Histological evaluation of critical size bone repair treated with xenogen graft in rats induced to hypothyroidism

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Abstract / This study aimed at assessing bone repair of critical-size defects by comparing normal animals with hypothyroid animals, with or without bone graft (Bio-Oss® Geistlich Pharma AG, Wolhusen, Switzerland) at two times of evaluation (30 and 60 days). Forty-two Wistar rats were used and divided into two major groups, namely: Group 1: Euthyroid, without graft, 30 days (G1E30); euthyroid, without graft, 60 days (G1E60); hypothyroid, without graft, 30 days (G1H30) and hypothyroid, without graft 60 days (G1H60). Group 2: Euthyroid, grafted, 30 days (G2E30); euthyroid, grafted, 60 days (G2E60); hypothyroid, grafted, 30 days (G2H30) and hypothyroid, grafted, 60 days (G2H60). The animals were induced to hypothyroidism by propylthiouracil (PTU) diluted with drinking water. Critical-size defects were created by trephine burs in the rats' calvarium. Treatment was performed to prepare histological slides and analysis as well as to carry out statistical tests. 95% confidence interval ($P < 0.05$) was employed. Results revealed no statistically significant differences in cortical repair between hypothyroid and euthyroid animals at both times of evaluation. However, statistically significant differences were found in comparing 30 x 60 days (G1E60> G1E30, p = 0.01 G1H60> G1H30 and p = 0.01 G2h60> G2H30). Bone formation around graft particles was not statistically different when groups with the same time of evaluation were compared. Nevertheless, animals with hypothyroidism had bone formation associated with graft particles statistically greater 60 days after repair (G2H60> G2H30 P = 0.03). Based on the results of this study it is reasonable to conclude that the systemic condition did not significantly affect bone repair. Additionally, graft seemed to positively contribute to bone formation in induced animals. / Keywords / Bone repair. Hypothyroidism. Biomaterial.

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The patients displayed in this article previously approved the use of their facial and intraoral photographs.

INTRODUCTION

There has been an increasingly search for new techniques and/or technologies that not only promote bone repair, but also result in normal anatomic pattern and implant rehabilitation. After bone fracture/trauma, coagulum and a fibrin network form, filling the remaining spaces. Inflammatory cells migrate to the area and release chemical mediators as well as growth factors that activate osteoprogenitor cells. Therefore, repair is subjected to proper vascularization and mechanical stability, given that osteoblasts only secrete bone matrix in high-oxygen areas.[2] However, in cases of extensive loss, bone repair may prove impossible, defective or incomplete.[21]

Bone graft proves necessary to aid or optimize repair in some areas. Bone graft is classified into autogenous, when tissue derives from sources within the same individual; homogeneous, when tissue is grafted between members of the same species; alloplastic material, foreign and inert bodies implanted into tissues; and xenograft, tissue taken from a donor of one species and grafted into a recipient of another species.[7]

Inorganic bovine bone graft is an osteoconductor that stands out among other types of biomaterial. The most commonly used is Bio-oss® (Geistlich Pharma AG, Wolhusen, Switzerland). It consists of a mineral bone matrix obtained by removing organic compounds from medullary bovine bone. It is recommended for alveolar ridge augmentation and socket filling after extraction.[6]

It is important to notice that, despite the efforts made to develop bone inducing material that when associated with surgical techniques provide the best repair, this regeneration cascade directly depends on patient's systemic conditions.

Hypothyroidism is highlighted in this context. It is most prevalent among adult women and characterized by reduction in T3 and T4 hormone levels. It may result from an autoimmune process that leads to enlargement of the thyroid gland (goiter) or from a deficiency of thyroid gland stimulus in normal conditions (hypothalamic or pituitary disease and poor TSH stimulation).[13,23] Hypothyroidism in adult patients is associated with reduced bone turnover, which can promote osteosclerosis — often corrected by means of thyroid hormone therapy.[4] Deficient thyroid hormones result in several metabolism alterations and complications, among which is the difficulty in bone repair.[20]

This article aims at comparing bone repair of normal animals (euthyroid) and hypothyroid animals, associated with Bio-oss® biomaterial graft (Geistlich Pharma AG, Wolhusen, Switzerland).

MATERIAL AND METHODS

This study was conducted at the Vivarium of the State University of Feira de Santana (UEFS). It respected the ethical principles of animal testing as well as the rules established for the didactic-scientific practice of vivisection in accordance with Law 6.638/79. The research protocol was subjected to the university Ethics Committee on Animal Use. Histological slides were prepared and analyzed at the Pathology Laboratory (School of Dentistry — Federal University of Bahia / UFBA).

Forty eight adult, male, clinically healthy, 400-g Wistar rats — species *Rattus Norvegicus Albinus* and *Rodentia Mammalia* — were randomly selected from the university Vivarium. They were divided into eight experimental groups with two different times of evaluation, as shown in Table 1.

Hypothyroidism was induced by the daily use of Propylthiouracil (PTU) 100 mg (Biolab®, Taboão da Serra, SP, Brazil) diluted with drinking water (0.05 g / 100 mL – 02 tablets ground in 400 ml of water) during five weeks. The condition remained until the end of the experimental period.[24] The euthyroid group had water without PTU *ad libitum*. The induction group's containers were shaken up twice a day so as to avoid decantation. Biochemical/laboratory confirmation of hypothyroidism was obtained after blood collection. A random sample was collected by puncturing the animals' jugular vein at the time of evaluation (T3 and T4 dosage). Animals were weekly subjected to weight assessment from the first induction procedures until they were killed.

After a 12-hour fast, the animals were anesthetized by an intramuscular injection of ketamine hydrochloride (0.08 mL / 100 g body mass) and subjected to sedation

and analgesia by a single-dose intramuscular injection of xylazine hydrochloride (0.04 mL/100 g body mass). Subsequently, they were positioned in ventral decubitus. Hair removal was performed in the calvarium, followed by antisepsis of the surgical site with alcohol iodine. A V-shaped full-thickness incision was performed with the flap exposing the skull bone. Defects (diameter: 10 mm, width: 1.5 mm) were created in the middle portion of each rat's skull, between parietal bones, using a 6-mm trephine bur (3i-Implants) (Fig 1) mounted in counter-angle with 1:20 reduction, with the aid of an implant motor system (Driller BLM 600, SP, Brazil) at 1500 rpm, under external irrigation with 0.9% saline solution. The dura mater was preserved. Biomaterial was placed into the bone defect of animals comprising the biomaterial group (Bio-oss®; Geistlich Pharma AG, Wolhusen, Switzerland). The bone defect of animals comprising the non-grafted group was filled with blood coagulum (Figs 2A and 2B). The amount of biomaterial to be inserted into the bone defect had been determined by a pilot study in which the defect was filled without overflowing and exceeding the limits of osteotomy. This amount was established by weighing the biomaterial on a precision balance (50.56 dg). Tissues were sutured with 5-0 nylon wire (Procare®, SP, Brazil). Sutures were not removed. No antibiotic, analgesic or anti-inflammatory therapies were performed during the experiment.

The rats were randomly killed by overdose of anesthetics. The portion of the skull where defects had been created was removed, with superficial soft tissues preserved, using diamond discs in low rotation with irrigation. The surgical specimens were stored in a closed container filled with 10% formaldehyde solution for 7 days. The specimens were sent to the Laboratory of Oral Surgery Pathology, at the Department of Propaedeutics and Integrated Clinics of the School of Dentistry — Federal University of Bahia.

Table 1: Experimental groups at two different times of evaluation.

GROUP	NUMBER OF ANIMALS	DESCRIPTION
G1E30	6	Animals without hypothyroidism, without graft, 30 days.
G1E60	6	Animals without hypothyroidism, without graft, 60 days.
G2E30	6	Animals without hypothyroidism, grafted, 30 days.
G2E60	6	Animals without hypothyroidism, grafted, 60 days.
G1H30	6	Animals with hypothyroidism, without graft, 30 days.
G1H60	6	Animals with hypothyroidism, without graft, 60 days.
G2H30	6	Animals with hypothyroidism, grafted, 30 days.
G2H60	6	Animals with hypothyroidism, grafted, 60 days.

Figure 1: Trephine bur with inner diameter of 6 mm and outer diameter of 7 mm.

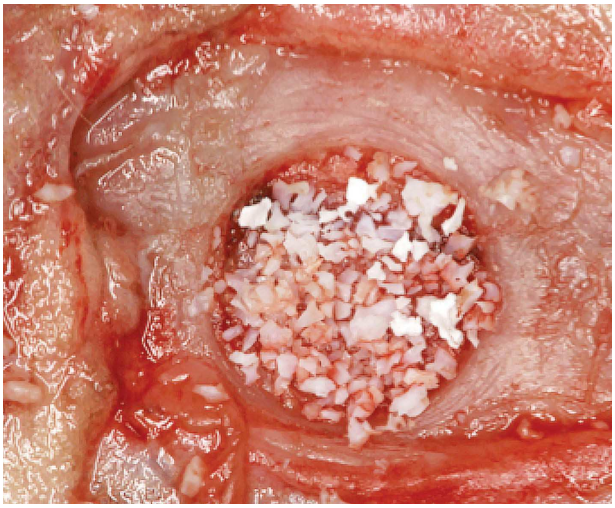

Figure 2A: Critical-size defect with biomaterial.

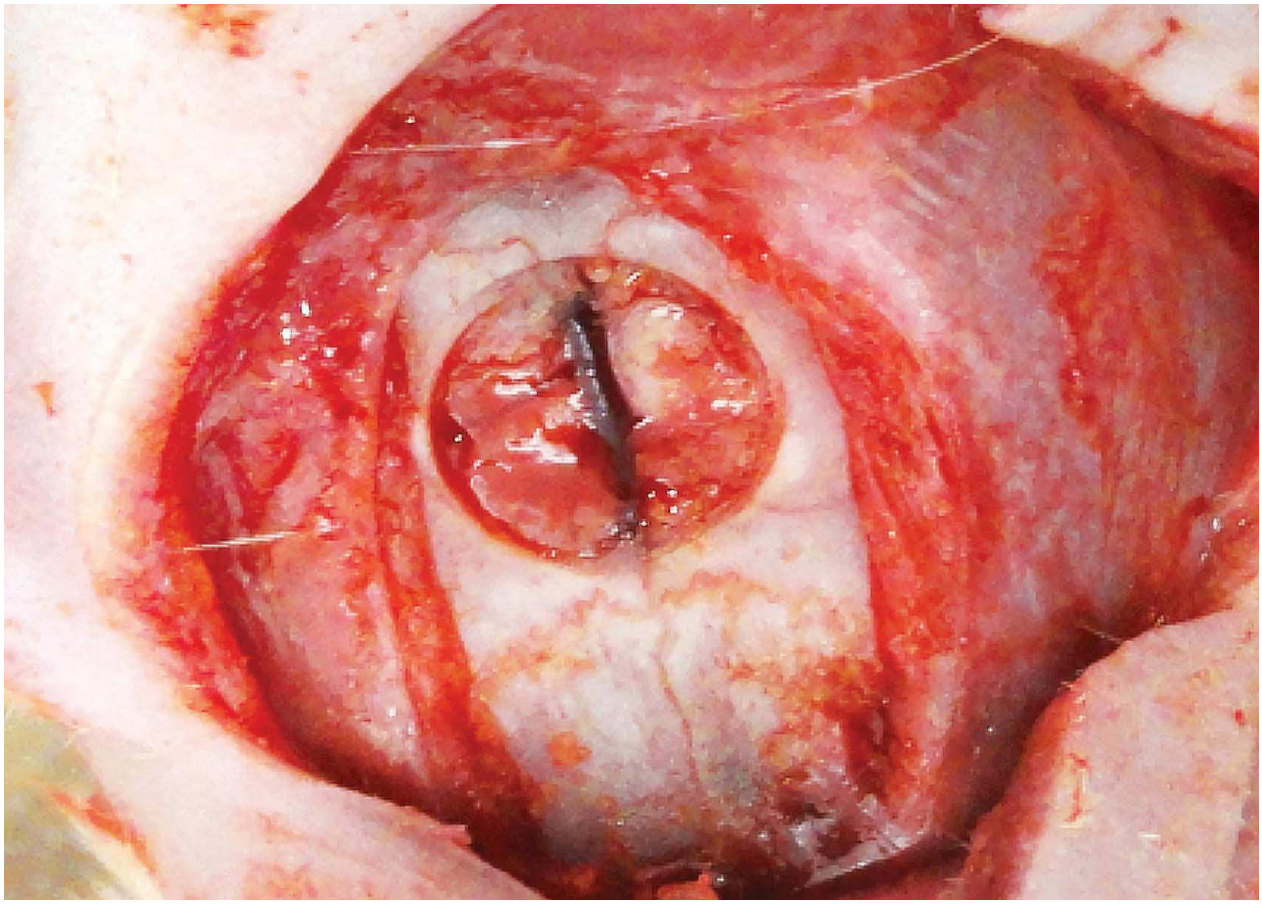

Figure 2B: Critical-size defect without biomaterial.

After fixation, they were decalcified with 5% nitric acid (two days). Histological slides were obtained through macroscopic sections performed by the same researcher in the region anterior to the eyes (snout) and laterally to the ears. A frontal section was also made to preserve the upper part of the brain and skull, with an axial section crossing the middle of the defect (Figs 3 and 4). Subsequently, the specimens were dehydrated in paraffin to obtain 5 µm histological sections. The sections were hematoxylin-and-eosin stained by Picrosirius staining.

The slides were analyzed by light microscopy performed by a skillful, blinded pathologist who read the slides recording their aspects according to the criteria established in a specific form.

Data were tabulated in Excel and statistically analyzed by Minitab®. Due to the sample size (4 to 6 animals per group), the nonparametric Fisher's exact test was employed with significance level set at 5% for intra as well as intergroup comparison.

RESULTS

With regard to bone neoformation, no statistically significant differences were found between groups for animals killed within 30 days ($P > 0.05$). Neoformation was clear in group G2E30, which yielded borderline results ($P = 0.08$) if compared to groups G1H30 and G2H30. However, no differences were found when G2E30 was compared to G1E30 (Table 1). Similar results were identified for animals killed within 60 days. This group did not yield borderline statistical results (Table 2) for disorders and graft, similarly to the 30-day group.

Data crossing between both times of evaluation revealed that greater bone neoformation was expected for the 60-day group. Statistically significant differences were found in 3 out of 4 comparisons. P-value was 0.03 for G1E60>G1E30, 0.01 for G1H60>G1H30 and 0.01 for G2h60>G2H30 (Table 3).

As for bone formation around the graft, no statistically significant differences were found between groups, which reveals that bone formation was not influenced by systemic disorders, whether at the initial or final phases of repair. Nevertheless, paired analyses revealed statistically significant differences between two groups: G2H60 > G2H30, $P = 0.03$ (Table 4).

DISCUSSION

Studies conducted with animal models contribute to performing trials on bone regeneration, given that the models allow variables to be properly controlled. Animal models in rats are suitable for studies on endocrine disorders, particularly those associated with the thyroid gland.[1,14,10,25]

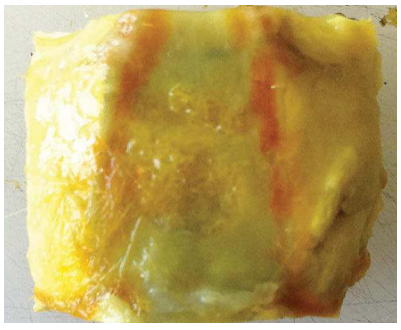

Figure 3: Macroscopic section: upper view. Note the central are with graft particles.

Figure 4: Axial section crossing the middle of the defect.

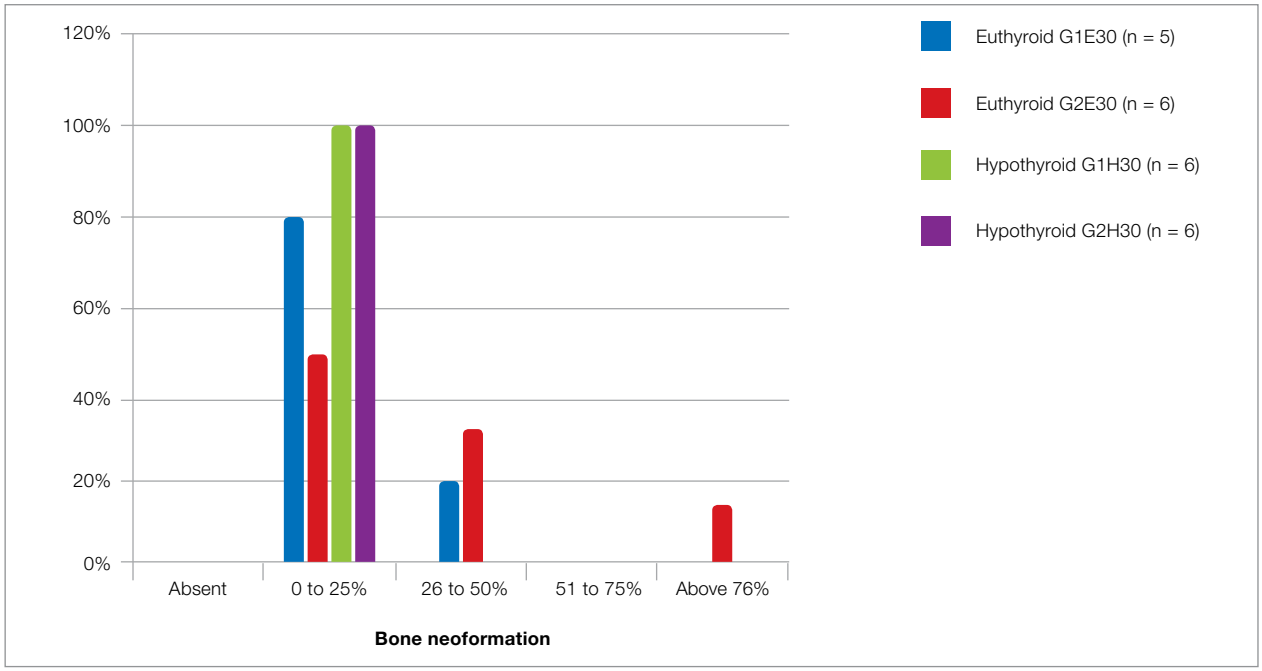

Figure 6: 30-day bone neoformation.

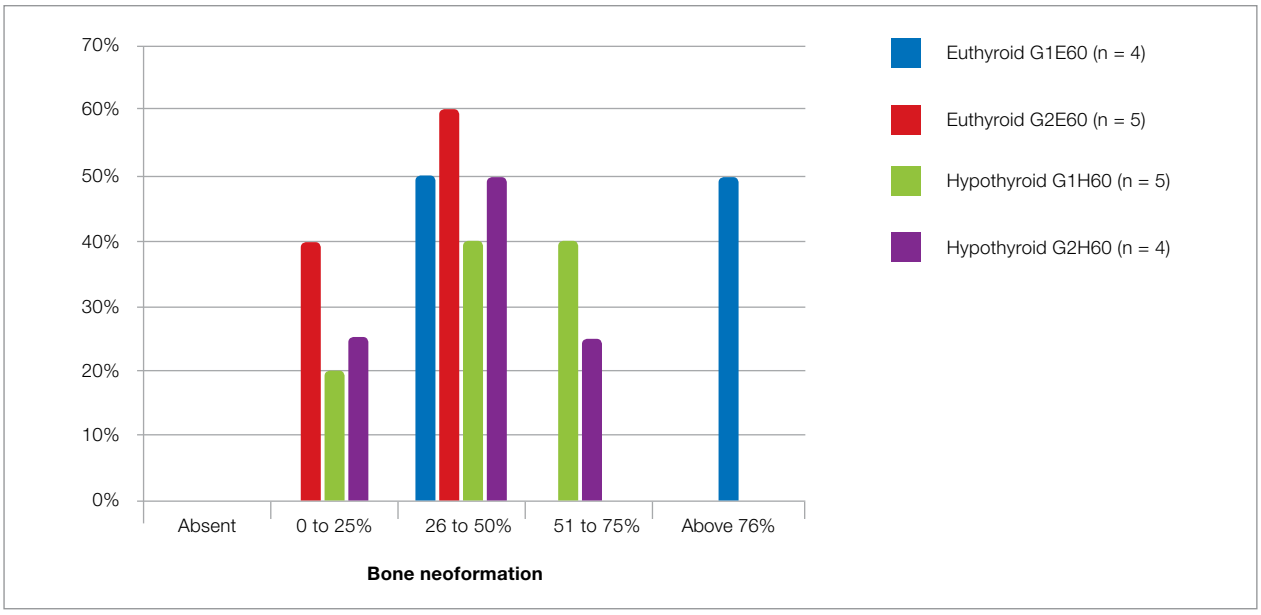

Figure 7: 60-day bone neoformation.

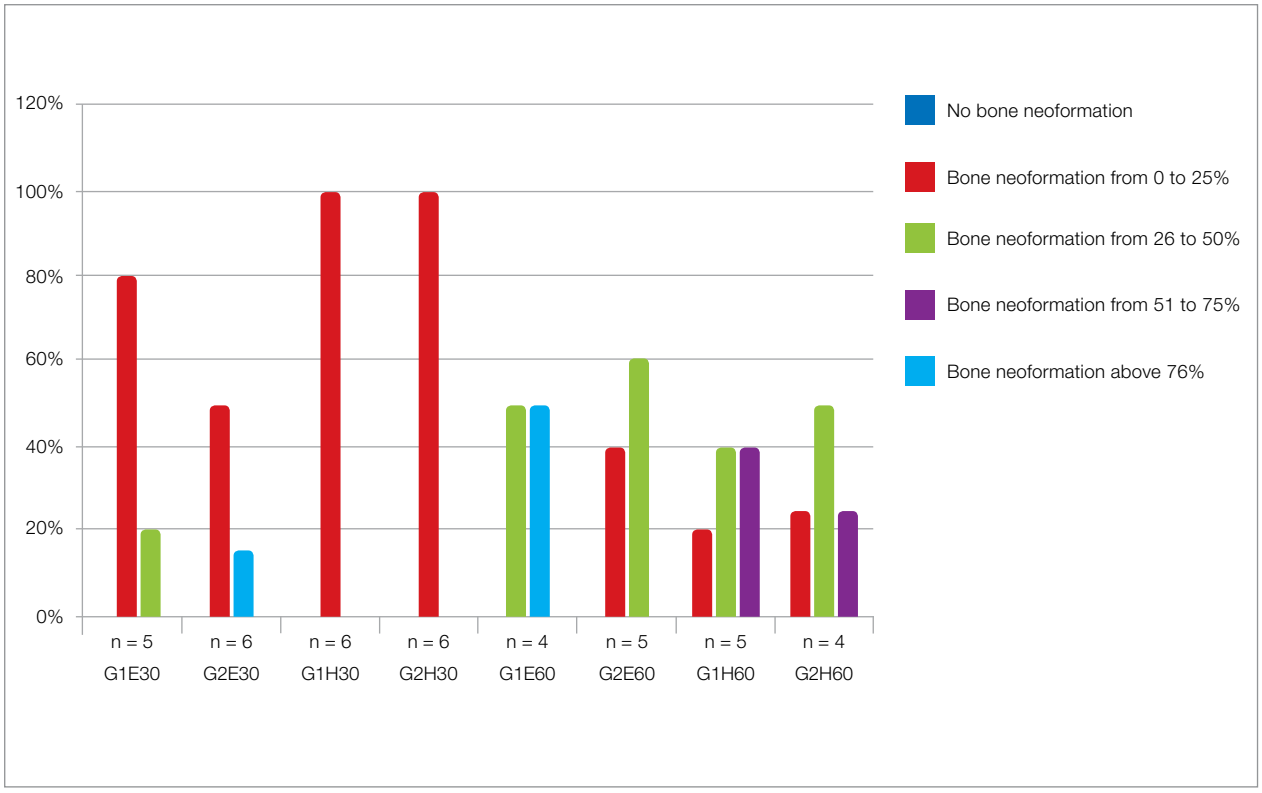

Figure 8: 30-day *versus* 60-day bone neoformation.

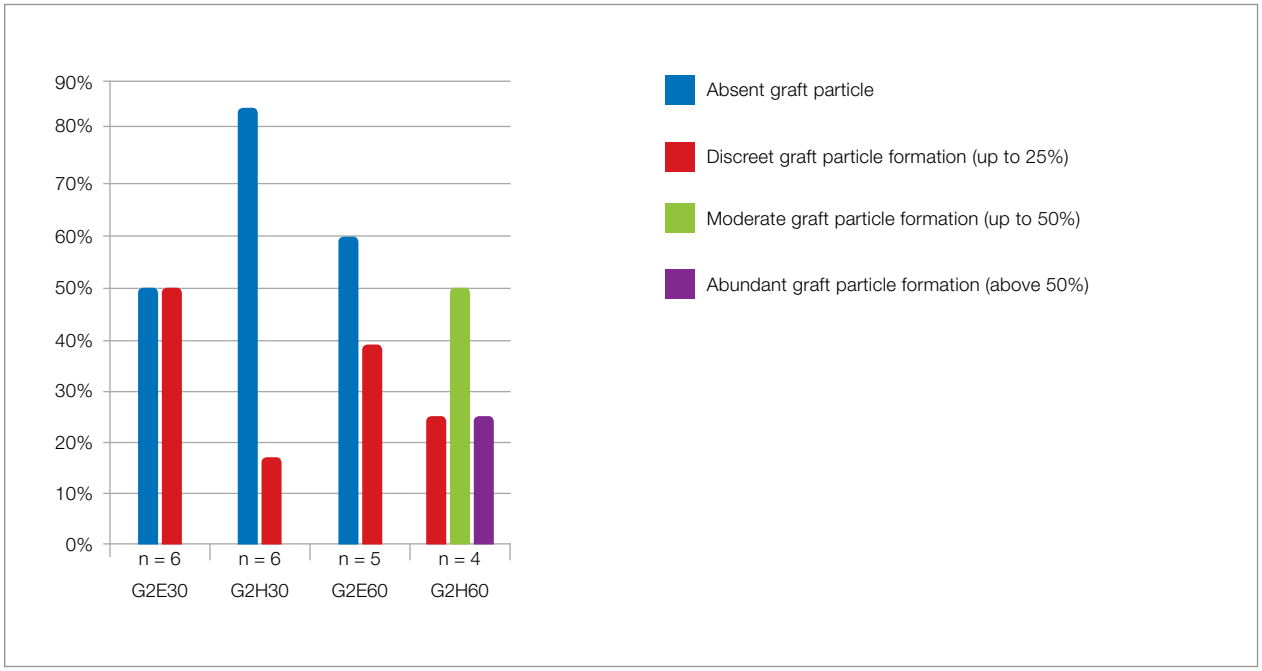

Figure 9: 30 *versus* 60-day bone formation around the graft.

Critical-size defects are those which an organism cannot spontaneously repair, unless an osteogenic, osteoinductive and/or osteoconductive material is added.[26] The defect investigated by this study is in accordance with the requirements[5] previously stated to evaluate bone regeneration. The authors[5] established that an experimental bone defect must be larger than the critical-size defects established for the species investigated; the implantation site must preferably include cortical and medullary bones; there must be local stability with a minimum risk of fracture; and that histological and radiographic analyses must be easily accessed. In the present study, a defect was created by a 6-mm trephine bur. By the end of the drilling process, the defect was 7-mm in diameter due to the thickness of the metal of which the bur was made.

The literature does not reach a consensus regarding the standard size of critical-size defects. Some authors[5,18,17] assert that critical-size defects in rats' calvarium are 5 mm in diameter, whereas others[8,19,22] defend a diameter of 8 mm. All researches evaluated revealed an inability to completely fill bone defect with bone tissue, which was also found by this analysis.

The weight assessment conducted by this study revealed a mean reduction in body weight of hypothyroid animals, associated with a general reduction in the growth of the group. This finding corroborates the literature regarding induced animal's slower development caused by low metabolism.[10,24] Some researches suggest that hypothyroidism be induced by removing the thyroid gland.[3,16] However, the removal of the thyroid gland is followed by the removal of parathyroid glands, which directly influences bone metabolism, making this protocol unfeasible for this study.

Analysis of euthyroid and hypothyroid animals control groups data revealed incomplete bone repair in the 30-day group. As for the 60-day group, euthyroid and hypothyroid animals presented statistically greater bone repair in comparison to the 30-day group (G1E60 > G1E30, P = 0.03 and G1H60 > G1H30, P = 0.01). However, the 60-day group presented incomplete bone neoformation. The defect was filled with collagenous fibers and inflammatory cell lineage. Microscopic evaluation data revealed that the defect used in the investigation was critical due to the absence of bone repair. The center part of the defect was filled with tissue other than bone.[5,9]

As for defects filled with regenerated bone tissue, this study does not reveal any statistically significant differences in bone repair 30 days after healing. Statistically significant differences were found when animals comprising the same group were compared. In other words, hypothyroid or euthyroid animals with or without graft, 30 days *versus* 60 days. Those results were already expected given the differences in repair time.

Comparison between hypothyroid and grafted animals, 30 days *versus* 60 days, was an exception. No statistically significant differences were found, which led us to conclude that graft contributed to the initial repair of normal

animals so as to be similar to the maximum repair time. These results corroborate the literature regarding bone repair hampered by hypothyroidism.[27] Fathabady et al[11] concluded that hypothyroidism-induced animals had delayed growth, development or bone repair. Feitosa[12] concluded that thyroid dysfunctions influenced cortical bone repair around titanium implants inserted in rats' tibia, and that hypothyroidism decreased the percentage of bone adjacent to the implant and within the limit of threads.

In case of biomaterial graft, euthyroid as well as hypothyroid animals had incomplete repair within 30 and 60 days. However, a larger amount of neoformed bone was found in the 60-day group, particularly in the hypothyroid group. In normal grafted animals, biomaterial sped up the initial repair process so as to prevent statistically significant differences between 30 and 60-day groups. This finding revealed that the hypothyroid animal model was more sensitive to graft, which considerably contributed to bone repair, in comparison to the euthyroid model (G2H60 > G2H30, P = 0.01).

This may be explained by the decreased bone resorption of animals comprising the hypothyroidism group. This finding may be associated to the fact that the decreased metabolism of hypothyroid animals may cause inflammatory cells to take longer and have difficulties in identifying the particles of the graft as foreign bodies.

Bone formation around the graft particle was absent or discreet in hypothyroid as well as euthyroid animals, thus demonstrating the biomaterial osteoconductive ability within the investigated times of evaluation. These data are confirmed by neoformed bone apposition directly related to the surface of the particle, which corroborates the findings of other authors assessing xenograft biomaterial.[15,28] Bone formation around the graft particle was not influenced by thyroid dysfunction when groups with the same time of evaluation were compared. However, as bone repair was established, bone formation was statistically greater for the 60-day hypothyroid group in comparison to other groups, including the euthyroid one (G2H60 > G2E60, P = 0.047).

This may be explained by the fact that, in this group, particles underwent decreased resorption, which caused them to remain in the site and, as a result, promote bone neoformation for a longer period of time.

CONCLUSIONS

The findings obtained for the model investigated in this study lead to the conclusion that:

- Cortical repair was incomplete for all the experimental groups assessed.
- Hypothyroidism did not significantly influence bone repair.
- Euthyroid rats' bone repair was not affected by xenograft.
- Hypothyroid rats' bone repair was affected by xenograft, with increased bone formation associated with the surface of graft particles within 60 days.

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