UNIVERSITY OF BELGRADE

FACULTY OF BIOLOGY

mr Njia Milad Ali Rajab

HISTOLOGICAL AND ULTRASTRUCTURAL ALTERATIONS OF RAT INTERSCAPULAR BROWN ADIPOSE TISSUE IN EXPERIMENTALLY INDUCED SYSTEMIC HYPERTHYROIDISM

Doctoral Dissertation

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mr Njia Milad Ali Rajab

HISTOLOŠKE I ULTRASTRUKTURNE PROMENE INTERSKAPULARNOG MRKOG MASNOG TKIVA PACOVA U EKSPERIMENTALNO INDUKOVANOM SISTEMSKOM HIPERTIROIDIZMU

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Beograd, 2015

MENTOR

Dr Maja Čakić-Milošević
Assistant professor at the Faculty of Biology, University of Belgrade

MEMBERS OF THE COMMITTEE

Dr Maja Čakić-Milošević
Assistant professor at the Faculty of Biology, University of Belgrade

Dr Radmila Glišić
Assistant professor at the Faculty of Science, University of Kragujevac

Dr Mirela Ukropina
Assistant professor at the Faculty of Biology, University of Belgrade

Defence date .....

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Researcher

mr Njia Milad Ali Rajab

Histological and ultrastructural alterations of rat interscapular brown adipose tissue in experimentally induced systemic hyperthyroidism

ABSTRACT

Overweight and obesity, caused by imbalance between energy intake and energy expenditure, are growing public health problem worldwide. Brown adipose tissue (BAT), as energy dissipating tissue, emerged as possible target for antiobesity therapy.

Thyroid hormones play an important role in regulation of temperature homeostasis and energy metabolism and they are involved in regulation of BAT activity. Thus, continuous cross-talk between thyroid gland/hormones and adipose tissue may be important for body weight control. While the importance of thyroid hormones for proper BAT function is well known, the influence of systemic hyperthyroidism on BAT activity is not yet fully understood.

This dissertation started from two directly confronted premises concerning the place of BAT in thyroid thermogenesis. The first of them implies that BAT thermogenic function in systemic hyperthyroidism is suppressed while according to the other one BAT is effectory organ of thyroid thermogenesis. Therefore, this dissertation aims to investigate the effects of experimentally induced systemic hyperthyroidism on structural remodelling and thermogenic capacity of BAT in order to shed more light on regulatory mechanisms controlling its function. Also, the aim was to analyse differences, if any, between effects of two thyroid hormones, T3 and T4.

The experiment was performed on a total of 18 male Wistar rats, kept under standard laboratory conditions, at the room temperature. Animals from the first experimental group received T3 in dose of 200 μg/kg b.w., *i. p.* while animals from the second experimental group received T4 in dose of 300 μg/kg b.w., *i. p.*, once a day, during five days. Rats from the third group were treated with vehicle only, according to the same schedule.

Histological and histochemical analyses of BAT were performed at the level of light microscopy using 5 μm thick paraffin sections of routinely processed tissue stained with hematoxylin-eosine, PAS, Bodian and Novelli methods. For detection of apoptosis, propidium iodide method was used.

Immunohistochemical detection of UCP1, Ki67 and TNFα, using LSAB (labelled streptavidin-biotin) method, served to estimate the effects of applied treatments on expression of proteins related to thermogenesis, cell population dynamics and signalling.

Ultrathin sections of Araldite-embedded BAT tissue samples were used for morphological analysis at the level of electron microscopy.

Stereological analysis was performed at the level of light and electron microscopy. Statistical analysis regarding differences between control and each of experimental groups was performed using Student's *t*-test.

Obtained results showed that systemic hyperthyroidism, regardless on which hormone induced it, caused growth of BAT. In T4-treated animals this growth was synchronised with remodelling of vascular and neural networks which was not the case in T3-treated group. Both hormones positively affected BAT thermogenic capacity, which was manifested by increased expression of UCP1. Possible transdifferentiation of unilocular cells toward thermogenically competent UCP1-positive cells was also suggested. Stereological analysis, however, did not confirm the existence of changes in abundance of main tissue components, most probably due to short duration of experiment.

At the ultrastructural level, thyroid hormones differently affected pattern of lipid accumulation in brown adipocytes. In both treated groups, lipid content was increased but in T4-treated animals multilocularity was well preserved, while after treatment with T3 it was reduced. Both T3 and T4 affected mitochondrial dynamics, which was manifested by the occurrence of mitochondria displaying unusual size and shape. After treatment with T4 mitochondria established more intensive contacts with surface of lipid bodies than it was noted in control and T3-treated animals. This was another important finding since topographic relation between mitochondria and lipid bodies in brown adipocytes reflects metabolic fuelling of mitochondria by fatty acids released from lipid bodies and thus may indicate that thermogenesis takes place in BAT of T4-hypethyroid rats.

Experimental design used in this study provided a rarely employed model for investigating the effects of exogenously induced hyperthyroidism on thyroid gland. Therefore, another aim of this dissertation was to investigate possible toxic effects of T3

or T4 on thyroid gland. Structurally, thyroid gland was subjected to involutive changes after treatment with both T3 and T4. Observed changes are most probably based on the absence of cytoprotective effects of TSH, together with reception of more pronounced proapoptotic signals. Given that structural alterations of the thyroid gland associated with dysfunction may require an unpredictable recovery period, current findings point to necessary caution needed when thyroid hormones are used as therapeuticals.

Keywords: brown adipose tissue, brown adipocytes, light microscopy, electron microscopy, thyroid gland, thyroid hormones, experimentally induced systemic hyperthyroidism, Wistar rats.

Scientific field: Biology

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Histološke i ultrastrukturne promene interskapularnog mrkog masnog tkiva pacova u eksperimentalno indukovanom sistemskom hipertiroidizmu

SAŽETAK

Povećana telesna težina i gojaznost prouzrokovane disbalansom između unosa energije i njenog trošenja, predstavljaju rastući zdravstveni problem u svetu. Mrko masno tkivo (MMT), kao tkivo koje rasipa energiju, postaje moguće ciljno tkivo za lečenje gojaznosti.

Tiroidni hormoni imaju važnu ulogu u regulaciji temperaturne homeostaze i energetskog metabolizma i uključeni su u regulaciju aktivnosti MMT. Prema tome, postojanje stalne komunikacije između tiroidne žlezde/tiroidnih hormona i masnog tkiva može biti značajno za kontrolisanje telesne težine. I dok je značaj tiroidnih hormona za ispravno funkcionisanje MMT dobro poznato, uticaj sistemskog hipertiroidizma na njegovu aktivnost još uvek nije u potpunosti shvaćen.

Ova disertacija bazirana je na dve međusobno suprotstavljene polazne pretpostavke koje se tiču mesta MMT u tiroidnoj termogenezi. Prva podrazumeva supresiju termogene funkcije MMT u sistemskom hipertiroidizmu, dok prema drugoj MMT predstavlja efektorski organ tiroidne termogeneze. Prema tome, cilj ove disertacije bio je ispitivanje efekata eksperimentalno indukovanog sistemskog hipertireoidizma na strukturno remodeliranje i termogeni kapacitet MMT, kako bi se doprinelo razjašnjavanju mehanizama njegove regulacije. Isto tako, cilj je bio analizirati razlike, ako ih ima, u efektima dva glavna tiroidna hormona, T3 i T4.

Eksperiment je izveden na ukupno 18 mužjaka Wistar pacova, držanih pod standardnim laboratorijskim uslovima, na sobnoj temperaturi. Životinje iz prve eksperimentalne grupe primale su T3 u dozi od 200 μg/kg b.w., *i. p*. dok su životinje iz druge eksperimentalne grupe primale T4 u dozi 300 μg/kg b.w., *i. p*., jednom dnevno, tokom pet dana. Pacovi iz treće grupe bili su tretirani samo prenosnikom, prema istom rasporedu.

Histološka i histohemijska analiza MMT obavljena je na nivou svetlosne mikroskopije, korišćenjem 5 μm parafinskih preseka rutinski sprovedenog tkiva, obojenih hematoksilinom-eozinom, PAS metodom, Bodian metodom i Novelli

metodom. Za detekciju apoptoze korišćen je metod bojenja propidijum-jodidom

Imunohistohemijska detekcija UCP1, Ki67 i TNFα, uz korišćenje LSAB (obeleženi streptavidin-biotin) metoda, služila je za procenu efekata primenjenih tretmana na ekspresiju proteina vezanih za termogenezu, dinamiku ćelijske populacije i signalizaciju.

Ultratanki preseci MMT ukalupljenog u Araldit korišćeni su za morfološku analizu na nivou elektronske mikroskopije.

Stereološka analiza rađena je na nivou svetlosne i elektronske mikroskopije. Statistička analiza razlika između vrednosti dobijenih za svaku od eksperimentalnih grupa i kontrolu vršena je Studentovim *t*-testom.

Dobijeni rezultati pokazali su da je sistemski hipertiroidizam, bez obzira na to koji je hormon do njega doveo, izazvao rast MMT. Kod T4-tretiranih životinja ovaj rast bio je usklađen sa remodeliranjem nervne i vaskularne mreže, što nije u potpunosti bio slučaj u T3-tretiranoj grupi. Oba hormona pozitivno su uticala na termogeni kapacitet MMT, povećavajući ekspresiju UCP1. Ukazano je i na moguću pojavu transdiferencijacije unilokularnih ka termogeno kompetentnim UCP1-pozitivnim ćelijama. Stereološka anliza, međutim, nije pokazala promene u zastupljenosti glavnih tkivnih komponenti, najverovatnije zbog kratkog trajanja eksperimenta.

Na ultrastrukturnom nivou, tiroidni hormoni različito utiču na obrazac akumulacije lipida u mrkim adipocitima: u obe tretirane grupe sadržaj lipida je povećan, ali je kod T4-tretiranih životinja multilokularnost dobro očuvana, dok se posle T3-tretmana ona smanjuje. I T3 i T4 utiču na dinamiku mitohondrija, što se manifestuje pojavom mitohondrija neobičnog oblika i veličine. Posle T4-tretmana, mitohondrije uspostavljaju intenzivnije kontakte sa površinom lipidnih tela nego što je to slučaj u kontrolnoj grupi i grupi T3-tretiranih životinja. Ovo je još jedan važan podatak, s obzirom na to da topografski odnosi mitohondrija i lipidnih tela u mrkim adipocitima odražavaju metaboličko "punjenje" mitohondrija masnim kiselinama oslobođenim iz lipidnih tela i mogu da ukažu na odvijanje termogeneze u MMT T4-tretiranih pacova.

Eksperimentalni dizajn korišćen u ovoj studiji obezbedio je inače retko korišćen model pogodan za ispitivanje egzogeno indukovanog hipertiroidizma na tiroidnu žlezdu. Stoga je još jedan cilj ove disertacije bio ispitivanje mogućih toksičnnih efekata T3 ili T4 na tiroidnu žlezdu. Strukturno, u tiroidnoj žlezdi je došlo do involutivnih

promena. Uočene promene najverovatnije su bazirane na odsustvu citoprotektivnih efekata TSH, uz prijem jače izraženih proapoptotskih signala. Imajući u vidu činjenicu da strukturne promene tiroidne žlezde udružene sa disfunkcijom mogu zahtevati period oporavka čija je dužina nepredvidiva, ovi rezultati ukazuju na neophodan oprez prilikom korišćenja tiroidnih hormona u terapijske svrhe.

Ključne reči: mrko masno tkivo, mrki adipociti, svetlosna mikroskopija, elektronska mikroskopija, tiroidna žlezda, tiroidni hormoni, eksperimentalno indukovani sistemski hipertiroidizam, Wistar pacovi.

Naučna oblast: Biologija

Uža naučna oblast: Biologija ćelija i tkiva

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1. Introduction

1. 1. Historical overview

Brown adipose tissue (BAT) was initially described by a Swiss naturalist Konrad Gessner (1551), in the hibernating marmot *Marmota alpina*. For a while it was also known as the hibernating gland, due to its compact organ-like appearance, rich vascularization and, as it was thought at that time, function exclusively related to hibernation (Rasmussen, 1923).

After recognizing its role in the thermoregulatory thermogenesis in non-hibernating mammals (Smith, 1961; Smith and Roberts, 1964) BAT became frequently used as a model system for studying physiology of thermoregulation. It attracted additional attention after the discovery that its metabolic activity could be stimulated not only by cold exposure, but also by certain aspects of diet (Rothwell and Stock, 1979; Bukowiecki *et al*., 1983; Saito *et al*., 1989).

Lately, BAT is again in the focus of interest of biological and biomedical sciences. In accordance to the concept proposed by Cinti (1999) BAT is today considered to be the part of integral multidepot adipose organ consisting of white and brown areas. Due to the fact that both brown and white adipocytes, as well as some other cells within the adipose organ, possess the ability to synthesize and release substances exerting autocrine/paracrine and endocrine effects, many authors currently consider the adipose organ as a part of endocrine system (Cannon and Nedergaard, 2004).

According to the classical point of view, BAT is present in humans and other mammals of large body mass (>10 kg) only during early period of life (Aherne and Hull, 1966; Sutter, 1969; Merklin, 1974). However, it has been demonstrated recently that BAT exists in healthy adult humans and that it is susceptible to activation and regulation in a similar way as in mammals of small body mass (van Marken Lichtenbelt *et al*., 2009; Virtanen *et al*., 2009; Yoneshiro *et al*., 2013). Thus, BAT is recognized as an important participant in the regulation of energy homeostasis and body weight control in humans and as a potential target for drug therapy of metabolic diseases including diabetes and obesity.

1. 2. Anatomy of BAT and developmental origin of brown adipocytes

In small rodents, adipose organ exists in the form of clearly distinguishable subcutaneous (interscapular, subscapular, dorso-cervical, suprasternal and axillary), intraperitoneal (perirenal, suprarenal) and intrathoracal (around esophagus, heart, aorta and large blood vessels) depots (Cinti, 2012; Klingenspor and Fromme, 2012) (Fig. 1. 2. 1). Relative proportion of brown *vs.* white adipocytes varies depending on specific location, condition and age of the animal (Frontini and Cinti, 2010), but quantitatively the most important depot of BAT is interscapular depot located subcutaneously, between the shoulder blades. Substantial depots are also found around large blood vessels and vital organs such as aorta, heart, kidney or adrenals (Afzelius, 1970; Klingenspor and Fromme, 2012).

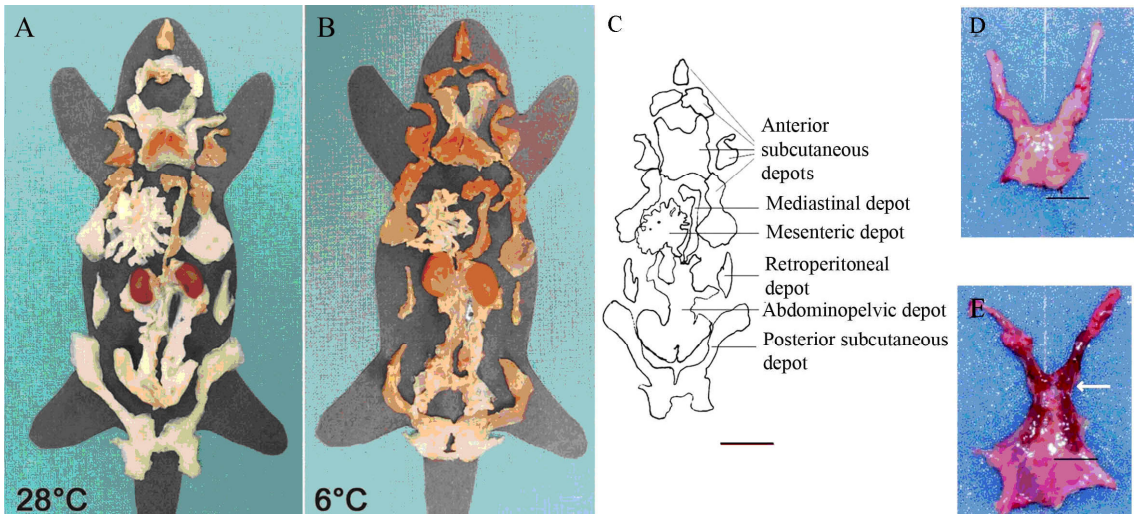


Figure 1. 2. 1: Gross anatomy of the adipose organ of adult mice kept at 28°C (A) and at 6°C (B); drawing of main adipose tissue depots (C), bar = 1 cm (taken and modified from Cinti, 2009) and macroscopic appearance of rat interscapular BAT in resting (D) and activated (E) state (taken and modified from Yo *et al*., 2013).

Macroscopically, interscapular BAT is discrete butterfly-shaped tissue pad consisting of two lobes subdivided into lobules by connective tissue septa (Ross and Pawlina, 2011a). In animals in which BAT activity is stimulated, it is easily discernible from surrounding tissue by its more intense (reddish) colour emerging primarily as the

result of dense vascularization, but also from the numerous mitochondria present in brown adipocytes (Yo *et al*., 2013).

In addition to "classical" BAT, it has been found that individual brown-like adipocytes exist within white adipose tissue WAT (Cinti, 2012). These cells, named brite adipocytes (or beige adipocytes or inducible brown adipocytes) arise in adrenergically stimulated WAT of the rat, through either transdifferentiation of white adipocytes or from common progenitors. Brite/beige adipocytes have morphological and some biochemical characteristics intermediate between brown and white adipocytes, but they possess a specific pattern of gene expression (Harms and Seale, 2013; Park *et al*., 2014; Rosenwald and Wolfrum, 2014).

Mesenchymal stem cell is precursor of three types of adipose cells as well as skeletal muscle cell (Fig. 1. 2. 2). Following first line of differentiation, mesenchymal stem cell gives the cell of myogenic line which further may develop to brown adipocyte or skeletal muscle cell, passing through the stage expressing myogenic marker myf5 (myogenic factor 5; $myf5^{+}$ cell). On the other hand, mesenchymal stem cell can give adipoblast from which white adipocyte or beige adipocyte may arise, passing through the stage negative for myogenic marker myf5 ($myf5^{-}$ cell) (Obregon, 2014). Conversions of white adipocytes to thermogenically active beige adipocytes (transdifferentiation) through the stage of paucilocular adipocyte and brown adipocytes to white adipocytes are also considered as possible (Barbatelli et al., 2010; Yao *et al*., 2011; Giralt and Villarroya, 2013; Harms and Seale, 2013; Obregon, 2014). Accordingly, it seems that brown adipocytes are closer by origin to skeletal muscle cells than to white and beige adipocytes.

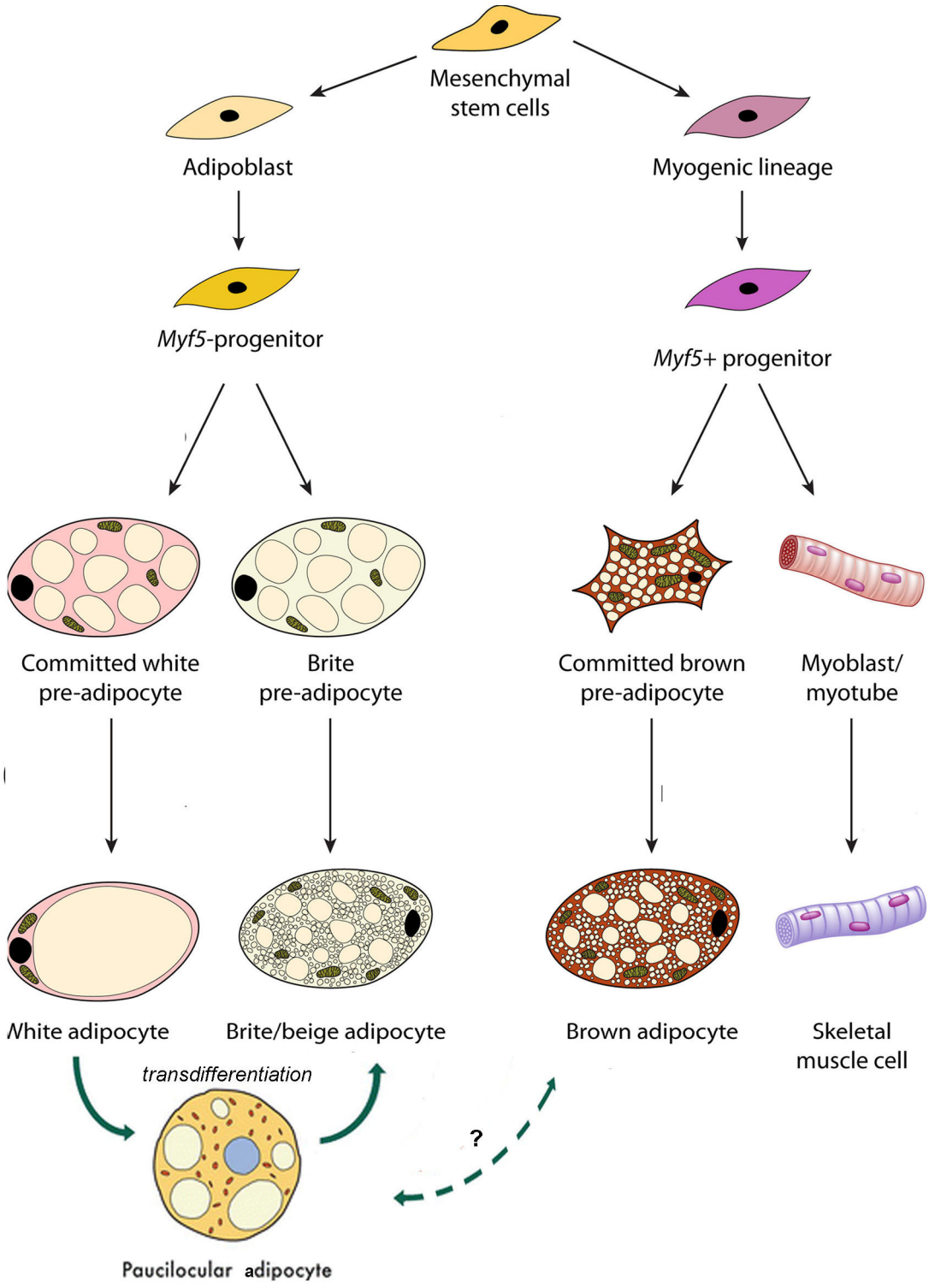


Figure 1. 2. 2: Currently accepted scheme of adipocytes differentiation from mesenchymal stem cell and white/beige/brown interrelationship (taken and modified from Giralt and Villarroya, 2013; Obregon, 2014).

1. 3. Histological organisation and cell types in BAT

BAT is extremely well vascularized and its thermogenic function is closely associated with blood flow (Baron *et al*., 2012). Thoracodorsal arteries passing through connective tissue septa bring blood with oxygen and metabolic substrate to the tissue and branch into the parenchyma where they form extensively developed capillary network (Cinti, 2001). Warmed blood leaves BAT medially by a Sulzer's vein, and bilaterally by the thoracodorsal veins, finally reaching *inferior* and *superior vena cava*, respectively. Each brown adipocyte is in contact with at least one capillary, while in activated BAT, more than one third of adipocyte surface area is in contact with the surface of endothelial cells (Aherne and Hull, 1966; Rauch and Hayward, 1969; Bukowiecki *et al*., 1980; Nechad, 1986). Thus, blood warmed by the activity of BAT may be efficiently distributed throughout the body protecting vital organs from cooling and providing survival. In addition, when closely apposed to some vital organs, BAT can warm them directly by convection (Rauch and Hayward, 1969).

BAT is extensively innervated by postganglionic sympathetic nerves which unilaterally innervate each of two lobes. Sympathetic innervation is responsible for strong and fast BAT activation upon receiving thermogenic stimulus. Efferent nerve fibres terminating onto brown adipocytes contain noradrenaline, while those terminating onto blood vessels contain noradrenaline and neuropeptide Y. Nerve fibres are unmyelinated and form synapses of "en passant" type (Himms-Hagen, 1991). In addition, BAT possesses sensitive nerve fibres containing substance P and CGRP (calcitonin gene-related peptide) (Néchad, 1986; Norman *et al*., 1988; De Matteis *et al*., 1998). According to current knowledge, it seems that the most of BAT depots including interscapular do not possess parasympathetic innervation (Bartness *et al*., 2010).

Quantitatively, BAT of adult rat consists mostly of brown adipocytes. However, brown adipocytes represent only approximately 40% of total cells in the tissue, while other cell types, including endothelial cells, interstitial cells, adipocyte progenitor/precursor cells, mast cells, macrophages, granulocytes, Schwann cells and fibroblasts constitute the remaining part of the tissue (Bukowiecki *et al*., 1982; Cinti, 2001).

During perinatal period several morphologically distinct developmental stages of brown adipocyte linage might be observed in rats: (a) adipoblast stage 1- small, blast-like cell with large nucleus, distinct nucleolus, mitochondria not yet typical for brown adipocytes and numerous polyribosomes, (b) adipoblast stage 2 - contains lipids in unilocular form, and usually small quantity of glycogen (c) adipocyte precursor - cell morphologically quite similar to mature brown adipocyte but smaller and (d) mature brown adipocyte. These stages may also be identified upon physiological stimulation of BAT (Bukowiecki *et al*., 1986; Cinti, 2001). Proliferation of brown adipocytes in stimulated BAT is tightly coupled with angiogenesis and some recently published data suggest possible origin of brown adipocytes from endothelial cells (Tran *et al*., 2012).

Presence of some unilocular adipocytes in BAT depots, usually at the periphery of lobules, but also among typical multilocular cells, is in line with the concept of adipose organ in which borders between brown and white areas are not clearly distinguishable (Klingenspor and Fromme, 2012) (Figs. 1. 3. 1 and 1. 3. 2).

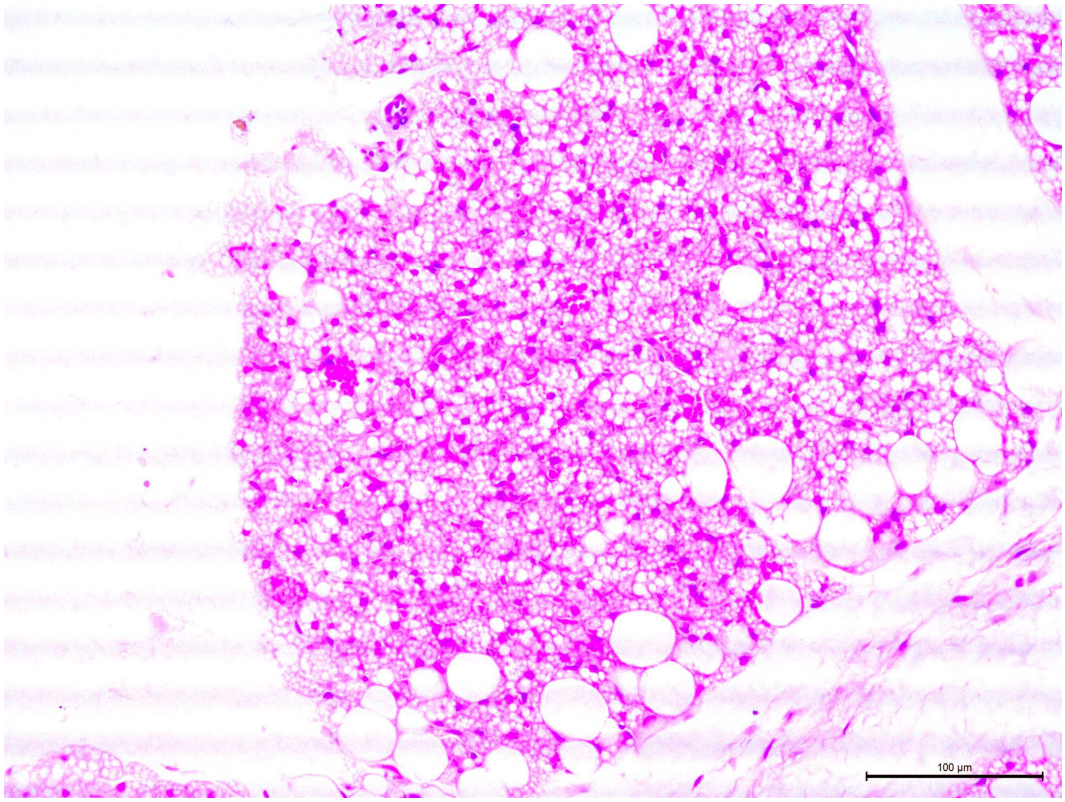


Figure 1. 3. 1: Light microscope low power view shows general histological appearance of interscapular BAT. Unilocular adipocytes are visible at the periphery and inside BAT lobules. Hematoxylin/eosin, 20x, original (bar = 100 µm).

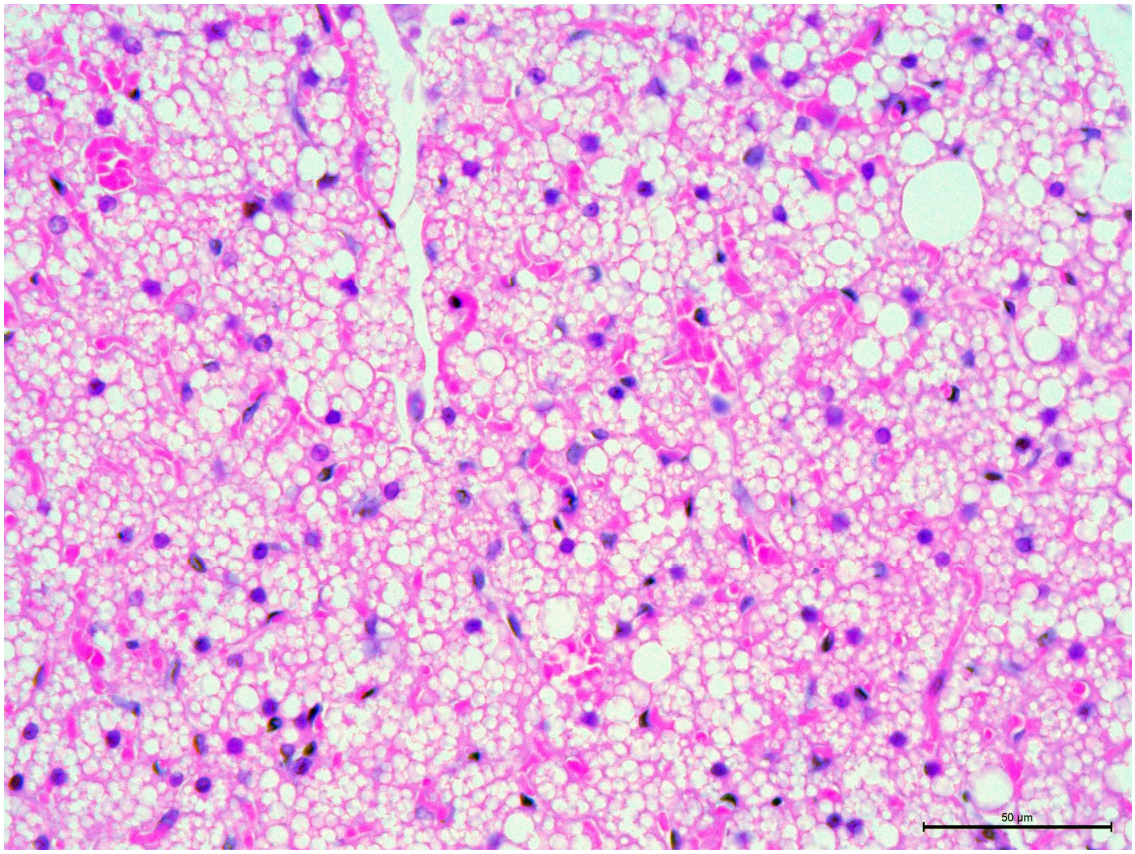


Figure 1. 3. 2: Interscapular BAT consists mainly of multilocular brown adipocytes and is very well vascularized. Haematoxylin/eosin, 40x, original (bar = 50 µm).

Several other cell types are also present in BAT (Fig. 1. 3. 3). Interstitial cells show some similarities with both fibroblasts and adipoblasts and they are considered to be a distinct cell type with still not completely understood function (Cinti, 2001). Schwann cells make a sheath around unmyelinated nerve fibres both in connective tissue septa and within the lobules (Umahara, 1968). Fibroblasts and mast cells are not different from those found in proper connective tissue. Mast cell number increases upon BAT physiological stimulation and it seems that their secretory products histamine and serotonin are involved in the regulation of blood flow and noradrenaline release (Steiner and Evans, 1976; Thureson-Klein *et al*., 1976; Cinti, 2001).

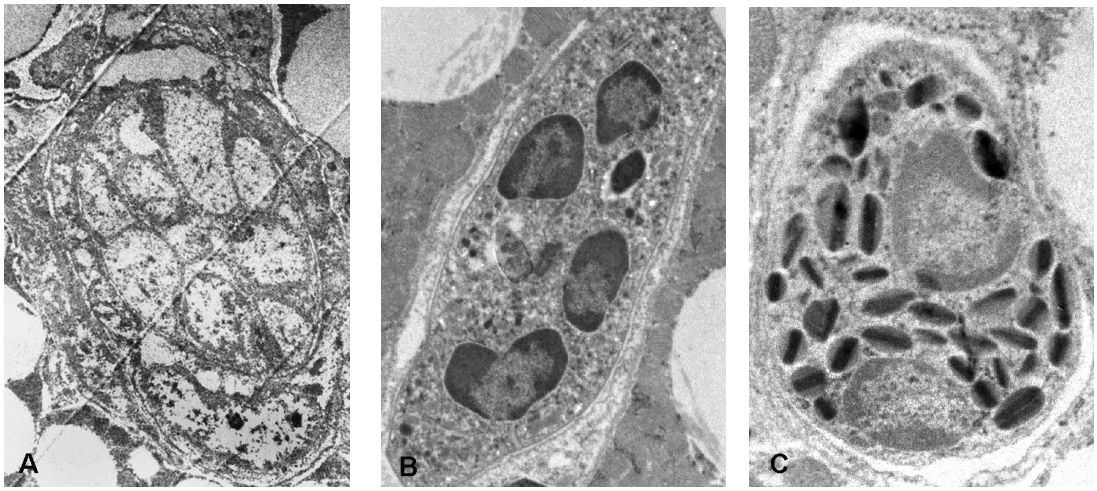


Figure 1. 3. 3: Some non-adipocyte cells in BAT: Schwann cell embracing nerve fibers (A), heterophil granulocyte (B) and eosinophil granulocyte (C). Magnification 2650x (A), 5600x (B) and 8800x (C), original.

Typical brown adipocyte (Fig. 1. 3. 4) is a large polygonal cell covered by thin external lamina. Its average diameter is usually 20-45 μm but it depends on species, age of the animal and state of BAT activity (Smith and Horowitz, 1969; Afzelius 1970; Schneider-Picard *et al*., 1980; 1984). Nucleus is spherical, euchromatic, with prominent nucleolus. Along the surface of the plasma membrane, especially in stimulated BAT, numerous small invaginations and cytoplasmic vesicles as signs of membrane transport are observable (Afzelius, 1970). Brown adipocytes are coupled by gap junctions which enable exchange of ions and small molecules between cells. Physiological stimulation of BAT leads to increase of gap junction area per cell volume (Schneider-Picard *et al*., 1980; 1984).

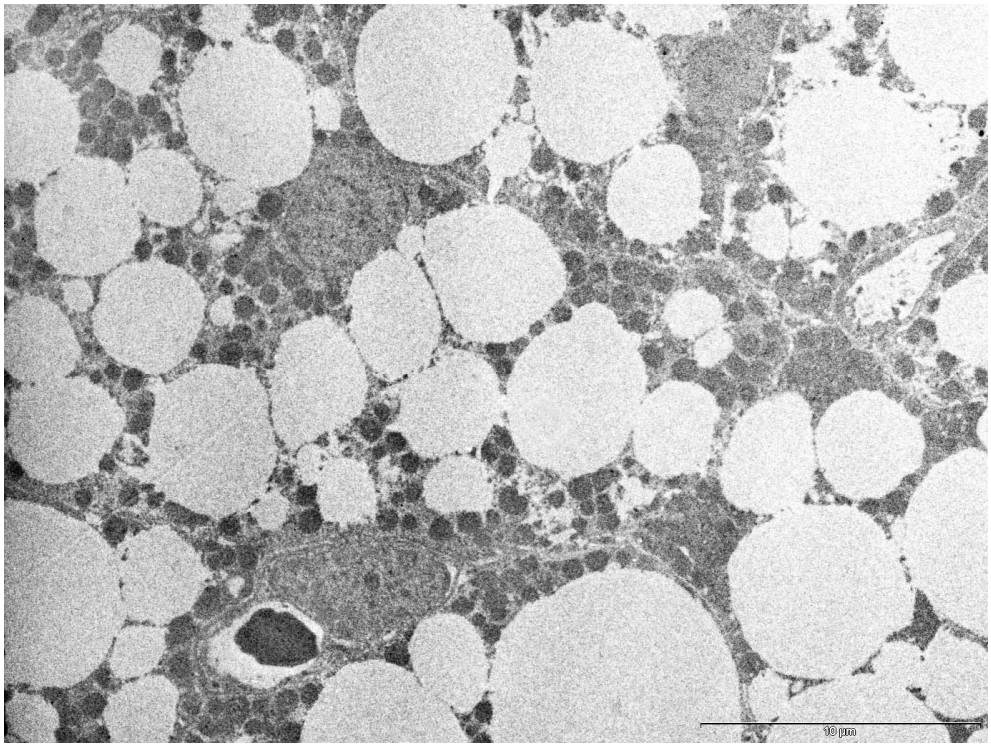


Figure 1. 3. 4: Ultrastructural appearance of brown adipocytes. Multilocular cells equipped with large spherical mitochondria, in close contact with capillaries, original, 2160x (bar = 10 µm).

Brown adipocyte stores fatty acids in the form of neutral lipids, mostly triglyceride, inside bodies of various sizes. The quantity of lipids in the cell and pattern of their distribution into lipid bodies depend on brown adipocyte state of activity. When the cell is thermogenically stimulated (for example during cold stimulation), lipid bodies are small and numerous, while in inactive tissue (for example at the temperature of thermoneutrality) lipids are contained within few large and sometimes fused lipid bodies. Since lipid bodies tend to coalesce due to their physicochemical characteristics, multilocularity is energetically unfavourable for the cell (Greenberg *et al*., 1991; Cinti *et al*., 2001).

The cytoplasm of brown adipocyte is densely packed with numerous mitochondria that represent distinct characteristic of BAT (Figs 1. 3. 5 and 1. 3. 6). They are mostly spherical and larger than mitochondria in other cell types. Numerous long straight cristae transverse the whole width of the mitochondria or form two or three separate, slightly curved systems. Many of the mitochondria are closely apposed to the surface of lipid bodies.

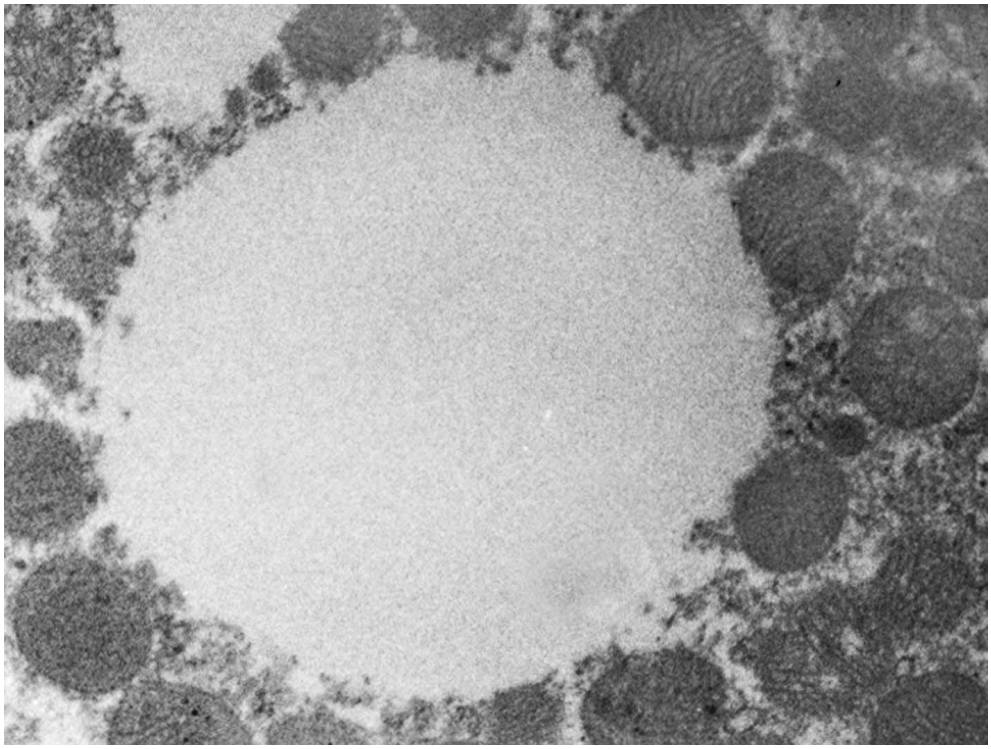


Figure 1. 3. 5: Large spherical mitochondria densely packed with cristae closely apposed to lipid body surface, original, 7100x.

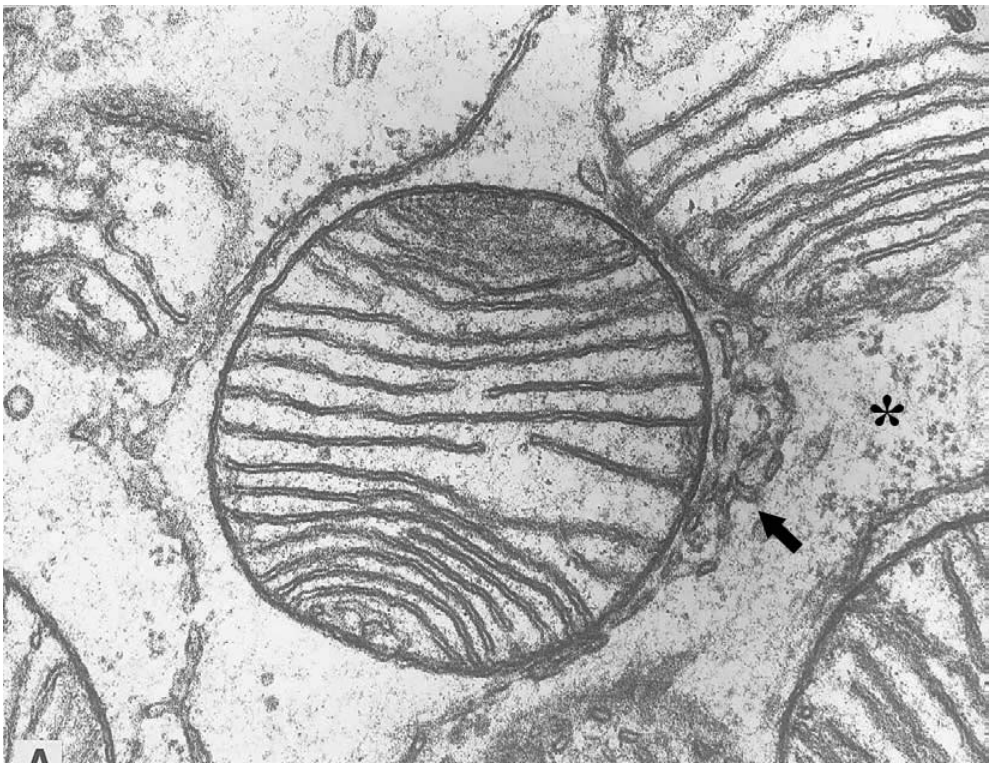


Figure 1. 3. 6: Typical mitochondria from brown adipocyte, 175000x. Cisterns of smooth endoplasmic reticulum (arrow), polyribosomes (asterisk) (taken and modified from Cinti *et al*., 2001).

Other cellular organelles are also present in brown adipocytes but they are less prominent except elongated cisterns of smooth endoplasmic reticulum which are often closely apposed to the surface of the lipid bodies (Ahlabo and Barnard, 1971). Peroxisomes are of typical appearance and it seems that they are involved in BAT response after thermogenic stimulation (Ahlabo and Barnard, 1971; Čakić-Milošević *et al*., 1997; Bagattin *et al*., 2010). Lysosomal system in brown adipocytes is relatively well developed at birth as well as later in life when BAT activity is stimulated. It is believed that lysosomes are involved in brown adipocyte remodelling after thermogenic stimulation (reviewed by Nedergaard *et al*., 1986).

1. 4. Brown adipocyte mitochondria - molecular mechanism of thermogenesis

After thermogenic stimulation brown adipocytes rapidly initiate oxidation of triglyceride lipid stores and release energy in the form of heat. Essential for BAT thermogenic function are morphologically and biochemically unique mitochondria of brown adipocytes.

The ability of BAT mitochondria to produce heat is dependent on the presence of the uncoupling protein 1 (UCP1) in the membrane of their cristae (Nicholls and Locke, 1984; Cinti *et al*., 1989; Matthias *et al*., 2000). Specific expression of UCP1 is accepted as biochemical marker of brown adipocytes (Nicholls *et al*., 1978; Park *et al*., 2014).

UCP1 is transmembrane protein made of 306 amino acids, with apparent molecular weight of 33 kDa. It is inserted into inner mitochondrial membrane with both ends oriented toward intermembrane space. UCP1 is made of three repeated domains each composed of two transmembrane α-helices. α-helices are interconnected by five extramembranous hydrophilic loops (Fig. 1. 4. 1) (Klingenberg and Huang, 1999; Nedargaard *et al*., 2001).

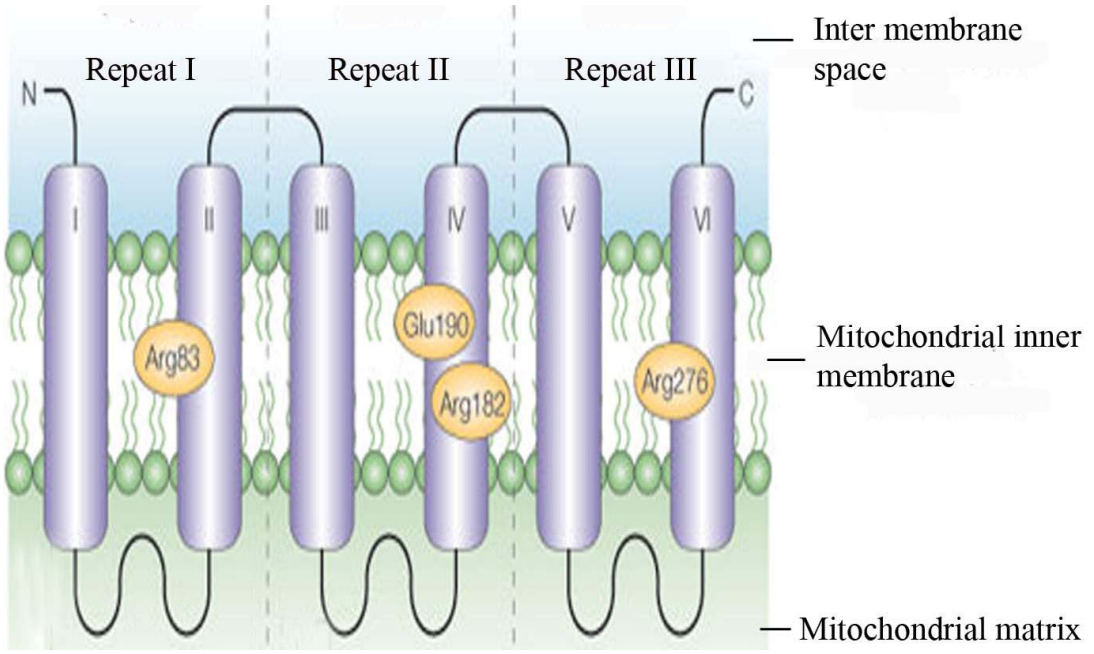


Figure 1. 4. 1: Model of the UCP1 molecule inserted in the inner membrane of mitochondria (taken and modified from Krauss *et al*., 2005).

UCP1 belongs to the family of mitochondrial anion carriers. It plays a role in the dissipation of the proton motive force, created from fatty acid β-oxidation and electron transport down the respiratory chain, thereby producing heat instead of ATP (Himms-Hagen, 1991; Cannon and Nedergaard, 2004). The exact mechanism of UCP1 function is still not completely understood. According to the simplest model, UCP1 acts as symporter for long chain fatty acids (LCFA) and H^+ (Fig. 1. 4. 2). However, LCFA cannot dissociate after binding, so only H^+ is effectively translocated while LCFA serves as the activator (Fedorenko *et al.*, 2012). Purine nucleotides such as ATP and GDP are inhibitors of UCP1 activity. It has also been found that in the membrane UCP1 tends to self-associate to form functional tetramers (Hoang *et al*., 2013).

Expression of UCP1 is controlled by noradrenaline-mediated pathways and influenced by several factors including PPARγ, thyroid hormones and some fatty acids (Sadurskis *et al*., 1995; Foellmi-Adams, 1996; Silva and Rabelo, 1997). The amount of UCP1 is particularly high in cold acclimated rodents (Needergaard and Cannon, 2013). Upon physiological stimulation mitochondrial heat generating activity occurs after 10-15 min, despite the fact that synthesis of UCP1 mRNA requires 1-2 hours and UCP1 synthesis takes an even longer period of time (Ricquier *et al*., 1984; Falcou *et al*., 1985).

This suggests that thermogenic capacity of BAT cannot always be correlated with its actual thermogenic activity, since BAT mitochondria contain a certain amount of "masked", inactive UCP1. It is more precise to say that the expression of UCP1 is proportional to BAT thermogenic capacity. As opposed to mitochondria in other tissues, BAT mitochondria possess only small amounts of ATP-synthase and thus they are not efficient in ATP synthesis (Cannon and Vogel, 1977; Houstek *et al*., 1991).

According to different studies, the half life of UCP1 inserted in the inner mitochondrial membrane is calculated to be from 30 hours to about five days (Puigserver *et al*., 1992; Rousset *et al*., 2007).

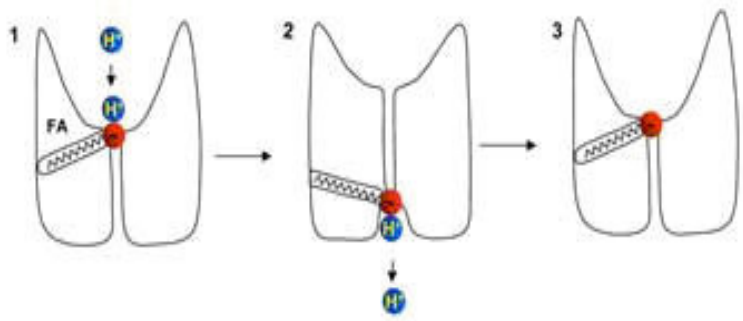


Figure 1. 4. 2: Model of LCFAs-dependent UCP1 activity: UCP1 acts as LCFA anion/H^+ symporter wherein LCFA can not dissociate from the binding site within UCP1 (taken from Fedorenko *et al*., 2012). FA - fatty acid.

1. 5. Control of BAT thermogenesis

BAT thermogenesis is dominantly controlled by noradrenaline released from sympathetic nerve endings innervating the tissue (Fig. 1. 5. 1) (Cannon and Nedergaard, 2004). Stimulation of β_3-adrenergic receptors on the membrane of brown adipocytes leads to increase of cAMP and activation of protein kinase A. This results in phosphorylation and activation/deactivation of target proteins. Upon phosphorylation, perilipin releases comparative gene identification 58-protein (CGI-58) which then activates adipose triglyceride lipase (ATGL). Protein kinase A also activates hormone sensitive lipase, which together leads to triacyl-glycerole hydrolysis (Himms-Hagen, 1991; Blanchette-Mackie *et al*., 1995; Granneman *et al*., 2007; 2009; Miyoshi *et al*., 2007; Souza *et al*., 2007). Released fatty acids serve as substrate for β-oxidation in

mitochondrial matrix and regulate UCP1-dependent proton conductance pathway on the inner mitochondrial membrane. In addition, stimulation of β_3-receptors promotes the expression of genes for UCP1 and lipoprotein lipase as well as mitochondrial biogenesis (Carneheim *et al*., 1988; Himms-Hagen, 1991; Uldry *et al*., 2006). After only few hours, in overstimulated brown adipocytes biogenesis of new mitochondria and UCP1 synthesis occur, by which brown adipocytes adapt their functional capacity to increased thermogenic requirements.

Beside β_3 receptors, other types of adrenergic receptors are also present in BAT. Thus, β_1-adrenoceptors on the membrane of brown adipocytes are mainly involved in proliferation of brown adipocytes and tissue hyperplasia, α_1-receptors contribute to positive regulation of thermogenic processes, while α_2-stimulation counteracts effects of β_3-stimulation. (Lafontan and Berlan, 1993; Cannon *et al*., 1996; Bronnikov *et al*., 1999). Receptors of β_2-type are predominantly localized to the vascular elements and are responsible for vascular response (Cannon and Nedergaard, 2004).

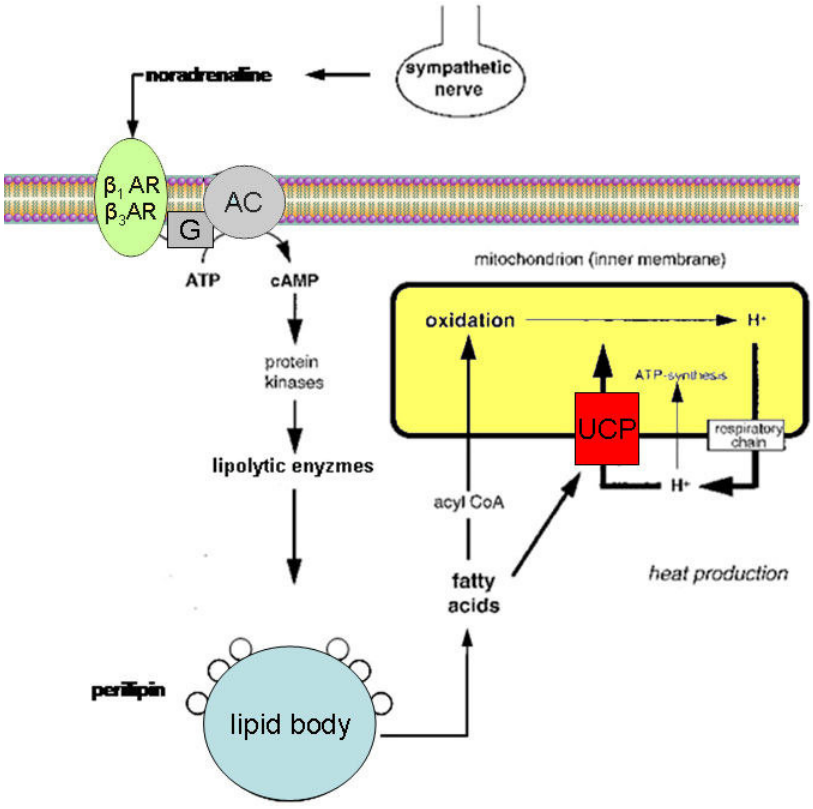


Figure 1. 5. 1. Schematic presentation of thermogenesis activation pathways in brown adipocytes after physiological stimulation.

1. 6. Hormones and mediators affecting BAT function

Beside noradrenaline, several hormones may modulate BAT thermogenic capacity and function, as it has been reviewed in details by Reddy *et al*. (2014). Glucocorticoids, aldosterone, testosterone and prolactin negatively affect BAT function, progesterone, growth hormone, and probably insulin and insulin growth factor-1 exert positive effects, while oestrogen seems to act dually.

For long time, the role of thyroid hormones in regulation of BAT thermogenesis has been considered as permissive (see Himms-Hagen, 1983 for review). Later, it has been accepted that they act synergistically with noradrenaline and that they are necessary for preparing for thermogenesis although not for its maintenance (Rubio *et al*., 1995; Ribeiro *et al.*, 2001; Zaninovich *et al*., 2002). Although studies are still incomplete, some recent findings suggest possible role for thyroid hormones in thyroid thermogenesis (Lopez *et al*., 2010).

TNFα belongs to the group of proinflammatory cytokines, pharmacologically active proteins of small molecular weight that are involved in autocrine and paracrine regulation of variety of cellular processes. It is produced primarily by macrophages and T-lymphocytes, but it may originate also from mast cells, B-lymphocytes, natural killer cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts and osteoclasts (Bradley, 2008). It is also produced in adipose tissue, both by adipocytes and stroma-vascular cells. Acting on white adipose tissue, TNFα exerts antilipogenic and antiadipogenic effects and induces insulin resistance. To date, there are few studies that have investigated the effects of TNFα on BAT. Mostly, they dealt with influence of TNFα on UCP1 expression and β3-adrenergic receptor level, yielding often conflicting results (Coombes et al., 1987; Nisoli et al., 1997; Sethi and Hotamisligil, 1999; Cawthorn and Sethi, 2008).

1. 7. Endocrine/paracrine/autocrine role of brown adipose tissue

BAT secrets various bioactive molecules with endocrine, paracrine and autocrine effects. These factors include but are not limited to fibroblast growth factor-2, bone morphogenetic protein-8b, insulin-like growth factor I, prostaglandins (autocrine);

NO, vascular endothelial growth factors, angiotensinogen (paracrine); and interleukin-1α, interleukin-6, adiponectin, fibroblast growth factor 21, leptin (endocrine) (Villarroya *et al*., 2013).

Interestingly, BAT is a poor source of endocrine factors normally secreted by WAT such as leptin and adiponectin, especially in thermogenically activated state (Cannon and Nedergaard, 2004). Some authors considered activated BAT as the important systemic source of triiodothyronine (T3) due to activity of deiodinase 2, expressed in brown adipocytes (Silva and Larsen, 1985).

Other important systemic effects of thermogenically activated BAT are related to its ability to uptake glucose and triglyceride from circulation and combust them for heat production. In this way, BAT takes a role in systemic protection from dyslipidemia and hyperglycemia (Gasparetti *et al*., 2003; Bartelt *et al*., 2011).

1. 8. Types of thermogenesis - obligatory and facultative

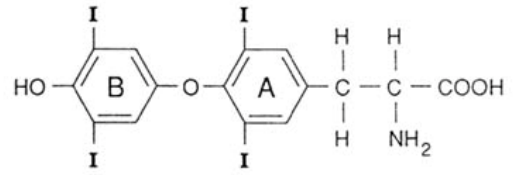
In homeothermic species two types of thermogenesis exist: obligatory and facultative. The obligatory thermogenesis is the constitutive heat production needed for maintaining cells alive. It represents heat produced by the body in the resting state, at the temperature of thermoneutrality, 12 hours after last food intake. Thyroid hormones are the main regulators of this type of thermogenesis (Silva, 2003).

In the cold environment when obligatory thermogenesis is not enough to maintain body temperature, body first activates energetically inexpensive heat-conserving mechanisms (cutaneous vasoconstriction, piloerection), followed by thermogenic mechanisms if required. These additional thermogenic mechanisms constitute adaptive thermogenesis which comprises shivering and, if cold is sustained, non-shivering facultative thermogenesis, with BAT as the main effector organ (Silva, 2003; 2006).

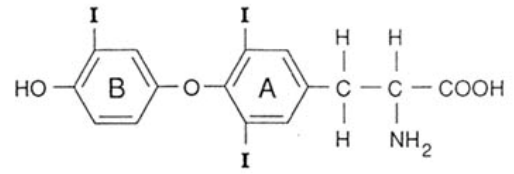
Defending temperature homeostasis at the environmental temperature above thermoneutrality also requires energy costs, but such considerations are beyond this study.

1. 9. Thyroid gland

Thyroid gland is an endocrine gland specialized for production and release of thyroid hormones, thyroxine (T4) and T3 (Fig. 1. 9. 1). T4 is a quantitative dominant hormone released from the gland, while T3 originates mainly from peripheral deiodination of T4 and has stronger biological activity (Silva 2003; Boelaert and Franklyn, 2005). Currently, increasing evidence has pointed to the physiological role for another thyroid hormone derivative, 3,5-diiodothyronine, mostly at the mitochondrial level (Goglia, 2005).



Thyroxine (3,5,3',5'-tetraiodo-L-thyronine)



Triiodothyronine (3,5,3'-triiodo-L-thyronine)

Figure 1. 9. 1: Thyroid hormones, thyroxine and triiodothyronine.

Thyroid gland (Fig. 1. 9. 2) is a brownish-red, butterfly-shaped gland that lies in the neck, in front of the upper part of the trachea. It consists of two large lobes connected by isthmus. Thyroid receives blood from the superior and inferior thyroid arteries, branches of external carotid artery and thyrocervical trunk, respectively. Venous drainage is provided by paired thyroid veins - superior, middle (both empty into jugular vein) and inferior (empties into innominate vein). Thyroid also possess complex lymphatic drainage. Principal innervation of the thyroid is derived from the autonomic nervous system (Schünke *et al*., 2010).

Connective tissue capsule at the surface of the thyroid makes projections into the parenchyma, dividing it in lobules. Parenchyma is organized into a number of follicles

whose walls are made of cuboidal epithelial cells, designated as thyrocytes or follicular cells, surrounding a lumen filled with colloid. The height of these cells, their internal organization, together with the size and shape of the follicle, depend on the activity of the thyroid gland (Ross and Pawlina, 2011b).

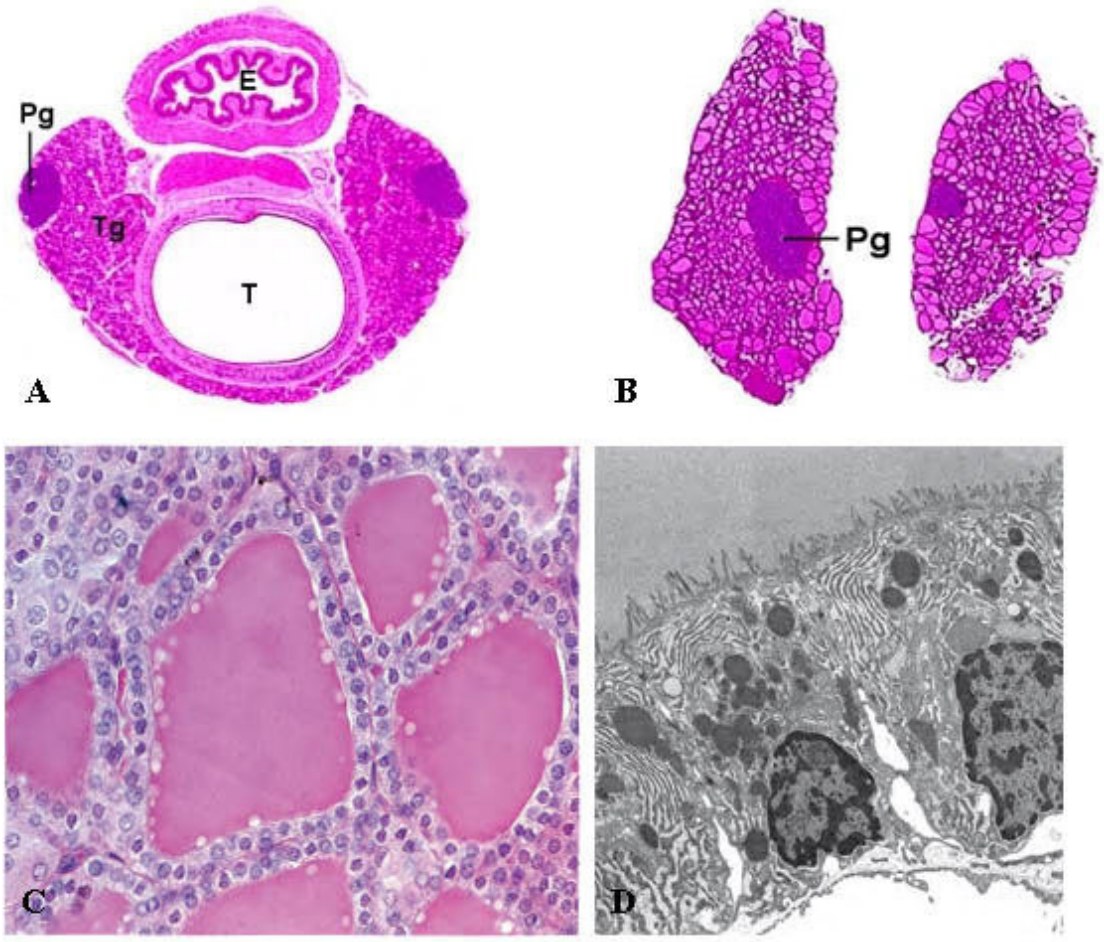


Fig. 1. 9. 2: Thyroid gland - light (a, b, c) and electron microscopical (d) images (taken from Kittel *et al*. 2004; Ross and Pawlina, 2011b). E - esophagus, Pg - parathyroid gland, T- trachea, Tg - thyroid gland.

Thyrocyte synthesizes thyroid hormones through following steps (Fig. 1. 9. 3):

a) uptake of iodide from the circulation (Na^+/I^- symporter localized in the basolateral membrane of thyrocytes), its transport through apical membrane of thyrocyte (pendrin) and oxidation to an active iodine (thyroid peroxidase);

b) synthesis of large glycoprotein thyroglobulin rich in amino acid tyrosine and its secretion into the follicular lumen;

c) iodination of tyrosyl residues along the thyroglobulin, resulting in either monoiodotyrosine or diiodotyrosine formation (thyroid peroxidase);

d) intramolecular coupling of monoiodotyrosine and diiodotyrosine resulting in formation of T3 and T4 (catalyzed by thyroid peroxidase); thyroglobulin containing T3 and T4 is stored inside the follicular lumen;

e) endocytosis of thyroglobulin by thyrocytes, its proteolytic cleavage and release of T3 and T4 into circulation.

Thyroid hormones reach their target cells by blood where they are found free or bounded to plasma proteins: thyroxine-binding globulin, thyroxine-binding prealbumin and albumin.

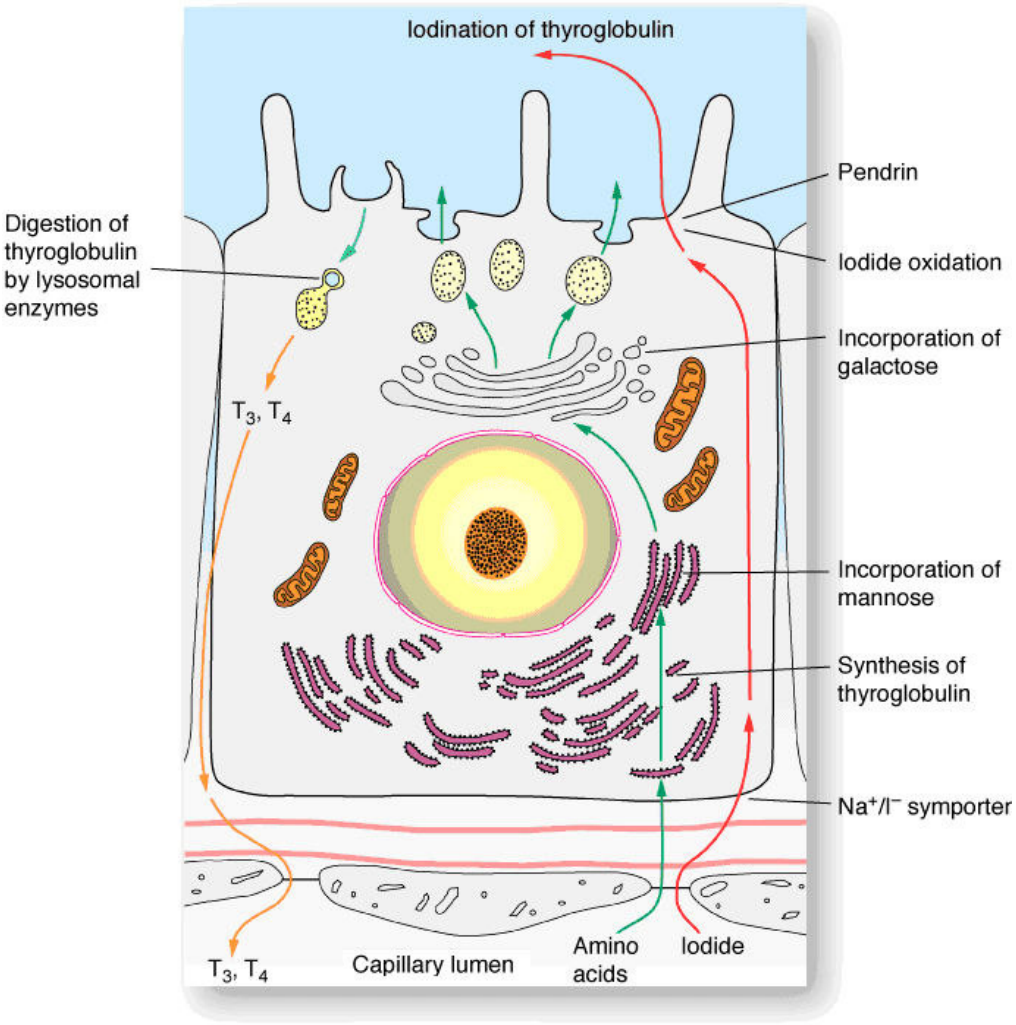


Figure 1. 9. 3: Synthesis and iodination of thyroglobulin and its absorption, digestion and hormone release (taken from Junqueira and Carneiro, 2003).

Synthesis and secretion of thyroid hormones are regulated by hypothalamus-pituitary-thyroid axis, with the negative feedback loop at the level of both hypothalamus and pituitary gland (Fig. 1. 9. 4) (Norman and Litwack, 1987; Williams and Bassett, 2011). Thyroid stimulating hormone (TSH) synthesized and secreted from anterior pituitary thyrotrophs regulates activity of thyroid gland. In turn, secretion from thyrotrophs is controlled by hypothalamic thyrotropin-releasing hormone (TRH). Circulating thyroid hormones suppress the release of thyroid stimulating hormone and thyrotropin-releasing hormone, providing so-called long negative feedback loop. In addition, there exist short and ultra-short feedback loops represented by suppressive activity of thyroid stimulating hormone on release of both thyrotropin-releasing hormone and thyroid stimulating hormone (Prummel *et al*. 2004).

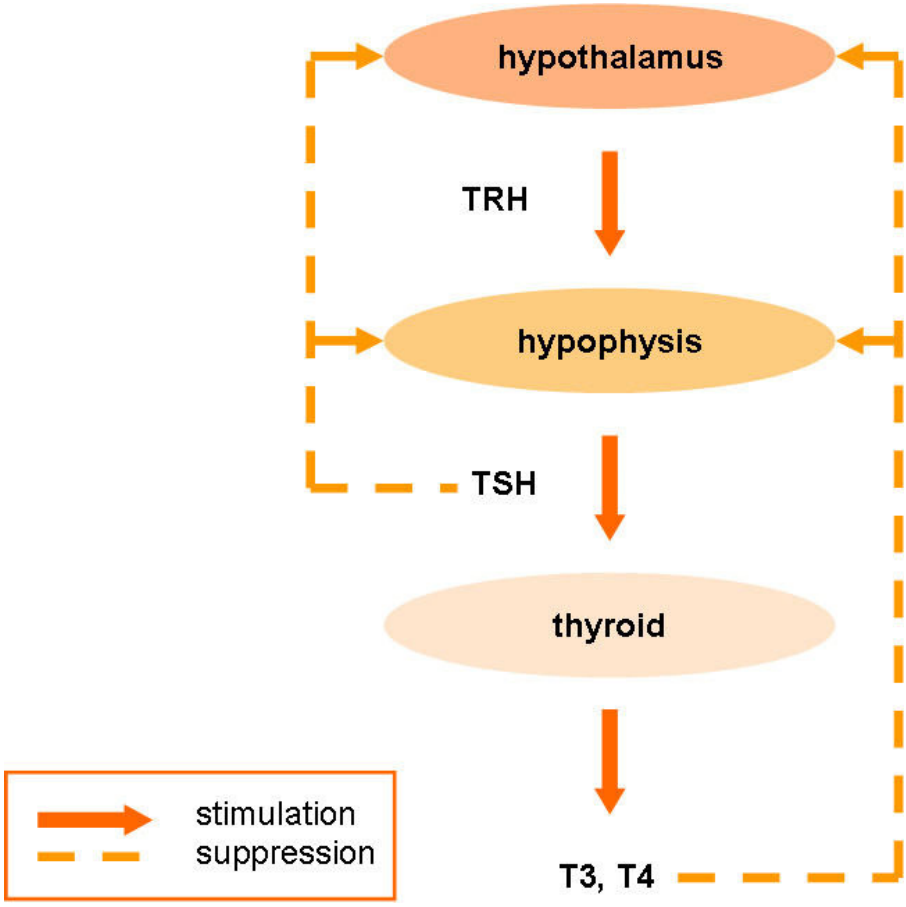


Figure 1. 9. 4: Control of TH synthesis. TH - thyroid hormones, TSH - thyroid stimulating hormone, TRH - thyrotropin-releasing hormone.

Thyroid gland contains another type of secretory cells called parafollicular cells (C cells). They are found between the follicles and synthesize and secrete calcitonin which regulates blood calcium level (Ahrén, 1991; Sawicki, 1995).

Peripheral metabolism of thyroid hormones is regulated by the activity of deiodinase enzymes type 1, 2 and 3 (Fig. 1. 9. 5). Deiodinase 2 removes iodine from the outer ring of iodothyronines, leading to the formation of T3 from T4 and diiodothyronine from reverse T3; deiodinase 1 transforms T4 into T3 but also removes iodine from inner ring of T4 forming inactive reverse T3; it also deiodinates reverse T3 into diiodothyronine; finally, deiodinase 3 transforms T4 into reverse T3 and T3 into diiodothyronine (Bianco and Kim, 2006).

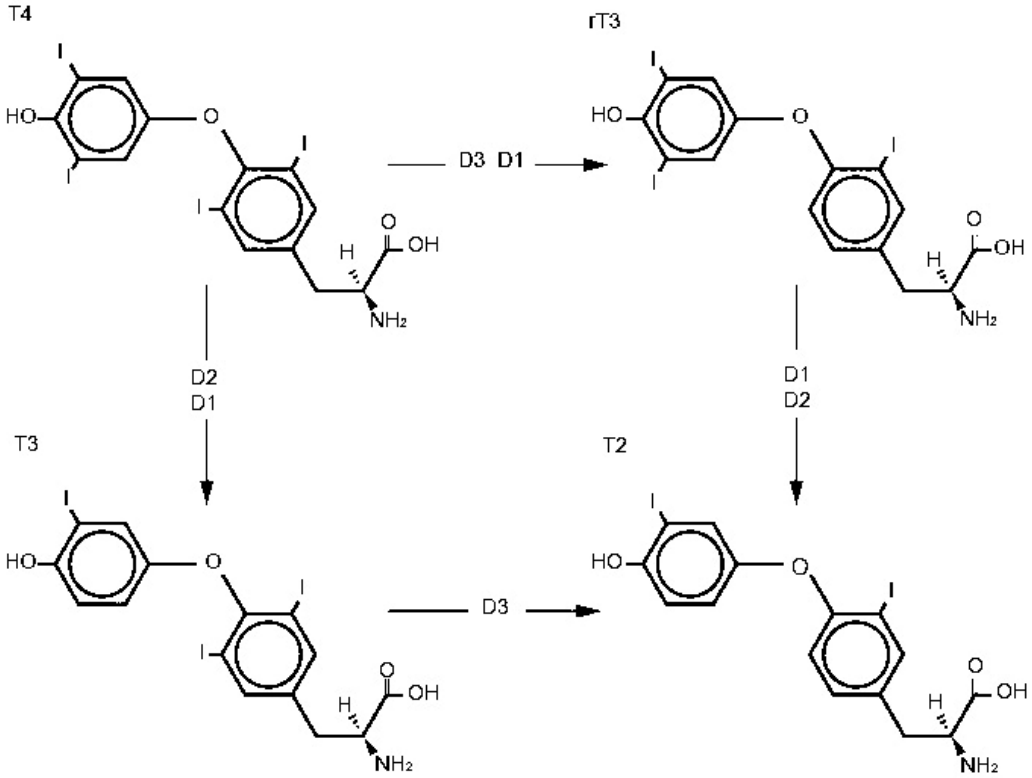


Figure 1. 9. 5: Peripheral metabolism of thyroid hormones catalyzed by deiodinases. (taken and modified from Bianco and Kim, 2006). D1, D2 and D3 - deiodinase 1, 2 and 3, T4 - thyroxine, rT3 - reverse triiodothyronine, T3 - triiodothyronine, T2 - diiodothyronine

In target cell, thyroid hormones (mostly T3) action is mediated through specific nuclear thyroid hormone receptor which usually forms a heterodimer with retinoid X receptor. This complex binds to thyroid hormone response elements and controls

transcription of large number of genes. Physiological effects of thyroid hormones include stimulation of metabolic activity, growth and differentiation of almost all tissues in the body. They stimulate oxygen consumption, glucose absorption, gluconeogenesis, lipolysis, protein synthesis, but also protein catabolism (Guyton and Hall, 2006; Brent, 2012). Thyroid hormones may also act on mitochondria, increasing their size, number and activity (Psarra and Sekeris, 2008).

1. 10. Effects of thyroid hormones on BAT activity

Regarding their physiological roles, both BAT and thyroid gland/thyroid hormones might be considered as participants in regulation of body energy metabolism and temperature homeostasis. Accordingly, these two systems are in complex regulatory and functional interrelationship.

Thyroid hormones are included in the regulation of the basal metabolic rate and heat production in processes which require minimal energy expenditure (basal thermogenesis) while BAT is responsible for the biggest part of heat production "on demand" (facultative thermogenesis) (Silva, 2003). Based on this, it could be said that these two systems are functionally complementary and that the increase of the level of thyroid thermogenesis reduces the need for BAT thermogenesis and *vice versa*.

Thyroid hormones are necessary for establishing thermogenic capacity of the BAT. Besides potentiation of noradrenaline β_3-mediated effects on synthesis of UCP1 mRNA, T3 directly induces expression of UCP1 (Rubio *et al*., 1995; Ribeiro *et al*., 2001). As it has been reported previously by others, rats adapted to room temperature and made hypothyroid fail to protect temperature homeostasis after cold exposure, fall into hypothermy and eventually die (Hsieh, 1961; Cageao *et al*., 1992; Zaninovich *et al*., 2003). However, in the case of moderate hypothyroidism, deiodinase 2 of brown adipocytes is able to deiodinate T4 and provide sufficient local quantity of T3 to meet BAT requirement (Bianco *et al*., 2006). Thyroid hormones are also required for expression of number of genes involved in proliferation and differentiation of brown adipocytes and mitochondriogenesis (Obregon, 2008).

BAT response on systemic hyperthyroidism is even less clear. Hyperthyroidism may induce central as well as systemic effects, both of which may interrelate with BAT

function. Central effect of hyperthyroidism leads to increase in "defended" body temperature (hyperpyrexia, hyperthyroid hyperthermia) with recruited and activated BAT as possible thermogenic effector organ (Cannon and Nedergaard, 2004). Several years ago, this was supported by the finding that T3, acting centrally, increases sympathetic input and upregulates several important thermogenic markers in BAT (López *et al*., 2010). On the other hand, acting peripherally, systemic hyperthyroidism provokes increase in basal thermogenesis, acting through mechanisms which are still not completely understood. This results in diminished need for facultative thermogenesis and thus deactivation and atrophy of BAT. According to these data, BAT functional status in systemic hyperthyroidism will be determined by shift in balance between central and peripheral effects, with some other factors that are probably also involved. Finally, thyroid hormones in systemic hyperthyroidism directly affect BAT, increasing thermogenic capacity but not necessarily its thermogenic function (Cannon and Nedergaard, 2004).

2. Aims and Objectives

Overweight and obesity are today's growing public health problem worldwide. They are caused by imbalance between energy intake and energy expenditure. BAT as energy dissipating tissue emerged as possible target for antiobesity therapy, especially since its existence has been recently confirmed in adult humans.

Thyroid hormones play an important role in regulation of temperature homeostasis and energy metabolism. They are also involved in regulation of BAT activity. Thus, continues cross-talk between thyroid hormones and adipose tissue may be important for body weight control. While the importance of thyroid hormones for proper BAT function is well known, the influence of hyperthyroidism on BAT activity is not yet fully understood.

This dissertation aims to explain the effects of experimentally induced systemic hyperthyroidism on structural remodelling and thermogenic capacity of BAT. In particular, this thesis investigates differences, if any, between effects of systemic hyperthyroidism induced by two thyroid hormones, T3 and T4.

Experimental treatment used in this study provided a rarely employed model for investigating the effects of exogenously induced hyperthyroidism on thyroid gland. Therefore, another aim of this dissertation is to investigate possible toxic effects of T3 or T4 on thyroid gland.

Specific assignments are to reveal possible changes in histological, ultrastructural and stereological parameters of BAT and expression pattern of proteins and mediators involved in its functioning, regulation and signalling, after short-term treatment with supraphysiological doses of thyroid hormones, either T3 or T4; to explain these differences in context of BAT function and overall body reaction to systemic hyperthyroidism; to investigate histological, ultrastructural and stereological parameters of thyroid gland in experimentally induced systemic hyperthyroidism.

In order to achieve these specific objectives both qualitative and quantitative methods were used: (a) routine histological and histochemical analyses at the light microscopy level were performed on paraffin, as well as on semi-fine sections of plastic embedded tissue; (b) detection of apoptosis on paraffin sections; (c) immunohistochemical detection of UCP1, Ki67 and TNFα on paraffin sections; (d)

electron microscopy ultrastructural analysis; (e) morphometry and stereology at the level of light and electron microscopy.

Results of this study should set forward the general knowledge of mechanisms regulating BAT activity and expand the basis for better understanding energy metabolism regulation, which would open new possibilities for developing strategies to resist obesity.

3. Material and Methods

3. 1. Animals and treatment

The experiment was performed on a total of 18 male Wistar rats, weighing 180–250 g. Animals were caged individually, at room temperature (22±1ºC), in 12:12 h light–dark cycle and had free access to food (commercial rat food, Subotica, Serbia) and tap water. Animal handling and treatment were carried out in accordance with The Serbian Laboratory Animal Protection Law proposed guidelines and protocols approved by The Ethics Committee of the Faculty of Biology, University of Belgrade.

The rats were divided into three equal groups and treated by intraperitoneal injections, once a day, for 5 days, as follows: T3-treated rats received injections of T3 (200 μg/kg b.w.) dissolved in 9 mM NaOH; T4-treated animals received T4 (300 μg/kg b.w.) dissolved in 9 mM NaOH; control (euthyroid) animals were injected with vehicle only (9 mM NaOH, 1 ml/kg b.w.). Body temperature was measured at the beginning and at the end of the experiment. In the course of the experiment, all animals were in good health and condition. After the last injection, body mass was measured and rats were sacrificed by decapitation using a guillotine (Harvard Apparatus, Holliston, MA, USA).

3. 2. Determination of thyroid hormones in the circulation

For determination of T3 and T4 concentration in the serum, blood samples were collected from the trunk during sacrificing. Total serum T3 and T4 concentrations were determined by the RIA method, at the laboratory of The Institute for the Application of Nuclear Energy (INEP, Belgrade, Serbia).

3. 3. Procedures used for thyroid gland

Processing of thyroids for light microscopy. After isolation and weighing, the left lobe of each thyroid gland was routinely processed for light and the right one for electron microscopy. For light microscopy, each thyroid lobe was fixed in 3.7%

phosphate-buffered formalin (pH=7.2), dehydrated through an ethanol series, cleared in xylene and embedded in paraffin. For general histological analysis, as well as for stereological measurements, 5 μm thick paraffin sections were obtained using rotary microtome Spencer (No. 820, American Optical Company, Buffalo, NY, USA). Sections, taken from the anterior, medial and posterior part of the thyroid lobe (five nonserial sections per each chosen part of a lobe) were stained with hematoxylin/eosin method, analyzed and photographed on Leica DMLB light microscope (Wetzlar, Germany), using Leica DFC 295 camera and LASCore software or Leica DMRB with JVC TK 1280E Video Camera (Leica) and QWin software (Leica).

Hematoxylin and eosin (HE) staining. Prior to staining in water soluble stains, paraffin had to be removed from tissue sections because it is not miscible with water. It is achieved by immersing microscopic slides, with tissue of interest on them, in xylene (2 times, 5 min. each). Sections were rehydrated by ethanol (100%, 96% and 70%, 5 min. each), brought to water, and stained in freshly filtered hematoxylin (5 min.), and eosin (1-2 min.). Tissue sections were dehydrated by ethanol (96% and 100%, 2 min. each), cleared in xylene and mounted in DPX. Tissue stained with hematoxylin and eosin is used for analysing the general histological organization of both BAT and thyroid gland.

Detection of cell death. Cell death was demonstrated by propidium iodide (PI) staining method (Scaglia et al., 1997; Markelic et al., 2011). Glycerol-mounted sections were examined with a Zeiss Observer.Z1 fluorescent microscope and photographed with AxioCam MR3 camera, using AxioVision Rel4.7 software. The occurrence of cell death was estimated by counting normal and apoptotic nuclei in the follicular wall, using three non-serial sections per each chosen part of the thyroid lobe (as described above). Results are presented as percentage of apoptotic nuclei.

Propidium iodide method. In order to demonstrate dead (apoptotic) cells in tissue, deparaffinized and rehydrated 5 μm thick sections were incubated in propidium iodide water solution (1 mg/ml, 10 minutes), washed in tap water and mounted in glycerol. Propidium iodide will bind to all nuclei because in the tissue specimen fixed in formalin all cells are dead. However apoptotic cells have more condensed chromatin, which stains vivid red.

Processing of the thyroids for electron microscopy. After initial fixation in 3.7% phosphate-buffered formalin, the right lobe of each thyroid gland was thoroughly rinsed

in tap water, diced into small pieces (~1 mm^3) and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, at pH 7.4, for 1 h, at 4ºC. This was followed by postfixation in 1% osmium-tetroxide in 0.1 M phosphate buffer at pH 7.4, for 1 h, at 4ºC. After dehydration through a series of cold alcohols and propylene oxide, tissue samples were embedded in Araldite. Ultrathin sections, gained after selection from semi-fine (1 μm) toluidine blue stained sections, were cut on Leica EM UC6 (Leicamicrosystem, Wetzlar, Germany) equipped with diamond knife (Diatome, Switzerland), contrasted with uranyl acetate and lead-citrate (Leica EM STAIN, Leica Microsystems, Germany), examined with a Philips CM12 electron microscope (Eindhoven, The Netherlands) and photographed with SIS MegaView III CCD digital camera (Olympus Soft Imaging Solutions).

Toluidine blue staining method. Semi-fine sections were used for histological analysis and for choosing the suitable part of tissue to be studied under the electron microscope. These sections were mounted on microscopic slides and stained with 1% toluidine blue mixed with Na-tetraborax, until the fluorescent green rim around the stain droplet appeared. The result of this staining was tissue coloured in different shades of blue.

Stereological analysis. Stereological analysis was performed by the point-counting method, using M42 multipurpose test grid, at a final magnification of 400x. Data obtained from 20 test fields of each sectioned part of the thyroid lobe (paraffin embedded samples stained with hematoxylin/eosin) served to calculate volume density of particular phases (colloid, follicular epithelium, interfollicular tissue) using standard equation $V_{vph} = P_{ph}/P_{tot}$, where P_{ph} is number of points on a particular phase and P_{tot} is the total number of points over tested area) (Weibel, 1979). Thyroid activation index (I_a) was expressed as the epithelial to colloid volume density ratio ($I_a = V_{ve}/V_{vc}$) (Kalisnik, 1972). All fully visible thyroid follicles seen on examined slides were included in the determination of mean diameter of thyroid follicles, which was calculated as: (max transverse diameter + max diameter perpendicular to the first one)/2. Follicular cell height was determined from toluidine blue-stained semi-thin sections of plastic embedded tissue. Measurement was carried out on 300 cells per animal, having a clearly defined nucleus, by the use of a micrometer scale inserted into the ocular, at a magnification of 1000x.

3. 4. Procedures used for BAT

Processing of BAT for light microscopy. After isolation and weighing, one half of each BAT pad from each animal was processed at the same manner as it is described for thyroid gland, above. Five micrometers thick sections were stained by variety of staining methods in order to demonstrate different aspects of histological architecture of the tissue:

- For general histological analysis, as well as for stereological measurements, sections stained with HE were used, taken from the anterior, medial and posterior part of the BAT lobe (five nonserial sections per each chosen part of a lobe).
- For demonstration of the erythrocytes and hence functional vascularization of the BAT, one section taken from each part of the BAT lobe was stained using Novelli staining method.

Novelli method. Routinely deparaffinized and rehydrated tissue sections were treated with warm (60 ºC) 1N HCl for 3 minutes, and washed in distilled water. The sections were firstly stained in 1% solution of acid fuchsin (1 min), washed under tap water, and then stained in 1% solution of light green (5 min), and rinsed. After short dehydration and clearing, sections were mounted in DPX. As a result of this staining erythrocytes are easily identified in tissue sections as their fuchsia stain pops up on the blue-greenish background.

- For demonstration of nerve fibers, one section taken from each part of the BAT lobe was stained using Bodian staining method.

Bodian method. After routine deparaffinization and rehydratation sections were placed in 1% protargol solution (silver proteinate), enriched with copper (4-5 g per 100 ml of solution), for 24 h at 37°C. Slides were rinsed in distilled water and placed in reducing solution (1% hydroquinone and 5% formalin, 10 min). After washing in distilled water, incubation in 1% gold chloride was done (10 min). Slides were rinsed and placed in 2% oxalic acid, until sections were grey and nerve fibers were black (3-5 min). After rinsing in distilled water sections were incubated in 5% sodium thiosulfate for 5 minutes, and finally rinsed. Tissue sections were dehydrated in ethanol, cleared in xylene and mounted in DPX. As the result of this staining method black nerve fibers can be seen either as dots or lines (in cross- or longitudinal-sectioned fibers, respectively) on the grey-blue background, with reddish nuclei.

- For demonstration of glycogen depositions, one section taken from each part of the BAT lobe was stained using PAS staining method (Periodic Acid Schiff staining).

Periodic Acid Schiff method. This method serves for demonstration of tissue components characterized by adjacent glycolic or aminohydroxylic groups, *i.e.* for macromolecules such as glycogen, glycans and mucins. All specific reagents applied were part of P.A.S. Periodic Acid Schiff Hotchkiss Mc Manus 04 – 130802 kit (Bio-Optica Milano). Tissue sections were first routinely deparaffinized and rehydrated, then immersed in periodic acid solution for 10 minutes, and washed in distilled water. Afterwards Schiff reagent Hotchkiss McManus was put on sections and left to act 20 minutes. After being washed in distilled water, potassium methabisulphite solution was left to act on sections for 2 minutes. Slides were just drained and fixative solution was put on sections (2 min), and rinsed in distilled water. As a final reagent, Mayer's hemalum was left to act on sections for 3 minutes. Slides were washed in running tap water for 5 minutes, dehydrated through ascending alcohols, cleared in xylene and mounted in DPX. Results of this staining are magenta red PAS-positive substances and blue nuclei.

Detection of cell death. Cell death was demonstrated by propidium iodide (PI) staining method, according to the same procedure as described for thyroid gland, above.

Immunohistochemistry. For immunohistochemical staining of tissue sections LSAB (labelled streptavidin-biotin) method was used. This is highly sensitive method in which antigen/primary antibody complex is linked by biotinylated secondary antibody with streptavidin-peroxidase conjugate, and this conjugate becomes visible after suitable chromogenic substrate is applied. From each animal, one 5 μm thick paraffine section taken from the medial part of BAT lobe was used for each of UCP1, Ki-67 and TNFα immunohistochemical detection.

Imunohistochemistry. Routinely deparaffinized and rehydrated tissue sections were treated with hydrogen peroxide and blocking serum in order to minimise background and unspecific staining. Afterwards sections were incubated with primary antibodies, appropriately diluted.

For the detection of the UCP1 in inner mitochondrial membrane of BAT, anti-UCP1 rabbit polyclonal antibody (ab10983, Abcam) was used (1/300, overnight, 4°C).

To visualise cells in different active phases of the cell cycle, mouse monoclonal antibody for nuclear protein Ki-67 (M7248, DakoCytomation) was used. Epitope revealing (in 10 mM citrate buffer, pH 6, 20 min, 100°C) preceded the incubation with primary antibody (1/30, 30 min, room temperature).

TNFα which can modulate many immune and inflammatory functions was detected using rabbit polyclonal antibody (sc-8301, Santa Cruz Biotechnology), in final dilution 1/300, overnight at 4°C.

Incubation with secondary antibody (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit, ab64264, Abcam) and streptavidin peroxidase were done afterwards. Staining was finished by incubating slides in solution containing both DAB chromogen and its substrate, and counterstaining in Mayer's hematoxylin. Sections were mounted in DPX after short dehydration and clearing. Positive result of immunohistochemical staining was evident as brown colour of tissue in areas in which proteins of interest/ antigens are present.

Processing of BAT for electron microscopy. Another half of each BAT pad was processed for electron microscopy according to the same procedure as described for thyroid gland, above.

Stereological analysis. Stereological analysis at the light microscopy level was performed by the point-counting method, using M42 multipurpose test grid, at a final magnification of 400x. Data obtained from 20 test fields of each sectioned part of the BAT half (paraffin embedded samples stained with hematoxylin/eosin) served to calculate volume density of particular phases (multilocular cells, unilocular cells, blood vessels, connective tissue) using standard equation, as described for thyroid gland, above.

Length of nerve fibers was determined from micrographs of paraffin sections stained with Bodian method (20 test fields of each sectioned part of the BAT half, final magnification of 400x), using ImageJ software freely available at http://rsb.info.nih.gov/ij/.

Stereological analysis at the electron microscopy level was performed using ImageJ software. Cell profile area, profile area and volume density of nucleus, mitochondria, lipid droplets and cytoplasm, number of lipid droplets per cell profile, number of mitochondria per cell profile and number of mitochondria/μm^2 of cell profile were determined from selected micrographs of brown adipocytes containing large nuclear profile, 15 micrographs per animal, at magnification of 3000x. Lipid droplets perimeter in contact with mitochondria and mitochondria perimeter in contact with lipid droplets were determined from selected micrographs (10 per each animal), at magnification of 7100x. Classification of mitochondria and lipid droplets in size ranges were made from analysing about 800 mitochondria per group and about 500 lipid droplets per group, from micrographs made at 7100 and 3000x, respectively.

3. 5. Statistics

All numerical results were expressed as mean ± standard error of the mean (SEM). Statistical analysis regarding differences between control and either of experimental groups was performed using Student's *t*-test. The level of significance was set at $p < 0.05$ (*), 0.01 (**) and 0.001 (***).

4. Results

4. 1. Effects of treatment with T3 or T4 on levels of thyroid hormones in serum and histological characteristics of thyroid gland

4. 1. 1. Serum total T3 and T4 concentration

Results for TH levels in control, T3- and T4-treated rats obtained using RIA method are presented in Table 4. 1. 1. 1.

Table 4. 1. 1. 1. Total serum T3 and T4 concentration in control, T3- and T4-treated rats.

	control	T3-treated	T4-treated
T4 total serum concentration (nmol/l)	133.7±8.27	20.6±3.10***	289.3±23.16***
T3 total serum concentration (nmol/l)	1.19±0.074	1.69±0.070**	1.87±0.130**

Statistics: * $p<0.05$ *vs*. control; ** $p<0.01$ *vs*. control; *** $p<0.001$ *vs*. control

After T3 treatment, level of T3 in circulation was increased compared to control (1.19±0.074 in control *vs*. 1.69±0.070 in T3 treatment), while circulating level of T4 was extremely reduced (133.7±8.27 in control *vs*. 20.6±3.10 in T3 treatment).

Treatment with T4 significantly increased both T4 (133.7±8.27 in control *vs*. 289.3±23.16 in T4-treated) and T3 (1.19±0.074 in control *vs*. 1.87±0.130 in T4-treated) concentrations in circulation.

4. 1. 2. Histological alterations of thyroid gland

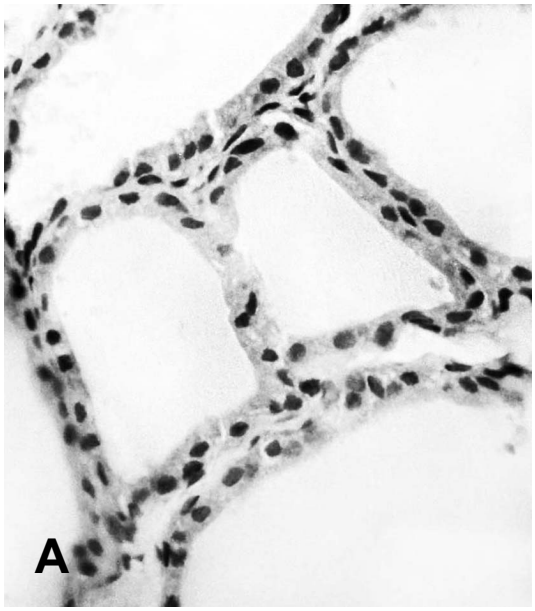
Absolute and relative thyroid mass remained statistically unchanged in both treated groups. Results are presented in Table 4. 1. 2. 1.

Table 4. 1. 2. 1. Absolute and relative thyroid gland mass

	control	T3-treated	T4-treated
Absolute thyroid mass (mg)	34.3 ± 8.46	28.6 ± 2.80	28.2 ± 3.90
Relative thyroid mass (mg/100g b.w.)	13.7 ± 1.12	11.6 ± 0.95	11.3 ± 1.36

Statistics: n. s.

General histological organization of thyroid gland was studied on both paraffin (5 μm) and semi-fine plastic sections (1-2 μm). In the control group (Fig. 4. 1. 2. 1A, D) thyrocytes lining small and medium-sized follicles (arbitrary determined) were cuboidal to low columnar, with rounded nucleus and sometimes bulging apical cell membrane, while larger follicles were lined with lower thyrocytes contained ovally flattened nucleus oriented parallel to the base of the epithelium. Between the follicles there was a connective tissue with blood capillaries and individual or clustered parafollicular cells. In T3 and T4 treated groups, thyroid parenchyma appeared less well ordered (Figs 4. 1. 2. 1B and E; C and F, respectively). The amount of interfollicular connective tissue was reduced, with collapsed blood vessels. Follicles were lined mostly with low follicular cells. Epithelium of some large follicles was extremely flattened, especially after T4-treatment. Remarkable presence of debris of exfoliated cells inside lumen of some follicles, particularly in T4 group, suggests more intensive cell elimination than is normally seen in control animals (Figs. 4. 1. 2. 1C). In regard to size and shape, thyroid follicles were generally enlarged after treatment with hormones and seemed distended. Large irregularly shaped follicles also can be seen indicating rupture of interfollicular barrier and fusion of neighbouring follicles. In places, the barrier between two follicles was presented by single row of thyrocytes (Fig. 4. 1. 2. 1C, E arrowhead, and F) or epithelial lining was interrupted, giving the impression of irregularly shaped follicles (Fig. 4. 1. 2. 1B and C, asterisk).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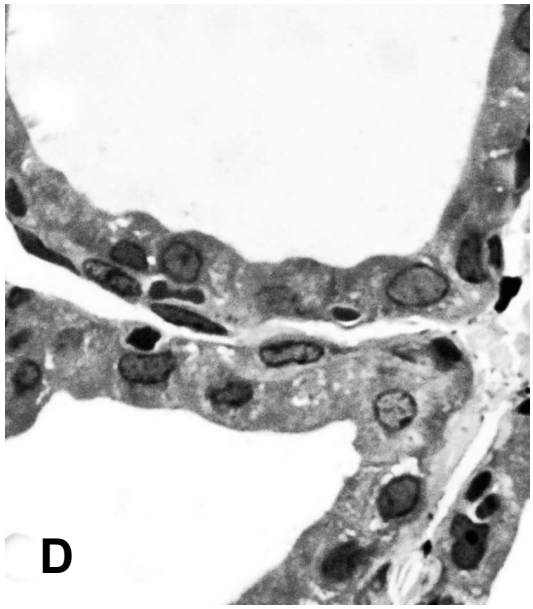
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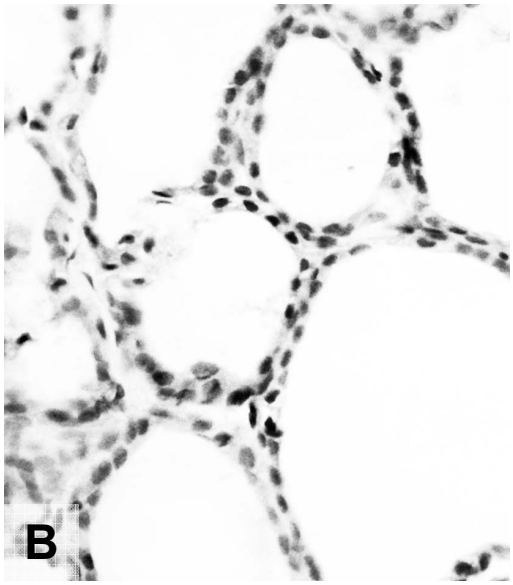
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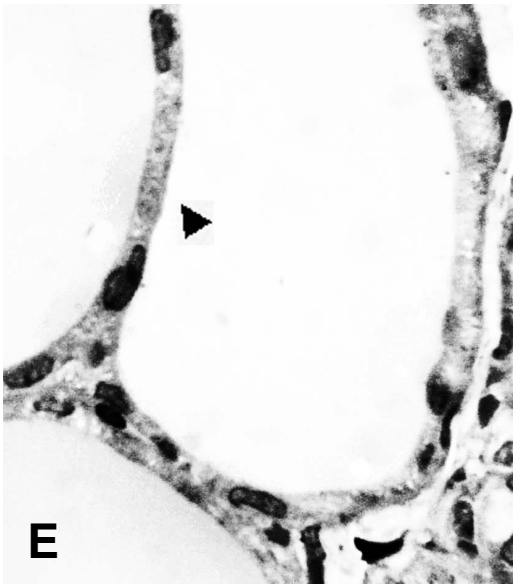
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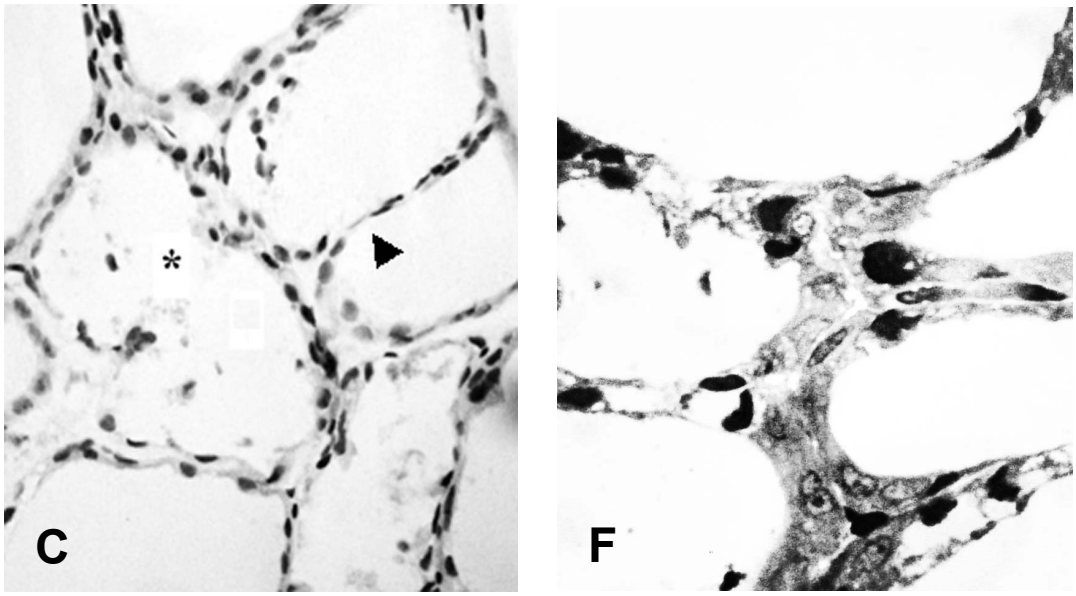


Figure 4. 1. 2. 1. General histological appearance of thyroids from control (A, D), T3- (B, E) and T4-treated (C, F) animals - light microscopy. A-C - paraffin embedded, hematoxylin/eosine stained sections, magnification 40x orig., D-F - plastic embedded tissue stained with toluidine blue, magnification 100x orig.

4. 1. 3. Detection of cell death in thyroid gland of control, T3- and T4-treated animals

PI served to label nuclei of dead cells. In control group, PI-positive nuclei were seen infrequently, mainly inside the follicular lumen (Fig. 4. 1. 3. 1A). In thyroids of T3-treated animals, in addition to lumen, PI-positive nuclei were seen individually or in small groups, within the follicular wall, as well as between follicles (Fig. 4. 1. 3. 1B). In T4-treated animals, PI-positive nuclei were characteristically present in the epithelium of remodelling follicles (Fig. 4. 1. 3. 1C).

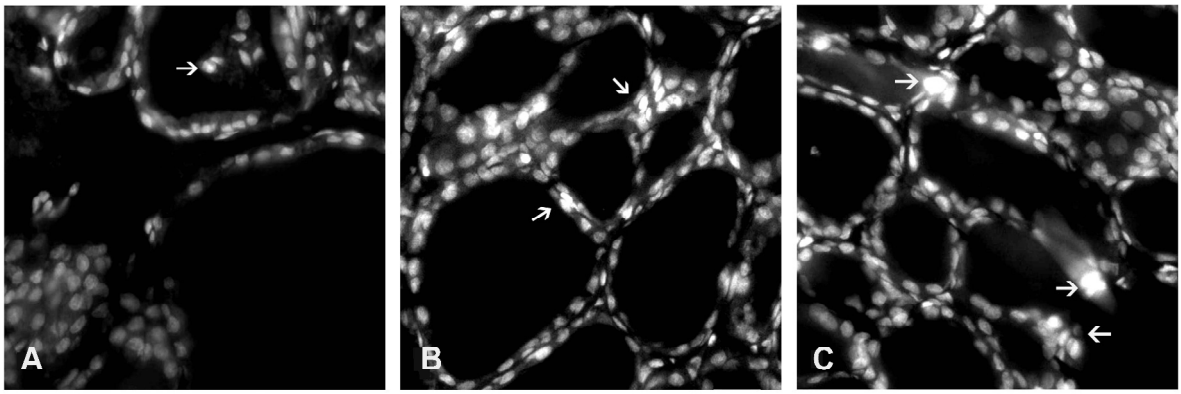


Figure 4. 1. 3. 1. PI-labelled nuclei (arrows) in the follicular lumen (control rats, A) and within follicular walls (T3- and T4-treated rats, B and C respectively). 20x orig.

The occurrence of cell death was estimated by counting normal and apoptotic nuclei in the follicular wall and results are presented in Fig. 4. 1. 3. 2. Percentages of PI-positive nuclei in thyroids of T3-treated rats (7.1±0.12, *p< 0.05) and T4-treated rats (9.1±0.44, **p<0.01) were significantly higher than in control animals (3.7±0.53).

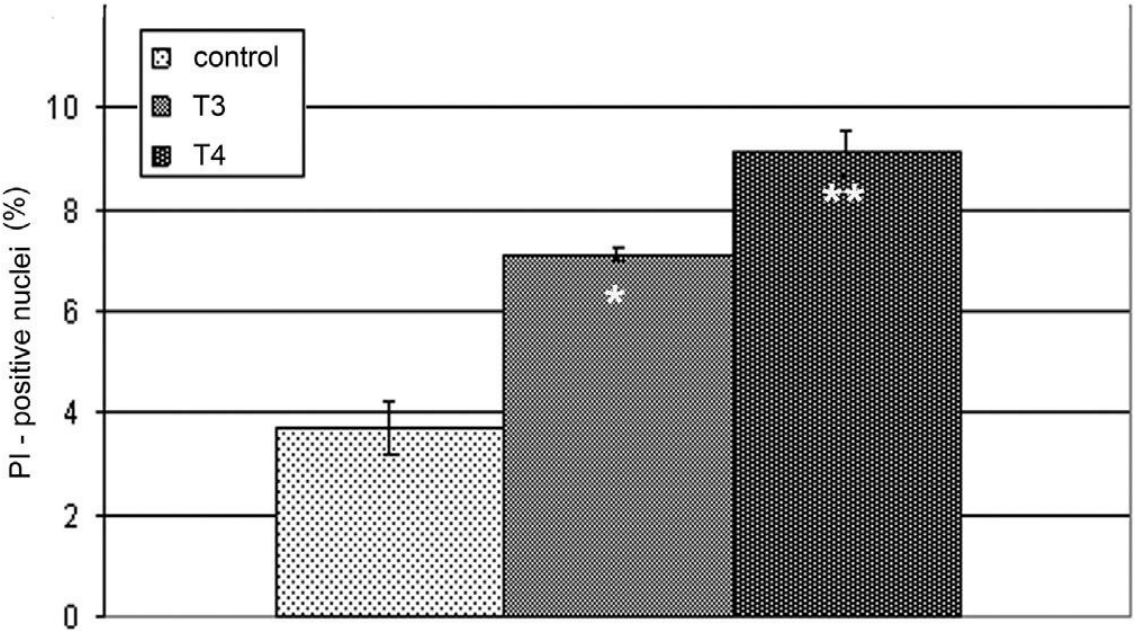


Figure 4. 1. 3. 2. Percentage of PI-positive nuclei in thyroids of control and TH treated animals.

4. 1. 4. Ultrastructural alterations of thyroid gland

Electron microscopy (Fig. 4. 1. 4. 1) revealed that thyrocytes from the control group in addition to a prominent nucleus, possessed endoplasmic reticulum, Golgi complex placed at the lateral margin of the cell and occasionally small dense granules usually identified as lysosomes. Apically, thyrocytes had numerous long, regular microvilli projecting into the follicular lumen (Fig. 4. 1. 4. 1a). After T3 treatment, thyrocytes became slightly flattened with irregular and fuzzy-like microvilli (Fig. 4. 1. 4. 1b). Treatment with T4 resulted in further flattening of follicular epithelium followed by shortening or even disappearing of microvilli (Fig. 4. 1. 4. 1c). Progressive stages of thyrocyte injury leading to gradual dissolution of interfollicular wall might be seen in places (Fig. 4. 1. 4. 2a–f). Some thyrocytes had irregularly shaped nuclei with condensed chromatin, and vesiculated cisterns of endoplasmic reticulum located in the apical region of the cell (Fig. 4. 1. 4. 3a). Increased quantity of cell debris (Fig. 4. 1. 4.

3a, inset) as well as individual erythrocytes within some follicles from T3-and T4-treated animals were also noted (Fig. 4. 1. 4. 3b).

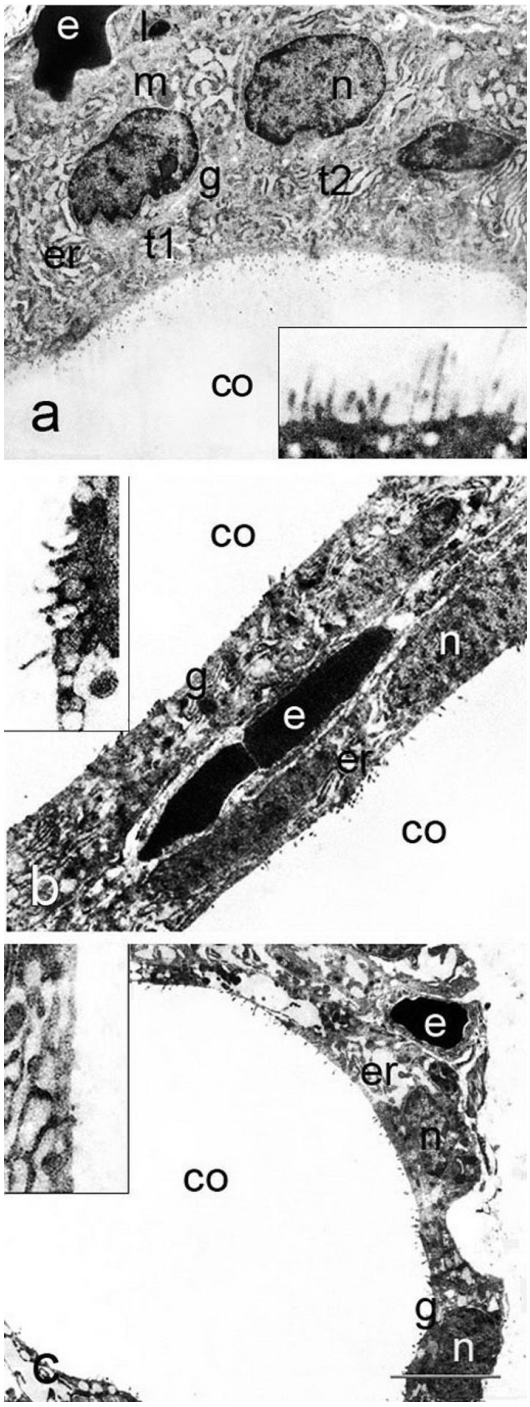


Figure 4. 1. 4. 1. Follicular epithelium of control, T3- and T4-treated rats (a, b and c); t – thyrocyte, n – nucleus, m – mitochondrion, l – lysosome, g – Golgi complex, er – endoplasmic reticulum, co – colloid, e – erythrocyte in the capillary lumen. Insets show apical pole of thyrocytes, with reduced and disorganized microvilli in T3- and T4-treated rats. Magnification 3000x; insets 7100x (bar = 4 μm (a–c) and 1.5 μm (insets)).

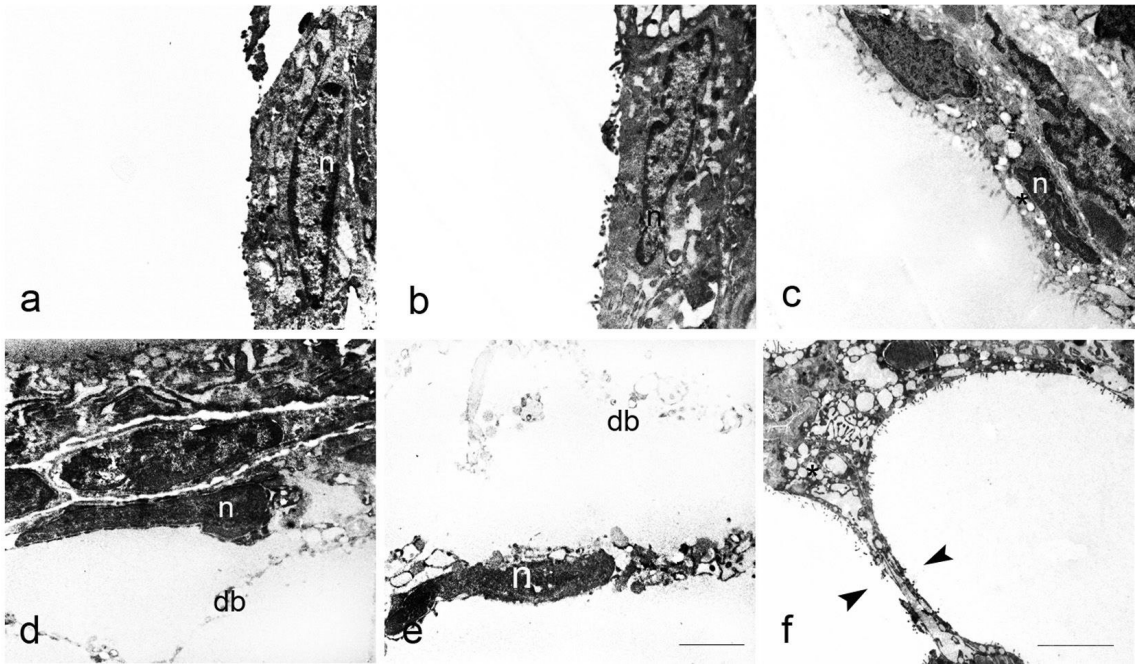


Figure 4. 1. 4. 2. Gradual injury of thyrocytes leading to dissolution of the interfollicular barrier. Note nuclear changes (n on a–e), intracytoplasmic vesiculation (asterisk on c) and cellular debris (db) in the follicular lumen (d and e); arrowheads show thinning and dissolution of interfollicular barrier (f). Bar 2 μm (a–e) and 5 μm (f) (magnification 5600x, 2650x).

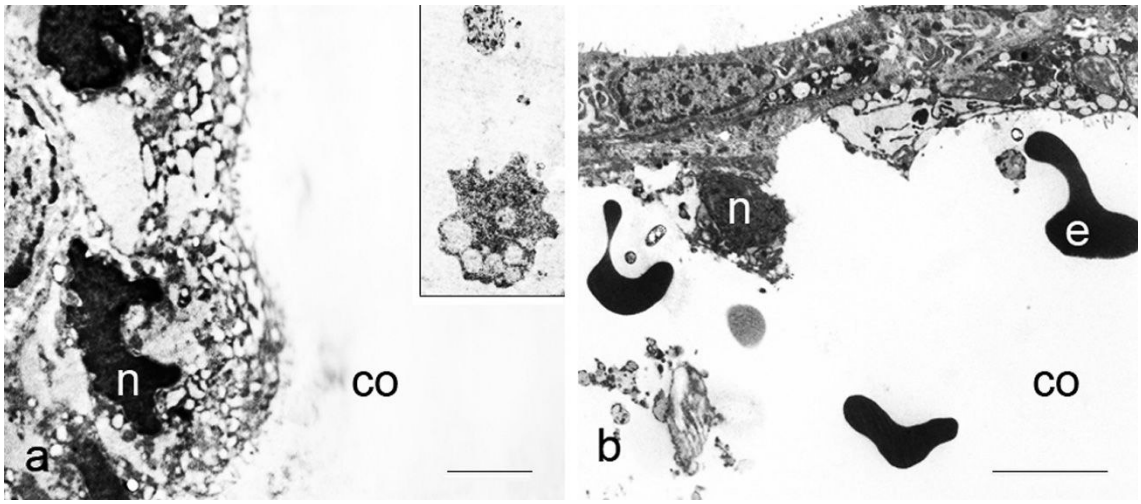


Figure 4. 1. 4. 3. Thyrocytes from animals treated with thyroid hormones, with deformed nuclei and vesiculated endoplasmic reticulum; inset shows cell debris in the follicular lumen (a); erythrocytes in the colloid observed after treatments with T3 or T4 (b); n – nucleus, co – colloid, e – erythrocyte. Bar 2 μm (a) and 5 μm (b) (magnification 7100x, 2650x).

4. 1. 5. Stereological analysis of thyroid gland

Stereological analysis confirmed the aforementioned findings (Table 4. 1. 5. 1). Volume density of colloid was increased in both T3 and T4 treated groups (0.45±0.032 in control *vs*. 0.54±0.021 in T3 and 0.61±0.025 in T4 treatment, $p<0.05$ and $p<0.01$, respectively), while volume density of follicular epithelium was decreased (0.34±0.017 in control *vs*. 0.24±0.003 in T3 and 0.21±0.011 in T4 treatment, $p<0.01$ and $p<0.001$, respectively). At the same time, volume density of follicles and interstitium remained nearly the same, although some tendency toward enlargement of follicles on the account of interstitum was noticed in T4-treated group. Height of follicular cells was reduced in both hormone treated groups (8.26±0.452 in control *vs*. 4.66±0.368 in T3 and 4.51 μm ±0.225 in T4 treatment, $p<0.001$ in both cases). Finally, thyroid activation index was significantly reduced in both groups under treatment 0.80±0.097 in control *vs*. 0.45 ±0.024 in T3 and 0.35±0.033 in T4 treatment, $p<0.01$ in both cases).

Table 4. 1. 5. 1. Results of stereological analysis - light microscopy

	control	T3-treated	T4-treated
V_v colloid (μm^0)	0.45±0.032	0.54±0.021*	0.61±0.025**
V_v follicular epithelium (μm^0)	0.34±0.017	0.24±0.003**	0.21±0.011***
V_v follicles (μm^0)	0.72±0.063	0.78±0.018	0.83±0.010
V_v interstitium (μm^0)	0.20±0.018	0.22±0.014	0.17±0.010
Thyroid activation index (I_a)	0.80±0.097	0.45±0.024**	0.35±0.033**
Follicular cells height (μm)	8.26±0.452	4.66±0.368***	4.51±0.225***

Statistics: * $p<0.05$ *vs*. control; ** $p<0.01$ *vs*. control; *** $p<0.001$ *vs*. control

4. 2. Effects of treatment with T3 or T4 on BAT

4. 2. 1. Body mass, BAT mass and body temperature

During the experiment, all animals were vital and in good health. Treatment with thyroid hormones exerted strong reducing effect on body mass gain. Absolute and relative BAT masses were significantly increased. Treatment with T4, but not with T3, produced elevation of body temperature.

Table 4. 2. 1. 1. Effects of treatment with T3 or T4 on body mass gain, BAT mass and body temperature.

	control	T3-treated	T4-treated
starting body mass (g)	221.7±9.54	230.5±4.61	231.0±7.76
final body mass (g)	256.3±9.12	244.7±4.73	247.7±5.73
body mass gain (g)	34.7±3.15	14.2±1.81***	16.7±2.72***
absolute BAT mass (mg)	339.7±8.81	366.2±5.82*	381.0±15.68*
relative BAT mass (mg/100 g b. m.)	134.5±8.99	151.3±2.22*	156.2±7.12*
starting body temperature (ºC)	37.9±0.08	37.8±0.17	37.8±0.17
final body temperature (ºC)	38.1±0.08	38.2±0.17	38.8±0.17*
body temperature change (ºC)	0.2±0.10	0.4±0.20	1.0±0.26*

Statistics: * $p<0.05$ *vs*. control; ** $p<0.01$ *vs*. control; *** $p<0.001$ *vs*. control

4. 2. 2. Light microscopical analysis of BAT from control, T3- and T4-treated animals

BAT from control rats (Fig. 4. 2. 2. 1) consists of several lobes interconnected with connective tissue containing large blood vessels and nerves. The most volume of the tissue is represented by brown adipocytes. WAT-like adipocytes, individual or in small groups, are visible at the periphery of BAT lobes. WAT-like adipocytes, usually individual, sometimes occur in the centre of the lobes. Typical brown adipocyte is characterized by the presence of numerous lipid bodies which size may be different from cell to cell and within the same cell. Nucleus is often roundish, centrally positioned and surrounded by lipid bodies. Usually it is euchromatic and possesses distinct nucleolus. However, in other cells nucleus can be removed from the cell centre and its form can be changed, which is sometimes accompanied with more or less heterochromatic appearance. Beside brown adipocytes, endothelial cells and WAT-like

adipocytes, other proper connective tissue cells are present (mast cells, macrophages, lymphocytes), but they are not numerous and some of them are not easily discernible on HE stained sections.

After T3-treatment (Fig. 4. 2. 2. 2), the lipid content of brown adipocytes is visibly increased. Nuclei of brown adipocytes appear darker and cytoplasm looks like compressed among lipid bodies. Lipid bodies in multilocular cells are larger and apparently less numerous than in the control. However there are also multilocular cells with small lipid bodies, thus difference in lipid bodies' size among cells is noticeable. WAT-like adipocytes are present at the periphery of the lobe and among multilocular cells.

Treatment with T4 also mostly affects pattern of lipid deposition in adipocytes (Fig. 4. 2. 2. 3). Lipid content is increased as in T3-treated group but multilocularity remaines preserved. Regions containing cells that can be described as paucilocular are usual finding in this group. Many cell profiles lack nucleus, meaning that cells are very large and plane of section did not pass through the nucleus.

These observations are confirmed on semi fine sections of plastic embedded tissue (Figs. 4. 2. 2. 4 - 4. 2. 2. 6). Additionally, it was noted that nuclei of brown adipocytes from T4-treated group are very large, euchromatic, with large nucleolus and in general correspond to nuclei of metabolically active cells.

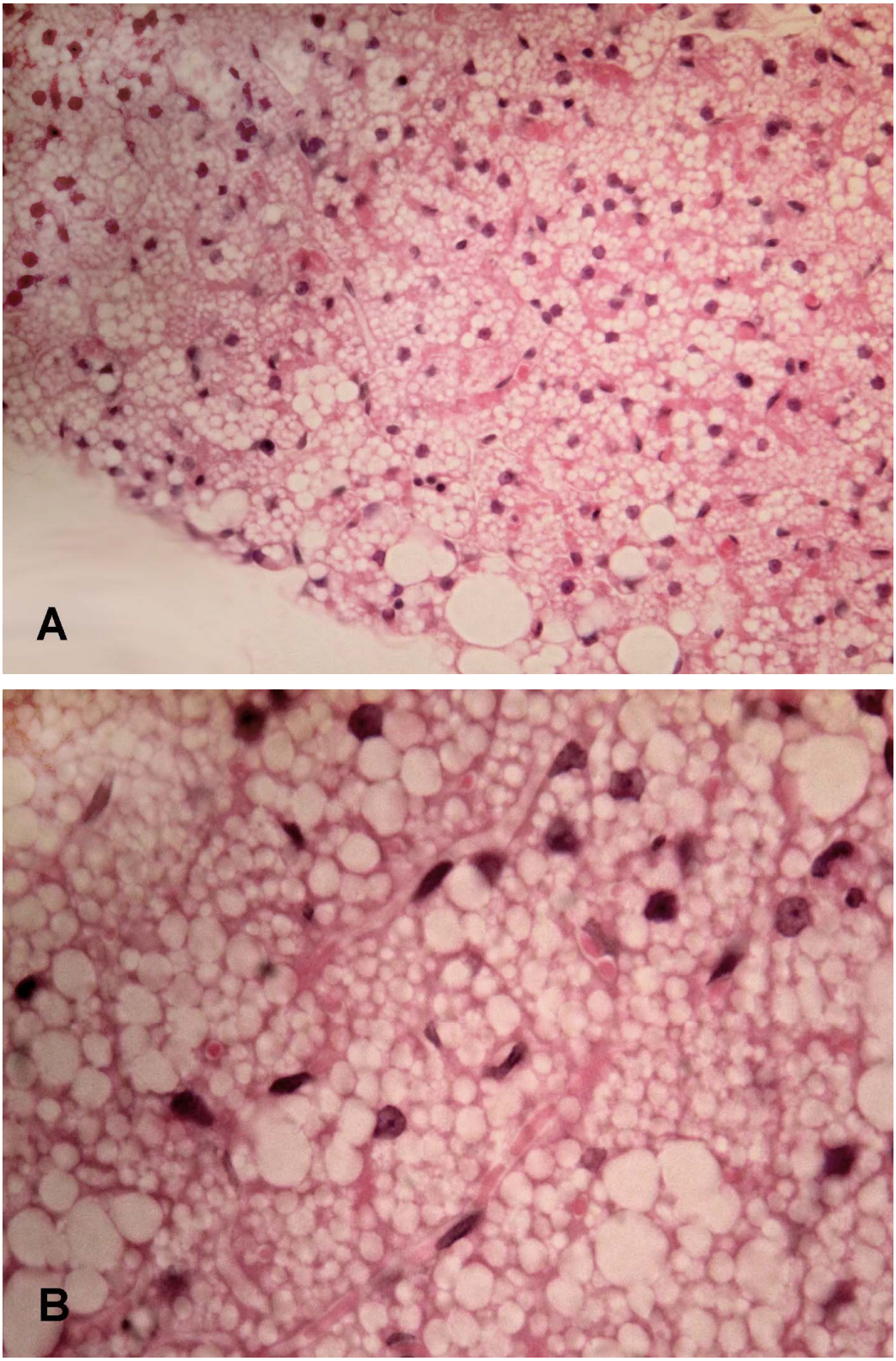


Figure 4. 2. 2. 1. BAT from control group of rats, paraffin embedded and HE-stained section. 40x (A) and 100x (B) orig.

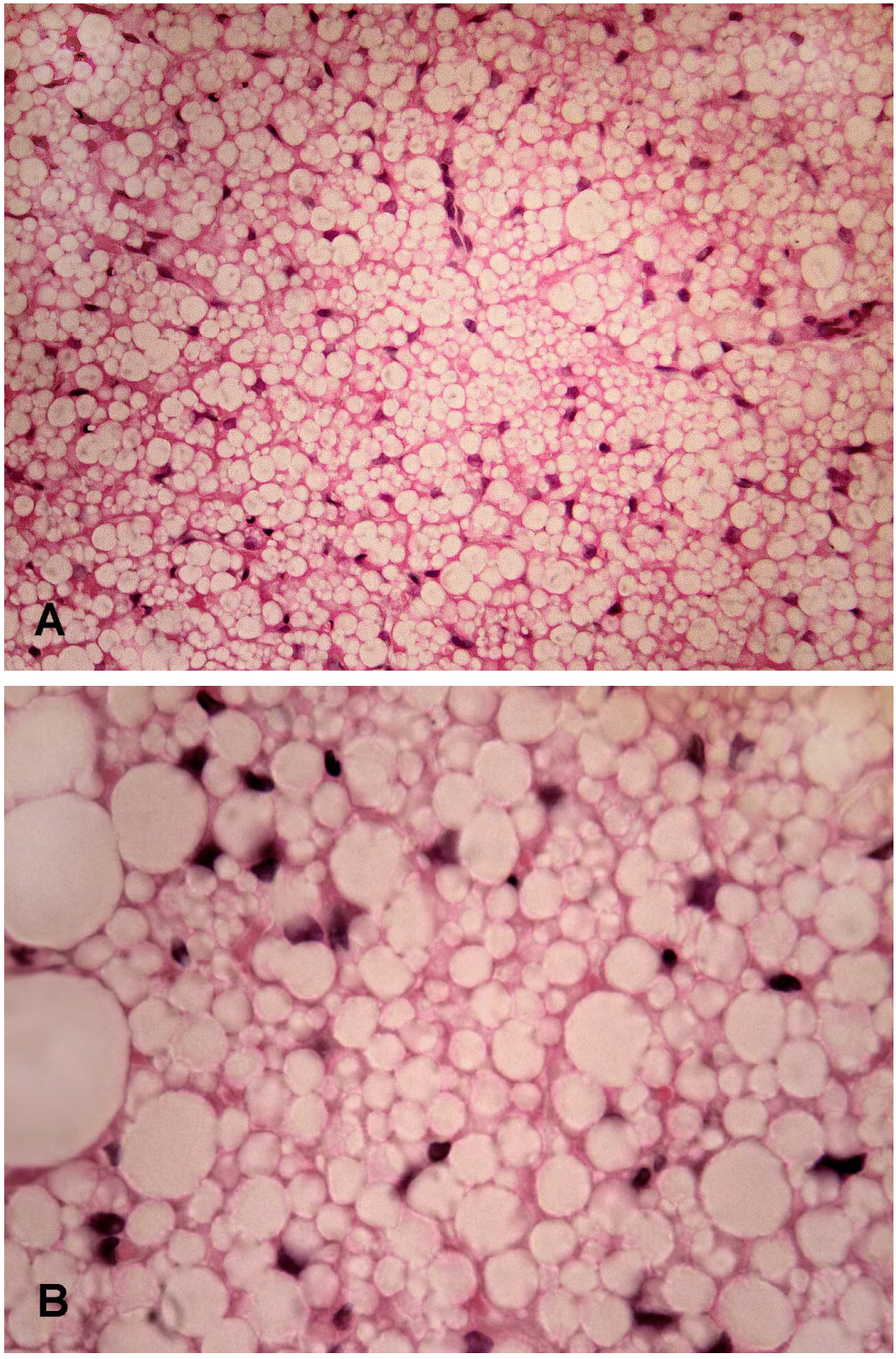


Figure 4. 2. 2. 2. BAT from T3 group of rats, paraffin embedded and HE-stained section. 40x (A) and 100x (B) orig.

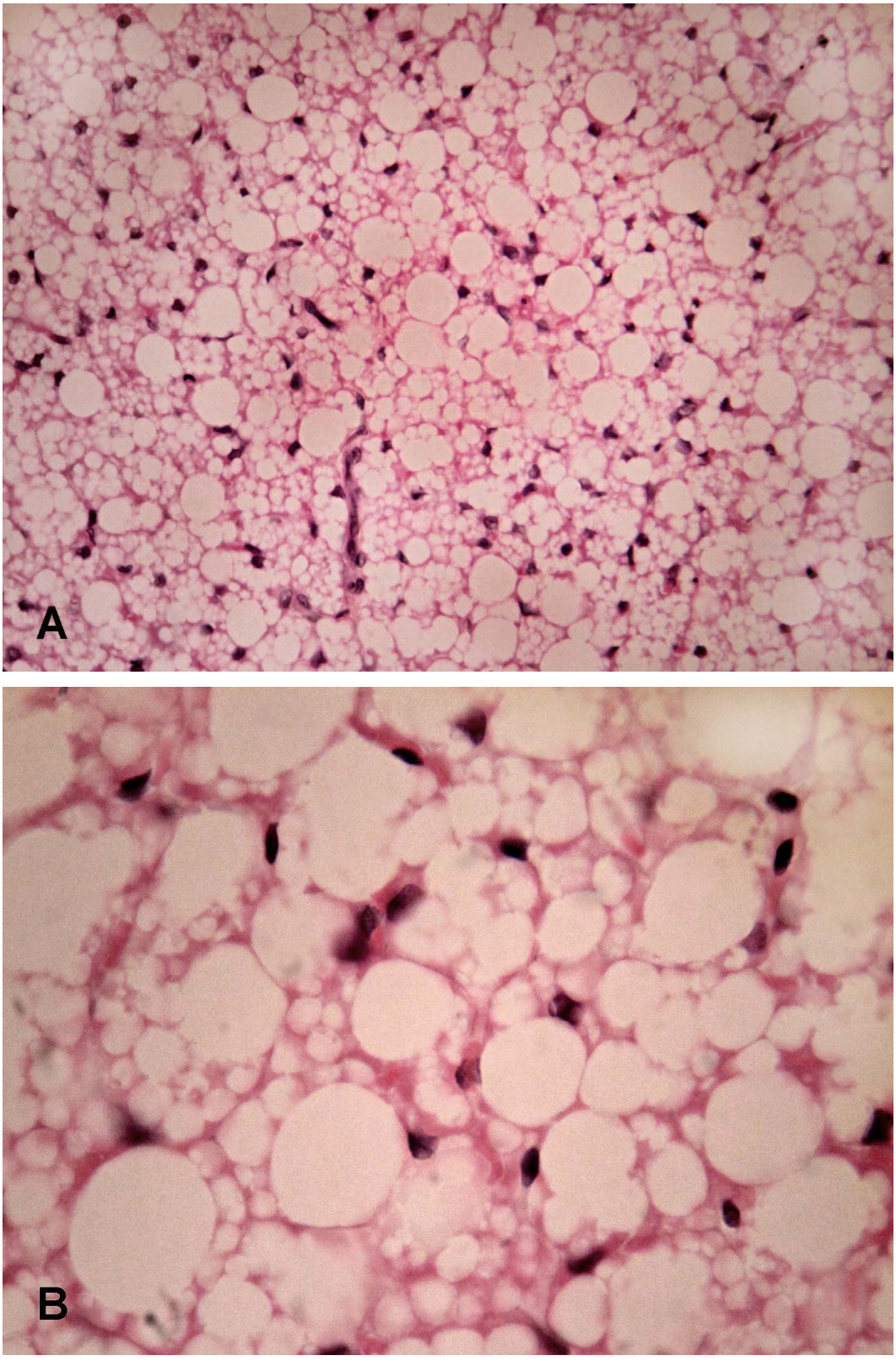


Figure 4. 2. 2. 3. BAT from T4 group of rats, paraffin embedded and HE-stained section - region with paucilocular adipocytes. 40x (A) and 100x (B) orig.

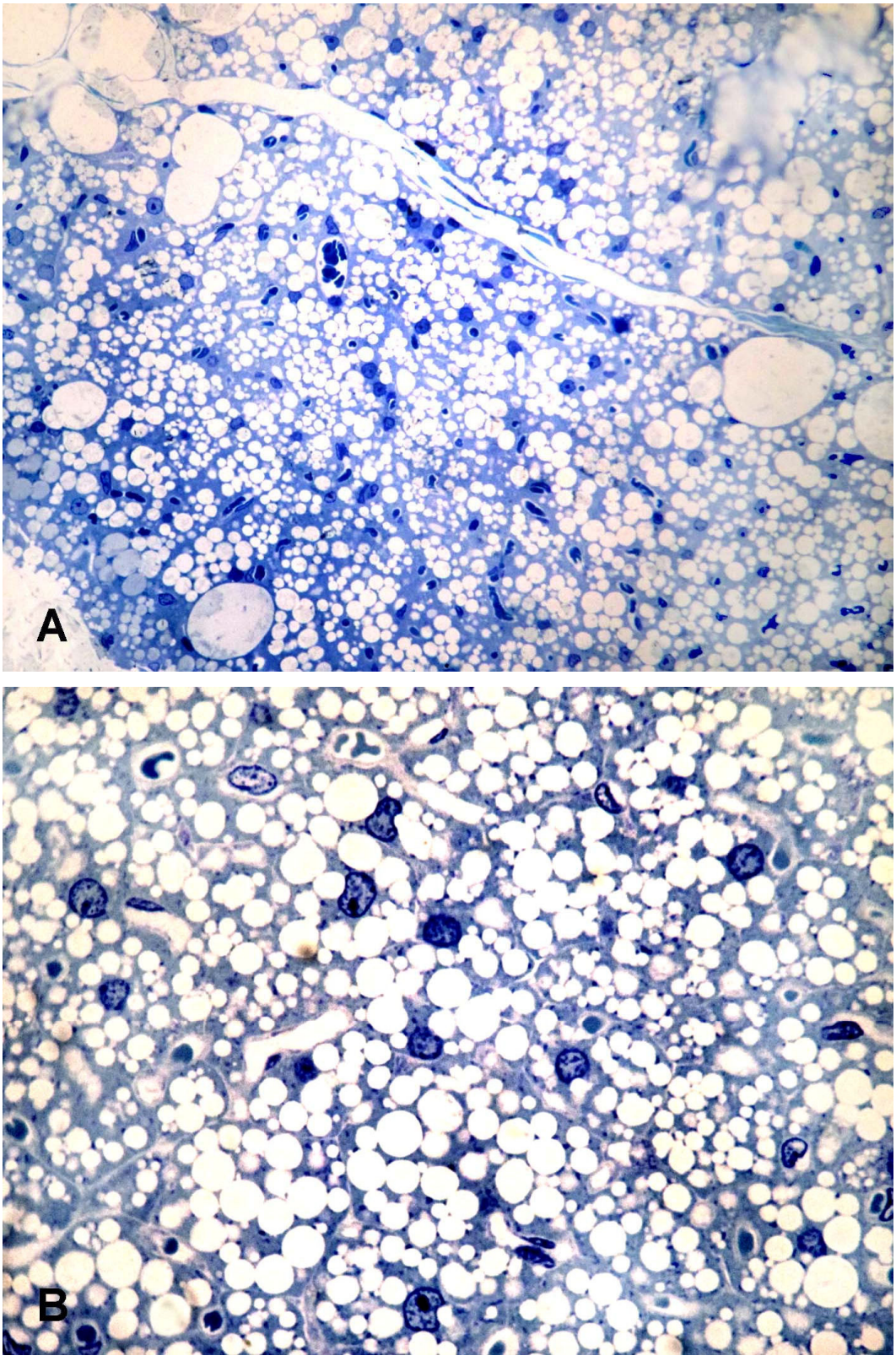


Figure 4. 2. 2. 4. BAT from control group of rats, plastic embedded and toluidine blue-stained semi-fine section. 40x (A) and 100x (B) orig.

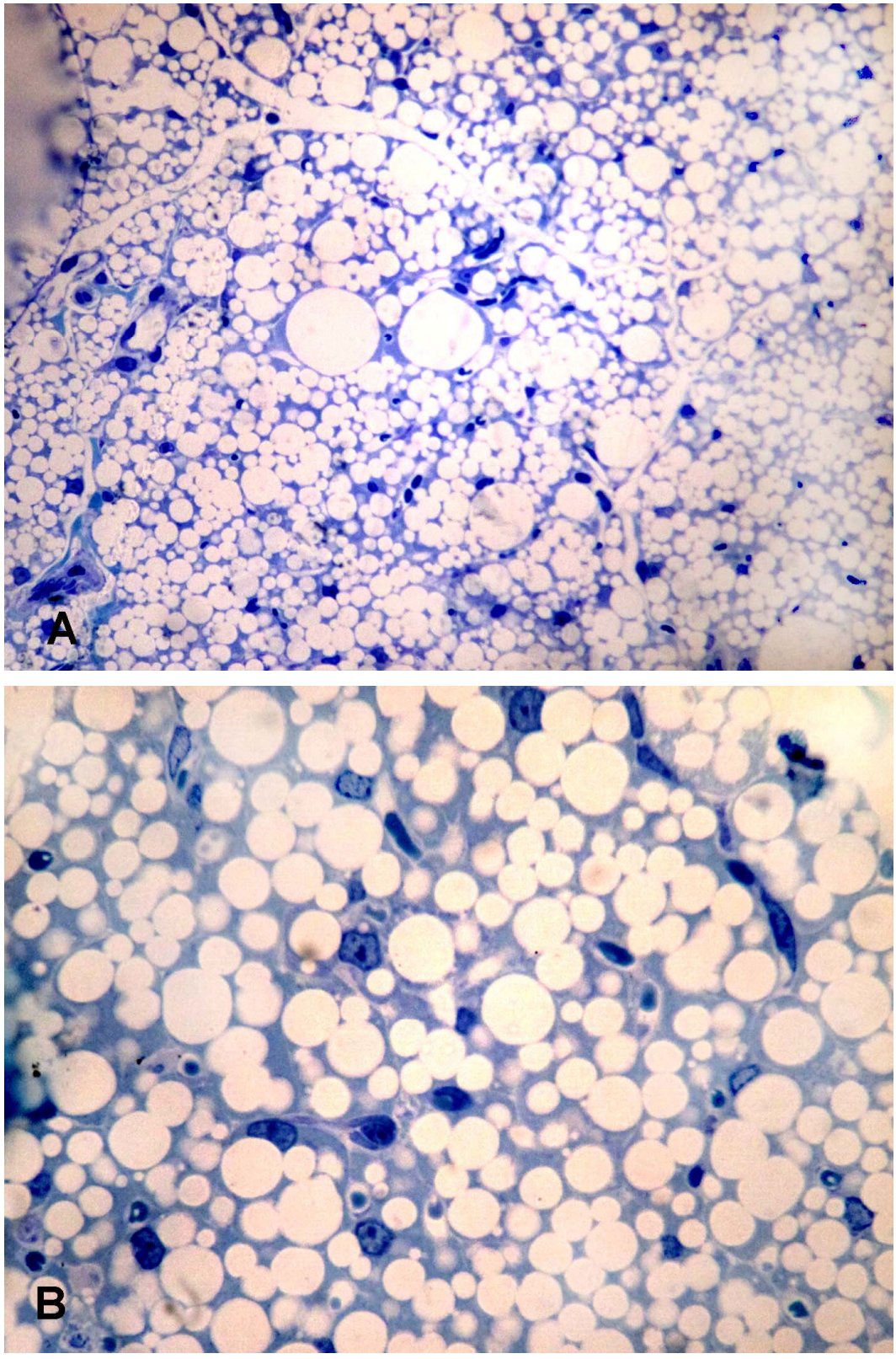


Figure 4. 2. 2. 5. BAT from T3 group of rats, plastic embedded and toluidine blue-stained semi-fine section. 40x (A) and 100x (B) orig.

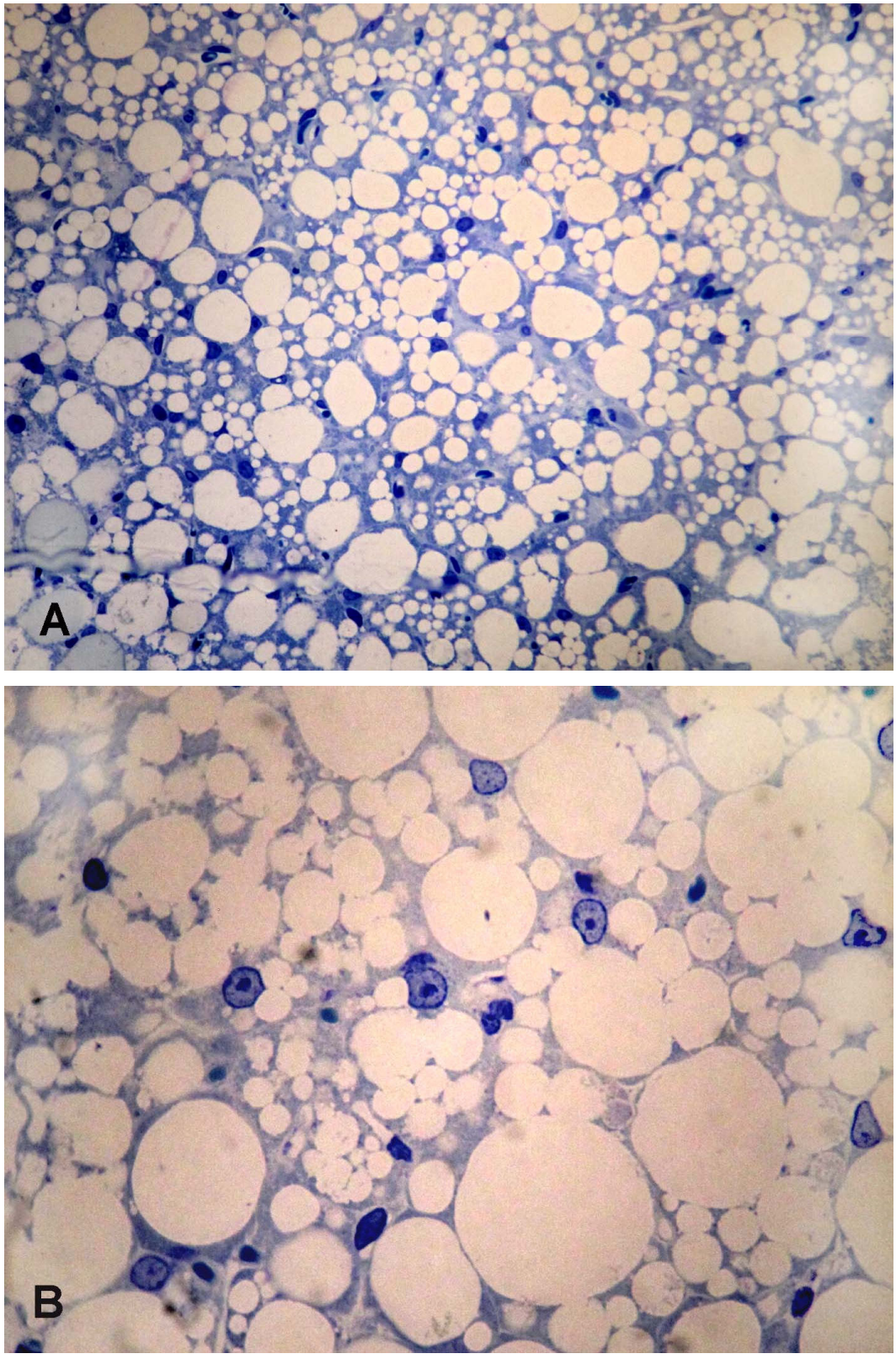
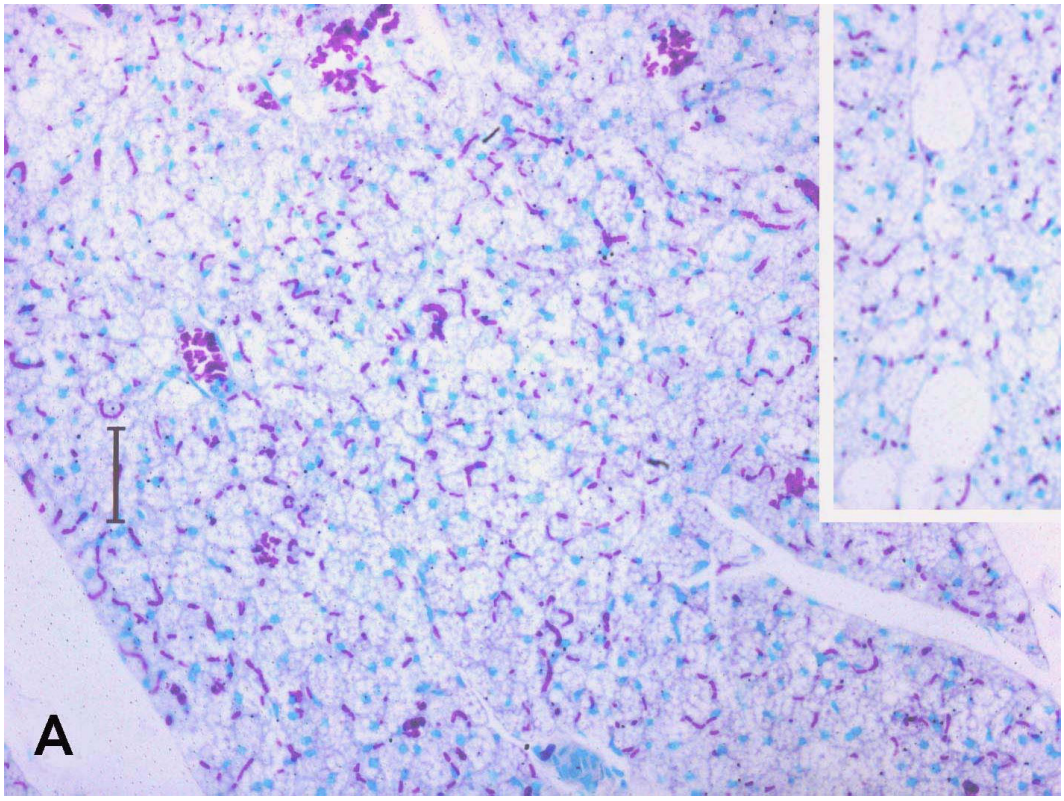


Figure 4. 2. 2. 6. BAT from T4 group of rats, plastic embedded and toluidine blue-stained semi-fine section. 40x (A) and 100x (B) orig.

Novelli method was used for demonstration of BAT vasculature (Fig. 4. 2. 2. 7). Erythrocytes stained by this method get purple coloration, thus making blood vessels (capillaries and larger vessels if present on section) distinctly visible against rather pale background. Nuclei are stained blue.

In control group (Fig. 4. 2. 2. 7A), BAT lobes are well vascularized and numerous longitudinally and transversally sectioned capillaries filled with erythrocytes are observed. Inset shows reduced presence of blood vessels in the vicinity of individual unilocular adipocytes. BAT after T3 treatment is apparently less vascularized and capillary profiles are mostly circular or, where are longitudinal, they are short (Fig. 4. 2. 2. 7B). After T4-treatment, BAT seems well vascularized, even better than BAT of control rats. Profiles of blood vessels are predominantly circular and of small diameter (Fig. 4. 2. 2. 7C).



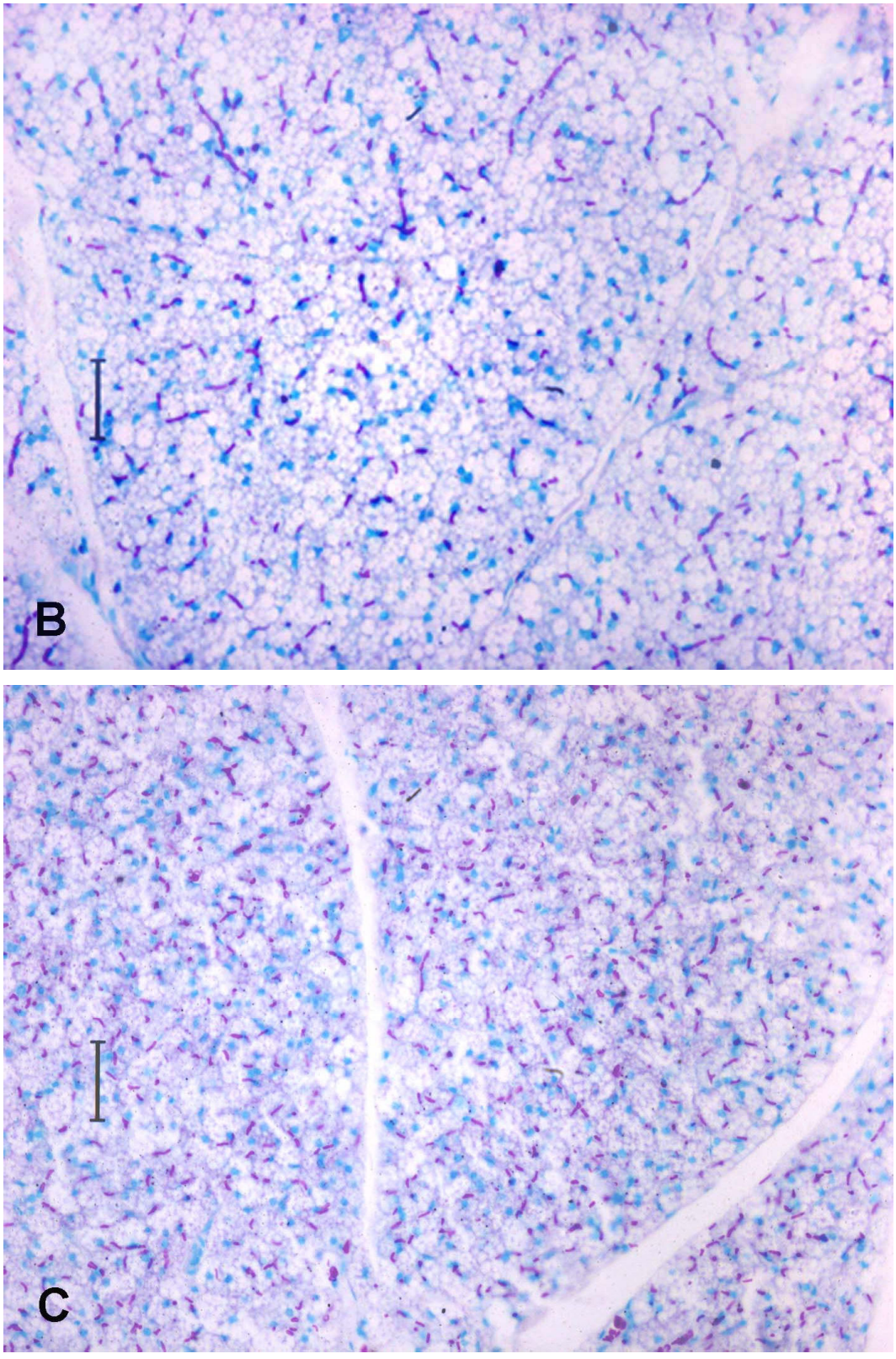
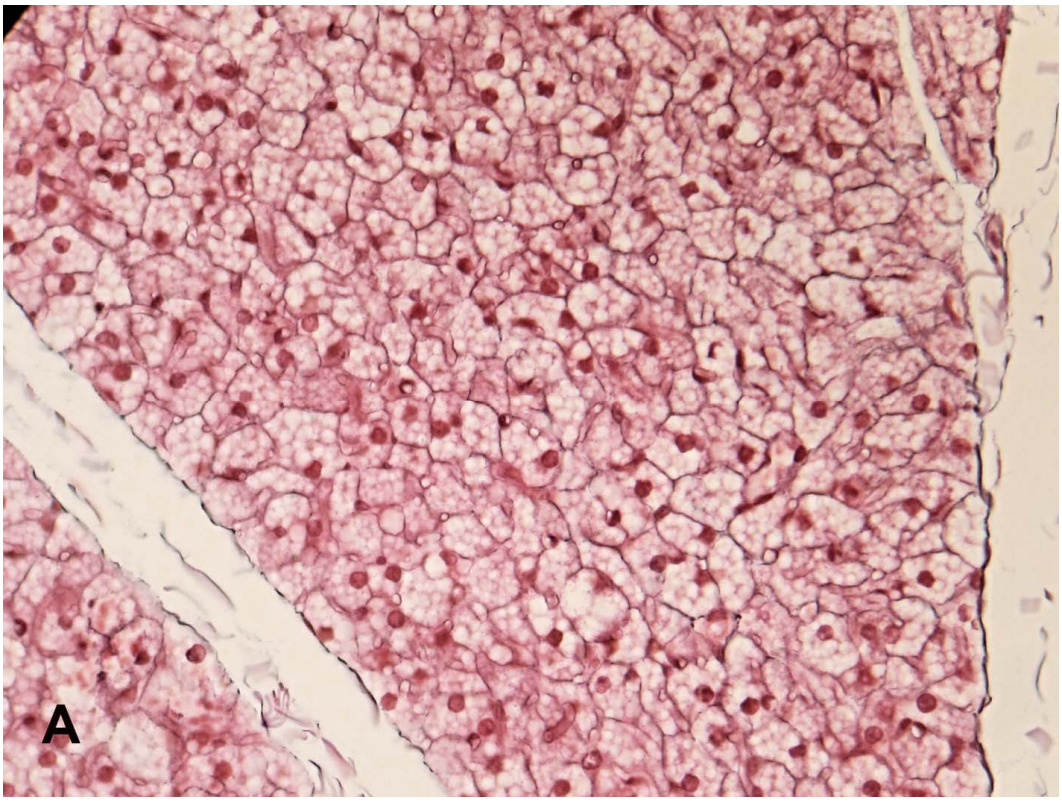
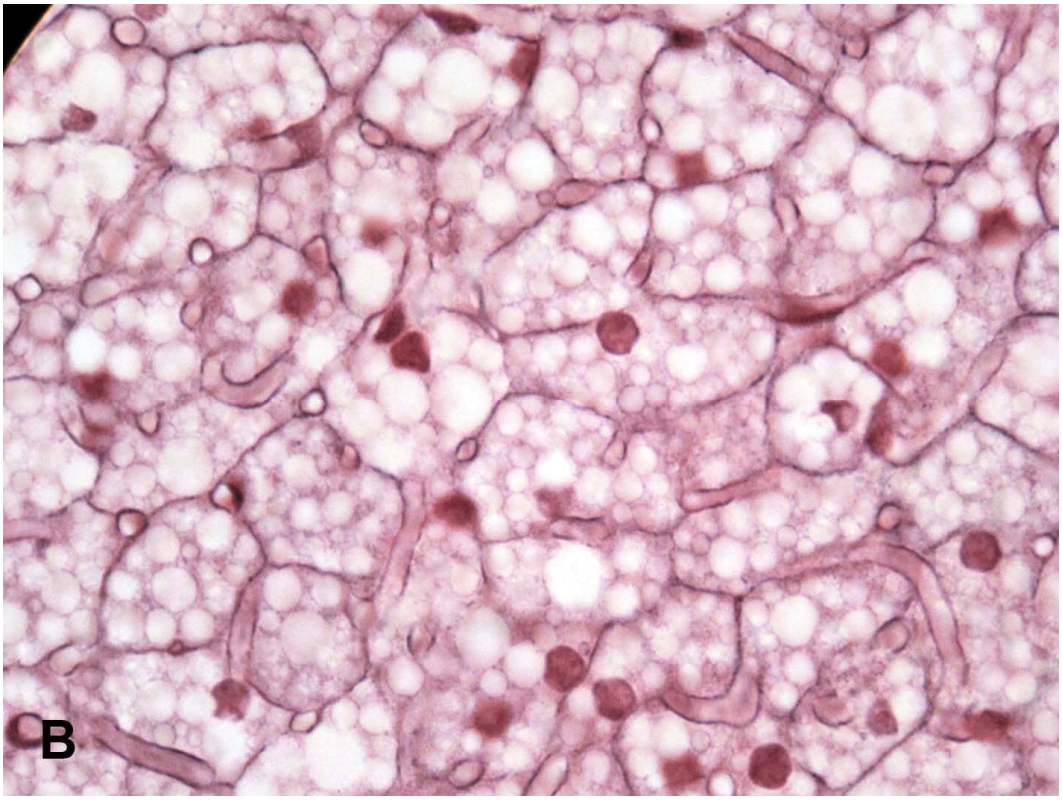


Figure 4. 2. 2. 7. Vascularization of BAT in control (A), T3- and T4-treated rats (B and C, respectively). Novelli method, 20x orig. (bar = 50 μm).

The extent of BAT innervation was studied using Bodian method for demonstration of nerve fibers (Fig. 4. 2. 2. 8). In control group brown adipocytes are well innervated. Nerve fibers usually surround the entire or almost entire cell. Nervature is also clearly visible around numerous capillary profiles (Fig. 4. 2. 2. 8A, B). Nerve fibers around blood vessels are more prominent than fibers around brown adipocytes. In T3-treated group, innervation is largely reduced. Nerve fibers are thin and often do not encircle the whole brown adipocyte. Even capillary associated nerve fibers are inconspicuous (Fig. 4. 2. 2. 8C, D). After T4-treatment, the extent of innervation closely resembles control innervation (Fig. 4. 2. 2. 8E, F).

Visual observations were confirmed measuring the length of nerve fibers on tissue sections. Obtained results are presented in Table 4. 2. 2. 1.

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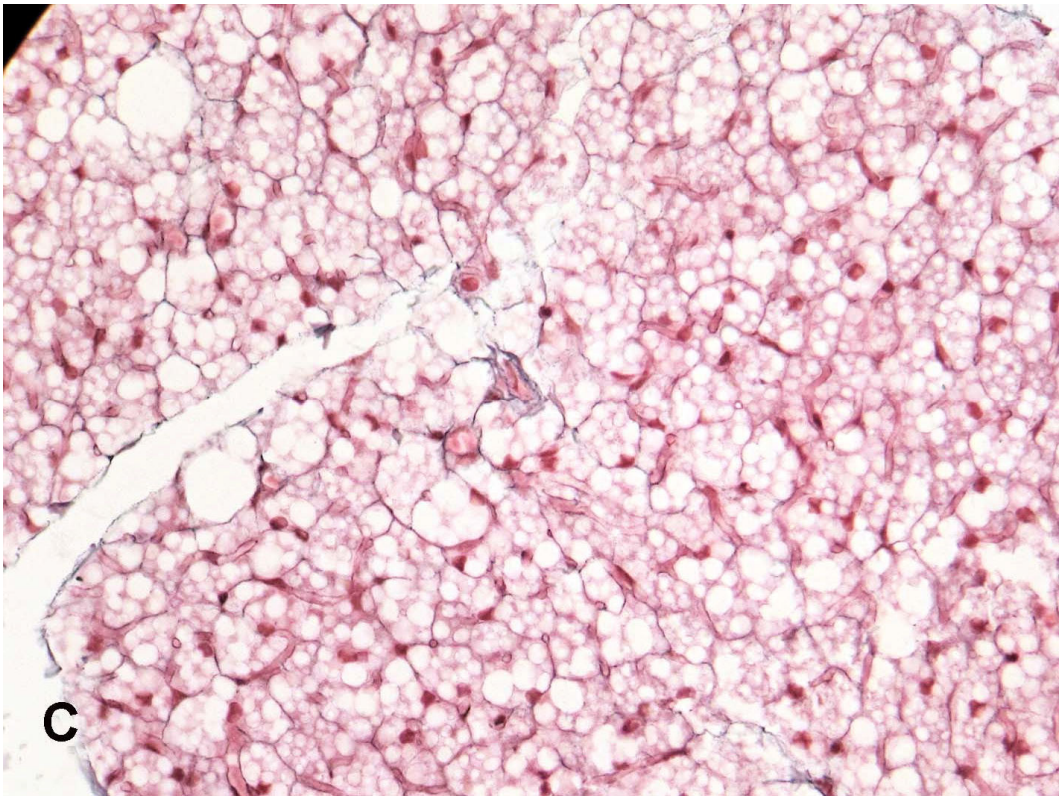
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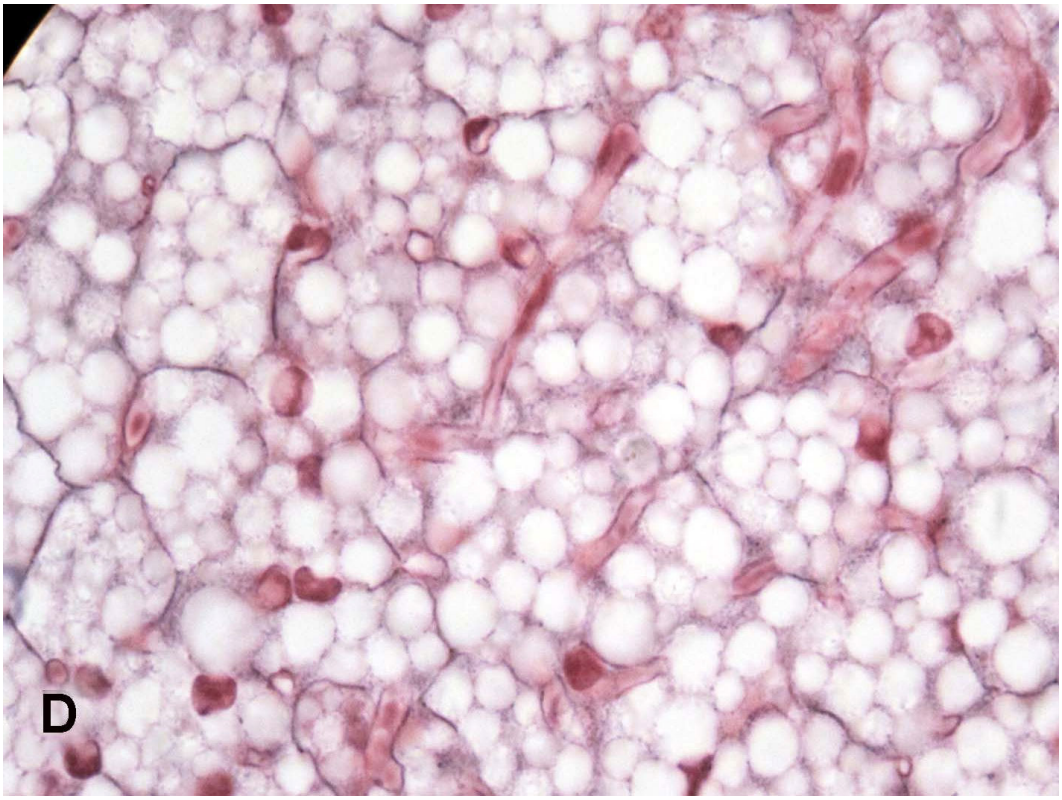
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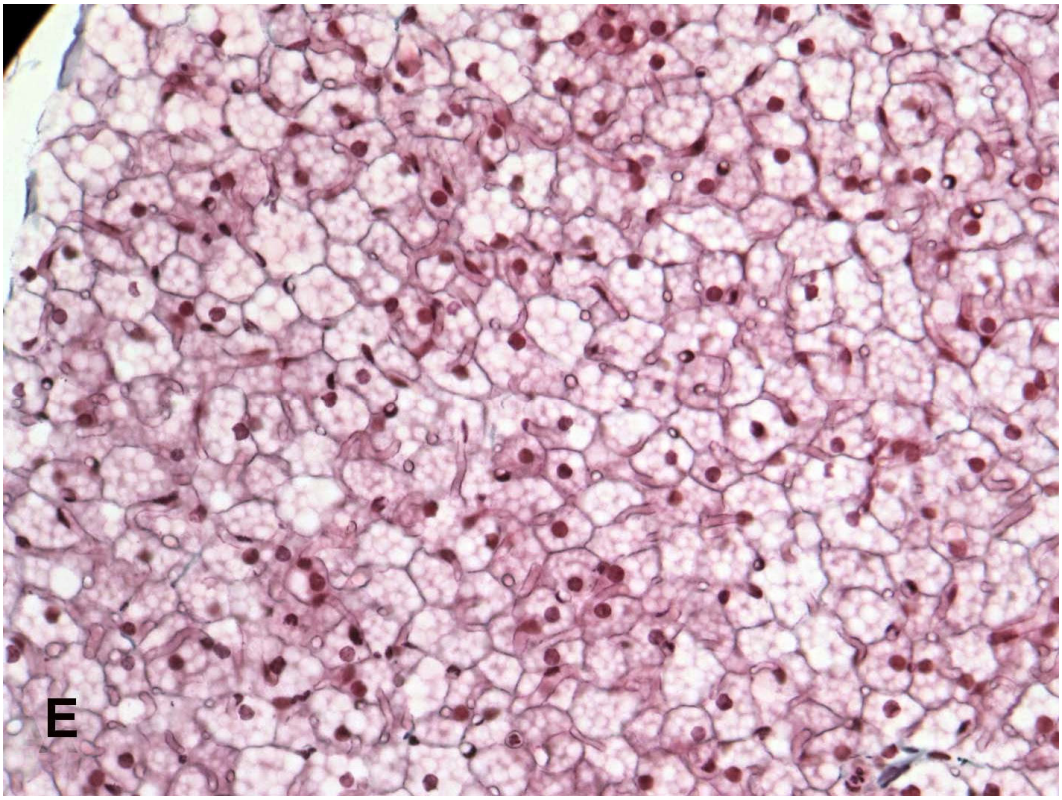
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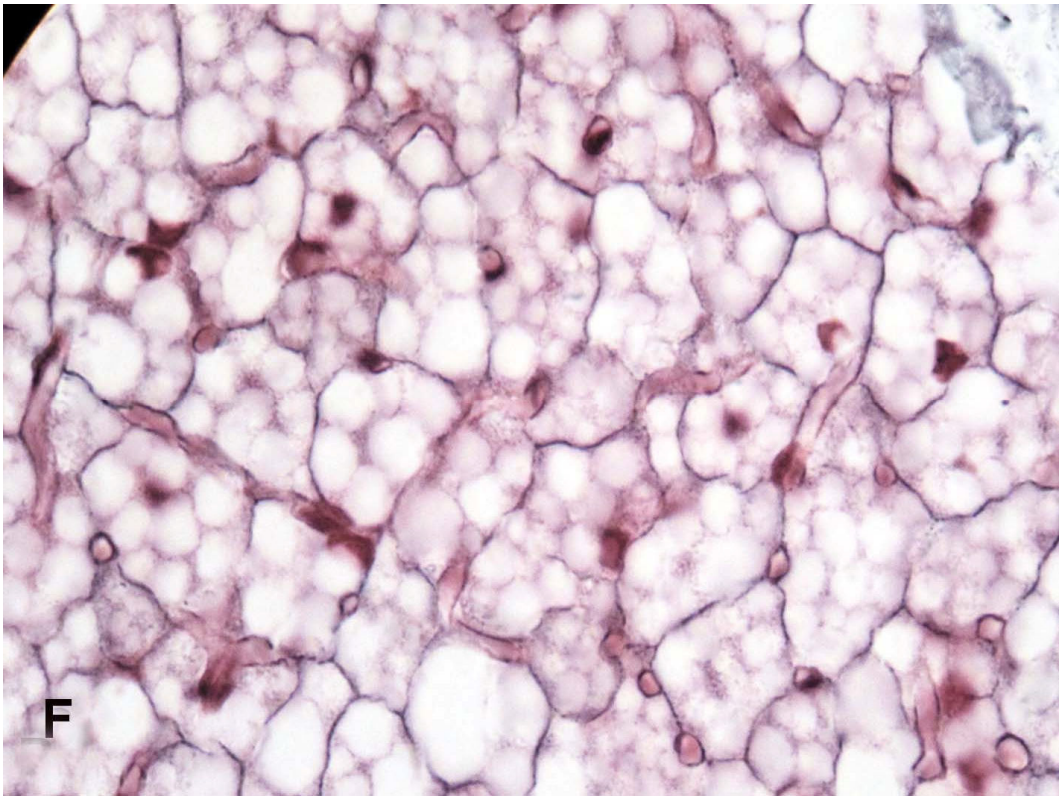


Figure 4. 2. 2. 8. Innervation of BAT in control (A, B), T3- and T4-treated rats (C, D and E, F, respectively). Bodian method, 40x orig. A, C, E and 100x orig. B, D, F.

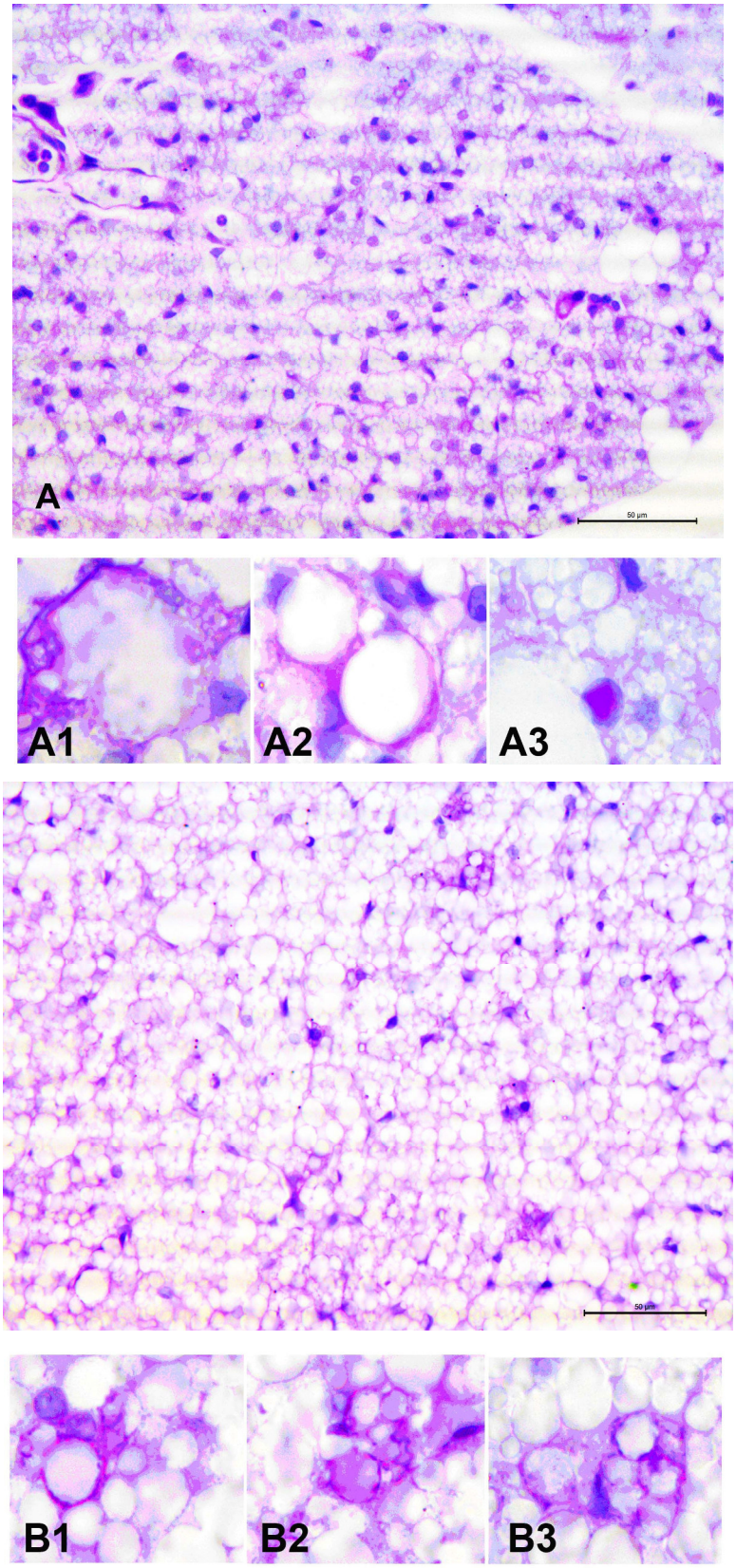
Table 4. 2. 2. 1. Quantitative data on innervation of BAT in control, T3- and T4-treated rats.

	Control	T3-treatment	T4-treatment
Length of nerve fibers around adipocytes (μm/mm^2)	37.12±2.283	25.52±0.190**	35.40±2.363
Length of nerve fibers around capillaries (μm/mm^2)	30.78±2.749	20.62±1.689*	26.33±5.744
Total length of nerve fibers (μm/mm^2)	67.90±4.020	46.15±2.384**	61.73±7.268

Statistics: * $p<0.05$ *vs*. control; ** $p<0.01$ *vs*. control; *** $p<0.001$ *vs*. control

PAS staining method was used to explore the extent of glycogen accumulation in BAT (Fig. 4. 2. 2. 9). This method also serves for demonstration of basal membrane surrounding many cell types. In control group, blood vessels are clearly demarcated by pink colour resulting from staining of basal membrane glycoproteins. Some cells, uni-,

multi- and paucilocular are also bordered by thin pink line. Intracytoplasmic positivity as indication of glycogen presence was visible only in some multilocular and paucilocular cells (Fig. 4. 2. 2. 9A1, A2). In several cases, glycogen deposition was found in nucleus of paucilocular cells (Fig. 4. 2. 2. 9A3). In thyroid hormones treated groups (Fig. 4. 2. 2. 9B, C), staining of basal membranes was less conspicuous. Intracytoplasmic PAS staining in T3-treated animals was clearly visible and rather frequent in multilocular cells (Fig. 4. 2. 2. 9B1-B3). In T4 group (Fig. 4. 2. 2. 9C1-C3), PAS staining was noted in multilocular, paucilocular and some immature multilocular cells, in which the reaction was very strong.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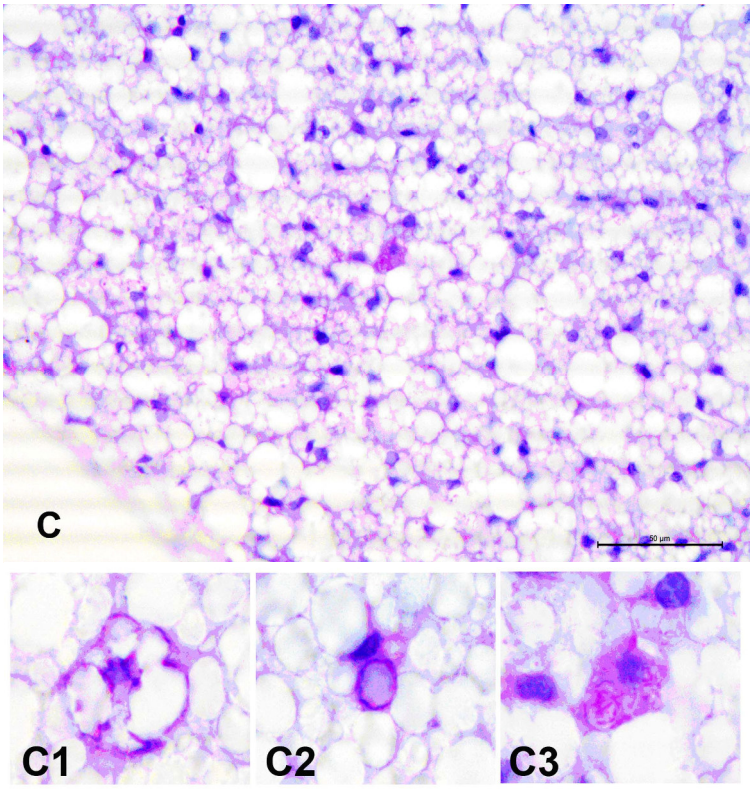
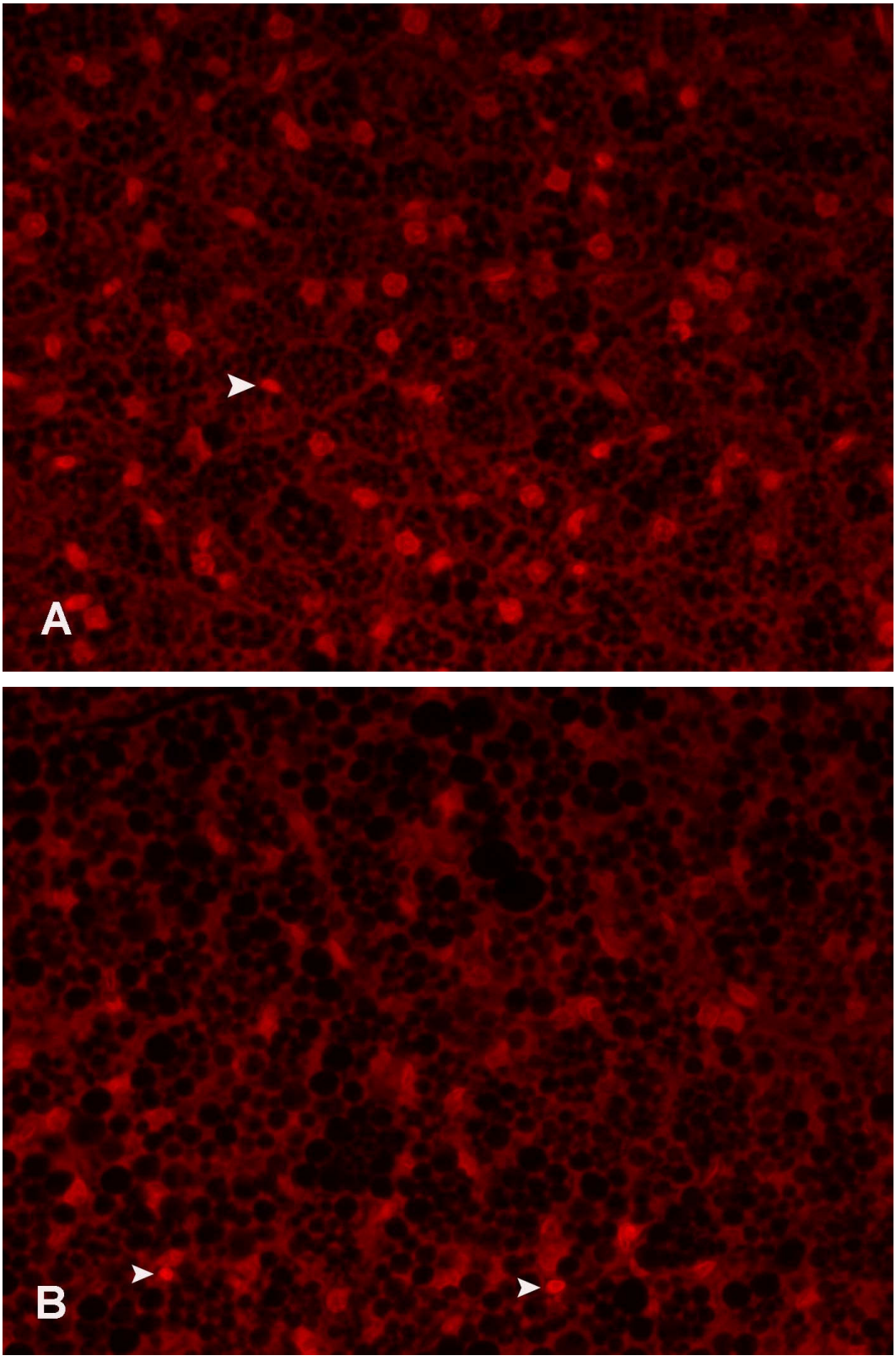


Figure 4. 2. 2. 9. Intracytoplasmic glycogen accumulation in BAT of control (A), T3- and T4-treated rats (B and C, respectively). PAS staining method, 40x orig. (bar = 50 μm); A1-A3, B1-B3, C1-C3 - 100x orig.

For detection of cell death, PI staining method was used. Compactly red stained nuclei belong to cells that were dead at the time of tissue fixation. In control group, PI-stained nuclei were rare and mostly belonged to cells other than adipocytes, such as endothelial cells (Fig. 4. 2. 2. 10A). After T3 treatment, PI reactive nuclei are still very rare. Although it is difficult to determine precisely, it seems that some of them belong to adipocytes, while the others are probably from non-adipocytes (Fig. 4. 2. 2. 10B). In T4-treated rats, PI-stained nuclei occur with similar frequency as in control group (Fig. 4. 2. 2. 10C).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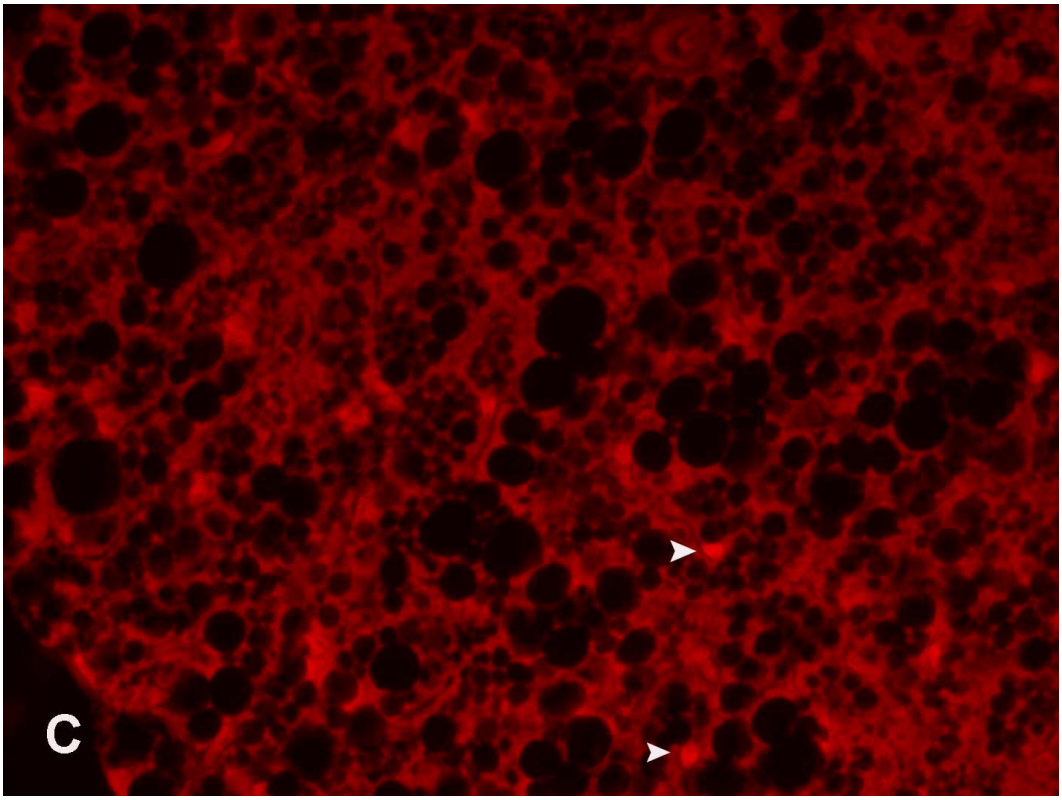
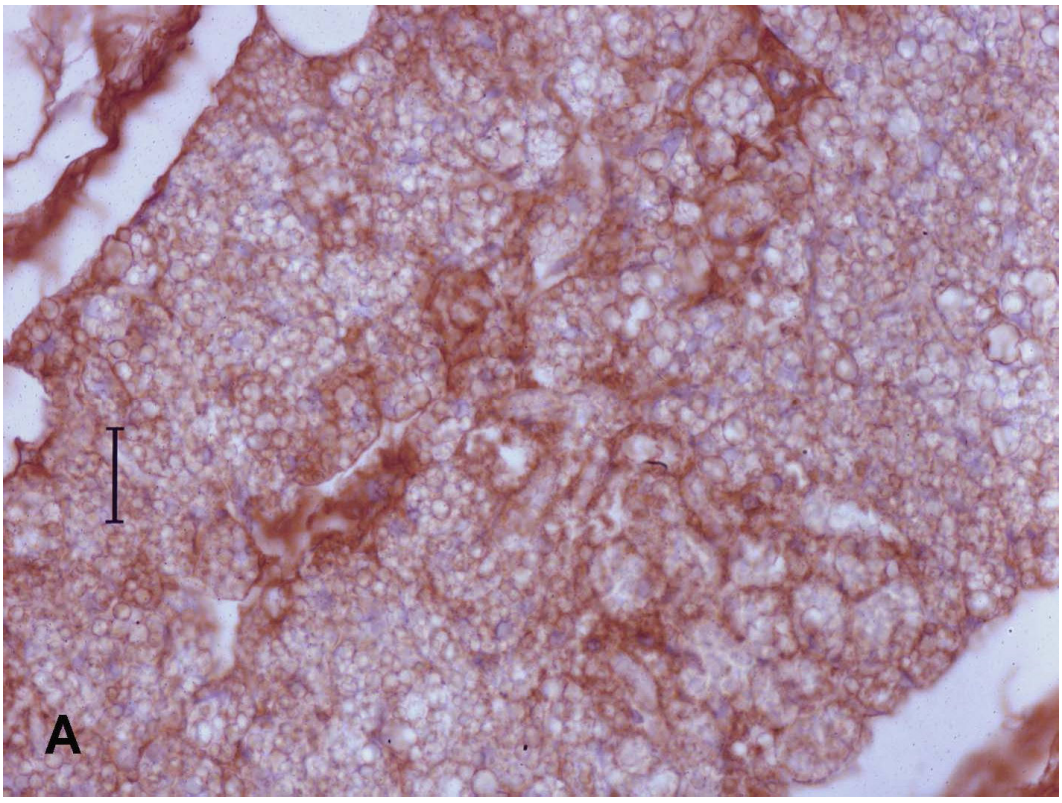


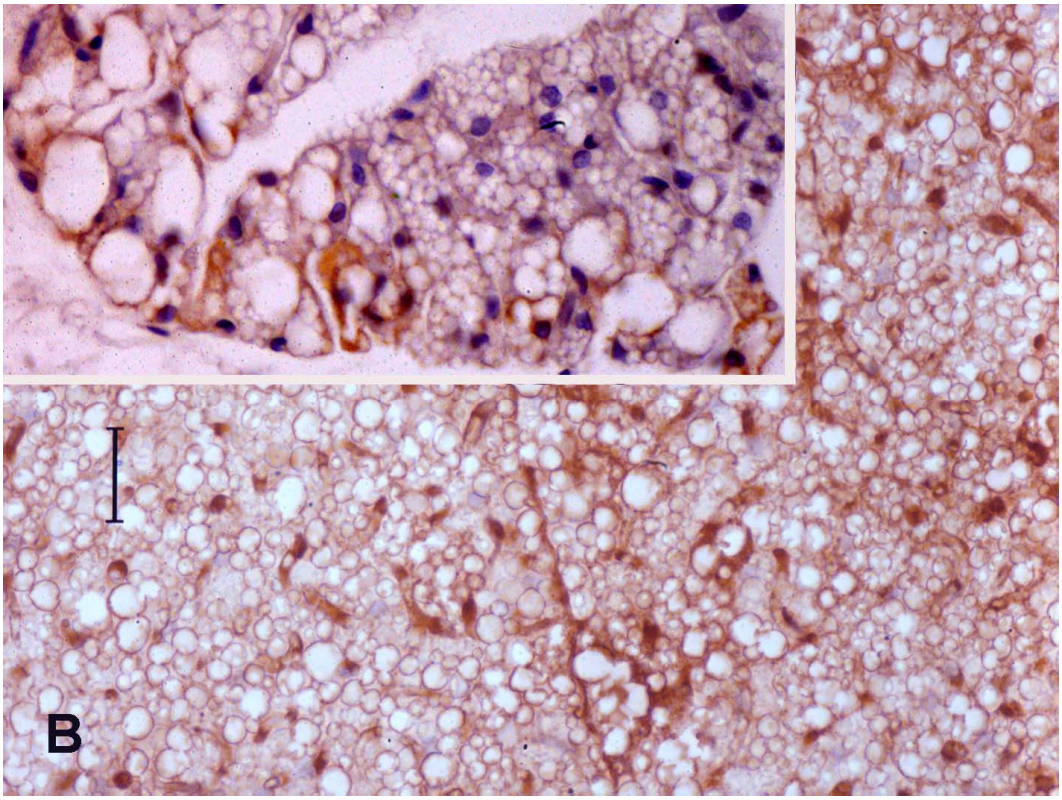
Figure 4. 2. 2. 10. Nuclei stained with PI demarcated dead cells. In control group they were very rare and usually belong to cells other than adipocytes such as endothelial cell (A). In T3 group, dead cells are also rarely seen and nuclei marked by PI are both from adipocytes as well as from non-adipocytes (B), while in T4 group frequency of PI-stained nuclei and type of cells they belong to, are similar to those from control group (C). 40x, orig.

4. 2. 3. Immunohistochemical analysis of BAT from control, T3- and T4-treated animals

Immunohistochemical detection of UCP1. Mitochondrial UCP1 is still the most reliable marker of brown adipocytes and demonstration of its presence is almost inevitable part of any immunohistochemical analysis of BAT, especially in cases considering BAT thermogenic capacity or activity. In control group, expectedly, UCP1-immunopositivity was detected in typical multilocular adipocytes (Fig. 4. 2. 3. 1A). Reaction shows characteristical "patchy" appearance and multilocular cells showing stronger immunopositivity are clustered in small groups intermingled with cells displaying mild immunoreactivity. All nuclei are counterstained blue. In T3-treated group, pattern of reaction in typical brown adipocytes is similar as in control group, although UCP1-immunoreactive clusters are slightly more frequent (Fig. 4. 2. 3. 1B). In areas where WAT-like adipocytes and adipocytes with small number of large lipid

bodies are present, strong UCP1-immunopositivity was observed (Fig. 4. 2. 3. 1B, inset). It seems that some nuclei of brown adipocytes show positive reaction. After treatment with T4, UCP1-immunopositivity was observed in typical multilocular adipocytes, but also in those in which several large lipid bodies dominate (Fig. 4. 2. 3. 1C). Nuclei of brown adipocytes are negative. Taken together, in thyroid hormone-treated groups, the main difference in comparison to control is occurrence of UCP1-immunodeposits in areas containing paucilocular adipocytes. Total UCP-immunopositivity is higher in T3 than in T4 group and in both hormone-treated groups than in the control.

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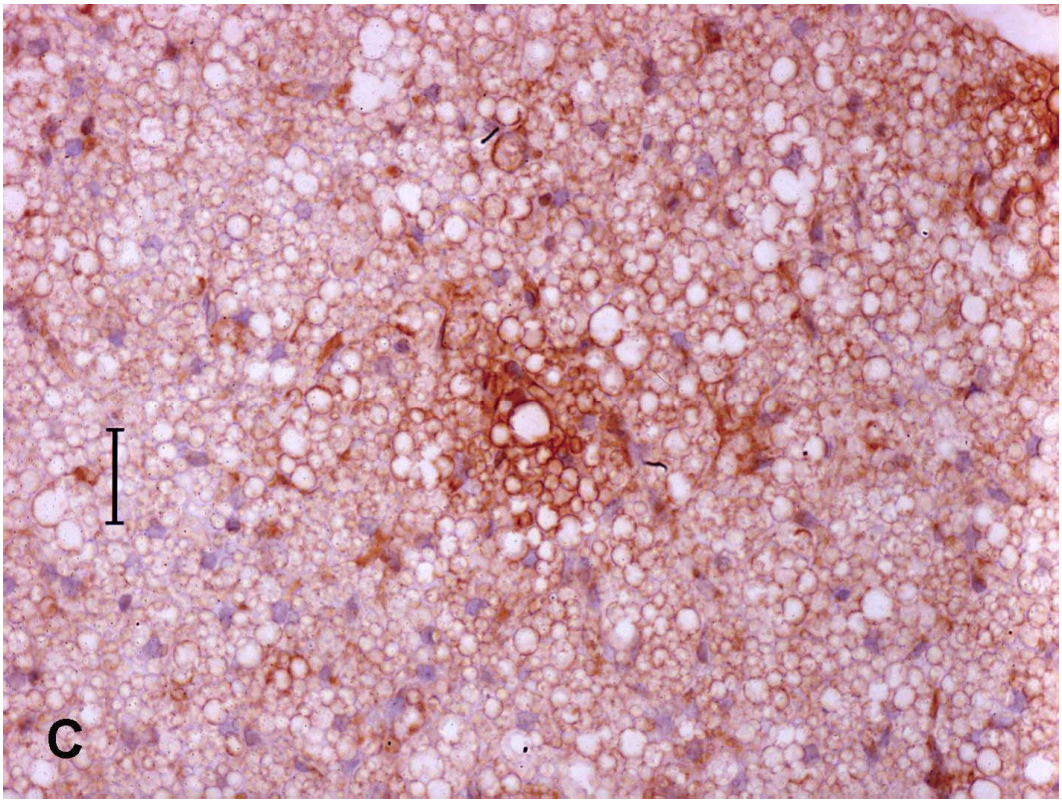
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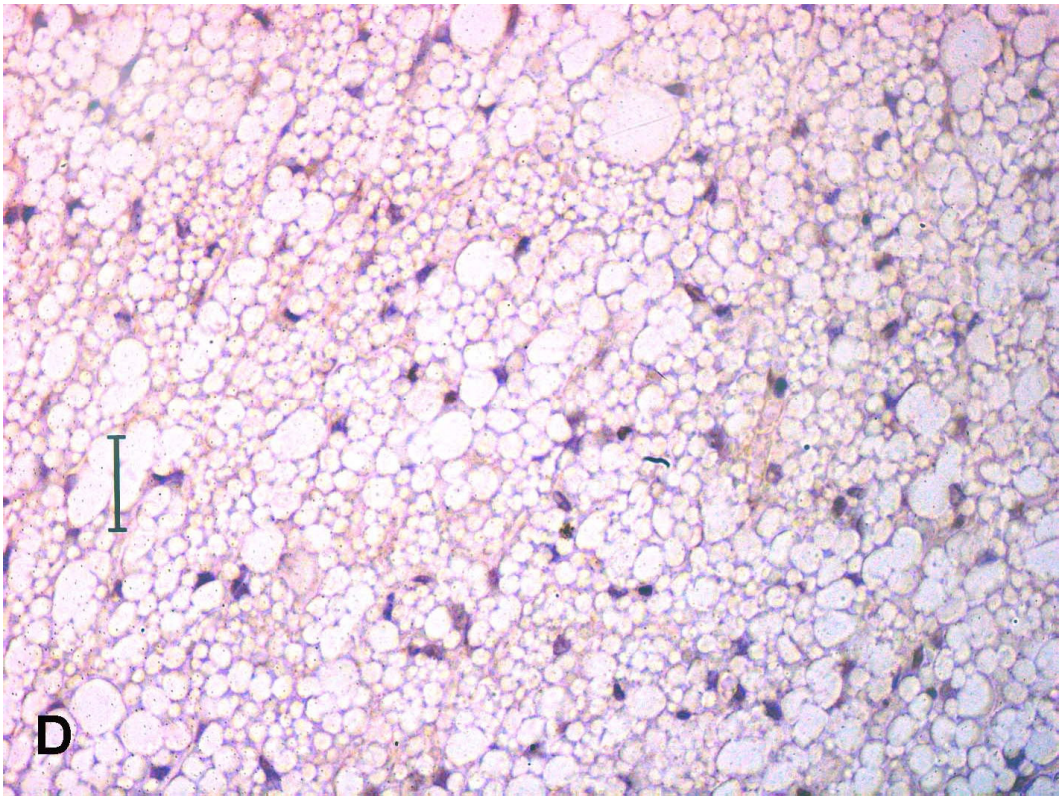
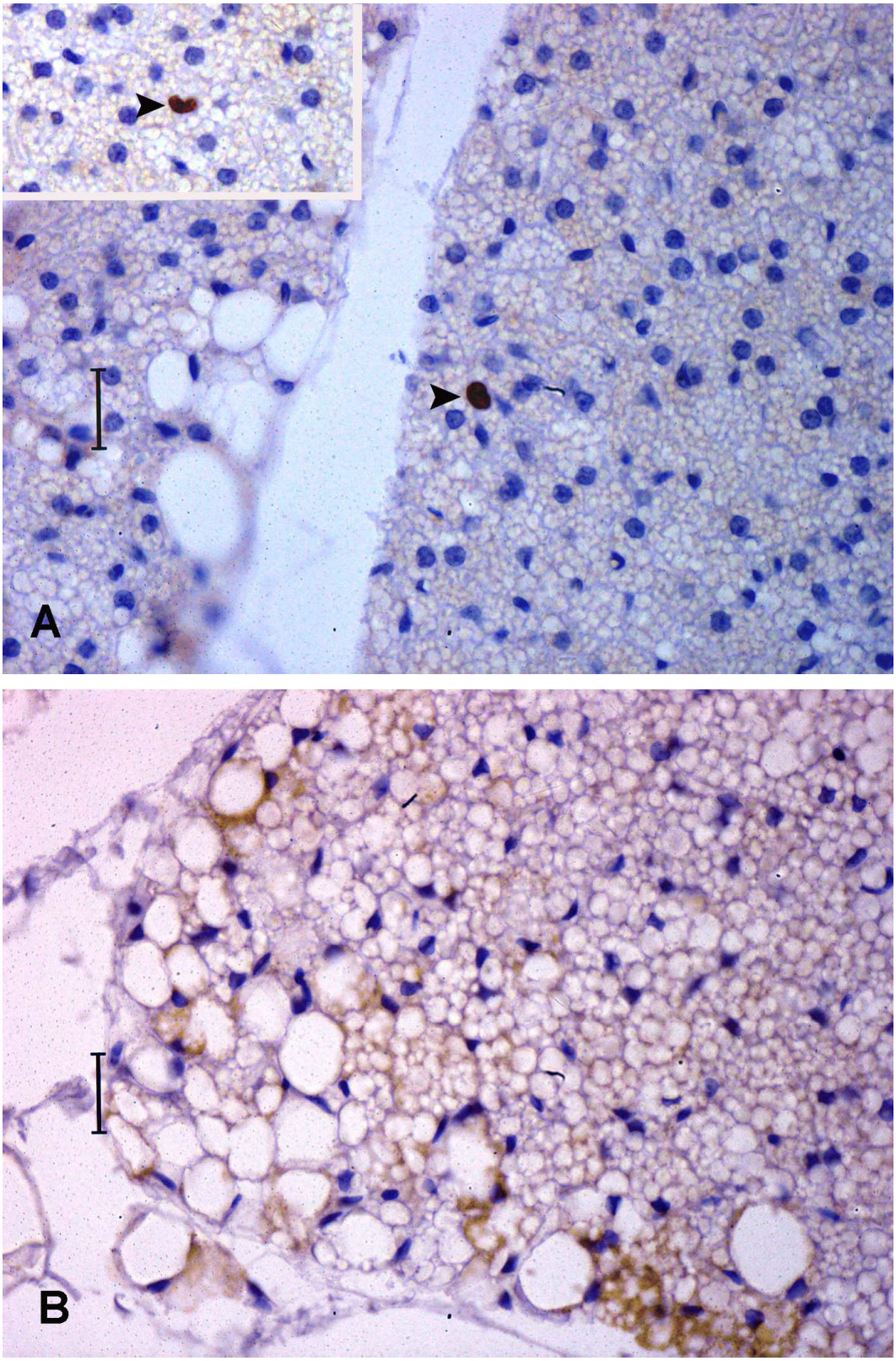


Figure 4. 2. 3. 1. Immunohistohemical reaction for UCP1 in BAT of control (A), T3- and T4-treated rats (B and C, respectively). Characteristic "patchy" pattern of UCP1-positive cells occurs in control animals. In T3 group, strong immunopositivity was noted in the areas with adipocytes with small number of large lipid bodies, while in T4 group immunopositive cells with one large and several small lipid bodies are surrounded by typical multilocular cells. For negative control primary anti-UCP1 antibody was omitted (D). 40x orig. (bar = 25 μm).

Immunohistochemical detection of Ki67. Ki67 is nuclear protein frequently used as cell proliferation marker. Ki67 immunopositivity was detected in small number of nuclei in control and both experimental groups (Fig. 4. 2. 3. 2). These nuclei were enlarged, roundish or had unusual shape, compared to Ki67-negative nuclei. This result indicates low level of cell proliferation and absence of effect of applied treatment on BAT proliferative processes. Unusual cytoplasmic localization of Ki67 was detected in adipocytes from hormones treated rats (Fig. 4. 2. 3. 2B, C). Adipocytes showing depositions of hromogene usually contain small number of larger lipid bodies, but some of them were typically multilocular.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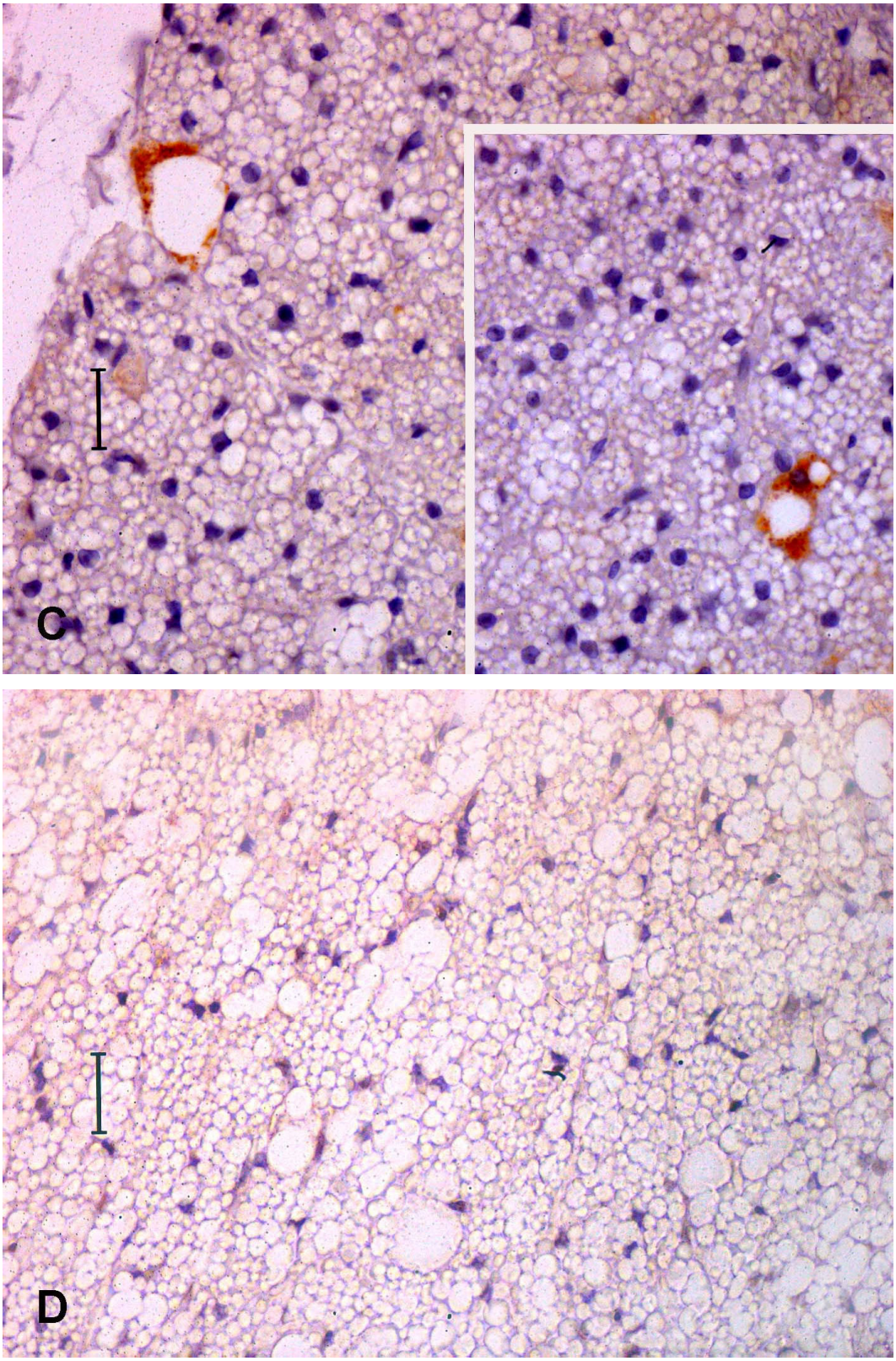


Figure 4. 2. 3. 2. Ki67-positive nuclei in BAT of control rat (A). Unusual intracytoplasmic Ki67-immunopositivity in the multilocular and paucilocular adipocytes detected in BAT of rats from groups T3 and T4 (B, C). For negative control primary anti-Ki67 antibody was omitted (D). 40x, orig. (bar = 25 μm).

Immunohistochemical detection of TNFα. The pattern of TNFα immunoexpression was analysed in order to reveal its possible involvement in regulation of brown adipocytes activity in systemic hyperthyroidism. TNFα is one of pyrogenic cytokines produced by many cell types, including adipocytes. As it could be seen from Fig. 4. 2. 3. 3, in all three studied groups TNFα-immunopositivity was localized within the cytoplasm of most brown adipocytes. TNFα-positive cells were evenly scattered throughout the tissue. In T3-group (Figure 4. 2. 3. 3B), reaction seemed to be slightly enhanced in comparison to control but the distribution pattern was similar (Figure 4. 2. 3. 3A). In T4-group (Figure 4. 2. 3. 3C), reaction was apparently more intensive than in T3 and control group and almost all brown adipocytes were more or less positive for TNFα.

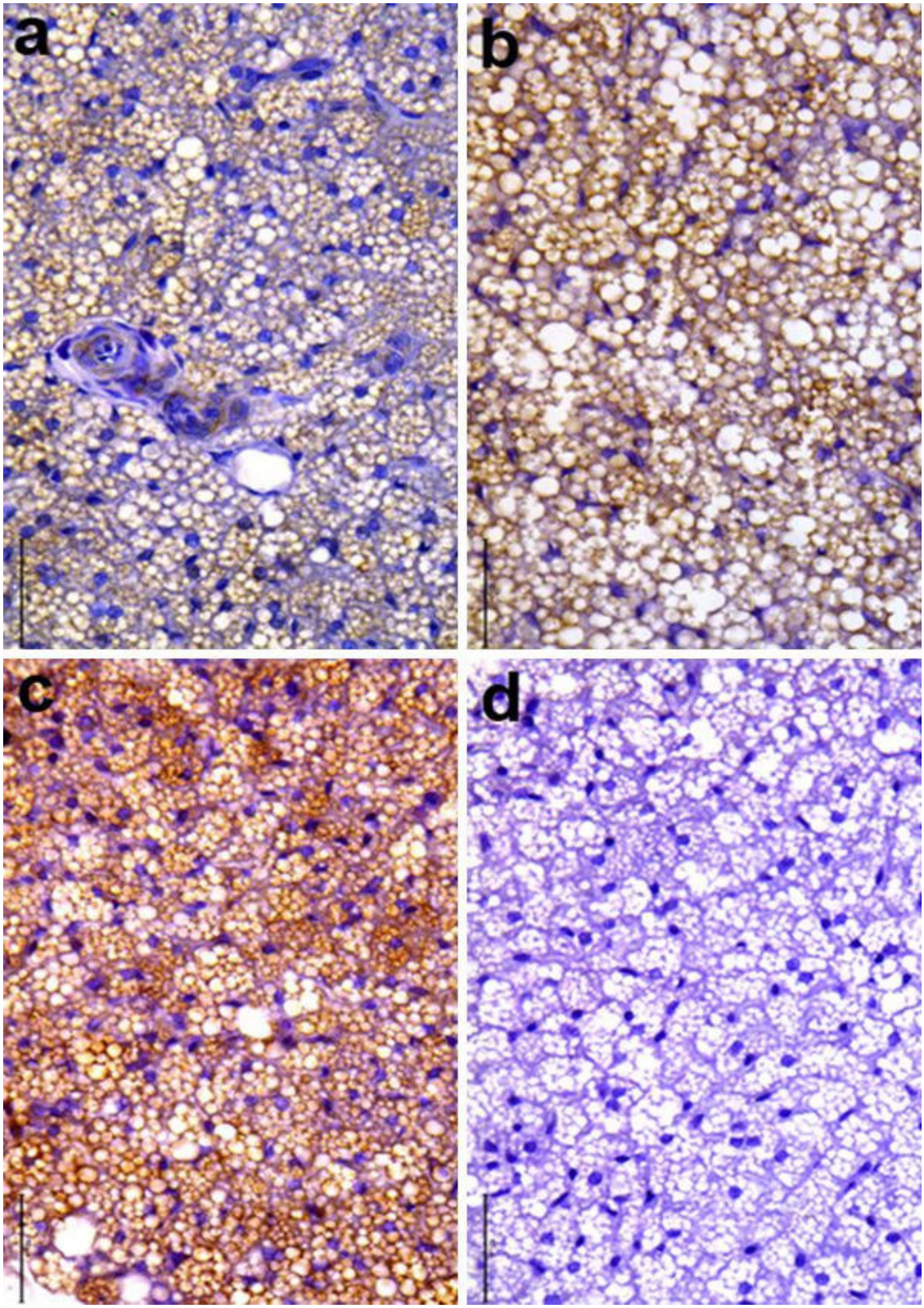


Figure 4. 2. 3. 3. TNFα-immunoexpression in interscapular BAT of control (a), T3-treated (b) and T4-treated rats (c); negative control - primary antibody omitted (d); magn. 40x orig. (bar = 50 μm).

4. 2. 4. Stereological analysis of BAT from control, T3- and T4-treated animals at the level of light microscopy

Stereological analysis performed at the level of light microscopy (Table 4. 2. 4. 1) did not show differences in volume density of multilocular and unilocular adipocytes, connective tissue and blood vessels in T3- and T4-treated rats in comparison to control animals.

Table 4. 2. 4. 1. Volume density of multilocular adipocytes, unilocular adipocytes, connective tissue and blood vessels in control, T3- and T4-treated rats.

	control	T3	T4
Vol. density multilocular adipocytes (%/μm^0)	88.6±0.89	86.6±0.73	87.6±1.31
Vol. density unilocular adipocytes (%/μm^0)	2.3±0.73	2.0±1.72	3.5±1.27
Vol. density connective tissue (%/μm^0)	4.3±0.18	3.7±0.63	4.0±0.16
Vol. density blood vessels (%/μm^0)	4.8±0.45	7.7±0.57	4.9±0.68

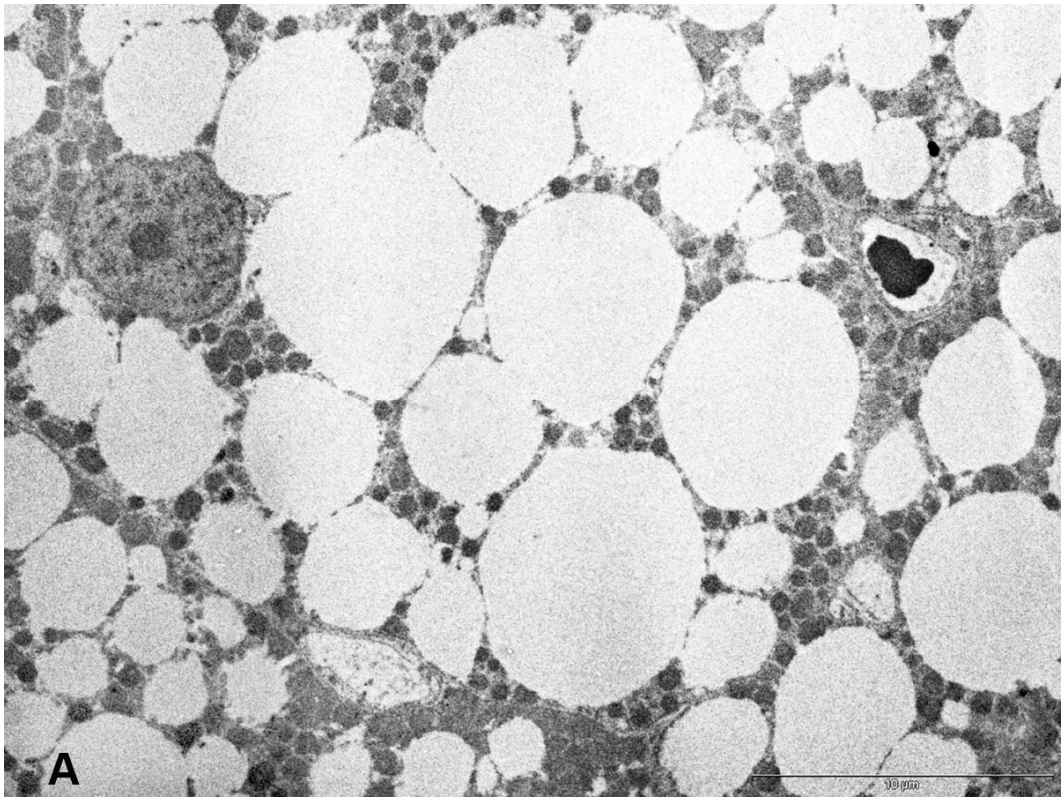
Statistics: n. s.

4. 2. 5. Electron microscopical analysis of BAT from control and T3- and T4-treated animals - general characteristics

Brown adipocytes of animals from control group (Fig. 4. 2. 5. 1A) are of typical appearance, with many lipid bodies, large nucleus and numerous mitochondria, while other organelles are usually not prominent. Nucleus is rounded or slightly altered in shape, euchromatic and with clearly visible nucleolus. "Free" cytoplasmic space is visible between mitochondria. Lipid bodies are rather uniform in size, sometimes they are closely apposed to each other or coalesced.

After treatment with T3 (Fig. 4. 2. 5. 1B), brown adipocytes are visibly enlarged, nucleus is deformed by lipid bodies and mitochondria are more closely apposed than in control group. Lipid bodies are usually very large and merged together.

In animals treated with T4 (Fig. 4. 2. 5. 1C) brown adipocytes also possess large quantity of lipids but they are distributed in lipid bodies which are clearly separated from each other. Lipid bodies differ in size and the least numerous are those of medium size. Mitochondria are closely apposed to each other, leaving only inconspicuous "free" cytoplasmic space. Large intercellular spaces between neighbouring brown adipocytes can be seen in this group (Fig. 4. 2. 5. 2).



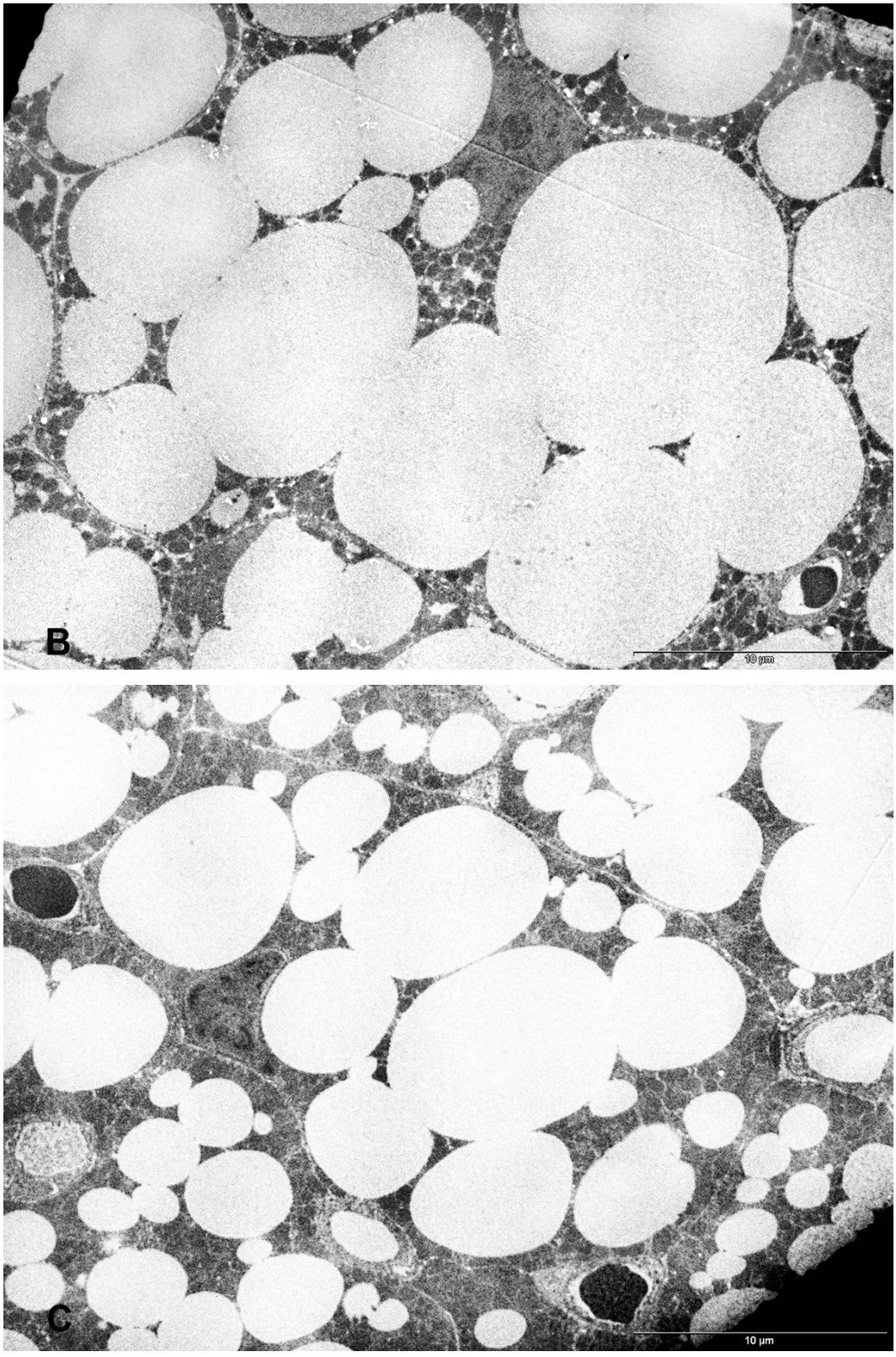


Figure 4. 2. 5. 1. Typical brown adipocytes from control (A), T3- and T4-treated rats (B and C, respectively). Magnification 2650x.

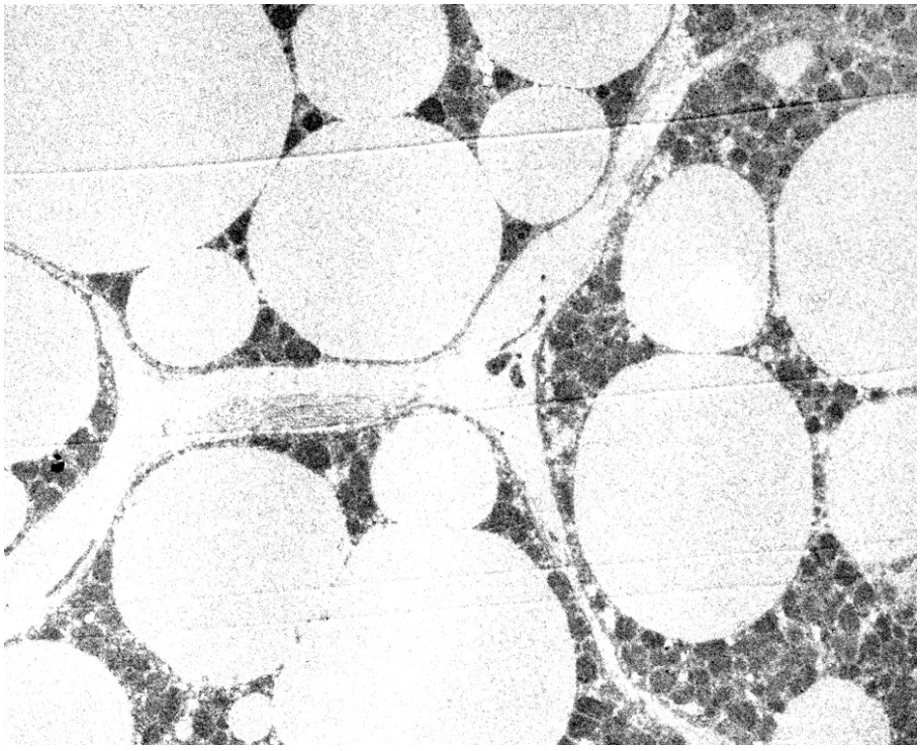


Figure 4. 2. 5. 2. Large intercellular spaces are present between brown adipocytes in some tissue samples from T4 group. Magnification 2650x.

In BAT of T3-treated rats, capillaries are mostly "empty" (without erythrocytes or some other blood cells). Endothelial cells are very thin and usually do not form protrusions at the luminal surface (Fig. 4. 2. 5. 3).

In BAT of T4-treated rats, cytoplasm of endothelial cells is thicker and appears undulated at luminal surface. Endothelial cell nuclei are often enlarged and euchromatic and cytoplasm contains numerous ribosomes (activated endothelium). In the capillary lumen one or more erythrocytes are usually present. In BAT of T4-treated rats, groups of several immature brown adipocytes were noted around capillaries (Fig. 4. 2. 5. 4). In some instances, adipoblasts can be noted in the vicinity of capillaries (Fig. 4. 2. 5. 5).

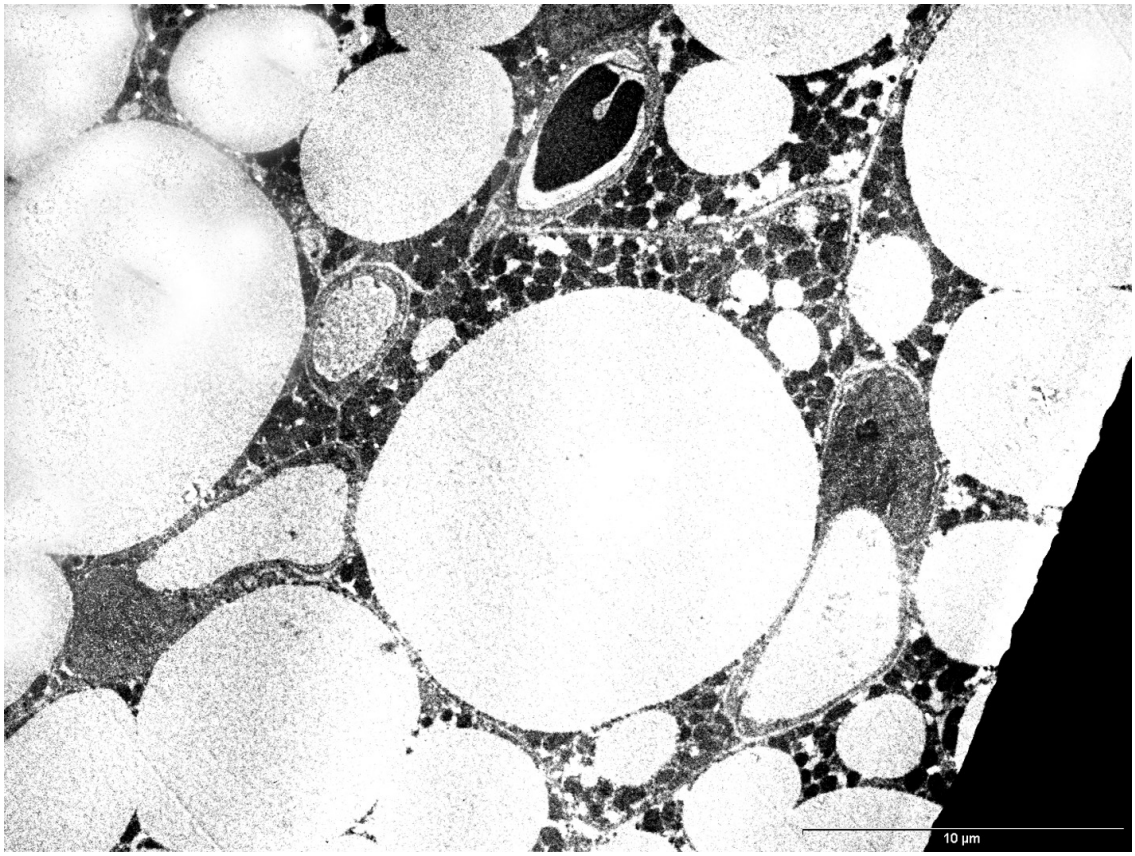


Figure 4. 2. 5. 3. In BAT of T3-treated rats, capillaries are often "empty" and appear collapsed. Magnification 2650x.

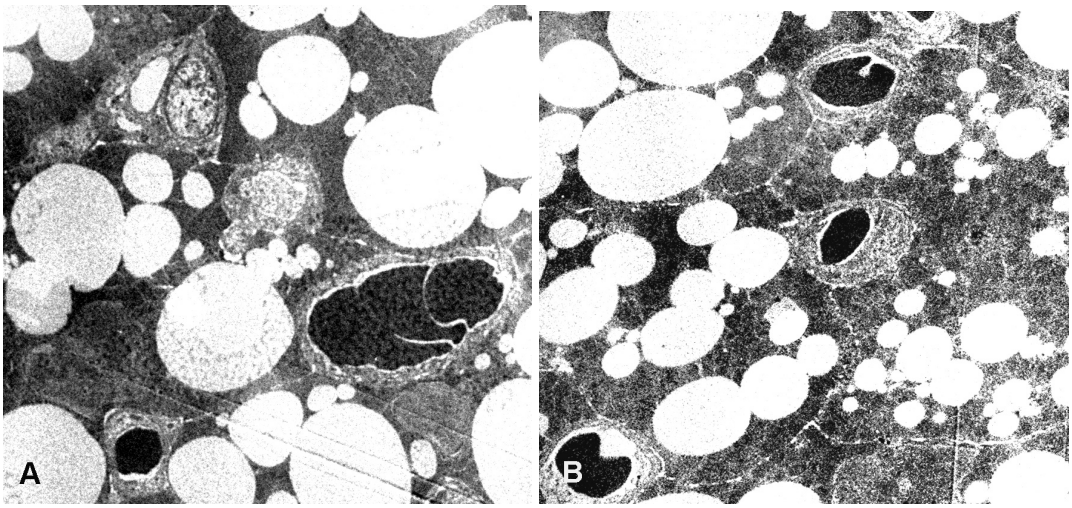


Figure 4. 2. 5. 4. In BAT of T4-treated rats capillary wall is thicker and there are usually one or more erythrocytes present in the capillary lumen. Some endothelial cells are activated. Several immature brown adipocytes can be seen around capillaries. Magnification 2650x.

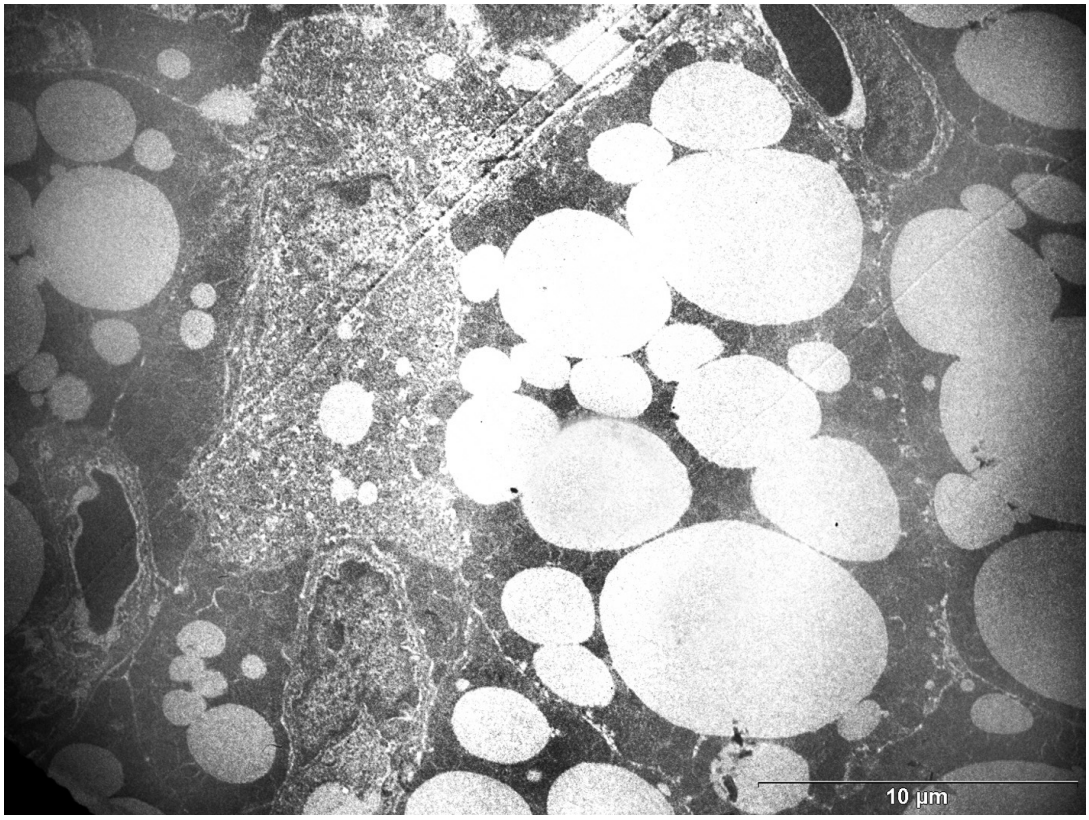


Figure 4. 2. 5. 5. Adipoblasts between brown adipocytes and capillaries. Magnification 2650x.

In BAT of rats it is possible to note some multilocular adipocytes with mitochondria which are morphologically more like mitochondria from white adipocytes - they are small, oval or elongated and not as numerous as those from brown adipocytes (Fig. 4. 2. 5. 6). However, most of them contain more cristae than mitochondria from WAT-like adipocytes. These cells are located closely to the capillaries or among typical brown adipocytes and are preferentially seen after treatment with T4. They probably represent cells differentiating from white-like to brown-like adipocytes.

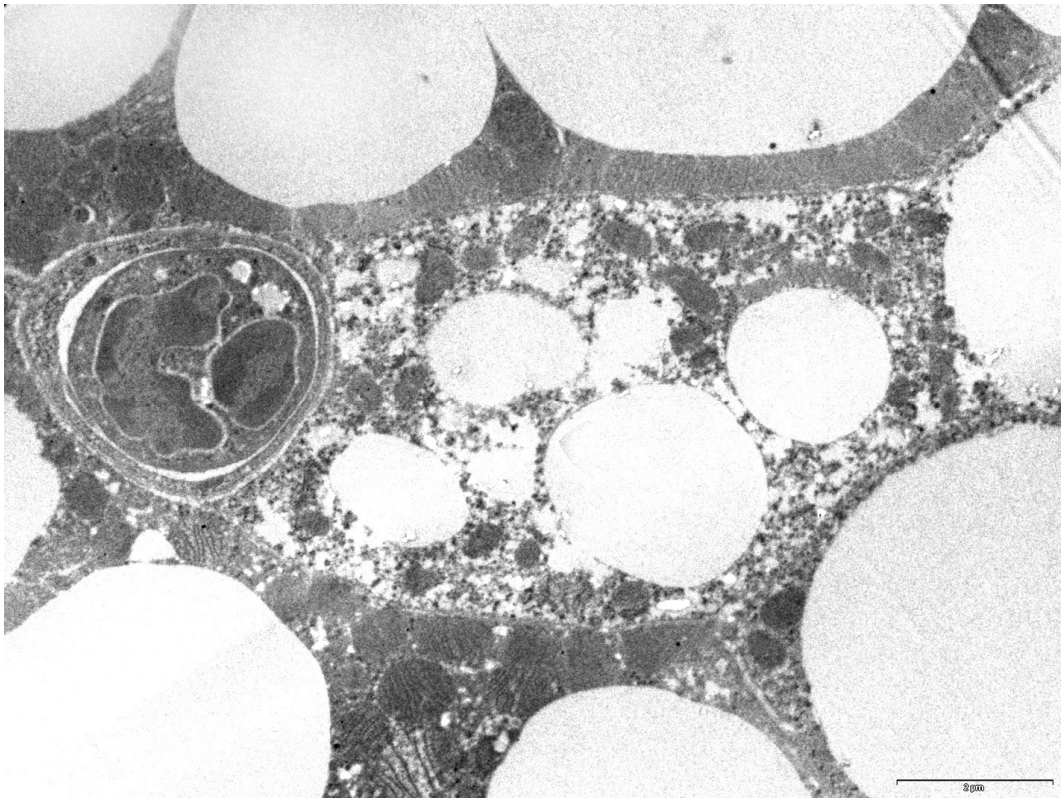


Fig. 4. 2. 5. 6. Multilocular adipocyte with atypical mitochondria, localized in the close proximity to capillary wall. Magnification 2650x.

Mitochondrial ultrastructure and their relationship with lipid bodies were analysed from electron micrographs of mitochondrial fields. In control group (Fig. 4. 2. 5. 7) mitochondria are mostly spherical, some are oval or elongated in shape. Spherical mitochondria have numerous straight and parallel cristae or cristae are grouped into two or three slightly curved systems. Oval mitochondria generally possess numerous straight and parallel cristae, but oval mitochondria with grouped and curved or short cristae also might be seen. Elongated mitochondria are rarely seen. Mitochondrial matrix is usually of similar density as cytoplasm only in some cells it is denser. Although there are many mitochondria around lipid bodies, they usually do not establish close contact with them. The most of mitochondria appear to be uniform in size.

In brown adipocytes of T3-treated rats (Fig. 4. 2. 5. 8) most numerous are again spherical mitochondria with straight and parallel cristae. In some of them cristae system is obviously reduced. Enlarged mitochondria, with straight or slightly woven cristae are also noted and some of them may be referred to as "giant" mitochondria. Occasionally, some very elongated mitochondria were noted, some of them being closely apposed to

the lipid body surface. Several mitochondria possess mixed cristae orientation. Mitochondrial matrix is usually denser than cytoplasm, but there are cells characterized by mitochondria with electron-translucent matrix.

After T4-treatment, brown adipocytes mitochondria show more pronounced pleomorphism (Fig. 4. 2. 5. 9). The most abundant are spherical and oval mitochondria, but elongated and irregular forms are also very frequent. Some very large slightly elongated mitochondria with a mixed system of cristae are visible. Mitochondrial matrix is denser than cytoplasm. Mitochondria-to-lipid body contact appears more intense than in control and T3-treated group. In places it looks like lipid body is absorbed by mitochondria.

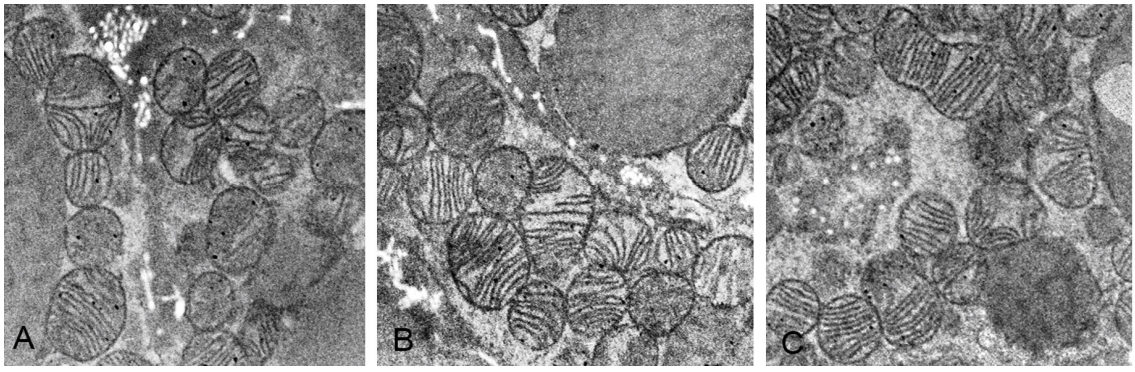


Figure 4. 2. 5. 7. Typical appearance of brown adipocyte mitochondria from control rats. Magnification 7100x.

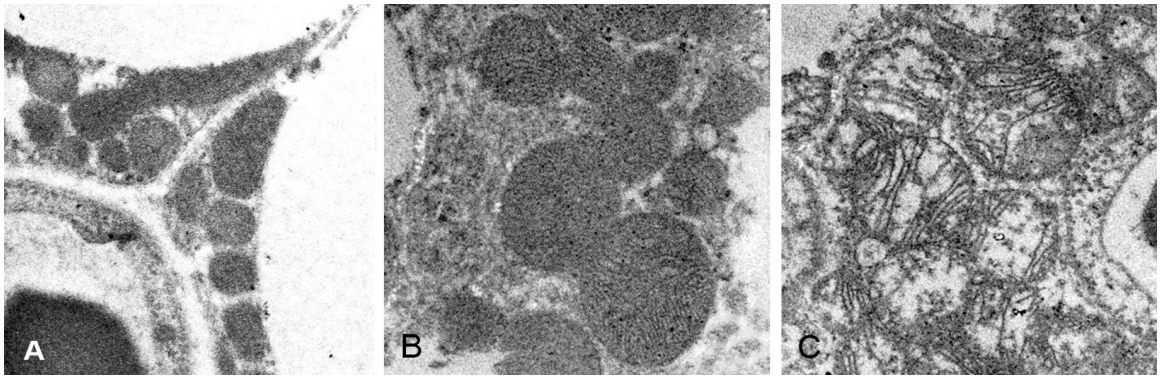


Figure 4. 2. 5. 8. Typical appearance of brown adipocyte mitochondria from T3-treated rats. Elongated mitochondria (A), "giant" mitochondria with woven cristae (B) and mitochondria with reduced and disorganised cristae and electron translucent matrix (C). Magnification 7100x.

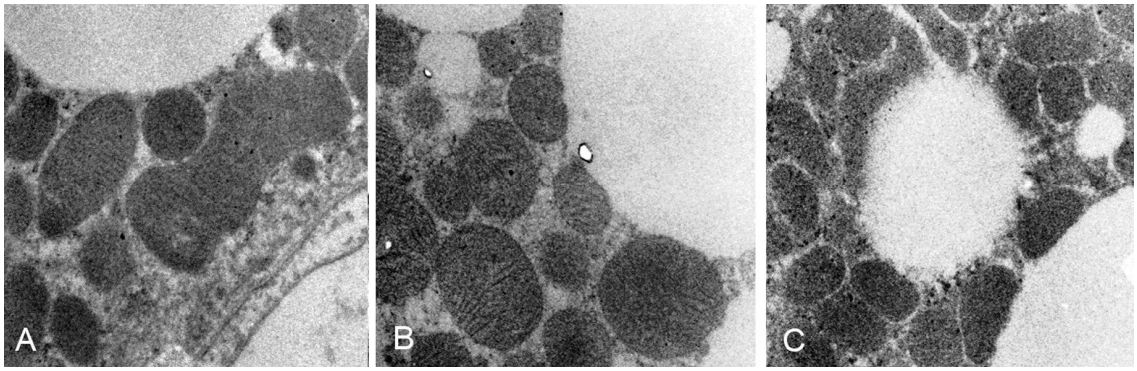


Figure 4. 2. 5. 9. Typical appearance of brown adipocyte mitochondria from T4-treated rats. Large irregularly shaped mitochondria and mitochondria generally variable in size and shape (A), abundant spherical mitochondria with well organised cristae (B) and mitochondria in close contact with lipid bodies (C).Magnification 7100x.

In some mitochondria of brown adipocytes from T3-treated rats, several small lipid bodies clustered inside the matrix might be seen (lipid inclusions) (Fig. 4. 2. 5. 10).

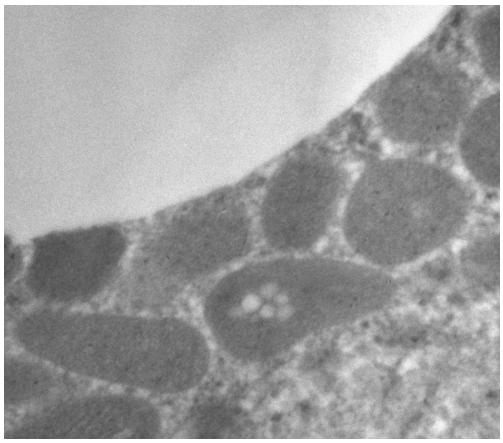


Figure 4. 2. 5. 10. Lipid inclusions in mitochondria of brown adipocyte from T3-treated rats. Magnification 19500x.

4. 2. 6. Stereological analysis of BAT from control, T3- and T4-treated animals at the level of electron microscopy

Results of stereological analysis are presented in Table 4. 2. 6. 1.

Analyses showed statistically significant increase of cell profile area (PA) in T3 group compared to control, while in T4 group it was increased but without significance.

Nuclear PA remained unchanged. At the same time, nuclear volume density was decreased in both treated groups compared to the control group.

Lipid bodies PA and volume density were increased in both treated groups. Lipid bodies number/cell profile was decreased in T3 group in comparison to control group. Mitochondrial PA was not changed with statistical significance, although slight

decrease was noted after both treatments. Mitochondrial volume density was decreased in T3 and T4 group, while mitochondrial number per cell remained unchanged.

Lipid bodies' surface in contact with mitochondria was increased in T3 and T4 groups. Mitochondrial perimeter in contact with lipid bodies was increased in T4-treated group. Cytoplasmic PA was significantly decreased in T4 group, and cytoplasmic volume density was decreased in T3 group and in T4 groups compared with control group.

Table 4. 2. 6. 1. Stereological analysis of brown adipocytes from control, T3- and T4-treated animals.

	control	T3	T4
cell PA (μm^2)	319.7±9.27	414.2±23.86*	370.7±22.18
nuclear PA (μm^2)	20.3±1.16	20.4±0.85	18.4±1.30
lipid bodies PA (μm^2)	185.4±11.36	292.7±15.80***	266.6±11.54***
mitochondrial PA (μm^2)	51.5±3.01	45.2±3.39	39.6±4.43
free cytoplasm PA (μm^2)	62.5±2.99	55.9±5.55	46.1±5.31*
Vv nucleus (%)	6.3±0.30	5.1±0.15**	5.0±0.27**
Vv of lipid bodies (%)	58.0±2.04	70.3±0.82***	71.9±1.14***
Vv of mitochondria (%)	16.1±1.01	11.2±0.47**	10.7±0.50***
Vv of free cytoplasm (%)	19.6±1.51	13.4±0.63**	12.4±0.87*
number of lipid bodies per cell profile (N)	19.8±1.57	11.5±0.72***	15.7±0.76
number of mitochondria per cell profile (N)	99.2±3.56	105.5±7.41	106.0±2.48
N of mitochondria/μm^2 of cell prof. (w/o nucl.)	0.34±0.020	0.28±0.010*	0.31±0.010*
lb perimeter in contact with mitochondria (%)	23. 5±1.94	30.2±1.27*	41.8±2.68***
mitochondria perimeter in contact with lb (%)	9.51±0.982	11.33±0.631	15.51±1.441***

Statistics: * $p<0.05$ *vs*. control; ** $p<0.01$ *vs*. control; *** $p<0.001$ *vs*. control

Electron micrographs also served for analysis of mitochondrial size in control and treated groups. According to their profile area, all analysed mitochondria were classified into class ranges as follows:

Class range	Mitochondrial profile area (μm^2)	Class range	Mitochondrial profile area (μm^2)
I	up to 0.099	VI	0.500-0.599
II	0.100-0.199	VII	0.600-0.699
III	0.200-0.299	VIII	0.700-0.799
IV	0.300-0.399	IX	0.800-0.899
V	0.400-0.499	X	0.900 and larger

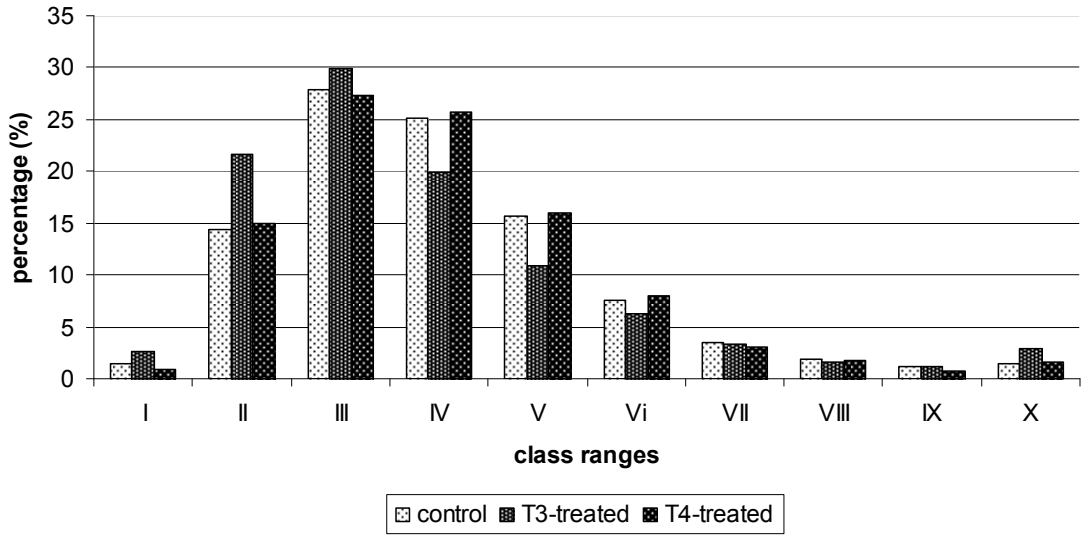


Figure 4. 2. 6. 1. Histogram shows distribution of mitochondria into different class ranges on the basis of profile area size. In all groups the most of mitochondria belong to categories II, III and IV. Distribution of mitochondria from control and T4-treated animals are almost parallel through the ranges. Mitochondria from T3-treated animals differ from other two groups since their distribution is shifted to the left side of the histogram, particularly to the class II, in comparison to control and T4-treated groups.

Similar analysis was done for lipid bodies. According to their profile area, all analyzed lipid bodies were classified into class ranges as follows:

Class range	Lipid body profile area (μm^2)	Class range	Lipid body profile area (μm^2)
I	0-0.9	VI	20-24.9
II	1-4.9	VII	25-29.9
III	5-9.9	VIII	30-39.9
IV	10-14.9	IX	40-49.9
V	15-19.9	X	50 and larger

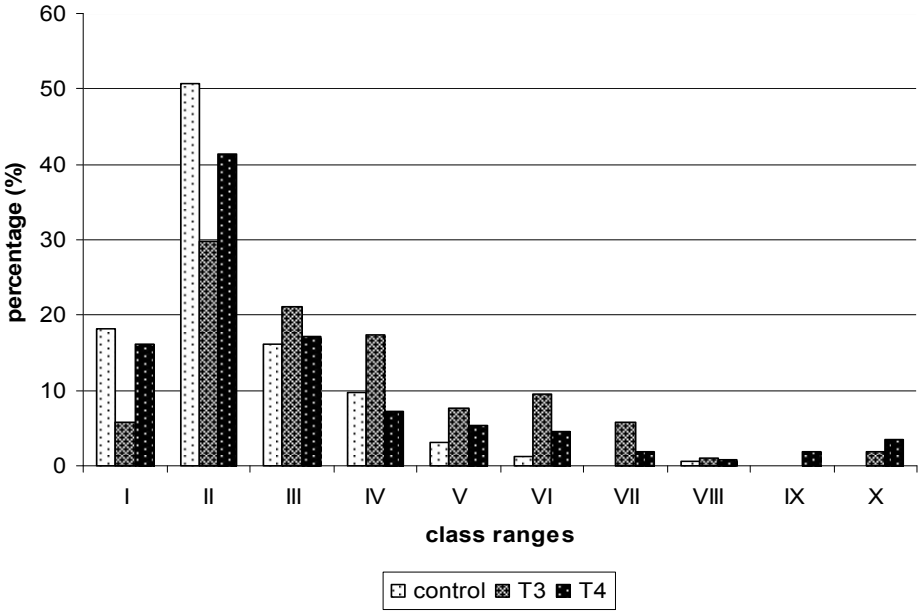


Figure 4. 2. 6. 2. Histogram shows distribution of lipid bodies into different class ranges on the basis of profile area size. In all groups the most of lipid bodies belong to category II. In larger classes (III to VII), predominate lipid bodies from T3 group, which means that in T3 group brown adipocytes contain mostly large lipid bodies.

4. 2. 7. Other observations

In one animal from control group, large fields of extracellular lipids were observed (Fig. 4. 2. 7. 1). In these areas, cell membranes were not visible and tissue appeared as a mass of lipid bodies, mostly of smaller size than in surrounding brown adipocytes. Inside these fields there were almost no nuclei, while nuclei at the border line with visibly normal tissue stained positively with PI, indicating dead cells (Fig. 4. 2. 7. 2). These fields do not contain nerve fibers (Fig. 4. 2. 7. 3) and UCP1 immunohistochemical reaction is absent (Fig. 4. 2. 7. 4).

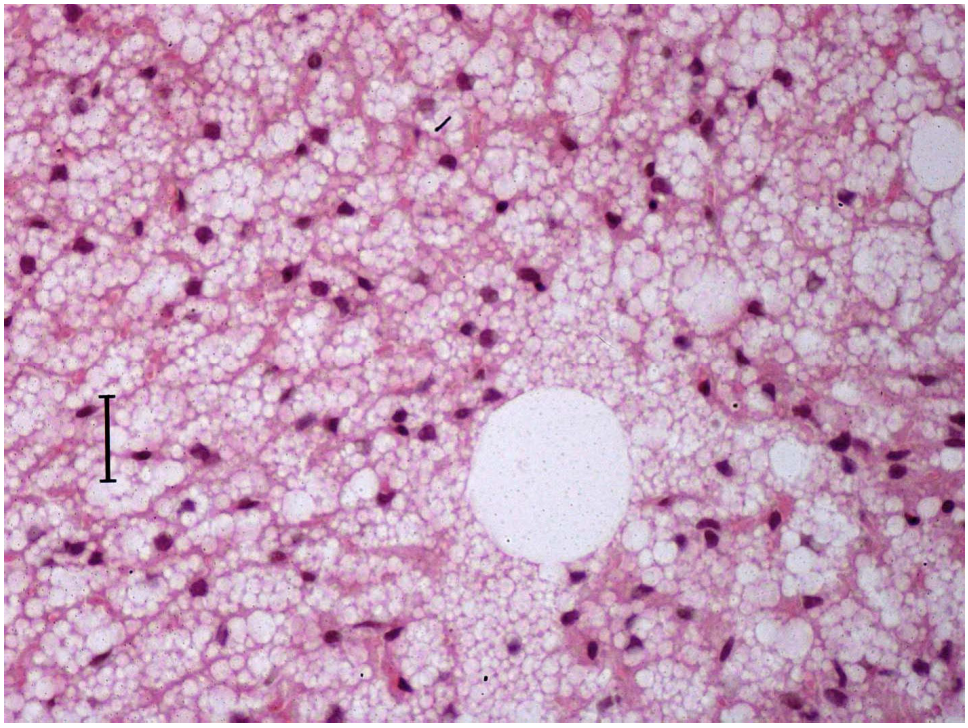


Figure 4. 2. 7. 1. Field of extracellular lipids, staining with HE. 40x, orig. (bar = 25 μm).

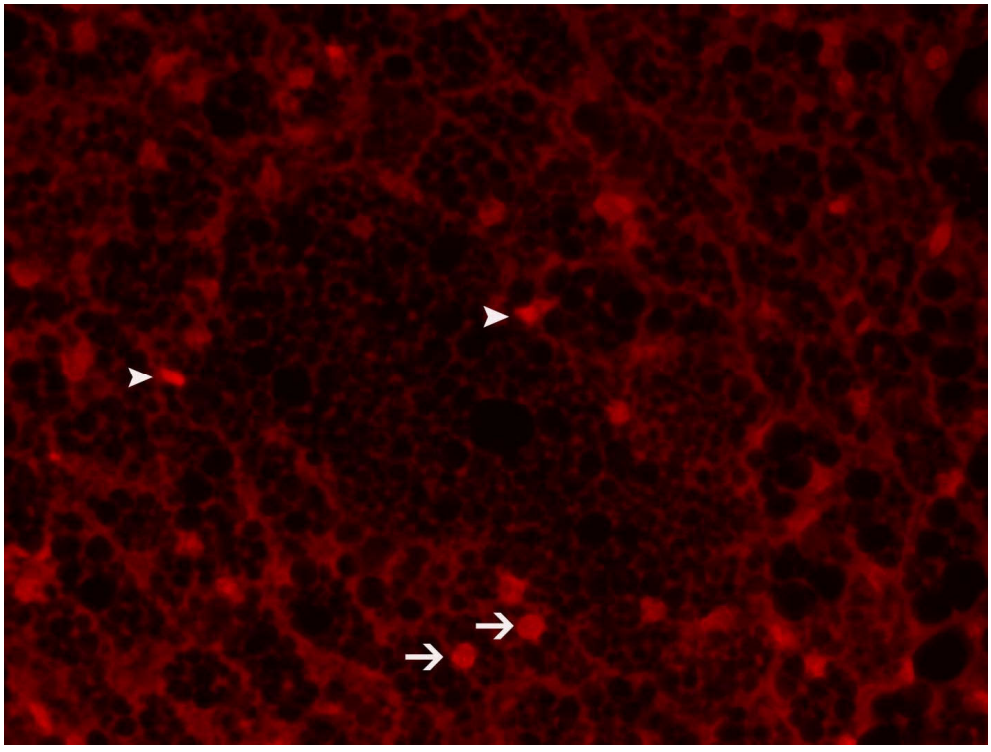


Figure 4. 2. 7. 2. Extracellular lipids are seen as dark area in the centre of the image. Nuclei marked with PI belong to dead cells at the borderline between necrotic field and "normal" tissue (nuclei of endothelial cells - arrowheads, nuclei of adipocytes - arrows). 40x, orig.

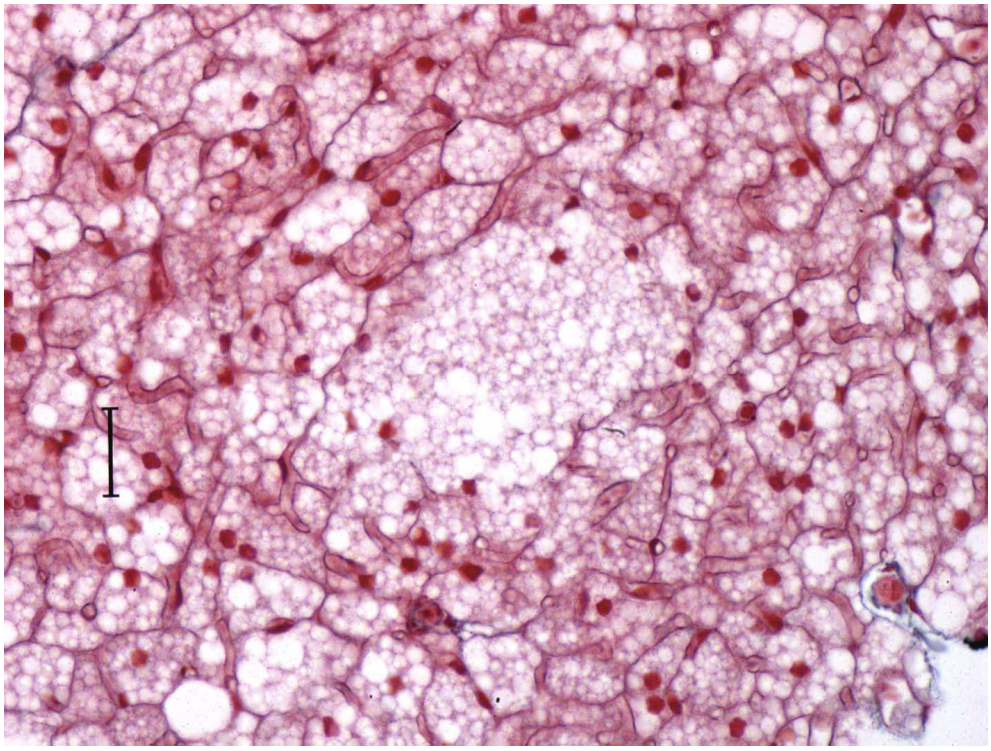


Figure 4. 2. 7. 3. Extracellular lipids. Bodian method for demonstration of nerve fibers. 40x, orig. (bar = 25 μm).

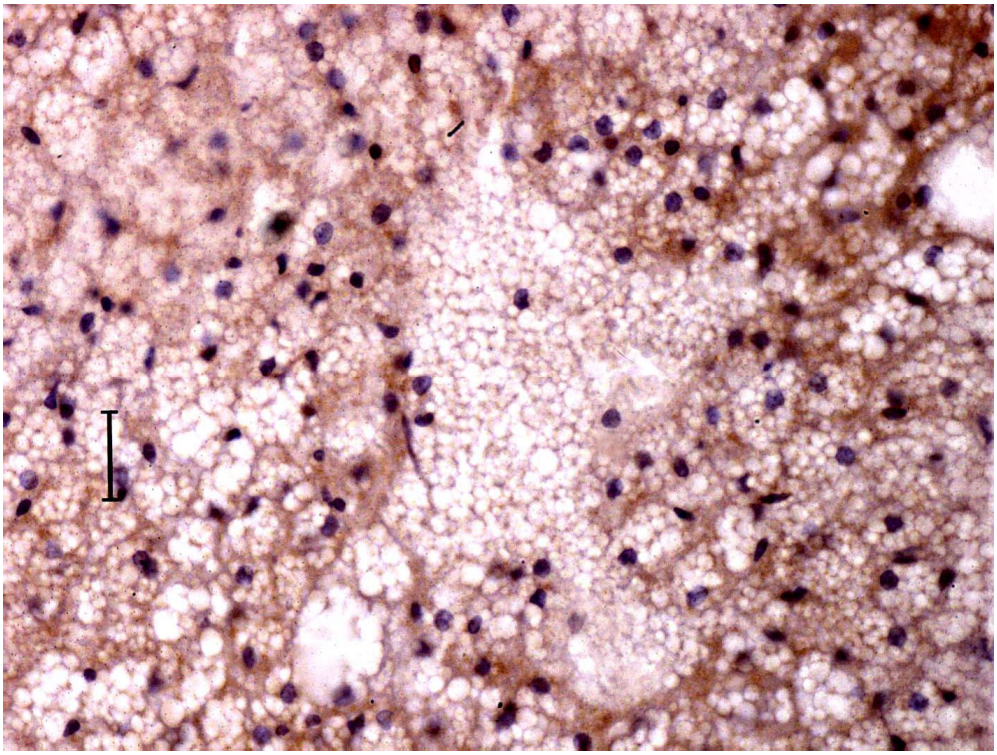


Figure 4. 2. 7. 4. Extracellular lipids, immunohistochemistry for UCP1. 40x, orig. (bar = 25 μm).

5. Discussion

5. 1. Effects of treatment with T3 or T4 on thyroid gland

Thyroid hormones are frequently used in human and veterinary medicine as replacement therapy for thyroid deficiency-related diseases (Wiersinga, 2001; Dixon et al., 2002; Escobar-Morreale et al., 2005) or in doses slightly or significantly above physiological (Bauer et al., 2002; Friedman et al., 2006; Brabant, 2008). Besides, some investigations regarding use of supraphysiological doses of thyroid hormones for heart repair after myocardial infarction are in progress (Pantos et al., 2007, 2008, 2009). Data on possible side effects of such therapies in humans are still inconsistent and vary depending on tissue, organ or function examined. Despite the fact that hyperthyroidism is commonly associated with insomnia, high doses of T4 used for treatment of mood disorders did not cause sleep impairment in otherwise healthy patients (Kraemer et al., 2011). Also, it seems that supraphysiological doses of T4 are not necessarily associated with bone mineral density loss even after a very long period of treatment (Ricken et al., 2012; but see also Chen et al., 2004). From the animal studies it is known that experimentally induced thyrotoxicosis causes impairment of some cognitive functions (Taşkin et al., 2011) and activates hypothalamic–pituitary–adrenal axis thus potentially compromising adrenal function (Johnson et al., 2005). Given the wide use of thyroid hormones as therapeutics in a number of diseases and conditions and rather good common knowledge on their undesired effects on various organs, there is a surprising paucity of studies dealing with the effects of supraphysiological doses of thyroid hormones on the thyroid gland.

In this experiment a previously established model of experimentally induced hyperthyroidism in rats (Petrovic et al., 2003) was used. Animals were treated with T3 or T4 in doses which were highly above physiological replacement doses estimated at 3 μg/kg b.w. for T3 (Dillmann et al., 1983) and 20 μg/kg b.w. for T4 (Berstein, 1980). Expectedly, treatment with T3 significantly elevated circulating T3. T4 was markedly decreased as the result of T3 suppressive effects on thyroid gland activity, mediated through feedback inhibition of TRH and TSH release, at levels of hypothalamus and pituitary, respectively (Belchetz et al., 1978; Maruta and Greer, 1988; Saleh et al.,

1998). At the same time, it means that T3 in serum of T3-treated animals was mainly exogenous. In rats treated with supraphysiological doses of T4, concentrations of both T3 and T4 were elevated but the elevation was not of the same magnitude. Namely, T3 increased for about 50%, while T4 concentration was more than doubled. This suggests that peripheral T4 to T3 deiodination mechanisms reached the level of saturation and could not be further intensified, even with excess of T4 as substrate. T4 concentration in the blood remained steadily increased due to the overloaded deiodination pathway.

Absolute and relative masses of the thyroid gland were unchanged after both treatments, while stereological analysis showed no differences in the relative abundance of main thyroid tissue compartments (parenchyma *vs*. interstitium) between control and experimental groups. These results are in disagreement with the data reported previously by Soukup et al. (2001) who found markedly atrophied thyroids in rats treated with high doses of thyroid hormones. However, considerably shorter duration of present experiment (5 days *vs*. 6 month) offers a good explanation for the absence of changes in mentioned parameters.

Within follicles, however, relative proportions of the thyroid epithelium and colloid were shifted in favour of colloid, while the height of follicular cells was markedly reduced in both treated groups. It is well known that the morphofunctional status of each follicle is controlled not only by the TSH level, but also by other factors including thyroglobulin contained within the follicle. Depending on the stage of the follicle physiological cycle, TSH may stimulate either expression of thyroid-specific genes involved in thyroglobulin synthesis, or reabsorption of colloid, its lysosomal degradation and release of thyroid hormones (Suzuki et al., 2011). Follicles containing large amounts of colloid, as noted in presented study in T3- and T4-treated groups, are lined with thyrocytes in which synthesis of thyroglobulin is low (Suzuki et al., 1998, 1999, 2011). Normally, under TSH-stimulation, such follicles should be engaged in colloid resorption and hormones release. Although TSH levels have not been measured, they were estimated indirectly, using the thyroid activation index, which is known to be positively correlated with the level of TSH in circulation (Kalisnik, 1981; Rajkovic et al., 2003, 2006). Thus, according to results obtained for thyroid activation index, thyroids in both hormone-treated groups were understimulated and in the state of arrest. Cytologically, the disorganization and/or reduction of microvilli, as well as significantly

reduced epithelial height, provide further evidence for the functional quiescence of the thyroid gland after thyroid hormone treatment.

In the control group the rare occurrence of desquamated cells inside the follicles was noted after PI-staining. This finding in the absence of hormonal treatment is related to normal basal thyroid cells turnover since it is known that, as in many other organs, thyroid gland maintains its mass homeostasis by sustained basal proliferation and apoptosis (Dremier et al., 1994; Okayasu et al., 1995; Tamura et al., 1998). Apoptotic cells are shed into the follicular lumen, subsequently being removed by macrophages and the remaining thyrocytes (Matsunaga et al., 1988). More frequently observed presence of cell fragments and debris inside some low-epithelial and irregularly shaped follicles of T3- and T4-treated rats, together with PI-positive condensed nuclei within walls of such follicles, point to loss of thyrocytes and remodelling/fusion of follicles. Such intensified shedding of thyrocytes and their membrane-bounded fragments was seen previously during involution of the hyperplastic thyroid gland (Tachiwaki and Wollman, 1982; Tachiwaki et al., 1990). Electron microscopy provided more detailed insights on cell damages after treatments with T3 or T4. Some thyrocytes within the follicular wall exhibited signs of reversible cell injury, such as nuclear condensation and vesiculation of cytoplasmic organelles. More advanced destruction of thyrocytes sometimes even with abruption of the follicular epithelium after thyroid hormone treatments might be considered as the high grade tissue injury. Occasionally, progressive damage expanded to nearby capillaries, resulting in microhemorrhage indicated by the occurrence of erythrocytes inside the follicular lumen. As it has been proposed earlier and mentioned above herein, thyroglobulin accumulated inside the follicle is involved in the regulation of thyrocyte activity, counterbalancing the TSH action (Suzuki et al., 1998, 1999, 2011). Excessive intrafollicular accumulation of thyroglobulin may produce increased pressure on thyrocytes, deforming their cytoskeleton and generating pro-apoptotic signals (Janmey, 1998; Field, 2010). Thus, beside attenuation of trophic TSH signals due to high circulating levels of thyroid hormones, thyrocytes in hyperthyroid rats may have received proapoptotic signals. The intensity of these signals, together with functional status of the particular follicle, determines the degree of epithelial damage ranging from mild degeneration of individual lining cells to total destruction of follicular wall and lumen fusion.

Investigating the period needed for thyroid recovery after cessation of treatment was out of the scope of this study. However, it has been shown previously that morphofunctional alterations of the thyroid gland caused by noxious stimulation were not completely resolved even after three months of repairing period (Rajkovic et al., 2003).

In summary, obtained results demonstrate that high doses of thyroid hormones cause serious damage of thyroid follicles in euthyroid rats, even after short-term treatment. Observed changes are most probably based on the absence of cytoprotective effects of TSH, together with reception of more pronounced proapoptotic signals. Given that structural alterations of the thyroid gland associated with dysfunction may require an unpredictable recovery period, current findings point to necessary caution needed when thyroid hormones are used as therapeuticals.

5. 2. Effects of treatment with T3 or T4 on BAT

Treatment with thyroid hormones expectedly caused significant reduction in **body mass gain**. This result is consistent with well established fact that thyroid hormones positively affect both energy intake and energy expenditure and lead to reduction in body mass gain, even despite increased energy intake (Iossa et al., 2001; Short et al., 2001; Upadhyay et al., 2004). Reduction in body mass gain observed in the present experiment, together with significant increase in thyroid hormones levels, may serve as indication of successfully established systemic hyperthyroidism.

The broadly accepted explanations on the mechanisms of increasing energy expenditure and heat production induced by thyroid hormones (primarily T3) in excess are based on their direct and indirect actions on cells in peripheral tissues. Direct peripheral effects are those that cause the increase in ATP utilization. Namely, thyroid hormones may stimulate so-called metabolic or futile cycles such as those consisted of reverse reactions regulated by enzymes operating in opposite manner (for example, glycolysis/gluconeogenesis or lipolysis/lipogenesis) and ion leaks and recovering (Na^+/K^+-ATPase and the sarco/endoplasmic reticulum Ca^{2+}-ATPase, for example). Indirect peripheral effects of thyroid hormones for increase in energy expenditure and heat production include stimulation of mitochondrial biogenesis, reduction of ATP

synthesis efficiency (by uncoupling oxidative phosphorylation in mitochondria). In addition, thyroid hormones may exert indirect peripheral effects acting through non-genomic pathways initiated at the plasma membrane, in the cytoplasm or mitochondria (for review, see Vaitkus et al., 2015). It is important to notice that these mechanisms of thyroid hormones action in hyperthyroid state are considered to diminish the need for thermogenesis in BAT and even reduced facultative BAT thermogenesis has been reported (Triandafillou et al., 1982).

However, mechanisms like proton leaks and futile cycles are seriously challenged since they have never been thoroughly explored and proven. In addition, considering this issue from the opposite point of view, the increased activity of some enzymes (such as those involved in futile cycles) may be the consequence rather than cause of elevated body temperature occurred in hyperthyroidism. Thus, López et al. (2010; 2013) offered another explanation for increased metabolic rate induced by thyroid hormones. They proposed that effects of thyroid hormones on energy expenditure and heat production may be mediated centrally and include stimulation of sympathetic input in BAT. According to this assumption, BAT may be the effector of thyroid thermogenesis.

Therefore, while waiting for further clarification, confirmation or rejection, these results provided two confronted starting points for this dissertation.

Another important and frequently observed effect of thyroid hormones action is elevation in **body temperature**. Contrary to the expectations, in this study significant increase in body temperature was found only after treatment with T4. This result is in contrast with earlier findings in which elevated body temperature was found in T3- but not in T4-treated rats (Petrovic et al., 2003). However, in both experiments at least the tendency for temperature elevation after T3 or T4 treatment has been shown. Therefore, this result may be explained by individual variations in response to elevation in circulating thyroid hormone levels, within particular experimental situation.

Supraphysiological doses of thyroid hormones used in this experiment significantly increased absolute and relative **BAT mass**. Increase in BAT mass after treatment with T3 or T4 was previously reported by others (Puerta et al., 1984; Masini et al., 1990; Abelanda and Puerta, 1992; Petrovic et al., 2003; López et al., 2010), with T3-effects being more consistently documented. Increase in absolute BAT mass may be

the result of brown adipocyte hypertrophy and/or brown adipocyte hyperplasia. In present work, proliferative activity in BAT was investigated using immunoexpression of proliferative marker Ki67. According to results obtained by that analysis, Ki67-immunopositive nuclei were observed only sporadically after five days of treatment with T3 or T4, similar as in control animals. However, electron microscopical observations showed the occurrence of immature brown adipocytes clustered around capillaries in both groups under treatment, but particularly in BAT from T4-treated animals. Accordingly, increase in absolute BAT mass as well as the presence of immature brown adipocytes support proliferative processes in BAT. However, proliferation of brown adipocytes is very early and transient process: it starts only few hours after beginning of stimulation, lasts in full extent for two days and then declines progressively (Bukowiecki et al., 1982; Masini et al., 1990). It means that at the end of five days-lasting experimental treatment proliferation was in late stages or completed and newly formed brown adipocytes were in different stages of maturation. At the same time, it is the explanation for the absence of Ki67 immunopositive reaction in nuclei of proliferating cells.

Mechanism of thyroid hormone action on proliferation in metabolically active tissues has not been discussed extensively before. Moreno et al. (1997) have suggested possible direct modulating effects on cellularity of metabolically active tissues including BAT, mediated through nuclear pathways. On the other hand, centrally mediated effects involving stimulation of sympathetic input also must be taken into account, at least in relation to BAT (López et al., 2010).

Another question is why immature brown adipocytes were not seen in BAT after T3-treatment in the similar extent as they were seen after T4-treatment or, in other words, why the effect of T4 on proliferation in BAT was more obvious then effect of T3. One explanation is possible difference in the rate of proliferation induced by these two hormones, which in turn may be the consequence of distinct mode of action of these two hormones in terms of directly or indirectly mediated effects and/or local BAT T3-concentration achieved by administration of T3 in comparison to that achieved by administration of T4. Obregon (2014) reviewed data on different requirements for T3 during proliferation and differentiation of BAT. According to these reports, proliferating brown adipocyte precursors need low concentration of T3, while higher

concentration is required for their differentiation into mature brown adipocytes. Thus it is possible that local regulation of T3 level in BAT, which depends on T3/T4 systemic concentration, creates conditions which differently affect dynamics of proliferation and differentiation in two groups of treatment.

Stereological analysis at the level of electron microscopy, presented herein, showed the enlargement of brown adipocytes in T3-treated group, on the account of lipid accumulation. Enlargement of brown adipocytes also can contribute to increase in absolute tissue mass.

Increased absolute BAT mass, regardless of how it has been reached, in association with decreased body mass gain, resulted in increased relative BAT mass, in both T3- and T4-treated rats. Accordingly, in systemic hyperthyroidism induced by both T3 and T4 the relative importance of BAT for organism as a whole is highlighted.

Discussion on hyperplastic alteration in BAT may be linked to results obtained studying apoptotic processes in the tissue after treatments with thyroid hormones. Apoptosis was not observed in any of treated groups with higher frequency than in the control group. It is well known that tissue/organ-size homeostasis is maintained by the dynamic equilibrium between two opposing processes - cell proliferation and cell apoptosis. During normal cell turnover, cell proliferation and apoptosis are balanced, while increased mitotic cell growth in concert with decreased apoptosis result in growth of the tissue/organ (Alenzi et al., 2004). In stimulated BAT, noradrenaline protects brown adipocytes from apoptosis. In addition, brown adipocytes synthesize and secrete various paracrine factors, some of which may have a role in promotion of cell survival and suppression of apoptosis, such as NGF, IGF-1, bFBF or VEGF (Lindquist and Rehnmark., 1998; Bagchi et al., 2013). Thus, according to results presented in this dissertation, systemic hyperthyroidism induced by T3 or T4, applied as specified above, favours the growth of BAT.

Rich **vascular network** is crucially important for delivery of oxygen, lipids and glucose to BAT and uptake of metabolic products. Not less important is uptake of heat generated by the activity of brown adipocytes and its distribution throughout the body, especially toward vital organs.

Any enlargement of BAT upon physiological or pharmacological stimulation, whether it occurred by hyperplasia, hypertrophy or their combination, requires precise

spatio-temporal coordination of adipogenesis and angiogenesis in order to maintain adequate tissue blood irrigation (Géloën et al., 1988; 1990). To investigate the intensity of blood flow within the tissue, light microscopical analysis of BAT sections stained by Novelli method was used. This method stains erythrocytes in pink, thus highlighting vascular components present in the tissue. In the control group, this method revealed highly branched network of small blood vessels containing erythrocytes, uniformly supplying all parts of the section. As it was estimated by visual observation, blood vessel density in T4 group was higher than in the control and their cross-sections were narrower. After treatment with T3, vascularization seemed attenuated in comparison to control.

Electron microscopical observations confirmed findings obtained at the light microscopy level and revealed more details on rearrangement of BAT vascular network after treatments with thyroid hormones. Endothelium in most of the capillaries in T3 group was in resting state, capillary walls were thin and lumens were usually collapsed. Capillaries usually did not contain erythrocytes or other blood cells. As opposed to that, capillaries in BAT from T4-treated group are often lined with activated endothelial cells and contain one or more erythrocytes. Adipocyte precursors were typically closely apposed to the capillary wall.

Vascular endothelial growth factor (VEGF) belongs to the group of paracrine factors secreted *inter alia*, by brown adipocytes. It stimulates proliferation and migration of endothelial cells, with concomitant degradation of extracellular matrix, and it is considered to be the most important proangiogenic signal in BAT (Asano et al., 1997; Bagchi et al., 2013). Endothelial cells stimulated by VEGF then secrete adipokines that in turn stimulate adipogenesis. This interaction supports coordinated adipogenesis and angiogenesis (Fukumura et al., 2003). Role of VEGF in protection of brown adipocytes from apoptosis has also been suggested earlier (Bagchi et al., 2013). It is also known from the literature that VEGF expression in brown adipocytes is controlled through β-adrenergic pathways (Asano et al., 1997).

Considering the results obtained for T4-treated group, BAT enlargement is synchronized with vascular remodelling that is necessary for maintaining adequate blood supply.

Increased blood flow is one of the most frequently mentioned peripheral effects of thyroid hormones and it is related to increased heart rate, cardiac contractility and output (Fazio et al., 2004). Blood flow can be roughly estimated via the erythrocytes found within blood vessels supplying some organ. In accordance with that, it seems possible that treatment with T4, but not with T3, slightly increases blood flow in BAT. However, it is also possible that erythrocytes are more abundant in BAT capillaries of T4-treated animals due to their retention in narrow capillary lumen, which creates artificial image of increased blood flow. Previous studies evaluating BAT blood flow did not observe any changes in T3-hyperthyroid rats, while effects of T4 were not studied (Rothwell and Stock, 1984).

In analyzing general histological alterations of BAT after treatment with T3 or T4, quantitative determination of main tissue components was of great help. Morphometric/stereological analysis has shown that relative abundance of multilocular adipocytes, unilocular adipocytes, connective tissue and blood vessels remained unchanged in both experimental groups. Taken together, it seems that current experiment did not last long enough to enable substantial alterations in arrangement of main tissue components.

Considering that noradrenaline is the principal factor triggering BAT thermogenesis, the estimation of the level of **innervation** may serve as indication of BAT activity or at least potential activity. Results presented in this work showed that T3 caused relative decrease of total nerve fibers length as result of decrease in nerves innervating both parenchyma and blood vessels. In similarly posted experiment, it was shown that treatment with T3 resulted in decrease of monoamine-oxidase activity (Petrovic et al., 2003). Monoamine-oxidase is catecholamine-degradative enzyme localized in the outer mitochondrial membrane. It is expressed in BAT and the level of its expression may serve as a measure of noradrenaline turnover (Barrand et al., 1984; Eisenhofer et al., 2004). Together, these results suggest that in T3-induced hyperthyroidism neural network and activity of sympathetic nervous system become reduced.

On the other hand, T4-treatment did not affect relative abundance of nerve fibers, meaning that density of nerve fibers followed the enlargement of BAT.

Thyroid hormones are important for normal neurogenesis, synaptogenesis, myelination, migration and plasticity during embryonal development of nervous system as well as during perinatal and adult period of life. In adults, thyroid hormone deficiency is related to some neurocognitive dysfunction and neurodegenerative diseases and it has been suggested that at least some of these problems may be caused by structural alterations resulted from thyroid hormones deficiency (reviewed by Remaud et al., 2014).

Effects of T3 on peripheral nerve growth have been investigated in context of regeneration after injury. These studies showed that T3, directly acting on injured neurons, lowered the degree of their apoptosis and promoted survival (Schenker et al., 2003). However, when it was given intraperitoneally in large doses, in order to reach high local concentration, T3 failed to produce any observable beneficial effect on nerve growth rate, probably because they were masked by adverse systemic effects of hyperthyroidism (Berenberg et al., 1977; Allpress and Pollock, 1986). While T3 effects on neuronal survival are mediated through thyroid receptors, this hormone may also promote outgrowth of new axons acting on close non-neuronal cells and stimulating them to synthesize neurotrophic factors (Barakat, 1999). Using these data for interpretation of results obtained from present experiment, it can be speculated that T3-induced hyperthyroidism did not produce visible effects on neural growth in BAT. On the other hand, owing to independent control of T3 levels in BAT, local conditions in T4-induced hyperthyroidism were more similar to those in situation when T3 directly acts on neural fibers. This local increase in T3 is sufficient to provide outgrowth of neural network that would follow BAT growth.

During development of BAT, **glycogen depositions** are present in adipoblasts, brown adipocyte precursors and brown adipocytes, sometimes in significant amount (Cinti 2001). Later during the life, glycogen/glucose serve for *de novo* lipogenesis and for generation of energy needed for adipocyte functions (Cannon and Nedergaard, 2004). Sudden and excessive accumulation of glycogen has been reported in brown adipocytes of rats in period of reacclimation after exposure to cold (Farkas et al., 1999). During reacclimation, increased level of insulin, as well as BAT glucose uptake and content of glycogen are strongly suggestive for glycogen synthesis (Jakus et al., 2008). Glycogen accumulation is particularly increased in BAT of cold-exposed rats on

carbohydrate-rich diet (Madar and Harel, 1991). Uptake of glucose in brown adipocytes is controlled by insulin-dependent pathway, during anabolic processes, or by insulin-independent pathway, during thermogenesis. Insulin-dependent pathway involves translocation of GLUT4 transporters from intracellular pool to plasma membrane (Huang and Czech, 2007; Zaid et al., 2008) while insulin-independent pathway involves β_3-mediated stimulation of GLUT1 synthesis and its translocation to the cell membrane (Olsen et al., 2014). The amount of glucose that can be uptaken in brown adipocytes from circulation can be significant and can improve glucose homeostasis and insulin sensitivity. Since BAT is able to consume high amount of glucose from circulation, it may affect the whole-body glucose homeostasis and improve insulin sensitivity. It has been additionally shown that this effect on glucose homeostasis is related to increase in BAT-derived IL-6 (Stanford et al., 2013).

In the present work, the pattern of glycogen accumulation in BAT was find to be slightly changed after both treatments (T3 and T4), compared to control animals: in control group PAS+ reaction was rare observation, in T3-group it was present quite frequently in multilocular cells, while in T4-group PAS-positive staining was detected in multilocular and paucilocular adipocytes as well as in immature brown adipocytes.

Response of tissues/organs on insulin action in hyperthyroid individuals is very complex and literature data are often contradictory. Studies conducted on human subjects with hyperthyroid disbalance showed impaired insulin secretion and reduced peripheral insulin sensitivity, resulting in glucose intolerance (Roubsanthisuk et al., 2006). On the other hand, Varnum et al. (1985) showed that in T4-hyperthyroid rats insulin secretion is decreased, insulin clearance is increased and insulin sensitivity is either normal or possibly enhanced. Results derived from the observational study based on the case report have suggested that levothyroxine, in TSH-supressive dose (for thyroid cancer therapy), may be involved in normalization of glucose metabolism in subject with severe hereditary impaired insulin signalling. It was hypothesized that this effect was achieved by activation of BAT and increase in insulin-independent glucose disposal in brown adipocytes (Skarulis et al., 2010).

Considering insulin-independent pathway of glucose uptake into brown adipocytes, linked to β_3-adrenergic stimulation, increased accumulation of glycogen in

T4 group may be related to increased sympathetic input in BAT, which in turn was in accordance with findings of López et al. (2010).

In the light of the fact that presence of glycogen in brown adipocytes is associated with process of lipogenesis (Cinti, 2001), its accumulation in T3-treated animals may be related to preparing of brown adipocytes for thermogenesis, while in T4 it may reflects preparing and/or activation.

The particularly increased PAS-positivity in immature brown adipocytes in T4 group was also in line with data on increased glycogen accumulation during development and maturation of brown adipocytes (Cinti, 2001).

Although previously proposed role for BAT in regulation of blood glucose by increased glycogen accumulation is attractive, its significance in this experimental model of systemic hyperthyroidism needs more detailed investigations.

Analysis of the **UCP1 immunoexpression** pattern served to explore the effects of thyroid hormones on metabolic capacity of the BAT. Generally, pattern of UCP1 expression in control and both treated groups corresponded to that previously described as Harlequin effect (Cinti, et al., 2002) when neighbouring brown adipocytes alternate in the levels of UCP1 protein expression, giving the "patchy" appearance to the tissue. Harlequin effect was particularly pronounced in acutely stimulated BAT (cold exposure or administration of a specific β_3-adrenoceptor agonist). Using electron microscopy Cinti and co-workers showed that stronger UCP1-immunoreactivity in some brown adipocytes was due to the higher content of UCP1 protein in mitochondrial cristae. Interestingly, this group did not observed further differences between mitochondria with higher *vs*. lower UCP1 content. Also, they did not reveal ultrastructural differences between brown adipocytes with higher or lower UCP1 content.

In the present study enhanced UCP1 immunoreactivity was observed after treatments with both thyroid hormones. These results were somewhat unexpected since they only partially corresponded to those obtained in earlier study performed by Petrovic et al., 2003, using similar experimental conditions. In that study, increase in amount of UCP1 was recorded in both treated groups, but statistical significance was shown only after treatment with T3. These differences can be explained in part by the particular methodology used for determination, in connection with exact subcellular localization of UCP1. Namely, Petrovic et al. (2003) determined UCP1 content of

mitochondrial fraction using Western blot method, so only intramitochondrial UCP1 was taken into account. However, it has been known for a while that, in noradrenergically stimulated BAT, UCP1 may exist in cytoplasm of brown adipocytes as preformed protein not yet incorporated in the membrane of mitochondrial cristae (Puigserver et al., 1992). Thus, it is reasonably to assume that amount of immunohistochemically labelled UCP1 (comprising both mitochondrial and cytoplasmic pool) may be higher than amount of UCP1 present in mitochondria alone. Extramitochondrial pool of UCP1 in T4-treated group, which could not be determined by Western blot, thus may significantly contribute to total UCP1 content. This also points to possibly distinctive intracellular dynamics of UCP1 synthesis and insertion in mitochondrial cristae in systemic T3- *vs*. T4-hyperthyroid rats.

UCP1 immunopositivity in T3-treated group was sporadically detected in nuclei of brown adipocytes. This finding is intriguing and point to possible role of UCP1 in intracellular signalling.

Strong UCP1-immunoreactivity observed in a number of adipocytes with several large lipid bodies in both treated groups was one of the most interesting findings obtained in the present work. After treatment with T3, reaction was clearly visible in adipocytes located at the boundary between typical multilocular adipocytes (centrally positioned within interscapular fat pad) and unilocular adipocytes (at the periphery of interscapular fat pad). According to morphology, these adipocytes correspond to previously described paucilocular cells of adipose organ. These paucilocular adipocytes show morphological characteristics that are intermediate between brown and white adipocytes and express UCP1 or not, depending on stage of differentiation (Barbatelli et al., 1993; Barbateli et al., 2010; Cinti, 2012). It is generally accepted that described cells represent cells that are in process of transdifferentiation from unilocular to multilocular thermogenically competent adipocytes. Detected UCP1-immunopositivity in them after treatment with T3 suggests later stadium of that process and points to increase in the overall thermogenic capacity of BAT.

In T4-treated group immunopositivity for UCP1 is also located in typical multilocular adipocytes and in multilocular adipocytes with several large lipid bodies. However, in this group, individual paucilocular adipocytes are located centrally in the brown fat pad, surrounded by multilocular cells. Currently, it is not known whether this

spatial relationship between two types of adipocytes is specifically caused by experimental treatment or it depends on selection of particular tissue sample. However, regular occurrence of such positional patterning suggests the possibility that paucilocular and multilocular adipocytes establish some kind of cross-talk through paracrine mediators, similarly as it was proposed earlier in BAT of hypothyroid rats (Čakić-Milošević et al., 2013). Assuming that paucilocular UCP1-positive adipocytes represent cells in the process of transformation from white-like to brown-like phenotype then, according to Cinti (2012) they can express leptin concomitantly with expression of UCP1. Otherwise, UCP1 and leptin are normally inversely regulated in typical brown and white adipocytes: typical brown adipocyte is leptin-negative and UCP1-positive, while typical white adipocyte is leptin-positive and UCP1-negative (Cinti et al., 1997). Speaking highly hypothetically, leptin produced in paucilocular adipocytes may diffuse toward surrounding multilocular (brown) adipocytes affecting, in paracrine manner, glucose utilization, lipid metabolism and possibly some signalling pathways, as it was reported previously by Siegrist-Kaiser et al. (1997).

Immunolocalization of proliferative marker Ki67 was used in order to examine possible hyperplasia in BAT of T3- or T4-treated rats, but results were negative, as described herein above. Instead, unusual cytoplasmic localization of Ki67 was detected in adipocytes from both hormone treated groups, particularly in paucilocular adipocytes.

Ki67 is nuclear protein expressed in all phases of cell cycle except G0 (Gerdes, 1990). It is widely used as marker of cell proliferation and important diagnostic and prognostic factor in different types of tumors (van Diest et al., 2004; Khan et al., 2013). Unusual pattern of membranous/cytoplasmic Ki67 expression in invasive breast carcinomas but also in normal heart tissue of rats during perinatal remodelling have also been described (Ciulla et al. 2009; Faratian et al., 2009). However, mechanisms underlying relocalization of Ki67 remained unexplained. Intracytoplasmic localization of Ki67 may correspond to a form of storage before translocation into nucleus, but also it can be considered as functional phenomenon reflecting the still unknown role of this protein.

In the present experiment ectopic localization of Ki67 was detected in regions of brown adipose tissue in which paucilocular cells predominate, mainly in paucilocular,

but also in some multilocular cells. Thus this result provide further support to the possibility that paucilocular adipocytes represent pool of adipocytes that are actively engaged in remodelling of BAT in animals treated with thyroid hormones. Further investigations will be necessary to explain the significance of intracytoplasmic localization of Ki67 protein in adipocytes as well as in other cell types.

Immunodetection and localization of TNFα served to explore at least in part, possible influence of applied treatments on some signalling pathways established and maintained between cells in BAT. As it is well known from the previous reports, TNFα belongs to the group of cytokines, pharmacologically active proteins of low molecular weight that affect cell functions in an autocrine or paracrine manner (McDermott, 2001). Initially, TNFα was recognized as a circulating factor displaying antitumor activity (Carswell et al., 1975) but its high proinflammatory activity also became evident (Shalaby et al., 1989). It is synthesized as transmembrane protein of 26 kDa (membrane form) and can be cleaved off and released from the cell surface by the activity of TNF*α*-converting enzyme. Released, soluble form (17 kDa) exerts its biological actions through TNF-receptors type 1 and 2 (TNF-R1, TNF-R2) coexpressed on almost all nucleated cells but in different proportions, with TNF-R2 being preferentially expressed on immune cells (Vandenabeele et al., 1995; Black et al., 1997). TNF*α* provides the defence from infections induced by microorganisms and parasites but its overproduction may be harmful for the host (for review see Bradley, 2008). Beside common role in controlling apoptotic cell death in target cells, it has been shown that TNF*α* affects intermediary metabolism regulatory pathways. Thus, several reports have shown that TNF*α* impairs lipid metabolism, glucose tolerance and insulin sensitivity (Grunfeld et al., 1991; Hotamisligil et al., 1993). In inflammation, it increases triglyceride and glucose content in serum thus providing excess of nutrients and facilitating their uptake in cells with increased metabolic needs (Popa et al., 2007).

TNF*α* is the best studied cytokine produced by cells present in adipose tissue, mainly adipocytes themselves. Prior studies regarding the expression of TNF*α* in adipose tissue were conducted mostly on WAT, while very little was found in the literature on the question of TNF*α* synthesis and action in BAT. Thus, it is well known that white adipocytes express both forms of TNF-receptors of which TNF-R1 are involved in apoptotic and lipolytic effects, while TNF-R2 signalization is related to

induction of mitogen-associated protein-kinases and insulin resistance (Hube and Hauner, 1999; Sethi et al., 2000). Although there exists an agreement that TNFα can modulate thermogenic capacity of BAT, link between this cytokine and BAT is still not completely understood. While some studies report that TNFα positively regulates BAT thermogenic activity by stimulating sympathetic outflow in BAT and UCP1 expression, the others suggest that it compromises BAT function promoting apoptosis of brown adipocytes and BAT atrophy (Coombes et al., 1987; Nisoli et al., 1997). These discrepancies could be attributed to (a) different experimental models, (b) different types and amounts of mediators present in the tissue in the same time, which can modulate action of TNFα, and (c) coordinated actions of two TNFα receptor types in mediating signal transduction.

The current study revealed that typical multilocular brown adipocytes are TNFα-producing cells. In hyperthyroidism induced by T4, TNFα-immunoexpression is increased, more cells are involved, and intensity of reaction was stronger in comparison to T3- and, particularly, control group. This finding suggests that two thyroid hormones differentially affect synthesis of TNFα in brown adipocytes. It has also been demonstrated previously that TNFα may be directly delivered into mitochondria and interact with receptor protein in the inner mitochondrial membrane which is related to TNF-R2 (Ledgerwood et al., 1998). The fact that mitochondrial morphological and biochemical abnormalities occurring from such interaction might be but are not necessarily related to autophagy and TNFα-induced cell death, points to complex relationship that exists between mitochondrial damage and cytotoxic effects on cell. In accordance with these findings, the possibility that TNFα may affect BAT thermogenesis acting directly on mitochondria (but through some other receptor protein) can not be completely excluded. However, more research on this topic needs to be undertaken to support or dismiss this assumption.

Apoptosis of brown adipocytes thus remains the best documented effect induced by TNFα. However, the results obtained from this study did not demonstrate any increase of apoptotic events after hormonal treatments in comparison to control group so TNFα immunopositivity, especially that in T4-treated group can not be related to induction of apoptosis. If one takes into account proposed involvement of BAT in thyroid thermogenesis discussed herein above, it is possible that TNFα plays a role in

the regulation of thyroid thermogenesis rather than induces apoptosis of brown adipocytes and BAT atrophy. This regulatory role may be linked to lipid metabolism and UCP1 expression.

As revealed both by light and electron microscopy, the most remarkable alteration of brown adipocytes after treatment with thyroid hormones were noted at the level of lipid content. Namely, lipid content (verified through measuring the profile area) in brown adipocytes of rats from both treated group was visibly increased, which was clearly confirmed by quantitative analysis. Moreover, significant enlargement of brown adipocytes in T3-treated rats can be attributed exclusively to intracellular lipid accumulation since profile areas of all other subcellular fractions remained unchanged but their relative amounts were decreased. These results on lipid accumulation after T3-teatment are generally in agreement with those obtained in earlier studies (Masini et al., 1990) which showed that administration of T3 in supraphysiological doses caused increased triglyceride content of BAT. In contrast to earlier findings, however, lipid accumulation in current study was already evident after five days of treatment, while in aforementioned study it was determined as late event (seventh day and later), even in spite of higher T3 doses applied. Although not essential, this difference in lipid accumulation dynamics may partly be explained by differences in activities of some enzymes involved in lipid metabolism between Sprague-Dawley and Wistar rat strains (Galan et al., 1994) which were used in these two experimental models.

The effect of T4 on lipid accumulation in brown adipocytes was comparable to that of T3: quantitatively, lipid content was increased in comparison to control group. However, there are some important differences in form of lipid stores in two hormone treated groups. Whereas in T3-treated group number of lipid bodies per cell was reduced in comparison to control, in T4 group multilocularity was well preserved despite its energy consuming nature. Multilocularity of brown adipocytes is generally thought to be related to the lipid breakdown and utilization when tissue is thermogenically active. However, multilocularity also can be associated with initial stages of lipogenesis which starts as multifocal process. In any case, multilocular aspect of brown adipocyte is indication of lipid turnover (Girardier, 1983) and its reduction may be considered as a sign of functional silencing.

Another issue related to increased lipid content in T4 group is unexpected lack of brown adipocyte enlargement on the account of lipid accumulation, noted after treatment with T3. An explanation for this may be simply technical due to limitation in selection of electron micrographs for stereological analysis. Namely, maximal size of cell profiles picked for quantitative analysis was limited to the size of view field at predetermined magnification used. As a consequence, many large multilocular cells, present in tissue section were excluded from the stereological analysis. On the other hand, newly differentiated brown adipocytes displaying all ultrastructural characteristics of mature cells, present in BAT of T4-treated rats, were included in the study although they possibly did not reach maximal size of fully mature cell.

Analysis of lipid bodies' distribution in class ranges additionally points to attenuated thermogenic processes in brown adipocytes from rats treated with T3, since lipid bodies from this group dominate in higher size ranges. Distributions of lipid bodies from control and T4-treated groups in class ranges are similar to each other. Considering increased lipid accumulation in T4 group, it could be argued that in this group multilocularization of brown adipocytes is slightly favoured.

Thermogenic function of BAT is directly based on highly specific **mitochondria** of brown adipocytes. Mitochondria of typical brown adipocyte have a number of ultrastructural and biochemical specificities that can be changed in adaptable manner upon stimulation of BAT thermogenic activity.

One of the mitochondrial alterations observed after treatment with thyroid hormones is the occurrence of giant mitochondria (megamitochondria) in some brown adipocytes in both T3- and T4-treated groups.

Phenomenon of giant mitochondria is well known from the literature. They were previously described in different cell types, in pathological situations or during aging (Coleman et al., 1987; Kanzaki et al., 2010; Sano et al., 2010). The detailed mechanisms of giant mitochondria formation and their impact on cell metabolism still remain to be elucidated. However, some evidences point to free radicals as crucial actors in formation of giant mitochondria. According to this assumption, formation of giant mitochondria is structurally and functionally reversible process of adaptation at the level of intracellular organelles by which cell tries to decrease production of reactive oxygen species (Wakabayashi, 2002). Given that production of reactive oxygen species can be

increased in hyperthyroid state, this explanation might be acceptable for giant mitochondria occurrence in experimental groups. The presence of giant mitochondria does not seem to be linked to some serious functional impairment at least as judged by morphological criteria. These results must be interpreted with caution because treatment was relatively short.

Another interesting finding of this study is mitochondrial pleomorphism occurred in both treated groups, which is more pronounced after treatment with T4. As it was postulated at the beginning of the 20th century and confirmed few decades ago, mitochondria do not exist in cell as isolated organelles floating in cytoplasm. On the contrary, they form intracellular network that change its shape by continual divisions and fusions of mitochondria, even in resting cells (Bereiter-Hahn and Voth, 1994). Mitochondria seen under electron microscope are in fact temporary fraction of the whole cellular chondrioma. Actual size, shape and number of mitochondria in one cell at the moment is dynamical function of fusion and fission events and represent the response of chondrioma on current requirements. In addition, mitochondria are able to relocate inside the cell, through interactions with cytoskeletal elements (Bereiter-Hahn et al., 2008). Mitochondrial fusion and fission are mediated by proteins that are members of dynamin protein family and are localized in inner and/or outer mitochondrial membrane, with the participation of some adaptor proteins, endoplasmic reticulum and cytoskeletal components (for review, see van der Blieck et al., 2013).

Wikstrom et al. (2014) showed that noradrenergic stimulation of brown adipocytes, both in culture and in BAT of mice transferred from thermoneutrality to cold environment, results in mitochondrial fragmentation with concurrent inhibition of fusion events, thus shifting dynamic balance between these two interrelated processes. They concluded that fragmentation represent a physiological response of mitochondria associated with enhancement of mitochondrial uncoupling. Mitochondrial fragmentation is required for thermogenic activation and control of energy expenditure in brown adipocytes.

Results obtained in present study showed that pleomorphism of mitochondria observed after treatment with T3 and particularly T4 was not associated with substantial changes in any of quantitative parameters related to mitochondria (unchanged mitochondrial profile area and number). Interpreting these results in the light of those

reported by Wikstrom et al. (2014) it is likely that conditions used in current experiment induced reorganisation of existing chondrioma, without biogenesis of new mitochondria. If previously demonstrated increased sympathetic activity in T4 treatment (López et al. 2010) is taken into account, the lack of expected mitochondriogenesis may be explained by the increased expression of TNFα. Namely, Valerio et al. (2006) showed that TNFα impairs mitochondrial biogenesis in metabolically active tissues including BAT. However, with a short experimental period caution must be applied, as the findings might be insufficiently conclusive regarding the effects of thyroid hormones on mitochondrial population in brown adipocytes.

After treatment with T4, the number of mitochondria establishing close contact with surface of lipid bodies was seemingly increased in the present study. This observation was supported by the results of morphometric analysis which showed that both percentage of mitochondrial perimeter in contact with lipid bodies and the percentage of lipid body perimeter in contact with mitochondria were increased.

Close topographic relation between mitochondria and lipid bodies in brown adipocytes is of functional importance and it was described after physiological stimulation of BAT thermogenesis (Yu et al., 2015). It reflects metabolic fuelling of mitochondria by fatty acids released from lipid bodies. Available evidences also show that lipid body coat proteins interact with enzymes of the lipolytic cascade, modulating fatty acid efflux from the lipid body to mitochondria. One of such proteins is perilipin 5, highly expressed in metabolically active tissues including BAT (Wolins et al., 2006). This protein is involved in regulation of fatty acid flux from lipid body to mitochondria and it protects mitochondria from excessive exposure to fatty acids (Aon et al., 2014).

Considering mitochondria-to-lipid body contacts in T3-treated animals, stereological analysis showed the increase in percentage of lipid body perimeter in contact with mitochondria. However, since number of lipid bodies in this group is reduced, their total surface available to interact with mitochondria is diminished, so mitochondria-to-lipid body contact at best remained the same. Thus, it can be suggested that fatty acid flux into mitochondria is not as intensive in T3 group as it is after treatment with T4. Moreover, intramitochondrial lipid inclusions, although as rare observation, may indicate that utilization of fatty acids as thermogenic substrate is not fully efficient.

In summary, results presented in this study clearly demonstrate that two main thyroid hormones, T3 and T4, applied separately, in supraphysiological doses, affect histological characteristics of BAT and change ultrastructural features of brown adipocytes. Their effects on some structural aspects are similar, but in some cases clear distinctions were noted.

Thyroid hormones exert positive effects on thermogenic capacity of BAT and brown adipocytes, which is manifested by tissue growth, increased expression of UCP1 and recruitment of new thermogenically capable multilocular adipocytes derived from unilocular/paucilocular adipocytes.

Based on the presented results, effects of T4 are more pronounced and are not related only to increase in thermogenic potential of BAT and brown adipocytes, but also possibly to induction of thermogenic activity. These effects probably depend on the activity of BAT deiodinase 2 (within non-blocking ranges of T4 concentration) in conjunction with differences in the affinity of thyroid hormone receptor types α and β for T3.

This dissertation opened many new questions and more research needs to be undertaken before the role of BAT in thyroid thermogenesis is more clearly understood.

6. Conclusions

- Treatment with thyroid hormones (T3 in dose of 200 μg/kg b.w. dissolved in 9 mM NaOH, *i.p.* or T4 in dose of 300 μg/kg b.w. dissolved in 9 mM NaOH, *i.p.* during 5 days) significantly affected levels of T3 and T4 in circulation. Structurally, thyroid gland was subjected to involutive changes. Observed changes are most probably based on the absence of cytoprotective effects of TSH, together with reception of more pronounced proapoptotic signals.

- Treatment with thyroid hormones caused significant reduction in body mass gain which is consistent with systemic hyperthyroid state.

- Absolute and relative masses of BAT were significantly increased, on the basis of hypertophic and hyperplastic processes in BAT.

- Growth of BAT in T4-treated animals was synchronised with remodelling of vascular and neural networks.

- Experimental treatment did not promote substantial rearrangement of main tissue components, probably due to short duration of experimental treatment.

- As judged by enhanced UCP1 immunoreactivity observed in both treated groups, thyroid hormones increased thermogenic capacity of BAT.

- Recruitment of inducible adipocytes contributed to increase in thermogenic potential of BAT.

- Thyroid hormones differently affected pattern of lipid accumulation in brown adipocytes. While in T4-treated animals multilocularity was preserved, after treatment with T3 number of lipid bodies per cell was reduced.

- Both treatments affected mitochondrial dynamics. This was manifested by the occurrence of mitochondria displaying unusual size and shape. Some of these changes might be associated with protection from reactive oxygen species, more extensively produced in hyperthyroid state.

- After treatment with T4 mitochondria established more intensive contacts with surface of lipid bodies than it was noted in control and T3-treated animals. This topographic relation between mitochondria and lipid bodies in brown adipocytes is of functional importance and it reflects metabolic fuelling of mitochondria by fatty acids released from lipid bodies.

Systemic hyperthyroidism induced by thyroid hormones (T3 or T4) positively affected thermogenic capacity of BAT, stimulating its growth, UCP1 expression and recruitment of new thermogenically capable adipocytes. Effects of systemic hyperthyroidism induced by T4 were more pronounced and were probably associated with slightly increased thermogenesis.

7. Literature

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Biography

Mr Njia Milad. A. Rajab was born in Abyar, Libya, on December 16/1974. She started her Bachelor studies in 1993-1994 at the Faculty of Science, Al-Mergeb University, Al-Khums, where she graduated in 1996-1997.

From 1998-2000 mr Njia Milad A. Rajab worked as a Demonstrator of biology of Al-Mergeb University, where she taught the following courses: Natural history, Anatomy and physiology, Histology, General biology.

In 2001 she started Master studies at the Department of Biological Science, Comparative Anatomy at the Faculty of Science, Al-Khums, University of Al-Mergeb, Libya and graduated in 2007.

From 2007 to 2008 she worked as Assistant lecturer at Al-Khums University of Al-Mergeb, Libya.

Прилог 1.

Изјава о ауторству

Потписани-а mr Njia Milad. A. Rajab

број индекса -

Изјављујем

да је докторска дисертација под насловом

Хистолошке и ултраструктурне промене интерскапуларног мрког масног ткива пацова у експериментално индукованом системском хипертироидизму

(Histological and ultrastructural alterations of rat interscapular brown adipose tissue in experimentally induced systemic hyperthyroidism)

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
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- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, 30. 10. 2015.

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора mr Njia Milad Ali Rajab

Број индекса -

Студијски програм Биологија; Биологија ћелија и ткива

Наслов рада Хистолошке и ултраструктурне промене интерскапуларног мрког масног ткива пацова у експериментално индукованом системском хипертироидизму

(Histological and ultrastructural alterations of rat interscapular brown adipose tissue in experimentally induced systemic hyperthyroidism)

Ментор др Маја Чакић-Милошевић

Потписани/а mr Njia Milad Ali Rajab

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**
Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.
Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, 30. 10. 2015.

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:
Хистолошке и ултраструктурне промене интерскапуларног мрког масног ткива пацова у експериментално индукованом системском хипертироидизму

(Histological and ultrastructural alterations of rat interscapular brown adipose tissue in experimentally induced systemic hyperthyroidism), која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.
Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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