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Research article

MATERNAL SUBCLINICAL AND CLINICAL HYPOTHYROIDISM EFFECTS ON RAT OFFSPRING: A STORY OF THE SKIN AND ITS DERIVATIVES

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Epidermis stem cells have a crucial role through the processes of proliferation and differentiation, to replace cells that are constantly lost during tissue turnover or following injury. On the other hand, thyroid hormones regulate the proliferation and differentiation of epidermal cells and thus significantly influence the homeostasis of the skin. It is well known that maternal hypothyroidism during pregnancy leads to impaired development of many organ systems in their offspring. However, there is a lack of data about the influence of maternal subclinical hypothyroidism during pregnancy and lactation on the development of the skin and its derivatives in the litter. The aim of this study was to investigate the effects of maternal thyroid dysfunction on the development of the skin and its derivatives in their offspring in the early postnatal period. Antithyroid substance 6-n-propyl-2-thiouracil was added into the drinking water to female Albino Oxfords rats from the beginning of pregnancy and during lactation, with the aim to induce subclinical and overt form of hypothyroidism. Skin samples were taken from male pups within twenty-four hours and seven days after birth. The main findings of this investigation were that both forms of maternal hypothyroidism lead to serious damage of the epidermis in pups in terms of pronounced hyperkeratosis and reduction of the germinal layer along with a reduced number of hair follicles and their delayed morphogenesis. Epidermal impairments were more pronounced in pups with the overt form of hypothyroidism while offspring with the subclinical form had impairments that were less pronounced and delayed in occurrence.

Keywords: apoptosis, hair follicle bulge, pluripotency, proliferation, regeneration, Nanog

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INTRODUCTION

The skin epidermis protects animals against major environmental stresses and has the remarkable ability to enrich the body surface with structures such as hair follicles, claws, sebaceous and sweat glands in mammals or scales and feathers in lower vertebrates [1].

The skin epidermis and its array of appendages undergo ongoing renewal, which is a key feature of skin tissue homeostasis. Stem cells (SCs) in the epidermis have a crucial role in the maintenance of this important barrier through the processes of proliferation and differentiation. Interestingly, the basic mechanisms and signaling pathways that orchestrate epithelial morphogenesis are reused during adult life to regulate skin homeostasis [2]. Proliferating cell nuclear antigen (PCNA) is a frequently used marker for the detection of epidermal cells proliferation [3] and caspase-3 (Casp3) is a critical executioner of apoptosis, as it is either partially or totally responsible for the activation of many key proteins in the apoptosis pathway, via the mechanism of proteolytic cleavage [4]. Different SCs reside in specific niches such as interfollicular epidermis, hair follicles and sebaceous glands, that provide the appropriate microenvironment and molecular cues to preserve their proliferative and tissue regenerative potential [5]. In the hair follicles (HFs), SCs reside in a relatively quiescent state within an anatomically distinct region known as the bulge. Bulge cells express the embryonic stem cell transcription factor NANOG, which helps maintain the capacity of self-renewal and multipotency in SCs [6,7]. In adult rats, bulge SCs can contribute to all three epithelial lineages of the skin [8,9]. During normal homeostasis, bulge SCs are periodically activated to fuel postnatal hair cycles. Interfollicular epidermis (IFE) and sebaceous gland (SG) both contain resident populations of unipotent progenitor cells [10].

By binding to their receptors, thyroid hormones regulate the proliferation and differentiation of epidermal cells and thus significantly influence the homeostasis of this largest organ [11]. Maternal hypothyroidism down regulates thyroid hormone receptors in the offspring's skin [12], thus disabling triiodothyronine (T3) effects, as one of the main regulators of epidermal growth and differentiation [13]. Numerous studies have shown that the altered function of the maternal thyroid gland during pregnancy and lactation can affect the development of various fetal organs [14,15]. However, scarce research has been conducted on the effect of maternal thyroid hormones on the development of skin and its derivatives during prenatal and early postnatal development. In particular, there is a lack of data about the influence of subclinical hypothyroidism of mothers during pregnancy on the development of the skin and its derivatives in the litter. It has been documented that subclinical and clinical hypothyroidism in mothers during pregnancy and lactation leads to offspring subclinical and clinical hypothyroidism [15-17]. Clinical hypothyroidism in mothers is characterized by rough coat, prolonged pregnancy and small litter size [15,18,19]. In the pups, the overt form of hypothyroidism results in reduced body mass, lower thyroid hormone levels and higher thyroid index of activation [15].

The aim of this study was to investigate the effects of maternal thyroid dysfunction on the development of the skin and its derivatives in their offspring in the early postnatal period.

MATERIAL AND METHODS

Animals

Ten-week-old female Albino Oxford rats (weight 140-160g), obtained from the Department of Laboratory and Experimental Care and Use of Animals Unit of the Institute of Medical Research, Military Medical Academy (Belgrade, Serbia), and their male pups were used in this experiment. Only male pups were used because of small litter size, higher number of males and the fact that female pups were used for investigating effects of maternal hypothyroidism on ovary development [16,17]. The animals were kept under a photoperiodic cycle of 12 h light / 12 h dark in an air-conditioned facility. The mean temperature was 21 ± 0.5°C. Pelleted rat feed (PA 20, Veterinary institute Subotica) and drinking water were available *ad libitum*. The experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine University of Belgrade (decision number 01-20/6) and by the Ministry of agriculture, forestry and water management – veterinary administration (decision number 323-07-00364/2017-05/8).

Experimental procedure

Antithyroid substance 6-n-propyl-2-thiouracil (PTU) (Sigma Chemical Co., St. Louis, MO, SAD) dissolved in drinking water, was used for inducing subclinical (low dose 1,5 mg/l) and overt hypothyroidism (high dose 150 mg/l). Water with PTU was administered from the beginning of pregnancy and during lactation.

Female Albino Oxford rats had a 7-day adaptation period, after which vaginal smears were made every day to determine the phase of the sexual cycle. After proestrus or estrus phase was determined, the females were mated. The presence of sperm in vaginal smears after mating or presence of the mating plug were considered as gestational day zero. Does were then separated from males and randomized into three groups, each consisting of six animals: control group (C), low dose PTU group (PTU_{low}), and high dose PTU group (PTU_{high}).

From each experimental group, six male pups were euthanized within 24h of delivery (neonatal pups) by decapitation using surgical scissors. Seven days after delivery, six more male pups (early infantile pups) from each group were euthanized by cervical dislocation. Does were euthanized at the same time point by using 100 mg/kg body weight Euthasol Euthanasia Solution (Produlab Pharma Production B.V.Raamsdonksveer, Netherlands).

Tissue collection and processing for light microscopy and histochemistry

To avoid wrinkling of the skin during the fixation process, the carcasses were placed in 10% neutral buffered formalin for six hours, and thereafter the back skin was taken and placed in formalin for eighteen more hours, after which processing, and paraffin embedding were done by routine laboratory procedures. Serial sections (5 μm thickness) were made using a rotary microtome (Reichert, Wien, Austria). Sections were stained with haematoxylin/eosin (H/E) (Merck Millipore, Darmstadt, Germany) and Van Gieson staining method, to ensure better visualization of the keratinized layer of the epidermis [20]. The sections were then mounted using DPX (phthalate-free) mounting media (Fisher Scientific, Loughborough, UK).

For semi-thin sections, skin samples were fixed in cold 4% glutaraldehyde, followed by fixation in 1% buffered osmium tetroxide, dehydrated in acetone, embedded in Araldite, and 1 μm thick sections were stained with toluidine blue. Cytological characteristics of the epidermis and hair follicles were examined using a light microscope (Olympus CX31, Münster, Germany).

Immunohistochemistry

All immunohistochemical analysis was done according to the following procedure: Antigen unmasking was performed with 0,1M citrate buffer (pH 6) in a microwave for twenty minutes. Inactivation of endogenous peroxidase was achieved with 3% H_2O_2 for 10 minutes. Sections were then washed in PBS and blocked using a protein block (5% goat serum) for 10 minutes. After the wash in PBS, sections were incubated with primary antibodies (Table 1) diluted in IHC Diluent (Novocastra, Leica Biosystems, Newcastle, UK) overnight at 4°C. For negative controls, primary antibodies were omitted. Primary antibodies signal amplification and visualization were performed using EnVision FLEX/HRP (RTU, Dako, Santa Clara, CA, USA) followed by Liquid DAB+ Substrate Chromogen System (Dako, Carpinteria, CA, USA). Counterstain was carried out with Mayer's haematoxylin.

Table 1. Primary antibodies used for immunohistochemistry

Antibodies	Manufacturer	Dilution	Role
PCNA (FL-261) (sc-7907)	*Santa Cruz, Biotech, USA*	1:1000	marker of cell proliferation
Cleaved Caspase-3 (Asp175) (5A1E) #9664	*Cell Signalling Technology, USA*	1:300	marker of cell apoptosis
NANOG (PA5-20889)	*Thermo Fisher Scientific, USA*	1:300	marker of stem cell

Histomorphometry and stereology

Histomorphometry and stereological analyses were done on serial sections using a microscope equipped with a digital camera and adequate software (Olympus CX31

with UC50 Soft Imaging Solutions camera and SensEntry 1.13 software, Münster, Germany).

The thickness of the non-cornified layer of the epidermis was measured using a vertical line drawn from the basal cell layer to the end of the granular layer of the epidermis. The thickness of the cornified layer was measured using a vertical line drawn through the cornified layer. These measurements were performed on three different sections, on 20 fields of view per section.

Stereological analysis of the dermis was done using a multipurpose stereological grid M42 [21]. The number of view fields per section was determined using a formula as reported by Kališnik [22]. The volume density of hair follicles (V_{vdf}) was calculated using the formula: $V_{vdf}=P_f/P_t$ (P_f- number of testing points hitting the hair follicle; P_t-total number of testing points in the multipurpose stereological grid M42). V_{vdf} is expressed as a percentage of hair follicles in relation to the entire volume of the dermis.

The number of PCNA-positive ($PCNA^+$) cells was expressed per $1mm^2$ of the epidermis area. The number of $PCNA^+$ cells, as well as Casp3-positive ($Casp3^+$) was expressed per $1mm^2$ of hair follicle area. The number of $Casp3^+$ hair follicles was also determined and expressed per $1mm^2$ of dermis area.

The number of NANOG-positive ($NANOG^+$) hair follicles was determined and expressed per $1mm^2$ of dermis area. The count of multipotent SCs which were $NANOG^+$ was determined and expressed per $1mm^2$ of hair follicle area.

All counting of primary antibody positive cells was performed on five different sections, on 10 fields of view per section.

Statistical analysis

Two independent researchers reviewed all slides twice for morphological and stereological analysis and three times for immunohistochemical evaluations. Values above and below the confidence interval (5-95%) were excluded from further processing of results. To determine statistically significant differences between the examined groups, within the framework of the general linear model, analysis of variance for repeated measurements was used. Results were expressed as mean ± standard deviation. Levels of significance bellow 0.05 were considerd significant.

RESULTS

Histology, morphometry and stereology

The skin sections of neonatal pups (T0) showed a formed epidermis and dermis with HFs (Fig.1A-F). Epidermis of C-T0 had a clear boundary between the epithelium and the dermis, a layer of basophilic basal keratinocytes was formed. Stratum spinosum was also formed and spines with desmosomes were observed between the polygonal

cells, and between the polygonal cells and the cells of the basal layer. In the stratum granulosum, the cells became flattened with many osmium-positive granules and keratohyalin granules. Upwards to the stratum corneum, as a result of terminal differentiation, the cells became irregularly shaped, and thinner, so that in the stratum corneum they became dehydrated corned coots (Fig.1G). In comparison with the C-T0 group, the thickness of non-cornified layers of the epidermis was not altered in PTU_{low}-T0 group (Table 2; Fig.1D, E), while there was a statistically significant increase in the thickness of the stratum corneum (Table 3; Fig.1D, E). The thickness of non-cornified layers was reduced, while stratum corneum was thicker in PTU_{high}-T0 group, compared to the same epidermal layers of the pups from the control group (Table 2 and 3, Fig.1D, F). Compared to the control, both treated groups, did not have a clear dermoepidermal junction and keratinocytes of the basal and spinous layers were irregular in size and shape (Fig.1G-I). While a perinuclear halo zone was observed in some cells of the stratum spinosum in the control and PTU_{low}-T0 groups, in the PTU_{high}-T0 groups a perinuclear halo zone was present in the majority of cells in this layer of the epidermis (Fig.1G-I). Abundance of keratohyalin and osmium-positive granules were present in the cells of the stratum granulosum (Fig.1G-I). In the dermis of the pups from the PTU_{low}-T0 group, as well as PTU_{high}-T0 there was a reduced number of HF, compared to the C-T0 group (Fig.1A-C). All HFs of hypothyroid pups from both groups were positioned solely in the papillary layer of the dermis (Fig.1B, C), whereas HFs of the pups from the control group extended from the surface of the epidermis to the subcutaneous muscle (Fig.1A). The marked delay of HF morphogenesis was present in both treated groups. Stereological analysis of the dermis of hypothyroid pups from both PTU_{low}-T0 and PTU_{high}-T0 groups, showed a statistically significant reduction of the V_{vdf} of HFs. in comparison with the C-T0 group (Fig.1J).

Table 2. The thickness of the non-cornified layer of the epidermis in neonatal and 7-day-old pups

	Experimental groups			
	Control (n=6)	**PTU_{low} (n=6)**	**PTU_{high} (n=6)**	**P**
		T0		
Mean (μm)	47.378	45.933	25.306***	<0.001 ***
SD	2.494	0.886	1.367	
		T7		
Mean (μm)	48.124	47.254	28.972 ***	<0.001 ***
SD	1.406	1.1267	1.518	

Control - control pups; PTU_{low} - pups from does treated with low dose of PTU; PTU_{high}- pups from does treated with high dose of PTU; T0 - within 24h of delivery; T7-7-day-old; SD-standard deviation; n - number of animals per group; *P*- significant difference; ***group with significant difference p<0.001.

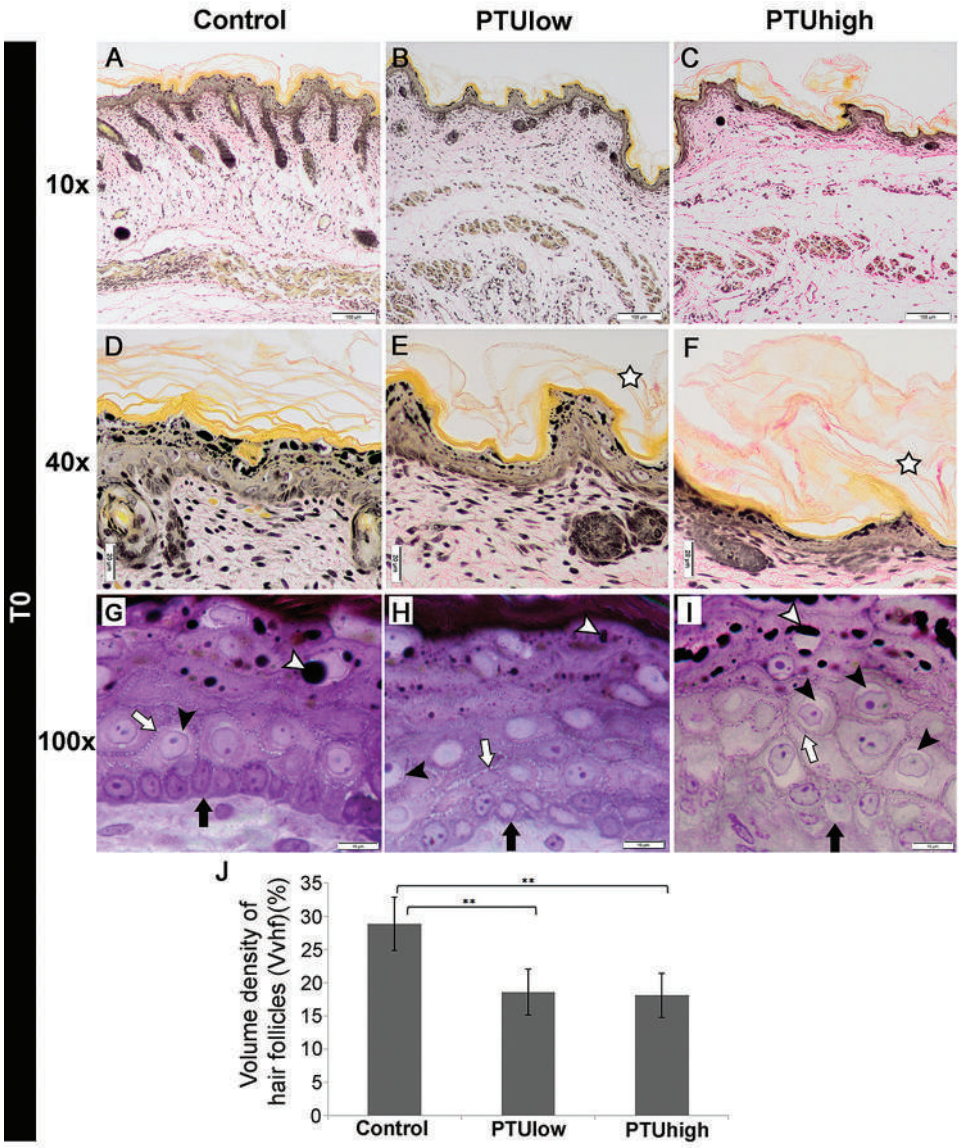

Figure 1. Representative photomicrographs of skin section and graph presenting mean ± standard deviation of stereological analysis in neonatal (within 24h of delivery) (T0) control pups (Control); pups from does treated with low PTU dose (PTU_{low}) and high PTU dose (PTU_{high}). Paraffin skin sections stained with Van Gieson staining method viewed with a low power (10x) (**A**, **B**, **C**) (bar: 100 μm) and a high power (40x) objective lens (**D, E, F**) (bar: 20 μm). Epidermis semithin araldite section stained with toluidine blue viewed with oil immersion (100x) objective lens (**G**, **H**, **I**) (bar: 10 μm) (black arrow - dermoepidermal junction; white arrow – desmosome; black arrow head - perinuclear halo; white arrowhead - keratohyalin and osmium positive granules; white star - hyperthickening of stratum corneum). Volume density of hair follicles (V_{vhf}) (**J**). (**$p<0.01$).

Table 3. The thickness of the stratum corneum in neonatal and 7-day-old pups

Experimental groups				
	Control (n=6)	**PTUlow (n=6)**	**PTUhigh (n=6)**	**P**
		T0		
Mean (μm)	22.392	24.024*	27.929***	<0.05* <0.001***
SD	1.215	0.917	0.788	
		T7		
Mean (μm)	53.103	54.460	61.544***	<0.001***
SD	1.260	0.914	0.556	

Control - control pups; PTU_{low} - pups from does treated with low dose of PTU; PTU_{high}- pups from does treated with high dose of PTU; T0 - within 24h of delivery; T7-7-day-old; SD-standard deviation; n - number of animals per group; *P*- significant difference; *group with significant difference p<0.05; ***groups with significant difference p<0.001

The skin sections of early infantile pups (T7) also showed a formed epidermis and dermis with HFs that are in the later stages of morphogenesis (Fig.2A-F). In PTU_{high}-T7 group there was a significant reduction in thickness of non-cornified layers of the epidermis, with an increase in thickness of the stratum corneum in comparison with both C-T7 and PTU_{low}-T7 groups (Table 2 and 3; Fig.2D-F). The epidermis of C-T7 had normal stratified organization of keratinocytes, like newborn controls (Fig.2G), while in the PTU_{low}-T7 group keratinocytes in the stratum basale were hypertrophic, irregular in shape, with a halo zone around the nucleus (Fig.2H). Towards the surface, keratinocytes, with a blebbing membrane and irregular nuclear envelope in stratum spinosum, became flattened and accumulated keratohyalin and osmium-positive granules in the stratum granulosum (Fig.2H). Flattening of stratum basale, a marked decrease in the number of layers of the stratum spinosum with altered morphology of keratinocytes and hyperthickening of stratum corneum are characteristics of PTU_{high}-T7 group (Fig.2I).

In the dermis of the pups from PTU_{low}-T7 group the number of HFs was reduced, and the pups from the PTU_{high}-T7 group had a significantly reduced number of HFs in comparison with both C-T7 and PTU_{low}-T7 groups (Fig.2A-C). Statistically significant reduction of the V_{vdf} was present in both PTU_{low}-T7 and PTU_{high}-T7 groups in comparison with the C-T7 group. There was also a statistically significant reduction of the V_{vdf} present in the PTU_{high}-T7 group in comparison with the PTU_{low}-T7 group (Fig.2J).

Immunohistochemistry

The proliferative activity of keratinocytes was determined based on the number of $PCNA^+$ cells in the germinative layer of the epidermis (Fig.3A-F). Proliferative activity in both PTU_{low}-T0 and PTU_{high}-T0 groups was not decreased in comparison with the

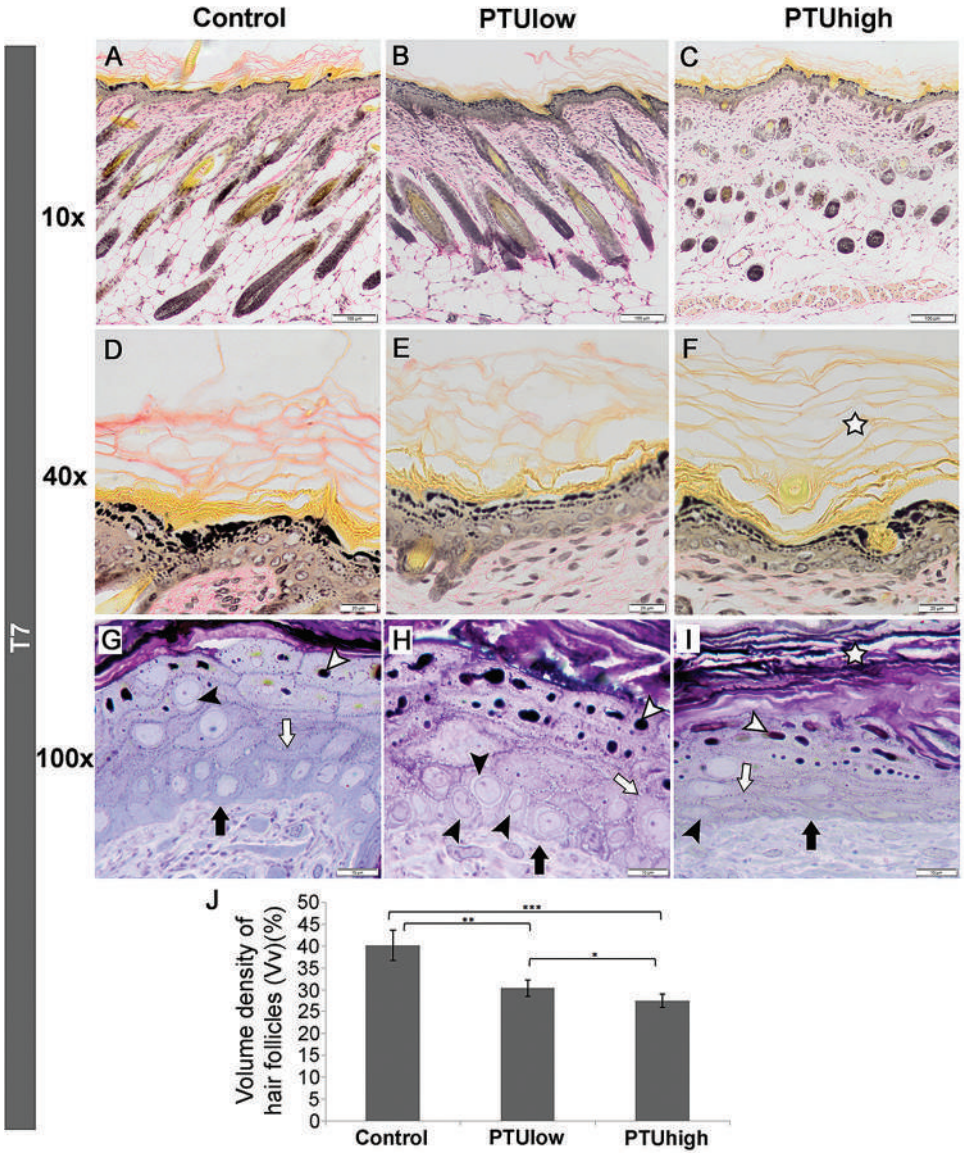

Figure 2. Representative photomicrographs of skin section and graph presenting mean ± standard deviation of stereological analysis in early infantile (7-day-old) (T7) control pups (Control); pups from does treated with low PTU dose (PTU_{low}) and high PTU dose (PTU_{high}). Paraffin skin sections stained with Van Gieson staining method viewed with a low power (10x) (**A**, **B**, **C**) (bar: 100 μm) and a high power (40x) objective lens (**D, E, F**) (bar: 20 μm). Epidermis semithin araldite section stained with toluidine blue viewed with oil immersion (100x) objective lens (**G**, **H**, **I**) (bar: 10 μm) (black arrow - dermoepidermal junction; white arrow - desmosome; black arrow head - perinuclear halo; white arrowhead - keratohyalin and osmium positive granules; white star - hyperthickening of stratum corneum). Volume density of hair follicles (V_{vhf}) (**J**). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

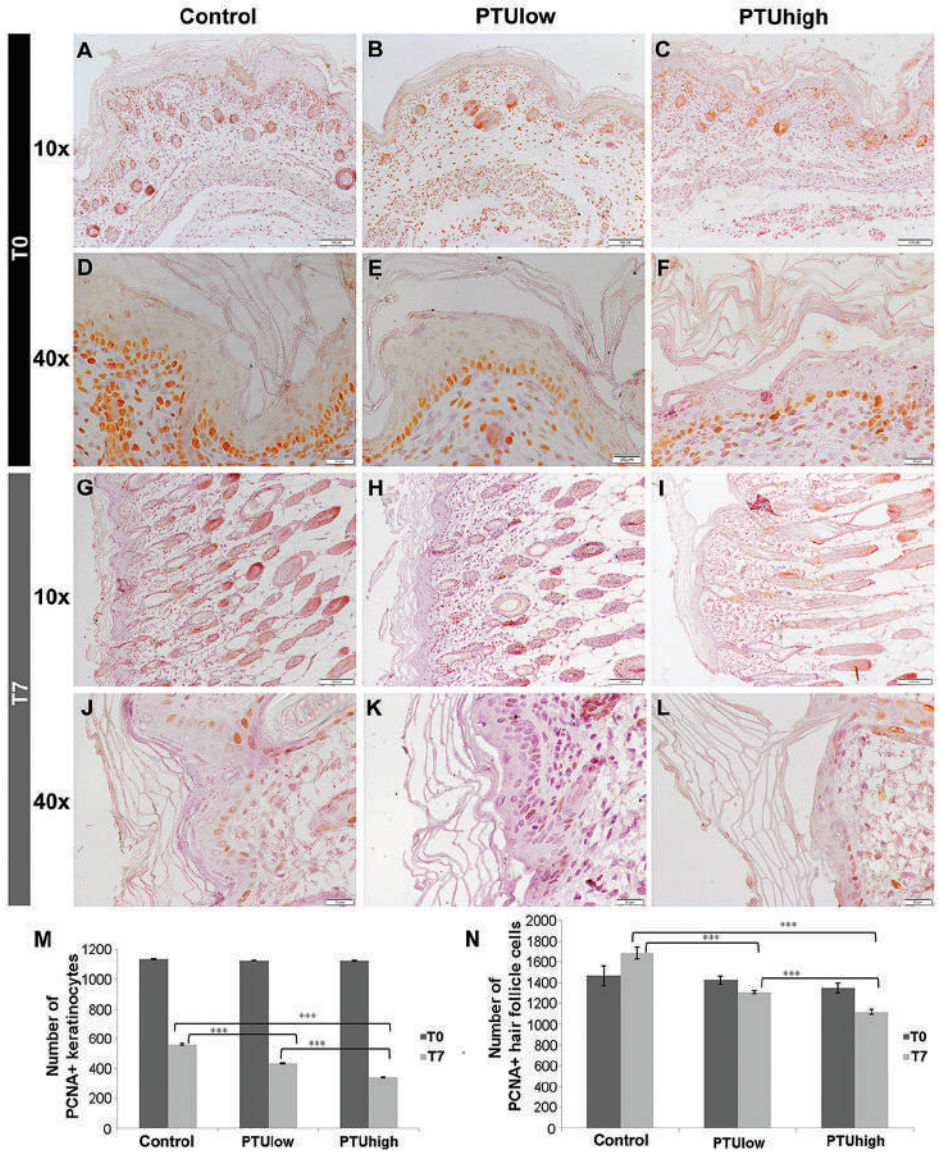

Figure 3. Representative photomicrographs of PCNA immunoexpression in neonatal (within 24h of delivery) (T0) and early infantile (7-day-old) (T7) control pups (Control); pups from does treated with low PTU dose (PTU_{low}) and high PTU dose (PTU_{high}). Immunoexpression of PCNA on paraffin skin sections of control (**A**), PTU_{low} (**B**) and PTU_{high} (**C**) T0 pups viewed with a low power (10x) (bar: 100 μm) and a high power (40x) objective lens (**D, E, F**) (bar: 20 μm). Immunoexpression of PCNA on paraffin skin sections of control (**G**), PTU_{low} (**H**) and PTU_{high} (**I**) T7 pups viewed with a low power (10x) objective lens (bar: 100 μm). The square with dashed lines represents the regions of the epidermis viewed with high power (40x) objective lens, which are shown in appropriate black bordered pictures (**J**, **K**, **L**) (bar: 20 μm) (3,3'-diaminobenzidine, counterstain haematoxylin). Graphs presenting mean ± standard deviation of number of PCNA positive keratinocytes (**M**) and hair follicle cells (**N**). (***$p<0.001$).

C-T0 group (Fig.3D-F, M). In the T7 pups there was a statistically significant decrease in the number of $PCNA^+$ cells in the germinative layer of the epidermis in both PTU_{low}-T7 and PTU_{high}-T7 groups in comparison with the C-T7 group (Fig.3J-M). Also, the number of $PCNA^+$ cells of the epidermis was decreased in the PTU_{high}-T7 group in comparison with the PTU_{low}-T7 group (Fig.3K-M).

The number of $PCNA^+$ cells in the HF of the control group pups was increasing with the pups' age (Fig.3A, G). There was no significant decrease in the number of $PCNA^+$ cells of the HF in both PTU_{low}-T0 and PTU_{high}-T0 groups in comparison with the C-T0 group (Fig3A-C, N). Still, in T7 pups from both treated groups the decrease in the number of $PCNA^+$ cells of the HF were statistically significant between those experimental groups, as well as in relation to the C-T7 group (Fig.3G-I, N).

The expression of Casp3 was not detected in HF of T0 experimental groups (Fig.4A-C). However, in treated groups PTU_{low}-T7 and PTU_{high}-T7 the number of

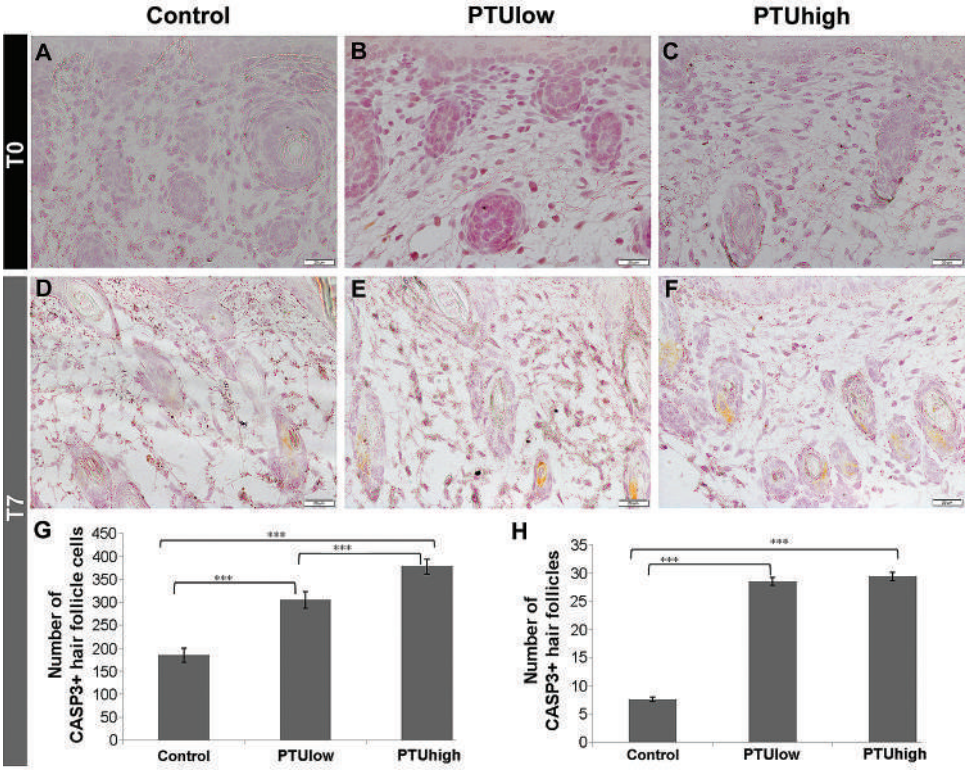

Figure 4. Representative photomicrographs of skin Caspase 3 immunoexpression in neonatal (within 24h of delivery) (T0) control pups (Control) (**A**); pups from does treated with low PTU dose (PTU_{low}) (**B**) and high PTU dose (PTU_{high}) (**C**) and early infantile pups (7-day-old) (T7) from control (**D**), PTUlow (**E**) and PTU_{high} (**F**) group. All photomicrographs viewed with high power (40x) objective lens (bar: 20 μm) (3,3'-diaminobenzidine, counterstain haematoxylin). Graphs presenting mean ± standard deviation of number of Caspase 3 positive hair follicle cells (**G**) and hair follicles (**H**). (***$p<0.001$).

Casp3$^+$ cell in the external hair sheet or in the associated sebaceus glands and the number of Casp3$^+$ HF per 1mm^2 of the dermis area were significantly increased in comparison to the C-T7 group (Fig.4D-H).

The overall number of HF in the dermis of the T0 and T7 pups from the PTU$_{low}$ and PTU$_{high}$ groups was decreased. However, the number of the NANOG$^+$ HFs was significantly increased in both treated T0 groups compared to the C groups (Fig.5A-C, G). The number of NANOG$^+$ HFs was also significantly increased in PTU$_{high}$T0 group, compared to the PTU$_{low}$T0 group (Fig.5B, C, G). Similar significant increases in number of NANOG+ HFs were observed in T7 treated groups (Fig.5D-G). Also, the expression of NANOG$^+$ HF bulge SCs significantly increased in treated groups of both age categories (Fig.5B, C, E-H), when compared to the C group (Fig.5A, D, H).

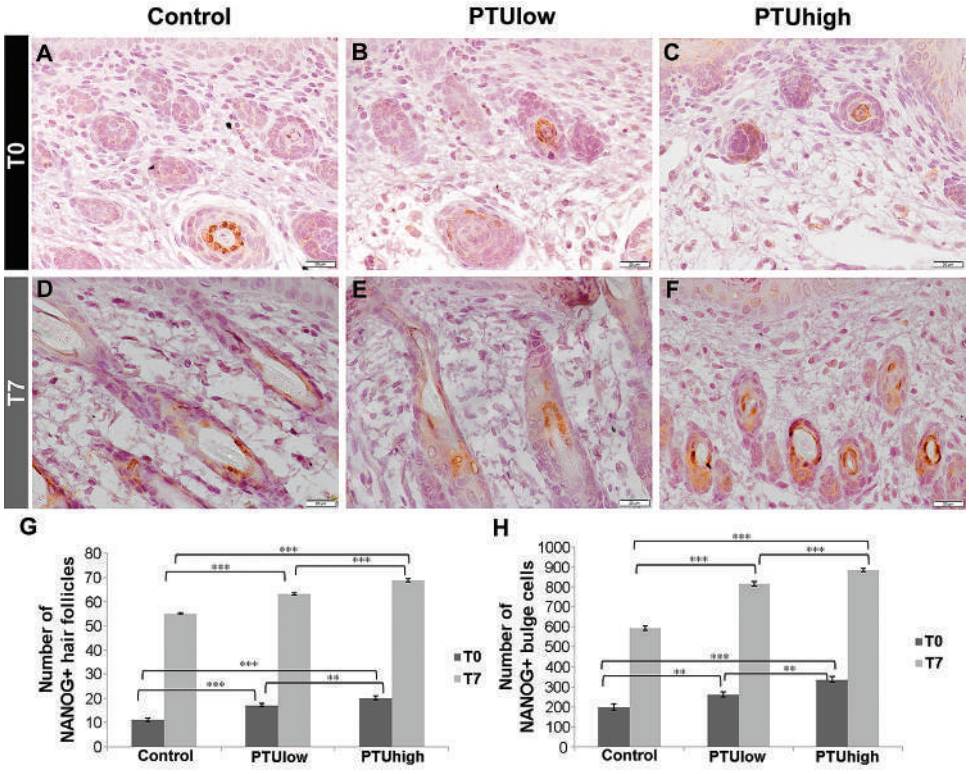

Figure 5. Representative photomicrographs of skin NANOG immunoexpression in neonatal (within 24h of delivery) (T0) control pups (Control) (**A**); pups from does treated with low PTU dose (PTU$_{low}$) (**B**) and high PTU dose (PTU$_{high}$) (**C**) and early infantile pups (7-day-old) (T7) from control (**D**), PTU$_{low}$ (**E**) and PTU$_{high}$ (**F**) group. All photomicrographs viewed with high power (40x) objective lens (bar: 20 μm) (3,3'-diaminobenzidine, counterstain haematoxylin). Graphs presenting mean ± standard deviation of number of NANOG positive hair follicles (**G**) and bulge cells (**H**). (**p<0.01, ***p<0.001).

DISCUSSION

This study used the rat as an animal model, previously proved appropriate for hypothyroidism investigation [23] and though histological and stereological approach is a basic way to access the subject, the obtained results might be a good starting point for further understanding the effects of the lack of thyroid hormones on skin cells at the ultrastructural and molecular level. The main findings of our investigation were that the subclinical and clinical form of hypothyroidism in pups lead to serious damage of the epidermis in terms of pronounced hyperkeratosis and reduction of the germinal layer along with reduced number of HFs.

Proliferation and differentiation are essential for normal skin structure and homeostasis and they take place alternately, diffusely and they overlap in some epidermal layers [24,25]. Proliferation is more pronounced in the germinal layer, with differentiation in the higher ones, while apoptosis is diffusely present in all layers of the epidermis [24]. On the other hand, T3 is proven as a suppressor of $Casp3^{+}$ activity and an important antiapoptotic factor [26]. The lack of T3 results in impaired synthesis of extracellular matrix proteins like laminin [23] and nuclear matrix proteins like PCNA, in charge for cell cycle control [27], both with a key role in connection, growth, proliferation and distribution of epithelial cells like keratinocytes [25]. Thus, the changes are understandably more pronounced in the pups from mothers with overt hypothyroidism (T0 and T7) where amplified apoptosis and decreased proliferation result in reduced thickness of the non-cornified layer. Furthermore, our observation of the cornified layer light thickening in the treated groups could be associated with the study of keratinocytes cell culture which indicated the promoting effect of T3 on terminal differentiation and shedding of these cells [28]. We presume that the reduced concentration of thyroid hormones thus leads to the decreased desquamation rate and since this is the only study to our knowledge associating the thyroid hormones and cornification, further insight to this subject is needed. No changes regarding epidermal thickness and cell morphology are detected in pups obtained from mothers with subclinical hypothyroidism immediately after birth. Still, it appears that there is a temporal demand for subtle changes like reduced proliferation and differentiation to develop, as confirmed at T7 pups from the same treatment. This could be of interest to further inspect from the aspect of possible long-term outcomes of maternal hypothyroidism, already observed for metabolic disorders and behavior [29].

A similar trend concerning the cells in the external and internal hair sheet or in the associated sebaceous glands was observed regarding reduced proliferation and increased apoptosis of HF cells. Safer et al. (2001) demonstrated that topical application of T3 stimulated hair growth in mice and rats, suggesting that thyroid hormones directly affect HF cell proliferation [13]. Tsujio et al. (2008) concluded that the diminishing growth of hair in hypothyroid animals is a consequence of reduced proliferation and accelerated apoptosis of HF cells, due to the reduced concentration of thyroid hormones, while the reduced number of HFs in hypothyroid animals could

be explained by their accelerated atrophy [30]. This lead to delay of HF morphogenesis in both groups of treated pups, confirmed in several previous studies [12,23,31].

Reduced number of HFs and their delayed morphogenesis could be discussed considering the regenerative potential of the skin. The skin is an organ with a compartment containing highly active pluri- and multipotent SCs [32,33]. As mentioned above bulge cells can be recognized by the expression of the embryonic stem cell transcription factor NANOG, which helps maintain the capacity of self-renewal and multipotency in SCs [6,7], promoting their proliferation and delaying senescence [6,34,35]. In our study, HF bulge region cells in the tissue samples from T0 and T7 pups originating from mothers with both types of hypothyroidism, showed an increase in the number of $NANOG^+$ HF, as well as $NANOG^+$ cells in HF bulge itself. It is known that under physiological conditions, apoptosis is suppressed in the epidermis, and that the Casp 3^+ cells cannot be observed [36,37], but within the bulge region, the activity of this enzyme is expressed. The increased NANOG immunopositivity seems to be in connection with the increase in the number of Casp 3^+ cells in the bulge of both hypothyroid T7 groups. The Caspase family role is involved in regulating a large cohort of cellular processes other than cell death, including regulation of SCs properties. The molecular mechanisms by which these enzymes promote regeneration in multicellular organisms have yet to be determined [38]. Our finding may indicate that the weaker signaling and slower mobilization of stem cells into the upper compartments of HF or impaired epidermis, lead to their consequent accumulation in the HF bulge [39]. Reduced regenerative potential of the skin was observed in hypothyroid individuals [39] and the impaired reepithelialisation is associated with a reduced contribution of bulge SCs [10,40].

Bulge SCs are multipotent and can also differentiate into epidermal and sebaceous gland cells [8,9]. Impaired differentiation of bulge cells due to the lack of thyroid hormones and the delayed morphogenesis of HF in treated pups could be partially explained by this fact. The decreased number of HF may be the consequence of the disrupted entry of HF into the anagen phase of growth and the persistence of HF of hypothyroid rats in the telogen phase [39]. Bulge cells divide several times during anagen [33,41], which enables and maintains an uninterrupted cycle of hair growth.

CONCLUSION

The results have shown that the intensity of the modifications on the skin and its derivates are dependent on the hypothyroidism form, as well as on the pups' age. The more pronounced alterations are detected on seven-day-old pups with overt form of hypothyroidism. In addition, this is the first study to the best of our knowledge that combines subclinical hypothyroidism with the clinical form, and sheds light on changes in epidermal morphology from the aspect of regenerative potential/stem cells and important processes such as proliferation and apoptosis. The study could be improved by examining the effects of maternal hypothyroidism in older (pubertal) offspring

and adults and assessing the reversibility of changes in skin and its derivates during growing. The obtained results indicate that there is significant impairment in epidermal morphology and thickness, and reduced hairiness in treated rats, especially in the overt form of hypothyroidism. In the offspring obtained from mothers with subclinical form, the observed changes were less pronounced and delayed in occurrence. The future prospective is to investigate the molecular mechanisms of these phenomena, in order to prevent the occurrence of more serious skin damage and to provide better insight into this controversial topic.

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Authors' contributions

IM, AR and MKF conceived and defined the research theme. TLB, IM, JDL, AN and MV performed the experimental part of the study and made a substantial contribution to conception, design, analysis, acquisition and interpretation of data and were involved in drafting the manuscript. IM, JDL and TLB took care of the health, well-being and welfare of the animals. DM, IM and TLB performed immunohistochemical analyses. TLB and AN did the statistical analysis. IM TLB and AN acquired new literature data and wrote the initial text. MKF, JDL, DM and AR were involved in drafting the manuscript and revising it critically for important intellectual content. IM and AR made substantial contribution to conception and design, analysis and interpretation of data. All authors discussed the results and contributed to the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UTICAJ SUPKLINIČKOG I KLINIČKOG HIPOTIREOIDIZMA MAJKI PACOVA NA POTOMSTVO: PRIČA O KOŽI I NJENIM DERIVATIMA

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Tokom regeneracije tkiva matične ćelije epidermisa imaju značajnu ulogu, jer kroz procese proliferacije i diferencijacije omogućavaju zamenu oštećenih ćelija. Navedeni procesi su pod uticajem tireoidnih hormona koji imaju značajnu ulogu u održavanju homeostaze kože. Dokazano je da smanjena funkcija štitaste žlezde majki tokom graviditeta utiče na razvoj različitih organskih sistema mladunaca. Međutim, nedostaju podaci o uticaju supkliničkog hipotireoidizma majki tokom graviditeta i laktacije na razvoj kože i njenih derivata kod mladunaca. Upravo zato cilj ovog istraživanja je bio da se ispitaju efekti smanjene funkcije štitaste žlezde majke na razvoj kože i njenih derivata kod potomaka tokom ranog postnatalnog perioda. Antitireoidna supstanca 6-n-propil-2-tiouracil je tokom trajanja graviditeta i laktacionog perioda, dodavana u vodu za piće ženkama pacova *Albino Oxford* soja, u cilju izazivanja supkliničkog i kliničkog oblika hipotireoidizma. Uzorci tkiva kože su zatim uzimani od muških potomaka u prvih dvadeset i četiri sata i sedam dana nakon rođenja. Osnovni rezultati ovog istraživanja su da obe forme maternalnog hipotireoidizma dovode do promene u građi epidermisa kod mladunaca, u smislu izražene hiperkeratoze i redukcije germinativnog sloja, uz smanjenje broja dlačnih folikula i njihovu odloženu morfogenezu. Kod klinički hipotireoidnih mladunaca promene su bile izraženije, dok su se kod supklinički hipotireodnih potomaka promene uočavale nešto kasnije i bile su slabijeg inteziteta.