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Research article

STEREOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF THE SPLEEN IN HYPOTHYROID JUVENILE RATS

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The purpose of this work was to investigate the influence of hypothyroidism on spleen tissue morphology and immune cell density in fourteen-day-old juvenile rats. Hypothyroidism in pups (n=10) was induced by administration of propylthiouracil (PTU) in drinking water (1.5 mg/L) to their mothers during pregnancy and period of lactation. Fourteen-day-old pups were sacrificed and the thyroid-stimulating hormone (TSH) serum concentration and thyroid activation index (Ia) were determined. Increased serum level of TSH and increased Ia showed that pups from PTU treated mothers were hypothyroid. White and red spleen pulp, marginal zone and connective tissue volume density has been assessed by using the stereological method. Using immunohistochemistry, the present CD3+ T lymphocytes, CD45RA+ B lymphocytes and CD68+ macrophages were quantified. A significant reduction of volume density of the periarteriolar lymphocyte sheath (Vv_{PALS}) and lymphatic follicles (Vvf) due to depletion of T and B lymphocytes respectively, was observed in the spleens of hypothyroid pups compared to controls. The volume density of the red pulp (Vvrp), marginal zone (Vvmz) and connective tissue (Vvct) was increased, as well as the number of CD68+ macrophages in the spleens of hypothyroid pups compared to controls. These results indicate that thyroid hormones might be important for normal development of both, specific and innate immune cells in the spleen during prenatal and early postnatal period.

Key words: spleen, hypothyroidism, stereology, immunohistochemistry, rat.

INTRODUCTION

The cross talk between the neuroendocrine and immune systems is vivid in developing organisms enabling spatial and temporal harmonization of cell proliferation and differentiation [1]. Humoral mediators, such as hormones, neurotransmitters and cytokines mediate the communication between these two major adaptive systems of the body, whose basic function is to maintain homeostasis. Thyroid hormones (TH)

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are critical for regulation of proliferation and differentiation of many types of cells as well as for growth, development and tissue homeostasis [2]. It has been demonstrated that leukocytes have receptors for T3 and T4 and it has been proposed that both hormones control the cell cycle and have an immunomodulatory role [3]. First findings concerning the influence of TSH, T3 and T4 on the immune system have shown that these hormones are necessary for the maintenance of the bone marrow cellularity – i.e. proliferation and differentiation of hematopoietic cells [4]. Further on, it became evident that the lack of TSH preferentially decreased the number of pre-B lymphocytes in the mice bone marrow but, curiously, only the innate immune response was impaired [5]. Furthermore, depending on the model used, and severity of hypothyroidism, various results have been reported including a normal or decreased number of T cells and macrophages [3,6,7]. No study reported the increase in other cellular types.

The spleen is a secondary lymphoid organ that functions as a blood filter and it contains about a fourth of all lymphocytes in the body. The spleen structure comprises white pulp, made of T lymphocytes organized in the periarteriolar lymphatic sheath (PALS) and B lymphocytes organized in primary and secondary follicles, red pulp made of macrophages and venous sinuses, and unique structure - marginal zone, with mutually dependent macrophages and B lymphocytes [8,9]. Although it is not a vital organ, the spleen has some peculiar functions that become obvious after splenectomy. Namely, splenectomised patients have an increased risk to develop encapsulated bacterial sepsis and meningitis probably due to lack of marginal zone immune cells and its ability to remove circulating pathogens [10]. Indeed, spleen functional microanatomy is still under investigation and exiting new data point to the diversity of its B lymphocyte and macrophage populations [9,11].

In humans, a lot of attention has been given lately to maternal hypothyroidism during pregnancy due to adverse effects on fetus development and mortality [12,13]. The domestic animal species that is most commonly diagnosed to have hypothyroidism is the dog, but there is no data on what would be the effect of canine maternal hypothyroidism on their pups and specifically on their immunological status. Although the association between hypothyroidism and immunity is demonstrated [3,6,7], the importance of TH for the maturation of the spleen tissue compartments in the period of prenatal development, neonatal and juvenile periods have received little attention. A normally developed spleen should be the prerequist for normal clearance of circulating pathogens and thus the protection of animal from sepsis. As neonatal animals are extremely prone to sepsis, all incongruity of the spleen tissue should be carefully investigated.

The aim of this study was to investigate whether subclinical hypothyroidism during pregnancy and lactation influence the spleen immune cell density and modulate the morphology of different spleen tissue compartments in the fourteen-day-old, juvenal pups.

MATERIALS AND METHODS

Animals and experimental protocol

Female Albino Oxford rats (n=12) were mated with three male rats. All animals were kept in animal colony under standard conditions with free access to food and water and on a cycle of 12h light: 12h darkness.

Two groups of animals were formed: (1) hypothyroid female pups (n=10) from PTU treated mothers (n=6) that were given 1.5 mg/L propylthiouracil in drinking water (PTU; Sigma Chemical Co. St. Louis, MO, USA) during pregnancy and lactation and (2) control female pups (n=10) from mothers that consumed water without PTU (n=6). Fourteen-day-old pups were sacrificed using an anesthetic overdose (Ketamine/ Xylazine). Blood was collected by heart puncture while the rats were anesthetized. The experimental protocol was in line with Directive 2010/63/EU and was approved by the Ethics Committee of the Faculty of Veterinary Medicine University of Belgrade.

Thyroid stimulating hormone level

The serum level of TSH was determined by the RIA method using rat TSH standards and immuno-reagents obtained as a gift from Dr. A. Parlow within the Harbor-UCLA Medical Center, Torrance, CA, USA. Samples were run in duplicate, the intra-assay coefficient of variation was 6.8%, while the assay sensitivity was 0.5 ng/mL.

Tissue processing and sampling for light microscopy and stereology

The spleen and thyroid gland were removed and fixed in 10% neutral-buffered formalin. The tissues were dehydrated in increasing ethanol concentrations, cleared in xylene and embedded in paraffin. From each block of spleen and thyroid gland tissue, sections were cut at 5μm thickness at 3 different levels of tissue 100 μm apart. Thereafter, all sections were stained with hematoxylin and eosin (H&E).

Stained sections of thyroid gland and spleen were analyzed using a multipurpose stereological grid M42 (The M42 testing system which had 21 straight-line segments and 42 testing points in a testing area equal to 36.36 d2). Model - based stereology [14] was used to determine the volume density of different thyroid and spleen compartments.

Thyroid gland: volume density (Vv) of interstitium (Vvi), Vv of epithelial tissue (Vve) and Vv of colloid (Vvc) was also determined.

The volume density of the epithelium and colloid was then used to calculate the activation index (Ia) of the thyroid gland according to the following formula:

$$Ia = Vve / Vvc$$

Spleen compartments: volume density (Vv) of periarteriolar lymphocyte sheath (Vv_{PALS}), follicles (Vvf), marginal zone (Vvmz), red pulp (Vvrp) and connective tissue zone (Vvct) was determined.

All spleen stereological analyses were made by the same researcher on 6 randomly chosen pups per group.

Tissue processing and sampling for immunohistochemistry

The spleen was removed and slices of spleen tissue (no larger than 3 mm in thickness) were fixed in "JB" fix [15] at 22ºC for 24h and afterwards embedded in paraffin. Tissue sections were blocked with 0.3% bovine serum albumin (BSA; Merck, Darmstadt, Germany) and 10% normal rabbit serum at 22ºC for 30 min. The following monoclonal antibodies were used: mouse anti-rat (CD3) T cell (clone R73, 1:100, Serotec, Oxford, UK); mouse anti-rat (CD45RA) B cell (clone OX-33, 1:100, Serotec, Oxford, UK) and pan-macrophage mouse anti-rat (CD68) macrosialin antibody (ED1, 1:300, Serotec, Oxford, UK). Sections with primary antibodies were incubated over night (16 hours) at 4°C. After washing the primary antibodies, sections were incubated with rabbit anti-mouse secondary IgG (1:100; Dako Cytomation, code No. P 0161, Glostrup, Denmark) coupled to a peroxidase labeled dextran polymer, for 30 min and the reaction was developed with 3-3'-diaminobenzidine-H_2O_2 medium (DAB; Merck, Darmstadt, Germany) at 22ºC. The sections were counterstained with Mayer's hematoxylin. Negative controls for immunostaining were performed by substituting the primary antibody with TBS.

Quantification of T lymphocytes, B lymphocytes and macrophages

To quantify the presence of T and B lymphocytes and CD68 positive macrophages in the spleens of control and hypothyroid animals an open source Fiji software (Image J) was used (http://fiji.sc/Fiji). DAB stained areas on each slide were measured and presented as a percentage of total area examined (total surface examined per slide was $2mm^2$). Four slides per animal were analyzed. The average calculated value per animal was used in further statistical analysis.

Statistical analysis

Results obtained from all analysis were expressed as mean ± standard deviation. Statistical significance of differences between data, was determined using Student's t-test. Levels of significance were: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

RESULTS

Thyroid status

Histological assesment showed that the thyroid gland of pups belonging to PTU treated mothers was symmetric and bilaterally hypertrophied in comparison to control pups. In control group, in macro- and microfollicles, both cuboidal and collumnar epithelium were present (Figure 1A). In pups from PTU treated mothers, microfollicles

were dominant containing exclusively collumnar epithelium with a small amount of colloid (Figure 1B).

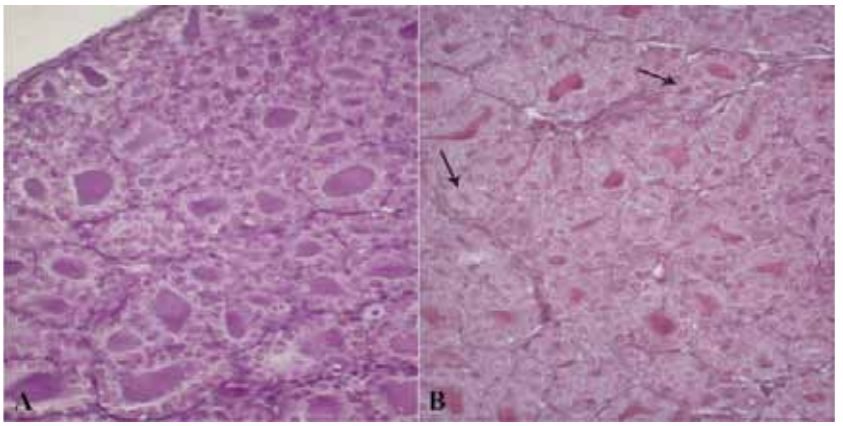

Figure 1. A. Thyroid gland of pup from control mother. **B.** Thyroid gland of pup from mother treated with propylthiouracil. Columnar thyreocytes in the microfollicles with small amount of colloid (arrows), H&E (x 40)

Volume density of individual structures in the thyroid gland of control and treated animals is shown in Figure 2. In the treated animals Vve was significantly increased ($p<0.01$), while Vvc and Vvi were significantly reduced with $p<0.001$ and $p<0.05$ respectively (Figure 2). Also, the Ia was significantly increased ($p<0.001$) in the treated group and it was 2.9 fold higher than in control animals (Table 1). The average concentration of serum TSH was 2.67 fold higher in the treated group in comparison to the control group ($p<0.001$). These results showed the hypothyroid status of pups derived from PTU treated mothers.

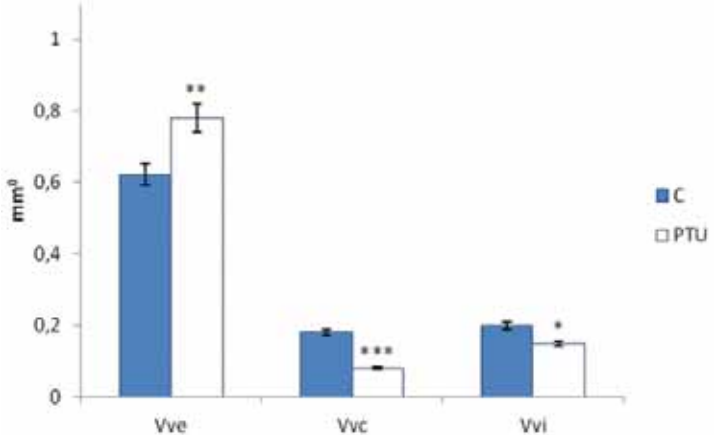

Figure 2. Volume density of epithelial tissue (Vve), colloid (Vvc) and interstitium (Vvi) of thyroid gland in control pups (n=10) and pups (n=10) from PTU treated mothers. Levels of significance were determined between groups (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Table 1. The serum concentration of TSH and the thyroid activation index (Ia) in the control group and group of pups from propylthiouracil treated mothers (hypothyroid pups). Results are presented as mean ± standard deviation (M ± SD)

Pups	TSH (ng/ml)	Ia
Control (n=10)	5.63±0.73	3.56±0.39
Hypothyroid (n=10)	15.06±2.25***	10.19±0.21***

Levels of significance: ***p<0.001 compared to control

Light microscopy and stereological analysis of the spleen

Results obtained by light microscopy and stereology of spleen tissue were consistent. White pulp area was reduced in hypothyroid pups, while red pulp, marginal zone and connective tissue was increased (Figure 3). Stereological analysis revealed a significant reduction of volume density of the PALS ($p<0.001$) and follicles ($p<0.001$) in the spleens of hypothyroid pups compared to controls. In contrast to white pulp, the volume density of the red pulp was increased ($p<0.001$) in hypothyroid animals compared to controls. The volume density of the marginal zone ($p<0.05$) and connective tissue ($p<0.05$) were also significantly increased in hypothyroid pups (Table 2).

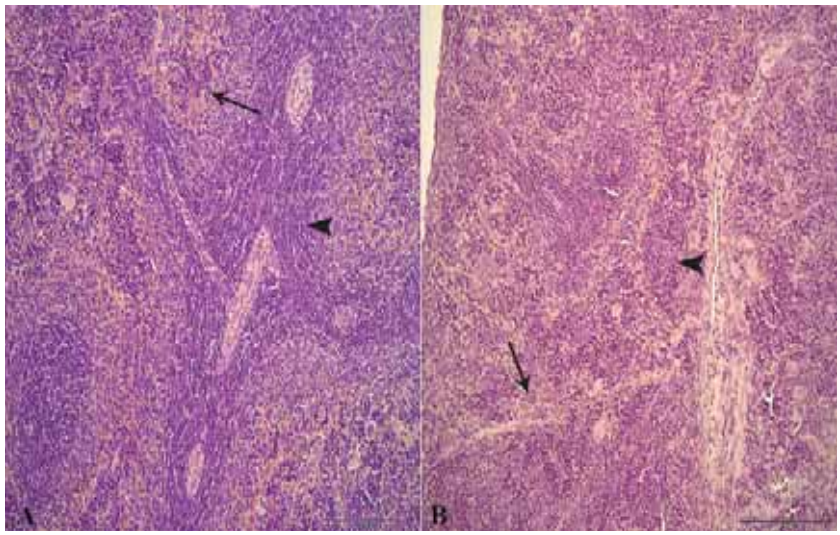

Figure 3. A. Spleen of control pup. Light microscopy examination shows the appearance of an adult-like PALS (arrow head) and marginal zone (arrow). **B**. Spleen of hypothyroid pup. Note the reduced PALS zone (arrow head). H&E, bar = 200 μm

Table 2. Volume density (Vv) of splenic tissue compartments of intact and hypothyroid pups. Results are presented as M ± SD

Pups	Vv_{PALS}	Vvf	Vvrp	Vvmz	Vvct
Control n=6	0.185±0.079	0.007±0.0005	0.640±0.101	0.089±0.056	0.078±0.053
Hypothyroid n=6	0.098±0.071***	0.005±0.0003***	0.692±0.113***	0.106±0.072*	0.096±0.061*

Vv_{PALS} – Vv of periarteriolar lymphocyte sheath, Vvf – Vv of follicles; Vvrp – Vv of red pulp, Vvmz – Vv of marginal zone; Vvct – Vv of connective tissue zone. Levels of significance: *p<0.05; ***p<0.001 compared to control

Immunohistochemistry

Using immunohistochemistry, a significant decrease of CD3+ T lymphocytes was found in the PALS area in the spleen of hypothyroid rats in comparison to control rats (p<0.001) (Figure 4). Also, a decrease of CD45RA+ B lymphocytes was noticed

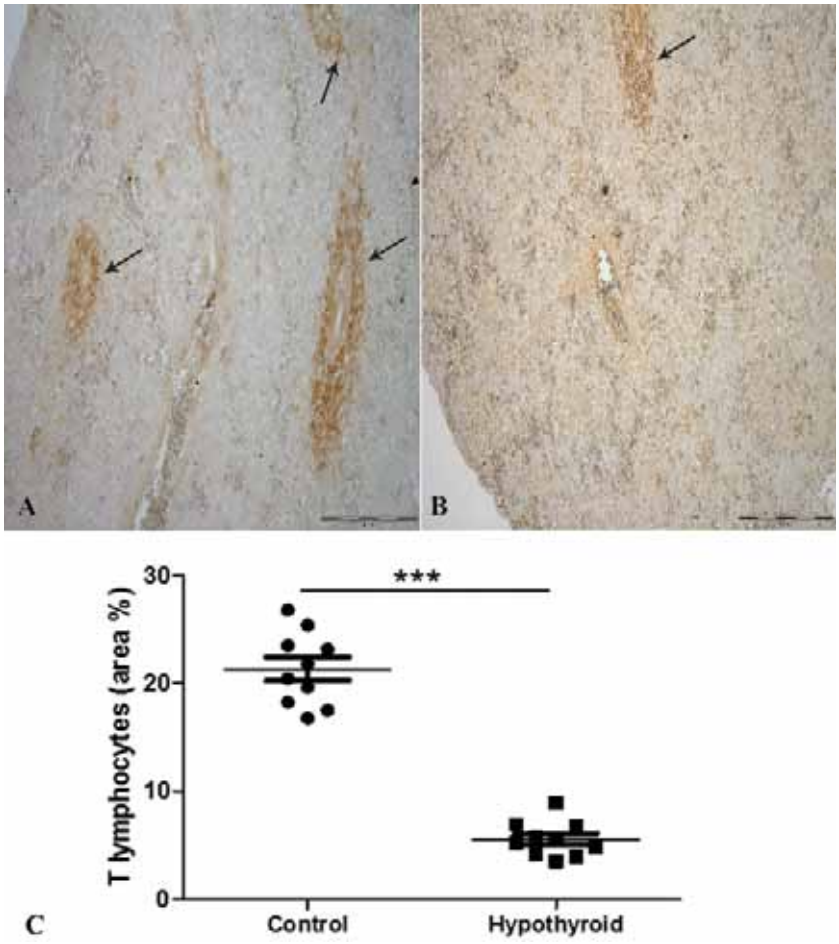

Figure 4 (A-C). **A** Spleen of control pup. Positive immunostaining of CD3+ T cells (arrows) in the majority of the PALS. **B**. Spleen of hypothyroid pup. CD3+ T cells (arrow) in the PALS. PALS area is reduced and exhibit severe depletion of T lymphocytes. Chromogen diaminobenzidine (DAB), counterstain hematoxylin. Bar = 500 μm. **C.** Quantification of T lymphocytes (area %) in the spleens of control and hypothyroid pups (*** $p<0.001$)

in the white pulp area in the hypothyroid group of animals ($p<0.001$) (Figure 5). Furthermore, the area of the spleen containing CD68+ macrophages was significantly increased ($p<0.001$) in hypothyroid animals and the majority of them were distributed in the red pulp in both groups of animals (Figure 6).

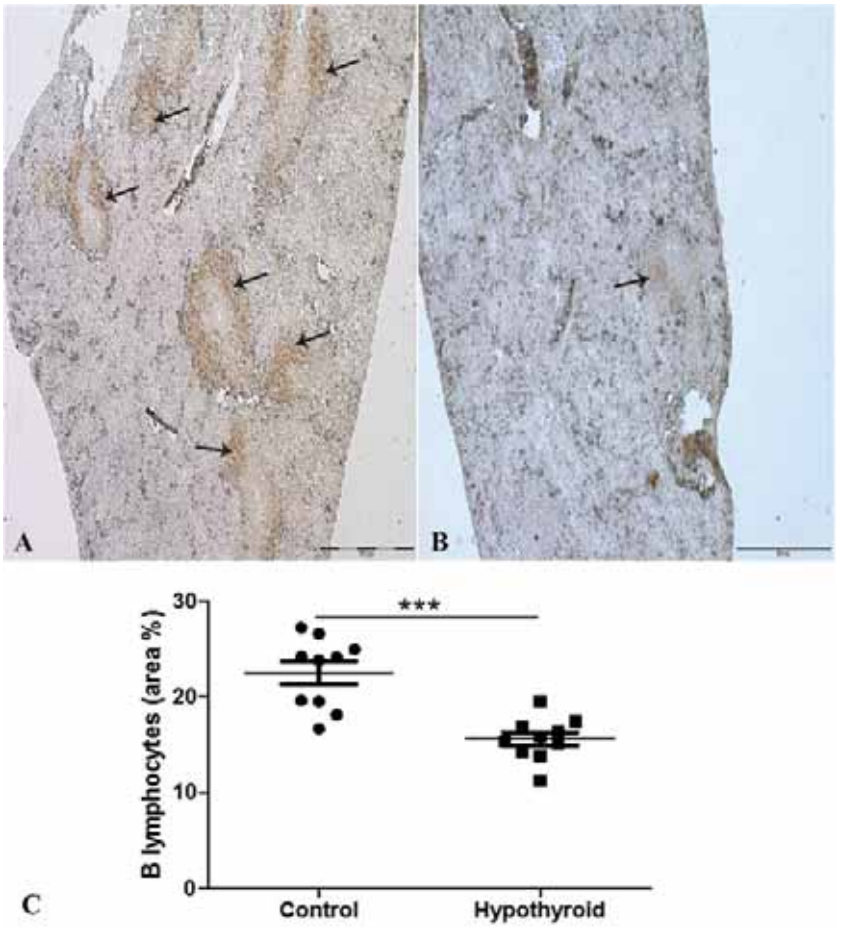

Figure 5. (A-C). A. Spleen of control pup. Positive immunostaining of CD45 RA+ B cells (arrows) in the white pulp area. **B**. Spleen of hypothyroid pup. CD45 RA+ B cells (arrow) in the white pulp area. White pulp area is reduced and shows considerable reduction of the amount of B lymphocytes. Chromogen diaminobenzidine (DAB), counterstain hematoxylin. Bar = 500μm. **C.** Quantification of B lymphocytes (area %) in the spleens of control and hypothyroid pups (*** $p<0.001$)

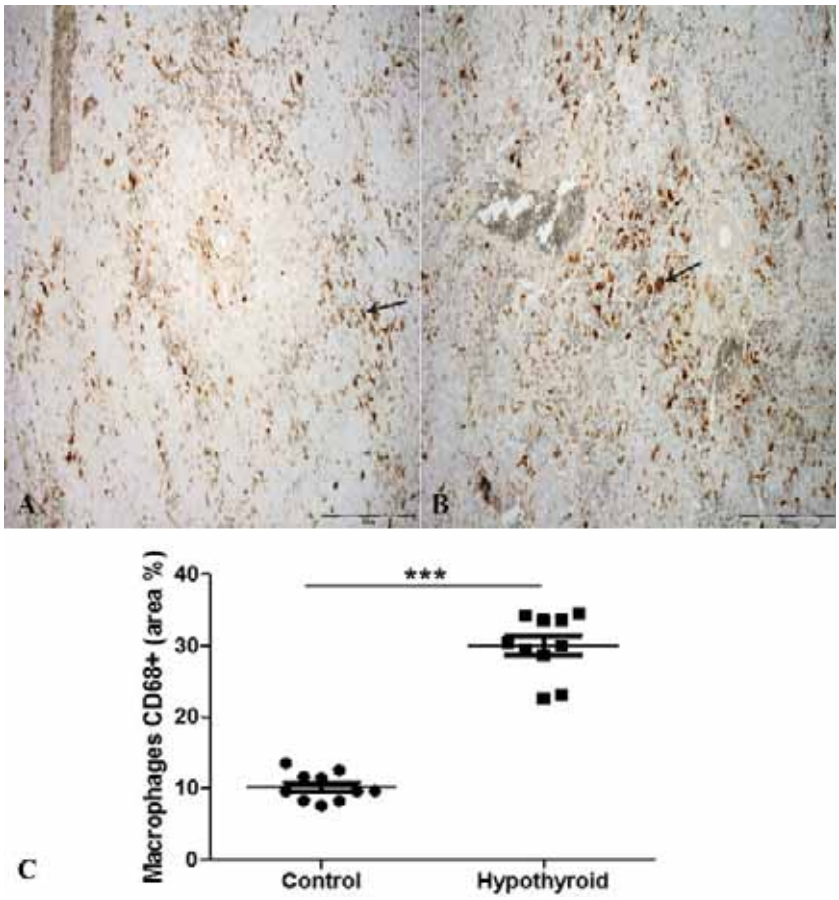

Figure 6. (A-C). A. Spleen of control pup. CD68+ macrophages (arrow). **B**. Spleen of hypothyroid pup. Note the increase of density of the CD68+ macrophages (arrow) in the red pulp. Chromogen diaminobenzidine (DAB), counterstain hematoxylin. Bar = 200μm. **C.** Quantification of CD68+ macrophage (area %) in the spleens of control and hypothyroid pups (*** $p<0.001$)

DISCUSSION

The main finding of this study is that in juvenile hypothyroid rats the volume of white pulp of the spleen is decreased because the number of T and B lymphocytes is reduced, while the volume of marginal zone and red pulp are increased, partly because the number of CD68+ macrophages is enhanced.

In this study, based on the concentration of TSH and significantly increased Ia, we concluded that pups exposed to PTU during the prenatal and suckling period have been hypothyroid. PTU inhibits thyroid hormone synthesis by inhibiting iodination of tyrosine residues in thyroglobulin and blocks the conversion of thyroxine to triiodothyronine within the thyroid and in peripheral tissues [16]. As the dose used is

one of the lowest reported that could induce hypothyroidism [17] it is supposed that other potential toxic effect of PTU were minimal and if present did not influence the results obtained.

The first important change that we observed in the spleens of the hypothyroid pups, was the decreased volume density of PALS and of primary lymphatic follicles that corresponded to a significant depletion of T and B lymphocytes, respectively. These results are in accordance to the data obtained by other authors that used slightly different methods of hypothyroidism induction [3,6]. The most convincing evidence that development of T and B lineage is thyroid hormone dependent came from two studies: one on Snell dwarf mice (multiple hormonal deficits), that recover B lymphocyte lineage after substitution of thyroid hormones [18] and the other on experiments demonstrating that deletion of genes for thyroid receptors result in an decreased number of T and B lymphocytes, macrophages and granulocytes in the spleen and other lymphoid organs [19]. Also, the mitogen induced proliferation of T and B lymphocytes were significantly reduced in hypothyroid mice [20]. However, it is not known if homing of T and B lymphocytes to the spleen is defective and what is the effect of hypothyroidism on the spleen stroma that has been shown to have a crucial effect on the recruitment of leukocytes into the secondary lymphoid organs, as well as into their activation and functional orientation [21]. As it was shown in the very beginning of thyroid hormones investigation, bone marrow cellularity is decreased in hypothyroid animals [4], making likely the possibility that the function of common lymphoid progenitor cells is also impaired. Another interesting observation is that macrophages and T lymphocytes in the spleen cannot be fully developed without adequate B lymphocytes compartment [9]. So, the question is, to what extent reduction of PALS is due to lack of TH action on T cells and to what extent this reduction is a consequence of B lymphocyte down regulation. Moreover, increased T and B lymphocyte death could not be ruled out as a mechanism of reduction in their number, although some investigations point that thyroid hormones, in fact, enhance apoptosis of human lymphocytes [22]. Altogether, it is possible to assume that the decrease in spleen T and B lymphocytes, in our model of juvenile hypothyroidism, has multiple etiologies and probably reflects the complex nature of immunological changes during naturally developed hypothyroidism.

The second important finding of this study is that volume density of the spleen marginal zone as well as CD68+ macrophage density was increased in comparison to the controls. Presence of an unimpaired marginal zone is crucial because its' unique ability to rise T independent response against blood borne pathogens, especially encapsulated bacteria [10]. The majority of cells in the marginal zone are B lymphocytes [23], but specialized Marginal Zone Macrophages (MZM) and Marginal Metallophilic Macrophages (MMM), dendritic cells, endothelial cells and fibroblasts are also present [9]. MMM derive from hematopoietic stem cells (HSC) and MZM are probably established prior to birth from elements present in the yolk sac, and not from HSC [24]. MZM could self-renew throughout adult life [25]. Recent data

show that lack of T3 in methimazole induced hypothyroid mice, increase the number of peritoneal macrophages during inflammatory stimulus [26]. Having that in mind, it is possible to speculate that the increased volume of the marginal zone is at least partly caused by an increased macrophage number that could be genuine spleen cells derived from yolk sac or could stem from blood monocytes [27]. At last, but not of less importance, it could be assumed that putative T and B lymphocytes death induced by lack of thyroid hormones, could enhance macrophage activation and proliferation and stimulate their phagocytic activity.

The last important result of this work is that in hypothyroidism the volume density of red pulp is increased. Red pulp is composed of reticular cells, endothelial cells and macrophages mainly functioning as phagocytic cells responsible for the removal of old and damaged erythrocytes [11]. It could be supposed that volume density of red pulp is probably due to the increased number of macrophages, but engorged blood vessels or their increased density could also contribute to this change. The volume of connective tissue was also increased in hypothyroid animals, the phenomenon that should be explored in more details knowing that different parenchymatous organs are prone to fibrosis [28]. Another attractive possibility to investigate connective tissue changes related to the lack of thyroid hormones is the accumulation of hyaluronic acid, the major glycosaminoglycan that is increased in myxedema connected with hypothyroidism. Myxedema was not clinically visible in the pups during our experiment, but some findings point that hyaluronic acid accumulates in internal organs during hypothyroidism [29]. This phenomenon could explain that spleen weights were increased in two and three weeks old hypothyroid pups in previously reported experiments [30].

All results presented, indicate that thyroid hormones might be important for the normal development of the spleen as a secondary lymphoid organ. Further studies should address the question whether hypothyroidism influence homing, proliferation or differentiation of T and B lymphocytes in the central and peripheral lymphoid organs and whether their lack stimulate macrophages proliferation or their number rise due to stimulation through enhanced phagocytosis of dying T and B lymphocytes.

Acknowledgements

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STEREOLOŠKA I IMUNOHISTOHEMIJSKA ANALIZA SLEZINE HIPOTIREOIDNIH JUVENILNIH PACOVA

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Cilj ovog istraživanja je bio da ispita uticaj hipotireoidizma na morfologiju tkiva slezine i zastupljenost ćelija imunskog sistema kod dve nedelje starih juvenilnih pacova. Hipotireoidizam mladunaca (n=10) je indukovan aplikacijom propiltiouracila (PTU) u vodi za piće (1,5 mg/L), majkama tokom perioda graviditeta i laktacije. Dve nedelje stari mladunci su žrtvovani i određena im je koncentracija tireostimulirajućeg hormona (TSH) u serumu, kao i indeks aktivacije štitaste žlezde (Ia). Na osnovu povišenog nivoa TSH i povišenog Ia, utvrđeno je da je PTU aplikovan majkama, doveo do pojave hipotireoidizma kod njihovih potomaka. Volumenska gustina bele i crvene pulpe, marginalne zone i vezivnog tkiva slezine mladunaca ispitivana je stereološkom analizom. Zastupljenost CD3+ T limfocita, CD45RA+ B limfocita i CD68+ makrofaga u slezini je određena primenom imunohistohemijske metode. Kod hipotireoidnih mladunca postojalo je značajno smanjenje volumenske gustine periarterijskog limfocitnog

omotača (Vv_{PALS}) i limfnih folikula (Vvf), kao i deplecija CD3+ T limfocita i CD45RA+ B limfocita, u poređenju sa mladuncima iz kontrolne grupe. Za razliku od bele pulpe, volumenska gustina crvene pulpe (Vvcp), marginalne zone (Vvmz) i vezivnog tkiva (Vvct), kao i broj CD68+ makrofaga je bio povećan kod hipotireoidnih mladunaca u odnosu na kontrolnu grupu. Ovi rezultati ukazuju da tireoidni hormoni mogu da budu važni za normalan razvoj ćelija specifičnog i nespecifičnog imunskog sistema tokom prenatalnog i ranog postnatalnog perioda razvoja.