THE EFFECTS OF MATERNAL HYPOTHYROIDISM ON THE IMMUNOREACTIVITY OF CYTOCHROME P450 AROMATASE IN THE POSTNATAL RAT TESTICLES

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Abstract

Introduction. Abnormal thyroid function affect spermatogenesis and male infertility. For men, the aromatase deficiency can cause infertility. In this study, the aim is to investigate the effect of maternal hypothyroidism on offspring testicular morphology and cytochrome-P450-aromatase (P450arom) immunoreactivity.

Materials and Methods. Eighteen Wistar albino pregnant rats were divided into three groups, namely A, B and K groups. Hypothyroidism was induced by adding 0.01% of propyl thiouracil (PTU) in drinking water. Hypothyroid mothers, group A: given PTU for 21 days during pregnancy, group B: given PTU for 21 days prior to pregnancy; control mothers, group K, given only water. Hypothyroid and control group mothers' pups at postnatal day (PND) 15 and 60 were sacrificed. We determined immunoreactivity intensity of P450arom and mRNA levels by RT-PCR performed in the testis tissues. ELISA method was used for thyroid function tests for T3, T4 and TSH. Structure of seminiferous tubule was evaluated by hematoxylin-eosin staining.

Results. It was seen that the aromatase expression in 15-day-old maternal hypothyroid groups was similar to the one in the control group while there was a decline in the aromatase expression of 60-day-old groups. As for mRNA, it was determined that it had a tendency to increase over time in all groups but this increase was not significant. The tubule diameter and Johnsen's Testicular Biopsy Score diminished in all hypothyroid groups in comparison to the control group.

Conclusion. The changes that occur in the early period of testis development due to maternal hypothyroidism negatively affect testis development in the next stages of life. This situation leads to a decline in aromatase expression in the following years.

Key words: testicles, aromatase, hypothyroidism, PCR.

INTRODUCTION

Gonadotropins (FSH, LH) and testosterone are responsible for sustaining spermatogenesis and

normal testis development. Estrogens considerably affects the regulation of male gonadal functions. It plays an important role in male pubertal development, spermatogenesis and the regulation of gonadal functions (1). The irreversible transformation of androgens in male gonads into estrogen occurs with the help of aromatase enzyme. Aromatase enzyme complex is a member of cytochrome P450 superfamily. P450arom is located on the long arm of human chromosome 15 (15q21.2). It is the product of a single gene called CYP19 (2-5). In humans, aromatase is expressed in a number of cells including ovary, testis, placenta, adipose tissue, skin and the brain (6). Its transcription is regulated by cytokines, cyclic nucleotides, gonadotropins, glucocorticoid and growth factors (7).

There are contradictory findings about the time of aromatase expression in the fetal period. However, in mature rats, P450arom is immunolocalized in pachytene stage spermatocytes, round and elongated spermatids and testicular spermatozoa as well as in Leydig cells (3, 8).

While it was thought that thyroid hormones did not play a role in male reproductive system, numerous experimental and clinical studies carried out in the past years have shown that thyroid hormone plays a critical role in testis growth and functions (9-11). The studies carried out using genetic models and/or goitrogen (e.g. propylthiouracil) have shown that there is a close, physiological relationship especially between Sertoli cell differentiation, mitotic activity, gap junction function, blood-testis barrier and thyroid hormone and testis functions (12). Thyroid hormones regulate the Sertoli cell proliferation and differentiation, formation of blood-testis barrier especially during testis development (13-15). They stimulate the differentiation of Leydig cells and steroidogenesis in rat testes. Recent studies have shown that the changes in thyroid hormone among adult males are related to

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abnormal spermatogenesis, decreased sexual activity and impairment of fertility (12). Moreover, the changes in thyroid hormone levels during early period of testes development affect testicular maturity and reproduction (9).

It is known that hypothyroidism has negative effects on testes development and maturity; however, there is not sufficient information about the interaction between thyroid hormones and aromatase. We think that hypothyroidism may have an effect on aromatase expression and thus can affect testes development and spermatogenesis. For this reason, the aim of the present study is to study the effect that maternal hypothyroidism has on cytochrome P-450 aromatase expression in the testes on rat pups.

MATERIALS AND METHODS

Animals

Eight to ten weeks pregnant female Wistar rats, which were obtained from the Hakan Cetinsaya Experimental and Clinical Research Center at Erciyes University, Kayseri, Turkey, were used for this study. They were housed in plastic cages in a well-ventilated rat house and allowed *ad libitum* access to food and water and kept at a 12-h light: 12-h dark cycle. All the animals received care according to the standard guidelines. Ethical approval for the study was obtained from the Erciyes University Animal Research Ethics Committee.

Experimental Protocol

Pregnant rats were divided into 3 groups being K, A and B. Group K became the control group and nothing was added into their drinking water. Group A had hypothyroidism during pregnancy (hypothyroidism was induced by adding PTU into their water for 21 days as of the first day of pregnancy). As for Group B, it was hypothyroidism before pregnancy (hypothyroidism was induced by adding PTU into their water for 21 days before the pregnancy). Hypothyroidism was induced in mother rats by adding 6-propyl-2-thiouracil (PTU, Sigma, P3755-25G) with a final concentration of 0.01% in their drinking water.

The vaginal smears of the rats to be used in the study were checked daily for 2 weeks. Female rats were taken into cages separately at proestrus stage. They were kept in the same cage with a male rat for one night and were separated in the morning. The day when vaginal smears of the female rats were taken was regarded as the day 0.5 thinking that the rats

with sperms in their smear could be pregnant and the experiment was launched.

After the mother rats gave birth, the sexes of the newborn pups in each group were specified and only male pups were used in the experiment. Male pups of each group were divided into 2 categories being 15-day-olds and 60-day-olds. The pups from the control and experimental groups were divided into 6 groups - 7 pups in each - as follows:

Group K15: 15-day-old male pups in group K;
Group K60: 60-day-old male pups in group K;
Group A15: 15-day-old male pups in group A;
Group A60: 60-day-old male pups in group A;
Group B15: 15-day-old male pups in group B;
Group B60: 60-day-old male pups in group B.

On 15th or 60th day of the experiment, the rats were anesthetized by giving intraperitoneally a mixture of 1 mg/100 g of Ksilazin (Rompun, Bayer) and 8 mg/100 g of ketamin. The anesthetized rats were laid back and their testicles were taken out with an incision on scrotal region. The rats whose testicles were taken out were sacrificed by cervical dislocation.

Histological Analysis

For light microscopic study, the testicular tissue samples that were taken were put into a formaldehyde solution of 10% concentration and were fixed for 48 hours. Afterwards, the tissue samples were dehydrated in series of alcohol and tissues were made transparent in xylol and embedded in paraffin. Five-micron thick sections were obtained from each paraffin-embedded testicular tissue. Sections were collected for histological staining (hematoxylin and eosin).

Histopathologic Assessment

Measurement of Mean Seminiferous Tubule Diameter (MSTD)

In order to evaluate the damage in testicles, mean seminiferous tubule diameter measurements were used. On the sections with hematoxylin and eosin stain, the seminiferous tubule diameters were measured using the Analysis LS Research programme in Olympus BX51 microscope and the mean seminiferous tubule diameters were calculated.

Johnsen's Testicular Biopsy Score (JTBS)

Johnsen's Testicular Biopsy Score was used in the evaluation of tubular damage (16). Olympus BX51 light microscope was used for the evaluation. On 25 seminiferous tubules in each testis section, the process of spermatogenesis was evaluated semi-

quantitatively. Each tubule was scored ranging from 1 to 10 according to the scoring criteria (16). In this system, 1 is the lowest score given when there is no cell in tubular section while 10 is the score when there is an entire process of spermatogenesis with excessive number of spermatozoa (Scoring was only made on 60-day testicular tissue sections, not on 15-day testicular tissues as the scoring system was not suitable for immature testicular tissues).

Immunohistochemistry

In order to determine the aromatase immunoreactivity in testicular tissue, immunohistochemical techniques were used through Avidin-Biotin-Peroxidase method. The section of 5 μ m thickness taken from paraffin blocks on poly-Lysine coated slides were oven-dried overnight at 60°C and then rehydrated by passing them through xylene and decreasing strengths of alcohol (100%, 96%, 80%, 70%). Later, the sections were washed in distilled water 3 times for 5 minutes. For antigen retrieval, they were put in a microwave oven by setting the power to 600W, with citrate buffer of 5% for 5 minutes. The sections that were rinsed with PBS were treated for 12 minutes with hydrogen peroxide of 3% in order to block endogenous peroxidase activity. In the following stages, Large Volume Detection System (Thermo Scientific, TP-125-HL) immunohistochemistry staining kit was used. In order to reduce background staining, the sections were treated with Ultra V block for 5 minutes followed by overnight primary antibody incubation (CYP19, sc-30086, SantaCruz Biotechnology) at 4°C in a humid environment. In the tissues that were prepared for negative control, PBS was used instead of primary antibody; the operations in the following stages were carried out in the same way. As a positive control, human placenta was used. After primary antibody incubation, the sections were rinsed. Reverse staining was performed using Gill's Hematoxylin after biotinylated secondary antibody, Streptavidin-HRP and DAB (3,3'-Diaminobenzidine) chromogen. Lastly, the sections that were dehydrated by immersion in graded series of alcohol (70%, 80%, 96%, 100%) were passed through xylene and covered with Entellan®, Merck, a rapid mounting medium and observed under Olympus BX51 microscope.

Based on the images taken from the slides, P450arom expression density measurements were performed using Image-J programme for immunoreactivity difference. The data obtained was evaluated statistically. As the seminiferous tubules

in 15-day-old groups were not stained for statistical analyses, only interstitial area was measured while both interstitial area and seminiferous tubular immunoreactivity were measured separately in 60-day-groups.

Quantitative Real-Time PCR (RT-PCR)

RNA isolation from the testis tissues was performed by using Trizol reagent according to the manufacturer's instructions. RNA quality and concentration were analyzed with NanoDrop (BioSpec-nano, Duisburg, Germany). Isolated RNA was kept frozen at -80°C.

One μ g RNA was used for cDNA synthesis using Transcriptor High Fidelity cDNA Synthesis Kit (REF:05091284001, Roche, Germany). Briefly, RNA, OligodT, random hexamers, and H₂O were mixed and incubated at 65°C for 10 min. Then, 2 $\times$ buffer, RNase inhibitor, DTT and RT enzyme were added on ice. The mix was incubated at 29°C for 10 min, 48°C for 60 min, and 85°C for 5 min. cDNAs were kept frozen at -20°C until gene expression analysis.

The cDNA was subjected to quantitative RT-PCR reaction using LightCycler® 480 Probes Master (Roche, REF: 04707494001) and detected with Universal ProbeLibrary Probes (Roche, Applied Sciences, Mannheim, Germany) for cyp19 (REF:05532957001) Roche, Germany) and the housekeeping gene, beta actin (REF:05532957001, Roche, Germany)

The protocol for real-time PCR was as the following: one cycle of preincubation for 10 min at 95°C, 45 cycles of amplification for 15 s at 95°C, for 1 min at 60°C, and for 30 s at 72°C. The expression of genes was normalized to the housekeeping β -actin gene. At the end of the experiment, the results were analyzed using $2^{-\Delta\Delta CT}$ method. Each experiment was performed twice.

ELISA

In order to find out T3, T4 and TSH levels, blood samples of 1.5 ml and 4 ml were taken from decapitated 15-day-old rats or 60-day-old rats respectively, into gel vacuum blood collection tubes. After being kept for half an hour, the blood samples were centrifuged and serum was extracted. The extracted serum was filled into Ependorf tubes and kept at -80°C until the analyses. The T3 (Sunred, Katalog no: 201-11-0256), T4 (Sunred, Katalog no: 201-11-0257) and TSH (YH Biosearch, Katalog no: YHB1050Ra) measurements made on the serums obtained at the end of the experiment were spectrophotometrically carried out using commercial

ELISA kits (Enzyme-Linked Immuno Sorbent Assay) according to the kit protocol. The normal range of values varies according to the applied Elisa test type. For this reason no reference interval is specified. The experimental groups were compared with the control groups.

Statistical Analyses

The data obtained in the present study was evaluated using SPSS 20.0 package program. Kolmogorov-Smirnov test was used to determine whether the obtained data was normally distributed. As the data indicated a normal distribution in seminiferous tubule diameter and immunoreactivity assessments, Anova test was administered while Tukey HSD test was used in order to find out significant differences between groups after Anova test. JTBS was used for statistical comparisons between groups; Kruskal Wallis H test from non-parametrical tests was administered since the data did not show a normal distribution and afterwards, Mann Whitney U test was used for paired comparisons between groups. As for RT-PCR analysis, Student's t-test was administered. The obtained $p < 0.05$ value was considered statistically significant.

RESULTS

Histological changes

In 15-day-old groups, it was observed that seminiferous tubules were composed of spermatogonium, Sertoli cells and primary spermatocytes. Secondary spermatocytes and spermatids were not observed. Unlike the control group, it was seen that there were dense, homogeneous nuclei that were about to become pycnotic beside degenerated cells in seminiferous tubules of the experimental group (Fig. 1).

In the cross section of testicular tissues belonging to 60-day-old control group rats (K60), it

was seen that the histological structures of seminiferous tubules and Leydig cells were normal (Fig. 2 A, B). In 60-day-old Group A and B rats (A60, B60), it was observed that spermatogenesis and germ cell arrangement in the seminiferous tubules were disrupted; there were cell loss and atrophy in some tubules (Fig. 2). We observed abnormalities in the chromosome structure of primary spermatocytes, spermatid loss, disorder in Sertoli-cell structure, undulations on the surface of the seminiferous tubule and multinucleated giant cells (Fig. 2 C,D). In group B60, cells poured into the lumen and few numbers of caudate spermatozoa were observed in the lumen while there were also normal-looking tubules (Fig. 2E). It was striking that there was a gap between spermatogonium cells besides condensation and shrinkage in their nuclei. It was observed that spermatids had an irregular shape and were very few (Fig. 2F). Multinucleated giant cells were not encountered in group B60.

Seminiferous tubule diameters of 15-day-old groups are shown in Table 2. The tubule diameters of groups A15 and B15 were similar but significantly lower in comparison to group K15 (Table 1). Seminiferous tubule diameters of 60-day-old groups are given in Table 4.1. Tubule diameters of rats in groups A60 and B60 were similar but significantly lower in comparison to group K60 (Table 1). According to JTBS analysis results, the scores belonging to the 60-day-old experimental groups were statistically significantly high in comparison to those of control groups while there is a similarity among the scores of the experimental groups (Table 1).

P450arom Immunoreactivity in Testes

In the sections belonging to the testicular tissues taken from the experimental subjects, P450 aromatase immunoreactivity was determined using the avidin-biotin-peroxidase method. In order to determine

Figure 1. K: Group K15, Sertoli cells (→), spermatogonia (↔) and primer spermatocytes (×) and Leydig cells (⇨); A: Group A15, Sertoli cells (→), spermatogonia (↔) and a degenerated cell (←) and Leydig cells (⇨); B: Group B15, Sertoli cells (→) spermatogonia (↔), primary spermatocyte (▶), Leydig cells (⇨) and degenerated cells (×), (X100, Bar 20.0 µm), (H+E).

the immunoreactivity difference among the groups, Image-J-Software programme was used and interstitial area and seminiferous tubule P450arom expression

Figure 2. A, B: Group K60, A: General testicular structure, B: Spermatogonia (↔), spermatocytes (▶), spermatids (→), spermatozoa in the lumen (Sz). C, D: Group A60, C: Seminiferous tubules with defects in the alignment of germ cells (*), An atrophic tubule (AT), D: Multinucleate cytotogenesis (→) and degeneration in spermatocytes (×). E, F: Group C60, E: Near normal seminiferous tubules (*), F: Gap between spermatogonial cells (→), germ cell loss, lumen (L). (A, C, E X20, Bar 100 µm, B, D, F X100, Bar 20.0 µm), (H+E).

levels were measured. With regard to P450arom immunoreactivity, 15-day-old and 60-day-old groups were separately evaluated among themselves.

In 15-day-old groups, P450arom was expressed especially in Leydig cells around interstitial areas. It was striking that some Leydig cells expressed more aromatase, while others expressed less (Fig. 3). There was no immunoreactivity in Sertoli cells and germ cells. The histoscore values belonging to the interstitial area measured using Image J programme in the testicular tissues belonging to 15-day-old rats are given in Figure 3.

In 60-day-old groups, P450arom was expressed in testicular tissues, Leydig cells, round and elongated spermatids and some spermatocyte cytoplasm belonging to the experimental group (Fig. 4). P450arom immunoreactivity was encountered in spermatogonia and Sertoli cells (Fig. 4K). Density measurements of aromatase expression were separately performed for seminiferous tubules and interstitial area and they were evaluated statistically. According to the analysis results, the aromatase expression in the interstitial area in groups A60 ($X=102.09$) and B60 ($X=102.93$) were similar, but significantly lower in comparison to group K60 ($X=111.63$) (Fig. 5A). As for the seminiferous tubule aromatase expression, there was not a statistically significant difference (Fig. 5B). Aromatase immunoreactivity was not encountered in the testicular tissue of negative control group A-60 (Fig. 4C). In human placenta tissue used as a positive control, there was P450arom immunoreactivity in

Figure 3. Testicular tissue of 15-day-old experimental groups, P450arom immunoreactivity in Leydig cells (→). K: Group K15, A: Group A15, B: Group B15, C: Group A15 negative control (No P450arom immunoreactivity), (X100, Bar 20.0 µm), immunohistochemistry, D: histoscore of P450arom in interstitial cells; * $p<0.05$.

Table 1. Mean seminiferous tubule diameter (MSTD) and Johnsen testicular biopsy score (JTBS) levels of groups

Groups	MSTD on 15 th day (n:140)	MSTD on 60 th day (n:140)	JTBS on 60 th day (n:175)
K	104.29±8.09	288.22±34.19	9.43±0.67
A	99.58±9.49*	262.22±35.37 *	8.50±1.56 *
B	99.79±10.83*	264.70±27.05 *	8.70±0.89*

Values are expressed as mean ± SD. * Significantly different from group K at p < 0.05.

Table 2. Thyroid hormone levels of mother rats at the end of pregnancy

Groups	TSH (mIU /L)	T4 (ng/mL)	T3 (ng /L)
K- Mother	3.10±0.53	59.84±4.23	602.34±173.80
A- Mother	4.08±0.58*	49.07±10.24	531.34±180.53
B- Mother	4.17±0.56*	47.43±2.67*	594.38±88.64

* Different from control group at p < 0.05. Values are expressed as mean ± SD.

Table 3. Thyroid hormone levels of pups on 15th and 60th days

Groups	TSH (mIU /L)	T4 (ng/mL)	T3 (ng /L)
15-day-old	K	1.96±0.82	30.85±2.33
	A	1.83±0.58	15.31±6.02*
	B	1.74±0.20	14.32±3.06*
60-day-old	K	4.02±1.65	52.84±10.09
	A	4.26±0.63	35.98±8.51*
	B	5.92±0.57	42.40±5.98

* Different from control group at p < 0.05. Values are expressed as mean ± SD.

Figure 4. Testicular tissue of 60-day-old experimental groups, P450arom expressions. K: group K60, A: group A60, B: group B60 C: group A60, negative control (no immunoreactivity). Leydig cells(⇨), Spermatids and spermatocytes (→), (X40, Bar 50.0 μm) D: Human placenta tissue as a positive control: P450 aromatase immunoreactivity in syncytiotrophoblasts (→), (X100, Bar 20.0 μm, immunohistochemistry).

syncytiotrophoblasts (Fig. 4D). In the testicular tissues belonging to 60-day-old rats, the Histoscore values measured using Image-J programme in the interstitial area and seminiferous tubules are given in Figure 5.

Thyroid Hormone and TSH Levels

The serum thyroid hormone values in the

blood samples taken from mother rats at the end of the pregnancy and from pups on the 15th and 60th days after pregnancy are given in Tables 2 and 3. It is seen that hypothyroidism was induced because the thyroid hormone levels of mother that were given PTU were low and their TSH values were high. The upper limit of TSH in pregnancy is lower than in normal individuals.

Figure 5. Comparison of P450arom immunoreactivity values in interstitial cells and seminiferous tubule of 60-day-old groups*, at $p < 0.05$, in comparison to the control group.

Figure 6. A: Normalized CYP19 in K15 and K60 B: A15 and A60 C: B15 and B60 (* $P < 0.001$).

The TSH levels of mothers are lower due to the effect of pregnancy compared to the 60-day-old male offsprings.

The thyroid hormone values of pups are given in Table 3. Thyroid functions are not normal in the experimental groups. TSH values in the 15-day groups were similar to those in the control group, whereas T3 and T4 values were lower than in the control group. TSH value in the rats; after 12-day peak, it declined rapidly at day-14 and remained at relatively low levels until the 30th day (17). Thus, it can be explained that the TSH values of the 15-day groups are lower than the 60-day groups.

In the 60-day groups; TSH values were similar in the control group or higher than in the control group, and values of T3 and T4 were found to be lower than the value in the control group. During the later ages, thyroid functions did not improve in group A60 and hypothyroidism occurred in group B60.

Cyp 19 Gene Expression Levels

In order to examine the P450arom (Cyp 19a1) gene expression in testicles, complement DNA (cDNA) was synthesized from RNA isolated from testicles and analyzed with Real time PCR. According to statistical analysis results, it was seen that the level of Cyp19 gene expression in 60-day-old groups was significantly high in comparison to 15-day-old groups ($P < 0.001$), (Fig. 6). When 15-day-old and 60-day-old groups were evaluated among themselves, Cyp19a1 gene expression levels of groups A and B tended to increase in comparison to those of the control group, but this increase was not statistically significant (Fig. 7).

Figure 7. A: Normalized CYP19 in 15-day-old groups and B: in 60-day-old groups.

DISCUSSION

In the present study, it was demonstrated that maternal hypothyroidism affected germ cells and spermatogenesis negatively in the adulthood. No obvious change was encountered in the testicular structure of 15-day-old pups. However, the negative effects of maternal hypothyroidism were seen on the testicles with the impairment in the seminiferous tubule and a decrease in germ cells, JTBS and tubule diameters. Though it was known that maternal hypothyroidism had negative effects on the general development and organogenesis of the pup, during pregnancy, there is no sufficiently informative study about the effects on the reproduction system of male pups. In the past, it was even believed that thyroid hormones did not have an effect on the male reproduction system. However, researchers' finding out the thyroid hormone receptors in testicle cells gave rise to thoughts about the possibility that thyroid hormones might affect the development and growth of testicles (12, 18). It was indicated that T3 had an effect on rat testicles while the effect of T4 was not entirely clear (19). It was demonstrated that thyroid hormone deficiency in the early stages of development and maturity affected the testis development and physiology negatively (11, 20) and these effects were not temporary but altered testicular development and reproduction in the future (21). In the present study, the most striking change in the seminiferous tubules in 15-day-old groups was the presence of degenerated cells that had not been seen before in the examinations (22). In general, the similarity of testis histology in the experimental groups to those of the control group demonstrated that maternal hypothyroidism could not have an

effect on seminiferous tubules in this period.

However, the impairment observed in tubules in the adulthood is affected by maternal hypothyroidism, but the negative effect does not come out due to the absence of spermatogenesis in the 15-day period. In relevant studies, thyroid hormones inhibited the proliferation of neonatal Sertoli cells (23, 24). According to these results, it can be concluded that thyroid hormones suppressed spermatogenesis directly or indirectly in the neonatal period. It was indicated in other studies that the suppression of Sertoli cell proliferation disappears in the early postnatal period in rats (14, 18). Thus, active Sertoli cells' being affected by maternal hypothyroidism is an expected result and in this case, adult testicles may also be affected from maternal hypothyroidism.

In the present study, low thyroid hormone levels in 15-day-old groups might account for the cellular degeneration in the testicles. There are studies on hypothyroidism carried out at different times. In these studies, it was seen that hypothyroidism developed before pregnancy, in neonatal and preadolescence period and in the adulthood. In the adulthood, the most common change in rats with a long-term hypothyroidism are atrophy in Leydig cells, edema in connective tissues, reduced levels of primary spermatocytes and a decrease in seminiferous tubule diameter (22, 25-27). As for the most common changes observed in the testicles of pups whose mothers had hypothyroidism are especially a decline in the number of primary spermatocyte (11, 12) and round spermatids (11, 27, 28), impairment of spermatogoniums, tubules filled with degenerated cells, puckered primary spermatocytes, thickened basal lamina and enlargement in interstitial tissue (22), deceleration in Sertoli cell development (11), decline in

the number of germ cells, degeneration and impairment of spermatogenesis (11, 27, 29). Similarly, we observed impairment of spermatogenesis, degeneration in cells and decrease in tubular diameter in 60-day-old experimental group in the present study. Additionally, it can be said that Group A-60 was affected more by hypothyroidism due to having multinucleated giant cells.

Also, estrogen and aromatase have important physiological roles in male fertility (30). In previous studies, there are contradictory findings about the time of occurrence of aromatase expression in the early period (5,31). In the present study, aromatase expression was observed in Leydig cells in 15-day-old groups while it was not observed in seminiferous tubule cells.

Whereas the localization of aromatase in the neonatal period was contradictory, it was demonstrated that aromatase expression occurred mainly in Leydig cells, spermatocytes, spermatids and spermatozoa in the adulthood. There was no aromatase expression in Sertoli cells and spermatogoniums (5). Similarly, it was seen in the present study that the aromatase expression in 60-day-old control and experimental groups occurred in the same cells. While there was a decrease in the aromatase expression in Leydig cells among the experimental groups in comparison to the control group, it was observed that the expression in the seminiferous tubule was similar to the one in the control group. When seminiferous tubule and interstitial area were evaluated together, it was seen that there was a decrease in the aromatase expression in the experimental groups in comparison to the control group.

Different studies have shown that several factors direct the expression of the aromatase gene and they have an effect on Sertoli, Leydig and germ cells (32). These factors are in interaction with one another and steroid hormones; they have an activating or inhibiting effect on Cyp 19 gene or lead to the fact that it remains either as biologically active aromatase or inactive protein (3). When a comparison is made among the groups according to PCR results, it was determined that there was no significant change in aromatase mRNA in the experimental groups in comparison to the control group, but it showed a tendency to increase. In previous studies, it was demonstrated that aromatase enzyme activity in testicular cells was down-regulated by T3 (33, 34). As a result of the studies on the effect of neonatal hypothyroidism on the interstitial cells in testicles, it was seen that Leydig cell proliferation increased and it was claimed that this increase could be a direct effect of hypothyroidism on these cells or it could

be stimulated directly due to Sertoli-specific factors or it is just the common effect of the both mechanisms (35-37). For this reason, it was thought that the increase in the Sertoli cells of rats that were administered PTU stimulated the increase in Leydig cell proliferation (35).

According to all the data obtained, Sertoli and Leydig cells were affected by the deficiency of thyroid hormones in pups whose mothers had hypothyroidism which led to structural changes in the testicles and impairment of spermatogenesis. It was seen that these effects were not obvious in the early period but lead to serious structural changes in testicles in the adulthood. It was observed that these effects were similar in all groups that were administered PTU both in the prenatal period and during the pregnancy.

It was seen that the aromatase expression of the pups whose mothers had hypothyroidism tended to increase in the neonatal period. The effects of the increase in the aromatase expression and/or the effects of maternal hypothyroidism may not have been seen in the histological structure because the shaping of seminiferous tubules was not completed and the spermatogenesis had not started. However, a decrease was observed in the aromatase activity in the adulthood. Since aromatase and estrogen played a role in gonadotrophin release, it was possible that it did not affect these mechanisms. The disorders in this hormonal balance might have reduced the aromatase expression which affected spermatogenesis negatively.

In conclusion, the present study has demonstrated that maternal hypothyroidism causes a permanent damage to testicles when induced in the prenatal period and during pregnancy. The deficiency of thyroid hormone in the early development period might result in negative effects on spermatogenesis by reducing the amount of aromatase enzyme in testicles in the following years. The study has also confirmed that thyroid hormones, aromatase and estrogen (indirectly) might have roles at least as important as those of androgens.

Conflict of interest

The authors declare that they have no conflict of interest concerning this article.

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