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The effect of different diclofenac doses on bones repairing and *col1a1* gene expression in *Poecilia latipinna* (Lesueur, 1821)

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ABSTRACT

Poecilia latipinna sailfish molly was exposed to diclofenac (DCF) at concentrations of 0 (control), 0.5, and 1.0 mg/L for 14 days with changing the water in which the DCF was daily dissolved during the experimental period. Morphology abnormalities in the jaws and spine, as well as erosion of the dorsal and caudal fins, were noted in the treated fish. Gene expression analysis showed a decrease in *col1α1* gene expression in treated fish with diclofenac. The *col1α1* fold change, for the analysis of fish with a total length of 20mm (0, 0.5 and 1.0mg/L), recorded values of 133± (0.04 a), 0.75 ± (0.01b), and 0.53± (0.01c), respectively. This showed a significant value (P<0.01), in comparison with the control group. For the adult fish, fold change of *col1α1* showed values of 1.38± (0.24 a), 0.83± (0.18 ab), and 0.47± (0.09 b) for the treatments 0, 0.5, and 1.0 mg/L, respectively, showing a significant decrease (P<0.05) in comparison with the control, although there was no significant difference between the diclofenac treatment at the concentration of 0.5 mg/L and the control treatment. In addition, the gene expression of female fish decreased significantly (P<0.05) through the treatments of 0.5 and 1.0 mg/L, compared to the control. Whole-mount of Alizarin and Alcian staining were used to discover the deformation of the skeleton showing the effect of DCF on bone remodeling in vertebrae, ribs between bones, cartilage, and the fins of fish.

INTRODUCTION

Diclofenac (DCF) is medical drug that has chemical formula $C_{14}H_{11}Cl_2NO_2$, its molecular weight = 296,148 g/mol and known commercially as voltaren (**da Silva Lyra *et al*., 2011**). DCF is one of the most widely used non-steroidal anti-inflammatory drugs (NSAIDs) in the world (**Aus der Beek *et al*., 2016**). The annual global consumption of DCF was estimated around 940 tons (**Zhang *et al*., 2008**).

Diclofenac as a pharmaceutical is used for many purpose including : anti-inflammatory, antipyretic, analgesic, and anti-rheumatic, it was first circulated in the UK in 1979, and used to treat the pain and inflammatory diseases such as gout (**Uzzaman *et***

***al*., 2021**). DCF is taken orally or rectally in the form of a suppository or by injection or applied on the skin, however it shows some side effects such as kidney, digestive, and cardiovascular injuries to humans and other organisms, and it also causes gastrointestinal bleeding if it is consumed at high dose for a long period and as a result, it has toxic effect on liver, in addition, it inhibits prostaglandin synthesis by blocking cyclooxygenase (**Uzzaman *et al*., 2021**).

In the aquatic environment, it was found that the level of DCF reached at concentration less than μg /L, and it is one of substances that were included in the European Union under surveillance (**Vieno and Sillanpää, 2014**). DCF is usually excreted to the sewage by patients undergoing treatment, then to aquatic environmental and some absorbed into the soil, so it is wide spread in freshwater environments, and it was detected that its concentration reached at levels ranged between 1 ng/L to 1 mg/L in Niagara River Natural Sciences and Engineering Research Center in Canada (**Metcalfe *et al*., 2003; Lonappan *et al*., 2016**).

Current study focused on the effect of DCF on bones defection through studying the activity of *col1a1* gene in charge of the cartilages and bones formation.

Collagen is the main protein in connective tissues, muscles, skin, ligaments, cartilage, and bones; it constitutes a large proportion of up to 25% of all proteins in mammals and some other species. Collagen proteins have a long fibrous structure and their function is different from globular proteins and other enzymes; collagen proteins together form firm bundles known as collagen fibrils (**Orgel, 2009**).

Collagen is a large, diverse family, dominating the external cytoskeleton, it constituent molecules collagen type I that is the most abundant fibrous collagen in vertebrates, encoded by two genes, *col1a1* and *col1a2* expressing α1 (I) and α2 (I) protein chains, respectively, in zebrafish (*Danio rerio*) (**Gistelinck *et al*., 2016**). There are three types of collagen genes, *col1a1a*, *col1a1b*, and *col1a2* encoding α1 (I), α3 (I) and α2 (I) chains, respectively, during early developmental stages, these types of collagen genes exhibited a similar spatio-temporal expression, indicating co-regulation and co-expression among them, production of glycosylated protein is necessary and a sign of normal cells during cell development, and this include all type of collagen, for example adult bone, skin, and scales contain equal amounts of α1 (I), α3 (I), and α2 (I) chains (**Gistelinck *et al*., 2016**).

Collagen plays an important role in the formation of the actinotrichia that is fibrils at the tips of the fins has role during their growth and regeneration (**Zhang *et al*., 2010**). The rays of fins are one of the basic structural parts that were formed through combining the actins (the non-calcified transparent membrane of Actinotrichia fibers between the fin spines) in the basement membrane of the epidermis, and the spines that are segmented

and calcified bony rays (**Zhang *et al*., 2010**). Microscopic examination in many fish species indicates that these fibers are hyper polymerized collagen (**Montes *et al*., 1982**).

Collagens Col2a1, Col1a1, and actinodin proteins engage in the skeletal structure of actinotrichia proteins that form radial filaments at the tips of the fins during fin growth and regeneration (**Konig *et al*., 2018**). Fish are the most familiar organisms in the study of molecular biology, and drug side effects. A unique fish models are used to study the relationships among genes and to find the effect of drug as inhibitor in some genes (**Alshami and Saud, 2021**). The sailfin molly, *Poecilia latipinna* is a rather small fish; its maximum length is 12.5 cm in length, sometimes may reach less than 15 cm in length (**Rohde *et al*., 1994**). The total length of Molly fish in Iraqi water reaches 7.5 cm in adult, while in adult females, total length reaches 15 cm and its length ranged from 10-12 cm in adult males in in southern Iraq (**Ghazwan, 2020**). Its body is oblong with a dorsally flattened head and superior mouth. Males have a modified anal fin called gonopodium, which is used as an insertion organ for reproduction. The male has a large sail-shaped dorsal fin with a distinctive color, the body color generally turns green and gray to black, with some rows of brown spots may mix on the sides together to create a striped appearance on the dorsal side (**Abu El-Regal and Al- Solami, 2020**). Some models can be used for molecular studies for their characters such as genome reference availability (**Saud *et al*., 2021**). Sailfin molly have the ability to reach sexual maturity in a short period of time not exceeding three months, and can tolerance harsh environmental conditions, so that it is considered a good model for genetics studies (**Schedina *et al*., 2018**). There is no local study on the concentration of DCF in Iraqi water. Sub-lethal concentrations were used in current study. The study goal is to find out the effect of DCF on skeletal bones through examination the bone morphology and collagen gene activity in fish.

MATERIALS AND METHODS

Drug source and preparation

Diclofenac sodium (DCF, concentration of 98%) was obtained from the General Company for Pharmaceutical Industries and Medical Appliances (Samarra, Iraq). DCF stock solutions (1.0 mg/L and 0.5 mg/L) were prepared by dissolving DCF powder in deionized water.

Fish breeding

Fish were reared in aquariums (30 cm × 30 cm × 30 cm) supplied with aerator system. Standard commercial pellets were used for feeding two times a day in the morning and afternoon. The water of tanks was being replaced weekly with new dechlorinated fresh water. Some parameters of water condition were measured during time of experiment

such as pH (7.2- 7.4), salinity (2-2.5 ppt), oxygen (5 mg/L) and water temperature (28 °C ± 2 °C).

Exposure with diclofenac

Fish were acclimated firstly in glass tanks 30-liter capacity for seven days after that they were constantly exposed to 1.0 mg/L or 0.5 mg/L of DCF during 14-days. The water of treatments was completely being replaced with new one every 24 hours. Survival rate and external symptoms were recorded through period of experiment. Samples were fixed in 10% neutral formaldehyde for staining and imaging process Above concentrations were used depending on tested doses and from our test 1.5mg/L was severe and lethal. Similar concentrations (0.5 and 1.0 mg/L) to current study were used by **Ajima *et al*.(2021)**.

Whole mount staining

Whole-mount staining for samples was applied depending on **Darias *et al.* (2010)** with some modifications describing below:

Washing: The samples (fish) were washed with tap water every 2 hours for one day in order to remove the fixative solution completely. .

Bleaching: Samples were transferred to bleaching solution consisting of 2% KOH and 3% hydrogen peroxide (1 volume of hydrogen peroxide and 9 volumes of potassium hydroxide), for 3 to 7 days (depending on fish size) until all dark spots disappeared, and skin color becomes yellow.

Cartilage staining: Samples were transferred to Alcian blue dye (10 mg of Alcian blue 8GN dye dissolved in a solution consisting of 80 ml of 95% ethyl alcohol + 20 ml of glacial acetic acid 50% for 28-24 hours).

Washing: Samples were washed with distilled water for 2-3 hours to remove excess dye.

Clearing: Clearing of fish is done by using saturated sodium borate solution. The solution is prepared by dissolving 5 g of borate in 150 mL of non-ionic water then placed it in water bath 55-60° C, then 0.2 mL of saturated borate was taken from stock solution and dissolved in 70 mL of distilled water and finally 1g of trypsin enzyme was mixed with the solution. Samples were incubated with sodium borate solution for 20 hours or more (depending on fish size) until the samples become transparent, as their bones can be clearly observed by eye.

Bone staining: Samples were transferred to Alizarin Red-S dye. Stock stain solution was prepared by dissolving 100 mg of alizarin dye in 5 mL of glacial acetic acid of 50% concentration + 10 mL of glycerin + 60 ml of Chloral hydrate at a concentration of 1%.

From Stock stain solution 1.0 mL was taken and added to 100 ml of KOH 2% concentration to prepare working stain that incubated with samples for 1-5 hours.

Washing: Sample was washed with 2% KOH for 2 minutes to remove excess dye.

Dehydration: The sample was incubated in the following increasing series of (2% KOH: Glycerol) 1:1, 1:3, 100% Glycerol

Stocking: Stained fish were preserved in 100% glycerol.

Quantitative real-time PCR technique (qRT-PCR)

Primers design:

The primers for the *col1α1*and housekeeping gene *eef1a1* were designed based on genome reference of *P. latipinna* in National Center for Biotechnology Information (NCBI) website. Forward and reversed primers were designed using Primer3 Plus software (Table 1).

Table 1. Primer Sequence of *col1α1*and *eef1a1*

Gene name	Primer sequence
col1α1	**F: 3'- AACATGGACACCGGAGAGAC-'5**
	R: 5'- CATGAGACGCAAGAAGGTCA-'3
eef1a1	**F: : 3'-GACACCTCATCTACAAGTGTG-5'**
	R: 5'-GTTTGTCCGTTCTTGGAGATGC-3'

Total RNA extraction

Approximately 100 mg tissue of jawbones and fins were taken and kept in liquid nitrogen until RNA extraction process beginning. Total RNA was extracted using the kit supplied by the Korean company Add Bio Inc. Product Code: 10119. Quality and quantity of total RNA were detected using Nanodrop Spectrophotometer (Thermo Scientific, ND-2000) at absorbance degree 260/280 nm.

cDNA synthesis

The extracted RNA was converted to cDNA to amplify the targeted genes for RT-PCR analysis. PCR process was carried out using Korean reagents (Accupower Rockscript RT Premix kit) and according the following protocol: 10 µL 20X RT AddScript Master mix was centrifuged for 5 seconds, then diluted with 3 µL molecular water after that 2 µL of Random Hexamer 10× primer was added to master mix then 5 µL of RNA template was

placed to the mixture. The final mixture was centrifuged for 5 seconds and transferred to PCR machine and operated under the condition referred in Table 2.

Table 2.The PCR condition of synthesis cDNA

Stage	Tm (°C)	Time	Cycles
Priming	**25°C**	**10min**	**1X**
Reverse transcription	**50°C**	**60min**	**1X**
RT inactivation	**80°C**	**5min**	**1X**

Quantitative Real-Time PCR (qRT-PCR)

RT-PCR of cDNA samples was performed using the Accupower Green Star Real-Time PCR kit and Exicycler™ 96 Real-Time Quantitative Thermal Block supplied by the Korean company Pioneer, with some necessary modifications, as shown in Table "3". SYBR® Green in a RT-PCR PreMix detection kit was used for amplification of the cDNA of the *col1α1* gene, using the designed primers and the *eef1a1* gene as a conservative gene (standard) to quantify the number of copies produced by PCR compared to the number of copies of the resulting standard curve of the resulting genome. The qPCR, the SYBR® Green-dye in the Kit binds to the target gene and the conservative gene, and then the fluorescent signals are recorded by the Real-Time PCR Thermo cycler.

Table 3. The real time PCR reagent materials used in the experiment

Components	Concentration	Volume (20µl)
GoTaq qPCR master mix, 2X	**1X**	**10 µl**
Forward primer	**10 µM/µl**	**2µl**
Reverse primer	**10 µM/µl**	**2 µl**
ddH_2O	-	**3.6 µl**
cDNA templete	**80 ng**	**2µl**

RT-PCR for *col1α1and eef1a1* process was subjected to the condition referred in Table 4.

Table 4. qRT-PCR condition for the *col1α1* gene and housekeeping gene

Stage	Tm (°C)	Time	Cycles
RT inactivation/ Hot-start activation	**95°C**	**10min**	**1X**
Denaturation	**95°C**	**10 sec.**	**40X**
Annealing/ collect data	**60°C**	**30 sec.**	**40X**
Extension	**72°C**	**30 sec.**	**40X**
Dissociation	**72°C**	**2 min**	**1X**

Real-time PCR data analysis

The ΔCT method using a reference gene 2-ΔΔCt which was developed by **Livak and Schmittgen (2001)** was used to analyze the data generated by the PCR in quantitative Real-time, as shown in equations below:

ΔCt (test) = Ct (target, test) – Ct (ref, test)

ΔCt (calibrator) = Ct (target, calibrator) – Ct (ref, test)

ΔΔCt= ΔCt (test) – ΔCt (calibrator)

Fold change = 2^-(ΔΔCt)

Statistical Analysis

The data were statistically analyzed using the program **Statistical Analysis System-SAS (2012)** to test the significant differences between the means using **Duncan's (1955)** multinomial test by applying the Least square means method.

RESULTS

The morphological defection by DCF

During the 14-days of exposure period, the treated fish showed many phenotypic abnormalities. The abnormalities represented by a regression in the jaw area (shortening) of the upper jaw of fish (Fig.1.B), and part of it were lost, especially the upper and lower jaws (Fig.1. D), compared with the control (Figs.1.A and C). Furthermore, deformation and curvature of the spine were observed, especially in the trunk area, some fish appeared clear and significant curvature in their spines (Figs. 2. B and D). Other deformation

showed weakness or erosion in the vertebrae of the spine close to the dorsal fin figure (2. E). In addition to a deformation of the rib bones or fusion of three vertebrae in the trunk (Fig.2.F). There were erosion of the fins, this extended to include loss part of the caudal fin (Fig. 3. B), and loss of ossification of the caudal fin rays (Fig. 3.D). Deferent shapes of damages occurred in caudal fin for example: fracture, deformation, and loss of ossification were happened (Fig. 3. E), sometime part of ossification occurred in anal fin (Fig. 3. H), There was also loss of the dorsal fin bones associated with the rib bones of the spine (Fig. 3. G). Malformation in gonopodium (reproductive organ) of the male fish was observed, which included impairment in the thickening in organ rays (Fis. 4. B and D), as well as the incorporation of the three small bones that support the anal fins (Figs. 4 A and C).

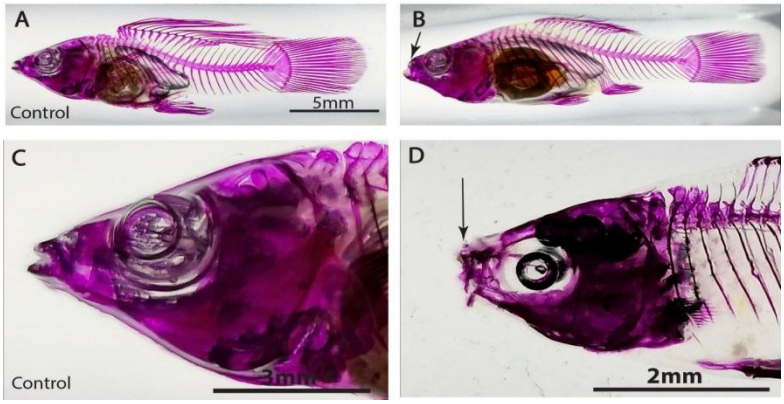

Fig. 1. Alizarin and Alcian stains in molly fish bones , shows jaws distortions in molly fish treated with diclofenac at concentrations 0.5 and 1.0 mg/L. (B and D), A and B normal jaws for untreated fish (control). Retraction upper jaw (B) and completely damage the mouth (D), scale bars: 5 mm in A and B , 3 mm in C and 2 mm in D.

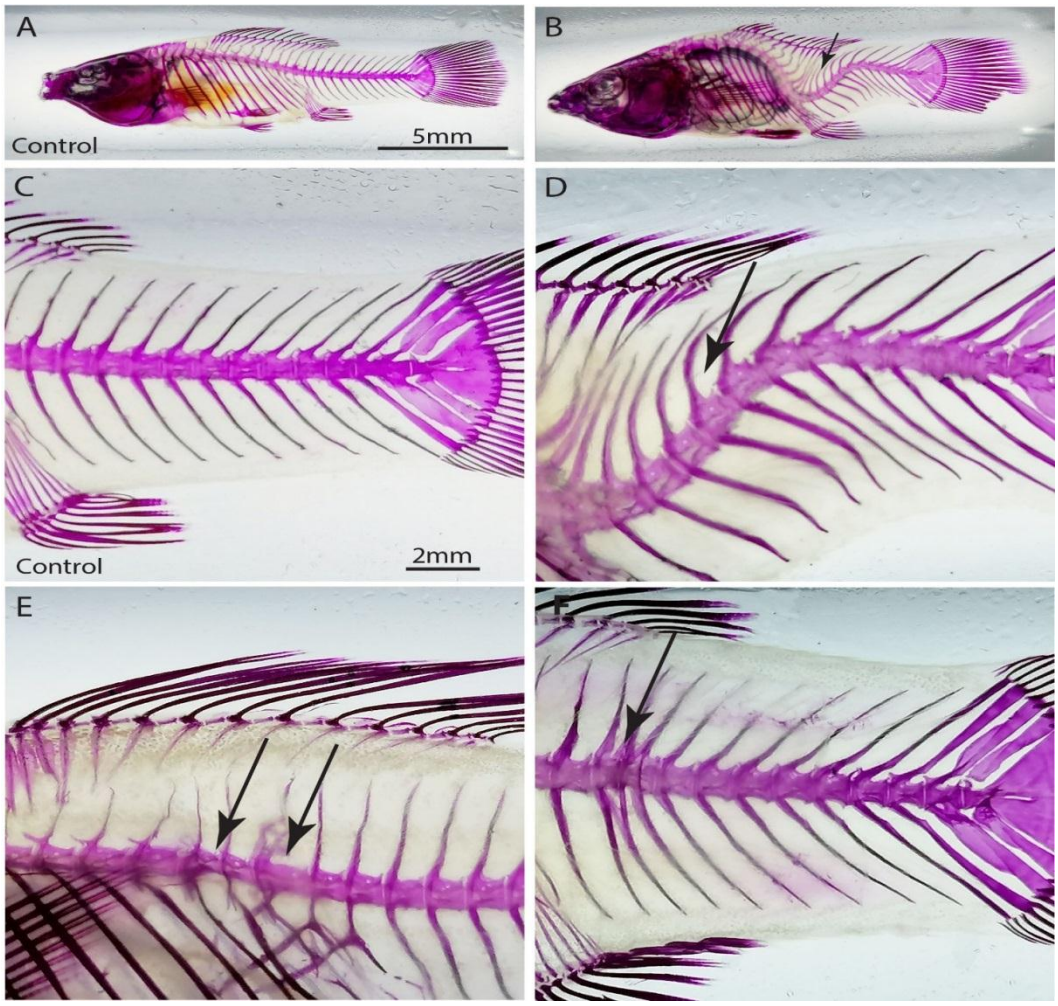

Fig.2. Alizarin and Alcian stains, shows spine defection treated molly fish with diclofenac at concentrations 0.5 and 1.0 mg/L. A and C untreated fish (control); E, disappearance of spine ribs; D, drooping in spine; F, distortion and fusion of vertebrates. Scale bars: 5 mm in A and B; 2 mm in C-F.

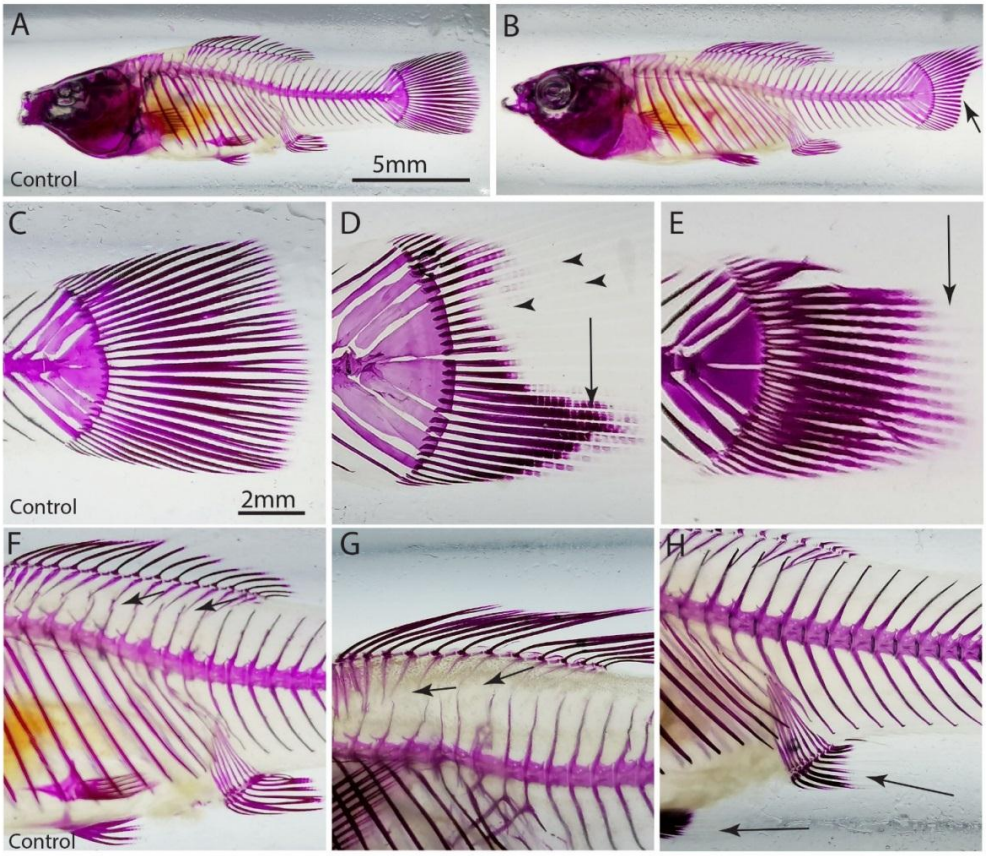

Fig.3. Alizarin and Alcian stains of molly fish bones. A, C and F untreated molly fish (control); D and D, Caudal fin affected by DCF (0.5 or 1.0 mg/L) arrows represent damages in caudal rays and arrow heads represent non-calcified caudal rays; G, loss of articulated ribs with vertebrae of dorsal fin; H, non-calcified pectoral and pelvic fins. Scale bars: 5 mm in A and B, 2mm in C- H.

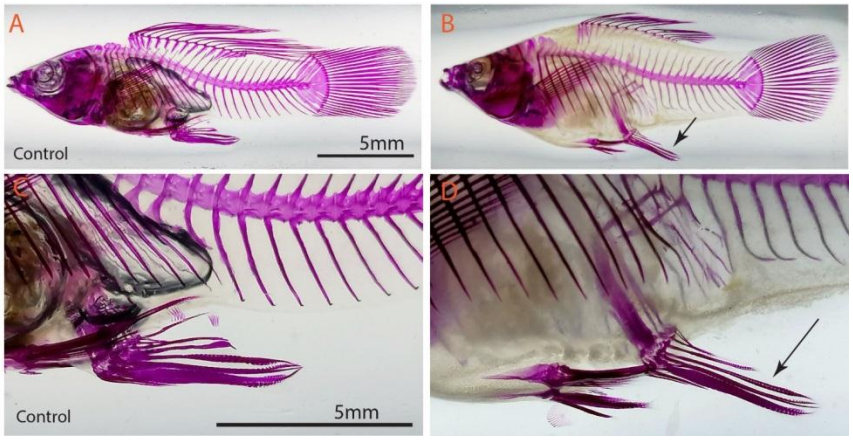

Fig.4. Alizarin and Alcian stains of molly fish bones. A and C untreated fish (control). Arrows represent the abnormal gonopodium in molly fish treated with DCF (0.5 or 1.0 mg/mL). scale bars: 5 mm in A and B; 5mm in C and D.

Gene expression of *col1α1* in treated sailfin molly with diclofenac

Table "5" shows the fold change of bmp gene expression for young fish (20 mm) r during 14 days of exposure period. There are significant differences (P<0.01) control and treatments. Fold change of treatments (0.5 mg/L and 1.0 mg/L) were 0.75± (0.01b) and 0.53±(0.01c), respectively, compared with untreated fish 1.33±(0.04a). From the previous results, DCF has made suppression of *col1α1*gene expression and more suppression has been observed in 1.0 mg/L of DCF.

Table 5. Represents the *col1α1* gene expression between untreated and treated fish with diclofenac at concentration of 1.0 mg/L and 0.5 mg/L for small fish (20 mm length) (mean ± standard error).

DCF treatment	CT	HKG	Δct	ΔΔct	Fold change
Control	**22.48 ±0.48**	**23.10 ±0.49**	**0.62 ±0.04**	**0.42 ±0.04**	**1.33 ±0.04 a**
0.5 mg	**23.09 ±0.50**	**22.89 ±0.51**	**0.20 ±0.01**	**0.40 ±0.01**	**0.75 ±0.01 b**
1 mg	**22.49 ±0.49**	**21.78 ±0.61**	**0.70 ±0.02**	**0.95 ±0.02**	**0.53 ±0.01 c**
Level of Sig.	-	-	-	-	**
	The lower case means that there are significant differences between the transactions ** (P≤0.01).				

Table 6 shows the gene expression levels in male sailfin molly fish. The results showed that there are significant differences (P<0.05) in fold change between untreated and treated fish. Fold change for control, 0.5 mg/L and 1.0 mg/L of DCF were 1.38± (0.24a), 0.83 ± (0.18ab) and 0.47± (0.09b). Similarly, the 1.0 mg/L of DCF has more suppressed than 0.5 mg/L.

Table 6. Represents col1α1 gene expression between control and diclofenac treatments at a concentration of 1.0 mg/L and 0.5 mg/L for male sailfin molly (mean ± standard error).

DCF treatment	CT	HKG	Δct	ΔΔct	Fold change
Control	**22.57 ±1.37**	**22.55 ±1.30**	**0.023 ±0.00**	**0.223 ±0.00**	**1.38 ±0.24 a**
0.5 mg	**22.64 ±0.49**	**22.49 ±0.46**	**0.153 ±0.37**	**0.353 ±0.37**	**0.83 ±0.18 ab**
1 mg	**22.74 ±0.00**	**21.56 ±0.00**	**0.180 ±0.00**	**0.380 ±0.00**	**0.47 ±0.09 b**
Level of Sig.	-	-	-	-	*
	The lower case means that there are significant differences between the transactions * (P≤0.05).				

Table 7 shows the results of the statistical analysis of fold change at a significant level P<0.05, for female sailfin molly treated with DCF at concentrations of 0.5 mg/L and 1.0 mg/L. The fold change of *col1α1*for untreated, treated with 0.5 mg/L and 1.0 mg/L of DCF were 0.99 ± (0.17a), 0.50 ± (0.00b) and 0.72± (0.05ab). 0.5 mg/L of DCF exhibited significant differences compared with control. While, at 1.0 mg/L significant effect was not observed. The values of fold change had repressed in exposed fish for both concentrations, confirming that there are no differences in *col1α1*expression between male or female or immature fish through treatment with DCF.

Table 7. Represents col1α1 gene expression of control and treated fish at concentrations of 1.0 mg/L and 0.5 mg/L for female sailfin molly fish (mean ± standard error).

DCF treatment	CT	HKG	Δct	ΔΔct	Fold change
Control	**22.58 ±0.71**	**21.73 ±0.95**	**0.14 ±0.95**	**0.50 ±0.08**	**0.99 ±0.17 a**
0.5 mg	**23.88 ±0.14**	**23.31 ±0.15**	**0.79 ±0.00**	**0.99 ±0.00**	**0.50 ±0.00 b**
1 mg	**21.58 ±0.67**	**21.31 ±0.78**	**0.26 ±0.11**	**0.46 ±0.11**	**0.72 ±0.05ab**
Level of Sig.	-	-	-	-	*
	The lower case means that there are significant differences between the transactions * (P≤0.05).				

DISCUSSION

Most medical products are generally used to treat human and animal's diseases, but the hidden side of its influence is the side effect on animal microenvironment and macro-environment. Many studied on medical products started to search the side effects of drugs especially through exposure treatments directly on animal models and detect its implication at the molecular level. The most defections parts of treated molly fish in this study were the skeletal bones so the study was focused on the skeletal morphology and choosing the important substrate involved in bone formation which is the collagen. Alpha-collagen gene plays an important role in bone development; itga and beta 1 subunit encode cell-surface receptor for collagen and laminin, in signaling pathways that is important for cells migration and adhesion associated with bone formation **(Li *et al*., 2020**). Collagen type I is the main organic component of extracellular matrices and several studies have been conducted on collagen aiming for improving the engineering of bone tissue regeneration (**Rico-Llanos *et al*., 2021**).

Diclofenac affects osteoclast cells that were grown *in vitro*, leading to inhibit cell proliferation and gene expression of the collagen I gene in the first two days after incubation, and the effect continued in the later stages and after transferring the stem cells to the body (*in vivo*) (**Blair and Athanasou, 2004**), This experiment explains the act of DCF in inhibition the bone regeneration through impede osteoblast functions subsequently prevention of proliferation, migration, and differentiation of mesenchymal stem cells (MSCs), during bone regeneration, producing deformed bones in organism (**Blair and Athanasou, 2004**).

Recently it was found that there is impaired in osteoblast proliferation and differentiation at early stages of bone regeneration; osteoblast-like cell line cells that were grown with 50 µg/mL diclofenac for 24 h, had presented a significant decrease in osteoblast proliferation and decline in collagen gene expression after two days of exposure and this effect has remained constant even after longer periods of exposure (9, 16 days of incubation), the study showed that the weak stage of fracture repair for immature osteoblasts happened during the first day after treated with NSAIDs diclofenac, in addition to that the effect was included the cancellous bone which is the second layer of bone and is so called spongy bone (**Kaspar *et al*., 2005**).

Most cartilage is the initial stage of bone formation, and the defection that affects the cartilage results in deformed bone at the end. Several studies have reported that there are two modes of bone formation in vertebrates, including endochondral ossification such as the neurogenic spine that undergoes a cartilaginous stage and eventually replaces cartilage with bone (**Nowak-Solińska *et al*., 2013**), and membranous ossification that has not undergone chondrocytes (**Xie *et al*., 2016**).

Current study clarified that DCF has clearly distortion effects on cartilages and bones that appeared on jaws, vertebrae and fins. **Buchan *et al*. (2014)** found that zebrafish larvae exposed to different concentrations of diclofenac had a curvature in their body, and abnormal growth in the spine concluding the fact that diclofenac affects the activity of various genes related to the development of the spine during embryonic development including those that belonging to members of the kinesin 6-kif6 family. In the same context, **Gray *et al*. (2014)** mentioned that zebrafish treated with diclofenac resulted mutated individuals having recessive genes of collagen (collagen, type VIII, alpha 1a (*col8a1a*) leading to generate individuals have phenotype of curvature spine.

The mechanisms by which NSAIDs influence the growth of osteoblasts are still under debate. It is thought DCF inhibit prostaglandin PG synthesis (**Li *et al*., 2011**). At others, its effect includes cell cycle or induction of programmed cell death (**Chang *et al*., 2009**). In rat, it was discovered that exposed to therapeutic doses of ketorolac, indomethacin, or diclofenac had led to the death of osteoblasts that divide to produce cells to differentiate into osteoprogenitors that necessary for calvaria bone cells that cover the cranial bone

cells, this happen through cell cycle arrest in the G0/G1 phase reducing formation or regeneration in bone remodeling (**Ho *et al*., 1999; Chang *et al*., 2007**). Both steroidal and non-steroidal drugs such as dexamethasone, can suppress the proliferation of cultured human osteoblasts cell at G0/G1 phase, NSAIDs have toxic effect on osteoblast cells at high concentrations resulting apoptosis and necrosis(**Chang *et al*., 2009**). Based on these data, it probably be suggested that NSAIDs suppress osteoblast proliferation by a different mechanism than PG synthesis.

The structure of the fins consists of Lepidotrichia and Actinotrichia, Lepidotrichia is a segmented and calcified bony ray arose from the bones of the dermis layer, and Actinotrichia is composed of collagen bound to non-collagen components (**Liedtke *et al*., 2019**). It has been found that rays are formed from collagen I and collagen II, the last one encode by *col2a1b* gene, and it is considered a second copy of collagen 2 genes that is found only in fish and is expressed in fins (**Liedtke *et al*., 2019**).

Collagen compounds, known as actinomycetes that lie under the epidermal cell layer at the distal tip of the fins are known to contribute to fin formation (**van den Boogaart *et al*., 2012**). In current study, it was observed that there are defection in the fins represented by obvious erosion, especially the caudal and pectoral fins, in addition to the occurrence of noticeable deformations in the dorsal fin bones for fish exposed to diclofenac, in males abnormalities in the gonopodium, was observed, these abnormalities included reduction number and size the rays that generate the gonopodium leading to a weakness in connective tissue ligaments subsequently affecting fertilization process. the generation of rays is related to the generation of collagen any defect in disrupting *col9a1c* gene leads to decrease the number of spines in the rays and fins gradually become shorter finally turn into abnormal shape and sometimes totally damage occurrence(**Nakagawa *et al*., 2022**). **Phan *et al*., (2019)** cited that capillaries are neatly arranged between the layers of basal keratinocytes and mesenchymal cells, and contain collagen (*col1a1*) and (*col2a1*).

Durán *et al*.(2011) indicated that a mutation in the *col1a1* gene leads to aberrant phenotypes in both Actinotrichia and Lepidotrichia during fin development and regeneration. This evidence supports that Actinotrichia consists of collagens I and II which are processed after translation by (Lh1) lysyl hydroxylase, and correct expression and assembly of collagen, this process is essential for fin formation, the unique collagen composition of the actinomycetes may play important role in the formation of the fins structure. From what was previously indicated, current study recorded reduction in *col1a1* gene expression after the exposure to DCF subsequently impact the fins and jaw parts in which cartilage is included in their construction.

CONCLUSION

The current study refers to influence the diclofenac (DCF) at concentration 0.5-1 mg/L on bones in sailfin molly fish, *Poecilia latipinna*, these effects including bones deformations and damages in fins and jaws chronically and temporary depending on the time and dose exposure. Gene expression of *col1a1* that is one of responsible genes for bones and cartilages formation has been detected to understand the relationship between the DCF and col1a1 . Our finding is that DCF suppress *col1a1*.

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