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ВПЛИВ ГІПОТИРОЗУ МАТЕРИНСЬКОГО ОРҐАНІЗМУ НА ПОСТНАТАЛЬНИЙ МОРФОҐЕНЕЗ ЯЄЧОК ЩУРІВ ЗА ДАНИМИ МОРФОМЕТРІЇ ТА ЛЕКТИНОВОЇ ГІСТОХІМІЇ

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Реферат

Захворювання щитоподібної залози нині належать до найпоширеніших ендокринних патологій, що впливають на різноманітні органи, у тому числі і яєчка. Разом із тим, вплив гіпотирозу материнського організму на постнатальний розвиток органів чоловічої репродуктивної системи потомства, включаючи їх гліком, у даний час залишається невивченим.

Мета. *Із використанням морфометричного аналізу та методів лектинової гістохімії дослідити вплив материнського гіпотирозу на постнатальний морфогенез яєчка потомства щурів.*

Матеріал і методи. *Експериментальний гіпотироз індукували у 15 самок щурів лінії Вістар шляхом додавання до їхнього добового раціону 5 мг/кг мерказолілу протягом 14 днів до запліднення та упродовж усього періоду вагітності. Яєчка отриманого від них потомства забирали на 20-й пренатальний, 1-й, 20-й, 40-й та 120-й постнатальні дні, фіксували в розчині Буена і заливали у парафін. Отримані дані морфометрії та лектинової гістохімії порівнювали з аналогічними параметрами потомства, отриманого від тварин контрольної групи.*

Результати й обговорення. *Гіпотироз материнського організму на 20-й день пренатального онтогенезу потомства обумовлював зменшення розмірів клітин Сертолі, збільшення їхньої кількості у складі сім'яних тяжів, зменшення вмісту інтерстиційної сполучної тканини у поєднанні з істотним збільшенням кількості клітин Лейдіга при одночасному зниженні реактивності лектинів з усіма структурними компонентами яєчка. На 1-й постнатальний день вміст клітин Лейдіга в інтерстиції яєчок тварин експериментальної групи також перевищував контрольні показники, хоча і в меншій мірі аніж на 20-й пренатальний день. На 20-й день постнатального розвитку розмір сім'яних трубочок потомства, отриманого від гіпотироїдних самок, значно перевищував аналогічні показники тварин контрольної групи, що було обумовлено підвищеною проліферативною активністю сперматогенних клітин. На 40-й постнатальний день сім'яні трубочки тварин експериментальної групи містили великі конгломерати дегенеративних і апоптичних сперматогенних клітин. У дорослих щурів було задокументовано зменшення розмірів сім'яних трубочок у поєднанні з підвищеним індексом сперматогенезу та вмістом клітин Лейдіга. Останні селективно маркувалися лектинами PSA, GNA та CCRA, мастоцити - лектином WGA, ранні акросоми - лектином SBA, акросоми на стадії шапочок і клинків - лектинами PNA і SNA.*

Висновки. *Материнський гіпотироз суттєво впливає на постнатальний морфогенез яєчок щурів. Відмінності у зв'язуванні лектинів зі структурними компонентами яєчок контрольних та експериментальних щурів були добре виражені на пренатальному етапі онтогенезу та нівелювались у дорослих тварин, тоді як морфометричні відмінності були достатньо чіткими у всіх вікових групах потомства. Задокументоване селективне маркування клітин Лейдіга лектинами PSA, GNA та CCRA, мастоцитів лектином WGA, акросом різного ступеня зрілості лектинами SBA, PNA та SNA може бути рекомендоване для подальшого використання у морфометричних дослідженнях.*

Ключові слова: *експериментальний гіпотироз, постнатальний морфогенез яєчок щурів, морфометрія, лектинова гістохімія*

Abstract

INFLUENCE OF MATERNAL HYPOTHYROIDISM ON THE POSTNATAL DEVELOPMENT OF RAT TESTES AS DETECTED BY MORPHOMETRIC AND LECTIN HISTOCHEMISTRY ANALYSIS

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Thyroid disorders are currently among the most widespread endocrine pathologies, influencing multiple organs, testes among others. However, the impact of maternal hypothyroidism on postnatal development of male reproductive system including its glycome currently remains obscure.

Aim. *By means of morphometric and lectin histochemistry analysis to investigate the influence of maternal hypothyroidism on postnatal morphogenesis of rat progeny testes.*

Material and Methods. *Experimental hypothyroidism was induced in 15 female Wistar rats by supplementation of their daily food allowance with 5 mg/kg of mercazolil during 14 days prior to fertilization and during whole gestational period. Testes of their progeny on prenatal day 20th, postnatal days 1st, 20th, 40th and 120th were excised, fixed in Bouin's fluid, embedded in paraffin and subjected to morphometric and lectin histochemistry investigation. The obtained data was compared with the same parameters of progeny from control group rats.*

Results and Discussion. *Maternal hypothyroidism in prenatal day 20th rats was associated with decreased size and enhanced count of Sertoli cells within the seminiferous cords; decreased amount of interstitial connective tissue combined with significant elevation of Leydig cells count and reduced lectin binding to all*

testicular components. On postnatal day 1st it was detected the increased Leydig cells content, though less prominent in comparison with prenatal period. On postnatal day 20th size of seminiferous tubules of hypothyroid rats progeny strongly exceeded control parameters due to the increased spermatogenic cells proliferation rate. On postnatal day 40th seminiferous tubules of experimental group rats contained large conglomerates of degenerating and apoptotic spermatogenic cells. In the adult rats, the decreased size of seminiferous tubules coincided with the increased spermatogenic index and content of Leydig cells. The latter were selectively labeled with PSA, GNA and CCRA, mast cells - with WGA, early acrosomes - with SBA, cap stage and wedged acrosomes - with PNA and SNA lectins.

Conclusions. *Maternal hypothyroidism has significant impact on postnatal morphogenesis of the rat testes. Differences in lectin binding to tissue samples of control and experimental rat testes were most significant in prenatal animals with certain levelling until adulthood, while morphometric indices were clearly distinct in all investigated progeny groups. Documented selective lectin labeling of Leydig cells with lectins PSA, GNA and CCRA, of mast cells with WGA, as well as different stages of acrosomal maturation with SBA, PNA and SNA can be recommended for further morphometric investigations.*

Key words: *experimental hypothyroidism, rat testes postnatal morphogenesis, morphometry, lectin histochemistry*

Introduction

Thyroid gland pathology occupies a significant place in the structure of endocrine pathologies with a trend to increase both in the world as a whole, and in Ukraine in particular [1]. Disorders of the thyroid gland affect about 3% of the world's population and the rate of increase in the number of these patients in the past 10 years remains invariably high. Hypothyroidism affects women roughly 20 times more often than men; it is often combined with pregnancy [2, 3], directly affecting children's health [3, 4, 5].

Infertility is an urgent medical and social problem, since according to the statistical data it affects about 20% of marriages, and in 50% of cases it is caused by male factor [6]. Formerly it was believed that thyroid hormones possess faint if any influence on the male reproductive system, but recent studies revealed the important role of triiodothyronine and thyroxine in testicular development and histophysiology [7]. In particular, it was established that hypothyroidism enhances proliferation and inhibits differentiation of Sertoli and Leydig cells, while hyperthyroidism has the opposite effect - inhibiting these cells proliferation and accelerating differentiation, which apparently has a significant impact on the process of testicular maturation and histophysiology of spermatogenesis [8].

Numerous publications demonstrate the important role of carbohydrate determinants - lectin receptor sites of tissue glycoconjugates - in normal histophysiology, as well as their involvement in the development of different pathologies [9-14]. In previous papers we described morphological parameters and testicular glycans profile of rat progeny that developed under physiological conditions [15, 16]. The purpose of this work - using morphometric and lectin histochemistry analysis to investigate the influence of maternal hypothyroidism on postnatal morphogenesis of offspring rat testes.

Material and Methods

The investigation was conducted on 25 female Wistar rats 180-200 g of weight, which were divided into two groups: first - control (10 animals), and second - experimental (15 animals), progeny from which included 40 and 35 rats respectively. The animals were kept under standard vivarium conditions, with maintenance of sanitary norms and diet. Experiments were conducted in agreement with the Bioethics Commission of LNMU (protocol № 2 of February 15, 2016) in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986), Council of Europe Directives 2010/63/ EU, Law of Ukraine № 3447-IV "On the Protection of Animals from Cruel Behavior".

Exogenous hypothyroidism was induced by daily supplementation of experimental group animals' food allowance with 5 mg/kg of mercazolil (Kharkiv, "Zdorovja") during 14 days prior to fertilization and throughout the whole gestational period. After the second week from the beginning of the experiment, the oestral cycle was controlled by the daily taking of vaginal smears from control and experimental rats. In the stage of oestrus rats were fertilized by intact males. The first day of pregnancy was identified by the presence of sperm in the vaginal smears. Verification of hypothyroid status included determination of T3 and T4 hormone levels in blood serum, carried out in the radioisotope laboratory of Lviv regional clinical hospital, as well as histological examination of the thyroid glands:

Table 1

Used lectins and their carbohydrate specificity

Lectin	Full name / Source	Carbohydrate specificity*
PSA	Pisum sativum agglutinin	DMan
GNA	Galanthus nivalis agglutinin	DMan
PNA	Peanut agglutinin	DGalβ(1-3)DGalNAc
CNFA	Clitocybe nebularis fungus agglutinin	DGalNAcβ(1-4)DGlcNAc > DGal
CCRA	Cyprinus carpio roe agglutinin	DGalNAc > DGal
SBA	Soybean agglutinin	αβDGalNAc
MPFA	Micena pura fungus agglutinin	DGlcNAcβ(1-2)DManα(1-6)
WGA	Wheat germ agglutinin	DGlcNAc > NeuNAc
SNA	Sambucus nigra agglutinin	NeuNAcα (2-6)DGal
LABA	Laburnum anagyroides bark agglutinin	αLFuc

** A more precise information concerning carbohydrate specificity of used lectins is presented in monographs [9, 10]. Abbreviations: DMan, D-mannose; DGlc, D-glucose; DGlcNAc, N-acetyl-D-glucosamine; DGal, D-galactose; DGalNAc, N-acetyl-D-galactosamine; NeuNAc, N-acetyl-neuraminic (sialic acid); LFuc, L-fucose*

thyrocytes of hypothyroid rats acquired columnar form; follicles contained trace amount or completely lacked colloid.

Testes from the offspring of experimental and control group animals were obtained on the 20th prenatal, 1st, 20th, 40th and 120th days of postnatal development. Histological material was fixed in Bouin's fluid and embedded in paraffin. For general morphology investigation sections 5-7 μm thick were stained with haematoxylin and eosin. Morphometric studies included evaluation of seminiferous cords (SC) and seminiferous tubules (ST) number per one field of observation, determining their area[1]), index of spermatogenesis[2]), Leydig cells covered area in one field of observation [6, 17, 18]. Micromorphology of rat testes on the subsequent stages of postnatal morphogenesis was characterized according to Parker and Picut [19], the stages of spermiogenesis were identified according to Leblond and Clermont scale [20].

Glycoconjugates of testicular structures were examined using 10 lectins of different carbohydrate specificity (Table 1), which were purified and conjugated to horseradish peroxidase by Dr.Pharm.Sci, Professor V. Antonyuk. Visualization of lectin receptor sites was performed by 3,3'-diaminobenzidine (Sigma, USA) in the presence of H_2O_2 as described earlier [15, 16]. Microscopic investigation and photographic work were performed using a "Granum R6053" photomicroscope equipped with an "Echoo-Imager 502000" camera and a "ToupView 3.7" computer program. Morphometric investigations were performed using ImageJ, statistical analysis - using Microsoft Office Excel software.

Results and Discussion

Fertility of hypothyroid rats was lower then that of control group animals: 10 rats of control group delivered 40 viable offspring, while 15 experimental rats delivered only 35 offspring (4.0 and 2.3 per individual respectively). 6% progeny of mercazolil treated group was delivered dead. Total body weight of hypothyroidism affected animals on prenatal day 20th was significantly higher (34.07±1.65 g in experimental versus 22.73±4.07 g in control group, $P<0.005$).

The obtained morphometric data are summarized in Table 2 and presented in Fig.1-7. On prenatal day 20th histological specimens of control rat testes contained 11.65±0.92 SC in one field of observation in comparison to 14.07±1.25 SC of experimental group animals. Diameters of SC were similar, namely, 57.55±2.80 μm in control and 56.56±2.37 μm in experimental group rats. The discrepancies in count of SC apparently rely on higher amount of connective tissue interstitium in control rats (Fig.1A, B). Moreover, cells filling SC of hypothyroid rat offspring were smaller and more numerous compared with control samples (Fig.1C, D). Since on this ontogenetic stage content of SC is represented primarily by Sertoli cells [19], this observation apparently relay on these cells increased proliferation rate and delayed maturation stipulated

[1]) Only cross cut seminiferous cords and tubules, which had a rounded form were taken into consideration.

[2]) Index of spermatogenesis was calculated according to the formula: IS = ΣA / N, where A was the number of spermatogenic epithelium layers in each seminiferous tubules, N - the number of tubules examined.

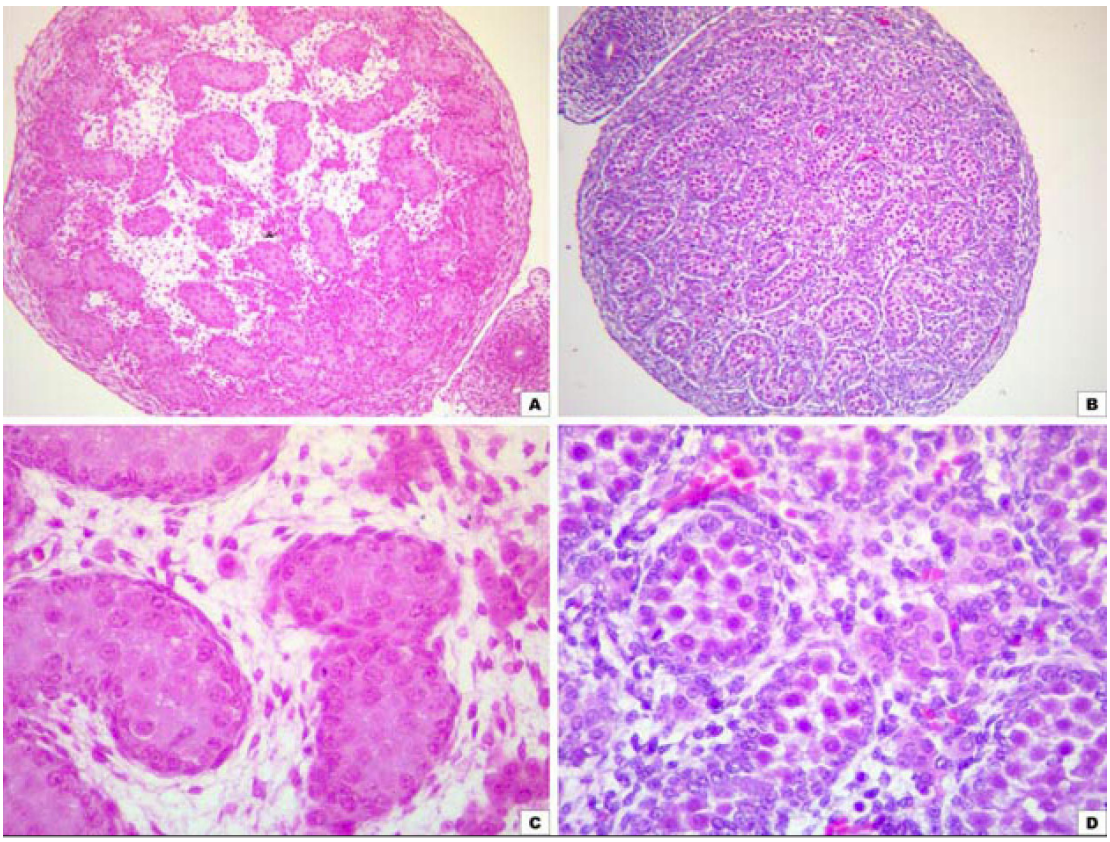

Fig. 1

Micromorphology of the rat testes on prenatal day 20th. Haematoxylin and eosin staining: control (A, C), hypothyroid rat (B, D). ×100 (A, B) and ×400 (C, D).

by thyroid hormones deficiency as reported by Wagner et al. [8].

Reactivity of hypothyroid rats testicular components with most of the lectins used, except for SBA and LABA, was significantly lower compared to control samples. However, in the previous publications [15, 16] we reported selective labeling of fetal Leydig cells with PSA and GNA. These same lectins, as well as CCRA, SBA, SNA and LABA distinctly demonstrated increased count of Leydig cells in hypothyroid rats compared to control group progeny (91.82±1.46 and 8.28±0.37 Leydig cells per one field of observation, respectively).

On the postnatal day 1st it was documented similar count and size of SC in testes of control and experimental group animals: one field of observation

Table 2

Results of morphometric measurements

		Seminiferous cord / tubule				* Count of Leydig cells per one field of observation M±m
		* Count of SC/ST per one field of observation M±m	Diameter (µm) M±m	Cross section area (µm^2) M±m	Index of spermatogenesis M±m	
Prenatal day 20th	control	11.65±0.92	57.55±2.80	2599.92±130.82	-	8.28±0.37
	experiment	14.07±1.25**	56.56±2.37	2511.24±122.18	-	91.82±1.46**
Postnatal day 1st	control	22.64±4.72	51.82±1.36	2107.97±63.78	-	10.84±0.86
	experiment	20.87±1.44	52.25±2.69	2134.64±88.72	-	45.22±4.75**
Postnatal day 20th	control	24.44±3.36	76.31±2.22	4571.22±135.88	-	9.84±1.46
	experiment	17.42±1.16**	101.73±2.60**	8123.96±167.41**	-	11.82±0.37
Postnatal day 40th	control	7.62±0.57	162.77±4.59	20797.85±618.26	3.45±0.18	11.84±1.07
	experiment	6.41±0.72	169.04±5.89	22431.00±670.18	3.82±0.22	18.81±1.35**
Postnatal day 120th	control	2.22±0.61	267.85±10.48	56318.74±2318.60	3.18±0.36	18.78±1.07
	experiment	2.00±0.46	224.67±7.48**	39624.14±1235.42**	3.84±0.41	24.22±1.46**

** Estimated at magnification ×200*

*** Results considered valid with P<0,05*

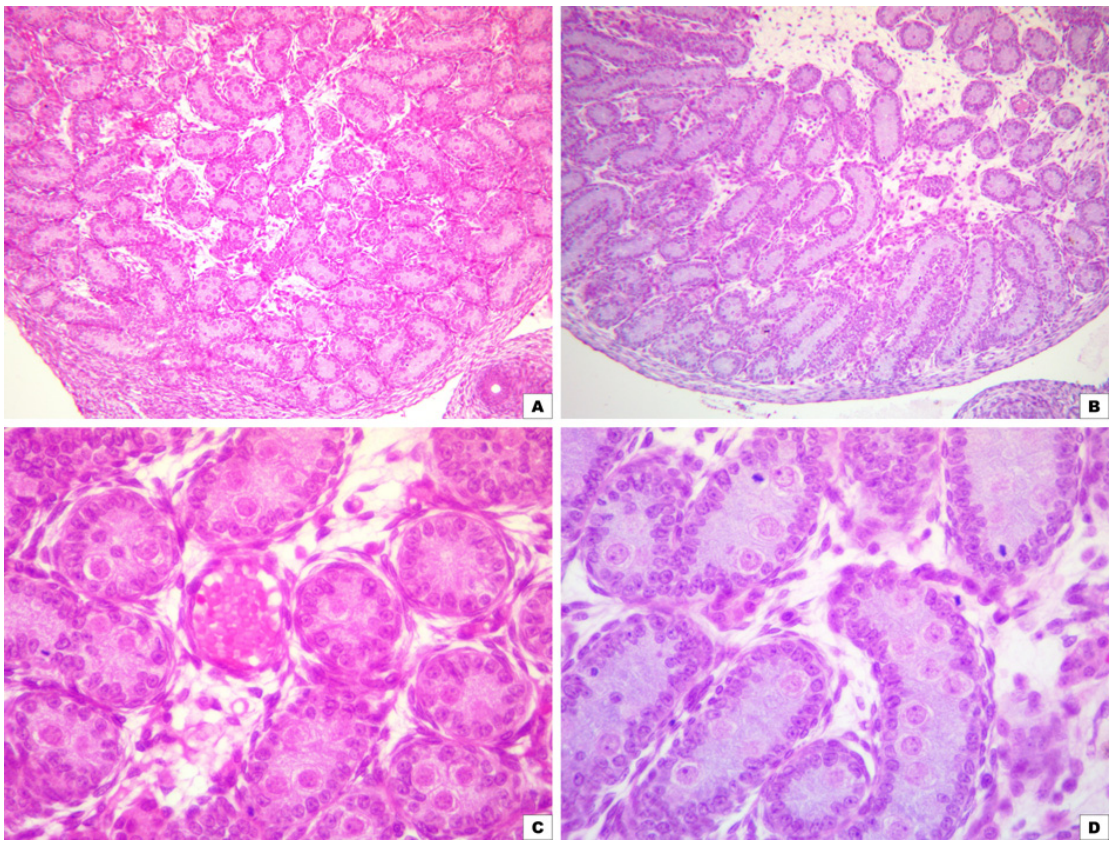

Fig. 2

Micromorphology of the rat testes on postnatal day 1st. Haematoxylin and eosin staining: control (A, C), hypothyroid rat (B, D). ×100 (A, B) and ×400 (C, D).

contained 22.64±4.72 SC in control and 20.87±1.44 SC in experimental rat specimens, diameters of SC corresponded to 51.82±1.36 μm in control and 52.25±2.69 μm in experimental group rats (Fig.2). Alongside with the increased content of Leydig cells in hypothyroid rats (Fig.3A, B), we detected also the increased count of mast cells, which were rather selectively labeled with CNFA and WGA (Fig.3C, D).

On the postnatal day 20th diameter of ST in experimental animals significantly exceeded the same parameter in control rats: 76.31±2.22 μm in control versus 101.73±2.60 μm in experimental rats. Lectin histochemistry studies revealed decreased lectin labeling of adluminal spermatocytes in within the ST, this phenomenon coinciding with early acrosomal granules staining, supplemented with the increased count and enhanced lectin reactivity of Leydig cells (Fig.4). Due to the enhanced proliferation rate of experimental rats spermatogenic epithelium, some of their enlarged size ST were lacking lumina, which were well presented in control specimens.

On the postnatal day 40th the diameter of ST was 162.77±4.59 μm in control and 169.04±5.89 μm in experimental group animals (difference insignificant). ST lumina of the latter were filled with large conglomerates of degenerating and apoptotic cells (Fig.5). Index of spermatogenesis corresponded to 3.45±0.18 in control versus 3.82±0.22 in experimental group rats. Differences in lectin binding included intensive CCRA reactivity with spermatogonia nuclei, as well as SBA and MPFA labeling of acrosomal caps of early spermatids in experimental, but not in control group animals.

In the adult rat testes there was detected certain reduction of ST diameter in experimental (224.67±7.48 μm) versus control group rats (267.85±10.48 μm) (Fig.6). Spermatogenic indices were as follows: 3.18±0.36 in control and 3.84±0.41 in experimental animals. ST demonstrated marked heterogeneity in lectin binding, apparently due to different stages of spermatogenic cycle presented in different segments of ST. Subsequent stages in acrosomal maturation were documented by lectin staining: SBA intensely labeled acrosomal granules and caps (stage 3-6 of spermiogenesis), while PNA and SNA labeled acrosomal caps (stage 7-8 of spermiogenesis) and wedged acrosomes (stage 10-14 of spermiogenesis) [20] (Fig.7). Majority of used

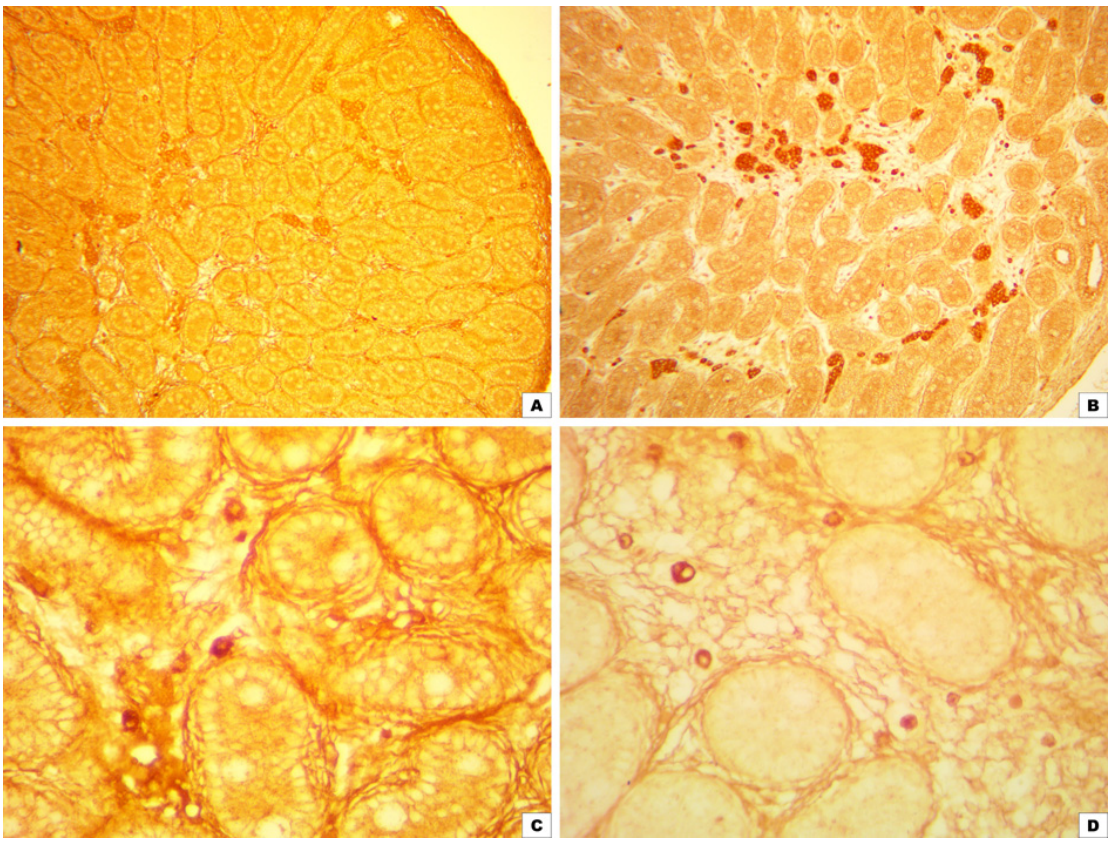

Fig. 3

Selective staining of Leydig (A, B) and mast cells (C, D) in within the connective tissue testicular interstitium of postnatal day 1st rats: GNA (A, B) and WGA (C, D) lectin labels. Control (A, C), hypothyroid (B, D) rats. ×100 (A, B), ×400 (C, D).

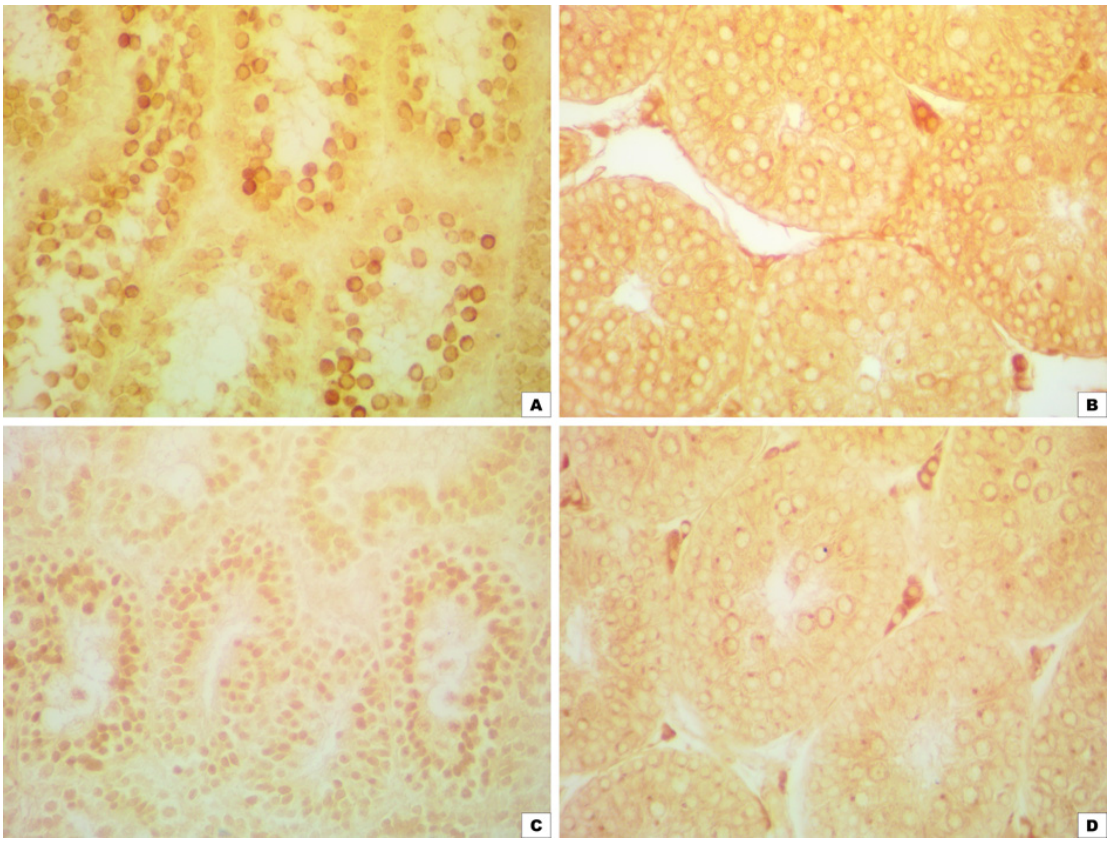

Fig. 4

Lectin histochemistry of rat testes on postnatal day 20th: spermatocytes in the adlumenal compartments of seminiferous tubules of hypothyroid rats (B, D) demonstrate reduced lectin reactivity in comparison to control rat samples (A, C): PSA (A, B) and CCRA (C, D) lectin labels. ×400.

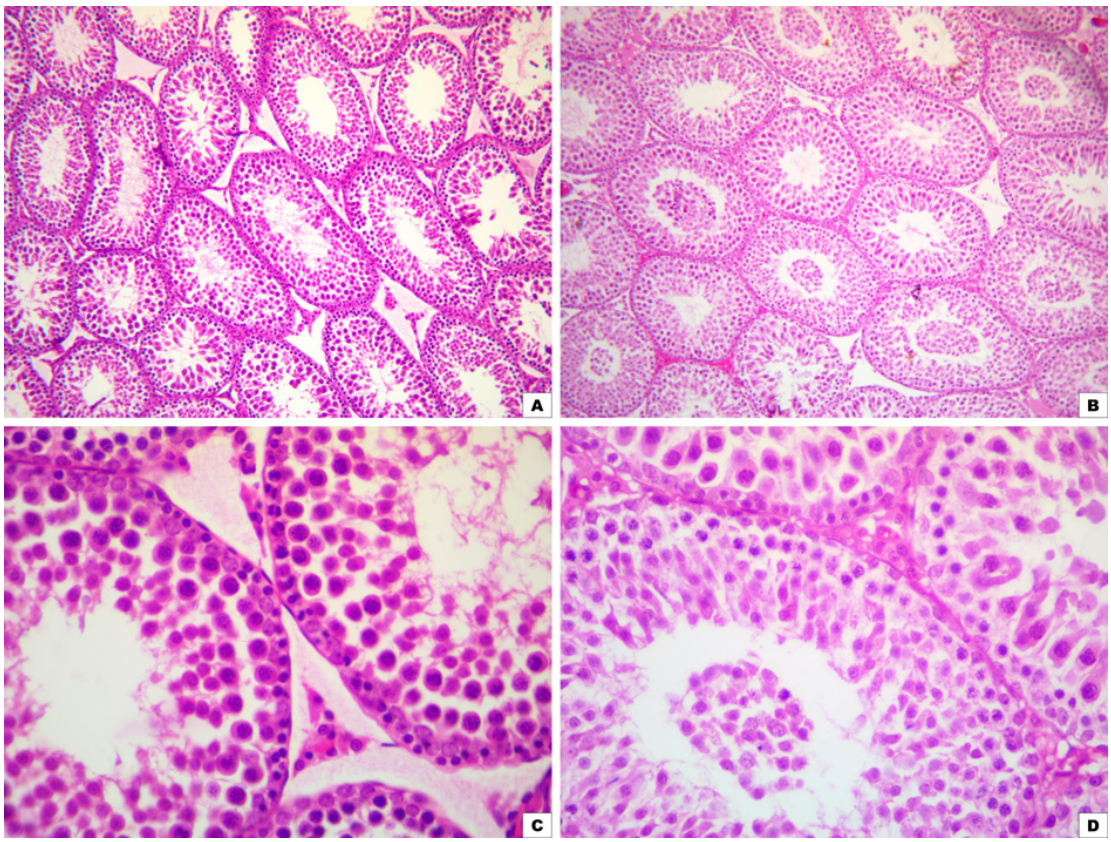

Fig. 5

Micromorphology of the rat testes on postnatal day 40th. Haematoxylin and eosin staining: control (A, C), hypothyroid rat (B, D). ×100 (A, B) and ×400 (C, D).

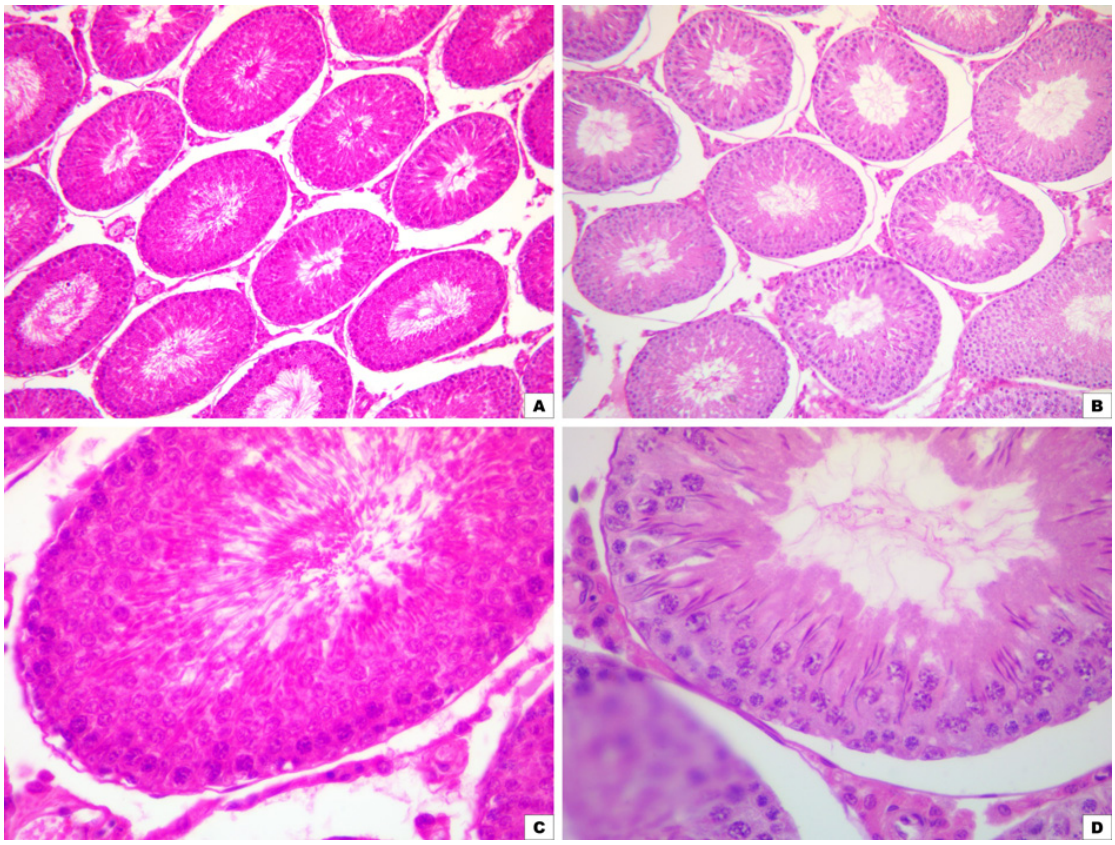

Fig. 6

Micromorphology of the adult rat testes. Haematoxylin and eosin staining: control (A, C), hypothyroid rat (B, D). ×100 (A, B) and ×400 (C, D).

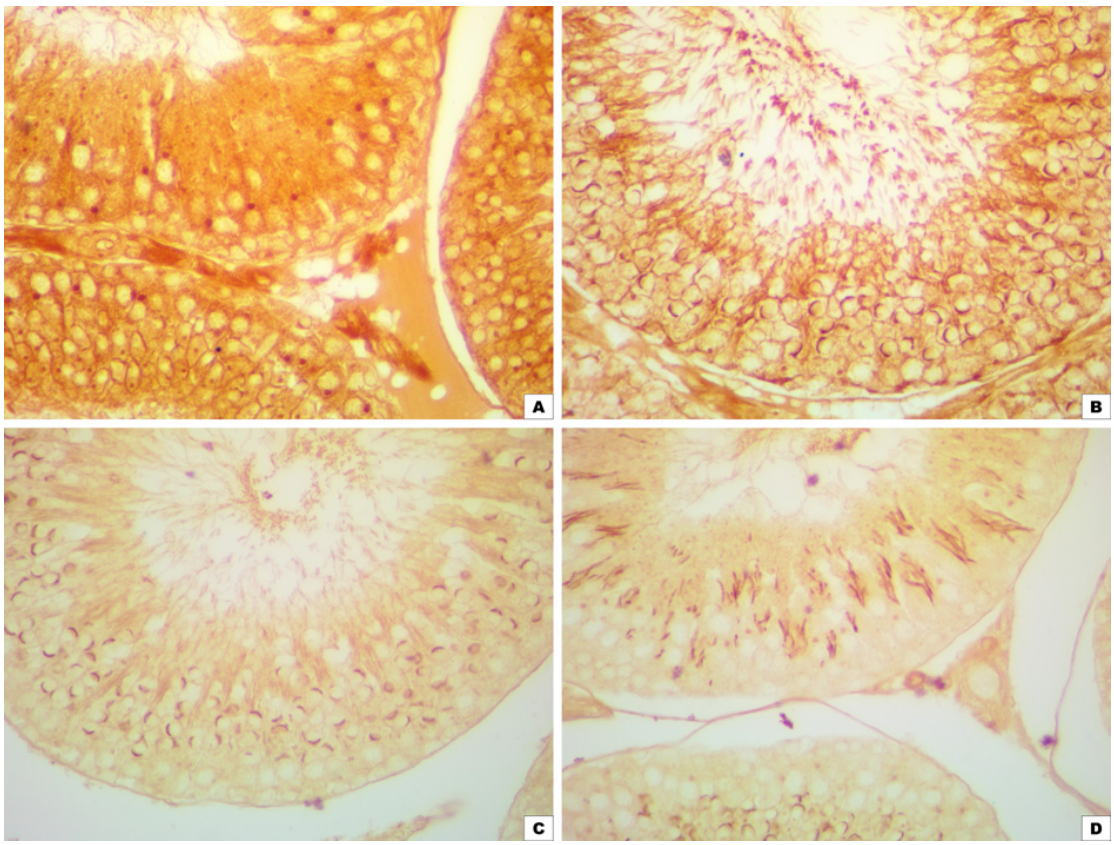

Fig. 7

Acrosomal granules (A) and caps (B, C), wedged acrosomes (D) detected by means of lectin histochemistry in spermatids and spermatozoa of experimental group adult rats. SBA (A), SNA (B), PNA (C, D) lectin labels. ×400.

lectins demonstrated high affinity to Leydig cells cytoplasmic glycoconjugates (Fig.3, 4, 7). We did not manage to detect significant differences in lectin binding to testicular specimens of adult control and experimental rats.

Our morphometric findings evidence that maternal hypothyroidism induces in progeny rats enhanced proliferation ratio of spermatogenic epithelium, increased size of seminiferous tubules and elevated index of spermatogenesis, these findings apparently coinciding with sperm cell delayed maturation. Offspring of hypothyroid rats demonstrated increased content of Leydig cells, which were selectively labeled with PSA, GNA and CCRA lectins. There was also detected decreased reactivity of experimental animals testicular structures with most of the lectins used in comparison to control rat samples.

Conclusions

1. Maternal hypothyroidism has significant impact on postnatal morphogenesis of rat testes, and the obtained data extend the existing knowledge on fine mechanisms of these influences.
2. Differences in lectin binding to tissue samples of control and experimental rat testes were significant in prenatal animals with levelling until adulthood, while morphometric indices were clearly distinct in all investigated progeny groups.
3. Documented selective labeling of Leydig cells with lectins PSA, GNA and CCRA, of mast cells with WGA, of early acrosomes with SBA, of cap stage and of wedged acrosomes with PNA and SNA can be recommended for further morphometric investigations.

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