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L–Thyroxine induced hyperthyroidism effect on Testicular endoplasmic reticulum stress and Perk–mediated Nrf2/HO–1 signaling axis of rats

Efecto del hipertiroidismo inducido por L–tiroxina sobre el estrés del retículo endoplásmico testicular y el eje de señalización Nrf2/HO–1 mediado por beneficios de ratas

***Gözde Arkalı*[1]*, *Şeyma Özer Kaya*[2], *Songül Çeribaşı*[3], *Edanur Güler Ekmen*[1], *Mesut Aksakal*[1], *Mehmet Çay*[1]

[1]*Firat University, Faculty of Veterinary Medicine, Department of Physiology. Elazığ, Türkiye.*
[2]*Firat University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. Elazığ, Türkiye.*
[3]*Firat University, Faculty of Veterinary Medicine, Department of Pathology. Elazığ, Türkiye.*
**Corresponding Author: garkali@firat.edu.tr / g.arkali38@gmail.com*

ABSTRACT

The effect of thyroid gland on the male reproductive system (neonatal–prepubertal and adult periods) has been investigated for many years. Hypertyhroidism may cause male infertility by effecting spermatological parameters such as loss of motility and decreased of sperm concentration. However, the mechanisms of male infertility caused by hyperthyroidism are still not fully explained. The aim of this study was to investigate the effect of hyperthyroidism on testicular endoplasmic reticulum stress and the protein kinase RNA–like endoplasmic reticulum kinase (PERK) mediated antioxidant pathway in adult male rats. 24 Sprague Dawley adult male rats were used. Rats were divided into two groups: control group (received intraperitoneal injections of saline solution 1 mL·day^{-1} for 8 week) and hyperthyroidism group (received intraperitoneal injections of l–thyroxine 0,3 mg·kg^{-1}·mL^{-1}·day^{-1} for 8 week). The serum free triiodothyronine (fT3) (P<0.01) and free thyroxine (fT4) (P<0.05) levels were increased, thyroid stimulating hormone (TSH) level (P<0.01) and final body weight (P<0.001) were decreased in the hyperthyroid groups. It was determined that tubulus seminiferus contortus diameters, germinal cell thicknesses, johnsen testicular score values significantly decreased in the hyperthyroid group (P<0.001). It was determined that it had a negative effect on reproductive organs weight and spermatological parameters. As per our results, hyperthyroidism significantly increased malondialdehyde level (P<0.01), glutathione level (P<0.001), glutathione peroxidase enzyme activity (P<0.001), PERK, GRP78 (P<0.01), ATF4 (P<0.05), Nrf2, HO–1 (P<0.05) protein expression levels and significantly decreased catalase activity (P<0.05). These results showed that increased thyroid hormones levels may be a negative factor in terms of testicular physiology as it causes endoplasmic reticulum stress in the testes, and PERK mediated antioxidant response may play an important role in testicular tissue in hyperthyroidism.

Key words: Hyperthyroidism; endoplasmic reticulum stress; Nrf2; HO–1; infertility

RESUMEN

El efecto de la glándula tiroides en el sistema reproductor masculino (períodos neonatal, prepuberal y adulto) se ha investigado durante muchos años. El hipertiroidismo puede causar infertilidad masculina al afectar parámetros espermatológicos como la pérdida de motilidad y la disminución de la concentración de espermatozoides. Sin embargo, los mecanismos de la infertilidad masculina causada por el hipertiroidismo aún no están completamente explicados. El objetivo de este estudio fue investigar el efecto del hipertiroidismo sobre el estrés del retículo endoplásmico testicular y la vía antioxidante mediada por PERK en ratas macho adultas. Se utilizaron 24 ratas macho adultas Sprague Dawley. Las ratas se dividieron en dos grupos: grupo control (recibió inyecciones intraperitoneales de solución salina 1 mL·día^{-1} durante 8 semanas) y grupo de hipertiroidismo (recibió inyecciones intraperitoneales de l–tiroxina 0,3 mg·kg^{-1}·mL^{-1}·día^{-1} durante 8 semanas). Los niveles séricos de fT3 (P<0,01) y fT4 (P<0,05) aumentaron, el nivel de TSH (P<0,01) y el peso corporal final (P<0,001) disminuyeron en los grupos de hipertiroidismo. Se determinó que los diámetros del túbulo seminífero contortus, el espesor de las células germinales y los valores de la puntuación testicular de Johnsen disminuyeron significativamente en el grupo de hipertiroidismo (P<0,001). Se determinó que tenía un efecto negativo sobre el peso de los órganos reproductivos y los parámetros espermatológicos. Según nuestros resultados, el hipertiroidismo aumentó significativamente el nivel de malondialdehído (P<0,01), el nivel de glutatión (P<0,001), la actividad de la enzima glutatión peroxidasa (P<0,001), PERK, GRP78 (P<0,01), ATF4 (P<0,05), Niveles de expresión de proteínas Nrf2, HO–1 (P<0,05) y actividad catalasa significativamente disminuida (P<0,05). Estos resultados mostraron que el aumento de los niveles de hormonas tiroideas puede ser un factor negativo en términos de fisiología testicular, ya que causa estrés en el RE en los testículos, y la respuesta antioxidante mediada por PERK puede desempeñar un papel importante en el tejido testicular en el hipertiroidismo.

Palabras clave: Hipertiroidismo; estrés del retículo endoplásmico; Nrf2; HO–1; infertilidad

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INTRODUCTION

Changes in THs levels affect many cellular processes in the organism, because thyroid hormones (THs) are very important for postnatal growth and development [1]. Hyperthyroidism is an endocrine disease characterized by hyperfunction of the thyroid gland or an increase in circulating extra thyroid-derived origin THs [2]. THs receptors are found in sperm, germ, sertoli, leydig and peritubular cells. For this reason, the effects of THs on the male reproductive system have been investigated histologically and steroidogenically for many years. The studies show that thyroid hormones play an important role in reproductive failure [3, 4].

In experimental animal models, ıt has been reported that the period during which sertoli cells are mitotically active is shortened, accompanied by a decrease in the number of germinal cells, testicular maturation is affected, and as a result, the sperm production capacity decreases [5, 6].

Sexual function in men with hyperthyroidism has been reported to be affected in several ways, such as decreased libido, erectile dysfunction and premature ejaculation. In other words it is stated that most of the patients with THs disorders have some kind of sexual dysfunction [7, 8]. However, the mechanisms involved in the relationship of THs dysfunctions with male infertility are still not fully elucidated.

Oxidative stress, which causes tissue damage, plays an important role in male infertility [9]. Hyperthyroidism triggers a hypermetabolic state in the organism that leads to excessive production of free reactive oxygen species (ROS) due to increased mitochondrial respiration. Increased ROS are toxic to cell membranes and leads to lipid peroxidation in the cell [10]. Germ cells are more susceptible to oxidative damage than somatic cells [11].

Therefore, the theory of testicular damage due to oxidative stress in hyperthyroidism is emphasized [12, 13]. Endoplasmic reticulum (ER) stress is expressed as long-term failure in protein folding and unfolded proteins accumulation in the ER. Oxidative stress, protein overload, Ca^{+2} leakage and hypoxia cause ER stress. After ER stress occurs, the cell uses the unfolded protein response (UPR) pathway to maintain cell survival or recover from the stress with minimal damage [14].

Activation of UPR results in the increased expression of the ER chaperone glucose-regulated protein 78 (GRP78), which increases the protein folding capacity of the ER. The Protein kinase RNA-like endoplasmic reticulum kinase (PERK) is an important protein that mediates the UPR under ER stress [15].

Additionally, Nuclear factor erythroid 2 (Nrf2) activation has an important role in the cell survival after ER stress. PERK-dependent phosphorylation of Nrf2 increases transcription of Nrf2 target genes. Studies have shown that Nrf2 is a direct substrate of PERK and that Nrf2 is a critical protein for PERK-mediated cell survival [14].

Based on this information, focused on the two questions. First, Could male infertility in hyperthyroidism be a result of impairment of the ER stress response? Second, what is the activity of the PERK signalling pathway associated with the antioxidant response in testicular tissue in hyperthyroidism?

MATERIAL AND METHODS

Animals and experimental design

Firat University Local Animal Use Committees (Elazığ, Türkiye) approved the animal experimental protocols (Protocol number 2021/06). 24 adult male Spraque Dawley rats (12 weeks/250–300 g) were procured and maintained from Firat University Experimental Research Centre (Elazığ, Türkiye). Rats (*Rattus norvegicus*) were individually tail marked and housed in a polycarbonate cage with 3 rats in each cage, and 4 cages were created for each group. Standard commercial pellet food (200 g·day^{-1}·cages^{-1}) and fresh drinking water (500 mL·day^{-1}·cages^{-1}) were given to animals ad libitum. A room temperature was 24 ± 3°C and light-dark-circle; 12 h light/12 h dark.

The hyperthyroidism model was performed with single intraperitoneal injection of 0.3 mg·kg^{-1} L-T4 (Sigma Aldrich, T2376) [6] for eight weeks (the time period of one spermatogenesis) [16]. Stock L-T4 was dissolved using 0.01 M sodium hydroxide. The final solution was prepared with saline solution (0.3 mg·kg^{-1} L-T4 per mL and 1 mL of the respective solution was administered). The rats were randomly divided into two groups. Control Groups (n:12); a single dose of saline (1 mL) was administered intraperitoneally every day for 8 weeks. Hyperthyroid Group (n:12); a single dose of LT4 0.3 mg·kg^{-1}·mL^{-1} was administered intraperitoneally once a day for 8 weeks. The final body weights at the end of the 8th week were evaluated. The animals were decapitation and the bloods was then collected into the serum tubes and centrifuged (Nüve NF800R, Türkiye) (3220 G for 10 min), serum was separated. Reproductive organs (testes, epididymis, cauda epididymis, seminal vesicles, ventral prostate) were removed and weighed (R-250AZ, Türkiye). Right testes were washed with cold 0.9% NaCl solution and then shocked with dry ice. The tissue samples and blood samples taken were transported to the laboratory in accordance with the cold chain and stored in deep freezer at -80°C (Haier biomedical, China) until analysed. The left testes were properly fixed in Bouin solution for histopathological analysis and Johnsen Score [17, 18].

Enzyme linked immunosorbent assay and oxidative stress analysis

The concentration of THs (TSH, fT3 and fT4) were measured in the serum of all animals using sandwich enzyme-linked immunosorbent assay kits according to the manufacturer's recommendations (sunred 201-11-0181; sunred 2010-11-0736; sunred 201-00-0087). The standard curve was used to determined the concentration of each hormone by using ELISA reader (Thermo Scientific Multiskan FC, USA). Testes tissue samples were weighed (R-250AZ, Türkiye) and homogenized with Tris Buffer (1/10,g/v) using a mechanical homogenizer (IKA Ultra-Turrax T25) at 4°C. After the homogenates were centrifuged (Nüve NF800R, Türkiye) (3220 G/60 min), the supernate was separated. Malondialdehyde (MDA) levels and antioxidant enzyme levels (GSH, GSH·Px and CAT) and total protein content were measured from the supernatant. Total protein content was determined according to the lowry method using spectrophotometer (Shimadzu, UV-1700 PharmaSpec, Kyoto Japan) [19].

The method described by Placer *et al*. [20] was used for MDA analysis using spectrophotometer. The MDA level was expressed as nmol·g^{-1} tissue. The method described by Sedlak and Lindsay [21] was used for GSH analysis using spectrophotometer. The GSH level was expressed as nmol/g tissue. The method described by Lawrence and Burk [22] was used for GSH·Px enzyme analysis using spectrophotometer. The GSH·Px enzyme activity was expressed as

IU·g^{-1} protein. The method described by Aebi [23] was used for CAT enzyme activities analysis using spectrophotometer. The CAT activity was expressed as k·g^{-1} protein.

Western blot analysis

The cold RIPA lysis buffer kit was used to homogenize tissue. The homogenate was centrifuged (Nüve NF800R, Türkiye) (3220 G at 60 min) and the supernatant was separated. Bradford protein assay kit (Thermo Scientıfic 1856209, USA) was used to determine total protein content. Supernatant was incubated at 95°C for 5 min (Supernatant was prepared with a 4x–laemmli buffer solution). Equal amount of protein was loaded into each well, and 1st well was loaded with protein marker, then the samples were electrophoresed. After SDS–PAGE, proteins were transferred to PVDF membrane. After these procedures, blocking with milk powder, washing and overnight primary antibody (PERK: sunred 201r–1399; 1/1000, GRP78: sunred 201r–0538; 1/1000, Activation transcription factor 4 (ATF4): sunred 201r–4224;1/1000, Nrf2: Fine test FNab05855;1/3000, HO–1: Santacruz sc–390991;1/1000, and betaactin: Santacruz sc–47778;1/1000) incubated were performed. The washing procedure was repeated and the membranes were incubated with secondary antibodies (anti mouse seconder antibody: Santacruz sc–516102: 1/5,000; anti rabbit seconder antibody: BBI D110058: 1/10000) for one hour using shaker (Thermo scientific, USA). The washing procedure was then repeated, and the membranes were incubated with enhanced chemiluminescence. Biorad ChemiDoc™ XRS+imaging device was used to band images. Bio–Rad Image Lab™ Software was used for band densities in the images. Betaactin was used to normalize the expression levels of proteins. The results was expressed as percent of control [24].

Spermatological parameters analysis

Epididymal sperm concentration was determined using the hemocytometer method. The right epididymis was minced in 1 mL of 0,09% NaCl and incubated for 4 h at room temperature. After incubation semen was drawn up to the 0.5 line of the pipette and 2% eosin solution was drawn up to the 101 line. The diluted sperm suspension was transferred to thoma lame counting chambers and counted at 400 magnification using a light microscope (Nikon eclipse Ci–L, Japan).

The result was expressed in million/right cauda epididymis. Motility analysis, samples were placed on a slide on the heating table of the light microscope (degree is 37°C). After few drops of tris buffer solution, the liquid obtained from left cauda epididymis was added to the slide and mixed. Sperm motility percentage was evaluated visually at 400 magnification. Three different areas were evaluated in each sample.

Motility rate was expressed as a percentage. To determine the percentage of abnormal sperm, tris buffer–spermatozoa suspension was mixed with eosin–nigrosine stain and peripheral smear slides were prepared, and examined under a light microscope (Nikon eclipse Ci–L, Japan) at 400 magnification. A total of 200 spermatozoa were examined on each slide and the total, tail and head abnormality rates of spermatozoa were expressed as a percentage [25].

Histopatological analysis and determination of Johsen Skoru

Testicular tissues were fixed in Bouin's solution for 48 h. They were dehydrated with ethanol, embedded in paraffin and sectioned at 5 µm. Sections were stained with hematoxylin and eosin [17]. Diameters of seminiferous tubules (TSC), germinal cell layer thicknesses (GCLT) and Johnsen's testicular score was evaulated using light microscopy at 200× magnification (Zeiss Primostar 1) [18]. All sectioned tubules were evaluated, and a score between 1 (very poor) and 10 (excellent) was given for each tubule according to Johnsen's criteria. 25 tubules were evaluated, for each animal TABLE I.

TABLE I
The table of Johnsen Score

Score Histologic Findings	Score Histologic Findings
10	Spermatogenesis is complete, germline epithelium is of regular height, and TSC lumens are of normal diameter.
9	Numerous spermatozoa in TSC, disorganization of germinal epithelium and sequestration of germinal cells, TSC lumen occluded
8	Less than 5–10 spermatozoa are present in each TSC lumen
7	No spermatozoa in TSC lumen, numerous spermatids, spermatocyte and spermatogonia present
6	No spermatozoa in TSC lumen, 5–20 spermatids, numerous spermatocytes and spermatogonia present
5	No spermatozoa and spermatids in the TSC lumen, numerous spermatocytes and spermatogonia present
4	There are no spermatozoa and spermatids in the TSC lumen, less than 5 spermatocytes, but numerous spermatogonia in each TSC.
3	TSCs have only spermatogonia.
2	Germinal cells completely absent, only Sertoli cells are present
1	All TSCs are cell–free.

Statistical analysis

SPSS package program (IBM SPSS Version 22.0) was used for statistical evaluations. Data were tested for normal distribution using the Shapiro–Wilk normality test. The difference between the two groups was compared using the independent *t* test. Study data were presented as mean ± standard deviation ($\bar{x} \pm SD$) for groups. Statistical significance level was accepted as $P<0.05$.

RESULTS AND DISCUSSION

The results of the this study showed that the hyperthyroidism model was successfully achieved with an increase in serum fT3 and fT4 levels ($P<0.01$; $P<0.05$) along with a decrease TSH levels ($P<0.001$), FIG. 1 (A, B, C). This is similar to several studies on hyperthyroidism [6, 26]. There are studies in which T3 was given to create a hyperthyroidism model in rats [5]. However, Triiodothyronine binds poorly to proteins, so its action is much faster but much shorter than T4. While thyroxine has a half–life of 6–8 days, T3 has a half–life of 8–12 h [27]. Therefore, L–T4 was chosen to induce hyperthyroidism in this study. The effects of THs begin with the entry of these hormones into the cell. Since only free fractions of THs enter the cell, measuring fT4 and fT3 is much more reliable in measuring TH [28]. For this reason, fT4 and fT3 measurements were preferred in this study to understand the formation of experimental hyperthyroidism. Feed intake and water intake values increased ($P<0.001$) in the hyperthyroidism group, and in this study, weight loss occured ($P<0.001$) despite hyperphagia in hyperthyroidism FIG. 2 (A, B, C). And the results are similar to several studies on hyperthyroidism [6, 13, 28].

It is well known that THs dysfunctions are associated with excessive ROS production. Researchers focused on oxidative stress in testicular

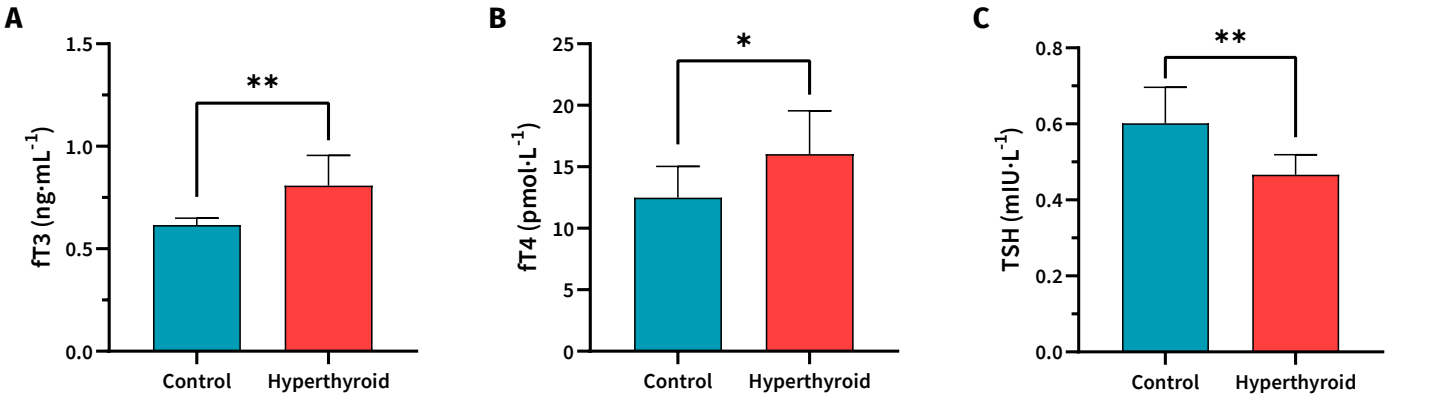

FIGURE 1. (A) Serum fT3 levels of groups (*P*<0.01). (B) Serum fT4 levels of groups (*P*<0.05). (C) Serum TSH levels of groups (*P*<0.01). Values were expressed as mean and standard deviation. (*; *P*<0,05; **: *P*<0.01)

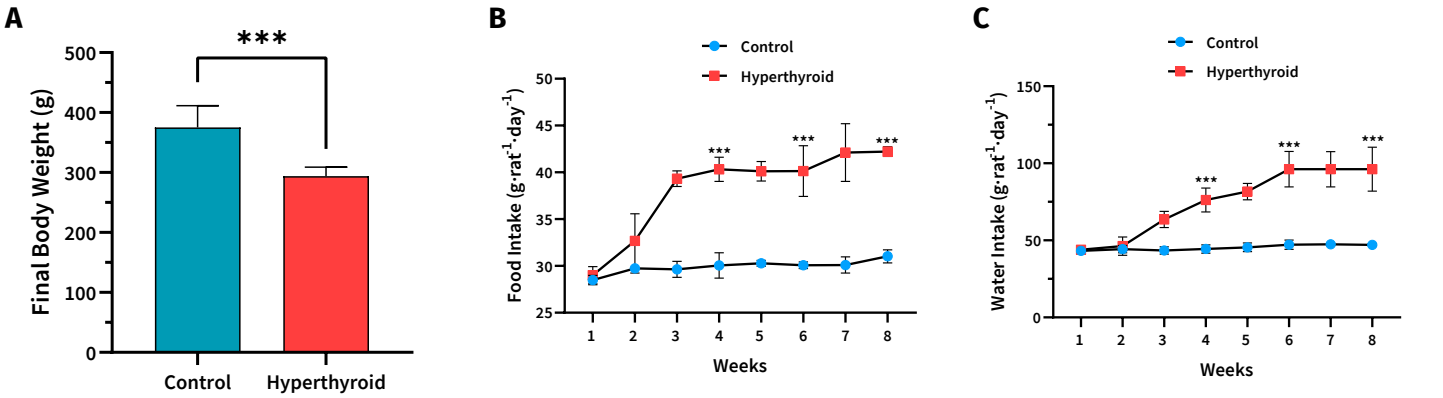

FIGURE 2. (A) Hyperthyroidism effect on Body Weight (*: *P*<0.001), (B) on food intake (***: *P*<0.001), (C) on water intake (***: *P*<0.001). Values were expressed as mean and standard deviation. (***: *P*<0.001)**

damage caused by hyperthyroidism [5, 6, 10, 12, 13]. Different TH isomers have been reported to cause different degrees of oxidative stress [10]. It was reported that this situation was dose dependent under in vitro conditions. In this study we administered 0.3 mg·kg^{-1}·day^{-1} L–thyroxine intraperitoneally for 8 weeks and found that oxidative stress increased. The this study, the testicular MDA levels increased (*P*<0.01)(TABLE II) significantly in the hyperthyroid group. The findings in this study are parallel to the results of studies reporting that hyperthyroidism causes oxidative stress in various tissues such as muscle [29], liver [30] and heart [31]. On the other hand, since antioxidants neutralize ROS, examining antioxidant enzyme levels in testicular tissue will be of great benefit in understanding oxidative stress in hyperthyroidism [5, 6, 12, 13]. In this study, GSH levels (*P*<0.001) and GSH·Px enzyme activities increased (*P*<0.001) significantly in hyperthyroidism group, while CAT enzyme activities decreased (*P*<0.05) significantly (TABLE II). The decreased in CAT enzyme activities may be due to the deactivation of excessive free radicals produced during the hypermetabolic state in hyperthyroidsm [32]. Sahoo *et al.* [33] reported that the decrease in SOD and CAT activities in rat testicular tissue in hyperthyroidism was due to the impairment of the testicular capacity to neutralize superoxide radicals and hydrogen peroxide produced in hypermetabolic state. Sahoo *et al.* [32, 33] suggested that the increase in GSH·Px enzyme activities may be an adaptive response to neutralise ROS generated

TABLE II
Effects of hyperthyroidism on testicular MDA, GSH levels and GSH.Px, CAT enzyme activity of the groups (Mean±SD)

Groups	MDA (nmol·g^{-1} tissue)	GSH (nmol·g^{-1} tissue)	GSH·Px (IU·g^{-1} prot)	CAT (k·g^{-1} protein)
Control	38.45±15.02	1.09±0.15	26.32±2.30	0.058±0.013
Hyperthyroid	67.83±23.18	1.52±0.21	32.72±2.18	0.045±0.007
Significant	*P*<0.01	*P*<0.001	*P*<0.001	*P*<0.05

due to disruption of the oxidant status of organ. Chattopadhyay *et al.* [34] concluded that CAT enzyme activity decreased and GSH·Px enzyme activity increased in hyperthyroid rat liver. Chattopadhyay *et al.* [34] suggested that these two antioxidant enzymes, which are important in ROS metabolism, respond oppositely to thyroid hormones in both transcription and translation. And this different pathway between CAT and GSH·Px mediated by thyroid hormones may be the rapid regulatory protective response [32, 33, 34].

When histopathological analyzes were evaluated in this study, it was observed that the testes in the control group had a normal histological appearance (FIG. 3A). Although its severity and prevalence vary, in

hyperthyroid group; the main lesions with decrease in GCLT (P<0.001) and TSC diameter (P<0.001), severe vacuolar degeneration in germinal epithelial cells, disorganization, dilatation, significant desumation and pause in spermatogenesis (FIG. 3B). Hypoplasia and syncytial cell formations were observed in some TSCs, but to a lesser extent. In addition; Johnsen testicular score values were found to be lower in the hyperthyroid group (P<0.001)(TABLE III). These results are similar to the results of the study of Abo-Elnour *et al*. and Özgüner *et al*. [35, 36]. Consequently, it was determined that there were changes in the germ cells and seminiferous tubule structure.

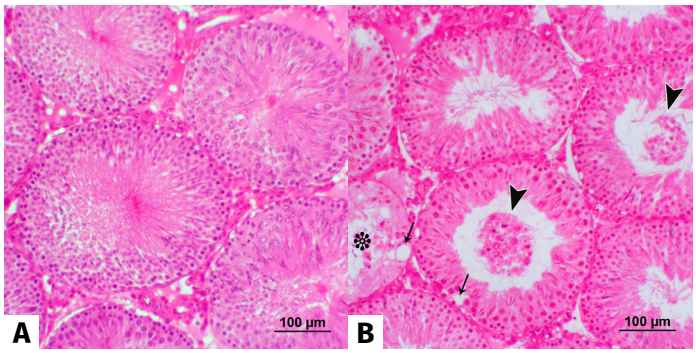

FIGURE 3. (A) Normal histological appearance of testes in control group rats, (B) Desquamation of germinal epithelial cells (arrow heads), vacuolar degeneration (arrows) and hypoplasia (asterix) in hyperthyroidism group, HE 100×

TABLE III
Mean TSC diameter, germinal cell thickness and Johnsen Testicular Score values in control and hyperthyroid groups (Mean ± SD)

Groups	TSC Diameter (µm)	Germinal Cell Thickness (µm)	Johnsen Testicular Score
Control	273.33±0.98	93.53±0.46	9.62±0.13
Hyperthyroid	261.20±1.37	66.25±0.86	8.36±0.15
Significant	***P*<0.001**	***P*<0.001**	***P*<0.001**

Many studies suggest that the reason for the deterioration in sperm quality is the increase in ROS formation due to increase in MDA level and the deterioration of the antioxidant defense system [5]. From this study, it was revealed that the increase in oxidative stress due to hyperthyroidism is associated wit a decrease in sperm count and motility. In this study, motility (P<0.001), spermatozoon concentration (P<0.05), testes weight (P<0.05), epididymis weight (P<0.01), right cauda epididymis weight (P<0.001), seminal vesicles weight (P<0.001) and ventral prostate weight (P<0.01) decreased, however there was no difference in abnormal spermatozoon rate (P>0.05) in hyperthyroid groups (TABLES IV and V).

TABLE IV
Effects of Hyperthyroidism on Spermatological Parameters (Mean±SD)

Groups	Motility (%)	Sperm concentration (million/right cauda epididymis)	Abnormal spermatozoon rate (%)		
			Head	Tail	Total
Control	74,00±9,66	121,20±25,80	1,80±0,78	2,20±0,91	4,00±1,05
Hyperthyroid	48,88±14,52	94,00±14,07	1,88±0,78	2,66±1,32	4,55±1,33
Significant	*P*<0.001	*P*<0.05	*P*>0.05	*P*>0.05	*P*>0.05

TABLE V
Effects of Hyperthyroidism on Absolute Reproductive Organ Weights (g) (Mean±SD)

Groups	Testes (Right +Left) 2	Epididymis (Right +Left) 2	Right Cauda Epididymis	Seminal Vesicles	Ventral Prostate
Control	1,53±0,17	0,67±0,09	0,27±0,02	1,71±0,22	0,52±0,11
Hyperthyroid	1,36±0,11	0,53±0,04	0,19±0,02	1,17±0,26	0,35±0,11
Significant	*P*<0.05	*P*<0.01	*P*<0.001	*P*<0.001	*P*<0.01

Sahoo *et al*. [33] observed that hyperthyroidism causes decreased sperm count, marked exfoliation of germ cells into the lumen of the seminiferous tubules, disorganization of the germinal epithelium, and increased interstitial space in the testicular compartment. Faraone-Mennella *et al* [37] reported that in sexually mature hyperthyroid rat testes, marked arrest in maturation of germ cells and germ cell apoptosis were observed. In another study, it was explained that the increase in oxidative damage caused by hyperthyroidism causes activation of poly(ADP-ribosyl)ationsystem, one of the biomarkers of DNA fragmentation in testis. Additionally, Sahoo *et al*. [33] reported that hyperthyroidism causes a decrease in sperm counts and the live sperms percentage. Khosrowbeygi *et al*. [38] reported that sperm motility and morphology were significantly associated with both catalase activity and total antioxidant capacity. In this study, it was reported that sperm motility decreased, but no significant difference was observed in sperm morphology. These data are similar to findings of poor semen quality and impaired motility reported in studies investigating the effect of hyperthyroidism on male fertility in humans [7]. Researchers suggest that THs may act as regulators of germ cell survival and possibly also play a role in spermatogenesis as well [39].

In the rat model, it has been shown that proper ER functions of the male reproductive organs are impaired inmany pathological conditions [15]. Consistent with the findings of many other researcher's studies on male infertility [40, 41], It was observed that protein expression levels of PERK (P<0.01), GRP78 (P<0.01), ATF4 (P<0.05), Heme oxygenase 1 (HO-1)(P<0.05) and Nrf2 (P<0.001) are increased in testicular tissue in hyperthyroidism (FIG. 4).

Nrf2, is an important transcription factor that can be induced by oxidative stress [14]. Likewise, it has been reported in many studies that Nrf2 is also affected by THs. Previous studies have suggested that hyperthyroidism increases Nrf2 protein expression in other tissue [42, 43, 44]. In this study, the protein expression level of Nrf2 and HO-1 increased in the hyperthyroid group. Therefore, the increase in HO-1 protein may be an adaptive response to high H_2O_2 levels caused by hyperthyroidism. Accumulation of unfolded proteins in the ER creates that a cellular survival response that triggers signaling events of both prosurvival and proapoptotic. PERK-dependent activation of Nrf2 is critical for cell survival signaling [14]. Although the importance of Nrf2 signaling in response to ER stress has been established, the mechanism by which Nrf2 promotes cell survival has not been defined [45]. Aykanat *et al* reported that ER stress is associated with cardiac hypertrophy [46] and kidney damage [47]. Liang *et al* reported that high T3 Induces beta-cell insulin resistance via ER Stress [48]. This study is similar to the effects of hyperthyroidism on ER stress described in various tissues [46, 47, 48]. In addition, some studies have reported that the degree of influence of UPR signaling pathways varies depending on the working time, and this affects the cell's decision whether to survive or not [49]. In this results, the increased ATF4 protein expression level was statistically

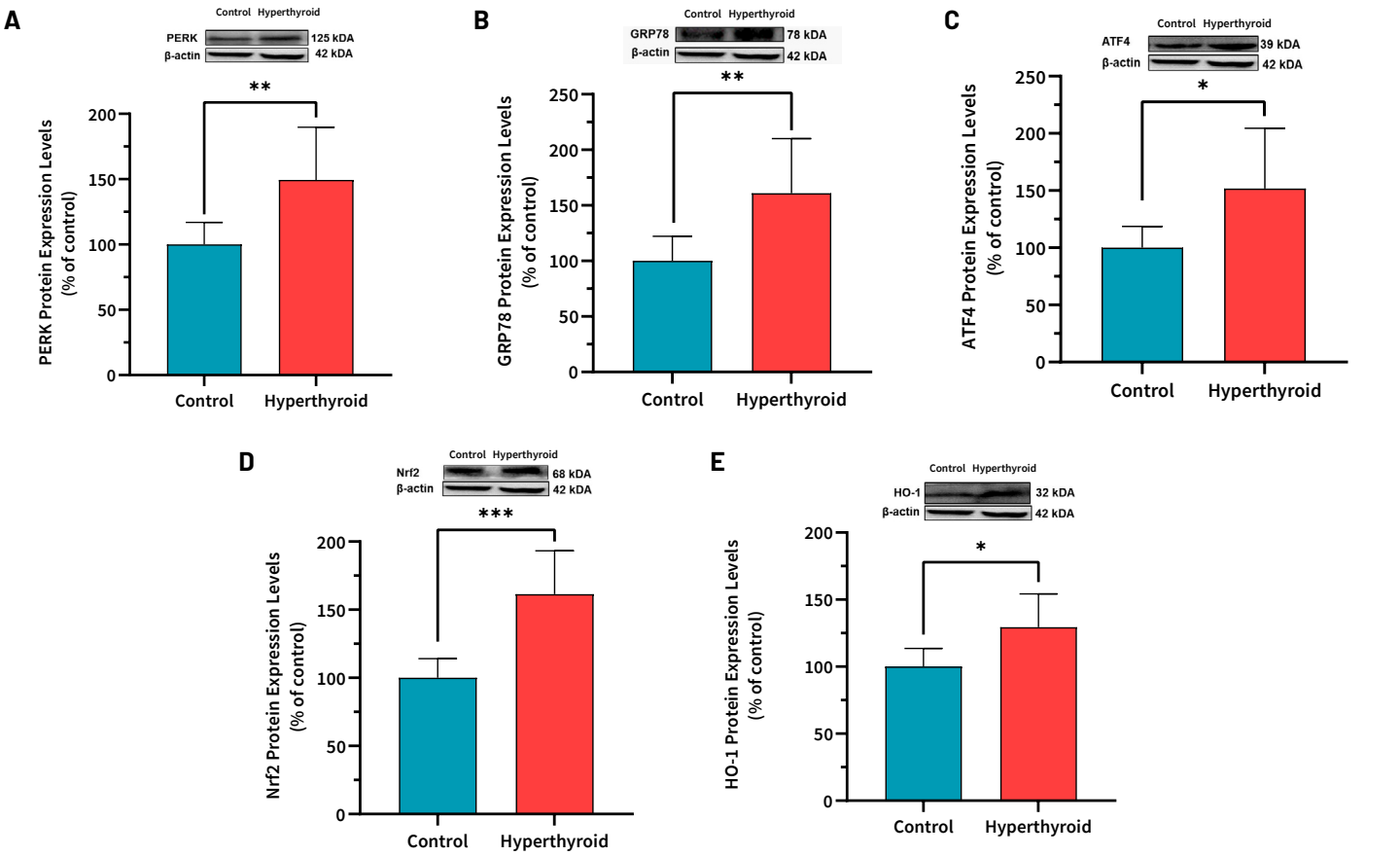

FIGURE 4. (A) Testes tissue PERK protein expression levels (;*P*<0.01), (B) Testes tissue GRP78 protein expression levels (**;*P*<0.01), (C) Testes tissue ATF4 protein expression levels (*;*P*<0.05), (D) Testes tissue Nrf2 protein expression levels (***; *P*<0.001), (E) Testes tissue HO–1 protein expression levels (*;*P*<0.05)**

significant. There is a paradox that pathways involved in ER stress lead to simultaneous activation of both adaptive and proapoptotic pathways. It has been reported that mild ER stress in the cell usually triggers cell adaptation rather than cell death [50]. The cell can develop an adaptive response to oxidative stress through ER stress.

CONCLUSIONS

Consequently, this findings underscore the widespread importance of the ER stress and PERK mediated Nrf2/HO–1 signaling axis described in the study of hyperthyroidism induced male infertility. Based on these results, hyperthyroidism induces testicular oxidative stress and ER stress, and subsequently activates the PERK/Nrf2 pathway. However, evaluating the activity of signaling pathways involved in ER stress at different times is important to better understand the effectiveness of this mechanism on male infertility in hyperthyroidism.

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Availability of data and materials

The authors declare that data supporting the study findings are also available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

- ***Gözde Arkali***: Conceptualization, Data curation, Experimental application, Western Blot Analysis, ELISA Analysis, Oxidative Stress Analysis, Project administration, Writing – original draft.
- ***Şeyma Özer Kaya***: Spermatological Analysis, Data curation
- ***Songül Çeribaşı***: Histopathological Analysis, Data curation.
- ***Edanur Güler Ekmen***: Experimental application, Western Blot Analysis, Oxidative Stress Analysis.
- ***Mesut Aksakal***: Oxidative Stress Analysis, Data curation.
- ***Mehmet Çay***: Writing – original draft, Data curation.

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