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Summary: In the present study was performed to evaluate the follicular growth and hormonal changes after ovarian artery ligation in rats. Fifty six virgin 90-day-old female rats were used. The rats were chosen from 100 female rats by a vaginal smear, which showed that they were synchronized in estrous phase of the menstrual cycle. Animals were kept in seven cages (n=8 in each) at a temperature of $22 \pm 2^{\circ}$ C and 12/12 hr light/dark conditions. The rats were divided into a treatment and control groups. On days 8, 14, 21, 30 and 60, after ligation of the right ovarian artery by laparotomy, nine rats were sacrificed for each test group on mentioned days and both ovaries were collected from each rat. Histological studies were the carried out on the ovaries. The ovaries on the ligated side had a significantly lower rate of ovulations ($p \le 0.01$) than the ovaries in the control group on the intact side. The right-sided ovary had small pea-sized follicles, which indicated polycystic ovarian syndrome, but no polycystic follicles were observed on the intact side and in the control group ovaries. There were no corpora lutea or corpora albicantia within the ovary subjected to ovarian artery ligation, which indicated that there had not been any recent ovulation. Furthermore, there was a greater degree of atresia in the ligated ovary than the control group. However, no abnormalities were detected in the left ovaries of the experimental group. Blood sampling was performed directly from the heart for hormonal analyses on days 5, 10, 20, 30, 40, 50 and 60 after ovarian artery ligation. In the experimental group, the serum analyses showed a significant (p<0.01) increase in the levels of estrogen and luteinizing hormone (LH), while the levels of progesterone and follicular stimulating hormone (FSH) decreased by the time in serum of the test group. Glucose levels were measured and found to be increased, as is seen in polycystic ovarian syndrome, which suggested the presence of insulin resistance.

Keywords: Polycystic follicles, ovary, corpora albicantia, corpus luteum.



Introduction

In female rats, the ovaries possess a dual arterial blood supply that includes the ovarian artery and uterine arteries (Ginther, 1976). It is crucial to understand the contribution of these vessels to the supply of blood to the ovary in the evaluation of treatments that result in the loss of blood flow to ovary by the uterine artery. For example, hysterectomies failed to shorten the life span of the corpus luteum (CL) in guinea pigs (Rowland, 1961). Deficiency in the circulation of blood to the ovary and any hormonal imbalance such as LH and FSH can create abnormalities within the ovary(Speroff et al., 1999). The disorder of polycystic ovarian syndrome (PCOS) is a constellation of symptoms and signs that seem to be a product of abnormal steroidogenesis. There are multiple types of dysfunction that collectively cause PCOS (Speroff et al., 1999). Cystic ovarian disease is common in dairy cattle and swine (Najati et al., 2006).

PCOS is a common causative factor for infertility in women and some species of animal. Five to ten percent of women are affected by PCOS during their reproductive life (Najati et al., 2006). PCOS has been studied from multiple different points of views for several decades as it causes permanent or temporary infertility and other secondary disorders (Stain and Leventhal, 1985). Previous studies have shown that women with PCOS have four different regions affected by this disorder: a) ovaries; b) adrenal glands; c) the hypothalamicpituitary-gonadal (HPG) axis; and d) insulin sensitivity in systemic tissues (Kabayashi et al., 2004; Delman et al., 1998). The precise cause of PCOS is unknown, but certain disorders, such as torsion of ovarian artery, can lead to abnormal hormonal levels within ovarian tissue in humans and mammals that can cause the clinical constellation of symptoms of PCOS.

The aim of this study was to produce an animal model that enabled the study of this kind of traumatic disorder to understand the effects of abnormal blood flow to the ovary. The object of this study was to determine the effect of altered blood flow to the ovary in the context of normal menstrual activity on



ovulation. The secondary aim was to investigate the effect of ovarian artery ligation on the etiology of PCO with the use of an experimental method for torsion of the ovary. The third aim of the study was to determine the changes in the serum levels of progesterone, free estrogen, luteinizing hormone (LH), follicular stimulating hormone (FSH) and glucose levels after the ligation of the right ovarian artery in rats.

Materials and Methods

Fifty-six virgin female 90-day-old rats (Rattus norvegicus) were used. A vaginal smear was performed initially on a total of 100 rats to detect the estrous phase of menstrual cycle in female mature rats, out of which these 56 rats were found to be synchronized (Najati et al., 2006). The mean \pm standard deviation (SD) weight of the rats was $140 \pm$ 1.1 g. Animals were kept in seven different cages (n=8 in each cage) at a temperature of $22 \pm 2^{\circ}$ C with 12/12 hr light/dark conditions. They were fed with a diet of wheat on a special rat plate and had free access to tap water. The rats were then divided into two groups: the treatment group (n=48) and the control group (n=8) rats had undergone a normal menstrual cycle with a length of four to five days in their previous cycle. The perineum of each rat was observed daily for the intensity of the skin color (Wehrenberg et al., 1979). The estimated day of ovulation was determined by the rapid and consistent decline from the most intense color of the perineum color (Wehrenberg et al., 1979).

Surgical procedure

The treatment group was anesthetized with 40 mg/kg 5% ketamine hydrochloride (Razak, Iran) and 5 mg/kg 2% xylazine (Germany) intraperitoneally (ip). The rats then underwent a laparotomy with a 2 cm incision across the lower third of their abdomen. The right ovarian artery was identified and ligated using 0'4 silk (Fleckwell, 1995). The artery was then checked to confirm that blood flow had been ligated completely. The control group consisted of rats that underwent a sham operation. They underwent a



laparotomy in the same fashion but the ovarian artery was not ligated. On postoperative days 8, 14, 21, 30 and 60, nine rats for each group were sacrificed with the use of carbon dioxide gas in an enclosed device. Both ovaries were then dissected out and cleaned.

Serum sampling and hormonal analyses

Blood samples from corresponding animals were collected on days 5, 10, 20, 30, 40, 50 and 60 directly from heart. The serum samples were then separated by centrifugation and subjected to hormonal analysis. The measurements of estrogen, progesterone, LH, FSH levels in serum are based on competition binding using the enzyme-linked immunosorbent assay (ELISA). Competition binding is between two non-classifieds antigen and a conjugated enzymatic antigen for the binding of limited antibody positions on a micro-well plate.

Furthermore, blood glucose was measured by Oncull now set with test strice glucose kits (Najati *et al.*, 2007). Blood samples were sampled from the caudal artery and measured for glucose immediately.

Histological analyses

For the histological morphological investigations, the ovaries were fixed in 10% physiological formalin solution and processed through paraffin embedding before being semi-serially sectioned into 5-7 μ m slices with a rotary microtome and stained with hematoxylin and eosin (H&E). The number of follicles and the relationship of healthy arteries and cystic follicles were assessed.

Statistical analyses

All of the results are presented as mean averages \pm SD. The numbers of cystic and attric follicles during the 60 days of the study were analyzed with the paired student t-test. A p-value of less than 0.01 was considered to be significant.

Results

On day eight after the intervention, type II follicles showed the start of cystic formation as they only had a few layers of granulosa cells around the

vast follicular cavity. Over the course of time, this reaction progressed (Figures 2 and 3). Type II follicles showed cystic formations from day 21 in the

experimental group (Figure 1), which was a statistically significant result ($p \le 0.01$). No polycystic ovaries (PCO) were observed in the control group on day 21 after laparotomy. The formation of cystic follicles and the progression of follicular atresia were obvious in the right ovary of the experimental group in comparison with the normally perfused left ovary of the experimental group and the control group.

This study showed no evidence of a corpus luteum or corpora albicantia, which indicated that there were no recent ovulations. Histological studies showed that on days 21, 30 and 60 after ligation, ovaries in the experimental group showed 2.00 \pm 0.51, 3.90 \pm 0.72 and 4.20 \pm 0.23 cystic follicles, respectively. Atretic follicles were detected within the ligated ovary. On days 21, 30 and 60 after the ligation of the ovarian artery, 10.20 \pm 1.47, 2.48 \pm 0.53 and 3.00 \pm 0.64 atretic follicles were revealed with the ovaries, respectively (Figure 4).

Moreover, ovulation significantly declined in the ligated ovary ($p \le 0.01$). There were no morphological differences between the ovary with intact vasculature and the ovary on the ligated side. Furthermore, numerous primary, secondary, tertiary follicles were observed by the microscopic evaluation of the ovaries that had been removed from control group. Atretic follicles were demonstrated on the ligated side, while no atretic follicles were demonstrated in the ovaries in the control group. Observations demonstrated that there were 132.05 \pm 3.42, 130.00 \pm 3.33, 127.12 \pm $3.19, 119.12 \pm 2.34$, and 100.44 ± 2.24 normal follicles in the ovaries on days 8, 14, 21, 30 and 60, respectively. The serum analyses revealed that free estrogen levels increased over time in the test groups (p<0.01). The level of serum progesterone was higher in the test group. The level of serum FSH decreased over time, while a significant (p<0.01) increase of LH was demonstrated in the hormonal analyses in the test group. Glucose measurements showed that high levels occurred in the experimental group that increased from days 40 to 60 (Figures 5, 6 and 7).



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Figure 1: A type II follicle stained with hematoxylin and eosin at x400 magnification: (1) thecal layer; (2) follicular antrum that has been created abnormally in this secondary follicle; and (3) the oocyte.



Figure 2: Cross-section from a polycystic ovary stained with hematoxylin and eosin at a magnification of x400. Note the white arrows that show small pea–sized follicles scattered throughout the ovary. The yellow arrow shows a large, atretic follicle.



Discussion

In the subsequent menstrual cycle of the cases of PCOS, significantly fewer ovulations were observed in the right ovary of the experimental group than in

the left-sided ovary with the intact vasculature (Myrovych, 1975). Our results corroborated the results of Myrovych, as our results showed an increase in the number of atretic and cystic follicles during days 21 to day 60 after surgery in the experimental group. Therefore, this suggests strongly that ovulation in cases with PCO decrease in a time dependent manner. According to Wehrenberg *et al.* (1980), significantly fewer ovulations occurred in the ovary with the ligated artery.

In the current study, as PCO occurred, the lower rate of ovulation can occur if the ovarian artery was ligated completely. Women with PCOS usually present with clinical symptoms of anovulatory infertility (Atiomo et al., 2000). The PCOS is a result of malfunction rather than an intrinsic ovarian or peripheral dysfunction; locally abnormal hormone levels can cause disruptions to the follicular pathways of ovaries (Chang, 1984; Calogera et al., 1987). Our study results were similar to those of Chang (1984) and Calogera (1987), which showed that if the ovarian artery was ligated completely, cases will have PCOS, and so they will have anovulatory infertility. This suggests that if any local deficiency occurs in the vessels that supply blood to the ovary, there may be problems with regards to the correct levels of hormone that reach the ovary, which could cause pathological condition in the development of follicles.

As ovaries appeared to be normal by histological examination, the loss of ovarian function on the ligated side cannot be explained by cellular atrophy. This fact led us to conclude that an ovarian artery ligation cannot completely produce an evaluation of the ovarian function because the uterine artery supplies 95% of the ovarian blood flow. (Wehrenberg *et al.*, 1979). At the same time, disruption in the blood supply from the uterine artery cannot generate PCOS because the results from histological studies showed that on the first menstrual cycle (on day 21 after ligation) type II follicles began to show cystic formation. The cystic follicles have characteristic features, which include 1-2 layers of granulosa cells and increased layers of

Figure 3: An attetic follicle stained with, A) tuloiden blue (x100 magnification) and B) hematoxylin and eosin (x400 magnification). Both images show the dissociation of granulosa cells and, (1) the external thecal layer; (2) the internal thecal layer; (3) granolosa cells with the appearance of macrophages in the antrum.



Figure 4: An atretic follicle. Black arrow: macrophage, Blue arrow: granulosa cell that has dissociated into the antrum. A) Disappearance of oocyte (x400 magnification); B) atretic oocyte stained with hematoxylin and eosin (x100 magnification).



Figure 5: Comparative means \pm SD of estrogen (E₂; mU/ml) and follicular stimulating hormone (FSH; pg/ml), which indicates an increasing level of E₂ and a decrease in the level of FSH over time.





Figure 6: Comparative means \pm SD of progesterone (ng/ml) and luteinizing hormone (LH; mU/ml), which indicates a significant decrease in the level of progesterone and increasing LH levels over time.



Figure 7: Comparative means \pm SD of glucose (mg/ml), which indicates a significant increase in the level of glucose on days 40, 50 and 60 of the study.



thecal cells (Liu *et al.*, 1993). In the light of the observations of Liu (1993), this study demonstrated the same reaction on the type II follicles on day 21 after ligation, that they had few granulosa cells. These follicles showed starting of cystic formation. Over time cystic follicles increased in number on the ligated side ovary, this suggest that deficiency in blood flowing to the ovary (caused by ligation) can lead to decreasing of necessary hormone acceptance by ovary, so it can cause cystic formation.

Over the time course of the study, the formation of cystic and atretic follicles progressed. Various atretic signs were demonstrated, which included precocious antrum formation, luteinization of the follicular wall, dissociation of granulosa cells and floating granulosa cells, the appearance of macrophages in the antrum and around the oocyte, deformation and pyknosis of the oocyte, nucleus and in some cases dilation of the nucleus of the oocyte.

Atretic follicles with same signs described by Cornver (1999) were detected within the ovary with the ligated ovarian artery. Complete vascular competence is necessary in rats for normal ovarian function (Peppler, 1976). There were no macroscopic differences observed between the ovary on the side with intact vasculature and the ovary on the ligated side. Furthermore, numerous primary, secondary, tertiary follicles were observed by the microscopic evaluation of the ovaries that were removed from the control group during different menstrual cycles that occurred during the duration of the study.

The control group had a normal follicular pathway during the cycles over 60 days after the sham laparotomy. With PCO, the body secretes far too much androgen, which counteracts the ability of the ovaries to make enough progesterone, which is necessary for a normal cycle. Estrogen levels are normal and the levels of LH are higher than usual, which works over time to try to "kick-start" the cycle (Rosenthal, 2007). As a result, follicles never develop and instead turn into small, pea-sized cysts on the ovaries (Franks, 1997). In the present study, follicles in the ovary on the ligated side were small in size or atretic. A high density of follicles was seen in the PCO ovaries of the test group. Furthermore, in the light of histological investigations, serum analyses showed a significant decrease in the level of progesterone over time in the test group. In a corroboration of the results of Rosenthal (2007), LH levels were high and this increased in test group over the duration of the study period.

High serum levels of androgens and decreasing levels of progesterone had been detected previously in cases of PCOS (Lara et al., 1993). In contrast to the findings of Rosenthal (2007), estrogen levels in the present study was not normal and were similar to the findings of Lara et al. (1993), who showed the estrogen level significantly increased over time (p<0.01). It has been discovered recently that insulin resistance and PCOS are associated. Women with insulin resistance are either at risk for, or have been diagnosed with, type 2 diabetes. Lowering the level of insulin in women with PCOS seems to help to restore the normal menstrual cycle and lower the levels of androgens (Rosenthal, 2007). Therefore, PCOS is considered not only as a reproductive endocrinological pathology but also as a metabolic deficiency. This syndrome is associated with insulin resistance, hyperinsulinemia, glucose intolerance, obesity and an altered lipid profile (Yildirim, 2003; Holte et al., 1999; Solmon, 1999). Some studies



(personal communication) demonstrated possible lesions between the gene that helps the body to use insulin and PCOS (Calogera *et al.*, 1978). For demonstrating and ensuring the presence of PCOS in the test group, another factor that was measured was glucose. In this study, the serum measurement of glucose showed an elevation of glucose level in rats with PCOS.

Conclusions

The ovarian artery plays a critical role in the supply of hormones to the ovary. If it is ligated experimentally to model conditions such as ovarian torsion in veterinary and human cases, this can lead to a deficiency in the blood flow to the ovary. This can then lead to severe disorders in the development of follicles, including the onset of PCOS. In cases of PCO, the follicular physiological pathways are affected, and no ovulation occurs. This study may suggest that ligation of the ovarian artery can increase the occurrence of follicular atresia in rats. As in PCOS in rats, the ligation of the ovarian artery resulted in alterations in the levels of hormones, including estrogen, progesterone, LH and FSH, which in turn changes over time.

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