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Research article

EFFECT OF SUBCLINICAL AND OVERT FORM OF RAT MATERNAL HYPOTHYROIDISM ON OFFSPRING ENDOCHONDRAL BONE FORMATION

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Maternal hypothyroidism in its overt form affects skeletal development of the offspring, but these data are not available for the subclinical form which is becoming very frequent among pregnant women. We hypothesized that the subclinical form of hypothyroidism in rat dams, influences the process of offspring endochondral ossification affecting proliferation and differentiation of chondrocytes, osteoclasts and osteoblasts in pups. Seven-day-old male pups (n=18) derived from control dams and dams treated with a low dose (1.5 mg/L) or high dose (150 mg/L) of propylthiouracil in drinking water during pregnancy and lactation were used. Histomorphometric analysis of pups' tibia proximal growth plate, expression of mRNA, immunohistochemical and histochemical visualization of extracellular matrix components was performed. The length of the tibia was reduced in hypothyroid pups. Secretion of type 2 and 10 collagens in the subclinical and overt form were lower while the amount of glycosaminoglycans was higher when compared with controls. Down-regulated tartrate resistant acid phosphatase mRNA indicated altered osteoclasts function while lower expression of dentin matrix acid protein–1 mRNA and reduced synthesis of type 1 collagen accentuated a compromised bone formation in the overt form of hypothyroidism. The subclinical form of maternal hypothyroidism had a negative effect on the differentiation of hypertrophic chondrocytes and calcified cartilage removal in 7-day-old pups. In addition, overt hypothyroidism had a negative effect on the proliferation of chondrocytes and deposition of osteoid.

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Both forms of hypothyroidism resulted in a decrease of tibia length due to changes in growth plate formation.

Key words: type 1, 2 and 10 collagens, TRAP, DMP-1, hypertrophic chondrocytes, offspring hypothyroidism

INTRODUCTION

Spatial and temporal regulation of proliferation, differentiation and maturation of fetal cells, tissues and organs is largely governed by the mothers' thyroid hormones (TH) during whole pregnancy [1]. The most severe and irreversible changes are seen on developing neural tissues and thus, the overt and subclinical form of maternal hypothyroidism are primary associated with intellectual and neurological problems of the offspring [2-4]. With increasing awareness that subclinical hypothyroidism is common among pregnant women [5], new investigations are prompted to define more subtle changes on tissues other than neural. Low birth weight is frequently reported as outcome of both subclinical and overt form of maternal hypothyroidism [6,7]. Although it is clear that the overt form of maternal hypothyroidism alters ossification in offspring [8] and influences the size of the fetal skeleton [9-11], all the consequences of subclinical form of maternal hypothyroidism on offspring bone formation are not described yet.

The developmental stage of rat pups during the suckling phase corresponds to the last trimester of human pregnancy and is marked with immaturity of TH/TSH axis in both species [12]. Thus, investigating effects of maternal hypothyroidism in a rat offspring appropriately mimics and replaces the need to investigate prenatal hypothyroidism in human neonates. Commonly used model for hypothyroidism investigation in laboratory rodents, is the application of 6-n-propyl-2-thiouracil (PTU) [13]. In the thyroid gland PTU reduces TH synthesis while in the peripheral tissues interferes with T3 binding to nuclear thyroid hormone receptors and also blocks conversion of T4 to T3 [14]. It is known that a very low dose of PTU is used for successful modeling of the subclinical form of hypothyroidism [15-17] and the high dose to induce the overt form of maternal hypothyroidism [18]. The comparative approach using both PTU doses could be helpful when subtle changes in cartilage and bone should be evaluated and interpreted.

The objective of this study was to investigate effects of subclinical and overt forms of maternal hypothyroidism on proximal epiphyseal growth plate of 7-day-old pups, at the beginning of growth spurt. Specific aims were: 1) to measure the length of the tibia and to describe the histological appearance of its proximal growth plate; 2) to test gene expression for type 2 and type 10 collagen in hypertrophic chondrocytes and to analyze the surrounding extracellular matrix (ECM) for the presence of both types of collagens and glycosaminoglycans; 3) to test gene expression for tartrate resistant acid phosphatase (TRAP) in osteoclasts and DMP-1 in osteoblasts, TRAP activity in lysosomes of osteoclasts, type 1 collagen protein expression in osteoblasts and ECM and to describe morphological changes in the trabeculae forming the ossification zone.

MATERIALS AND METHODS

Animals

Eighteen 10-week-old female Albino Oxford rats (weighting 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 eighteen of their male offspring were used in the experiment. 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 ± 0.5ºC and the relative humidity 40 ± 7%. Pelleted rat feed (PA 20, Veterinary Institute Subotica, Serbia) and drinking water were available *ad libitum*.

Animal handling and treatment were performed with the approval of the Ethical Committee of the Faculty of Veterinary Medicine University of Belgrade (number 01-20/6), and Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate approval (number 323-07-00364/2017-05/8) and according to the Serbian Animal Welfare Protection Law and Directive 2010/63/EU. The procedures with rats are described according to the ARRIVE guidelines [19]. In concern with 3R practice, organs from pups of both sexes that were not investigated in this paper were preserved for future investigation on maternal hypothyroidism. Also, female pups were used for studying investigating effects of maternal hypothyroidism on ovary development [16,17].

Animal model

The first step in our experimental design was to induce subclinical and overt form of hypothyroidism in pregnant dams using low (1.5 mg/L) and high (150 mg/L) dose of PTU (Sigma Chemical Co., St. Louis, MO, USA) dissolved in drinking water. PTU was available *ad libitum* from day zero of dams' pregnancy until the end of the first week post-partum. Gestation day zero was considered as the day after mating characterized with the presence of the vaginal plug. Pregnant females were randomly divided into three groups – control (C), low dose (H-1) and high dose PTU (H-2). Each group consisted of six animals.

Experimental procedure

Seven days after birth, six male pups of dams from each group were euthanized using cervical dislocation and their body weights were recorded. Dams were euthanized at the same time point by using 100 mg/kg body weight Euthasol Euthanasia Solution (Produlab Pharma Production B.V. Raamsdonksveer, Netherlands). Blood samples from dams and pups were collected during the euthanasia protocol in polypropylene tubes with heparin (32 U/tube). After separation (1500 g, 10 min), plasma samples were stored at -20ºC for up to 2 months before further analyses were done.

Tissue collection and processing

After euthanasia thyroid glands were removed from the dams, and the thyroid glands and tibiae were removed from the pups. Tibiae were measured with a sliding mechanical caliper (accuracy 10 μm). Fixation, paraffin embedding of all tissues, deparaffinization, rehydration and staining were done by routine laboratory procedures. After fixation, tibiae were decalcified in 10% EDTA for 10 days. Every fifth 5-μm-thick section of thyroid glands and tibiae was cut on Superfrost Plus Slides (Menzel-Glaser, Saarbruckener, Germany).

Hematoxylin/eozin (Merck Millipore, Darmstadt, Germany) was used for routine morphometry and stereology. Alcian blue (SERVA Electrophoresis GmbH, Heidelberg, Germany) for visualization of glycosaminoglycans and Masson-Goldner trichrome (Merck Millipore, Darmstadt, Germany) for visualization of calcified cartilage and bone matrix. Activity in TRAP in osteoclast lysosomes was assessed with leukocyte acid phosphatase kit according to the manufacturer's protocol (Sigma-Aldrich, Poole, UK).

Thyroid status assessment

Thyroid status of dams was assessed through the determination of TH concentration in plasma and calculation of the thyroid activation index (Ia) which indirectly demonstrated the activity of TSH. Higher Ia values stand for higher blood TSH levels and reversely [20]. The form of hypothyroidism was defined as following: 1) dams were considered to have the subclinical form of hypothyroidism if their TH concentration was not changed, while Ia was higher compared with control animals [16,17] and 2) dams were considered to have an overt form of hypothyroidism if TH concentration was lower, and Ia higher compared with control animals. Thyroid gland stereology and Ia were assessed as described previously [16,20]. Briefly, thyroid glands were analyzed using a multipurpose stereological grid M42 (42 testing points in a testing area equal to 36.36 d^2). The volume density (V_v) of different thyroid compartments, interstitium (V_{vi}), epithelial tissue (V_{ve}) and colloid (V_{vc}) were determined. The Ia of thyroid glands was calculated: $Ia = V_{ve}/V_{vc}$[20].

Radioimmunoassay for T3 and T4

Triiodothyronine (T3) and thyroxine (T4) concentrations in blood plasma of dams and pups were determined using commercial radioimmunoassay kits (INEP, Zemun, Serbia).

Histomorphometry

Histomorphometric analyses were done on serial sections obtained from medial third part of pups' tibiae parallel to longitudinal axis. On each section, measurements were performed choosing the middle part of the proximal tibial growth plate.

Sections were examined 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) by two observers who were blinded for the group assignment. The height measurements: the proliferative zone (PZ) and hypertrophic zone (HZ) height were measured parallel to the chondrocyte columns. The border between PZ and HZ was defined by the presence of the first hypertrophic chondrocytes (height ≥10 μm) [21]. The growth plate height was determined as the sum of the PZ and HZ. The resting zone (RZ) was not included in the measurements as the uneven development of the secondary ossification centre (SOC) in the control, H-1 and H-2 pups would introduce a large spurious variation between groups. The number of hypertrophic chondrocytes was determined per 1 mm^2 of tissue area (objective X40) in HZ. The terminal hypertrophic chondrocytes were defined as the last ones in the lacunae that were not invaded by metaphyseal blood vessels and their average height was obtained after 250 measurements per tibia.

To define the characteristics of the newly formed trabeculae in the ossification zone (OZ), histomorphometric analysis was performed on 10 serial sections of the tibia stained with Masson-Goldner. The complete trabecular volume (TV), calcified cartilage volume (CCV) and bone tissue volume (BTV) was calculated as the number of pixels representing the corresponding tissue area divided by the number of the pixel representing the total tissue area [22]. These measurements were made using Photoshop (Adobe, San Jose, CA).

TRAP positive area was measured just below the last hypertrophic chondrocyte in the ossification zone (OZ) of the proximal tibial epiphysis. Alcian blue staining for quantification of glycosaminoglycan components of the extracellular matrix was performed according to the protocol described by Ribeiro et al. [23]. Examined areas (pixels) on histological sections were measured using Image J software (Media Cybernetics Manufacturing, Rockvill, MD).

Immunohistochemistry

Immunohistochemistry (IHC) on paraffin embedded tissue sections was used to determine the presence of type 1, 2 and 10 collagens. Antigen retrieval was achieved by Trypsin Enzymatic Antigen Retrieval Solution (ab970, 1:3 dilution; Abcam, Cambridge, UK) at 37ºC and blocked with 3%H_2O_2. Slides were washed two times in PBS and 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. After four washes, the secondary antibody was used (EnVision FLEX/HRP, RTU, Dako, Santa Clara, CA, USA). For visualization Liquid DAB+ Substrate Chromogen System (Dako, Carpinteria, CA, USA) was used, according to the manufacture's protocol. Counterstaining was carried out with Mayer's haematoxylin and slides were mounted with DPX (Sigma-Aldrich, Poole, UK). The immunopositive areas in pixels were determined using Image J software.

Table 1. Primary antibodies used for immunohistochemistry

Antibodies	Manufacturer	Dilution
Anti-Collagen I antibody ab34710	Abcam, Cambridge, UK	1:500
Anti-Collagen II antibody ab34712	Abcam, Cambridge, UK	1:200
Anti-Collagen X antibody ab58632	Abcam, Cambridge, UK	1:300

In Situ Hybridization

Serial 5-μm-thick sections from paraffin-embedded tissues were processed for *in situ* hybridization (ISH) as previously described [24]. Riboprobes were generated from PCR amplification of cDNA by using specific primer flanked by T7 and T3 promoters (Table 2.). Antisense and sense digoxigenin-labeled RNA probes were prepared by *in vitro* transcription from a T7 or a T3 promoter (DIG RNA labeling kit, Roche). Deparaffinized sections were treated with 20 μg/ml proteinase K for 10 min at 37°C and incubated with 1 μg/ml labelled probe overnight at 60°C. Labeled cells were visualized using a DIG nucleic acid detection kit (Roche Applied Science). Sections were mounted in aqueous solution (Mowiol®) and scanned using a Hamamatsu NanoZoomer HT digital scanner. The number of osteoblasts/osteocytes expressing mRNA for DMP-1 in OZ was determined per 1 mm^2 of tissue area (objective X40).

Table 2. Sequences of primer pairs, gene bank accession numbers used for generation riboprobes and size of PCR products

Gene	GeneBank N°	Forward T3 primer	Reverse T7 primer	Amplicon size (b p)
Col 2a1	NM_031163	5'–GAGAATTAACCC TCACTAAAGGGTCT CCTGCCTCCTCCTG CTC–3'	5'–GAGTAATACG ACTCACTATAGG GCTCCATCTCTG CCACGGGGT–3'	584
Col 10a1	NM_009925	5'–GAGAATTAACCC TCACTAAAGGGCGG GTCTGCCTGGATCC CCT–3'	5'–GAGTAATACG ACTCACTATAGGG GCTATGCCAGC TGGGCCTGG–3'	442
ACP5	NM_001102404	5'–GAGAATTAACCC TCACTAAAGGGCAG CTCAGTTGGGTAGC ACA–3'	5'–GAGTAATACG ACTCACTATAGG GACGGTTCTGGC GATCTCTT–3'	223
DMP-1	NM_016779	5'–GAGAATTAACCC TCACTAAAGGGAGC AACAGCAGGGAAA CC–3'	5'–GAGTAATACG ACTCACTATAGG GGCAAAACTGAG CCTGAAGCAC–3'	1074

Col2a1 – type 2 collagen; Col10a1 – type 10 collagen; ACP5 – tartrate resistant acid phosphatase (TRAP); DMP-1 – dentin matrix acid protein–1

Statistical analysis

Depending on the values of coefficients of variation (cv), an appropriate method was chosen to test the difference between the groups. For homogenous datasets (cv<30%) the groups were compared using one-way ANOVA followed by Tukey´s multiple comparison test, and for heterogeneous datasets (cv >30%) the groups were compared using Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test. Significant difference was estimated at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ significance levels. Numerical data for homogenous datasets are presented as mean ± standard deviation (Mean ± SD) and for heterogeneous datasets as median values with corresponding interquartile range (IQR). Statistical analysis of the results obtained in the experiment was carried out using statistical software GraphPad Prism version 6 (GraphPad, San Diego, CA, USA).

RESULTS

Thyroid status of mothers and pups

In H-1 dams, duration of pregnancy was not changed (21.8 ± 0.45 days) and in H-2 dams was prolonged (23 ± 0.71 days, $p<0.01$) compared with controls (21.40 ± 0.55). Litter size was smaller in H-2 dams when compared to H-1 or control ones (C: 9±1.73; H-1: 8.8±1.92; H-2: 5.4±1.52; C:H-2 $p<0.05$). Nesting behavior was preserved in both groups of PTU treated dams. In H-2 dams a rough hair coat was noticed during the second week of pregnancy until euthanasia. Histologically, the thyroid gland of dams in both PTU treated groups was characterized by high columnar epithelium and low colloid volume (Figure 1A, B, C). As a consequence, Ia of the thyroid gland was significantly increased in both, H-1 and H-2 dams (D). Concentration of T3 and T4 was significantly lower only in H-2 dams (Figure 1E, F).

Seven-day old pups from H-1 and H-2 dams were viable and with normal behavior regarding the suckling reflex and color of the skin. Body weight was lower in H-1 and even lower in H-2 pups when compared with controls (Figure 1G). Stereology of the thyroid gland matched the one seen in dams of the corresponding group (Figure 1H, I, J). In H-1 and H-2 pups, Ia was higher (Figure 1K), while concentrations of T3 and T4 were significantly lower only in H-2 pups (Figure 1L, M).

Morphometry of the tibia and its proximal epiphyseal growth plate

Tibiae were 12.18% shorter in H-1 and 18.11% shorter in H-2 pups compared with controls (Figure 2A). SOC of proximal tibia epiphysis were clearly formed in the control, poorly formed in H-1and not present in H-2 pups (Figure 2B, C, D).The height of PZ and HZ differed between groups (Figure 2B, C, D, E). PZ was significantly lower only in H-2 pups, while HZ was lower in both, H-1 and H-2 pups (Figure 2E). Also, the number of the hypertrophic chondrocytes per mm^2 was not changed in H-1, while it was significantly higher in H-2 pups (Figure 2F). The height of terminal hypertrophic chondrocytes in H-1 and H-2 pups was significantly lower compared with controls (Figure 2G).

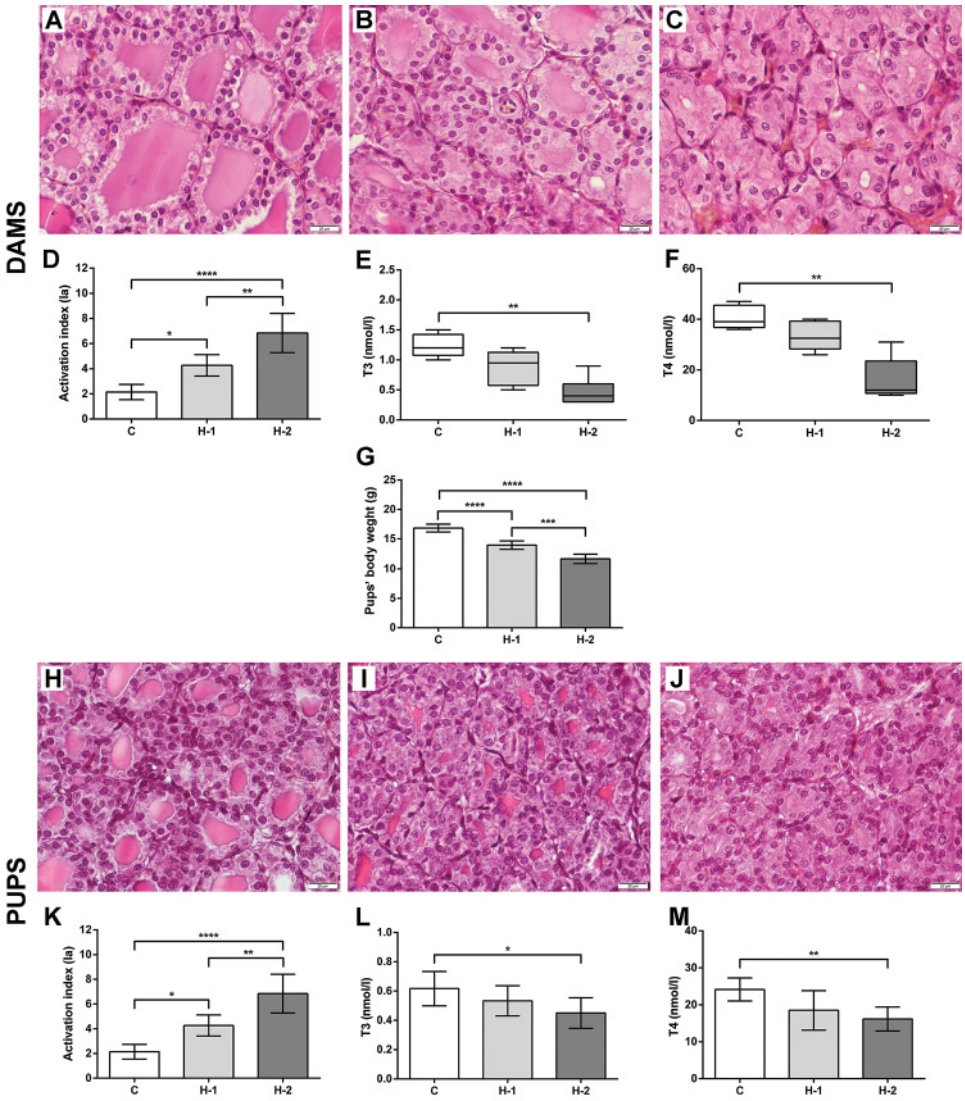

Figure 1. Thyroid status of dams and their 7-day-old pups from the control group and 6-n-propyl-2-thyouracil (PTU) treated groups and pups body weight: Thyroid gland histology of control dams (n=6, C) **(A)** and dams treated with low dose PTU (n=6, H-1) **(B)** and high dose PTU (n=6, H-2) **(C)** seven days after delivery. Note the high columnar follicular epithelium and low colloid volume in H-1 and H-2 dams (A, B, C) (haematoxylin/eosin, bar: 20 μm). Activation index (Ia) **(D)** and concentration of T3 **(E)**, T4 **(F)** in control, H-1 and H-2 dams. Body weight (g) was lower in pups from dams treated with the low PTU dose (n=6, H-1) and high PTU dose (n=6, H-2) as compared with control pups (n=6, C) **(G)**. Note the thyroid gland follicles in control pups **(H)** and a decrease in colloid and high columnar epithelial cells in follicles of H-1 **(I)** and H-2 **(J)** pups (haematoxylin/eosin, bar: 20 μm). Ia **(K)** and concentration of T3 **(L)**, T4 **(M)** in control, H-1 and H-2 pups. Boxes indicate the lower to upper quartile (25th-75th percentile) and median value. Whiskers extend to minimum and maximum values (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

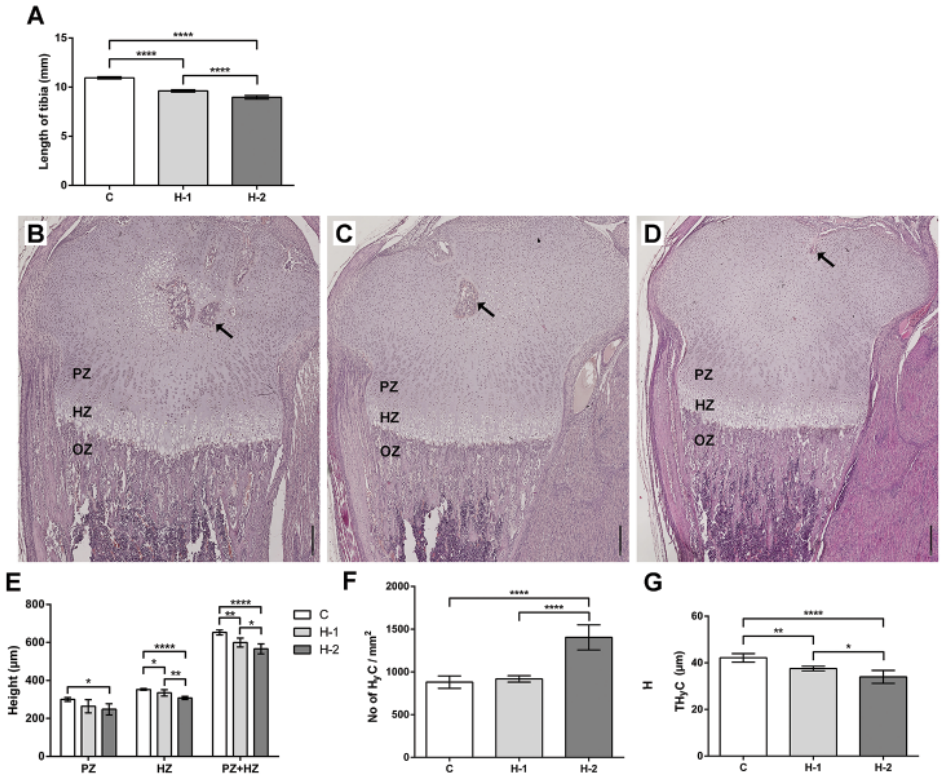

Figure 2. Length of tibia, representative photomicrographs of longitudinal section (medial third part) and morphometry of proximal tibial epiphysis growth plate in 7-day-old control pups (n=6, C) and in pups from dams treated with low PTU dose (n=6, H-1) and high PTU dose (n=6, H-2): Tibia is reduced in length in H-1 and H-2 pups as compared with control pups **(A)**. Secondary ossification centre (SOC) (arrow) of proximal tibial epiphysis is well developed in control pups **(B)**, less developed in H-1 **(C)** and undeveloped in H-2 pups **(D)** (haematoxylin/eosin, bar: 250 μm). Proliferation zone (PZ), hypertrophic zone (HZ) and ossification zone (OZ) are well distinguished in all three groups of pups (B, C, D). Height of PZ is lower in H-2 pups, and height of HZ and PZ+HZ is lower in H-1 and H-2 pups as compared with control pups **(E)**. Number (No) of hypertrophic chondrocytes (HyC) per mm^2 is higher in H-2 pups **(F)** and terminal hypertrophic chondrocytes height (THyC) is lower in H-1 and H-2 pups **(G)**. (*$p<0.05$, **$p<0.01$, ****$p<0.0001$).

The type 2 collagen, type 10 collagen and Alcian blue staining in proliferative and hypertrophic zones

The type 2 collagen mRNA expression was weak in PZ and HZ chondrocytes of control pups, while it was strong in the chondrocytes of H-1 and H-2 pups (Figure 3A, B, C). IHC showed that the amount of type 2 collagen in PZ and HZ ECM was lower in H-1 pups when compared with controls and also, it was lower in H-2 pups when compared with H-1 pups (Figure 3D, E, F G). Inversely, the area stained with Alcian blue was higher in H-1 pups when compared with controls and higher in H-2 pups when compared with C and H-1 pups (Figure 4H, I, J K).

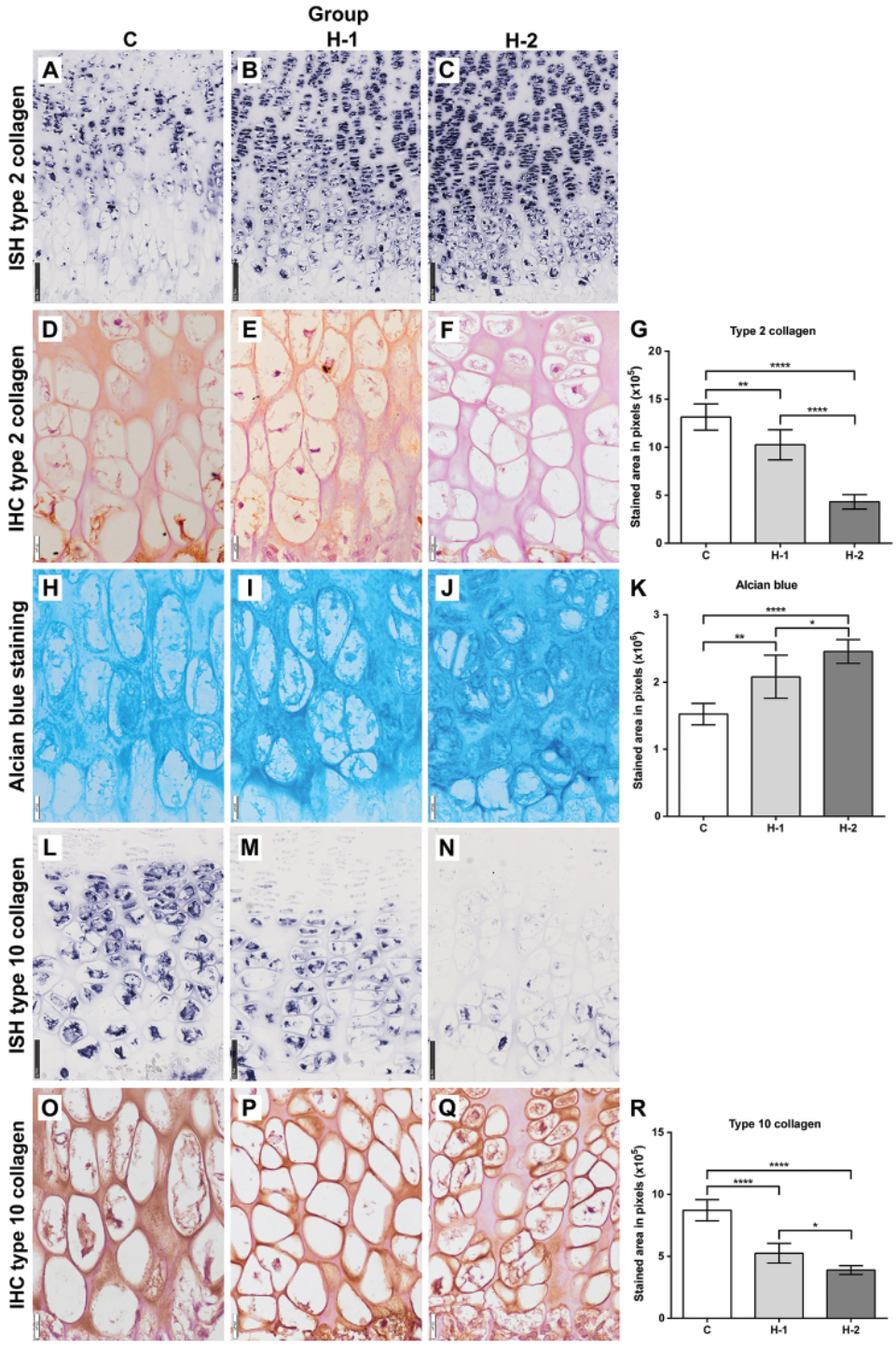
Group
C
H-1
H-2
ISH type 2 collagen
IHC type 2 collagen
Alcian blue staining
ISH type 10 collagen
IHC type 10 collagen
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Type 2 collagen
Stained area in pixels (x10^5)
Alcian blue
Stained area in pixels (x10^6)
Type 10 collagen
Stained area in pixels (x10^5)

Figure 3. Representative photomicrographs of proliferating zone (PZ) and hypertrophic zone (HZ) and graphs presenting mean ± standard deviation of morphometric analysis: *In situ* hybridization (ISH) for type 2 collagen mRNA in PZ and HZ in 7-day-old control pups (n=6, C) **(A)** and in pups from dams treated with low PTU dose (n=6, H-1) **(B)** and high PTU dose (n=6, H-2) **(C)** (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, bar: 100 μm). Note the weak expression of mRNA in HZ chondrocytes in control pups and very strong expression reaching the chondro-osseous junction in H-1 and H-2 pups (C). Immunohistochemical (IHC) detection of type 2 collagen shows its presence in the extracellular matrix (ECM) in control pups **(D)** and reduction in H-1 **(E)** and H-2 **(F)** pups (3,3'-diaminobenzidine, counterstain haematoxylin, bar: 20 μm). Area of HZ with type 2 collagen positive signal is lower in H-1 and H-2 pups as compared with control pups **(G)**. Area of HZ stained with Alcian blue was increased in H-1 **(I)** and H-2 **(J)** pups as compared with control pups (H, I, J, K) (bar: 20 μm). Note the strong expression of type 10 collagen mRNA in HZ of control pups **(L)**, weak expression in H-1 **(M)** and H-2 **(N)** pups (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, bar: 50 μm). Note that expression of type 10 collagen in ECM of HZ is strong in control pups **(O)**, moderate in H-1 **(P)** and weak in H-2 pups **(Q)** (3,3'-diaminobenzidine, counterstain haematoxylin, bar:20 μm). Area with type 10 collagen positive signal is lower in H-1 and H-2 pups as compared with control pups **(R)**. (*$p<0.05$, **$p<0.01$, ****$p<0.0001$).

The type 10 collagen mRNA expression was strong in HZ chondrocytes of control pups, and it was weak in chondrocytes of H-1 and H-2 pups (Figure 3L, M, N). IHC showed that the amount of type 10 collagen in ECM was lower in HZ in H-1 pups when compared with controls and also it was lower in H-2 pups when compared with H-1 pups (Figure 3O, P, Q, R).

TRAP, trabecular morphology, type 1 collagen and DMP-1 expression in the ossification zone

With severity of hypothyroidism, TRAP mRNA expression in osteoclasts (Figure 4A, B, C) and the area covered with lysosomes expressing TRAP activity declined in H-1 and H-2 pups (Figure 4D, E, F, G).

On Masson-Goldner stained sections it was possible to distinguish green-blue osteoid – bone tissue matrix, and colourless calcified cartilage together forming primary bone trabeculae (Figure 4H, I, J). In H-1 and H-2 pups they were wider when compared with controls (Figure 4H, I, J). Morphometric analysis showed that when compared with controls, TV and BTV were not different while CCV was increased in H-1 pups (Figure 4K); TV and CCV were increased in H-2 and H-1 pups, while BTV was lower only in H-2 pups (Figure 4K).

Osteoblasts in OZ had a well distinguished morphology that was cuboidal on the interface of the newly synthesized bone in control and H-1 pups, while it was spindle shaped in H-2 pups (Figure 4L, M, N). IHC showed that amount of type 1 collagen in osteoblasts and ECM was lower in H-1 and H-2 pups when compared to the control (Figure 4L, M, N, O). Also, it was lower in H-2 pups when compared with H-1 pups (Figure 4M, N, O). The number of osteoblasts/osteocytes expressing mRNA for DMP-1 was decreased in H-1 and H-2 pups when compared with the controls and between H-1 and H-2 pups (Figure 5P, Q, R, S).

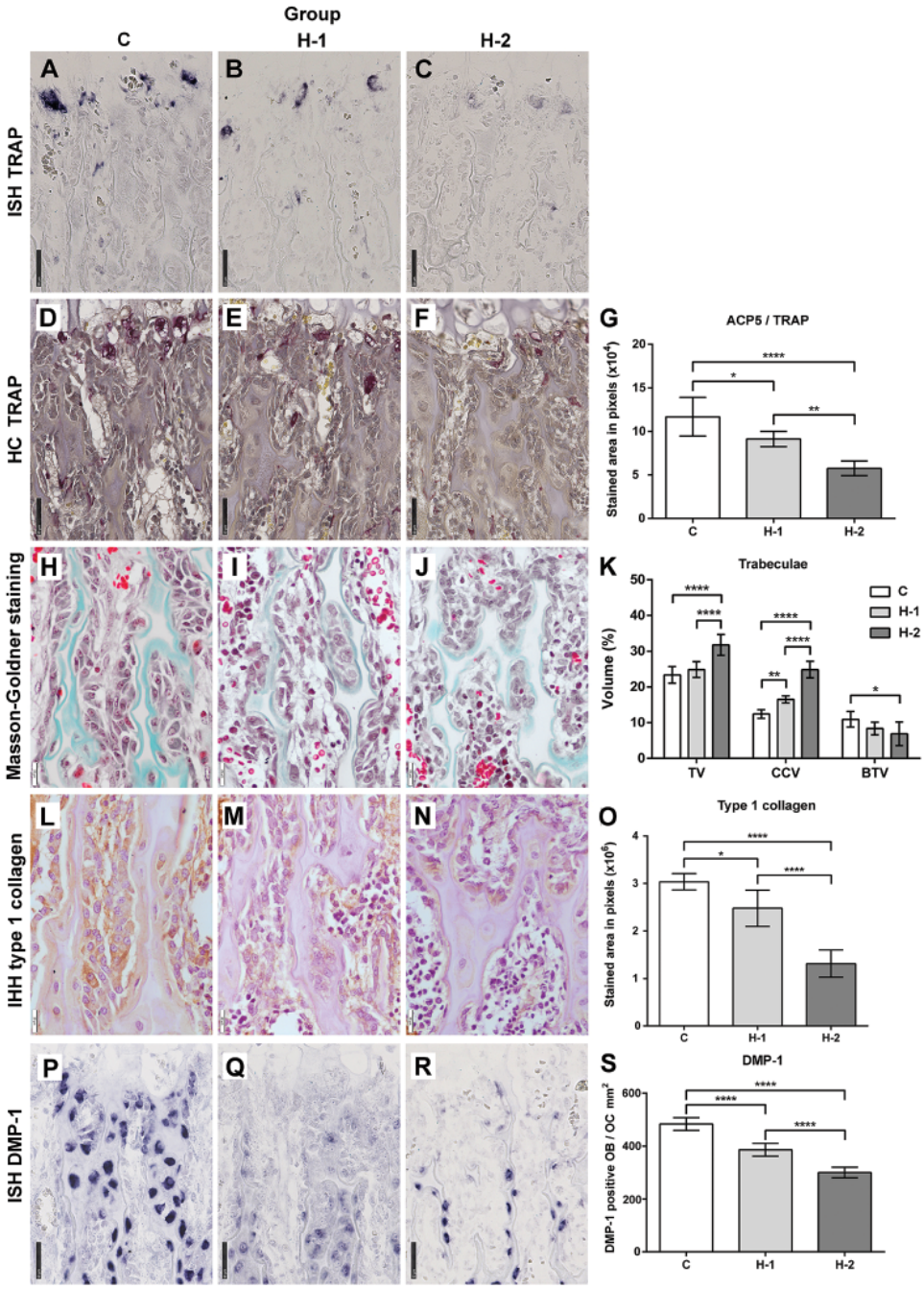

Figure 4. Representative photomicrographs of ossification zone and graphs presenting mean and standard deviation of morphometric analysis: *In situ* hybridization (ISH) for acid phosphatase 5 tartrate resistant (TRAP) mRNA in ossification zone in 7-day-old control pups (n=6, C) **(A)** and in pups from dams treated with low PTU dose (n=6, H-1) **(B)** and high dose PTU (n=6, H-2) **(C)** (5-bromo-4-chloro-

3-indolyl phosphate/nitro blue tetrazolium, bar: 50 μm). Note a strong signal next to chondro-osseous junction in control pups (A) and weak signal in H-1 (B) and practically absent signal in H-2 pups (C). Histochemistry (HC) demonstrates strong purple-violet TRAP activity of osteoclasts in control pups **(D)**, lower signal in H-1 pups **(E)** and weak signal in H-2 pups **(F)** (bar: 50 μm). Area stained in purple-violet was lower in H-1 and H-2 pups as compared with control pups **(G)**. Masson-Goldner staining shows green osteoid and colourless calcified cartilage that together form primary trabeculae in the ossification zone of the control **(H)**, H-1 **(I)** and H-2 **(J)** pups (bar: 20μm). Morphometric analysis shows that trabecular volume (TV) and bone tissue volume (BTV) are not changed in H-1 pups. In H-2 pups, TV was higher while BTV was lower compared with controls **(K)**. Calcified cartilage volume (CCV) was higher in H-1 and H-2 pups compared with controls **(K)**. Immunohistochemical (IHC) detection of type 1 collagen shows its presence in osteoblasts/osteocytes and extracellular matrix of ossification zone in control pups **(L)** and reduction in H-1 **(M)** and H-2 **(N)** pups (3,3'-diaminobenzidine, counterstain haematoxylin, bar: 20 μm). Fibroblast-like morphology of osteoblasts could be seen in H-2 pups (N). Area with type 1 collagen positive signal is lower in H-1 and H-2 pups as compared with control pups **(O)**. ISH for dentin matrix protein-1 (DMP-1) mRNA shows high expression in control pups **(P)** and lower expression in H-1 **(Q)** and H-2 pups **(R)** (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, bar: 50μm). Control animals have more DMP-1 positive osteoblasts/osteocytes (OB/OC) **(S)**. (*$p<0.05$, **$p<0.01$, ****$p<0.0001$).

DISCUSSION

The main findings of this study are that the subclinical and overt form of hypothyroidism in 7-day-old pups lead to 1) lower body weight and bone length and 2) changes in growth plate of proximal tibia epiphysis that include: a) lower hypertrophic chondrocytes size; b) lower amount of synthesized type 2 and type 10 collagen; c) higher HZ glycosaminoglycan content; d) lower TRAP synthesis and activity, lower number of mature osteoblasts (DMP-1 positive cells) and amount of type 1 collagen in osteoblasts and bone ECM. All these changes lead to the formation of shorter and wider primary bone trabeculae.

In this study it was demonstrated that the subclinical and overt form of maternal hypothyroidism lead to the subclinical and overt form of hypothyroidism in 7-day-old pups, respectively. Although literature defines both forms of hypothyroidism in humans using reference values for TH and TSH [25], in the rat model of maternal hypothyroidism the reference values are not available. Thus, clinical signs in conjunction with TH concentration and Ia are used to define the thyroid status of dams as well as pups. Main clinical signs in the overt form of hypothyroidism in dams are rough hair coat, prolonged pregnancy and small litter size. These results are in accordance with previously described models [6,18]. The main clinical sign in the overt form of hypothyroidism in 7-day-old pups is low body weight. We confirmed that pups with overt hypothyroidism had TH values lower and Ia higher than those with the subclinical form of the disease and the control. Although TSH concentrations were not measured, they were estimated indirectly, using the Ia [16,20]. According to high Ia in both groups of hypothyroid pups, we could assume that pups had supra-physiological levels of TSH.

Low body weight and the length of tibia in both groups of pups might be the consequence of prenatal changes caused by the hypothyroid state of dams. This is in

accordance with low birth weight in human neonates born from subclinical and overt hypothyroid mothers [26]. Early infantile rat pups 7-day-old are at the beginning of growth spurt [27,28] meaning that all components of the growth plate are vulnerable to hormonal changes. Previously, it was shown that TSH, independently of TH, acts on different components of growth plate [29]. Thus, it could be considered that endochondral ossification in early infantile hypothyroid rat pups is affected with a decrease in TH and increase in TSH levels or the balance between these two hormones (TH/TSH).

Subclinical hypothyroid pups maintained nearly normal height of PZ and nearly equal number of hypertrophic chondrocytes in HZ, while those with the overt form had underdeveloped, short PZ and increased number of hypertrophic chondrocytes in HZ. Terminal hypertrophic chondrocytes' height gradually decreased with the severity of hypothyroidism. Thus, in the overt form of hypothyroidism, the spatial and temporal balance between proliferation and differentiation of chondrocytes is altered leading to accumulation of chondrocytes that did not acquire adequate hypertrophic morphology. In addition, it was previously demonstrated that the TSH receptor was expressed on growth plate chondrocytes and that over expression of TSH in mice lead to a decreased height of the growth plate [30]. These findings point that in the overt form of hypothyroidism, TH/TSH balance does not favor the proliferation of chondrocytes, while in the subclinical form the proliferation capacity of chondrocytes is preserved at a nearly normal rate. In both forms differentiation was slowed, but in a severity-dependent manner.

This study suggests that PZ chondrocytes contained an increased amount of type 2 collagen mRNA in the subclinical and even more in the overt form of hypothyroidism. The amount of type 2 collagen in ECM was decreased in both treated groups. Similarly, the mice model of hypothyroidism in the early infantile period, has shown that the amount of type 2 collagen is decreased in ECM surrounding epiphyseal chondrocytes [8]. Taken together these findings suggest that the deficit of TH and possible excess of TSH stimulate the transcription of type 2 collagen or increase mRNA stability, and possibly interfere with post-translation mechanism of type 2 collagen modification and/or its secretion in ECM.

This study also showed that ECM of HZ in the subclinical and overt form hypothyroid pups had increased amount of glycosaminoglycans. A previous study showed that the hypothyroid state was characterized with increased amount of heparan sulfate in growth plates of early infantile mice [31]. It was also suggested that TH regulates the synthesis of aggrecanase-2, an enzyme involved in proteoglycan degradation, and expressed by hypertrophic chondrocytes [32]. Thus, it could be hypothesized that the steady state TH/TSH limits the synthesis and/or degradation of glycosaminoglycans. Apparently, subclinical and overt hypothyroidism disrupt the fine regulation of ECM synthesis leading to decrease in type 2 collagen and increase in the amount of glycosaminoglycans.

The major chondrocyte morphological change in both forms of hypothyroidism was a decrease in the terminal hypertrophic chondrocyte height, i.e. their volume, suggesting the alteration in their terminal differentiation. It has been shown that IGF-1 mediates signals leading to maximal chondrocyte hypertrophy increasing proportionately the dry mass and fluid volume in an already swelled pre-hypertrophic stage of differentiation [33,34]. Thus, it is possible that in the steady-state TH/TSH act in combination with IGF-1 to optimise the process of chondrocytes hyperthrophy. Also, large amounts of glycosaminoglycans in ECM could be seen as a compensatory mechanism needed for water retention and at least partially compensating the lack of chondrocyte hypertrophy. It is also possible to hypothesize that the large amount of glycosaminoglycans with their strong water-binding capacity is not favorable for the influx of water into the chondrocytes.

Alteration in terminal differentiation of chondrocytes was related to decreased expression of type 10 collagen mRNA and to decreased secretion of this protein. However, in the overt form of hypothyroidism there was a visible increase in ECM between chondrocytes in HZ possibly reflecting high amount of glycosaminoglycans. It is well-known that TH are important for type 10 collagen synthesis and final differentiation of the growth plate chondrocytes to hypertrophic ones [35]. Independently, TSH has the same effect [30]. It is also known that an adequate amount of type 10 collagen is important for cartilage calcification [36]. Thus, we can conclude that in both forms of hypothyroidism, TH/TSH inhibit the differentiation of chondrocytes in the growth plate and that in the overt form there is a substantial accumulation of ECM that does not contain the proper amount of type 10 collagen and possibly is not adequately calcified.

Down-regulation of mRNA for TRAP as well as the lysosomes TRAP activity was influenced by the severity of hypothyroidism. With methods used, it was not possible to assess the number of osteoclasts, so the unresolved questions are if TH/TSH are important for osteoclastogenesis or for TRAP mRNA transcription. Several studies demonstrated that hyperthyroidism is characterized with an increased number of osteoclasts followed with osteoporosis [37]. It could be presumed that there is an inverse effect on osteoclastogenesis in hypothyroidism. Thus, our findings could point that alteration in TH/TSH during hypothyroidism disrupt normal osteoclast function during endochondral ossification.

This study showed that in overt hypothyroid pups the osteoblasts in OZ retained a spindle shaped morphology indicating their immaturity. Also, the number of mature osteoblasts expressing mRNA for DMP-1 was decreased in both groups of hypothyroid pups, in a severity dependent manner. ECM surrounding the osteoblasts contained less type 1 collagen. These findings are associated with a decrease in osteoid and high increase in calcified cartilage volume that was also reflected in an increased whole trabecular volume measured in this study. Previously, using micro-computed tomography, it was shown that ovine foetuses have an increase in the bone trabecular volume when subjected to intrauterine thyroidectomy [38]. So, the findings of this study

demonstrated that trabecular volume increases due to increased amount of calcified cartilage volume, which could be the consequence of decreased osteoclast number/activity. It was already hypothesized that bone length reduction in newborn mice could be a result of compromised calcified cartilage removal due to reduced expression of TRAP [39]. It could be assumed that mineralization of newly formed bone tissue was also altered as mRNA for DMP-1 was decreased. The described changes suggest that inadequate functioning of osteoclasts and immaturity of osteoblasts during the subclinical and overt form of hypothyroidism disturb the formation of the normal ossification zone.

CONCLUSION

The subclinical form of maternal hypothyroidism had a negative influence on the differentiation of hypertrophic chondrocytes and removal of calcified cartilage by osteoclast in 7-day-old pups. In addition, the overt form of maternal hypothyroidism had a negative influence on the proliferation of chondrocytes and osteoblast function related to the deposition of osteoid. The changes in growth plate influenced the tibia length in both forms of hypothyroidism.

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Authors' contributions

AR, JG and MKF conceived defined a research theme. IM done the experimental part of the study and made a substantial contribution to conception and design and analysis, acquisition and interpretation of data and was involved in drafting the manuscript. IM, JDL and TLB carried out animal welfare. IM and TLB performed immunohistochemical analyses. SBC, SSP, and IM performed molecular analyses. BV has done statistical analysis. IM and JDL acquired new literature data and were writing the initial text. MKF, JG, JDL and AR were involved in drafting the manuscript and revising it critically for important intellectual content. MKF and AR have made a 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 SUBKLINIČKE I KLINIČKE FORME HIPOTIREOIDIZMA MAJKI PACOVA NA ENDOHONDRALNO FORMIRANJE KOSTIJU MLADUNACA

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Dobro je poznato da klinički hipotireoidizam majki utiče na razvoj skeleta njihovih potomaka, međutim, ne postoji dovoljno podataka o uticaju supkliničke forme koja je sve učestalija u toku graviditeta. Pretpostavili smo da supklinička forma hipotireoidizma ženki pacova utiče na proces endohondralnog okoštavanja tako što remeti proliferaciju i diferencijaciju hondrocita, osteoklasta i osteoblasta kod mladunaca. U eksperimentu su korišćeni mladunci muškog pola (n=18) potomci majki iz kontrolne grupe i dve tretirane grupe koje su tokom graviditeta i laktacije dobijale nisku (1.5 mg/L), ili visoku dozu (150 mg/L) propiltiouracila u vodi za piće. Promene na proksimalnoj epifiznoj ploči tibije mladunaca su određene uz pomoć morfometrije, *in situ* hibridizacije, imunohistohemije i histohemije. Osim skraćenja tibije, zabeležena je smanjena sekrecija kolagena tipa 2 i 10, kao i povećana količina glikozaminoglikana u supkliničkom i kliničkom hipotireoidizmu u odnosu na kontrolnu grupu. Smanjena ekspresija iRNK za tartarat rezistentnu kiselu fosfatazu ukazuje na promene u funkciji osteoklasta, dok smanjena ekspresija iRNK za kiseli protein matriksa dentina–1 i smanjena sinteza ko-

lagena tipa 1 ukazuje na poremećaj u formiranju kosti kod klinički ispoljene forme hipotireoidizma. Supklinički hipotireoidizam majki negativno utiče na diferencijaciju hipertrofičnih hondrocita i resorpciju kalcifikovane hrskavice kod mladunaca starih 7 dana. Zaključak je da klinički hipotireoidizam majki ima negativan uticaj na proliferaciju hondrocita i depoziciju osteoida. Smanjena dužina tibije, kod obe forme hipotireoidizma, posledica je promena nastalih u toku formiranja epifizne ploče.