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# Experimental model of heterotopic ossification in Wistar rats

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## Abstract

Heterotopic ossification (HO) is a metaplastic biological process in which there is newly formed bone in soft tissues adjacent to large joints, resulting in joint mobility deficit. In order to determine which treatment techniques are more appropriate for such condition, experimental models of induced heterotopic bone formation have been proposed using heterologous demineralized bone matrix implants and bone morphogenetic protein and other tissues. The objective of the present experimental study was to identify a reliable protocol to induce HO in Wistar rats, based on autologous bone marrow (BM) implantation, comparing 3 different BM volumes and based on literature evidence of this HO induction model in larger laboratory animals. Twelve male Wistar albino rats weighing 350/390 g were used. The animals were anesthetized for blood sampling before HO induction in order to quantify serum alkaline phosphatase (ALP). HO was induced by BM implantation in both quadriceps muscles of these animals, experimental group (EG). Thirty-five days after the induction, another blood sample was collected for ALP determination. The results showed a weight gain in the EG and no significant difference in ALP levels when comparing the periods before and after induction. Qualitative histological analysis confirmed the occurrence of heterotopic ossification in all 12 EG rats. In conclusion, the HO induction model was effective when 0.35 mL autologous BM was applied to the quadriceps of Wistar rats.

Key words: Heterotopic ossification; Autologous bone marrow; Serum alkaline phosphatase

## Introduction

Heterotopic ossification (HO) is a metaplastic biological process in which newly formed bone is found in soft tissues adjacent to large joints (1-3), possibly leading to substantial disability (4).

The pathophysiology of HO is not completely understood, but osteoid formation commonly follows an inflammatory phase characterized by local swelling, pain, erythema, and sometimes fever and joint restriction (1,2,4). It is associated with spinal cord and brain injuries, burns, fractures, muscle contusion, joint arthroplasty, lower motor neuron disorders, and hereditary disorders (1,5-7). HO induction was first reported in the 1930s (8,9), and since then animal models have been used in different forms of HO induction in order to define techniques that may aid in the prevention and treatment of the condition.

The implantation of demineralized bone matrix is often used to induce ectopic bone formation (10-13), but this procedure is time-consuming and expensive and is not free of rejection risks. Another option is to induce HO by bone morphogenetic protein implantation; however, although this

is an effective technique for bone tissue formation, not all laboratories have sufficient resources and technology for material extraction.

Thus, the purpose of the present study was to identify a reliable protocol for the induction of HO in Wistar rats by autologous bone marrow (BM) implantation based on the literature for larger laboratory animals.

## Material and Methods

This study was approved by the University Ethics Committee in Animal Experiments, Pontifícia Universidade Católica do Paraná, No. 499/09. The experiment was divided into two phases. The first was a pilot study carried out to determine an effective induction protocol, and the second was the experiment itself.

The animals were kept in standard plastic cages under controlled environmental conditions (12/12-h light-dark cycle) with food and water *ad libitum* in the Pontifícia Universidade Católica do Paraná vivarium. The study was

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conducted in accordance with international ethics standards for animal experimentation.

A total of 28 Wistar albino rats were used. The pilot study (first phase) used 16 adult male Wistar albino rats (*Rattus norvegicus*) weighing 300-350 g, approximately 3 months of age, divided into four groups of 4 rats each. Three groups were used for the induction of heterotopic bone, but with different volumes of BM, and the 4th group was used as control. Twelve animals were used in the experimental study (second phase).

### Pilot study

*Method for the induction of heterotopic ossification.* All animals were anesthetized by intramuscular injections of 80 mg/kg ketamine and 8 mg/kg xylazine, receiving a booster dose if necessary. BM was then collected bilaterally from the iliac crest of the animal with a 25 x 17-mm puncture needle.

After collection, BM was implanted bilaterally in the quadriceps. For the implant, a thin needle (0.3 x 16 mm) of the type used for insulin injection was inserted perpendicularly to the ventral side of the thigh.

The animals were divided into three groups, receiving 0.2, 0.35, and 0.5 mL BM, respectively. The minimum implant volume was adopted from published studies (5,11). The maximum volume of BM was determined as the maximum volume supported by the animal as long as there was no leakage of liquid during the implant.

All animals were given oral doses of 20 mg·kg<sup>-1</sup>·24 h<sup>-1</sup> Dipyron® (500 mg/mL Eurofarma®, Brazil) during the first 3 days for pain relief after the BM collection procedure.

The animals were euthanized 35 days after BM implantation, a period considered to be sufficient for the development of heterotopic bone formation (5,11).

*Evaluation methods.* The rats were anesthetized for blood collection for serum alkaline phosphatase (ALP) determination and for the dissection of the left and right quadriceps. During the dissection, the muscles were periodically dripped with normal saline solution to prevent tissue desiccation. Still under anesthesia, the animals were given a lethal intracardiac dose of an anesthetic drug. Four tissue fragments were collected from each group for histological analysis, and another four (of the left quadriceps) were collected for spectrophotometric analysis.

The following parameters were evaluated in the pilot study group: mortality rate, mean serum ALP, and calcium percentage in the left quadriceps (Table 1). In addition to the histological analysis, a qualitative evaluation of the presence of heterotopic bone was performed.

The results obtained in the experimental study are reported as mean, median, minimum, maximum, and standard deviation. Repeated measure analysis of variance was used to evaluate the effect of time on weight (pre x 8 x 43 days). Multiple comparisons were made by the least significant difference test. ALP data obtained before the experimental procedure were compared to data obtained 43

days after the procedure using the Student *t*-test for paired samples. The normality of the data was evaluated by the Shapiro-Wilk test, with *P* values less than 0.05 considered to be statistically significant. Data were analyzed using the computer program Statistica v. 8.0 (Table 1).

### Experimental study

Based on the results of the pilot study, we determined the most effective protocol for HO induction by correlating the volume of BM deployed, the death rate, levels of ALP, the amount of calcium in muscle, and bone formation rate. The volume of 0.35 mL was the most effective and thus chosen for the induction protocol to be tested.

The sample group consisted of 12 adult male Wistar albino rats (*R. norvegicus*) weighing 350-390 g, a size that would facilitate BM collection. The anesthetic induction was performed by an intramuscular injection of 80 mg/kg ketamine and 8 mg/kg xylazine before all the procedures (blood sampling for ALP evaluation, BM collection and HO induction, muscle sampling, and euthanasia).

HO induction was performed 1 week after blood sampling for ALP determination, so that the animals would have time for recovery after blood collection. The experiment lasted 43 days: on day 1, blood was collected for ALP determination; on day 8, HO induction was carried out by BM sampling from the iliac crest and re-injection in the quadriceps muscles, and on day 43, the animals were euthanized and blood and muscles were collected for analysis. The period between BM implantation and euthanasia was 35 days.

The methods used to detect induced heterotopic bone formation were the same as those in the pilot study. For histological analysis, slides were stained with HE. To confirm heterotopic bone formation, counterproof slides with Mallory's trichrome were performed.

## Results

### Results of the pilot study

The mortality rate was highest in the 0.5-mL BM group (Table 1), a result related to the large volume of organic material collected from the iliac crest of these animals, and in the 0.2-mL group 1 animal died.

ALP levels increased with the 0.2-mL group showing a higher ALP level than the average of the other groups that received the implant of BM. The amount of calcium in the quadriceps was assessed by flame photometry, and the values are reported as mg Ca<sup>2+</sup>/g muscle. The 0.35-mL group obtained more mg Ca<sup>2+</sup>/g than all the other groups (Table 1).

The percentage of heterotopic bone formation was assessed by histological analysis. The 0.2-mL group showed 33.3% bone formation, whereas the 0.35-mL group showed 100% heterotopic bone formation. The 0.5-mL group was not suitable for analysis since only one of the animals survived and showed bone formation while the rest of the

**Table 1.** Effect of bone marrow volume on mortality, serum alkaline phosphatase (ALP) and calcium levels in the left quadriceps.

Bone marrow volume	N	Mortality rate	ALP (IU)	Muscular calcium (mg/g)	Muscle weight of EG (g)	Weight of EG (g)
Control	5	0%	264 ± 7.87	0.235 ± 0.06	-	-
0.2 mL	4	25%	239.3 ± 135.1	0.120 ± 0.006	-	-
0.35 mL	4	0%	230 ± 39.1	0.4286 ± 0.617	-	-
0.5 mL	4	75%	135 ± 0	0.1391 ± 0	-	-
Experimental group (EG, 0.35-mL)	12	0%	191.64 ± 71.5	0.16 ± 0.01	3.31 ± 0.21	381.3 ± 14.78*

Data are reported as means ± SD. \*P < 0.05 when the weight obtained on the 8th and 43rd days was compared (ANOVA). Before and after the induction of HO, the data had normal distribution (Shapiro-Wilk test).

group suffered premature deaths before this phase could be completed.

### Results of the experimental study

The assessment was performed on 12 Wistar rats. Their weights increased significantly between the beginning and the end of the experiment (Table 1). ALP levels were compared between the 8th and 43rd day showing no statistical significance.

The amount of calcium in the quadriceps was assessed by flame photometry, and the values are reported as mg Ca<sup>2+</sup>/g muscle. The data obtained were compared to those of the pilot study indicating no statistical significance.

No statistical significance was detected for the weight of the quadriceps muscle; however, despite these results, histological analysis revealed heterotopic bone formation in all animals of the experimental group.

### Discussion

At the end of the experimental study there was an increase in body weight, whereas ALP, muscle weight and Ca<sup>2+</sup> values did not differ significantly (Table 1), although 100% heterotopic bone formation was observed in the animals of the 0.35-mL BM group as assessed by histological analysis.

Heterotopic bone formation is usually generated by the implantation of demineralized bone matrix (10-13), more specifically by implantation of bone morphogenetic protein in ectopic sites (10,14), or by implantation of BM stromal cells (15-17). Most of these models have variable repeatability, but the implantation of bone marrow to induce heterotopic bone formation can be demonstrated simply without the demand of time needed to prepare the material to be deployed. The induction procedure used in the present study was adopted from studies (5,10,11) that have documented that 35 days are sufficient for heterotopic bone formation in the quadriceps of rabbits following implantation of 2 mL BM collected from the iliac crest of the animal. Namazi and

Mozaffarian (5) observed that after 35 days of BM implant in rabbits, heterotopic bone formation occurred in 83.3% of control animals. Dudkiewicz et al. (11), using the same methodology for HO induction, observed 97% heterotopic bone formation. In the present study, we detected 100% heterotopic bone formation in the quadriceps of Wistar rats.

A texture liquid, which is deposited in the recipient site by percutaneous injection (16), has osteogenic cells, and the resulting implantation forms bony capsules (18) when injected into the muscle tissue or the peritoneal cavity. This type of implant is a sufficient stimulus for osteogenesis (19,20).

It was found that BM implantation in the quadriceps of animals induces heterotopic bone formation; however, care must be taken about the volume implanted. For this reason, the implantation of 0.35 mL BM was more suitable for the induction of heterotopic bone in Wistar rats as indicated by the low mortality rate and the level of ALP. This is important for the initiation of calcification (21) since it is associated with inflammatory processes and their modulation/regulation (22-31) of Ca<sup>2+</sup> in muscle and bone formation demonstrated by histological analysis.

Loss of animal weight due to stress suffered as a result of manipulation, anesthetic procedures and collection of material for analysis is usually reported in the literature (32,33). However, the animals used in the present study showed a weight gain, as expected for normal animals. We may infer that, despite the manipulation, anesthesia and BM implant, there was little or no interference creating stress in the animals and a weight loss (Table 1).

It is known that bone alkaline phosphatase (BAP) can be specifically used to assess osteoblast differentiation and that mineralization increases with decreasing BAP values. This leads to an increase in osteocalcin, a specific biochemical marker for bone formation (34). Despite the specificity of these tests, in the present study we chose ALP, which is used as a tracer of osteoblast activity and is indicated as a diagnostic method of HO (35,36). Besides being present in bone, ALP is also present in the intestine,



liver, kidneys, and placenta but, despite its presence in these organs, its increased levels may be related to the increase in the amount of calcium in the joints and muscles (37), and ALP levels show 97.5% specificity for detecting bone activity (24). ALP levels in the experimental study did not differ significantly between the beginning and the end of the experiment.

In a study that used an implant of demineralized bone matrix to induce HO in 50 Wistar rats (28), evaluation of calcium concentration by flame spectrophotometry showed a significant increase compared to control. In the present study, calcium concentration in muscle was determined by flame spectrophotometry, assuming that, in case of the existence of bone, the calcium concentration of muscle would be higher, which was not detected in the present study (Table 1).

We chose histological analysis for the visualization of bone formation. Although radiography is commonly used as a diagnostic method for HO in experimental studies (4, 10), we choose not to use this method because the amount of BM implant was small and bone formation would be difficult

to identify in a conventional radiography. Radiographic findings in experimental research can be questionable because the volume of implant is usually small. Regarding the visualization of bone formation, when some opacity is radiographically present it is difficult to determine whether new bone is present or simply tissue edema resulting from material implant for HO induction. In studies that used a BM implant in the quadriceps of rabbits to study the influence of anti-inflammatory therapies, bone formation was observed in the muscle of control animals that received the implant of BM (5,10), in agreement with the histological findings of the present experimental study.

Reliable animal models of HO that mimic pathologies in humans would be invaluable for the development of new treatments to combat heterotopic ossification (2,5,8-12,28). Therefore, autotransplantation of 0.35 mL BM in the quadriceps of Wistar rats can be an alternative induction protocol for HO studies since the procedure is simple and inexpensive, and the induction rate of bone formation within 35 days was 100%.

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