Excess Maternal Thyroxine Alters the Proliferative Activity and Angiogenic Profile of Growth Cartilage of Rats at Birth and Weaning

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Abstract

Objective. The aim of this study was to unravel the mechanisms by which thyroxine affects skeletal growth by evaluating proliferative activity and angiogenic profile of growth cartilage of neonatal and weanling rats. **Methods.** Sixteen adult Wistar rats were equally divided into 2 groups: control and treated with thyroxine during pregnancy and lactation. The weight, measurement of plasma free T_4 and thyroids, femurs' histomorphometric analysis, and proliferative activity and angiogenic profile by immunohistochemical or real-time reverse transcriptase-polymerase chain reaction in growth cartilage was performed. Data were analyzed using Student's *t* test. **Results.** The free T_4 was significantly higher in the treated rats. However, the height of the follicular epithelium of the thyroid in newborns was significantly lower in the treated group. The excess maternal thyroxine significantly reduced the body weight and length of the femur in the offspring but significantly increased the thickness of trabecular bone and changed the height of the zones of the growth plate. Furthermore, excess maternal thyroxine reduced cell proliferation and vascular endothelial growth factor (VEGF) expression in the growth cartilage of newborn and 20-day-old rats ($P < 0.05$). There was also a reduction in the immunohistochemical expression of Tie2 in the cartilaginous epiphysis of the newborns and FLK-1 in the articular cartilage of 20-day-old rats. No significant difference was observed in Ang2 expression. **Conclusions.** The excess maternal thyroxine during pregnancy and lactation reduced endochondral bone growth in the progeny and reduced the proliferation rate and VEGF, Flk-1, and Tie2 expression in the cartilage of growing rats without altering the mRNA expression of Ang1 and Ang2.

Keywords

maternal hyperthyroidism, growth cartilage, angiogenesis, rat

Introduction

Thyroxine (T_4) and triiodothyronine (T_3) are essential for endochondral bone formation and growth as well as for bone and mineral metabolism.^{1–3} Thus, both hypothyroidism and hyperthyroidism may cause several bone damage.^{3,4} The synthesis of T_3 and T_4 and the regulatory mechanisms of these hormones are similar in humans and animals,⁵ and the exogenous administration of thyroid hormone to rats has served as a model of human hyperthyroidism.⁶ Thus, the rat has been used by many researches as a comparative model for thyroid dysfunction and metabolic changes in several tissues,^{7–9} including the effects *in vivo* and *in vitro* on bone metabolism and growth.^{3,10–13} Similarly to human, in adult rats the excess thyroid hormone stimulates both bone apposition and resorption, with supremacy of the catabolic process.^{11,14} So rats have become a widely accepted model of human bone disease conditions.¹⁵

In maternal hyperthyroidism, fetal tissues are exposed to excessive amounts of thyroid hormone,¹⁶ which may disrupt the fetal and newborn ability to regulate thyroid-stimulating hormone (TSH) and T_4 levels.¹⁷ But, although less common than hypothyroidism, if the congenital hyperthyroidism is not recognized and treated it can also have serious effects on bone growth, particularly in the first 2 years of life.¹⁸ Preliminary results from our group showed that the offspring of rats with hyperthyroidism have reduced endochondral bone formation and growth at birth and weaning. Furthermore, we observed increases in the growth plate

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height with an increased hypertrophic zone in these offspring.¹⁹ One hypothesis is that the reduction in bone growth is caused by the failure of vascular invasion of the growth cartilage.

Angiogenesis is important for the endochondral bone formation and growth that occurs prenatally and postnatally. During this process, there is progressive replacement of the growing cartilage by bone via interactions between the chondroblasts and the cells of the vascular system.²⁰ Vascular endothelial growth factor (VEGF) is expressed in the growing cartilage, predominantly in hypertrophic chondrocytes.²¹ VEGF acts by binding to the tyrosine kinase receptors Flt-1 (VEGFR1) and Flk-1 (VEGFR2)²² and is essential for the development of the skeleton.^{21,23} The inactivation of the VEGF by systemic administration of a soluble receptor chimeric protein (Flt-(1-3)-IgG) suppressed the invasion of blood vessels, impaired trabecular bone formation, expanded the chondrocyte hypertrophic zone of the growth plate, and led to abnormal differentiation of the chondroblasts.²⁴ In addition to VEGF, other regulators are also important for angiogenesis and maturation of the blood vessels, such as angiopoietins 1 and 2 (Ang1, Ang2). Angs are highly homologous peptides that bind with a high affinity to the tyrosine kinase receptor Tie2.²⁵ Ang1 and Ang2 are coexpressed with VEGF during local endochondral ossification, and their expression in the cartilaginous template increases with chondroblast differentiation.²⁶ The aim of this study was to unravel the mechanisms by which thyroxine affects skeletal growth of newborn and 20-day-old rats by evaluating proliferation by CDC-47 expression and angiogenic profile by VEGF, Flk-1, Ang1, Ang2, and Tie2 expression.

Methods

All procedures were approved by the Ethics Committee on Animal Experiments of UFMG (Protocol No. 47/2014).

Mating and Administration of Thyroxine

Sixteen 2-month-old adult female Wistar rats were used for this study and were housed at a density of 4 rats per cage with a 12-hour light-dark cycle. The rats were fed commercial rat chow. Food and water were provided *ad libitum* to all the animals. After a 2-week adaptation period, all females were subjected to vaginal cytology to monitor the estrous cycle.²⁷ Rats in proestrus and estrus were kept in plastic cages with adult male rats for 12 hours at a ratio of 2 females to each male. After this period, vaginal smears were obtained daily to detect spermatozoa. Copulation was confirmed by the presence of spermatozoa in vaginal cytology, and that day was considered to be Day 0 of gestation. Then, females were kept individually in plastic cages.^{28,29}

On the first day of gestation, the rats were randomly divided into 2 groups, treated and control, with 8 animals

each. Treated rats received daily administration of L-thyroxine (Sigma-Aldrich, St. Louis, MO) diluted in 5 mL of distilled water through an orogastric tube using previously established protocols (50 µg/animal/day)^{3,29} during pregnancy and lactation. For female control rats, 5 mL of distilled water was administered through an orogastric tube throughout the experimental period.

For each female rat, 2 pups were euthanized per litter, one at birth and another in the weanling stage (20 days of age). Thus, 4 groups were formed: (1) newborns from rats treated with L-thyroxine ($n = 8$), (2) newborns from control rats ($n = 8$), (3) weanling rats from rats treated with L-thyroxine ($n = 8$), and (4) weanling rats from control rats ($n = 8$). The animals were sacrificed by cardiac puncture preceded by anesthesia with xylazine (40 mg/kg; Vetnil, São Paulo, Brazil) and ketamine (10 mg/kg; König, São Paulo, Brazil).

Plasma Levels of Free T_4

Blood from breastfeeding rats and offspring at 20 days was collected by cardiac puncture in tubes with heparin. The plasma was separated by centrifugation and stored at -20°C for measurement of free T_4 . The measurements were performed with a chemiluminescent ELISA commercial kit according to the manufacturer's instructions (IMMULITE, Siemens Medical Solutions Diagnostics, Malvern, PA) (sensitivity: 0.4 ng/dL) in an automatic system.^{3,30}

Processing and Histomorphometric Analysis of the Thyroid

The thyroids of newborn rats and 20-day-old rats were dissected and fixed in 10% neutral buffered formalin solution and processed using routine techniques for paraffin inclusion. Histological sections of 4 µm were stained with hematoxylin-eosin (HE) for morphometric analysis. Thirty follicles/thyroid were randomly measured, including the largest and smallest diameters, to obtain the average of these measurements. An ocular micrometer with a ruler attached to the microscope at 40× objective was used. The epithelial height was measured in 20 follicles/thyroid. In each follicle, 4 different and equidistant points were measured to obtain the average value of 4 measurements. This measurement was performed using an ocular micrometer with a ruler attached to the microscope at 100× objective. The values, obtained for the diameter and height of the epithelium, were converted into micrometers using the scale of a micrometer slide.³¹

Measurement of Body Weight and Femoral Length

The average individual body weight (g) was determined in randomly selected newborns and 20-day-old rats of female

rats. In these animals, the right femur was dissected of adjacent muscle and connective tissue and measured with a caliper ruler as well as the length from the proximal to the distal epiphysis, while the width was measured at the femoral diaphysis.

Histological Processing and Histomorphometric Analysis of Bones

The samples were subjected to decalcification using 2 different solutions. The first solution comprising ethylenediaminetetraacetic acid (EDTA; 0.7 g), potassium sodium tartrate (8 g), sodium tartrate (0.14 g), hydrochloric acid (120 mL), and distilled water (900 mL) was incubated with the bones for 24 hours. Subsequently, the bones were placed in the second 10% EDTA solution dissolved in distilled water, and the solution was changed once a week until complete decalcification³² or every 3 days for the bones of newborns and 15 days for the bones of 20-day-old rats. After the decalcification was completed, the bones were longitudinally sectioned, processed by routine inclusion in paraffin, and subjected to microtomy similar as previously described.

In the femurs of newborn rats, we measured the percentage of trabecular bone in the distal metaphyseal in a field, which included the entire length of the histological section. The percentage was determined with the aid of a graticule of 121 points coupled to an optical microscope with a 10× objective. The thickness of 10 bone trabecula in 2 fields at 40× magnification of the metaphyseal region was measured at 3 points equidistant from each trabecula, with the aid of an eyepiece micrometer. In newborns, due to the presence of a fully cartilaginous epiphysis indistinguishable from the growth plate, we measured the height of the entire cartilaginous epiphysis and the height of only the hypertrophic zone of the growth plate, which was easily distinguished from the other regions. The values obtained were then converted to micrometers using the scale of a micrometer slide.

In 20-day-old rats, we determined the percentage of trabecular bone in both the distal epiphysis and metaphysis in femurs in 3 fields with a 20× objective. These assessments were performed using a graticule with 121 points coupled to the optical microscope eyepiece. The thickness of 20 bone trabecula in 4 fields of the metaphyseal region below the growth plate was measured at 3 points equidistant from each trabecular with a 40× objective. The height of the articular cartilage and the growth plate, as well as the resting, hypertrophic, and proliferative zones of the growth plate were determined by the average of the height taken at 15 equidistant points with a 10× objective. These evaluations were performed with the aid of an eyepiece with a ruler coupled to the microscope. The obtained values were converted into millimeters.

Immunohistochemistry Analysis of Growth Cartilages

Histological sections of the epiphysis and metaphysis of distal femurs in newborn and 20-day-old rats were placed on gelatinized slides. Histological sections were subjected to immunohistochemistry to assess proliferation with an anti-CDC-47 antibody (47DC14; Neomarkers, Fremont, CA; 1:100 dilution in newborn bones and 1:50 dilution in bones of 20-day-old rats). The angiogenic profile was assessed using anti-VEGF antibody (sc-152; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50 dilution), anti-Flk-1 antibody (sc-6251; Santa Cruz Biotechnology; dilution 1:50), anti-angiopoietin-2 antibody (sc-14403; Santa Cruz Biotechnology; 1:50 dilution), and anti-Tie2 antibody (sc-31268; Santa Cruz Biotechnology; 1:50 dilution).

We used the streptavidin-biotin-peroxidase technique (Streptavidin Peroxidase, Dako, St Louis, MO), and antigen retrieval was performed in a water bath at 98°C for 20 minutes. The slides were incubated overnight in a humid chamber with primary antibody and for 30 minutes during the steps for blocking endogenous peroxidase, blocking serum (Dako), and streptavidin peroxidase (Dako). Diaminobenzidine was the chromogen (Dako); it was used for 30 minutes. Sections were counterstained with Methyl Green for CDC-47 and Harris hematoxylin for the other antibodies. A negative control was performed by replacing the primary antibodies with IgG. Sections of rat spleen were used as a positive control for CDC-47, VEGF, and Tie-2. For angiopoietin, we used fish ovary.³³

Immunohistochemical analyses were performed in all the femoral growth cartilages from the treated and control groups and evaluated in the isolated hypertrophic zone of the growth plate. The immunostained cells were evaluated in 10 random fields/histological section with a 40× objective using images from the Leica DM 4000B coupled with a digital camera. The percentage of CDC-47-positive cells was determined by considering the total number of cells per field using Image Pro-Plus software (Media Cybernetics, Rockville, MD). For angiogenic factors, we determined the area and intensity of immunostaining with WCIF J Image software (Media Cybernetics). The evaluation was performed in both cartilage and the growth plate of the femur, obtaining an average of 3 histological sections for each animal. The analysis was conducted in the superficial, intermediate, and deep zones of the articular cartilage and also in all areas of the cartilage of the epiphyseal plate (proliferation, maturation, and hypertrophy).

Expression of Gene Transcripts by Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assays in Growth Cartilages

The quantification of VEGF, Flk-1, Ang1, Ang2, and Tie2 expression was performed by real-time RT-PCR using all cartilaginous epiphysis from the left femur of newborns and

Table 1. List of Genes with Primer Sequences.

Genes	Primer Sequences	Accession Number
VEGF	Forward primer: GCCCAGACGGGGTGGAGAGT Reverse primer: AGGGTTGGCCAGGCTGGGAA	NM_001110336.1
<i>Flk-1</i>	Forward primer: GTCCGCCGACACTGCTGCAA Reverse primer: CTCGCGCTGGCACAGATGCT	NM_013062.1
Ang1	Forward primer: GTCAGCCTTTGCACAAAAGAAGTTT Reverse primer: TCCAGCCCCTCTGGAAATCT	NM_053546.1
Ang2	Forward: TGCCTGCAAGTTTGCTGAAC Reverse: GGCTGAGGCCAAGACAAGAT	NM_134454.1
Tie2	Forward: CGGCTTAGTTCTCTGTGGAGTC Reverse: GGCATCAGACACAAGGTAGG	NM_001105737.1
Actina- β	Forward primer: TCCACCCGCGAGTACAACCTTCTT Reverse primer: CGACGAGCGCAGCGATATCGT	NM_031144.2

fragments of articular cartilage from the left femur of 20-day-old rats. These cartilages were collected in TRIzol, immediately frozen in liquid nitrogen for 2 hours, and then stored at -80°C . The extraction of cartilage mRNA was performed by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA concentration in each group was determined by absorbance at 260/280 nm. For the reverse transcription reactions, the commercial kit SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) was used, and each reaction contained 10 μL of $2\times$ RT Reaction Mix, 2 μL RT Enzyme Mix, 6 μL of RNase-free DEPC-treated water, and 2 μL of the RNA at 0.5 mg RNA/ μL . Reverse transcription was performed in a thermal cycler, and the cycling protocol consisted of 25°C for 10 minutes, 42°C for 50 minutes, and 85°C for 5 minutes. Subsequently, 1 μL of RNase H was added to the microtubes, which were again placed in the thermal cycler for 20 minutes at 37°C . The real-time PCR reactions contained 2.5 μL of cDNA, 1.0 μL of each primer, 1.0 μL of ROX, 7 μL of RNase-free DEPC-treated water, and 12.5 μL of SYBR Green reagent in a final volume of 25 μL . Data acquisition and analysis of the real-time RT-PCR assay were performed using the 7500 Real-Time PCR System (Applied Biosystems, Life Technologies). The parameters used for amplification were 50°C for 120 seconds, 95°C for 150 seconds, and 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The primers were designed based on the sequence of *Rattus norvegicus* mRNA (Table 1). Gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method, where the results for each group were compared quantitatively after normalization based on β -actin.

Statistical Analysis

The design was completely random. The mean and standard deviation for each variable were determined. Statistical analysis was carried out using Student's *t* test with GraphPad Prism 6. The gene expression data from real-time RT-PCR

were compared by Student's *t* test after logarithmic transformation of the data. Differences were considered significant if $P < 0.05$.³⁴

Results

Levels of plasma thyroxine and thyroid histomorphometry

Litters ranged from 10 to 14 pups per rat, with an average of 11 pups per rat. There was no significant difference in the litter size in this study (data not shown). The thyroxine treatment increased free T_4 levels in the female rats after 20 days of lactation compared with control rats. Treated rats showed clinical characteristics of hyperactivity and aggressiveness. In the 20-day-old rats, there was no significant difference between the groups in free T_4 plasma levels (Fig. 1).

Thyroids of newborns and 20-day-old rats in all groups showed follicles of different sizes ranging from round to oval. In the control group, the follicles were surrounded with predominantly cuboidal epithelium and filled with dense or sometimes vacuolated colloid. In the offspring of rats treated with L-thyroxine, thyroids presented several follicles surrounded by flattened epithelium. By morphometry, there were no significant differences in the diameter of the follicles between the treated and control groups (Fig. 2). However, newborns and 20-day-old rats in the treated groups showed a significant reduction in the follicular epithelial height compared to the control group of same age (Fig. 2).

Body Weight and Femoral Length

The offspring of female rats treated with thyroxine, at birth and at 20 days of age, showed a significant reduction in body weight (Fig. 3A) and femoral length (Fig. 3B) compared to the control group. However, the femoral width did not differ significantly between the groups (Fig. 3C).

Figure 1. Free T_4 plasma levels (mean $\pm$ SD) in lactating control rats, rats treated with L-thyroxine, and 20-day-old offspring of female rats control and treated with L-thyroxine. Plasma levels of free T_4 are higher in rats treated with thyroxine compared to the control group (*** $P < 0.001$), and plasma levels of free T_4 are similar between 20-day-old control rats and rats treated with L-thyroxine ($P > 0.05$).

Bone Histomorphometry

In newborns of both groups, the distal femoral epiphysis was completely cartilaginous, with no secondary ossification centers and no distinction between the articular cartilage and the growth plate. Therefore, we measured the height of all cartilaginous epiphyses and only the hypertrophic zone of the growth plate. There was no significant difference in the height of the cartilaginous epiphysis between the groups; however, the height of the hypertrophic zone was significantly larger in the treated animals compared to the control group (Fig. 4A, B, and E).

In 20-day-old rats, no significant difference was observed in the height of the articular cartilage, the growth plate, and the resting and hypertrophic zones. However, the height of the proliferative zone was significantly lower in treated animals compared to the control group (Fig. 4A, B, and F).

The femoral trabecular bone was thicker and confluent in newborns and 20-day-old rats in the treated group. Osteoblasts were predominantly bulky and cuboid and had large nuclei. On the surface of trabecular bone, there was one or multiple layers of osteoblasts (osteoblast hyperplasia focus). Osteocytes were active, with large nuclei and large lacunae. Confirming the morphological analysis, the thickness of the trabecular bone and the percentage of trabecular bone in the femoral epiphysis and metaphysis of 20-day-old animals and metaphysis of newborn rats were significantly higher in the treated group compared to the control group ($P < 0.05$) (Fig. 4C, D, E, and F).

Immunohistochemistry Analysis of CDC-47, VEGF, Flk-1, Tie2, and Ang2

The proliferation rate as determined by CDC-47 expression was significantly lower in the chondrocytes of the femoral

cartilaginous epiphysis of newborns and the femoral articular cartilage and growth plate of 20-day-old rats compared to control animals ($P < 0.05$) (Fig. 5A and B).

Immunohistochemical analysis of angiogenic factors showed a significant reduction in area and intensity of VEGF expression in the cartilaginous epiphysis and the hypertrophic zone of the growth plate in newborns in the treated group compared with the control group. We observed a reduction in the area of VEGF expression in the growth plate and hypertrophic zone in 20-day-old rats in the treated group. There was no significant difference between groups in VEGF expression in the articular cartilage (Fig. 6A and B). Flk-1 expression was similar in both newborn groups (stained area: control— $11,764 \pm 1,141$ and treated— $14,050 \pm 410.9$ /integrated density: control— $1,642 \times 10^6 \pm 0.192 \times 10^6$ and treated— $1,643 \times 10^6 \pm 0.216 \times 10^6$). In the 20-day-old rats, there was no Flk-1 expression in the articular cartilage and growth plate of the distal femur (data not shown).

There was no Ang1 staining in the growth cartilage of newborn and 20-day-old rats in the treated and control groups (data not shown). However, Tie2 expression was observed in the newborns. Newborns in the treated group showed a reduced area and intensity of Tie2 expression in the cartilaginous femoral epiphysis compared to the control group (Fig. 7A and B).

Expression of Gene Transcripts for VEGF, Flk-1, Ang1, Ang2, and Tie2

Newborn rats in the treated group showed a significant reduction in VEGF mRNA expression (Fig. 8), and 20-day-old rats in the treated group showed a significant reduction in mRNA expression of VEGF and Flk-1. There was no significant difference between the groups in gene expression of Ang1, Ang2, and Tie2 (Fig. 8).

Discussion

High doses of thyroxine may promote iatrogenic hyperthyroidism.³⁵⁻³⁷ In this study, there was a significant increase in plasma levels of T_4 in female rats treated with L-thyroxine. In weanling rats, there was no significant difference in thyroxine levels between the groups. The hormone analysis was performed only in lactating rats and 20-day-old rats due to the small blood volume that can be collected in newborns; therefore, it was not possible to examine this group. Regarding litter size influencing the plasma concentration of thyroxine, there was no significant difference in the litter size in this study (data not shown). In a research to study the concentrations of thyroid hormones in euthyroid nulliparous rats and in lactating rats with different litter sizes, the nulliparous and postpartum rats not lactating did not differ in serum thyroxine or triiodothyronine concentrations. But the authors observed that litter size increased maternal

Figure 2. Thyroid histomorphometry (mean $\pm$ SD) of newborns and 20-day-old offspring of female rats control and treated with L-thyroxine. **(A)** Follicular diameter (μm) was similar between the groups ($P > 0.05$). **(B and C)** Height reduction of the follicular epithelium (μm) of the thyroid of newborns ($*P < 0.05$) and 20-day-old rats in the treated group compared to the control ($**P < 0.01$). Hematoxylin-eosin; bar: 13.8 μm .

Figure 3. Body weight (g) and length (mm) of the femur of newborns and 20-day-old offspring of female rats control and treated with L-thyroxine. **(A)** Reduced weight of newborns ($***P < 0.001$) and 20-day-old rats ($*P < 0.05$) in the treated group compared to the control. **(B)** Reduction of femur length of newborns ($*P < 0.05$) and 20-day-old rats ($**P < 0.01$) in the treated group compared to the control. **(C)** Similar width of the femur of newborns and 20-day-old rats in the treated and control groups ($P > 0.05$).

Figure 4. Bone histomorphometry of distal femurs of newborn and 20-day-old offspring of female rats control and treated with L-thyroxine. **(A)** Height of the growth cartilage of the femurs of newborns and 20-day-old rats was similar in the treated and control groups ($P > 0.05$). **(B)** Increased hypertrophic zone of the growth plate of the newborns ($*P < 0.05$) and reduced proliferative zone of the growth plate of 20-day-old rats in the treated group compared to control ($*P < 0.05$). **(C)** Increased percentage of metaphyseal trabecular bone of femurs of newborns ($**P < 0.01$) and epiphyseal and metaphyseal trabecular bone of femurs of 20-day-old rats in the treated group compared to the control ($*P < 0.05$). **(D)** Increased thickness of the metaphyseal trabecular bone of femurs of newborns ($****P < 0.0001$) and epiphyseal ($**P < 0.01$) and metaphyseal ($****P < 0.0001$) trabecular bone of femurs of 20-day-old rats. **(E and F)** Images of the hypertrophic zone of the femur of newborns demonstrating increased height of the hypertrophic zone and the growth plate of the femur of 20-day-old rats demonstrating reduced proliferative zone in the treated group (Bar). Images of trabecular bone of the metaphysis of the femur of newborns and epiphysis and metaphysis of the femur of 20-day-old rats demonstrating the increase of the thickness of trabecular bone (arrow), which is surrounded with a layer of cuboidal osteoblasts in the treated group compared to the control, which has trabecular bone surrounded with a layer of flattened osteoblasts. Hematoxylin-eosin; bar: 13.8 μm. EP = epiphysis; GP = growth plate; AC = articular cartilage; MT = metaphysis; RZ = resting zone; PZ = proliferative zone; HZ = hypertrophic zone; SZ = surface zone; ZM = middle zone; DZ = deep zone.

Figure 5. Immunohistochemical detection of CDC-47 expression in the growth cartilage of the distal region of the femurs of newborn and 20-day-old offspring of female rats control and treated with L-thyroxine. **(A)** Reduction in the immunostained cells in the cartilaginous epiphysis of the femurs of newborns (** $P < 0.001$) and the growth plate (** $P < 0.001$) and articular cartilage (* $P < 0.05$) of the femurs of 20-day-old rats in the treated group compared to the control. **(B)** Images of the cartilaginous epiphysis of the femurs of newborns and the growth plate and articular cartilage of the femurs of 20-day-old rats demonstrating more positive cells for CDC-47 (with nuclei stained brown) in all zones in the treated group compared to the control (streptavidin-biotin-peroxidase, Methyl Green; bar: 40 μ m). EP = epiphysis; GP = growth plate; AC = articular cartilage.

Figure 6. Immunohistochemical detection of VEGF expression in the growth cartilage of distal femurs of newborn and 20-day-old offspring of female rats control and treated with L-thyroxine. **(A)** Reduction of the area and integrated density of VEGF expression in the cartilaginous epiphyseal and hypertrophic zone of the growth plate ($P < 0.001$) and decreased VEGF expression in the hypertrophic zone and growth plate in 20-day-old rats from the treated group compared to the control ($P < 0.05$). **(B)** Images of the cartilaginous epiphysis of the femurs of newborns and the growth plate and articular cartilage of the femurs of 20-day-old rats in control and treated groups demonstrating brown cytoplasmic immunostaining of VEGF, illustrating the results shown in **A** (streptavidin biotin-peroxidase, Harris hematoxylin; bar: 40 μ m). EP = epiphysis; GP = growth plate; AC = articular cartilage; HZ = hypertrophic zone.

Figure 7. Immunohistochemical detection of Tie2 expression in cartilaginous epiphysis of the distal femur of newborns of female rats treated with L-thyroxine and control. **(A)** Intense reduction of the area and Tie2 expression intensity in the hypertrophic zone and cartilaginous epiphysis of the femoral group of newborns treated compared to the control group ($***P < 0.0001$). **(B)** Images of the hypertrophic zone of growth plate and cartilaginous epiphysis brown cytoplasmic immunostaining of Tie2 illustrating the results presented in **(A)** (streptavidin-biotin-peroxidase, Harris hematoxylin; bar: 40 μ m). EP = epiphysis; HZ = hypertrophic zone.

Figure 8. Expression of gene transcripts for VEGF, Flk-1, Ang1, Ang2, and Tie2 by real-time RT-PCR of the cartilaginous epiphysis of newborns and the articular cartilage of the distal femurs of 20-day-old offspring of female rats control and treated with L-thyroxine. Reduction in VEGF mRNA expression in the cartilaginous epiphysis of newborns ($P < 0.05$) and in the articular cartilage in 20-day-old rats ($P < 0.001$) and reduced FLK-1 expression in the articular cartilage of 20-day-old rats ($P < 0.05$).

serum thyroxine and triiodothyronine concentrations. In pups of 12 days, serum triiodothyronine concentrations decreased as litter size increased, but serum thyroxine concentrations were not affected.³⁸

It is known that the half-life of thyroxine administered in thyroidectomized rats is 1.2 days.³⁹ Thus, the dose administered in female rats is sufficient to maintain the elevated status of thyroxine; however, maternal transfer through the placenta and milk cannot be sufficient to increase the placental thyroxine, but it changed the morphology of thyroid epithelium and the growth plate, which proves the action of the hormone received from mother.

Thyroxine treatment of lactating female rats had an effect on the thyroid epithelium of newborns and weanling rats because these animals showed significantly reduced follicle epithelial height compared to the controls. The morphological appearance of thyroid follicles is closely related to their functional activity.⁴⁰ For this reason, histomorphometric analysis of the thyroid is important in assessing the morphological variations, and the height of the follicular epithelium is a significant marker for determining changes in glandular function.³⁰

The excess maternal thyroxine may result in neonatal hyperthyroidism depending on the dose, followed by atrophy of the thyroid, reduced size and number of follicles, and flattening of the follicular epithelium.⁷ Fetal hyperthyroidism as a result of maternal hyperthyroidism is uncommon^{41,42} and may cause morphological changes in the fetal thyroid by reducing TSH in the offspring, resulting in a loss of the stimulatory effect of this hormone in the thyroid.⁷ Furthermore, exposure of the fetal hypothalamic-pituitary-thyroid system to high concentrations of maternal thyroxine, even in the absence of hyperthyroidism in the fetus, can impair the physiological maturation of the fetal gland because there is a continuous decrease in T_4 and TSH during development.⁴³ In this study, we cannot confirm that there was hyperthyroidism in offspring, but based on the morphometric analysis of the thyroid, we showed the effect of excess maternal thyroxine on the thyroid of newborns and 20-day-old rats due to the passive transfer of the mother's thyroxine through the placenta and milk.

In this study, newborn and 20-day-old rats from mothers with hyperthyroidism showed reduced body weight. Apparently, the association between thyrotoxicosis and the increased risk of low birth weight in newborns and children may be similar to that observed in adults with excess thyroid hormones and an increase in metabolism.^{44,45}

In humans, the excess maternal thyroid hormone, even if it is not accompanied by hyperthyroidism in the offspring, has harmful effects on fetal growth.^{44,46} Several metabolic changes have been reported to be induced by maternal thyroid dysfunction during pregnancy and lactation.^{45,47-50} Similar to the results reported in this study, several authors have reported fetal growth retardation and low birth weight

associated with maternal hyperthyroidism, but the factors that may be involved in these changes have not been identified.^{45,47,48,50}

As shown by the femoral morphometric analysis, newborn rats treated with thyroxine showed a significant increase in the height of the hypertrophic zone and at 20 days had a reduction in the height of the proliferative zone of the growth plate. Physiologically, the effects of thyroid hormones can occur in different areas of the growth cartilage, for example, stimulating clonal expansion of resting chondrocytes and the subsequent differentiation of chondrocytes in the hypertrophic zone.⁵¹ Thus, the excess maternal thyroxine affected the maturation of the cartilage, which showed an increase in the hypertrophic zone of the growth plate in the offspring of rats treated with thyroxine. Conversely, rats with a mutated TR α 1 receptor (TR α 1 L400R) that has a dominant-negative phenotype in chondrocytes similar to hypothyroidism show a disorganized and reduced growth plate, mainly in proliferative and hypertrophic zones, likely due to reduced clonal expansion of progenitor cells.⁵²

We observed that the excess maternal thyroxine resulted in a significant reduction in the proliferation rate of chondrocytes in the cartilaginous epiphysis of newborns and reduced the height of the proliferative zone of 20-day-old rats. These actions are either direct or mediated through interactions with other signaling pathways. In particular, T_3 has been shown to control or interact with pathways involved in the pace of chondrocyte proliferation and differentiation.⁵² For example, in embryonic chick limb-bone rudiments the effect of T_3 on the radius, which is slowly growing, is to stimulate growth, whereas the same amount of T_3 decreased the growth rate of the third metatarsus, which is normally a fast growing bone. In the other words, T_3 can cause stimulation or retardation of bone growth, which may differ from bone to bone.⁵³

In vitro, T_3 decreased the growth of chondrocyte colonies and inhibited cell proliferation.^{51,54-56} T_3 stimulates clonal expansion of chondrocyte progenitor cells but inhibits subsequent cell proliferation,^{51,57} while promoting hypertrophic chondrocyte differentiation and cell volume expansion. However, the mechanisms responsible for these effects are still poorly understood due to the complex interactions of chondrocytes with other cells, as well as the interactions of thyroid hormones with other molecules with autocrine and paracrine actions.⁵⁸ Thus, the inhibition of chondrocyte proliferation in the growth cartilage may be the cause of the reduced femur length of the offspring of rats treated with thyroxine.

Excess maternal thyroxine showed opposite effects on the bone and cartilage tissue of the offspring. An increase in the percentage and thickness of trabecular bone was observed in the offspring of treated rats. Similar to the results of this study, hyperthyroidism in children is

associated with increased bone apposition, inhibition of chondrocyte proliferation, and early closure of the growth plates, resulting in short stature.⁵⁹ In adult skeletons, excess thyroid hormone results in increased bone remodeling, stimulating both bone apposition, and bone resorption, with the catabolic process predominating.^{11,14,60} Thus, it is possible to suggest that bone remains shorter even with the trabecular thickness increasing in the treated group because there may have been supremacy of the bone apposition process compared to bone resorption. *In vivo* experiments demonstrated that thyroxine administration in rats reversed osteopenia caused by castration or lactation, along with activation of osteoblasts and an increase of the percentage of trabecular bone.^{3,30}

Excess thyroid hormones can cause negative effects and diseases in the skeletons of children and adults.⁶¹ It is likely that the thickness and percentage increase of trabecular bone in the offspring of rats treated with thyroxine may be due to increased osteogenic differentiation of stem cells promoted by thyroid hormones as demonstrated *in vitro* and *in vivo*.^{13,62,63} So the increased bone apposition can also be explained by the direct influence of T₃ and T₄ on osteoblasts by stimulating the expression of proteins that are important for bone apposition, such as collagen X, alkaline phosphatase,⁶⁴ and osteocalcin.⁶⁵ In this study, excess thyroid hormone during pregnancy and lactation decreased VEGF expression in chondrocytes as shown by immunohistochemical and molecular analyses. This result differs from what has been observed in other tissues, where thyroid hormones were shown to have pro-angiogenic effects in the heart⁹ corpus luteum,⁸ and placenta⁶⁶ as well as in tumors.⁶⁷ However, the mechanisms by which thyroid hormones contribute to angiogenesis are not fully understood.⁶⁸ Our results demonstrate that lower expression of VEGF on chondrocytes may be one of the mechanisms involved in the reduction of femur length in the treated group. The excess maternal thyroxine affected the gene expression of Flk-1 only in 20-day-old rats. One explanation for this result is that longer exposure to excess maternal thyroid hormones can reduce the VEGF receptor expression. However, newborn rats treated show less growth even in the absence of changes in Flk-1 expression.

The relationship between reduced VEGF expression, shorter bone length, and the increased hypertrophic zone of the growth plate has been reported by other researchers. Rats with induced traumatic injury in the growth plate of the tibia and local administration of VEGF inhibitors showed a reduction in the longitudinal bone growth and increases in the hypertrophic zone without a change in the size of the resting and proliferative zones. It was also demonstrated that VEGF-A knockout mice or VEGF inhibition also delayed endochondral bone growth with reduced proliferation and expansion of the hypertrophic zone,⁶⁹ similar to the changes observed in this study.

VEGF is critical for angiogenesis, increased capillary permeability, and the subsequent differentiation of chondrocytes during endochondral bone formation,⁷⁰⁻⁷² promoting the oxygen supply and aiding in the transport of nutrients.⁷³ Therefore, it is possible that the inhibition of VEGF expression in the chondrocytes in combination with excess maternal thyroxine can contribute to the reduction in cell proliferation observed in the treated group.

Although Ang2 mRNA expression was detected, no protein expression was observed in the growth cartilage of rats by immunohistochemistry. In pig fetuses, Ang2 staining was found in regions of endochondral ossification, while Ang1 was not detected.⁷⁴ Expression of Ang1 and Ang2 was detected in the growth plate of newborns using indirect immunofluorescence, but they were absent in the rest of the region and were increased in differentiated chondrocytes, which were mainly adjacent to blood vessels.²⁶ Ang2 expression is the most intense in the proliferation and hypertrophic zones. However, Ang1 and Ang2 expressions in the growth cartilage of rats has not been studied. VEGF showed enhanced expression during bone growth, and both Ang1 and Ang2 are coexpressed with VEGF in the endochondral ossification sites and in areas of bone remodeling as shown by indirect immunofluorescence analysis.²⁶ We observed mRNA expression of Ang1, Ang2, and Tie2, but it was not significantly different between the control and treated groups. No Ang2 immunostaining was observed in the growth cartilage, but mRNA levels do not always coincide with the protein levels. The amount of protein present in a cell depends not only on the level and frequency of transcription and translation but also on protein degradation and transport out of the cell.⁷⁵

In newborn rats treated with thyroxine, there was a reduction in Tie2 immunoreactivity. Although the angiopoietins are ligands of the Tie2 receptor, it was demonstrated that in endothelial cells from human umbilical veins, VEGF activates Tie2 via a mechanism involving proteolytic cleavage of the associated tyrosine kinase Tie1, leading to transphosphorylation of Tie2.⁷⁶ It has been shown that Ang1 and Ang2 could modulate the postnatal neovascularization induced by VEGF.⁷⁷ However, the VEGF-VEGFR and Ang-Tie signaling pathways regulate different aspects of angiogenesis.⁷⁸ Tie2 assists in maintaining the integrity of mature vessels; thus, loss of Tie2 signaling can lead to destabilization of the blood vessels.⁷⁹ Therefore, it can be suggested that reduced expression of VEGF and Tie2 in the growth cartilage is associated with reduced angiogenesis. Studies in zebra fish demonstrated the synergistic action between Tie2 and VEGF to control angiogenesis and the importance of Tie2 expression in the induction of Flk-1 by VEGF.⁸⁰ However, this relationship has not been fully elucidated in bone growth, and studies of angiopoietin-Tie signaling in growth cartilage and its influence in the presence of VEGF are needed.

It was shown that excess maternal thyroxine during pregnancy and lactation reduced endochondral bone growth in the progeny and reduced the proliferation rate and VEGF, Flk-1, and Tie2 expression in the cartilage of growing rats without altering the mRNA expression of Ang1 and Ang2.

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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.

Ethical Approval

Ethical approval for this study was obtained from the Ethics Committee on Animal Experiments of UFMG (Protocol No. 47/2014).

Animal Welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

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