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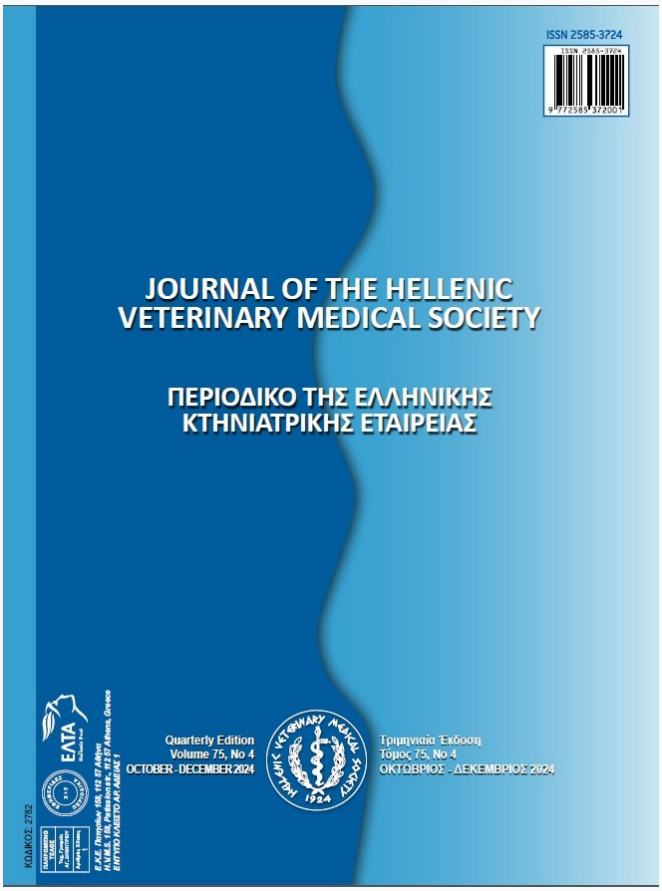

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Research article
Ερευνητικό άρθρο

Beneficial effects of metformin on high fructose-induced ovarian cycle abnormalities and antioxidant activity

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ABSTRACT: This study aimed to evaluate the effect of metformin on the regulation of the ovarian cycle through serum antioxidant activities in female rats with fructose-induced metabolic syndrome. Thirty-four, immature female Wistar rats (21 days-old) were randomly divided into five groups (n: 4-8/group) control, carboxymethylcellulose (CMC), fructose, metformin, and fructose+metformin groups. The metabolic syndrome model was induced by fructose solution (20% w/v) for 15 weeks. Metformin was administrated by oral gavage in CMC solution for the last final 6 weeks. Vaginal cytology was evaluated before (the 3rd week after fructose administration; 49 days old) and at the 9th and 15th weeks of the experiment. Estrogen, progesterone, testosterone, aromatase, total antioxidant status (TAS), total oxidant status (TOS), and paraoxonase (PON)-1 levels were assessed in serum samples. The statistical analysis was performed using a one-way ANOVA test. Results are expressed as the mean ± standard error of the mean (SEM). Metformin restored the regularity of the estrous cycle, which was disturbed by a high fructose diet. Moreover, dietary high-fructose markedly increased serum testosterone, TOS, and OSI values, but significantly decreased serum estrogen, progesterone, aromatase, TAS and PON-1 levels than the other groups ($p < 0.05$). Metformin, through its antioxidant and hormone regulation activity, may have had a beneficial effect on the impaired ovarian cycle associated with oxidative stress induced by high fructose diet.

Keywords: High-Fructose Diet; Metformin; Ovarian Cycle; PON-1; Steroid Hormone; OSI.

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INTRODUCTION

Metabolic syndrome (MetS) is a clustering of abnormalities that include insulin resistance, glucose intolerance, atherogenic dyslipidemia, hypertension, endothelial dysfunction, inflammation, and visceral obesity (Wang et al., 2020-a). MetS is closely linked to polycystic ovarian syndrome, which includes ovulation failure and hyperandrogenism, according to earlier research (Wang et al., 2020-b; Khan et al., 2023). In addition, it is known that hepatic steatosis, inflamed adipose tissue, increased coagulation factor activity, and oxidative stress can develop in MetS (Fahed et al., 2022). Oxidative stress results from an imbalance between the production and scavenging of reactive oxygen species (ROS) during many pathological processes (Pizzino et al., 2017; Singh et al., 2022). Uncontrolled production of ROS or a lack of antioxidants plays a vital role in the development of clinical complications of MetS (Tan et al., 2018; Vona et al., 2019). High fructose-induced chronic hyperglycemia and hypertriglyceridemia cause an increase in oxidative stress, systemic low-grade inflammation, and lipid accumulation which contributes to the development of MetS (Ko et al., 2017; Zhang et al., 2017). Paraoxonase (PON)-1, an antioxidant enzyme associated with high-density lipoprotein, has also been shown to be affected by high-fructose consumption (Dornas et al., 2012). Evidence suggests that excessive ROS production can also induce reproductive problems such as deterioration of oocyte quality and a decrease in estrogen level (Sugino, 2005; Lu et al., 2018). The link between the MetS and the diseases such as polycystic ovary syndrome (PCOS), endometriosis, and infertility is represented by oxidative stress (Johnson, 2014; Pasquali, 2018; Akintayo et al., 2021). Therefore, oxidation is considered as both a cause and a consequence of MetS (Tangvarasittichai, 2015).

Metformin, a biguanide-derived drug widely used to treat of type 2 diabetes, obesity, MetS, and PCOS, is known to improve glucose uptake by enhancing insulin sensitivity (Ahmed Mobasher et al., 2020; Lv and Guo, 2020; Bai and Chen, 2021; Mohammed et al., 2021; Gasser et al., 2022). Metformin is thought to alleviate hyperandrogenism by reducing hyperinsulinemia-induced excessive ovarian androgen production, thereby improving menstruation, steroidogenesis, and ovulation function(Rice et al., 2009; Viollet et al., 2012; Zhao et al., 2017). However, the molecular mechanism underlying this effect is not fully understood yet. Moreover, metformin administration has been shown to reduce the imbalance between peroxidation and antioxidant defences (Apostolova et al., 2020; Astiz et al., 2020; Ren et al., 2020; Liu et al., 2022).This beneficial effect of metformin on oxidative stress is thought to be due to the reduction in cellular ROS production (Algire et al., 2012). The effects of metformin on oxidative stress markers and PON-1 activity remain under debate in metabolic syndrome (Sentí et al., 2003; Torun et al., 2011; Meaney et al., 2012; Camps et al., 2016; Adhe-Rojekar et al., 2018). The aim of the current study was to investigate the possible beneficial effects of metformin on the ovarian cycle and serum antioxidant activities in high-fructose-fed rats. We hypothesized that metformin would improve ovarian activity by regulating oxidative balance in rats with metabolic syndrome.

MATERIALS AND METHODS

Animals

Thirty-four immature, healthy, 3-week-old female (75-85 g) Wistar albino rats (*Rattus norvegicus*) were provided from the Laboratory Experimental Animals, Kobay, Turkey. The rats were quarantined for one week. During the experiment, the animals were kept in polysulfone cages (425 × 266 × 185 mm in size; three animals in each cage) at 21-24 °C and 40-45% humidity and under light-controlled conditions (12 h light/12 h dark) at the Laboratory Animals Breeding and Experimental Research Center of the Faculty of Pharmacy, Gazi University (Ankara, Turkey). The animals were fed with a standard pellet diet (5.5% raw fat, 23% raw proteins, 3.5% raw cellulose, 8% raw ash, 11% neutral detergent fiber; 12.5 kJ/g; Optima Yem, Turkiye) and water ad libitum. All the animals were maintained in accordance with the directions of the Guide for the Care and Use of Laboratory Animals (Guide for the Care and Use of Laboratory Animals 2011), and the experimental procedures were approved by the Experimental Animal Ethics Committee of Gazi University (G.U.ET-22.030).

Induction of metabolic syndrome model

All the rats were randomly divided into five groups, as follows: control group (n=6), carboxymethylcellulose (CMC) group (n=4), fructose group (n=8), metformin group (n=8), and fructose+metformin group (n=8). The metabolic syndrome model was not applied to the control, CMC, and metformin groups. To induce the metabolic syndrome model, fructose (Danisco Sweeteners, Finland) was added to the drinking water of the rats in

20% solution (w/v) and administered *ad libitum* for 15 weeks (Korkmaz et al., 2019) The drinking water with fructose was daily prepared.

The treatment protocols

Metformin was used for the treatment of rats with metabolic syndrome. The treatment was started in the 9th week of the experimental procedure and continued for 6 weeks. While no treatment was applied to the animals of the control group, to create gavage stress, only gavage was administered. The CMC and fructose groups were given carboxymethyl cellulose (0.5%; 1 mL) by oral gavage. The rats in metformin and fructose+metformin groups were treated with metformin. Metformin was prepared in 0.5% CMC (Sigma-Aldrich Corporation, MO, USA) solution at 200 mg/kg doses (Quaile et al., 2010) and given by oral gavage (1 mL/rat/day).

Vaginal cytology

To determine the stages of the estrous cycle, vaginal smear samples were obtained by a sterile swab before (the 3rd week after fructose administration; 49 days old) and at the 9th and 15th weeks of the experiment. The cells of the vaginal lumen were carefully collected and transferred to a slide. After the slides were air-dried, they were stained by Giemsa stain and then assessed under a light microscope. The stages of the estrous cycle were characterized as proestrus (oval nucleated epithelial cells), estrus (irregular-shaped cornified squamous epithelial cells), metestrus (fragmented, cornified epithelial cells and smaller darker stained leukocytes), and diestrus (nucleated epithelial, predominate leukocytes (Cora et al., 2015). Treatment efficacy was evaluated by the regularity of the estrous cycle.

Termination of the experimental procedure

At the end of the treatment protocol, all the rats were sacrificed by cardiac puncture under general anesthesia (10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride). After blood samples were collected into a serum separator gel tube, they were centrifuged at 3000 rpm/15 min. The obtained serum samples were stored at -20°C until analysis.

Serum levels of steroid hormones and aromatase

Serum estrogen, progesterone, testosterone, and aromatase concentrations were assayed by enzyme-linked immunosorbent assay (ELISA; Azure Biosystems Microplate Reader) using a commercial rat kit according to the manufacturer's instructions.

Measurement of paraoxonase (PON)-1 activity, total antioxidant status (TAS), total oxidant status (TOS), and calculation of the oxidative stress index (OSI)

Serum PON-1 level was assayed by enzyme-linked immunosorbent assay (Rel Assay Diagnostics kit, Turkey) using a commercial rat kit. PON-1 activity was investigated by using paraoxon as a substrate and analyzed by increases in the absorbance at 412 nm due to the formation of 4-nitrophenol as described. Briefly, the PON-1 level was measured at 25°C by adding 50 µl of serum to 1 ml Tris-HCl buffer (100 mM at pH 8.0) containing 2 mM $CaCl_2$ and 5.5 mM paraoxon. The rate of formation of 4-nitrophenol was determined at 412 nm. The enzymatic activity of PON-1 was calculated by using the molar extinction coefficient 17,100 $M^{-1}cm^{-1}$ (Verit et al., 2008). The results were presented as U/L.

Total antioxidant-oxidant levels of homogenized uterine tissue were determined using a novel automated measurement method (Rel Assay Diagnostics kit, Turkey). The antioxidant effect of the sample against the potent free radical reactions, triggered by the produced hydroxyl radical, is measured. This assay relies on the ability of antioxidants in the sample to inhibit the formation of 2,2′-azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid]. The values of TAS are expressed in millimoles Trolox equivalent/liter (Erel, 2004). The color intensity is related to the total amount of oxidant molecules present in the sample. The test is based on the oxidation of ferrous ions to ferric ions in the presence of various oxidative species in an acidic medium and the measurement of the ferric ion by xylenol orange. The assay is calibrated with hydrogen peroxide (H_2O_2). The results are expressed in terms of micromoles H_2O_2 equivalent/liter (Erel, 2005). The OSI was defined as the ratio of the TOS level to the TAS level. TAS levels were converted to micromoles. Specifically, OSI (arbitrary unit) = TOS (µmol H2O2 equivalent/L) / TAS (µmol Trolox equivalent/L) × 100.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0. Results are expressed as the mean ± standard error of the mean (SEM). The one-way ANOVA test was used to determine the significance of differences among groups. Statistical significance was assumed at the level of $p < 0.05$.

RESULTS

The microscopic findings of vaginal smear

The vaginal cytology findings are given in Figure 1. Before the experimental procedure, all rats showed a regular estrous cycle of 4-5 days. In control, CMC and metformin groups, the estrous cycle continued to be seen regularly until the last day of the experimental procedure. However, in fructose and fructose+metformin groups, it was observed that the regularity of the estrous cycle was disturbed in the 7th week. All rats in these groups were generally in the luteal (metestrous-diestrous) phase of the estrous cycle. An irregular cycle was over 15 days in both the fructose and fructose+metformin groups. In the fructose+metformin group, irregular-shaped cornified squamous epithelial cells began to be observed every 4-5 days at the end of the experimental procedure (15th week).

The effects of dietary high-fructose and metformin on serum levels of steroid hormones and aromatase

The results of serum steroid hormones and aromatase levels are presented in Figure 2. Serum estrogen (8.20±2.10 pg/mL), progesterone (6.80±1.70 ng/mL), and aromatase (11.00±0.68 pg/mL) levels of the fructose group were found to be lower than the other groups. On the contrary, the testosterone value (1.20±0.24 pg/ml) of the fructose group increased compared to the other groups ($p<0.05$). Estrogen level was decreased in the fructose group as compared to

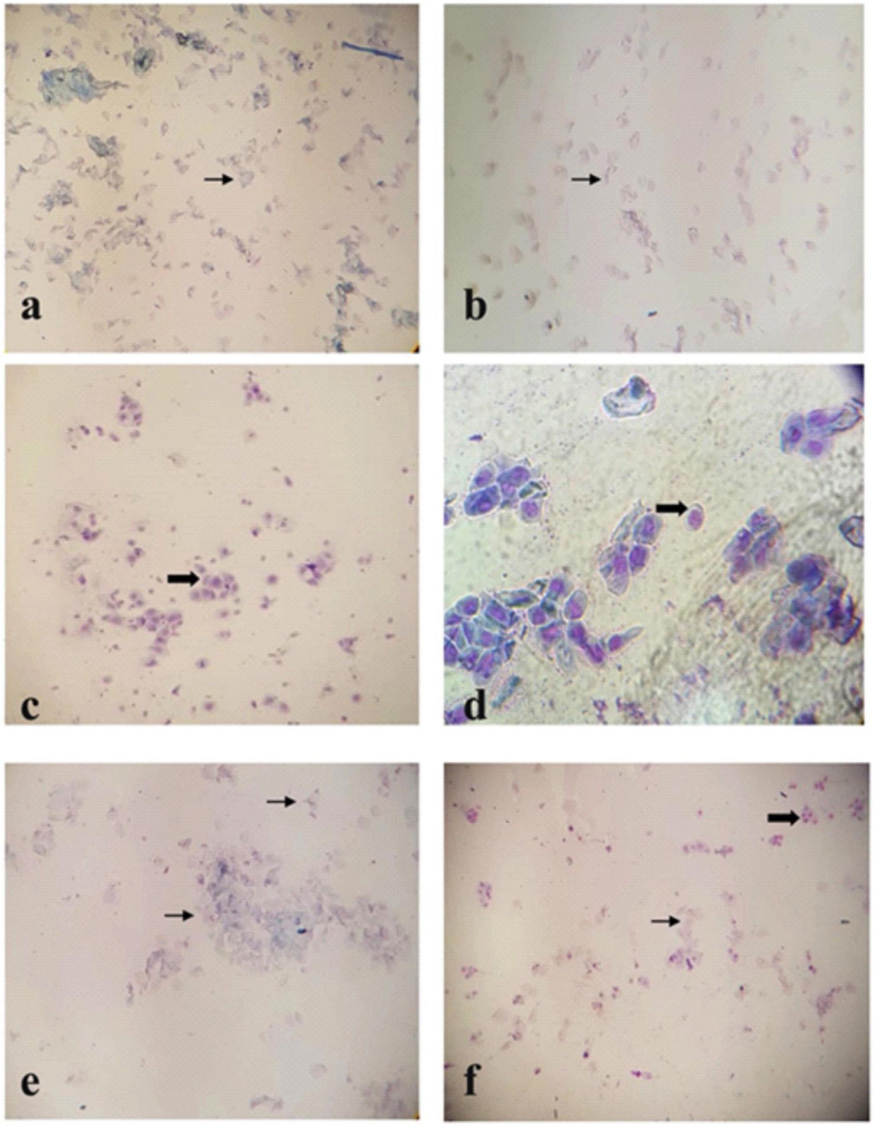

Figure 1. Vaginal cytology findings in the 15th week of the experimental procedure. Anucleate superficial cell in control (a), CMC (b), and metformin (e) groups x10; nucleated basal cell (c; x10 and d; x40) in fructose group; anucleate superficial and decreasing nucleated basal cells in fructose+metformin group. Thick arrow: nucleated basal cell; thin arrow: anucleate superficial cell.

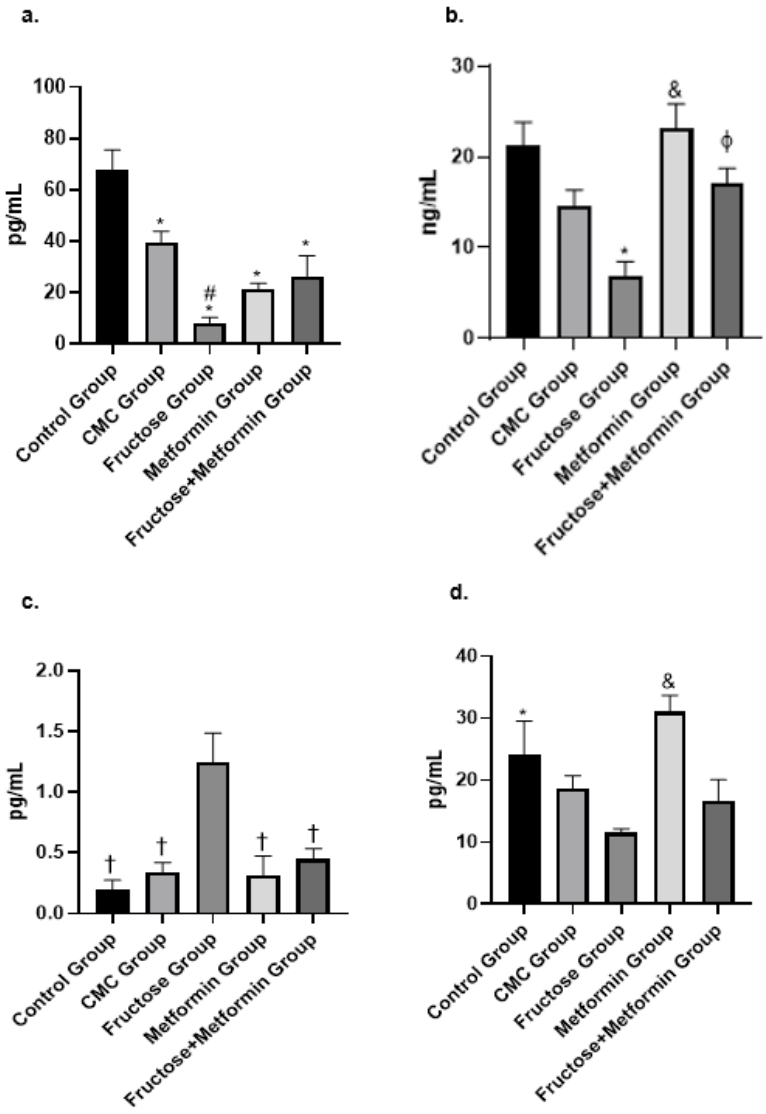

Figure 2. Effects of metformin on serum estrogen (a), progesterone (b), testosterone (c), and aromatase (d) levels. *p < 0.05, significantly different from the control; #p < 0.05, significantly different from the CMC group; &p < 0.05, significantly different from the metformin group; ϕp < 0.05, significantly different from the fructose+metformin group; † p < 0.05, significantly different from the fructose. Values are expressed as mean ± SEM.

those of the control and CMC groups (p<0.05). Progesterone level was decreased in the fructose group as compared to those of the control, metformin, and fructose+metformin groups (p<0.05). The aromatase level in the fructose group significantly reduced when compared to the control and metformin groups (p<0.05).

Oxidative stress index

The serum TAS level was the lowest in the fructose group (0.86 ± 0.03 mmol/L) and significantly decreased compared to the control (1.15 ± 0.06 mmol/L) and carboxymethyl cellulose (1.20 ± 0.04 mmol/L) groups (p < 0.05). Serum TAS levels were enhanced by metformin administration. There was also a difference between the fructose and metformin (1.16 ± 0.05 mmol/L) groups (p < 0.05). Although it was determined that there was no difference between the fructose+metformin group (1.07 ± 0.10 mmol/L) and the other groups, it was observed that the serum TAS levels increased compared to the fructose group. Serum TOS level was determined to be the highest in the fructose group (13.23 ± 1.36 μmol/L). This value of the fructose group was found to be statistically different from that of the other groups (p < 0.05). It was noticed that the OSI value was the lowest in the control (0.43 ± 0.06 AU) and the CMC groups (0.35 ± 0.02 AU). OSI value in the fructose group (1.50 ±

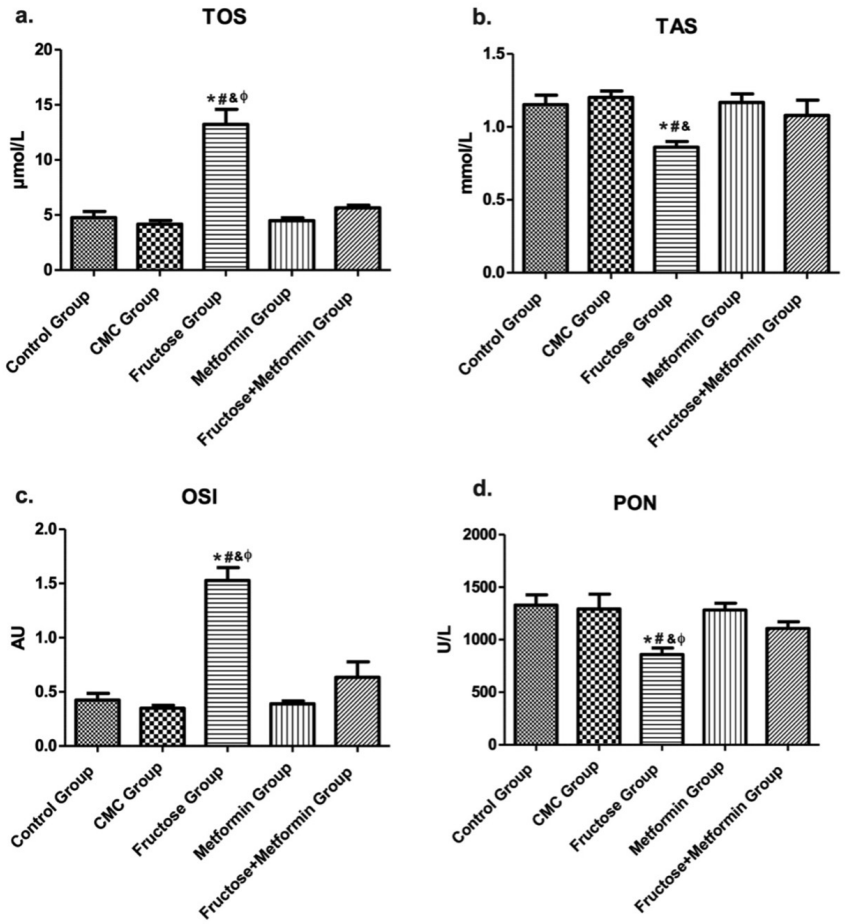

Figure 3. Effects of metformin on serum TAS (a), TOS (b), OSI (c), and PON (d) levels.
*p < 0.05, significantly different from the control; #p < 0.05, significantly different from the cmc group; &p < 0.05, significantly different from the metformin group; ϕp < 0.05, significantly different from the fructose+metformin group. Values are expressed as mean ± SEM.

0.12 AU) was significantly higher when compared to the other groups ($p < 0.05$) (Figure 3).

PON-1 level

Regarding serum PON-1 levels, the lowest values were found in the fructose group (859.60 ± 60.40 U/L) compared to the other groups. It was observed that the serum PON-1 level was at similar levels in the control, CMC, and metformin groups (1329.00 ± 98.39 U/L, 1292.00 ± 141.80 U/L, 1282.00 ± 65.44 U/L: respectively). The serum PON-1 level of the fructose group was significantly lower than that of the other groups ($p<0.05$) except for the metformin group. In the fructose+metformin group (1107.00 ± 63.63 U/L), it was determined that serum PON-1 level was increased by metformin treatment after fructose administration ($p<0.05$) (Figure 3).

DISCUSSION

The impact of dietary high fructose on female gender function has been relatively less studied compared to those of males. Therefore, there are findings on the adverse effects of a high-fructose diet on the female reproductive system. A study reported that high-fructose corn syrup feeding for 28 days in adult female rats changed the length of the estrous cycle and ovarian histology (Ko et al., 2017). Another study revealed that high-fructose consumption increased ovarian weight, and luteinizing hormone levels, but decreased follicle-stimulating hormone in rats with letrozole-induced polycystic ovarian syndrome (Akintayo et al., 2021). In the present study, we investigated the therapeutic effects of metformin on the ovarian cycle and serum oxidation parameters in female rats with high-fructose diet-induced metabolic syndrome. Rats fed a high-fructose diet (20%) showed impaired ovarian activity because this diet caused the distribution

of steroid hormones and an oxidative imbalance. Our results suggest that metformin can regulate the ovarian cycle in female rats with metabolic syndrome by increasing the activities of serum levels of TAS and PON-1.

The effects of metabolic syndrome on ovarian hormones have not been fully proven. However, it is known that hormones play an important role in the development and pathogenesis of metabolic syndrome. Many features of the metabolic syndrome, including glucose tolerance, lipid metabolism, and blood pressure, are correlated with estrogens. Numerous polymorphisms in the estrogen receptor genes have been linked to metabolic syndrome (Finan et al., 2012). In the light of this information, estrogen is known to be effective in the treatment of metabolic syndrome. Furthermore, insulin has been shown to stimulate ovarian androgen synthesis directly or indirectly via the pituitary gland. Additionally, insulin resistance contributes to the development of metabolic syndrome and leads to severe metabolic and endocrine disorders. A high-fructose diet causing insulin resistance and altered steroidogenesis can cause the development of PCOS. Therefore, the term metabolic syndrome is used to describe the clinical characteristics of PCOS, including insulin resistance, obesity, dyslipidemia, and hyperandrogenism in women. Compensatory hyperinsulinemia and insulin resistance are thought to be the main pathogenic causes of PCOS. Clinical signs of PCOS include hyperandrogenism, oligomenorrhea, persistent anovulation, and hirsutism. Accordingly, metabolic syndrome is present in 43% of adult women and almost a third of adolescents with PCOS (Chen and Pang, 2021; Poojari et al., 2022).

The elevated plasma levels of LH, and the abnormal ovarian and adrenal androgen production typical of PCOS may be explained by hyperinsulinemia and insulin resistance. Recent studies have demonstrated that insulin-sensitizing medications such as metformin decrease plasma levels of LH, as well as ovarian and adrenal androgen production. A cytochrome P450 enzyme complex known as aromatase, which is found in adipocytes and adipocyte stromal tissue, can convert androgens into estradiol. This enzyme is thought to be directly stimulated by insulin. Therefore, excessive stimulation of androgen production, hyperinsulinemia, and insulin resistance may be the underlying cause of the hyperandrogenism associated with PCOS. Insulin increases the effects of FSH by stimulating aromatase activity (la Marca et al., 2002). In the present study, the serum estrogen and progesterone levels were markedly decreased in the fructose group when compared to those of other groups. The testosterone level exhibited a higher concentration in the fructose group when compared to that of the other groups ($p<0.05$). The serum aromatase levels in the fructose group were lower than in the control and metformin groups ($p<0.05$). Metformin treatment was found to lower serum testosterone levels. Similar to other studies (Wang et al., 2020-b; Khan et al., 2023), our results also provide evidence that high fructose diet increased serum testosterone levels and decreased steroid hormones and metformin treatment was effective in improving these parameters. The findings confirmed that metformin could be used as a treatment option through hormone regulation in high-fructose diet-induced metabolic syndrome.

The stages of the estrus cycle can be identified by the vaginal cytology method. This method, which is simple, inexpensive, and practical, is performed by evaluating vaginal smear samples under a light microscope. Vaginal cytology is regarded as the "gold standard" for estrus staging of female rats. It is known that there is a relationship between the appearance of the exfoliated vaginal epithelium layer on vaginal smear samples and the estrus cycle (Cora et al., 2015). In this study, vaginal cytology was performed to evaluate the effects of both metabolic syndrome and metformin treatment on the ovarian cycle. In the vaginal smear samples in rats of the fructose group, nucleated epithelial cells were observed during the experimental procedure. As indicated in the literature, metabolic syndrome alters the secretion of ovarian steroid hormones and therefore, this finding of vaginal cytology was the result of an imbalance of ovarian hormones (Gurka et al., 2016). Previous studies indicated that steroid hormones appear to be affected by metformin. The effect of metformin on androgen and estrogen indicators has been the focus of these investigations (Tang et al., 2006; Rice et al., 2009). However, it was exhibited that metformin treatment decreased the stimulated activity of aromatase in women with PCOS (Zhao et al., 2017; Gasser et al., 2022). In the vaginal smear samples of the fructose+metformin group, the presence of irregular-shaped cornified squamous epithelial cells was evidence for the onset of the estrous cycle at the end of the treatment procedure. These findings are thought to be because of metformin on steroid hormones.

Oxidative stress plays an important role in the

pathogenesis of complications caused by metabolic syndrome, including vascular disease, nephropathy, retinopathy, hypertension, atherosclerosis, diabetes mellitus, and even diabetes-related neuropathy (Pizzino et al., 2017). ROS are known to function in multiple physiological systems under conditions of oxidative stress and eventually contribute to cellular dysfunction. Furthermore, oxidative stress leads to the development of insulin resistance by causing lipid peroxidation in muscle cell membranes (Pizzino et al., 2017; Vona et al., 2019). It has been shown that women with metabolic syndrome produce significant amounts of proinflammatory cytokines in adipose tissue. Therefore, this syndrome may compromise the effect of insulin on the cellular uptake of glucose and induce the endothelial inflammatory response in women (Mohammed et al., 2021). High-fructose diet results in ATP depletion, which sets off an inflammatory response and oxidative stress, thus impairing the functions of organs (Zhang et al., 2017). In the present study, oxidation was found to be increased after the induction of metabolic syndrome and the serum TOS value was significantly higher (13.23 ± 1.36 μmol/L) in the fructose group compared to the other groups ($p < 0.05$). This finding demonstrated that inflammation-related diseases, including metabolic syndrome, are associated with increased oxidation.

Antioxidants can be useful in controlling oxidative stress in metabolic syndrome. Antioxidants are known to protect the cell from ROS through both enzymatic and non-enzymatic mechanisms (Tan et al., 2018). In recent years, studies on the possible role of oral hypoglycaemic agents, including metformin, have increased in reducing oxidative stress in metabolic syndrome. Metformin has been reported to have a potential role in modulating of oxidative stress (Ahmed Mobasher et al., 2020; Astiz et al., 2020; Bai and Chen, 2021; Liu et al., 2022). Previous studies also indicated that metformin improved hyperinsulinemia and hyperandrogenemia and restored ovulatory function (Faure et al., 2018; Pasquali, 2018). Although metformin has been demonstrated to increase ovulation and pregnancy rates in women with PCOS, controversial results have also been reported on the effect of metformin on fertility in PCOS (Johnson, 2014; Faure et al., 2018; Wu et al., 2020). The findings of this study showed that serum TAS values in the fructose+metformin group were markedly higher than the fructose group ($p < 0.05$). Metformin, which is known to have a potential for antioxidant activity, showed an increase in the rate of serum TAS value in rats with metabolic syndrome. It was thought that metformin may be able to contribute to the treatment by using this pathway. In addition to this, the serum OSI value, which is a marker of the degree of oxidative stress, was significantly decreased in the fructose+metformin group when compared with that of the fructose group ($p < 0.05$). As a result, it was recommended that metformin administration is effective in reducing OSI in rats with metabolic syndrome.

PON-1, an antioxidant enzyme associated with high-density lipoprotein, has been shown to decrease due to metabolic syndrome(Sentí et al., 2003; Dornas et al., 2012; Adhe-Rojekar et al., 2018). In the current study, like other studies, serum PON-1 values were determined to be the lowest in the fructose group (859.60 ± 60.40 U/L). Serum PON-1 value was found to be a significant difference between fructose and fructose+metformin groups ($p < 0.05$). Previous studies suggest that increased PON-1 activity has been observed in patients treated with metformin (Meaney et al., 2012; Camps et al., 2016). In this study, it was seen that serum PON-1 activity increased after metformin administration and there was a difference between the fructose and fructose+metformin group (1107.00 ± 63.63 U/L) ($p<0.05$).

ROS are important for the ovarian process and follicular survival and are also physiological regulators of ovarian functions (Sugino, 2005). The intraovarian environment is directly damaged by oxidative stress. Increased levels of ROS in the ovaries impair oocyte quality, cause granulosa cell death, and degradation of the corpus luteum. Additionally, it affects preovulatory oocyte development by reducing communication between oocytes and granulosa cells (Yang et al., 2017; Acar et al., 2019). In the present study, it was thought that ovarian functions were impaired due to oxidative stress in rats with metabolic syndrome and reversed with metformin treatment.

CONCLUSION

The results of the present study evidence that metabolic syndrome in high-fructose-fed rats causes ovarian disruption and irregular cycles due to oxidation. We determined that serum OSI and PON-1 values could be used as diagnostic markers in the pathogenesis of metabolic syndrome. In addition, this study revealed that vaginal cytology, which is convenient, cheap, and feasible way, can be used to monitor ovarian activity in metabolic syndrome. Metformin is involved in the regulation of the ovarian cycle by in-

creasing the activities of antioxidants.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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