Effects of excess maternal thyroxin on the bones of rat offspring from birth to the post-weaning period

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ABSTRACT

Objective: To evaluate, in rat offspring, bone changes induced by excess maternal thyroxin during pregnancy and lactation, and to assess the reversibility of these changes after weaning. **Material and methods:** Twenty Wistar rats were distributed in two groups, hyperthyroid and control, that were treated daily with L-thyroxin (50 mcg/animal) and placebo, respectively. The treatment was initiated seven days before mating and continued throughout pregnancy and lactation. From every female of each of the two groups, two offspring were euthanized after birth, two at 21 days of age (weaning), and two at 42 days of age (21 days after weaning). In newborns, the length of pelvic and thoracic limbs were measured, and in the other animals, the length and width of the femur and humerus were measured. Bones were dissected, decalcified, embedded in paraffin, and analyzed histomorphometrically. **Results:** Excess maternal thyroxin significantly reduced the length of the pelvic limb in neonates. In 21-day-old individuals, excess maternal thyroxine reduced the length and the width of the femur and the humerus. It also increased thickness of the epiphyseal plate and the percentage of trabecular bone tissue. In 42-day-old individuals, there were no significant differences between groups in relation to the parameters evaluated in the previous periods. **Conclusion:** Excess maternal thyroxine reduced growth in suckling rats both at birth and at weaning, and it also increased the percentage of trabecular bone tissue in 21-day-old animals. These changes, however, were reversible at 42 days, i.e., 21 days after weaning. Arch Endocrinol Metab. 2016;60(2):130-7

Keywords
Maternal thyroxin; bone growth; reversibility; rats

INTRODUCTION

Thyroid hormones, represented by thyroxin (T4), and triiodothyronine (T3), have important roles in the different body tissues since embryonic stages (1), particularly in bone metabolism in processes such as pre-natal bone formation, post-natal bone growth (2), and balance between bone deposition and resorption (3).

Hypothyroidism and hyperthyroidism are common disorders, and are normally observed in women at reproductive age. Although less frequent, thyroidal dysfunctions are increasingly being diagnosed in pregnant women (4). As a consequence, maternal hormone changes may lead to abnormal fetal development, once thyroidal maternal hormones have a critical action in all systems, particularly in pre-natal bone formation (5).

During pregnancy, embryonic and fetal tissues are exposed to maternal thyroidal hormones (1) and in spite of the autonomy of the fetal thyroid, excess maternal thyroidal hormones may affect the ability of the fetus to regulate TSH and T4 levels (6). Therefore, maternal hyperthyroidism may be associated with intra-uterine death, spontaneous miscarriage, premature delivery, and low birth weight (5,7), as well as changes in bone maturity and early interruption of bone growth with premature fusion of growth plates and bone sutures, and low stature (8).

Excess thyroid hormones are also shed in milk, and offspring may be exposed to maternal hormone dysfunction during lactation (9). Because of that, thyroidal dysfunctions in pregnant and lactating females may change the normal course of fetal and post-natal offspring development, once these hormones have critical roles in several systems, mainly in skeleton formation and maturation (2).

The effects of congenital hypothyroidism on bone growth have been widely studied, mainly due to the higher frequency and more evident consequences, such

as dwarfism and mental retardation, among others (10). However, congenital hyperthyroidism also has important impacts on growth (8). In children and mice it increases bone maturation and causes early growth interruption of epiphyseal plates and bone sutures (2,8,11).

Although hyperthyroidism is a common endocrinopathy in women at reproductive age, with possible severe changes in the skeleton of animals in the first years of life (12), the effects of excess maternal thyroidal hormones on offspring bone formation and growth are little understood. It is know that the administration of T4 to pregnant rats affects the thyroid of the fetus, reducing the levels of T4 and T3 and the size of the offspring (13). However, bone changes caused by maternal hyperthyroidism in offspring at different ages, as well as the possible reversibility of these effects, have to be better understood. Therefore, the objective of this study was to evaluate, using histomorphometry, the occurrence of bone changes in the offspring of female rats that had excess thyroxin during pregnancy and lactation, and to assess if these changes are reversible after weaning, when offspring are not in contact with maternal thyroidal hormones anymore.

MATERIAL AND METHODS

Thyroxin treatment and mating

Twenty 2-month-old Wistar rats weighting 200 g in average were divided into two groups (hyperthyroid, n = 10; and control, n = 10). Rats were placed in plastic cages and received water *ad libitum* and commercial feed (22% crude protein, 1.4% calcium, 0.6% phosphorus and micronutrients). Animals were kept under a 12-hour light and 12-hour dark light cycle. All experimental procedures were approved by the Ethics Committee In animal Research at Universidade Federal de Minas Gerais (UFMG) (protocol no. 47/2014).

After seven days of adaptation, rats in the treated group received L-thyroxin (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/animal, diluted in 5 mL distilled water. The control group received 5 mL of distilled water as a placebo treatment. In both groups, animals were treated daily at the same time of the day using an orogastric tube, according to protocols determined elsewhere (14,15). Before mating, five rats of each group were anesthetized (2.5% Thionembuthal), followed by intracardiac puncture for serum samples that were used in free T4 determination in commercial chemiluminescence kits (Immulite Free T4, Siemens Medical Solutions Diagnostics, Malvern, PA, USA).

After it was confirmed that treated rats showed free T4 levels significantly higher than the control animals, the other rats of the group were analyzed by vaginal cytology to determine phase of the estrus cycle. Animals in estrus or pro-estrus were placed in cages with adult male rats (three females for each male) for 12 hours. In the following morning, mating was confirmed by the identification of spermatozoa in vaginal smears, which was considered day zero of pregnancy. Thyroxin treatment began seven to nine days before mating and was maintained during the whole pregnancy and lactation, that is, until 21 days after delivery.

At the moment of weaning, 21 days after delivery, five females per group were again subjected to general anesthesia (2.5% Thyonembutal) followed by blood collection for free T4 determination using the same method described above, in order to prove that rats treated with thyroxin presented free T4 levels significantly higher than those of the control animals.

From each female of the two groups, two offspring were euthanized after birth, two at 21 days of age (weaning), and two at 42 days of age (21 days after weaning). Offspring were euthanized with an association of ketamine (40 mg/kg) and Xylazine hydrochloride (10 mg/kg) by subcutaneous route. Female rats were euthanized using the same association of anesthetics. Therefore, six groups were formed: 1) neonates of thyroxin-treated females (n = 8); 2) neonates of control rats (n = 8); 3) 21-day-old offspring of thyroxin-treated females (n = 8); 4) 21-day-old offspring of control females (n = 8); 5) 42-day-old offspring of thyroxin-treated females (n = 8); and 6) 42-day-old offspring of control females (n = 8).

In the three moments of analysis, that is, at birth, at 21 days of age (weaning), and at 42 days of age, body weight of the offspring was determined and means of the different groups were compared.

In neonates (groups 1 and 2), the length of the thoracic limb was measured from the proximal end of the humerus to the distal end of the phalanges, and the length of the pelvic limb was measured from the proximal end of the femur to the distal end of the phalanges. In the rest of the offspring, length was measured from the head of the humerus to the articular surface of the humeral condyles, and from the proximal to the distal epiphysis of the femur, whereas the width was measured in the central diaphysis of the bones. All measurements were carried out with a pachymeter.

Histomorphometric analysis

Thoracic and pelvic limbs were collected from the euthanized neonates. Bones were dissected without being disarticulated. In animals that were 21 and 42 days old, the right femur and left humerus were individually dissected. Bones were fixed in neutral, buffered formalin 10% for 24 hours. Later on, they were decalcified in formic acid solution 21% for about 30 days. The decalcifying solution was changed every 48 hours. After decalcification, bones were processed by paraffin embedding, and cut in a microtome in 4 µm-sections that were stained by hematoxylin-eosin (HE).

In neonates of both groups, the percentage of condrocytes and the cartilaginous matrix was measured in three fields of the distal epiphysis of the femur, and in three fields of the proximal epiphysis of the tibia. In the proximal metaphysis of the tibia, the percentage of trabecular tissue was determined in a field that took up this whole area. These assessments were carried out with the aid of a 121-point grid coupled to the ocular of an optical microscope at 40x magnification.

In 21 and 42-day-old offspring, the percentage of trabecular bone tissue was determined in the distal epiphysis and diaphysis of the femur, and in the proximal epiphysis and diaphysis of the humerus. These assessments were carried out with the aid of a 121-point grid coupled to the ocular of an optical microscope at 40x magnification. In these animals, the thickness of articular cartilages and epiphyseal plates of the humerus and femur were also determined using the mean thickness of 15 equidistant points. The thickness of the proliferative, columnar, and hypertrophic zones were determined by the mean thickness of 10 equidistant points throughout the epiphyseal plate. This assessment was carried out with the aid of a micrometric objective provided with a ruler and coupled to the microscope at 10x magnification. These values were later on transformed in millimeters using the scale of a microscopic ruler.

Statistical analysis

A completely randomized design was used in the study, and means and standard deviations were determined for each variable. The analysis of variance and comparison of the means was carried out with Student *t* test. Differences were considered to be significant at $p < 0.05$ (10). Statistical analysis was carried out in GraphPad InStat® software.

RESULTS

Rats in the treated group showed T4 levels significantly higher than the control group, both before mating and at weaning (Table 1).

Body weight of offspring of females treated with thyroxin, at birth and at 21 days, was significantly lower than that of offspring of control females (Tables 2 and 3). However, at 42 days, that is, 21 days after weaning, there was no statistical difference between the groups (Table 4).

Table 1. Mean, standard deviation, and p value of free T4 serum levels in the control females and those treated with thyroxin before mating and at weaning

Free T4 (ng/dL)	Control group (n = 10)	Group treated with thyroxin (n = 10)	p value
Before mating	1.20 ± 0.12	4.36 ± 1.36	0.0003*
At weaning	0.91 ± 0.02	1.26 ± 0.06	0.0002*

Values expressed as means ± standard deviation.
*: significant, by Student t test ($p \leq 0.05$).

Table 2. Mean, standard deviation, and p value of the variables analyzed in neonates born of control females and females treated with thyroxin

Variables	Control group (n = 10)	Group treated with thyroxin (n = 10)	p value
Body weight (g)	6.79 ± 0.14	5.50 ± 0.26	0.0007*
Length of the pelvic limb (cm)	2.19 ± 0.11	2.04 ± 0.12	0.0130*
Length of the thoracic limb (cm)	2.05 ± 0.07	2.02 ± 0.10	0.3955 NS
Chondrocytes of distal femur CE (%)	60.12 ± 6.35	60.97 ± 4.78	0.7863 NS
Chondrocytes of proximal tibia CE (%)	61.70 ± 7.64	65.43 ± 3.2	0.2839 NS
Trabecular bone tissue tibia PM (%)	42.12 ± 10.16	47.75 ± 28.67	0.5925 NS

Values expressed as means ± standard deviations; CE: cartilaginous epiphysis; PM: proximal metaphysis.
*: significant, NS: non-significant, by Student *t* test ($p \leq 0.05$).

Table 3. Mean, standard deviation, and p value of the variables analyzed in 21-day-old rats (at weaning) born of control females and females treated with thyroxin

Variables	Control group (n = 10)	Group treated with thyroxin (n = 10)	p value
Body weight (g)	43.15 ± 1.56	37.54 ± 1.36	0.0168*
Length of the femur (mm)	16.90 ± 0.30	16.50 ± 0.44	0.0205*
Width of the femur (mm)	2.13 ± 0.23	1.86 ± 0.23	0.0127*
Length of the humerus (mm)	14.95 ± 0.15	14.70 ± 0.42	0.0962 NS
Width of the humerus (mm)	1.60 ± 0.21	1.15 ± 0.24	0.0003*
Femur AC thickness (mm)	0.55 ± 0.10	0.58 ± 0.04	0.5988 NS
Humerus AC thickness (mm)	0.59 ± 0.12	0.67 ± 0.07	0.1531 NS
Femur EP thickness (mm)	0.77 ± 0.10	0.98 ± 0.15	0.0359*
Humerus EP thickness (mm)	0.59 ± 0.08	0.69 ± 0.03	0.0099*
Femur PZ thickness (mm)	0.14 ± 0.02	0.23 ± 0.05	0.0070*
Femur CZ thickness (mm)	0.23 ± 0.02	0.34 ± 0.05	0.0036*
Femur HZ thickness (mm)	0.33 ± 0.05	0.47 ± 0.04	0.0026*
Humerus PZ thickness (mm)	0.13 ± 0.02	0.25 ± 0.03	0.0001*
Humerus CZ thickness (mm)	0.17 ± 0.02	0.21 ± 0.02	0.0111*
Humerus HZ thickness (mm)	0.18 ± 0.02	0.28 ± 0.02	0.0001*
Trabecular bone tissue of femur DE (%)	23.56 ± 2.80	43.04 ± 2.17	0.0001*
Trabecular bone tissue femur DM (%)	36.70 ± 5.20	49.08 ± 2.81	0.0016*
Trabecular bone tissue humerus PE (%)	24.38 ± 7.92	41.68 ± 6.95	0.0010*
Trabecular bone tissue humerus PM (%)	38.11 ± 4.11	51.58 ± 4.94	0.0001*

Values expressed as means ± standard deviations; AC: articular cartilage; EP: epiphyseal plate; DE: distal epiphysis; DM: distal metaphysis; PE: proximal epiphysis; PM: proximal metaphysis.
*: significant; NS: non-significant, by Student *t* test ($p \leq 0.05$).

Table 4. Mean, standard deviation, and p value of the variables analyzed in 42-day-old rats born of control females and those treated with thyroxin

Variables	Control group (n = 10)	Group treated with thyroxin (n = 10)	p value
Body weight (g)	143.90 ± 4.89	145.20 ± 4.24	0.8334 NS
Length of the femur (mm)	27.66 ± 0.50	27.45 ± 0.85	0.5181 NS
Width of the femur (mm)	3.05 ± 0.16	3.04 ± 0.15	0.8885 NS
Length of the humerus (mm)	21.22 ± 0.44	21.70 ± 0.63	0.0762 NS
Width of the humerus (mm)	2.05 ± 0.16	2.001 ± 0.003	0.3137 NS
Femur CA thickness (mm)	0.25 ± 0.04	0.29 ± 0.06	0.1568 NS
Humerus CA thickness (mm)	0.29 ± 0.04	0.32 ± 0.04	0.1882 NS
Femur EP thickness (mm)	0.60 ± 0.22	0.56 ± 0.08	0.5456 NS
Humerus EP thickness (mm)	0.39 ± 0.05	0.40 ± 0.06	0.8072 NS
Trabecular bone tissue of femur DE (%)	34.25 ± 4.91	36.66 ± 5.59	0.3454 NS
Trabecular bone tissue of femur DM (%)	44.31 ± 3.58	45.44 ± 8.44	0.7265 NS
Trabecular bone tissue of humerus PE (%)	29.66 ± 5.55	33.33 ± 6.71	0.2024 NS
Trabecular bone tissue of humerus PM (%)	39.83 ± 7.26	43.01 ± 11.18	0.5246 NS

Values expressed as means ± standard deviation CA: articular cartilage; EP: epiphyseal plate; DE: distal epiphysis; DM: distal metaphysis; PE: proximal epiphysis; PM: proximal metaphysis.
NS: non-significant, by Student *t* test ($p \leq 0.05$).

In neonates, there was no significant difference between the groups in the length of the thoracic limb. However, the pelvic limb of the offspring of females treated with thyroxin was significantly shorter than the pelvic limb of control animals. This difference, though, was not reflected in the histomorphometric analysis, once the mean percentage of chondrocytes in the cartilaginous epiphyses of the femur and tibia were si-

milar between the groups. Similarly, there was no significant difference between the groups in relation to the percentage of trabecular bone tissue in the proximal metaphysis of the tibia (Table 2). Offspring of females treated with thyroxin that remained with their mothers during the whole lactation showed significant differences both in bone length and in all histomorphometric variables, except in relation to the thickness of the articular cartilage of the femur and the humerus (Table 3).

The length and width of the long bones were significantly smaller in the group treated with thyroxin. In these animals, the epiphyseal plates were much thicker, with significant difference between the groups, as proven by the histomorphometric analyses (Table 3). The proliferative, columnar, and hypertrophic zones were larger, with many hypertrophic chondrocytes (Figure 1).

Bone trabeculae of the metaphysis region immediately below the epiphyseal plate showed to be thicker and more confluent, and were covered by large osteoblasts and foci of osteoblastic hyperplasia (Figure 2). The percentage of trabecular bone tissue both in the metaphysis and the epiphysis of the bones was significantly greater in offspring of females treated with thyroxin (Table 3). Thickness of the articular cartilage was not significantly different between the groups, but similar to the epiphyseal plate, the articular cartilage of the humerus and femur of the offspring of females treated with thyroxin showed increased number of chondrocytes in the hypertrophic zone (Table 3).

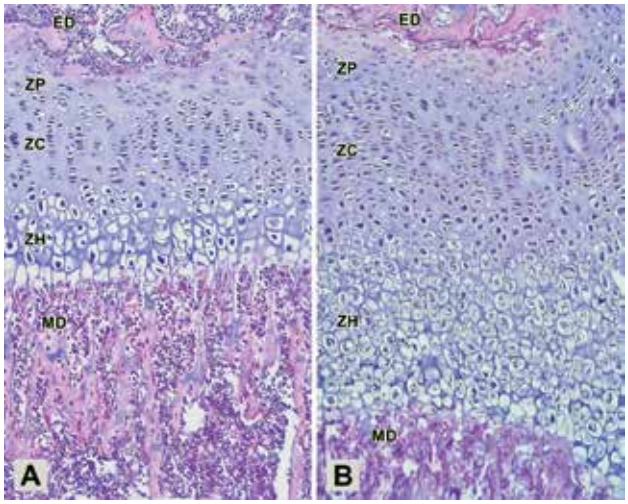

Figure 1. Photomicrograph of a transversal section of the epiphyseal plate/metaphysis of the distal femur of rats at 21 days of age, born of control females (**A**) and of females treated with thyroxin (**B**), HE, 25x. **A**) Control group, showing the distal epiphysis (DE), proliferative zone (PZ), columnar zone (CZ), hypertrophic zone (HZ), distal metaphysis (DM). **B**) Group treated with thyroxin showing increased thickness of the epiphyseal plate and increased number of chondrocytes in the hypertrophic zone (HZ).

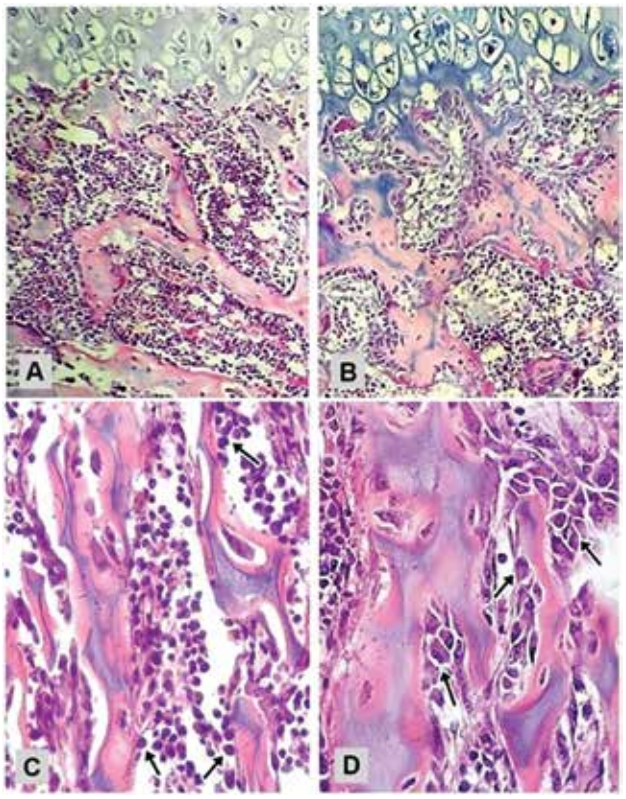

Figure 2. Photomicrograph of proximal epiphysis of the humerus (**A**, **B**) and the distal metaphysis of the femur (**C**, **D**) of 21-day-old rats born of control females (**A** and **C**), and females treated with thyroxin (**B** and **D**), HE, 25x (**A** and **B**) and 100x (**C** and **D**). **A**) Control group showing proximal epiphysis with thin and disconnected trabeculae; **B**) Groups treated with thyroxin showing thick and confluent trabeculae; **C**) Control group with trabeculae covered with cuboid osteoblasts (arrows); **D**) Groups treated with thyroxin with large osteoblasts and foci of osteoblastic hyperplasia (arrows).

In animals that were 42 days old, that is, 21 days after weaning, there were no significant differences between the groups in the length and width of the bones, as well as in the other variables studied (Table 4).

DISCUSSION

This study seems to be the first one to evaluate, by histomorphometry, bone changes in offspring of rats with excess thyroxin during pregnancy and lactation, and that assessed the reversibility of these changes after weaning.

Bone changes caused by excess maternal thyroxin were more expressive in offspring at 21 days of age, that is, at weaning. It is known that bone responses related to experimental hyperthyroidism depend on the dose of thyroxin and the course of the disease (14). Therefore, this result is probably due to the fact that these animals were affected by excess maternal thyroid

hormones throughout pregnancy and lactation, once the passage of these hormones to the placenta (16) and milk (9) has been proven, with exposure of the offspring to the interference caused by these hormones.

In neonates, due to the difficulty in dissecting and isolating each long bone, the measurement of total length of the limbs was chosen instead. The shorter length of pelvic limbs in offspring of rats treated with thyroxin reflects intrauterine changes in bone formation and development. Besides, in neonates, the epiphyses of long bones are still cartilaginous, without a distinction between the articular cartilage and the epiphyseal plate. Because of that, the thickness of each of these structures was not measured. Therefore, more studies based on molecular biology techniques are necessary to determine the mechanisms by which maternal hyperthyroidism changes neonate bones, once it has been established that any interference in chondroblast proliferation, differentiation, and maturation may affect endochondral formation and growth.

At 21 days of age, the bones of offspring of rats treated with thyroxin were shorter, with thicker epiphyseal plates, and increased number of chondrocytes in the hypertrophic zone. These findings demonstrate the sensitivity of the developing skeleton, particularly of growth cartilages, to maternal thyroid hormones, in spite of the apparent absence of hyperthyroidism in the offspring.

Maternal hyperthyroidism may also be associated with fetal and neonatal transitory hyperthyroidism. But this change is not frequent, generally occuring in 1% of the neonates of hyperthyroid mothers (17,18). Low casuistry of persistent congenital hyperthyroidism has also been described. The results of this study, in relation to 21-day-old animals, are different from those observed in congenital hyperthyroidism, in which early closing and maturation of epiphyseal plates led to short stature and differences in bone constitution (8). In the present study, epiphyseal plates were not closed with the fusion of epiphysal and metaphyseal trabeculae.

The epiphyseal plate is made up by two zones that have different morphofunctional characteristics (19-21), and is affected by several hormones, growth factors, and genes (22-27). However, in spite of the years of research on bone formation and growth, the mechanisms by which specific transcription factors affect the development of the epiphyseal plate and formation of trabecular bones are still unclear, and the role of hypertrophic chondrocytes in the morphogenesis of the trabecular bone is still controversial. As corroborated by data in the literature, the increased number of cells in the hypertrophic zone may be a reflex of increased cell maturation. However, the mechanisms by which excess maternal thyroxin causes these changes also need to be elucidated, once several factors act in the hypertrophic zone, including angiogenic ones, such as VEGF, which stimulates vascular invasion of the epiphyseal plate, and Runx, important in the synthesis of the bone matrix that replaces the cartilaginous matrix (25,26,28,29).

Another change was a significant increase in the percentage of trabecular bone tissue in weaned animals that were born of rats treated with thyroxin. This finding was described in adults rats with hyperthyroidism (14). According to some researchers, thyroid hormones stimulate osteoblast synthesis and, thus, the voluminous aspect observed in the osteoblasts of offspring of rats treated with thyroxin may be a reflex of this action (30,31). Besides, a recent study demonstrated that stem cells of the bone marrow of hyperthyroid female rats show greater potential for differentiation in osteoblasts (31), justifying the presence of areas of hyperplasia and the consequent increase in the percentage of trabecular bone tissue observed in animals in this study.

The significant reduction in body weight of the neonates and 21-day-old offspring of rats treated with thyroxin corroborates literature data, once maternal hyperthyroidism is detrimental to fetal development, changing body weight and size of the offspring after birth, both in humans (1,32) and rats (13). In some cases, reduced bone growth in the offspring may be a result of the inadequate milk intake that is a consequence of behavioral changes in females rats with hyperthyroidism. However, this possibility was ruled out in this study as rats treated with thyroxin showed hyperactive behavior but offspring did not show changes caused by undernutrition, such as osteopenic disease, that would be evidenced by the microscopic analysis of the bones. This analysis, instead, showed increased percentage of trabecular bone tissue in the suckling rats. Therefore, it is suggested, once more, that changes in body and bone mass evidenced in offspring of rats treated with thyroxin may be caused by contact with excess maternal thyroid hormones.

Surprisingly, animals that were 42 days of age and were weaned at 21 days, thus having had no contact with maternal thyroid hormones for 21 days, showed means equivalent to those of offspring of control mothers. Therefore, it may be considered that changes ob-

served in neonates and mainly in suckling rats that were 21 days old were reversible at 42 days of age, when contact with the mother was not occurring anymore. There are few studies on the long-term effects of congenital hyperthyroidism (32) and, apparently, there is no difference in body growth and intellectual development of humans born from hyperthyroid or euthyroid mothers (33,34). However, little has been studied in relation to bone changes. It is known that the length and width of bones are good parameters to analyze possible changes in bone growth (35). However, the absence of differences between the two groups in relation to these and the other parameters studied does not rule out the presence of some constitutional or conformational bone disease caused by failure in the formation of delicate bone structures or in skeleton biodynamics. These factors were not studied and need to be investigated before confirming the complete reversibility of the consequences of excess maternal thyroxin on the bone of the offspring.

It is concluded that excess maternal thyroxin reduced growth in rat offspring at birth and at weaning, increased the percentage of trabecular bone tissue of suckling rats, and that these changes were reversible 21 days after weaning.

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