/PD indices is depicted in Figure 6. Table 4 shows the AUC_{0-24h}/MIC ratios required to achieve various efficacy targets. The AUC_{0-24h}/MIC values for bacteriostatic activity, bactericidal action, and virtual eradication in the ileum were 36.79, 52.67, and 62.71 h, respectively.

4 Discussion

Given the increasing apprehension among consumers regarding the presence of antibiotic residues in poultry products and the emergence of antibiotic-resistant strains, substitutes for antibiotics are necessary (28, 29). Previous studies have confirmed that the active ingredients, derived or separated from hops, exhibit a substantial inhibitory effect on various pathogenic microorganisms [*Escherichia coli*, *S. aureus*, *Listeria monocytogenes* (21), and *C. perfringens* (14), and they are considered safe and reliable (12)]. The antibacterial activity of hexahydrocolupulone was evaluated using the broth microdilution method to determine the MICs of hexahydrocolupulone against 26 strains of *C. perfringens*. The MIC range of hexahydrocolupulone against clinical *C. perfringens* strains was found to be 2–32 mg/L, with more than half of the strains exhibiting MICs in the range of 2–4 μg/mL. This result indicated the strong sensitivity of *C. perfringens* to hexahydrocolupulone. This study also investigated the effect of different *ex vivo* and *in vitro* conditions, such as MH broth and ileal content, on bacterial growth and determination of MIC. The MIC of hexahydrocolupulone against *C. perfringens* ATCC13124 was found to be 4 mg/L in MH broth and 32 mg/L in the ileum. To assess the effectiveness of hexahydrocolupulone against a broad range of *C. perfringens* isolates, we determined MICs in MH broth and ileum

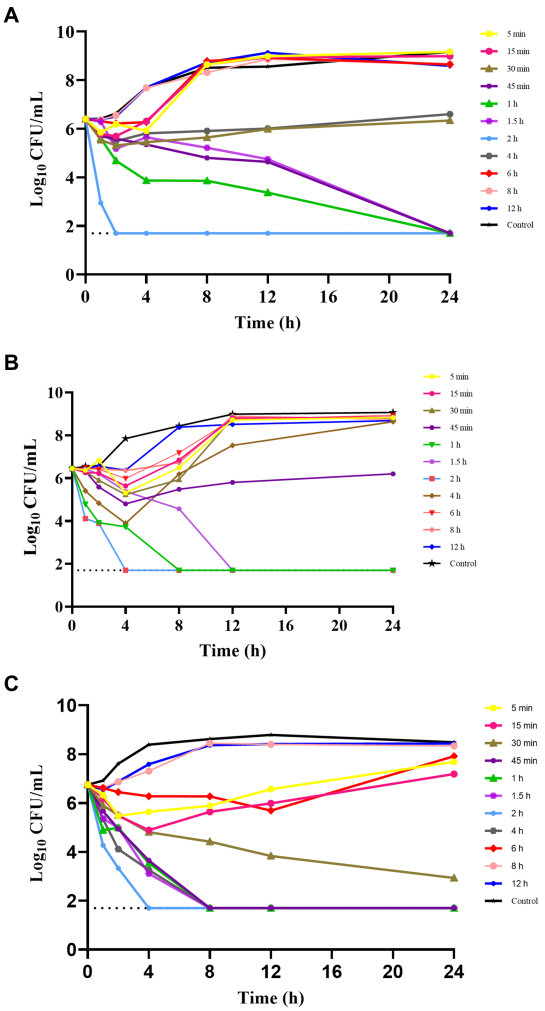


FIGURE 4
Ex vitro time-kill curve of hexahydrocolupulone against *C. perfringens* ATCC13124 **(A)**, GDZ21C61W **(B)**, and GDZ21C222W **(C)**.

for 26 selected isolates. The geometric means of the MICs differed significantly between MH broth and ileal content, with an ileal content/MH ratio of 5.57 for MICs. This result suggested that the ileal content has a substantial attenuative effect on the efficacy of hexahydrocolupulone. The utilization of the PK/PD index, specifically the AUC_{0-24h}/MIC ratio, is highly suitable when the MIC is based on the ileal content.

On the basis of the PK findings of hexahydrocolupulone, the absorption and distribution of hexahydrocolupulone in broiler intestine were found rapid after oral administration, as evidenced by peak concentrations attained within 1.5 h; this pattern was similar to that observed with non-oral absorption medications like cyadox (30). Thus, the utilization of hexahydrocolupulone as a potential therapeutic agent against *C. perfringens* is justified, given that this bacterium predominantly targets the intestinal tract of humans and animals. AUC_{last} and C_{max} ranged from 478.88 μg h/g to 3,121.41 μg h/g and from 291.42 μg/g to 3,519.50 μg/g, respectively. This study aimed to investigate the PK data of hexahydrocolupulone in the ileum of healthy broiler chickens for PK/PD studies. Following intragastric administration, the concentrations of hexahydrocolupulone in the

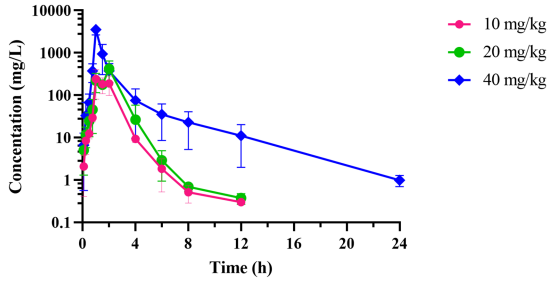


FIGURE 5
The time-concentration profile of hexahydrocolupulone in intestinal contents of broiler chickens following a single oral administration of 10, 20, and 40 mg/kg ($n = 4$).

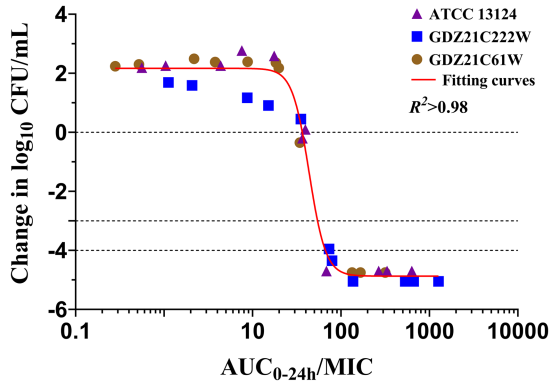


FIGURE 6
Relationships between the effect of hexahydrocolupulone against *C. perfringens* and PK/PD indices AUC_{0-24h}/MIC in the *ex vivo* model. R^2 is the coefficient of determination.

broiler chickens' ileum exhibited a rapid decrease due to chyme transport, which was consistent with the PK properties observed in other orally administered non-absorbable drugs.

The approach to drug development has evolved from an empirical methodology to a modeling and simulation-based methodology, wherein the interplay between PK and PD governs the correlation between dosage and response (31). In this study, a strong correlation ($R^2 > 0.98$) was observed between the PK/PD index of AUC_{0-24h}/MIC and antibacterial activity in the *ex vivo* model. The AUC_{0-24h}/MIC targets necessary to achieve bacteriostatic, bactericidal, and virtual eradication effects were determined to be 36.79, 52.67, and 62.71 h, respectively. To calculate the dosage, we multiplied the MIC distribution in MH broth by a scaling factor of 5.57 to account for the differences between MH and ileal content, and the Cl/F in ileal content was measured to be 28.01 ± 9.06 mL/kg h. The use of fu was not necessary for the utilization of PD data generated in the small intestine (32). The recommended therapeutic dosage of hexahydrocolupulone for the treatment of *C. perfringens* with an MIC of ≤4 mg/L was 32.9 mg/kg, to be administered every 24 h.

Some compounds of hops have potential to be used as feed additives to broiler chickens. Some references in the literature indicate that some compounds of hops can replace antimicrobial performance enhancers in the diets of broiler chickens (33). Nevertheless, hop supplementation at the highest concentration influenced the performance of broiler chickens (34). The potency of hop as an antimicrobial agent has also been shown in poultry. The results have demonstrated that hop ß-acid lupulone supplementation to drinking water decreased caecal *C. perfringens* counts in challenged chickens in both jejunal and caecal sampling sites across all lupulone dosages tested (14). On the other hand, no significant changes were noted in the overall microbiota of the caecum or the midgut when lupulone was added to the water (35). Therefore, it is significant to investigate the PK and PD properties hexahydrocolupulone in broiler chickens.

In this study, we successfully demonstrated the efficacy of hexahydrocolupulone against *C. perfringens* through *in vitro* and *ex vivo* experiments. Additionally, we determined the AUC_{0-24h}/MIC targets of hexahydrocolupulone in simulated broiler intestines. Although HBA exhibit lipophilicity, rendering them insoluble in water, which hinders homogenous dispersion (36), Lu et al. (36) successfully prepared the inclusion complex of HBA/M-β-CD so that the water solubility of HBA was enhanced by CD inclusion. These findings strongly suggested that hexahydrocolupulone holds significant promise as a novel therapeutic agent for the treatment of *C. perfringens* infection in broiler chickens.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of South China Agricultural University, with the assigned approval number of 2022A016. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WZ: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Methodology. YL: Conceptualization, Data curation, Formal analysis, Writing – review & editing, Investigation, Methodology. MM: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. JY: Methodology, Writing – review & editing. HH: Methodology, Writing – review & editing. XP: Project administration, Writing – review & editing. ZZ: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. DZ: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research

was supported by the National Key R&D Program of China (grant 2022YFD 1802100).

Conflict of interest

XP was employed by Guangzhou Insighter Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1362292/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Chromatogram of a standard solution of hexahydrocolupulone.

SUPPLEMENTARY FIGURE S2
Blank ileal content chromatogram.

SUPPLEMENTARY FIGURE S3
Chromatogram of addition to the blank ileal contents with 10 μg/g of hexahydrocolupulone.

SUPPLEMENTARY FIGURE S4
Chromatogram of ileal contents sample after 1.5 h of oral administration.

References

1. Emami NK, Dalloul RA. Centennial review: recent developments in host-pathogen interactions during necrotic enteritis in poultry. *Poult Sci*. (2021) 100:101330. doi: 10.1016/j.psj.2021.101330

2. Yang Q, Liu J, Robinson KJ, Whitmore MA, Stewart SN, Zhang G. Perturbations of the ileal mycobiota by necrotic enteritis in broiler chickens. *J Anim Sci Biotechnol*. (2021) 12:107. doi: 10.1186/s40104-021-00628-5

3. Lee KW, Lillehoj HS, Jeong W, Jeoung HY, An DJ. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. *Poult Sci*. (2011) 90:1381–90. doi: 10.3382/ps.2010-01319

4. Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. Necrotic enteritis in broilers: an updated review on the pathogenesis. *Avian Pathol*. (2011) 40:341–7. doi: 10.1080/03079457.2011.590967

5. Haque MH, Sarker S, Islam MS, Islam MA, Karim MR, Kayesh MEH, et al. Sustainable antibiotic-free broiler meat production: current trends, challenges, and possibilities in a developing country perspective. *Biology*. (2020) 9:110411. doi: 10.3390/biology9110411

6. Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci*. (2005) 84:634–43. doi: 10.1093/ps/84.4.634

7. Castanon JI. History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci*. (2007) 86:2466–71. doi: 10.3382/ps.2007-00249

8. Bacanli M, Basaran N. Importance of antibiotic residues in animal food. *Food Chem Toxicol*. (2019) 125:462–6. doi: 10.1016/j.fct.2019.01.033

9. Darwish WS, Eldaly EA, E-Abbasy MT, Ikenaka Y, Nakayama S, Ishizuka M. Antibiotic residues in food: the african scenario. *Jpn J Vet Res*. (2013) 61:S13–22.

10. Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, et al. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *Antimicrob Chemother*. (2004) 172:136–52. doi: 10.1136/vr.f624

11. Efenberger-Szmechtyk M, Nowak A, Czyzowska A. Plant extracts rich in polyphenols: antibacterial agents and natural preservatives for meat and meat products. *Crit Rev Food Sci Nutr*. (2021) 61:149–78. doi: 10.1080/10408398.2020.1722060

12. Ahmadifar E, Yousefi M, Karimi M, Raieni RF, Dadar M, Yilmaz S, et al. Benefits of dietary polyphenols and polyphenol-rich additives to aquatic animal health: an overview. *Rev Fish Sci Aquac*. (2021) 29:478–511. doi: 10.1080/23308249.2020.1818689

13. Karabin M, Hudcova T, Jelinek L, Dostalek P. Biologically active compounds from hops and prospects for their use. *Compr Rev Food Sci Food Saf*. (2016) 15:542–67. doi: 10.1111/1541-4337.12201

14. Siragusa GR, Haas GJ, Matthews PD, Smith RJ, Buhr RJ, Dale NM, et al. Antimicrobial activity of lupulone against *Clostridium perfringens* in the chicken intestinal tract jejunum and caecum. *J Antimicrob Chemother*. (2008) 61:853–8. doi: 10.1093/jac/dkn024

15. Van Cleemput M, Heyerick A, Libert C, Swerts K, Philippe J, De Keukeleire D, et al. Hop bitter acids efficiently block inflammation independent of gralpha, pparalpha, or ppargamma. *Mol Nutr Food Res*. (2009) 53:1143–55. doi: 10.1002/mnfr.200800493

16. Saugspier M, Dorn C, Thasler WE, Gehrig M, Heilmann J, Hellerbrand C. Hop bitter acids exhibit anti-fibrogenic effects on hepatic stellate cells in vitro. *Exp Mol Pathol*. (2012) 92:222–8. doi: 10.1016/j.yexmp.2011.11.005

17. Duarte LM, Adriano L, de Oliveira M. Capillary electrophoresis in association with chemometrics approach for bitterness hop (*humulus lupulus L.*) classification. *Electrophoresis*. (2018) 39:1399–409. doi: 10.1002/elps.201700420

18. De Almeida NEC, Nascimento ESPD, Cardoso DR. On the reaction of lupulones, hops β-acids, with 1-hydroxyethyl radical. *J Agric Food Chem*. (2012) 60:10649–56. doi: 10.1021/jf302708c

19. Ruckle L, Senn T. Hop acids can efficiently replace antibiotics in ethanol production. *Int Sugar J*. (2006) 108:139–47.

20. Yajima H, Ikeshima E, Shiraki M, Kanaya T, Fujiwara D, Odai H, et al. Isohumulones, bitter acids derived from hops, activate both peroxisome proliferator-activated receptor alpha and gamma and reduce insulin resistance. *J Biol Chem*. (2004) 279:33456–62. doi: 10.1074/jbc.M403456200

21. Tian B, Xu D, Li W, Wang J, Cheng J, Liu Y. Proteomic analysis of hexahydro-beta-acids/hydroxypropyl-beta-cyclodextrin inhibit *Listeria monocytogenes*. *Appl Microbiol Biotechnol*. (2022) 106:755–71. doi: 10.1007/s00253-022-11764-x

22. Stirting L, Mcconathy L, Bettardo B, Yasbar S, Franco J, Saverson E, et al. *Code of federal regulations*. (2001)

23. CLSI. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved standard. 5th* ed. Wayne: CLSI supplement VET01S (2020).

24. Kong L, Lu Y, Yang L, Zhang W, Zuo B, Peng X, et al. Pharmacokinetics and pharmacodynamics of colistin combined with isopropoxy benzene guanidine against mcr-1–positive *Salmonella* in an intestinal infection model. *Front Microbiol*. (2022) 13:907116. doi: 10.3389/fmicb.2022.907116

25. Riviere Je TP. Chapter 13: simultaneous pharmacokinetic pharmacodynamic modeling. Riviere Je, (Ed.) *Comparative pharmacokinetics: principles, techniques & applications. 2nd edn*, Ames, IA: Wiley-Blackwell, John Wiley & Sons, Ltd., 255–294. (2011)

26. Toutain PL, Del CJ, Bousquet-Melou A. The pharmacokinetic-pharmacodynamic approach to a rational dosage regimen for antibiotics. *Res Vet Sci*. (2002) 73:105–14. doi: 10.1016/s0034-5288(02)00039-5

27. Toutain P, Bousquet-Melou A, Martinez M. AUC/MIC: a PK/PD index for antibiotics with a time dimension or simply a dimensionless scoring factor? *J Antimicrob Chemother*. (2007) 60:1185–8. doi: 10.1093/jac/dkm360

28. Hosny RA, Gaber AF, Sorour HK. Bacteriophage mediated control of necrotic enteritis caused by *C. perfringens* in broiler chickens. *Vet Res Commun*. (2021) 45:409. doi: 10.1007/s11259-021-09821-3

29. Parent E, Archambault M, Charlebois A, Bernier-Lachance J, Boulianne M. A chicken intestinal ligated loop model to study the virulence of *Clostridium perfringens* isolates recovered from antibiotic-free chicken flocks. *Avian Pathol*. (2017) 46:138–49. doi: 10.1080/03079457.2016.1228825

30. Wang F, Mi K, Ahmad I, Xie S, Hussain HI, Yuan Z, et al. Antibacterial activity of cyadox against *Clostridium perfringens* in broilers and a dosage regimen design based on pharmacokinetic-pharmacodynamic modeling. *Microb Pathog*. (2020) 141:103981. doi: 10.1016/j.micpath.2020.103981

31. Dingemanse J, Appel-Dingemanse S. Integrated pharmacokinetics and pharmacodynamics in drug development. *Clin Pharmacokinet*. (2007) 46:713–37. doi: 10.2165/00003088-200746090-00001

32. Lu Y, Yang L, Zhang W, Li J, Peng X, Qin Z, et al. Pharmacokinetics and pharmacodynamics of isopropoxy benzene guanidine against *Clostridium perfringens* in an intestinal infection model. *Front Vet Sci.* (2022) 9:4248. doi: 10.3389/fvets.2022.1004248

33. Cornelison J, Yan F, Watkins S, Rigby L, Segal J, Waldroup P. Evaluation of hops (*Humulus Lupulus*) as an antimicrobial in broiler diets. *Int J Poultry Sci*. (2006) 5:134–6. doi: 10.3923/ijps.2006.134.136

34. Rezar V, Levart A, Cerenak A, Salobir J, Pirman T. Effect of hop (*Humulus Lupulus*) supplementation on performance, oxidative stress, and oxidative stability of broiler chicken meat. *Eur Poult Sci*. (2020) 84:e299. doi: 10.1399/eps.2020.299

35. Tillman GE, Haas GJ, Wise MG, Oakley B, Smith MA, Siragusa GR. Chicken intestine microbiota following the administration of lupulone, a hop-based antimicrobial. *FEMS Microbiol Ecol*. (2011) 77:395–403. doi: 10.1111/j.1574-6941.2011.01119.x

36. Lu N, Xu H, Liu Y. Characterization and antimicrobial activity of a 2-o-methyl-β-cyclodextrin inclusion complex containing hexahydro-β-acids. *Mater Sci*. (2019) 54:4287–96. doi: 10.1007/s10853-018-3148-9

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 21 May 2024
DOI 10.3389/fvets.2024.1387853

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Yanzhu Zhu,
Jilin Agricultural Science and Technology College, China
Jianzhu Liu,
Shandong Agricultural University, China
Xu Yang,
Henan Agricultural University, China

*CORRESPONDENCE
Hao Lu
luhao@nwsuaf.edu.cn

RECEIVED 18 February 2024
ACCEPTED 09 April 2024
PUBLISHED 21 May 2024

CITATION
Zhang S, Zhang Y, Yin H, Liu Y, Tang L, Zhu Y, Sun P, Wu K, Zhao B and Lu H (2024) Metabolomic analysis of swainsonine poisoning in renal tubular epithelial cells. *Front. Vet. Sci.* 11:1387853. doi: 10.3389/fvets.2024.1387853

COPYRIGHT
© 2024 Zhang, Zhang, Yin, Liu, Tang, Zhu, Sun, Wu, Zhao and Lu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Metabolomic analysis of swainsonine poisoning in renal tubular epithelial cells

Shuhang Zhang, Yingqingqing Zhang, Hai Yin, Yiling Liu, Lihui Tang, Yanli Zhu, Pinzhi Sun, Kexin Wu, Baoyu Zhao and Hao Lu*

College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi, China

Locoweed is a poisonous plant widely present in grasslands around the world. Swainsonine (SW), an indole alkaloid that, is the main toxic component of the locoweed. To understand the mechanism of SW-induced toxicity and to delineate the metabolic profile of locoweed poisoning we performed the LC–MS/MS untargeted metabolomic study to analyze metabolites in SW-treated renal tubular epithelial cells (0.8 mg/mL, 12 h) and in order to identify the SW-induced metabolomic changes. The analysis identified 2,563 metabolites in positive ion mode and 1,990 metabolites in negative ion mode. Our results showed that the metabolites were mainly benzenoids, lipids and lipid-like molecules, nucleosides, nucleotides, and analogs, organic acids, and derivatives. The differential metabolites were primarily enriched in pathways involving bile secretion, primary bile acid biosynthesis, riboflavin metabolism, ferroptosis, drug metabolism-cytochrome P450, and primidine metabolism. We have screened out substances such as swainsonine, 3alpha,7alpha-Dihydroxy-5beta-cholestanate, 2-Hydroxyiminostilbene, and glycochenodeoxycholate, which may have the potential to serve as biomarkers for swainsonine poisoning. This study provides insights into the types of metabolomic alteration in renal tubular epithelial cells induced by swainsonine.

KEYWORDS

swainsonine, metabonomics, rat renal tubular epithelial cells, bile secretion, cytochrome P450

1 Introduction

Locoweed, a plant species with a global distribution (1), has been documented in various countries including the United States (2), Brazil (3), Argentina (4), Australia (5), and China (6). The earliest instances of poisonous plant poisoning diseases in the western region of the country were documented in the United States in 1873 (7). In China, the distribution of locoweed is mainly concentrated in Qinghai, Xinjiang, Tibet, Gansu, and other regions (8). Swainsonine (SW) is an indolizidine alkaloid, which is the main toxic component of locoweed (9–11). SW-induced toxicity is primarily characterized by the inhibition of α-mannosidase activity and the induction of widespread cellular vacuolar degeneration (12, 13). Previous studies have unequivocally demonstrated that SW can inflict damage on multiple organs, encompassing the cerebrum, cerebellum, liver, kidneys, pancreas, and thyroid gland (1). Notably, renal injury is observed at relatively lower dosages or at earlier time points (4, 14).

Metabolites are important characteristics of an organism's phenotype, understanding the metabolomics provides important insights for the biological processes and their underlying mechanisms. Metabolomics is the systematic quantification and analysis of all small molecules (15) present in biological samples such as cells, tissues, or biological fluids. It involves the qualitative and quantitative characterization of these substances and their comprehensive analysis. Metabolomics has the ability to directly capture the molecular phenotype of a species (16) as well as the functional state of an individual (17–19). Through this analysis, metabolites of biological significance and statistically significant differences can be identified thus facilitating disease diagnosis and metabolite analysis. Metabolomics technology has been extensively employed in the identification of biomarkers for disease diagnosis and screening. Studies have utilized this technology to screen for specific metabolite associated with depression (20), neurodegenerative diseases (21), venous thromboembolism (22), osteoporosis (23), and diabetes mellitus (24). Currently, the metabolomics research surrounding locoweed and SW remains scant, with the bulk of studies focusing on elucidating the intricate relationship between the secondary metabolites of endophytic fungi and the biosynthesis of SW. Unfortunately, research papers exploring the intoxication mechanisms of SW are highly limited, with only a solitary study identified. In this study, the author cleverly harnessed targeted metabolomics and high-throughput sequencing techniques to reveal that SW has the capacity to profoundly alter bile acid metabolism and disrupt the delicate balance of intestinal microbiota in mice, ultimately triggering inflammatory reactions in the liver (25). Nevertheless, our understanding of the kidney toxicity and associated metabolites triggered by SW remains tenuous, necessitating further investigation in this vital area.

Currently, the prevention and control of livestock poisoning primarily rely on the implementation of preventive measures, as there is currently no specific antidote drug available for affected animals. As the early stage of locoweed poisoning is reversible, timely intervention can help mitigate the symptoms of locoweed poisoning by feeding non-toxic pasture grasses. Therefore, timely identification of the metabolic changes due to the locoweed poisoning is important for effective intervention. At present, studies on SW primarily focuses on its biosynthesis and toxicity mechanisms, with limited reports on the metabolic pathways it participates in after entering cells. Previous research has indicated that locoweed poisoning primarily affects the kidney, causing severe damage to this organ and the renal epithelial cells, especially the proximal tubular epithelial cells, are the most significantly affected (4, 14). In this study, rat primary renal tubular epithelial cells were utilized as an experimental model to investigate the metabolomic perturbation following locoweed poisoning. Untargeted metabolomics technology was employed to analyze the metabolic profile of SW-induced alteration in renal tubular cells in order to aid the diagnosis of locoweed poisoning. The identification of these metabolomic changes is important for the early detection and prevention of locoweed poisoning in livestock.

2 Materials and methods

2.1 Ethical approval

All procedures were conducted in accordance with the Code of Ethics and approved by the Laboratory Animal Management and Ethics Committee of Northwest A&F University, Project number: XN2023-1007. All efforts were made to minimize the suffering of animals.

2.2 Chemical reagents and instrumentation

Swainsonine (provided by the Laboratory of Animal Toxicology Team, Northwest A&F University, purity >98%), methanol (Merck, Germany), acetonitron (Merck, Germany), L-2-chlorophenylalanine (Aladin, China), formic acid (TCI, Japan), ultra-high performance liquid phase (Waters, United States), High resolution mass spectrometry (Waters, United States), chromatographic column (Waters, Unites States), and Cell incubator (Thermo, United States).

2.3 Cell culture and treatment

Primary rat renal tubular epithelial cells were obtained by tissue block culture, the detailed operational steps are as follows.

Euthanize the rats by decapitation, and aseptically extract the kidneys. Delicately remove the capsule and separate the cortical portion. Cut the cortical tissue into 1 mm^3 tissue blocks. Thoroughly rinse the tissue blocks with PBS buffer until the supernatant becomes clear and free of turbidity. Centrifuge the tissue blocks at 1,000 rpm for 3 min. Add 0.1% type IV collagenase to the tissue blocks and incubate the mixture at 37°C for 1 h to disperse the tissue. To terminate the enzymatic digestion, add an equal volume of culture medium to the mixture. Pass the content of the centrifuge tube through a 100-mesh screen to collect the filtered solution. Repeat the filtration process using a 400-mesh screen and collect the upper layer material, which represents the renal tubule segments. Resuspend the renal tubule segments in an appropriate amount of culture medium. Transfer the resuspended renal tubule segments to culture dishes and incubate them in a cell culture incubator. Replace the culture medium every 48 h to maintain the optimal growth conditions.

When the cells were fused to 80%, SW is dissolved in the culture medium to achieve a concentration of 0.8 mg/mL. Subsequently, the SW-containing culture medium is filtered through a 0.22 μm filter membrane to ensure sterility. The sterilized medium is then applied to the cells and incubated for 12 h.

The cell culture medium used in our study was Dulbecco's Modified Eagle Medium (Gibco, America, 12800-017), supplemented with 15% Fetal Bovine Serum Standard (Newzerum, New Zealand, FBS-S500) for cultivation, without the addition of any other substances.

2.4 Metabolites extraction

The extraction solution with internal label (Vmethanol:Vacetonitrile = 1:1, internal standard concentration 20 mg/L) was prepared, and the extraction solution was added three times (300, 300, and 400 μL), and the cell samples were completely transferred into the EP tube, and vortex mixed for 30 s. Add steel beads, process for 10 min on a 45 Hz grinder, sonicate for 10 min (ice-water bath); leave at −20°C for 1 h; centrifuge at 12,000 rpm for 15 min at 4°C, and extract the supernatant (500 μL); dry the extract in a vacuum concentrator; after drying, add 160 μL of the extract solution (V water:V acetonitrile = 1:1) to reconstitute the extract solution;

vortex for 30 s, and sonicate for 10 min in an ice-water bath. The extract was centrifuged at 12,000 rpm for 15 min at 4°C. 120 μL of the supernatant was taken into the injection bottle, and 10 μL of each sample was mixed to form quality control samples (QC) for the assay.

2.5 LC–MS/MS analysis

The liquid-mass spectrometry system for metabolomics analysis consisted of an Acquity I-Class PLUS (Waters, United States) ultra-high performance liquid chromatography (UHPLC) tandem with a Waters Xevo G2-XS QTOF (Waters, United States) high-resolution mass spectrometer. The column used was Acquity UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm) (Waters, United States). The mobile phase of positive ion mode (POS) and negative ion mode (26) were the same: mobile phase A: 0.1% formic acid aqueous solution; mobile compositions B: 0.1% formic acid acetonitrile, and the injection volume was 1 μL. The elution gradients were: 98% mobile phase A, 2% mobile phase B, 0–0.25 min; 2% mobile phase A, 98% mobile phase B, 10–13 min; 98% mobile phase A, 2% mobile phase B, 13.1–15 min; and the flow rate was 400 μL/min. The high-resolution mass spectrometer (Xevo G2-XS QTOF, Waters, United States) is capable of primary and secondary mass spectrometry data acquisition in MSe mode under the control of acquisition software (MassLynx V4.2, Waters, United States). Dual-channel data acquisition for both low and high crash energy is performed in each data acquisition cycle. The low collision energy was 2 V, the high collision energy interval was 10–40 V, and the scanning frequency was 0.2 s. The parameters of the ESI ion source were as follows: capillary voltage: 2,500 V (positive ion mode) or −2,000 V (negative ion mode); cone-well voltage: 30 V; temperature of the ion source: 100°C; temperature of the desolvent gas was 500°C; flow rate of the blowback gas was 50 L/h; flow rate of the desolvent gas was 800 L/h; and mass-to-nucleus ratio (m/z) acquisition range was 50–1,200. Flow rate: 800 L/h; mass-to-core ratio (m/z) acquisition range 50–1,200.

2.6 Characterization and quantification of metabolites

The raw data collected by MassLynx V4.2 were processed by Progenesis QI software for peak extraction and peak alignment, and then identified based on the Progenesis QI software online METLIN database, Human Metabolome Database (HMDB), and Biomark's self-built library, and the theoretical fragments were also identified, and the deviation of mass number of parent ions is 100 ppm, and that of fragments is 50 ppm or less. The deviation of the parent ion mass number is 100 ppm and the deviation of the fragment ion mass number is 50 ppm or less (27).

2.7 Classification and functional annotation of metabolomes

The metabolites were annotated using the HMDB database[1] to obtain superclass and class information; the pathways in which the identified metabolites were found were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[2] to annotate the pathways in which the identified metabolites were found.

2.8 Data processing and analysis

Principal component analysis, which downscales the dimension of high-throughput metabolic data and categorizes them by the similarity of their principal components, is an unsupervised classification mode that responds to the overall distribution of samples among groups and the magnitude of differences between samples within groups. The supervised discriminant analysis statistical method of partial least squares regression orthogonal projections to latent structures-discriminant analysis (OPLS-DA) was used to visualize within-group differences by filtering out orthogonal variables unrelated to categorical variables. To check the reliability of the OPLS-DA model, a permutation test is required. The groupings of the samples were randomly disrupted and the OPLS-DA modeling was performed and the evaluation parameters coefficient of determination for Y (R^2Y) and predictability for Y (Q^2Y) were obtained according to the new groupings. Variable importance in projection (VIP) reflects both the loading weight of each metabolite in the model and the variability of the response explained by that metabolite, and can be used for ANOVA. In order to analyze the metabolic patterns of metabolites under different experimental conditions, all the metabolites with differences between the obtained comparison pairs were clustered into classes with the same or similar metabolic patterns for hierarchical clustering analysis.

3 Results

3.1 Quality control of survey data

The base peak intensity (BPI) charts monitor the strongest peaks in each chromatogram. The strongest peak intensity at each point in the analysis is shown (Figure 1). The BPI from the QC sample has a good overlap of peak retention time and peak area, indicating good instrument stability. The positive ion mode 17,312 peaks were identified and 2,563 metabolites were annotated, of which 94.25% had relative standard deviation (RSD) ≥ 0.7; in the negative ion mode 7,825 peaks were identified and 1,990 metabolites were annotated, of which 86.81% had RSD ≥ 0.7 (Table 1). If the RSD is greater than 60%, it is an indication that the sample is reliable and can be used for follow-up testing.

3.2 Principal component analysis

By performing principal component analysis (PCA) on the samples, we can preliminarily understand the overall metabolic differences between the samples of each group and the degree of variation between the samples within the group. After PCA analysis of the total samples, the results (Figure 2) showed that the two groups

1 https://hmdb.ca/

2 http://www.genome.jp/kegg/

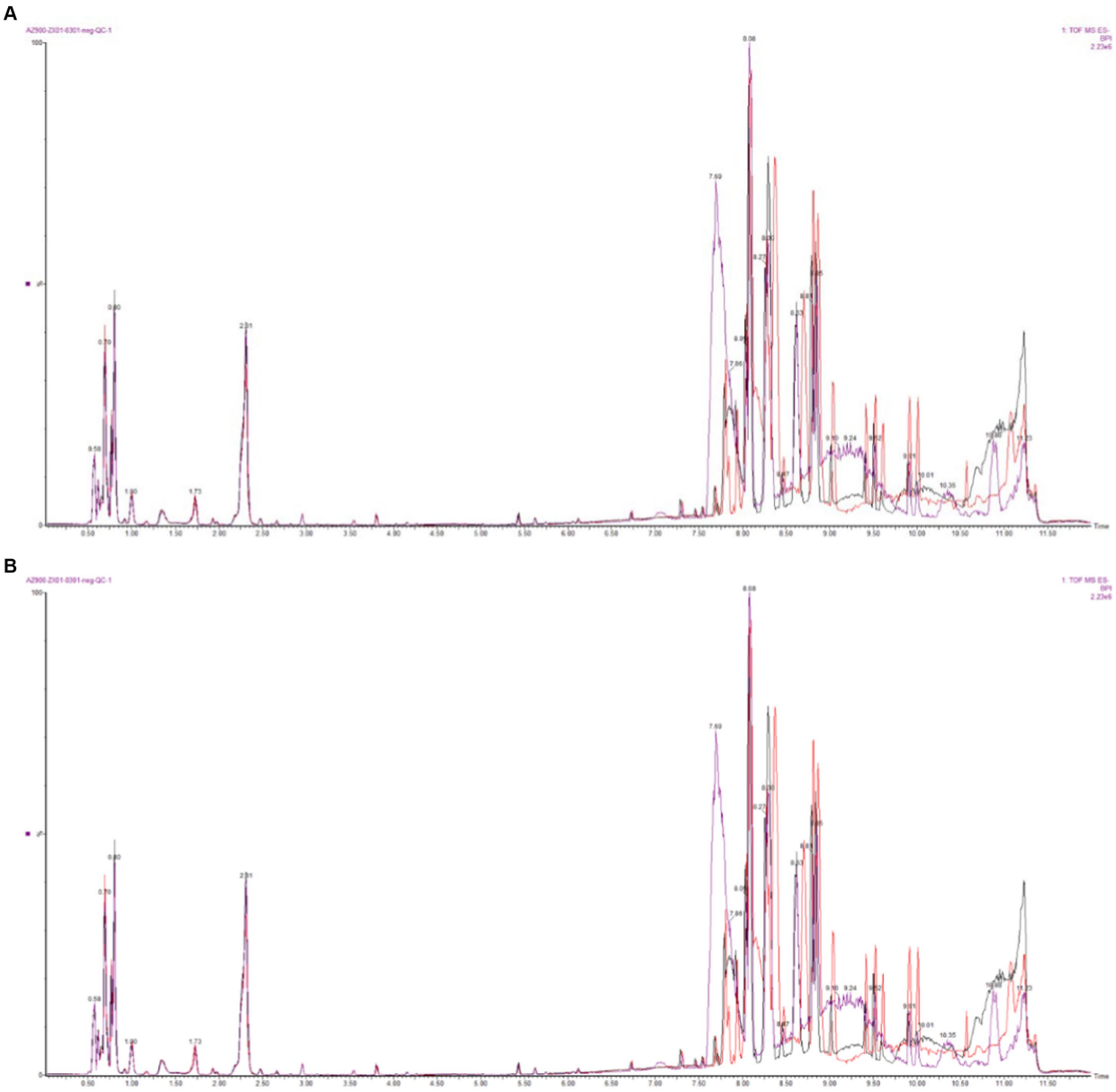


FIGURE 1
Base peak intensity (BPI) overlapping diagram of QC samples (**A**: positive ion mode; **B**: negative ion mode).

TABLE 1 List of quality control indicators.

Mode	Number_of_peaks	Number_of_metabolites	QC_RSD_percent (≥0.7)
pos	17,312	2,563	94.25%
neg	7,825	1,990	86.81%

of samples were divided into two different groups, and each sample was within the ellipse of the 95% confidence interval, indicating that the samples in the group had good repeatability. The two ellipses were independent of each other and there was no overlapping area, indicating that there was a large difference between the two groups of samples.

3.3 Orthogonal partial least squares discriminant analysis

Orthogonal partial least squares discriminant analysis (OPLS-DA) is a supervised analysis that can better screen for differential metabolites by excluding influences that are not relevant to the study. OPLS-DA analysis was performed on the control and SW treatment groups and the results are shown in Figure 3. Under the two modes, the samples of the two groups were clearly grouped, indicating that the metabolites of the two groups were significantly different.

To check the reliability of the OPLS-DA model, a replacement test is required. This means that the grouping of the samples is randomly scrambled, and the OPLS-DA is modeled according to

10.3389/fvets.2024.1387853

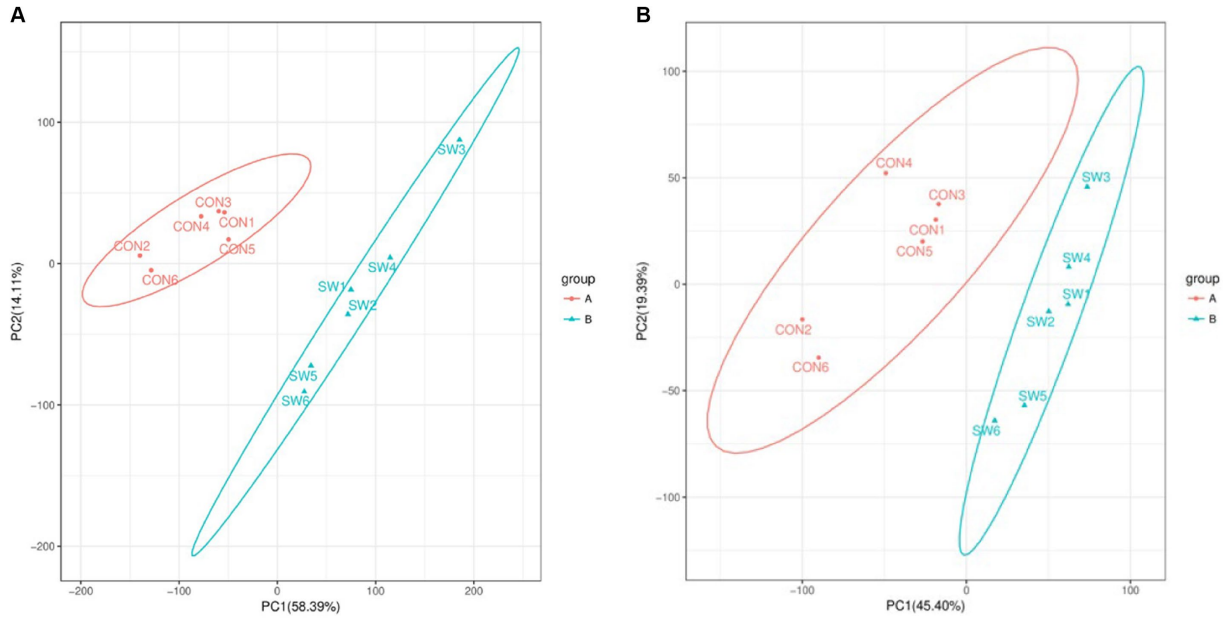


FIGURE 2
Principal component analysis (PCA) of all samples (**A**: positive ion mode; **B**: negative ion mode). Where the *x*-axis indicates the first principal component, the *y*-axis indicates the second principal component, and the percentage axis indicates the percentage contribution of that principal component to the sample variance. Each point on the graph represents a sample, samples in the same group are shown in the same color and samples in different subgroups are shown in different colors. Ellipses represent 95% confidence intervals.

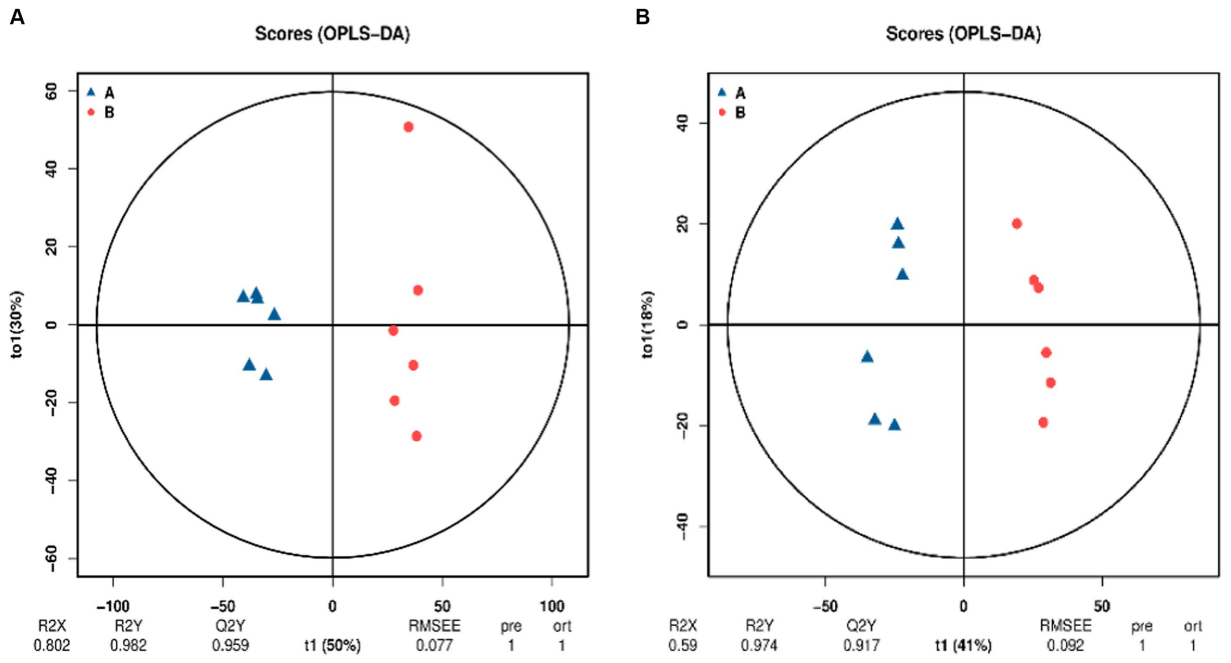


FIGURE 3
Orthogonal projections to latent structures–discriminant analysis (OPLS-DA) score chart (**A**: positive ion mode; **B**: negative ion mode). The blue point A in the image is the control group and the red point B is the SW treatment group. The *x*-axis (t1) represents the prediction component (the between-group variance component), the *y*-axis (t2) represents the orthogonal component (the within-group variance component), and the cross *y*-axis percentage represents the component's proportion of the total variance. The model parameters are listed below, including R^2X, R^2Y, Q^2Y, root mean square error (RMSEE), pre (number of predicted components), and ort (number of orthogonal components).

the scrambled grouping and its R^2Y and Q^2Y are calculated. After many iterations, the results of multiple modeling are plotted on a scatterplot, as shown in Figure 4, where $Q^2Y > 0.9$ indicates that the model is excellent. The intercept of the Q^2Y fitting regression line is negative, indicating that the model does not overfit; the slope of the Q^2Y fitting regression line is positive, indicating that

the model is meaningful, and the blue point is mostly above the red point, indicating that the independence of the modeling training set and test set is good. In the negative ion mode, the blue point overlaps the red point. This shows that the independence of the negative ion mode is not as good as that of the positive ion mode.

3.4 Metabolite classification and functional annotation

Metabolites in positive and negative ion mode are combined and annotated in HMDB as shown in Figure 5. The metabolites mainly include benzenoids, lipids and lipid-like molecules, nucleosides, nucleotides and analogs, organic acids and derivatives, organic nitrogen compounds, organic oxygen compounds, organoheterocyclic compounds, phenylpropanoids and polyketides, eterocyclic compounds, and pids and lipid-like molecules.

The KEGG database is a major public database related to metabolic pathways, which can be used for metabolic analysis *in vivo*. KEGG database is used to annotate all identified metabolites. Figure 6 shows the most annotated information (top 20 displayed) of Knock Out (KO) pathway level 2. The main metabolic pathways affected include amino acid metabolism, cancer: overview, digestive system, lipid metabolism, membrane transport, metabolism of cofactors and vitamins, overview, digestive system, lipid metabolism, membrane transport, metabolism of cofactors and vitamins, metabolism of other amino acids, metabolism of terpenoids and polyketides, nervous system, metabolism of other amino acids, metabolism of terpenoids and polyketides, nervous system, Nucleotide metabolism, and Xenobiotics biodegradation and metabolism.

3.5 Differential metabolite analysis

Based on the results of OPLS-DA, the VIP of the OPLS-DA model can be obtained by multivariate analysis, and the metabolites of different varieties or tissues can be initially screened out. The screening criteria are ① FC ≥ 1; ② VIP ≥ 1; and ③ $p < 0.05$.

By combining metabolites in positive and negative ion mode, a total of 2,170 differential metabolites were identified, of which 1,342 were upregulated and 828 were downregulated. Details of the differential metabolites are shown in Supplementary Table 1.

3.5.1 Difference multiple analysis

The Fold Changes were compared after qualitative and quantitative analysis of the detected metabolites Table 2 shows the results of upregulated and downregulated $\log_2$FC of the top 20 metabolites in the test group compared with the control group after log conversion treatment for differential metabolite multiple. Among them, the top 5 metabolites with the highest upregative difference multiple were Ttricosanoylglycine, 1-Tricosanol, Sambacolignoside, Kanzonol T, and Nemonoxacin, respectively. The top 5 metabolites with the largest down-variance were 2-(Ethylamino)-4,5-dihydroxybenzamide, Cinobufotalin, Undecylprodigiosin, Hypochoeroside A, and Muzanzagenin.

3.5.2 Volcanic map of differential metabolites

The volcano plot can directly show the overall distribution of the difference in metabolite content in the two groups, and the statistical significance of the difference in metabolite content. As shown in Figure 7, blue dots represent downregulated differentially expressed metabolites, red dots represent upregulated differentially expressed metabolites, and gray dots represent metabolites with insignificant differences. After sorting by p value, the first five metabolite names identified are shown in the Figure 7.

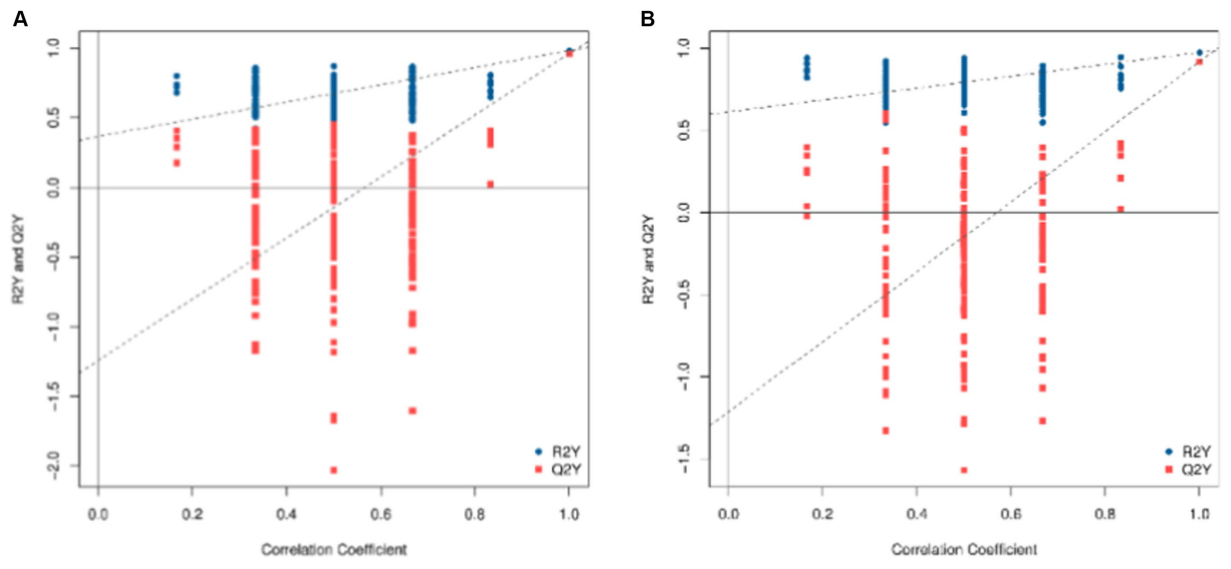


FIGURE 4
Replacement test diagram of OPLS-DA model (**A**: positive ion model; **B**: negative ion mode). In this image, the *X*-axis represents the correlation between the replacement group and the original model group, the *Y*-axis represents the value of R^2Y or Q^2Y (where Q^2Y and R^2Y of 1 are the values of the original model), the blue point and the red point represent the R^2Y and Q^2Y of the model after replacement, respectively, and the two dotted lines represent the regression line fitted by R^2Y and Q^2Y.

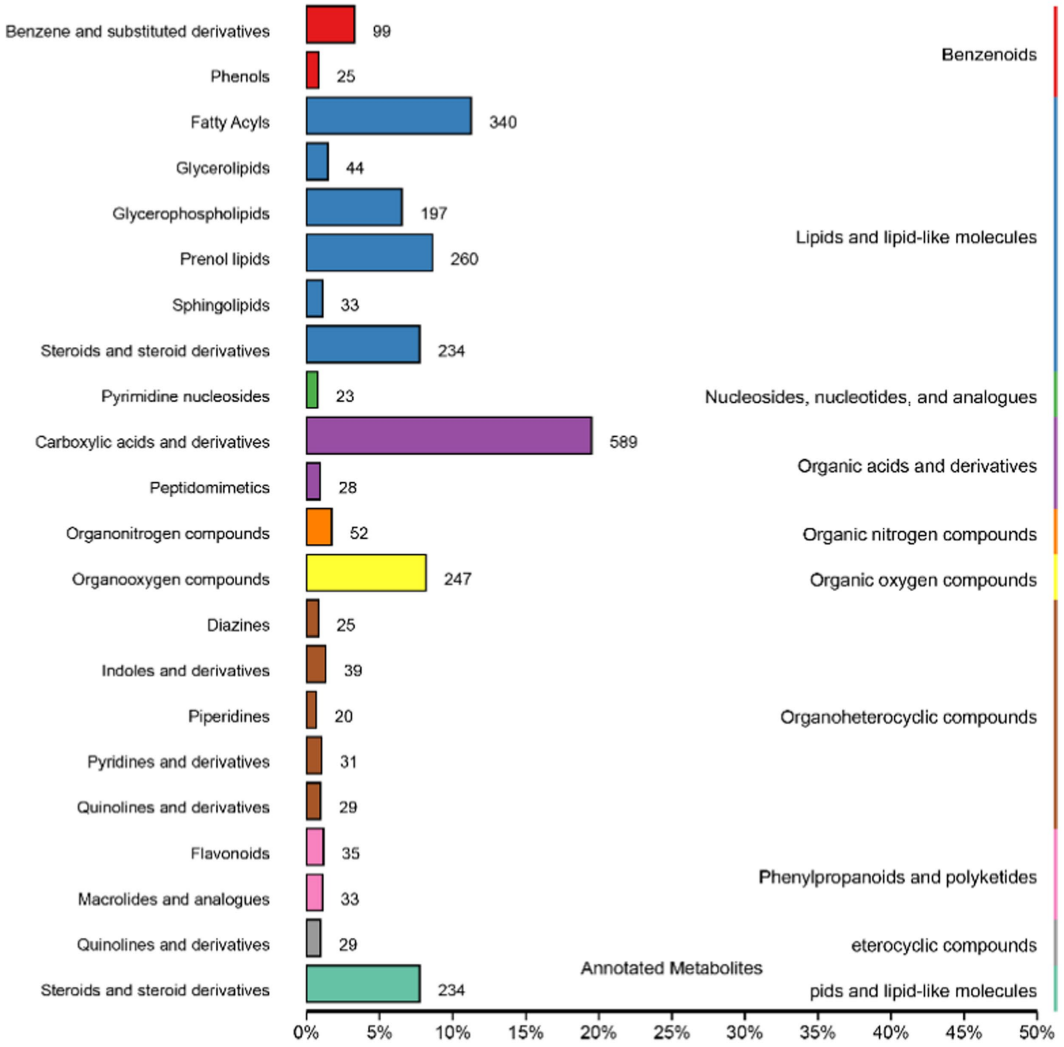


FIGURE 5
Human Metabolome Database (HMDB) classification summary. Items in the same box in the image represent HMDB level classification information, corresponding to the super class and class information of the HMDB database. Column length represents the number of metabolites annotated by the classification.

3.5.3 Differential metabolite cluster analysis

Cluster Analysis is a common multivariate statistical analysis method, the quantification of metabolites is expressed by color, the redder the color, the higher its content in a sample, and conversely, the greener the color, the lower the content, which is usually simple and intuitive to observe the overall characteristics of the data. Hierarchical cluster analysis was performed on the differential metabolites, and the results were shown in Figure 8. As can be seen from the figure, the color difference of the selected metabolites in the group is small, indicating that the content is similar, and the color difference between different treatment groups is large and the change is obvious, indicating that these different metabolites can clearly distinguish the control group and the SW group.

3.6 Enrichment analysis of differential metabolite metabolic pathways

The cluster profiler was used to analyze the annotation results of the selected differential metabolites in KEGG by means of hypergeometric test, and the differential metabolite enrichment diagram was drawn (only the top 20 results were displayed). The results were shown in Figure 9, where the size of the points represented the number of enriched differential metabolites. The color of the dots represents *p* value, and the redder the color, the more significant the enrichment. The top 6 channels enriched to Bile secretion, Primary bile acid biosynthesis, Riboflavin metabolism, Ferroptosis, Drug metabolism-cytochrome P450, and pyrimidine metabolism. The five pathways with the most significant differences are Primary bile acid biosynthesis, Ferroptosis, Riboflavin metabolism and Valine, leucine, and isoleucine degradation.

3.7 Screening for specific metabolites associated with the SW toxicity

Based on metabolic specificity and the size of VIP value and log-fold change (LFC) of the difference multiple was taken into account. Among them, we have identified five metabolites associated

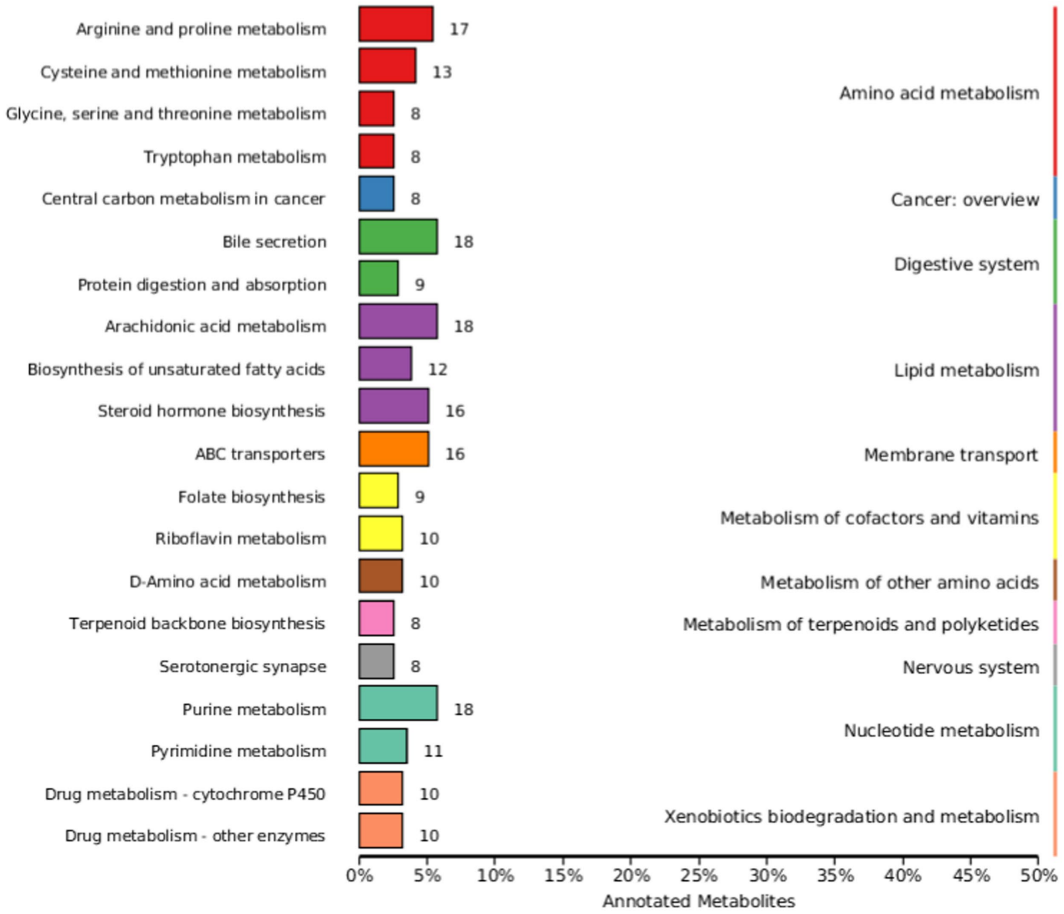


FIGURE 6
Kyoto Encyclopedia of Genes and Genomes (KEGG) database classification summary. The entries under the same box in the image represent the level classification notes of the KEGG pathway, corresponding to KO pathway Level 1 and KO pathway Level 2. The length of the column indicates the number of metabolites annotated by the pathway.

with SW toxicity which were shown in Table 3 and diagnostic accuracy was evaluated by the area under the curve.

4 Discussion

This study utilized untargeted metabolomics to analyze the differential metabolites in renal tubular epithelial cells treated with SW compared to the control group. A total of 2,170 significantly different metabolites were screened, most of which were not annotated due to limited related research. The annotated differential metabolites were mainly enriched in the following pathways: Bile secretion, Primary bile acid biosynthesis, Riboflavin metabolism, Ferroptosis, Drug metabolism-cytochrome P450, and Primidine metabolism. Among these differential metabolites, SW is the most distinctive one. However, due to its short half-life of only 20 h (9, 12), it may not be detectable, thus requiring corroboration with other indicators. Therefore, combining VIP values and Log_2FC, we have screened out substances such as 3alpha,7alpha-Dihydroxy-5beta-cholestanate, 2-Hydroxyiminostilbene, and glycochenodeoxycholate as potential diagnostic values for SW poisoning.

Bile secretion and Primary bile acid biosynthesis were the pathways with the highest number of differentially expressed metabolites. Among them, glycochenodeoxycholate, a component of primary bile acids and a raw material for bile acid synthesis, 3α,7α,12α,26-tetrahydroxy-5β-cholestane, and 7a-Hydroxy-cholestene-3-one, intermediates in the bile acid synthesis pathway (28), were significantly elevated. Previous results genomic study showed consistent enrichment in bile metabolism and drug metabolism-cytochrome P450 when rat renal tubular epithelial cells were exposed to the same conditions as in this study. Additionally, Fu et al. (25) demonstrated that SW induced liver inflammation by altering bile acid metabolism and intestinal microbiota in mice. Thus, these results suggest that exposure to SW may significantly alter the bile acid metabolism whether these changes is associated with the SW-induced cellular damage remain to be investigated.

Cytochrome P450 is one of the most common enzyme families involved in metabolism of the xenobiotics, playing a crucial role in the metabolism of drugs, toxins, and endogenous substrates (29). It plays a crucial role in the clearance of compounds such as drugs, toxins, and endogenous substrates. Our results have found significant increases in 2-Hydroxyiminostilbene, carbamazepine iminoquinone, carbamazepine-O-quinone, morphine-6-glucuronide, and 6-Methylmercaptopurine.

10.3389/fvets.2024.1387853

TABLE 2 Results of differential metabolite multiple analysis.

ID	Name	Formula	m/z	Retention time (min)	log_2FC	Regulated
neg_6622	Tricosanoylglycine	$C_{25}H_{49}NO_3$	456.37	7.88	37.11	Up
pos_7083	1-Tricosanol	$C_{23}H_{48}O$	379.33	3.28	36.80	Up
neg_1289	Sambacolignoside	$C_{43}H_{54}O_{22}$	921.29	0.70	36.29	Up
pos_1086	Kanzonol T	$C_{25}H_{26}O_7$	915.31	0.70	35.38	Up
pos_12100	Nemonoxacin	$C_{20}H_{25}N_3O_4$	354.18	4.14	35.34	Up
pos_11933	2-Benzylidene-1-heptanol	$C_{14}H_{20}O$	222.19	4.08	35.22	Up
pos_11705	Lotaustralin	$C_{11}H_{19}NO_6$	279.15	4.02	35.19	Up
pos_13943	FMNH2	$C_{17}H_{23}N_4O_9P$	481.11	5.43	35.07	Up
neg_1292	Probenecid Glucuronide	$C_{19}H_{27}NO_{10}S$	981.29	0.70	35.06	Up
pos_14727	(Z)-7-Hexadecen-1,16-olide	$C_{16}H_{28}O_2$	527.41	6.94	35.01	Up
neg_1212	(13Z,16Z)-Docosadienoyl-CoA	$C_{43}H_{74}N_7O_{17}P_3S$	1120.39	0.70	34.86	Up
pos_14363	Herculin	$C_{16}H_{29}NO$	234.22	6.30	34.58	Up
pos_13944	Coriandrin	$C_{13}H_{10}O_4$	483.11	5.43	34.37	Up
pos_14513	N2-Galacturonyl-L-lysine	$C_{12}H_{22}N_2O_8$	340.17	6.67	34.19	Up
pos_17309	Pyridinoline	$C_{18}H_{28}N_4O_8$	451.18	9.52	34.13	Up
pos_13603	3-Heptylpyridine	$C_{12}H_{19}N$	195.19	5.06	34.10	Up
pos_13914	4-Amino-3-hydroxybutanoylcarnitine	$C_{11}H_{22}N_2O_5$	280.18	5.37	34.03	Up
pos_13931	Pantoyllactone glucoside	$C_{12}H_{20}O_8$	331.08	5.41	33.75	Up
pos_11369	2-Amino-5-formylamino-6-(5-phospho-D-ribosylamino)pyrimidin-4(3H)-one	$C_{10}H_{16}N_5O_9P$	399.11	3.93	33.68	Up
neg_1285	(1-(2-(Methylsulfonamido)ethyl) piperidin-4-yl)methyl 5-fluoro-2-methoxy-1H-indole-3-carboxylate	$C_{19}H_{26}FN_3O_5S$	899.31	0.70	33.60	Up
neg_4335	Linalool oxide D 3-(apiosyl-(1->6)-glucoside)	$C_{21}H_{36}O_{11}$	463.22	3.27	−29.65	Down
neg_1374	Ile-Val-Val	$C_{16}H_{31}N3O_4$	364.20	0.75	−29.76	Down
neg_3616	Rotundifoline	$C_{22}H_{28}N_2O5$	421.17	2.63	−29.96	Down
neg_1905	Labetalol	$C_{19}H_{24}N_2O_3$	349.15	1.91	−30.15	Down
neg_4644	Bursin	$C_{14}H_{25}N_7O_3$	374.17	3.52	−30.19	Down
neg_1726	2-Isopropyl-5-methoxypyrazine	$C_8H_{12}N_2O$	349.19	1.14	−30.21	Down
neg_5099	Periplocin	$C_{36}H_{56}O_{13}$	741.37	3.97	−30.51	Down
neg_1744	Histidyltryptophan	$C_{17}H_{19}N_5O_3$	340.14	1.20	−30.55	Down
neg_4478	LysoPE (20:4(5Z,8Z,11Z,14Z)/0:0)	$C_{25}H_{44}NO_7P$	536.25	3.38	−30.62	Down
neg_5581	CDP-DG [a-21:0/20:4(5Z,8Z,11Z,14Z)-OH (20)]	$C_{53}H_{91}N_3O_{16}P_2$	1108.56	4.85	−30.65	Down
neg_1177	2′-C-Methylcytidine	$C_{10}H_{15}N3O_5$	513.21	0.68	−31.05	Down
neg_4257	N-Docosahexaenoyl Aspartic acid	$C_{26}H_{37}NO_5$	442.27	3.20	−31.07	Down
neg_4772	Asp Phe Val Glu	$C_{23}H_{32}N_4O_9$	507.21	3.63	−31.25	Down
neg_21	Solithromycin	$C_{43}H_{65}FN_6O_{10}$	889.46	0.47	−31.37	Down
neg_5587	Dioncophylline A	$C_{24}H_{27}NO_3$	799.40	4.89	−31.38	Down
neg_3144	Muzanzagenin	$C_{27}H_{38}O_5$	477.25	2.06	−31.50	Down
neg_3128	Hypochoeroside A	$C_{21}H_{32}O_9$	449.18	2.02	−32.02	Down
neg_3713	Undecylprodigiosin	$C_{25}H_{35}N_3O$	428.25	2.73	−32.17	Down
neg_3530	Cinobufotalin	$C_{26}H_{34}O_7$	493.21	2.52	−32.48	Down
neg_1823	2-(Ethylamino)-4,5-dihydroxybenzamide	$C_9H_{12}N_2O_3$	437.17	1.64	−32.48	Down

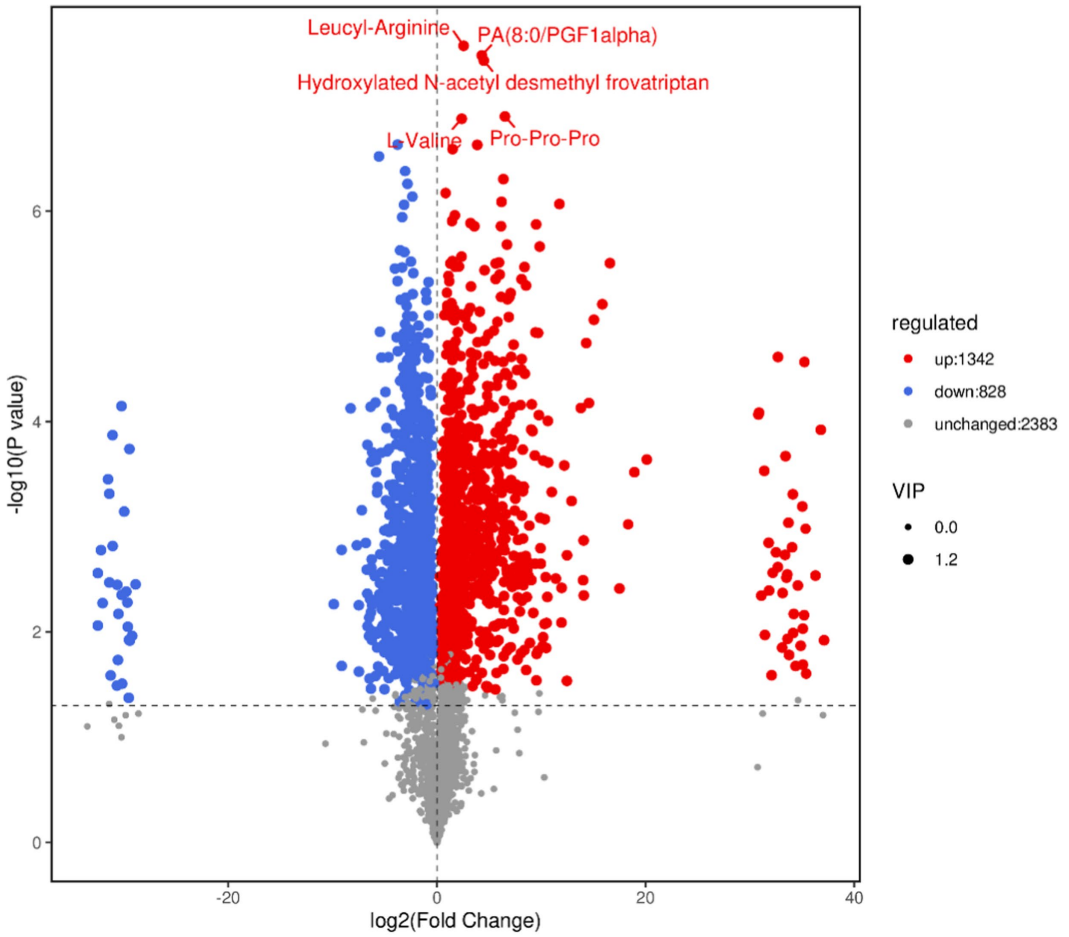


FIGURE 7
Volcano map of differential metabolites. In the volcano map, each point represents a metabolite. The *x*-axis represents the fold change of different substances in the comparison group (log_2 transformed), and the *y*-axis represents the *p* value (log_{10} transformed). The size of the scatter point indicates the VIP value of the OPLS-DA model. Larger scatter points correspond to higher VIP values, indicating more reliable selection of differentiated expression metabolites.

These metabolites are associated with the metabolism of Carbamazepine, morphine, and capecitabine, and they rely on P450 enzymes for catalysis (30), suggesting that cells may activate drug metabolism-cytochrome P450 to facilitate the clearance of drugs after exposure to SW. The particular cytochrome P450 induced by the SW however need to be further investigated.

Ferroptosis is a new type of cell death discovered in recent years. Many studies have shown that ferroptosis is related to kidney damage (31–33). A notable elevation in glycochenodeoxycholate (GCDA) levels has been observed in patients suffering from chronic kidney disease. Wang et al. (34) demonstrated that that glycochenodeoxycholate GCDA has the capacity to activate the expression of hepcidin, profoundly influencing the iron homeostasis within the body. Disruption of this iron balance can lead to an excessive accumulation of iron ions, particularly Fe^{2+}, which can subsequently trigger ferroptosis (34). Nevertheless, the intricate relationship between GCDA and ferroptosis remains to be thoroughly elucidated and validated.

There are several documented research on related toxicants. For example, another indole alkaloid, vincristine, its impact on plasma metabolites in human children was mainly enriched in the pathways of purine metabolism, arginine biosynthesis, sphingolipid metabolism, glutathione metabolism, glycerophospholipid metabolism, and lysine degradation (35) This differs significantly from the metabolic pathways affected by SW, possibly due to differences in species and tissues of the samples. Rao et al. (36) found that exposure to Matrine in mice resulted in significant changes in metabolites such as cholic acid, taurocholic acid, L-tyrosine, flavin mononucleotide (FMN), UDP-glucuronate, urea, sulfate, and inosine monophosphate, where riboflavin metabolism, purine metabolism, and ascorbate and aldarate metabolism were the most significant pathways. In our results, we also observed significant changes in FMN and UDP, which are involved in riboflavin and pyrimidine metabolism, respectively, suggesting the potential importance of these pathways in the metabolism of alkaloid toxins.

Riboflavin is a component of vitamin B complex and plays an important role in maintaining body health. Flavin mononucleotide (FMN), a coenzyme of riboflavin, is involved in reactions, including drug, lipid and heterogeneous metabolism, energy metabolism, and cellular processes such as protein folding and cell signaling (37, 38). Cytochrome P450 is one of the most common enzyme families involved in drug metabolism, most

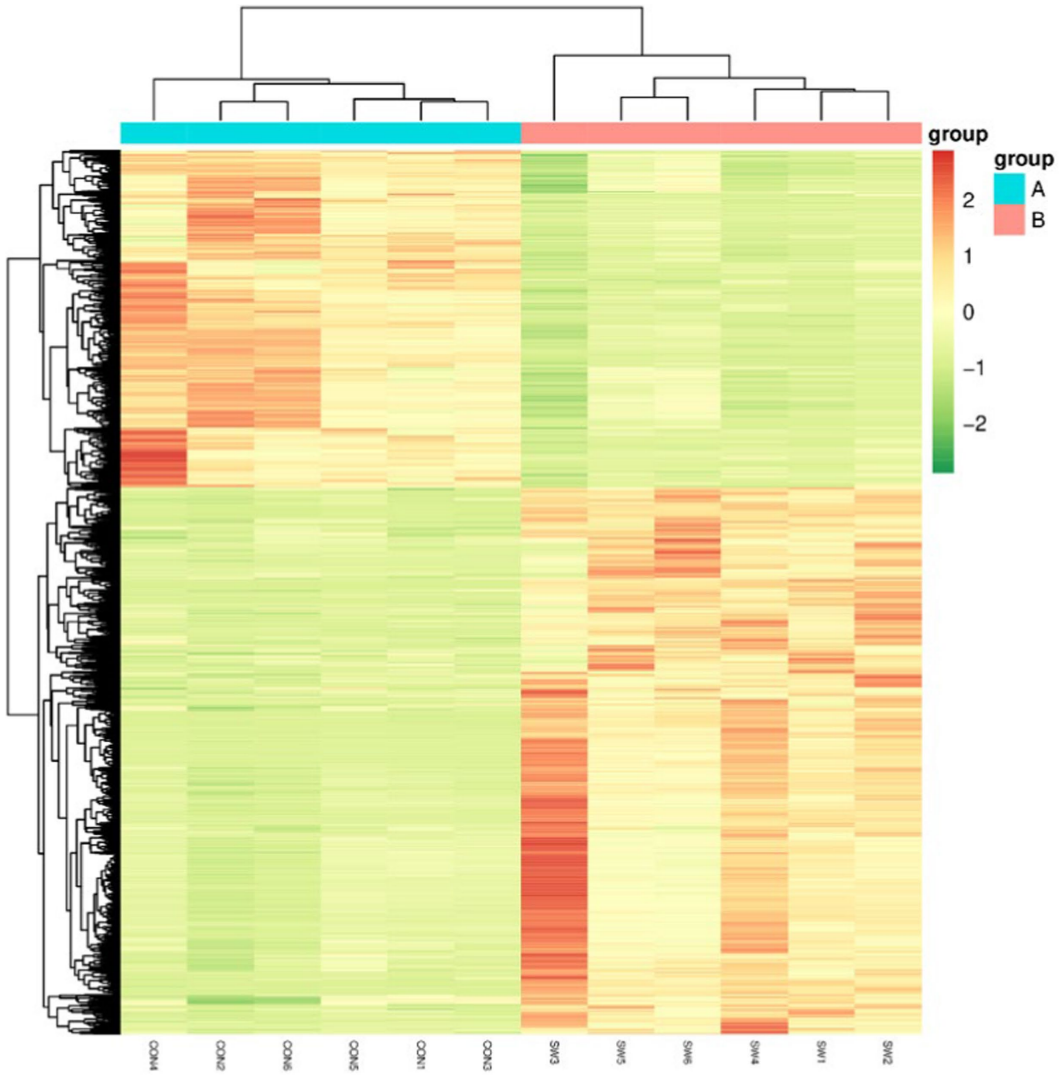


FIGURE 8
Differential metabolism clustering heat map. Group A served as the control group, while Group B was treated with SW. On the *x*-axis, each sample is represented, and the *y*-axis represents the quantitative value of metabolites standardized by *Z*-score after hierarchical clustering.

P450 enzymes use NAD(P)H as a coenzyme, which undergoes dehydrogenation to form NAD(P) and release electrons. The electron is then transferred to FMN, converting it to FMNH+2 (39). These results suggested that SW might induce cell death by inducing ferroptosis, riboflavin metabolism, and drug metabolism-cytochrome P450.

Untargeted metabolomic study can provide the broadest coverage of metabolites and help undiscovered specific metabolites associated with SW toxicity, giving us an objective and comprehensive understanding of the link between the body's metabolism and external stimuli. It has been shown to be useful in studying the complex diseases. For example, metabolomics has now become a good exploration tool in psychiatry (40, 41). In veterinary medicine, these characteristic metabolic changes related to livestock plant poisoning have great practical application. Clinical symptoms in livestock often have certain specificity but only show when poisoning is severe enough, while these specific changes in the metabolites can help diagnosis of earlier and faster (7), so as to respond more quickly taking corresponding measures to minimize losses. Our study shows that after SW treatment, based on metabolic specificity and the VIP values and Log_2FC of differential metabolites, we have identified five metabolites which may be used for diagnosis for SW poisoning.

In summary, the findings of this study reveal that the primary metabolites encompass benzenoids, lipids and lipid-like molecules, nucleosides, nucleotides, and analogs, along with organic acids and derivatives. Notably, the differential metabolites are predominantly enriched in specific pathways such as bile secretion, primary bile acid biosynthesis, riboflavin metabolism, ferroptosis, drug metabolism-cytochrome P450, and primidine metabolism. Upon screening for potential biomarkers of SW poisoning, it has been observed that each metabolite exhibits an area under the ROC curve (AUC) value were 1, indicating a significant distinction between the control group and the SW group. Consequently, these metabolites are viable candidates for potential biomarkers of SW poisoning. However, the diagnostic values of these metabolites for SW poisoning need to be further

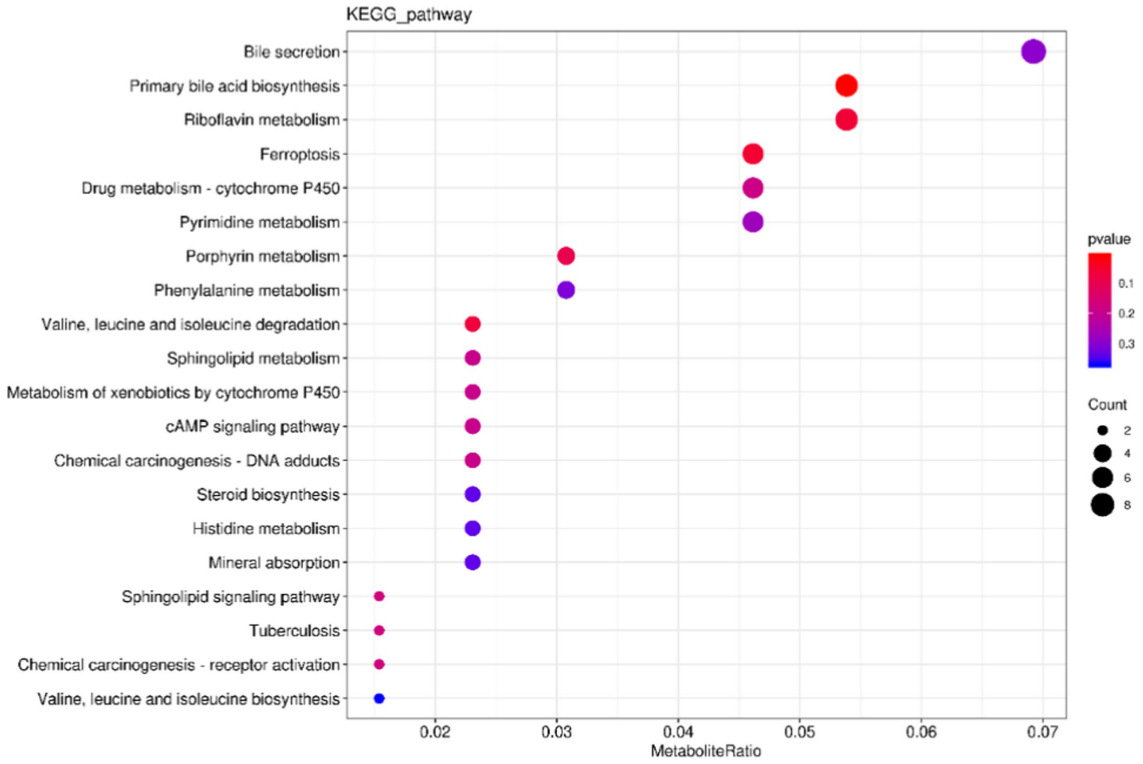


FIGURE 9
Kyoto Encyclopedia of Genes and Genomes enrichment map of differential metabolites. The *X*-axis represents the ratio of the number of differentially expressed metabolites in the corresponding pathway to the total number of metabolites detected and annotated by the pathway. The *Y*-axis corresponds to the pathway names. The color depth of the dots represents the log *p* value, with a deeper shade of red indicating a more significant enrichment. The size of the dots indicates the number of enriched differentiated metabolites.

TABLE 3 **Differential metabolites associated with renal tubular epithelial cells treated by SW.**

Number	#ID	Name	VIP	*p*-value	log_2FC	Regulated	AUC
1	pos_789	Swainsonine	1.4512	0.0000044	5.6368	UP	1
2	pos_17585	3alpha,7alpha-Dihydroxy-5beta-cholestanate	1.4468	0.0000013	3.2384	UP	1
3	pos_1797	2-Hydroxyiminostilbene	1.4176	0.0000458	4.8365	UP	1
4	pos_14725	Glycochenodeoxycholate	1.3034	0.0015693	3.4498	UP	1

validated in a variety of livestock and to rule out the potential effects of species, feeding environment, grazing practices, and other factors including the coexisting diseases.

Certainly, this study has certain limitations as it was conducted solely as an *in vitro* experiment, unable to fully simulate the *in vivo* environment. In the future, *in vivo* experiments could be conducted on the target animal, sheep, with various time gradients set to more precisely screen for early biomarkers of SW poisoning, thereby improving the detection and prevention of locoweed poisoning.

Data availability statement

The data presented in the study are deposited in the EMBL-EBI MetaboLights database with the identifier MTBLS9951.

Ethics statement

The animal study was approved by Laboratory Animal Management and Ethics Committee of Northwest A&F University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SZ: Formal analysis, Methodology, Validation, Writing – original draft. YiZ: Methodology, Writing – original draft. HY: Methodology, Writing – original draft. YL: Formal analysis, Writing – original draft. LT: Software, Writing – original draft. YaZ: Software, Writing – original draft. PS: Formal analysis, Writing – original draft. KW: Software, Writing – original draft. BZ: Conceptualization, Writing

– review & editing. HL: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by the grants from the National Natural Science Foundation of China (Nos. 32072929 and 32072928) and National Key R&D Program of China (No.2023YFD1801100).

Acknowledgments

Widely targeted/non-target metabolomics experiment was carried out at Biomarker Technologies (Beijing, China). We extend our sincere gratitude to them for providing invaluable technical support throughout the process. And we deeply appreciate the insightful and invaluable feedback provided by Yanan Tian, which has significantly contributed to the improvement of our manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1387853/full#supplementary-material

References

1. Marin RE, Micheloud JF, Vignale ND, Gimeno EJ, O'Toole D, Gardner DR, et al. Intoxication by Astragalus garbancillo var. garbancillo in llamas. *J Vet Diagn Invest*. (2020) 32:467–70. doi: 10.1177/1040638720914338

2. Cook D, Gardner DR, Lee ST, Pfister JA, Stonecipher CA, Welsh SL. A swainsonine survey of North American Astragalus and Oxytropis taxa implicated as locoweeds. *Toxicon*. (2016) 118:104–11. doi: 10.1016/j.toxicon.2016.04.033

3. Riet-Correa F, Medeiros RM, Schild AL. A review of poisonous plants that cause reproductive failure and malformations in the ruminants of Brazil. *J Appl Toxicol*. (2012) 32:245–54. doi: 10.1002/jat.1754

4. Marin RE, Gardner DR, Armien AG, Fortunato RH, Uzal FA. Intoxication of llamas by Astragalus punae in Argentina. *J Vet Diagn Invest*. (2022) 34:674–8. doi: 10.1177/10406387221094272

5. O'Sullivan BM, Goodwin JA. An outbreak of Swainsona poisoning in horses. *Aust Vet J*. (1997) 53:446–7. doi: 10.1111/j.1751-0813.1977.tb05496.x

6. Zhao M, Gao X, Wang J, He X, Han B. A review of the most economically important poisonous plants to the livestock industry on temperate grasslands of China. *J Appl Toxicol*. (2013) 33:9–17. doi: 10.1002/jat.2789

7. Green BT, Welch KD, Lee ST, Stonecipher CA, Gardner DR, Stegelmeier BL, et al. Biomarkers and their potential for detecting livestock plant poisonings in Western North America. *Front Vet Sci*. (2003) 10:1104702. doi: 10.3389/fvets.2023.1104702

8. Zhou KS, Tian W, Zhang Z, Tan CJ. Research advances on chemical constituents of Oxytropis ochrocephala Bunge and its bioactivities. *Nat Product Res Dev*. (2020) 32:161–71. doi: 10.16333/j.1001-6880.2020.1.022

9. Li B, Zhang C, Zhu Y, Sun P, Fan S, Wang W, et al. Development of novel formulation for sustained release of drug to prevent Swainsonine-containing plants poisoning in livestock. *Animals*. (2023) 13:2646. doi: 10.3390/ani13162646

10. Wang S, Tan P, Wang H, Wang J, Zhang C, Lu H, et al. Swainsonine inhibits autophagic degradation and causes cytotoxicity by reducing CTSD O-GlcNAcylation. *Chem Biol Interact*. (2023) 382:110629. doi: 10.1016/j.cbi.2023.110629

11. Wu C, Han T, Lu H, Zhao B. The toxicology mechanism of endophytic fungus and swainsonine in locoweed. *Environ Toxicol Pharmacol*. (2016) 47:38–46. doi: 10.1016/j.etap.2016.08.018

12. Stegelmeier BL, James LF, Panter KE, Ralphs MH, Gardner DR, Molyneux RJ, et al. The pathogenesis and toxicokinetics of locoweed (Astragalus and Oxytropis spp.) poisoning in livestock. *J Nat Toxins*. (1999) 8:35–45.

13. Cholich LA, Martinez A, Micheloud JF, Pistán ME, García EN, Robles CA, et al. Alpha-mannosidosis caused by toxic plants in ruminants of Argentina. *An Acad Bras Cienc*. (2021) 93:e20191496. doi: 10.1590/0001-3765202120191496

14. Wu CC, Wang W, Liu XX, Ma F, Cao D, Yang XW, et al. Pathogenesis and preventive treatment for animal disease due to locoweed poisoning. *Environ Toxicol Pharmacol*. (2014) 37:336–47. doi: 10.1016/j.etap.2013.11.013

15. Ramírez J, House LK, Karrison TG, Janisch LA, Turcich M, Salgia R, et al. Prolonged pharmacokinetic interaction between capecitabine and a CYP2C9 substrate, celecoxib. *J Clin Pharm Ther*. (2019) 59:1632–40. doi: 10.1002/jcph.1476

16. Zhan Q, Wang L, Liu N, Yuan Y, Deng L, Ding Y, et al. Serum metabolomics study of narcolepsy type 1 based on ultra-performance liquid chromatography-tandem mass spectrometry. *Amino Acids*. (2023) 55:1247–59. doi: 10.1007/s00726-023-03315-z

17. Witting M. (Re-)use and (re-)analysis of publicly available metabolomics data. *Proteomics*. (2023) 23:e2300032. doi: 10.1002/pmic.202300032

18. Wang Z, Zhu H, Xiong W. Advances in mass spectrometry-based multi-scale metabolomic methodologies and their applications in biological and clinical investigations. *Sci Bull*. (2023) 68:2268–84. doi: 10.1016/j.scib.2023.08.047

19. Liu ML, Zheng P, Liu Z, Xu Y, Mu J, Guo J, et al. GC-MS based metabolomics identification of possible novel biomarkers for schizophrenia in peripheral blood mononuclear cells. *Mol BioSyst*. (2014) 10:2398–406. doi: 10.1039/c4mb00157e

20. Rimti FH, Shahbaz R, Bhatt K, Xiang A. A review of new insights into existing major depressive disorder biomarkers. *Heliyon*. (2023) 9:e18909. doi: 10.1016/j.heliyon.2023.e18909

21. Dong R, Lu Q, Kang H, Suridjan I, Kollmorgen G, Wild N, et al. CSF metabolites associated with biomarkers of Alzheimer's disease pathology. *Front Aging Neurosci*. (2023) 15:1214932. doi: 10.3389/fnagi.2023.1214932

22. Della Corte V, Riolo R, Scaglione S, Pecoraro R, Tuttolomondo A. The role of biomarkers, metabolomics, and COVID-19 in venous thromboembolism-a review of literature. *Int J Mol Sci*. (2023) 24:13411. doi: 10.3390/ijms241713411

23. Li Y, Si Y, Ma Y, Yin H. Application and prospect of metabolomics in the early diagnosis of osteoporosis: a narrative review. *Bioanalysis*. (2023) 15:1369–79. doi: 10.4155/bio-2023-0131

24. Shahisavandi M, Wang K, Ghanbari M, Ahmadizar F. Exploring metabolomic patterns in type 2 diabetes mellitus and response to glucose-lowering medications-review. *Gene*. (2023) 14:1464. doi: 10.3390/genes14071464

25. Fu K, Chen X, Shou N, Wang Z, Yuan X, Wu D, et al. Swainsonine induces liver inflammation in mice via disturbance of gut microbiota and bile acid metabolism. *J Agric Food Chem*. (2023) 71:1758–67. doi: 10.1021/acs.jafc.2c08519

26. van Zogchel LMJ, Zappeij-Kannegieter L, Javadi A, Lugtigheid M, Gelineau NU, Lak NSM, et al. Specific and sensitive detection of neuroblastoma mRNA markers by multiplex RT-qPCR. *Cancer*. (2021) 13:150. doi: 10.3390/cancers13010150

27. Wang J, Zhang T, Shen X, Liu J, Zhao D, Sun Y, et al. Serum metabolomics for early diagnosis of esophageal squamous cell carcinoma by UHPLC-QTOF/MS. *Metabolomics*. (2016) 12:116. doi: 10.1007/s11306-016-1050-5

28. Deo AK, Bandiera SM. Identification of human hepatic cytochrome P450 enzymes involved in the biotransformation of cholic and chenodeoxycholic acid. *Drug Metab Dispos*. (2008) 36:1983–91. doi: 10.1124/dmd.108.022194

29. Zhao M, Ma J, Li M, Zhang Y, Jiang B, Zhao X, et al. Cytochrome p450 enzymes and drug metabolism in humans. *Int J Mol Sci*. (2021) 22:12808. doi: 10.3390/ijms222312808

30. Pearce RE, Uetrecht JP, Leeder JS. Pathways of carbamazepine bioactivation in vitro: II. The role of human cytochrome P450 enzymes in the formation of 2-hydroxyiminostilbene. *Drug Metab Dispos*. (2005) 33:1819–26. doi: 10.1124/dmd.105.004861

31. Li J, Cao F, Yin HL, Huang ZJ, Lin ZT, Mao N, et al. Ferroptosis: past, present, and future. *Cell Death Dis*. (2020) 11:88. doi: 10.1038/s41419-020-2298-2

32. Tonnus W, Meyer C, Steinebach C, Belavgeni A, von Mässenhausen A, Gonzalez NZ, et al. Dysfunction of the key ferroptosis-surveilling systems hypersensitizes mice to tubular necrosis during acute kidney injury. *Nat Commun*. (2021) 12:4402. doi: 10.1038/s41467-021-24712-6

33. Ni L, Yuan C, Wu X. Targeting ferroptosis in acute kidney injury. *Cell Death Dis*. (2022) 13:182. doi: 10.1038/s41419-022-04628-9

34. Wang LJ, Zhao GP, Wang XF, Liu XX, Li YX, Qiu LL, et al. Glycochenodeoxycholate affects Iron homeostasis via up-regulating hepcidin expression. *Nutrients*. (2022) 14:3176. doi: 10.3390/nu14153176

35. Verma P, Devaraj J, Skiles JL, Sajdyk T, Ho RH, Hutchinson R, et al. A metabolomics approach for early prediction of vincristine-induced peripheral neuropathy. *Sci Rep*. (2020) 10:9659. doi: 10.1038/s41598-020-66815-y

36. Rao SW, Duan YY, Zhao DS, Liu CJ, Xu SH, Liang D, et al. Integrative analysis of transcriptomic and metabolomic data for identification of pathways related to matrine-induced hepatotoxicity. *Chem Res Toxicol*. (2022) 35:2271–84. doi: 10.1021/acs.chemrestox.2c00264

37. Lee TY, Farah N, Chin VK, Lim CW, Chong PP, Basir R, et al. Medicinal benefits, biological, and nanoencapsulation functions of riboflavin with its toxicity profile: a narrative review. *Nutr Res*. (2023) 119:1–20. doi: 10.1016/j.nutres.2023.08.010

38. Powers HJ. Riboflavin (vitamin B-2) and health. *Am J Clin Nutr*. (2003) 77:1352–60. doi: 10.1093/ajcn/77.6.1352

39. Wang L, Xu Z, Zhang Y, Wang R, Wang J, Yang S, et al. Recent insights into function, structure, and modification of cytochrome P450 153 a family. *Mol Biol Rep*. (2023) 50:6955–61. doi: 10.1007/s11033-023-08553-8

40. Pedrini M, Cao B, Nani JVS, Cerqueira RO, Mansur RB, Tasic L, et al. Advances and challenges in development of precision psychiatry through clinical metabolomics on mood and psychotic disorders. *Prog Neuro-Psychopharmacol Biol Psychiatry*. (2019) 93:182–8. doi: 10.1016/j.pnpbp.2019.03.010

41. Quinones MP, Kaddurah-Daouk R. Metabolomics tools for identifying biomarkers for neuropsychiatric diseases. *Neurobiol Dis*. (2009) 35:165–76. doi: 10.1016/j.nbd.2009.02.019

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 05 June 2024
DOI 10.3389/fvets.2024.1390304

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Pavulraj Selvaraj,
Louisiana State University, United States
Jinxin Xie,
Xinjiang Agricultural University, China

*CORRESPONDENCE
Tongtong Wang
✉ wangtongtong@lcu.edu.cn
Changfa Wang
✉ wangchangfa@lcu.edu.cn
Muhammad Zahoor Khan
✉ zahoorkhattak91@yahoo.com

†These authors have contributed equally to this work

RECEIVED 23 February 2024
ACCEPTED 13 May 2024
PUBLISHED 05 June 2024

CITATION
Li L, Cui X, Yu Y, Sun Q, Li W, Li Y, Li S, Chen L, Khan MZ, Wang C and Wang T (2024) Blebbistatin as a novel antiviral agent targeting equid herpesvirus type 8. *Front. Vet. Sci.* 11:1390304. doi: 10.3389/fvets.2024.1390304

COPYRIGHT
© 2024 Li, Cui, Yu, Sun, Li, Li, Li, Chen, Khan, Wang and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Blebbistatin as a novel antiviral agent targeting equid herpesvirus type 8

Liangliang Li†, Xiu Cui†, Yue Yu†, Qi Sun, Wenjing Li, Yubao Li, Shuwen Li, Li Chen, Muhammad Zahoor Khan*, Changfa Wang* and Tongtong Wang*

College of Agronomy, Liaocheng University, Liaocheng, China

Introduction: Equid herpesvirus type 8 (EqHV-8) poses a significant threat to equine health, leading to miscarriages and respiratory diseases in horses and donkeys, and results in substantial economic losses in the donkey industry. Currently, there are no effective drugs or vaccines available for EqHV-8 infection control.

Methods: In this study, we investigated the *in vitro* and *in vivo* antiviral efficacy of Blebbistatin, a myosin II ATPase inhibitor, against EqHV-8.

Results: Our results demonstrated that Blebbistatin significantly inhibited EqHV-8 infection in Rabbit kidney (RK-13) and Madin-Darby Bovine Kidney (MDBK) cells in a concentration-dependent manner. Notably, Blebbistatin was found to disrupt EqHV-8 infection at the entry stage by modulating myosin II ATPase activity. Moreover, *in vivo* experiments revealed that Blebbistatin effectively reduced EqHV-8 replication and mitigated lung pathology in a mouse model.

Conclusion: Collectively, these findings suggest that Blebbistatin holds considerable potential as an antiviral agent for the control of EqHV-8 infection, presenting a novel approach to addressing this veterinary challenge.

KEYWORDS

EqHV-8, blebbistatin, myosin II ATPase inhibitor, antiviral activity, animal model

1 Introduction

Equid herpesvirus type 8 (EqHV-8), also known as Asinine Herpesvirus 3 (AHV-3), is a pathogen that causes severe respiratory disease and miscarriages in equines, posing a persistent threat to horse and donkey farming worldwide (1, 2). The EqHV-8 belongs to the Alpha Herpesviridae family, It is characterized as a double-stranded DNA virus with a genome length of approximately 150 kilobases and comprising 76 open reading frames (3). In recent years, EqHV-8 has shown a significant increase in prevalence, particularly in large-scale donkey farms in China. For instance, Wang et al. conducted a study indicating an EqHV-8 infection rate of approximately 38.7% (457/1180) in donkey farms in Shandong province, China (4). Additionally, cases of abortion and neurological diseases in donkeys induced by EqHV-8 infections have been documented (5, 6), highlighting the potential threat to the donkey industry. While the molecular mechanisms underlying EqHV-8 infections have been well documented in previous studies (7–10), it is crucial to note the limited availability of effective drugs for combating EqHV-8 infections (9, 10).

Blebbistatin, known for its inhibitory properties on myosin II ATPase activity, has gained significant attention and utility in biochemical, cell biological, and physiological research (11, 12). Recent studies have also explored its antiviral properties against various viruses. Notably, Antoine et al. demonstrated Blebbistatin's ability to significantly reduce the entry of Herpes Simplex Virus Type-1 (HSV-1) into human corneal epithelial (HCE) cells by interfering with myosin light chain kinase (13). In a study by Gao et al. (14), Blebbistatin exhibited inhibitory effects on Porcine reproductive and respiratory syndrome virus (PRRSV) infection. This inhibitory effect was observed in multiple contexts, including *in vitro* experiments using MARC-145 cells and PAM cells, as well as in swine (14). Moreover, Li et al. highlighted Blebbistatin's potential to mitigate PRRSV infection in other susceptible cell lines, such as PK-15^{CD163}, HEK-293T^{CD163}, and BHK-21^{CD163} (15). Additionally, Blebbistatin has demonstrated the capacity to suppress the replication of Murine Gammaherpesvirus 68 (MHV-68) both *in vitro* and *in vivo* (16). Despite the growing body of evidence regarding Blebbistatin's antiviral properties, its effectiveness against EqHV-8 remains unexplored.

In this study, we aimed to investigate the anti-EqHV-8 activity of Blebbistatin and elucidate its underlying mechanism. Our results demonstrate that Blebbistatin significantly reduces EqHV-8 replication in Rabbit kidney (RK-13) and Madin-Darby Bovine Kidney (MDBK) cells. Furthermore, Blebbistatin exerts its inhibitory effects at the adsorption and internalization stages of EqHV-8. Notably, Blebbistatin also displayed efficacy in decreasing EqHV-8 replication in lung tissue within a mouse model. In summary, our research highlights Blebbistatin as a promising candidate for controlling EqHV-8 infections.

2 Materials and methods

2.1 Cells, EHV-8 strain and reagents

The RK-13 and MDBK cells were cultured in Modified Eagle's medium (MEM, Gibco, United States) supplemented with 10% fetal bovine serum (FBS, Gibco, United States) and 1% penicillin–streptomycin (Servicebio, Wuhan, China) at 37°C and 5% CO_2. The EHV-8 SDLC66 strain (GenBank: MW816102.1), SD2020113 (GenBank: MW822570.1), and donkey/Shandong/10/2021 (GenBank: OL856098.1) were propagated in RK-13 cells. Additionally, Blebbistatin was obtained from Shandong Sikejie Biotechnology Co., LTD (Jinan, China) and dissolved in dimethyl sulfoxide (DMSO) (Solarbio, Beijing, China).

2.2 Cell viability detection

The cytotoxicity of Blebbistatin was detected using the Cell Counting Kit-8 (CCK-8) assay (Beyotime, Nanjing, China) as previously described (17). Briefly, RK-13 or MDBK cells were seeded into a 96-well plate at a density of 1 × 10^4 cells per well and incu-bated with Blebbistatin at various concentrations (0, 2.5, 5, 10, 20, 40, and 80 μM) for 24 h. Subsequently, the CCK-8 reagent was added to each well according to the manufacturer's instructions (10 μL/well) and incubated at 37°C for 2 h. The viability of the cells was determined by measuring the absorbance at 450 nm and analyzed us-ing the formula "cell survival rate (%) = [OD (sample) - OD (blank)/OD (control) -OD (blank)] × 100%."

2.3 DNA/RNA extraction and qPCR analysis

The DNA supernatant virus from different samples was extracted using the DNA Viral Genome Extraction Kit (Solarbio, Beijing, China). Absolute quantification PCR (qPCR) assay was performed to detect the supernatant DNA copies of EqHV-8 with recombinant plasmids pMD18-T-gD and *ORF72*-F and *ORF72*-R primers, as previously established method (10), and calculated by normalization to the standard curve.

The isolation of total RNA from cellular samples was accomplished employing TRIzol reagent (Sparkjade, Jian, China), following established procedures (10). Subsequently, the RNA was reversed to complementary DNA (cDNA) by All-in-One First Strand cDNA Synthesis Kit (GENENODE, Wuhan, China). The ensuing reactions were conducted on a Step One Plus real-time PCR system, wherein the SYBR™ Green PCR Master Mix (Genstar, Beijing, China) was employed in conjunction with specific primers designed to target the gD gene of EqHV-8. For the purpose of normalizing the gene expression data, mRNA expression levels of Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) were utilized as an internal reference, in accordance with well-established practices (14). The quantification of the target gene expression was carried out employing the $2^{-\Delta\Delta Ct}$ method, a widely accepted methodology previously described in the literature (14). The primer sequences are listed in Supplementary Table S1.

2.4 Western blot assay

The western blot assay was conducted in accordance with established protocols (18). In brief, cellular samples were collected at designated time intervals and subsequently lysed using NP40 lysis buffer. The lysates were mixed with 5 × protein loading buffer and separated via 12% SDS-PAGE gels. Following gel separation, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were subjected to blocking with 5% nonfat dry milk for a duration of 1 h. Subsequently, they were incubated with primary antibodies, specifically mouse anti-EqHV-8 gD, or mouse anti-α-Tubulin antibodies, and washed using PBST. Following the primary antibody incubation, the membranes were treated with a secondary antibody, the HRP-conjugated goat anti-mouse antibody. Ultimately, the visualization and imaging of specific protein bands were accomplished utilizing the ChemiDoc MP Imaging System (BioRad, California, United States).

2.5 Virus titration

The quantification of viral progeny production was executed through a titration procedure as previously documented (6). In brief, RK-13 cells were cultured in 96-well plates at a density of 1×10^4 cells per well and allowed to incubate overnight. The viral supernatant was serially diluted by a factor of 10, with each dilution

replicated eight times and added at a volume of 100 μL per well. These cells were then incubated at 37°C for a duration of 3–5 days, during which the cytopathic effect (CPE) was observed daily. The 50% cell culture infectious dose ($TCID_{50}$) was subsequently calculated employing the Reed–Muench method.

The virus titers in lung samples of EqHV-8-infected mice were detected using RK-13 cells. Briefly, all mice from 3 groups were sacrificed at 7 dpi, and their lungs were collected. The lung tissues (0.1 g) were mixed with PBS (1 mL), further crushed, homogenized, frozen and thawed 3 times. The supernatant was obtained from lung tissues by centrifugation, and filtered to remove bacteria using 0.22 μm syringe filter. Finally, the virus titer was determined in RK-13 cells as above.

2.6 Antiviral activity analysis

RK-13 and MDBK cells were seeded in 12-well plates and pre-treated with various concentrations of Blebbistatin (0, 2.5, 5, 10, and 20 μM) for 1 h. Subsequently, they were infected with EqHV-8 SDLC66 at a multiplicity of infection (MOI) of 0.1 for 1 h. Following infection, the cells were cultured in 3% FBS MEM containing Blebbistatin at the indicated concentrations. Cells were collected at 24-h post infection (hpi) to assess EqHV-8 replication via western blot analysis. Additionally, the cellular supernatants were also harvested for the detection of progeny virus titer using $TCID_{50}$.

2.7 Indirect immunofluorescence assay

To assess the antiviral efficacy against various EqHV-8 strains, an IFA was employed. In brief, RK-13 cells were pretreated with Blebbistatin at concentrations of 0, 5, 10, and 20 μM for a duration of 1 h. Subsequently, these cells were infected with EqHV-8 strains, including SDLC66, SD2020113, or Donkey/Shandong/10/2021, each at0.1 MOI for 1 h. Following infection, the cells were washed with PBS and then incubated in Modified Eagle Medium (MEM) containing 3% fetal bovine serum (FBS) and the indicated dosage of Blebbistatin. At 36 hpi, these cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and subjected to washing with PBS. These cells were subsequently treated with mouse anti-EqHV-8-positive serum (produced in-house) and incubated with a secondary Rhodamine-conjugated goat anti-mouse IgG antibody. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized using a DMi8 microsystems (Leica, Germany).

2.8 Analysis of temporal dynamics in EqHV-8 life cycle perturbation by Blebbistatin

To elucidate the specific phase of the EqHV-8 life cycle affected by Blebbistatin, RK-13 cells were cultured in 12-well plates under varying treatment conditions. These included distinct stages of exposure to EqHV-8 (MOI = 0.1) and 20 μM Blebbistatin, such as pre-treatment, co-treatment, post-treatment, and exposure throughout all stages. Subsequent to these treatments, the cells were harvested for the assessment of viral replication, utilizing quantitative PCR (qPCR) and western blot analyses at 24 hpi.

2.9 Assays for virus adsorption and internalization

In the virus adsorption assay, RK-13 cells, pre-seeded in 12-well plates, were treated with either 20 μM Blebbistatin or DMSO for 1 h at 37°C. Following treatment, the cells were cooled on ice and exposed to EqHV-8 SDLC66 (MOI = 1) for 1 h. Then, cells were washed thrice with pre-chilled PBS to remove non-adherent virions. The *mRNA* expression levels of bound EqHV-8 particles were quantified via qPCR.

For the virus internalization assay, RK-13 cells in 12-well plates were pre-treated with 20 μM Blebbistatin or DMSO at 37°C for 1 h, followed by incubation with EqHV-8 SDLC66 (MOI = 1) at 4°C for 1 h to facilitate virus binding. Unbound viruses were removed with chilled PBS, and the cells were incubated in 3% FBS MEM containing either Blebbistatin or DMSO at 37°C for 1 h to initiate internalization. Post-internalization, non-internalized viruses were eliminated using citrate buffer (pH 3.0), and the mRNA levels of EqHV-8 gD expression were detected by qPCR.

2.10 *In vivo* anti-EqHV-8 efficacy assay

Fifteen SPF (Specific Pathogen-Free) male BALB/c mice, aged 6 weeks, were procured from Pengyue (Jinan, Shandong Province, China), and were assigned randomly into three distinct experimental groups (n = 5/group). The first group, termed the 'mock group,' received intranasal administration of MEM in a volume of 50 μL (including 0.1% Dimethyl Sulfoxide, DMSO). The second group, designated as the 'EqHV-8 group,' underwent a pre-inoculation process in which they received intranasal administration of a solution consisting of 0.1% DMSO in MEM (50 μL). Subsequently, they were subjected to EqHV-8 inoculation at a viral concentration of 1 × 10^5 PFU (Plaque-Forming Units) per mouse. The third group, denoted as the 'Blebbistatin+EqHV-8 group'. These mice were pre-inoculated intranasally with Blebbistatin at a dosage of 30 μM/kg in MEM (50 μL) at three specific time points: 1 day pre-infection, 1-day post-infection (dpi), and 3 dpi. Following this, they were subjected to EqHV-8 inoculation at the same viral concentration of 1 × 10^5 PFU per mouse. All inoculations were performed under profound anesthesia induced using Zoletil 50(Virbac in Nice, France). Throughout the duration of the study, the mice were provided with *ad libitum* access to food and water, and were individually housed to prevent potential cross-contamination. Clinical symptoms were meticulously monitored throughout the experimental period. On the 8th dpi, humane euthanasia was conducted via cervical dislocation, and lung tissues were harvested for subsequent histopathological analysis and evaluation of viral replication efficiency.

2.11 Histopathological evaluation

The protective effect of Blebbistatin against lung damage induced by EqHV-8 was evaluated through hematoxylin and eosin (H&E) staining methodology, as outlined in previous study (7). Lung tissues were meticulously preserved by immersing them in a 10% formalin solution. Following fixation, the tissues were embedded in paraffin

wax, and subsequently, thin sections measuring 4 μm in thickness were meticulously prepared. These sections were then carefully mounted onto glass slides and subjected to the H&E staining procedure. The stained tissues were subsequently examined using light microscopy.

2.12 Statistical analysis

Data were processed and analyzed using GraphPad Prism 8.0 (San Diego, CA, United States). Differences between groups were assessed using the unpaired Student's t-test. Statistical significance was denoted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3 Results

3.1 Assessment of Blebbistatin's impact on cellular viability

The chemical structure of Blebbistatin is represented in Figure 1A. To assess the potential cytotoxic effects attributed to Blebbistatin, we conducted experiments involving RK-13 and MDBK cells. These cells were exposed to different concentrations of Blebbistatin, and subsequently, the CCK-8 reagent was introduced. Our findings revealed that the concentrations of Blebbistatin up to 20 μM exhibited no discernible impact on cell viability ($p > 0.05$) in either MDBK or RK-13 cell lines, as depicted in Figure 1B.

3.2 Inhibition of EqHV-8 infection by Blebbistatin in susceptible cells

To assess the potential anti-EqHV-8 effect of Blebbistatin, the RK-13 and MDBK cells were subjected to pretreatment with Blebbistatin at different concentrations for 1 h. Following this pre-incubation period, these cells were exposed to EqHV-8 SDLC66 (0.1 MOI) for 1 h. Subsequently, these cells and cellular supernatants were meticulously collected to facilitate the evaluation of EqHV-8 replication through western blot and $TCID_{50}$ analysis. Our findings revealed that Blebbistatin exerts a profound inhibitory influence on gD expression level and progeny virus titer in RK-13 cells, as elucidated in Figures 2A,B. Remarkably, Parallel observations were noted in MDBK cells (Figures 2C,D).

3.3 Antiviral activity of Blebbistatin against diverse EqHV-8 strains

In order to ascertain whether Blebbistatin exhibits antiviral properties against various strains of EqHV-8, a comprehensive investigation was conducted. RK-13 cells were pre-treated with Blebbistatin at 20 μM for 2 h, followed by their exposure to three distinct EqHV-8 strains, namely EqHV-8 SDLC66, EqHV-8 SD2020113, and EqHV-8 donkey/2021, each at 0.1 MOI for 1 h. Subsequently, the culture medium was replaced with 3% FBS MEM containing Blebbistatin, and these cells were fixed at 36 hpi to assess EqHV-8 replication via IFA. Meanwhile, those cellular supernatants were also collected to detect progeny virus titers by $TCID_{50}$. Our results showed that Blebbistatin significantly reduces the infection efficiency of diverse EqHV-8 strains in RK-13 cells in a dose-dependent manner, as represented in Figure 3. These data indicated that Blebbistatin exerts a broad spectrum of inhibitory effects against different EqHV-8 strains.

3.4 Blebbistatin attenuates EqHV-8 infection during early stages

To gain a deeper insight into the specific stage of the EqHV-8 life cycle that is perturbed by Blebbistatin, we performed a one-time course analysis experiment in RK-13 cells. As depicted in Figure 4A, our

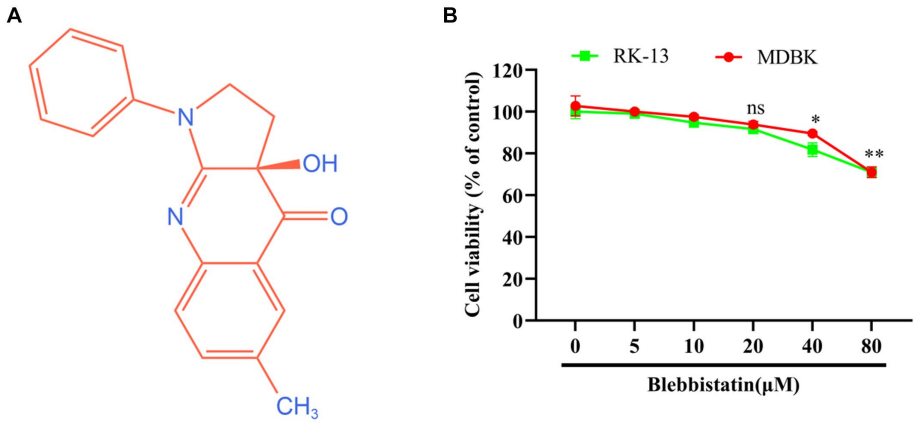


FIGURE 1
The chemical structure and cytotoxicity of Blebbistatin. **(A)** The chemical structure of Blebbistatin. **(B)** The cytotoxicity of Blebbistatin at different concentrations was determined in RK-13 and MDBK cells by CCK-8 kit and was expressed as relative cell viability by comparing with the viable cells in the absence of Blebbistatin (set up as 100%). These data shown are representatives from three independent experiments. *$p < 0.05$; **$p < 0.01$, ns: no significant.

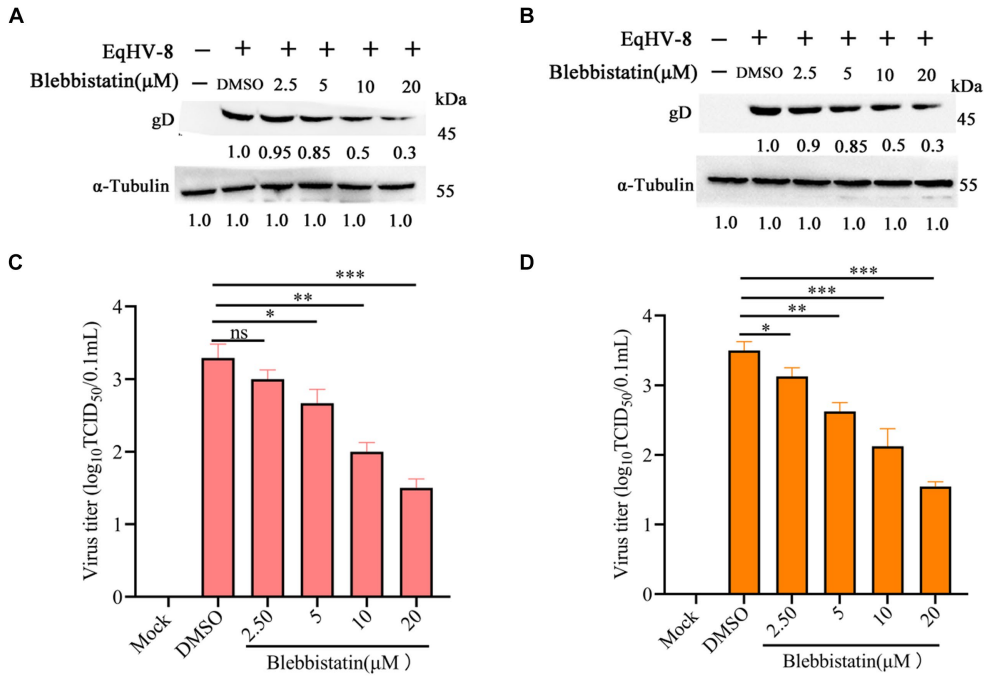


FIGURE 2
Blebbistatin inhibited EqHV-8 replication in susceptible cell. RK-13 **(A,B)** or MDBK **(C,D)** cells were pretreated with Blebbistatin at different concentrations (0, 2.5, 5, 10, and 20 μM) for 1 h, then infected with EqHV-8 SDLC66 (0.1 MOI) for 1 h at 37°C, then these cells were collected to detect gD expression by western blot at 24 hpi. Meanwhile, those cellular supernatants were harvested to assess virus titer using $TCID_{50}$. Data were presented as the means of normalized data ± standard deviations (error bars) based on at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns: no significant.

experiment was divided into four groups: R1, which represents the Blebbistatin pretreated group (Pre); R2, signifying the Blebbistatin and EqHV-8 co-treated group (Co); R3, representing the Blebbistatin post-treated group (Post); and R4, denoting the Blebbistatin treated group (All-stage). These cellular samples were harvested at 24 hpi to assess the gD expression. Our data demonstrate a significant reduction in gD protein expression in both R1 (Pre) and R2 (Co) groups when compared to the R3 (Post) group, as visually depicted in Figure 4B. This substantial decrease in gD expression suggests that Blebbistatin exerts an anti-EqHV-8 activity at an early stage of the viral life cycle. Furthermore, we examined the progeny virus production at 24 hpi by $TCID_{50}$ assay. Our data reveals that a clear dose-dependent reduction in the copy number of EqHV-8 in both R1 (Pre) and R2 (Co) groups, as illustrated in Figure 4C. However, there were no visible changes observed in the R3 (Post) group.

3.5 Blebbistatin substantially reduces EqHV-8 entry into RK-13 cells

To investigate the impact of Blebbistatin treatment on the EqHV-8 binding and internalization processes within RK-13 cells, we conducted both virus adhesion and internalization assays. Our experimental findings yielded noteworthy results. At the adsorption stage, it was observed that RK-13 cells subjected to Blebbistatin treatment exhibited a notable reduction in gD expression in comparison to the group treated with DMSO (Figure 5A). This observation implies that Blebbistatin treatment significantly affects EqHV-8 infection during the initial adsorption phase. Similarly, during the internalization stage of EqHV-8 in RK-13 cells, we encountered analogous outcomes. The gD expression was notably decreased in RK-13 cells treated with Blebbistatin as compared to those treated with DMSO (Figure 5B). This provides further evidence that Blebbistatin exerts its influence on EqHV-8 infection not only at the adsorption stage but also during the internalization process.

3.6 Blebbistatin inhibits EqHV-8 infection *in vivo*

To assess the potential antiviral properties of Blebbistatin *in vivo*, we conducted an experiment involving BALB/C mice (Figure 6A). Our primary objective was to evaluate the extent of EqHV-8 replication within the lungs of these mice at 8 dpi. This assessment was achieved by titrating lung samples from different groups of mice on RK-13 cells, with the resulting data providing valuable insights into the antiviral effects of Blebbistatin. Our findings revealed a distinct contrast in viral replication between the groups. Specifically, the mean viral titers in the EqHV-8 group were notably elevated, measuring at 1.8×10^3 $TCID_{50}$, in contrast to the Blebbistatin+EqHV-8 group, where viral titers were substantially reduced, measuring at 1.1×10^2 $TCID_{50}$, as depicted in Figure 6B. These results underscore the significant inhibitory effect of Blebbistatin on EqHV-8 replication within the lungs of the mouse model. Moreover, histopathological examination of lung tissue specimens from the EqHV-8 group unveiled severe alveolar wall thickening, resulting in the compression and collapse of alveolar cavities, accompanied by a marked infiltration of inflammatory cells. In contrast, the lungs of BALB/C mice treated with

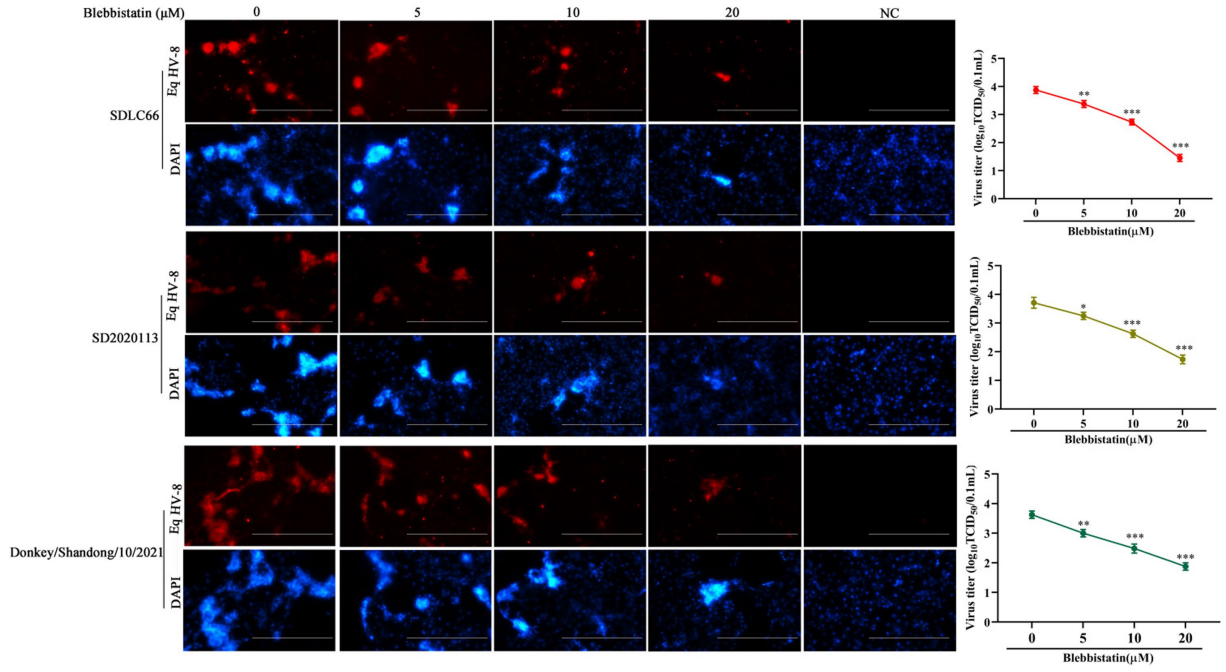


FIGURE 3
Blebbistatin show antiviral activity against other EqHV-8 strains. RK-13 cells were pretreated with Blebbistatin at different concentrations (0, 5, 10, or 20 μM) or DMSO for 1 h at 37°C, respectively, infected with EqHV-8 SDLC66, SD2020113, or Donkey/Shandong/10/2021 at 0.1MOI for 1 h in the presence of Blebbistatin at indicated concentrations. These cells were fixed with paraformaldehyde and stained using mouse anti-EHV-8 positive serum to detect EHV-8 proteins at 36 hpi (red), and the nucleocapsid was counterstained with DAPI (blue). Images were captured using Leica microsystems (DMi8, Germany). Scale bar, 100 μm. The mock-infected cells were served as negative control. Additionally, the progeny viral titer in cellular supernatants was analyzed by $TCID_{50}$. Data were presented as the means of normalized data ± standard deviations (error bars) based on at least three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Blebbistatin exhibited only minimal to mild alveolar wall thickening and inflammatory cell infiltration (Figure 6C). These histological observations further reinforce the notion that Blebbistatin exerts a substantial mitigating effect on EqHV-8-induced lung pathology in the mouse model. In addition, our findings strongly suggest that Blebbistatin could be considered a potential and promising antiviral drug candidate against EqHV-8 infection.

4 Discussion

In recent years, EqHV-8, a virus that affects donkeys in China, has caused significant economic losses in the donkey industry. Several cases of EqHV-8 infections in donkeys have led to issues like abortion, respiratory diseases, and viral encephalitis. Unfortunately, there are limited effective drugs against EqHV-8 infection. Our research has found that Blebbistatin can inhibit EqHV-8 in susceptible cells and under controlled *in vitro* conditions. This inhibition is due to Blebbistatin's ability to modulate myosin II ATPase activity.

Blebbistatin is a compound known for inhibiting ATPase in myosin II, affecting processes like micropinocytosis and cellular blebbing (19–21). Recent studies show its potential in fighting viral infections. Recent research has unveiled Blebbistatin's ability to suppress various viral infections. For instance, Veettil et al. demonstrated that Blebbistatin significantly reduces KSHV internalization into human dermal microvascular endothelial (HMVEC-d) cells (22). Similar antiviral effects have been reported against HSV-1, PRRSV, and MHV-68 in previous studies (13, 14, 16). In our experiments, Blebbistatin markedly decreased EqHV-8 infection in RK-13 and MDBK cells with dose-dependent properties (Figure 2). Additionally, our investigation delved into the intricate antiviral mechanisms underlying the action of Blebbistatin. Our findings revealed that Blebbistatin exerts its inhibitory influence on EqHV-8 replication during the early entry stage, as elucidated in Figure 4. Furthermore, our findings substantiate that Blebbistatin hampers EqHV-8 replication during the adsorption and internalization stages (Figure 5). Importantly, *in vivo* experiments confirmed the protective effect of Blebbistatin (30 μM/kg) against EqHV-8 in a mouse model, where it reduced viral replication and mitigated lung damage (Figure 6).

The involvement of myosins, a family of motor proteins with common features including ATP hydrolysis (ATPase enzymatic activity), kinetic energy transduction potential, and actin binding, is noteworthy in the context of viral infections (23). Previous studies have shown that nonmuscle myosin heavy chain IIA (NMHC-IIA) plays a crucial role in mediating HSV-1 entry, with Blebbistatin significantly reducing HSV-1 infection by inhibiting myosin II ATPase activity (24). Similar observations were made for PRRSV, where NMHC-IIA facilitates viral entry, and Blebbistatin notably reduces PRRSV replication via affecting myosin II ATPase activity (14, 15). In the current study, Blebbistatin significantly attenuated EqHV-8 replication in RK-13, MDBK cells, and in a mouse model (Figures 2, 6). Further research into the intricate mechanisms of Blebbistatin's action and its interaction with non-muscle myosin heavy chain IIA is imperative for a comprehensive understanding of its antiviral properties. Understanding the role of myosin II and related host factors in EqHV-8 pathogenesis could pave the way for novel therapeutic strategies against EqHV-8 infection. Furthermore,

10.3389/fvets.2024.1390304

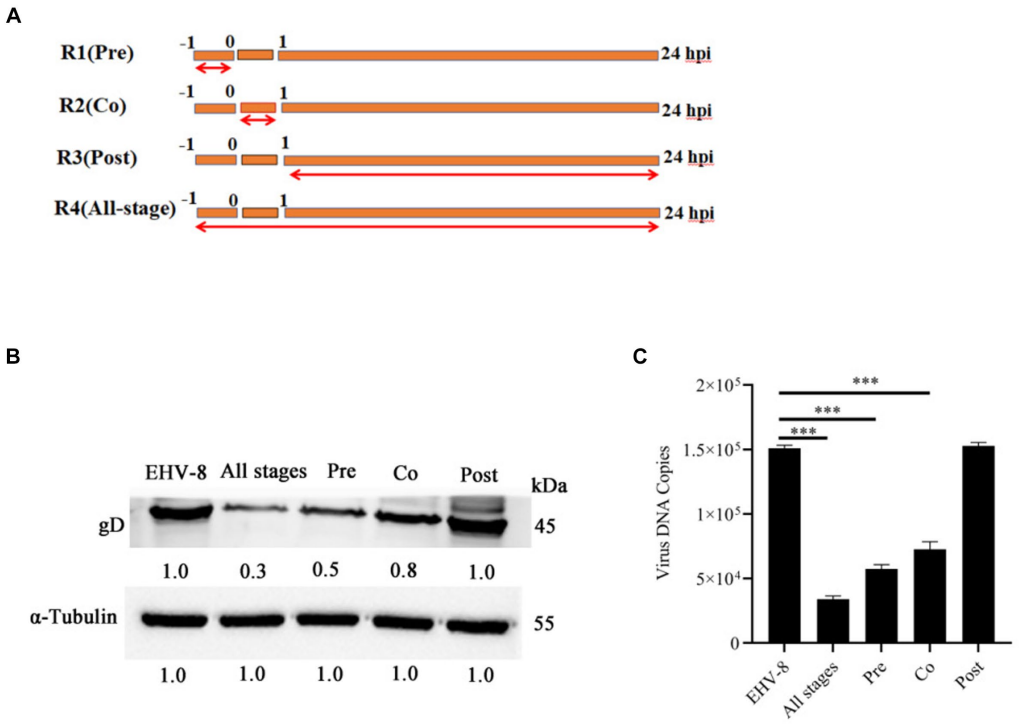


FIGURE 4
Blebbistatin inhibits EqHV-8 infection mainly at initial stage. Time-of-addition schematic **(A)**. The RK-13 cells were infected with EqHV-8 SDLC66 (MOI = 0.1) and treated with Blebbistatin at different time points, including before infection (Pre-treatment), during infection (Co-treatment), after infection (Post-treatment), and All-stage treatment. The RK-13 cells were treated with Blebbistatin (20 μM) as Pre-treatment, Co-treatment, Post-treatment and All-stage treatment for 24 hpi, and infected with EqHV-8 SDLC66 (MOI = 0.1), the cells were collected to detect gD expression for western blot **(B)**, meanwhile, the cell supernatants were also harvested to progeny virus generation by qPCR **(C)**. α-Tubulin acts as loading control, the data represent as mean ± SD. from three independent experiments, $^{**}p < 0.01$; $^{***}p < 0.001$, ns: no significant.

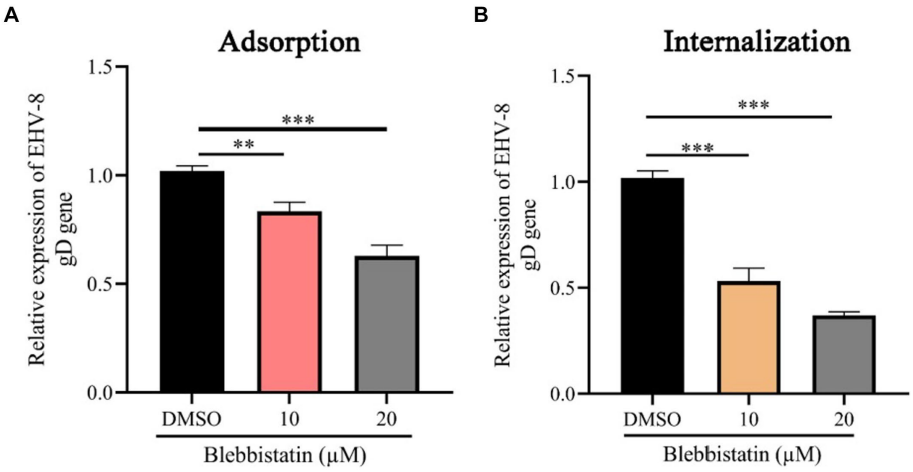


FIGURE 5
Blebbistatin suppress EqHV-8 replication at adsorption and internalization stage. The RK-13 cells were incubated with a mixture of Blebbistatin (20 μM) or DMSO and EqHV-8 (1 MOI) for 1 h at 4°C, and washed by PBS and incubated with 3% FBS MEM at 37°C for 24 h before the gD expression was tested using qPCR **(A)**. RK-13 cells were pretreated with Blebbistatin (20 μM) for 1 h and then incubated with EqHV-8 (MOI = 1) at 4°C for 1 h. The cells were washed with PBS and incubated with Blebbistatin (20 μM) or DMSO for another 1 h at 37°C, then washed by citrate buffer. The gD expression was detected using qPCR at 24 hpi. **(B)** $^{**}p < 0.01$; $^{***}p < 0.001$; ns: not significant.

future studies using primary equine cells or *in vivo* models are warranted to validate the findings. While the current study utilized an *in vivo* mouse model to assess the anti-EqHV-8 activity of Blebbistatin's, it is important to note that mice may not fully elucidate the pathogenesis and immune response observed in horses and donkeys infected with EqHV-8. Therefore, additional research using

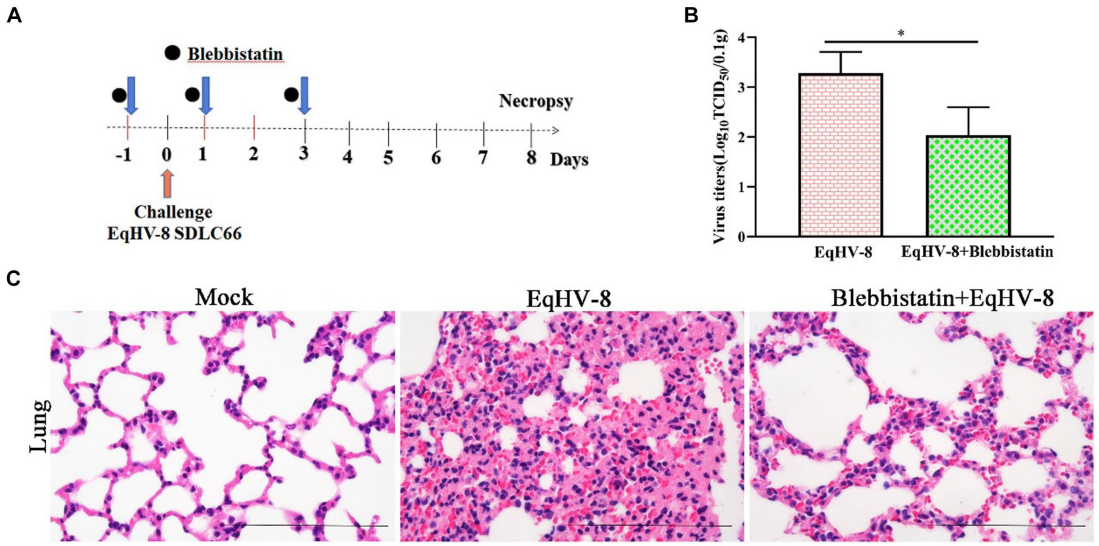


FIGURE 6
Blebbistatin inhibits EqHV-8 replication *in vivo* **(A)** The pattern diagram of the animal experiments. **(B)** The titration of EqHV-8 in lung from different group. RK-13 cells were used to titrate in lung from EqHV-8 infected or Blebbistatin treated EqHV-8 infected mice using the Reed–Muench method. The results are presented as the mean ± SD (error bars). *$p < 0.05$. **(C)** Representative images of hematoxylin and eosin (H&E) in the lungs derived from mice in indicated groups. Bar, 100 μm.

equine-specific models is necessary to confirm the drug's efficacy and safety. Altogether, our study not only reinforces the antiviral potential of Blebbistatin against EqHV-8 but also highlights the need for continued research into the mechanistic aspects of its action by utilizing equine-specific models.

5 Conclusion

In conclusion, our study provides compelling evidence of Blebbistatin's significant inhibitory effect on EqHV-8 replication, demonstrated in both *in vitro* and *in vivo* experimental models. While our results highlight Blebbistatin's potential as a novel antiviral agent against EqHV-8, further research using equine-specific models is warranted to validate its efficacy and safety in horses and donkeys.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by all experimental protocols were approved by the Liaocheng University Animal Care and Use Committee (permit number: LC2023-11). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LL: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. XC: Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. WL: Data curation, Software, Writing – review & editing. YL: Data curation, Methodology, Writing – review & editing. SL: Methodology, Software, Writing – review & editing. LC: Data curation, Software, Writing – review & editing. MK: Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing. CW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. TW: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. YY: Methodology, Formal analysis, Validation, Writing – review & editing. QS: Methodology, Formal analysis, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by the Project of Shandong Province Higher Educational Science and Technology Program for Youth (2022KJ287), the Innovation and Entrepreneurship Program for College Students (CXCY2022434, CXCY2023275 and S202310447042), the National Natural Science Foundation of China (32002248), and the Shandong Province Modern Agricultural Technology System Donkey Industrial Innovation Team (SDAIT-27-18).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1390304/full#supplementary-material

References

1. Garvey M, Suarez NM, Kerr K, Hector R, Moloney-Quinn L, Arkins S, et al. Equid herpesvirus 8: complete genome sequence and association with abortion in mares. *PLoS One*. (2018) 13:e0192301. doi: 10.1371/journal.pone.0192301

2. Yoon J, Park T, Kim A, Song H, Park BJ, Ahn HS, et al. First report of equine parvovirus-hepatitis and equine hepacivirus coinfection in horses in Korea. *Transbound Emerg Dis*. (2022) 69:2735–46. doi: 10.1111/tbed.14425

3. Liu C, Guo W, Lu G, Xiang W, Wang X. Complete genomic sequence of an equine herpesvirus type 8 Wh strain isolated from China. *J Virol*. (2012) 86:5407. doi: 10.1128/JVI.00445-12

4. Wang T, Xi C, Yu Y, Liu W, Akhtar MF, Li Y, et al. Characteristics and epidemiological investigation of equid herpesvirus 8 in donkeys in Shandong, China. *Arch Virol*. (2023) 168:99. doi: 10.1007/s00705-023-05704-x

5. Wang T, Hu L, Wang Y, Liu W, Liu G, Zhu M, et al. Identification of equine herpesvirus 8 in donkey abortion: a case report. *Virol J*. (2022) 19:10. doi: 10.1186/s12985-021-01738-2

6. Wang T, Hu L, Liu M, Wang T, Hu X, Li Y, et al. The emergence of viral encephalitis in donkeys by equid herpesvirus 8 in China. *Front Microbiol*. (2022) 13:840754. doi: 10.3389/fmicb.2022.840754

7. Li S, Xi C, Geng Y, Tian W, Li L, Wang T, et al. Pathogenicity and host cytokines response of EqHV-8 infection in C57BL/6J mice. *Microb Pathog*. (2024) 186:106506. doi: 10.1016/j.micpath.2023.106506

8. Wang T, Li S, Hu X, Geng Y, Chen L, Liu W, et al. Heme oxygenase-1 is an equid alphaherpesvirus 8 replication restriction host protein and suppresses viral replication via the PKCβ/ERK1/ERK2 and NO/cGMP/PKG pathway. *Microbiol Spectr*. (2024) 12:e03220–3. doi: 10.1128/spectrum.03220-23

9. Li C, Li S, Li W, Chen W, Zhou H, Wang C, et al. Rutin prevents EqHV-8 induced infection and oxidative stress via Nrf2/HO-1 signalling pathway. *Front Cell Infect Microbiol*. (2024) 14:1386462. doi: 10.3389/fcimb.2024.1386462

10. Li L, Hu X, Li S, Li Y, Zhao S, Shen F, et al. Cobalt Protoporphyrin blocks EqHV-8 infection via IFN-α/β production. *Animals*. (2023) 13:2690. doi: 10.3390/ani13172690

11. Rauscher AA, Gyimesi M, Kovacs M, Malnasi-Csizmadia A. Targeting myosin by blebbistatin derivatives: optimization and pharmacological potential. *Trends Biochem Sci*. (2018) 43:700–13. doi: 10.1016/j.tibs.2018.06.006

12. Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, Sellers JR, et al. Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. *Science*. (2003) 299:1743–7. doi: 10.1126/science.1081412

13. Antoine TE, Shukla D. Inhibition of myosin light chain kinase can be targeted for the development of new therapies against herpes simplex virus type-1 infection. *Antivir Ther*. (2014) 19:15–29. doi: 10.3851/IMP2661

14. Gao J, Xiao S, Xiao Y, Wang X, Zhang C, Zhao Q, et al. MYH9 is an essential factor for porcine reproductive and respiratory syndrome virus infection. *Sci Rep*. (2016) 6:25120. doi: 10.1038/srep25120

15. Li L, Sun W, Hu Q, Wang T, Zhu G, Zhao Q, et al. Identification of MYH9 key domain involved in the entry of PRRSV into permissive cells. *Front Microbiol*. (2022) 13:865343. doi: 10.3389/fmicb.2022.865343

16. Han X, Clark JJ, Sharma P, Bentley EG, Kipar A, Alsayer M, et al. Amino acids 1811-1960 of myosin heavy chain 9 is involved in murine gammaherpesvirus 68 infection. *Virology*. (2023) 587:109849. doi: 10.1016/j.virol.2023.109849

17. Li L, Xue B, Sun W, Gu G, Hou G, Zhang L, et al. Recombinant MYH9 protein C-terminal domain blocks porcine reproductive and respiratory syndrome virus internalization by direct interaction with viral glycoprotein 5. *Antivir Res*. (2018) 156:10–20. doi: 10.1016/j.antiviral.2018.06.001

18. Wang T, Du Q, Wu X, Niu Y, Guan L, Wang Z, et al. Porcine MKRN1 modulates the replication and pathogenesis of porcine circovirus type 2 by inducing capsid protein ubiquitination and degradation. *J Virol*. (2018) 92:351. doi: 10.1128/JVI.01351-18

19. Roman BI, Verhasselt S, Stevens CV. Medicinal chemistry and use of myosin II inhibitor (S)-blebbistatin and its derivatives. *J Med Chem*. (2018) 61:9410–28. doi: 10.1021/acs.jmedchem.8b00503

20. Mercer J, Helenius A. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science*. (2008) 320:531–5. doi: 10.1126/science.1155164

21. Kovacs M, Toth J, Hetenyi C, Malnasi-Csizmadia A, Sellers JR. Mechanism of blebbistatin inhibition of myosin II. *J Biol Chem*. (2004) 279:35557–63. doi: 10.1074/jbc.M405319200

22. Valiya Veettil M, Sadagopan S, Kerur N, Chakraborty S, Chandran B. Interaction of c-Cbl with myosin IIA regulates bleb associated macropinocytosis of Kaposi's sarcoma-associated herpesvirus. *PLoS Pathog*. (2010) 6:e1001238. doi: 10.1371/journal.ppat.1001238

23. Chen J, Fan J, Chen Z, Zhang M, Peng H, Liu J, et al. Nonmuscle myosin heavy chain IIA facilitates SARS-CoV-2 infection in human pulmonary cells. *Proc Natl Acad Sci USA*. (2021) 118:11118. doi: 10.1073/pnas.2111011118

24. Arii J, Goto H, Suenaga T, Oyama M, Kozuka-Hata H, Imai T, et al. Non-muscle myosin IIA is a functional entry receptor for herpes simplex virus-1. *Nature*. (2010) 467:859–62. doi: 10.1038/nature09420

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 11 June 2024
DOI 10.3389/fvets.2024.1399815

Check for updates

OPEN ACCESS

EDITED BY
Arturo Anadón,
Complutense University of Madrid, Spain

REVIEWED BY
Gregg Mitchell Griffenhagen,
Colorado State University, United States
Claudia Interlandi,
University of Messina, Italy

*CORRESPONDENCE
Guilherme de Camargo Ferraz
guilherme.c.ferraz@unesp.br

RECEIVED 12 March 2024
ACCEPTED 21 May 2024
PUBLISHED 11 June 2024

CITATION
Carvalho JRG, Del Puppo D, Littiere TO, Sales NAA, Silva ACY, Ribeiro G, Almeida FN, Alves BG, Gatto IRH, Ramos GV and Ferraz GC (2024) Functional infrared thermography imaging can be used to assess the effectiveness of Maxicam Gel® in pre-emptively treating transient synovitis and lameness in horses.
Front. Vet. Sci. 11:1399815.
doi: 10.3389/fvets.2024.1399815

COPYRIGHT
© 2024 Carvalho, Del Puppo, Littiere, Sales, Silva, Ribeiro, Almeida, Alves, Gatto, Ramos and Ferraz. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Functional infrared thermography imaging can be used to assess the effectiveness of Maxicam Gel® in pre-emptively treating transient synovitis and lameness in horses

Júlia Ribeiro Garcia de Carvalho[1], Debora Del Puppo[2], Thayssa de Oliveira Littiere[1], Nathali Adrielli Agassi de Sales[1], Ana Carolina Yamamoto Silva[1], Gesiane Ribeiro[3], Ferdinando Nielsen de Almeida[2], Bruna Gomes Alves[2], Igor Renan Honorato Gatto[2], Gabriel Vieira Ramos[4] and Guilherme de Camargo Ferraz[1]*

[1]Laboratory of Equine Exercise Physiology and Pharmacology (LAFEQ), Department of Animal Morphology and Physiology, School of Agricultural and Veterinary Sciences, São Paulo State University, FCAV/UNESP, São Paulo, Brazil, [2]Research and Development Department, Ourofino Animal Health Company, São Paulo, Brazil, [3]Veterinary and Animal Research Centre (CECAV), Faculty of Veterinary Medicine, Lusófona University - Lisbon University Centre, Lisbon, Portugal, [4]Equine Sports Medicine Laboratory, Department of Veterinary Medicine and Surgery, School of Agricultural and Veterinary Sciences, São Paulo State University, FCAV/UNESP, São Paulo, Brazil

Introduction: Diagnosing and treating lameness in horses is essential to improving their welfare. In equine orthopedic practice, infrared thermography (IRT) can indirectly detect soreness. Non-steroidal anti-inflammatory drugs can treat painful and inflammatory processes in horses. Using IRT, the efficacy of meloxicam (Maxicam Gel®) was evaluated in pre-treating transient synovitis in horses induced by a middle carpal joint injection of lipopolysaccharides (LPS) from *E. coli* 055:B5 at a dose of 10 endotoxin units.

Methods: In a cross-over design, six healthy horses were randomly assigned to receive either 0.6 mg/kg of oral Maxicam Gel® (MAXVO) or a mock administration (control group, C) following a two-week washout period. IRT of the middle carpal joint, visual lameness assessment and joint circumference were recorded over time. Clinical and hematological evaluations were performed. Synovial fluid aspirates were analyzed for total nucleated cell count, total protein, and prostaglandin E_2. A mixed effects analysis of variance was performed for repeated measures over time, followed by Tukey's test. A multinomial logistic regression was conducted to determine whether there is a relationship between a thermography temperature change and the lameness score.

Results: There were no changes in joint circumference. The MAXVO group showed a lower rectal temperature 4 h after synovitis induction. The C group presented an increase in neutrophils and a decrease in total hemoglobin and hematocrit 8 h after induction. No changes were observed in the synovial fluid between groups. The horses that received meloxicam did not show clinically significant lameness at any time, while the C group showed an increase in lameness 2, 4, and 8 h after synovitis induction.

Discussion: IRT indicated that the skin surface temperature of the middle carpal joint was lower in horses who received meloxicam, suggesting a reduction in the inflammatory process induced by LPS. It was observed that the maximum temperature peaks in the dorsopalmar and lateropalmar positions can be utilized

to predict the severity of lameness, particularly when the temperature rises above 34°C. Horses pre-treated with meloxicam showed either reduced or no indication of mild to moderate pain and presented a lowehr thermographic temperature, which indicates the effectiveness of Maxicam Gel® as an anti-inflammatory.

KEYWORDS

analgesic, lameness, lipopolysaccharide, meloxicam, nonsteroidal anti-inflammatory drug, soreness, cyclooxygenase, osteoarthritis

1 Introduction

Diagnosing and treating lameness in horses is crucial for ensuring their welfare in the equine field practice. One helpful approach to diagnosis is thermal imaging, which estimates the pathophysiological response to a noxious stimulus through heat production (1). To manage inflammatory and painful conditions in horses, non-steroidal anti-inflammatory drugs (NSAIDs) are commonly administered to treat musculoskeletal diseases (2). These drugs are potent inhibitors of cyclooxygenase enzymes (COXs), which are responsible for converting arachidonic acid into eicosanoids such as prostaglandins, leukotrienes, and thromboxanes. These chemical mediators play an essential role in the inflammation cascade, including increased vascular permeability and heat. Blocking the production of these mediators causes an anti-inflammatory and analgesic effect (3, 4).

COX-1 is constitutively expressed in almost all body tissues, acting in routine and physiological functions, including gastrointestinal homeostasis, through cytoprotection of the gastric epithelium, and maintenance of renal blood perfusion. COX-2 is an enzyme up-regulated by cytokines that show increased activity during pro-inflammatory states. Unlike COX-1, COX-2 has relatively low activity under physiological conditions (3, 5, 6). In recent decades, drugs with greater selectivity for COX-2 have been developed, such as meloxicam, an NSAID belonging to the enolic acid class. The main advantage of COX-2 selective NSAIDs is the reduction in adverse effects associated with the gastrointestinal tract, such as gastric ulcerations and colitis (6). In rats, ulcerogenicity in the stomach is weak compared to anti-inflammatory potency, resulting in a high therapeutic index (7).

Meloxicam effectively relieves postoperative pain in human patients undergoing several types of surgeries, such as knee osteoarthritis surgery. For instance, a trail showed that two oral doses preemptively administered of meloxicam improved postoperative pain control in patients receiving arthroscopic knee surgery (8). According to these authors, meloxicam's potential as a preemptive analgesic in postoperative pain control is preliminary but promising. In equine medicine, more information on the preemptive use of meloxicam that can be applied clinically needs to be present in the literature.

Abbreviations: IRT, Infrared thermography; MAXVO, Group treated with meloxicam; C, Control group; LPS, Lipopolysaccharides; NSAID, Non-steroidal anti-inflammatory drug; COX, Cyclooxygenase enzyme; ML, Mediolateral; LM, Lateromedial; DP, Dorsopalmar; PD, Palmarodorsal; TP, Total protein; TNCC, Total nucleated cell count; PGE_2, Prostaglandin E_2; EU, Endotoxin units; HR, Heart rate; RR, Respiratory rate; RT, Rectal temperature; EDTA, Ethylenediaminetetraacetic acid; CL, Contralateral limb.

Some studies have shown that meloxicam is effective in treating lameness in horses caused by synovitis induced by intra-articular administration of lipopolysaccharides (LPS), principally model of inflammatory pain. These studies have used various methods and biomarkers to test the clinical effectiveness of meloxicam. The administration of meloxicam has been found to reduce clinical lameness scores (5, 9–11), mitigate the asymmetry of head movement (5), and suppress inflammatory markers such as total protein (TP), total nucleated cell count (TNCC), and prostaglandin E_2 (PGE_2) in synovial fluid (11, 12). Thus, there is scientific evidence to suggest that meloxicam can reduce musculoskeletal pain of inflammatory origin in horses.

Another technique that demonstrates potential for detecting NSAIDs' effectiveness is infrared thermography (IRT), a noninvasive, radiation free, practical, fast, and low-cost method. Evaluation of the surface temperature can be used as a diagnostic tool to accurately estimate the thermal, metabolic, and vascular conditions of the equine body. Changes in local perfusion, such as vasodilation, can cause alterations in the body surface temperature. Increases in vascularization and blood supply to tissues are the basis for thermography diagnosis. Temperature is a crucial physical property that can directly reflect joint inflammation. Thus, IRT can be used to map classic inflammatory clinical signs such as heat, which indicates changes in skin surface temperature caused by vascular or inflammatory alterations (1, 13–15).

Heat is one of the key signs of inflammation, as the increased blood supply caused by inflammation leads to an increase in local temperature (16). IRT has been used in various studies involving horses, such as detection of jugular venipuncture for anti-doping control in equestrian events (17), monitoring of musculoskeletal adaptation to training (18), evaluation of the welfare in athlete horses (19), monitoring the effects of training (20, 21), assessment of thermoregulation during exercise (22), validation for the use of eye temperature as an indicator of well-being (23), detection of pregnancy in mares (24), evaluation of the oral administration of meloxicam or flunixin meglumine in an inflammatory response induced by the administration of systemic LPS (25), evaluation of thermoregulation using two methods of post-exercise cooling (26), evaluation of biocompatibility of polymers (27, 28), among others.

IRT has been used in humans to screen soft tissue and measure joint skin temperature to diagnose conditions such as rheumatoid arthritis, osteoarthritis, and ankle sprains (15, 29–31). In bovines, IRT was used to compare the effectiveness of oral meloxicam and intravenous flunixin meglumine in controlling lameness-associated pain in lactating dairy cattle (32). This technique was also used to evaluate dogs with hip osteoarthritis (33).

More research is still needed on the use of IRT to indirectly assess pain and inflammation in domestic animals (1). However, IRT is a currently available method to detect soreness in horses (34). When it comes to using IRT to prove the effectiveness of NSAIDs, there is a relative scarcity of studies in horses. Some studies have used IRT to assess the vascular component of the inflammatory response and examine the effects of anti-inflammatory drugs on experimentally induced acute inflammation (35, 36). Recent studies have induced systemic inflammation by administering LPS intravenously and used IRT to evaluate the effectiveness of NSAIDs by analyzing the temperature of the hoof wall of horses (25, 37). Furthermore, IRT has been shown to be effective in detecting intrasynovial injections in horses (38).

The purpose of this study was to investigate the effectiveness of non-steroidal anti-inflammatory drugs (NSAIDs) using IRT as a non-invasive diagnostic method. The authors report on the use of IRT to quantify the degree of inflammation in horses with experimentally induced transient synovitis with LPS. These horses had previously received meloxicam, an older COX-2 selective inhibitor.

2 Materials and methods

2.1 Ethics statement

The study adhered to the Ethical Principles in Animal Experimentation as established by the National Council for Control in Animal Experimentation (CONCEA). The protocol underwent review and approval by the Ethics Committee on the Use of Animals—CEUA—UNESP, Jaboticabal, Brazil (Protocol No. 2887/2021).

2.2 Horses

Six crossbreed horses, three males and three females, were used for the experiment. They weighed an average of 395 ± 35 kg and aged between 12 and 20 years. These horses belong to the didactic herd of the Equine Exercise Physiology and Pharmacology Laboratory (LAFEQ), Department of Animal Morphology and Physiology, School of Agricultural and Veterinarian Sciences, São Paulo State University (FCAV/UNESP), Jaboticabal, São Paulo, Brazil. They were kept in a paddock and fed with 0.05% of body weight in concentrate once a day, along with hay, hay silage, mineral salt, and water *ad libitum*. Before the experiment began, the horses underwent a complete physical examination to ensure their health status. Haematological and biochemical tests were also conducted. The horses were previously treated with anthelmintics and vaccinated against rabies, tetanus toxoid, eastern and western equine encephalomyelitis, and equine influenza types A1 and A2.

2.3 Experimental groups

Based on a former study (11), the study's sample size was six horses, all housed under the same condition. The horses were distributed into two groups: the control group (C, $n = 6$) and the group treated with meloxicam (MAXVO, $n = 6$). The meloxicam was formulated and manufactured by Ourofino Animal Health Company for commercial use in Brazil, and the study was designed to meet Brazilian regulations. The study was conducted in a controlled cross-over design in a paired, blinded, randomised experiment, with a two-week washout between phases. The MAXVO group was given meloxicam orally at a dose of 0.6 mg/kg of body mass, once per day for 3 days, at 48 h (D-2), 24 h (D-1), and 1 h (0) before synovitis induction, according to a previously adapted protocol (11). If the horses still showed lameness, an additional dose was given 24 h after synovitis induction. The horses in the control group received a saline solution orally at the exact times as the MAXVO group to simulate the same conditions as the meloxicam administration. As mentioned, the study was conducted blindly, and the researchers responsible for evaluations were not aware of which group each horse belonged to. Only one researcher (JC) was responsible for administering treatments to the horses.

2.4 Preparation of LPS

Standardization of laboratory procedures for preparing the LPS solution is crucial. A previously established LPS model, described in Standardbred, was followed (11). The solution of *E. coli* 055:B5 (L2880, Sigma Aldrich, lot 059N4031V) was prepared using sterile materials, and all stages of solution preparation were carried out in a laminar flow, under refrigeration. From the stock solution, which had a concentration of 5 mg/mL of LPS in RPMI 1640 Medium (Gibco™, Thermo Fisher), a new dilution was prepared by adding 1 µL of the stock solution to 5 mL of RPMI. This intermediate solution had a concentration of 3,000 EU/mL and was stored in an appropriate flask. The working solution was obtained by diluting 90 µL of the intermediate solution in 27 mL of sterile PBS, resulting in a desired concentration of 10 EU/mL. The working solution was stored in microtubes containing 1.5 mL each, and the material was thawed for a maximum of 1 h before intra-articular injection to induce the inflammatory process. The stock and intermediate solutions were vortexed for 10 min at a speed of 1,500 rpm before dilution to ensure a homogeneous solution (11). Before intra-articular administration, the working solution was vortexed again for 2 min at 1,500 rpm.

2.5 Induction of the inflammatory process

The horses underwent a procedure that induced a temporary inflammatory process by applying *E. coli* 055:B5 LPS to their middle carpal joint. A random drawing determined which joint (left or right) would receive the 1 mL solution containing 10 endotoxin units (EU) of LPS. To ensure the safety of the procedure, the horses were sedated with detomidine (0.01 mg/kg i.v.) and then given yohimbine (0.12 mg/kg i.v.) to reverse the sedation after the procedure was completed.

The initiation of inflammation was designated as the baseline time point (0). Before the arthrocentesis procedure, the application site was cleaned with chlorhexidine degermant and 70% alcohol to ensure antisepsis. A 30 × 8 needle and a 1 mL syringe were used to collect the synovial fluid. After that, the entire LPS solution was injected into both groups. Following the induction of transitory synovitis, the horses were kept in individual paddocks with an area of approximately 75.9 m^2. These paddocks were surrounded by a wooden fence and smooth wire protected with plastic insulation and had a roof to protect

against precipitation and intense sunlight. This type of management ensured the maintenance of regular habits of the equine species, such as grazing, moving, and socializing.

2.6 Assessment methods

2.6.1 Clinical and hematological evaluation

Throughout the experiment the horses were being closely monitored by veterinarians to detect any signs of systemic clinical issues that might arise due to the administration of LPS. The horses were evaluated clinically by a physical examination, and blood samples were collected for hematological analysis (complete blood count). Heart rate (HR), respiratory rate (RR), rectal temperature (RT), degree of hydration, the color of the apparent mucous membranes (1 = pink, 2 = light pink, 3 = reddish, 4 = white, 5 = yellowish, 6 = bluish), behavior (0 = normal, 1 = apathetic; 2 = restless), and appetite (0 = normal, 1 = hyporexia; 2 = anorexia) were all measured. The horses were evaluated at several time points: five days (D-5), two days (D-2), one day (D-1), and immediately before the induction of the inflammatory process, and 0, 2, 4, 6, 8, 12, 24, and 48 h after synovitis induction with LPS. Blood samples were collected by venipuncture of the jugular vein in negative pressure tubes containing ethylenediaminetetraacetic acid (EDTA). The samples were kept in isothermal boxes with recyclable ice and transported to the laboratory. A hematological analyzer (ABX Micros 60, Horiba) was used. Blood collection was performed at the following time points: 0, 8, 24, and 48 h after the induction of the inflammatory process.

2.6.2 Synovial fluid

Synovial fluid was obtained via aseptic arthrocentesis of the middle carpal joint. The animals were chemically restrained using detomidine (0.01 mg/kg i.v.) and the application site was made aseptic using chlorhexidine degermant and 70% alcohol. Synovial fluid was collected using a 30 × 8 needle. Samples were divided into 0.5 mL pediatric tubes containing EDTA and plain microtubes and were immediately stored at 4°C. Samples in EDTA tubes were analyzed, whereas plain tubes were processed within 1 h after collection. Synovial fluid was collected at 0, 8, 24, and 48 h after induction of the inflammatory process. The animals were chemically reverted from sedation using yohimbine (0.12 mg/kg i.v.). Aliquots of synovial fluid collected in tubes with EDTA were used to perform TNCC and TP assays. The plain tubes were centrifuged at 2,000 G for 20 min at 4°C. The supernatant was then collected and distributed into 500 µL aliquots, placed in microtubes without anticoagulant, and stored at −80°C until the moment of quantification of PGE_2. The quantification of PGE_2 was performed using the Prostaglandin E_2 EIA Kit (Elabscience, Texas, United States) through ELISA. The intra-assay coefficient of variation for PGE_2 was 1.2%.

2.6.3 Joint circumference

To assess inflammation and oedema, joint circumference was measured using a measuring tape placed immediately distal to the accessory carpus. The measurements were taken at the following time points: 0, 2, 4, 6, 8, 12, 24, and 48 h after the inflammation was induced.

2.6.4 Lameness

During the study, the horses were evaluated to detect movement asymmetries for their degree of lameness. Visual examination was done by inspecting the horses while they trotted in a straight line over a hard surface for approximately 40 meters. The evaluation was conducted by an experienced clinician (GCF) unaware of the experimental groups. The clinician assigned scores to the horses based on the following criteria: 0 for absence of visible lameness, (1) for discrete asymmetry that was occasionally inconsistent, (2) for visible lameness that was rarely inconsistent, (3) for visible lameness always, and (4) for complete inability to bear weight (39). The evaluations were performed at 0, 2, 4, 8, 24, and 48 h after the induction of the inflammatory process.

2.6.5 Infrared thermography

Functional IRT imaging is a useful diagnostic tool in veterinary medicine for examining inflammation. The proper use of thermography requires a controlled environment and adherence to imaging protocol to eliminate errors. Thermographic examinations were conducted on the joint that received LPS and the opposite joint, which was used as a negative control. Four images were taken for each limb of the carpal anatomic region: mediolateral (ML), lateromedial (LM), dorsopalmar (DP), and palmarodorsal (PD) views (Figure 1). The horses were led into a closed, airy environment that was free from drafts and direct sunlight (14). Before taking the images, the carpal anatomic region on both limbs was gently cleaned with dry gauze, and the area was not touched again after cleaning. These procedures were carried out to allow the horse to acclimate to the ambient environmental factors. The temperature and humidity of the environment were controlled to standardize the thermal measurements. The horses were not sedated during the evaluations. It is worth noting that before the examinations, the cutaneous area of the carpal joint was clipped at least 24 h before induction of synovitis to minimize errors caused by reflection or refraction.

An infrared thermographic camera from Flir® Systems (model i50, Wilsonville, Oregon, United States) was used for the assessment. Based on the technical specifications provided by the manufacturer, the camera's sensitivity value is less than 0.1°C, with an emissivity of 0.98, temperature range from −20 to 350°C, image frequency of 9 Hz, resolution of 140 × 140 pixels, and an accuracy of ±2%. The thermographic camera was placed perpendicular to the assessment site at a 0.4 m distance to capture infrared thermographic images. All thermographic images were taken by the same examiner (TOL). Image analysis was performed using FLIR Tools software from FLIR Systems Inc. (Wilsonville, OR, United States). An area was marked on the thermographic image across the entire carpal region. It should be noted that the middle carpal joint, where the LPS was injected, communicates with the small carpometacarpal joint situated between the third and fourth carpal bones (40). The software calculated the delimited area's minimum, maximum, and average thermographic temperatures. The assessments were carried out at 0, 2, 4, 6, 8, 12, 24, and 48 h after the induction of the inflammatory process.

2.7 Statistical design and analysis

The data was analyzed using SigmaPlot software (v.12.5) and tested for normality using the Shapiro–Wilk test. The experimental design was a factorial design with three paired replications in a split-split-plot scheme. Each split-plot was a treatment (C and MAXVO) and each split-split-plot was an examination time point (eight-time

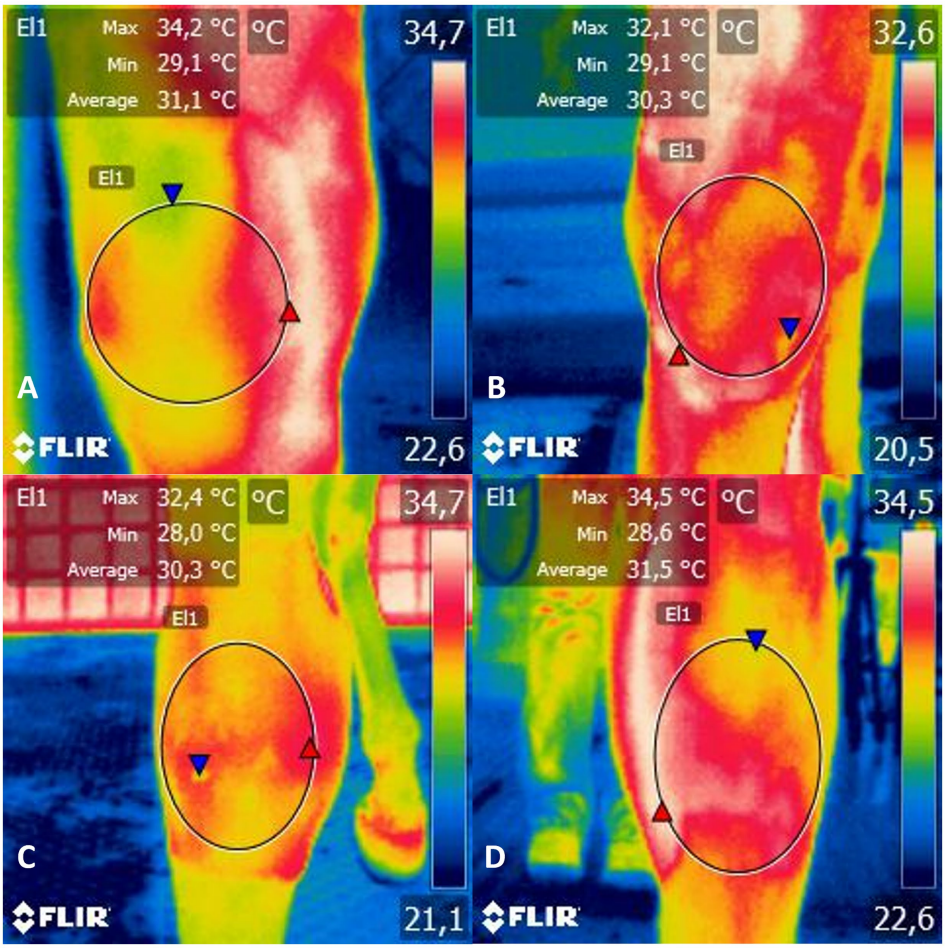


FIGURE 1
Infrared thermogram of the **(A)** mediolateral, **(B)** lateromedial, **(C)** dorsopalmar and **(D)** palmarodorsal view of the carpal joint of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO). C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection. Observe temperature scale on the right. The circle delimits the evaluated area.

points for thermographic examination). In the next phase of the experiment, a cross-over design was applied, where the three horses that received Maxicam Gel® in the first phase became controls in the second phase and vice versa, resulting in a total of six animals per experimental group. The model $Y_{ijkl}=\mu+\alpha_i+\beta_{ij}+tk+(\alpha\beta)_{ik}+\epsilon_{ijk}$ was used, in which Y_{ijkl} is observation at the meloxicam factor level I; μ is the overall mean common to all observations; α is the effect of treatment (meloxicam); β is the effect of time point (in hours); and the $\alpha\beta$ = effect of the interaction between treatment i and time k. A mixed model was applied with the horse as a random effect and time point, treatment, and their interaction as categorical fixed effects. The treatment effect was also tested at different times separately, according to the time points of each variable for the studied variables after synovitis induction. A mixed effects analysis of variance was performed for repeated measures over time, and means were compared using Tukey's test. For the degree of lameness, the Wilcoxon non-parametric test was used for the intergroup comparison. For the intragroup comparisons, the Kruskal–Wallis one-way analysis of variance on ranks was used, and the means were compared using the Tukey test. A multinomial logistic regression (MLR) classification algorithm was conducted with R Programming Language (version 2024.04.0 + 735 "Chocolate Cosmos" release for windows) using multinon function ["nnet" package (version 7.3–19)]. To evaluate the predictive potential of thermography variables, such as minimum peak, maximum peak, and average peak thermographic temperatures, and anatomic regions ML, LM, DP, and PD, the MLR was employed. The goal was to accurately classify and predict the lameness score based on these variables. This model was used given the classification of lameness into five scores (0 to 4, considered the outcome variables) and the ability of the MLR to evaluate outcome variables with more than two categories. Before applying the MLR, the independence of irrelevant alternatives (IIA), the independence of errors (IE) and the absence of multicollinearity were checked. The first two premises were evaluated through visual inspection of the data, ensuring two similar categories in the outcome variable (for IIA assumption) and the absence of clusters within the experimental group used (for IE assumption). The assessment of multicollinearity was evaluated using the variance inflation factor (VIF) using vif function ["car" package (version 3.1–2)] with a threshold of 5. Variables that presented VIF >5 had a high correlation with the other variables included in the model and were removed from the analysis. To create the graphs, the functions ggplot ["ggplot2" package (version 3.4.4)], ggeffect ["ggeffects"

package (version 1.5.2)] and ggarrange ["ggpubr" package (version 0.6.0)] were used. A Monte Carlo simulation was performed to create hundred temperature samples using the normal distribution. The means and standard deviation of each treatment and time point were taken from the original data. The simulation was also adjusted for ±2% accuracy. We applied a paired *t*-test to compare the generated data points to better understand the dispersion of the temperature data. Overall significance was set at 5% ($p < 0.05$).

3 Results

3.1 Clinical and hematological evaluation

Clinical and hematological variables were measured to detect possible signs of systemic sepsis caused by the intra-articular administration of LPS. The C group showed a higher RT 4 h after synovitis induction. Upon intragroup comparison, it was observed that in both groups the RR initially increased and then returned to its initial values. Additionally, an increase in RT was noted, especially between 4 and 12 h after synovitis induction, for both groups (Table 1). It is worth noting that most of the average values of RR and RT remained within the reference range for the equine species (41). Furthermore, there were no changes observed in the degree of hydration, colouration of the apparent mucous membranes, behaviour, or appetite of the animals. Table 2 shows no changes in erythrocytes, leukocytes, and lymphocytes. Group C exhibited a decrease in hemoglobin and hematocrit 8 h after induction. Neutrophilia was observed only at the eight-hour mark for group C.

3.2 Synovial fluid

No significant differences in the synovial fluid variables were displayed between groups (Table 3). A rise in TNCC was observed 8 h post-injection in both groups but returned to baseline concentrations after 48 h. Similarly, TP concentrations were elevated in both trials 8 and 24 h after the induction of transient synovitis but decreased after 48 h. Over time, PGE_2 concentrations increased 24 and 48 h in C, and 48 h in MAXVO after the induction of transient synovitis compared to baseline.

3.3 Joint circumference

During the evaluation period, joint circumference was measured to indirectly assess joint swelling, which is indicated by effusion and periarticular edema. No statistically significant differences in joint circumference were observed within or between groups (Figure 2).

3.4 Lameness

Table 4 presents the median lameness scores 0, 2, 4, 8, 24, and 48 h after synovitis induction. A difference was observed for group C at 2, 4, and 8 h after synovitis induction, with the peak of lameness at 4 h. The median clinical lameness score was 3 (with a range of 3–3) in the C group, while it was 1 (with a range of 0–2) in the MAXVO group. The group that received meloxicam showed a reduction in lameness at all time intervals (2, 4, 8, 24, and 48 h) after synovitis induction (Supplementary Figure S1). As stated in the material and methods under item 2.3, if the horses continued to show signs of lameness, they would receive an additional dose of treatment 24 h after the LPS injection. Only one horse from the MAXVO group and three horses from the control (placebo) group received this additional dose.

3.5 Infrared thermography

IRT was used to indirectly detect inflammation by measuring heat radiation. In this study, it was found that horses who received meloxicam had lower average, maximum, and minimum cutaneous

TABLE 1 Means ± standard deviation values of heart rate (HR), respiratory rate (RR), and rectal temperature (RT) values of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO).

Assessment times (days/hours)	HR (bpm)		RR (mpm)		RT (°C)	
	C	MAXVO	C	MAXVO	C	MAXVO
D-5	46 ± 9	42 ± 6	18 ± 8^{ab}	15 ± 5^{ab}	37.1 ± 0.5^{a}	37.0 ± 0.3^{ab}
D-2	49 ± 11	39 ± 7	18 ± 6^{ab}	18 ± 8^{ab}	37.0 ± 0.4^{a}	37.0 ± 0.4^{ab}
D-1	49 ± 8	48 ± 17	19 ± 4^{ab}	17 ± 7^{ab}	37.0 ± 0.6^{a}	36.7 ± 0.5^{ad}
0	44 ± 10	53 ± 16	15 ± 9^{ab}	14 ± 6^{ab}	37.2 ± 0.4^{ab}	37.3 ± 0.3^{bcd}
2	45 ± 8	51 ± 14	16 ± 7^{ab}	21 ± 11^{a}	37.4 ± 0.2^{abc}	37.3 ± 0.3^{bc}
4	46 ± 6	48 ± 11	20 ± 10^{a}	19 ± 7^{ab}	38.0 ± 0.4^{cd}	37.5 ± 0.3^{*bc}
6	49 ± 17	44 ± 12	19 ± 7^{a}	15 ± 7^{ab}	37.6 ± 0.7^{ad}	37.6 ± 0.2^{bc}
8	46 ± 13	48 ± 13	15 ± 7^{ab}	11 ± 4^{b}	37.9 ± 0.3^{bd}	37.5 ± 0.2^{bc}
12	42 ± 6	43 ± 10	12 ± 4^{ab}	13 ± 5^{ab}	37.8 ± 0.4^{bd}	37.8 ± 0.3^{c}
24	43 ± 9	40 ± 4	12 ± 6^{ab}	14 ± 6^{ab}	37.1 ± 0.3^{a}	37.0 ± 0.2^{ab}
48	36 ± 5	43 ± 12	10 ± 2^{b}	13 ± 7^{ab}	37.0 ± 0.3^{a}	37.3 ± 0.4^{abc}

C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection. Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. Lowercase letters indicate intragroup differences over time. *Indicates difference between experimental groups.

TABLE 2 Means ± standard deviation values of erythrocytes, hemoglobin, hematocrit, leukocytes, segmented neutrophils, and lymphocytes of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO).

Variable	Groups	Assessment times (hours)			
		0	8	24	48
Erythrocytes (×10^6/μL)	C	6.83 ± 0.23	6.70 ± 1.39	6.52 ± 0.59	6.42 ± 0.52
	MAXVO	6.67 ± 0.59	6.23 ± 0.66	6.42 ± 0.57	6.43 ± 0.69
Hemoglobin (g/dL)	C	11.72 ± 0.52^a	10.38 ± 0.85^b	11.1 ± 0.73^{ab}	11.22 ± 0.80^{ab}
	MAXVO	11.28 ± 0.83	10.85 ± 1.05	11.13 ± 0.75	11.15 ± 0.69
Hematocrit (%)	C	34.83 ± 1.72^a	31.50 ± 2.43^b	33.50 ± 2.35^{ab}	32.67 ± 2.73^{ab}
	MAXVO	34.17 ± 2.32	32.33 ± 2.50	33.50 ± 2.07	33.33 ± 2.07
Leukocytes (μL)	C	12,550 ± 3,352	13,483 ± 2,221	11,050 ± 2,268	10,700 ± 908
	MAXVO	10,600 ± 2,261	12,133 ± 2,734	10,267 ± 2,998	10,367 ± 3,092
Segmented neutrophils (μL)	C	7,599 ± 1,640^a	9,782 ± 1,632^b	7,084 ± 1,602^a	6,467 ± 725^a
	MAXVO	6,050 ± 1,021	7,871 ± 2,008	6,524 ± 2,185	6,215 ± 1,970
Lymphocytes (μL)	C	4,069 ± 1,805	2,937 ± 934	3,159 ± 590	3,293 ± 493
	MAXVO	3,592 ± 1,343	3,110 ± 587	3,023 ± 748	3,265 ± 1,242

C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection. Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. Lowercase letters indicate intragroup differences over time.

TABLE 3 Means ± standard deviation of synovial fluid total nucleated cell count (TNCC), total protein (TP), and prostaglandin E_2 (PGE_2) of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO).

Assessment times (hours)	TNCC (×10^3/mm^3)		TP (mg/dL)		PGE_2 (pg/mL)	
	C	MAXVO	C	MAXVO	C	MAXVO
0	0.21 ± 0.15^a	0.25 ± 0.16^a	1.27 ± 0.38^a	1.18 ± 0.42^a	179 ± 116^a	136 ± 25^a
8	96.7 ± 58.69^b	113.07 ± 77.66^b	4.78 ± 0.78^c	5.22 ± 1.07^c	281 ± 296^{ab}	188 ± 71^{ab}
24	30.13 ± 21.27^a	13.25 ± 13.08^a	4.22 ± 0.73^c	4.33 ± 0.64^c	355 ± 306^b	404 ± 524^{ab}
48	0.82 ± 0.73^a	0.57 ± 0.16^a	2.83 ± 1.15^b	2.73 ± 0.46^b	367 ± 267^b	339 ± 265^b

C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection. Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. Lowercase letters indicate intragroup differences over time.

temperatures 4 h post-injection in ML and DP views. Similarly, in the LM view, the MAXVO group showed lower average and minimum temperatures 4 h post-injection of LPS and lower average temperature 24 h post-injection compared to the control group. In the PD view, the MAXVO group showed lower average temperature 4 h post-injection of LPS. These findings suggest that meloxicam may have a positive impact on reducing cutaneous temperatures in horses post-LPS injection. It is worth noting that the cutaneous temperature in the group that received meloxicam was the same as the CL group most of the time, except at 24 h (average and maximum temperatures) after synovitis induction in ML, LM and DP views. In other words, the MAXVO group had the same cutaneous temperature as the contralateral limbs, which were not subjected to any invasive procedure (Figures 3–5 and Supplementary Tables S1–S3). In the study, it was found that the regions farthest from the LPS infiltration site, such as the ML and PD views, showed a higher temperature at the C group, when compared to the CL group only for the average temperature at 4 and 24 h. The PD view showed higher minimum and maximum temperatures only at 8 and 24 h, respectively, for the control group when compared to CL. At the ML view the C group showed higher maximum and minimum temperature than the CL only at 24 h. The Monte Carlo simulation was used to generate means and standard deviations, adjusted for ±2% accuracy, for each treatment and moment to ensure the reliability of our findings from the original data (Supplementary Tables S4–S15).

3.6 Multinomial logistic regression

Our MLR analysis revealed an association between the peaks of thermographic temperatures and lameness scores. The relative log odds, an equivalent to log odds in the logistic regression, demonstrated that a one-unit rise in the maximum temperature obtained by DP thermographic imaging is associated with an increase of 20 in the logarithm of the chances of having score 3 lameness compared to horses that presented lameness of score 0 ($p < 0.0001$). A significant association existed between a unit increase in maximum peak temperature obtained through LM thermographic imaging and a reduction of 18.8 in the logarithm of the odds of having a score 3 lameness compared to horses without lameness ($p = 0.009$). One unit increase in maximum peak temperature obtained by PD thermographic imaging is associated with an increase of 11.7 in the logarithm of the odds of having score 3 lameness compared to horses that do not lame ($p < 0.0001$). One unit increase in maximum temperature obtained by PD thermographic imaging is associated with a reduction of 18.7 in the logarithm of the

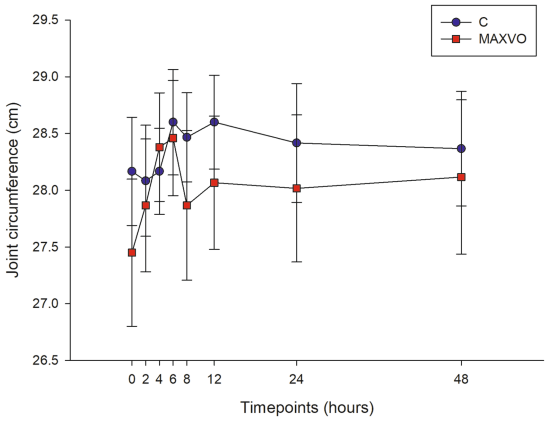


FIGURE 2
Graphical representation of means ± standard error of joint circumference of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO). C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection. Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h.

TABLE 4 Medians (interquartile range) of the degree of clinical lameness of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO).

Assessment times (hours)	Degree of lameness		*p*
	C	MAXVO	
0	0 (0–0)[a]	0 (0–0)[a]	NS
2	3 (2–3)[b]	0 (0–0.25)[a]*	0.003
4	3 (3–3)[b]	1 (0–2)[a]*	0.002
8	3 (1.75–3)[b]	1 (0–1.25)[a]*	0.016
24	1.5 (0.75–2)[ab]	0 (0–0.25)[a]*	0.024
48	1 (1–1)[ab]	0 (0–0)[a]*	0.001

C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection. Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. Lowercase letters indicate intragroup differences over time. *Indicates difference between experimental groups. NS, not significant.

odds of having score 3 lameness compared to horses that do not lame ($p < 0.0001$). The group of horses that received meloxicam orally is associated with a reduction of 76.2 in the logarithm of the odds of having grade 3 lameness compared to animals that do not lame ($p < 0.0001$). The relative risk ratios, equivalent to odds ratios in logistic regression, showed that one unit increase in the maximum temperature obtained by the thermographic image obtained from the DP view multiplies the chances of lameness classification 3 concerning horses that do not lame (score 0) by 4,528 (353%). Regarding the LM view, one unit increase in maximum temperature, obtained by thermal imaging, induced the chances of lameness, score 3, concerning horses that do not lame in 47.8%. The horses that received meloxicam, when compared with the control group, multiplied the chances of having a score of 1 lameness compared to animals that do not have lameness (score 0) by 146% ($p < 0.0001$). Lastly, the predicted probabilities, the same approach as logistic regression, but it is the probability of falling in a specific category, showed that the DP and LM positions' maximum temperature peaks were able to predict degree 3 of lameness, especially above 34°C (Figure 6).

4 Discussion

Infrared thermography can be essential in diagnosing and treating inflammatory processes (15). This diagnostic technique indirectly determines the presence of a nociceptive stimulus through the activation of the nervous system and its vasomotor responses, leading to an inflammatory process and increased heat radiation (1). Thermography can be an important tool to measure the reduction of inflammation or to check the effectiveness of anti-inflammatory medication (14). Changes in local temperature of the middle carpal joint, in addition to the results of synovial fluid variables, such as TNCC, TP, and PGE_2, confirmed the induction of synovitis. The present study showed that injecting 10 EU of *E. coli* O55:B5 LPS into the middle carpal joint of horses consistently induced transient synovitis and lameness. These findings are compatible with the results obtained in a previous study (11), which showed that a 10 EU dose of LPS induced lameness of clinically acceptable intensity, eliminating the need for additional doses of LPS.

It is important to highlight the use of the EU instead of mass units when processing and preparing LPS to ensure direct comparisons with other studies can be made (11). Standardizing laboratory procedures for preparing the LPS solution is also essential. Endotoxin molecules have micelle formation, which can affect their activity, so in this study, the stock and intermediate solutions were vortexed for 10 min at 1,500 rpm before dilution to break up LPS micelles and achieve a homogeneous solution. Additionally, the working solution was vortexed for 2 min at a speed of 1,500 rpm before induction, to disrupt any existing micelles (11).

This study evaluated the efficacy of oral preemptive meloxicam for inflammatory pain relief in horses with transient lameness and synovitis induced by the LPS model. The study findings suggest that this medication is a possible option for managing inflammation in horses in a preventative manner. Based on former studies, meloxicam treatment was initiated 3 days before LPS injection (10, 11). The researchers guided that the model is advantageous in providing information regarding the preemptive effects of treatments attributable to the concise duration of inflammation induced by LPS injection. This model provides relative data on whether it treats the signs of inflammation. Indeed, some of it is prevented when used in this manner. Therefore, the horses were given treatment for a few days before inducing synovitis. This decision was based on information regarding meloxicam pharmacokinetics, which indicated a maximum concentration time (T_{max}) of 2.62 ± 1.88 h (range: 1.5–8 h) and an elimination half-life of 10.2 ± 3.0 h (42). Besides, after injection, the LPS effect can vary widely, so the decision was made to consider the peak lameness reported in the literature (9–11, 43). This preventive treatment is likely to be clinically applicable for pain management in horses undergoing arthroscopic surgery (8) or surgical trauma, especially in the presence of pre-operative pain. It may prevent the initiation of a cascade of events that sensitize peripheral and central pain networks, leading to long-lasting maladaptive pain (44).

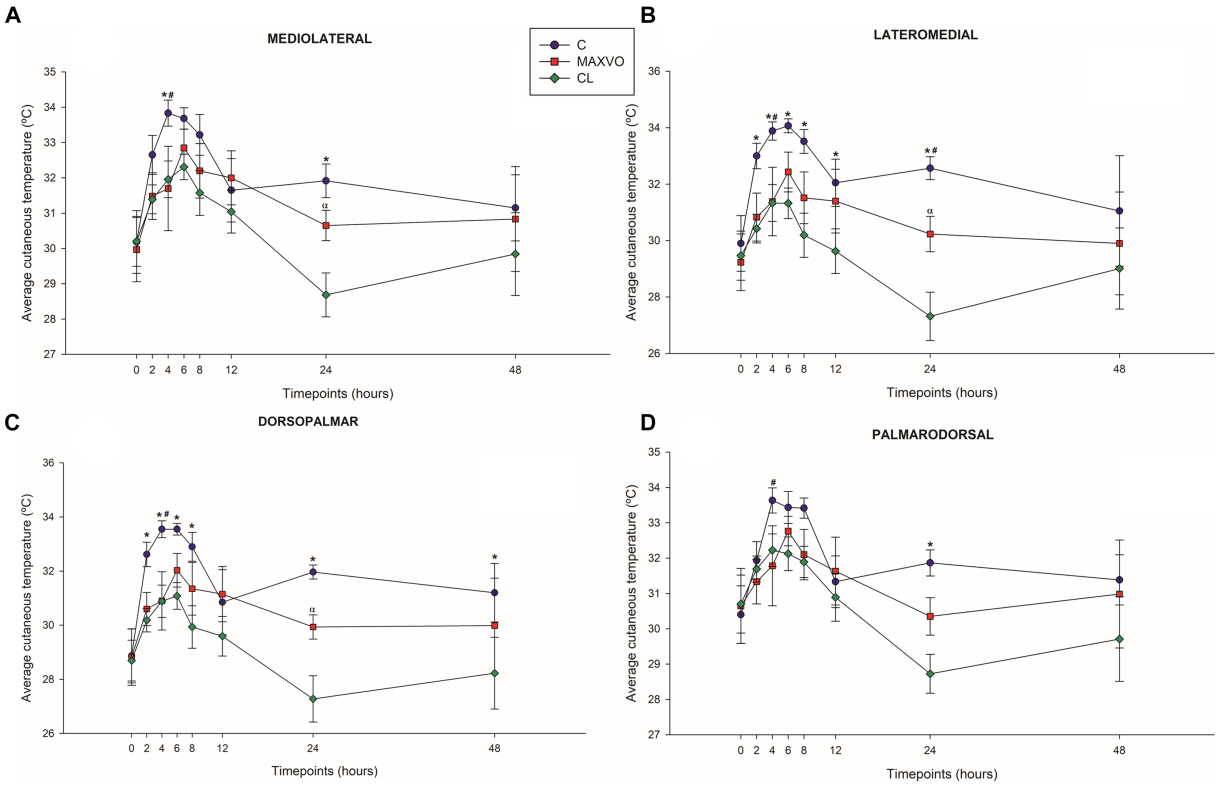


FIGURE 3
Graphical representation of means ± standard error of average cutaneous temperature of the **(A)** mediolateral, **(B)** lateromedial, **(C)** dorsopalmar and **(D)** palmarodorsal view of the carpal joint of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO). C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection; CL, contralateral limbs (negative control). Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. #Indicates difference between C and MAXVO. *Indicates difference between C and CL. αIndicates difference between CL and MAXVO. CL, contralateral limb (negative control). $p < 0.05$.

During the trial, clinical, cardiorespiratory, and hematological assessments were conducted to ensure the horses' health and to check for any signs of systemic illness, such as fever, tachycardia, and tachypnea, which may have been caused by the intra-articular administration of LPS. Physical examination variables remained within the reference range for horses, indicating no exacerbated systemic inflammatory response due to LPS infiltration. As expected, there was an increment in RT for both groups, with the MAXVO group showing lower RT 4 h after synovitis induction. This finding suggests that meloxicam may have potential antipyretic action. Variations in RR values were probably related to the time of day of the collection. HR, RR, and RT increases could be related to the release of inflammatory mediators due to LPS injection or pain (39, 43, 45, 46). However, these parameters alone are non-specific for determining the presence and severity of pain and can be influenced by external factors (47, 48). Some studies that induced synovitis in horses with LPS injection found no changes in HR, RR, and RT (10–12, 49). These differences may be related to the dose of endotoxin used (11). Also, systolic arterial pressure could be added to check for signs of systemic LPS or meloxicam effect (39).

The results of hematological exams revealed that the group of horses who did not receive meloxicam displayed neutrophilia. This finding may indicate the presence of inflammatory conditions and pathophysiologic feedback in the body (50–52). Furthermore, a decrease in total hemoglobin and hematocrit was also observed. These hematological responses in humans with septic arthritis suggest that the hematopoietic system may undergo changes during an infectious process (53). Further studies are necessary to determine whether similar results occur in horses.

The assessment of joint circumference did not indicate a clinical detection of effusion. This may be due to the inconsistent doses of LPS found in previous studies (11). A wide range of dosages have been used to induce synovitis and lameness with LPS, ranging from 0.125 ng to 5,000 ng of the same serotype of *E. coli* O55:B5. Some authors have reported swelling after the induction of synovitis by intra-articular administration of LPS (45, 46, 49, 54). It should be noted that the absence of effusion does not necessarily mean the absence of inflammation. Additionally, it is important to highlight that the behavior was similar between the groups.

When diagnosing synovitis, TNCC, TP, and PGE_2 concentrations are the most essential variables to assess. In both groups, the dose of LPS used increased these inflammatory biomarkers over time, and there was no difference between groups. This lack of difference between groups may be attributed to the lower frequency of meloxicam administration in the current therapeutic protocol compared to former studies (10, 11). Another factor that could contribute to the increase in these synovial inflammatory markers is the impact of successive arthrocentesis over time, despite the mild

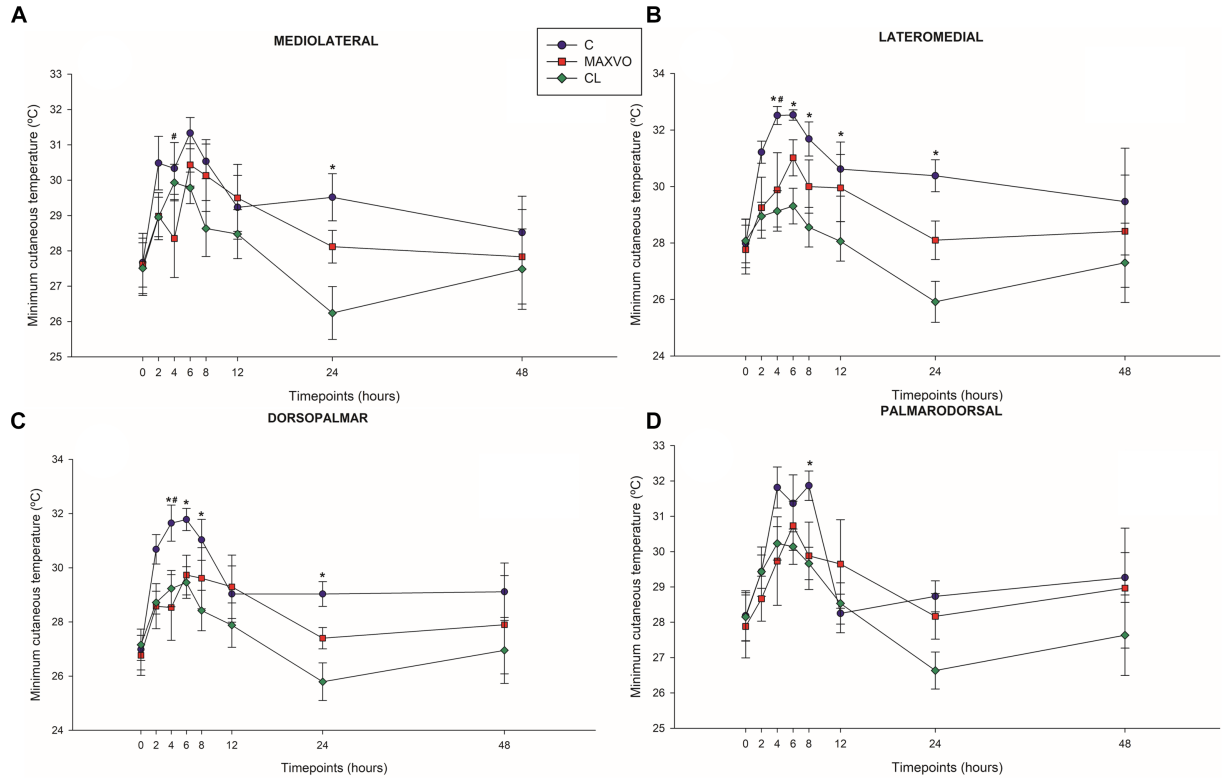


FIGURE 4
Graphical representation of means ± standard error of minimum cutaneous temperature of the **(A)** mediolateral, **(B)** lateromedial, **(C)** dorsopalmar and **(D)** palmarodorsal view of the carpal joint of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO). C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection; CL, contralateral limbs (negative control). Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. #Indicates difference between C and MAXVO. *Indicates difference between C and CL. αIndicates difference between CL and MAXVO. CL, contralateral limb (negative control). $p < 0.05$.

impact of this repetitive procedure on synovial fluid cytology, even without clinical significance (11, 55). The intra-articular injection of LPS had a more significant impact on the synovial fluid composition than successive arthrocentesis (11, 43, 56). However, it has been suggested by former studies that the repeated aspiration of synovial fluid could potentially serve as a confounding factor when using synovial inflammatory biomarkers to diagnose joint diseases (57, 58).

The study followed established criteria (39) for determining lameness, using blinded subjective evaluation. The study found that the peak of lameness occurred 4 h after induction in the control group. However, for the horses that received meloxicam, there was no significant change in the degree of lameness after the injection of LPS. This result is consistent with another study that evaluated the experimental induction of transient lameness and synovitis in trotter breed horses treated with oral administration of meloxicam (11). This suggests that the investigational veterinary product tested in this study effectively reduces inflammation and pain. Additionally, quantitative gait analysis could complement visual lameness assessment, as it provides objective information to support clinical decision-making during lameness evaluations (59, 60).

Detecting and quantifying inflammation and joint pain in horses has been a topic of research for many years. The scores of lameness and severity of synovitis were reflected in the skin surface temperature of the middle carpal joint obtained via thermography. Temperature is a key physical property that can be quantified using infrared thermography. It directly provides information about the inflammatory component of joint diseases (15). The present study focuses on using blinded infrared thermographic examination to detect changes in skin temperature surface pattern as an indicator of the inflammatory response induced by transient synovitis. The study found that joint temperatures increased significantly in horses with synovitis, while the horses that did not receive meloxicam showed a marked increase in temperature. The skin surface temperature can be used as an accurate indicator of the physiological state, with changes in temperature being caused by changes in local perfusion. Thermographic representation is based on tissue vascularization and blood supply and inflammation causes an increase in blood supply, which increases the local temperature (13, 30).

The MAXVO group showed lower surface temperatures in comparison to the control group, which indicates less intense inflammation. Increased heat is one of the classic signs of the inflammatory process. This is because tissue injuries can cause vasodilation and an increase in exothermic cellular metabolism, leading to an increase in temperature at the site of the injury (61). Hence, it is possible to obtain quantitative data on the degree of joint inflammation by thermographically evaluating the cutaneous surface of the joints. This is done by measuring the infrared radiation emitted spontaneously by the site of the joint injury. Several studies have used this technique to evaluate joint inflammation (1, 15, 30, 31, 33, 38).

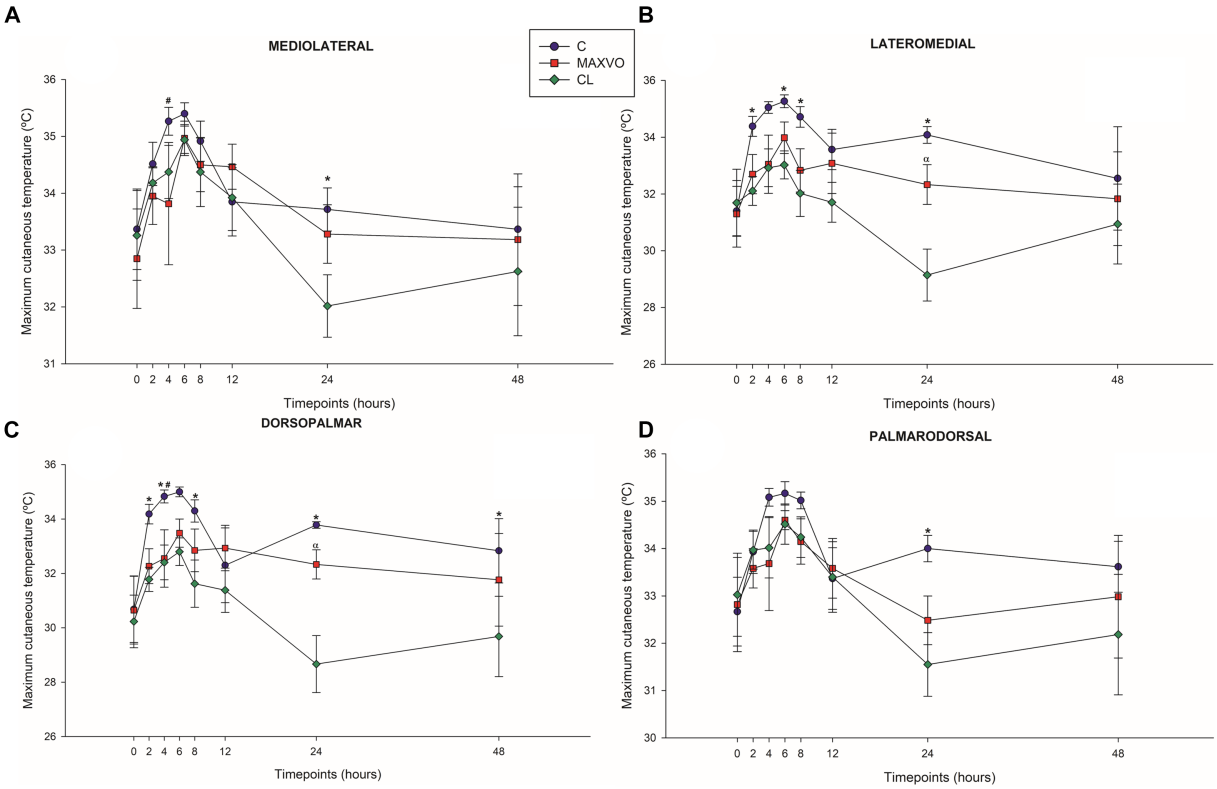


FIGURE 5
Graphical representation of means ± standard error of maximum cutaneous temperature of the **(A)** mediolateral, **(B)** lateromedial, **(C)** dorsopalmar and **(D)** palmarodorsal view of the carpal joint of horses submitted to experimental induction of transient synovitis for both trials (C and MAXVO). C, control group; MAXVO received meloxicam (Maxicam Gel®), administered orally at 0.6 mg/kg daily 3 days before LPS injection; CL, contralateral limbs (negative control). Synovitis induction by injection of the middle carpal joint with 10 EU LPS occurred at 0 h. #Indicates difference between C and MAXVO. *Indicates difference between C and CL. αIndicates difference between CL and MAXVO. CL, contralateral limb (negative control). $p < 0.05$.

To accurately compare the left and right sides, capturing images of contralateral joints at an equal imaging distance in separate images is crucial (14). The contralateral joint was used as a negative control for both C and MAXVO. After inducing synovitis, the group that received meloxicam had a temperature of the middle carpal joint that was like that of the contralateral joint (negative control), which did not receive LPS and was always clinically normal. Only 24 h after the induction of synovitis MAXVO group presented higher cutaneous temperature than the negative control in all regions of interest, except in the PD view. This result indicates the effectiveness of meloxicam as an anti-inflammatory, as the skin temperature of the middle carpal joint that received the induction of inflammation in the group medicated with meloxicam remained like the joint that was not infiltrated with LPS at most timepoints.

MLR, a precise and reliable extension of binary logistic regression, applies to a categorical outcome variable with more than two categories. It was found that the predicted probabilities, like the approach used in logistic regression, can be used to determine the likelihood of falling into a specific category. In this case, this study found that the maximum temperature peaks of the DP and LM positions can be used to predict the severity of lameness with a high level of accuracy, especially when the temperature exceeds 34°C.

The results of this study have wide-ranging implications for the field of functional infrared thermography imaging and its use in lameness management in horses. Thermography could be a justifiable option to aid in the diagnosis of clinical or experimental synovitis, as its use could avoid sequential invasive procedures, such as sedation and intra-articular collection. Additionally, using thermography can prevent the promotion of an additional inflammatory process promoted by repeated aspiration of synovial fluid that could mask the actual effects of LPS or the action of NSAIDs. In this regard, IRT examination combined with lameness checking proved to be a more sensitive diagnostic tool to detect the effectiveness of the NSAID used herein. These findings could pave the way for future research and practical applications in this area, offering potential benefits for both human and animal health.

5 Conclusion

Horses that were given meloxicam orally before the induction of synovitis with LPS showed a notable decrease in the temperature of the middle carpal joint surface detected by infrared thermography. This reduction in temperature was accompanied by a significant mitigation of lameness. In conclusion, the use of Maxicam Gel® proved to be an effective preventive anti-inflammatory medication for horses.

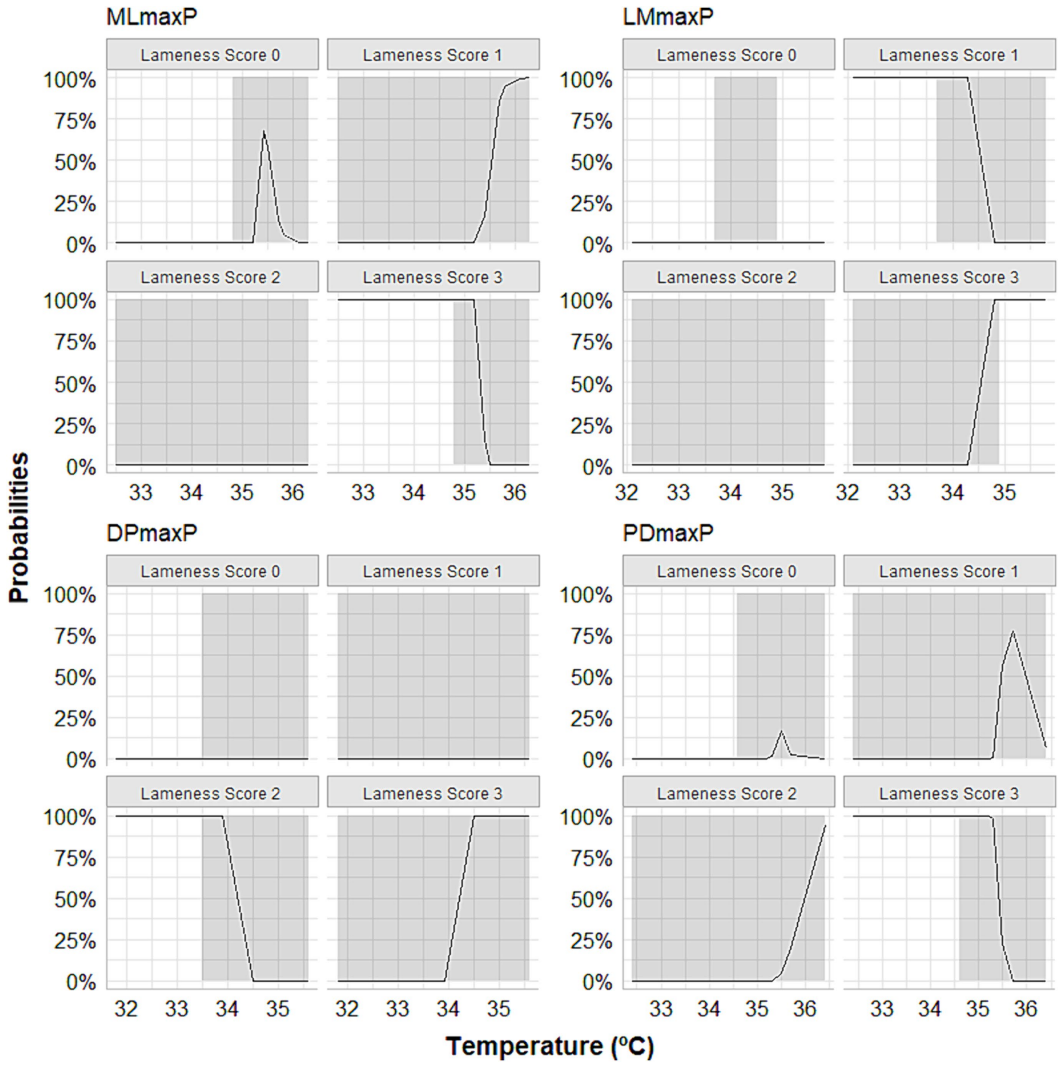


FIGURE 6
Curves depicting the calculation of predicted probabilities following multinomial logistic regression showing that the maximum temperature peaks observed in the lateromedial (LMmaxP) and dorsopalmar (DPmaxP) views could predict score 3 of lameness, especially when they exceeded 34°C. The horses were submitted to experimental induction of transient synovitis with 10 EU LPS and treated with meloxicam administered orally at 0.6 mg/kg daily 3 days before LPS injection.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The study adhered to the Ethical Principles in Animal Experimentation as established by the National Council for Control in Animal Experimentation (CONCEA). The protocol underwent review and approval by the Ethics Committee on the Use of Animals—CEUA—UNESP, Jaboticabal, Brazil (Protocol No. 2887/2021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JC: Software, Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. DD: Writing – review & editing, Writing – original draft, Methodology, Conceptualization, Supervision. TL: Writing – original draft, Software, Investigation, Formal analysis, Data curation. NS: Writing – original draft, Software, Investigation, Formal analysis, Data curation. AS: Writing – original draft, Software, Investigation, Formal analysis, Data curation. GRi: Writing – original draft, Investigation, Formal analysis. FA: Writing – review & editing, Conceptualization. BA: Writing – review & editing, Conceptualization. IG: Writing – original draft, Conceptualization. GRa: Writing – review & editing, Methodology, Formal analysis. GF: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by a grant from the São Paulo Research Foundation (FAPESP, Process No. 2019/16779-3 and Process No. 2020/01172-3). Additionally, this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES)—Finance Code 001.

The authors declare that this study received funding from Ourofino Animal Health Company. The funder was involved in the study's design, writing of this article, and the decision to submit it for publication. The meloxicam was formulated and manufactured by Ourofino Animal Health Company for commercial use in Brazil, and the study was designed to meet Brazilian regulations.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors thank the School of Agricultural and Veterinary Sciences, São Paulo State University, (FCAV/UNESP) for the academic support. The authors also thank the Graduate Course Program in Veterinary Medicine (FCAV/UNESP), the Graduate Course Program in Animal Science (FCAV/UNESP), and the Department of Animal Morphology and Physiology (FCAV/UNESP) for their academic support.

Conflict of interest

DD, FA, BA, and IG were employed by company Ourofino Animal Health Company.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1399815/full#supplementary-material

References

1. Whittaker AL, Muns R, Wang D, Martinez-Burnes J, Hernández-Ávalos I, Casas-Alvarado A, et al. Assessment of pain and inflammation in domestic animals using infrared thermography: a narrative review. *Animals*. (2023) 13:2065. doi: 10.3390/ani13132065

2. Jacobs CC, Schnabel LV, Mcllwraith CW, Blikslager AT. Non-steroidal anti-inflammatory drugs is equine orthopaedics. *Equine Vet J*. (2022) 54:636–48. doi: 10.1111/evj.13561

3. Knych HK. Nonsteirodal anti-inflammatory drug use in horses. *Vet Clin N Am Equine Pract*. (2017) 33:1–15. doi: 10.1016/j.cveq.2016.11.001

4. Bonnesen K, Schmidt M. Recategorization on non-aspirin nonsteroidal anti-inflammatory drugs according to clinical relevance: abandoning the traditional NSAID terminology. *Can J Cardiol*. (2021) 37:1707. doi: 10.1016/j.cjca.2021.06.014

5. UCVM Class of 2016Banse H, Cribb AE. Comparative efficacy of oral meloxicam and phenylbutazone in 2 experimental pain models in the horse. *Can Vet J*. (2017) 58:157–67.

6. Ziegler A, Fogle C, Blikslager A. Update on the use of cyclooxygenase-2-selective nonsteroidal anti-inflammatory drugs in horses. *J Am Vet Med Assoc*. (2017) 250:1271–4. doi: 10.2460/javma.250.11.1271

7. Khalil NY, Aldosari KF. Meloxicam In: HG Brittain, editor. *Profiles of drug substances, excipients and related methodology*. London: Academic Press (2020). 159–97.

8. Yuan Y, Cui D, Zhang Y. Preemptive meloxicam achieves a better effect on postoperative pain control and similar tolerance compared with postoperative meloxicam in patients receiving arthroscopic knee surgery. *Inflammopharmacology*. (2019) 27:1091–100. doi: 10.1007/s10787-019-00614-4

9. Toutain PL, Cester CC. Pharmacokinetic-pharmacodynamic relationships and dose response to meloxicam in horses with induced arthritis in the right carpal joint. *Am J Vet Res*. (2004) 65:1533–41. doi: 10.2460/ajvr.2004.65.1533

10. de Grauw JC, van de Lest CHA, Brama PAJ, Rambags BPB, van Weeren PR. *In vivo* effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine Vet J*. (2009) 41:693–9. doi: 10.2746/042516409x436286

11. van de Water E, Oosterlinck M, Korthagen NM, Duchateau L, Dumoulin M, van Weeren PR, et al. The lipopolysaccharide model for the experimental induction of transient lameness and synovitis in Standardbred horses. *Vet J*. (2021) 270:105626. doi: 10.1016/j.tvjl.2021.105626

12. van de Water E, Oosterlinck M, Dumoulin M, Korthagen NM, van Weeren PR, van den Broek J, et al. The preventive effects of two nutraceuticals on experimentally induced acute synovitis. *Equine Vet J*. (2017) 49:532–8. doi: 10.1111/evj.12629

13. Redaelli V, Bergero D, Zucca E, Ferrucci F, Costa LN, Crosta L, et al. Use of thermography techniques in equines: principles and applications. *J Equine Vet*. (2014) 34:345–50. doi: 10.1016/j.jevs.2013.07.007

14. Soroko M, Howell K. Infrared thermography: current applications in equine medicine. *J Equine Vet*. (2018) 60:90–6. doi: 10.1016/j.jevs.2016.11.002

15. Schiavon G, Capone G, Frize M, Zaffagnini S, Candrian C, Filardo G. Infrared thermography for the evaluation of inflammatory and degenerative joint diseases: a systematic review. *Cartilage*. (2021) 13:1790S–801S. doi: 10.1177/19476035211063862

16. Kruljc P. Thermographic examination of the horse. *Acta Vet*. (2023) 73:289–316. doi: 10.2478/acve-2023-0023

17. Daglish J, Le Jeune SS, Pypendop BH, Ramirez EM, Turner TA. Use of infrared thermography to detect jugular venipuncture in the horse. *J Equine Vet*. (2017) 59:1–6. doi: 10.1016/j.jevs.2017.09.003

18. Prochno HC, Barussi FM, Bastos FZ, Weber SH, Bechara GH, Rehan IF, et al. Infrared thermography applied to monitoring musculoskeletal adaptation to training in thoroughbred race horses. *J Equine Vet*. (2020) 87:102935. doi: 10.1016/j.jevs.2020.102935

19. Silva CJFL, Trindade KTG, Cruz RKS, Vilela CF, Coelho CS, Ribeiro-Filho JD, et al. Association between infrared thermography, blood count and creatine kinase in the evaluation of the welfare of Vaquejada horses. *Open J Vet Med*. (2023) 13:53–67. doi: 10.4236/ojvm.2023.136006

20. Witkowska-Piłaszewicz O, Masko M, Domino M, Winnicka A. Infrared thermography correlates with lactate concentration in blood during race training in horses. *Animals*. (2020) 10:2072. doi: 10.3390/ani10112072

21. Janczarek I, Wiśniewska A, Tkaczyk E, Wnuk-Pawlak E, Kaczmarek B, Liss-Szczepanek M, et al. Effect of different water-cooling treatments on changes in rectal and surface body temperature in leisure horses after medium-intensity effort. *Animals*. (2022) 12:525. doi: 10.3390/ani12040525

22. Verdegaal ELJMM, Howarth GS, McWhorter TJ, Delesalle CJG. Thermoregulation during field exercise in horses using skin temperature monitoring. *Animals*. (2024) 14:136. doi: 10.3390/ani14010136

23. Kim SM, Cho GJ. Validation of eye temperature assessed using infrared thermography as an indicator of welfare in horses. *Appl Sci*. (2021) 11:7186. doi: 10.3390/app11167186

24. Domino M, Borowska M, Kozlowska N, Zdrojkowski L, Jasinski T, Smyth G, et al. Advances in thermal image analysis for the detection of pregnancy in horses using infrared thermography. *Sensors*. (2022) 22:191. doi: 10.3390/s22010191

25. Urayama S, Tanaka A, Kusano K, Sato H, Muranaka M, Mita H, et al. Oral administration of meloxicam and flunixin meglumine have similar analgesic effects after lipopolysaccharide-induced inflammatory response in thoroughbred horses. *J Equine Vet*. (2023) 121:104205. doi: 10.1016/j.jevs.2022.104205

26. Lisboa BRF, Silva JAR, Silva WC, Barbosa AVC, Silva LKX, Lourenço-Júnior JB. Evaluation of thermoregulation of horses (*Equus caballus*) submitted to two methods of post-exercise cooling, in hot and humid climate conditions, in the Eastern Amazon. *Front Vet Sci*. (2023) 10:1150763. doi: 10.3389/fvets.2023.1150763

27. Carvalho JRG, Conde G, Antonioli ML, Dias PP, Vasconcelos RO, Taboga SR, et al. Biocompatibility and biodegradation of poly (lactic acid)(PLA) and an immiscible PLA/poly (ε-caprolactone) (PCL) blend compatibilized by poly (ε-caprolactone-b-tetrahydrofuran) implanted in horses. *Polym J*. (2020) 52:629–43. doi: 10.1038/s41428-020-0308-y

28. Carvalho JRG, Trindade PHE, Conde G, Antonioli ML, Funnicelli MIG, Dias PP, et al. Facial expressions of horses using weighted multivariate statistics for assessment of subtle local pain induced by polylactide-based polymers implanted subcutaneously. *Animals*. (2022) 12:2400. doi: 10.3390/ani12182400

29. Ioannou S. Functional infrared thermal imaging: a contemporary tool in soft tissue screening. *Sci Rep*. (2020) 10:9303. doi: 10.1038/s41598-020-66397-9

30. Branco JHL, Branco RLL, Siqueira TC, Souza LC, Dalago KMS, Andrade A. Clinical applicability of infrared thermography in rheumatic diseases: a systematic review. *J Therm Biol*. (2022) 104:103172. doi: 10.1016/j.jtherbio.2021.103172

31. Marziani L, Boffa A, Angelelli L, Andriolo L, Di Martino A, Zaffagnini S, et al. Infrared thermography in symptomatic knee osteoarthritis: joint temperature differs based on patient and pain characteristics. *J Clin Med*. (2023) 12:2319. doi: 10.3390/jcm12062319

32. Warner R, Kleinhenz M, Ydstie J, Schleining J, Wulf L, Coetzee J, et al. Comparison of analgesics for control of lameness-associated pain in lactating dairy cattle. *J Anim Sci*. (2019) 97:162–3. doi: 10.1093/jas/skz122.287

33. Alves JC, Santos A, Jorge P, Lavrador C, Carreira LM. Evaluation of digital thermography imaging to assess and monitor treatment of police working dogs with naturally occurring hip osteoarthritis. *BMC Vet Res*. (2021) 17:180. doi: 10.1186/s12917-021-02876-z

34. National Academies of Sciences, Engineering, and Medicine; Division on Earth and Life Studies; Board on Agriculture and Natural Resources; Committee on a Review of Methods for Detecting Soreness in Horses. *A review of methods for detecting soreness in horses*. Washington, DC: National Academies Press (2021). 624–6242.

35. Purohit RC, McCoy MD. Thermography in the diagnosis of inflammatory processes in the horse. *Am J Vet Res*. (1980) 41:1167–74.

36. Higgins AJ, Lees P, Sedgwick AD, Buick AR, Churchus R. Use of a novel non-steroidal anti-inflammatory drug in the horse. *Equine Vet J*. (1987) 19:60–6. doi: 10.1111/j.2042-3306.1987.tb02584.x

37. Urayama S, Tanaka A, Kusano K, Sato H, Nagashima T, Fukuda I, et al. Oral administration of meloxicam suppresses low-dose endotoxin challenge-induced pain in thoroughbred horses. *J Equine Vet*. (2019) 77:139–43. doi: 10.1016/j.jevs.2019.03.001

38. Figueiredo T, Dzyekanski B, Pimpão CT, Silveira AB, Capriglione LG, Michelotto PV Jr. Use of infrared thermography to detect intrasynovial injections in horses. *J Equine Vet*. (2013) 33:257–60. doi: 10.1016/j.jevs.2012.07.003

39. Carregaro AB, Freitas GC, Ribeiro MH, Xavier NV, Dória RGS. Physiological and analgesic effects of continuous-rate infusion of morphine, butorphanol, tramadol or methadone in horses with lipopolysaccharide (LPS)-induced carpal synovitis. *BMC Vet Res*. (2014) 10:1–8. doi: 10.1186/s12917-014-0299-z

40. Fails AD. Functional anatomy of the equine musculoskeletal system In: GM Baxter, editor. *Adams and Stashak's lameness in horses*. Hoboken: John Wiley & Sons, Inc. (2020). 1–65.

41. Byars TD, Gonda KC. Equine history, physical examination, records, and recognizing abuse or neglect in patients In: BP Smith, editor. *Large animal internal medicine*. Riverport Lane: Elsevier (2015). 14.

42. Noble G, Edwards S, Lievaart J, Pippia J, Boston R, Raidal SL. Pharmacokinetics and safety of single and multiple oral doses of meloxicam in adult horses. *Vet Intern Med*. (2012) 26:1192–201. doi: 10.1111/j.1939-1676.2012.00976.x

43. Lucia JL, Coverdale JA, Arnold CE, Winsco KN. Influence of an intra-articular lipopolysaccharide challenge on markers of inflammation and cartilage metabolism in young horses. *J Anim Sci*. (2013) 91:2693–9. doi: 10.2527/jas.2012-5981

44. Guedes A. Pain management in horses. *Vet Clin North Am Equine Pract*. (2017) 33:181–211. doi: 10.1016/j.cveq.2016.11.006

45. Andreassen SM, Vinther AML, Nielsen SS, Andersen PH, Tnibar A, Kristensen AT, et al. Changes in concentrations of haemostatic and inflammatory biomarkers in synovial fluid after intra-articular injection of lipopolysaccharide in horses. *BMC Vet Res*. (2017) 13:182. doi: 10.1186/s12917-017-1089-1

46. Martinez RE, Leatherwood JL, Arnold CE, Glass KG, Walter KW, Valigura HC, et al. Responses to an intra-articular lipopolysaccharide challenge following dietary supplementation of *Saccharomyces cerevisiae* fermentation product in young horses. *J Anim Sci*. (2021) 99:1–8. doi: 10.1093/jas/skab272

47. Wagner AE. Effects of stress on pain in horses and incorporating pain scales for equine practice. *Vet Clin North Am Equine Pract*. (2010) 26:481–92. doi: 10.1016/j.cveq.2010.07.001

48. de Grauw JC, van Loon JPAM. Systematic pain assessment in horses. *Vet J*. (2016) 209:14–22. doi: 10.1016/j.tvjl.2015.07.030

49. Kahn MK, Coverdale JA, Leatherwood JL, Arnold CE, Dabareiner RA, Bradbery AN, et al. Age-related effects on markers of inflammation and cartilage metabolism in response to an intra-articular lipopolysaccharide challenge in horses. *J Anim Sci*. (2017) 95:671–80. doi: 10.2527/jas2016.1078

50. Anderson SL, Singh B. Equine neutrophils and their role in ischemia reperfusion injury and lung inflammation. *Cell Tissue Res*. (2018) 371:639–48. doi: 10.1007/s00441-017-2770-1

51. Sheats MK. A comparative review of equine SIRS, sepsis, and neutrophils. *Front Vet Sci*. (2019) 6:69. doi: 10.3389/fvets.2019.00069

52. Meleiro MCZ, Carvalho HJC, Ribeiro RR, Silva MD, Gomes CMS, Miglino MA, et al. Immune functions alterations due to racing stress in thoroughbred horses. *Animals*. (2022) 12:1203. doi: 10.3390/ani12091203

53. Borzio R, Mulchandani N, Pivec R, Kapadia BH, Leven D, Harwin SF, et al. Predictors of septic arthritis in the adult population. *Orthopedics*. (2016) 39:e657–63. doi: 10.3928/01477447-20160606-05

54. Neuenschwander HM, Moreira JJ, Vandruscolo CP, Fülber J, Seidel SRT, Michelacci YM, et al. Hyaluronic acid has chondroprotective and joint-preserving effects on LPS-induced synovitis in horses. *J Vet Sci*. (2019) 20:e67. doi: 10.4142/jvs.2019.20.e67

55. Rinnovati R, Bonelli F, Tognetti R, Gallo C, Bassini RF, Marchetti V, et al. Effect of repeated arthrocentesis on cytology of synovial fluid. *J Equine Vet*. (2017) 57:112–5. doi: 10.1016/j.jevs.2017.07.008

56. Ross TN, Kisiday JD, Hess T, McIlwraith CW. Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1 beta and lipopolysaccharide. *Osteoarthr Cartil*. (2012) 20:1583–90. doi: 10.1016/j.joca.2012.08.008

57. van den Boom R, van de Lest CHA, Bull S, Brama PAJ, van Weeren PR, Barneveld A. Influence of repeated arthrocentesis and exercise on synovial fluid concentrations of nitric oxide, prostaglandin E2 and glycosaminoglycans in healthy equine joints. *Equine Vet J*. (2005) 37:250–6. doi: 10.2746/0425164054530740

58. NCR TM, van Weeren PR. How exercise influences equine joint homeostasis. *Vet J*. (2017) 222:60–7. doi: 10.1016/j.tvjl.2017.03.004

59. Bosch S, Serra Bragança F, Marin-Perianu M, Marin-Perianu R, van der Zwaag BJ, Voskamp J, et al. EquiMoves: a wireless networked inertial measurement system for objective examination of horse gait. *Sensors*. (2018) 18:850. doi: 10.3390/s18030850

60. Hardeman AM, Egenvall A, Serra Bragança FM, Swagemakers JH, Koene MHW, Roepstorff L, et al. Visual lameness assessment in comparison to quantitative gait analysis data in horses. *Equine Vet J*. (2022) 54:1076–85. doi: 10.1111/evj.13545

61. McManus C, Tanure CB, Peripolli V, Seixas L, Fischer V, Gabbi AM, et al. Infrared thermography in animal production: an overview. *Comput Electron Agric*. (2016) 123:10–6. doi: 10.1016/j.compag.2016.01.027

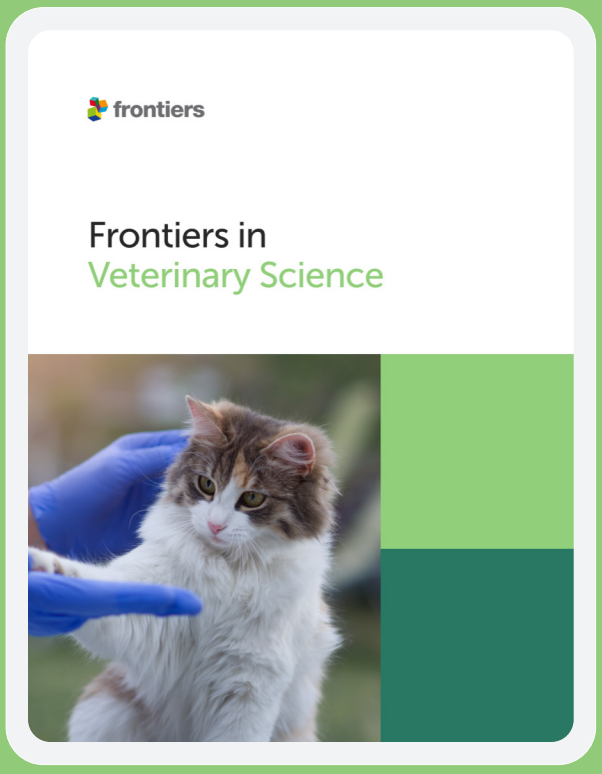
Frontiers in Veterinary Science

Transforms how we investigate and improve animal health

The third most-cited veterinary science journal, bridging animal and human health with a comparative approach to medical challenges. It explores innovative biotechnology and therapy for improved health outcomes.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact