Influence of Diabetes on Morphometric Index of Ovarian Follicles in Streptozocin-Induced Rats

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Abstract: The study aimed to determine the effect of diabetes on follicle development by measuring the diameters of rats' ovarian follicles. The structure and function of many organs change by being affected by diabetes. The ovary is an essential organ of the reproductive system affected by diabetes. The size of the ovarian follicles and corpus luteum can also be affected by diabetes. For this reason, body mass, blood glucose level, and rat ovarian follicles, and corpus luteum diameters were measured in this study. Ten healthy female rats were kept as the control group. In the other rat group, experimental diabetes was induced with streptozotocin (STZ)(60 mg/kg). The rats in both groups were killed after 30 days and their ovaries removed. 5-6 µm sections were made using paraffin embedding techniques and stained with hematoxylin-eosin. On the fifth day of STZ administration to rats, the mass loss of rats was 10%, and the diabetogenic index was 330%. Compared with the control group, diameters of diabetic rats significantly decreased the diameter of the primordial, primary and Graafian follicles, and corpus luteum. The decrease in the diameter of secondary follicles of diabetic rats was not significant. The percentage shrinkage index was the highest in the corpus luteum with 37%. As a result, it can be said that diabetes influences the size of the ovarian follicles and especially the corpus luteum, thereby negatively affecting the ability to improve oocyte quality. Diabetes-related follicle diameter may shrink and cause infertility. It may be essential to measure the diameters of the follicles in vitro fertilization studies in patients with diabetes.
Keywords: Diabetes; ovarian follicle; streptozotocin; diabetogenic index; shrinkage index.

INTRODUCTION

The primary function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species. Additionally, the ovary produces steroids that allow the development of female secondary sexual characteristics and support pregnancy. In mammalian ovaries, the individual follicles consist of an innermost oocyte, surrounding granulosa cells, and outer layers of thecal cells. The fate of each follicle is controlled by endocrine as well as paracrine factors. The follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas a few of them, under the cyclic gonadotropin stimulation that occurs after puberty, reach the preovulatory stage. These Graafian follicles are the major source of the cyclic secretion of ovarian estrogens in women of reproductive age. In response to preovulatory gonadotropin surges during each reproductive cycle, the dominant Graafian follicle ovulates to release the mature oocyte for fertilization,

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whereas the remaining theca and granulosa cells transform to become the corpus luteum (Hirshfield, 1991; Lunenfeld and Insler, 1993; Richards et al., 1995; Gougeon, 1996; McGee, & Hsueh, 2000; Stouffer and Hennebold, 2015; McCulloh et al., 2020).

Like radiation (such as radiotherapy), various chemicals, drugs (such as those used in chemotherapy), diabetes also affects the development of sexual characteristics of women and pregnancy (Ribeiro et al., 2012).

Diabetes is a complex and multifarious group of disorders characterized by hyperglycemia. Diabetes is associated with an increased risk of neuropathy and cardiovascular diseases. Still, it is also linked to reproductive problems such as spontaneous abortions, neonatal morbidity and mortality, congenital malformation, and poor embryo development (Shima et al., 2011). Nowadays, the death rate of those who died with COVID-19 is around 7-9% in people with diabetes. Patients with diabetes against COVID-19 are in the leading risk group (second place) (Wu and McGoogan, 2020).

Diabetes causes alterations in the timing of the estrous cycle, associated with modifications in ovary function, which induces a decrease or even absence of ovulated oocytes and oocyte maturation in female rats. Suppressed ovarian folliculogenesis and steroidogenesis, enhanced endometrial adiposity, hypo-vascularization, and utero-ovarian compartmental metabolic shifts from normal oxidative to lipogenic non-oxidative dominance promote female reproductive incompetence (Shima et al., 2011; Songsasen and Nagashima, 2020).

Streptozotocin (STZ), which is widely used to create a diabetic rat model, was used in the study. Similar to glucose as a chemical structure, STZ is a glucosamine-nitrosourea compound. STZ disrupts DNA and other mechanisms, causing toxicity for cells. The first effect of STZ is the activation of poly-ADP ribosylation, which is more critical for the induction of DM than DNA damage. STZ is like glucose in function as long as it is transported by the glucose transport protein GLUT2 into β cells, but it is not recognized by other glucose transporters. Therefore, β cells have relatively high GLUT2 levels; this explains the specific STZ toxicity for β cells. STZ was applied not only to rats but also to many other mammals. Diabetic models caused by STZ are available for many studies (Sakata et al., 2012; Furman, 2015; Hu et al., 2018). Although there are many studies on the effect of diabetes on the ovaries (McGee and Hsueh, 2000; Dorostghoal et al., 2011; Shima et al., 2011; Uslu et al., 2017), no study evaluating the effect of diabetes on the diameter of the ovarian follicles in rats with mass loss index, diabetogenic index, and shrinkage index have not been found.

This study aims to determine the effect of diabetes on follicle development by measuring the diameters of ovarian follicles in rats with diabetes created with streptozotocin and comparing these values with each other. For this purpose, the mass losses and blood glucose levels of rats in the groups and the diameter of the ovarian follicles over histological sections were measured. A statistical comparison of these values of diabetic and non-diabetic groups was made.

MATERIALS AND METHODS

Streptozotocin (STZ), a diabetogenic compound, is designed by Sigma Chemical Co., St. It was purchased from Louis, Missouri, USA. The animals were obtained from the Sivas Cumhuriyet University Medical Faculty Experimental Animal Research Laboratory. Sivas Cumhuriyet University local committee approved all test protocols (Approval No: B.30.2. CUM.0.01.00.00-50 /29). Twenty healthy female Wistar albino rats weighing 250 ± 20 g and 8-10 weeks old were used in the study. Rats were bred at room temperature in 12 hours light-12 hours dark periods. The

animals were allowed free and unlimited access to standard laboratory food and drinking water. There was no rat death during the study period.

Measurement of body mass and blood glucose level of rats

Body masses of the rats in the groups were measured with a balance ($\pm$ 0.1 g) at the beginning of the experiment and five days after STZ administration, and blood glucose levels were determined with a glucometer (RA1001WF; Rheamed Biotechnology Co., Changhua, Taiwan) immediately after mass measurements. While normal blood glucose value is accepted as 90-110 mg dL^{-1}, those with blood glucose levels above 250 mg dL^{-1} are considered diabetic (Jaouhari et al., 2000).

The mass loss index (mLI) measures the weakening of rats under the influence of the diabetogenic substance, and the percentage mLI can be calculated from the given equation.

$$\text{mLI\%} = \frac{m_a - m_b}{m_b} \times 100$$

Where m_b and m_a are the masses of rats before and after STZ administration, respectively.

The diabetogenic index (DI) shows the effect of any substance that causes diabetes. Percent diabetogenic index (DI%) was calculated from the equation below;

$$\text{DI\%} = \frac{G_a - G_b}{G_b} \times 100$$

Where G_b and G_a are the blood glucose levels of rats before and after STZ application, respectively.

Experimental protocol

Rats were divided into control (n = 10) and STZ-induced experimental (n = 10) groups. STZ was used to produce diabetes (Özdenoğlu and Ünver Saraydın 2016). A single dose of STZ (60 mg/kg) in 0.1 M of citrate buffer at pH 4.5 was given intramuscularly to an animal in the experimental group (Hu LL et al. 2018). Citrate buffer (vehicle) was given intramuscularly to the animals of the control group. STZ-induced experimental and control group animals were sacrificed on day 30 (6-7 times the menstrual period in rats (Embryology, 2020)) with 200 mg/kg sodium Penta-barbital intraperitoneally.

Histology

After fixation of the ovarian tissues in 10% buffered neutral formalin for 48 hours, the fixed tissues were dehydrated in a series of different alcohol concentrations, followed by clearing in xylene and embedding in paraffin wax. Paraffin blocks were then made from the tissues, and sections were prepared using a microtome at a thickness of 5 µm on glass slides. The sectioned tissues were then deparaffinized and stained with hematoxylin and eosin using the standard protocol. The stained slides were coverslipped with a mountant, labeled, and examined under a light microscope (BX51, Olympus, Japan), and photographs were taken from appropriate areas.

Measurement of follicle diameter

Diameter (d) measurements were made over each of five sections selected randomly from serial sections taken from ovary tissues of each animal belonging to control and experiment groups. Lengths of ellipsoid-shaped ovarian follicles on the horizontal (x) and vertical (y) axes were measured by using the program Zen on a microscope (Zeiss, Axiocam, Germany). The average lengths of the short and long axes are assumed to be the diameter of the ovarian follicles.

The shrinkage index (SI) measures the shrinking of rat ovarian follicle diameter under the influence of the diabetogenic substance, and the percentage SI can be calculated from the given equation.

$$SI\% = \frac{d_a - d_b}{d_b} \times 100$$

Where d_b and d_a are the diameters of the ovarian follicles before and after STZ application, respectively.

Statistics

Nonparametric statistical methods were used when the numerical data in this study did not have a normal distribution, and Mann Whitney U nonparametric test (Mat Roni et al., 2019) was used because of the prerequisites of the Student t-test were no fulfilled. Statistical significance between the control group and the STZ-induced experimental group was analyzed by Mann Whitney U test by using Sigma Plot 12.5 software. The results are expressed as means ± standard deviation (sd), which was used for the statistical treatment of the data. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

The masses (m) and blood sugar levels (G) of the rats in control (n = 10) and STZ-induced experimental (n = 10) groups were measured, and the mean values (m_{mean} or G_{mean}) are given in Table 1 together with their standard deviations (sd). The calculated mLI% and DI% values were added to the last lines of Table 1.

H&E staining was performed to determine the type of ovarian follicles in the ovarian tissue of the control and STZ-induced experimental rat groups. Micrographs of the primordial, primary, secondary and Graafian follicles and corpus luteum determined in control and experimental groups are given collectively in Figure 1.

Follicles were classified as primordial if they contained an oocyte surrounded by a partial or complete layer of squamous granulosa cells. Primary follicles showed a single layer of cuboidal granulosa cells. Follicles were classed as secondary (antral) if they possessed more than one layer of granulosa cells with visible antrum. Graafian follicles (mature) had a rim of cumulus cells surrounding the primary oocyte (Sighinolfi et al., 2018).

The length measurement for determining the diameter of the ovarian follicles is shown in the secondary follicle of the experimental group as an example representation (Figure 2). The mean diameters, standard deviations, and percentage SI values of ovarian follicles were calculated and presented in Table 2. The sign '−' in front of the SI% values indicates a decrease in the diameter of the ovarian follicle. In addition, these mean values are shown in the bar graph in Figure 3 for ease of seeing and understanding.

Table 1. Body Mass and Blood Glucose Levels with Index Values in Control and Diabetes Groups

Parameter	Control Group mean ± sd	STZ-Induced Rat Group mean ± sd
Body Mass of Rats / g	249.9 ± 18.50	224.4± 25.44
Blood Glucose Level of Rats / mg dL^{-1}	96.4 ± 3.143	412.4± 52.19
mLI %	−	−10.32
DI %	−	327.65

Table 2. Follicle and Corpus Luteum Diameters in The Ovaries of Control and Diabetes Groups

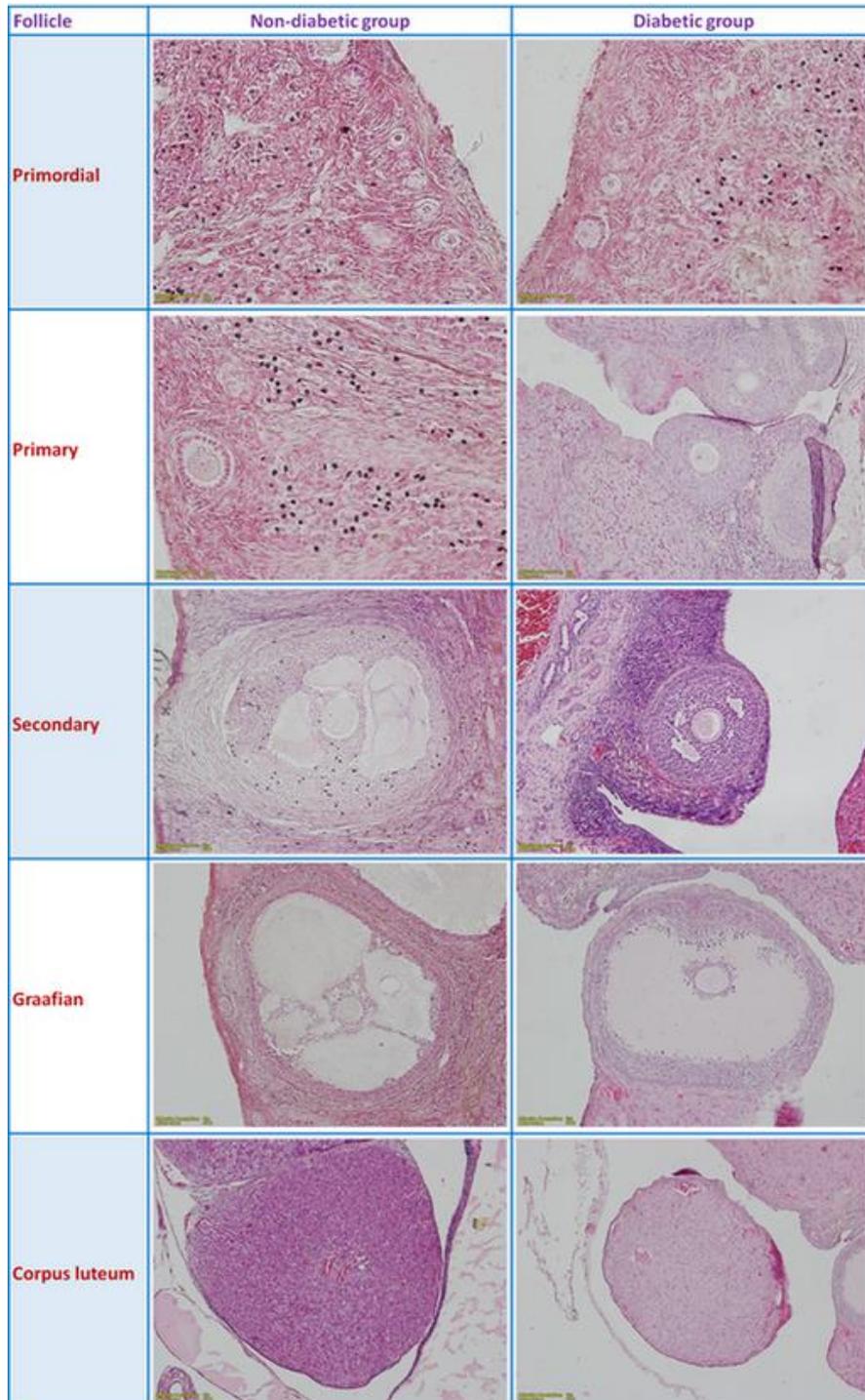| Follicle	d_{ndia} mean ± sd / µm	d_{dia} mean ± sd / µm	SI %
Primordial	24.38 ± 3.324	18.80 ± 3.143	−22.89
Primary	85.52 ± 17.014	63.35 ± 17.374	−25.92
Secondary	142.73 ± 28.474	126.56 ± 17.172	−11.33
Graafian	244.58 ± 17.758	201.65 ± 26.746	−17.55
Corpus luteum	240.91 ± 74.930	151.47 ± 8.529	−37.13

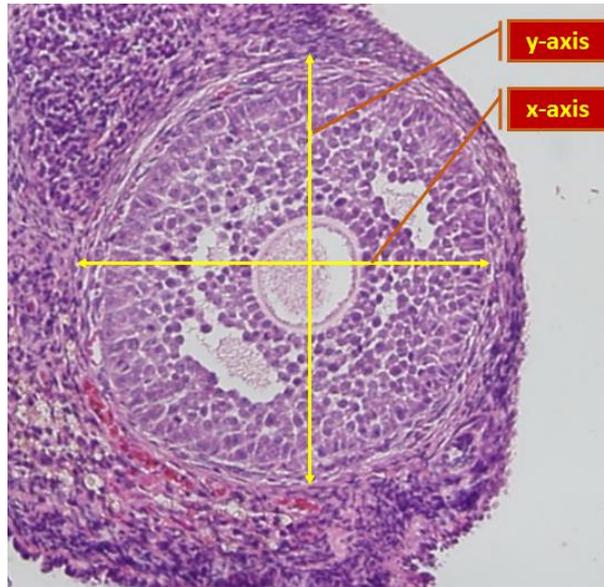Figure 1. Ovarian Follicles in Non-Diabetic and Diabetic Groups.

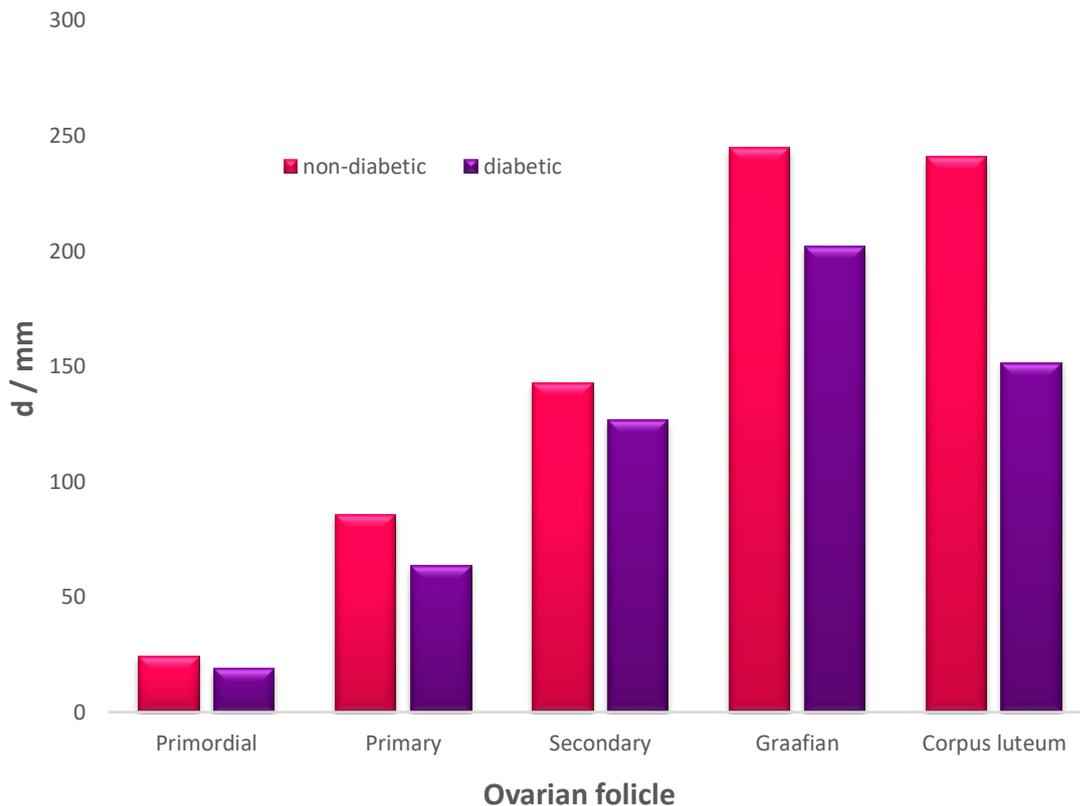Figure 2. Diameter Measurement of an Ovarian Follicle.

Figure 3. The Bar Graph of Ovarian Follicles of Non-Diabetic and Diabetic Groups.

In recent years, concerns have been raised regarding the increases in human reproductive disorders. Exposure to environmental and occupational toxicants and progressive changes in many aspects of lifestyle, including dietary habits, has been shown to deteriorate reproductive health, thus, affecting the ability of couples to conceive and maintain a healthy pregnancy (Dorostghoal et al., 2011). One of the most important of these effects is diabetes.

Diabetes is a disease considered to be a worldwide epidemic. According to International Diabetes Federation data, the prevalence among adults in 2019 was around 463 million cases (IDF, 2020). This number is expected to increase to 693 million in 2045. Although diabetes mainly affects developed countries, its prevalence is increasing significantly in developing countries. Since it is a lifelong condition, it creates a huge economic burden on health systems worldwide (Burgos-Morón et al., 2019).

Diabetes is caused by insufficient insulin secretion of the pancreas or the deterioration of the tissues' response to insulin and affects protein, fat, and carbohydrate metabolism (Jung and Choi, 2014). Diabetes is a complex and multifarious group of disorders characterized by hyperglycemia (Pradhan and Mahapatra, 2016) and has various adverse effects on the ovary.

As seen in Table 1, at the end of the first five days of the experiments, a decrease in the mass of the rats and an increase in blood glucose levels were observed. The mLI% values, which measure the weakening of the rats, decreased to −10% after STZ administration (the minus sign in front of the mLI% values indicates mass loss). This negative value shows that STZ administration causes a significant weakening in rats. Excess glucose (or STZ) destroys beta cells of the pancreatic gland. Thus, the body has to use its proteins and fats for energy needs. Especially with excessive destruction of fats, mass loss and eventually a weakening of people with diabetes begins.

On the fifth day of STZ application to rats, the Mann Whitney U test was applied to test whether the effect of STZ on mass change showed a statistically significant difference, and finally, the p-value was found to be $p \leq 0.001$. Thus, the difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the control and STZ-induced experimental groups ($p \leq 0.001$).

With STZ application, the blood glucose level of the rats was examined to determine whether the rats had diabetes. The mean blood sugar level of the rats in the control group was found in 90-110 mg dL−1, which is a normal value; this indicates that the control group rats did not have diabetes at the beginning and during the experiments (Table 1).

As can be seen from Table 1, the blood glucose level in rats increased to about 100 mg dL−1 before STZ administration, while it increased above 400 mg dL−1 five days after administration. Blood glucose level above 250 mg dL−1, STZ-induced rats, indicates diabetes. Also, the fact that the% DI value was about 330% after the administration of STZ to rats indicates that STZ is an effective diabetogenic substance, and the experimental group rats have diabetes. Therefore, the control group will be referred to as the non-diabetic group and the experimental group as the diabetic group.

On the fifth day of STZ application to rats, the Mann Whitney U test was applied to test whether the effect of STZ on blood glucose level showed a statistically significant difference. Finally, the p-value was found to be $p \leq 0.001$. Thus, the difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the non-diabetic and diabetic groups ($p \leq 0.001$).

When STZ is applied to the rats, it creates reactive oxygen species (such as NO, ONOO). It disrupts the structure of the insulin through oxidative stress, and the glucose level rises in the blood because insulin cannot regulate the glucose levels in the blood. Thus, hyperglycemia may occur due to increased glucose in the blood. In

this study, the glucose in their blood reached high levels by applying STZ to rats, and they developed diabetes due to hyperglycemia.

As a result of the long-term effects of diabetes mellitus, various organ failure, kidney, eye, vascular, and heart damage (Chawla et al., 2016) can be seen, and damage to the ovary (Rosenfield and Ehrmann, 2016) and morphological disorders. Histological sections from the ovarian tissue of the rats are shown in Figure 1 to show the effects of diabetes on the ovarian follicles and corpus luteum.

As seen in Figure 1, there are differences between the size of primordial, primary, and secondary, Graafian follicle and corpus luteum. When groups are compared, there are also size differences between the same follicles. To better understand these differences, the mean and standard deviations of the follicle diameters are given in Table 2 and Figure 3. The diameters of the ovarian follicles of rats in both non-diabetic and diabetic groups increase in the following order:

$$d_{primordial} > d_{primary} \text{ and } d_{secondary}, d_{graafian\ follicle}$$

And the diameter of the corpus luteum was smaller than the diameter of Graafian follicles in both groups (Table 2 and Figure 3).

On the other hand, when looking at the SI% values, the secondary and Graafian follicles shrinkage less (11% and 15%, respectively), while the primary and the primary follicles shrinkage (23% and 26%, respectively) due to the effect of STZ (or diabetes). The tertiary follicle excludes the oocyte as a result of ovulation and is laid in the Corpus luteum as collapse so that the diameter of the Corpus luteum shrinks. The Corpus luteum was the most shrinkage (37%). Compared to the ovarian follicular, the most shrinkage is the Corpus luteum (37%).

In addition, the diameters of the ovarian follicles and Corpus luteum in both groups were statistically compared. p values for primordial, primary, secondary, Graafian follicle and corpus luteum were found to be $p \leq 0.001$, $p = 0.021$, $p = 0.180$, $p \leq 0.001$, $p = 0.007$, respectively. Thus, the difference in the mean values of the two groups is greater than expected by chance for primordial, primary, Graafian follicle, and corpus luteum; There is a statistically significant difference between non-diabetic and diabetic groups. The difference in the mean values of the two groups for the secondary follicle is not large enough to reject the probability that the difference depends on random sampling variability. There is no statistically significant difference between the input groups.

These evaluations show that oocyte quality significantly negatively affects the ability of diabetes to develop. However, depending on the increase in the follicle diameter, it may be related to the follicular fluid of the incremental increase values. Ovarian follicle fluid contains many biochemical substances, such as the development of follicles, growth, and maturation of oocytes, ovulation, and steroid synthesis, and these substances are effective on reproduction (Bódis et al., 2018). Glucose, an extremely important energy source metabolized by anaerobic ways to create lactate in ovarian metabolism, is of great importance. It is an energy source in ovarian follicular fluids, as in many tissues in the organism (Nandi et al., 2008).

It is stated that glucose, which forms the basis of the energy source in the follicle fluid, can also change to the follicle diameter. Eissa found that follicle fluid glucose concentrations varied between the types of follicles in cattle according to the various stages of the cycle. In small follicles, the glucose concentration levels of follicle fluid are determined in about half the serum, while in large follicles this increases by about 21% (Eissa, 1996). Ali et al., on the other hand, has associated the increase in follicle diameter with the increase in the rate of water in the follicle (Ali et al., 2008).

In addition, ROS level elevation in diabetes may be due to a decrease in the destruction or/and increase in the production by catalase (CAT–enzymatic/non-enzymatic), superoxide dismutase (SOD), and glutathione peroxidase (GSH–Px) antioxidants. The variation in the levels of these enzymes makes the tissues susceptible to oxidative stress, leading to diabetic complications (Lipinski, 2001; Asmat et al., 2016; Jabeen et al., 2017).

We can say that diabetes causes water loss an increase in reactive oxygen species in the follicular fluid so that the ovarian follicles of rats induced by STZ shrink. The reduction in the diameter of the follicles due to diabetes, primarily the reduction in the corpus luteum, may have a negative effect on implantation. The corpus luteum is a source of progesterone, which is essential in early pregnancy. Studying hormone levels together with follicle diameters in future diabetes model studies will give healthier results.

CONCLUSION

Mammalian ovarian follicles develop from primordial follicles to the antral follicles necessary for the normal reproductive function of the female. Diabetes and related complications are the most critical health problems in the world. It can be thought that hyperglycemia decreases the diameter of the follicles may have a detrimental effect on development and consequently leads to infertility. The fact that the rat ovarian follicle diameter differs semantically between the control and experimental groups supports that diabetes is essential in determining follicular diameter values in the ovary. In fertilization, in fertilization and IVF studies of patients with diabetes, it may be crucial to measure the diameter of the ovarian follicles.

CONFLICT OF INTEREST

All authors have contributed to this manuscript, reviewed and approved the current form of the manuscript to be submitted. None of the authors has any financial interest related to this study to disclose.

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