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The effect of levothyroxine on the changes of sex steroid hormones' and the ultrastructure of ovarian tissue in Three Spot Gourami (Trichogaster trichopterus)

Amirreza Samieimanesh[1], Tahereh Naji[2*], Homayoun Hosseinzadeh Sahafi[3]

[1]Pharmacy student, Department of Basic Sciences, Faculty of pharmacy and pharmaceutical Sciences, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.
[2]Associate Professor, Department of Basic Sciences, Faculty of pharmacy and pharmaceutical Sciences, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran, tnaji2002@gmail.com
[3]Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran
Corresponding Author: Tahereh Naji, tnaji2002@gmail.com,

naji_t@iaups.ac.ir

Abstract

Today, the high consumption of levothyroxine in society has made this drug used as one of the best-selling and well-known drugs for hypothyroid patients. The long-term use of this drug can cause side effects. In this study, the effects of levothyroxine on ovarian tissue were examined. The purpose of this study was to investigate sex steroid hormones and the ultrastructure of ovarian tissue in Three Spot Gourami.The number of 75 mature female Three Spot Gourami(3to6 month age) were released in 5 separate groups (3 treatment groups, intact group, and solvent group) each group including 5 pieces of fish and 3 repetitions totaling 15 pieces in the aquariums; Intramuscular injection was done every other day and at last dissection was done. The results showed that there was a significant difference between the levels of sex hormones among the control and treatment groups ($P<0.05$). It should be noted that the stimulating effect of levothyroxine drug on maturation and sex steroid levels was shown as a significant increase in testosterone and 17-beta-estradiol and 17-hydroxyprogesterone in mature female Three Spot Gourami. And finally, the results of this study showed that levothyroxine had a dose-dependent effect in increasing maturation.Also levothyroxine increased membrane thickness and gap junctions; therefore intercellular exchanges were increased.

Keywords: Levothyroxine, Three Spot Gourami, Sex steroid hormones, Ovarian tissue.

INTRODUCTION

Levothyroxine balances thyroid hormones in the body and is structurally very similar to thyroxine or (T4), one of the hormones produced naturally in the body (Costa et al., 2017). Most researchers have investigated the effects of this drug on the body's metabolism, for example, according to its formulation and clinical results, this drug can affect different pathways of the body, And considering that the organ under our research is the ovary and this organ receives orders under the influence of the hypothalamus and pituitary. Interactions of levothyroxine with progesterone hormone and its effects on ovarian cells, the role of thyroid hormones in fish attracted the opinions of researchers, considering the high reproduction rate of fish and on the other hand, part of human food comes from marine organisms (Mossadegh et al., 2012; Benunga et al., 2019). The effect of levothyroxine drug on the extracellular structure of the ovary and the changes in the levels of sex hormones, as well as finding the mechanism of the drug's effect and knowing the path of its effect is the main

issue of this research. There have not been many experiments in this field, but since physical and environmental factors cause hormonal fluctuations and changes in the reproductive process of fish. In this research, it is intended to investigate the effects of levothyroxine on the level of sex steroid hormones and also the extracellular changes of the ovarian tissue of Three Spot Gourami fish. Due to the fact that the reproductive endocrine control system in fish is based on the hypothalamus-pituitary-gonadal axis, similar to mammals, and also the period of development and maturity of fish is short. The Three Spot Gourami fish has been investigated as a model in this research (Vishnupriya et al., 2022; Costa et al., 2017). Considering that hypothyroidism reduces the sensitivity of dopamine receptors and has an antagonistic effect on its receptors. Also, the effects of levothyroxine use in women on pregnancy and during pregnancy show the mechanism of the effect of this drug on sex hormones (Degani et al., 1995). According to previous studies, in the upcoming research, instead of the usual use of laboratory mice, Three Spot Gourami fish should be used to investigate the effect of levothyroxine on the ultrastructure of ovarian tissue (Ghebar et al., 2010).

In 2012, Scoccia et al showed that women with treated hypothyroidism (n=21) had significantly lower rates of implantation, clinical pregnancy, and live birth than those without hypothyroidism (Scocia et al., 2012).

In 2018, Pelliccione et al showed that treating hypothyroidism with levothyroxine usually restores the normal menstrual pattern, reverses hormonal changes, and improves fertility (Pelikyon and Hamaran, 2018).

Literature and record findings indicate that levothyroxine is the drug of choice for hypothyroidism. Most researchers have investigated the effects of this drug on the body's metabolism, for example, according to its formulation and clinical results, this drug can affect different pathways of the body (Junklas et al., 2014). According to the experiments conducted on mice or similar models, the effect of testosterone on thyroid function can help us a lot in the work process (Prior, 1987). The effects of levothyroxine-induced hyperthyroidism on LH and FSH hormones and testosterone and elements of calcium, magnesium, copper and zinc in rat serum and studies of the histometric effects of thyroid hormones on the reproductive system of adult male rats, It shows that there are interactions in the way this drug works with steroid hormones (Kong et al., 2014). The role of thyroid hormones in fish attracted the opinions of researchers, due to the high reproduction rate of fish and also thyroid disorders in women also cause uterine dysfunction and bleeding caused by the uterine wall of women and cause early menopause symptoms (Kong et al., 2014).

Due to the similarity of the process of ovulation and fertilization of eggs in fish and mammals, like the gonadotropins secreted from the hypothalamus, as well as the secretion of LH and FSH from the hypophysis, the secretion of estrogen and progesterone from the gonad, and due to the similarity of reproductive control by the hypothalamus-pituitary-gonadotropin axis in fish and mammals. (Safi, 2012).

In this research, the aim was to investigate the effect of levothyroxine drug on the changes of sex steroid hormones and ovarian tissue ultrastructure in Three Spot Gourami.

Materials and Methods

In this study, it was intended to investigate the effects of different doses of levothyroxine on sex steroid hormonal changes and ovarian tissue changes using light microscope (ECLIPSE Si ,Japan,Nikon Co,2010) and electron microscope (Netherlands, Philips Co,2007).The effects of levothyroxine drug were investigated by measuring and comparing the amount of hormones as well as cutting from the ovarian tissue after the experiment. This research was conducted on the Three Spot Gourami, which was one of the most abundant fish families with an average weight of 7±1 grams, which reaches maturity in females at the age of 3 to 6 months, was selected as a laboratory sample.

This research was reviewed and approved with the ethical identifier IR.IAU.PS.REC.1400.442 in March 2022 at the Faculty of Pharmacy and Pharmaceutical Sciences of Tehran Islamic Azad University of Medical Sciences.

One gram of levothyroxine drug powder (Batch no:2009947) from Aburihan Pharmaceutical Company were prepared.

After calculating the weight of the fish and calculating the dose of the treatments in terms of mg/kg, the desired dose values in each treatment were weighed with a digital scale with an accuracy of 0.001. The amount of powder calculated in each treatment was dissolved in normal saline as a solvent and after preparing the doses (20, 50, 100 µg/kg) were selected.

The fishes were anesthetized with a concentration of 0.1 ml/l of clove flower's extract. The method of injection was intramuscular(IM). The drug, which was previously drawn by an insulin syringe (with a volume of 1 cc) and filled with the desired concentration, was slowly injected between the dorsal fin and the lateral line of the muscle at an angle of 30 degrees. Became After the drug injection, the fish were placed in the water that had been dechlorinated and balanced with room temperature, resuscitation was performed, then all the fish were transferred to the aquarium. All groups were injected ten times for twenty days every other day. After finishing the injections, there was no activity on the fish for 24 hours, and then the dissection of the fish began.

The fishes were fixed on the dissection tray from the head and tail area, and a longitudinal cut was made from the mouth side to the belly side along the midline with a scissor until under the gill cover. Then, two transverse slices, one at the beginning in the vicinity of the fish head and the other along the slice up to the top of the lateral line, were connected together. Then slowly and carefully, it was removed with scissors and the internal organs and other viscera were emptied and the ovaries were separated. After separating the secretions from around the ovary, each one was selected separately with a digital weight scale and a number of each treatment was selected and placed in 10% formalin solution for study with a light microscope. In order to study with electron microscope, some ovaries were placed in 2.5% glutaraldehyde solution and finally, to prepare the grid in the next steps, the samples were kept in the refrigerator.

The results obtained from the tests are analyzed using spss program by Duncan's method and analysis of variance is done using one way ANOVA test.

Results

Length of fish

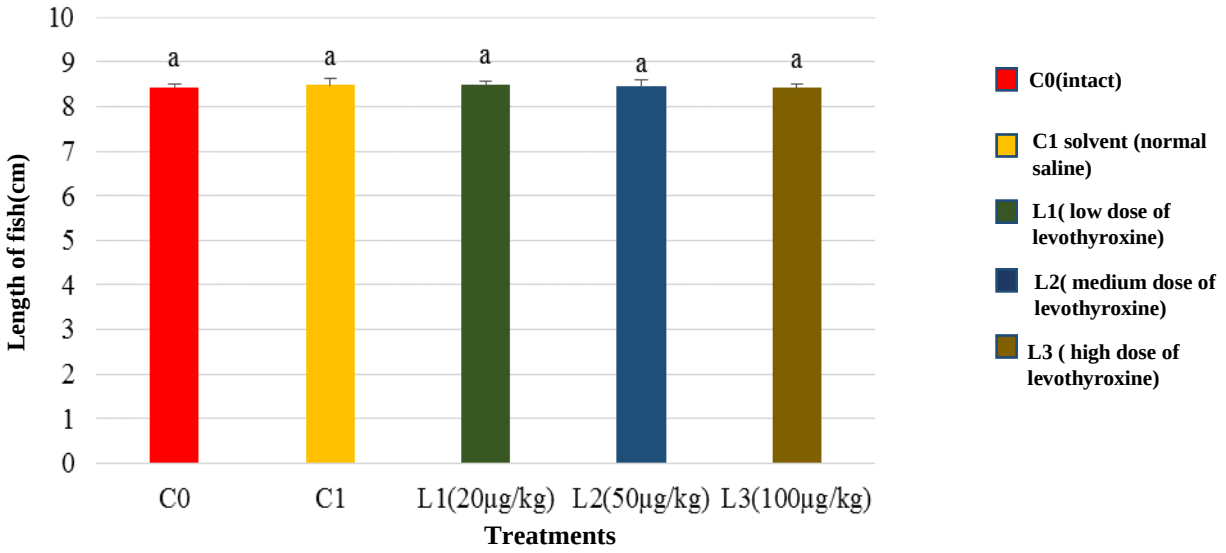

Figure 1. The average values of the length of the fish before the injection (the same letters mean that there was no difference at the 0.05 significance level.)

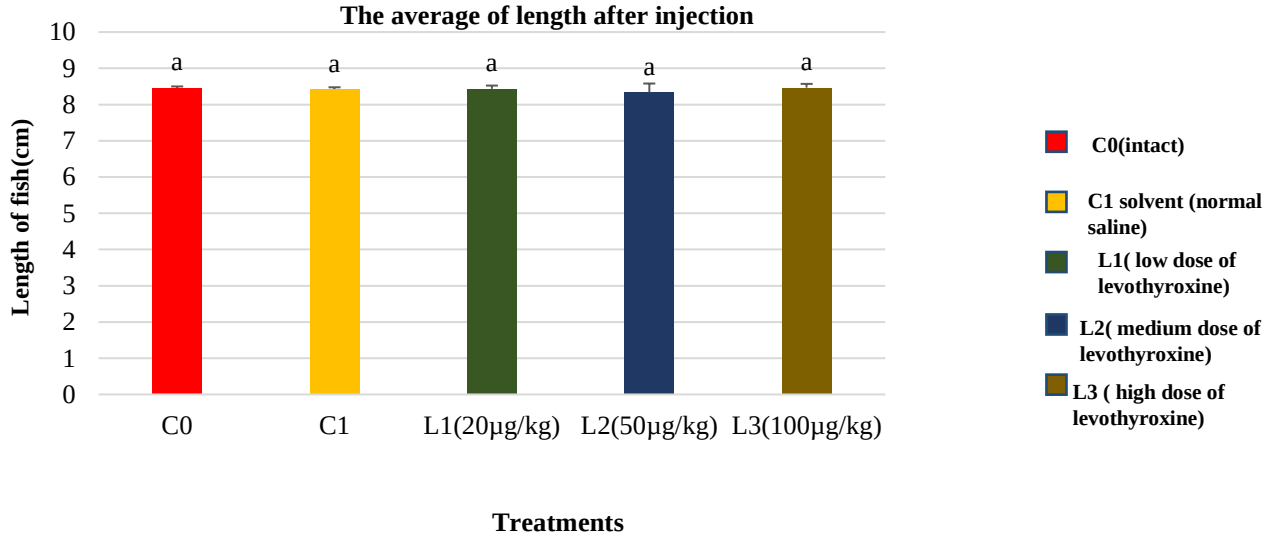

Figure 2. The average length of the fish after injection (the same letters mean there was no difference at the 0.05 significance level.)

The results of the average length of the fish in the treatment and control groups in before injection phase showed that there was no significant difference between the groups

($p<0.05$). Also, there was no significant difference in the statistical comparison of the fish length between the control groups (intact and solvent) and after injection treatment ($p<0.05$).

Examining the results of length of fish before injection in Figure2 showed that there was no W

weight of fish

significant difference between the control and treatment groups ($p>0.05$). Also, examining the results of length's fish after levothyroxine injection in Figure2 showed that there was no significant difference between the control and treatment groups ($p>0.05$).

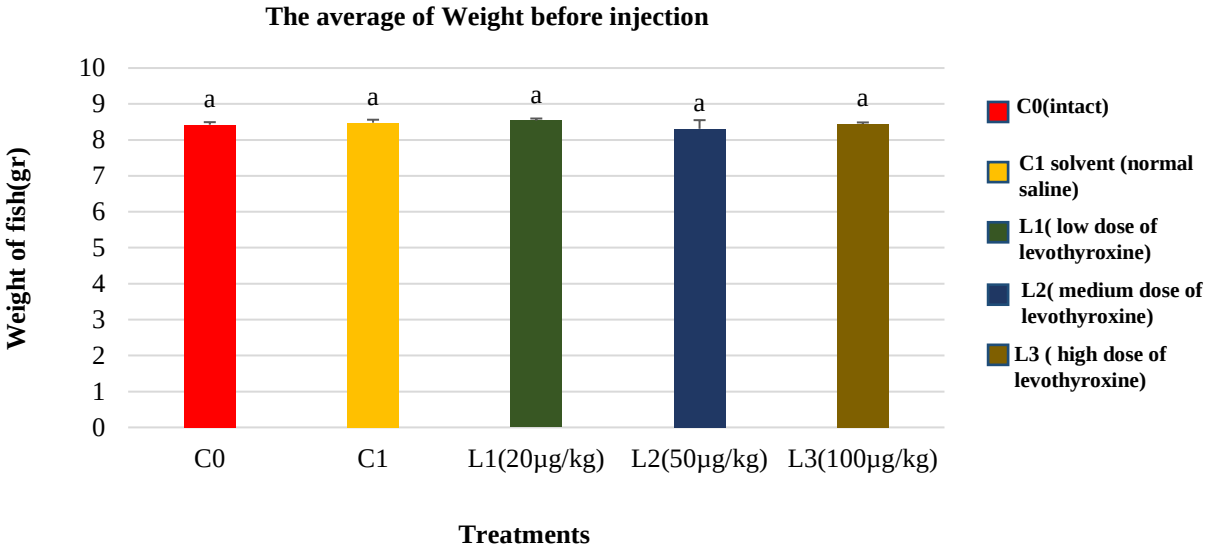

Figure 3. The average weight of fish before injection (the same letters mean there was no difference at the 0.05 significance level.)

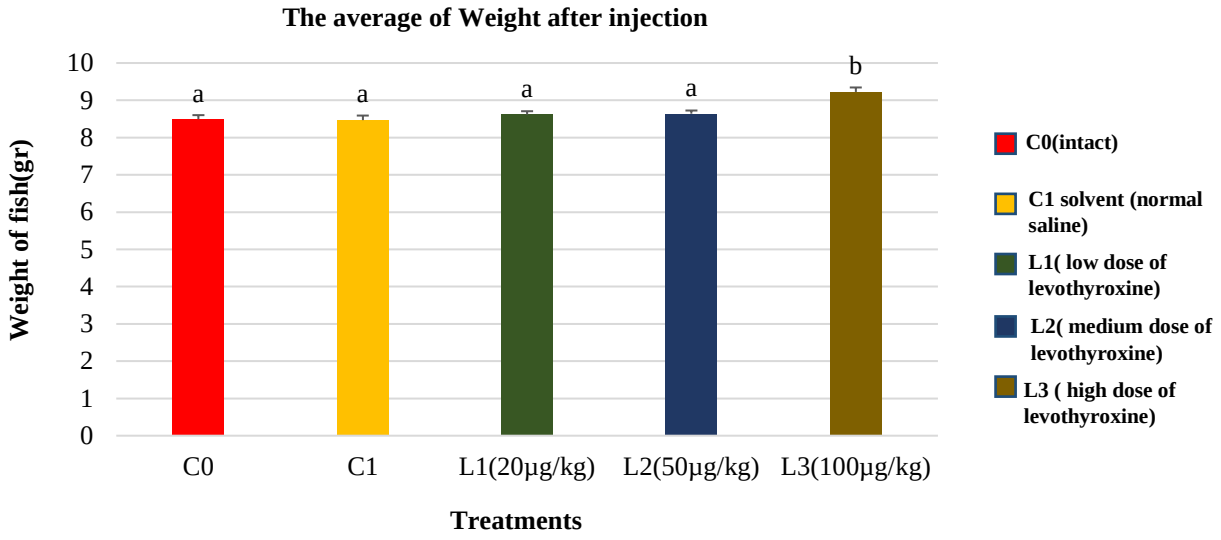

Figure 4. The average weight of fish before injection (the same letters mean there was no difference at the 0.05 significance level.)

The results of the average weight of the fish in the treatment and control groups in before injection phase showed that there was no significant difference between the groups ($P<0.05$). Also, the statistical comparison after injection showed that there was no significant difference in the weight between the control groups (intact and solvent), but there was no significant difference between the control and treatment groups ($P<0.05$). Thus, weight gain occurred in the high dose levothyroxine injection group.

Examining the results of weight of fish before injection in Figure3 showed that there was no significant difference between the control and treatment groups ($P>0.05$). Examining the results of weight of fish after levothyroxine injection in Figure4 showed that Only in the treatment group with high dose, a significant difference was observed with other treatment groups (medium dose and low dose) as well as control groups (intact and solvent).

Gonadosomatic index

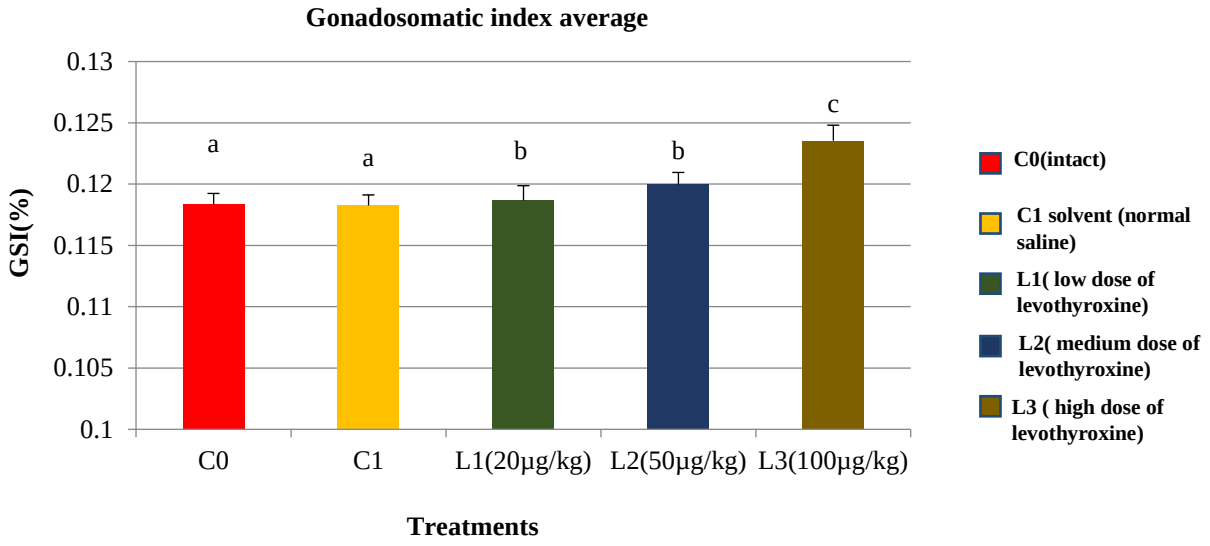

Figure 5. Average Gonadosomatic index of fish (similar letters mean no difference at the 0.05 significance level.)

The results of the Gonadosomatic index showed that there was no significant difference between the control groups (intact and solvent) (P>0.05). But this comparison showed that there was a significant difference between the control groups and all three injection doses of the treatments. Also, this comparison showed that there was no significant difference between the low dose treatment groups (P>0.05). And there was a significant difference between the low and medium

Testosterone hormone

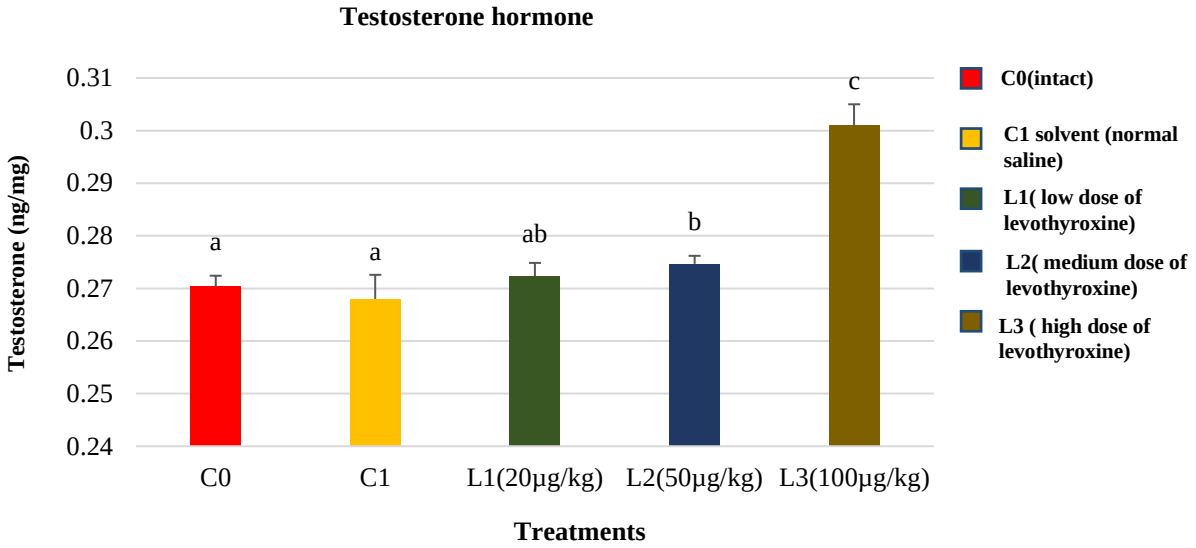

Figure 6. The average values of testosterone hormone in fish (similar letters mean no difference at the significance level of 0.05.

In the analysis of the results of testosterone hormone shown in Figure6 it had been shown that there was no significant difference between the control groups (intact and solvent) ($P>0.05$). But this study showed that there was some difference between the control and low dose treatment groups. However, there was a significant difference between the control groups, the medium dose treatment group and the high dose treatment group. Also, there was a significant difference between the treatment groups with increasing dose ($P<0.05$).

17-hydroxyprogesterone hormone

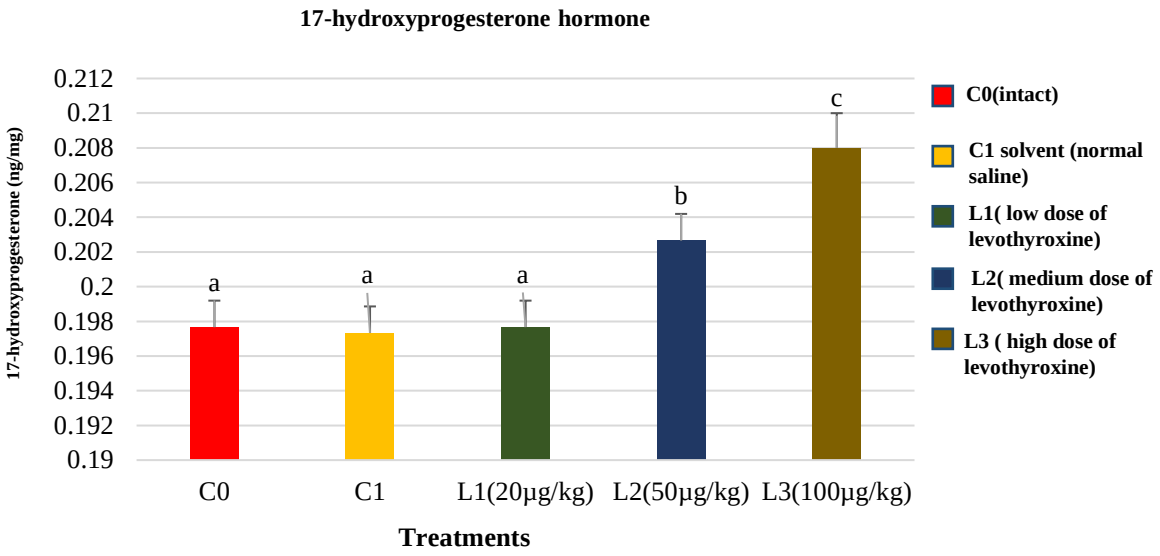

Figure 7. Mean values of progesterone hormone in fish (similar letters mean no difference at the 0.05 significance level.)

There was no significant difference between the control groups (intact and solvent) in examining the results of 17-hydroxyprogesterone hormone shown in Figure7 (P>0.05). Also, there was no significant difference between the control group and the low dose treatment group (P>0.05). But there was a significant difference between the control and the treatment groups with medium and high doses.

17-beta stradiol hormone

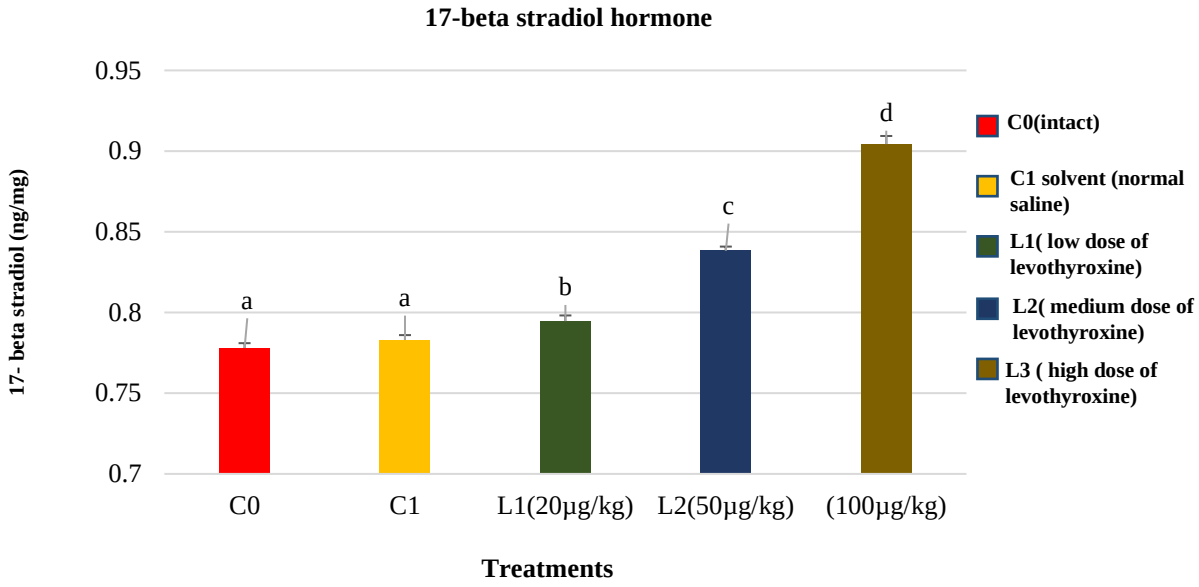

Figure 8. Average values of 17-beta estradiol hormone in fish (similar letters mean no difference at the 0.05 significance level.)

The results of 17-beta estradiol hormone in Figure8 showed that there was no significant difference between intact and solvent groups (P>0.05). But there was a significant difference between the control and the treatment groups.

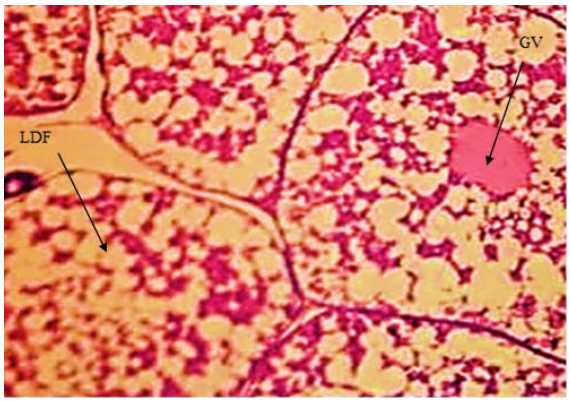

Figure 9. A cross section of the ovarian tissue in intact group. The dominant stage of vitellogenin (V) was the beginning of the movement of the Germinal Vesicle (GV) towards the animal pole.

The results of this study showed that most of the oocytes in the intact group were in the vitellogenesis stage. The beginning of the movement of the germinal vesicle towards the animal pole was shown.

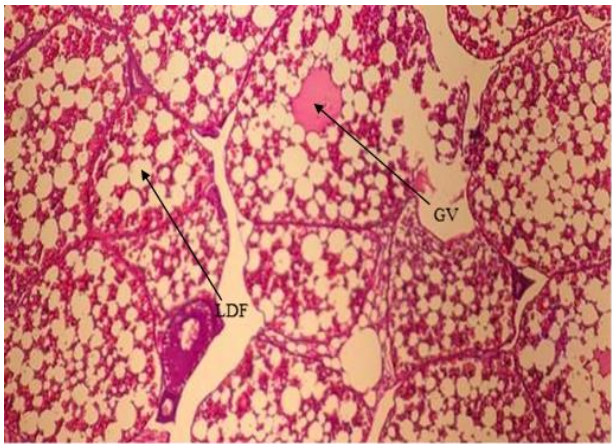

Figure 10. A cross section of the ovarian tissue of the solvent group (normal saline), The dominant phase of vitellogenin (V) was the beginning of the movement of the Germinal Vesicle (GV) towards the animal pole.

In the solvent group, like the intact group, most of the oocytes were in the vitellogenesis stage. The beginning of the movement of the germinal vesicle towards the animal pole was shown and

the binding of lipid particles to each other was visible in the ooplast.

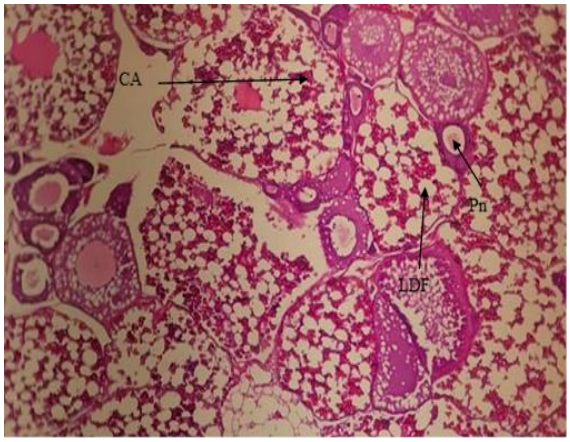

Figure 11. A cross section of the ovarian tissue of a fish treated with levothyroxine in a low dose (20µg/kg), the presence of oocytes in the cortical alveolar stage CA (Cortical Alveolar) and LDF(Lipid Droplet Fusion) Pn(prenucleus).

The results of the images of levothyroxine treatment with a low dose (20µg/kg) showed that the number of cells in the cortical alveolar stage generally increased compared to the control group and most of the oocytes were mature. In this group, a number of oocytes in the prenuclear stage were also observed.

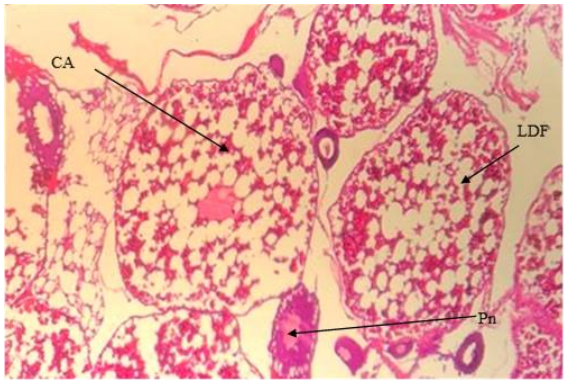

Figure 12. A cross section of the ovarian tissue of a fish treated with levothyroxine in an average dose (50 µg/kg), the presence of oocytes in the alveolar cortical stage CA (Cortical Alveolar) and LDF (Lipid Droplet Fusion), Pn (prenucleus).

The results of the figures of levothyroxine treatment with a medium dose (50µg/kg) showed that the number of cells in the cortical alveolar stage increased compared to the control group and the low dose treatment group. Also, the number of prenuclear cells decreased compared to the low-dose treatment groups.

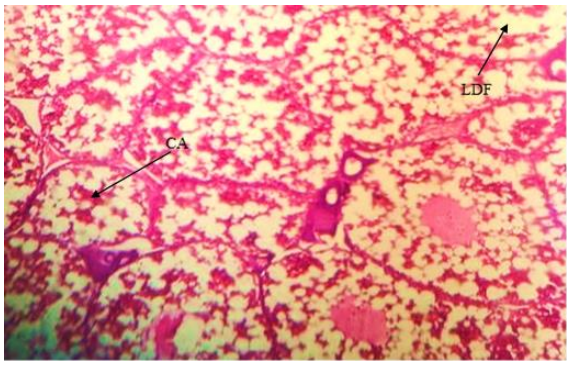

Figure 13. A cross-section of the ovarian tissue of a fish treated with levothyroxine in a high dose (100 µg/kg), the presence of oocytes in the cortical alveolar stage CA (Cortical Alveolar) and LDF (Lipid Droplet Fusion)

The results of the figures of levothyroxine treatment with medium dose (100µg/kg) showed that the number of oocyte cells in the cortical alveolar stage increased greatly and also the number of mature cells was more than other treatment groups. Examination of ovarian sections in the control groups showed that the cells were in cortical alveolar and maturation stages and the movement of the germinal vesicle towards the animal pole indicated the beginning of the maturation stage. Also, fat particles form fat droplets by connecting to each other, and before, the nuclear membrane was opened, and as a result, its contents were dispersed into the cytoplasm, and so the accumulation of these particles in the cytoplasm, the nucleus was closer to the membrane.

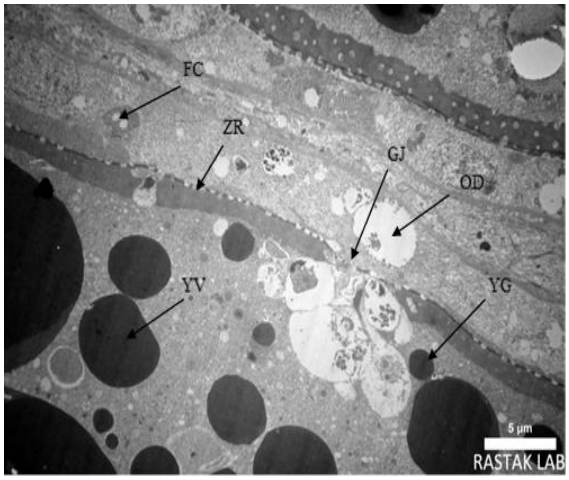

Figure 14. Electron microscope image of a section of an oocyte cell in an intact. In this image, Follicular Cell FC. Zonradiata layer ZR, Fat droplets OD. (Droplet Oil), yolk particles YG. (Granules Yolk), Yolk vesicle YV. (Vesicle Yolk), and intercellular gap GJ. (Junction gap) has been specified. (Scale bar 5 µm).

An abnormal trend was observed in the ovary in intact group. The structure of the membrane was discrete and the layers of the ovary are normal, yolk particles and fat particles do not penetrate into the cell and membrane exchanges were established.

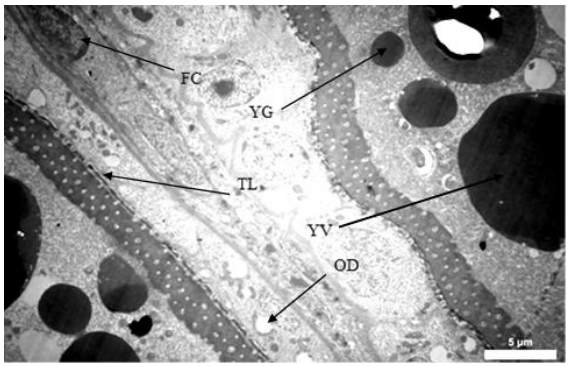

Figure 15. Electron microscope image of a section of an oocyte cell in In low-dose (20 µg/kg). In this image, Follicular Cell FC, Zonradiata layer ZR, Fat droplets OD. (Droplet Oil), yolk particles YG. (Granules Yolk), Yolk vesicle YV. (Vesicle Yolk), and intercellular gap with GJ. (Junction gap) has been specified. (Scale bar 5 µm).

According to the image of the ovary of fish in low dose (20µg/kg): Membrane exchanges were increased compared to the control group, and yolk and fat particles penetrate into the cell at a faster rate.

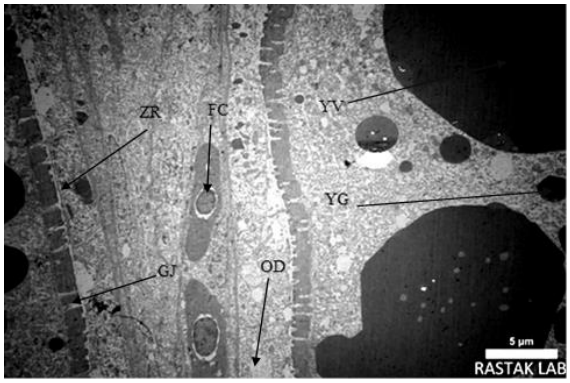

Figure 16. Electron microscope image of a section of an oocyte cell in In high-dose (100 µg/kg). In this image, Follicular Cell FC,Zonradiata layer with ZR, Fat droplets OD. (Droplet Oil), yolk particles YG. (Granules Yolk), Yolk vesicle YV. (Vesicle Yolk), and intercellular gap GJ. (Junction gap) has been specified. (Scale bar 5 µm).

According to the figure of the ovary of the fish treated in high dose (100µg/kg): the membrane was completely discrete in some parts and the penetration of particles and fat was easily done. Mature oocyte cells were completely visible and the way of oocyte maturation was done as follows. The covering layers of the egg from the outside to the inside include the theca layer, follicular epithelium layer or the granulosa layer, zonaradiana layer and membrane.

Discussion and conclusion

Considering the reports of the results of levothyroxine drug injection to Three Spot Gourami, The process of changing test variables such as lenght and weight index, GSI, HSI and the level of testosterone, 17-hydroxyprogesterone, 17-beta estradiol Changes have been made in the histological structure of the gonads in the light microscope and the ultrastructural changes of the Three Spot Gourami ovaries in the electron microscope. According to the study conducted by Mohaghegpour and his colleagues conducted on Japanese quail, the drug Levothyroxine was able to increase the high weight of this sample. This issue is completely

consistent with our observations and shows that levothyroxine increases the weight of fish and this change is quite noticeable and visible in the high dose treatment (Porkavos et al., 2016). According to Gerdon et al.'s study, increasing the plasma level of T4 can reduce the conversion of testosterone to androstenedione, This action can be seen in hyperthyroidism and leads to an increase in the level of testosterone in the plasma (Gerdon et al., 1969) In this research, according to the electron microscope figures,increase in the theca layer was observed, because this layer is the producer of testosterone, so with the increase in the thickness of this layer, the production of testosterone hormone increases. According to the study of Spicer and his colleagues, who investigated the effect of thyroid hormones on gonadotropin, it showed that increasing the plasma level of T4 can increase the progesterone hormone (Spicer et al., 2001). Considering that progesterone is produced in the final stages of ovarian development, according to the observations of this research, the growth and development of the ovary in the figures of the light microscope confirms this issue. Examining the figures of levothyroxine treatment groups in figures (11, 12, and 13) showed that the number of cells in the cortical alveolar phase increased with the increase in the dose of levothyroxine and the cells entered the next stage from prenuclear Also, the growth of fish ovaries in clinical evidence indicates that the number of mature eggs increased with increasing dosage. According to Muderris study, with the increase in T4 serum level in plasma, the amount of precursors of androgen hormones has increased, and these precursors are from stimulating the production of oocytes in the early prenuclear stages to the full maturity of the cell, it is clearly shown in the pictures (Muderris et al., 2011). The results of examining the electron microscope figures (14, 15 and 16) showed that in the intact group, the cells were in the cortical alveolar stage, Also, the theca and granulosa follicular layer were distinct and the cell membrane had a normal process. The penetration of the yolk particles into the cytoplasm as well as their joining together and the formation of yolk granules were observed in the figures, which emphasizes that the cell was in the cortical alveolar stage, On the other hand, the fat particles join together and form fat vacuoles, and at this stage, the egg size increases due to the entry of yolk and fat particles and the accumulation of their vesicles. According to Kabudmehri and his colleagues, it was found that by increasing the dose of levothyroxine, the normal process of the membrane has changed, the disintegration of the membrane has increased, and the exchange rate of yolk and fat particles has increased, And this indicates the stimulation of the ovary to reach puberty (Kabudmehri et al., 2021). Which can be seen based on our obtained images in figures (24 and 25). In addition to the growth of the yolk cells and their transformation from the granular state to the vesicle, the rate of maturation of the oocyte cells increases with the increase in membrane rupture.

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