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# IMMUNOHISTOCHEMICAL PARAMETERS OF TGAB AND FOX–1 EXPRESSION IN THE THYROID GLAND OF RATS AFTER PRENATAL ANTIGEN EXPOSURE

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The results were obtained about features of thyroglobulin and Fox–1 expression in rat's thyroid, as immunomorphological markers of thyroid morphogenesis, the formation of the synthetic apparatus and its functional activity, after prenatal antigenic action by staphylococcal toxoid. In the process of morphogenesis and establishment of the synthetic function of the thyroid gland after the prenatal action of staphylococcal toxoid, two aberrant intersections in nuclear and cytoplasmic Fox–1 expression were detected among themselves and with indicators of proliferative activity. In the thyroid gland, such aberrant intersections occur from the 3–7 days and the 14–21 days, which indicates the intensification of the processes of establishment and normalization of structural and functional units of the thyroid gland. These data correlate with the expression of thyroglobulin, both cytoplasmic and colloidal, which is respectively expressed by signs of secretory inversion of thyroglobulin first with morphological signs of hypofunction up to 14 days, which then change to hyperfunctional.

**Keywords:** proliferation, thyroid follicles, thyrocytes, antigen prenatal load, experiment, rats, thyroglobulin.

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# ІМУНОГІСТОХІМІЧНІ ПОКАЗНИКИ ЕКСПРЕСІЇ TGAB ТА FOX–1 У ЩИТОПОДІБНІЙ ЗАЛОЗІ ЩУРІВ ПІСЛЯ ПРЕНАТАЛЬНОГО ВПЛИВУ АНТИГЕНУ

Отримані результати щодо особливостей експресії тироглобуліну та Fox–1 у щитоподібній залозі щурів, як імуноморфологічних маркерів морфогенезу щитоподібної залози, формування синтетичного апарату та його функціональної активності після пренатальної антигенної дії стафілококового анатоксину. У процесі морфогенезу та встановлення синтетичної функції щитоподібної залози після пренатальної дії стафілококового анатоксину було виявлено 2 аберрантні перехрести ядерної та цитоплазматичної експресії Fox–1 між собою та з показниками проліферативної активності. У щитоподібній залозі такі аберантні перехрести відбуваються з 3–го по 7–й день та з 14–го по 21–й, що свідчить про активізацію процесів встановлення та нормалізації структурно–функціональних одиниць щитоподібної залози. Ці дані корелюють з експресією тиреоглобуліну, як цитоплазматичною, так і колоїдною, що виражається відповідно ознаками секреторної інверсії тиреоглобуліну спочатку з морфологічними ознаками гіпофункції до 14 доби життя, які потім змінюються на гіперфункціональні.

**Ключові слова:** проліферація, фолікули щитоподібної залози, тироцити, пренатальне антигенне навантаження, експеримент, щури, тироглобулін.

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Thyroid gland is an important endocrine organ. The action of its hormones variously aimed at all the metabolic processes of many organs and tissues, including fetal development, the growth and differentiation of tissues. Thyroglobulin is a large preprotein containing multiple glycosylation sites. Located in the thyroid gland, thyroglobulin is the precursor of the iodinated thyroid hormones thyroxine and triiodothyronine. Thyroglobulin monomers undergo conformational maturation in the endoplasmic reticulum, prior to forming dimers. Defects in thyroglobulin are known to cause some types of goiter (an enlargement of the thyroid gland). This condition is thought to result from defective dimerization and transport of thyroglobulin to the Golgi complex [2, 9].

Fox-1, also known as A2BP, A2BP1 or HRNBP1, is a 397 amino acid protein that localizes in both the nucleus and the cytoplasm and contains one RRM domain. Expressed predominately in muscle and brain tissue, Fox-1 interacts with Ataxin-2 and functions as an RNA-binding protein that regulates alternative splicing events during erythropoiesis, specifically by binding to 5′-UGCAUGU-3′ DNA elements. Multiple isoforms of Fox-1 exist due to alternative splicing events. The gene encoding Fox-1 maps to human chromosome 16, which encodes over 900 genes and comprises nearly 3 % of the human genome [6, 8], but the features of its immunohistochemical expression in the thyroid gland, especially under morphological changes are insufficiently studied.

According to the literature of recent years, special attention of scientists is focused on studying the effects of various infectious factors on the body of a pregnant woman, which would affect the health and development of the child after birth [3, 4, 7]. Antigenic load at critical moments of ontogenesis can cause significant "failures" in the child's immune system [7, 11]. The consequences of infections transmitted by

the mother may be the initiation of morphological changes in organs and barrier structures in the child, as these infections may not lead to permanent changes in structure, but leave a permanent immunological "background" in the body and the body as a whole, initiate pathological conditions, including autoimmune diseases, etc. [12, 14, 15]. This effect is achieved in various ways: immunologically, by modifying the subsequent immunologically dependent development of structures, or morphologically, by direct damage caused by infection. Features of the interaction of the fetus or newborn with exogenous antigens may be crucial for the formation of their immune status in the future. Under the influence of prenatal antigenic load there is a premature release of T–lymphocytes from the thymus, which in the tissues change not only the timing of development of structural elements, but also their immunological tolerance, etc. [5, 10, 13]. Recently, many works have been published on the development of various organs under prenatal antigenic loads, including staphylococcal toxoid, but the morphogenesis and features of the formation of the synthetic apparatus of thyrocytes in these works have not yet been disclosed [1, 2, 14].

**The purpose** of the study was to evaluate the immunohistochemical parameters of TgAb and Fox–1 expression in the thyroid gland of rats after prenatal exposure to staphylococcal toxoid.

**Materials and methods**. In the experimental study, the material were thyroid glands of Wistar rats aged 1 to 21 days of postnatal development (108 animals), 6 animals in each group, which were kept in the standard conditions of the vivarium of Zaporizhzhia State Medical University. All manipulations were performed in compliance with the basic principles of experimental animals managing in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1985), the Helsinki Declaration of the World Medical Association General Assembly (2000), "Ethical Principles of Animal Experiments", adopted by the First National Congress on Bioethics (Kyiv, 2001), the Law of Ukraine "On Protection of Animals from Cruelty" (dated 21.02.2006 № 3447–IV, edition dated 09.12.2015, grounds 766–19 ).

The animals of three groups were studied on 1, 3, 7, 11, 14, 21 days after birth: group I – intact animals (norm); group II – control, animals which were subject to intrauterine injections of 0.9 % NaCl solution; group III – experimental animals injected with staphylococcal toxoid liquid purified adsorbed (10–14 units of binding in 1 ml, diluted 10 portions) by operation intrauterinely on the 18th day of dated pregnancy. Injections of antigen or 0.9 % NaCl solution for fetus were performed surgically during laparotomy, by intrauterine, transdermal subcutaneous in interscapular region at the dose of 0.05 ml to each fetus.

Using standard methods, the material was imbedded in paraffin blocks, of which sections 4 μm thick were made and stained with hematoxylin and eosin. Immunohistochemical study was performed according to the protocol recommended for a particular antibody of the manufacturer with restaining of the nuclei with Mayer hematoxylin. Thyroglobulin Antibody (1D4) (Tg Ab) was used for visualization localization of thyroglobulin, Fox-1 Antibody (A-12) (Fox-1 Ab) was used for detection of the Fox-1 protein by Santa Cruz Biotechnology, Inc. (USA).

The result was considered positive in the precipitation of chromogen salts in the form of a specific reaction (nuclear, cytoplasmic reaction depending on the location of the antigen). The intensity of benzidine label deposition was assessed by photographic digital morphometry using Image J in each case in 5 standardized microscope spaces with magnification of 400 (lens x40, eyepiece x10).

A set of morphometric studies was performed by microscope Carl Zeiss Primo Star (Germany) with the Axiocam (Germany) digital microphoto using software complex Zeiss Zen 2011 (Germany). Statistical processing of the results was performed in the "STATISTICA"® software for Windows 6.0" (StatSoft Inc., USA). Hypothesis about the distribution normality of the studied indices were checked by the Shapiro–Wilk test. The median lower and the upper quartile was calculated, the data were presented as Me (Q1 ; Q3). For all types of analysis differences were considered significant at $p<0.05$.

**Results of the study and their discussion**. Newborns (early dairy) period (1–7days). In the thyroid glands of neonatal animals that were prenatal immunologically stimulated with staphylococcal toxoid, the follicular structure was morphologically better developed (fig. 1 b) comparing to the animals from the control and intact groups (fig. 1 a). The total number of thyrocytes per unit area at birth did not differ significantly in the experimental and control groups and were 129.53 (118.07; 133.64) and 131.21 (127.48; 135.81), respectively, but the number of proliferative clusters (9.33 (8.68; 10.04) and 6.43 (5.58; 6.92) respectively) and cells with Fox-1 expression were statistically significant (fig. 3). Expression of antibodies to thyroglobulin in thyrocytes was much more pronounced than in colloid of the thyroids in animals of an experimental group. In some thyrocytes in peripheral follicles, intensive nuclear and cytoplasmic reaction with Fox-1 Ab was visualized (fig. 1 e). On the 3rd day after birth in the peripheral parts of rat's thyroid glands of control group follicles of colloidal type secretion began to appear. They were characterized by

well-defined reaction with Tg Ab and parietal vacuolation of colloid (fig. 1 c). In experimental animals of 3 days life there was a decrease in the specific area of the thyroid epithelium, due to the appearance of flattened thyrocytes and large follicles on the periphery of the thyroid lobes (fig. 1 d). The total number of thyrocytes per unit area decreased in the control (126.07 (123.69; 129.31)) and experimental (97.58(92.88; 99.72)) groups of animals due to the increase in the number and size of hollow follicles per unit area. An increase in the relative percentage of the area of the colloid due to an increase in the number of large and medium-sized follicles containing dense colloid, non-intensive $Tg^+$ and desquamated cells were detected.

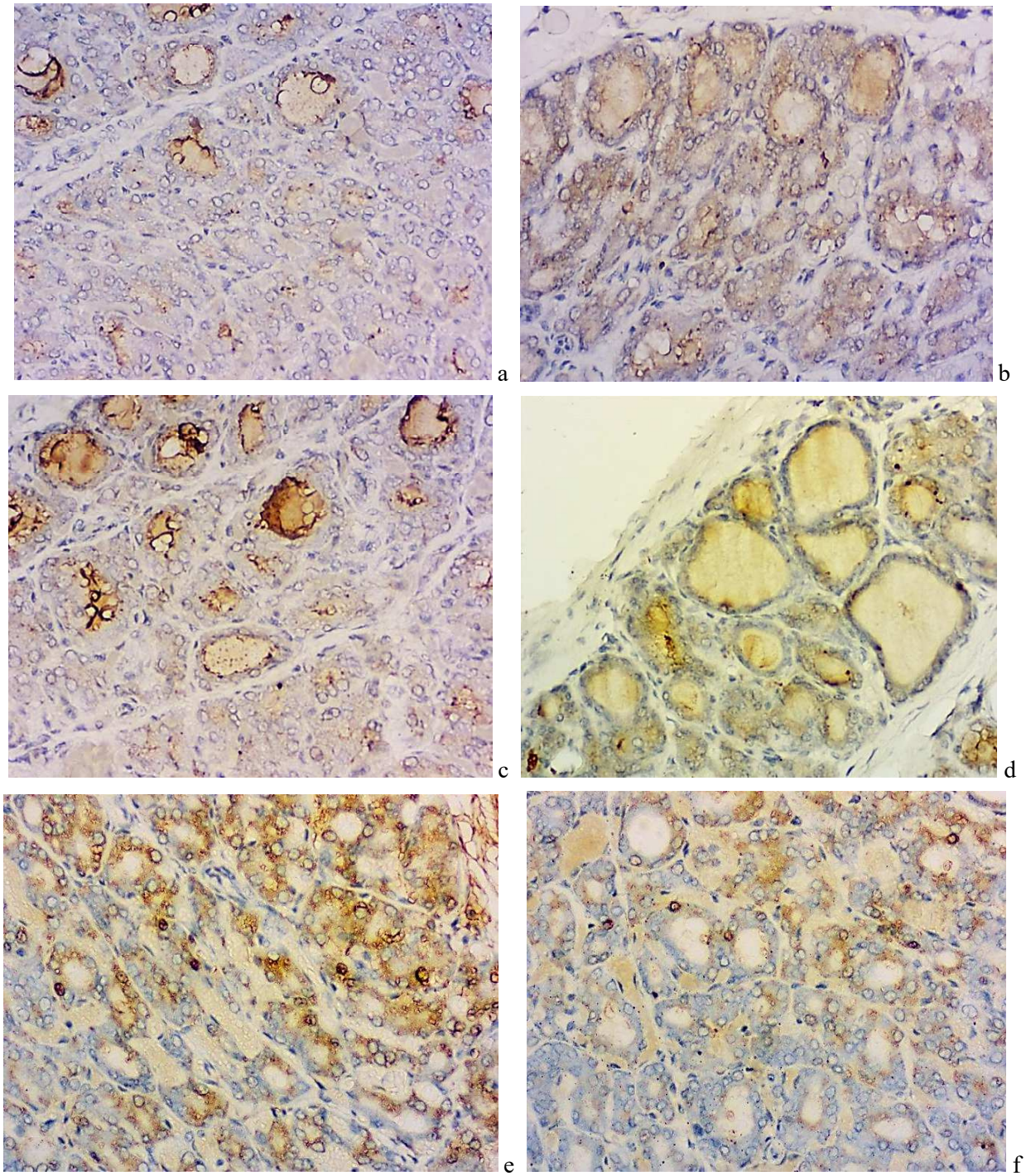


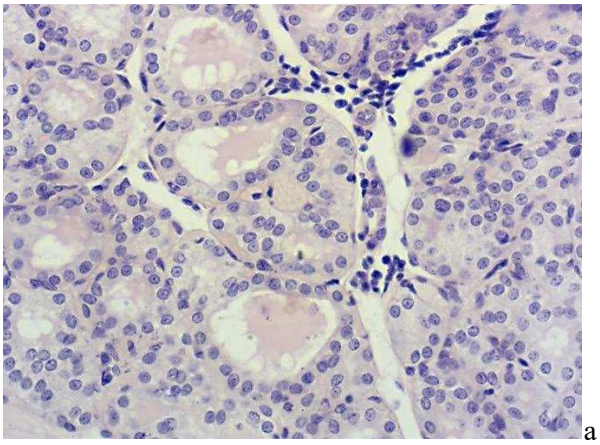
Fig. 1. Immunohistochemical reaction with thyroglobulin and Fox–1 antibodies in rat's thyroid glands of control and experimental groups. a – control group 1st day after birth, Tg Ab; b – experimental group 1st day after birth, Tg Ab; c – control group 3rd day after birth, Tg Ab; d – experimental group 3rd day after birth, Tg Ab; e – control group 1st day after birth, Fox–1 Ab; f – experimental group 1st day after birth, Fox–1 Ab. Magnification × 600.

The thyrocytes of such follicles were $Fox–1^-$(fig. 1 f). In such follicles pre-wall vacuolation of colloid is absent. On 7th day of the postnatal period of life in the peripheral part of the thyroid gland appears lymphoid infiltration, which is formed by diapedesis of lymphocytes from the venules of the subcapsular and interparticle layers of connective tissue. Extrafollicular proliferation of thyrocytes began in the follicles

surrounded by lymphocytes (fig. 2 a). Cytoplasmic expression of Fox-1 Ab and nuclear expression in single thyrocytes were detected in extracellular clusters of thyrocytes. Cytoplasmic reaction with TgAb was absent in the extrafollicular clusters. The cells of such proliferative clusters were cylindrical, their nuclei were located in the basal part, and had large depths of premembrane chromatin and several nucleoli. Due to this, the total number of thyrocytes per unit area increased compared to the previous period of the experiment by 1.18 and in comparison with the control – by 1.09 and amounted to 114.51 (112.73; 116.08). In such follicles weak expression of antibodies to thyroglobulin was found both in thyrocytes, and in the colloid.

At the same time, taking into account the area of the entire thyroid gland in serial sections, it should be noted that Fox-1 expression in the thyroid glands of intact and control groups was uniform over the entire area (fig. 1 e), and in the experimental group was mosaic, mainly on the periphery of the gland and local groups of follicles (fig. 1 f).

Sucking (mean dairy) period (11–21 days). In the animals of the experimental group from the 7$^{th}$ to the 21$^{st}$ day of the postnatal period of life there was a more rapid rate of folliculogenesis and the development of the synthetic apparatus of thyroid as in the control group. Follicles of mostly medium and large size without colloid vacuolisation, lined with cuboid thyroid epithelium, were either laced with epithelial strands or formed intrafollicular clusters, but in both cases there was an intense cytoplasmic and nuclear reaction with Fox-1 Ab in such follicles. On the 14$^{th}$ day, clearly formed, mostly single, lymphoid nodules were visible (fig. 2 b), which contained vaguely formed capsule. In the particles adjacent to the lymphoid nodules in the colloid of follicles, desquamated cells of the thyroid epithelium were often visible, while the shape of the follicles, the integrity of the epithelium and the basement membrane were not impaired.


a

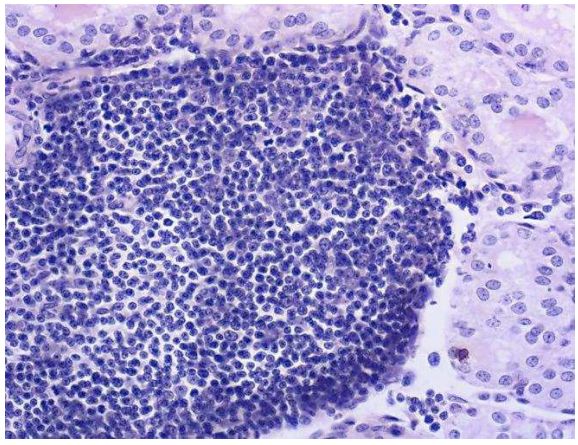

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Fig. 2. Thyroid gland of rats prenatally immunized with staphylococcal toxoid at the 7$^{th}$ (a) and 14$^{th}$ (b) days after birth. Stain by hematoxilin & eosin. a – magnification: × 600. b – magnification: × 300.

On the 11$^{th}$ day of postnatal life, the total number of thyrocytes and proliferative clusters were maximum (134.38 (130.95 – 136.89) and 28.40 (25.63 – 32.04) respectively) in the thyroid glands of experimental animals that prenatally received antigenic load on the body with staphylococcal toxoid, and greater than identical values by 1.37 and 5.6 times, respectively.

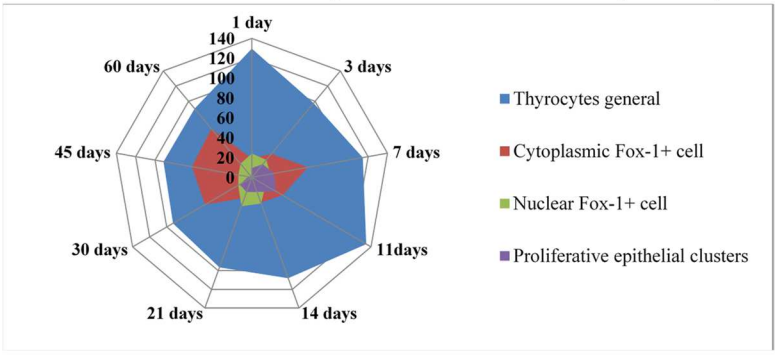
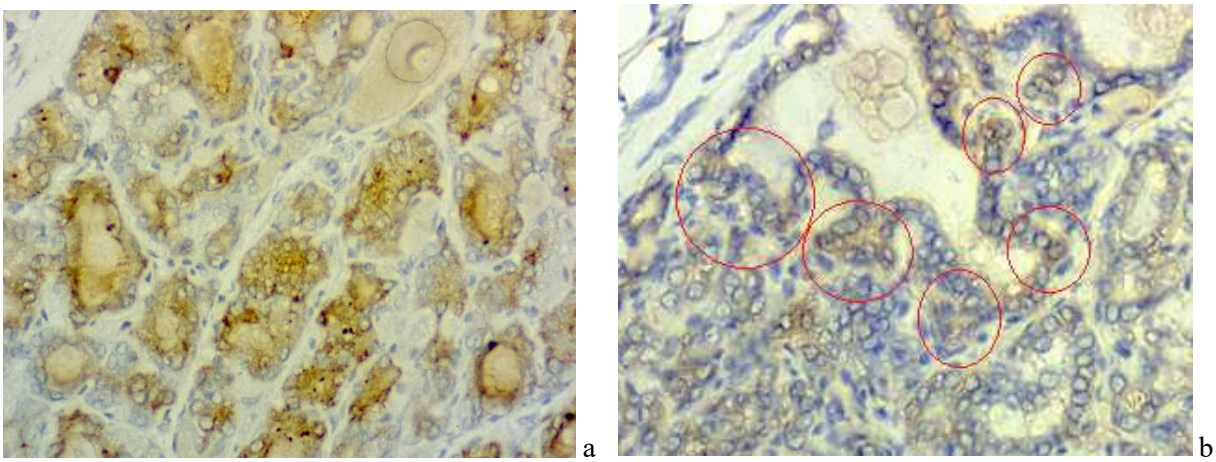



Fig. 3. Indices of cell composition, proliferation and expression in the peripheral part of the thyroid gland after prenatal exposure to staphylococcal anatoxin per unit area of 10000 μm, M±m; p<0.05.

Also on the periphery of the gland appeared large follicles, the wall of which was represented by flattened thyrocytes, with low $Tg^{+}$ colloid without signs of resorption and negative Fox-1 Ab reaction. The correlations of the values of the studied features of the thyroid gland in this period can be clearly seen in fig. 3.

Part of such follicles from the morphologically unchanged follicular epithelium was laced with strands of thyrocytes with a basement membrane that grew along the axis of cell division (fig. 4 a). In such epithelial intrafollicular intussusception, cytoplasmic and single nuclear Fox-1 expression were determined. Immunohistochemical reaction with antibodies to thyroglobulin in such thyrocytes and follicles was negative or weak (fig. 4 a). By the end of this period, in the thyroid glands of animals that

prenatally received immunostimulation with staphylococcal toxoid, the ratio of the expression intensity of thyroglobulin antibodies was stronger in the colloid than in the cytoplasm of thyrocytes throughout the gland. On the 21st day of life in large follicles, the squamous epithelium changed to cuboid, intense vacuolation of colloid and intussusception in the wall of the follicle, which acquired a folded structure (fig. 4 a). Mostly in the locations of extrafollicular intussusception in thyrocytes a strong cytoplasmic Fox-1 Ab reaction was visualized (fig. 4 b). At the same time, these thyrocytes and the adjacent colloid had a negative immunohistochemical reaction with antibodies to thyroglobulin.



a b

Fig. 4. Immunohistochemical reaction with thyroglobulin and Fox–1 antibodies in rat's thyroid glands after prenatal antigenic load with staphylococcal toxoid at the sucking (average dairy) period. a – immunohistochemical reaction with Tg Ab; b – immunohistochemical reaction with Fox-1 Ab. Magnification × 600.

After prenatal antigenic action of staphylococcal toxoid on the body there is a restructuring and proliferation of constituent structural elements at both organ cellular and subcellular levels. The most intense changes in the proliferation and intracellular formation of the synthetic apparatus takes place from the 7th to the 14th day, which persists with the terms of intensive formation of intra–organ lymphoid tissue and lymphoid nodules. Thus, the total number of thyrocytes per unit area increased and the number of prolirative buds with maximum values on the 11th day after birth after prenatal immunostimulation by staphylococcal toxoid. This trend is confirmed by studies of other scientists who fit into the general concept of organ morphogenesis under prenatal antigenic stimulation and the role of lymphocytes and in this process as a factor of morphogenesis [1, 2, 14]. The occurrence of positive cytoplasmic and partially nuclear expression of Fox-1 Ab in newborns after prenatal influense of staphiloccocal toxoid in thyrocytes of extracellular proliferative clusters indicates the formation of a synthetic apparatus of thyrocytes aimed at the production of specific thyroid protein thyroglobulin. Fox-1 interacts with ataxin 2 and functions as an RNA-binding protein that regulates splicing processes during thyrocyte differentiation [2, 6, 12]. That is, when mRNA matures, exons that encode thyroglobulin synthesis bind due to Fox-1. Thus, immature pre–mRNA is converted into mature mRNA from which thyroid proteins, in particular thyroglobulin, are translated. The fact, that Tg Ab positive expression is more intense intracytoplasmic than in colloid, indicates a inversion of synthetic producing of thyroglobulin [9]. A similar phenomenon of aberrant expression of thyroglobulin under experimental conditions in the literature is not described, as the peculiarities of the distribution of Fox–1 in the thyroid gland, but although the peculiarities of Fox-1 expression are described by researchers in other tissues and organs. In such processes, thyroglobulin positive reaction in cytoplasmic vacuoles is associated with glycoprotein complexes formed as a result of degradation of thyroglobulin. Expression of Fox–1 is mosaic in mainly follicles on the periphery of the gland. These changes indicate different intensities of RNA protein accumulation, and, consequently, a change in the functional properties of follicles of different diameters. Thus, summarizing the data on morphofunctional changes in the thyroid gland in the experimental intrauterine action of staphylococcal toxoid, we can say that the transformations at the tissue, cellular and subcellular levels are unidirectional inverse and reflect the reactive changes occurring in the body.

Thyroid folliculogenesis accelerates, but despite this, in the early dairy period on serial sections revealed morphological signs that indicate a decrease in the functional activity of the organ. Among them: an increase in the number of large and medium–sized follicles, a decrease in the height of the follicular epithelium, the lack of marginal vacuolation of the colloid. These data correlate with transformations at the cellular level, namely, there is a decrease in the number of nucleoli and a decrease in the concentration of RNA in the cytoplasm of thyrocytes, which indicates the suppression of synthetic processes occurring in

the cell. With the advent of lymphocytic infiltration with the onset of the middle dairy period in the thyroid gland in large and medium follicles increases the height of the thyroid epithelium, the number of nucleoli in the nuclei of thyrocytes, increases regenerative– desquamation processes, begins resorption of colloid and active production of hormones in the blood by morphologically formed structures. Such abrupt changes in the thyroid gland of experimental animals are due to systemic prenatal antigenic influence of the body as a whole and has an adaptive–compensatory nature, both on the part of the immune system and the thyroid gland, because its hormones directly affect the body's development under endogenous and exogenous factors confirmed by other studies [1, 2, 4, 8, 9]. Thyroid transformation under prenatal antigenic influence on the fetus is an immunologically dependent dynamic process with sequential deployment and change of regular stages with the release of a polymorphnoplastic variant of the thyroid gland in dairy rats, and coincides with the concept of studies of various organs under antigenic conditions.

## Conclusion

In the process of establishing synthetic function during thyroid morphogenesis after prenatal action of staphylococcal toxoid, two aberrant intersections of nuclear and cytoplasmic Fox-1 expression were detected with each other and with indicators of proliferative activity, which indicates activation of processes of establishment and normalization of structural function. These data correlate with the expression of thyroglobulin, both cytoplasmic and colloidal, which is respectively expressed by signs of secretory inversion of thyroglobulin. Thus, there is an adaptogenic immune–stimulated intracellular and structural rearrangement of the thyroid gland, which is configured to normalize and maintain intraorganic homeostasis and compensatory adaptive functional activity of the thyroid gland.

We suggest that this change in the rate and pathways of organ morphogenesis and aberrant morphogenetic crosses may increase the risk of developing pathological conditions of the thyroid gland under the influence of trigger factors during these periods, which is the purpose of further study. The obtained data are important for pediatricians, endocrinologists, immunologists in explaining certain etiopathogenetic aspects of autoimmune thyroiditis in children.

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