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THE PATHOGENETIC ROLE OF STRESS IN THE COURSE OF THYROID GLAND CELLULAR RENEWAL

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Abstract

The goal of the study was to identify the relationship between external stressors of different origin and the development of proliferative - apoptotic changes in the thyroid gland at the cellular-molecular level in the experiment, taking to account the gender.

The study were carried out on 40 white Wistar rats of 130-150 g. body weight. Immunohistochemical method was used to study the dynamics of proliferative thyrocytes index and the severity of apoptosis reactions in the cell population after the influence of alimentary and immobilization stress in males and females rats separately.

At the age of 3-3.5 months, the thyroid gland of the White rat had a low degree of differentiation due to the significant rate of the inter-follicular epithelium. This corresponds to the normal value of the age-related structure in small laboratory rodents and reflects ontogenetic patterns. The active growth and development of the organ is reflected by Ki-67 marker of proliferation. Rats of the control group fed with normal diet, showed the level of Ki-67 expression onaverage 2.4% in females and 4.25% in males. In females rats, that were within a month on a special diet (alimentary stress), the level of Ki-67 increased up to 3.4%, but that value could be estimated as low. In males, on the contrary, the level of glandular epithelial cell proliferation decreased up to 2.75%. Animals exposed to immobilization stress within 7 days, had significant ($p \leq 0.05$) changes in Ki-67 expression with the opposite vector depending on gender. The expression of Ki-67 in stressed females reached on average 11.25%, which is 5 times higher than the control. In males, on the contrary, its value fell up to 1.5%.In the group of animals with combined stress, females did not survive until the end of the experiment, and in males the Ki-67 index fell up to 1.3%.The determination of the apoptosis marker expression FAS-R on thyrocytes revealed certain regularities. Multiple comparisons between groups of animals showed significant differences between the group of females exposed to stress and on a normal diet, from all groups, except for a similar group of males (by the Kraskel-Uollis method at a value of $p \leq 0.05$). Multivariative analysis ANOVA showed a high degree of influence (96.7%) of animal gender on the proliferation of glandular epithelium, as well as stress (54.28%).

There is a direct link between external stressors and the development of functional and organic compensatory changes in the thyroid gland, which indicates the leading pathogenetic role of adaptation syndrome in the development of organ pathology. Changes in Ki-proliferation index and CD- 95 or FAS-R in experimental animals significantly indicates the role of multiple stress in the development of thyroid disease.There is a sexual dimorphism of the studied parameters, which can cause different morbidity of males and females in the population under stress.

Keywords: *thyroid gland, proliferation index, apoptosis, stress, immunomorphology.*

Introduction

Much attention is paid to the study of the role of stress in the development of thyroid pathology [*Jasenjavskaja A.L., 2010; Fliers E. et al., 2015*]. This is due to the direct relationship of aetiopathogenesis, traced in patients, and a combination of global trends such as the growth of thyroid disease and the life intensity of the individual and society [*Stjazhkina S.N. et al., 2018*]. Psychological, environmental and other types of stress prevail in different countries in combination and differentially.

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The literature describes the dynamics of serum thyroid hormone levels in animals and humans under acute and chronic stress. Activation of thyroid function and increased thyroxine concentration (T4) observed after acute thermal stress in rats within 7 days [*Bil'zhanova G.Zh. et al., 2016*]. Inhibition of the glandfunctional activity was found at 14-day stress [*Jasenjavskaja A.L. et al., 2014*]. 60-day stress induces an increase in T3 concentration and a decrease in T4 concentration [*Zhang J. et al., 2018*]. In patients after severe intraperitoneal operations, there was a decrease in total and free T3, but an increase in T4 and thyroid-stimulating hormone (TSH). There is also a correlation between the degree of thyroid hormone level reduction and the severity of prognosis in patients in intensive care units with a high degree of reliability [*Fliers E. et al., 2015*]. Under psychological stress people with anxiety and depressive disorders and insomnia develop hypothyroidism [*Krishna V. N. et al. 2013*]. Micro elements chronic environmental stress (changes in the content of copper, bromine, calcium and iodine in water and soil, vitamins in food) also showed a correlation with the formation of the adaptation-associated thyroid pathology [*Bezrukov O. F., 2011, Ye J. et al., 2017*].

It is proved the fact not only functional, but also morphological changes in thyroid tissue under stress. The development of the general adaptation syndrome is greatly influenced by the functional state of the hypothalamic-pituitary-thyroid axis, sensitive to stress. This hierarchical regulatory chain reacts to stress not only by changing the level of thyroid hormones, but also by compensatory morphological rearrangement of the follicular compartment of the thyroid gland and interfollicular structures [*Uribe R.M. et al., 2014*]. Under severe stress, the height of thyrocytes decreases significantly, and the area of follicles increases significantly [*Smirnova T.S. et al., 2009*]. The effect of stress on the thyroid gland can have profound consequences, including the development of autoimmune pathology and cancer [*Asif F.et al., 2018*].

Thyroid cancer is the most common malignant tumor of the endocrine system. According to the Ministry of health of the Russian Federation, for the period from 2013 to 2016, the incidence of thyroid diseases increased by 12.1%: from 2037.1 cases for 100 thousandpopulations up to 2283.5. The curve showing the number of patients suffering from thyroid problems has been steadily creeping up for over a decade. This is evidenced by the data of the unified interdepartmental statistical information system (UISIS), which collected indicators since 2005 [*Fliers E. et al., 2015*]. About 90% of all thyroid cancers are differentiated forms [*Nadol'nik L.I., 2010*], and 75-80% of them are papillary cancers [*Korshunova D.V. et al., 2014*].

Malignant alterations of the cell renewal vector are of the greatest interest in our opinion [*Pompella A., 2013*]. Features of morphological and functional alteration of the thyroid gland in different types of stress are not detailed enough depending on gender and age, predominant dynamics of cell populations, expression of protein markers of the functional state of different types of cells [*Gevorkian V.S., Gevorkian I.S., 2017*]. At present, immunohistochemical (IHC) research is becoming more and more widespread in the study of the thyroid gland. IHC-method as antigen-antibody reaction, realized in tissue, is one of the specific methods of protein detection with the establishment of their tissue localization and quantitative parameters. This type of research allows to evaluate the degree of proliferative activity of thyroid cells.

The aim of our work is to show the relationship between external stressors of different nature (alimentary deficiency and immobilization) and the development of proliferative – apoptotic changes in the thyroid gland at the cellular-molecular level in the experiment taking into account the gender.

Materials and Methods

To establish regularities of changes in the expression of Ki-marker of proliferation and CD 95-FAS-R -receptors of apoptosis in thyrocytes of animals, subjected to chronic stress, an experiment was conducted on 40 white Wistar rats,females and males aged 3-3.5 months (early puberty) with a body weight of 130-150 *g*. Animals were divided into 4 groups: control, with alimentary stress and immobilization stress, a combination of two types of stress. Each group included 10 rats, 5 females and 5 males. The animals were kept in vivarium in cages with 5 animals in compliance with the standards of microclimate and natural light of the late summer and early autumn of the Crimean region. Males and females were placed in adjacent cells separately from the age of 2 months.

Rats of the first group were subjected to handling only during feeding, change of bedding, weighing. All manipulations were performed by one employee.

To simulate the alimentary stress of rats of the second group within 4 weeks fed with calcined grain and watered of low iodine content and high bromine content, in the free regime of drinking and feeding, but without giving other food and water. For calcination, wheat was placed in an oven AT 300 °C for 1 hour. Cooked water containing 10 *μg* of NaBr for 5 litres, which corresponds to the content of bromine in the water of west Crimea. Thus, the concentration of bromine in fresh waters of the Crimea (for example, in the city of Saki and Saki district) is 0.02 *μg* per liter, which exceeds the maximum allowable concentration [*Bezrukov O.F., 2015*].

Immobilization stress was simulated by placing the animals for 20 hours in the fixators (animal-restrainers) AE1001-R1 (LLC "NPK Open Science", Russia) for rats of medium size. The fixator is made of transparent acrylic plastic, has internal dimensions of 165×55×55 *mm* and designed for fixing laboratory animals of different sizes, due to a customizable system of two movable stops. The stress simulation mode was chosen in such a way that the immobilization time was 20 continuous hours per day, including the night period of activity of rats (from 14^{00} to 10^{00} hours) and 4 hours of free movement of rats in the cell (respectively, in the midday time). The duration of the experiment was 7 days, which, in our opinion, corresponds to acute and sub-acute stress.

The animals were removed from the experiment by decapitation after introduction into drug induced sleep by means of ether. The thyroid gland was isolated by dissection of the anterior region of the neck and by cutting off the larynx. Laboratory rats RattusNorvegicus have the thyroid gland with two lobes, located on the sides of the trachea, without isthmus. The lobes were cut off with a scalpel and placed in a fixator for light microscopy. The tissue was fixed in 10% neutral buffered formalin within 18 hours. The wiring and impregnation with paraffin were carried out, followed by the manufacture of multiblock for serial paraffin sections with a thickness of 4 microns. Used histological laboratory equipment: cutting station LEEC ltd (Leica, Germany), hybrid histological processor LOGOS (Millestone, Italy), modular center for pouring Leica EG 1150 (Leica, Germany), automatic rotary microtome Leica RM 2255 (Leica, Germany).

Three sectios were prepared from each block, one was stained with hematoxilin and eosin (Bio-Vitrum, Russia) and IGX reaction with monoclonal mouse antibodies to Ki-67 (Leica, Germany) and Fas-R (Abcam, USA). Concentrated anti-FAS-R antibodies (ab82419) were diluted with Bond solution for antibody dilution (Leica, Germany) 100 times, incubated at room temperature for 60 minutes. HIER-unmasking was performed using Bond solution for high-temperature unmasking pH 6.0 at a temperature of 96ºC for 20 minutes. For imaging, a NovolincPolimer Detection System based polymer detection system (Leica, Germany) was used. For setting IHC reaction with the Ki-67 marker (MM1), the staining protocol recommended by the manufacturer was used.

The laboratory microscope Leica DM2000 (Leica,Germany), digital scanner Aperio CS2 (Leica,Germany), immunohistosteiner of semi - closed type BOND - MAX (Leica,Germany) were used to obtain images.

We also used methods for calculating the number of cellular components of thyroid tissue and biostatistical methods to identify the significance of quantitative parameters of expression of the studied proteins-markers of cell renewal. The proliferation index considered by the percentage of the thyroid cells with nuclear staining for Ki-67, cytoplasmic marker of Fas-R was determined semiquantitatively by translation of quality reaction into a numeric way of identifying cases of different colors and compare them in groups according to the method Kraskel-Uollis, adopted with significant value $p \leq 0.05$ was also used multivariative analysis ANOVA.

The studies were conducted in the Histological laboratory with immunohistochemistry and electron microscopy of the Central research laboratory of the medical Academy named after S. I. Georgievsky and Center for Collective Use of Scientific Equipment "Molecular Biology" of the medical Academy named after S. I. Georgievsky (structural unit) V.I. Vernadsky Crimean Federal University by the initiative of surgeons of the Crimean medical multidisciplinary center named after St. Luka (structural units) of V.I. Vernadsky Crimean Federal University.

Results and discussion

At the age of 3-3.5 months, the thyroid gland of the white rat has a low degree of differentiation due to a significant proportion of the interfollicular epithelium as a reserve pool of thyrocytes in the gland parenchyma and poor development of the follicular apparatus (Fig. 1-4). This corresponded to the age norm of the structure of this organ in small laboratory rodents. In our opinion, this feature of the gland morphology in three-month rats reflects a number of ontogenetic patterns.

1. Despite the fact that rats at this age are already sexually mature, they grew intensively with a positive protein (nitrogen) balance and a predominance of anabolic processes.
2. Reproductive function in this period was unstable and the functional relationship of the thyroid gland and gonads was one of the reasons.

The active growth and development of the organ is reflected in the marker of Ki-67 proliferation. In rats of the control group on a normal diet, the level of Ki-67 expression averaged 2.4% in females and 4.25% in males (Fig.1), such difference can be explained by more intensive growth of males in the given period in comparison with females.

In females, contained within a month on a special diet composed of calcined grain and bromine water (alimentary stress), the level of Ki-67 increased up to 3.4%, but still its value can be estimated as low. In males, on the contrary, the level of glandular epithelial cell proliferation decreased up to 2.75% (Fig.2).

Animals subjected to immobilization stress within 7 days had significant ($p \leq 0.05$) changes in Ki-67 expression with opposite orientation depending on gender. Thus, in stressed females the degree of Ki-67 expression reached an average of 11.25%, which is 5 times higher than the control. In males, on the contrary, this value fell up to 1.5% (Fig. 3).

In the group of animals with a combination of

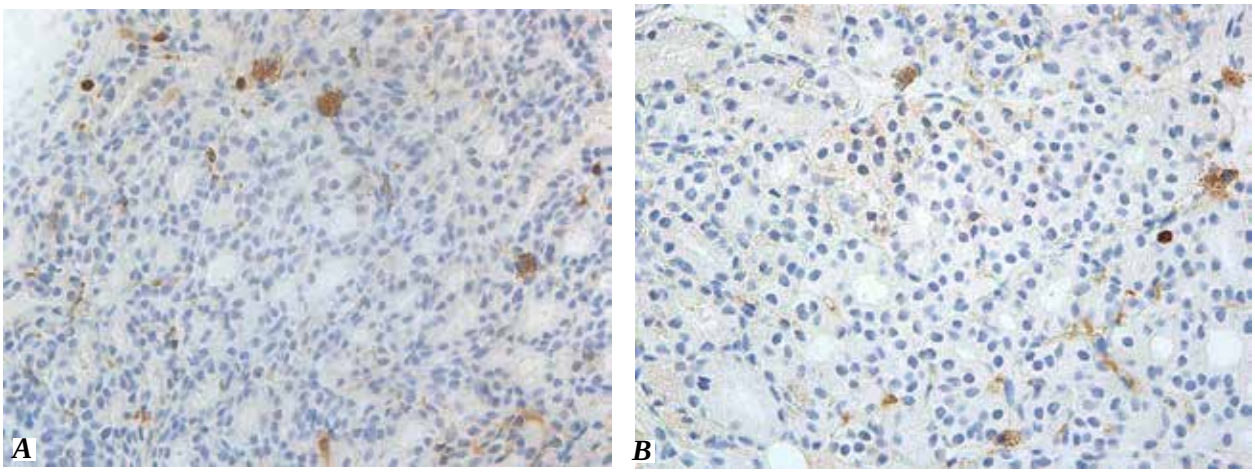

Figure.1. *Fragments of the thyroid gland of a white rat at the age of early puberty. Paraffin sections. HC. 200x. IHC reaction with antibody to Ki-67. Control group.* ***A****. Male.* ***B****. Female.*

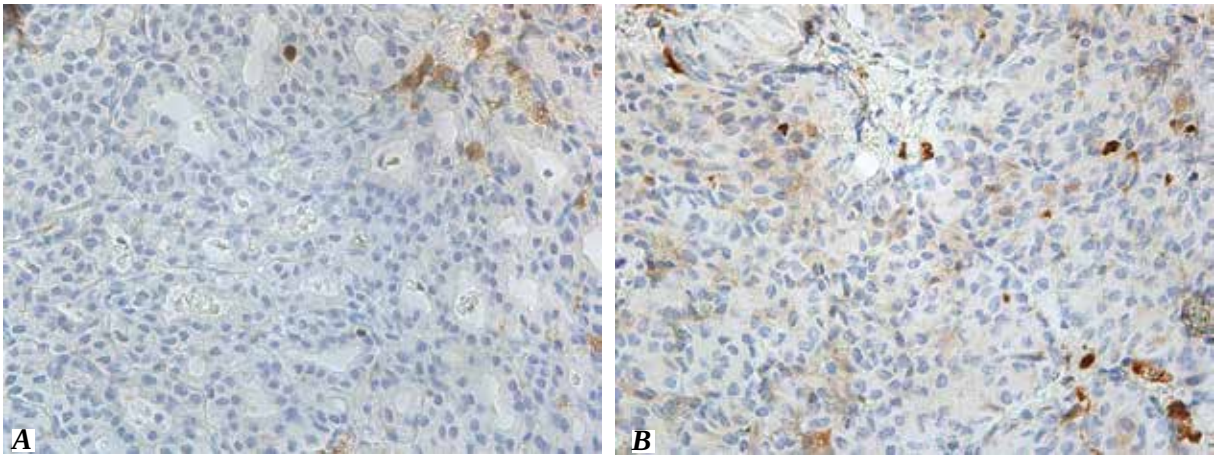

Figure. 2. *Fragments of the thyroid gland of a white rat at the age of early puberty. Paraffin sections. HC. 200x. IHC reaction with antibody to Ki-67. The experimental group-alimentary stress.* ***A****. Male.* ***B****. Female.*

stress contained in a deficit diet and immobilization, females did not live to the end of the experiment, and in males the Ki-67 index fell even lower: up to 1.3 %(Fig. 4).

Multiple comparisons between groups of animals showed significant differences between the group of females subjected to stress and on a normal diet, from all groups, except for a similar group of males (by the Kraskel-Uollis method at a value of $p \leq 0.05$). Multivariative analysis ANOVA showed a high degree of influence (96.7%) of animal gender on the proliferation of glandular epithelium, as well as stress (54.28%).Determining the expression of apoptosis markers FAS-R on the thyrocytes allowed us to reveal certain regularities.In groups of animals kept on a special diet, the expression of FAS receptors in females decreased by 40% relative to the control group (mainly cytoplasmic weak staining 60% of cells, up to 10% of the nuclei had a positive color), while in males the indices practically did not change and amounted to about 90% of poorly stained cells. After a 7-month immobilization stress, 100% of the glandular epithelium cells had a medium intensity color of the cytoplasm and nuclei.(Fig. 5).

Comparison of the results of the study of proliferative activity (Ki-67 marker) and apoptotic activity markers of CD 95-FAS-R receptors of thyrocytes in laboratory animals subjected to 7-day combined stress allows us to draw conclusions about the different response of the follicular epithelium to the experimental conditions in males and females. Apparently, such differences are due to different developmental tasks set for the individual. Moreover, if Ki67- reflects the degree of adaptation of the thyroid tissue by active division in females subjected to experimental influence, CD 95-FAS-R reflects the degree of tension of the

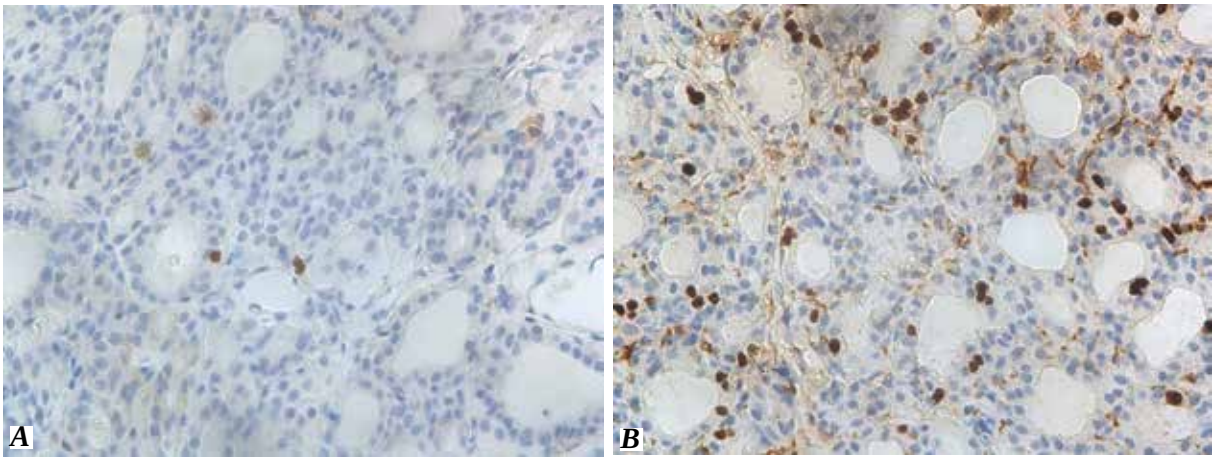

FIGURE. 3. *Fragments of the thyroid gland of a white rat at the age of early puberty. Paraffin sections. HC. 400x. IHC reaction with antibody to Ki-67. The experimental group-alimentary stress.* ***A****. Male.* ***B****. Female.*

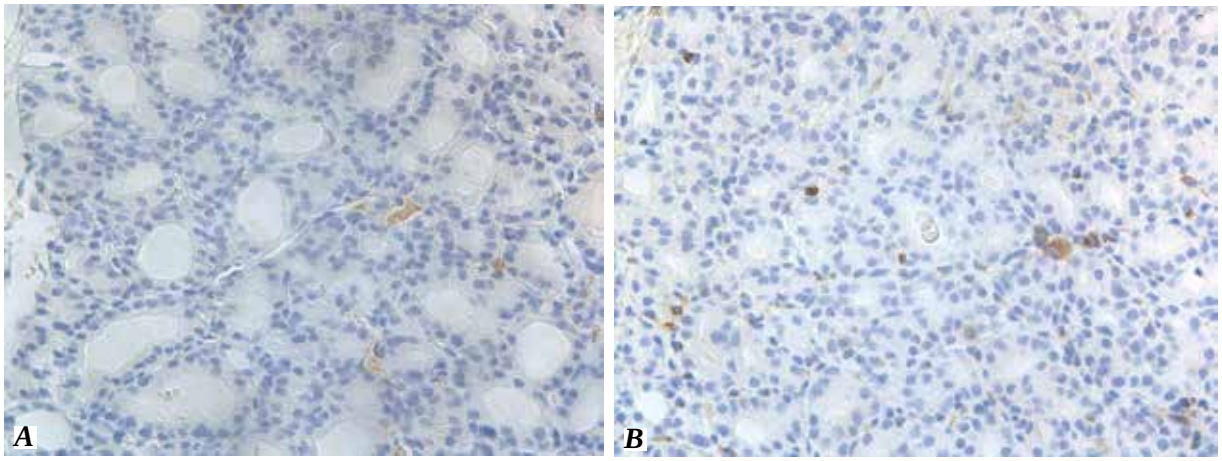

FIGURE. 4. *Fragments of the thyroid gland of a white rat at the age of early puberty. Paraffin sections. HC. 400x. IHC reaction with antibody to Ki-67. The experimental group-alimentary stress.* ***A****. Male with a higher degree of differentiation of the thyroid parenchyma.* ***B****. Male with lower degree of epithelial differentiation.*

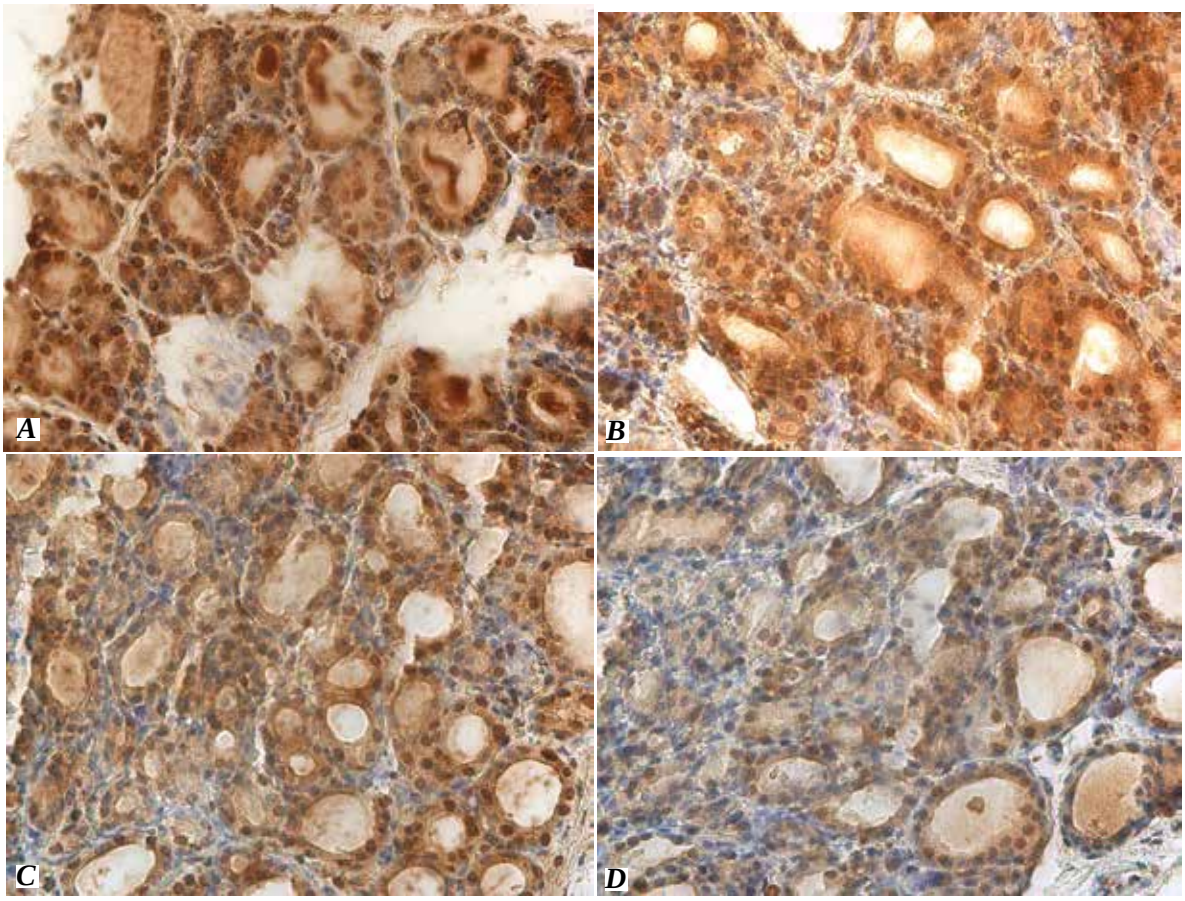

Figure. 5 *Fragments of the thyroid gland of the white rat at the age of early sexual maturity. Paraffin sections. HC. 400x. IHC reaction with antibody to FAS-R. Experimental group - alimentary stress:* ***A****. Male.* ***B****. The female. Experimental group - immobilization stress:* ***C****. Male.* ***D****. Female.*

metabolic processes in thyrocytes and readiness for apoptosis arising under the experimental conditions [*Cruz A.C. et al., 2016*]. It is likely that the prolongation of the timing of the impact of stress factors will make it possible to further deplete and strain the compensatory mechanisms in the thyroid gland, up to the development of failures in the genetic apparatus of the thyrocyte and the appearance of autoimmune and oncopathologies [*Markosyan R et al., 2015*]. These findings are planned to be tested in an experiment on laboratory animals and operating material of patients with various thyroid pathologies.

Conclusion

There is a direct link between external stressors and the development of functional and organic compensatory changes in the thyroid gland, which indicates the leading pathogenetic role of adaptation syndrome in the development of organ pathology.

High sensitivity of the thyroid gland to many aggressive environmental factors is manifested by functional and morphological changes in its tissues and causes the growth of thyroid pathology in the population.

There is a pronounced sexual dimorphism of the studied parameters, which can cause different morbidity in men and women.

The change in the indices of Ki-proliferation index and CD - 95 or FAS-R in experimental animals significantly indicates the role of multiple stress in the development of thyroid pathology.

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REFERENCES

1. *Asif F, Noor-Ul-Amin M, Ahmad MR.* Identification of the risk factors of the thyroid cancer by using logit model in Lahore. Pakistan Journal of Pharmacutical Science. 2018; 31(6): 2391-2396.
2. *Bezrukov OF.* [Nodular goiter in the Crimean region: etiological problems and diagnostic prospects] [Published in Russian]. Krymskii zhurnal eksperimental'noi I klinicheskoi meditsiny. 2011; 3-4: 171-178.
3. *Bezrukov OF.* [Operations on the thyroid gland. Tasks and unsolved problems] [Published in Russian]. Vestnik khirurgii imeni I.I. Grekova. 2015; 1: 73-74.
4. *Bil'zhanova GZh, Chekurov IV, Vishnevskaia T.* [Morphofunctional profile of the thyroid gland of male wistar rats in the framework of the experimental model "hypothyroidism – stress"] [Published in Russian]. Izvestiia Orenburgskogo gosudarstvennogo agrarnogo universiteta. 2016; 2(58): 177-180.
5. *Cruz AC, Ramaswamy M, Ouyang C., et al.* Fas/CD95 prevents autoimmunity independently of lipid raft localization and efficient apoptosis induction. Nature Communications. 2016; 23(7): 13895.
6. *Fliers E, Bianco AC, Langouche L, Boelen A.* Thyroid function in critically ill patients. Lancet Diabetes Endocrinol. 2015; 3(10): 816-825.
7. *Gevorkian VS, Gevorkiana IS.* [Current research on the effects of various stressors on rats and mice] [Published in Russian]. Al'manakh Prostranstvoi Vremia. 2017; 15(1): 9.
8. *Jasenjavskaja AL, Samotrueva MA, Luzhnova SA.* [The effect of antioxidants on the morphometric parameters of the thyroid gland of different age rats under immobilization stress] [Published in Russian]. Biomedicina. 2014; 3: 78-82.
9. *Jasenjavskaja AL.* [Study of the effect of immobilization stress and antioxidants on the hormonal activity of the thyroid gland of white rats at different stages of ontogenesis] [Published in Russian]. Vestnik Nizhegorodskogo universiteta im. N.I. Lobachevskogo. 2010; 2: 689-693.
10. *Korshunova DV, Gizzatullina EA, Bryndina IG.* [The impact of chronic psychosocial stress on the intensity of degenerative changes in the retina in rats with different stress-resistance] [Published in Russian]. Izvestiia Samarskogo nauchnogo tsentra Rossiiskoi akademii nauk. Sotsial'nye, gumanitarnye, mediko-biologicheskie nauki. 2014; 16(5-4): 1215-1218.
11. *Krishna V.N. et al.* Association between bipolar affective disorder and thyroid dysfunction. Asian Journal of Psychiatry. 2013; 6(1): 42-45.
12. *Liu H, Hou C, Zeng Q., et al.* Role of endoplasmic reticulum stress-induced apoptosis in rat thyroid toxicity caused by excess fluoride and/or iodide. Environmental Toxicology and Pharmacology. 2016; 46: 277-285.
13. *Markosyan RL. Aghajanova EM, Bayburdyan GM, Navasardyan LV.* Combination of autoimmune endocrinopathy and non-endocrine disease of autoimmune genesis: a rare case in pediatric practice. The New Armenian Medical Journal. 2015; 9(3): 89-92.
14. *Nadol'nik LI.* [Stress and Thyroid] [Published in Russia]. Biomeditsinskaia khimiia.2010; 56(4): 443-456.
15. *Pompella A.* Review Agents and Mechanisms of Oxidative Stress in Human Pathology. The New Armenian Medical Journal. 2013; 7(2): 4-14.
16. *Stiazhkina SN, Kolesnikova ET, Liukina AN.* [The incidence of thyroid cancer in comparison with other thyroid pathologies in women] [Published in Russian]. Sinergija Nauk. 2018; 22: 1287-1289.
17. *Uribe RM, Jaimes-Hoy L, Ramírez-Martínez C.* Voluntary exercise adapts the hypothalamus-pituitary-thyroid axis in male rats. Endocrinology. 2014; 155(5): 2020-2030.
18. *Ye J, Zhong X, Du Y, Cai C, Pan T.* Role of levothyroxine and vitamin E supplementation in the treatment of oxidative stress-induced injury and apoptosis of myocardial cells in hypothyroid rats. Journal of Endocrinological Investigation. 2017; 40(7): 713-719.
19. *Zhang J, Huang J, Aximujiang K, Xu C, Ahemaiti A, Wu G, Zhong L, Yunusi K.* Thyroid Dysfunction, Neurological Disorder and Immunosuppression as the Consequences of Long-term Combined Stress. Scientific Reports. 2018; 8(1): 4552.