Research Article

Journal of Applied Toxicology

Received: 19 March 2012, Revised: 20 May 2012, Accepted: 20 May 2012 Published online in Wiley Online Library: 9 August 2012

(wileyonlinelibrary.com) DOI 10.1002/jat.2787

Exposure time to caffeine affects heartbeat and cell damage-related gene expression of zebrafish *Danio rerio* embryos at early developmental stages

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ABSTRACT: Caffeine is white crystalline xanthine alkaloid that is naturally found in some plants and can be produced synthetically. It has various biological effects, especially during pregnancy and lactation. We studied the effect of caffeine on heartbeat, survival and the expression of cell damage related genes, including oxidative stress (HSP70), mitochondrial metabolism (Cyclin G1) and apoptosis (Bax and Bcl2), at early developmental stages of zebrafish embryos. We used 100 μM concentration based on the absence of locomotor effects. Neither significant mortality nor morphological changes were detected. We monitored hatching at 48 h post-fertilization (hpf) to 96 hpf. At 60 and 72 hpf, hatching decreased significantly ($P < 0.05$); however, the overall hatching rate at 96 hpf was 94% in control and 93% in caffeine treatment with no significant difference ($P > 0.05$). Heartbeats per minute were 110, 110 and 112 in control at 48, 72 and 96 hpf, respectively. Caffeine significantly increased heartbeat – 122 and 136 at 72 and 96 hpf, respectively. Quantitative RT-PCR showed significant up-regulation after caffeine exposure in HSP70 at 72 hpf; in Cyclin G1 at 24, 48 and 72 hpf; and in Bax at 48 and 72 hpf. Significant down-regulation was found in Bcl2 at 48 and 72 hpf. The Bax/Bcl2 ratio increased significantly at 48 and 72 hpf. We conclude that increasing exposure time to caffeine stimulates oxidative stress and may trigger apoptosis via a mitochondrial-dependent pathway. Also caffeine increases heartbeat from early phases of development without affecting the morphology and survival but delays hatching. Use of caffeine during pregnancy and lactation may harm the fetus by affecting the expression of cell-damage related genes. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: caffeine; heartbeat; cell damage; gene expression; q RT-PCR; embryos; zebrafish

Introduction

Caffeine is a white crystalline xanthine alkaloid that acts in human as a stimulant drug for the central nervous system and metabolism. It is a commonly used medicine indicated for headache and other pain relied and is considered the most widely consumed psychoactive drug in the world (Carlsen *et al*., 2005). Caffeine is naturally found in varying quantities in the seeds, leaves and fruit of some plants and also can be produced synthetically (Chiranjib *et al*., 2011). It is also used as an additive in many products. There are many beverages containing caffeine, such as coffee, tea, soft drinks and energy drinks (Lovett, 2005). It was found that a cup of coffee containst 60–80 mg caffeine; however, this varies according to cup size, coffee preparation method and filtration (Cook *et al*., 1996). A recent review of national dietary consumption between 1994 and 1998 estimated that 87% of the population consumes caffeine, with an average of 193 mg per day. Among adults 18 years and older, the daily intake ranges between 166 and 336 mg per day (Frary *et al*., 2005). The caffeine level is estimated as 21.22 μM in the blood circulation of people imbibing 2427 mg caffeine for 36 weeks (Cook *et al*., 1996).

Caffeine has shown a wide array of pharmacological and biological effects. The manner in which caffeine triggers pleiotropic effects is still largely unknown (Calvo *et al*., 2009). It was found that caffeine induces cell death in human osteoblasts, attributed primarily to apoptosis (Pin-Zhen *et al*., 2008). Sometimes caffeine is prescribed to pregnant woman, and it has been reported that it can cause problems to the fetus as well as the mother (Carlsen *et al*., 2005).

Recently, zebrafish embryos and larvae have been one of the most extensively used tools for investigating the negative impact of chemicals and pharmaceuticals (Kristensen, 1995; Luckenbach *et al*., 2001). The zebrafish is a small, freshwater, aquarium species. It has a short generation time and breeds almost all year round. They are easy to grow, very sensitive and can be maintained in different environments. Furthermore, as the developmental stages of embryos are external and transparent, the effectof treatments can be observed clearly and distinctly (Chiranjib *et al*., 2011). Thus, zebrafish is considered an excellent model for studying the effect of drugs and

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pharmaceuticals (Ulrike 2003; Holmberg *et al.*, 2006; Bello *et al.*, 2004 and Brion *et al.*, 2004), especially at the early development stages.

The aim of our research is to study the effect of different exposure durations of caffeine on zebrafish embryos at the first developmental stages: 24, 48 and 72 hpf. In this study, we adopted a real-time polymerase chain reaction (PCR) to determine the effects of caffeine on the expression levels of some cell damage-related genes in embryo–larval zebrafish exposed to different durations at about 2 hpf. We selected a linked package of genes that included oxidative stress gene (HSP70), mitochondrial metabolism (Cyclin G1) and apoptosis (Bcl2 and Bax), to study the effect of caffeine on the expression behavior of these genes. Also, we observed the effect on survival rate, hatching rate and heartbeat of the developing zebrafish embryos.

Materials and Methods

Zebrafish Maintenance and Egg Collection

Zebrafish (*Dario rerio*) were obtained locally (Green Fish, Korea) and were raised at the laboratory housing system (Daejong Instrument Industry, Korea). Adult zebrafish were maintained in glass aquaria with a continuous re-circulating system and a 14:10 h. light–dark photocycle. The temperature was 28.5 °C during all stages of the experiment. Adult zebrafish were fed three times a day with a combination of dried blood worms (TetraWerke, Melle, Germany) and newly hatched brine shrimp, *Artemia* (San Francisco Bay Brand Inc., Newark, CA, USA). Two pairs of males and females were separated with a barrier in a spawning box containing a mesh bottom to prevent the spawned eggs from being cannibalized and then the boxes were incubated overnight in a 28.5 °C incubator. In the next day, the barrier was removed at the beginning of light period and the zebrafish started spawning. Fertilized eggs were obtained from matured zebrafish according to the manual by Westerfield (1995). We followed the Care and Treatment of the Animals guidelines established by Institutional Animal Care and Use Committee, Seoul National University (approval no. SNU-050418-2).

Chemical Preparation and Exposure

Caffeine, 1,3,7-trimethylxanthine (ReagentPlus®, powder), was purchased from Sigma-Aldrich (CAS no. 58-08-2). The exposure solution was prepared in a concentration of 100 μM by dissolving caffeine powder in distilled water. The tested concentration was selected according to Katiucia *et al.* (2011). We washed the fertilized eggs twice and started exposure at the cleavage stage 32–64 cells (about 2 hpf). For the monitoring assays – hatching rate, survival and heartbeat – a six-well cell culture plate was used. We put 30 eggs in each well filled with 3 ml of 100 μM caffeine solution or distilled water, in three replicates, to give a total of 90 eggs per treatment. About 30% of the solution was replaced daily with fresh one, for 96 hpf. For the molecular analysis, the remained fertilized eggs were separated and handled carefully under temperature control to avoid heat-shock. We divided them into two groups and exposed them immediately to caffeine solution or distilled water. We allocated a separate control for each treatment.

Embryo–Larvae Monitoring Assay

The morphological changes were observed using an Olympus IX70 microscope during the experiment. Embryo and larvae were counted for survival rate and hatching rate at 36, 48, 60, 72, 84 and 96 hpf. The heartbeat was counted at 48, 72 and 96 hpf. The Petri dishes were placed under a stereomicroscope (SZ-PT, Olympus, Japan) to count the heartbeat rates per minute for larvae using a stop watch and counter (Zhu *et al.*, 2007) under temperature control. About 30 embryos from each treatment were used in this study.

Total RNA Extraction and cDNA Synthesis

At 24, 48 and 72 hpf, about 60–70 embryos from caffeine treatment and control were pooled and prepared for total RNA extraction using the following protocol provided by Chen Laboratories, Department of Chemical and Systems Biology, Stanford University. Briefly, the embryos were washed using E3 buffer then phosphate-buffered saline. They were flash frozen in liquid nitrogen then homogenized in 500 μl Trizol reagent (Invitrogen, USA). A 150 μl aliquot of chloroform was added and the mixture shaken vigorously for 30 s then centrifuged at 10 000 rpm at 4 °C for 15 min. The RNA-containing upper aqueous phase was transferred to a new tube and the RNA precipitated by adding 0.8 vols of isopropanol. The solution was mixed well and incubated for 10 min at room temperature, then centrifuged at 10 000 rpm for 15 min at 4 °C. The supernatant was removed and the RNA pellet washed with 750 μl of 75% RNase-free ethanol by centrifuging at 8000 rpm for 15 min at 4 °C. The pellet was air-dried for 10 min and the RNA re-suspended in 50 μl RNase-free H_2O. cDNAs were synthesized using an M-MLV cDNA synthesis kit (Enzynomics, Korea) following the supplier's instructions.

Relative Quantification of Transcripts by Real-time RT-PCR

Real-Time RT-PCR (qPCR) was performed according to the Takara Bio Inc. guidelines. A 22 μL PCR reaction was made by adding 2 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 8 μL SYBR Premix Ex Taq, 0.4 μL ROX Reference (Takara Bio Inc. Shiga, Japan) and 9.6 μL of Nuclease-free water (Ambion Inc., Austin, TX, USA). The reaction was performed using a 7300 Real Time PCR System (Applied Biosystems, Forest City, CA, USA) according to the company's instructions. The thermal profile for real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 40 s. We used GAPDH as a housekeeping gene to normalize the results by eliminating variations in mRNA and cDNA quantity and quality, and each mRNA level was expressed as a ratio to GAPDH mRNA. Three replicates (pools of embryos) and three technical replicates of each RNA sample were performed. Relative mRNA expression for each gene was calculated as a fold change compared with the control group. All primers are described in Table 1.

Statistical Analysis

The effects of caffeine treatment on the hatching rate, survival rate, heartbeat rates and relative quantitative expression of tested genes were determined using one-way analysis of variance (ANOVA) followed by Tukey's test. Significant difference occurred for a given parameter when $P < 0.05$. The entire statistical analysis was carried out using Graphpad Prism (version. 5).

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Table 1. Gene-specific primers used for quantitative real time qRT-PCR

Gene name	Function	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number
HSP70	Oxidative stress	CATCGACGCCAACGGG	CCAGGGAGTTTTTAGCAGAAATCTT	AB062116
CyclinG1	Mitochondrial metabolism	TCTCTCCTTGACTCGATTCTTTG	AATATTCAACCAGGCACTTAGCA	BC052125
Bcl2	Apoptosis	TTGTGGAGAAATACCTCAAGCAT	GAGTCTCTCTGCTGACCGTACAT	BC133848
Bax	Apoptosis	GAGCTGCACTTCTCAACAACTTT	CTGGTTGAAATAGCCTTGATGAC	BC055592

Results

Morphological Changes and Monitoring Assays

We did not observe morphological changes during the experiment. There was no significant difference ($P > 0.05$) in the survival rate between control and caffeine treatment from 24 to 72 hpf. The final survival rate (%) at 72 hpf was 94% in the control group and 92.6% in the caffeine treatment group. The hatching rate (%) started at 48 hpf and the overall hatching rate was calculated at 96 hpf (Fig. 1). At 60 and 72 hpf the hatching rates were 50 and 80% in the control group but they were 30 and 60% in the caffeine treatment group, respectively, with significant difference ($P < 0.05$) from the control. Overall hatching rate was 94% in the control group and 93% in the caffeine treatment group with no significant difference ($P > 0.05$). Therefore, there was no significant difference between the control and caffeine treatment in the overall hatching rate (96 hpf), but there was a significant decrease in hatching rate in the caffeine treatment at 60 and 72 hpf.

Effect of Caffeine Treatment on Heartbeat of Larvae

The effect of caffeine exposure on the heartbeat of embryos and larvae was studied at 48, 72 and 96 hpf. We allocated controls for each treatment. In the control groups, the heartbeats per minute were 110, 110 and 112 at 48, 72 and 96 hpf, respectively. Significantly higher heartbeat rates ($P < 0.05$) were observed with caffeine treatment at 72 and 96 hpf: 122 and 136 heartbeats per minute, respectively (Fig. 2).

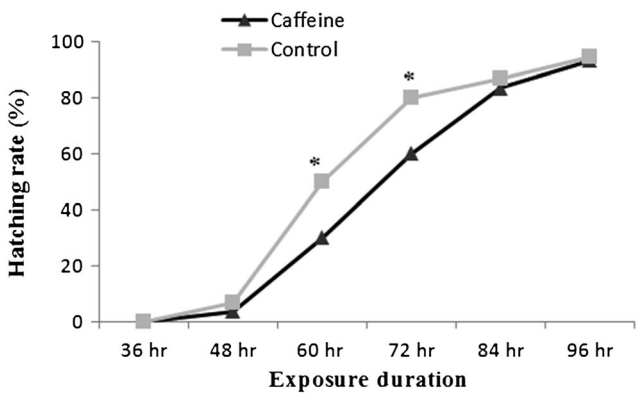

Figure 1. Hatching rate of zebrafish embryos exposed to 100 μM caffeine for different durations. Hatching rate was observed at 48 hpf, and the overall hatching was calculated at 96 hpf. Effect of caffeine treatments appeared at 60 and 72 hpf by a delay of hatching rates with a significant difference from control ($P < 0.05$). Data are presented as means ± SD. The asterisk indicates a significant difference ($P < 0.05$) at the same point as the control.

Gene Expression

The effect of caffeine different exposure durations on cell damage-related genes expression was studied using real-time PCR. The quantitative mRNA levels of the genes related to oxidative stress (HSP70), mitochondrial metabolism (Cyclin G1) and apoptosis (Bcl2, Bax) were determined at early development stages of zebrafish embryos, 24, 48 and 72 hpf after exposure to 100 μM caffeine. The mRNA level of HSP70, one of the oxidative stress-related genes, showed slight down-regulation at 24 hpf with no significant difference from the control. Up-regulation of mRNA level, 1.43- and 11.75-fold, was found at 48 and 72 hpf treatments, respectively. There was no significant difference ($P > 0.05$) between control and 48 hpf treatment but there was significant difference ($P < 0.01$) at 72 hpf (Fig. 3A).

The mRNA expression level of the mitochondrial metabolism-related gene, CyclinG1, increased significantly directly after exposure to caffeine at 24, 48 and 72 hpf treatments (1.34-, 1.2- and 1.35-fold increase, respectively; Fig. 3B). The mRNA expression levels of apoptosis-related genes, Bax and Bcl2, were also affected in caffeine treatments. Bax expression increased directly after caffeine exposure but with significant difference from the control only at 48 and 72 hpf treatments (1.7- and 2.5-fold increase, respectively; Fig. 3C). The mRNA expression level of Bcl2 gene slightly increased at 24 hpf treatment (1.05-fold) with no significant difference from control ($P > 0.05$). The expression level decreased significantly ($P > 0.01$) at 48 and 72 hpf treatments (0.65- and 0.45-fold decrease, respectively; Fig. 3D).

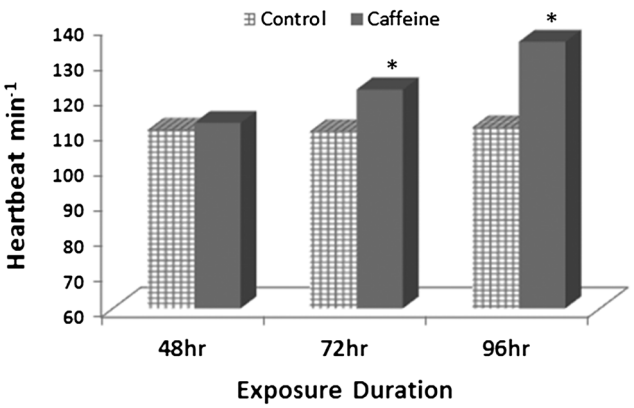

Figure 2. Heartbeats per minute in zebrafish embryos exposed to 100 μM caffeine solution at different durations: 48, 72 and 96 hpf. We counted the heartbeats in 1 minute for the treatment and control at each point. The heartbeats in the control groups were 110, 110 and 112 at 48, 72 and 96 hpf, respectively, with no significant difference ($P > 0.05$). Significantly highere heartbeat was found in caffeine treatment: 122 and 126 heartbeats per minute at 72 and 96 hpf, respectively. Values are relative to the average of control group at each point and expressed as means ± SD. * Values are significantly different from control ($P < 0.05$).

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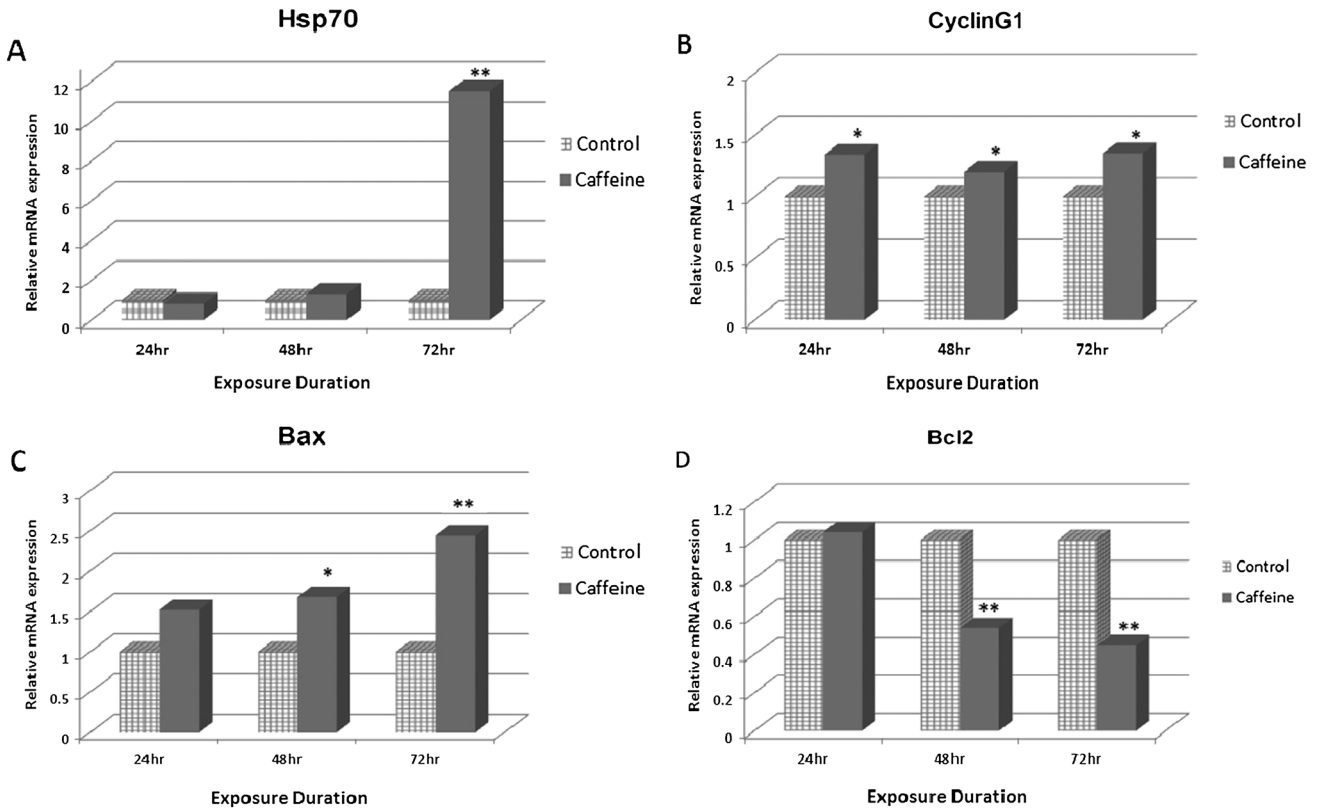

Figure 3. Relative quantitative mRNA of cell damage-related genes; oxidative stress, Hsp70 (A); mitochondrial metabolism, Cyclin G1 (B); apoptosis, Bax (C); and Bcl2 (D) of the zebrafish embryos after exposure to 100 μM caffeine solutionfor different durations at early developmental phases: 24, 48 and 72 hpf. The control values at each point were adjusted to equal 1, and the treatments values were multiplied in the same factor as the control. Values are relative to the control group and expressed as a fold-change, means ± SD. Values significantly different from the control: * $P < 0.05$; ** $P < 0.01$.

We also analyzed the Bax/Bcl2 ratio. We found that all values of Bax/Bcl2 ratio increased at 24, 48 and 72 hpf treatments with significant difference from control at 48 and 72 hpf treatments (3.05- and 4.7-fold increase, respectively), but there was no significant difference at 24 hpf treatment (1.54-fold) from the control (Fig. 4).

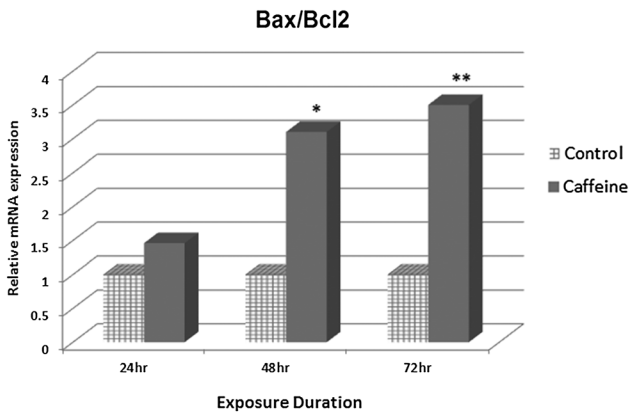

Figure 4. Bax/Bcl2 ratio. mRNA isolated from zebrafish embryos exposed to 100 μM caffeine solution for different durations: 24, 48 and 72 hpf. Values are relative to the control group and expressed as means ± SD. Mean values significantly different from the control group: * $P < 0.05$; ** $P < 0.01$.

Discussion

We investigated the effect of different exposure durations of caffeine on zebrafish embryos at early development stages 24, 48 and 72 hpf. Zebrafish embryos and larvae are extensively used for investigating the negative impact of chemicals (Kristensen, 1995; Luckenbach *et al.*, 2001). As they are easy to grow, very sensitive, can be maintained in different environments and all the developmental stages are transparent and *ex-utero*, they are very suitable organisms for toxicological studies. We studied the effect of caffeine on the expression of some cell damage-related genes. We also observed the effect of caffeine on heartbeat, survival and hatching rate. We started exposure at the cleavage stage, 32–64 cells (about 2 hpf). Samples were pooled for molecular analysis at 24, 48 and 72 hpf. Zebrafish are unable to eat until about 7 days post fertilization because of the late development of the mouth and the diminishment of the yolk ball. Therefore, the most suitable way to expose them to chemical is by diluting it in the maintenance water (Katiucia *et al.*, 2011). We used a concentration of caffeine of 100 μM (about 19.4 μg ml^{-1}) in our experiment. The rationale for using this concentration was based on the absence of locomotor effects, especially in regard to the fact that this concentration does not promote significant embryo toxicity or effects on heartbeat, blood circulation, number of somites, hatching, tactile sensibility and other phenotypic features (Chen *et al.*, 2008; Selderslaghs *et al.*, 2009).

In this study, under our experimental conditions, there was no visible morphological alteration in caffeine treatment. Also,

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there was no significant difference ($P > 0.05$) between control and treatment groups in survival rate and overall hatching rate. Although it is reasonable to assume that caffeine cannot enter in complete concentration the embryo during the chorionated phase (Katiucia *et al*., 2011), we observed that a delay of hatching rate occurred at 60 and 72 hpf in the caffeine treatment with significant difference from control ($P < 0.05$). This finding is in agreement with Chiranjib *et al*. (2011), who found that increased caffeine concentration decreased the hatching rate of zebrafish embryos. Therefore, the small amount of caffeine affected the developing embryos even though the complete concentration did not reach the embryos inside the eggs.

Although previous studies found that the concentration of 100 μM caffeine did not affect the heartbeat of zebrafish larvae (Chen *et al*., 2008; Selderslaghs *et al*., 2009), we found that continuous exposure to this concentration increased the heartbeat at all durations, 48, 72 and 96 hpf, with significant difference from the control at 72 and 96 hpf.

Previous studies, in which caffeine had various biological effects on all the tested organisms, showed that caffeine induces various cell responses, including cell death (He *et al*., 2003). In our experiment, we studied the expression of some cell damage-related genes in order to determine the mechanism of caffeine toxicity after exposure at different durations during the early development stages. We selected a linked package of genes related to cell damage, including oxidative stress (SP70), mitochondrial metabolism (Cyclin G1) and apoptosis (Bax and Bcl2). Our results showed significant changes in all tested genes. Several studies reported an induced expression of these genes after exposure to different genotoxic compounds (Gonzalez *et al*., 2006; Jung *et al*., 2011). HSP70 is a molecular biomarker of cellular stress that induces denaturation of other proteins (Sanders, 1990; Lewis *et al*., 1999) because it is involved in protecting and defending cells from environmental insults (Sanders, 1990); its induction is much more responsive than traditional indices of contaminant effects (Feder and Hofmann, 1999). There is no documented evidence to show that caffeine directly provokes oxidative stress (Pin-Zhen *et al*., 2008). In our results, we offer evidence that caffeine directly provokes oxidative stress. First, we found a down-regulation of HSP70 at 24 hpf after treatment (0.84-fold). The reason of this phenomenon is that the chorionated phase prevented the complete concentration of caffeine reaching the embryos. Therefore, the small amount that entered the embryos acted as an anti-oxidant. It is known that caffeine has anti-oxidant properties if taken in moderate doses (Nikolic *et al*., 2003; Jaya *et al*. 2010). Also, it has been reported that it acts as a protective substance against cellular damage (Kamat *et al*., 2000; Krisko *et al*., 2005). Therefore, the first embryo-response to caffeine may enhance a natural tolerance after exposure to caffeine for a short time as if taken in a small amount (Kristensen, 1995; Luckenbach *et al*., 2001; Chiranjib *et al*., 2011). At 48 hpf treatment, the up-regulation of HSP70 occurred (1.35-fold increase), but with no significant difference ($P > 0.05$) from control. Highly significant up-regulation expression of HSP70 (11.75-fold increase) was found after continuous exposure to caffeine up to 72 hpf, as an indicator of the high stress that occurred to the cells of the developing embryo.

Our results agree with and confirm the results obtained by Pin-Zhen *et al*. (2008), who demonstrated that caffeine stimulates intracellular oxidative stress in human osteoblasts. One of the main targets of toxicity is mitochondria, and there is a close relationship between oxidative stress and proper mitochondrial function (Krunschnabel *et al*., 2005; Pourahmad and O'Brien, 2000; Risso-de-Faverney *et al*., 2004). The expression behavior of a gene related to mitochondrial metabolism, Cyclin G1, was also studied in order to demonstrate the role of mitochondria in the pathway of caffeine toxicity and apoptosis. Cyclin G1 is a gene that is involved in cell cycle arrest to allow time for DNA repair when DNA is damaged (Ford, 2005; Kohn, 1999). Mitochondrial membrane potential change is directly associated with apoptosis (Li *et al*., 1997; Weber *et al*., 2003). Our results showed direct up-regulation of Cyclin G1 expression with significant difference from the control immediately after exposure and at all durations – 24, 48 and 72 hpf (1.37-, 1.21- and 1.35-fold increase, respectively). The slight decrease in Cyclin G1 expression at 48 hpf treatment was not significant ($P > 0.05$), possibly because embryos can tolerate this amount (Katiucia *et al*., 2011; Chiranjib *et al*., 2011). Alternatively, zebrafish embryos may have developed a natural tolerance of toxicity from natural systems (Kristensen, 1995; Luckenbach *et al*., 2001). There is evidence that the cytotoxicity of caffeine may be due to its ability to trigger apoptosis (Fernandez *et al*., 2003). Our finding regarding the expression of Cyclin G1 may support the idea of that caffeine triggers apoptosis via a mitochondria-dependent pathway (Pin-Zhen *et al*., 2008). In order to verify this, two genes involved in apoptotic mechanisms were selected, Bax and Bcl2 (Fernandez *et al*., 2003; Sandrini *et al*., 2009). Bax and Bcl2 are the major effectors of mitochondria-mediated apoptosis. Both Bcl2 and Bax are transcriptional targets for the tumor suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage Basu and Haldar (1998)). These genes regulate changes in the mitochondrial membrane potential and permeability, which play important roles in apoptotic processes (Shimizu *et al*., 2000). Bcl2 plays an important role in apoptosis, similar to cysteine proteases that regulate mitochondrial membrane potential changes and the release of cytochrome C by modulation of the outer mitochondrial membrane permeability (Martins *et al*., 1997; Nicholson and Thornberry, 1997). It is located in the inner membrane of the mitochondria and plays an important role in the regulation of ROS production and anti-oxidation. It localizes to sites where free radicals are generated and functions as an apparent antioxidant against oxidative stress to prevent apoptosis (Hockenbery *et al*., 1993). The negative impact occurs when Bax expression increases and Bcl2 expression decreases (Basu and Haldar, 1998; Elizabeth *et al*., 2000; Miyashita *et al*., 1994). Our results showed significant down-regulation of Bcl2 with the increase of exposure time at 48 and 72 hpf. The other gene, Bax, affects mitochondria-mediated apoptosis. Bax is a pro-apoptotic Bcl2 member gene, which triggers a mitochondrial pro-apoptotic pathway by promoting the mitochondrial release of cytochrome C (Bernardi *et al*., 2001; Gottlieb 2001). The up-regulation expression of Bcl2 shows the activation of apoptotic cell death. However, many researchers have presented not expression data for both Bax and Bcl2 genes but the ratio of Bax/Bcl2 as an indicator for cell apoptosis (Jung *et al*., 2011; Basu and Haldar, 1998). The up-regulation of Bax/Bcl2 ratio shows cell apoptosis (Pepper *et al*., 1997; Pavlovic *et al*., 2006; Salakou *et al*., 2007; Jin *et al*., 2011). Therefore, the change in the Bax/Bcl2 ratio is an important issue to demonstrate the occurrence of apoptosis (Pavlovic *et al*., 2006). Specifically, a high Bax/Bcl2 ratio is associated with a lower threshold of apoptosis, while a low ratio represents a higher apoptotic threshold (Chan *et al*., 2007; Chan, 2007). Our results demonstrate that the

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Bax/Bcl2 ratio increased with the increase in exposure time in caffeine treatment and was significantly higher than the control. Thus, the results in Fig. 4 indicate a potential increase of apoptosis, because of oxidative stress, which was induced by increasing the exposure time of caffeine (Deng *et al*., 2009; Echtay *et al*., 2002; Nègre-Salvayre *et al*., 1997).

In conclusion, linking the above results together and with the previous studies, we can deduce that the increase in the exposure time of caffeine increases the heartbeat and affects the expression of cell damage-related genes of the developing zebrafish embryos and may cause damage to the cell. By analyzing the expression of all the selected genes, we can illustrate that the oxidative stress caused by caffeine exposure affects mitochondrial metabolism, which may induce cell apoptosis. Therefore, our results demonstrate that caffeine may induce apoptosis by modulating the Bax/Bcl2 ratio, the major effector of mitochondria-mediated apoptosis via the mitochondria-dependent pathway. Use of caffeine during pregnancy and lactation may affect the developing fetus and cause cell damage by affecting the expression of cell damage-related genes. Finally, zebrafish embryos as a model for examining and analyzing the negative impact of chemicals and pharmaceuticals are promising and have many advantages over other laboratory animals in this regard.

Acknowledgment

This study was partially supported by the Research Institute for Veterinary Science, Seoul National University. The authors are grateful for the scholarship provided by the Islamic Development Bank, scholar's file no. 13-EGT-P29.

References

Basu A, Haldar S. 1998. The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. *Mol. Human Reprod.* **4**(12): 1099–1109.

Bello SM, Heideman W, Peterson RE. 2004. 2,3,7,8-Tetrachlorodibenzo *p*-dioxin inhibits regression of the common cardinal vein in developing zebrafish. *J. Toxicol. Sci.* **78**: 258–366.

Bernardi P, Petronilli V, Di Lisa F, Forte M. 2001. A mitochondrial perspective on cell death. *Trends Biochem. Sci.* **26**: 112–117.

Brion F, Tyler CR, Palazzi X, Laillet B, Porcher JM, Garric J, Flammarion P. 2004. Impacts of 17 beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo, larval, juvenile and adult-life stages in zebrafish (*Danio rerio*). *Aquat. Toxicol.* **68**: 193–217.

Calvo IA, Gabrielli N, Iglesias-Baena I, Garcia-Santamarina S, Hoe KL. 2009. Genome-wide screen of genes required for caffeine tolerance in fission yeast. *PLoS One* **4**: e6619.

Carlsen SM, Jacobsen G, Vatten L, Romundstad P. 2005. In pregnant women who smoke, caffeine consumption is associated with an increased level of homocysteine. *Acta Obstet. Gynecol. Scand.* **84**: 1049–1054.

Chan WH. 2007. Citrinin induces apoptosis via a mitochondria-dependent pathway and inhibition ofsurvival signals in embryonic stem cells, and causes developmental injury in blastocysts. *Biochem. J.* **404**: 317–326.

Chan WH, Wu HJ, Shiao NH. 2007. Apoptotic signaling in methylglyoxal-treated humanosteoblasts involves oxidative stress, c-Jun N-terminal kinase, caspase-3, and p21-activatedkinase 2. *J. Cell. Biochem.* **100**: 1056–1069.

Chen YH, Huang YH, Wen CC, Wang YH, Chen WL, Chen LC. 2008. Movement disorder and neuromuscular change in zebrafish embryos after exposure to caffeine. *Neurotoxicol. Teratol.* **30**: 440–447.

Chiranjib C, Hsu CH, Wen ZH, Lin CS, Govindasamy A. 2011. Effect of caffeine, norfloxacin and nimesulide on heartbeat and VEGF expression of zebrafish larvae. *J. Environ. Biol.* **32**: 179–183.

Cook DG, Peacock JL, Feyerabend C, Carey IM, Jarvis MJ, Anderson HR, Bland JM. 1996. Relation of caffeine intake and blood caffeine concentrations during pregnancy to fetal growth: prospective population based study. *Br. Med. J.* **313**: 1358–1362.

Deng J, Yu L, Liu C, Yu K, Shi X, Yeung LW, Lam PK, Wu RS, Zhou B. 2009. Hexabromocyclododecane induced developmental toxicity and apoptosis in zebrafish embryos. *Aquat. Toxicol.* **93**: 29–36.

Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD. 2002. Superoxide activates mitochondrial uncoupling proteins. *Nature* **415**: 96–99.

Elizabeth MB, Song C, Shawn B, Dennis JJ, Jeffrey RG, Norman G, Timothy JM. 2000. The impact of bcl-2 expression and bax deficiency on prostate homeostasis in vivo. *Oncogene* **19**: 2404–2412.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**: 243–282.

Fernandez MJ, Lopez A, Santa-Maria A. 2003. Apoptosis induced by different doses of caffeine on Chinese hamster ovary cells. *J. Appl. Toxicol.* **23**: 221–224.

Ford JM. 2005. Regulation of DNA damage recognition and nucleotide excision repair: another role for p53. *Mutat. Res.* **577**: 195–202.

Frary CD, Johnson RK, Wang MQ. 2005. Food sources and intakes of caffeine in the diets of persons in the United States. *J. Am. Diet. Assoc.* **105**: 110–113.

Gonzalez P, Baudrimont M, Boudou A, Bourdineaud JP. 2006. Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). *Biol. Metals* **19**: 225–235.

Gottlieb RA. 2001. Mitochondria and apoptosis. *Biol. Sign. Recept.* **10**: 147–161.

He Z, Ma WY, Hashimoto T, Bode AM, Yang CS, Dong Z. 2003. Induction of apoptosis bycaffeine is mediated by the p53, Bax, and caspase 3 pathways. *Cancer Res.* **63**: 4396–4401.

Hockenbery D, Oltvai Z, Yin X, Milliman C, Korsmeyer SJ. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**: 241–251.

Holmberg A, Olsson C, Holmgren S. 2006. The effects of endogenous and exogenous nitric oxide on gut motility in zebrafish, *Danio rerio* embryos and larvae. *J. Exp. Biol.* **209**: 2472–2479.

Jaya RP, Prasanthi BD, Gurdeep M, Tyler L, Xuesong C, Jonathan DG, Othman G. 2010. Caffeine protects against oxidative stress and Alzheimer's disease-like pathology in rabbit hippocampus induced by cholesterol-enriched diet. *Free Radic. Biol. Med.* **49**: 1212–1220.

Jin Y, Zheng S, Pu Y, Shu L, Sun L, Liu W, Fu Z. 2011. Cypermethrin has the potential to induce hepatic oxidative damage and apoptosis in adult zebrafish (*Danio rerio*). *Chemosphere* **82**: 398–404.

Jung H, Seok SH, Han JH, Abdelkader TS, Kim TH, Park JH. 2011. Effect of fluorescent whitening agent on the transcription of cell damage-related genes in zebrafish embryos. *J. Appl. Toxicol.*; doi: 10.1002/jat.1665

Kamat J, Boloor K, Devasagayam T, Jayashree B, Kesavan P. 2000. Differential modification by caffeine of oxygen-dependent and independent effects of gamma-irradiation on rat liver mitochondria. *Int. J. Radiat. Biol.* **76**: 1281–1288.

Katiucia MC, Fabiano PM, Luiza RN, Julhana BP, Giovanna MT, De Oliveira LF, Maurício RB, Carla DB, Rosane SD. 2011. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (*Danio rerio*). *J. Neurotoxicol. Teratol.* **33**: 680–685.

Kohn KW. 1999. Molecular interaction map of the mammalian cell cycle control and DNA repair systems. *Mol. Biol. Cell* **10**: 2703–2734.

Krisko A, Kveder M, Pifat G. 2005. Effect of caffeine on oxidation susceptibility of human plasma low density lipoproteins. *Clin. Chim. Acta* **355**: 47–53.

Kristensen P. 1995. Sensitivity of embryos and larvae in relation to other stages in the life cycle of fish: a literature review. In *Sub-lethal and Chronic Effects of Pollutants on Freshwater Fish*, Muller R, Lloyd R (eds). FAO: Oxford; 155–166.

Krunschnabel G, Manzl C, Berger C, Hofer B. 2005. Oxidative stress, mitochondrial permeability transition and cell death in Cu-exposed trout hepatocytes. *Toxicol. Appl. Pharmacol.* **209**: 62–73.

Lewis R, Handy RD, Cordi Z, Billinghurst Z, Depledge MH. 1999. Stress proteins (HSP's): Methods of detection and their use as an environmental biomarker. *Ecotoxicology* **8**: 351–368.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of

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Apaf-1/caspase-9 complex initiates anapoptotic protease cascade. *Cell* **91**: 479–489.
Lovett R. 2005. Coffee: the demon drink. *New Scientist Mag.* **2518**: 577–579.
Luckenbach T, Kilian M, Triebskorn R, Oberemm A. 2001. Fish early life stage tests as a tool to assess embryotoxic potentials in small streams. *J. Aquat. Ecosyst. Stress Recov.* **8**: 355–370.
Martins LM, Kottke T, Mesner PW, Basi GS, Sinha S, Frigon N, Tatar E, Tung JS, Bryant K, Takahashi A, Svingen PA, Madden BJ, McCormick DJ, Earnshaw WC, Kaufmann SH. 1997. Activation of multiple interleukin-1beta converting enzyme homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis. *J. Biol. Chem.* **272**: 7421–7430.
Miyashita T, Krajewski S, Krajewska M, Hong GW, Lin HK, Liebermann DA, Hoffman B, Reed JC. 1994. Tumor suppressor p53 is regulator of bcl-2 and bax gene expressino in vitro and in vivo. *Oncogene* **9**: 1799–1805.
Nègre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, Penicaud L, Casteilla L. 1997. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J.* **11**: 809–815.
Nicholson DW, Thornberry NA. 1997. Caspases: killer proteases. *Trends Biochem. Sci.* **22**: 299–306.
Nikolic J, Bjelakovic G, Stojanovic I. 2003. Effect of caffeine on metabolism of L-arginine in the brain. *Mol. Cell. Biochem.* **244**: 125–128.
Pavlovic V, Cekic S, Kocic G, Sokolovic D, Zivkovic V. 2006. Effect of monosodium glutamate on apoptosis and bcl-2/bax protein level in rat thymocyte culture. *Physiol. Res.* **56**: 616–626.
Pepper C, Hoy T, Bentley DP. 1997. Bcl-2/bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. *Br. J. Cancer* **76**(7): 935–938.
Pin-Zhen L, Ching-Yu L, Wen-Hsiung C. 2008. Caffeine induces cell death via activation of apoptotic signal and inactivation of survival signal in human osteoblasts. *Int. J. Mol. Sci.* **9**: 698–718.
Pourahmad J, O'Brien P. 2000. A comparison of hepatocyte cytotoxic mechanisms for Cu^{2+} and Cd^{2+}. *Toxicology* **143**: 263–273.
Risso-de-Faverney C, Orsini N, De Sousa G, Rahmani R. 2004. Cadmium induced apoptosis through the mitochondrial pathway in rainbow trout hepatocytes: involvement of oxidative stress. *Aquat. Toxicol.* **69**: 247–258.
Salakou S, Kardamakis D, Tsamandas AC, Zolota V, Apostolakis E, Tzlepi V, Papathanasopoulos P, Bonikos DS, Papapetropoulos T, Petsas T, Dougenis D. 2007. Increased bax/bcl2 ratio up-regulates caspase-3 and increases apoptosis in the thymus of patients with myasthenia gravis. *In Vivo* **21**(1): 123–132.
Sanders BM. 1990. Stress proteins: potential as multi-tiered biomarkers. In *Environmental Biomarkers*, Shugart L, McCarthy J (eds). Lewis Publishers: Boca Raton, FL; 165–191.
Sandrini JZ, Bianchini A, Trindade GS, Nery L, Marins L. 2009. Reactive oxygen species generation and expression of DNA repairrelated genes after copper exposure in zebrafish (*Danio rerio*) ZFL cells. *Aquat. Toxicol.* **95**(4): 285–291.
Selderslaghs IW, Van Rompay AR, De Coen W, Witters HE. 2009. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. *Reprod. Toxicol.* **28**(3): 308–320.
Shimizu S, Konishi A, Kodama T, Tsujimoto Y. 2000. BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changesand cell death. *Proc. Natl Acad. Sci. USA* **97**: 3100–3105.
Ulrike L. 2003. Zebrafish: a new model on the pharmaceutical catwalk. *Bioessays* **25**: 904–912.
Weber T, Dalen H, Andera L, Negre-Salvayre A, Auge N, Sticha M, Lloret A, Terman A, Witting PK, Higuchi M, Plasilova M, Zivny J, Gellert N, Weber C, Neuzil J. 2003. Mitochondria play a central role in apoptosis induced by alpha-tocopheryl succinate, an agentwith antineoplastic activity: comparison with receptor-mediated pro-apoptotic signaling. *Biochemistry* **42**: 4277–4291.
Westerfield M. 1995. *The Zebrafish Book: Guide for the Laboratory Use of Zebrafish, Brachydanio rerio*. University of Oregon Press: Eugene, OR.
Zhu X, Zhu L, Li Y, Duan Z, Chen W, Alvarez PJJ. 2007. Developmental toxicity in zebrafish (*Danio rerio*) embryos after exposure to manufactured nanomaterials: Buckminsterfullerene aggregates (nC60) and fullerol. *Environ. Toxicol. Chem.* **26**: 976–979.

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