

## Ovarian Histomorphometry at Puberty in Rat Offspring from Diabetic Mothers

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**Abstract:** Purpose: In pregnant mothers, maternal diabetes occurs when the pancreas cannot produce enough insulin, which 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. 60 days after birth, the female offspring were terminated, the body weight and blood glucose of the animals measured and their ovaries removed. Various histological parameters were determined using histological 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 controls. The weight, volume and diameter of the ovary and ovarian capsule thickness were decreased in the ODM group. The number and diameter of primary, pre-antral, antral and pre-ovulatory follicles and corpora lutea were decreased in ovaries in the ODM. *Conclusion:* Maternal hyperglycaemia exhibited deleterious effects on the reproductive system of their offspring.

**Key words:** Maternal Diabetes • Rat • Offspring • Ovary • Follicle

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### INTRODUCTION

Diabetes mellitus is the most common metabolic and endocrine disorder [1, 2]. Diabetes mellitus is characterized by hyperglycemia and is associated with disturbances in carbohydrate, protein and fat metabolism [3]. In diabetic subjects, the pancreas produces insufficient amounts of insulin, causing blood sugar levels to rise [4]. Diabetes mellitus is usually associated with glycosuria, polyuria and polydipsia [5]. In diabetic mothers, during pregnancy placental transport of glucose and other nutrients increases due to increased availability at the maternal site, resulting in foetal and neonatal macrosomia [6]. The elevated glucose concentration in the mother is accompanied by hyperglycaemia in the foetus, leading to degranulation of the foetal B-cells, resulting in foetal hypoinsulinaemia [7]. Data has indicated that pre-gestational maternal diabetes is associated with strong teratogenic effects and major congenital anomalies are two to four times more common in diabetic pregnancies than in normal pregnancies [8]. However, it has been

reported that abnormalities are unlikely to impair reproductive function significantly in isolation [9].

Diabetes has deleterious effects on female reproductive functions [10] and on the development of the blastocysts [11]. Diabetes is associated with increased risk of reproductive problems such as spontaneous abortions, neonatal morbidity and mortality, congenital malformation and poor embryo development [12]. Polycystic ovary syndrome, the main androgen disorder in women, has been suggested to be associated with a high risk of developing type 2 diabetes [13]; this disorder is probably the most common hormonal abnormality in women of reproductive age and certainly is a leading cause of infertility [14]. 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 [15]. Data also have indicated 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 sensitivity to LHRH [16]. Diabetes mellitus may cause the enhancement of prostaglandin F2 alpha (PGF2 $\alpha$ ) -induced responses in small blood vessels [17]. One study demonstrated that there are acute actions of PGF2 $\alpha$  in micro dialyzed bovine corpus luteum *in vitro* [18]. Moley and co-workers have 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 [19].

The most convincing evidence comes from the commonly observed association of premature ovarian failure with some autoimmune disorders in diabetes mellitus [20]. Data have suggested that diabetes mutation-induced ovarian structural and functional involution is a direct reflection of the cellular metabolic shift towards lipogenesis [21]. The diabetes mutation (leptin-receptor defect) induced a hyperglycaemic-hyperinsulinaemic endometabolic environment that promotes hypercytolipidaemic, utero-ovarian involution in mice, resulting in reproductive sterility and eventual organoatrophy [22]. Maternal diabetes increased apoptosis in mice oocytes [23]. Ovarian dysfunction in a diabetic mutant mouse is associated with follicular atrophy, adiposity, impaired steroidogenesis and imbalanced glucose utilization [24]. The purpose of this investigation is to evaluate the possibility of congenital ovarian malformations in the offspring of diabetic rats at day 60 after birth.

## MATERIALS AND METHODS

Sixteen adult female Sprague Dawley rats (200–230 g and 4–5 months old) were housed in an air-conditioned room (22 $\pm$ 2 $^{\circ}$ C) and supplied with standard pellet food, with tap water *ad libitum*. Animals were separated 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.

Diabetes was induced in eight rats by a single intraperitoneal injection (150 mg/kg) of alloxan tetra hydrate (Sigma, St. Louis, MO) according to our previous study [25]. 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, were considered to be diabetic and were selected for the experiment [26].

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 [27]. Female offspring of both groups were reared in similar conditions in an animal house for 60 days. 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 [28] and the ovaries were fixed in 10% buffered formalin solution.

Formaldehyde-fixed samples were embedded in paraffin and then sectioned at 4–5 $\mu$ m. They were further deparaffinised with xylol and histologic observations were performed after staining by the Haematoxylin-Eosin or Green Masson's trichrome method [29]. 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 ( $\mu$ m), (2) The ratio of medulla to cortex, (3) The diameter of primary, pre-antral, antral and pre-ovulatory follicles and the diameter of the corpora lutea ( $\mu$ m), (4) The number of primary, pre-antral, antral and pre-ovulatory follicles and the number of corpora lutea(/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. Morphometric data are presented as the mean  $\pm$  SD and were analyzed by Student's t test using SPSS software. Significant differences were considered when P value was  $\leq$ 0.05.

**RESULTS**

Body weight of the offspring of diabetic mothers (ODM) was significantly greater than that of controls 60 days after birth ( $p < 0.05$ ) (144.2±6g in ODM and 126.8±3.4g for controls). Blood glucose in the ODM was also significantly greater than the controls ( $p < 0.05$ ) - 120.3±6.8mg/dl and 92.5±4.3mg/dl respectively.

Values for weight, 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 decreased in ODM compared to that of controls. Ovarian weight was 42g in ODM but 43.5g in the controls. Volume of ovary was decreased in ODM in comparison to control, which was 39.5 mm<sup>2</sup> in ODM but 40.9 mm<sup>2</sup> in the control group.

Figure 1 has compared the diameter of different follicles of the ovary in ODM and controls 60 days after birth. The diameter of the primary (51.2µm vs. 53.07µm), pre-antral (142.8µm vs. 149.83µm), antral (297.94µm vs. 306.96µm) and pre-ovulatory (514.5µm vs. 535.1µm) follicles showed a non-significant decrease in ODM ovaries compared to that of controls. The diameter of the corpora lutea showed a non-significant decrease in ODM ovaries compared to that of controls (801.3µm in ODM and 884.7µm in control group).

Figure 2 has demonstrated the comparison between the number of different follicles in ODM ovaries and controls 60 days after birth. The number of primary and pre-ovulatory follicles showed a non-significant decrease in ovaries in ODM compared to that of controls. The number of pre-antral and antral follicles showed a significant decrease ( $p < 0.05$ ) in ODM ovaries. The numbers of primary, pre-antral, antral and pre-ovulatory follicles were 0.14, 0.26, 0.15 and 3.1 in

Table 1: Ovarian histomorphometric parameters in ODM and controls 60 days after birth. Values are presented as mean± SD.

Group	ODM	Control
Volume of ovary (mm <sup>2</sup> )	39.5±3.5	40.9±4.1
Diameter of ovary (mm)	4.2±0.4	4.3±0.4
Weight of ovary (mgr)	42±3.8	43.5±4.4
Ovarian capsular thickness (µ)	10.7±1.1	11.1±1.2
Medulla to cortex ratio	0.27±0.02	0.26±0.02

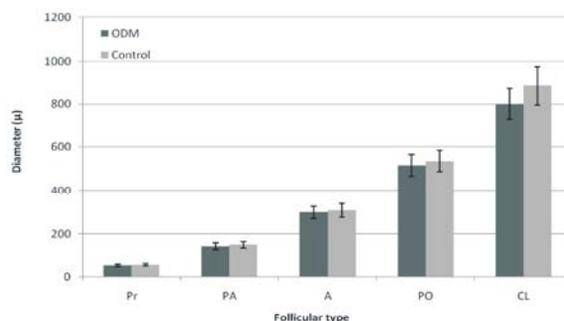


Fig. 1: Diameters of different follicles of the ovaries of ODM and controls 60 days after birth. Pr (Primary follicle), PA (Pre-antral follicle), A (Antral follicle), PO (Pre-ovulatory Follicle), CL (Corpus Luteum).

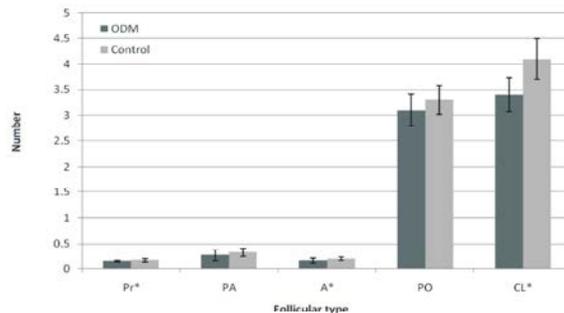


Fig 2: Number of different follicles in ODM and control ovaries 60 days after birth. Pr (Primary follicle), PA (Pre-antral follicle), A (Antral follicle), PO (Pre-ovulatory Follicle), CL (Corpus Luteum). Asterisk (\*) represents a significant difference at  $p < 0.05$ .

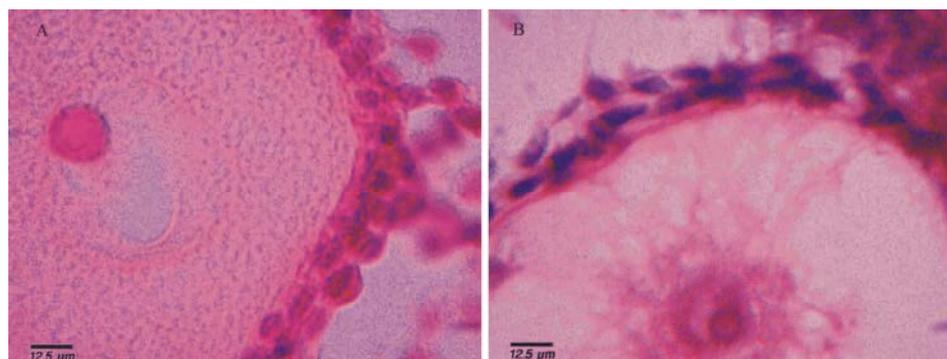


Fig. 3: Comparison of the antral follicle in ovary at 60 days after birth in control (A) and ODM(B) groups (Staining: Haematoxylin-Eosin).

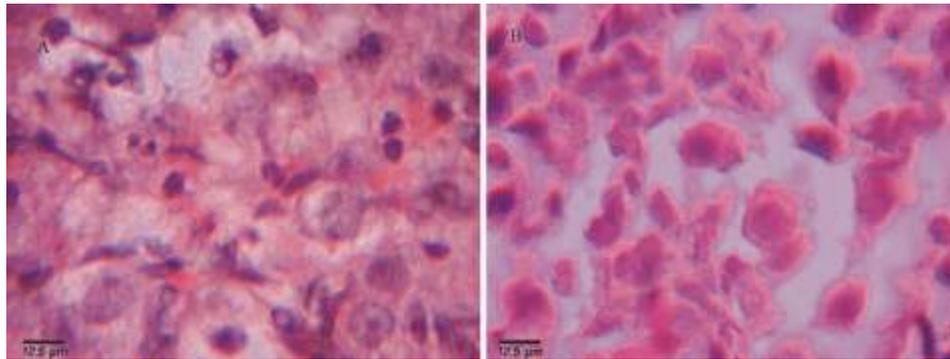


Fig. 4: Comparison of the corpus luteum in an ovary, 60 days after birth in control (A) and ODM(B) groups (Staining: Haematoxylin-Eosin).

ODM ovaries and 0.16, 0.32, 0.19 and 3.3 in control ovaries, respectively. The number of corpora lutea was decreased in ODM ovaries compared to that of controls (3.4 in ODM and 4.1 in the control group).

Figures 3 and 4 show sections of the antral follicle and corpus luteum in ODM and control groups 60 days after birth.

## 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 [4]. 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 [25].

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) [21]. Diabetes can play a role in ovarian atrophy, suggesting that ovarian involution in these mutants is directly related to an impaired follicular ability to properly metabolize the elevated intracellular glucose concentrations that develop in the diabetic mice [24]. 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 [23] and follicular atrophy [24] in mice.

A reduction in the diameters (Figure 1) and number (Figure 2) of primary, pre-antral, antral and pre-ovulatory follicles as well as corpora lutea was observed in ODM. It has been indicated that 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 [12]. Garriss and co-workers indicate that follicular atrophy appears in diabetes [24]. 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 [30]. Lin and co-workers reported that maternal diabetes increase oocyte apoptosis [23]. 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 [31]. 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 [32]. 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 [11]. Garris and Garris demonstrated that enhanced lipid deposition and cellular metabolic indices promote a very non homeostatic, 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 [21].

We are concluded 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.

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