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Effect of diclofenac on *bmp1* gene expression and its influence on bone distortion in sailfin molly *Poecilia latipinna* (Lesueur, 1821)

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Abstract

Importance of the work: Diclofenac (DCF) causes bone deformities when animals are exposed for a long period. The effect of DCF on bone genes was investigated.

Objectives: To identify the malformations of affected bone and the level of gene expression after treatment with DCF.

Materials & Methods: Sailfish molly (*Poecilia latipinna*) fish were exposed to DCF at concentrations of 0.5 mg/L and 1 mg/L for 14 d. Morphological abnormalities were detected using radiological examinations. Quantitative real-time polymerase chain reaction was used to detect *bmp1* expression after DCF exposure. Phenotypic deformities were observed in the jaws, spine, eye socket and iris, as well as hemorrhagic areas behind the eye and in the head.

Results: The mean (± SD) relative expression levels of *bmp1* were 1.053 ± (0.02), 0.760 ± (0.03) and 0.595 ± (0.05) following 0 mg/L, 0.5 mg/L and 1 mg/L DCF treatments, respectively ($p < 0.05$). Considering the sex, these values for mature males were 1.016± (0.01), 0.720± (0.03) and 0.616 ± (0.02) ($p < 0.05$) while those in mature females were 1.042 ± (0.03), 0.735 ± (0.12) and 0.625 ± (0.15), respectively ($p < 0.05$).

Main finding: DCF has effects on bone remodeling in the jaws and other bone parts due to the disturbance in osteoclast function affected by bone gene disturbance.

Introduction

Diclofenac (DCF) is an important drug used to reduce pain, inflammation and stiffness caused by many conditions, such as osteoarthritis, rheumatoid arthritis and arthritis that commonly affect the joints (Francio et al., 2017). It is widely used and its annual production has been estimated in the hundreds of tonnes for use in tablets, capsules, suppositories and intravenous solutions, as well as in ointments and gels for dermal application (Buser et al., 1998).

A study in Finland reported found that the level of DCF in the aquatic environment was at concentrations of less

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than a microgram per liter (parts per trillion; ppt) and it is one of the substances under surveillance in the European Union (Vieno and Sillanpää, 2014). Since DCF is usually excreted in the waste from patients undergoing treatment, the primary sources of environmental influx are effluents from wastewater treatment systems (Fatta-Kassinos et al., 2011), It is generally dissolved in water and likely absorbed in soil and clay sediments; consequently, DCF is dispersed widely in freshwater environments but at detected concentrations between 1 ng/L and 1 mg/L, based on a study conducted at the Natural Sciences and Engineering Research Center in Canada (Lonappan et al., 2016). As DCF is in the aquatic environment, it forms part of the food chain of aquatic organisms and subsequently may cause health damage after a long period of exposure. The skeletal formation is a vital developmental process in the embryonic stages of vertebrates when skeletal growth and development are completed (Grünbaum et al., 2012). The developmental process includes the differentiation and proliferation of different types of cells, with one of the most active being the chondrocytes and osteoblasts that are responsible for bone formation; specifically, osteoclasts digest excess and useless bone according to bone shape, size and mineral composition (Fernández et al., 2011).

Studies of bone gene expression reflect the level of skeletal development and cell proliferation and differentiation; however, many factors can be involved, such as individual genetic characteristics, biotic factors and biotic components, whereas abiotic components include water, soil, air, temperature and sunlight, with all of these factors playing major roles in genetic activity so that knowledge of bone gene expression during skeletal formation is useful for determining possible causes of skeletal malformation (Cloutier et al., 2010).

Bone morphogenetic proteins (BMPs) are members of growth factors and are also defined cytokines, belonging to the family of transforming growth factor-β (TGF-β) (Chen et al., 2004). BMP is considered one of the functional proteins involved in regulating tissue development (Alshami and Saud, 2021). It was discovered that *bmp* are specific molecules that stimulate bone formation after implantation in rodent muscle (Chen et al., 2004), with *bmp*s being conserved genes that are highly structural in all animals and their biological role is reflected by the functional and structural redundancy of different *bmp*s in the same species (Yang et al., 2016). In general, the BMP1 and BMP2 proteins can stimulate osteoblasts and play an important role in bone fracture repair (Grgurevic et al., 2011). Both the BMP2 and BMP4 proteins are involved in skeletal formation, particularly in the differentiation of chondrocytes to form cartilage, and in both cell differentiation and maturation in the progenitors of bone-forming cells (Wan and Cao, 2005). During *bmp* activity, the pathways for cartilage and bone formation are initiated *in vivo*; this activity leads to a series of processes, including chemotaxis, proliferation and differentiation in cells, which result in transformation of cartilage into living bone tissue complete with hematopoietic marrow (Wozney et al., 1988).

Many studies have been conducted on *bmp* gene expression in different fish species, focusing on the embryonic stage of development (Palomino et al., 2014). The expression of *bmp* genes after the hatching stage and their potential biological significance was studied in farmed fish, such as Atlantic salmon, *Salmo salar* larvae (Ytteborg et al., 2010) and European seabass, *Dicentrarchus labrax* larvae (Villeneuve et al., 2006). Fish model species are used for genetic and molecular studies for their characteristics, such as ease of rearing in the laboratory, available genome references and short time of generation (Saud et al., 2021).

The sailfish molly, *Poecilia latipinna* (Lesueur, 1821), was chosen as the model species in current study. It is native to the northeastern coasts of the USA and South America (Markle, 2015). It was introduced to many countries and has been recorded in 29 countries mainly in Asia, Oceania and Central and South America (Koutsikos et al., 2018). *P. latipinna* has been introduced to some countries in the Middle East, such as Iraq, where it has been recorded in the southern marshes (Hussain et al., 2009), *P. latipinna* is considered an ornamental fish and can survive in a temperature range of 20–26 °C and a pH range of 7.5–8.5. *P. latipinna* is found in many aquatic environments such as ponds, swamps and rivers, where it can withstand harsh environmental conditions (Coad, 2017).

The current study aimed to detect the effect of the diclofenac drug on skeletal malformations of fish and via genetics through testing the bone morphogenetic protein (*bmp*) gene.

Materials and Methods

Diclofenac sodium

Diclofenac sodium (DCF, 98% pure) was obtained from the General Company for Pharmaceutical Industries and Medical Appliances (Samarra, Iraq).

Fish breeding

The molly fish used in this study were collected from the city of Qurna, Iraq and transferred to the laboratories of the College of Agriculture at the University of Basra for this study, where they were placed in glass tanks supplied with dechlorinated water (pH = 7.2–7.4, salinity and 2.5 ppt oxygen flow during the experimental period at 7 mg/ L) at 28 ± 2 °C.

Exposure to diclofenac

Two concentrations (0.5 mg/L and 1 mg/L) of DCF (98% pure) were prepared by dissolving the DCF in deionized water to make up the treatment solutions.

At the beginning of the experiment, 24 mature fish (aged 3 mth, length 12–15 cm) and 24 young fish (aged 20 d, length 20 mm) were placed in 30 L tanks and acclimated for 7 d to their new environment (glass tanks), after which they were constantly exposed to either 1 mg/L or 0.5 mg/L DCF for 14 d. The water treated with the drug was completely replaced with similar treated water every 24 h. The survival rate and external symptoms were recorded throughout the experiment. The fish were fed twice daily on artificial suspension pellets prepared in the laboratory of the Fish Culture Unit of the College of Agriculture. The dead fish resulting from exposure to the drug were fixed in 10% neutral formaldehyde for further image processing.

Photography and radiography

Morphological analysis was carried out using 16 individual fish. Photographs were taken under a dissecting microscope (Mariobrunoroma Srl; Rome, Italy) for detecting any skeletal malformations, X-ray images were taken using 2.5 mA and 40 kV for 90 s in a soft X-ray machine (CR30-X; Baghdad, Iraq).

Quantitative real-time polymerase chain reaction technique

Design of primers

Primers for the expression analysis of *bmp1* were designed according to sequences available in GenBank (Sequence ID: XM_015029074.1). Forward and reverse primers were designed using the Primer3 Plus software (Primers were supplied by the Korean macrogen company). The size of the amplified *bmp1* gene was 242 bp. The primer sequences are shown in Table 1.

Total RNA extraction

Jawbone and fin tissue (approximately 100 mg) were taken and kept in liquid nitrogen until the RNA extraction process. Total RNA was extracted using an AddPrep Total RNA extraction kit (Add Bio Inc.; Addbio, Korea). The DNA was removed by DNase I at 37 °C for 15 min. The quality and quantity of the total RNA were detected using a nanodrop spectrophotometer (Analytik jena, Germany) with the absorbance degree at 260/280 nm.

cDNA synthesis

The extracted RNA was converted to cDNA to amplify the targeted genes for real-time polymerase chain reaction (RT-PCR) analysis. Reverse transcription was carried out using an AddScript cDNA Synthesis Kit (Add Bio Inc.; (Addbio, Korea) according the following protocol: 10µL 1X RT master mix was centrifuged for 5 s, then, the master mix was diluted with 3µL molecular water. After that, 2 µL of Random Hexamer 10X primer was added and then 5µL of RNA template for each sample was added to the mixture. The final mixture was centrifuged for 5 s and transferred to a PCR thermocycler and incubated at 25 °C for 10 min followed by the reverse transcription at 50 °C for 60 min. The reaction was terminated by incubation at 80 °C for 5 min.

Quantitative real-time polymerase reaction

Quantitative RT-PCR of cDNA samples was performed using an AddScript cDNA Synthesis Real-Time PCR kit run in the Mx3005P Real-Time PCR System (Agilent; USA), with some necessary modifications, specifically, 20 µL reaction mixture containing 1X qPCR Master Mix, 10 pM each of forward and reverse primers and 320 ng of the first-strand cDNA.

The relative expression level of *bmp1* was estimated using *eef1a1* as the reference gene. Quantitative real-time PCR for *bmp1* and *eef1a1*was performed with the following thermal cycling program: hot-start activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The dissociation was performed at 72 °C for 2 min.

Table 1 Primer sequences of *bmp1* and *eef1a1*

Gene name	Primer sequence
bmp 1	F : 5'- GTGCAAGTCTGAGCTGAACG-3' R : 5'- GGTAACGGCTCTCTGTCGAG -3'
eef1a1	F: 5'-GACACCTCATCTACAAGTGTG-3' R: 5'-GTTTGTCCGTTCTTGGAGATGC-3'

Real-time polymerase chain reaction data analysis

The ΔCT method using the reference gene 2-ΔΔCt as developed by Livak and Schmittgen (2001) was used to analyze the data generated using the quantitative RT-PCR, as shown in Equations 1–3:

$$\Delta Ct\ (test) = Ct\ (target, test) - Ct\ (ref, test) \quad (1)$$

$$\Delta\Delta Ct = \Delta Ct\ (test) - \Delta Ct\ (calibrator) \quad (2)$$

$$Fold\ change = 2^{-(\Delta\Delta Ct)} \quad (3)$$

Statistical analysis

The data were analyzed using the Statistical Analysis System software (SAS, 2012) to conduct one-way analysis of variance to identify significant differences between treatments using Duncan's 1955 multinomial test and applying the least square means method. Results were presented as the mean ± SD.

Ethics statements

The fish were anesthetized using cloves (400 mg/L) before conducting any physical examinations. The animal experiments carried out conformed to the Regulations for Animal Care and Use for Scientific Research of the University of Basrah.

Results

Diclofenac and fish mortality

At the beginning of the experiment, 12 pairs of adult fish and 6 juvenile fish were exposed to DCF to evaluate their tolerance to different concentrations and to measure the survival rate and determine non-fatal concentrations. They were on low numbers of fish death recorded during the period of exposure (14 d). There was mortality with both DCF concentrations (0.5 mg/L and 1 mg/L); however, it was greater with 1 mg/L than with 0.5 mg/L and greater in males than females. The differences in the death ratio between the sexes were not clear (Table 2).

Morphological defects due to diclofenac

During the exposure period (14 d), the treated fish showed many phenotypic abnormalities, representing deformities in the jaws, curvature of the spine and damage to the fins, with some losses of the complete dorsal fin. Furthermore, bleeding in the caudal fin and operculum was observed. In the eyes, increased pigmentation was observed, with some individuals having the whole pupil darker, while in another case, eye socket erosion appeared. Damage in the operculum was observed (Fig. 1).

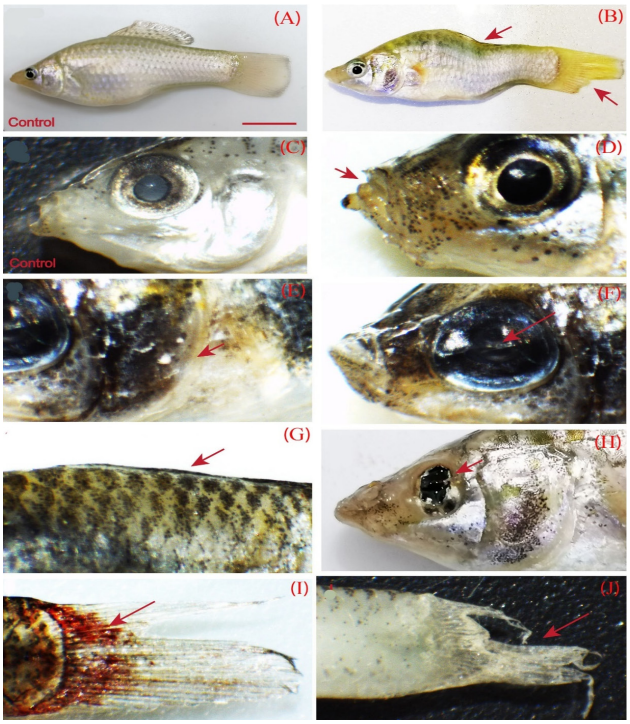

Fig. 1 Morphological deformation of sailfin molly caused by diclofenac treatments: (A) side view of untreated fish (control); (B) bending of the spine (0.5 mg/L treatment); (C) normal jaws (control); (D) damaged jaws (1 mg/L treatment); (E) incisions in operculum (0.5 mg/L treatment); (F), deformation of eyes (scotoma) and retraction of upper jaw (1 mg/L); (G) dorsal fin loss (0.5 mg/L); (H) eye deformities (1 mg/L treatment); (I) hemorrhage in caudal and peduncle fins (0.5 mg/L treatment); (J) caudal fin erosion (1 mg/L treatment), where scale bar = 5 mm and arrows indicate abnormal appearances.

Table 2 Number of surviving and dead fish exposed to 0.5 mg/L or 1mg/L of diclofenac

Treatment (mg/L)	Number of fish (male +female)	Number of small fish (20 mm)	Mortality (number)		
			Male	Female	Small fish
Control	12	6	0	0	0
0.5	12	6	0	0	1
1	12	6	3	1	4

Radiological examination of fish treated with diclofenac

Radiographic imaging was taken of fish exposed to 0.5 mg/L or 1 mg/L DCF for 14 d. The radiological examination clearly showed the skeletal structure with malformed parts of bones, clearly visible as curvature of the spine (Figs. 2B and 2F) and jaw abnormalities and fin erosion (Figs. 2D and 2F, respectively), in addition malformation in the gas bladder (Figs. 2B and 2F) and calcification in some vertebrae (Fig. 2E).

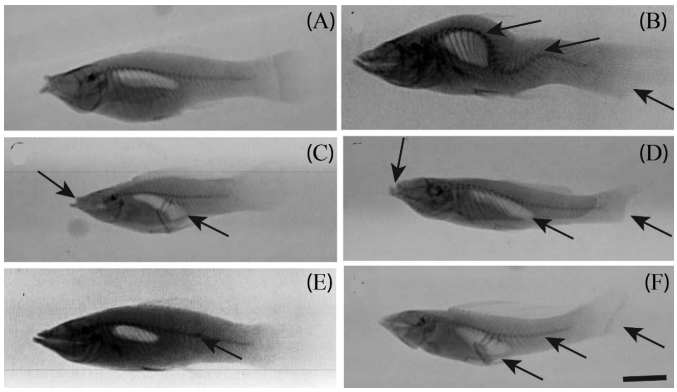

Fig. 2 Radiographic images of sailfin molly caused by diclofenac (DCF) treatment: (A) control fish; (B) fish treated with DCF (0.5 mg/L) showing spinal curvature and caudal fin erosion; (C) deformation of upper jaw and gas bladder (1 mg/L treatment); (D) deformation of upper jaw and gas bladder and caudal fin erosion (0.5 mg/L treatment); (E) calcification in vertebrae (1 mg/L treatment); (F) deformation in gas bladder and caudal fin erosion with spinal curvature (1 mg/L treatment), where scale bar = 5 mm and arrows indicate abnormal appearances.

Gene expression of bmp1 in treated sailfin molly with diclofenac

The current results indicated that there were significant ($p < 0.05$) differences between the control and treatments in the *bmp1* relative gene expression for small fish (20 mm) and for male and female mature fish during the 14 d of exposure. Relative gene expression levels in small fish for 0.5 mg/L and 1 mg/L were 0.760 ± (0.03) and 0.595 ± (0.05), respectively, compared to the untreated fish level of 1.053 ± (0.02) (Fig. 3A). In male sailfin molly fish, relative gene expression levels for *bmp1* were 1.016 ± (0.01), 0.720 ± (0.03) and 0.616 ± (0.02) for the control, 0.5 mg/L and 1 mg/L respectively (Fig. 3B). Relative gene expression levels for *bmp* in female sailfin molly were 1.042 ± (0.03), 0.735 ± (0.12) and 0.625 ± (0.15) for the control, 0.5 mg/L and 1 mg/L respectively (Fig. 3C). In all treated fish groups, DCF suppressed *bmp1* expression and the suppression was more with 1 mg/L DCF, confirming the effect of the drug on the level of *bmp1* expression.

Discussion

DCF is classified as a non-steroidal anti-inflammatory drug and is used for the treatment of inflammation, as a painkiller and to lower a fever (Parolini, 2020). The current study sought to reveal the side effects of this drug at the molecular level, and to describe morphological distortions, in sailfin molly *P. latipinna* as a target species. The current research confirmed

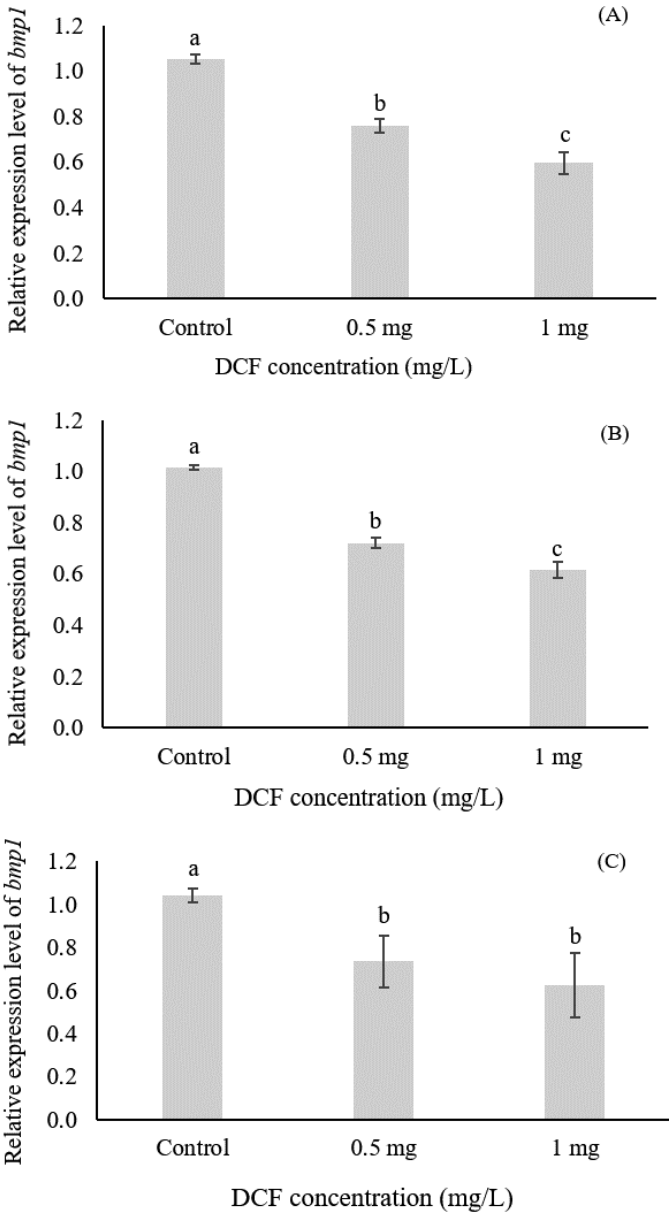

Fig. 3 Relative expression level of *bmp1* (mean ± SD) in sailfin molly fish treated with 0.5 mg/L or 1 mg/L DCF: (A) small fish; (B) male fish; (C) female fish where different lowercase letters above bars indicate significant ($p < 0.05$) differences among means and error bars indicate SD.

that DCF has a clear effect on skeletal tissue. The *bmp* gene is involved in growth and maturation of osteoblast cells and their regeneration in bone tissue (Yokota et al., 2017). The *bmp1* activity analysis showed a decrease in its expression in sailfin molly exposed to DCF at 0.5 mg/L and 1mg/L concentrations for fish of different size and sex compared to the control. A study on medaka (*Oryzias latipes*) fish detected morphological defects due to DCF at concentrations 0.608 µg/L, 2.15 µg/L, 7.29 µg/L, 26.5 µg/L and 94.8 µg/L (Yokota et al., 2018).

Manzano-Moreno et al. (2018) reported that therapeutic doses have a physiological effect on osteoblasts without causing any cellular abnormalities (necrosis), on osteoblasts. BMP is an important differentiating factor capable of inducing bone regeneration through the enhancement of endochondral ossification by chemotaxis, migration, proliferation and differentiation of mesenchymal stem cells, with potential role as a stimulating agent for new bone growth as well as in the treatment of bone fractures and defects and in bone formation (Rosen, 2009).

NSAIDs are reversible nonselective inhibitors that function by inhibiting cyclooxygenase enzymes (Costela-Ruiz, 2019). *Bmp1* has major role in osteoblast growth and differentiation, by targeting genes in the pathways of the *runx-2*, and *osx9* and genes, which have a regulation role in the differentiation of osteoblasts (Grgurevic et al., 2011; Long, 2012). Studying the genes affected by these treatments contributes to complementing knowledge on the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the molecular, cellular and functional processes of osteoblasts (Luna-Bertos et al., 2015). In addition, such studies have clarified the mechanisms that underlie the effects of NSAIDs on these bone-forming cells.

Several studies, such as Morthorst et al. (2013), have indicated that treatments with NSAIDs significantly reduce the production of prostaglandins (PGs). In zebrafish, at the cellular level, PGs have a direct effect on osteoclasts by increasing their cleavages and functional activity, leading to increased bone resorption (Lin et al., 1995). On the other hand, PGs can exert an anabolic effect on bone by increasing osteoclast proliferation and differentiation; with this dual role, they can protect the balance between bone resorption and bone formation (Kawaguchi et al., 1995). The exact mechanism of PGs on bone cells is not yet clear; however, it has been found that PGE2 regulates *bmp2*, *bmp7* and *rank1* gene expression (Arikawa et al., 2004). Coetzee et al. (2007) indicated that PGE2 increases the number of osteoclasts by suppressing apoptosis without a direct effect on cell proliferation. PGs act through their binding action in a variety of receptors on the cell surface. For example, the G-protein-c receptor family involves coupling of the EP1, EP2, EP3 and EP4 subtypes, although the role of each receptor has not been fully explored (Sugimoto and Narumiya, 2007). PGE2 binds to EP4 and can stimulate osteoclast genesis and osteoblast differentiation, with the role of the EP2 and EP4 receptors emerging through their inactivation, which in turn led to defects in bone metabolism (Zhang et al., 2011).

The current results showed that the use DCF led to deformities in the bones of fish, with these distortions represented by curvature of the spine, fin erosion, loss of some ribs and abnormalities in the jaws and eyes. DCF had a toxic influence during treatment and affected abnormal bone growth through its effect on osteoblasts and osteoclasts (Yokota et al., 2017). BMP regulated the activation or inactivation of gene transcriptional for bone growth and bone differentiation (Bonilla-Claudio, 2012). DCF reduced *bmp* expression by blocking the binding of the receptors that regulate bone growth and differentiation and affect bones, with deformities occurring in different areas of the skeleton (Melguizo-Rodríguez et al., 2018). DCF also reduced gene expression of *runx2*, *col1*, *osx* and *osc* cultivars in direct association with bone maturation and differentiation (Luna-Bertos et al., 2013).

Mechanisms on the effects of the drug may be explained by the inhibition of osteoclast activity, as it has been indicated that it inhibits the process of bone formation and calcium replacement or calcium deposition in bones. For example, in a study on the offspring of pregnant mice treated with the drug, underdevelopment and deformity of a cleft palate occurred (Shahin et al., 2011). Significant bone retardation, growth retardation and abnormalities in skeletal elements were due to the exposure of mice to DCF, through the inhibition of NF-kB signaling, a transcription factor that consists of a complex protein that control the transcription process of targeting genes activating osteoclasts in mice (Kotake et al., 2010). Some enzymes associated with the presence of osteoclast cells play an important role in bone remodeling by degrading bone to begin bone remodeling, including the enzyme tartrate-resistant acid phosphatase (TRAP). This enzyme inhibits the work of osteoclasts cells; thus, enzyme activity is very low after exposure to the drug compared to untreated fish (Nemoto et al., 2007). another study indicated the effect of DCF on the differentiation and activation of stem cells responsible for the formation of red blood cells, along with a decrease in the concentration of the TRAP enzyme, in the bones of mice (Oka et al., 2012).

Conclusion

The study confirmed that DCF affected bone remodeling and subsequently disturbed the osteoclast function that was affected by bone gene disturbance.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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