Histochemical study of estradiol valerate-induced polycystic ovary syndrome in the rat

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Summary

For the present study, and follow-up of histochemical changes in estradiol valerate-induced polycystic ovary syndrome (PCOS), 48 virgin 90-day-old female rats were used. The rats were divided into the treatment and control groups. For each rat of the treatment group, 4 mg estradiol valerate was injected through intramuscular route for the induction of PCOS. The control group was taken the same amount of sterile sesame oil. After 63 days of hormone administration, all rats were sacrificed and their ovaries were collected immediately and processed for histochemical studies. These studies were consisted of localization of carbohydrate using PAS method, saturated and unsaturated lipids using Oil-Red-O and Sudan Black B, lipase and alkaline phosphatase. The PAS reaction was seen in some structures of the atretic and cystic follicles such as on zona pellucida (ZP), basement membrane of granulosa cells, connective tissue fibers of ovarian stroma, follicular fluid and granulose cells. This reaction was enhanced in basement membrane of granulose cells of cystic follicles. The lipid droplets were seen in follicular structures of both the atretic and cystic follicles. The presence of macrophages was confirmed in cystic ovaries by this study. The lipase reactions were seen in granulose and theca cells of atretic and cystic follicles. The lipase reaction in the theca layer of cystic follicles was stronger than in the granulose cell layer of such follicles. The alkaline phosphatase reaction was seen in the theca and granulose cells of atretic and cystic follicles and by the progression of these processes and cystic follicle formation, the reaction was increased accordingly. We concluded that, during follicular atresia and cystic follicle formation, histochemical alterations are occurred in follicular structures.

Key words: Polycystic ovaries, Estradiol valerate, Histochemistry, Rat

Introduction

Polycystic ovary syndrome (PCOS) is not merely a disease, but is referred to the sum of some clinical findings. There are lots of dysfunctions which collectively cause PCOS. After publishing materials on clinical signs by the scientists (Stein and Leventhal) in (1935), this disorder was called "Stein-Leventhal" (Speroff *et al*., 1999).

By the introduction of ultrasonography in medical fields, this disease was referred to as PCOS. This name was coined for the characteristic appearance of ovaries in ultrasonography. Other names such as polycystic ovary, sclerocystic ovary, nonovulatory ovary associated with increased androgen, and insulin resistant syndrome are commonly used in previous literatures (Rayan *et al*., 1995; Speroff *et al*., 1999).

The PCOS is a common causative factor for infertility in women and some animals and 5–10% of women get affected by PCOS during their reproductive lives (Konchehauer *et al*., 1998; Legro *et al*., 1998; Speroff *et al*., 1999). Cystic disease is very common in veterinary medicine and presents a number of specific features (Devance *et al*., 1975; Hafez, 1993; Pineda *et al*., 2003). Cystic ovarian disease or cystic ovary is common in dairy cattle and swine but is rarely encountered in beef cattle or other species (Holte *et al*., 1999). According to recent reports, cystic follicular degeneration or cystic ovarian disease is more common in dairy cows (Solmon, 1999).

This disease is an endocrine-metabolic disorder and its characteristic features are

increased androgen, hirsutism, oligomenorrhea, amenorrhea, anovulation, and infertility (Dargent *et al*., 1984; Sterner-victorin *et al*., 2000; Teixeira *et al*., 2000; Sabuncu *et al*., 2003). In this disease, obesity and increased body fat is commonly seen and some of disorders such as type II diabetes mellitus, cardiovascular disorders, endometrial and breast cancers are more likely to happen (Dahgra *et al*., 1994; Franks, 1995; Dunaif, 1997; Franks, 1997; Ehramann *et al*., 1999; Medissa and Hunter, 2000).

In spite of numerous intensive investigations which were carried out on PCOS, so far, a definite single etiology of this disease is remained unclear (Diamante-Kandorakis and Dunaif, 1996; Henmi *et al*., 2001), and that is why its treatment is quite difficult. For this reason, the recent investigations are directed towards the finding of the etiology of this disease. The precise studies to figure out its signs, clinical and microscopic findings, and appropriate treatments are therefore necessary. One of the creditable studies would be the investigation of histochemical changes which are more likely to occur in ovaries and follicular tissues in PCOS. We induced PCOS in rats with estradiol valerate (Brawer *et al*., 1996). In the previous studies PCOS were induced by the use of dihydroxy-epiandrostendione (DHEA) (Lee *et al*., 1998) and letrozole (Kafali *et al*., 2003).

The objective of this study was to determine the probable histochemical changes occur in estradiol valerate-induced cystic and atretic follicles.

Materials and Methods

Forty-eight virgin female 90-day-old rats (*Rattus norvegicus*) were used. The mean ± SD weight of rats was 140 ± 1.1 g. Animals were kept in 12 cages with four rats in each, and reared at 22 ± 2°C temperature and 12/12 hrs of light/dark conditions. They feed on special rat plates, wheat, and tap water. The rats were divided into two groups, i.e., the treatment (n=32) and control (n=16) groups. The treatment group was taken 4 mg/rat (0.4 ml) estradiol valerate intramuscularly, for the induction of PCOS. The control group was received 0.4 ml/rat of sterile sesame oil through the same route.

After 63 days, the rats were sacrificed, by CO_2 gas in a special device. Both the ovaries were dissected out, and cleaned. They were processed for the histochemical studies, as follows:

1- For the investigation of lipase enzyme in the ovarian tissue, i.e., ovarian follicles and ovarian interstitial tissues, the fresh unfixed specimens were sectioned with cryostat microtome (frozen sections), and stained through special techniques for the detection of this enzyme (Gretchen, 1979).

2- For the study of lipids, we adapted Oil-Red-O and Sudan Black B techniques. In this procedure, the tissue specimens were fixed in 10% neutral formaldehyde solution and sectioned with cryostat microtome and stained through special techniques for each of them separately (Gretchen, 1979).

3- For the alkaline phosphatase enzyme, we adopted routine paraffin method and sections prepared through rotary microtome and stained with special alkaline phosphatase technique (Gomeri's technique) (Gretchen, 1979).

4- For the PAS, we adopted paraffin sectioning procedure and stained through special technique for the study of carbohydrates (Gretchen, 1979).

Results

Alkaline phosphatase reaction: the study of this reaction revealed that the theca and granulosa cells of atretic follicles were slightly reactive for this enzyme. In the advanced atretic follicles, the reaction was seen in all the follicular structures such as theca interna cells, granulosa cells and oocyte. In the theca cells of healthy follicles the reaction was fairly weak. In the granulosa cells of such follicles, however, it was very weak. This reaction was observed in the ooplasm, zona pellucida (ZP), and follicular fluid (Figs. 1A and B).

Summary of results obtained from alkaline phosphatase reaction: a) This reaction was seen in the theca and granulosa cells of atretic and cystic follicles. b) By the progression of atresia in follicles and cystic follicle formation, the reaction was

increased accordingly. c) The alkaline phosphatase reaction was positive in ooplasm of oocytes of atretic follicles. d) In the theca cells of healthy follicles, the alkaline phosphatase reaction was moderate, but in the granulosa cells of such follicles it was very weak.

PAS method: this reaction was positive in structure of atretic and cystic follicles such as ZP, basement membrane of granulosa cells, connective tissue stroma of follicles, follicular fluid and granulosa cells (Fig. 2B). The PAS reaction was positive in the aforementioned structures of both the non-cystic atretic follicles, and cystic atretic follicles. In the cystic follicles, however, it was enhanced at basement membranes of attenuated granulosa cells (one layer) and cytoplasm of these cells (Fig. 2A). In the small follicles (multilayer secondary follicles) which were committed to the precautious atresia, and in the way of becoming cystic forms, the recently formed antrum was filled with PAS-positive material. The PAS reaction was also seen in ovarian stromal connective tissue, intima of blood vessels and ovarian macrophages. In the process of follicular atresia and follicular cyst formation, by the enlargement of such follicles, the PAS reaction was getting weaker and weaker progressively.

Lipid foci were seen in the form of droplets in the cytoplasms of some cells such as follicular granulosa cells, follicular theca cells, oocyte and macrophages. In the granulosa cells of advanced atretic follicles, the Oil-Red-O reaction was weaker than those in the primary state of atresia. Some ovarian interstitial cells of the rat which were affected experimentally by estradiol valerate (the treatment group) had numerous cystic follicles which were strongly reacted to the O-R-O stain. Colonies of large cells with orange colour were observed with this technique. The distribution of these cells was higher in the treatment than in the control group.

For the confirmation of results in O-R-O method, the Sudan Black B technique revealed black granules (oil droplets) in the cytoplasm of oocyte, granulosa cells and theca cells of cystic and non-cystic follicles. In the advanced cystic follicles the dark Sudan Black-positive granules were observed in granulosa cells in higher intensity, whereas this reaction was weaker at the theca rather than granulosa layer (Fig. 3A). The presence of ovarian macrophages was confirmed by this staining technique. These cells were highly reactive to this stain (Fig. 3B).

In conclusion, the results for the histochemistry of lipids: a) The healthy follicles were negative for the saturated lipids, but by the onset of follicular atresia and in the cystic follicles, the reaction got positive. b) By the progression of atresia and formation of cystic follicles, the staining for saturated lipids in the granulosa cells in comparison to the theca cells become more intensive. c) In the process of atresia and in the way of cystic follicle formation, the oocyte becomes non-reactive to these stains.

Lipase reaction: the atretic follicles, granulosa and theca cells were reactive for this enzyme. This was seen in the cystic follicles too. However, by progression of atresia and cystic follicle formation, this reaction became weak. The interstitial tissue cell showed weak lipase reaction. The lipase reaction in the theca layers of cystic follicles was quite stronger than the granulosa cell layers of these follicles.

Discussion

The PCOS is a common problem in reproduction and other veterinary and medical fields. Study of this syndrome has been started since many years ago (Stein and Leventhal, 1935) and is strongly going on (Speroff *et al*., 1999). This problem is studied from different points of views because, it causes definite or temporary infertility and some other secondary disorders. The PCOS is considered not only as a reproductive endocrinopathy but also as a metabolic disorder. It is associated with insulin resistant, hyperinsulinemia, glucose intolerance, obesity, and altered lipid profile (Holte *et al*., 1999; Solmon, 1999; Yildirim, 2003). The main peripheral and visceral fat thicknesses get increases in patients with polycystic ovaries. Women with PCOS have abnormalities in the metabolism of androgens and estrogen production (Yildirim, 2003). High serum concentrations

Fig. 1: (A) An early atretic follicle, granulosa layer (→), theca layer (↑). Alkaline phosphatase reaction is observed in both the layers (light brown granules). Alkaline phosphatase (×400). (B) A cystic follicle (▲). In this follicle granulosa layer showing weak alkaline phosphatase reaction (↑), but in the theca layer, the reaction is not seen. Few macrophages are seen in antrum of cystic follicle (↑). Alkaline phosphatase (×250)

of androgenic hormones such as testosterone, androstendione may be encountered in these patients (William, 2003).

Women with PCOS usually present with clinical symptoms of anovulatory infertility and/or hyperandrogenism (Atiomo *et al*., 2000). A positive association has been

Fig. 2: (A) A cystic follicle. In its granulosa and theca cell layers relatively strong PAS reaction is present (▲). In the follicular fluid, ZP of degenerated oocyte (↑) PAS positive reaction is present. In the interstitial tissue some cells are PAS positive (↑). PAS staining (×100). (B) In the right side of figure, a large cystic follicle is present. A small atretising follicle is present at left side, in all the layers, PAS reaction is present. PAS staining technique (×100)

revealed between PCOS and a family history of breast cancer and heart disease. These associations may be genetic in origin or secondary to a complex interplay of genetic, intrauterine, and environmental factors (Speroff *et al*., 1999; Medissa and Hunter, 2000). Numerous models have been developed to study PCOS in rats (Lara *et al*., 1993; Brawer *et al*., 1996; Sterner-victorin *et al*., 2000). Some scientists have induced

Fig. 3: (A) a cystic follicle. In the theca layer Sudan Black B reaction is weak (→), but in the granulosa layer this reaction is quite strong (↑). Macrophages with black granules in their structure are seen (↑). Sudan Black B staining (×250). (B) In this figure numerous large cells in the ovarian interstitium which are highly reactive to Sudan Black B staining are seen. These cells are ovarian macrophages, which are engulfed lipids. Sudan Black B staining (×400)

PCOS by exposing rats to constant light (Natali *et al*., 2003). A form of polycystic ovary resembling with some aspects of the human PCOS can be induced in rats by a single injection of estradiol valerate. An increase in sympathetic outflow to the ovary proceeds by several weeks the appearance of cysts, suggesting the involvement of a

neurogenic competent pathology of this ovarian dysfunction (Devance *et al*., 1975; Lautikainen *et al*., 1980; Lawerence *et al*., 1984; Lara *et al*., 1990; Erickson, 1991; Lara *et al*., 1993; Urbanek *et al*., 1999). We adapted this model of study. The PCOS is a result of malfunction rather than a special central or peripheral dysfunction. In the patients with PCOS, the mean daily production of estrogen and androgen are increased. This depends on the LH stimulation (Chang, 1984; Calogera *et al*., 1987). This condition is associated with increased level of blood testosterone, androstendione, DHEA, dehydroandrosten-dione sulfate, 17-hydroxy-progesterone and estrogen (Devance *et al*., 1975; Atiomo *et al*., 2000; Lara *et al*., 2000). Testosterone, androstendione, and dehydroandrostendione are produced by the ovary. Nevertheless, in 50% of patients with PCOS, DEHS is produced only by the adrenal gland. In patients with PCOS, the relative production of follicular androgen to the estradiol is increased which makes the role of aromatization dysfunction and gene mutation in p450 aromatase more likely as the causes of this syndrome. Increase in intra-ovarian androgen has important role in anovulatory process (Medissa and Hunter, 2000; Kafali *et al*., 2003). Generally, the polycystic ovaries are larger than the normal ovaries and have pearl appearance in colour (Tanabe *et al*., 1983). Our results revealed that the size of ovary in PCOS is diminished.

According to the previous reports (Natali *et al*., 2003), histochemical alterations are taken place in extracellular matrix, follicular wall of induced polycystic ovaries of rats in comparison to the controls. Other changes such as alterations in the amount of collagens of theca externa, the neutral carbohydrates and acidic glycosaminogly-cans might take place following cystic follicle formation (Natali *et al*., 2003).

The histochemical study of polycystic ovaries may help to come close to answer some questions in this regard. Our study revealed that PAS reactions were present in atretic and cystic follicular structures such as ZP of oocytes, basement membrane of granulosa cells, stromal tissue, follicular fluid, and granulosa cells (Fig. 2A). The PAS reaction was higher in granulosa cells basement membrane of cystic follicles than in healthy and atretic follicles. It can be concluded that in cystic follicles, this reaction is present which shows that accumulations of glycoproteins or the carbohydrates contents are present in such follicles. In the process of atresia and cystic follicle formation, PAS reaction gradually became weaken, and the reason for this is still obscure.

Our results in histochemical studies of lipids in the ovarian tissue, especially ovarian follicles, by the Oil-Red-O and Sudan Black B techniques revealed that lipid droplets with different sizes are present in the cytoplasms of some granulosa cells, oocytes and especially in the theca cells, and ovarian macrophages. The Oil-Red-O reactions were weaker in advanced cystic and atretic follicles than the early stages of these follicles. It is a clue for the concept that in apoptotic process, an accumulation of lipids takes place in follicular cells, a process which does not continue in advanced stages of atresia because of massive cell destruction and removal. In the polycystic ovaries, some structures had strong reactions to the Oil-Red-O and Sudan Black B. The increased androstendione and testosterone levels in the process of PCOS and the reactiveness of cystic follicle granulosa cells to the FSH hormone could be the reason for this phenomenon.

Interestingly, the presence of ovarian macrophages was revealed by this method, and their population was higher in the treatment than in the control group. In response to the increased cell death in atretic and cystic follicles and for the removal of these cells, the population and activity of macrophages were increased accordingly.

The study of activity of lipase enzyme in atretic and cystic follicles revealed that this enzyme presented in the granulosa and theca cells of such follicles. By advancement of follicular atresia and cystic follicle formations, however, the reaction for this enzyme was decreased. It is concluded that the biological activities of follicular cells in the processes of atresia and cyst formation were decreased and accordingly, the normal life of them was also affected. Furthermore, by this way, the activity of the lipase enzyme is decreased. This finding could

have applications in the detection and diagnosing of cystic follicles.

The alkaline phosphatase activity was detected in the cystic, atretic, and healthy follicles of the treatment and control groups. The activity of this enzyme was demonstrated in the granulosa and theca cells of atretic follicles. By advancement of atresia, this reaction was persisted in structures of atretic follicles including oocytes. Nevertheless, such reactions were weak in healthy follicles in comparison to the atretic follicles. This reaction was also seen in cystic follicles, but it was very weak. We concluded that, by the destructive processes of follicular cells, and decreasing biological activities of such cells, the alkaline phosphatase enzyme activity is increased accordingly. The definite reason for this observation is still not known.

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