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Excess DHA Induces Cell Cycle Arrest by Activating the P53/Cycling Pathway in Blunt Snout Bream (*Megalobrama amblycephala*)

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A previous study showed that diets with high levels of docosahexaenoic acid (DHA) cause growth retardation in *Megalobrama amblycephala*. In order to explore the mechanisms involved, a feeding trial and a primary hepatocyte culture experiment were designed. *In vivo*, fish (average weight 26.40 ± 0.11 g) were randomly divided into groups and were fed three levels of DHA (0, 0.2, and 1.6% diets, respectively.) for 8 weeks. The results showed that the final body weight and weight gain of fish fed 1.6% DHA were significantly lower than those of fish fed 0.2% DHA ($P < 0.05$). P53 gene and protein expression levels were significantly increased ($P < 0.05$), while expression of downstream Cyclin D1 and Cyclin E1 was significantly inhibited in the 1.6% DHA group when compared with the 0 and 0.2% DHA groups ($P < 0.05$). *In vitro*, primary hepatocytes isolated from *Megalobrama amblycephala* incubated with 500 μM DHA showed significantly increased cell cycle arrest and apoptosis ($P < 0.05$), significantly increased P53 gene and protein levels ($P < 0.05$), and significantly decreased Cyclin D1 and Cyclin E1 levels ($P < 0.05$) when compared with other groups. When primary hepatocytes were incubated with DHA and a P53 inhibitor (pifithrin-α), P53 expression and P53-mediated signaling were inhibited, cell cycle progression recovered, and apoptosis was reduced. In summary, high levels of DHA activated the P53/Cyclin pathway to induce cell cycle arrest. Inhibition of P53 activity may be a potential way of reducing the side effects of DHA on the growth of *Megalobrama amblycephala*.

Keywords: docosahexaenoic acid, cell cycle arrest, apoptosis, P53, cycling family, *Megalobrama amblycephala*

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INTRODUCTION

Docosahexaenoic acid (C22:6n-3; DHA) is an essential n-3 polyunsaturated fatty acid (n-3 PUFA) that is commonly used as a feeding supplement for aquatic animals (Hossain et al., 2012; Hong et al., 2017; Wang et al., 2019), pigs (Gabler et al., 2007; Li et al., 2009), chickens (Allen and Danforth, 1998; Howe et al., 2002), and other species. DHA is also an important health product for human use (Holub, 2001). Conversion of α-linolenic acid (C18:3n-3, ALA) into DHA is almost negligible in the human body, so DHA must be obtained directly from foods (Plourde and Cunnane, 2007).

DHA supplementation at appropriate levels is beneficial to animals and humans, but an excess may produce adverse effects. DHA, a highly unsaturated fatty acid, is susceptible to attack by active free radicals, resulting in lipid peroxidation, cell membrane damage, and various physiological abnormalities and disease (Tappel, 1973). Some studies have focused on the damage caused by the peroxidation of DHA. A previous study reported that high DHA levels led to increased plasma urea concentration and fish redness (Glencross and Rutherford, 2011). MedihaYildirim-Aksoy et al. (2007) showed that fish fed excessive amounts of fish oil had abnormally high red and white blood cell counts and suffered from infection or disease. However, there have been very few studies focusing on the damage caused by unoxidized DHA. Our previous study found that a high level of DHA reduced growth performance, indicating that excessive DHA, not oxidized, may be harmful to fish growth (Wang et al., 2019). Investigating whether there is possible damage from high levels of unoxidized DHA is important for human and animal nutrition.

P53, a tumor suppressor, is a key transcriptional regulator that affects various physiological functions, such as apoptosis (Yonish-Rouach et al., 1991; Chipuk and Green, 2004), cell cycle progression (Livingstone et al., 1992; Agarwal et al., 1995) and antiviral immunity (Takaoka et al., 2003; Munoz-Fontela et al., 2008), by regulating relevant genes (Lane, 1992; Francis et al., 2007). When cells are stimulated by the external environment, P53 can become activated to maintain a stable internal environment by regulating a series of related genes (Lane, 1992). P53 has been widely studied in mammals, where it plays an indispensable role (Levine, 1997; El-Deiry, 1998), but there have been few studies on the physiological functions of P53 in lower vertebrates (such as fish). Mai et al. (2012) reported that P53 is cooperatively activated via the ataxia telangiectasia mutated (ATM)-P53 pathway in response to stress and DNA damage signals in Tilapia. On the other hand, Guo et al. (2016) showed that P53 plays a critical role in the immune and antiviral responses of mandarin fish (*Siniperca chuatsi*).

The blunt snout bream (*Megalobrama amblycephala*) is an herbivorous freshwater fish and teleost species (Wang et al., 2019) that is vulnerable to oxidative stress and hepatocyte apoptosis when it is fed high-fat diets (Lu et al., 2017). This is because it has an herbivorous instinct and a relatively small hepatosomatic index (HSI) (Zhang et al., 2014; Cao et al., 2019a). Hence, this species is a suitable model to study fatty acid toxicity. To investigate why high levels of DHA reduced the growth of *M. amblycephala* (Wang et al., 2019) and to explore the causes of problems caused by excessive fish oil in fish farming, in this study we exposed fish and hepatocytes to high levels of DHA and investigated its effects *in vivo* and *in vitro*, which will be beneficial for elucidating the DHA nutrition of other animals and humans.

MATERIALS AND METHODS

All experimental procedures involving animals were conducted following the Guidance for the Care and Use of Laboratory Animals in China. This study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) [Permit number: SYXK (Su) 2011–0036].

Diets, Feeding Trial, and Sample Collection

Three diets containing different DHA levels were prepared. The dietary DHA levels were 0, 0.2, and 1.6%, respectively. The 0% DHA diet was given to the control group. The three diets contained 31% crude protein and 6% crude lipids. Proteins were supplied by defatted fishmeal (3.5% lipid), soybean meal, rapeseed meal, and cottonseed meal. Lipids were supplied by soybean and DHA oils (DHA content, 50% of oil). Ethoxyquin was used as an antioxidant. Freshly prepared diets were kept out of direct sunlight, dried by ventilation, and stored at –20°C. The fatty acid composition of soybean oil and DHA oil and the formulation of six diets is referenced in our previous study (**Supplementary Table S1, S2**; Wang et al., 2019).

M. amblycephala was obtained from the Fish Hatchery of Yangzhou (Jiangsu, China). The fish were fed commercial diets for 2 weeks to allow them to adapt to the environment. A total of 240 fish (average initial body weight 26.40 ± 0.11 g) were randomly distributed into 12 floating net cages (2 × 1 × 1 m, L: W: H), with four cages per group and with 20 fish in each cage. All fish were hand-fed to satiation thrice a day (8:00, 12:00, and 16:00) for 8 weeks. The presence of dead fish, water temperature (28–34°C), dissolved oxygen (5.0 mg L^{-1}), and pH (7.4–7.6) were recorded daily.

After 8 weeks, fish were anesthetized with MS-222 (100 mg L^{-1}, tricaine methanesulfonate, Sigma, United States). The quantity and weight of the fish in each cage were recorded. Three fish were randomly sampled per cage (total per group, 12 fish). Livers were collected, frozen in liquid nitrogen, and stored at –80°C for molecular analyses.

Primary Hepatocyte Culture

The primary hepatocyte culture was performed according to Cao et al. (2019a). Briefly, the liver was carefully removed under sterile conditions and rinsed with sterile phosphate buffer saline (PBS) supplemented with streptomycin (100 μg mL^{-1}) and penicillin (100 IU mL^{-1}). The liver was cut into pieces (1 mM^3) with sterilized tweezers and scissors, and the liver pieces were digested with 0.25% trypsin in a thermostatic water bath at 28°C for 40 min; the liquid was shaken every 5 min. The digested liver solution was filtered with a nylon cell strainer (200 mesh) and collected into a sterilized 50-mL centrifugal tube. Next, the filtrate was centrifuged (300 × g, 10 min) to obtain the cell pellet. The pelleted hepatocytes were resuspended in DMEM/F12 medium containing glutamine (1 mmol L^{-1}), 10% FBS, streptomycin (100 μg mL^{-1}), and penicillin (100 IU mL^{-1}). The hepatocytes were cultured at a density of 10^6 cells mL^{-1} in a 5% CO_2 incubator at 28°C for 48 h. After 24 h of DHA treatment, the cells were collected for subsequent analysis. The reagents were prepared as follows: DHA (Sigma, D2534, United States) and the P53 inhibitor, pifithrin-α (Apexbio, A4206, United States), were dissolved in dimethyl sulfoxide

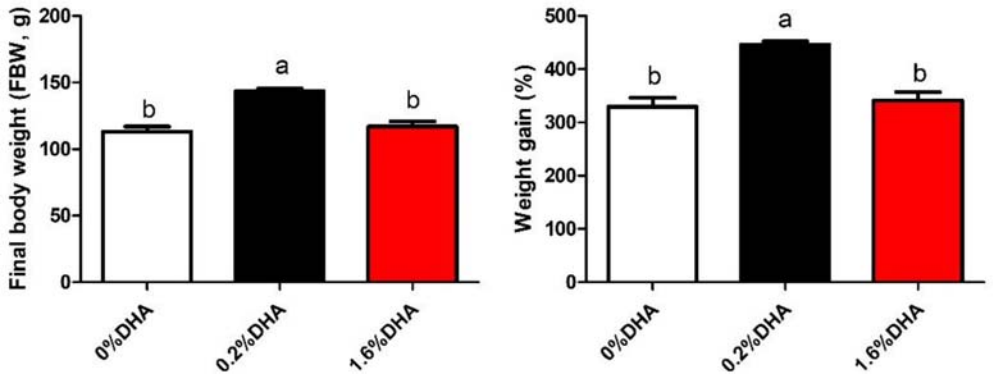

FIGURE 1 | Growth of *M. amblycephala* fed the experimental diets. Values are means ± SEM of four replicates (n = 4) represented by vertical bars. Different superscript letters indicate significantly different values ($P < 0.05$). Final weight (g) = total final weight/total number. Weight gain rate (WGR,%) = (Final body weight - initial body weight) × 100/initial body weight.

(DMSO) and mixed thoroughly with cell media containing FBS, following the procedure described by Ferguson et al. (2018). The final concentrations of DHA were 100, 500, and 1,000 μM, and the final concentration of pifithrin-α was 10 μM. Media and vehicle alone were added to the controls. All the solutions were stored in the dark at –20°C.

We designed a small experiment to test whether DHA was oxidized. Cells were collected and lysed 0 h and 24 h after being exposed to 500 μM DHA. Media and vehicle were the control groups. The DHA content was measured with the GC/MS (Trace ISQ, United States) method. The results are shown in **Supplementary Figure S1**.

Quantitative Real-Time PCR

Total RNA was extracted from liver and hepatocytes with Trizol reagent (Invitrogen, United) and treated with RQ1 RNase-free DNase (Promega, United States) to eliminate genomic DNA contamination. The concentration of the extracted RNA was determined by spectrophotometric analysis, and the purity was assessed based on the OD260/OD280 ratio (1.8–2.0). cDNA was generated from 500 ng of DNase-treated RNA using the ExScriptTM RT-PCR kit (Takara, Japan). The PCR protocol is described in Cao et al. (2019b), and the target cDNA was diluted and amplified using the SYBR Green II Fluorescence Kit (Takara, Japan). Following Livak and Schmittgen (2001), relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

The normalized gene expression of the control groups (0% DHA *in vivo*; media alone *in vitro*) was set to 1. Normalized gene expression of the control group (0% DHA *in vivo*; Media group *in vitro*) was set to 1. The *ATM*, Checkpoint kinase 2 (*Chk-2*), *P53*, *Cyclin D1*, *Cyclin E1*, *Cyclin A2*, *Cyclin B1,* and Elongation factor 1α (*EF1*α) primers used are shown in **Table 1**. *EF1*α was used as the reference for relative quantification.

Western Blot Analysis

RIPA lysis buffer (#ab156034, Abcam, United Kingdom) was added to the liver and hepatocytes, and total proteins were extracted on ice with a glass tissue grinder (Kimble Chase, United States). Protein concentration was quantified using a BCA protein assay kit (Tiangen, China). Heat-denatured protein lysates (20 μg of protein) were separated by reducing SDS-PAGE electrophoresis and transferred to a PVDF membrane. The primary antibodies used had the following specificities: β-Actin (ABclonal, AC004, China), ATM (ABclonal, A18257, China), Chk-2 (ABclonal, A2145, China), P53 (ABclonal, A0263, China), Cyclin D1 (ABclonal, A11310, China), Cyclin E1 (ABclonal, A14225, China), Cyclin A2 (ABclonal, A2891, China), and Cyclin B1 (ABclonal, A2056, China). The secondary antibodies used were the following: goat anti-rabbit (IgG H + L: ABclonal, AS003, China) and goat anti-mouse (IgG H + L: ABclonal, AS014, China). The bands were visualized by adding electrochemiluminescence (ECL) reagents (GoodHere, China) and with a luminescent image analyzer (Fujifilm LAS-3000, Japan). Protein levels were quantified using Image J software (National Institutes of Health, United States).

Cell Cycle Assay

Five treatments (Media, Vehicle, Pifithrin-α, 500 μM DHA, and 500 μM DHA + Pifithrin-α) were added to hepatocytes for 24 h, then the hepatocytes were collected and rinse twice with cold PBS. The hepatocytes were resuspended in 400 μL PBS, after which 300 μL cold absolute 75% ethanol was added to the cell suspensions, which were then fixed overnight at 4°C. Precipitated cells were collected by centrifugation the next day. Next, 400 μL propidium iodide (50 μg mL^{-1} PI) solution was added to incubate for 30 min (4°C, light avoided). Cells were immediately detected with an Accuri C6 II flow cytometer (Beckman, United States). Hepatocytes were exposed to five different conditions (media alone, vehicle, pifithrin-α, 500 μM DHA, and 500 μM DHA + Pifithrin-α) for 24 h. After 24 h, hepatocytes were collected, rinsed twice with cold PBS, and resuspended in 400 μL PBS. Next, 300 μL of ice-cold 75% ethanol was added to the cell suspensions, for overnight fixation at 4°C. The precipitated cells were collected by centrifugation the next day, 400 μL of propidium iodide (PI) solution was added, and the cell suspensions were incubated for another 30 min at 4°C in

TABLE 1 | Nucleotide sequences of the primers for real-time quantitative PCR.

Target gene	Forward (5′–3′)	Reverse (5′–3′)	Accession number
ATM	ATGGGAAGAAACCTCAGC	CAAGCATCCAGGTCCAA′	NW_017542480.1
Chk2	CAGGGATAGAAAAGAAACG	AGGTGTATCATTGTCCGAG	XM_019072142.1
P53	CCATCCTCACAATCATCAC	TGCTCTCCTCAGTTTTCCT	NM_131327.2
Cyclin E1	GGGCTGAAGTGGTGTGATTTG	GAGCTGCCTGCTTCACGAA	NM_130995
Cyclin D1	CAAGCCCTCCCTCCATGAT	GCAACTGTCGGTGCTTTTCAG	NM_131025
Cyclin A2	GCTTTTGGCTTCGAAGTTTGA	TTGTGTACGTGTCGTCAGTGATG	NM_152949
Cyclin B1	GCTTATGCCCTGACCCTGAA	GCATCACAGGAACCAGCTCAT	NM_131513
EF1α	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	X77689.1

ATM, Ataxia telangiectasia mutated; Chk2, Checkpoint kinase 2; EF1α, Elongation factor 1α.

the dark. Cells were analyzed with an Accuri C6 II flow cytometer (Beckman, United States).

Apoptosis Assay

Cellular apoptosis was measured by annexin V-FITC/PI staining and flow cytometry (FCM) analysis. Hepatocytes were exposed in triplicate to five different conditions (media alone, vehicle, pifithrin-α, 500μM DHA, and 500μM DHA + pifithrin-α) for 24 h. After removing the medium, the hepatocytes were rinsed twice with cold PBS and collected in a flow tube, and 400 μL of 1X annexin V binding buffer was added to resuspend the cells (10^6 cells mL^{-1}). Next, 5 μL of annexin V-FITC solution was added, and the cells were incubated for 15 min at 4°C in the dark, followed by 10 μL of PI and an additional 5 min incubation at 4°C in the dark. Cells were immediately analyzed with the Accuri C6 II flow cytometer (Beckman, United States). After fluorescence compensation, the annexin V-FITC (FL1) fluorescence was set as the abscissa, and the PI (FL2) fluorescence was set as the ordinate. Results are shown as quadrant dot plots and percentage of stained cells.

Statistical Analysis

The normality and homogeneity of the data were evaluated by the Kolmogorov-Smirnov and Levene tests. All data were analyzed by one-way ANOVA using SPSS 16.0 (SPSS, United States) for Windows. Significant differences between groups were determined by the Tukey multiple range test. The level of significance was set at $P < 0.05$. All results are expressed as the mean ± S.E.M. (standard error of the mean) of four replicates ($n = 4$) *in vivo* experiments and three replicates ($n = 3$) f *in vitro*.

RESULTS

Results *in vivo*

Growth Performance

As shown in **Figure 1**, the final body weight and weight gain of fish fed 1.6% DHA was significantly lower than that of fish fed 0.2% DHA ($P < 0.05$).

Gene Expression in Liver

As shown in **Figure 2**, *ATM*, *Chk-2*, and *P53* gene expression levels were significantly higher (**Figures 2A–C**), while *Cyclin D1* and *Cyclin E1* gene expression levels were significantly lower, in the 1.6% DHA group when compared with the control and 0.2% DHA groups ($P < 0.05$; **Figures 2D,E**). There were no significant differences in *Cyclin A2* and *Cyclin B1* gene expression between treatments ($P > 0.05$; **Figures 2F,G**).

Protein Expression in Liver

Compared with the control group, fish fed 1.6% DHA showed significantly higher hepatic ATM and P53 protein levels (**Figures 3A,C**), whereas Cyclin D1, Cyclin E1, and Cyclin A2 protein levels were significantly lower ($P < 0.05$; **Figures 3D,E,G**). No significant differences were observed in Chk-2 and Cyclin B1 protein levels between the three groups ($P > 0.05$; **Figures 3B,F**).

Results *in vitro*

Three concentrations of DHA (100, 500, and 1,000 μM) were tested for toxic effects in hepatocytes (**Supplementary Figure S2**). DHA significantly influenced the *ATM* and *P53* gene expression levels, which peaked at 500 μM DHA and decreased when the DHA concentration was increased to 1,000 μM.

Cell Cycle Arrest

Treatment with 500 μM DHA significantly increased the ratio of hepatocytes in the G1 phase and decreased the ratio of hepatocytes in the S phase ($P < 0.05$; **Figure 4**). On the other hand, treatment with both 500 μM DHA and pifithrin-α significantly decreased the ratio of hepatocytes in the G1 phase when compared with the 500 μM DHA group ($P < 0.05$; **Figure 4**).

Apoptosis in Primary Hepatocytes

Figure 5 shows apoptosis in DHA-treated hepatocytes. The apoptosis rate (early and late) was significantly increased in hepatocytes treated with 500 μM DHA when compared with other groups ($P < 0.05$; **Figures 5-1D**). Treatment of cells with pifithrin-α significantly decreased the DHA-induced apoptosis rate ($P < 0.05$; **Figures 5-1E**, **5-2**).

Gene Expression in Hepatocytes

ATM and *P53* gene expression levels were significantly increased (**Figures 6A,C**), while downstream *Cyclin D1* and *Cyclin E1* gene expression levels were significantly reduced, in hepatocytes treated with 500 μM DHA when

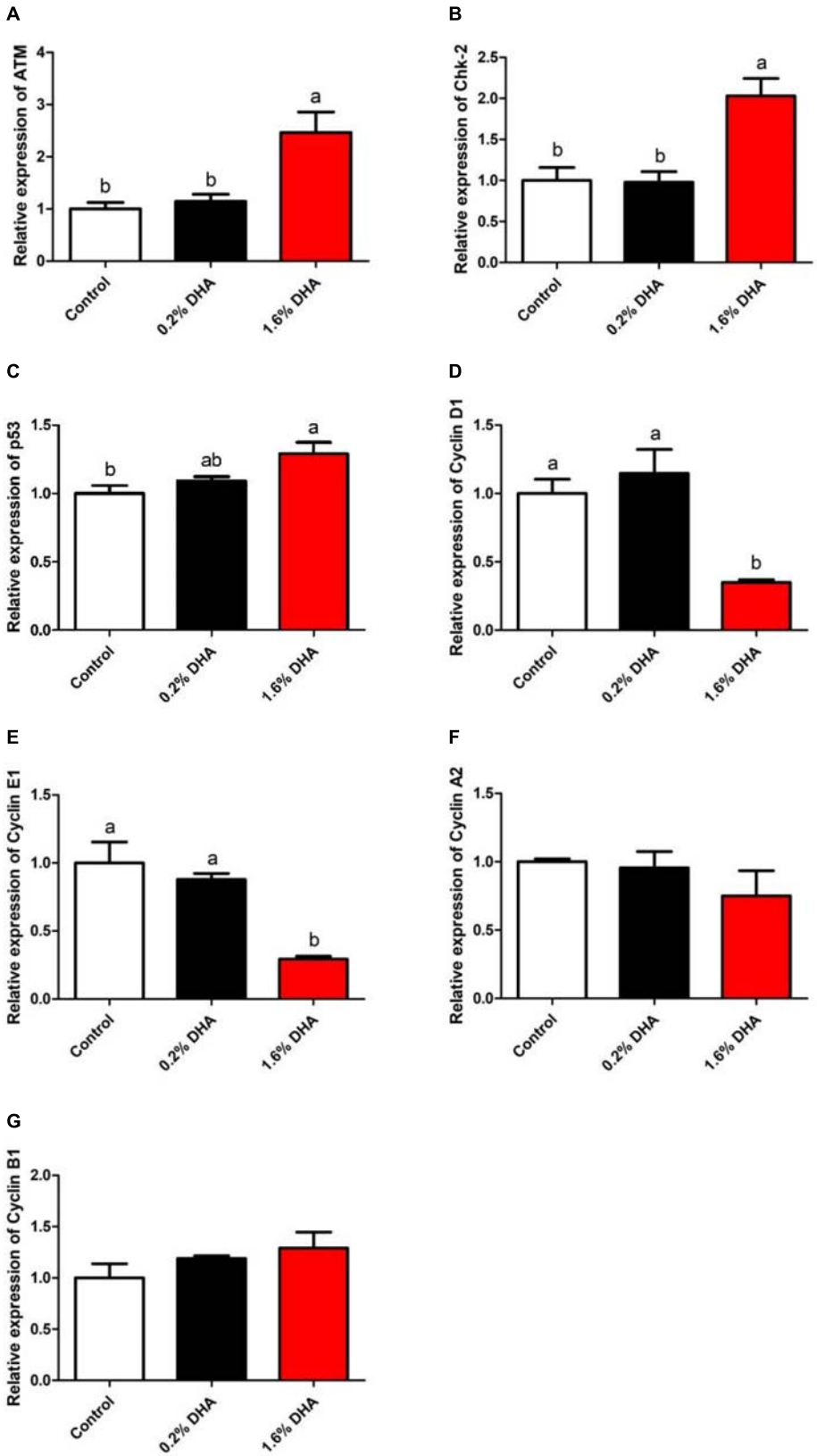

FIGURE 2 | Relative gene expression of *ATM*, *Chk-2*, *P53*, *CyclinD1*, *CyclinE1*, *CyclinA2*, and *CyclinB1* in the liver of *M. amblycephala* fed the experimental diets **(A–G)**. Values are means ± SEM of four replicates ($n = 4$) represented by vertical bars. Different superscript letters indicate significantly different values ($P < 0.05$).

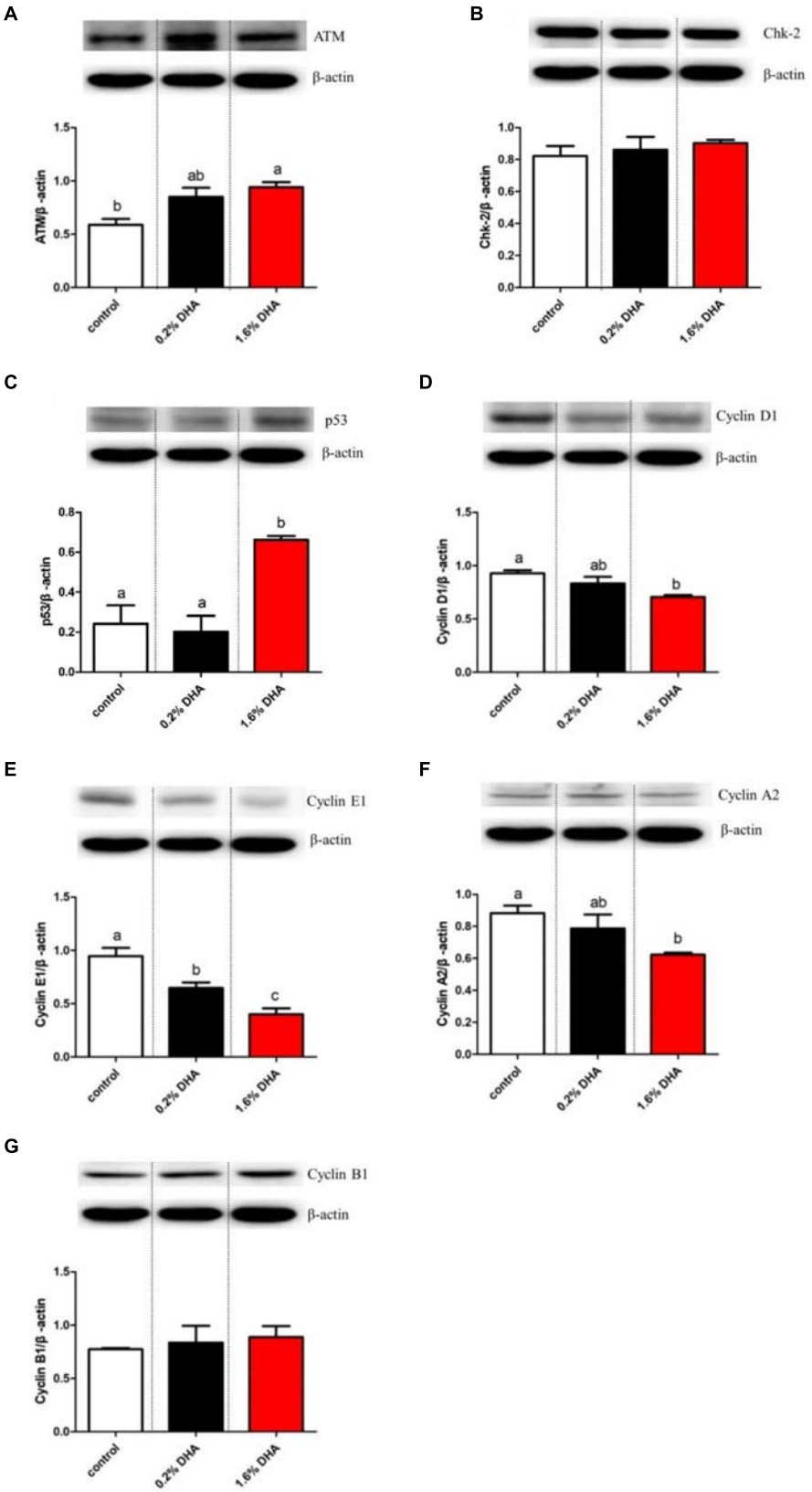

FIGURE 3 | Western blot analysis of *ATM, Chk-2, P53, CyclinD1, CyclinE1, CyclinA2*, and *CyclinB1* in the liver of *M. amblycephala* fed the experimental diets **(A–G)**. Values are means ± SEM of four replicates ($n = 4$) represented by vertical bars. Different superscript letters indicate significantly different values ($P < 0.05$).

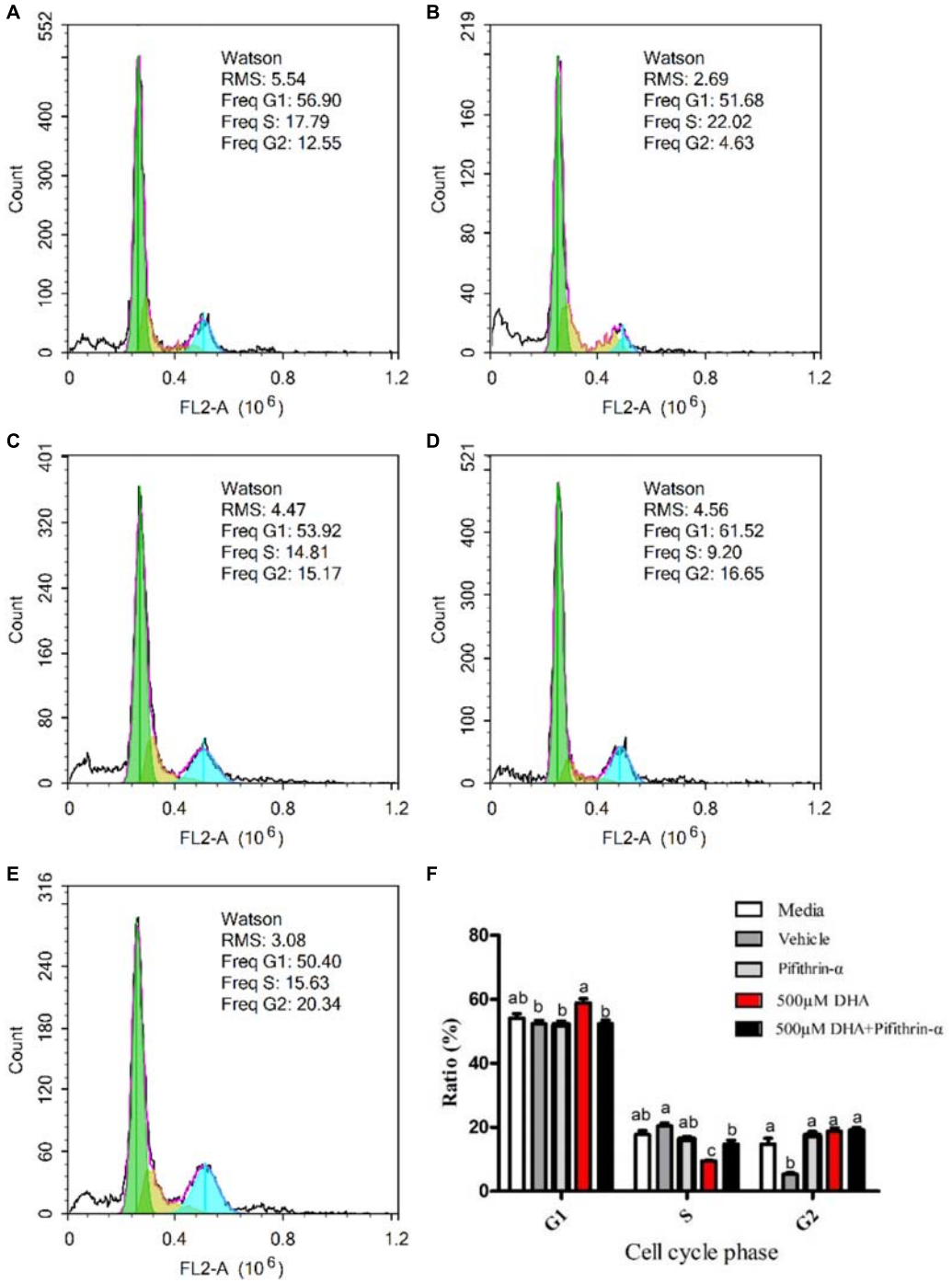

FIGURE 4 | Effects of DHA on hepatocytes cell cycle. The cell cycles of the hepatocytes exposed to Media, Vehicle, Pifithrin-α, 500 μM DHA, and 500 μM DHA + Pifithrin-α, respectively **(A–E)**. The ratio of cells at the G1, S, and G2 phases were measured by flow cytometry **(F)**. Values are means ± SEM of three replicates (n = 3) represented by vertical bars. RMS: root mean square. Different superscript letters indicate significantly different values ($P < 0.05$).

compared with the control and co-treated (500 μM DHA + pifithrin-α) groups ($P < 0.05$; **Figures 6A,C–E**). There were no significant differences in *Chk-2* and *Cyclin B1* gene expression levels between treatments ($P > 0.05$; **Figures 6B,F**).

Protein Expression in Hepatocytes

P53 protein levels were significantly upregulated ($P < 0.05$; **Figure 7B**), while Cyclin D1 and Cyclin E1 protein levels were significantly downregulated, in hepatocytes treated with 500 μM DHA when compared with other groups

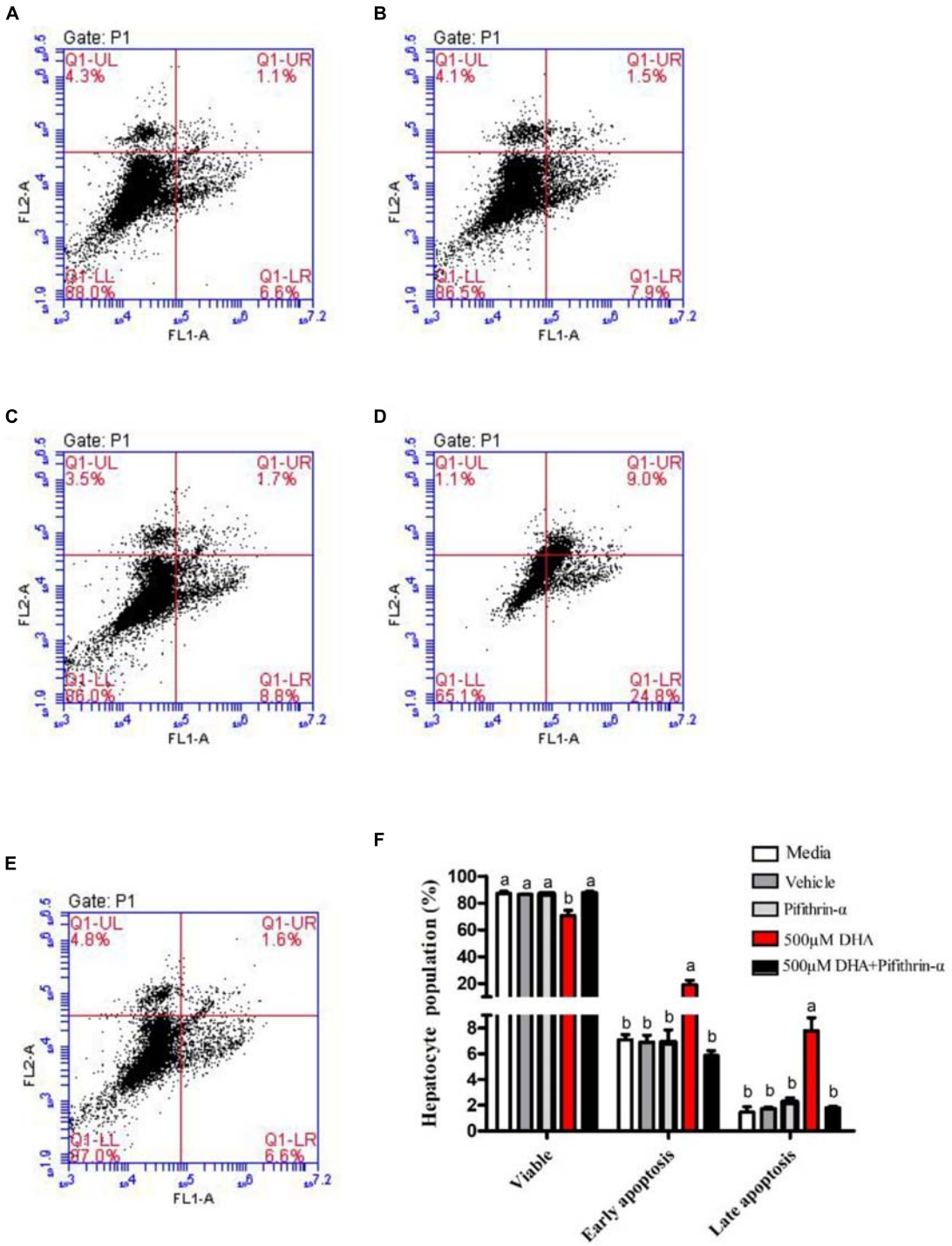

FIGURE 5 | Effects of DHA on hepatocyte apoptosis. The apoptosis of hepatocytes exposed to Media, Vehicle, Pifithrin-α, 500 μM DHA, and 500 μM DHA + Pifithrin-α, respectively **(A–E)**. The percentages of apoptotic cells were measured by flow cytometry **(F)**. Values are means ± SEM of three replicates ($n = 3$) represented by vertical bars. Different superscript letters indicate significantly different values ($P < 0.05$).

($P < 0.05$; **Figures 7C,D**). On the other hand, P53 protein levels were significantly reduced (**Figure 7B**), whereas Cyclin D1 and Cyclin E1 protein levels were significantly increased, in hepatocytes treated with both 500 μM DHA and pifithrin-α when compared with the 500 μM DHA group ($P < 0.05$; **Figures 7C,D**). There were no significant differences in Chk-2 and cyclin B1 protein levels ($P > 0.05$; **Figures 7A,E**).

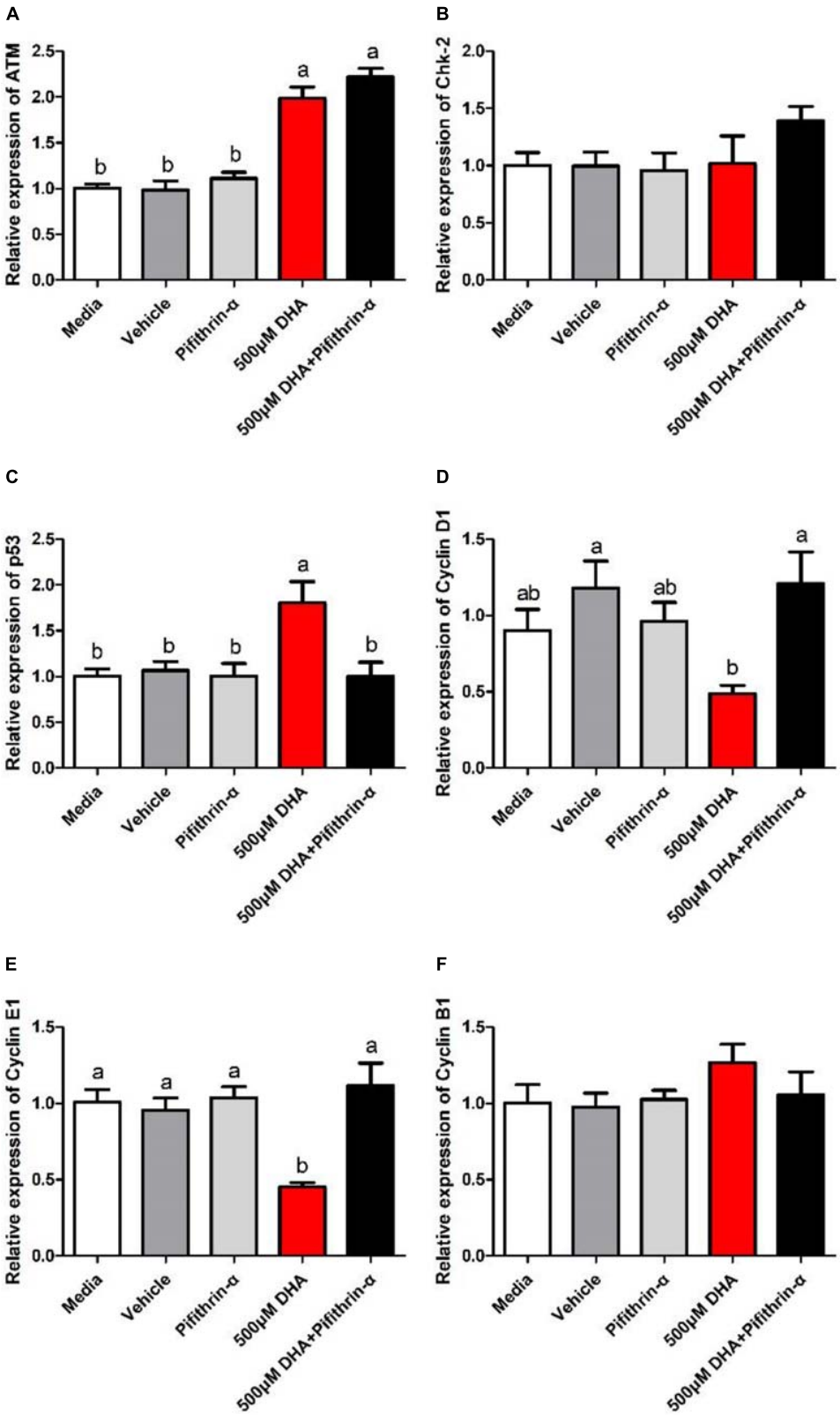

FIGURE 6 | Relative gene expression of *ATM*, *Chk-2*, *P53*, *CyclinD1*, *CyclinE1,* and *CyclinB1* in the hepatocytes exposed to Media, Vehicle, Pifithrin-α, 500 μM DHA, and 500 μM DHA + Pifithrin-α, respectively **(A–F)**. Values are means ± SEM of three replicates ($n = 3$) represented by vertical bars. Different superscript letters indicate significantly different values ($P < 0.05$).

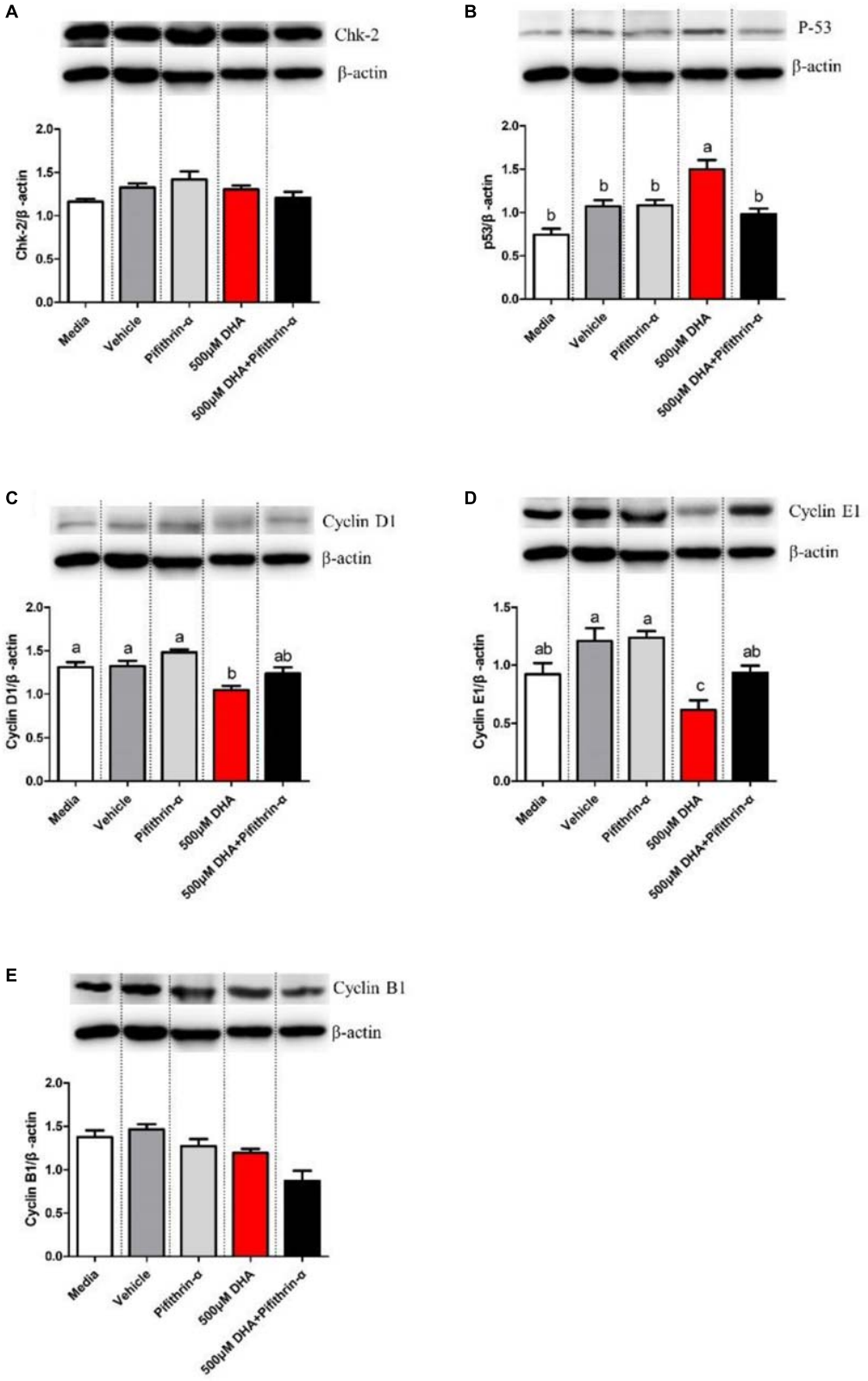

FIGURE 7 | Western blot analysis of *Chk-2, P53, CyclinD1, CyclinE1*, and *CyclinB1* in the hepatocytes exposed to Media, Vehicle, Pifithrin-α, 500 μM DHA, and 500 μM DHA + Pifithrin-α, respectively **(A–E)**. Values are means ± SEM of three replicates ($n = 3$) represented by vertical bars. Different superscript letters indicate significantly different values ($P < 0.05$).

DISCUSSION

DHA plays an important role not only regulating the growth (Villalta et al., 2005; Moran et al., 2018), dietary oil source selection (Haak et al., 2008), and nutritional qualities (Raes et al., 2004; Moreno-Indias et al., 2012) of commercially important species but also in human health (Conquer and Holub, 1998; Holub, 2001). This study and our previous study found that excess DHA caused growth retardation in blunt snout bream (Wang et al., 2019). Glencross and Rutherford (2011) also found that high doses of DHA decreased growth and led to an increase in plasma urea concentration and fish redness in juvenile barramundi (*Lates calcarifer*). DHA has been well investigated as a health supplement, but why would fish fed high levels of DHA show such effects? This is an interesting result that needs to be explored.

The cell cycle is divided into cellular interphase and mitosis. In turn, the cellular interphase is divided mainly into gap1 (G1), DNA synthesis (S), and gap2/mitosis (G2/M) phases (Ishikawa et al., 2006; Chen et al., 2017; Dai et al., 2019). The cyclin family plays a pivotal role in regulating the timing and progression of the cell cycle (Ohki et al., 2000). P53 is a potent cyclin-dependent kinase inhibitor that regulates the expression of genes associated with cell growth and the cell cycle (Ohki et al., 2000; Francis et al., 2007). Previous studies reported that multiple inflammatory factors could activate the *ATM-Chk2-P53* signaling pathway to induce cell cycle arrest (Chen et al., 2017; Dai et al., 2019). Also, Mai et al. (2012) reported that P53 was cooperatively activated by the ATM-P53 pathway in response to stress and DNA-damaging signals in *Oreochromis niloticus*. In this study, the effects of DHA on cell cycle regulation in *Megalobrama amblycephala* were analyzed, including the P53 pathway (P53, Chk2, and ATM), G1 phase (Cyclin D1, Cyclin E1), S phase (Cyclin A2), and G2/M phase (Cyclin B1) (Huang et al., 2010). The feeding trial showed that ATM and P53 were activated in response to 1.6% DHA, indicating the possibility of DNA damage and apoptosis. On the other hand, downstream Cyclin D1 and Cyclin E1, which promote G1/S phase progression (Cunningham et al., 2009), were inhibited. These results indicate that high levels of DHA affect the cell cycle, resulting in decreased growth of *M. amblycephala*. To better understand the negative effects of high levels of DHA, we conducted *in vitro* experiments with primary hepatocytes.

When primary hepatocytes were incubated with DHA, 1,000 μM DHA has a lower effect on the gene expression of ATM and P53 than 500 μM, but they were all significantly higher than the control group. This result may indicate that 500 μM caused cell damage. The reason for the downward trend at 1,000 μM is still worthy of further study. When primary hepatocytes were incubated with 500 μM DHA, the ratio of cells in the G1 phase was significantly increased, a result that was consistent with the observed reduction of cyclin D1 and cyclin E1gene and protein expression levels. These results indicate that high levels of DHA induce cell cycle arrest in the G1 phase. Other compounds that can influence the cell cycle have been reported. For example, Chen et al. (2017) reported that cyclin E1 and cyclin A1 mRNA levels decreased in male zebrafish treated with 229.05 μg/L TDCPP, suggesting cell cycle arrest at the G1 to S phase. Cell cycle arrest is usually accompanied by increased apoptosis (Slotkin and Seidler, 2012). For example, a previous study found that tris (1,3-dichloro-2-propyl) phosphate (TDCPP) induced cell cycle arrest and caused cell apoptosis in zebrafish (Huang et al., 2010). Saquib et al. (2012) showed that it induced cell cycle arrest and apoptosis, adversely affecting normal cellular function in rats. In this study, 500 μM DHA induced cell cycle arrest and apoptosis, indicating that excess DHA could affect normal cellular function in *Megalobrama amblycephala*. Similar results have been reported in previous studies using tumor cell lines, demonstrating that apoptosis is a common response to DHA (Fasano et al., 2012; Sam et al., 2016; Song and Kim, 2016). However, very few studies have investigated the negative effects of high DHA levels on normal organisms or cells. Although DHA is a common health supplement and has excellent anti-cancer effects (Conquer and Holub, 1998), our results indicate that excessive DHA is likely to have adverse effects on normal cellular function. This is worthy of attention and should be verified in other animal species.

P53 and downstream (cyclin D1 and cyclin E1) gene and protein expression levels were significantly affected by 500 μM DHA in our *in vitro* experiments, which was consistent with the results of the *in vivo* trial. When primary hepatocytes were incubated with a P53 inhibitor, P53-mediated signaling was inhibited, cell cycle progression recovered, and apoptosis decreased, suggesting that DHA-induced cytotoxicity is mediated by P53. Hence, high levels of DHA caused negative effects in *Megalobrama amblycephala*, which may be attributed to the activated P53/cyclin pathway. Similarly, Zand et al. (2007) documented how DHA induced apoptosis through P53. Another study with zebrafish also showed that cell cycle arrest and apoptosis were induced through the activation of P53 (Zhu et al., 2015). Thus, inhibition of P53 activity could be a potential approach to reduce cell damage in fish fed DHA.

CONCLUSION

In summary, the feeding trial showed that 1.6% DHA in the diet has no "growth-promoting" effect in *Megalobrama amblycephala* when compared with 0% DHA. In primary hepatocytes, 500 μM DHA treatment significantly induced cell cycle arrest in the G0/G1 phase and, consequently, apoptosis. *In vivo* and *in vitro*, high levels of DHA significantly upregulated P53 gene and protein expression and decreased downstream cyclin D1 and cyclin E1 expression. Thus, DHA can activate the P53/cyclin pathway to induce cell cycle arrest. Inhibiting P53 activity could be a potential way of reducing the side effects of DHA on the growth of *Megalobrama amblycephala*.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was approved by the Animal Care and Use. Committee of Nanjing Agricultural University (Nanjing, China) (permit number: SYXK (Su) 2011–0036).

AUTHOR CONTRIBUTIONS

CW was responsible for doing experiments, analyzing data, and writing the article. GJ provided substantial contributions to the conception and design of experiments. WL provided valuable advice. YH, XW, and KX assisted in experiments. XC, XL, and DZ provided writing assistance.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00286/full#supplementary-material

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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.