The evaluation of Laminin α5 and Collagen IV alterations in the mouse testis and epididymis following experimental hypothyroidism, Running title: Hypothyroidism on Laminin α5 and Collagen IV in the testis and epididymis

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Abstract: Background: the normal function of the thyroid is necessary for maintaining male fertility. It is well known that the thyroid malfunction has a negative impact on male reproductive system. There is some evidence indicating that extracellular matrix (ECM) components involve profoundly in the testis development and function. Adversely, hypothyroidism affects the basement membrane (BM) molecules of various tissues. In this regard, this study was designed to determine whether hypothyroidism alters BM proteins in the mouse testis and epididymis. **Materials and Methods**: 20 Balb/C male mice were randomly divided into (1) the control group, & (2) the hypothyroid group that were received 0.05% 6-n-propyl-2-thiouracil (PTU). 35 days later, after the confirmation of hypothyroidism, Real-Time PCR, Immunohistochemistry and PAS staining methods were carried out to evaluate the Collagen IV and Laminin α5 alterations in BM of seminiferous tubules in the testis and epididymis. **Results**: There was a significant increase in the Laminin α5 and Collagen IV mRNA expression in the hypothyroid group compared to the control group (p). Moreover, a remarkable increase was also observed in the immunoreactivity of BM of the hypothyroid group in comparison with the control group. However, no significant differences were observed between the two groups in terms of Periodic Acid-Schiff (PAS) staining. In addition, the mRNA levels of Laminin α5 and Collagen IV in the epididymis of the hypothyroid mice increased significantly compared to the control group (p). Nonetheless, no significant differences were observed between the groups in terms of immunohistochemistry and PAS staining. The data suggests that hypothyroidism may increase the thickness of seminiferous tubules BM which is possibly due to some abnormal increase in the amount of the Collagen IV and Laminin α5 proteins.
[Fatemeh Alipour, Mehdi Jalali, Mohammad Reza Nikravesh, Alireza Fazel, Mojtaba Sankian, Elnaz khordad. **The evaluation of Laminin α5 and Collagen IV alterations in the mouse testis and epididymis following experimental hypothyroidism, Running title: Hypothyroidism on Laminin α5 and Collagen IV in the testis and epididymis.** *Biomedicine and Nursing* 2018;4(1): 42-52]. ISSN 2379-8211 (print); ISSN 2379-8203 (online). http://www.nbmedicine.org. 8. doi:10.7537/marsbnj040118.08.

Keywords: Hypothyroidism, basement membrane, laminin α5, collagen IV, testis, epididymis.

Introduction

Hypothyroidism, is one the most common endocrine disorders which its incidence increases with age, associated with decreased thyroid hormones (Bensenor, 2012; Gesing, 2012). The thyroid hormones play a vital role in regulating the development, differentiation and metabolism of different tissues of mammals (Fadlalla, 2017; Gao, 2014; Ulisse, 1998; Wagner, 2008; Wajner, 2009). It was for a long time thought that the testis is an unresponsive tissue to the thyroid hormones; however, the studies of the past two decades identified the presence of the thyroid hormone receptors (TRs) in the testis as well as in the epithelial cells of the epididymis which exert their effects on the organs through these receptors (Dittrich, 2011; Gao, 2014; Krajewska-Kulak, 2013; Wagner, 2008). The thyroid hormones are important in the testis functions such as, the regulations of growth, the proliferation and differentiation of the sertoli cells, the germ cells, the leydig cells and the spermatogenesis (Gao, 2014; Krajewska-Kulak, 2013; Krassas, 2010; A. Kumar, 2014; Sarkar, 2017). On the other hand, it has been reported that the altered thyroid status, negatively affects the spermatogenesis, reducing the sexual activity and preventing fertility (Aiceles, 2017; Nikoobakht, 2012; Rajender, 2011; Wagner, 2008). Following neonatal hypothyroidism, a delay occurs in the differentiation as well as an increase in the number of the sertoli and leydig cells which consequently causes an increase in the number of the germ cells (Auharek, 2010; Bunick, 1994; Buzzard, 2000; Chang, 2007; Simorangkir, 1997; Wagner, 2008). It has also been demonstrated that both the morphology and the secretary activity of the epididymis are affected by the low thyroid hormone levels (Krajewska-Kulak, 2013; A. Kumar, 2014). As the sperm transit through the epididymis, undergo post-testicular maturation. In

hypothyroid rats, the altered sperm features may be in correlation with the modified structure and function of this organ (Alasmari, 2013; Jin, 2007). A few studies have shown that hypothyroidism causes a morphological and biochemical impairment in the epididymis such as chromatin decondensation, the epithelial cells, the depression of internal lumen, and the loss of stereocilia (Kala, 2002; P. N. Kumar, 1994). It has been reported that the sperm maturation, sperm number and sperm motility have also impaired in the epididymis of the hypothyroid rats (Krajewska-Kulak, 2013; Krassas, 2008; Krassas, 2010; A. Kumar, 2014). Since the epididymis is the site of the post-testicular sperm maturation, any change in the function of this organ may affect male fertility (Kala, 2002; P. N. Kumar, 1994). There is some evidence that ECM has a great impact on the growth, development, and differentiation of the tissues and organs in vivo and in vitro, as well as on promoting the attachment and polarization of many cell types (Auharek, 2010; Bunick, 1994; Rajender, 2011). Besides, it has been demonstrated that ECM molecules are also involved in the testis development, function and spermatogenesis (Buzzard, 2000; Enders, 1995; Hadley, 1985; Siu, 2004b, 2008; Yazama, 1997). The BM, as a part of ECM that underlies the seminiferous tubules is synthesized by a cooperative interaction between the sertoli and peritubular myoid cells so that Collagen IV and laminin are produced by the sertoli cells, and collagens I, IV and fibronectin are secreted by the myoid cells (Davis, 1990; Gesing, 2012; Gulkesen, 2002; Loveland, 1998; Ulisse, 1998; Yazama, 1997). BM surrounding of the seminiferous tubules has a foundation of laminin, collagen IV, entactin/nidogen, and heparan sulfate that are localized directly below the sertoli cells (Davis, 1990; Gulkesen, 2002; Hadley, 1987; Loveland, 1998). Laminin is one of the most important ECM components with a large and flexible structure composed of three non-identical polypeptide chains called α, β and γ. These chains are arranged in the shape of an asymmetric cross held together by disulfide bonds. Laminin regulates the cell adhesion and signaling via integrin binding, and it is also very active in promoting migration and differentiation. Laminin is thought to have an organizing function in BM assembly because of its early appearance in the embryo and many interactions (Davis, 1990; Rafighdost, 2010; Siu, 2004a, 2004b, 2008). Collagen IV, which is one of the major structural components of seminiferous tubules BM, is composed of three α chains forming a triple helical structure by the bandings of the monomers. Genetically, there are six different α chains. αl (IV) and α2 (IV) chains are ubiquitously present in BM, the α3-α6 (IV) chains generally have a more restricted distribution (Enders, 1995; Siu, 2004a, 2004b, 2008). Several reports have demonstrated that the disruption in the BM function can perturb the cross-talks between the ECM and the sertoli cells; moreover, the antibodies functioning against the components of the seminiferous tubule BM like laminin and collagen IV disturbed the spermatogenesis (Enders, 1995; Gulkesen, 2002; Siu, 2004a, 2008). It has, however, been shown that the postnatal hypothyroidism affects the maturation of the peritubular myoid and sertoli cells; which consequently causes a delay in the formation BM components (Loveland, 1998; Ulisse, 1998). Due to the importance of these components of BM in the function of the testis and epididymis in the one hand, and because of our little knowledge and understanding about the impact of hypothyroidism on the structure and function of the epididymis, in the other hand, this study was to investigate the changes of collagen IV and laminin in the testis and epididymis, following experimental hypothyroidism.

Materials and Methods

The Animals and the study design

20 Balb/C male mice, about 2 months old and weighing 20-25g was obtained from Experimental Animal House, of the Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. The animals were housed under the standard conditions (12 hours in light, 12 hours in darkness), a controlled temperature (22 to 24 ^{o} C), and free access to water and food. The study's experimental procedures were in accordance with the principals and guidelines of Animal Laboratory Care, of Mashhad University of Medical Sciences, Mashhad, Iran. After their acclimation to the laboratory condition, the animals were randomly divided into 2 groups (n=10): (1) The hypothyroid group were administrated with 0.05% 6-n-propyl-2-thiouracil (PTU) through the drinking water for 35 days, and (2) The control group without any intervention. At the end of the experiment period, the animals of the two groups were anesthetized with chloroform and the blood samples were taken from heart, centrifuged to obtain sera. The levels of T4, TSH in each serum were measured by using chemiluminescent immunoassay. The animals were scarified by the cervical dislocation and their right testis and epididymis were prepared for immunohistochemistry method and PAS staining. For real-time PCR study, the left testis and epididymis were preserved in RNA stabilization reagent (RNA later, Qiagen, Germany) and kept at –70°C until use.

RNA extraction and Real- time PCR

Total RNA was isolated from the testis and epididymis by using the RNA Extraction Kit (Parstous Corporation, Iran) according to the manufacturer's recommendations. To put it briefly, 20 mg of the tissue, cut into small pieces, then was lysised in 750 µl

of the RL solution. Thereafter, 150µl of the chloroform was added to the solution and centrifuged for 12 min at 4°C. Next, 400 µl of the upper phase containing RNA was transferred to another tube, and an equal volume of 70% ethanol was also added to it. The mixture was then transferred to the spin column and centrifuged for 1 min. In the next step, 700 µl of PW was also added to the mixture and centrifuged for 2 min. Finally, 50 µl of Diethylpyrocarbonate (DEPC) water was used and centrifuged for 1 min to wash RNA off the column. The purity of the extracted RNA was evaluated by nanodrop spectrophotometer (260/280nm ratios) as well as by the visual observation on electrophoresed agarose 1% gel. cDNA was synthesized by using a cDNA synthesis kit (Parstous Corporation, Iran) according to the manufacturer's instructions. Briefly, 0.5 µl of total RNA, 1 µl of oligo (dt) and 8.5 µl of DEPC water was incubated at 65° C for 5 min and immediately made cold on ice, next 10 µl of RT-premix was added to the mixture and it was again incubated at 25°C for 10 min followed by another 60 min at 50°C. For the last step, the enzyme was inactivated at 70°C for 10 minutes. The cDNA samples were kept at -70°C.

Real time PCR

The Real time PCR reactions were set up in duplicate for collagen IV, laminin and GAPDH as a reference gene by SYBER Green/ROX master mix (Parstous Corporation, Iran) kit. The reaction mixture was consisting of 10 µl of master mix, 1µl of each of the forward and the reverse primers (Table.1), 7.1µl distilled water, 0.4 µl of Rox dye and 0.5 µl of the cDNA templates (volume of 20 µl in each reaction tube). The conditions of the amplification cycles were as follows: an initial denaturation step (94° C for 10 min) followed by 35 cycles (denaturation at 95°C for 30sc; annealing at 60°C for 1 min, and extension at 72°C for 30 sc). The relative gene expression was calculated by using the comparative CT method (Aldaghi, 2014)

Immunohistochemistry method

To assess laminin α5 and collagen IV proteins changes in BM of the seminiferous tubules, the testis and epididymis sections were deparaffinized, and rehydrated through the descending grades of ethanol then washed in the phosphate buffer saline solution (PBS) (pH 7.4) 3 times for 15 min. Heat-induced antigen retrieval was done by PBS / Ethylene diamine tetra acetic acid (EDTA) solution buffer at 100°C for 30 min, which was then rinsed in PBS for 10 min. To inhibit endogenous peroxidase activity, the sections were immersed in 0.3 % H2O2 and methanol for 20 min. After being washed in PBS, the nonspecific binding antibodies were blocked in 10% goat serum and bovine serum albumin (BSA) 1% in PBS for 30 min. Thereafter, the sections were incubated overnight at 4°C by the laminin α5 primary antibody diluted 1:100 in PBS and the collagen IV primary antibody (1:200 in PBS) on the separate sample slides. On the second day, the sections were at first washed by PBS for 10 min; subsequently, the goat polyclonal secondary antibody diluted 1:400 was applied for 2 hours at 37°C. To visualize the reactions, the slides were then treated with 0.03% solution of 3-diamianobenzidine containing 0.01 % H2O2 (DAB) for 15 min. After being washed in running as well as distilled water, the sections were counter-stained by hematoxylin. At the end, the sections were again washed, dehydrated, cleared and mounted. After taking some photomicrographs, the distribution of laminin α5 and collagen IV which were presented in brown was considered as the positive staining. The intensity of the staining was assessed semi quantitatively by the scales as follows: +: weak reaction, ++: moderate reaction, +++: strong reaction, ++++: very strong reaction (Aldaghi, 2014).

Periodic Acid-Schiff (PAS) staining

The slide samples of the testis and epididymis tissues were deparaffinized, rehydrated and washed by distilled water for 3 min and the slides were then placed into periodic acid 1% for 10 min. After being rinsed in distilled water, the slides were stained with Schiff' s reagent for 5 min and they were again rinsed, in turn, in running tap water for 5 min and distilled water for 3 min. In the next step the slides were counterstained in Harris Hematoxylin for 2 min. After being washed in running tap water for 3 min, the slides were again rinsed in distilled water. Then slides were dehydrated in alcohol, cleared with xylene, and cover-slipped as well. After imaging with Olympus BX51 light microscope, the reactions were scored semi-quantitatively as follows: weak +, moderate ++, strong +++ and highly strong ++++ by two persons (Pujar, 2015).

Statistical analysis

All data was done by using SPSS software (v.16). The results of immunohistochemistry and PAS staining were analyzed by Mann-Whitney non-parametric test and the results of real-time PCR by the utilization of T independent test. Meanwhile, P-value<0.05 was considered as significant.

Results

Expression of laminin α5 and collagen IV in mice testis

In this study, quantitative real-time PCR was performed to investigate the expression of laminin α5 and collagen IV mRNAs. Our findings indicated that the levels of laminin α5 mRNA in the hypothyroid group increased significantly compared to the control group (p<0.001) (Figure1). Meanwhile, the mRNA levels of collagen IV in the hypothyroid mice

increased significantly compared to the control group ($p<0.001$) (Figure 2). However, the real-time PCR results also support the immunohistochemistry findings.

Immunoreactivity and PAS staining of basement membrane in mice testis

Immunohistochemistry was applied to determine the distribution of laminin α5 and collagen IV proteins in BM of seminiferous tubules. The intensity of the reaction was scored according to color opacity. The findings indicated that the laminin α5 reaction increased significantly in the BM of the hypothyroid mice compared to that of the control group ($p<0.001$) (Figure 3, 4). Furthermore, a significant increase was observed in the immunoreactivity of collagen IV of the seminiferous tubules BM in the hypothyroid group in comparison with the control group ($p<0.001$) (Figure 5, 6). However, there was no remarkable difference between the two groups in terms of the intensity of PAS staining (Figure7).

Expression of laminin α5 and collagen IV in the epididymis of mice

The mRNAs levels of laminin α5 had a remarkable increase **i**n the epididymis of the hypothyroid group in comparison with the control group ($p<0.05$) (Figure 8). Besides, the expression of collagen IV in the hypothyroid group increased significantly compared to the control group (p $p<0.05$) (Figure 9).

Immunoreactivity and PAS staining of basement membrane in mice epididymis

No changes in the intensity of immunostaining of the laminin α5 and collagen IV were observed between the hypothyroid and the control groups. Besides, no significant change in the intensity of PAS staining was seen between two groups (Figure10, 11, 12).

Discussion

The present study was to examine the correlation between hypothyroidism and collagen IV and laminin α5 changes in BM of the seminiferous tubules and epididymis. The thyroid hormones have been reported to regulate the expression of ECM components (Ulisse, 1998). BM contains the growth factors that influence the cellular behavior, the cellular activity and the epithelial cells regeneration (Yazama, 1997). BM plays an important role in regulating the seminiferous tubules formation; it particularly influences the sertoli cell proliferation and function, as well as spermatogenesis (Davis, 1990; Gulkesen, 2002; Hadley, 1985; Raychoudhury, 2011). Loveland and et al. demonstrated that BM components were uniformly distributed in the postnatal seminiferous tubule BM. They also mentioned that there was a direct correlation between the developmental changes in the BM composition and the delay in the development of germ cell and sertoli cell maturation in the testis of the hypothyroid rats (Loveland, 1998). Moreover, in this study, the thyroid hormones deficiency was found to be associated with the alteration in the regulation of laminin α5 and collagen IV expression so that hypothyroidism can lead to large changes in the laminin α5 and collagen IV expression. Nikravesh, Jalali and et al. showed that hypothyroidism, during pregnancy, caused a significant increase in the laminin expression of different parts of rat newborns' skin (Amerion, 2013). In consistent with the results of the above-mentioned study, our study indicated that the laminin α5 expression increased significantly in the hypothyroid group compared to the control group. Furthermore, we found a significant increase in the expression of collagen IV in the hypothyroid group compared to the control group. In several cases of the impaired spermatogenesis, the lamina propria of seminiferous tubule had become thick by an increase in the ECM components along with the differentiation of the myoid cells to fibroblastic nature (Buzzard, 2000; Gulkesen, 2002). Hypothyroidism has been shown to cause the fibrosis and hyalinization of the seminiferous tubules walls, the proliferation of fibroblasts, edema in the interstitium, as well as the thickening of seminiferous tubules BM that prevents the normal development of the germ cells and subsequently the male fertility (Wajner, 2009). In agreement with the findings of the several past researches, hypothyroidism, in the present study, caused a remarkable increase in the immunostaining and up-regulation of laminin α5 and collagen IV in the seminiferous tubules BM. The increase in the amounts of these proteins may be associated with the thickening of BM and may impair spermatogenesis; however, the results of PAS staining illustrated that there was no significant difference between the hypothyroid and the control groups. Meanwhile, according to the results we obtained from the epididymis, a significant increase was observed in the expression of collagen IV and laminin in the hypothyroid group compared to the control group. However, there was found no significant difference between the two groups in the immunostaining and PAS staining results. This can suggest that the level of RNA transcription does not necessarily correlate with the protein levels. A few studies have shown that hypothyroidism negatively changes the structure and function of the epididymis of adult rats (Kala, 2002; P. N. Kumar, 1994). The results of this study are consistent with several other studies as well as our previous study (in press) showing hypothyroidism alters the epididymal sperm parameters (A. Kumar, 2014; La Vignera, 2017; Singh, 2011; Wajner, 2009)

which could be due to the negative changes in the structure and function of the epididymal BM and epithelium, however more precise studies are needed. In addition to the above findings, the data analysis suggested that an increase occurred in the thickness of BM in the hypothyroid state, which may be partially due to an abnormal increase in the amount of collagen IV and laminin α5 proteins. Consequently, hypothyroidism may increase the synthesis of these components of BM or reduce the turnover of these proteins. Since the ECM of most tissues is not well protected against oxidative stress, it increases the susceptibility of tissues to the proteolytic cleavage, the ECM permeability and the altered function of the organ (Sahoo, 2008; Singh R, 2011). It has been found that the testis has limited enzymatic and nonenzymatic antioxidant defense systems and the thyroid hormones are important in preserving the balance between reactive oxygen species (ROS) and the antioxidant molecules in the testis (Chakraborty, 2016; Choudhury, 2003; Sahoo, 2012). Therefore, hypothyroidism can alter the antioxidant defense parameters in the testis and epididymis. Probably, changes in the levels of laminin α5 and collagen IV proteins might, in Hypothyroidism, be associated with the increased ROS and the impaired antioxidant defense systems.

Conclusions

In summary, the present study indicated that hHypothyroidism up-regulates the expression of laminin α5 and collagen IV of the testis and epididymis. Accordingly, the thyroid hormones may regulate the synthesis of these key molecules in BM.

Figure 1. Comparison between the lamininα5 mRNA level expressions in the hypothyroid group compared to the control ones. Values are expressed as mean±SD. * Significant differences compared with control group (*p<0.05).

Figure 2. Comparison between the collagen IV mRNA level expressions in the hypothyroid group compared to the control ones. Values are expressed as mean±SD. *Significant differences compared with control group (*p<0.05).

Figure 3. Photomicrographs show immunoreactivity of laminin α5 protein in BM of seminiferous tubules in the control group (A) and hypothyroid group (B). Positive immunoreaction was shown in different brown color (Arrows). Scale bar = 50 µm.

Figure 4. Graph shows the effect of hypothyroidism on immunoreaction of laminin α5 protein in BM of seminiferous tubules. Significant differences compared with control group (*p<0.05).

Figure 5. Photomicrographs show immunoreactivity of collagen IV protein in BM of seminiferous tubules in the control group (A) and hypothyroid group (B). Positive immunoreaction was shown in different brown color (Arrows). Scale bar = 50 µm.

Figure 6. Graph shows the effect of hypothyroidism on immunoreaction of collagen IV protein in BM of seminiferous tubules. Significant differences compared with control group (*p<0.05).

Figure 7. Photomicrographs show the BM of seminiferous tubules with PAS staining (Arrow) in Control (**A**) hypothyroid (**B**) groups, Scale bar=100 µm.

Figure 8. Comparison between the lamininα5 mRNA level expressions in the hypothyroid group compared to the control ones. Values are expressed as mean±SD. * Significant differences compared with control group (*p<0.05).

Figure 9. Comparison between the collagen IV mRNA level expressions in the hypothyroid group compared to the control group. Values are expressed as mean±SD. * Significant differences compared with control group (*p<0.05).

Figure 10. Photomicrographs show immunoreactivity of laminin α5 protein in BM of epididymis (Arrow). control (**A**) hypothyroid (**B**) groups, Scale bar = 100 µm.

Figure 11. Photomicrographs show immunoreactivity of collagen IV protein in BM of epididymis (Arrow). control (**A**) hypothyroid (**B**) groups, Scale bar = 100 µm.

Figure 12. Photomicrographs show the BM of epididymis with PAS staining (Arrow) in control (**A**) hypothyroid (**B**) groups, Scale bar = 100 µm.

Table 1. Sequences of primers used for real-time PCR

Gene primer sequences product size (bp) Annealing temperature (°C)
Collagen IV 5'-AAGCTGTAAGCATTCGCGTAGTA-3'(R) 5'- ATTCCTTTGTGATGCACACCAG-3'(F) 107 58
Laminin α 5 5'-TACCAACGAAGGGCTGCG- 3'(R) 5'-CGTCCCACAGGAATAGGCT- 3'(F) 109 58
[a]GAPDH 5'- CTGTAGCCATATTCATTGTCATACCA-3'(R) 385 58 5'-AACTCCCATTCTTCCACCTTTG-3' (F)

[a]GAPDH. Glyceraldehyde 3-phosphate dehydrogenase.

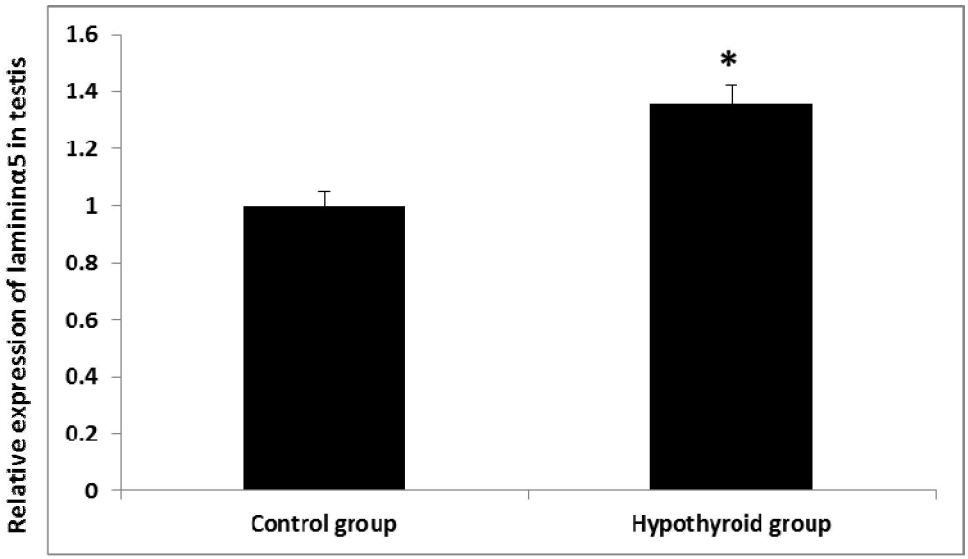


Figure 1.

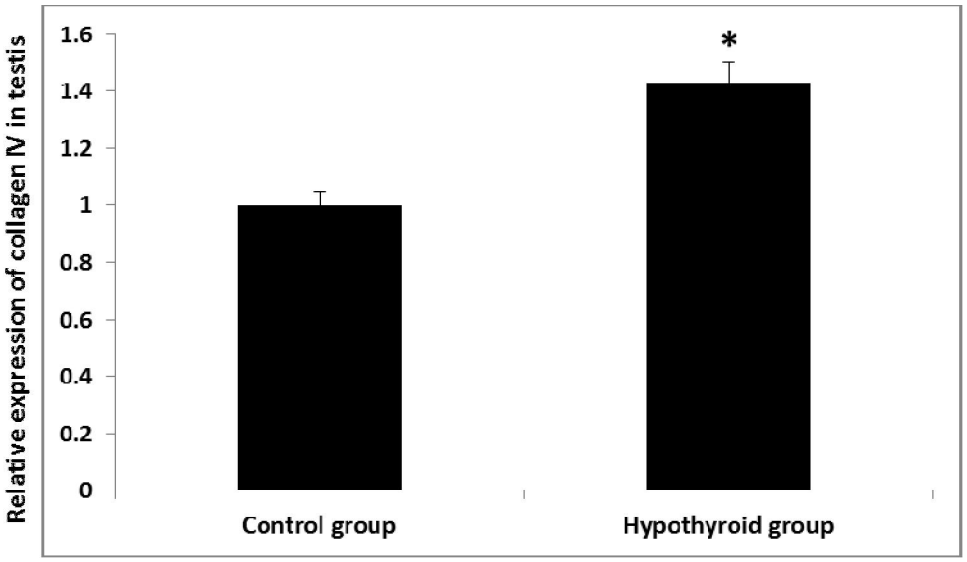


Figure 2.

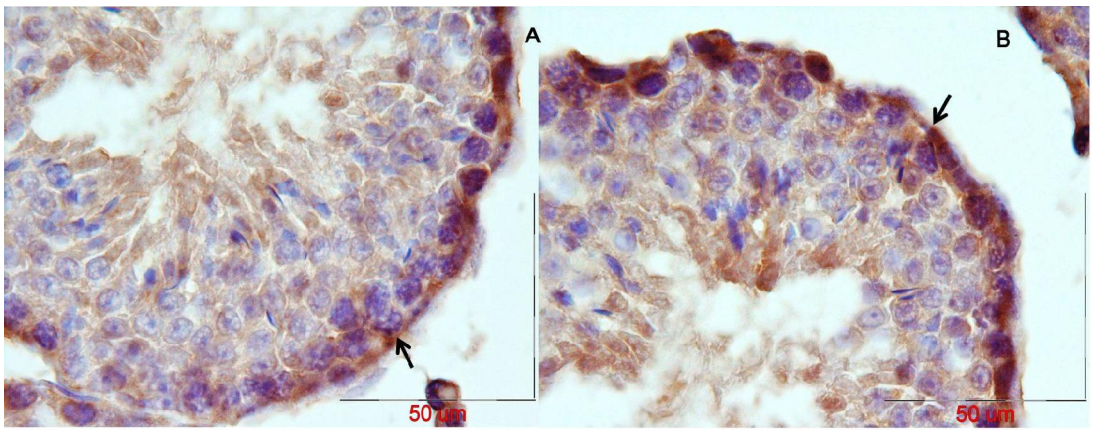
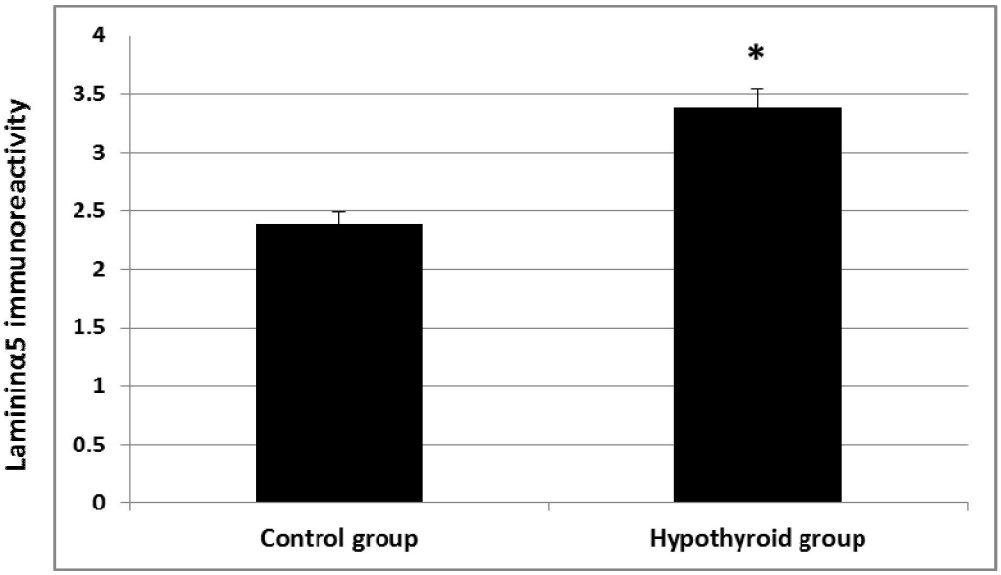


Figure 3.



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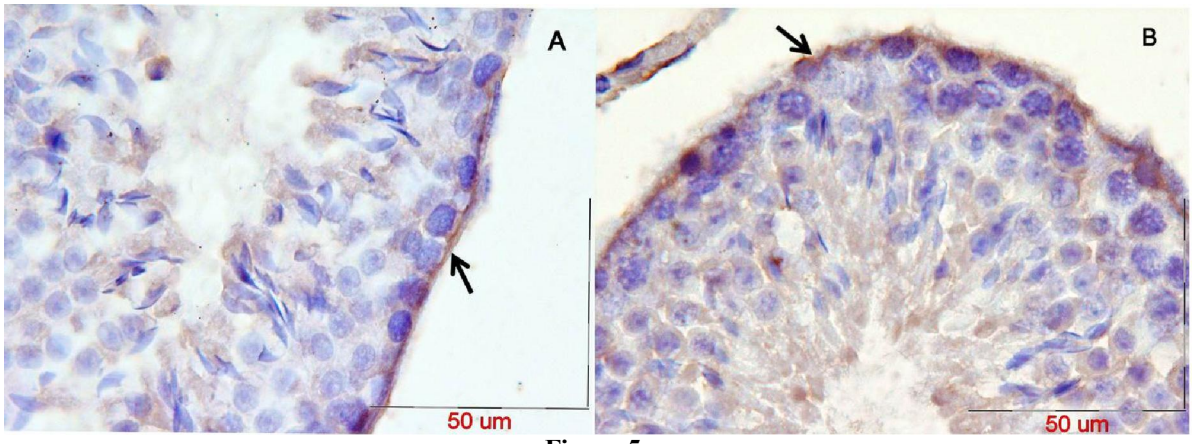
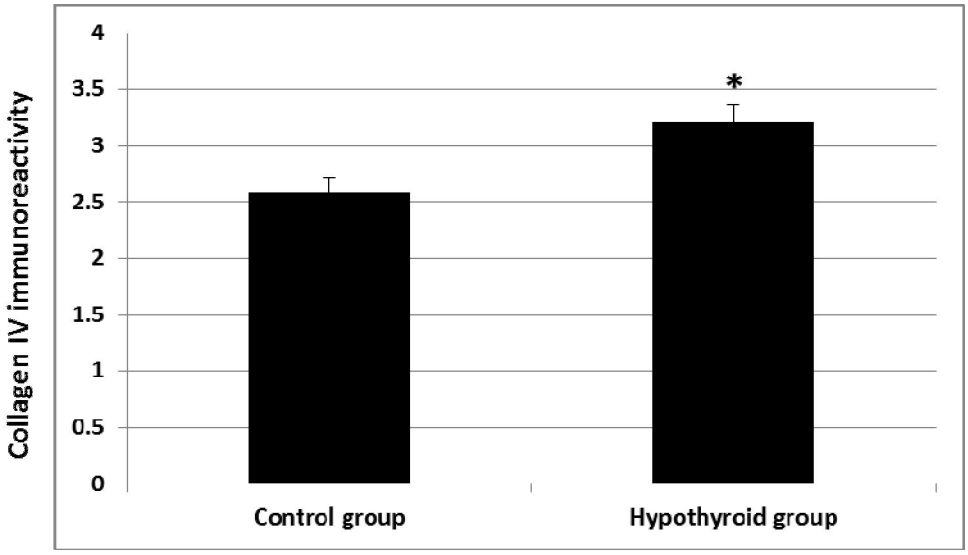


Figure 5.



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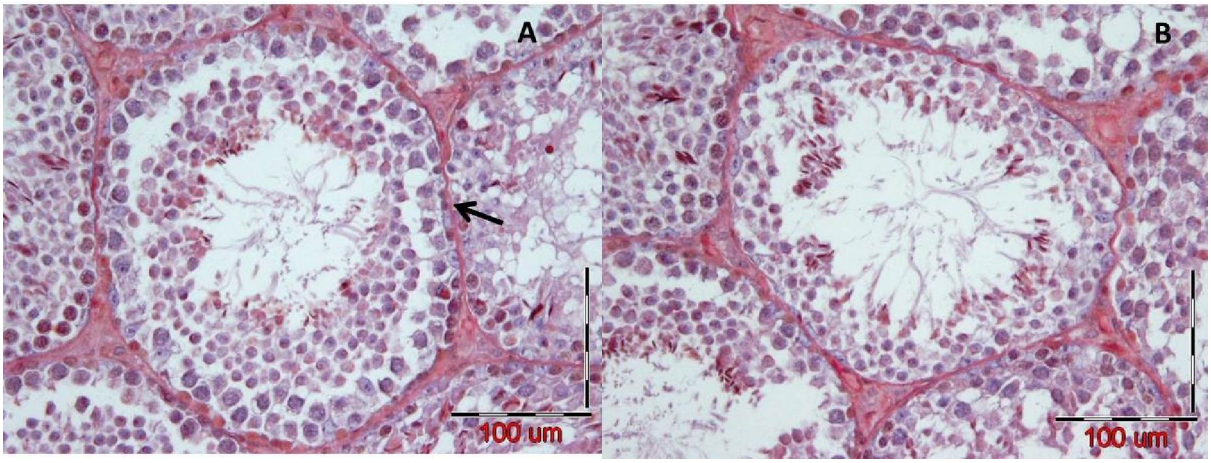

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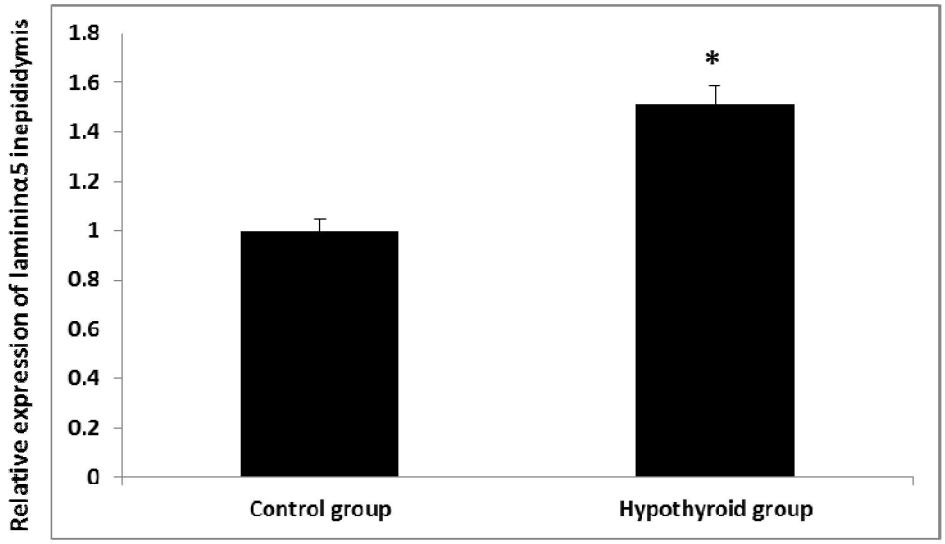
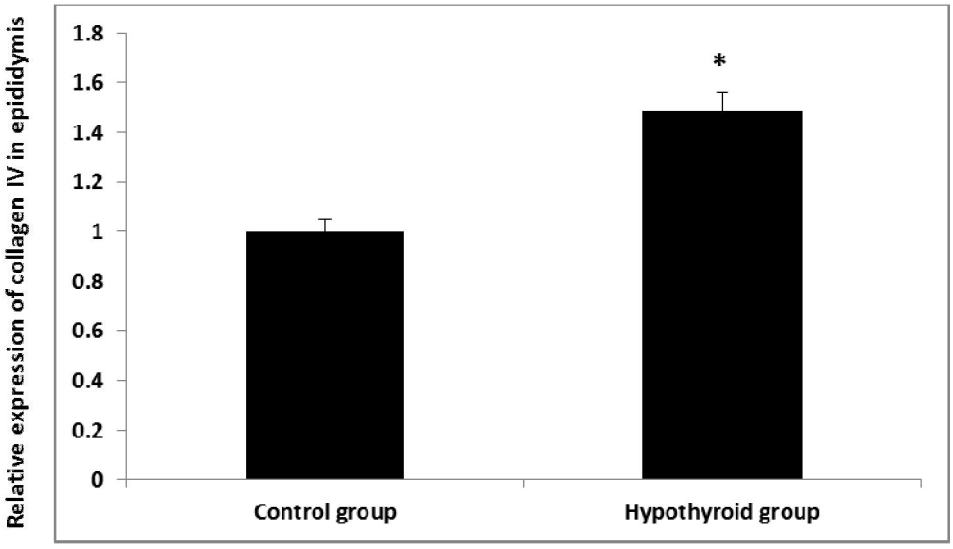


Figure 8.



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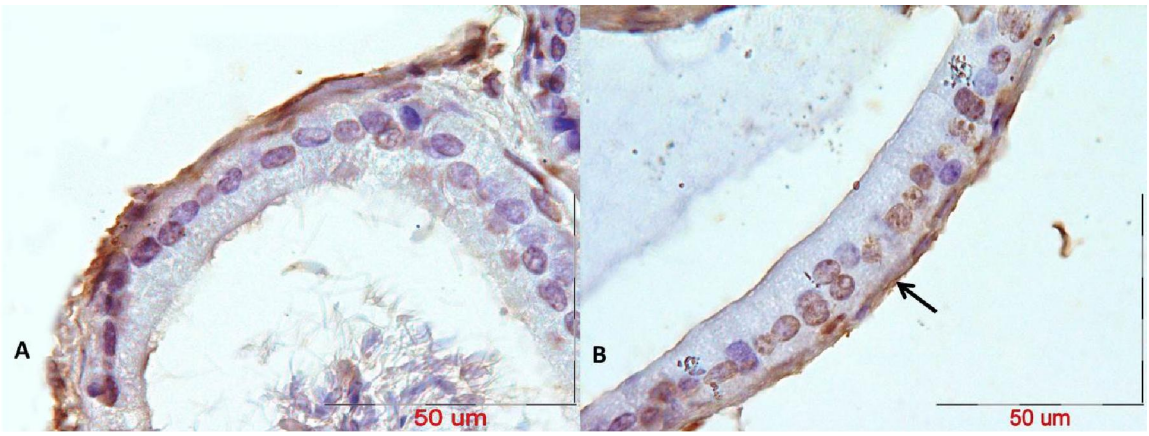

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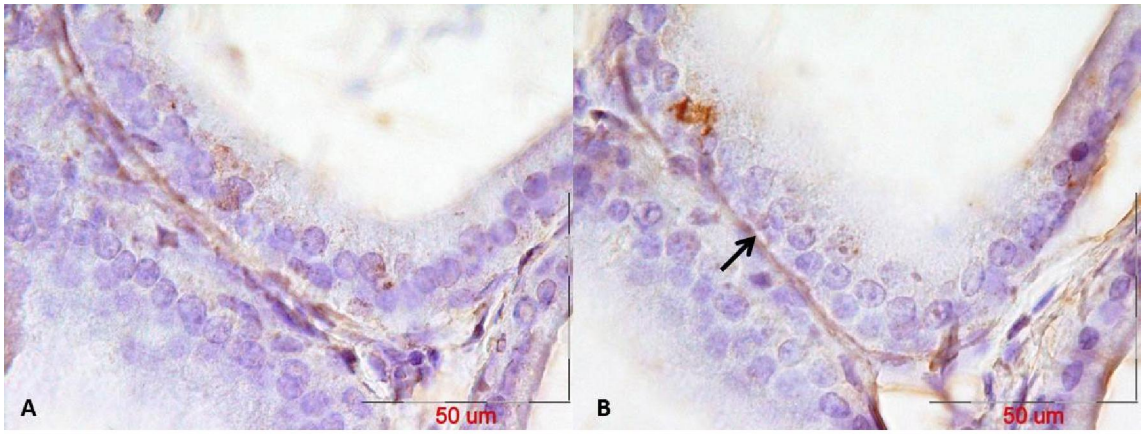

Figure 11.

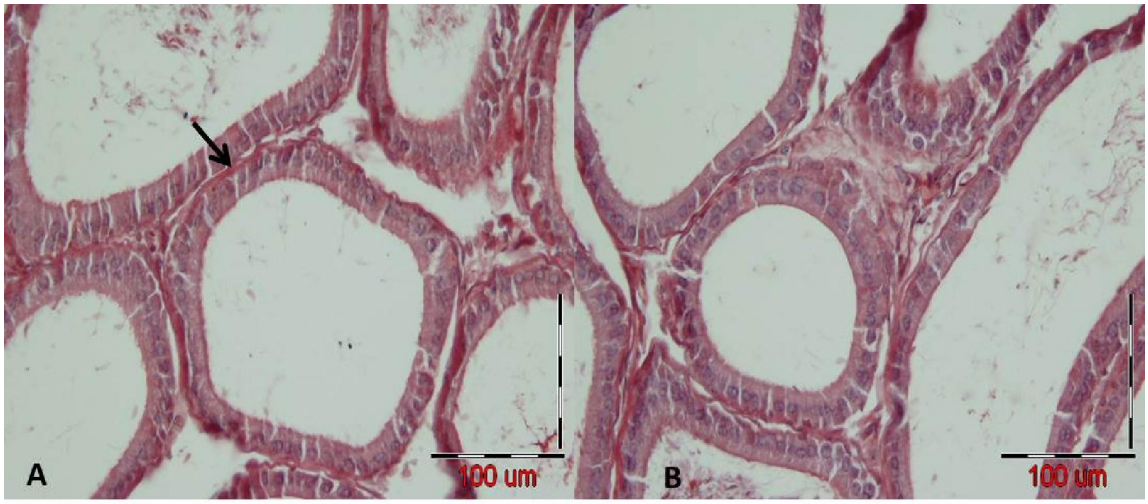

Figure 12.

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Acknowledgements
The data used in this paper is from a Ph.D. student thesis (NO: 931752). This work supported by

the funding from Vice-Chancellor for researches of Mashhad University of Medical Sciences, Mashhad, Iran. The authors would like to thank Mrs. F. Motejadded and Mrs. F. Tajik for their technical assistances.

Conflict of interest

The authors declare that there is no conflict of interests associated with the publication of this paper.

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