AMLS Archives of Medical Laboratory Sciences

Original Article

Sitagliptin Suppresses Apoptotic Cell Death and Histological Changes in the Ovaries of Rats with Polycystic Ovary Syndrome

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Received: May 20, 2021; Accepted: August 23, 2021

Abstract

Background and Aim: Polycystic ovary syndrome (PCOS) is the most typical endocrine disorder affecting reproductive-aged women. The patients with PCOS show decreased follicular granule cells' maturation in their ovaries, probably associated with cell apoptosis. This study was designed to investigate Sitagliptin's protective effect against the apoptotic mechanism in PCOS via evaluating apoptosis rate and the mRNA expressions of apoptotic and anti-apoptotic molecules in PCOS rats.

Methods: PCOS was induced by injection of estradiol valerate (4 mg/kg, I.M.). Twenty-two female rats divided into four groups: Control (n=5), PCOS+Vehicle (n=5), PCOS+ Sitagliptin 25 mg/kg (n=6) and PCOS+ Sitagliptin 50 mg/kg (n=6). Hematoxylin and eosin staining were used to determine qualitative changes in the ovary follicles. The apoptotic index was examined by TUNEL assay, and the quantitative polymerase chain reaction was performed to detect expression levels of Bax and Bcl-2.

Results: Bax mRNA expression was up-regulated (1.38-folds), and Bcl-2 mRNA expression was down-regulated (0.45-folds) in PCOS rats' ovarian tissues. Sitagliptin did not change Bax expression but increased the Bcl-2 mRNA expression (1.95-folds). The apoptosis index was increased in the PCOS group compared with the control group. The number of cystic follicles and pre-antral follicles increased the number of corpus luteum was decreased in PCOS rats compared to control rats. Sitagliptin decreased apoptosis rate and prevented the increase of cystic and pre-antral follicle numbers compared to the PCOS group.

Conclusion: In conclusion, Sitagliptin might have a major role in preventing PCOS development due to its anti-apoptotic properties on PCOS rats' ovaries.

Keywords: Polycystic Ovary Syndrome; Apoptosis; Bcl-2; Bax; Sitagliptin.

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Please cite this article as: Safaeian A, Zare Mehrjerdi F, Yadegari M, Ebrahim Rezvani M. Sitagliptin Suppresses Apoptotic Cell Death and Histological Changes in the Ovaries of Rats with Polycystic Ovary Syndrome. Arch Med Lab Sci. 2021;7:1-9 (e22). https://doi.org/10.22037/amls.v7.34952

Introduction

Polycystic ovary syndrome (PCOS) is a complicated endocrine and metabolic disturbance affecting reproductive-aged females worldwide, with a prevalence between 6% and 21% (1, 2). This syndrome is known as one of the major causes of infertility in females and is characterized by hyperandrogenism, chronic anovulation, and polycystic ovaries morphology on ultrasound scans (3). On the other hand, PCOS is also associated with a wide range of metabolic disorders, including insulin resistance, type 2 diabetes mellitus (T2DM), dyslipidemia, hypertension, and coronary heart disease (4). Clinical studies have shown that PCOS is associated with ovulation disturbance in 75% of cases (5, 6). Despite numerous studies, the exact cellular and molecular mechanisms of PCOS are not yet fully understood. In the mammalian ovaries, each follicle contains an oocyte surrounded by granulosa cells, and an outer layer contains theca cells (7). One of the characteristics of women with

PCOS is impaired folliculogenesis, which is associated with failure in the maturation of antral follicles resulting in extensive follicular atresia (4). However, large numbers of small follicles were found in women's ovaries with PCOS (8). Previous studies have shown that apoptosis of follicular granulosa cells can lead to abnormal folliculogenesis and follicular atresia because granulosa cells are responsible for controlling oocyte maturation (9). In line with these findings, high rates of apoptosis in follicular granulosa cells have been reported in women with PCOS. Several studies have demonstrated that a balance between survival and apoptotic factors in granulosa cells is critical to a follicle (10, 11). The B-cell lymphoma-2 (Bcl-2) family of proteins is involved in the apoptosis of follicular granulosa cells and PCOS development (9, 10). In an animal model study of PCOS, immunohistochemical staining of cystic follicles showed low expression of Bcl-2 (anti-apoptotic protein) and high expression of BCL2 Associated X (Bax) (pre-apoptotic protein) in granulosa cells (9). Overall, an increase in ovarian apoptosis through an imbalance between the Bcl-2 family members may be involved in the transformation of developing follicles into cystic follicles in the ovary in PCOS (10). Therefore, by inhibiting apoptosis, atresia of ovarian follicles may be prevented, and PCOS can be improved.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from the gut when glucose is consumed and stimulates insulin secretion from pancreatic beta cells (12). In the pancreas, GLP-1 increases beta-cell proliferation and inhibits apoptosis (13). The secreted incretins are rapidly degraded by the dipeptidyl peptidase 4 (DPP-4), which reduces the insulinotropic effects of GIP and GLP-1. DPP-4 enzyme inhibitors are a new class of anti-hyperglycemic agents used to treat type 2 diabetes (14, 15). Sitagliptin was the first commercial DPP-4 inhibitor to be approved in 2006 by the US Food and Drug Administration (FDA) and in 2007 by the European Medicines Agency (EMA) at a daily dose of 100 mg (16). Numerous studies have indicated that Sitagliptin also has protective effects against apoptosis in various pathologic conditions, including T2D and cardiovascular disorders (17, 18). Here, we hypothesized that Sitagliptin might have anti-apoptotic impacts in polycystic ovaries. The present study was designed to determine the effect of Sitagliptin at doses of 25 or 50 mg/kg against apoptosis in estradiol valerate induced PCOS rats by measuring apoptosis rate and the gene expression of pro-apoptotic and anti-apoptotic markers.

Methods

Animals: Twenty-two female Wistar rats weighing 175 to 200 gr were prepared from the Laboratory Animal House of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The animals were maintained under controlled laboratory temperature (22±2°C) and a 12-hours light/dark cycle with free access to food and water.

Vaginal Smear Test: Before the PCOS induction, the rats' regular estrous cycle was confirmed. For this purpose, a vaginal smears examination was performed every morning for four consecutive days. To perform this test, 50 µl of 0.9% normal saline was injected into the vagina of each rat, and then the same amount was sucked and placed on a slide. After drying, the smear samples were evaluated under a light microscope (magnification 10 ×), and different stages of the estrous cycle were identified based on cellular characteristics of cornified cells, nucleated epithelial cells, and leukocytes (19). A vaginal smear test was done every morning for four consecutive days, and the rats with three or four regular estrous entered the intervention.

Experimental design and treatment: After confirming regular estrous cycles for each rat, PCOS was induced by 4 mg/kg estradiol valerate (EV) injection (intraperitoneally, I.P.). The first injection of EV was done in the proestrus phase. Three days after PCOS induction animal drug administration was started and lasted for 30 days. The powder of Sitagliptin was purchased from Abidi Pharmaceutical Company, Karaj, Iran. Sitagliptin was dissolved in distal water daily and gavaged to the animals. The animals were randomly distributed into four groups: Control group, (n=5): normal rats were gavaged with distilled water daily. PCOS + Vehicle (Veh) group, (n=5): PCOS rats

were orally gavaged with distilled water daily. PCOS + Sita25 group, (n=6): PCOS rats were orally received 25 mg/kg/day of Sitagliptin. PCOS + Sita50 group, (n=6): PCOS rats were orally received 50 mg/kg/day of Sitagliptin.

Sample Collection: Before tissue sample collection, rats were anesthetized (a mixture of 90 mg/mL ketamine and 10 mg/kg xylazine), intraperitoneally. After that, rats were sacrificed, and the ovaries were immediately removed. One of the ovaries was placed in 8% formaldehyde for histological studies, and the other ovary was frozen in liquid nitrogen and then stored at −80°C for molecular studies.

Histopathological evaluation: Haematoxylin and eosin (H&E) staining was used for the routine histopathological study of ovarian tissue. For this purpose, ovarian tissue samples were first fixed in 8% formaldehyde buffer immediately after removal from the animal. The samples were then washed, dehydrated (with ascending concentration of alcohol), clarified with xylene, and then placed in paraffin. Five µm thick sections were cut from paraffinized samples, stained, and finally examined by light microscopy (Nikon, Japan). The healthy antral and cystic follicles were evaluated qualitatively in every fifth section. Healthy antral follicles were considered as follicles with an obvious antrum and nucleus, more than three granulosa layers and a fine theca cell layer.

Cystic follicles were defined as follicles without an ovum, with a large antral space and a thickened theca cell layer. To estimate the mean thickness of theca and granulosa cell layers in the follicles, the Micrometrics SE Premium 4 software was used. The tissue and follicle characteristics were assessed by a histologist blinded to the experimental group.

Apoptosis Assay: Apoptotic cell death was detected by the TUNEL technique, using the in-situ Cell Death Detection Kit (Roche, Germany) based on the manufacturer's protocols. Briefly, the ovarian tissue sections were digested with proteinase K at 37°C for 30 minutes. The samples were washed with PBS and then incubated with 3% H2O2 for 10 minutes in the dark. The sections were rewashed with PBS, and then a 500 µl TUNEL reaction mixture (450 µl Lable Solution+50 µl Enzym Solution) was added to the tissue sections and incubated at 37°C for 60 minutes. After washing with PBS and incubated with horseradish peroxidase (HRP) for 10 min, the cells were incubated with DAB solution for 5 minutes in the dark. The sections were then stained with hematoxylin and evaluated by light microscopy (Nikon, Japan). The apoptotic cells were counted (3 windows per section/animal; 4 animals/group) and the mean values for each group were used for analysis. The percentage of apoptotic cells was calculated as TUNEL-positive cells/total numbers of cells (20).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The mRNA levels of Bax and Bcl-2 from the ovarian tissue samples were determined with a Rotor-Gene Q (Qiagen, Germany) system. Briefly, total RNA from ovarian tissue samples was extracted by RNA Extraction Kit (Cinnagen Company, Iran), and its amount was quantified with a 2000 Nanodrop spectrophotometer.

One µg of total RNA from each sample was reverse transcribed to single-stranded cDNA synthesis according to the reverse transcriptase kit (Parstous, Iran) and finally amplified based on the instructions of the qRT-PCR using a Rotor-Gene Q Detection System. A Real-time PCR reaction was carried out with one µl cDNA sample, one µl specific primers (10 pmol/µl each of forward and reverse primers) (Table 1), 10 µl SYBR green qPCR master mix (Amplicon, Denmark), and 8 µl sterile distal water with a final volume of 20 µl.

Finally, q-RT-PCR reactions were performed with the following thermal cycle: 15 min at 95 C plus 40 cycles including 95°C for 30 s and 58-64°C for 30 s, and finally, one cycle of 70°C for 30 s. At the end of the reaction and after normalization with Ribosomal protein L13 (RPL13 gene) as house Keeping gene, each gene's mRNA level was determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Table 1. The sequences of the primers of Bax, Bcl-2, and RPL13.

Gene	Forward Primer	Reverse Primer
Bax	5′-CGA GTG TCT CAG GCG AAT TG-3′	5′-CCA GTT GAA GTT GCC GTC TG-3′
Bcl-2	5′-GGG ACG CGA AGT GCT ATT G-3′	5′-CTC CAG TAT CCC ACT CGT AGC -3′
RPL13	5′-ATT GTG GCC AAG CAG GTA-3′	5′-GTT GGT ATT CAT CCG CTT CC-3′

Statistical Analysis: GraphPad Prism software (version 8) was utilized for the statistical data analysis, and the obtained data were expressed in the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to find significant differences between all experimental groups. P <0.05 is considered statistically significant in all experiments.

Results

Vaginal smears: Vaginal smears were evaluated on Days 30 and 45–60 after EV injection to confirm PCOS induction. Persistent vaginal cornification (PVC) was identified as ≥5 consecutive days in the oestrous phase of the oestrous cycle (21, 22).

Effect of different doses of Sitagliptin on mRNA expression of apoptotic factors: Bax mRNA expression was significantly increased in the PCOS group compared to the control group (1.38-folds, P<0.05). Treatment with different doses of Sitagliptin (25 and 50 mg/kg) did not significantly alter the mRNA expression of Bax. Also, the mRNA expression of Bcl-2 in the_PCOS rats was significantly decreased compared to the control rats. Also, treatment with Sitagliptin had a significant effect on the mRNA expression of Bcl-2 (1.95-folds). The ratio of Bax/Bcl-2 mRNA expression indicated a significant increase in PCOS + Veh group (P<0.001) and Sitagliptin could decrease this elevated level of Bax/Bcl-2 mRNA expression (P<0.01).

Results of the TUNEL study

As shown in Figure 2, TUNEL staining revealed the high density of apoptotic cells in ovary sections in the PCOS + Veh group. Also, the apoptosis index increased in the PCOS group compared to the control group. Sitagliptin at 50 mg/kg decreased the apoptosis index compared with the PCOS group (p<0.05). The effect of Sitagliptin at dose of 25 mg/kg on the apoptotic index was found to be marginally significant (p=0.061).

Results from the histological study by H&E staining: The qualitative evaluation indicated that in the control group, ovarian tissue appears normal, and no cystic follicles were observed in this group. The ovary has antral follicles, and the corpora lutea are also seen. Normal ovarian follicles are seen at different stages of development (Figure 3A). Several cystic follicles are seen in the PCOS group with a thin layer of granulosa (one or two cell layers). In PCOS + Veh group, the number of small, primary, and preantral follicles was qualitatively higher than that of in other groups. The number of corpora lutea was less than that of in the normal group, which is probably a sign of ovulation (Figure 3B).

In PCOS + Sita (25 or 50) treatment groups, morphological studies showed that the number of cysts decreased especially in a higher dose of Sitagliptin, and a corpus luteum was seen. Different follicles in the group with a dose of 50 mg/kg Sitagliptin, including more antral follicles, graph follicles in smaller numbers, and corpus luteum can be seen. It seems that the number of graph follicles in dose 50 mg/kg is more than the of dose 25 mg/kg. At 25 mg/kg dose, a small number of cystic follicles are still visible (Figure 3C and Figure 3D).

Comparison of theca and granulosa layers in different experimental groups: As shown in Figure 4, the theca layer's diameter increased in the PCOS group compared to the normal group. Conversely, the granulosa layer's diameter in the PCOS rats is reduced compared to the normal rats. To some extent, Sitagliptin reduced the diameter of the theca layer and increased the diameter of the granulosa layer in the treated groups compared with the PCOS group (Figure 4).

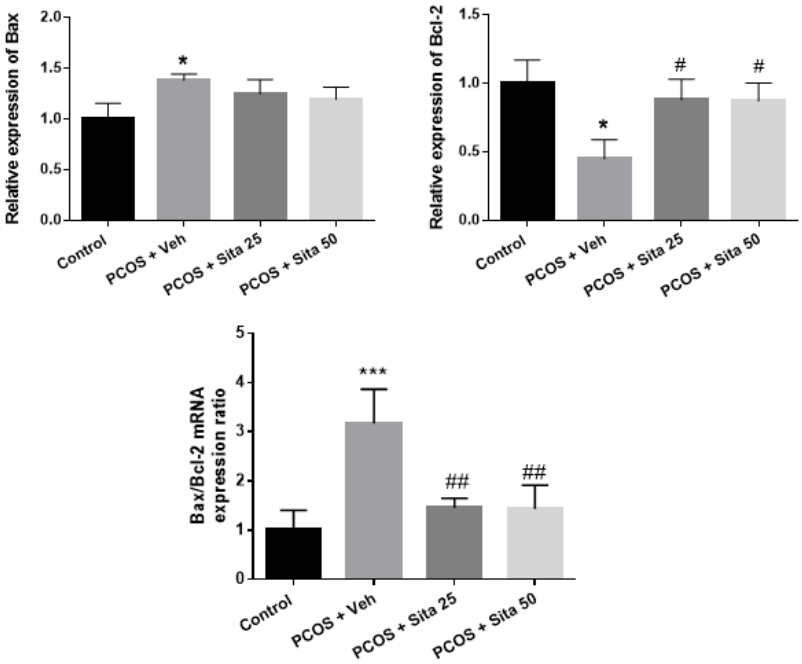

Figure 1. Effect of Sitagliptin on the mRNA expression of Bax, Bcl-2 and Bax/ Bcl-2 ratio in ovarian tissue. Data from all experiments are shown as mean±standard deviation (mean±SEM). * , *** P<0.05 and P<0.001 significant difference compared to the control group. ##P<0.05 significant difference compared to the PCOS + Veh group**.**

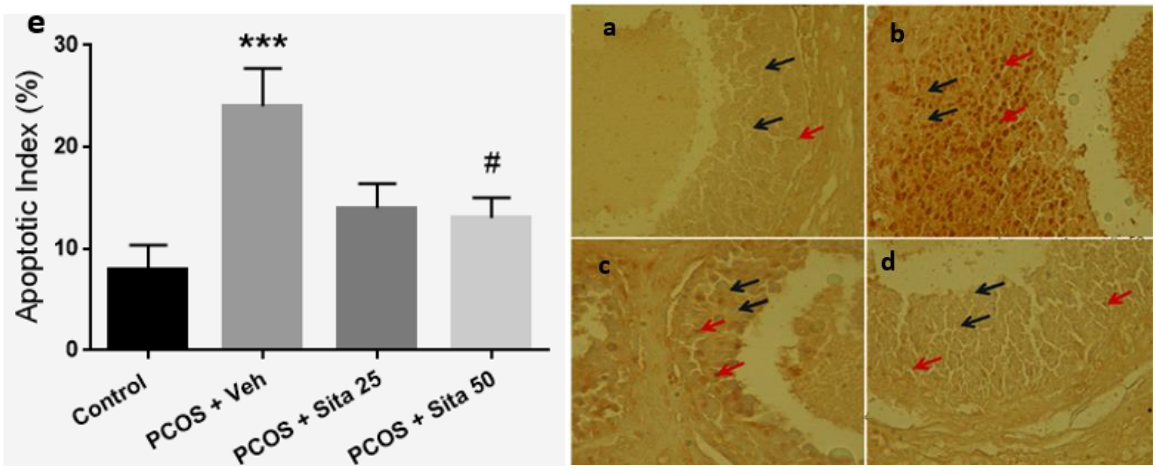

Figure 2. Results of the TUNEL staining in different experimental groups (400x magnification**).** a) control group, b) PCOS + Veh group, c) PCOS + Sita 25 group, and d) PCOS + Sita 50 group. e) the graph indicating the percentages of the apoptotic index. ***P<0.001 significant difference compared to the control group and #P<0.05 significant difference compared to the PCOS + Veh group. Red arrow: Apoptotic cells, Black arrow: Healthy cells.

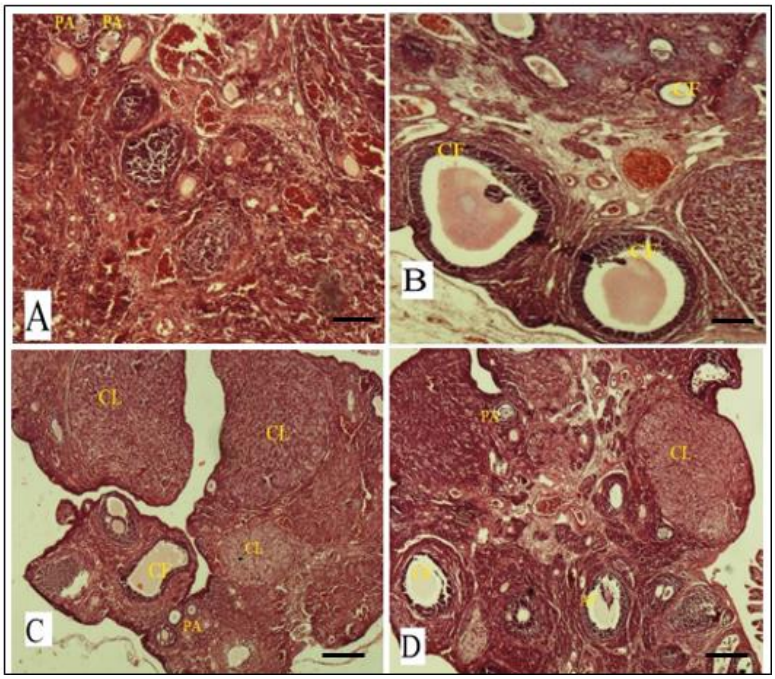

Figure 3. Morphological examination of ovaries in different experimental groups by H&E staining (200x magnification). A) Control group, B) PCOS + Veh group, C) PCOS+ Sita25 group, and D) PCOS+ Sita50 group. CL: corpus luteum, A: Antral follicle, PA: Preantral follicle, FC: Follicular cyst. Scale bars: 150 µm.

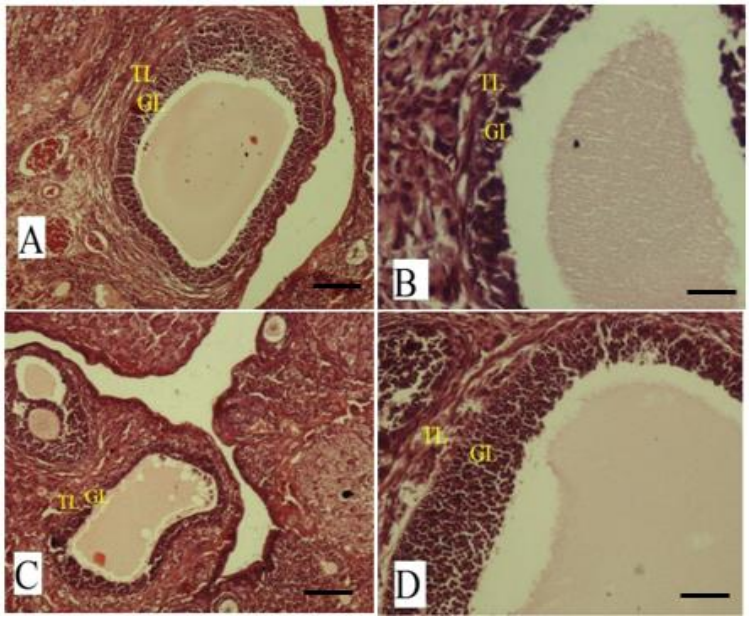

Figure 4. Comparison of theca and granulosa layers in different experimental groups by H&E staining (400x magnification). A) control group, B) PCOS + Veh group, C) PCOS+ Sita25 group, and D) PCOS+ Sita50 group. GL: Granulosa layer, TL: Theca layer.

Discussion

In the present study, Sitagliptin attenuated the rate of apoptosis and ovarian histological abnormalities. The TUNEL test results showed that the apoptosis index in the polycystic ovaries was increased, and Sitagliptin can reduce this index in the treatment groups compared to the PCOS group. Histological

studies by H&E staining showed that many cysts were visible in polycystic ovaries, and the number of immature follicles increased compared to the control group. Sitagliptin at both doses reduced ovarian morphological abnormalities in the treated groups compared with the PCOS group.

PCOS is one of the significant endocrine disorders and the most common cause of infertility due to women's anovulation (23). This syndrome's most common clinical features include irregular menstruation, polycystic ovaries, infertility, hyperandrogenism, hirsutism, obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (8). Due to PCOS's heterogeneity, the exact molecular mechanism of this disease's progression is still unknown. In 2017, Hua Wei et al. conducted a study to investigate apoptosis's mechanism in women with PCOS. The TUNEL study results showed that the apoptosis index was higher in the PCOS group than in the control group in all follicular stages. Also, their immunohistochemical staining results showed that Bax expression was higher in the PCOS group than the normal group, and in contrast, BcL2 expression in the PCOS group was lower than normal group (9). This finding was consistent with the results of our TUNEL and molecular studies. Our molecular study results also showed an increase in Bax gene expression and a decrease in BcL2 gene expression in polycystic ovaries compared to healthy rat ovaries. Previous studies have shown the presence of undifferentiated pre-antral follicles in polycystic ovaries. Laboratory and clinical findings show that dysregulation in the apoptotic molecules increases the number of preantral follicles in women with PCOS. Granulosa cells are the first target cells to apoptosis in the follicle and then the theca cells (10, 24). In a histological study, Bas et al. showed cystic follicles, atretic follicles, and the absence of corpus luteum in polycystic ovaries. Their results were in agreement with the results of the H&E staining of our study. In their study, western blot findings showed that ovarian Bcl-2 levels decreased in the PCOS group compared to the control group. Also, no change in the Bax level was reported between groups (25). The results of our study confirmed these findings in the PCOS rat model.

Several experimental studies have shown that various therapeutic strategies improve the PCOS feature, suggesting that the apoptotic processes' modulation is a potential therapeutic target to prevent PCOS in animal models. Zheng and his co-worker reported that atrial natriuretic peptide (ANP) improved ovary function and morphology in PCOS rats by promoting proliferation and reducing apoptosis of ovarian granulosa cells (26). Also, previous studies have shown that DPP-4 inhibitors have a protective role in PCOS patients. Sitagliptin, as a DPP-4 inhibitor and a new anti-diabetic drug, inhibits DPP-4 activity and lead to an increase in GLP-1 concentrations and inducing insulin secretion into the circulation (14, 15). Besides the hypoglycemic effects of Sitagliptin, it also has protective effects against apoptosis in various pathologic conditions, including T2D and cardiovascular disorders (17, 18). Marques et al. revealed that Sitagliptin's daily oral administration decreased the levels of Bax/Bcl-2 ratio and Bid protein levels. TUNEL study confirmed the protective effects of Sitagliptin against the pro-apoptotic condition in the diabetic rat's kidney (27). However, no studies have investigated Sitagliptin's beneficial impact against the apoptosis mechanism in PCOS patients' ovaries. In the present study, we showed that Sitagliptin at both doses could attenuate apoptosis in PCOS rats' ovarian tissue. TUNEL study showed t of rats' treatment rats with Sitagliptin decreased the number of apoptotic cells in PCOS animals' ovaries. Also, Sitagliptin reduced cystic follicles, increased the number of corpus luteum, antral and adult follicles in the treatment groups, and reduced the diameter and thickness of the theca layer in the treatment compared with the PCOS group.

Conclusion

Our results showed that PCOS induction with estradiol valerate could cause tissue changes (increase in the number of cystic follicles, decrease in the number of corpus luteum, absence of the antral follicle, increase in diameter and thickness of the theca layer, and thinning of the granulosa layer in the polycystic ovary) and changes in the expression level of apoptotic factors (increased

expression of pro-apoptotic factor Bax and decreased expression of anti-apoptotic factor Bcl-2) in the ovarian tissue. Also, PCOS rats' treatment with Sitagliptin can improve these histological changes and reduce ovarian tissue apoptosis index. Overall, this study suggests that Sitagliptin can improve the condition of polycystic ovaries by reducing apoptosis. Further studies with higher sample sizes and considering other aspects of Sitagliptin effects in PCOS treatment are suggested.

Acknowledgments

Notably, our colleagues at the Department of Physiology of Shahid Sadoughi University of Medical Sciences would be greatly acknowledged for their support.

Conflict of Interest

The authors declared that they have no conflict of interest associated with this work.

Funding/Support

The present research was not supported by any funding organization of the commercial, nonprofit, or public sectors.

Ethics

The Ethics Committee of Shahid Sadoughi University of Medical Sciences (Yazd, Iran) approved all procedures for the care and use of laboratory animals (Ethics approval No: IR.SSU.MEDICINE.REC.1396.144).

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