

# THE EFFECT OF LOW-INTENSITY THERAPEUTIC ULTRASOUND IN INDUCED FRACTURE OF RAT TIBIAE

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## ABSTRACT

**Objective:** To analyze the possible effects of low-intensity ultrasound on induced tibia fracture of rats in a dose commonly used in physical therapy treatments. **Methods:** Twenty male Wistar rats were distributed into two groups with 10 animals each. In the ultrasound group (USG), the animals were submitted to bone fracture and treatment with therapeutic ultrasound (TUS). Ultrasonic parameters are: frequency of 1.0 MHz, intensity of 0.2 W/cm<sup>2</sup>, pulsed mode at 20%, applied in stationary form during 10 minutes on the fracture region, for five weeks. The control group (CG) was submitted to bone fracture but not treated with ultrasound. **Results:** The

radiographies showed better consolidation in USG compared to CG. The statistical tests for alkaline phosphatase and serum calcium did not show significant difference between groups. **Conclusion:** According to this study, TUS, applied with these parameters (not commonly used for bone therapy) accelerates bone healing, confirmed by radiography, yet the biochemical analysis was not conclusive. One reason for this inconsistency may have been some inadequacy of the biochemical protocol, currently under investigation. **Level of Evidence II, Prospective Comparative study.**

**Keywords:** Fracture healing. Ultrasonic therapy. Physical therapy. Tibia. Rats.

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## INTRODUCTION

Fracture healing is a complex process that involves cell proliferation and differentiation, chemotaxis and synthesis of extracellular matrix,<sup>1</sup> responsible for the reestablishment of the mechanical, and consequently functional, integrity of the bone tissue.<sup>1,2</sup> However, this bone repair process may occur slowly (retarded healing) or even fail to develop (pseudoarthrosis), resulting in the impairment or disability of individuals.<sup>3</sup>

The electrical properties of bone tissue, particularly piezoelectricity, motivated several researchers to develop techniques that would have a repercussion on the alteration of the bone metabolism, if the consolidation did not occur in the expected time or even to accelerate this metabolism in recent fractures.<sup>1,4-7</sup>

The acceleration of the fracture healing process by low-intensity pulsed ultrasound (US), is well documented in scientific literature.<sup>1,4,5,8</sup> However, the cellular and molecular mechanisms, triggered in treatment with therapeutic ultrasound (TUS), are

still not fully understood, and neither are the parameters used in the equipment during the treatment.<sup>1,3,7,9,10</sup>

Low-intensity pulsed TUS, in propagating as mechanical energy, reaches the bone surface and generates electrical signals that stimulate the bone metabolism.<sup>1,9,10</sup> The direct action of low-intensity TUS on mechanoreceptors is probable, but it can also act through the release of agonists of osteoblast, inducing their proliferation and differentiation<sup>1</sup> and the release of prostaglandins, through activation of the P2X<sub>7</sub> receptor in bone cells, allowing the inflow of ions, such as calcium.<sup>1</sup>

Specific low-intensity (30mW/cm<sup>2</sup>) pulsed ultrasound equipment, which costs around 4 times more than therapeutic ultrasound equipment,<sup>4,7</sup> is used frequently in studies on animals.

Thus, the aim of this study is to ascertain the possible effects of low-intensity US, used in physical therapy treatments, on induced fracture of rat tibiae. The originality of the present study lies in the investigation of an intensity that is common in

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commercial ultrasound equipment in Physiotherapy, since the specific equipment uses 30mW/cm<sup>2</sup>.

## MATERIALS AND METHODS

### Ethical regulations

The study was conducted, after approval by the Institutional Review Board of Universidade do Estado do Pará, under protocol n.13/2009, in compliance with the precepts of Colégio Brasileiro de Experimentação Animal (COBEA) and with the Brazilian National Animal Vivisection Legislation in force (Federal Law 11,794 of October 8, 2008).

### Samples

The study subjects were twenty male Wistar rats (*Rattus norvegicus albinus*) from the Vivarium of UFPa (Universidade Federal do Pará), weighing between 300 to 350g or minimum age of 90 days. After a 15-day period of adaptation to the Experimental Surgery Laboratory (LCE) of Universidade do Estado do Pará (UEPA), they were accommodated in cages measuring 45 x 15 x 30cm, with the bottom covered with autoclavable rice straw, changed on alternate days, in a controlled environment and receiving water and feed *ad libitum*. The animals were randomly distributed into 2 groups, with 10 animals each. (Figure 1)

In the ultrasound group (USG), the animals were submitted to bone injury and treatment with low-intensity TUS. In the control group (CG), the animals were submitted to bone injury and were not treated with TUS.

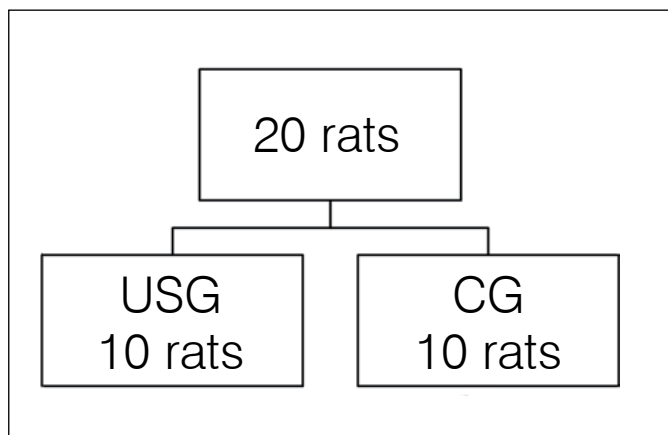


Figure 1. Organization chart of the groups.

### Establishment of the fracture

The 20 rats were weighed on digital scales then anesthetized with Ketamine® (0.9 mL/kg) and Xylazine® (0.5 mL/kg), administered intraperitoneally. After clinical confirmation of anesthesia, the animals were positioned in right lateral decubitus and had the right tibia fractured in the middle third, with the fracture equipment adapted<sup>11</sup> from the system described by Matheus *et al.*<sup>12</sup>, (Figure 2) which caused a closed fracture. There was no type of immobilization of the segment or pharmacological treatment afterwards.<sup>13</sup>

### Treatment with ultrasound

Before the start of the treatment, the TUS equipment was gauged and calibrated by the Electrical Engineering sector

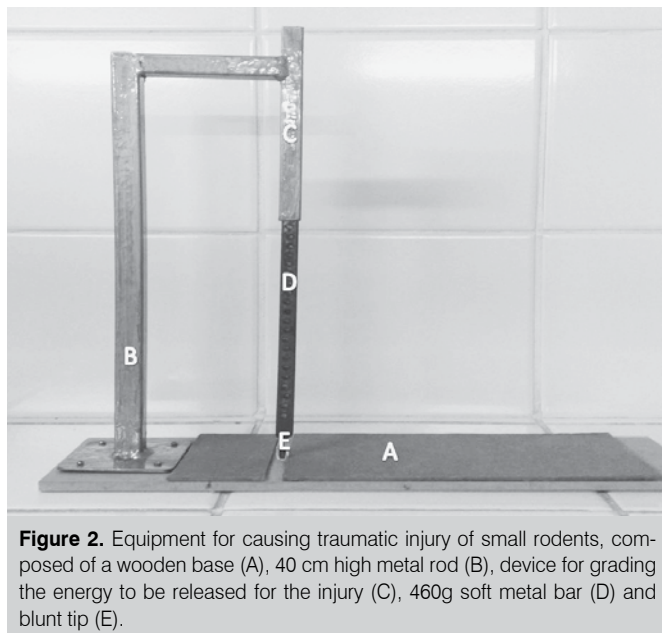


Figure 2. Equipment for causing traumatic injury of small rodents, composed of a wooden base (A), 40 cm high metal rod (B), device for grading the energy to be released for the injury (C), 460g soft metal bar (D) and blunt tip (E).

of Universidade Federal do Pará. The treatment with therapeutic ultrasound began 24 hours after the induction of the bone injury. The SONACEL PLUS® model BIOSET ultrasound apparatus was applied at the fracture site, with frequency of 1 MHz, intensity of 0.2W/cm<sup>2</sup> (SATA), pulsed mode, duty cycle of 20%, and ERA (Effective Radiating Area) of 0.8cm<sup>2</sup>. Commercial water-soluble ultrasound gel was used as a contact material. The treatment was carried out for 10 minutes, once a day, over five consecutive days and two days of interval, until 25 sessions were completed, simulating the physical therapy treatment.

### Post-treatment procedure

On the 34<sup>th</sup> day, the animals were once again anesthetized with Ketamine® (0.9 mL/kg) and Xylazine® (0.5 mL/kg), administered intraperitoneally. After the anesthesia, the animals were submitted to exsanguination through cardiac puncture (5 mL) and then decapitated. The hind limb of each animal was carefully removed and submitted to radiological and biochemical analysis.

### Radiological analysis

The evaluation of the bone injury was performed using the same radiographic technique (40kV x 2mAs) and always at the same distance from the X-ray tube (1m).

### Biochemical analysis

The levels of alkaline phosphatase (ALP) and serum calcium (SC) in the animals' blood were determined after 12 hours of fasting on the 34<sup>th</sup> day after establishment of the fracture. After coagulation, the serum was separated by centrifugation in Eppendorf tubes at 1000G.

The serum ALP dosing was performed with a laboratory kit (Labtest) and enzyme activity was estimated through absorbance at 590 nm. The SC was dosed using the laboratory kit (Labtest) and its concentration was estimated through absorbance at 570 nm. Both analyses were carried out in the automated Labtest system - VITALAB SELECTRA E® (Vital Scientific N.V.) and analyzed in the biochemistry laboratory of UEPA.

## Statistical analysis

The statistical analysis consisted of the application of the Student's t-test, considering a significance level  $\alpha=0.05$ , using Bioestat 5.0 software.

## RESULTS

The study subjects were 20 male Wistar rats (*Rattus norvegicus albinus*), with average age of 150 days and bodyweight of  $284.95 \pm 48g$ , (Table 1), with 18 rats constituting the final sample: CG (n=8) and USG (n=10). The sudden death of 2 animals occurred in the CG prior to the start of treatment.

Transverse diaphyseal fracture of middle third of the tibia and absence of signs of osteomyelitis were observed in the radiographs in both groups. Bone callus in formation is observed in USG, (Figure 3) while interpenetration of fragments is exhibited in CG, characterizing them as impacted fractures. (Figure 4) This therefore defines the acceleration of consolidation in USG in comparison to CG. As regards the data from the biochemical analysis, it was possible to evaluate two markers for bone remodeling: ALP and SC. (Table 2) In relation to the analysis of ALP and SC, no statistically significant effect was evidenced. (Table 3)

**Table 1.** Data on the weight of each rat from the sample (n=20), in grams.

Rats	Weight (in grams)
1	250
2	269
3	330
4	350
5	340
6	220
7	270
8	370
9	245
10	350
11	335
12	250
13	250
14	310
15	240
16	240
17	250
18	260
19	240
20	330

## DISCUSSION

The treatment with ultrasound has been widely used in bone repair. However, there is still controversy over its biological potentials and its effects on tissue repair, and its use is often neglected or based on practical experience, which results in erroneous procedures.<sup>3</sup>

The small temperature increase produced by TUS has a repercussion on the action of some enzymes, namely, matrix



**Figure 3.** Radiography of USG, evidencing transverse in diaphyseal fracture of the middle third of the tibia.



**Figure 4.** Radiography of CG, evidencing diaphyseal fracture in the middle third of the tibia.

**Table 2.** SC and ALP results of the sample, USG (n = 9), CC (n= 8).

Group/rats	Serum calcium (mg/dL)	Alkaline phosphatase (U/L)
USG 1	14.59	353
USG 3	14.93	335
USG 4	16.73	419
USG 5	14.41	630
USG 6	14.20	775
USG 7	14.39	343
USG 8	14.53	306
USG 9	14.73	354
USG 10	14.31	312
CG 1	13.74	290
CG 2	14.39	649
CG 3	14.34	322
CG 4	14.71	228
CG 5	15.08	220
CG 6	15.13	468
CG 7	14.90	612
CG 8	14.98	533

**Table 3.** Comparative data of USG (n=9) and of CC (n=8), in relation to the variables SC and ALP.

Biochemical analysis	USG		CG		p*
	Mean	sd	Mean	sd	
Serum calcium	14.76	0.77	14.66	0.48	0.76
Alkaline phosphatase	425.22	164.52	415.25	172.20	0.91

p ≥ 0.05 (Student's t-test).

metalloproteinase-1 and collagenase.<sup>1,14</sup> Accordingly, TUS can serve to effectively reestablish or normalize metabolic temperatures in the tissue healing regions.

Moreover, the treatment with low-intensity pulsed TUS is a good stimulator of the different cells of the osseous system,<sup>1</sup> accelerating the healing of the clinical fracture and increasing bone formation through osteoblast activity.<sup>1,15</sup> In addition, it increases the activity of ALP and SC.<sup>1,8,16</sup> These actions thus enable the use of pulsed ultrasound in therapeutic applications.<sup>1</sup> In this study, the bone of choice was the tibia, as it is the most frequently fractured long bone and associated with a high incidence of fracture healing retardation and bone nonunion.<sup>2</sup> The fracture was initially executed in a pilot study, making a transverse cut in the tibia with a scalpel. Once the osteotomy was confirmed, the fracture site was reduced and the skin was sutured. After the surgery, the area was disinfected with bactericide agent. Five days after the osteotomy, it was observed that the animals evolved to develop infection in the sectioned area and compartment syndrome, which was also described in other studies.<sup>13</sup> In an attempt to avoid this syndrome, a new study model was subsequently applied with immobilization using a plaster splint. However, the experiment was not successful, as the animals also developed compartment syndrome, corroborating the

findings of other authors.<sup>13,17</sup> Another problem identified was the presence of infection at the site of the surgical incision, which would alter the bone healing process.<sup>13</sup> For this reason, the closed fracture model is justified as it greatly reduces the risks of infection, besides causing minimum damage to soft tissues.<sup>13,18</sup> Zacharias *et al.*<sup>17</sup> assert that models for the production of fractures by invasive means can lead to complications such as suture dehiscence and, consequently, deep infection, as was the case in their study.

Due to the controversy over the fracture method, it was proposed that equipment adapted<sup>11</sup> from the system described by Matheus *et al.*<sup>12</sup> be created, obtaining low-cost, easily reproducible handmade equipment, which standardizes the fracture. It was tested previously and successfully on 16 female and 18 male Wistar rats (*Rattus norvegicus albinus*), with average age of 90 days and mean bodyweight of 250g. This equipment was used to obtain the fractures in this study.

Invasive fracture stabilization methods can also result in complications, which happened to Pelker and Friedlaender,<sup>19</sup> who used Kirschner wires and excluded 87 animals from the experiment, due to several complications, including deaths in the perioperative period and femoral fracture, during the assembly of the wires.

Complications can also occur when noninvasive methods are employed to stabilize fractures.<sup>13</sup> These facts, together with the different descriptions of fracture models that do not employ any type of immobilization, justify the decision of the investigators not to use them.<sup>13,17,18</sup>

After the random distribution of the groups, USG was treated with TUS. According to the principles of Wolff's law, ultrasonic stimulation in bone repair transmits micromechanical forces and stress to the fracture site, resulting in bone formation. Consequently, several studies with TUS for the repair of injured bone tissues can be found in the literature.<sup>1,2,12</sup> Low-intensity pulsed therapeutic ultrasound was used as it stimulates bone metabolism in propagating as mechanical energy.<sup>1,9,10</sup>

The intensity of 0.2W/cm<sup>2</sup> was used as it is commonly found in commercial ultrasound equipment in Physiotherapy, since the specific equipment uses 30mW/cm<sup>2</sup> and costs about 3 to 4 times more. The demonstration of the feasibility of using more accessible equipment would allow better diffusion of the treatment to accelerate bone healing.

Albertin<sup>20</sup> compared different treatment times (5, 10, 20 and 40 minutes) applying low-intensity ultrasound to the bone defects of rabbits and concluded that, with the exception of 5 minutes, the other times caused an increase in ossification. Due to this, and seeking similarities with the physical therapy treatment, the time of 10 minutes was chosen.

To elucidate the effect of the TUS, a biochemical analysis was conducted by means of the alkaline phosphatase activity (since this enzyme indicates osteoblast activity that determines bone formation or reabsorption<sup>1,8,16,21</sup>) with an analysis of serum calcium as this indicates bone matrix synthesis process.<sup>1,8,16,21</sup> Radiographs were taken to ratify the recommendations indicated by the other exams, with an analysis of the basic parameters, such as bone callus, fracture type and location.<sup>13</sup>

In the present study, the biochemical analysis did not reveal a statistically significant effect between the groups. This could have happened for three reasons: (I) the long period between



the fracture and the biochemical analysis, since there is a tendency for both groups to present equivalent values, after a long post-fracture period; (II) the inadequacy of the biochemical protocol; or (III) the sample size was not sufficient to reveal significant difference.

The persistence of the fracture line in the radiographs up to the fifth week is consistent with data from the literature. Castro *et al.*<sup>13</sup> verified that, at 6 weeks after the fracture, the fracture line still remained clear. Utvag and Reikeras<sup>22</sup> verified that, 20 days after the fracture in rats, there was still a visible fracture line, while after 40 days, the line was hardly perceptible. The persistence of this continuity solution could be attributed to the absence of immobilization.

The presence of bone callus in a more accelerated process of formation in USG than in CG suggests that TUS can be used as adjuvant treatment in bone fractures.<sup>1</sup> A study described by Chan *et al.*<sup>4</sup> in tibia fractures in 17 rabbits (New Zealand) treated with TUS (30mW/cm<sup>2</sup>, 1.5MHz), for 20 minutes per day over 4 weeks also verified the efficacy of TUS in the experimental group, when being analyzed by radiography in the 2<sup>nd</sup> and 4<sup>th</sup> weeks, proving more effective in the initial phase of the treatment. According to the findings of the present study, a survey conducted by Takikawa *et al.*<sup>5</sup> with rats, inducing pseudoarthrosis with the promotion of low-intensity ultrasound treatment (30mW/cm<sup>2</sup>) for 20 minutes per day over 6 weeks, discovered

a significant improvement in the radiographic analysis of the group treated with ultrasound.

No complications were observed in this survey, although it is known that several factors are capable of interfering in the bone healing process, including: irradiation, form of immobilization, type of fracture, fracture line, nerve section, presence of bone tumor, soft tissue interposition and infection at the fracture site.<sup>13</sup> New studies are necessary to expand knowledge of the effect of therapeutic ultrasound on the bone tissue. Hence it is suggested that the bone healing process evaluation analysis method be adopted throughout the treatment period, using consolidation process analysis methods such as scanning electron microscopy, histopathological analysis, ultrasound diagnosis and histomorphometry.

## CONCLUSION

Intervention by means of low-intensity pulsed TUS (0.2 W/cm<sup>2</sup>), with a duty cycle of 20%, applied in stationary form during 10 minutes in the fracture region, for 5 weeks, accelerated healing, confirmed by radiography. The biochemical analysis did not reveal significant difference between the groups, but the levels of ALP and SC were higher in USG. Therefore, these data suggest that therapeutic ultrasound (in doses differing from those of the equipment normally used for this therapy) can accelerate the bone healing process.

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