

EXPERIMENTAL STUDY

Alterations in the immunoreactivity of laminin, type IV collagen and $\alpha3\beta1$ integrin in diabetic rat ovarian follicles

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ABSTRACT

AIM: In order to determine the possible effects of diabetes, we aimed to investigate the expression of extracellular matrix proteins in the theca and granulosa layers in different follicular stages.

METHODS: Thirty-two adult Wistar albino male rats were divided into 4 groups as control and sampled groups. Four, eight and twelve weeks after inducing diabetes with an intraperitoneal injection of streptozotocin (40 mg/kg), the expressions of laminin, type IV collagen and $\alpha3\beta1$ integrin in ovarian tissues were evaluated by immunohistochemical method.

RESULTS: In our study, in the first month of diabetes, a significant increase was observed in laminin, type IV collagen and $\alpha3\beta1$ integrin expressions in all follicle types compared to the control group in both the theca and granulosa layers. Laminin and type IV collagen immunoreactivity tended to increase in D2 and D3 groups also. Integrin expression did not change in the newly formed follicles in the D2 and D3 groups, however, it tended to change and increase in the developing follicles.

CONCLUSIONS: The changes in the expression of laminin, type IV collagen and $\alpha3\beta1$ integrin, which are the extracellular matrix proteins in the follicle, along with diabetes, show that diabetes plays a role in the regulation of follicular development (Tab. 4, Fig. 36, Ref. 29). Text in PDF www.elis.sk.

KEY WORDS: $\alpha3\beta1$ integrin, laminin, type IV collagen, diabetes, IHC, ovarian follicles.

Introduction

Diabetes mellitus is considered to be a metabolic disorder which is characterized by insufficient secretion or endogenous insulin action and hyperglycemia (1).

Diabetes is related with an increased risk of diseases such as cardiovascular disorders and neuropathy. At the same time however, it is associated with reproductive problems such as poor embryo development, congenital malformation, impaired folliculogenesis, spontaneous abortions, and anovulation in women (2, 3). Folliculogenesis is an extremely regulated process where various autocrine, endocrine and paracrine factors act spatially and temporally in order to coordinate and regulate the development and growth of the oocyte as well as the surrounding theca and granulosa cell layers (4). The extracellular matrix has different roles in the cellular development in the ovary and in many

systems. The extracellular matrix (ECM) provides the structural support to the follicle, provides biochemical signals which promote maturation and follicular development, as well as provides connectivity and cellular organization (5, 6). As the ovary is reshaped by continuous follicle growth, development and atresia, it is important to detect the cells in which ECM components are expressed at different stages of rat ovarian follicle. In addition, immunohistochemical studies conducted on the effect of diabetes on type IV collagen, laminin and $\alpha3\beta1$ integrin expressions in these stages are very limited.

Laminin was localized to theca cell compartment primarily, with a characterized ring outside the follicular granulosa cells that mark the basement membrane. Lower levels of laminin were likewise clear in the granulosa, and stroma cell compartment. Collagen IV was found to be abundant in theca cell compartment, with a low-level expression in the granulosa and stroma cells. The distribution of collagen was consistent during the follicular development (4). Integrins are considered to be the major receptors which mediate adhesion to ECM (7). They are trans-membrane heterodimeric glycoproteins that are composed of subunits α and β ; 16 different α and 8 β subunits have been determined (8), and according to β subunits, they have been divided into subfamilies. The $\beta1$ superfamily contains specific receptors for laminin. Some $\beta1$ integrins may bind collagens or laminin. However, the most versatile receptor is the $\alpha3\beta1$ integrin, which binds collagen molecules (9) to structures such as laminin (10) and fibronectin (8, 11, 12).

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The follicular microenvironment varies significantly throughout folliculogenesis because of the follicle movement along the ovary, changing extracellular milieu and development of the follicle. The folliculogenesis may be regulated by ECM through the composition which changes with both cellular compartment and follicle stage (6). ECM components that are present in follicular fluid, follicular basement membrane and around the follicular cells of the ovary, take part in the follicular development regulation, and the role of integrins is highly suggested in this process (7). The determining of ECM protein distributions and understanding the changes of ECM with diabetes in different ovarian follicle stages in rats will contribute to the understanding of the possible effects of diabetes on follicles.

In this study, streptozotocin (STZ) was administered to 12-week-old female mice to establish the type 1 diabetes model. The aim of this study was to investigate the laminin, type IV collagen and $\alpha 3\beta 1$ integrin expressions in theca and granulosa layers in different follicular stages using immunohistochemical methods in order to reveal the possible effects of diabetes on rat follicular development stages.

Material and methods

Animals

This study was conducted in the Erciyes University, Hakan Çetinsaya Experimental and Clinic Research Center. Thirty-two adult twenty-week-old Wistar albino rats were used in this study. The rats were placed in a well-ventilated plastic cage rat house, kept on a 12-h light and dark cycle, while the feed and water were provided *ad libitum*. All animals were given good care according to the standard guidelines. Ethical approval from the Erciyes University, Animal Research Ethics Committee was obtained for the study (no: 12/105/2012). Ethical regulations were strictly followed according to the national and institutional guidelines. The rats were assigned randomly to four groups of eight rats per group. Group C (control) served as control; groups D1, D2 and D3 were composed of diabetic rats according to the duration of diabetes, namely one, two or three months, respectively.

Diabetes induction and tissue sampling

Diabetes induction was performed in 12-week-old female Wistar rats after an overnight fast ($n = 24$) using STZ (40 mg/kg) intraperitoneal injection (Sc-200719, Santa Cruz Biotechnology, CA, USA), while physiological saline ($n = 8$) was injected in the control rats. Seventy-two hours after the injection of streptozotocin, hyperglycemia was confirmed by measurements of glucose levels in the blood, which was obtained from the tail vein. The measurements were done by using a glucometer. Animals whose mean plasma glucose levels were higher than 250 mg/dL, were considered to be diabetic. After streptozotocin injection, glycemia was checked as well at sacrifice, i.e. at weeks 4, 8 or 12. The animals were decapitated under intraperitoneal xylazine (10 mg/kg) + ketamine (75 mg/kg) anesthesia. The ovarian tissues were removed quickly after decapitation, and they were fixed in a fixative of 4 % formaldehyde for histological examination.

Immunohistochemistry

The Avidin-Biotin-Peroxidase Complex (ABC) method and a couple of immunohistochemical methods were used to determine laminin, type IV collagen and $\alpha 3\beta 1$ integrin immunoreactivities in the ovarian tissue. Paraffin sections (5 μm thick) were placed on poly-(L-lysine)-coated slides and stored overnight in an oven at 60 °C. Then they were deparaffinized with xylene and rehydrated with a series of decreasing grades of alcohol (100 %, 96 %, 80 %, 70 %). After rehydration, the sections were washed in distilled water 3 times for 5 minutes. For antigen retrieval, the tissues were heated for 5 minutes in 5 % citrate buffer in a microwave oven at 600 W. The sections were washed with phosphate buffered saline (PBS) and treated with 3 % hydrogen peroxide (H_2O_2) for 12 minutes in order to block endogenous peroxidase. For the next stages, a Large Volume Detection System (Thermo Fisher Scientific, Waltham, MA, USA, Catalog no: TP-125-HL) immunohistochemistry staining kit was used. To prevent background staining, the sections were treated with ultra V block for 5 minutes and then incubated overnight at +4 °C in a humid environment with appropriate primary antibodies (Laminin (Anti-Laminin antibody (abcam), ab11575, 1/100 dilution), Type IV collagen (Anti-Collagen IV antibody (abcam), ab6586, 1/100 dilution) and $\alpha 3\beta 1$ integrin (Integrin Alpha3, Beta1 antibody, ABIN737034, 1/50 dilution). Negative controls were treated with PBS in place of the primary antibodies. Processes in the other stages mentioned above were carried out in the same way. After primary antibody incubation, the sections were rinsed. Reverse staining was performed using appropriate biotinylated secondary antibodies, Streptavidin-HRP (Horse Radish Peroxidase), DAB (3,3'-diaminobenzidine) chromogen, and Gill's Hematoxylin. Finally, the sections were dehydrated in a series of increasing grades of alcohol (70 %, 80 %, 96 %, 100 %) and cleared in xylene before being cover-slipped with Entellan® (Merck, Kenilworth, NJ, USA) (13). The slides were viewed and photographed using an Olympus BX51 microscope with a digital camera (DP71).

Evaluation of immunohistochemical staining

Based on images that were taken from the slides, laminin, $\alpha 3\beta 1$ integrin and type IV collagen immunoreactivity measurements were taken using Image-J program for immunoreactivity difference. Immunoreactivity differences were measured separately in granulosa and theca layers in each of the primary, secondary and tertiary follicles in all groups. Measurements were taken homogeneously from different follicles in each follicle type in the tissue. The data obtained were evaluated statistically.

Tab. 1. Blood glucose levels of control and streptozotocin (STZ)-induced diabetic rats.

Groups	Blood glucose (mg/dl)
Group C	118.87±2.18
Group D1	544.40±33.84*
Group D2	517.25±42.30*
Group D3	538.33±36.10*

* significantly differed from control, by $p < 0.05$ (according to ANOVA results)

Follicle classification

Follicles were classified in accordance with the commonly used terms for rats. Primary follicles contained one or fewer than two layers of the cuboidal granulosa cells. Secondary follicles (preantral follicles) had an oocyte and were categorized as two-layered or multi-layered secondary follicles according to the number of present granulosa cell layers, and had no visible antrum. In tertiary follicles (antral follicles), also, an oocyte, which might be surrounded by multiple layers of cuboidal granulosa cells and

containing one or more *cumuli oophori*, antral spaces and theca layer, might have been evident (6, 14).

Statistical analysis

The obtained data were analyzed using SPSS 23.0. Firstly, to determine normality, the values of skewness and kurtosis were examined. ANOVA was used in order to determine statistical significance.

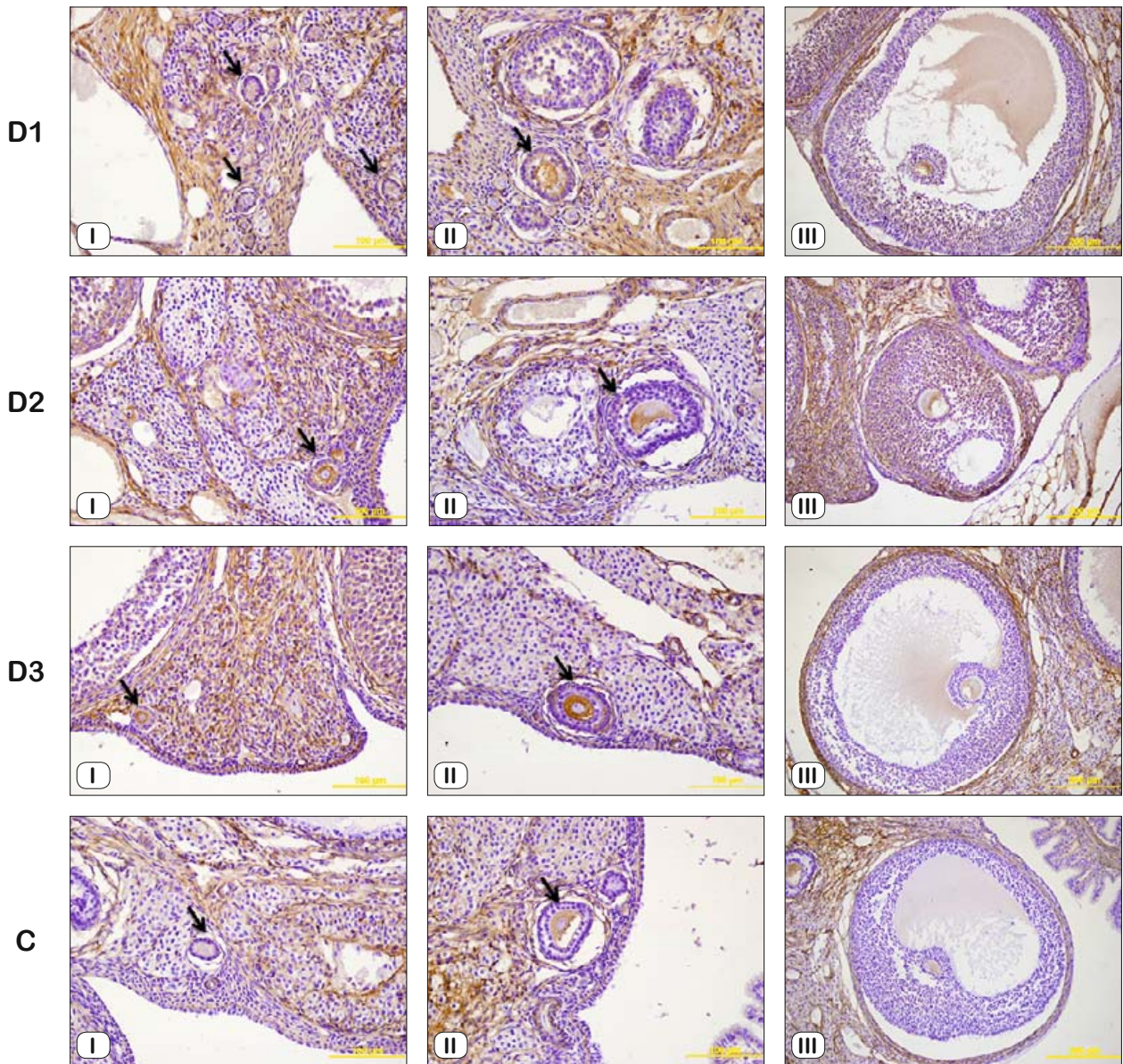


Fig. 1. Laminin immunostaining in experimental groups. Groups D1, D2, D3, and C: I (Primary Follicles), Laminin expression in the primary follicles (arrow), II (Secondary Follicles), Laminin expression in the secondary follicles (arrow), III (Tertiary Follicles). I, II X20, Bar 100 μ m; III X40, Bar 200 μ m.

Tab. 2. Immunoreactivity intensity values of Laminin in the follicles.

LAMININ		$\bar{X} \pm SD$			
		Control	D1	D2	D3
Primary fol.	Granulosa	93.44±6.68	103.58±10.08**	102.22±5.22**	100.81±8.43**
Secondary fol.	Granulosa	94.80±4.73	102.39±7.19**	100.05±5.24**	99.22±6.00**
	Theca	103.16±5.29	110.75±7.90**	105.85±6.92	104.57±7.83
Tertiary fol.	Granulosa	90.82±4.23	100.33±5.71**	95.64±5.98**	96.76±4.64**
	Theca	106.51±5.54	115.45±8.15**	107.82±7.29	110.28±9.02

* significantly differed from control, by $p < 0.05$ (according to ANOVA results)

** significantly differed from control, by $p < 0.01$ (according to ANOVA results)

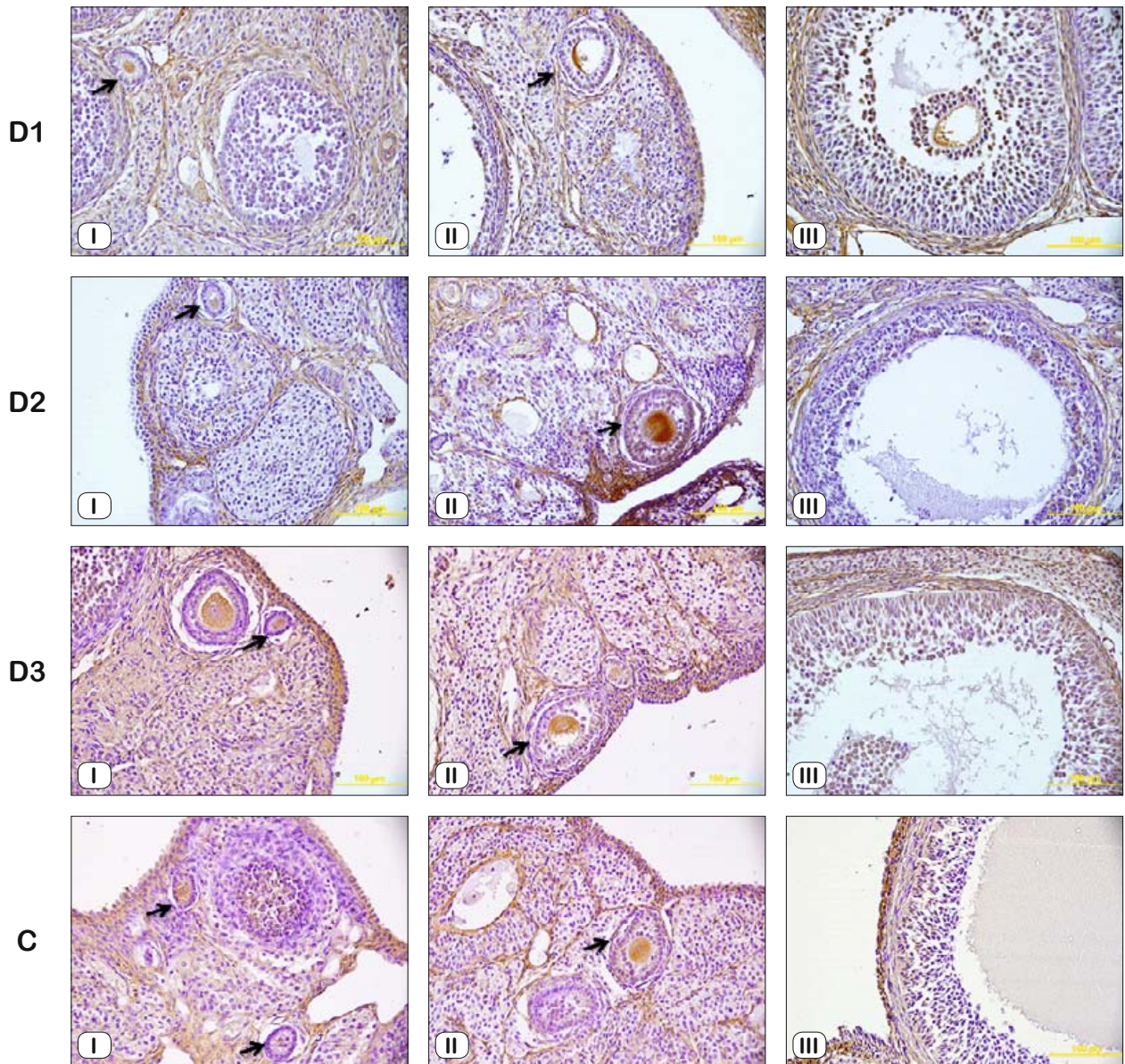


Fig. 2. Type IV collagen immunostaining in experimental groups. Groups D1, D2, D3, and C: I (Primary Follicles), II (Secondary Follicles), Type IV collagen expression in the primary follicles (arrow), III (Tertiary Follicles). I, II, III X40, Bar 100 μ m.

Tab. 3. Immunoreactivity intensity values of Type IV collagen in the follicles.

TYPE IV COLLAGEN		$\bar{X} \pm SD$			
		Control	D1	D2	D3
Primary fol.	Granulosa	90.93±7.28	102.43±6.88**	93.87±6.49	96.54±5.79**
Secondary fol.	Granulosa	92.14±7.11	105.51±8.57**	98.71±6.01**	96.35±5.13*
	Theca	93.29±5.68	103.07±7.69**	98.73±7.43**	95.68±7.63
Tertiary fol.	Granulosa	94.18±7.00	108.23±7.37**	104.05±6.63**	103.33±7.78**
	Theca	100.07±8.76	110.45±8.65**	102.20±6.34	104.32±10.19

* significantly differed from control, by $p < 0.05$ (according to ANOVA results)

** significantly differed from control, by $p < 0.01$ (according to ANOVA results)

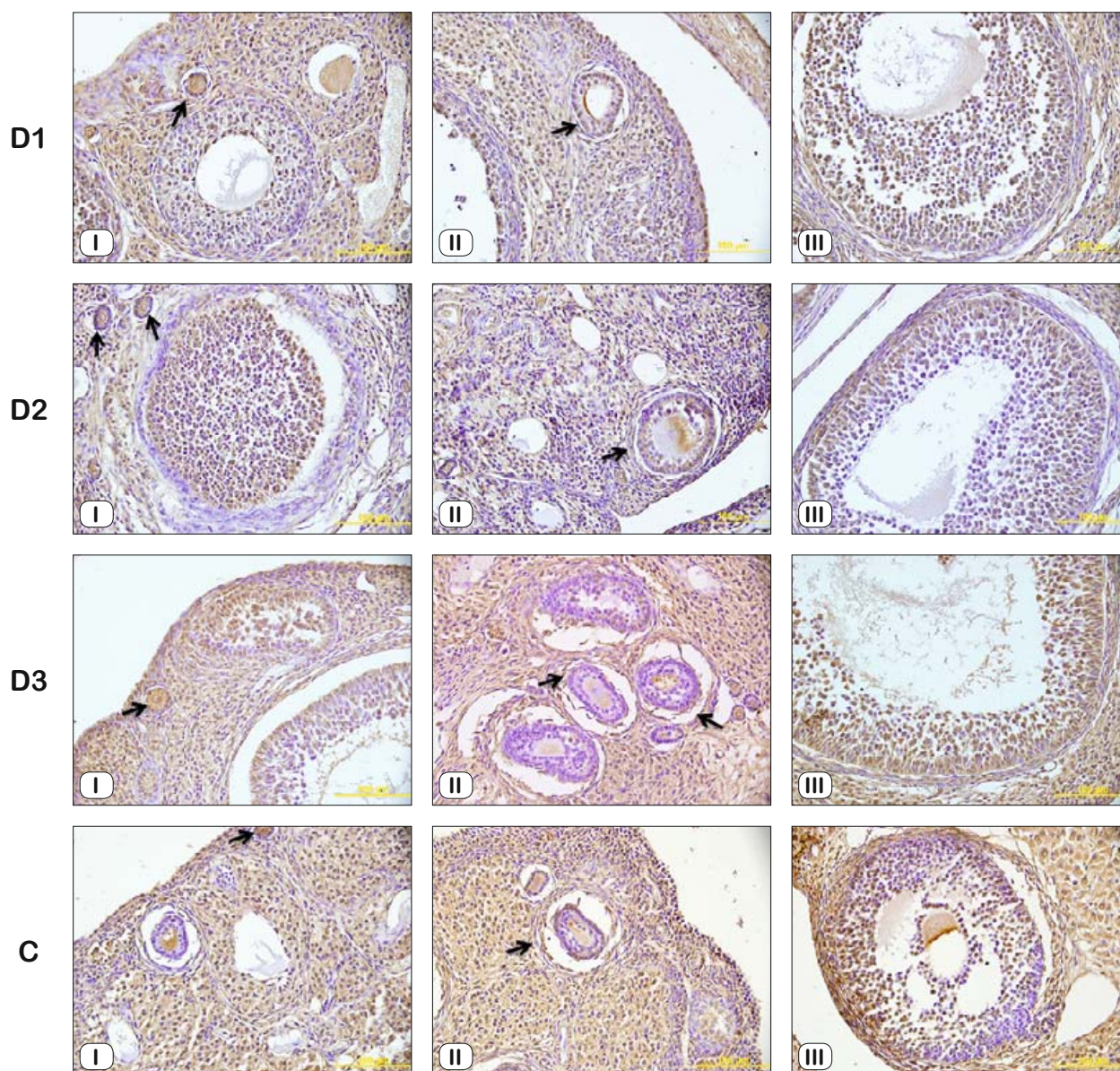


Fig. 3. Integrin $\alpha 3 \beta 1$ immunostaining in experimental groups. Groups D1, D2, D3, and C: I (Primary Follicles), $\alpha 3 \beta 1$ integrin expression in the primary follicles (arrow), II (Secondary Follicles), $\alpha 3 \beta 1$ integrin expression in the secondary follicles (arrow), III (Tertiary Follicles). I, II, III X40, Bar 100 μm .

Tab. 4. Immunoreactivity intensity values of $\alpha 3\beta 1$ integrin in the follicles.

$\alpha 3\beta 1$ INTEGRIN		$\bar{X} \pm SD$			
		Control	D1	D2	D3
Primary fol.	Granulosa	96.01 \pm 9.67	108.50 \pm 10.36**	95.86 \pm 9.06	97.81 \pm 13.09
	Theca	92.45 \pm 5.71	98.14 \pm 7.48**	95.30 \pm 9.36	94.11 \pm 9.48
Secondary fol.	Granulosa	96.21 \pm 9.06	103.66 \pm 7.31**	93.87 \pm 9.90	97.18 \pm 7.09
	Theca	92.45 \pm 5.71	98.14 \pm 7.48**	95.30 \pm 9.36	94.11 \pm 9.48
Tertiary fol.	Granulosa	96.55 \pm 5.43	109.12 \pm 9.73**	98.06 \pm 7.96	103.47 \pm 7.77**
	Theca	94.21 \pm 4.69	103.10 \pm 12.63**	100.18 \pm 9.55*	100.61 \pm 10.24**

* significantly differed from control, by $p < 0.05$ (according to ANOVA results)

** significantly differed from control, by $p < 0.01$ (according to ANOVA results)

Results

Induction of diabetes

At the end of the experiment, blood glucose levels were measured. The increase in blood glucose levels were statistically significant in the diabetic groups as compared to the control group (Tab. 1).

Immunohistochemistry

Laminin immunoreactivity

Laminin was immunolocalized to granulosa cells, theca cell compartment and stroma for all the follicular stages (Fig. 1). According to the results of statistical analysis, laminin immunoreactivity in primary follicles containing granulosa cells showed a significant increase in D1, D2 and D3 groups as compared to the control group. There was a significant increase in granulosa layer of the secondary and tertiary follicles in D1, D2 and D3 groups compared to the control group. In the theca layer, a significant increase was observed in the D1 group, compared to the control group, whereas, there was no significant increase in the D2 and D3 groups compared to the control group. According to these data, as the diabetes progressed in rats, there was an increase in laminin immunoreactivity in granulosa cells in all follicular stages, whereas the immunoreactivity of the theca layer was observed to be increased in the initial stages of diabetes. In later stages however, it was observed to be similar to that in the control group (Tab. 2).

Type IV collagen immunoreactivity

Type IV collagen was immunolocalized to granulosa cells, theca cell compartment and stroma for all the follicle stages (Fig. 2). According to the results of statistical analysis, Type IV collagen immunoreactivity in primary follicles containing granulosa cells showed a significant increase in D1 and D3 groups as compared to the control group. The D2 group was similar to the control group. A significant increase was observed in the granulosa layer of secondary follicles in D1, D2 and D3 groups as compared to the control group. In theca layer, a significant increase was observed in D1 and D2 groups as compared to the control group. The D3 group was similar to the control group. In the tertiary follicles, there was a significant increase in the granulosa layer in D1, D2 and D3 groups as compared to the control group. D1 was the only group to show a significant increase in the theca layer as compared to the control group, whereas D2 and D3 groups were similar to the control group. According to these data, as the diabetes progressed

and follicle developed in rats, type IV collagen immunoreactivity was observed to be increased in granulosa cells, whereas the theca layer immunoreactivity was observed to be similar to that in the control group (Tab. 3).

Integrin $\alpha 3\beta 1$ immunoreactivity

Integrin $\alpha 3\beta 1$ was immunolocalized to granulosa cells, theca cell compartment and stroma for all follicular stages (Fig. 3). According to the results of statistical analysis, $\alpha 3\beta 1$ integrin immunoreactivity in primary and secondary follicles containing granulosa layer and secondary follicle theca layer showed a significant increase in D1 group, whereas no significant change was observed in D2 and D3 groups as compared to the control group. In D1 and D3 groups, the tertiary follicle granulosa layer showed a significant increase compared to the control group, whereas the D2 group was similar to the control group. There was a significant increase in tertiary follicle theca layer in D1, D2 and D3 groups as compared to the control group. According to these data, while the increase in $\alpha 3\beta 1$ integrin immunoreactivity in granulosa and theca layers was significant in the first month of diabetes and in the initial stages of follicular development in rats, this increase was observed not to be significant as diabetes progressed. However, there was a significant increase in $\alpha 3\beta 1$ integrin immunoreactivity in the theca layer of follicles in the advanced stage of development as compared to the control group (Tab. 4).

Discussion

Diabetes has a negative effect on ovarian follicular development, and increases the morbidity of infertility (15–17). However, the mechanisms responsible for the pathogenesis of diabetes-induced infertility are not fully clear (17). Folliculogenesis and steroidogenesis deterioration, anovulation and stromal fibrosis may develop due to hyperglycemia (18, 19). It is very important to elucidate the molecular mechanisms underlying such diabetic disorders. Extracellular matrix (ECM) is a macromolecular network found in a three-dimensional form and without cells (20). ECM components are found in the ovary, follicular basement membrane, around follicular cells, and in the follicular fluid (6). The changes in the ECM expression in the normal state and in the state of diabetes inside the follicle, as well as the role of these changes in the regulation of follicular development are very important. Studies conducted on the expression and function of ECM proteins in ovarian tissue are quite limited in the case of diabetes.

In this study, we aimed to investigate the immunohistochemical changes of ECM proteins such as laminin, type IV collagen and cell adhesion molecule $\alpha 3\beta 1$ integrin in ovarian follicles at different stages of the development of diabetes in rats. In the light of available data, we would like to discuss whether the immunoreactivity of laminin, type IV collagen and $\alpha 3\beta 1$ integrin, functions of these proteins, and their effects on follicular growth in ovarian granulosa and theca cells change with diabetes.

Although the molecules present in the ECM are classified in many ways, it includes molecules such as collagen, elastin, and proteoglycan, which are generally found to be structural, as well as some specialized structures such as integrin, fibronectin, etc. (20). Integrins are an important adhesion protein family (21). Integrin molecules and other receptors play a role in the transfer of ECM signals of cells to the intracellular skeletal structure. Ligand structures for integrins can be expressed as collagen, laminin and fibronectin. Interactions of these ligands and integrin help in the regulation of processes such as cell survival, cell migration and proliferation. Thus, the ECM components play a significant role in the ovarian function (20, 22, 23).

Little is known regarding the stages of folliculogenesis and integrins in oogenesis (especially $\alpha 3\beta 1$ integrin). Clearly, their function is thought to be more complex than a simple adhesion receptor or a signaling molecule that stimulates proliferation or differentiation in case of contact with ECM (24). In many cases, it is known that they primarily regulate events mediated by other integrins and even other non-integrin adhesion systems (24). Integrin $\alpha 3\beta 1$ is expressed in granulosa cells throughout the follicular development and atresia in sheep. Ovine granulosa cells express integrin receptors for laminin ($\alpha 6\beta 1$, $\alpha 3\beta 1$) and collagen ($\alpha 3\beta 1$), suggesting that these integrins can mediate *in vivo* activities of ECM components (7). In addition, in another study conducted on integrin subtype $\alpha 6\beta 1$ integrin, it was reported that laminin- $\alpha 6\beta 1$ integrin interaction increased the cell survival and proliferation and modulated the steroidogenesis of ovine granulosa cells (25).

Type VI collagen, which is one of the main structural components of ECM, plays a role in determining the organization and architecture of ECM (26), whereas, laminin glycoprotein allows the attachment of epithelial cells in the tissue and provides stability to the basal lamina structure with the associated type IV collagen (20, 27). ECM is also important for granulosa cell proliferation during folliculogenesis. In *in vitro* studies, the presence of ECM supports granulosa cell growth and proliferation (4). The effect of ECM proteins on granulosa cell proliferation depends on the size of follicle, and this suggests that the ECM composition in each follicle changes during the folliculogenesis (4). We observed that the expression of ECM proteins, which we investigated in the theca and granulosa layers of follicles in different developmental stages, changed in our study.

In the literature, laminin was immunoreactive in primary, secondary and antral follicles in granulosa and theca cells and was stained darker in antral follicles. Type IV collagen showed a low-level expression in granulosa cells, whereas it was abundant in the theca cell layer. The collagen distribution was consistent throughout the follicle maturation (6). In another study, type IV

collagen expression was observed only in the theca layer of follicles and there was no expression observed in granulosa layer (16).

In our study, there was a significant increase in laminin, type VI collagen and $\alpha 3\beta 1$ integrin expressions in all follicle types in both theca and granulosa layers compared to the control group in the first month of diabetes (in group D1). There was an increase observed in the immunoreactivity of laminin and type IV collagen in D2 and D3 groups. This increase was significant in some follicular stages and layers, but insignificant in others.

It was noteworthy that integrin expression did not change in the newly formed follicles (primary and secondary follicles) in the D2 and D3 groups, however, it tended to change and increase in the developing follicles (tertiary follicles). Also, there was a significant increase in $\alpha 3\beta 1$ integrin immunoreactivity in all stages of diabetes in the theca layer of tertiary follicle.

Studies have reported differences in the number of ovarian follicles in the case of diabetes. Tatewaki et al (28) observed a reduction in the percentages of primary follicles in diabetic mice (28). In another study, the mean primordial, secondary and tertiary follicle counts decreased significantly in diabetic rats compared to the control group, whereas, atretic follicles increased significantly in diabetic rats (29).

Along with diabetes, the changes in the expression of laminin, type IV collagen and $\alpha 3\beta 1$ integrin, which are ECM proteins inside the follicle, show that diabetes plays a role in the regulation of follicular development. Furthermore, it is thought that cellular interactions with ECM proteins through $\alpha 3\beta 1$ integrin may be impaired with diabetes. In order to better understand the role of ECM in the effects of diabetes on the ovary, studies need to be conducted to elucidate the molecular mechanisms in folliculogenesis.

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