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Cellular, molecular and genomic alterations in the hatchlings of *Labeo rohita* after exposure to Triclosan

Sunil Sharma[1], Owias Iqbal Dar[1,2], Megha Andotra[1],
Simran Sharma[1], Ankeet Bhagat[1], Sharad Thakur[3],
Anup Kumar Kesavan[3] and Arvinder Kaur[1]*

[1]Aquatic Toxicology Lab, Department of Zoology, Guru Nanak Dev University, Amritsar, Punjab, India,
[2]Department of Biosciences, University Institute of Biotechnology, Chandigarh University, Mohali,
Punjab, India, [3]Molecular Microbiology Lab, Department of Molecular Biology and Biochemistry, Guru
Nanak Dev University, Amritsar, Punjab, India

Triclosan 5-chloro-2-(2, 4-dichlorophenoxy) phenol (TCS) is widely used as a biocide in human and veterinary medicines, personal care products and household articles. To obtain biomarkers for the acute stress of Triclosan, the hatchlings of *Labeo rohita* were exposed for 96 h to 0.06, 0.067 and 0.097 mg/L TCS. Morphological deformities, cell viability, frequency of micronucleated and aberrant cells, transcriptomic and biomolecular alterations were recorded after exposure and a depuration period of 10 days. The exposed hatchlings had a pointed head, curved trunk, lean body, deformed caudal fin, haemorrhage, hypopigmentation and tissue degeneration at 0.067 and 0.097 mg/L only. The frequency of viable cells declined but that of necrotic, apoptotic, micronucleated and abnormal cells increased ($p \leq 0.01$) in a concentration dependent manner after exposure as well as the depuration period. After recovery, the frequency of viable and micronucleated cells increased, but that of necrotic, apoptotic, and aberrant cells declined in comparison to their respective 96 h values. The mRNA level of HSP47, HSP70, HSc71 and α-tropomyosin increased ($p \leq 0.01$), while that of HSP60, HSP90, DHPR, myosin light polypeptide 3, desmin b and lamin b1 declined ($p \leq 0.01$) after exposure. Ten days post exposure, a significant increase ($p \leq 0.01$) over control was observed in the expression of all the heat shock and cytoskeletal genes and the values (except for HSc71) were higher than the respective 96 h values also. Infrared spectra showed that band area of amide A, amide I, amide II and phospholipids increased significantly ($p \leq 0.01$) but peak intensity of lipid, glycogen and nucleic acids decreased after exposure. After recovery, area of the peaks for most of the biomolecules [except lipids (2924–2925, 1455–1457 cm^{-1}) and glycogen (1163–1165 cm^{-1})] declined significantly over control and 96 h values. Collectively these changes seem to be responsible not only for the onset of paralysis but also for the concentration dependent increase in larval and cellular abnormalities as well as no/sporadic swimming movement in exposed hatchlings. It is evident that HSP60, HSc71, HSP90, α-tropomyosin and DHPR were strongly affected but DHPR can be used as the most sensitive marker for the toxicity of TCS. This is

the first study reporting effect of TCS on the selected heat shock and cytoskeletal genes in a single model.

Introduction

Triclosan [TCS, 5-chloro-2-(2, 4-dichlorophenoxy) phenol] is a non-antibiotic antimicrobial biocide that is commonly used in personal, household and healthcare products (Lu et al., 2018). It mainly enters water bodies and land through the wastewater and biosolids that are discharged from the wastewater treatment plants (Dann and Hontela, 2011). Excessive use of triclosan containing sanitizers and antiseptics during Covid-19 pandemic has raised concern about its risks to the environment and public health (Lu and Guo, 2021). High bioaccumulation factor (2.7–90) and octanol/water partition coefficient (4.8) of TCS increase the possibility of its build up in non-target aquatic organisms (Dhillon et al., 2015; Yao et al., 2018). High concentrations of TCS ranging between 13 and 1040 µg/kg and 91.1–589 µg/kg were detected in Gomti river (Uttar Pradesh) and Torsa river (West Bengal) of India, respectively (Nag et al., 2018; Sarkar et al., 2020). Presence of TCS in water has been reported to cause abnormal and delayed embryonic development (Dar et al., 2019), neurological and endocrine disruption (Wang and Tian, 2015; Kim et al., 2018), oxidative stress (Dar et al., 2020a; Dar et al., 2020b), histological changes (Gyimah et al., 2020), transcriptomic alterations and DNA damage in fish (Sharma et al., 2021a). TCS impairs excitation-contraction of skeletal and cardiac muscles by altering the interaction between dihydropyridine receptors (DHPRs) and Ca^{2+} release channels of sarcoplasmic reticulum (Ahn et al., 2008; Cherednichenko et al., 2012). These skeletal proteins play an important role in dendritic growth and synaptic plasticity (Pessah et al., 2010; Wayman et al., 2012) while disruption of Ca^{2+} homeostasis in fish and mammals leads to abnormal neuronal development and muscle contraction. Earlier reports show that TCS induces alterations in the expression of cytoskeletal genes in fresh water mussel *Dreissena polymorpha* (Riva et al., 2012), fathead minnow (Fritsch et al., 2013), zebra fish larvae (Falisse et al., 2017) and early life stages of zebrafish (Ma et al., 2019).

Previous studies reported cytotoxic and genotoxic effects of TCS in *Closterium ehrenbergii* (Ciniglia et al., 2005), *Mytilus galloprovincialis* (Canesi et al., 2007), *Haliotis tuberculate* (Gaume et al., 2012), *Dreissena polymorpha* (Binelli et al., 2009a; 2009b), *Oreochromis niloticus* (Vijitha et al., 2017), *Carassius auratus* (Wang et al., 2018), *Danio rerio* (Parenti et al., 2019), *Pangasianodon hypophthalmus* (Paul et al., 2019, 2020) and *Clarias gariepinus* (Jimoh and Sogbanmu, 2021). Cell viability and micronucleus/aberrant cell assay are the most commonly used approaches to assess cyto-genotoxic potential of such xenobiotics.

During stress, the most common type of cellular response in fish is the production of heat shock proteins (HSPs) for maintenance of protein integrity and function (Iwama et al., 1999). HSPs are the highly conserved families and play an essential role in folding, repair and transportation of proteins (Liu et al., 2013). They are also called "stress proteins" because HSPs show marked variations in their expression under the influence of stressors like reactive oxygen species, heavy metals, xenobiotics, pathogens *etc.* (Ponomarenko et al., 2013). TCS has been observed to alter mRNA copy number of HSPs in zebra mussel (Riva et al., 2012), *Eisenia fetida* (Lin et al., 2014), *Brachionus koreanus* (Han et al., 2016), zebrafish larvae (Falisse et al., 2017), rainbow trout (Capkin et al., 2017) and *Caenorhabditis elegans* (García-Espiñeira et al., 2018). Fourier transform infrared spectroscopy (FT-IR) is an important and sensitive technique for observing cellular changes at molecular level (Cakmak et al., 2006). It can detect structural and functional alterations in biomolecules (protein, lipid and nucleic acids) and the results can be used to predict the possible effects of stressors on the vital functions of an organism (Palaniappan and Vijayasundaram, 2008a). FT-IR has been successfully used to highlight changes in the biomolecules due to 17β-estradiol in the juveniles of rainbow trout (Cakmak et al., 2006), arsenic in the fingerlings of *Labeo rohita* (Palaniappan and Vijayasundaram, 2008a; Palaniappan and Vijayasundaram, 2008b; Palaniappan and Vijayasundaram, 2009a; Palaniappan and Vijayasundaram, 2009b) and TCS in the embryos of four food fishes (Dar et al., 2022).

TCS affects development and functioning of skeletal muscles by impairing the excitation-contraction coupling of skeletal muscles. HSPs participate in stabilization of cytoskeleton (Pijanowska and Kloc, 2004) and HSCs play significant role in support and repair of damaged cytoskeleton and maintenance of membrane lipoproteins of nuclear and cellular membrane (Roberts et al., 2010). Expression of HSPs is much more sensitive to stress than traditional health indices (Eder et al., 2009). Therefore, the present study envisaged to evaluate acute toxicity of TCS by taking into account larval abnormalities, cell viability, nucleo-cellular aberrations, expression of cytoskeletal and heat shock genes and secondary structure of functional groups of the hatchlings of *Labeo rohita*, an important fresh water food fish of India, Nepal, Pakistan, Vietnam, Bangladesh and Myanmar. Although small amount of TCS enters in aquatic ecosystems from domestic and industrial sectors but accidental release of TCS from manufacturing plants or waste water treatment plants cannot be ruled out and it warrants studies

in non-target organisms like fish which are at the top of aquatic food chains and an important link in the food chain of man. SCHER et al. (2012) that prediction of the toxic effects of a pollutant based on the results of a single model species may not adequately represent the extremely diverse group Pisces. In addition, there is hardly any report wherein the expression of selected heat shock protein genes and cytoskeletal genes have been studied in one model fish. The current study also holds importance because early life stages display a vast array of morphological, cellular and molecular changes on exposure to even minute concentrations of toxic pollutants (Hallare et al., 2005), and are therefore considered to be effective indicators of aquatic pollution (Osman et al., 2007).

Materials and methods

Test chemical

Triclosan (TCS, cat. No. 72779-25G-F and purity ≥97%) was purchased from Sigma Aldrich, and stock solution of TCS (10 mg/ml) was prepared in acetone.

Experimental fish and treatment

Fertilized eggs/embryos of *L. rohita* were obtained from the Government Fish Seed Farm, Rajasansi, Amritsar and maintained in 40 L plastic pools containing dechlorinated tap water at $25 \pm 1.2°C$, under 14:10 h light/dark photoperiod. 3 days post hatching (3 dph), the larvae were exposed for 96 h in 8 L plastic tubs to control, solvent control (acetone), 0.06 (LC_0), 0.067 (LC_{10}) and 0.097 (LC_{30}) mg/L TCS (selected according to Sharma et al., 2021a). Exposures were given in pentuplicate (50 hatchlings/tub, 250/exposure group and total 1250 hatchlings). The test water was replaced daily and the hatchlings were fed with boiled egg yolk, 1 h before changing the water. After 96 h, about half of the hatchlings from each treatment group were pooled separately and used for cell viability assay, micronucleus/aberrant cell frequency assay, expression profiling of heat shock and cytoskeletal genes and FT-IR analysis. The other half was kept for observing the reversal or remedial effect of a recovery period of 10 days on the selected parameters.

Cell viability test

It was performed by the protocol of Sharma et al. (2021b). Cell suspension was prepared by homogenizing 10 hatchlings in 500 µl of phosphate buffer saline (7.4) followed by centrifugation at $500 \times g$ for 10 min. 5 µl cell suspension and 25 µl dye mixture (100 µg/ml acridine orange and ethidium bromide) were taken

on a slide. Three types of cells i.e., green (viable), red (necrotic) and yellow (apoptotic) were scanned under fluorescent microscope (Nikon ECLIPSE E200), photographed with Nikon D5300 camera. 1500 cells/exposure group (500cells/replicate) were recorded for this assay.

Micronucleus/aberrant cell frequency assay

This assay was performed by the method of Sharma et al. (2021b) with slight modifications. Twenty hatchlings were kept in 1 ml of Carnoy's fixative (6: 3: 1: ethanol: chloroform: acetic acid) for 1 h, homogenized, centrifuged and finally cell suspension was prepared in 100 µl of acetic acid. The cells were smeared on a clean glass slide, dried properly at room temperature and stained for 30 min with 5% Giemsa. 1500 cells/ treatment group and 500 cells/replicate were scanned under microscope (Olympus BX43) at 100X magnification for calculating micronucleated (MN) and aberrant cell (AC) frequency.

Gene expression

The primers used in the present study were designed according to NCBI and EMBL database and are listed in Table 1. The protocols used for RNA isolation, DNase treatment, cDNA preparation and thermocycling conditions were same as in our previous study (Sharma et al., 2021a). EF-1α was used as housekeeping gene for normalizing the expression of target genes. Samples were run in triplicate. Expression of the housekeeping gene showed non-significant variation throughout the experiment. The results of gene expression were assessed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Fourier transform infrared spectroscopy

Around 50 larvae from each treatment were freeze dried with Heto Power Dry (Allerod, Denmark) at $-55°C$ and powdered with mortar and pestle. FT-IR spectra were recorded from 1 mg of sample with IR-Tracer-100 spectrometer (Shimadzu, Japan) equipped with diamond attenuated total reflectance (ATR) cell. The samples were scanned in triplicate at room temperature in the spectral range $3300–900\ cm^{-1}$. The variations in band areas were analysed using Origin 8.6 software.

Statistical analysis

One-way ANOVA and student's t-test were used to find out significance level ($p \leq 0.05$ and $p \leq 0.01$) between treatments and

TABLE 1 Primer sequences, accession numbers and optimum annealing temperatures for various genes.

Gene	Forward primer sequence	Reverse primer sequence	Accession no.	Tm (^{0}C)
Myosin light polypeptide 3	5′-TAGGGAGTGGTGAAGAAGGC-3′	5′-TGACTGCATGAGGACGATGT-3′	NM_131619.3	53
Lamin b1	5′-AACGTCTCCATCGATGAGCT-3′	5′-TGCCCAGATCGTCACTTTCT-3′	NM_152972.2	53
Desmin b	5′-AATGTGAAGATGGCCCTGGA-3′	5′-CACCTCTCCATCACGTGTCT-3′	NM_001077452.1	52
Alpha tropomyosin	5′-ACATTGCTGAGGAGGCTGAT-3′	5′-GGCCTCCAGAGACTTCATGT-3′	AF180892.1	51
DHPR	5′-TAACAACCCCAGCAAACACA-3′	5′-GATTGGTGCCAGTGAGGTCT-3′	NM_001002652.1	50
Hsp47	5′-AAGGCTGGAGAAACTGCTCA 3′	5′-CGGAAATGTTGGACAGGTCT-3′	KM091922	54
Hsp60	5′-CACTAACGAGGAGGCTGGAG 3′	5′-TAGCCACCTGAGCAATTTCC-3′	KM091921	54
Hsp70	5′-TGGTGAACCACTTTGTGGAA 3′	5′-AGTCGATGCCCTCGTACAGT-3′	KM369886.1	54
Hsc71	5′-GAGGCCTACCTGGGAAAGAC 3′	5′-TTCCTCTCAGCACCAACCTT-3′	KM091923	55
Hsp90	5′-ACGCCTCTGATGCTCTTGAC 3′	5′-AAGGCCTTTGTCCCAGACTT-3′	KM091924	53
EFL1- α	5′-ATCATTGATGCCCCTGGACA 3′	5′-TGACTCCAACGATCAGCTGT-3′	L23807.1	53

FIGURE 1
Normal larva **(A)** and TCS induced deformities in the hatchlings of *L. rohita*, dt, degeneration of tissue; ddt, degenerated digestive tract; dsb, deflated swim bladder; ssb, small swim bladder; hp, hypopigmentation; dcf, deformed caudal fin; he, hemorrhage; ct, curved trunk; ph, pointed head; lb, lean body **(B−F)**.
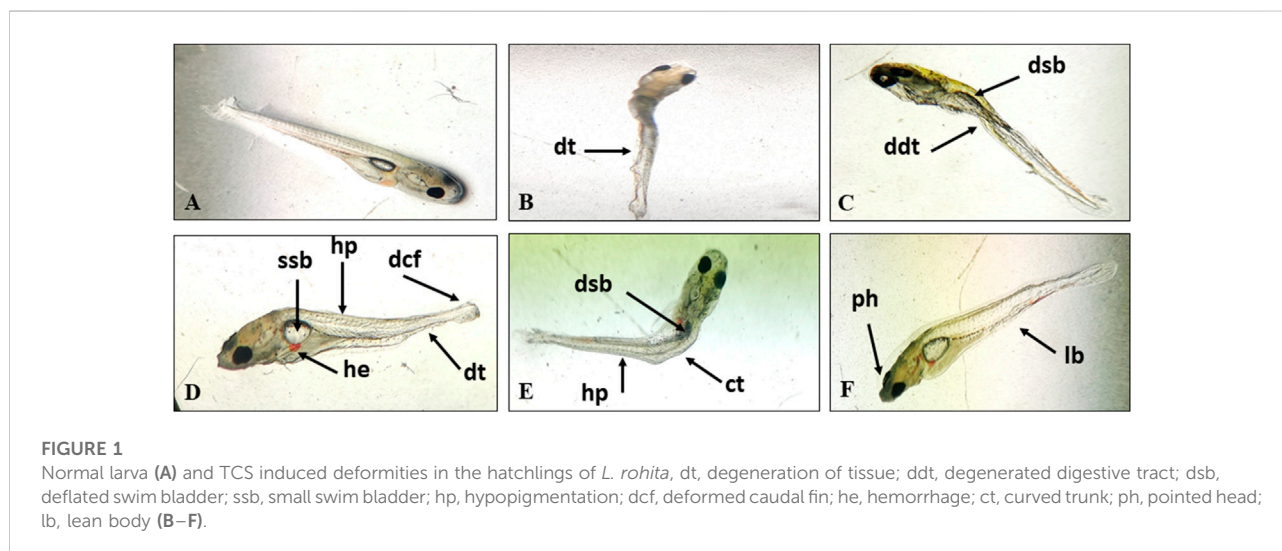
durations, respectively. Tukey's HSD *post hoc* test was performed to obtain differences among the means. The results have been presented as Mean ± SD and all statistical analyses were carried with the help of SPSS 16.0 version.

Results

After 96h, haemorrhage, hypopigmentation, deformed caudal fin, curved trunk, pointed head, lean body, withering of muscles, degeneration of digestive tract, paralysed hatchlings (could not swim or showed erratic swimming) and a small and deflated swim bladder were observed to increase with concentration at 0.067 and 0.097 mg/L (Figure 1). No abnormality was observed in control, solvent control and 0.06 mg/L.

The frequency of viable cells decreased but that of necrotic and apoptotic cells increased significantly ($p \le 0.01$) in a concentration dependent manner after 96 h exposure and 10 days of the recovery period (Figures 2, 3). Comparison of recovery values with the 96 h values showed a significant increase ($p \le 0.05$) in viable cell frequency but a significant decrease ($p \le 0.05$) in necrotic and apoptotic cell frequency at 0.067 and 0.097 mg/L. However, at 0.06 mg/L, the frequency of viable and apoptotic cells increased non-significantly while the frequency of necrotic cells decreased non-significantly in comparison to their respective 96 h values.

Figure 2 shows the micronucleated and aberrant cells. The frequency of binucleated cells was maximum after exposure as well as recovery period. Significant ($p \le 0.05$) concentration dependent increase was observed in the frequency of MN and AC after 96 h exposure and 10 days of the recovery period (Figure 3).
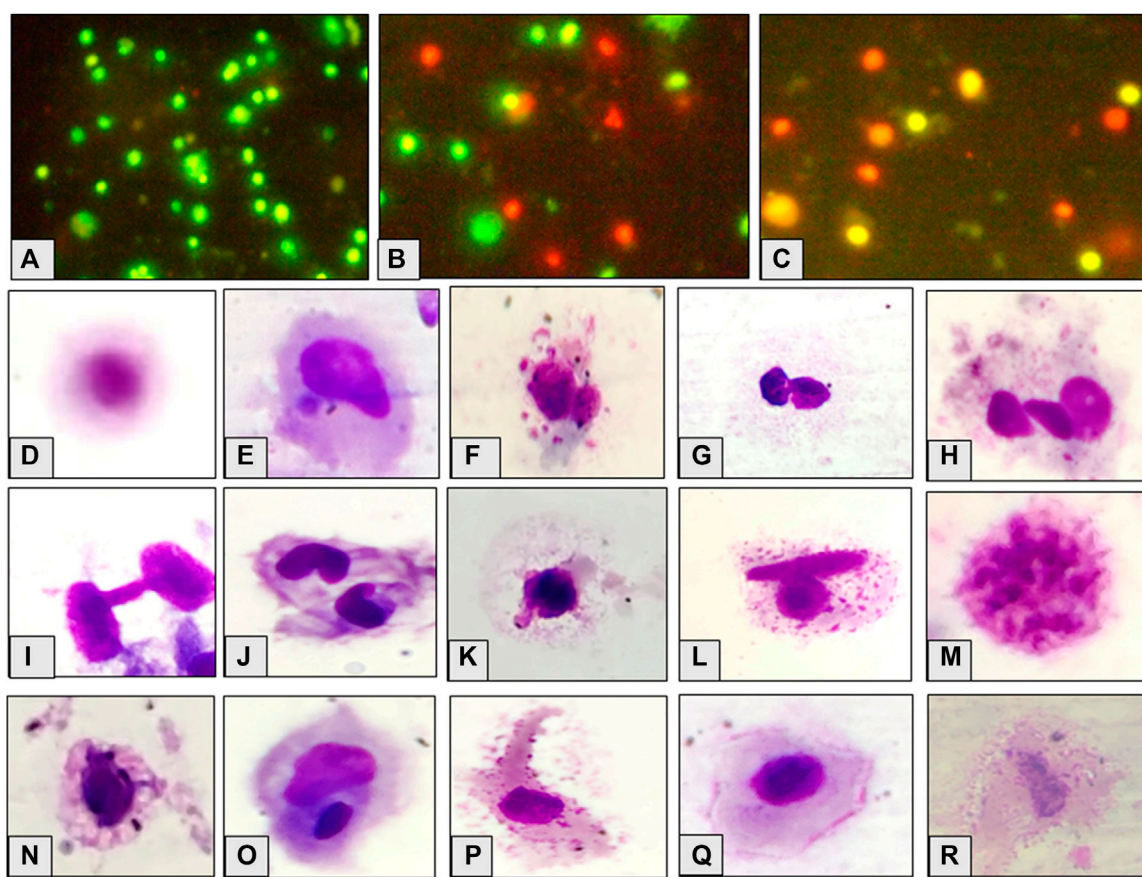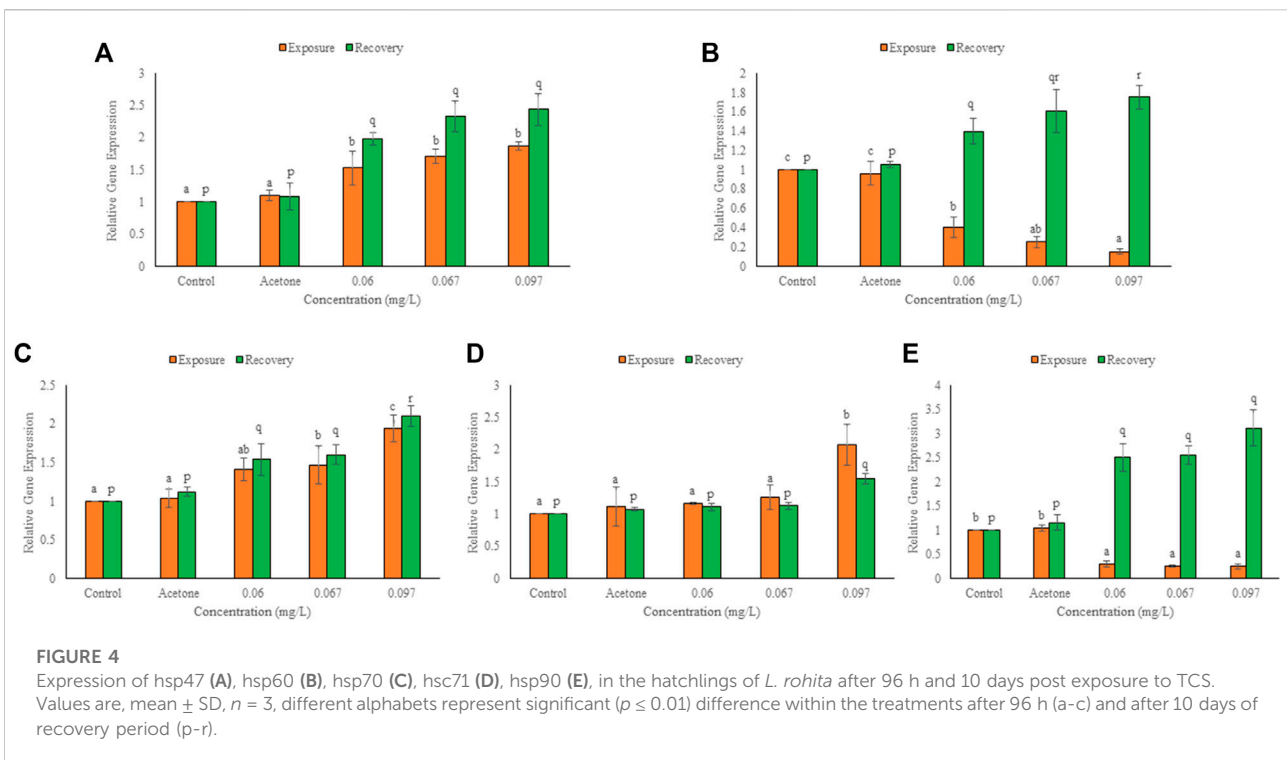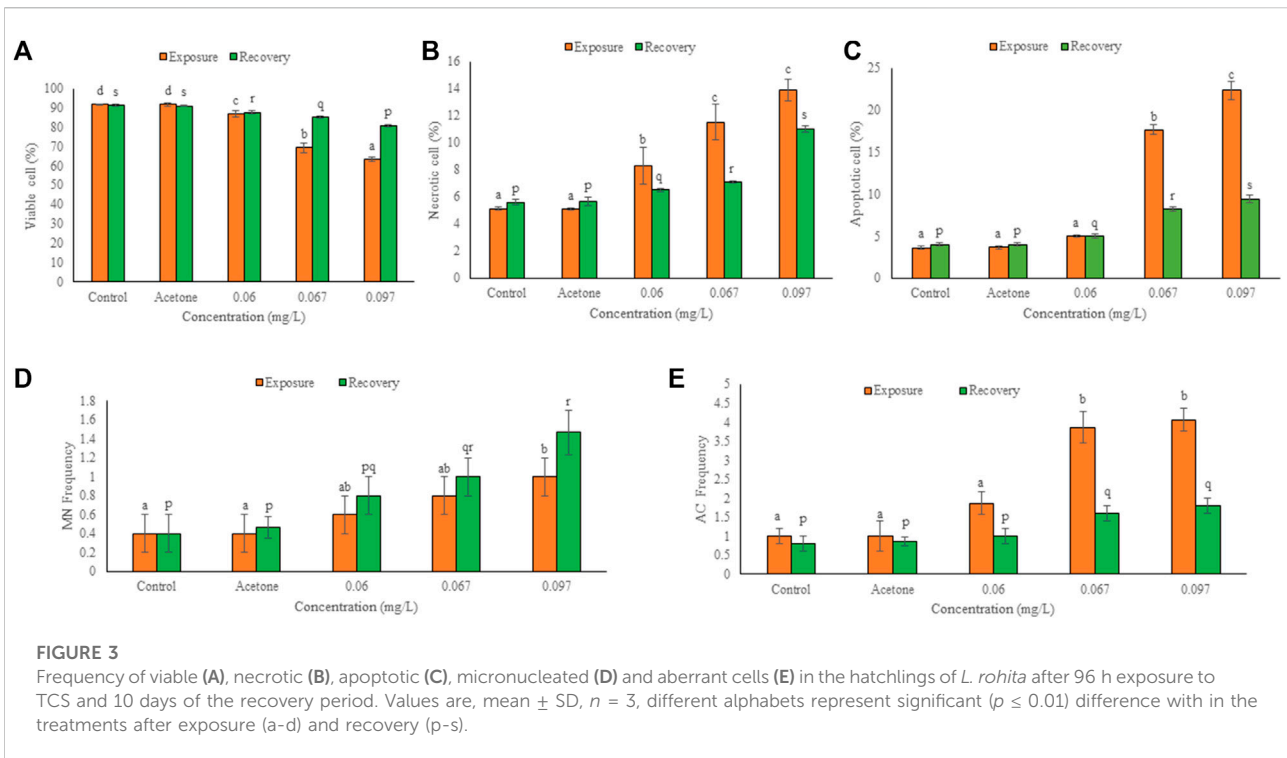
FIGURE 2
Appearance of cells, viable **(A)**, necrotic **(B)** and apoptotic **(C)**; observed cellular abnormalities, normal cell **(D)** micronucleated cell **(E)** multi-micronuclei **(F)**, binucleate **(G)**, trinucleate **(H)**, cytoplasmic bridge **(I)**, notched nuclei **(J)**, nuclear bud **(K)**, binucleate and vesicles **(L)**, nuclear fragmentation **(M)**, vacuolation **(N)**, degmacyte **(O)**, finger like projection **(P)**, membrane folding **(Q)**, necrotic cell **(R)** in the hatchlings of *L. rohita* after 96 h exposure to TCS and 10 days of the recovery period.

Respective increase over control in the frequency of MN and ACs at the highest concentration was 1.50 and 3.07-fold after exposure, but 2.67 and 1.25-fold after recovery, respectively. Compared to 96 h values, MN increased non-significantly while ACs decreased significantly ($p \leq 0.01$) after 10 days of recovery period.

Significant transcriptomic alterations over control were observed in HSPs and cytoskeletal genes after 96 h exposure and 10 days of the recovery period. In solvent control, non-significant change over control was observed in the expression of selected genes after both the durations. There was a concentration dependent significant ($p \leq 0.01$) increase over control in the expression of HSP47, HSP70, and HSc71 but a significant decrease ($p \leq 0.01$) in the expression of HSP60 and HSP90 (Figure 4), after exposure. At 0.06, 0.067 and 0.097 mg/L, the respective elevation in gene expression was 53, 70 and 87% for HSP47, 41, 47 and 94% for HSP70, and 17, 26 and 108% for HSc71. However, the respective decline in gene expression was

60, 70 and 85% for HSP60, and 70, 74 and 75% for HSP90. After exposure, minimum and maximum decline in expression was observed for HSP60 at 0.06 and 0.097 mg/L, respectively whereas HSc71 showed minimum and maximum elevation at 0.06 and 0.097 mg/L TCS, respectively. During the recovery period, mRNA copy number of all the heat shock proteins increased significantly ($p \leq 0.01$) in a concentration dependent manner. The elevation in expression at 0.06, 0.067 and 0.097 mg/L TCS was 98, 133 and 144% for HSP47, 54, 60 and 110% for HSP70, 11, 13 and 56% for HSc71, 43, 61 and 75% for HSP60, and 151, 125 and 211% for HSP90, respectively. HSc71 (0.06 mg/L) and HSP90 (0.097 mg/L) showed minimum and maximum induction, respectively. In comparison to 96h, HSP60 and HSP90 increased significantly ($p \leq 0.05$), HSP47 and HSP70 increased non-significantly, while HSc71 showed non-significant decline after recovery.

After 96 h exposure, mRNA copy number increased ($p \leq 0.01$) over control for α-tropomyosin but declined ($p \leq 0.01$) over

FIGURE 3
Frequency of viable **(A)**, necrotic **(B)**, apoptotic **(C)**, micronucleated **(D)** and aberrant cells **(E)** in the hatchlings of *L. rohita* after 96 h exposure to TCS and 10 days of the recovery period. Values are, mean ± SD, *n* = 3, different alphabets represent significant (*p* ≤ 0.01) difference with in the treatments after exposure (a-d) and recovery (p-s).

FIGURE 4
Expression of hsp47 **(A)**, hsp60 **(B)**, hsp70 **(C)**, hsc71 **(D)**, hsp90 **(E)**, in the hatchlings of *L. rohita* after 96 h and 10 days post exposure to TCS. Values are, mean ± SD, *n* = 3, different alphabets represent significant (*p* ≤ 0.01) difference within the treatments after 96 h (a-c) and after 10 days of recovery period (p-r).

control for the other cytoskeletal genes in a concentration dependent manner (Figure 5). At 0.06, 0.067 and 0.097 mg/L TCS, percent change over control was 40, 66 and 73 for DHPR, 26, 31 and 58 for myosin light peptide, 38, 42 and 44 for desmin b, 46, 50 and 66 for laminb1 and 44, 99 and 128 for α-tropomyosin, respectively. Reduction was minimum for

FIGURE 5
Expression of DHPR **(A)**, myosin light polypeptide 3 **(B)**, α-tropomyosin **(C)**, desmin b **(D)**, lamin b1 **(E)** in the hatchlings of *L. rohita* after 96 h and 10 days post exposure to TCS. Values are, mean ± SD, $n = 3$, different alphabets represent significant ($p \leq 0.01$) difference within the treatments after 96 h (a-c) and after 10 days of recovery period (p-r).
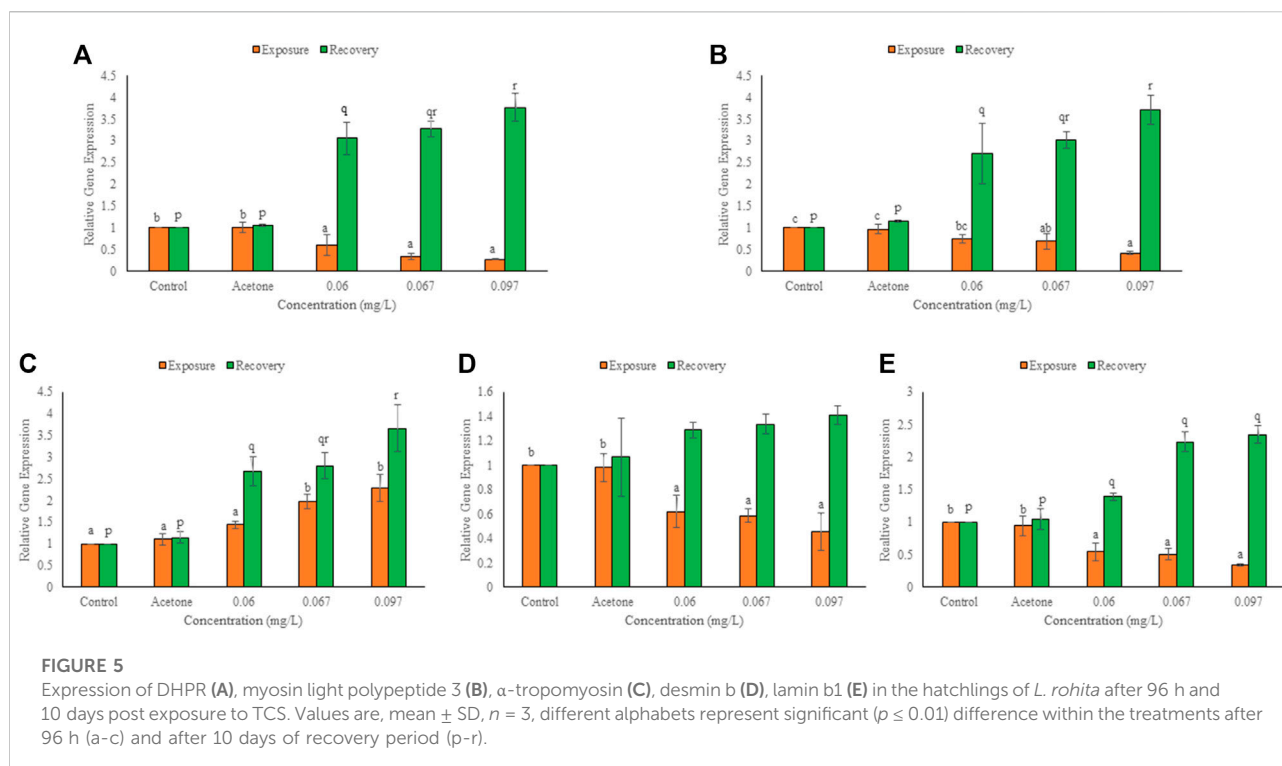
myosin light polypeptide (0.06 mg/L) but maximum for DHPR (0.097 mg/L). After 10 days of the recovery period, expression of α-tropomyosin increased ($p \leq 0.01$) further, and the expression of other cytoskeletal genes also increased significantly ($p \leq 0.01$) in a concentration dependent manner (except for a non-significant increase in desmin b). The induction after recovery at 0.06, 0.067 and 0.097 mg/L TCS, was 206, 228 and 277% for DHPR, 171, 202 and 272% for myosin light peptide, 167, 180 and 266% for α-tropomyosin, 29, 34 and 41% for desmin b and 39, 123 and 134% for lamin b1, respectively. Desmin b showed minimum (0.06 mg/L) while DHPR (0.097 mg/L) showed maximum increase in expression after recovery. Expression of all cytoskeletal genes during recovery was significantly ($p \leq 0.01$) higher than the respective 96 h values.

FT-IR spectra of secondary structure of the biomolecules of hatchlings after 96 h exposure and 10 days of the recovery period have been presented in Figure 6 and Table 2 and the tentative assignment of different peaks is given in Table 3. After 96 h exposure, there was a significant ($p \leq 0.01$) concentration dependent decrease in band area (%) at wave no. 1082–1083 and 1398–1401 cm^{-1} while a significant increase ($p \leq 0.01$) was observed at wave no. 1235–1237, 1543–1546, 1651–1653 and 3290–3296 cm^{-1}. Peak no. 1163–1165, 1455–1457, 2852–2855 and 2924–2925 cm^{-1}, on the other hand, showed a non-significant decline in percent band area after exposure. Significant concentration dependent decrease in percent band area ($p \leq 0.01$) was observed for all the peaks

(except at peak 1163–1165, 1455–1457 and 2924–2925 cm^{-1}), after 10 days of recovery period. At 0.097 mg/L, decline in the band area of wave no. 1082–1083 cm^{-1} was 66.28% after exposure and 75.67% after recovery. However, maximum increase in band area, after exposure and recovery period was in wave no. 1543–1546 cm^{-1} (33.45%) and 1455–1457 cm^{-1} (100.75%), respectively. After recovery, a significant ($p \leq 0.05$) difference was observed in the percent band area of all the peaks compared to the respective 96 h values. Area of all the bands (except for a significant ($p \leq 0.05$) increase in peaks 1163–1165, 1455–1457 and 2924–2925 cm^{-1}) decreased significantly ($p \leq 0.05$) over 96 h values, after recovery.

Discussion

Probable toxicity of a particular pesticide can be easily evaluated through observational studies related to morphological and/or biochemical alterations in fish. Embryos and larvae/early developmental stages of fish are highly sensitive to the effects of even small quantities of xenobiotics and the changes observed in them can be used as important toxicity biomarkers (Dar et al., 2019).

The concentration dependent increase in larval abnormalities in the present study seems mainly due to the high octanol water coefficient (4.8) and organic carbon water partition coefficient (3.8–4.0) of TCS that lead to its
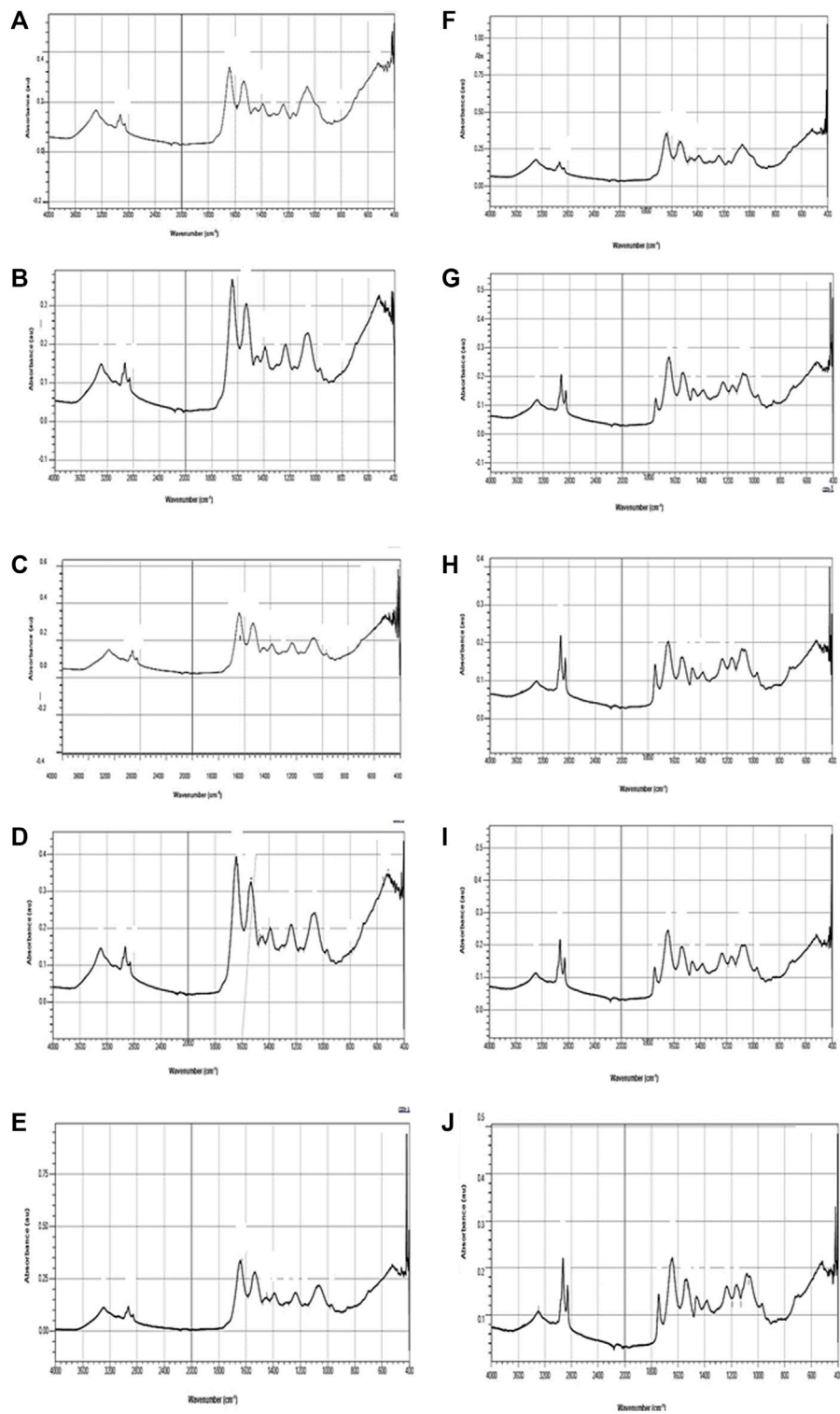
FIGURE 6

FT–IR spectra of *L. rohita* hatchlings after 96 h exposure **(A–E)** to TCS and 10 days of the recovery period **(F–J)**. Control **(A,F)**, acetone **(B,G)**, 0.06 mg/L **(C,H)**, 0.067 mg/L **(D,I)**, 0.097 mg/L **(E,J)**.

TABLE 2 Variations in the percent band area of *L. rohita* hatchlings after 96 h (E) and 10 days post exposure to TCS (R).

Concentration (mg/L)		3290–3296	2924–2925	2852–2855	1651–1653	1543–1546	1455–1457	1398–1401	1235–1237	1163–1165	1082–1083
Control	E	9.33 ± 0.25[a]	5.95 ± 1.03	9.67 ± 1.03	13.20 ± 0.18[b]	5.86 ± 0.30[a]	1.46 ± 0.02	8.71 ± 0.02[b]	11.19 ± 0.16[a]	4.26 ± 0.06	30.79 ± 0.56[b]
	R	13.95 ± 0.68[q]	5.53 ± 0.06[p]	11.05 ± 0.97[r]	12.30 ± 0.19[r]	5.22 ± 0.22[r]	1.33 ± 0.02[p]	8.19 ± 0.16[q]	10.23 ± 0.05[q]	4.03 ± 0.10[p]	28.20 ± 0.06[r]
Acetone	E	9.15 ± 0.36[a]	5.77 ± 0.08	9.46 ± 1.11	13.21 ± 0.57[b]	5.53 ± 0.35[a]	1.42 ± 0.03	8.74 ± 0.21[b]	11.18 ± 0.53[a]	4.24 ± 0.60	30.31 ± 1.03[b]
	R	13.83 ± 0.43[q]	5.49 ± 0.10[p]	10.71 ± 0.11[r]	12.18 ± 0.21[r]	5.31 ± 0.18[r]	1.31 ± 0.04[p]	8.29 ± 0.55[q]	10.36 ± 0.21[q]	4.30 ± 0.28[p]	28.55 ± 0.37[r]
0.06	E	10.59 ± 0.34[b]	5.72 ± 0.23	9.62 ± 1.03	11.82 ± 0.46[a]	6.15 ± 0.50[a]	1.40 ± 0.07	4.73 ± 0.26[a]	10.97 ± 0.55[a]	4.15 ± 0.10	11.42 ± 0.31[a]
	R	6.53 ± 0.44[p]	9.17 ± 0.97[q]	8.56 ± 0.38[q]	10.09 ± 0.59[q]	3.79 ± 0.19[q]	2.50 ± 0.34[q]	4.43 ± 0.36[p]	7.96 ± 0.21[p]	5.71 ± 0.10[p]	7.76 ± 0.19[q]
0.067	E	10.68 ± 0.22[b]	5.63 ± 0.11	9.51 ± 0.84	14.20 ± 0.31[bc]	7.54 ± 0.13[b]	1.49 ± 0.02	4.90 ± 0.08[a]	12.21 ± 0.12[b]	3.82 ± 0.10	11.28 ± 0.66[a]
	R	6.62 ± 0.12[p]	9.45 ± 0.20[qr]	8.55 ± 0.38[q]	9.41 ± 0.43[q]	3.37 ± 0.24[pq]	2.57 ± 0.12[q]	4.37 ± 0.09[p]	7.77 ± 0.42[p]	5.80 ± 0.39[q]	7.67 ± 0.30[pq]
0.097	E	11.02 ± 0.70[b]	4.83 ± 0.61	8.07 ± 0.84	14.65 ± 0.24[c]	7.82 ± 0.39[b]	1.40 ± 0.09	4.70 ± 0.29[a]	12.47 ± 0.31[b]	3.48 ± 0.24	10.38 ± 0.26[a]
	R	5.65 ± 0.07[p]	9.68 ± 0.21[r]	6.39 ± 0.54[p]	7.24 ± 0.15[p]	3.20 ± 0.07[p]	2.67 ± 0.18[q]	4.05 ± 0.07[p]	7.49 ± 0.33[p]	5.89 ± 0.05[q]	6.86 ± 0.27[p]
F- value	E	18.90**	ns	ns	24.95**	21.11**	ns	290.93**	20.26**	ns	756.53**
	R	256.71**	658.09**	25.70**	77.55**	91.04**	43.18**	125.96**	66.40**	50.07**	502.23**

Values are, mean ± SD, $n = 3$, different alphabets represent significant ($p \leq 0.01$) difference within the treatments after 96 h (a-c) and after 10 days of recovery period (p-r).

bioaccumulation in organisms. This also indicates its potential for biomagnification in the food chain (Zhou et al., 2017). The abnormal development of caudal fin, trunk, head, digestive tract and swim bladder in the larvae of *L. rohita* can be linked with the observed alterations in mRNA and/or protein expression of cytoskeletal genes under the stress of TCS and gets support from the studies of Fritsch et al. (2013), Falisse et al. (2018) and Ma et al. (2019). Teratogenic effects of TCS have also been reported in zebra fish (Oliveira et al., 2009), Japanese medaka (Nassef et al., 2010) and four carps (Dar et al., 2019).

The concentration dependent decrease in cell viability while increase in the percentage of necrotic and apoptotic cells confirm the cytotoxic potential of TCS and the observation is directly supported by Binelli et al. (2009a, 2009b), Parenti et al. (2019) and Guidony et al. (2020) who observed similar effects of TCS in zebra mussel, zebrafish embryos and zebrafish hepatocytes, respectively. Apoptosis or necrosis is induced by TCS either due to its action as an intercalating agent (through dioxins, the degraded product of TCS) directly or through induction of oxidative stress indirectly (Aranami and Readman, 2007). Schweizer (2001), suggested that TCS inhibits synthesis of biomolecules and activity of membrane bound ATPase, which promote cell lysis and membrane perturbations. TCS has also been reported to induce apoptosis by the activation of caspase 3, fragmentation of DNA and formation of apoptotic bodies (Szychowski et al., 2015). Oxidative stress may also damage the biomolecules and organelles that in turn also activate apoptotic machinery (Redza-Dutordoir and Averill-Bates, 2016). 10–100 µM TCS for 24 h is reported to cause dose dependent cell death in B cell acute lymphoblastic leukemia cell lines by Alfhili et al. (2021). The authors associated the cell death with impaired membrane permeability, cell shrinkage, increased concentration of intracellular Ca^{2+}, redox imbalance, chromatin condensation and fragmentation of DNA. Canesi et al. (2007) observed that TCS at low µM (1–100) range destabilized the lysosomal membrane of *Mytilus galloprovincialis* hemocytes. Gaume et al. (2012) observed that 2–10 µm TCS induced dose dependent cytotoxicity in marine gastropod *Haliotis tuberculate* and Ciniglia et al. (2005) reported that TCS decreased cell viability up to 25 and 40% in *Closterium ehrenbergii* at 0.187 and 0.25 mg/L, respectively. TCS induced necrosis has also been reported in the gill lamellae of *L. rohita* (Hemalatha et al., 2019) and muscle tissue of *Oreochromis mossambicus* (Deepika et al., 2021).

The micronucleus assay is the microscopic assessment of genotoxic potential of a xenobiotic in the exposed organisms. Micronucleus is a laggard chromosome or chromosomal fragment formed due to aneugenesis or clastogenesis (Emery, 2012), while binucleated cells are formed due to abnormal cell division or blocked cytokinesis (Kaur and Kaur, 2015; Kaur and Dua, 2016). Clastogenic pollutants like TCS impair DNA replication and normal cell division that may lead to formation of aberrant cells (Sharma and Chadha, 2020).

TABLE 3 Tentative Assignment of the observed peaks.

Wavenumber (cm^{-1})	Assignments	Source
3290–3296	N-H stretching of proteins (Amide A)	Palaniappan and Vijayasundaram (2009a)
2924–2925	CH$_2$ asymmetric stretching (lipids)	Velmurugan et al. (2018)
2852–2855	CH$_2$ symmetric stretching (mainly lipids)	
1651–1653	C=O stretching of proteins (Amide I)	
1543–1546	N-H bend, C-N stretching of proteins (Amide II)	
1455–1457	CH$_2$ bending (mainly lipids)	
1398–1401	COO – symmetric stretching (fatty acids and amino acids)	
1235–1237	PO^{2-} asymmetric stretching (phospholipids)	
1163–1165	CO–O–C symmetric stretching (glycogen)	
1082–1083	PO^{2-} asymmetric stretching (nucleic acids)	

Significant concentration dependent rise in the frequency of MN and ACs in the present study is supported by the findings of Binelli et al. (2009a), Emery (2012), Vijitha et al. (2017), Wang et al. (2018) and Jimoh and Sogbanmu (2021). These workers gave exposure of TCS to *Dreissena polymorpha* (1, 2 and 3 nM TCS for 96 h), *Lithobates catesbeianus* (0.0023, 0.023, 0.23 mg/L TCS for 1, 8 and 15 days), *Oreochromis niloticus* (0.28 and 0.56 mg/L TCS for 96 h), *Carassius auratus* (0.14, 0.28 and 0.56 mg/L TCS for 14 days) *and* Clarias gariepinus *(0.027 mg/L TCS for 28 days), respectively.*

HSPs are intracellular chaperones that remain conserved from bacteria to humans (Lindquist et al., 1988) and are involved in cellular defense during stressful conditions (Sanders, 1993). Due to their role in stability, translocation and folding of proteins (Xing et al., 2015), HSPs can be used as an important biomarker to monitor the effect of environmental stressors on animals including fish (Lewis et al., 1999). These proteins, important for immune function are generally altered during stress (Xing et al., 2013). Results of the present study show that expression of HSP47, HSP70 and HSc71 increased after exposure and recovery period, while the expression of HSP60 and HSP90 declined after exposure but showed reverse trend after recovery. Change in the expression of HSPs is an indicator of TCS induced oxidative stress in the hatchlings of *L. rohita*. Transcription of HSPs in eukaryotes is regulated by heat shock transcription factor (HSF) and the oxidative stress generated by the pesticides has been reported to directly activate HSF and induce the expression of HSPs (Lee and Corry, 1998). HSP47 is a resident of endoplasmic reticulum and generally acts as a molecular chaperone for folding of collagen fibrils (Pearson, 1996). The elevated expression of HSP47 was consequent with the TCS induced deformities in the head, trunk, fins and gut of the hatchlings in the present study. Elevated expression of HSP47 has been reported in gill and liver of *Epinephelus guaza* after exposure of polycyclic aromatic hydrocarbons by Abdel-Gawad and Khalil (2013). Heavy metals and hydro soluble organic pollutants have also been observed to

elevate the expression of HSP47 in Chinese hamster (Guizani et al., 2012). HSP70 plays multiple roles under stress conditions, such as avoidance of protein aggregation, uncoupling of protein aggregates and suppression of apoptosis (Wiech et al., 2012). So, the increased frequency of necrotic and apoptotic cells in the present study can be related with altered expression of HSP70. This family comprises of both stress inducible (HSP70) and constitutively expressed (HSc70) genes. Xing et al. (2013) observed that HSP70 levels were strongly induced while HSc70 was slightly induced in liver, brain, kidney and gill of *Cyprinus carpio* under the stress of atrazine and chlorpyrifos individually and their mixture. TCS upregulated the expression of HSP70 in *Eisenia fetida* (Lin et al., 2014) and *Caenorhabditis elegans* (García-Espiñeira et al., 2018) also. HSP60 is an important chaperone involved in assembly, stability and folding of proteins and their import into endoplasmic reticulum while HSP90 is necessary for assembly of oligomeric proteins, folding of monomeric proteins and formation of steroid receptor (Cechetto et al., 2000; Roberts et al., 2010; Liu et al., 2013). Decline in HSP60 and HSP90 in our study is corroborated by Eder et al. (2009), who reported that 96 h exposure of 1.2 and 7.3 μg/L chlorpyrifos declined the proteomic expression of both these genes in *Oncorhynchus tshawytscha*.

Cytoskeletal proteins are involved in various cellular functions such as maintenance of cell shape, transport, locomotion, intracellular organization etc. (Nawaz et al., 2005). These proteins are generally differentially expressed after exposure of xenobiotics and reflect general stress response of organisms (Groh and Suter, 2015). Expression of these genes was observed to be highly dynamic under the stress of TCS by Loh et al. (2000) and Costa et al. (2002), they suggested that TCS disrupted the organization of cytoskeletal proteins. No swimming or erratic swimming of TCS exposed hatchlings in the present study may have been due to the alterations in the expression of cytoskeletal genes that gradually caused paralysis. Fritsch et al. (2013) also observed same behaviour in

TCS exposed fathead minnow. We observed that after exposure, mRNA levels of all cytoskeletal genes except for α-tropomyosin declined but a reverse trend was observed after recovery. An increase in the expression of α-tropomyosin has also been reported by Lebreton et al. (2021) in oxazepam exposed gastropod *Radix balthica.* DHPR plays a key role in the development of trunk musculature (Ma et al., 2019), therefore the observed decline can be linked with the concentration dependent increase in curved trunk, lean body, pointed head and abnormal swimming in the exposed hatchlings in the present study. DHPR is a voltage gated Ca^{2+} channel and is responsible for intracellular Ca^{2+} release, its lower expression could have reduced the level of Ca^{2+} in the cells (Ma et al., 2019). In the absence of Ca^{2+}, tropomyosin blocks interaction of actin with myosin that could have resulted in erratic swimming under the stress of TCS. MLP-3 was observed to be the second most sensitive gene, this is the master regulator of muscle function. Reduced mRNA levels of myosin regulatory light chain A have been reported by Lebreton et al. (2021) in fresh water gastropod, *Radix balthica* after exposure of 0.8 and 10 μg/L oxazepam for 28 days. Desmin and lamin, the two important cytoskeletal proteins are susceptible to the stress of a variety of xenobiotics (Groh and Suter, 2015). Decline in desmin expression in the hatchlings of the present study may also be related to muscular degeneration and deformed caudal fin as desmin is a component of intermediate filaments in muscles (skeletal, cardiac and smooth) and its expression is correlated with the development of fin muscles during embryogenesis (Loh et al., 2000). Lamin is an important constituent of nuclear lamina which supports nuclear envelope (Peter and Stick, 2012) while lamin b1 maintains the structure and stability of chromosomal territories (Camps et al., 2015). Decline in the expression of lamin b1 seems to be the probable cause of observed concentration dependent nuclear abnormalities in the present study. Along with it, lamin b1 also plays a key role in the development of neurons and decrease in its expression has been linked with impaired neuronal development (Razafsky et al., 2016). Our results are consistent with the study of Falisse et al. (2017), who also observed elevated proteomic expression of α-tropomyosin and decline in myosin light polypeptide 3 and lamin D1 in the larvae of zebrafish after exposure of 50 and 100 μg/L of TCS for 7 days.

Fourier transform infrared spectroscopy is a physical method used to study alterations in functional groups and biomolecules (Cakmak et al., 2006). The non-significant decline in stretching (symmetric and asymmetric) and bending of CH_2 after exposure while significant elevation in lipid related peaks (except 2852–2855 cm^{-1}) after recovery indicate impaired lipid metabolism. Significant induction in the band area of amide A, amide I and amide II in TCS exposed larvae suggests that the increased concentration of protein during stress was a general adaptive response for synthesizing important enzymes for detoxification of the

xenobiotic and gets support from Bharti and Rasool (2021) who observed a significant decline in protein content of liver and kidney tissue of *C. punctatus* after exposure to 0.4 mg/L malathion for 4, 8 and 12 days. Significant decrease in the intensity of these peaks after recovery may be linked with either oxidation of proteins under the influence of accumulated ROS or their metabolism to fulfil higher energy demand in the stressed hatchlings. In our previous studies (Sharma et al., 2021a; 2021b), we observed a significant decline in protein content in the TCS exposed hatchlings and this directly supports the present observations. Similarly, altered intensity of amide A, amide I and amide II has also been reported in the TCS exposed embryos/larvae of four food fishes (Dar et al., 2022) and in liver, muscle, brain and kidney of arsenic exposed fingerlings of *L. rohita* (Palaniappan and Vijayasundaram, 2008a; Palaniappan and Vijayasundaram, 2008b; Palaniappan and Vijayasundaram, 2009a; Palaniappan and Vijayasundaram, 2009b). Increased area of the band at 1235–1237 cm^{-1} (phospholipids and amino acids) after exposure and its decline after recovery while decline in the band area at 1398–1401 cm^{-1} till the end of recovery clearly suggests that the fluctuations in the contents of fatty acids and amino acids in the hatchlings could have been for maintaining homeostasis or repair of wear and tear during stress. These results are supported by the TCS induced lipid peroxidation and proteolysis in carps by Dar et al. (2020a); Dar et al. (2020b). Non-significant decline in the intensity of CO-O-C symmetric stretching (1163–1165 cm^{-1}) after exposure and significant induction after recovery suggests that TCS affected metabolic performance and energy levels in the larvae by altering the level of glycogen and disruption of carbohydrate metabolism and is corroborated by Velmurugan et al. (2018). The band at 1082–1083 cm^{-1} specifies asymmetric stretching of phosphodiester group of nucleic acids. Significant decline in the area of this band indicates low contents of nucleic acids (DNA and RNA) in the hatchlings and is also indicative of the genotoxic potential of TCS (Hemalatha et al., 2019; Gyimah et al., 2020; Sharma et al., 2021a).

Conclusion

The increasing global use of insecticides and pesticides raises concern due to their persistence in the environment and several negative impacts on humans and other non-target terrestrial and aquatic organisms. Significant alterations in the secondary structure of biomolecules and the expression of heat shock and cytoskeletal genes seem to be the underlying cause for an increase in morphological abnormalities along with a significant decline in the viable cell frequency but an increase in the frequencies of necrotic, apoptotic, micronucleated and aberrant cells in the TCS exposed hatchlings. The results highlight that out of the selected genes, DHPR (cytoskeletal

genes) and HSP90 (heat shock genes) may be used as the highly sensitive markers for the acute stress of TCS.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The above statement is replaced with All data generated during this study are included in the manuscript.

Ethics statement

Ethical review and approval was not required for the animal study because embryos of food fishes do not come under the purview of animal ethics in India.

Author contributions

SS: Conceptualization, experimentation and writing the original draft. OD, MA, SS, and AB: Experimentation and writing. ST and AK: RT-PCR study. AK: conceptualization, supervision, writing, review and editing.

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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.

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