Effects of Metformin in High Concentrations on the Fertility of Female Sailfin Molly Poecilia latipinna

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Abstract

Metformin is a medicine that is used to treat type 2 diabetes and polycystic ovary syndrome (PCOS). This study investigated the effects of exposing the sailfin molly fish to sub-lethal concentrations of 0.008 mol.L⁻¹ of metformin. The results showed that metformin caused disturbances in the endocrine glands, especially the pituitary hormones (GTHs). Metformin increased the expression of the Fshb and inhibited the Lhb genes in the pituitary gland, which led to higher levels of FSH and lower levels of LH hormones in the blood of fish. These hormonal changes led to irregularities in the ovarian development of female fish, such as an increase in the first stage of primary eggs and a delay in the final stage of embryo production.

Key words: FSH, LH, metformin, Poecilia latipinna.

I. INTRODUCTION

Metformin is a medicine that is used to treat type 2 diabetes and polycystic ovary syndrome (PCOS). This study investigated the effects of exposing the sailfin molly fish to sub-lethal concentrations of 0.008 mol.L⁻¹ of metformin. The results showed that metformin caused disturbances in the endocrine glands, especially the pituitary hormones (GTHs). Metformin increased the expression of the Fshb and inhibited the Lhb genes in the pituitary gland, which led to higher levels of FSH and lower levels of LH hormones in the blood of fish. These hormonal changes led to irregularities in the ovarian development of female fish, such as an increase in the first stage of primary eggs and a delay in the final stage of embryo production.

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I. INTRODUCTION

Metformin lowers blood sugar by affecting the way of metabolism in the liver, intestines, and cells handle glucose. It does not change the amount of insulin produced in the body, but it does make cells more sensitive to insulin and lowering the sugar absorption (Rena et al., 2017). Metformin also reduces the absorption of sugar by the gastrointestinal tract and reduces the production of glucose by the liver (Aungst, 2022). Some studies suggested that metformin should be included in a group of polyactive, such as penicillin and aspirin, due to its characteristics in treating many diseases (Shmerling, 2021). In addition, it is considered the first choice for patients with type 2 diabetes. It has been used to treat several conditions, including cysting of varies (Lashen, 2010.), as well as to reduce body weight and elimination of triglycerides in the blood (Yerevanian, & Soukas, 2019). Improving and treating infections, protecting the heart and blood vessels (Bai & Chen, 2021), delaying the aging (Cheng et al., 2022), as well as helping to treat HIV (Planas et al., 2021). Promising treatment in types of cancers in humans improved by metformin therapy (Wu et al., 2020). Also, it use for treating Polycystic Ovary Syndrome (PCOS), a hormonal disorder that affects women of childbearing age and can cause irregular menstruation, infertility, excessive hair growth, acne, improve menstrual cycles, ovulation, and insulin resistance in women with PCOS (Hashim, 2016).
Metformin can also improve male reproductive function, especially in obese men who have low fertility (Tseng, 2022). It can improve sperm quality, motility, and morphology by increasing testosterone production and reducing oxidative stress (Ye et al., 2019). According to two studies by Liu et al. (2018) and Faure et al. (2018), metformin can act against prostate cancer by reducing its growth and spread.

However, metformin did not have serious risks or side effects. It can cause gastrointestinal trouble, such as nausea, diarrhea, and abdominal pain (Drugs.com 2022). Metformin can also interact with other drugs or supplements, such as alcohol, iodinated food, or vitamin B12 (Davies 2022; Gershman, 2022). Moreover, metformin can have long-term effects on the offspring exposed to it in the womb, causing increased body weight, altered metabolism, and epigenetic changes (Tseng, 2022).

Other studies cited that metformin has effects on endocrine in aquatic organisms. Niemuth, & Klaper, (2015) studied the effect of metformin at same concentrations recorded in the environment 40µg.L⁻¹ on fathead minnows (Pimephales promelas) from early life stages to adulthood. They found that exposure to metformin has transformed male fish to female and reduced the size and fertility of male fish. The study concluded that metformin acts as an endocrine disruptor at environmentally relevant concentrations and they suggested that it may be possible cause hermaphroditism in fish. So that they suggested that it may have negative effects on fish populations and ecosystems.

Mudumbi, et al. (2018) indicated that metformin can influence on the growth, reproduction, and survival of aquatic organisms such as fish, algae, bacteria, and zooplankton. Their study concluded that metformin can alter gene expression in aquatic organisms, especially genes involved in the endocrine hormone pathways.

Lee et al. (2019) indicated that when applying different concentrations (40, 60, and 360) µg.L⁻¹ of metformin on Oryzias latipes fish for two consecutive generations, it caused endocrine disruption, hermaphroditism in males, and oxidative stress in fish. Subsequently, it reduced vitellogenin (Vtg2) expression, indicating that metformin has adverse effects on aquatic organisms. Ambrosio-Albuquerque, et al. (2021) described the effects of metformin on aquatic organisms showing that metformin can affect on gene expression related to endocrine hormone pathways. Phillips et al., (2021) reported that metformin has negatively effects on the development of fish embryos for example, long term exposure to metformin reduced survival rate and caused malformations in zebrafish embryos and larvae. They also found that exposure to metformin for a short period affected on fish movement and genes expression in zebrafish associated with brain and heart development.

It was found that metformin disrupts the gonadal development and reproduction cycle of zebrafish at low concentrations (Barros et al., 2022a). Generally fish have been used in medical experiments because they have high matching of genetic similarity with humans, making us use them as useful model for human diseases and understanding the molecular mechanisms of cells (Schaeck, et al., 2013). Moreover, fish have several advantages over rodent models, such as large numbers of offspring, visual clarity of embryos, lower maintenance costs, and ease of generation of transgenic animals (Bailone et al., 2020; Choi et al., 2021; Adhish, & Manjubala 2023). The Sailfin molly, Poecilia latipinna is a small viviparous fish that can live in fresh, brackish, and coastal saltwater, they habitats the regions from North Carolina to Mexico (Florida Museum of Natural History, 2023). It is a tolerant species that can survive at low level of oxygen and high salinity (Gonzalez, et al., 2005). The Sailfin molly fish have short life period, they become mature and differentiate embryos from four to five weeks for one batch, separating eight to ten weeks between one batch and other, depending on the surrounding conditions (Costa, and Schlupp, 2020). P. latipinna used commonly in biological experiments as a model for many disciplines including genetics, ecology, and biochemistry (Yang et al., 2009).

The aim of our study is to determine the effect of metformin on the reproductive path of the sailfin molly fish, Poecilia latipinna, through which we can determine the level and type of effects resulting from the release of hospital effluent containing drug residues and sterilization solutions on the environment and its environmental components.

II. Materials and Methods

Fish collection and treatment with metformin

96 fish Poecilia latipinna were collected from Basrah body water then they kept in an aquarium and acclimated before experiment start. As a preservative process, all fish were treated with 40 g.L⁻¹ of sodium chloride (NaCl) in laboratory.
aquariums to eliminate any potential parasites (Dewi et al., 2018). The captured fish comprised of half males with a total length of 65-81 mm and half females with total length of 59-74 mm. They were acclimated in 100-liter glass tanks for two weeks. For feeding, it was used standard pellets once a day. Fish were divided into 12 groups each contain eight individuals (four males and four females) separated in 30-liter tanks (35 × 30 × 35 cm). Four groups were fixed as control samples (C), four groups of the first treatment were exposed for three hours per day (T1), and other four groups for the second treatment were exposed for five hours per day (T2). It was used one concentration (1320µg.L⁻¹)(0.008 mol. L⁻¹) of metformin hydrochloride in all treatments. Water system was changing with new prepared concentrations of metformin daily for period of experiment that extended five weeks. Fish were reared under environmental conditions included photoperiod: 12-hour light/dark, temperature of 26°C (± 2), pH of 8.8-2, and continuous aeration.

**Quantitative real-time PCR (qRT-PCR)**

**Primer Design**

We designed primers for *Lhb* and *Fshb* based on the GenBank sequences (Sequence ID: Fshb: XM_015020261.1, Lhb: XM_015023733.1, and eef1a1: housekeeping gene: XM_015020125.1). We used Primer 3 Plus software to design forward and reverse primers, primers synthesized by Macrogen Korea laboratory (Table 1).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fshb</td>
<td>F: 5-TGCTTCAGCTGCAACCTGAA-3</td>
</tr>
<tr>
<td></td>
<td>R: 5-ATCCTCTGTGTGGCATGGTGT-3</td>
</tr>
<tr>
<td>Lhb</td>
<td>F: 5-TAACGTCATCTCTGCTGATAA-3</td>
</tr>
<tr>
<td></td>
<td>R: 5-CAACTGAGGTAGGTACGACA-3</td>
</tr>
<tr>
<td>eef1a1</td>
<td>F: 5-GACACCTCATCTACAAGTG-3</td>
</tr>
<tr>
<td></td>
<td>R: 5-CTTTGTCCGTTCTGGAGATGC-3</td>
</tr>
</tbody>
</table>

**Total RNA extraction**

Total RNA was extracted from the brain of four female fish (Four fish from each group. fish were anesthetized with cloves (400 mg.L⁻¹) following the Basrah University regulations for animal research. The upper part of the head (about 100 mg) from each individual was cut off and stored it in liquid nitrogen until initiation of RNA extraction. Addp rep Total RNA extraction kit (Add Bio Inc.; Addbio, Korea) was applied and DNaseI used to degrade DNA. Quality and quantity of extracted RNA were measured with a nanodrop spectrophotometer (Analytic Jena Germany) at 260/280 nm absorption.

**cDNA synthesis**

Reverse transcription was performed using AddScript cDNA Synthesis Kit (Add Bio Inc.) to convert the extracted RNA to cDNA for RT-PCR analysis. 5 µL RNA template was mixed with 10 µL of 1X RT master mix, 3 µL of water molecules, 2 µL of random hexamer 10X primer, then the mixture was centrifuged for 5 seconds and transferred it to a PCR thermal cycler. the mixture was incubated at 25°C for 10 minutes, then mixture incubate at 50°C for 60 minutes for reverse transcription, and finally at 80°C for 5 minutes to stop the reaction.

**Quantitative real-time polymerase chain reaction analysis**

AdScript cDNA Synthesis real-time PCR kit and the Mx3005P real-time PCR system (Agilent; USA) were employed to perform quantitative RT-PCR for the cDNA samples. 20 µL of reaction mixture was mixed with 1X qPCR master mix, 10
pM of each forward and reverse primer, and 320 ng of cDNA product. We used eef1a1 as a reference gene to standardize the relative expression levels of Lhb and fshb. We ran quantitative real-time PCR of Lhb, fshb, and eef1a1 with the following thermal cycling scheme: 95°C for 10 minutes for hot-start activation, then 40 cycles of 60°C for 10 sec for annealing and 72°C for 30 sec for extension, and finally 72°C for 2 min for denaturation (Schmittgen & Livak, 2008). Equation was used to analyze the fold change of the target genes.

ELISA Test

LH and FSH hormone concentrations were measured in the blood plasma of the fish treated with metformin. We used 1.0 mL syringe moistened with EDTA to draw blood from the heart. Then the blood was transferred to 1.0 mL micro-centrifuge tubes and left at room temperature for 10-20 minutes. Tubes were centrifuged at 2000-3000 rpm to separate the plasma from the red blood cells and solid particles. ELISA Kits from SunLong Biotech Co., LTD of China (Fish LH ELISA Kit Cata N: SL0024FI and Fish FSH Elisa kit Cata N: SL0019FI) was applied to measure the LH and FSH hormones. samples were read with an absorbance machine (HUMAREADER HS 16670 from Human GmbH, Germany) at 450 nm. Then it was obtained the straight line equation from the x-value curve in terms of y for the calibration concentrations. We multiplied the values by 5 to get the final concentrations of the samples. SPSS statistical analysis was utilized to find out the significant differences among the treatments for FSH and LH hormones concentrations.

2-4 Gonado Somatic Index (GSI) and Actual Fertility

For measure GSI, the fish were anesthetized using clove (0.8µg.L⁻¹), then the fish were measured with a sensitive scale, to the nearest 0.001 grams, and their ovaries were removed and weighed with a sensitive balance with an accuracy of 0.0001 grams.

\[
GSI = \frac{GW}{BW}, \\
GW = \text{Gondal Weight (g)} \\
BW = \text{Body Weight without entrails (g)} \quad (Bahamonde, \ et\ al., \ 2013; \ Rizzo \ and \ Bazzoli \ 2020).
\]

The actual fertility was calculated by keeping the ovaries in a 10 ml test tube, Gilson's solution (100 ml alcohol 60%, 880 ml distilled water, 18 ml glacial acetic acid, 15 ml nitric acid 80%, and 20 g HCl mercury) them. After several days, until the eggs had hardened, they were emptied into an earthenware beaker, and the number of eggs counted under a magnifying glass (Muchlisin, 2014; Chen et al., 2022).

Statistical Analyses.

Statistical analysis software (SPSS) version 20 was used to identify statistically significant differences between the values obtained (mean ± SD) by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparison test. P value < 0.05 was considered significant.

Results

Gene Expression of Fshb

fshb exhibited relative increase for both treatments (1.20 and 1.23) compared with control, but the increase was not significant (p>0.05) Fig. 1. These results shows that metformin stimulate fshb expression
Fig. (1): showed the mean values of fold change and standard deviation of \textit{fsrb} expression in the pituitary gland of females sailfin molly (\textit{Poecilia latipinna}), C: control, T1: treatment with 1320 mg.L\(^{-1}\) of metformin for three hours per day, T2 treatment with 1320 mg.L\(^{-1}\) of metformin for five hours per day. Similar letters mean there were no statistically significant differences between treatments (P\leq0.05).

\textbf{Gene Expression of \textit{lhb}}

\textit{Lhb} expression has been showed dramatically decrease for both treatments (T1: 0.26 and T2: 0.19) of exposure to metformin in comparison with control, and statistically were significant (p\leq0.002). Fig. (2) indicating that metformin has inhibition effectiveness on \textit{lhb} expression.

Fig. (2): showed the mean values of fold change and standard deviation of \textit{lhb} expression in the pituitary gland of females sailfin molly (\textit{Poecilia latipinna}), C: control, T1: treatment with 1320 mg.L\(^{-1}\) of metformin for three hours per day, T2 treatment with 1320 mg.L\(^{-1}\) of metformin for five hours per day. Different letters mean that there are significant differences between the treatments (P\leq0.05).

\textbf{ELISA test of FSH Hormone}
ELISA test and statistical analysis (SPSS) using the LSD test at a level of 0.05 confirmed a significant (p≤0.0204) increasing in FSH hormone secretion that tested in the plasma of females fish *Poecilia latipinna*, as it was noticed that there is observed increase in the hormone level with increase the length of the exposure period C (6.379± 0.562 ng.mL⁻¹), T1 (8.707 ±2.56 ng.mL⁻¹) and T2 (18.288 ± 6.079 ng.mL⁻¹) as (Table 2).

**Table (2): FSH hormone concentration (ng.mL⁻¹) for control and treatments with metformin in the plasma of females fish *Poecilia latipinna* (mean ± SD).**

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (ng.mL⁻¹)</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1</td>
<td>6.767</td>
<td>6.37875</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.897</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.164</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.687</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>1</td>
<td>7.586</td>
<td>8.707</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.543</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.371</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.326</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>1</td>
<td>22.457</td>
<td>18.288</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.129</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.341</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>24.224</td>
<td></td>
</tr>
</tbody>
</table>

**ELISA test of LH Hormone**

The ELISA test of LH hormone in the blood plasma of *Poeclia latipinna* that treated with metformin showed that there were a strong significant increasing (P≤0.0001) in comparison with the control. Table (3) shows the concentrations of the LH hormone in the blood plasma of *Poeclia latipinna* in units of ng/ml (the mean ± S.D).

**Table (3): LH hormone concentration (ng.mL⁻¹) for control and treatments in the plasma of females fish *Poeclia latipinna* (mean ± SD), showing the drop of hormone with increase of metformin concentrations.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (ng.mL⁻¹)</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1</td>
<td>8.0423</td>
<td>6.374</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.670</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.381</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.404</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>1</td>
<td>4.165</td>
<td>3.521</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.328</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.489</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.103</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>1</td>
<td>1.925</td>
<td>1.978</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.318</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.531</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.138</td>
<td></td>
</tr>
</tbody>
</table>
Gonado Somatic Index (GSI) and Fecundity

However, the results of GSI exhibited differences between the control treatment (average ± standard deviation) (0.173 ± 0.05), and the treatments (T1 = 0.149 ± 0.09, T2 = 0.121 ± 0.07). Statistical analysis did not show significant differences (P ≥ 0.175, table 4). And between the specialist analysis and the presence of significant differences for the actual fertility between. At the same time, the fecundity of treated groups showed significantly high levels (table 4).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>fish No.</th>
<th>GSI mean(± S.D)</th>
<th>Actual Fecundity, mean (± S.D)</th>
<th>embryos number</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>12</td>
<td>0.173(±0.05)</td>
<td>65(±18.56)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>9</td>
<td>0.149(±0.09)</td>
<td>134.67(±79.46)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>14</td>
<td>0.121(±0.072)</td>
<td>109.43(±75.98)</td>
<td></td>
</tr>
</tbody>
</table>

Through the morphological examination of eggs for the ovaries of exposed fish to metformin, it observed different stage of embryonic development (Figs. 4 and 5) in comparison with control that showed a certain developmental stage of embryos (Figs. 3). Variety of stages reached %42 in the first treatment (T1), and %44 in the second treatment (T2).

Statistical analysis for the number of embryos between the control and treatments showed high significant (P≤0.018) whereas there was low significant in the number of embryos between treatments (P≤0.047) table (4).
Fig. (3): The ovary of the sailfin molly fish, *Poecilia latipinna*, taken from a control fish, shows one stage of development. A: Post-vitelligenic oocyte (PVO). B: Embryo with yolk (EY).

Fig. (4): showed the ovary of the sailfin molly fish *Poecilia latipinna* exposed to metformin, indicating the variety of developmental stages due to effects of metformin on the development process, Embryo with yolk: EY, post-vitelligenic oocyte: PVO, primary oocytes: P.O
Fig. (6): showed the ovary of the sailfin molly fish Poecilia latipinna that exposed to metformin, pointing out early stages and late stage of embryonic development, post-vitellogenic oocyte: PVO, primary oocytes: P.O

III. Discussion

GHTs (FSH and LH) are two hormones responsible for regulating reproduction in fish, like all vertebrates. FSH stimulates meiosis of germ cells (production of eggs and sperm), while LH triggers maturation (final development and release of eggs and sperm ( Hatef, & Unniappan. 2019; Blanco, 2020). FSH and LH regulate the gonads development by activation of signaling pathway and synthesis of sex steroids (Zhang et al., 2015; Hollander-Cohen et al., 2021). The roles of FSH and LH may vary depending on fish species, sex, and stage of reproduction (Ramos-Júdez et al., 2022). It is equally important for males as female (Zhang et al., 2015).

Current study showed that exposing molly fish, Poecilia latipinna, to high concentrations (0.008 mol.L$^{-1}$) of metformin, leads to disturbances in the endocrine glands that is responsible for the reproductive, e.g. hypothalamus-pituitary-glands (HPG) which are the main organs for regulating the reproduction process in vertebrates in general, including fish (Niemuth & Klaper 2015; Lee et al., 2019; Elizalde-Velázquez et al 2020). According to a recent study by Ambrosio-Albuquerque et al. (2021), metformin, a drug that affects the pituitary gland, can increase the levels of FSH in fish, FSH is a hormone that helps female fish develop their eggs and produce estrogen, a female sex hormone, FSH also affects the production of vitellogenin, a protein that is important for egg formation (Cabrita et al., 2008; Biran & Levavi-Sivan, 2018).

Metformin increases the incidence of hermaphroditism in males, the presence of eggs in the testicular tissue of male fish Pimephales promelas, and the increase in the production of vitellogen from male liver, as a result of increase in the FSH hormone (Niemuth et al., 2015). Exposing male fish of Betta splendens to metformin reduces their hostility as a result of decrease in the level of LH hormone (MacLaren et al., 2018).

Metformin is also a treatment for polycystic ovary syndrome (PCOS), which is a hormonal disorder in FSH/LH hormone levels, a decrease in the level of FSH leads to the cessation or delay of ovulation in the ovary, while an increase in the level
of LH causes an increase in androgen and testosterone (Saadia, 2020; Mitrašinović-Brulić et al., 2021). Metformin regulates hormone levels by increasing the secretion of FSH, and reducing of LH (Genazzani et al., 2010; Tso, et al, 2020; Jiang et al., 2022). The current result corresponds the previous authors' results. Recent study proved that via gene expression and ELISA analyses. The morphological test of the ovary also reflected the gene expression and ELISA result, showing presence different stage of embryonic development at variance untreated fish (control) that showed up ovary contain a similar stage of embryonic development. This explains the relationship between GHTs and metformin, where FSH stimulates germ cells division and LH has an important role in reproduction by regulating the final maturation of gametes (eggs and sperm). LH is secreted by the pituitary gland targeting the gonads to stimulate other production hormones, such as MIH or MIS that control oocyte maturation. It plays an important role in the final stages of ovulation (Hollander-Cohen et al., 2021; Kumar et al., 2021; Zohar et al., 2021)

Endocrine-disrupting chemicals can interfere with the normal function of the endocrine system by mimicking the action of hormones, preventing their stimulation through binding to hormone receptors, or altering the normal action of hormones in stimulating hormones (Yilmaz et al., 2020). Endocrine-disrupting chemicals (EDCs) can affect some organs or endocrine glands, such as the thyroid, ovaries, testes, adrenal glands, and pituitary gland. Endocrine-disrupting chemicals can also disrupt energy metabolism and cause obesity and metabolic syndrome (Lauretta et al., 2019; Lacouture et al., 2022).

The most common mechanism of action for metformin is inhibition of mitochondrial respiratory complex 1, this leads to reduced oxidative phosphorylation, reduced ATP production, and an increase in the AMP to ATP ratio that activates AMP-activated kinase (AMPK), which inhibits mechanistic target of rapamycin (mTOR) and activates the catabolism of ATP for energy (Wang et al., 2019; Agius et al., 2020; Ma et al., 2022). Metformin-induced activation of AMPK, inhibition and decrease in expression of mTOR, a protein kinase belonging to the family of phosphatidylinositol-3 kinases (PIKKs). Which regulates various cellular processes such as metabolism, growth, and reproduction (Murugan, 2019), that is, it stops the building processes of proteins and fats and accelerates their metabolism (Lamming et al., 2013; Weichhart, 2018). This suggest the reduction of GSI in treated fish. Despite the increase in the hormone FSH, the volume of the egg yolk derived from vitellogen did not increase as a result of the effect of mechanistic target of rapamycin (mTOR). A study on fish about the effect of metformin on the reproduction activity found that it interferes with the energy pathway, which is an important in the regulation and success of the reproduction process (Volkoff & London, 2018; Barros et al., 2022b).

Another hypothesized pathway by which metformin may influence on endocrine secretions is DNA methylation. This process means adding a methyl group (carbon with three hydrogen atoms) to a DNA base, usually cytosine To activate or suppress specific genes (Moore et al., 2013). Hassler et al. (2016) and Lin et al. (2020) cited that metformin accumulated in zebrafish tissues, acting DNA methylation by its interaction with DNA methyltransferase 1 (DNMT1) that is an enzyme regulates DNA methylation and alters the state of gene expression.

We conclude that metformin leads to disrupt the endocrine glands, especially the pituitary gland hormones (GTH), as it increased the level of the hormone FSH, subsequently lead to change cycle and inhibit LH hormone. The latter in turn act to delay the ovulation. The metformin reproductive effectiveness also can be influenced by the species and the physiological state of fish, as it acts to interference with the energy metabolism pathway via other genetic pathways.
IV. Conclusion

Our study shows that metformin affects the reproductive cycle of female molly fish by enhancing the expression of Fshb gene and suppressing that of Lhb gene. These two effects lead to ovarian changes in the process of egg formation and maturation.

Acknowledgments

We would like to express our sincere gratitude to our colleagues from the Department of Fisheries and Marine Resources, College of Agriculture, University of Basrah, for their support and assistance.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical approval

This study was conducted in accordance with all relevant institutional, national and international ethical guidelines for the care and use of animals.

V. REFERENCES


