Available online at http://bjas.bajas.edu.iq
https://doi.org/10.37077/25200860.2023.36.2.06
College of Agriculture, University of Basrah

Basrah Journal of Agricultural Sciences

ISSN 1814 – 5868 Basrah J. Agric. Sci., 36(2), 68-80, 2023 E-ISSN: 2520-0860

Effect of Dexamethasone on Tail Regeneration in the Electric Black Ghost Knifefish *Apteronotus albifrons* (Linnaeus, 1766)

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Received 16th February 2023; Accepted 5th July 2023; Available online 27th December 2023

Abstract: The study was conducted to find out the impact of dexamethasone of different doses on tail regeneration in the electric black ghost knifefish *Apteronotus albifrons* (Linnaeus, 1766) after two weeks of amputation. Fish were distributed into five equal groups; Dexamethasone drug was applied at concentrations of 0, 1, 2, 3 and 4 mg.l^{-1}. Water was exchanging every 48 hours with new prepared drug to ensure its stability in treatments. Concentrations 1, 2 and 3 mg.l^{-1} were not lethal , while the concentration 4 mg.l^{-1} was lethal, It was observed that fish were dead after four days for concentration 4 mg.l^{-1}.The study showed that the group of control was increased considerably ($P < 0.05$) (4.5mm± 0.866) in the tail after 14 days of amputation in comparing with treated groups. Treated groups (1, 2 and 3 mg.l$^{-1)}$ showed slowly increase with an average 2.83mm ±0.763, 2.50mm ±0.500, and 2.33mm ±0.577 respectively. The mean of relative gene expression of the *junb* was 1.109 in control group that is significant ($P < 0.05$) compared with treated groups that showed a decrease in *junb* expression (0.074 for 1 mg.l^{-1}, 0.050 for 2 mg.l^{-1} and 0.006 for 3 mg.l^{-1}). Current study concludes that dexamethasone inhibit tissues regeneration after amputation via suppress stem cells growth, and *junb* gene specific for stem cells enhancing and the drug reduced its expression.

Keywords: Dexamethasone, Gene expression, Ghost knifefish, Regeneration.

Introduction

Mammals for instance human and adult mice can recover from injuries; however most deterioration tissues do not regenerate after wound (Stoick-cooper *et al*., 2007); in contrast several species of teleost fish have the capability to renew their fins and restore full action in cases of injury (Yoshinari *et al*., 2009). The regeneration is a complex mechanism for example amphibians can regenerate their cutting parts after first hours of injury (Murciano *et al*., 2007). Regeneration of tissue is essential for multicellular organisms to preserve their integrity, on the other hand efficiency of tissue recovery varies with various species, ages and type of tissues (Ishido, 2009).

The electric fish (*Apteronotus albifrons*) is an equatorial fish relating to the family Apteronotidae that live and breed in the fresh water of South America. This type of fish is distributed in Venezuela, Paraguay-Parana River, and the Amazon basin. The dominant colour of *A. albifrons* is a black colour except for two white stripes in the tail, and there is a white halo on its nose, which can sometimes expand into a strip down its back. Fish movement is fundamentally by a long undulating tail on its underside, reaching a maximum length of 50 cm (Froese & Pauly, 2005).

Regeneration assay was studied on electric fish by many researchers for example: *S. macrurus* (Weber *et al*., 2012); *Brachyhypopomus gauderio* (Alshami *et al*., 2020). Caudal fin generally was used for regeneration studies because it renews clearly and easily to observe its growth (Rolland-Lagan *et al*. 2012).

The regeneration of fins is affected by many chemical and physical factors, for example differences of temperature, the environmental pollutants, and some drugs that can act as inhibitor for collagen synthesis and renewal (Bechara *et al*., 2000; Bechara *et al*., 2003). Alshami & Saud (2021) showed the action of some inhibitors (DM, SB431542, SU5402 and cyclopamine) on mesodermal induction during early developmental stages in zebrafish. In teleost fish, the regeneration of fin is very ticklish to the activity of certain drugs, such as dexamethasone, beta-aminopropionitrile, acetylsalicylic acid, and penicillamine; they can overlap with the regeneration of fish fins (Bechara *et al*., 2000; Bechara *et al*., 2003).

Dexamethasone is a synthetic glucocorticoid that exceedingly utilized in numerous disciplines (Wang *et al*., 1999), and is commonly used in surgeries to decrease tumescence and pain after surgery by decreasing the inflammatory response (Fleischli & Adams, 1999). The side effect of use the Dexamethasone for long term can cause osteoporosis, metabolic diseases and cardiovascular diseases (Vegiopoulos & Herzig, 2007). Hamouda & Yasear (2009) documented a steady raise in the number of osteoclasts, meaning a decrease in bone material, and this is the inevitable result associated with dexamethasone drug, even used for a short term (two weeks). Glucocorticoid drugs, for instance dexamethasone and prednisolone have a strong impact on metabolism of bone; it is reducing the density of bone mineral, causing osteoporosis and rising the danger of bone shatter (Coutinho & Chapman, 2011; Briot & Roux, 2015). Effects of Dexamethasone have been conducted on fish regeneration for example Sharif *et al*. (2015) discussed zebrafish fin regeneration exposed to different doses of Dexamethasone, they concluded Dexamethasone reduced fin regeneration in comparison with the regeneration without treatment with the drug.

Regeneration at cellular level is a type of renovation that begins with the formation of a blastema (Akimenko *et al*., 2003; Gurtner *et al*., 2008). Moreover it is a bloc of undifferentiated progenitor cells, pursued by their differentiation to achieve tissue recovery, also cell preparation strategies vary with tissues kinds or size of injury, so repair of small damage is by cell division from fibroblasts and surrounding epithelial cells, while big tissue loss demands several distinct processes to occur namely i- wound closure, ii- Epithelial hurt recovering and forming from the injury epidermis, (iii) formulation of blastema at the distal end of the mesenchyme and (iv) blastema cell

propagation, differentiation, and tissue reconstruction (Akimenko *et al*., 2003; Gurtner *et al*., 2008). The regulation of skeletal muscle is by the transcription factor AP-1 Junb, which is necessary to take part the construction of muscle sarcomere, and any defect in it leads to failure of skeletal muscle (Meder *et al*., 2010).

Junb is a subunit of the transcription factor complex AP-1. It participates in varied biological operations for example: cell propagation, and differentiation in response to a huge number of extracellular stimuli. It also acts as a critical positive regulator of vascular development, tail regeneration process, moreover regulating the expression of differentiation-related genes involved in the cell cycle (Zenz, *et al*., 2008; Kristin *et al*., 2015; Yoshida *et al*., 2016). *Junb* was found to be participatory in erythroid and T-cell differentiation, and it is primarily required for cell migration, for example in *Xenopus* tadpole tail regeneration is through positive regulation of cell proliferation, and embryonic fibroblasts (Nakamura *et al*., 2020; Katagiri, *et al*., 2021).

The current study aims to understand the impact of Dexamethasone as one of the medical treatments on the regeneration of cut tail to prove that it reduced the process of healing in injured tissues through its effect on specific gene expression.

Materials & Methods

Fish rearing

Black Ghost Knifefish (24 immature individuals, less than one year in age) was collected from a local aquarium supplier in Baghdad/Iraq, then transferred to the laboratory (Genetics and developmental biology), College of Agriculture, University of Basrah. Fishes identified by specialist; prof. Dr. Atheer H. Ali, Department of Fisheries and Marine Resources, College of Agriculture, University of Basrah. They were placed in clean glass tanks supplied with dechlorinated fresh water at a temperature of 26°C and provided with air pump. Fish were acclimatized for before conducting the experiment. Fish were fed twice a day, in the morning and in the evening, with dried blood worms and fish eggs (roe).

Experimental Design

In a current study, Dexamethasone (9-fluoro-11β, 17, 21-trihydroxy-16α-methylpregna-1, 4-diene, 3, 20-dion) was obtained from Sama Al-Fayhaa Pharmaceutical Industries Factory in Basrah. Different concentrations of drug were used in experiment by dissolving it with non-ionic water to study the impact of different doses on the regeneration of the tail.

Electric black ghost knifefish were divided into five groups distributed to small glass aquariums, and each treatment contained three immature individuals, each fish was separated in one aquarium. The treatments doses were 0 $mg.l^{-1}$ (control), 1 $mg.l^{-1}$ 2 $mg.l^{-1}$, 3 $mg.l^{-1}$, and 4 $mg.l^{-1}$

Fish were anesthetized with clove (500 $mg.l^{-1}$) (Hassan, 2016) before start the amputation that began after one minute then tail was amputated. The fish were returned to their own ponds sterilized with methylene blue solution (two drops per 15 litres).Water were replaced every 48 hours. The experiment was carried out under laboratory conditions (26°C, aerated with air pump). After 14 days, the new regenerated tail in all treatments were measured using a wooden board with a measuring ruler then cut using sharp blade, and preserved in liquid nitrogen (-80◦C) until RNA extracting start to estimate relative gene expression.

Length of regeneration tail measurement

The length of each fish was measured from the front of the head to the end of the tail before and after tail amputation. After 14 days of amputation they were measured again to determine the growth of the tail using a wooden board with a measuring ruler attached.

Quantitative real-time PCR technique (qRT-PCR)

Primers design

The primers for the *junb* and housekeeping gene *eef1a1a* were designed based on available genome reference of neighbor electric fish in National Center for Biotechnology Information (NCBI) website. Forward and reversed primers were designed using Primer 3 Plus software (Table 1).

Total RNA extraction

For gene expression analysis, the regenerated tissues from three individuals of each treatment were amputated and kept in liquid nitrogen until RNA extraction, Total RNA was extracted utilizing the kit supplied by the Korean company Add Bio Inc. Product Code: 10119. Quality and quantity of RNA were measured using Nanodrop spectrophotometer (Thermo Scientific, ND-2000) at absorbance wave length 260/280 nm.

Synthesis of cDNA

The Total RNA transferred to cDNA utilizing. Korean kit (Accupower Rock script RT Premix kit) representing the following steps: 10 µl 1X master mix was centrifuged for 5 seconds, then, 2 µl of master mix was taken and mixed with 3 µl free RNA distilled water, after that Random Hexamer primer (10x) was placed to diluted master mix and mixed with 5 µl of total RNA, finally the final solution was centrifuged for 5 seconds and transferred to PCR machine applying the conditions showed in table (2).

Quantitative Real-Time PCR (qRT-PCR)

Depending on the protocol and material that supplied by the Korean company Pioneer (Accupower Green Star Real-Time PCR kit and Exicycler™ 96 Real-Time Quantitative Thermal Block) (Table 3), quantitative Real-Time PCR was analysed from cDNA product (four cDNA samples were converted from RNA samples). It was used SYBR® Green in a RT-PCR Pre Mix detection kit for amplification of the cDNA of the *junba* gene. Primers of *junba* and the house keeping gene *eef1a1a* were used to quantify gene expression of *junba.* The condition of quantitative Real-Time PCR was shown in table (4).

Analysis of gene expression

It has been followed Livak and Schmittgen (2001) to analyze real time PCR according to the formula :(1): ΔCt (test) = Ct (target, test) – Ct (ref, test) ,(2): ΔΔCt= ΔCt (test) – ΔCt (calibrator) ,(3): Fold change = 2^-(ΔΔCt) . Statistical Analysis System (SAS (2012) one way ANOVA utilized for testing the significant among treatments.

Table (1): Primer Sequence of *junb* and *eef1a1a*.

Gene name	Primer sequence
Junb	F: 3'- CGAACCTTACCGGAATCTCA -5'
	R: 5'- TGGTTCATCTTGTGCAGCTC-3'
eef1a1a	F: : 3'- CCAGGCATGGTAGTGACCTT -5'
	R: 5'- GTTTCCACGACGGATGTCTT-3'

Table (2): The condition used in PCR machine for cDNA synthesis.

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

Table (3): The real time PCR reagents used in the current study.

Components	Concentration	Volume (20µl)
qPCR master mix, 2X	1X	10 µl
Forward primer	10 µM/µl	2µl
Reverse primer	10 µM/µl	2µl
ddH2O	-	3.6 µl
cDNA template	120 ng	2µl

Table (4): The working conditions of qRT-PCR used for *junb* gene analyses.

Stage	Temperature (°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

Results

Different concentrations including 1, 2, 3 and 4 mg.l^{-1}were exposed for fish to elucidate the sub lethal concentration (Table 5). At 4 mg.l^{-1}, all fish were dead, so we selected less than 4 mg.l^{-1} to test the effect of drug on tail regeneration process.

Fig. (1) shows the regeneration of tail after amputation (5mm) of untreated group of electric black ghost knifefish with Dexamethasone (control) was significantly ($P<0.05$) faster (higher) when compared with all treatments. One mg. l^{-1} of Dexamethasone in treatment 1 gave rise to regeneration growth by 3 mm (Fig. 2), while the regeneration of tail was dramatically slow at concentrations of 2mg.l^{-1} and 3 mg. l^{-1} (Figs. 3 and 4), concluding the drug at1- 3 mg. l^{-1} concentrations has obvious effect on regeneration process, and when its concentration raises, the process of regeneration become more suppressed.

Fig. (5) shows the mean regeneration tissue in the tail of electric black ghost knifefish after 14 days of amputation for the control group (4.5mm ± 0.866). The treated groups with Dexamethasone doses 1, 2 and 3 mg.l^{-1} exhibited a decrease in the tail regeneration (2.83mm ± 0. 763, 2.50mm ± 0.500 and 2.33mm ±0.577, respectively).

Fig. (6) shows mean relative expression of *Junb* gene after 14 days of regeneration in the tail of *A. albifrons* in the control group that was significantly higher (1.109 ± 0.527), in comparing with all treatments exposed to 1, 2 and 3 mg.l^{-1} of Dexamethasone (0.074 ± 0.064, 0.050 ± 0.007, and 0.006 ± 0.001 respectively).

Table (5): The concentrations of Dexamethasone, the total length and the number of fish used in the experiment showing the lethal and sub lethal concentrations for 14 days of exposure.

Group	Concentration	Total length (cm) of fish (Mean± SD)	Number of immature fish	Number Of fish dead	Number of regeneration of tail
Control	-	7.9 ± 0.45	3	-	3
Dexamethasone	1 mg.l^{-1}	7.9 ± 0.45	3	-	3
Dexamethasone	2 mg.l^{-1}	7.6 ± 0.51	3	-	3
Dexamethasone	3 mg.l^{-1}	8± 0.25	3	-	3
Dexamethasone	4 mg.l^{-1}	8.1 ± 0.3	3	All died after 4 days	-

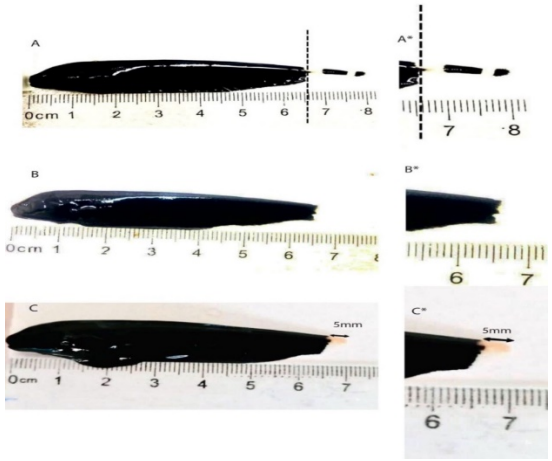

Fig. (1): Untreated group of electric black ghost knifefish with Dexamethasone (control). The increase in the length of the tail after cutting (5 mm). A: Tail before amputation, B: Tail after amputation, C: Tail growth after 14 days of amputation.

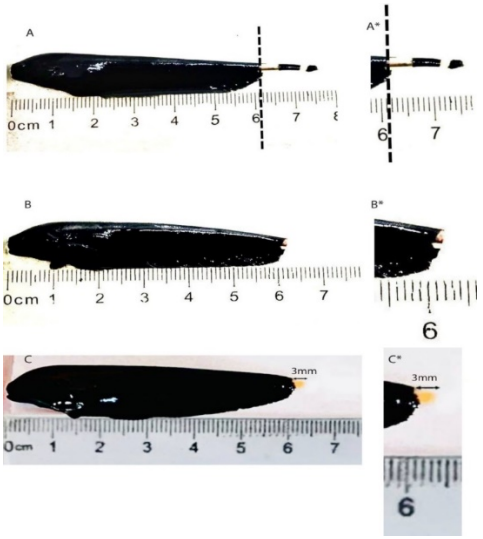

Fig. (2): The treated group of electric black ghost knifefish with Dexamethasone (1 mg. l^{-1}). The increase in the length of the tail after amputation (3 mm). A: Tail before amputation, B: Tail after amputation, C: Tail growth after 14 days of amputation.

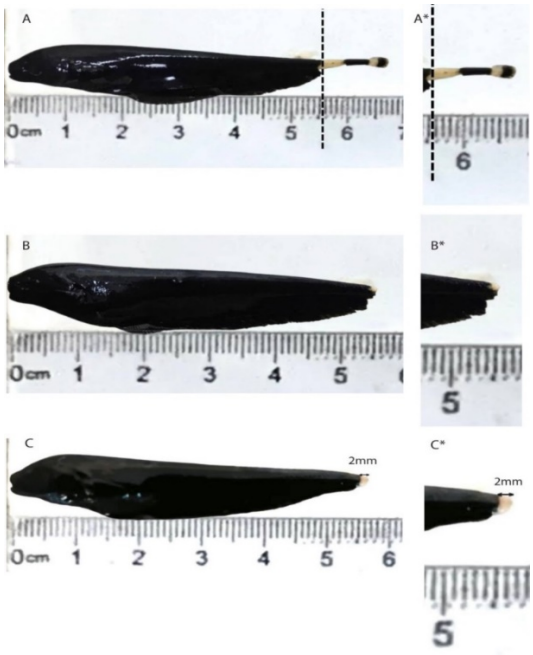

Fig. (3): The treated group of electric black ghost knifefish with Dexamethasone (2 mg.l^{-1}). The increase in the length of the tail after amputation (2 mm). A: tail before amputation, B: tail after amputation, C: Tail growth after 14 days of amputation.

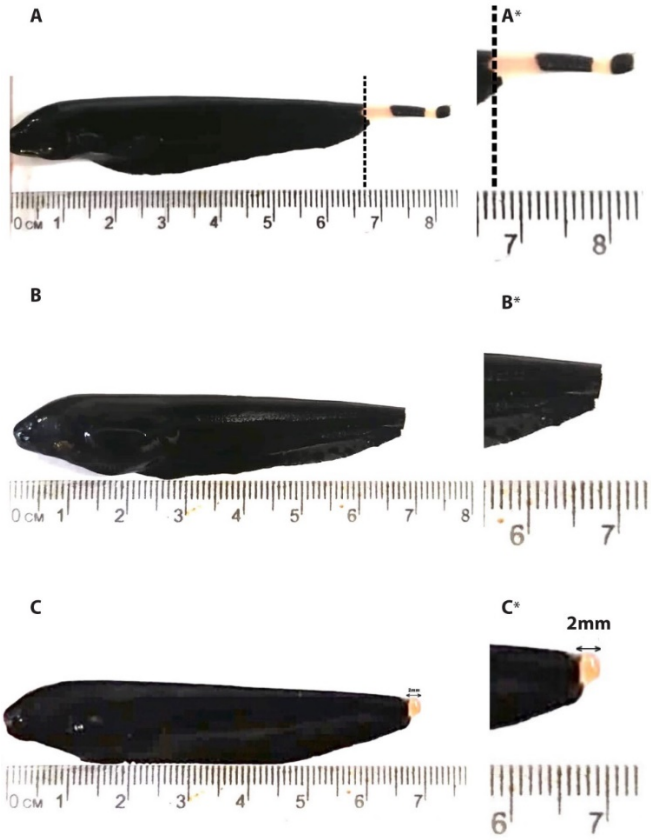

Fig. (4): The treated group of electric black ghost knifefish with Dexamethasone (3 mg.l^{-1}). The increase in the length of the tail after amputation (2 mm). A: tail before amputation, B: tail after amputation, C: Tail growth after 14 days of amputation.

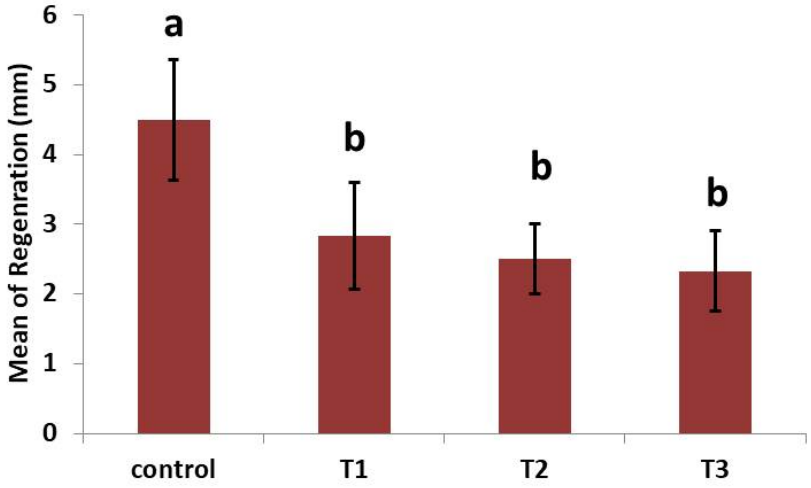

Fig. (5): Mean of tail regeneration in the *A. albifrons* exposed to different concentrations of Dexamethasone after 14 days of amputation. Control =0 mg. l^{-1}, T1=I mg. l^{-1}, T2=2 mg. l^{-1}, and T3=3 mg. l^{-1}; bars=±SD, Lower case refers to significance among treatments, P< 0.05 (0.0176).

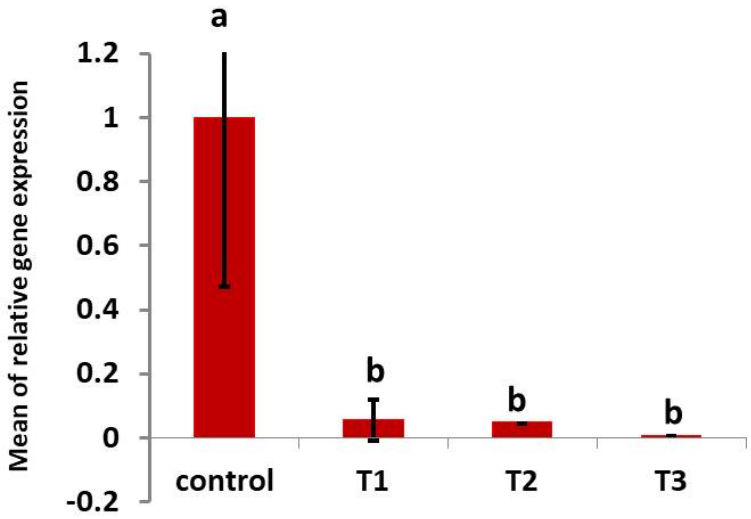

Fig. (6): The mean of expression of *junb* in regenerated tail after 14 days for *A. albifrons* exposed to different concentrations of Dexamethasone. Control=0 mg.l^{-1}, T1=1 mg.l^{-1}, T2 = 2 mg.l^{-1}, T3 = 3 mg.l^{-1}; bars=±SD, Lower case refers to significance among treatments, P< 0.05 (0.0024).

Discussion

Regeneration was studied by many researchers for its important to understand the mechanisms of healing and the damage tissues. By understanding how cells sustain damaged tissues, we may use this biological process for medical treatment and identify the variables that influence surgical healing. Fish served as a suitable model for studies on regeneration because many of its body parts may grow back after being cut or wounded. Appendages such as fins and limbs in amphibians and teleost fish used to study the renovated ability of tissues and organs (Yin & Poss, 2008), although fish fins renew readily in natural cases, they are affected by many environmental factors like differences in the temperature, some pollutants and light density (Nechiporuk & Keating, 2002). Also, the tail can regenerate after amputation specifically in

fish; so they are the best among the vertebrates in the process of regenerating organs not only the tail or fins ,but this process includes the spinal cord , retina and many internal organs including heart and pancreas (Kawakami, 2010).

Christian *et al*. (1999) reported that Dexamethasone inhibits the secretion of growth hormone, as the drug works to inhibit the pituitary Somatostatin hormone that stimulates physical growth. Bechara *et al*. (2000) cited that some species of fish had weak lepidotriquias regeneration after treated with Dexamethasone (at a dose of 0.25 mg. l^{-1}), indicating the effect of this Medication on lepidotrichia collagen synthesis. Furthermore, Hamouda & Yasear (2009) mentioned that Dexamethasone activated the formation of osteoclast cells via increasing in the cells number, and this increase may lead to a decrease in bone substance even if they were given for a short period (14 days). Sharif *et al*. (2015) showed that Dexamethasone caused weaknesses and slow fin regeneration of zebra fish; as it caused the death of a huge amount of cells through necrosis, apoptosis and an important reduction in the number of macrophages in the site of the wound. Moreover, Cea *et al*. (2016) stated that high doses for long term of exposure with Dexamethasone caused skeleton and muscles atrophy.

The injection of Dexamethasone in zebrafish peritoneum over five days suppressed fin regeneration, and reduced the circular diameter of newly regenerated scales compared to the group of control (Chaichit *et al*., 2021).

The effect of Dexamethasone extend to activity of different genes related to the growth of many tissues. It was defect the bone formation through inhibition of osteoblast cells division and disturb differentiation of osteoblasts into mature cells in rabbits treated with Dexamethasone for two weeks (Hamouda, & Yasear, 2009). However, previous studies comprised the regrowth tissues and fins in fish exposed to Dexamethasone, current study focused on the regeneration at the molecular level using gene expression as indictor for the effect of this drug on healing tissues. Furthermore, stem cells of injured area were reduced and the wound delayed, so regeneration process was slowly.

Several studies cited that some steroidal anti-inflammatories such as Dexamethasone and non-steroidal anti-inflammatory treatments have suppression effect on the propagation of cultured human osteoblasts by arresting the cell cycle in the phase G0/G1 (Chang *et al*., 2009). Johnson & Weston (1995) and Akimenko *et al*. (2003) explained that when the caudal fin is partially amputated in teleost or when hardly wounded, the fins undergo a regeneration process called epimorphic restoration; during this regenerative process, lepidotrichia and actinotrichia are formed and described by the following processes: forming of a multipotent epidermal stratum, disorganization, distal migration of pluripotent mesenchymal cells, and proliferation of these cells to generate blastemal; continuous propagation of distal blastema to ease growth, and differentiation of proximal blastema in order to renew the missing part. Poss *et al*. (2000a), Poss *et al*. (2000b), Nechiporuk & Keating (2002) showed that Dexamethasone affected on expression in some essential genes such as *lef1*, *fgf* and *msxb* that have crucial role in blastemal formation.

Kawashima *et al*. (2003) explained that Dexamethasone may inhibit collagen-producing cells by inhibiting mRNA translation of type II collagen in cultured cells. Ishida *et al*. (2010) documented that the phosphorylation of the protein resulting from the expression of *Junb* by Junb N-terminal Kinase after the

injury recovering stage is a unique and needful characteristic for renewal process. Bone development, immunity, wound healing, and the epidermis have been influenced by the loss of *Junb* (Zenz *et al*., 2005; Florin *et al*., 2006). When *Junb* was blocked in mice, fetal blood vessel abnormalities appeared, and died due to placental failure (Schorpp-Kistner *et al*., 1999). Nakamura *et al*. (2021) studied the function of *Junb* in tail restoration, they cited that after tail cutting the *junb* was expressed immediately in the injured tail, and when it was Knocked out, it led to suppress the tail regeneration because the decrease in the number of cells during their proliferation in the stage of division before tissue differentiation, in addition inhibition of TGF-β signals tended to reduce *junb* expression during tail regeneration, so *Junb* is important for tail renovation by regulating cell propagation. From above, the study proved that Dexamethasone has crucial effect on regrowth of healing tissues and prolong the time of healing in the cut tissues.

Conclusion

Our findings conclude that Dexamethasone has suppression effect on *Junb* gene tending to delay of stem cell division subsequently the delay of regeneration in imputation tissues. This can be attributed to disrupt the signaling pathway of maintaining cell division through its effect on cell receptors, and blocking the pathway of *Junb* activation.

Acknowledgments

We thank staff of Department of Fisheries and Marine Resources, College of Agriculture, University of Basrah for supporting the study. Dr. Hussein A. Saud, Department of Pathological Analyses, College of Science, University of Basrah for advices. Sama Al-Fayhaa Pharmaceutical Industries Factory in Basrah for their providing the Dexamethasone.

Contributions of authors

IJJA: Suggest a title of the research, equation design, graphs, and editing revision, Statistical analysis and Interpretation of results.

AAI & IJJA: Sample collection and reviewed the final manuscript.

All authors: Laboratory methodology, and writing the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All ethical guidelines related to fish and care issued by national and international organizations were implemented in this report.

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تأثير الديكساميثازون على تجدد الذنب في سمكة الشبح السوداء الكهربائية

Apteronotus albifrons (Linnaeus, 1766)

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المستخلص: اجريت الدراسة لمعرفة تأثير الديكساميثازون على تجدد الذنب في سمكة الشبح السوداء الكهربائية. تم توزيع الاسماك على خمس مجموعات متساوية ، تم تطبيق عقار الديكساميثازون بتراكيز 0،1، 2، 3، 4 ملغم. لتر$^{-1}$. تم استبدال الماء كل 48 ساعة بجرعات جديدة من العقار لضمان ثباتها في ماء الحوض خلال فترة التجربة. كانت التراكيز 1 و 2 و3 ملغم. لتر$^{-1}$غير مميتة، بينما كانت التركيز 4 ملغم. لتر$^{-1}$ مميت، لوحظ موت الأسماك بعد 4 أيام للتركيز 4 ملغم . لتر$^{-1}$. أظهرت الدراسة بعد 14يوم من قطع الذنب أن مجموعة السيطرة كانت مرتفعة معنويا ($P < 0.05$) في متوسط الزيادة (4.5 ملم) في نمو الذنب بعد 14 يوم من البتر بالمقارنة مع المجموعات المعاملة بالعقار للتراكيز 1 و 2 و 3ملغم. لتر$^{-1}$، وبمتوسط زيادة 2.83 و2.50 و2.33 على التوالي. كان متوسط التعبير الجيني النسبي 1.109 للجين *junb* في مجموعة االسيطرة وهو معنوي ($P < 0.05$) مقارنة بالمجموعات المعاملة (1 و 2 و 3ملغم . لتر$^{-1}$) التي أظهرت انخفاضاً في التعبير الجيني (0.074 و 0.050 و0.006على التوالي).

الكلمات المفتاحية: دكساميثازون، التعبير الجيني، سمكة السكين الشبحية، إعادة التجدد.