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Caffeine exposure causes downregulation of the expression of genes related to osteogenesis and chondrogenesis in Zebrafish (*Danio rerio*)

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Abstract

With the aim of evaluating the effects of caffeine on the expression of gene transcripts related to osteogenesis and chondrogenesis, during zebrafish embryonic development the embryos were divided into five groups: control (without caffeine), 0.25 mM caffeine, 0.5 mM caffeine, 1.0mM caffeine, and 2.0mM caffeine. Embryos were placed in cell culture plates with aquarium water at 28^{0}C for 72 hours post-fertilization (hpf). Embryonic development was followed at times of six, 12 hpf, 24 hpf, 30 hpf, 48 hpf, 54 hpf, and 72 hpf. At 72 hpf, the expression of Sox9a, runx2b, osteocalcin, osteopontin, collagen 2a1, and bmp2a was analyzed using real-time RT-PCR. The 0.25mM caffeine group showed embryonic development that was visually similar to the control group despite having a significantly lower relative expression of bmp2a, runx2b, and collagen 2a1. In the 0.5mM and 1mM caffeine groups, morphological alterations such as tail folding, pericardial edema, and yolk sac deformation were observed, and the relative expression levels of bmp2a, runx2b, and collagen 2a1 were significantly lower. In contrast, osteocalcin expression was significantly higher than in the control group. In the 2mM caffeine group, the embryos did not hatch until 72 hpf and showed tail docking and pericardial and yolk sac edema. The relative expression of bmp2a and runx2b was significantly lower, whereas that of osteocalcin, osteopontin, and collagen 2a1 did not differ from that of the control group. We conclude that caffeine altered the expression of gene transcripts related to osteogenesis and chondrogenesis in zebrafish embryos, even in embryos without visible morphological changes.

Keywords: 1,3,7-trimethyl xanthine, contaminants, embryos, fish, morphology

Introduction

Pharmaceuticals, personal care products, and industrial chemicals are being increasingly identified in aquatic environments worldwide. They have aroused increasing concern about preserving aquatic ecosystems because they can damage ecosystems, even at residual levels (21). Caffeine (1,3,7-trimethyl xanthine) is one of the most emerging contaminants in natural aquatic environments and is an indicator of anthropogenic contamination. It is an alkaloid present in coffee, tea, soft drinks, beverages, and various pharmaceutical and personal care products. Caffeine can contaminate freshwater and saltwater ecosystems through sewage, improper disposal of drugs, and medical waste. Although wastewater

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treatment is efficient for caffeine removal, it is insufficient to prevent caffeine from being transported through aquatic systems because of its relative stability under different environmental conditions and significant and increasing consumption (34, 62).

In freshwater ecosystems, caffeine concentrations have been found in concentrations of up to 1500 ng/L. The highest concentration detected in marine environments was 11,000 µg/L in the Port of Darwin, Northern Australia (25). In US marine waters, caffeine concentrations range from 2 to 5860 ng/L (62). In Europe, the highest concentrations of caffeine were found in the Aegean Sea (3068 ng/L) (39) and the Arade River in Portugal (600–804 ng/L) (27). Caffeine has also been identified in marine environments in several other countries and various aquatic organisms, including corals, algae, mussels, and fish (62).

Research has shown that caffeine present in aquatic environments can influence the health of organisms, affecting the reproduction and survival of species and, consequently, compromising the food chain of ecosystems. Caffeine concentrations similar to those found in natural ecosystems cause adverse effects in several aquatic species maintained under laboratory conditions (62). Embryos of *Palaemonetes pugio* shrimp exposed to 20 mg/L caffeine for five days showed delayed development and egg hatching (26). The effect of caffeine exposure (0.5–18 µg/L) on the regenerative capacity of the polychaete *Diopatra neapolitana* was evaluated, demonstrating a significant delay in regeneration with reduced formation of new segments (41). Growth inhibition was observed in marine algae when *Isochrysis galbana* was exposed to 100 or 500 mg/L of caffeine (2). Polychaetes represented by *Hediste diversicolor, D. neapolitana*, and *Arenicola marina,* when exposed to caffeine concentrations between 0.5 and 18.0 µg /EU for 28 days, showed 8.3%, 12.5%, and 22.2% of lethality, respectively (41,42).

In the presence of caffeine, aquatic organisms increase their metabolic activity and reduce their energy reserves, such as glycogen, which are used to combat oxidative stress and prevent cell damage (15, 34, 41, 42). In addition to stimulating oxidative stress, caffeine can trigger apoptosis, as evidenced by the increased expression of Bax and Bcl2 (1). Exposure to caffeine, associated with other emerging pollutants, also alters the expression of genes in zebrafish that are used as markers of exposure to pollution (58). However, several questions remain regarding the genomic responses of aquatic organisms exposed to caffeine. For example, studies on the effects of caffeine on the expression of genes that control and participate in the development of aquatic organisms, including bone and cartilage development, are lacking.

Our team has been studying the effects, in mammals, of caffeine consumption by mothers on the bones of their offspring for several years. These studies have shown that caffeine inhibits postnatal bone growth and alters *in vivo* and *in vitro* expression of several gene transcripts related to osteogenesis and chondrogenesis (48-52). It has also been shown that caffeine consumption by the mother can have teratogenic effects in the offspring (12). However, the effects of caffeine on embryogenesis have been poorly studied in mammals (65). In rats, because the endochondral bone formation is intrauterine, studies aiming to evaluate the effect of caffeine on this process supply the drug to the mother so that it reaches the fetus transplacentally. However, the lack of control over the amount of caffeine that reaches the fetus is the main limitation in using mammals as models to study the effects of caffeine or any other drug on endochondral bone formation. In this context, zebrafish are a more suitable model for studying bone changes resulting from failures in the bone formation process during embryonic development.

Despite the Zebrafish being widely used as a model to study the genesis of several human diseases, including cardiovascular, neuromuscular, and bone diseases (9, 11, 13, 36, 40, 46) and also be used as a model for studying the action of various drugs, including pollutants such as caffeine (2, 37, 43, 54), this seems to be to the best of our knowledge the first study that evaluated the effects of caffeine on the expression of gene transcripts related to osteogenesis and chondrogenesis, during zebrafish embryonic development.

Material and Methods

The procedures described below were approved by the Animal Use Ethics Committee of *Universidade Federal de Minas Gerais* under protocol nº. 115/2016.

Zebrafish maintenance and egg collection

Adult Zebrafish AB (*Danio rerio*) were raised in rearing rack systems using a filter system and water recirculation. The animals received granulated feed three times a day (4, 32) and were ordered in a regime of 12 hours of light and 12 hours of dark (13) at 28 ±1°C. The pairs were placed in breeding tanks overnight so that newly laid eggs could be collected the next morning. Unfertilized or dead eggs were removed. Embryo staging was performed as described by Kimmel et al. (30).

Caffeine exposure

Fertilized eggs (embryos/5hpf) were collected and randomly divided into five experimental groups (128 eggs/embryo per group). Group 1: control (without caffeine); Group 2:0.25 mM caffeine; Group 3:0.5 mM caffeine; Group 4:1 mM caffeine; and Group 5:2 mM caffeine. The embryos were placed in 6-well cell culture plates containing water from the aquarium where spawning occurred and placed in greenhouses at a temperature of 28ºC for up to 72 h post-fertilization. In the exposed groups, caffeine (Sigma-Aldrich,

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St. Louis, MO, USA) in different concentrations was added to the water (35, 47, 61), and the embryos were exposed by immersion at 6 hpf. The medium was changed once daily (29).

External morphological changes in developing zebrafish embryos after caffeine exposed were carefully examined under a stereomicroscope (Stemi 508, Zeiss) at 6, 12, 24, 30, 48, 54, and 72 hpf, and images were captured using a digital camera (Axiocam 105 color, Zeiss).

Evaluation of gene transcript expression by real-time RT-PCR

The larvae of the five groups, 72hpf, were submitted to RT-PCR in real time for the quantitative evaluation of the expression of Sox9a, runx2b, osteocalcin, osteopontin, collagen 2a1 and bmp2a through protocols previously established (31). Larvae were macerated in Trizol. Specific PCR primer sequences were obtained from GenBank.

Gapdh: *forward*: CGCTGGCATCTCCCTCAA, *reverse*: TCAGCAACACGATGGCTGTAG (10.1111/j.1745-7270.2007.00283.x.); EFLa1: *forward*: CTGGAGGCCAGCTCAAACAT, *reverse*: ATCAAGAAGAGTAGTACCGCTAGCATTAC (10.1111/j.1745-7270.2007.00283.x.); collagen 2a1: *forward*: CCTCTGAAATCCAGCCATGT, *reverse*: GACTGCTGTGGTTCCAGTCA (https://doi.org/10.1016/j.gep.2015.07.004); bmp2a: *forward*: AGTGTTAGGACGACGCAGC, *reverse*: CGGCCTCATTTGAGACCAC (doi:10.1016/s0378-1119(97)00292-8.); runx2b: *forward*: ATGGCCGAGATCATCGCCGATCAC, *reverse*: CGGGGCCACCTGGTTCTTCATAACC (doi.org/10.3389/fendo.2017.00125); sox9a: *forward*: CGGTGAAGAACGGCCAGAGC, *reverse*: CTGTAGAGTCAGCAATGGGT (doi.org/10.1006/dbio.2000.0129); osteocalcin: *forward*: TGGCCTCTATCATCATGAGACAGA, *reverse*: CTCTCGAGCTGAAATGGAGTCA (doi:10.1016/j.jot.2015.07.002); osteopontin: *forward*: GCCACCTCCTATTCTTCG, *reverse*: TGCCTCCTCAGTGTCATCT (doi: 10.1007/s00223-013-9817-4).

Subsequently, lysis and homogenization were performed for 5 min at room temperature to completely dissociate the nucleoprotein complexes. The lysate was transferred to a 1.5mL microtube, with the addition of 0.2mL of chloroform, being homogenized for 15 seconds and incubated for 3 minutes at room temperature, followed by centrifugation at 12,000g for 15 minutes at 4°C, aiming to separate into three phases, the colorless and superficial phase, which contains the RNA. The aqueous phase was transferred to a new tube, with the addition of 0.5mL of isopropyl alcohol and incubation for 15 minutes at room temperature, followed by centrifugation at 12,000g for 10 minutes at 4°C for RNA precipitation. The pellet was washed with 1mL of 75% ethanol, homogenized, and centrifuged at 7,500g for 5 minutes at 4°C. The RNA was solubilized in RNAse-free water and immediately stored at -80°C. The quality of the RNA samples was analyzed using spectrophotometry.

Reverse transcription reactions were performed using a commercial Taqman reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA). PCR was performed using 2µg of cDNA, 600nM of each primer, and 17µL of Syber Green reagent (Applied Biosystems, Foster City, CA, USA) in a final volume of 25µL of reaction in a 7900HT Fast Real-Time PCR System. The optimal conditions for the real-time PCR of the amplified cDNAs were determined in preliminary assays. The results obtained for each group were quantitatively compared after normalization based on GAPDH and EFLa1 danio rerio expression. The analysis was performed in quadruplicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0.2 software to statistically compare the experimental values between the control and each treatment group. The study design was randomized. Means and standard deviations were determined. Averages were compared using the Student's t-test. Data were tested for normality and homoscedasticity. Differences were considered statistically significant at $P < 0.05$.

Results

Effect of caffeine on the external appearance of developing zebrafish embryos/larvae

As previously described, normal embryonic development occurred in the control group (Fig. 1). At 6 hpf, the yolk sac, blastoderm, and chorion were observed. At 12 hpf, the beginning of the division of the embryonic planes (displacement of the head from the tail) was observed, concomitant with the appearance of the yolk syncytial layer. At 24 hpf and 30 hpf, the optic placode, pericardial cavity, somites, notochords, and olfactory placodes were observed. At 48 hpf stage, the embryo begins the process of melanization, the optic placode gives rise to the eyes, and the heart is observed. At 54 hpf the embryos started to hatch. At 72 hpf, all animals hatched were active and maintained a constant swimming position with spontaneous movement.

There was no significant difference in embryonic development in the exposed group with 0.25 mM of caffeine compared to the control group. The formation and development of embryo body structures followed the same temporal dynamics observed in the control group (Fig. 1). Spontaneous movement was unaffected by caffeine.

Morphological deformities in the embryos were observed in the 0.5 mM caffeine group at 12 hpf, where only the yolk sac, blastoderm, and chorion were observed. There

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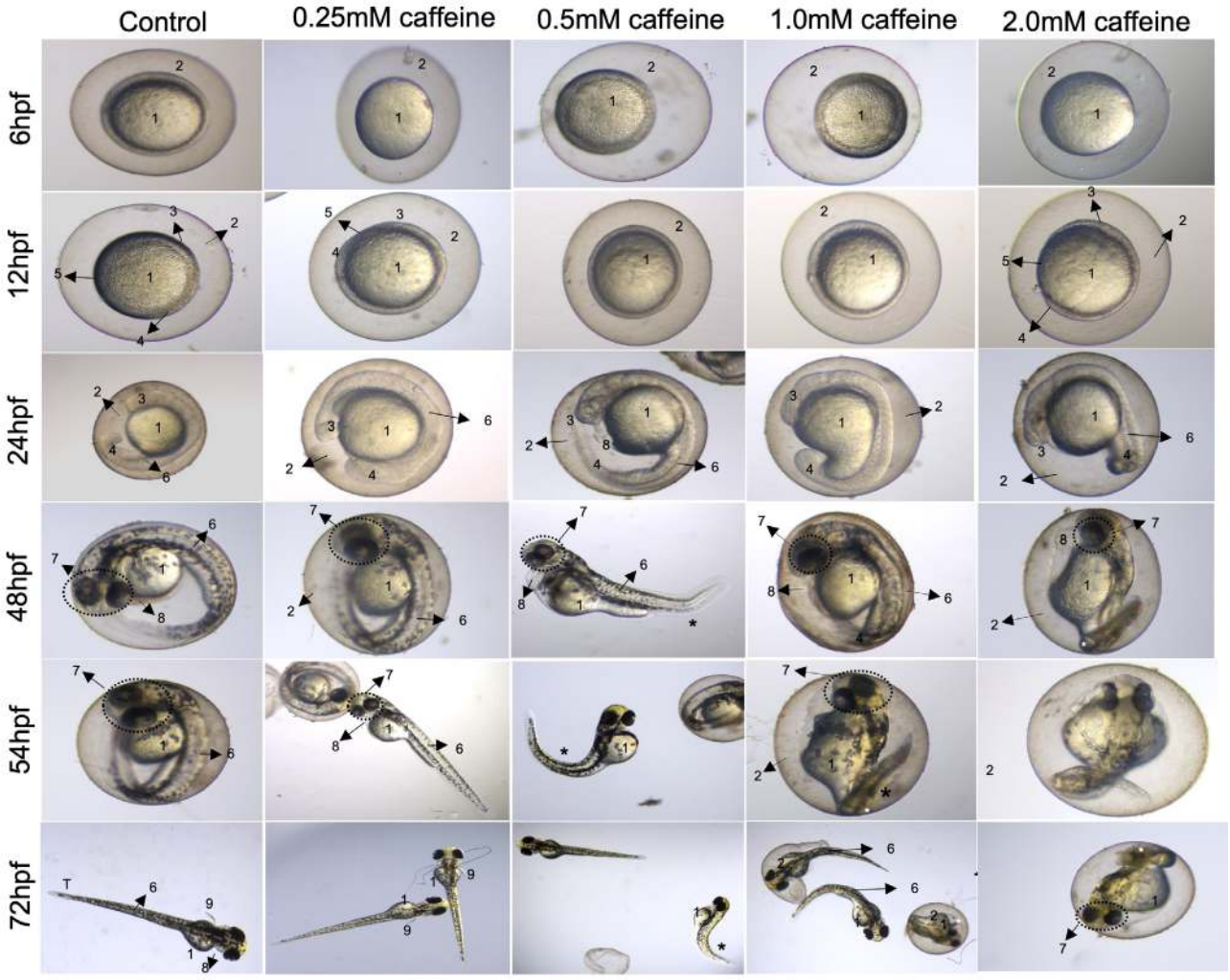

Figure 1. Gross morphological changes after caffeine treatment. The embryos were exposed to 0.25, 0.5, 1, and 2 mM caffeine from 6 to 72 hours post fertilization (hpf).1: yolk sac; 2: corion; 3: head region; 4: tail region; 5: yolk syncytial layer; 6: notochord; 7: eye; 8: heart; 9: fin; T: tail. * axial-tail curvature; white arrow: pericardial edema; and red arrow: yolk sac malformations (edema).

was a delay at the beginning of the division of the embryo planes, which was different from that observed in the group with 0.25 mM of caffeine. However, at 24 hpf and 30 hpf, the optic placode, pericardial cavity, somites, notochords, and olfactory placodes were observed. At 48 hpf and 54 hpf, the embryos began the melanization process, the optic placode gave rise to the eyes, and the heart was observed. Some embryos began to hatch, and some animals displayed significant axial tail curvature. At 72 hpf, all animals hatched, and larvae without deformation were active in the swimming position with spontaneous movement. However, larvae with an axial tail curvature remained in a side-to-side position with less movement.

In the 1 mM group, external morphological malformations were more evident. Embryonic development was similar to that observed in animals treated with 0.5 mM up to 48 hpf. At 54 hpf, the animals did not start hatching and exhibited significant axial tail curvature, pericardial edema, and yolk sac malformations (edema). At 72 hpf, these malformations were observed in all animals, such as those that hatched and those that did not (Fig. 1).

In the 2mM group, external morphological malformations were more evident. Embryonic development was similar to that observed in animals treated with 1 mM. Significant axial tail curvature, pericardial edema, and yolk sac malformation (edema) were observed as early as 48 hpf. At 72 hpf, the animal did not hatch (Fig. 1).

Expression of transcription factors associated with osteogenesis and chondrogenesis

Embryos exposed with 0.25 mM caffeine showed significantly lower relative expression of bmp2a, runx2b, and

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collagen 2a1 compared to the control group; however, there was no significant difference in the expression of osteocalcin, osteopontin, and Sox9a (Fig. 2). In the 0.5 mM caffeine group, the relative expression levels of bmp2a, runx2b, and collagen 2a1 were significantly lower than those in the control, while osteocalcin expression was significantly higher. No significant differences were observed in the expression of osteopontin and sox9 (Fig. 2). In the 1 mM caffeine group, the relative expression levels of bmp2a, runx2b, and collagen 2a1 were significantly lower than those in the control group, and the osteocalcin expression was significantly higher. No significant differences were observed in the expression of osteopontin and sox9a (Fig. 2). In the 2mM caffeine group, the relative expression of bmp2 and runx2 was significantly lower, whereas that of osteocalcin, osteopontin, sox9a, and collagen 2a1 did not differ from that of the control group (Fig. 2).

Discussion

Based on the results presented, the genomic effects of caffeine on the expression of gene transcripts related to chondrogenesis and osteogenesis can occur independently of embryonic morphological changes; that is, the addition of caffeine to the medium containing zebrafish embryos can alter the expression of gene transcripts, even without altering embryonic morphology.

This result was visible at 0.25 mM caffeine, which significantly reduced the expression of bmp2a, runx2b, and collagen 2a1, without altering the embryonic morphology, as observed at higher concentrations of caffeine. Caffeine significantly reduced the expression of bmp2a and runx2b transcripts at all concentrations. At concentrations lower than 2 mM, there was also a reduction in collagen 2a1. The transcriptional alteration of genes involved in chondrogenesis and osteogenesis promotes a better understanding of the mechanisms involved in changes in bone development caused by caffeine.

The caffeine concentrations were determined based on previous studies that tested similar concentrations in zebrafish (16, 22, 45, 60) and rat chondrocytes. In addition to changes in the notochord and tail, the embryonic morphological changes found at concentrations of 0.5 mM, 1 mM, and 2 mM caffeine were not directly related to the process of chondrogenesis and osteogenesis but reaffirmed the toxic and teratogenic potential of caffeine (9, 45, 46, 59). Alterations in gene transcripts related to caffeine-induced chondrogenesis and osteogenesis during embryonic development have not been previously described. One of the interesting results of this study is the evidence that caffeine at a concentration of 0.25 mM can alter the profile of these gene transcripts in apparently healthy embryos.

Caffeine-induced changes in the expression of gene transcripts without phenotypic changes are interesting because some studies have shown that zebrafish embryos descending from parental exposure to environmental pollutants may exhibit developmental abnormalities, even if they did not occur in the parents. These subsequent generational effects, mediated by mothers and fathers exposed to toxicants, may occur due to the bioaccumulation and transfer of toxicants in adult zebrafish germ cells. However, there is evidence that developmental changes occur through epigenetic modification

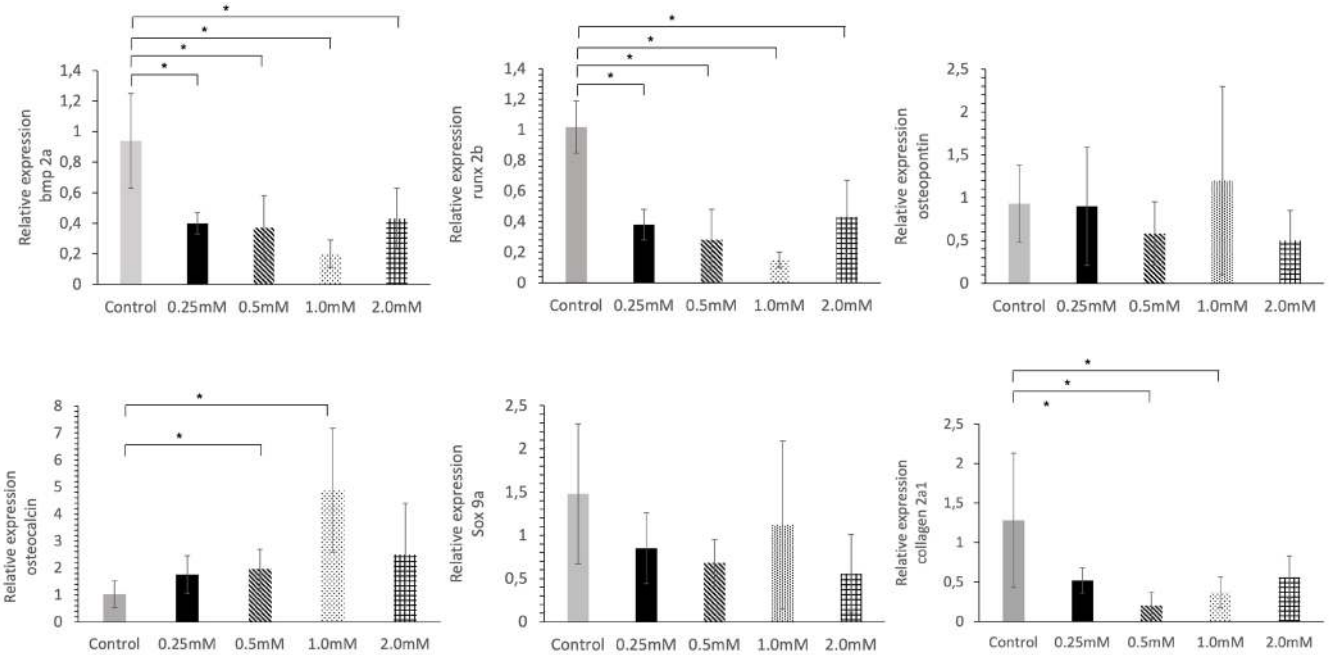

Figure 2. Expression of gene transcripts (mean ± standard deviation) for bmp2a, runx2b, osteopontin, osteocalcin, sox9a, and Col21a by the RT-qPCR in zebrafish embryos treated with 0.25; 0.5; 1, and 2 mM caffeine at 72hpf. p≤0.05*

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of genes transferred from F0 to F1 larvae (67). Thus, complementary transgenerational studies are required to verify whether the transcriptional changes induced by caffeine can be passed on to subsequent generations.

The effect of caffeine on gene expression has been studied in the embryo, as chondrogenesis and osteogenesis begin in the embryonic phase of mammals and teleosts. In zebrafish, this process begins during embryonic development before hatching. The zebrafish skeleton has a simple organization composed of cartilage and bone, with the craniofacial cartilage being one of the first to form during development. The first elements, represented by the branchial arch and opercular bones, develop 48 h after fertilization, generating a fully functional cartilaginous skeleton on the fifth day after fertilization, that is, two days after hatching of the larva (23, 30, 55, 64). Research on gene deletion in zebrafish has shown that several genes, including those studied here, such as runx2ab, sox9a, collagen 2a1, and bmp2a, also participate in chondroblast differentiation in the craniofacial region of zebrafish, similar to that in mammals (10, 24, 44, 63).

All caffeine concentrations used significantly reduced gene transcript expression for runx2b. Runx2 is an essential transcription factor for osteoblast, chondrocyte differentiation, and cartilage and bone development in vertebrates (17). In mammals, runx2 has two distinct functions: one is essential for the differentiation of mesenchymal progenitor cells into osteoblasts, and the other stimulates the differentiation of hypertrophic chondrocytes (14) and is therefore considered a key regulator of osteogenesis (8).

In zebrafish, the runx genes exhibit a hierarchy of functions during skeletogenesis. runx2b plays a dominant role in differentiating pre-cartilaginous cells into chondrocytes. Cephalic endochondral osteogenesis begins 48 h after fertilization via focal mesenchymal condensation (55). At this stage, both runx2a and runx2b are expressed in the primordia of dermal and cartilaginous bones. However, unlike mammals, runx1 is not important in the early stages of chondrocyte differentiation. In contrast, runx2b depletion seriously compromises the formation of the craniofacial ethmoid cartilage (24). Therefore, it is possible that the reduction in expression of the gene transcript for runx2b caused by all caffeine concentrations subsequently determines some type of alteration in the bones that develop from the structures in which it is expressed.

Caffeine effects on runx2 expression in mice por rat differ from those in zebrafish. Osteoblasts extracted from the calvariae of rats born to mothers who received caffeine during pregnancy significantly increased runx2 expression. Increased runx2 expression is associated with increased osteoblastic activity and the expression of other gene transcripts such as osteocalcin, osteopontin, sialoprotein, and collagen 1 (50). However, the expression of runx2 in chondrocytes extracted from rats born to mothers who received caffeine during pregnancy did not differ from that in the untreated group (control) (52). The effects of caffeine on mammalian and zebrafish expression of runx 2 differ, possibly due to several reasons besides the inherent differences in animal species. In rats, the effect of caffeine has been studied after birth (50, 52) but not during embryonic development, as in the present study. Furthermore, in studies by Reis et al. (50, 52), caffeine was transferred to the fetus through the placenta, and the effect of caffeine was studied on a specific population of cells, in this case, osteoblasts, and chondrocytes.

In addition to reducing runx2b expression, all the caffeine concentrations significantly reduced gene transcript expression for bmp2a. Bmp2 plays an important role in regulating osteoblast proliferation and differentiation. In zebrafish, bmp2a and bmp2b are primarily expressed in calcified tissues (38). Knockdown of the antisense morpholino oligonucleotide gene mediated by bmp2a causes a delay in early bone development and decreases the expression of osteogenic genes, such as runx2a (28). Mutations in bmp2a in zebrafish compromise complete rib formation and cause vertebral deformities (66). It is suggested that the reduced expression of the bmp2a transcript may be one of the reasons why zebrafish larvae show defects, such as tail curling. However, this hypothesis warrants further investigation.

At caffeine concentrations lower than 2 mM, there was a reduction in the transcript of the gene encoding collagen 2a1. Collagen is the most abundant constituent of the chondrogenic matrix. During chondrogenesis in mammals, the expression of different types of collagens occurs temporally according to the level of cell differentiation, with the initial expression of collagen type 1 by undifferentiated cells, followed by collagen type 2 by differentiated cells, and finally, by collagen type 10 in mature cells (5, 56, 57). In zebrafish, no studies have demonstrated the temporal profile of collagen expression during development. However, 2a1 collagen gene expression has been identified in the craniofacial cartilage, ear, notochord, floor plate, hypochondrium, and zebrafish fin. In this fish, 2a1 collagen expression is a marker of cartilage and notochord development (18). It is likely that, in addition to the reduction of the gene transcripts for runx2b and bmp2a, the reduction of the transcript for collagen 2a1 is also involved in the genesis of the alterations caused by caffeine, not only in the skeletal system but also in other tissues and organs containing this type of collagen.

None of the caffeine concentrations studied altered the gene transcript expression for sox9a. Sox9 stimulates the differentiation of chondroprogenitor cells into chondroblasts (19, 33). The inactivation of sox9 in mice results in the absence of expression of chondrogenic markers, such as collagens 2, 9, and 11, and aggrecan, which prevents cartilage formation (7). Like mammals, sox9a and sox9b are regulators of cartilage development and are necessary for the expression of collagen 2a1; therefore, they are essential for endochondral bone formation in zebrafish (17). Based on the results of the present study, it can be concluded that at the concentrations studied and up to 78 h after fertilization, caffeine did not alter the expression of sox9a.

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However, additional studies are required to verify whether caffeine alters the expression of this transcript in the later stages of post-embryonic development. In mammals, we found antagonistic effects of caffeine on sox9 expression in chondrocytes. The addition of caffeine to a culture medium with cartilaginous epiphyses of the femurs of newborn rats significantly increased the expression of the transcript for sox9 in chondrocytes (48). In contrast, the articular cartilage of rats born to mothers who received caffeine during pregnancy significantly reduced sox9 expression (52).

None of the caffeine concentrations significantly altered osteopontin expression. However, treatment with 1 mM caffeine significantly increased osteocalcin expression. In mammals, osteopontin is expressed during cell proliferation and at the beginning of mineralization (68), promoting the premature precipitation of calcium phosphate crystals in the collagen matrix (20). Osteocalcin, on the other hand, is found later in the course of the bone mineralization process (3, 53). In zebrafish, although osteocalcin is expressed in the vertebral bodies, one of the osteocalcin isoforms, oc1, is expressed in the notochord before osteoblastic differentiation and bone formation. Thus, it is likely that oc1 is involved in processes before bone formation, that is, in the mineralization of the notochord sheath.

The notochord is important in the early stages of life because its mechanical function is only replaced by the vertebral column in the post-embryonic phase (6). Based on this study, it is difficult to understand why only 1 mM caffeine significantly increased osteocalcin expression, despite significantly reducing the expression of transcripts for bmp2, runx2, and collagen 2. The implications of this caffeine-induced increase in osteocalcin expression on notochord mineralization and subsequent skeletal development require further study. Osteoblasts extracted from the calvariae of rats born to mothers who received caffeine during pregnancy showed a significant increase in osteocalcin expression. However, contrary to the results, increased osteocalcin expression was associated with increased expression of other gene transcripts, such as osteopontin, sialoprotein runx-2, and collagen 1 (50).

Conflict of Interest

The authors declare no competing interests.

Acknowledgments

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