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The Effect of Metformin on the Reproductive Pathway of the Male Sailfin Molly *Poecilia latipinna* (Lesueur, 1821)

Abdul-Ameer M.F. Al-Hachamei[1] , Hussein A. Saud[2], Sajed S. Al-Noor[2,*]
Department of Fisheries and Marine Resources, College of Agriculture, University of Basrah, Iraq
[*]**Corresponding Author: pgs.abdulameer.mohareb@uobasrah.edu.iq**

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ABSTRACT

The use of metformin has significantly increased over the past years in the treatment of many health symptoms. This research investigated the effects of metformin on the reproductive of male sailfin molly fish (*Poecilia latipinna*) by exposing them to high concentrations of 1320 mg/L (0.08 M) of metformin hydrochloride for different periods and tracking pituitary hormones. The mRNA gene expression and ELISA techniques were used to measure the level of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) hormones. In addition, testis sections were prepared to detect the level of germ cell development in the testis. The *Fshb* gene expression witnessed an increase after metformin exposure (1.39, 1.63 for T1 and T2, respectively, compared to C: 1.0), whereas *Lhb* decreased (0.51 and 0.47 for T1 and T2, respectively). ELISA analysis showed increases (T1: 85.99, T2: 104.74, C: 64.23)ng/mL in FSH hormones and decreases (T1: 28.685, T2: 11.535, C: 59.975).ng/mL Metformin effects reflected the development of germ cell stages tending to elevate the number of early stages of sperms but slowing down the mature stages.

INTRODUCTION

Metformin (C4H11N5; 1,1-dimethylbiguanide) belongs to the group of biguanides that includes phenformin and buformin, and this class of drugs is originate from herbs (**Bailey, 2017**). It is the first drug treatment for type 2 diabetes (T2D) (**Morales-Barragán *et al.*, 2021**). Commonly, it was prescribed as a drug since the 1950s in the European countries, and then it was introduced in the UK in 1957 (**Flory, 2019**). Metformin was also withdrawn from the US market due to concerns about lactic acidosis, but was later proven to be safe and effective in lowering glucose levels and was reintroduced in 1995 (**Bailey & Turner 1996; Tahrani *et al.*, 2007**). Since then, possibilities for treating many diseases have begun to be explored, including treating women for obesity-related polycystic ovary disease (**Guan *et al.*, 2020**). Some recent studies reported that metformin has anti-cancer properties and inhibits the growth of cancer cells (**Deng *et al.*, 2021**). Some studies have been conducted on the effect of metformin on aquatic organisms; for instance, it was found that metformin stimulates the expression of certain genes, particularly genes of endocrine hormone pathways

(**Ambrosio-Albuquerque *et al*., 2021**). **MacLaren *et al*. (2018**) mentioned a change in the aggressive behavior of male Siamese fighting fish (*Betta splendens*), as it became less aggressive to males exposed to concentrations of metformin similar to those recorded in the polluted aquatic environment (40 µg/L) with the metformin, and the response to external stimuli decreased with an increase in concentration (80 µg / liter). **Niemuth *et al*. (2015**) studied the effect of metformin on metabolism and endocrine system in a concentration of 40 µg/L similar to concentration found in sewage treatment plants in the city of Milwaukee, Wisconsin, USA, on adult large-headed anchovies (*Pimephales promelas*). For the level of gene expression, it was found that there is a significant increasing in the gene expression of ribonucleic acid which is responsible for encoding the egg protein (vitellogenin) in males, indicating pathogenesis of significant endocrine disruptions **(Niemuth *et al*., 2015**). In this respect, **Niemuth and Klaper (2015**) tested the effect of metformin on the reduction of male size in big head anchovies (*Pimephales promelas*) upon using a concentration similar to that found in wastewater. It is obvious that metformin is associated with the decreasing fertility and generating hermaphrodite gonads in males. In the study of **Al-Kuraishy and Al-Gareeb (2016)**, diabetics treated with metformin were compared to those treated with glibenclamide, the results of 64 patients treated with metformin showed that the group that used metformin had a low level of testosterone, which led to sexual weakness. **Yan *et al*. (2015)** indicated that metformin improved sperm formation, semen quality, sex hormones, and testicular apoptosis; it increased testicular volume and improved leydig cell and sertoli cells in obese rats fed high fat. **Cai *et al*. (2021)** in a real-world study on patients with type 2 diabetes mellitus (T2DM) consumed metformin, the authors reported that patients had low total testosterone, free and bioavailable testosterone. Among the vertebrate, **Tseng (2022)** elucidated the role of metformin in the treatment of erectile dysfunction in men, its results were controversial and contradictory between positive in the treatment or harmful to the testicular functions of testosterone synthesis and sperm formation. Fish are generally used as effective models in scientific research since they are characterized by several advantages such as small size, high fertility and ease of monitoring embryonic development (**Shin & Fishman, 2002**), as well as great symmetry with mammals in endocrine glands, hormones and their receptors. The role of endocrine glands and reproductive hormones is inactive before reproduction, unlike other mammals, which allows checking and measuring its role easily **(Busby *et al*., 2010**)

The sailfin molly (*Poecilia latipinna*) is a small tropical fish, reaching to 15cm in total length; it belongs to the Poeciliidae family. A member of this family inhabit the continent of South and North America (**Baensch & Riehl, 1985; Smith, 1997**). It is found in ponds, marshes, low-flowing streams and estuaries (**Allen *et al*., 2002**). It is able to survive in a wide range of salinities, thus it can live in brackish, freshwater and estuarine environments (**Nordley, 1992; Florida Museum of Natural History, 2005**). This species' individuals can tolerate oxygen levels as low as 1mg/ L (**Timmerman &**

Chapman, 2004). Life cycle of molly fish is short, especially males that do not exceed one year of age, and males inseminate females internally through the gonopodium, which is a modified anal fin (**Farr & Travis, 1986**). Fertilized eggs develop into embryos around three to four weeks, and reproduction is repeated every eight to ten weeks, depending on typical environmental conditions (**Wischnath, 1993; Yamamoto & Tagawa, 2000**). Molly fish have been introduced to tropical and subtropical regions in more than 29 countries around the world, either for biological control of mosquitoes or for commercial purposes (**Koutsikos *et al*. 2018**). For the characteristics of this species, it is considered a typical model for molecular studies. **Yang *et al*. (2009**) used molly fish as a model for laboratory experiments in genetics, ecology and biochemistry. Due to the previous data, the current study aimed to understand the effect of metformin on the maturation and development of gonads in male molly fish by using fish as a model for such studies to deeply understand the effects of some drugs on other vertebrates such as humans.

MATERIALS AND METHODS

1. Design of experiment

Specimens of sailfin molly *Poecilia latipinna* were collected from river waters in Basrah Governorate, adapted in laboratory aquariums, and treated with a concentration of 40g/ liter of sodium chloride (NaCl) to get rid of any prospective parasites. They were acclimated in laboratory conditions in glass tanks of 100 liters for two weeks. The fish were fed once a day with standard pellets. Approximately, 96 fish samples were used and half of them were males. The total length of males was between 65-81 mm. The fish samples were divided into 12 groups, each group consisted of four males and four females, kept in tanks with dimensions of 35 × 30 × 35 cm and a water capacity of 30 liters. The groups of animals were four groups representing the control sample (C) and four others for the first treatment, exposed for three hours a day (T1) in addition to another four groups for the second treatment, exposed for five hours per a day (T2). All treatments of exposure were with solution metformin hydrochloride at a concentration of 0.08 M that contained approximately 1320 mg/L. The system water was daily changed using new prepared concentrations of drug for treated groups during the period time that reached five weeks. Conditions of experiment included 12 hours of light and 12 hours of darkness photoperiod, temperature of 26° C (± 2), pH of 8-8.2 and continuous aeration.

2. Quantitative real-time PCR (qRT-PCR)

2.1. Primer design

Primers of *Lhb* and *Fshb* were designed based on the sequences available in GenBank (Sequence ID: *Fshb*: XM_015020261.1, *Lhb*: XM_015023733.1, and *eef1a1*: housekeeping gene: XM_015020125.1). Forward and reverse primers were designed

using Primer 3 Plus software (primers products were generated by Macrogen Korea). (Table 1).

Table 1. Primer sequences of *Lhb, Fshb* and *eef1a1*

Primer sequence	Gene name
F: 5-TGCTTCAGCTGCAACCTGAA-3	***Fshb***
R:5-ATCCTCTGTGTTGCATGTGGT-3	
F:5-TAAGCTCATCTCTGCTGTATAA-3	***Lhb***
R:5- CAACTGGGTAGGTGACGACA-3	
F: 5-GACACCTCATCTACAAGTGTG-3	***eef1a1***
R: 5-GTTTGTCCGTTCTTGGAGATGC-3	

2.2. Total RNA extraction

Fish were anesthetized using cloves (400 mg/L) before any physical test. Animal experiments were performed according to Basrah University regulations for the care and use of animals in scientific research. Four male fish were sacrificed from treatment group and control for gene expression analysis.

The upper part of the head containing the brain (about 100 mg) was removed and stored in liquid nitrogen until the RNA extraction process. Total RNA was extracted using the AddPrepTotal RNA extraction kit (Add Bio Inc.; Addbio, Korea). DNA was removed by DNase I at 37°C for 15min. The quality and quantity of total RNA were detected using a nanodrop spectrophotometer (Analytic Jena Germany), with absorption at 260/280 nm.

2.3. cDNA synthesis

The extracted RNA was converted to cDNA for real-time PCR (RT-PCR) analysis of the gene of interest. Reverse transcription was performed using the AddScript cDNA Synthesis Kit (Add Bio Inc.) according to the following protocol: 10 µL of 1X RT master mix was centrifuged for 5 seconds, followed by 3µL of water molecules. After adding 2µL of random hexamer 10X prime, 5µL of template RNA was added to the mixture for each sample. The final mixture was centrifuged for 5 seconds, transferred to a PCR thermal cycler incubated at 25°C for 10 minutes, followed by the reverse transcription at 50°C for 60 minutes and incubated at 80°C for 5 minutes to terminate the reaction.

2.4. Quantitative real-time polymerase reaction

The cDNA samples were performed by quantitative RT-PCR using the AdScript cDNA Synthesis real-time PCR kit on an Mx3005P real-time PCR system (Agilent; USA), with some necessary modifications. The 20 μL reaction mixture contains 1X qPCR master mix with 10 pM of each forward and reverse primers also 320 ng of the first-strand cDNA. Evaluation of the relative expression levels of *Lhb* and *fshb* were standardized with *eef1a1* as a reference gene. Quantitative real-time PCR of *Lhb*, *fshb*, and *eef1a1* were performed using thermal cycling scheme: hot-start activation at 95°C for 10 minutes, followed by 40 cycles at 60°C, denaturation at 60°C and 10 sec annealing in 30 sec. Extension at 72°C and denaturation at 72°C for 2 min. The equation in the study of **livak and Schmittgen (2001**) was used to analyze fold change of targeted genes.

3. ELISA test

Blood plasma was extracted to measure the concentration of the hormones (LH and FSH) for treated fish with metformin. Blood was extracted from the heart using a syringe of 1.0 mL capacity after moistening it with EDTA. Then, blood was transferred to a 1.0 mL micro-centrifuge tubes and left for 10-20 minutes at room temperature; after that, samples were centrifuged at 2000-3000 revolutions per minute until the plasma was isolated from red blood cells and solid particles. ELISA Kits of LH and FSH hormones measurement were used from SunLong Biotech Co., LTD of China; Fish LH ELISA Kit Cata N: SL0024FI and Fish FSH Elisa kit Cata N; SL0019FI

Samples were read by an absorbance machine (German-made HUMAREADER HS 16670 from Human GmbH) at 450nm. After obtaining the absorbance value of the calibration concentrations, the straight line equation was obtained from the x-value curve in terms of y, then the values were multiplied by 5 to obtain the final concentrations of the samples. The SPSS statistical analysis was used to find out the significant values among the treatments of FSH and LH concentrations.

4. Sperm extraction

The method described depended on that of **Aspbury and Gabor (2004a, b)**; the males were transferred to a container containing 400mL of water, with an addition of two drops of clove oil for the anesthesia of fish; the standard length of males were measured and dried with cotton. A gently massage was done from the end of the gill cover near the eye towards the modified fin of gonopodium organ; this process was done several times to stimulate of semen secretion, after the semen came out, it was sucked out with a plastic pipette, then transferred to a 1.0mL micro-centrifuge tube containing 500μmL of 0.9% saline solution (0.9 grams of sodium chloride NaCl per 100 mL of water), then withdrawn with a pipette and returned several times inside the micro-centrifuge tube to homogenize the sperm. Hemocytometer slide was used to count the sperm volume. The average of sperm number was calculated in five squares (calculated on the basis of five

replicates), then the total number of sperm was calculated via multiplying the average by 50000. German-made Leica (DM1000) microscope was used to examine and take photograph of sperm.

5. Histology examination

The testes were extracted from fish cavity using fine-pointed dissection sharp scissors. Then, they were transferred to fixative solution (10% of formalin) for 24 hours; after that, they were rinsed well with distilled water and treated with series of solutions to make a wax block according to **Humason (1967)**. Sections were made with a thickness of 7 micrometers using semiautomatic microtome (MRS3500) Italian; the tissues were stained with aqueous eosin and Harris hematoxylin dyes.

RESULTS

1. Gene expression

Results of gene expression showed that *Fshb* recorded a significant increase ($P \leq 0.008$) in treatment samples (T1=1.39 ± 0.362, T2=1.63 ± 0.171), compared to the control (1 ± 0.134) (Fig.1). SPSS statistical analysis program of one-way analysis of variance (ANOVA) selecting the LSD test was used.

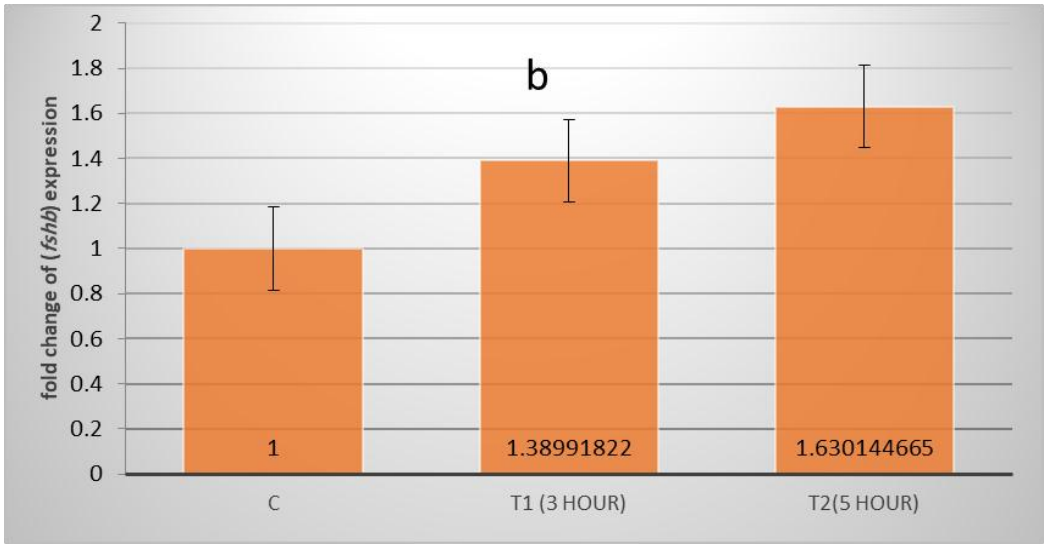

Fig.1. The mean values of fold change and standard deviation of *fshb* expression in the pituitary gland of male sailfin molly (*Poecilia latipinna*), C: control, T1: treatment with 1320 mg/liter of metformin for three hours per day, T2 treatment with 1320mg/ liter of metformin for five hours per day. Different letters mean that there are significant differences between the treatments ($P \leq 0.05$).

Fig. (2) shows the fold change and the standard deviation of *Lhb* expression for the control sample (1 ± 0.49), treatment T1 (0.51 ± 0.14) and treatment T2 (0.47 ± 0.215). Statistical analysis affirmed the significant decrease (P≤0.041) of *lhb* gene for both treatments with metformin.

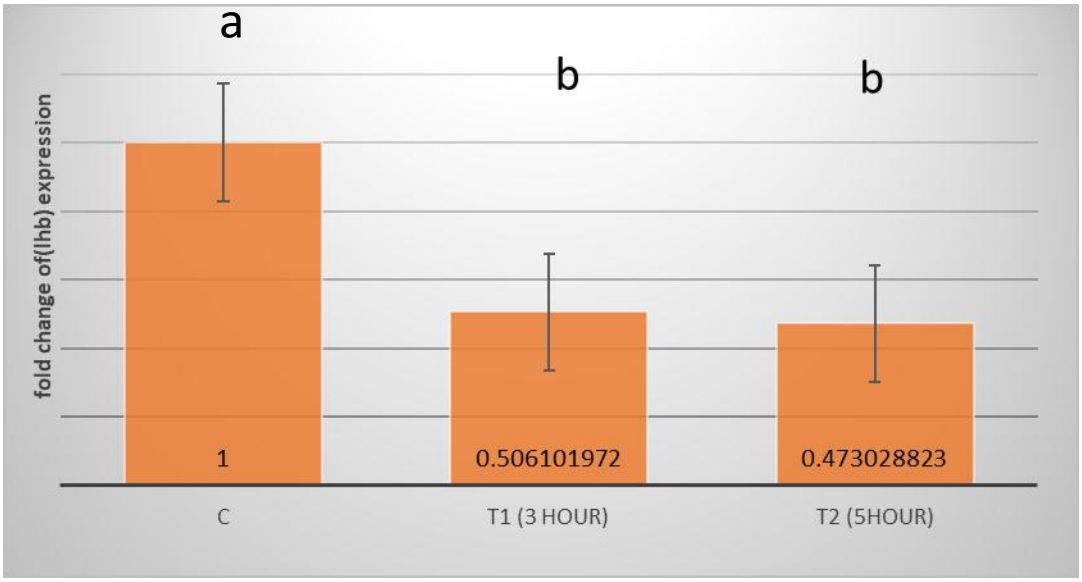

Fig. 2. The mean values of fold change and standard deviation of *Lhb* expression in the pituitary gland of male sailfin molly (*Poecilia latipinna*), C: control, T1: treatment with 1320 mg/liter of metformin for three hours per day, T2 treatment with 1320 mg/liter of metformin for five hours per day. Different letters mean that there are significant differences between the treatments ($P \leq 0.05$).

2. Male fertility (sperm numbers)

Table (2) displays the number of spermatozoa, standard length, average for sperm number for the treatments, standard deviation, and the correlation of standard length with sperm number. These data revealed that the number of spermatozoa is inversely proportional to standard length in control samples, while it is positively proportional in the treatment samples of T1 and T2. Statistical analysis by LSD test clarified significant differences ($P = \leq 0.045$) between the control (C) and the treatment T2.

Table 2. The number of spermatozoa, the standard length of the male sailfin molly (*Poecilia latipinna*), the mean of sperm number, the standard deviation, and the correlation between the number of spermatozoa and the standard length of males

Group	Standard length (mm)	Sperm number	Mean	Standard deviation	Correlation between standard length and sperm number
control	42	6675000	4715857.143	1368384.	-0.79
	54	4650000			
	55	4166000			
	58	5025000			
	54	4582500			
	58	2250000			
	44	5662500			
T1	42	2150000	3311000	1439709.	0.77
	55	4070000			
	50	2655000			
	52	5770000			
	40	1910000			
T2	39	2646000	2803000	1022282.	0.82
	41	1425000			
	55	3441000			
	56	3700000			

3. ELISA test

Statistical analysis (SPSS) using the LSD test at a level of 0.05 confirmed a significant ($P \leq 0.024$) increase in FSH hormone secretion tested in the plasma of male fish of *Poecilia latipinna*. An increase was observed in hormone secretion with an increase the length of the exposure period of drug C (64.23± 6.12ng/L), T1 (85.99 ±5.99 ng/L) and T2 (104.74 ± 27.72 ng/L), as indicated in the Table (3).

Table 3. FSH hormone concentration (ng/mL) for control and treatment samples in the plasma of *Poecilia latipinna* male (mean ± SD).

Group	Concentration (ng/mL)		Mean	SD
C	1	60.34	64.23	±6.12
	2	73.3		
	3	62.5		
	4	60.78		
T1	1	90.95	85.99	±5.99
	2	78.88		
	3	83.19		
	4	90.95		
T2	1	74.57	104.74	±27.72
	2	92.67		
	3	112.5		
	4	139.22		

On the contrary, LH hormone secretion for male sailfin molly treated with metformin manifested a significant decrease ($P \leq 0.02$) in treatments T1: 28.685 ±12.1 ng/L; T2: 11.535 ±3.44 ng/L, compared to the control (C: 59.975± 8.05 ng/L), as shown in Table (4).

4. Histology examination

The control group (Fig. 3) showed different stages of sperms included spermatogonia, primary and secondary spermatocytes stages that represent early immature stages of sperm, spermatids and spermatozoa. Spermatozoa represent the late stage of sperm, which are ready for fertilization. High percentage of mature sperm (spermatozoa) was observed in control group. In treatment 1 (T1), the deterioration of spermatozoa and atresia was observed in the morphology of the tubule, which deferred the increase of the connective tissue among the tubes (Fig. 4). In treatment 2 (T2), a decrease in sperm number was detected; distortion in seminiferous tubules morphology was evident. In addition, in the atresia tubular structure, an increase in distance among tubules and connective tissue was noticed (Fig. 5).

Table 4. LH hormone concentration (ng/mL) for control and treatment samples in the plasma of *Poecilia latipinna* male (mean ± SD).

Group	Concentration (ng/mL)		Mean	SD
C	1	50.90	59.975	±8.05
	2	66.52		
	3	66.99		
	4	55.49		
T1	1	40.93	28.685	±12.1
	2	13.03		
	3	25.94		
	4	34.84		
T2	1	11.50	11.535	±3.44
	2	14.09		
	3	13.86		
	4	6.69		

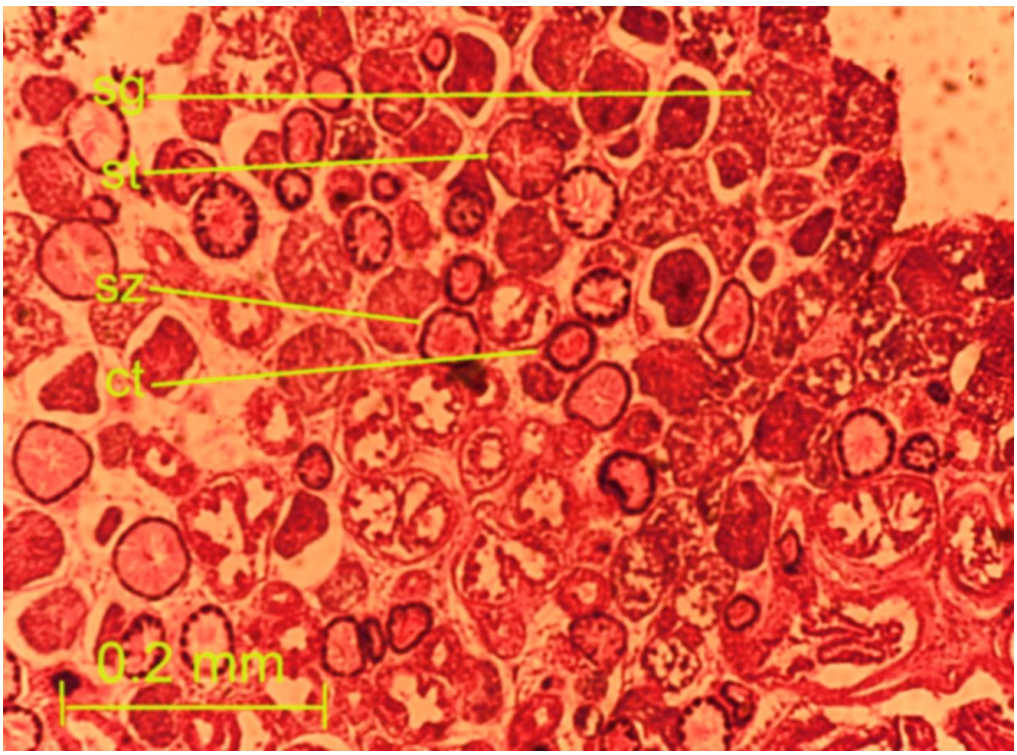

Fig. 3. Histological section in control testis (C) stained with hematoxylin and eosin stains showing the different stages of sperm; sg: spermatogonium, st: spermatid, sz: spermatozoa, ct: connective tissue (Magnification 40X).

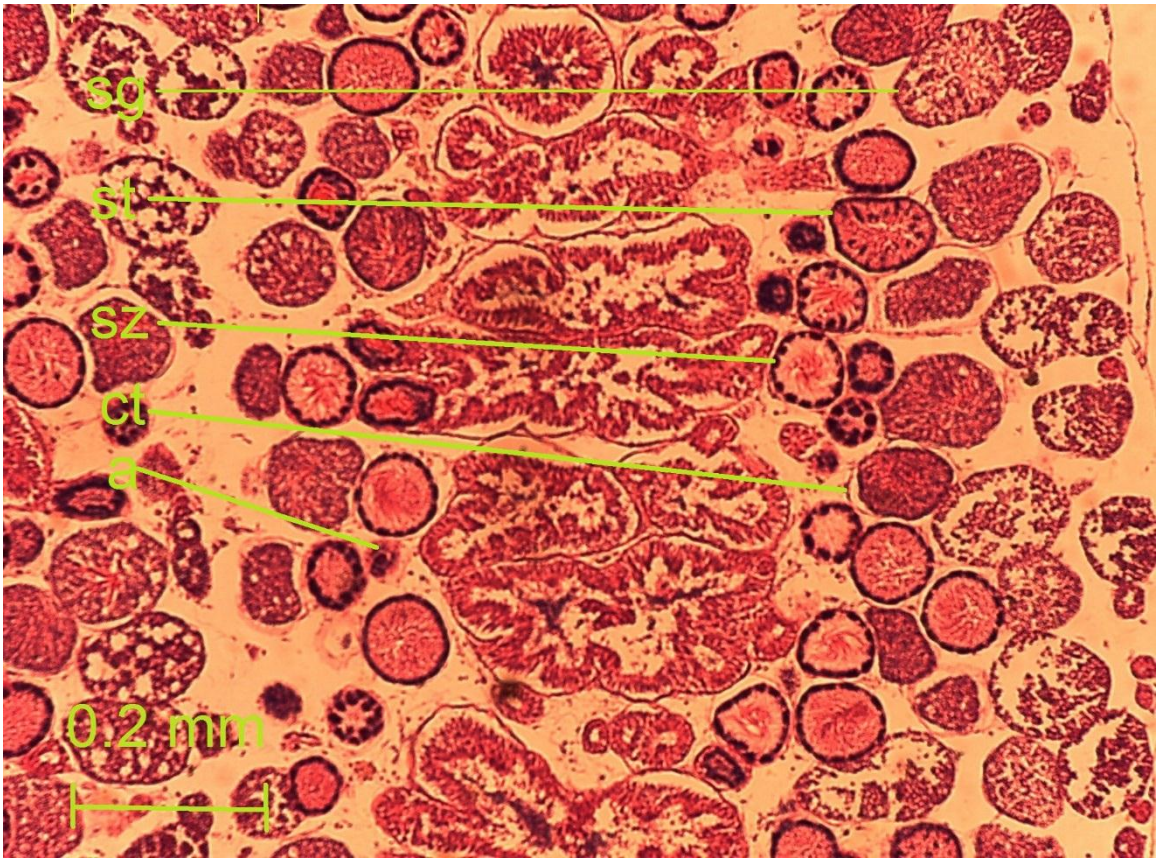

Fig. 4. Histological section in testis obtained from fish exposed to metformin for three hours a day (T1) stained with hematoxylin and eosin stains showing seminiferous tubules and openings among them; the different stages of sperm; sz: spermatozoa, st: spermatid, a: atresia. ct: connective tissue (Magnification 40X).

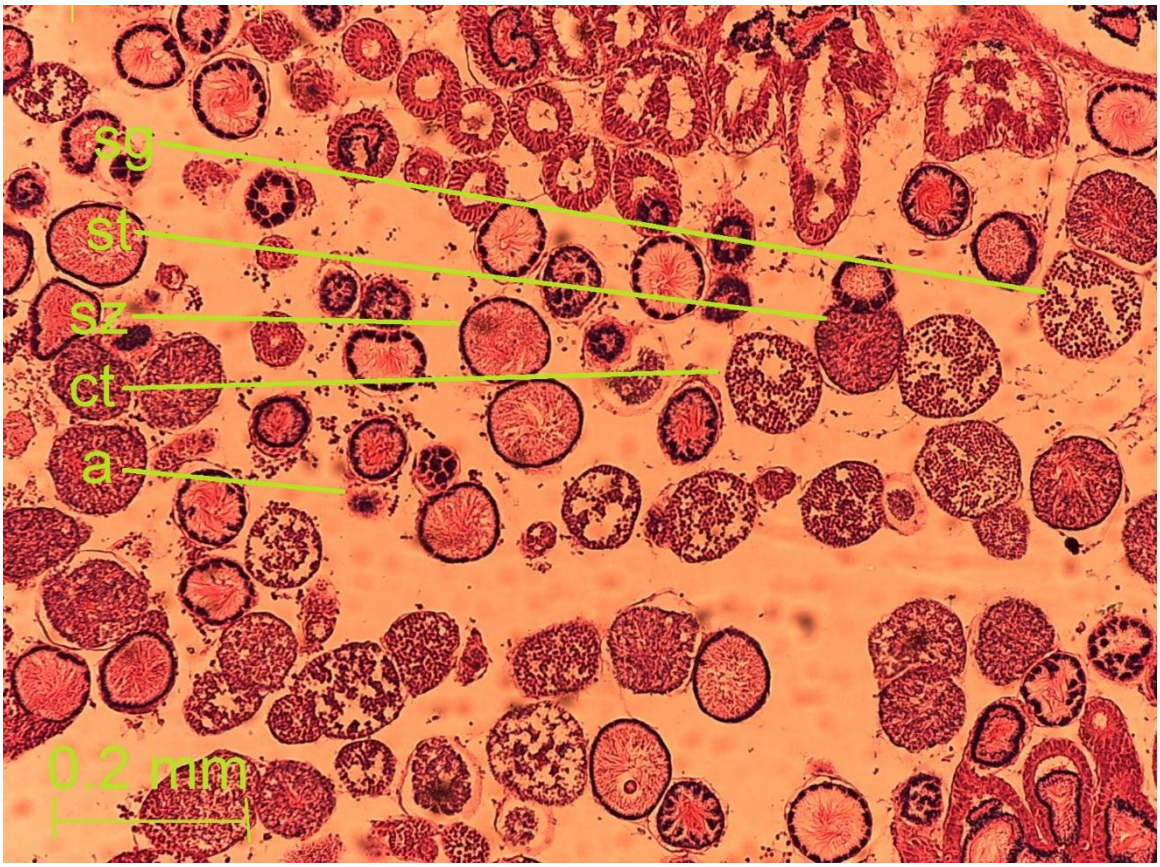

Fig. 5. Histological section in testis obtained from fish exposed to metformin for five hours a day (T2) stained with hematoxylin and eosin stains showing deterioration in seminiferous tubules and openings among seminiferous; the different stages of sperm; sz: spermatozoa, st: spermatid, a: atresia. ct: connective tissue (Magnification 40X).

5. Survival rates

The survival result showed that exposing male sailfin molly (*Poecilia latipinna*) to metformin (0.08 mL M) increased the mortality ratio, as the mortality rate in the second treatment (T2) reached 75%, where 12 out of 16 fish died in the first two weeks of exposure. In treatment (T2), the death rate was 69%, while the death rate in the control (C) did not exceed 0.06%. It was also noted that males lost their bright colors and aggressive behavior of fights and competition for females.

DISCUSSION

The current study dealt with the effect of metformin at high doses on the fertility of male fish sailfin molly (*Poecilia latipinna*) by tracking pituitary hormones GTHs and their secretion through reading the mRNA of *fshb* and *lhb* levels, using the usual method quantitative real time PCR (qRT-PCR). Hormones were measured in blood plasma by enzyme-linked immunosorbent assay (ELISA); sperm count and histological sections of gonad were considered to evaluate the effectiveness of metformin on reproductive

activity. Gene expression of *fshb* exhibited a significant increase, while the expression of *Lhb* was suppressed, and those results matched with hormones levels measured by ELISA method. This change in the hormonal level enhanced the number of sperms at early stage (spermatogonia). At the same time, metformin inhibited the development of sperm to late stages (spermatids and spermatozoa), and this reflects the role of hormones in the generation and development of gonadal products. The reproduction process in fish is controlled by the act of hormones of the hypothalamic pituitarygonadal (HPG) through external environmental influences and internal physiological processes that are integrated and controlled via endocrine secretions; among the external and internal physiological influences are the availability of food and its representation within the body of the fish (**Bronson, 1985; Volkoff & London, 2018; Sumpter, 2019**). The pituitary gland secretes two types of hormones, the first is FSH, which is a glycoprotein hormone that stimulates the early stages of gametogenesis and stimulates the initial development of spermatozoa in males, and LH is a binuclear glycoprotein hormone secreted by the anterior pituitary gland which acts through the gonadal receptors to stimulate steroidogenesis and gamete formation at the final stages (**Yaron & Levavi-Sivan, 2011**). Metformin has influential role on the endocrine glands of the pituitary gland (**Niemuth *et al*., 2015; Niemuth & Klaper, 2015; MacLaren *et al*. 2018; Phillips *et al*. 2021**)

The reproduction of fish species coincides with the food abundance at time of hatching. Deficiency of food availability can be an inducer for hormones signals to be a start point for the development of the gonads in fish. Thus in most fish, the reproduction is subjected to seasonal fluctuations even in the subtropical area (**Guerrero *et al*., 2009**). Initial stages begin with the release of FSH hormones from the pituitary gland (**Cabrita *et al*., 2008**). For present results, it can be expected that the main effect of the metformin is a stimulator for the cellular energy sensor AMPK kinase. This sensor reduces anabolic processes and increases metabolic processes including lipid phosphorylation to increase the amount of ATB in cells, as well as reducing glucose uptake in the intestine (**Kaneto *et al*., 2021)**. **Jacob *et al*. (2018)** stated that the exposue of brown trout larvae to metformin reduced the growth and caused weight loss. The effect of metformin results from energy effects and its metabolism in the body, and subsequently, signals inside the body are generated via endocrine secretions (**Zhang, *et al*., 2015**). Diet and energy have effective signals' pathway in the body of the fish which enhances the differentiation and formation of reproductive organs at the early and middle stage of development (**Luquet & Watanabe, 1986**).

Anterior part of pituitary gland releases gonadotropins hormones (GTHs) including FSH and LH, with significant role in gonads maturation and spermatogenesis at the early stages of development, but it does not have a strong effect on spermatogenesis at later stages (**Zhang, *et al*., 2015**), **Shpakov (2021**) showed the positive effect of metformin on men with metabolic disorders by improving steroid synthesis and formation of spermatozoa, thus improving the reproductive state by absorption and metabolism of food. ELISA analysis confirmed the results of gene expression for *fshb* and *Lhb*, where FSH hormone increased and LH decreased. An increase in sertoli cells is associated with

the increase of FSH, thus metformin acted to increase the cellular energy sensor AMPK, and this action acted as an inhibitor for FSH in male gonads (**Riera *et al*., 2012**). Current results supported this conclusion through the histological tissues of testis, showing the lack of serratori cells in exposed fish, compared to the control sample that showed the accumulation of sertoli cells at all stages of maturity testis that included spermatogonium, spermatid and spermatozoa, and the connective tissues of Leydig-cells. In five hours a day of metformin exposure, the testis tissues showed lack of sperm sac cells, tissue hypertrophy and increase in atresia of the sperm sacs that resulted in lowering mature sperm for the reproduction. This suggestion is consistent with that of **Tartarin *et al*. (2012)** who cited that a decrease in testicular volume was associated with a decrease in the number of Leydig and Sertoli cells in embryos of mice treated with metformin. Additionally, a decrease was detected in testosterone resulted from a decrease in gene expression of the LH hormone, leading to a significant increase in lactate after the treatment for three days. Similar results are those of **Yon and Akbulut (2013**) who reported a similar conclusion in male guppy fish (*Poecilia reticulata*) treated with BPA 2 bis(4-hydroxiphenyl) at concentrations of 4 and 8mg/ L, which showed clear changes in testicular tissue structures in Leydig and Sertoli cells.

Wedell *et al*. (2002) also showed that spermatogenesis can be energy costly, and any defect in energy supply will harm male reproduction and limits reproductive success, as well as causing a decrease in the mRNA gene expression of the gene (*lhb*) in the pituitary gland responsible for the hormone LH.

In males, LH stimulates the gonads to secrete androgens, mainly 11-ketotestosterone (11-KT) that induces spermiogenesis and regulate spermatogenesis. DHP has another role through initiating meiotic division of spermatogonia and controlling the maturation of spermatogonia (**Yaron & Levavi-Sivan, 2011**). DHP in its free or conjugated forms works like pheromones; it also regulates spermatogenesis and spermiogenesis by 11-ketotestosterone (11-KT) (**Themmen & Huhtaniemi, 2000**). This suggests the lost bright colors **(Houde, 1987)** in treated male indicating that males are ready to release sperm, mate, courtship, and competition with the other males. There is a strong connection between the concentration of androgens and the hostile state of males (**Wingfield *et al*., 1990; MacLaren *et al*., 2018**). Sexual behavior of fish is also under the control of hormones;, for example testicular androgenization is required for sexual behavior to implement the reproduction in most fish species (**Munakata & Kobayashi, 2010**). The increased LH level results in producing more semen in mature males ready for mating (**Kobayashi *et al*., 2002**). Our result showed that there is a significant ($P \leq 0.045$) difference in the number of sperms between control group and treated groups; however, the correlation factor between the standard length of males and the number of sperms was negative ($r = -0.79$) in the control group (C). On the other hand the correlation between sperm number and stander length was positive in treatment groups (T1, $r = 0.76$; T2, $r = 0.82$). This can happen owing to the dominance of the old males in the control group among the young ones, thus the reproduction and sperm supply was at the lowest level, unlike the younger ones, who can supply more amount of sperms, but they did not have opportunity for mating with females due to the dominance of older males in both treatment groups. Therefore, for the lack of LH hormone, lack of male

hormones, and lack of reproductive effectiveness in males, the correlation was positive between the standard length of males and the number of sperms. This results is consistent with those of **Robinson *et al*. (2011)**. **Aspbury and Gabor (2004b)** also explained that the presence of older males in abundance in fish community mean greater number of sperms at the beginning of the breeding season; however, during the late breeding season, the amount of sperms become low. This, in turn, suggests that the size of the males is important in the courting behaviors; the older males have good chance for fertilization than younger ones. Mixed of old and young males lead the older behave as the young males through forced insemination **(Travis & Woodward, 1989)**. Additionally, the bright color of males stimulates females and makes them more ready to reproduce (**Butler *et al*., 2019**). **AL-Saeed *et al*. (2019)** found that Carob plant (*Ceratonia siliqua*) is more effective in improving the quality of sperm and the health status of guinea pigs, compared to metformin since the authors confirmed that the ethanol extracted from the fruits of carob plant was more effective in improving sperm quality, some biochemical parameters, lowering blood sugar, and improving the health status of diabetic, compared to metformin and glimefan. Further studies are required to understand the mechanisms and signal the pathways that belong to the effects of these groups of medicine.

CONCLUSION

The current study concluded that, metformine has significant effect on FSH and LH. It increases the levels of FSH hormone tending to accelerate sperms production and causes a decrease in the LH level that subsequently delay the production of matuture sperms.

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