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ORIGINAL ARTICLE

Alterations of the ovarian histomorphometry at pre-puberty in rat offspring from diabetic mothers

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Abstract
Purpose Maternal diabetes leads to increased blood glucose concentration in the mother and consequently in the foetus, causing various neonatal problems. This study was conducted to evaluate the effects of maternal diabetes on foetal ovarian structure.
Methods Sixteen adult female rats were allocated into two equal groups. Diabetes was induced in one group by alloxan. Both groups became pregnant by natural mating. Thirty days after birth, the female offspring were terminated, the body weight and blood glucose of the animals measured and their ovaries removed. Various histological and cellular parameters were determined using histological and electron microscopy techniques.
Results Results revealed a significant increase in body weight and blood glucose in the offspring of the diabetic mothers (ODM) compared to that of the controls. The weight, volume and diameter of the ovary and the ovarian capsule thickness were inclined to decrease in ODM compared to that of controls. The number and diameter of primary, pre-antral and antral follicles were decreased in ovaries in the ODM. The electro-micrographs have demonstrated the organelle alterations in oocytes and granulosa cells that suggest the apoptosis progress and oxidative stress.
Conclusions Maternal hyperglycaemia exhibited deleterious effects on the female reproductive system in the offspring.

Keywords Follicle · Maternal diabetes · Offspring · Ovary · Rat

Introduction

Insulin is a hormone, produced by the pancreas, which allows the body to use glucose efficiently. However, in diabetic subjects, the pancreas produces insufficient amounts of insulin, causing blood sugar levels to rise [1]. In diabetic mothers, placental transport of glucose and other nutrients increases during pregnancy due to increased availability at the maternal site, resulting in foetal and neonatal macrosomia [2]. So, the elevated glucose concentration in the mother is accompanied by hyperglycaemia in the foetus [3].

Diabetes has deleterious effects on female reproductive functions [4] and on the development of the blastocysts [5]. It has been reported that diabetic females had fewer luteinizing hormone gonadotroph changes than diabetic males. Luteinizing hormone-releasing hormone (LHRH) axonal lesions might play a primary pathogenic role in the hypothalamo-pituitary disorder [6]. Data also indicates that streptozotocin-induced diabetes mellitus inhibits the feedback action of gonadal steroids and this could account for both the loss of oestrous cyclicity and the reduced pituitary

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sensitivity to LHRH [7]. Moley and co-workers [8] reported that hyperglycaemic conditions, either in vivo or in vitro, modulate the expression of an apoptosis regulatory gene as early as the pre-implantation blastocyst stage in the mouse. The most convincing evidence comes from the commonly observed association of premature ovarian failure with some autoimmune disorders in diabetes mellitus [9]. Data suggests that diabetes mutation-induced ovarian structural and functional involution is a direct reflection of the cellular metabolic shift towards lipogenesis [10]. The diabetes mutation (leptin-receptor defect) induces a hyperglycaemic-hyperinsulinaemic endometabolic environment that promotes hypercytolipidaemic, utero-ovarian involution in mice, resulting in reproductive sterility and eventual organoatrophy [11]. Maternal diabetes increases apoptosis in mice oocytes [12]. Ovarian dysfunction in a diabetic mutant mouse is associated with follicular atrophy, adiposity, impaired steroidogenesis, and imbalanced glucose utilization [13]. These data suggest that there might be a relationship between maternal diabetes and ovarian structural alterations in offspring. The hypothesis of this study is "Maternal diabetes can affect the offspring ovarian structure". The purpose of this investigation is to study the relation of maternal diabetes and the ovarian functional and structural alterations in diabetic rats' offspring at day 30 after birth.

Materials and methods

Animals

Sixteen adult female Sprague–Dawley rats (200–230 g and 4–5 months old) were housed in an air-conditioned room (22 ± 2 °C) and supplied with standard pellet food, with tap water ad libitum. Animals were allocated into two equal groups: diabetics and normal (control). The animals were cared for and treated in accordance with the guidelines for laboratory animals established by the National Institute of Health as well as by the local ethical committee.

Induction of diabetes mellitus

Diabetes was induced in eight rats by a single intra-peritoneal injection (150 mg/kg) of alloxan tetra hydrate (Sigma, St. Louis, MO, USA) according to our previous study [14]. The animals were fasted 12 h before and after alloxan injection. Rats with blood glucose above 200 mg/dl, as well as with polydipsia, polyuria and polyphagia for at least 1 week (such as increased moistness of the cage bottom, increased water and food consumption compared to before the injection), were considered to be diabetic and were selected for the experiment [15]. The glucose and insulin levels of the mothers were measured during the pregnancy by taking blood from the tail and measuring the glucose and insulin by glucometer and radioimmunoassay [16], respectively.

Experimental design

Females in both groups at the oestrus stage of the reproductive cycle were caged with male rats for mating. Mating was confirmed by observation of vaginal plugs [17]. Four female offspring of each rat from both groups were reared in similar conditions in an animal house for 30 days. The offspring of diabetic mothers were cross-hosted with the normal mothers to prevent the effects of their mothers' high blood glucose concentrations on the results which were to appear in their milk.

Although puberty in rats occurs at 35–40 days, the puberty was checked in the female offspring of both groups by vaginal smear.

At the end of the experiment, the animals were anaesthetized with diethyl ether and terminated by whole blood collection via cardiac puncture. Body weight and blood glucose of the offspring were measured in both the control and the test groups. The volume, diameter and weight of the freshly isolated ovaries were measured [18], and the ovaries were fixed in 10 % buffered formalin solution. Ten random samples of ovaries were taken for electron microscopy of both the ODM and the control groups.

Histomorphometric study

Formaldehyde-fixed samples were embedded in paraffin and then sectioned at 4–5 μm. They were further deparaffinised with xylol, and histologic observations were performed after staining by the H&E or Green Masson's trichrome method [19]. For histomorphological and histomorphometric study, the sections were observed under a light microscope, and the average of the following parameters were evaluated in ovaries of both control and test groups: (1) thickness of the ovarian capsule (μm), (2) the ratio of medulla to cortex, (3) the diameter of primary, pre-antral and antral follicles (μm), (4) the number of primary, pre-antral and antral follicles (/mm^2).

The thickness of the ovarian capsule was measured at 9100 magnification using Olysia software (Olysia soft imaging system provided by Olympus 2000) and an Olympus BX51 light microscope. At least six points of the capsule sections were chosen randomly and measured for each test.

The diameters of the ovarian follicles were measured at 9100 magnification using Olysia software (Olysia soft imaging system provided by Olympus 2000) and an

Olympus BX51 light microscope. At least six points of the cortex sections were chosen randomly and measured for each test. Ovarian follicles were counted at 940 magnification using a 441-intersection grid placed in the ocular of the light microscope (Olympus BX51). Ten sections were chosen at random from each ovary, and the number of round or nearly round ovarian follicles in square millimeters (mm^2) was obtained.

Ten random samples of both groups were fixed in 2 % buffered glutaraldehyde and were subsequently embedded in resin. Sections were obtained with ultra-microtome as semi-thin (1 μm thickness) and ultra-thin (60 nm thickness). Semi-thin sections were stained with toluidine blue and ultra-thin sections with uranium acetate and lead citrate. Ultra-thin sections were observed with a transmission electron microscope (CM10) for evaluation of ultra-structure morphological changes such as the size and structure of the cells, nuclei and cytoplasmic organelles.

Statistical analysis

Morphometric data are presented as the mean ± SD, and were analyzed by Student's *t* test using SPSS software. Significant differences were considered when the *p* value was ≤0.05.

Results

Blood glucose of diabetic mothers was increased significantly ($p < 0.05$) compared to control mothers (245.3 ± 11.4 mg/dl in diabetic mothers and 85.4 ± 4.3 mg/dl in control mothers). Insulin level was significantly ($p < 0.05$) decreased in diabetic mothers (29.4 ± 1.5 μU/ml) compared to that of the control (3.9 ± 0.9 μU/ml). As we checked puberty in the same female animals with a vaginal smear, offspring of the diabetic mothers (ODM) and control groups were matured at 38.1 ± 2.7 and 38.5 ± 3.2 days, respectively. Body weight of ODM was significantly greater than that of controls 30 days after birth ($p < 0.05$, 71.4 ± 2.5 g in ODM and 60.1 ± 2.7 g in controls). Blood glucose in the ODM was also significantly greater than the controls ($p < 0.05$, 118.2 ± 5.9 mg/dl in ODM and 93.2 ± 5.1 mg/dl in control).

Values for ovary weight and volume, ovarian diameter, medulla to cortex ratio and ovarian capsular thickness for both groups are presented in Table 1. The weight, volume and diameter of ovary and ovarian capsule thickness were inclined to decrease in ODM compared to that of controls.

Figure 1 shows the diameter of different follicles of the ovaries in ODM and control groups at 30 days after birth. There were no significant differences between diameters of the primary (50.3 vs. 56.9 μm), pre-antral (137.9 vs. 148.6 μm) and antral (301 vs. 316.4 μm) follicles in the ODM and control groups.

Table 1 Ovarian histomorphometric parameters in ODM and controls at 30 days after birth

Histomorphometric parameters	ODM	Control
Volume of ovary (mm^2)	10.3 ± 1.1	11.9 ± 1.5
Diameter of ovary (mm)	2.7 ± 0.2	2.8 ± 0.2
Weight of ovary (mg)	10.9 ± 1.1	12.7 ± 1.3
Ovarian capsule thickness (μm)	10.5 ± 1.3	10.6 ± 1.3
Medulla to cortex ratio	0.22 ± 0.02	0.2 ± 0.02

Values are presented as mean ± SD

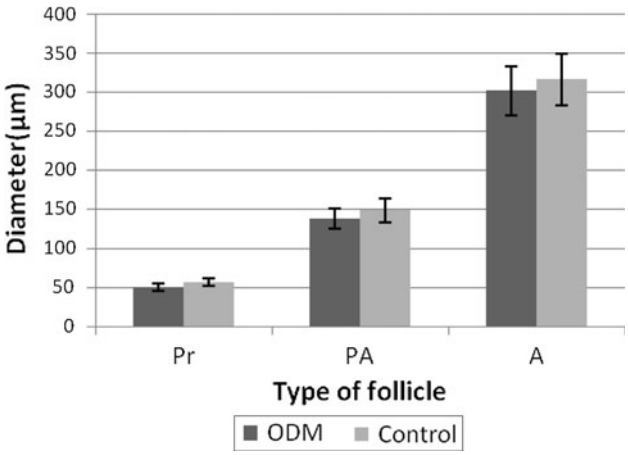

Fig. 1 Diameters of different stage follicles in ODM and control groups at 30 days after birth. *Pr* primary follicle, *PA* preantral follicle, *A* antral follicle

Figure 2 shows the number of different follicles in ODM and control mothers at 30 days after birth. There was no significant difference between the number of antral follicles in the ODM and control groups. The number of primary and pre-antral follicles showed a significant decrease ($p < 0.05$) in ODM ovaries. The numbers of primary, pre-antral, antral follicles were 0.9, 1.4 and 0.4 in ODM ovaries and 1.2, 1.9 and 0.6 in control ovaries, respectively.

Figure 3 demonstrates an electro-micrograph of the typical granulosa cells of both control (a) and ODM (b) groups from a follicle in the preantral stage at day 30 after birth. In the electro-micrographs of the ovaries from ODM, the granulosa cells were morphologically changed as their nucleoplasm demonstrated more heterochromatin compared to that of the control. The nuclei in granulosa cells of ODM ovaries were darkened and condensed compared to those of the control group. Cytoplasm of the granulosa cells was also not clear and the borders of the cells weren't distinguished. Mitochondrial abnormalities were noticed as mitochondrial cristae had been destroyed, and the mitochondria showed vacuolation. Endoplasmic

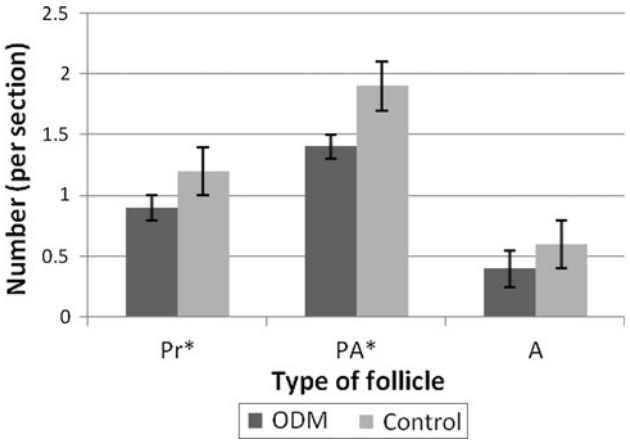

Fig. 2 Numbers of different stage follicles in ODM and control groups at 30 days after birth. *Asterisk* represents a significant difference at $p < 0.05$. *Pr* primary follicle, *PA* preantral follicle, *A* antral follicle

reticulum of the granulosa cells was destroyed and vacuolated in the ODM group. In addition, the nuclei of oocytes in primordial follicles of ODM were amorphous, their mitochondria destroyed and their cytoplasms were watery compared to those of the control.

Discussion

The body weight of ODM was significantly greater than that of controls (macrosomia), which is due to an increase in placental transport of glucose and other nutrients to the foetus [1]. Excessive gestational weight gain and hyperglycaemia may overstimulate foetal pancreatic β cells and, consequently, bring about foetal hyperinsulinism. Insulin itself is a growth hormone for the foetus, resulting in higher birth weight and in impaired glucose tolerance and obesity in adolescence [20]. Our previous study showed the body weight of neonates of diabetic rats was significantly increased [21]. The blood glucose of ODM was significantly higher than that of the controls. This condition, accompanied by a moderate increase in fasting blood glucose in ODM, may be due to maternal hyperglycaemia, leading to foetal hyperglycaemia and hypoinsulinaemia [14].

There was a decrease in the weight, volume and diameter of ovary and thickness of ovarian capsule in ODM. Ovarian atrophy and reproductive tract incompetence are recognized consequences of the progressive expression of the overt, diabetes-obesity syndrome (DOS) in diabetic mutant mice. In both humans and experimental models, utero-ovarian structural, functional, and metabolic parameters are altered in response to the progressive hyperglycemic–hyperinsulinemic systemic conditions that characterize noninsulin dependent (Type II) [10]. Diabetes can result in ovarian atrophy, suggesting that ovarian involution in these mutants

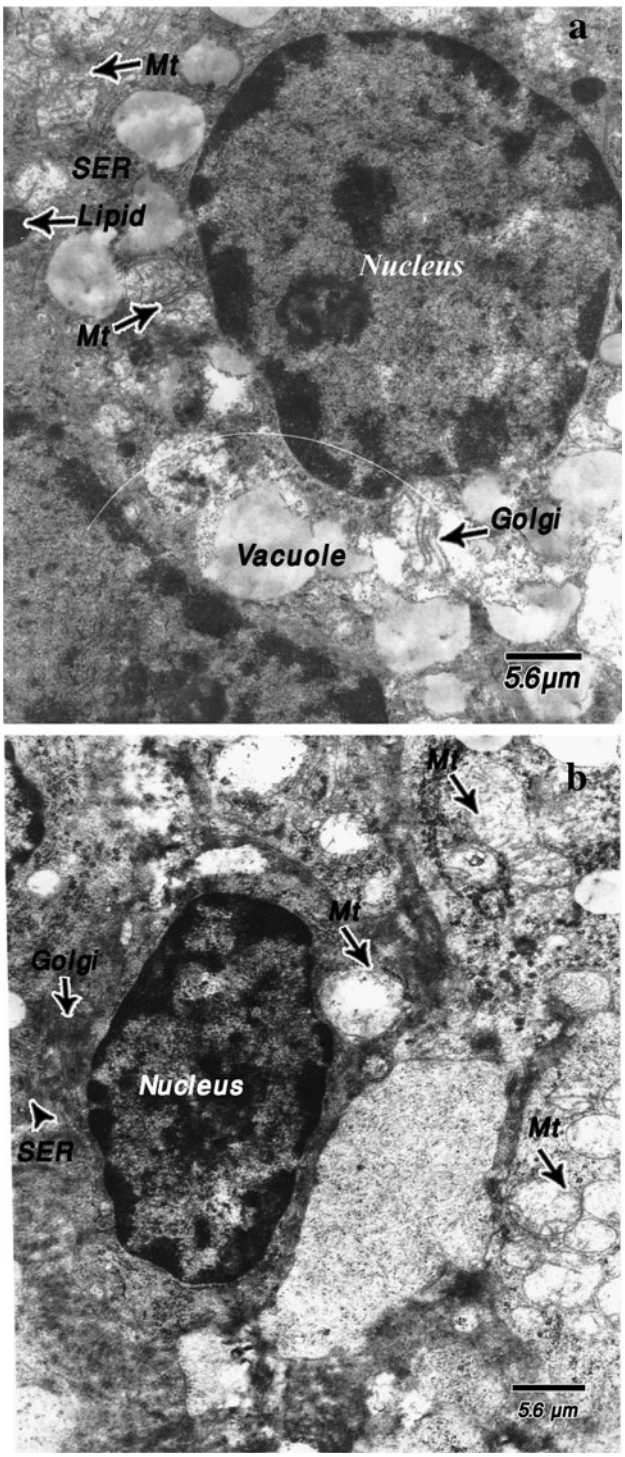

Fig. 3 Typical electro-micrograph of the granulosa cells of both control (**a**) and ODM (**b**) groups from follicle in the preantral stage at day 30 after birth. *MT* mitochondria, *SER* soft endoplasmic reticulum. As the figure demonstrates, the mitochondria and nuclei of the granulosa cells in ODM were altered in comparison to the control

is directly related to an impaired follicular ability to properly metabolize the elevated intracellular glucose concentrations that develop in the diabetic mice [13]. The increase in

medulla to cortex ratio seen in the ODM group may be due to a decline in cortical elements such as oocytes and follicles. Previous studies demonstrated that maternal diabetes increases oocytes apoptosis [12] and follicular atrophy [13] in mice.

There was a reduction in the diameters (Fig. 1) and number (Fig. 2) of primary, pre-antral and antral follicles observed in ODM compared to control. Garris and co-workers [13] indicate that follicular atrophy appears in diabetes. One study demonstrated that both models of maternal hyperglycemia and hypoinsulinemia may have a detrimental effect on oocyte maturation and development as detailed by the smaller sizes of oocytes and developing ovarian follicles and the greater amount of apoptosis [22]. The electro-micrographs have demonstrated the nuclei and mitochondrial alterations in the oocytes and granulosa cells. Lin and co-workers reported that maternal diabetes increase oocyte apoptosis [12]. Wang et al., demonstrated that the mitochondrial impairments induced by maternal diabetes lead to cumulus cell apoptosis, at least in part through the release of cytochrome *c*. Together, the deleterious effects on cumulus cells may disrupt trophic and signalling interactions with the oocyte, contributing to oocyte incompetence and, thus, poor pregnancy outcomes in diabetic females [23]. Cumulus cells and the oocyte are metabolically coupled throughout follicular development by membrane specializations known as gap junctions. An important point, particularly in relation to diabetes, is that oocytes are deficient in their ability to use glucose as an energy substrate and require cumulus cell-provided products of glycolysis like pyruvate for their own development [24]. Egg, zygote or blastocyst derived from diabetic parents may develop into offspring with high risk of any type of diabetes, even if placed in a normal uterus, producing developmental delay, embryopathy, geno- and cyto-toxicity, teratogenic changes, free radicals and apoptosis. These early insults may then lead to an increased rate of miscarriage and congenital anomalies depending on free radicals signaling and cell-death pathways involved with the diabetogenic agents. Furthermore, egg, zygote or blastocyst from normal parents will have an increased risk of diabetes if placed in a diabetic uterus [5]. Garris and Garris demonstrated that enhanced lipid deposition and cellular metabolic indices promote a very nonhomeostatic, hyperlipogenic metabolic environment within all ovarian compartments of a diabetic mutant rat. The progressive deposition of enhanced interstitial and follicular lipid pools compromises the functional and structural characteristics of all ovarian cellular and tissue compartments, ultimately inducing a hypercytolipidemia, which contributes to premature tissue involution and ovarian failure [10]. Ishikawa et al. [25] reported there were definite histopathological alterations such as mitochondrial abnormalities, dense bodies and lamellar structures at the nerve terminal innervating the dilator muscle. Schramm et al. [26] reported that intra-axonal mitochondria were more condensed or hydropic in diabetic patients' compared to the nondiabetics.

We conclude that female foetal gonads may be affected by maternal hyperglycaemia which remains after birth such as reduction in their ovarian weight, volume and diameter, number of ovarian follicles and follicular diameter. These changes might occur as a result of apoptosis.

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Conflict of interest We have no conflict of interest.

References

1. Jones CW. Gestational diabetes and its impact on the neonate. Neonatal Netw. 2001;20:17–23.
2. Persson B, Hanson U. Neonatal morbidities in gestational diabetes mellitus. Diabetes Care. 1998;2:79–84.
3. Van Assche FA, Aerts L, De Prins FA. Degranulation of the insulin-producing B cells in an infant of a diabetic mother. Br J Obstet Gynecol. 1983;90:182–5.
4. Ballester J, Muñoz MC, Domínguez J, Palomo MJ, Rivera M, Rigau T, Guinovart JJ, Rodríguez-Gil JE. Tungstate administration improves the sexual and reproductive function in female rats with streptozotocin-induced diabetes. Hum Reprod. 2007;22:2128–235.
5. Ahmed RG. Evolutionary interactions between diabetes and development. Diabetes Res Clin Pract. 2011;92:153–67.
6. Bestetti G, Locatelli V, Tirone F, Rossi GL, Müller EE. One month of streptozotocin-diabetes induces different neuroendocrine and morphological alterations in the hypothalamo-pituitary axis of male and female rats. Endocrinology. 1985;117:208–16.
7. Blades RA, Bryant KR, Whitehead SA. Feedback effects of steroids and gonadotrophin control in adult rats with streptozotocin-induced diabetes mellitus. Diabetologia. 1985;28:348–54.
8. Moley KH, Chi MM, Knudson CM, Korsmeyer SJ, Mueckler MM. Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. Nat Med. 1998;4:1421–4.
9. Goswami D, Conway GS. Premature ovarian failure. Hum Reprod Update. 2005;11:391–410.
10. Garris DR, Garris BL. Diabetes (db/db) mutation-induced ovarian involution: progressive hypercytolipidemia. Exp Biol Med (Maywood). 2003;228:1040–50.
11. Garris DR, Garris BL. Diabetes (db/db) mutation-induced female reproductive tract hypercytolipidemia: estrogenic restoration of utero-ovarian indices. Reprod Toxicol. 2004;18:641–51.
12. Lin S, Lin K, Li W, Zhou X, Huang T. Maternal diabetes increases apoptosis in mice oocytes, not 2-cell embryos. Endocrine. 2010;37:460–6.
13. Garris DR, Williams SK, West L. Morphometric evaluation of diabetes-associated ovarian atrophy in the C57BL/KsJ mouse: relationship to age and ovarian function. Anat Rec. 1985;211:434–43.
14. Jelodar G, Khaksar Z, Pourahmadi M. Endocrine profile and testicular histomorphometry in adult rat offspring of diabetic mothers. J Physiol Sci. 2009;59:377–82.

15. SzkuDelski T. The mechanism of Alloxan and Streptozotocin action in B Cells of the rat pancreas. Physiol Res. 2001; 50: 536–46.
16. Damas J, Hallet C, Lefebvre PJ. Changes in blood glucose and plasma insulin levels induced by bradykinin in anaesthetized rats. Br J Pharmacol. 2001;134:1312–8.
17. Turner CD, Bagnara JT. General endocrinology. Philadelphia: WB Saunders Company; 1971.
18. Elias H, Hyde DM. An elementary introduction to stereology (quantitative microscopy). Am J Anat. 1980;159:411–7.
19. Luna LG. Manual of histologic staining methods of the Armed forces institute of pathology. USA: McGraw Hill; 1968.
20. Wrotniak BH, Shults J, Butts S, Stettler N. Gestational weight gain and risk of overweight in the offspring at age 7 y in a multicenter, multiethnic cohort study. Am J Clin Nutr. 2008;87: 1818–24.
21. Khaksar Z, Jelodar G, Hematian H. Cerebrum malformation in offspring of diabetic mothers. Comp Clin Pathol. 2011; 10:1160–9.
22. Chang SA, Dale NA, Oley HK. Maternal diabetes adversely affects pre-ovulatory oocyte maturation, development, and granulosa cell apoptosis. Endocrinology. 2005;146:2445–53.
23. Wang Q, Frolova AI, Purcell S, Adastra K, Schoeller E, Chi MM, Schedl T, Moley KH. Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice. PLoS ONE. 2010; 5:e15901.
24. Sugiura K, Eppig JJ. Control of metabolic cooperativity between oocytes and their companion granulosa cells by mouse oocytes. Reprod Fertil Dev. 2005;17:667–74.
25. Ishikawa S, Bensaoula T, Uga S, Mukuno K. Electron-microscopic study of iris nerves and muscles in diabetes. Ophthalmologica. 1985;191:172–83.
26. Schramm E, Wagner M, Nellessen U, Inselmann G. Ultrastructural changes of human cardiac atrial nerve endings in diabetes mellitus. Eur J Clin Invest. 2000;30:311–6.