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Análise de propriedades biomecânicas em falhas ósseas de tíbias após tratamento com ozônio

Analysis of biomechanical properties of tibias after bone failure and ozone treatment in rats

Análisis de las propiedades biomecánicas en las faltas huesas de tibasa después del tratamiento con ozono

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Resumo

Introdução: O ozônio é um oxidante potente que age como precursor diversos radicais, sendo indicado com terapêutico potente com auxiliar no processo de reparação tecidual. Objetivos: A proposta do presente estudo foi analisar as propriedades materiais e estruturais por meio de ensaio mecânico em tíbias após aplicação de ozônio em falha óssea produzidas cirurgicamente. Metodologia: Foram utilizados 10 ratos albinos Wistar, machos, com 40 dias de vida, divididos em dois grupos: grupo controle e grupo ozônio, sendo este tratado com água ozonizada na concentração de 25 µg/ml até a data da eutanásia. Na perna dos animais foi realizada tricotomia e incisão longitudinal na pele, expondo a da diáfise da tíbia de ambos antimeros e com auxílio de uma caneta de alta-rotação, produzindo uma falha óssea. Após 60 dias da cirurgia os animais foram eutanesiados, as tibias foram coletadas para análise biomecânica. Resultados: as propriedades biomecânicas (estruturais e materiais) evidenciaram interações significativas mediante a exposição ao ozônio, evidenciando uma diminuição na

resistência óssea dos animais do grupo controle, observado por meio da diminuição na força máxima (N) necessária para romper o osso quando comparado aos valores necessários para quebrar os ossos dos animais do grupo ozônio e as análises das propriedades morfométricas não revelaram diferenças entre os grupos experimentais. Conclusão: O uso do ozônio não alterou as propriedades morfológicas das tíbias e que o grupo que usou ozônio exibiu mais resistência no ensaio mecânico, pois a força máxima para ruptura da tíbia foi maior neste grupo.

Palavras-chave: Tibias; Biomecanica; Ozônio; Resistência.

Abstract

Introduction: Ozone is a potent antioxidant that acts as a precursor of various radicals, being indicated as a powerful therapy, assisting in the process of tissue healing. Objectives: The proposal of this study was to analyze material and structural properties via mechanical testing in tibias after application of ozone in bone defects produced surgically. Methods: Ten male 40-day old albino Wistar rats have been used, divided in two groups: control group and ozone group, this last one being treated with ozonized water in 25 µg/mL of concentration until the day of euthanasia. Trichotomy and longitudinal incision was conducted in the animals' leg skin, exposing the tibia's diaphysis of both antimers, and with help of a high rotation pen a flaw has been produced on the bone. After 60 days of surgery the animals were euthanized, and tibias were collected for biomechanical analysis. Results: The results of the biomechanical properties – structural and material – evidenced significant interactions through exposure to ozone, showing a diminished bone resistance in animals from the control group, observed by the decrease of the maximum force (N) needed to rupture the bone when compared to the value needed to break the bones of the animals from the ozone group, and the analysis of the morphometrical properties did not show any difference between both experimental groups. Conclusion: The use of ozone did not alter the morphological structures of the tibias, and the group which used ozone presented more resistance during mechanical testings, because the maximum force for the rupture of tibia was greater in this group.

Keywords: Tibias; Biomechanical; Ozone; Resistance.

Resumen

Introducción: El ozono es un potente oxidante que actúa como precursor de varios radicales, siendo indicado con potente terapéutico con auxiliar en el proceso de reparación de tejidos. Objetivos: El propósito de este estudio fue analizar las propiedades materiales y estructurales

mediante ensayos mecánicos en tibias después de la aplicación del ozono en la insuficiencia ósea producida quirúrgicamente. Metodología: Se utilizaron diez ratas wistar albinas macho con 40 días de vida, divididas en dos grupos: grupo de control y grupo de ozono, que fue tratado con agua ozonizada a una concentración de 25 g/ml hasta la fecha de la eutanasia. En la pierna de los animales, se realizó tricotomía e incisión longitudinal en la piel, exponiendo la diáfisis de la tibia de ambos antimémeros y con la ayuda de una pluma de alta rotación, produciendo una falla ósea. Después de 60 días de cirugía, los animales fueron eutanasiados, y las tibias fueron recolectadas para análisis biomecánicos. Resultados: las propiedades biomecánicas (estructurales y materiales) mostraron interacciones significativas a través de la exposición al ozono, evidenciando una disminución en la resistencia ósea de los animales del grupo de control, observadas por la disminución de la fuerza máxima (N) necesaria para romper el hueso en comparación con los valores necesarios para romper los huesos de los animales del grupo de ozono y los análisis de propiedades morfométricas no revelaron diferencias entre los grupos experimentales. Conclusión: El uso del ozono no alteró las propiedades morfológicas de las tibias y que el grupo que utilizó ozono exhibiera más resistencia en el ensayo mecánico, porque la fuerza máxima para la ruptura tibial fue mayor en este grupo.

Palabras clave: Tibias; Biomecánica; Ozono; Resistencia.

1. Introduction

In 1840, Christian Friedrich Schönsein discovered a more allotropic and active variant of oxygen (O_2): ozone (O_3) (Grootveld M, et al., 2004) (Veranes XG, et al., 1999). The first ozone generator was developed by Werner von Siemens in Germany around 1854, and the first report of its therapeutical use was made by C. Lender in 1870, with the proposal of blood purification. Ozone is a potent antioxidant that can act as a precursor of a series of other radicals, with both in vitro and in vivo actions (Grootveld M, et al., 2004) .

Ozone is a gas that stirs controversies because even though it has great potential of therapeutic utilization, it is also a highly toxic gas, very useful in the stratosphere as an absorber of ultraviolet radiation (Grootveld M, et al., 2004) (Bocci VA, 2005) (Buliés JCE, et al., 1997).

The contact of this molecule with organic fluids – saliva, plasma, urine and lymph – results in the formation of reactive molecules of oxygen, which influence biochemical events

of cellular metabolism and that can, according to some authors, provide beneficial effects to tissue healing (Cardoso CC, et al., 2000) (Re L, et al., 2008).

Good results have been observed with the ozone therapeutical use in treating patients with diabetic neuropathy (Batista AD, et al., 2001), post-traumatic bone exposures (Buliés JCE, 1996), degenerative arthropathy (Buliés JCE, et al., 1997), respiratory illnesses (Gent JF, et al., 2003), avascular osteonecrosis(Agrillo A, 2006) and other diseases (Martínez-Sánchez G, et al., 2005).

Numerous indication possibilities exist, with well proved indicatives for the treatment of some clinical situations. Furthermore, many of these therapies are referred to in literature, i.g. potent antimicrobial action, easy local or systemic application, low cost, no collateral effect, intolerance, or contraindication (Martínez-Sánchez G, et al., 2005) (Baysan A & Whiley RA & Lynch E, 2000) (Bocci VA, 2006).

There are many ways to administer ozone therapy. Besides the endovenous path that should be avoided due to the high risk of embolia (Bocci VA, 2005), literature describes four main means of administration: autohemotherapy, rectal insufflation, hermetical bag of ozone, and topic application with ozonized water and oil (Bocci VA, 2006). The most used ozone therapy method is a autohemotherapy. (MAH), in which a predetermined volume of blood is drawn from the patient (200-270 mL), mixed with the proportional gas volume (O_3 + O) and reinfused endovenously.

Ozone concentration must respect the therapeutic window between 10 and 80 µg/mL (Bocci VA, 2006). Due to high instability and ozone toxicity, its gaseous form is the safest to be incorporated to fluids like blood, water, isotonic solutions, and oils for clinical application (Cardoso CC, et al., 2000). It can be mixed with oils – e.g. sunflower, which has high affinity due to being rich in unsaturated acids – through electric discharges. When ozonized, these acids form ozonides and, with hydrolysis, aldehydes, ketones, and hydrogen peroxides can be formed, which are responsible for triggering biochemical reactions (Lincheta LF, et al., 2000) (Siqueira JF, et al., 2000).

Tissue healing consists in progressive and ordered events, characterized by various concomitant phases regulated by specific biological events, that initiate in the moment of trauma and remain carious for a period (Kandler B, et al., 2005). All the healing phases are coordinated by cytokines and specific growth factors. For example, prostaglandins, interleukins (IL), and nitric oxide actuate in inflammations; epithelial growth factor (EGF) actuates in re-epithelization; fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) actuate in angiogenesis; and platelet derived growth factor (PDGF), FGF,

tumor growth factor β (TGF-β), IL-1, and IL-4 (Kwon YB, et al., 2006) actuate in migration and proliferation of fibroblasts, and in deposition and remodeling of extracellular matrix (collagens type I, II, and III).

Kandler et al. emphasized that in the whole healing process, including in bone tissue and systemic diseases, needs of an equilibrium between the production of oxygen reactive species (ORS) and its neutralization to avoid toxicity. The hydrogen peroxides neutralization by platelets, protecting the tissue from granulation during bone remodeling, has been exemplified.

Osseous consolidation is composed by coordinated interaction among different types of cells and the liberation of various cytokines (Samee M, et al., 2008). Histomorphological evaluations have shown that ozonized water can alter the process of osseous repair, improving and amplifying vascular neoformation, and expanding the number of osteoclasts next to the injured region, but was not able to stimulate neoformation of osseous trabeculae (Kim HS, et al., 2009).

Considering the great number of accidents that involve extensions bone fractures and the corrective surgical interventions, the study of substances that can assist in bone repair is high relevance for orthopedics and odontological clinics. This study evaluated structural – maximum break up force, displacement at maximum force, and extrinsic bone rigidity – and material biomechanical properties – elasticity module, maximum tension, and deformation at maximum tension – by mechanical testings in tibias after application of ozone in bone defects surgically produced.

2. Material and Methods

From the point of view of the problem approach, this is a qualitative and quantitative research, exploratory and experimental, regarding the objective of data collection respectively. Garcia, J.A.D., et al, 2015 was used for the methodology of scientific research

Animals: there have been used 10 male 40-day old albino rats given by the animal house of the Federal University of Alfenas (UNIFAL-MG). The animals have been divided into two groups of five animals each, one being a control group (CT) and the other ozone group (OZ). The animals were maintained in conventional cages and sawdust. All animals were given the same solid feed (Autoclavable Nuvilab CR-1) and ad libitum water. The animals' weight was checked weekly and every three days solid and liquid consumption by the animals were measured.

Animal experimentation was carried out according to the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA) and was approved by the Ethics Research Institutional Committee of Federal University of Alfenas (UNIFAL), protocol number 669/2015.

Experimental protocol: CT group (control) – all animals were given Autoclavable Nuvilab CR-1 feed as solid diet and ad libitum water as liquid diet; OZ group (ozone) – all animals had the same diet as CT group and treatment using injectable ozone at 25 mg/mL (León Fernández OS, et al., 2016) of concentration, injecting 1 mL by each application until the day of the animal euthanasia. The consumption of liquid and solid diet was measured every 48 hours and the animals' weight was measured daily to determine the increase of mass.

Surgical procedure: The animals were anesthetized with Ketamine solution (Francotar®) and xylazine chlorhydrate (Virbaxyl 2%) at the dose of 90 mg/kg and 12 mg/kg, via intraperitoneal injection. There have been conducted trichotomy and longitudinal incision in the skin of tibia's diaphysis of both antimers, with the help of a high rotation pen using a 3 mm drill producing a flaw on the bone (Figure 1). The periosteum of the tibia on the surgical area was repositioned through the suture of both borders with a 8.0 silk thread and the skin was sutured with a 4.0 cotton thread. All animals were treated with analgesic acetominophenol (Tylenol®) at the dose of 110 mg/kg, administered via intraperitoneal injection every 12 hours for 48 hours, totaling 4 postoperative doses (FELASA, 2016). At the completion of 60 days of experiment every animal was euthanized by intraperitoneal injection overdose of sodium thiopental (100 mg/kg).

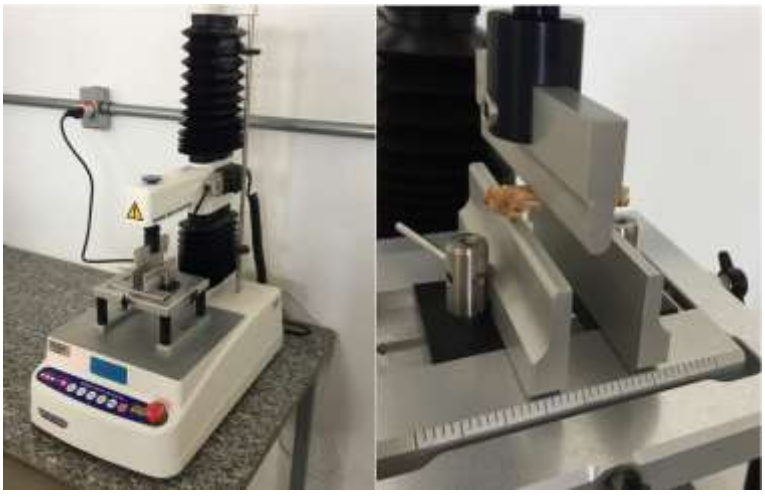
Figure 1. Surgical procedure - 3 mm bone failure produced in the proximal tibial epiphysis.

Source: Collection of article.

In Figure 1 it observed the photo of the ossea failure performed in the right tibia to illustrate how the procedure was performed.

Mechanical test: Animals' right tibias were removed and stored in a freezer (–20 °C) until the day prior to mechanical test. For mechanical test, tibias (n=10 per group) were submitted to three-point bending test until complete fracture at a velocity of 1.3 mm/min. An TA.XT plus (Texture Analyser) set with a load cell of 50 Kgf was used. Each tibia was tested in the anterior-posterior plane (concave-up position), with the anterior surface of the bone facing upwards. The distance between the two bone ends was 50 mm, and in order to obtain the resistance value, a load was applied to the middle third of the bone (Soares EV, et al., 2010) (diaphysis at the site of the surgical intervention) by means of a tip, coupled to the equipment. The load and displacement data were obtained directly from the TA.XT system and recorded with a computer coupled to the testing machine (Figure 2a e 2b). These data were used for the acquisition and calculation of the structural properties: maximum load, displacement at maximum load, and extrinsic stiffness. The extrinsic stiffness was calculated as the slope of the most linear portion of the elastic region of the load-displacement curve.

Figure 2. Three-point bending test TA.XT system until complete fracture the tibias. 2a) TA.XT system; 2b) Tibia in the anterior-posterior plane (concave-up position), with the anterior surface of the bone facing upwards.



Source: Collection of article.

Figure 2 shows the equipment used to perform the biomechanical test in which the right tibias were submitted.

After testing of the specimens in three-point bending, the failure sites of all bone specimens were photographed, together with a measurement standard, by a high resolution digital camera at a standardized distance according to Huang, Lin, Chang et al.

The parameters of cross-sectional cortical bone area of the diaphysis were measured on the images using NIS-Elements 3.0 software (Advanced Research, USA). The cross-sectional moment of inertia (CSMI) at the point of failure was calculated by the method of Turner and Burr (1993):

$$I = \frac{\pi}{64}\left[ab^3 - (a-2t)(b-2t)^3\right]$$

The material properties were obtained from the structural properties(Soares, EA, et al., 2015) (Weisbroth SH, et al., 1977). The following material properties were evaluated: maximum stress, strain at maximum stress and elastic modulus. On the basis of the load-displacement data, these parameters were calculated using the following equations:

$$\sigma = \frac{força \cdot L \cdot c}{4I} \qquad \varepsilon = \frac{12 \cdot c \cdot d}{L^2} \qquad E = \frac{rigidez \cdot L^3}{48I}$$

Where σ is the stress, L is the distance between the two lower supports, c is the maximum distance from pixels to the line that crosses the center of the mass, Є is the strain, d is the displacement, and E is the elastic modulus. The mechanical tests were carried out at the Department of Anatomy of Federal University of Alfenas.

Statistical analysis - Final weight (g), daily liquid intake (ml), daily solid intake (g), biomechanical tibias analysis, and morphological and morphometric comparison were statistically compared between the two groups by analysis of variance followed by Tukey's test, with the level of significance set at 1 and 5%, respectively. Means with different letters were significantly different (5%) from each other.

Anatomical dimensions of the tibia - After removal of all soft tissue from the tibia, the following four measurements were obtained with a digital caliper and magnifying glass: 1) tibia length; 2) tibia diaphysis width (measured at the narrowest point of the mid-tibia); 3) width of proximal tibia; 4) width of distal tibia.

3. Results and Discussion

Laboratory animals help in the study of various humane diseases and their treatments, because they surpass the clinical studies limitation. Knowledge regarding the process of osseous cicatrization and osseous biomechanics are majorly obtained from experimental studies, which have helped to comprehend osseous reparation (Soares EV, et al., 2010).

In this study the animals have been submitted to surgical procedure in order to generate an osseous defect in the tibia and in order to guarantee the animals' health during the experiment, the animals' weight, liquid intake (water), and solid intake (feed) were all controlled. Weisbroth et al. wrote that variations in the amount of solid and liquid intake can provoke modifications on biological responses in animal experimentation.

Solid intake inferior to 25 g/day and significant losses of mass during the experiment characterized malnutrition in rodents (Palencia G, et al., 1994). However, our results demonstrated that the rats from the CT group (299,12±8,5) and OZ group (313,37,12±8,5) gained weight during the experiment and there was no significant differences between these groups.

According to Svendsen and Hau (Svendsen P, 1984), the rats should be fed approximately 25 g of feed and from 15 to 80 mL of water. In our experiment, all the animals had solid and liquid intake between ideal limits, not characterizing protein malnutrition or dehydration.

Animals from group CT and OZ had adequate solid and liquid intake according to Table 1, and this data corroborate the confirmation that during the experiment the animals didn't present malnutrition or dehydration. Our study demonstrated that the maximum force to break the tibias of the animals from the group OZ was greater than the force needed to cause the rupture of the bones of the animals from group CT.

Gain in weight (g), solid intake (g) and liquid intake (mL) were satisfactory, with no occurrence of significant interactions in experimental groups (Table 1).

Table 1. Comparison of averages among experimental groups for the gain in weight (g), solid intake (g) and liquid intake (mL) (measured every two days). Results expressed in average ± standard error.

Variable	CT	OZ
Weekly weight gain (Δ, g)	299,12±8,52[a]	313,37±8,50[a]
Solid intake (g)	88,62±1,99[a]	89,93±1,97[a]
Fluid intake (ml)	64,12±1,39[a]	62,56±1,32[a]

Averages followed by the same lower case letter in line are statistically the same by the Tukey test (P ≤ 0,05). Source: Authors.

All animals showed weight gain, solid and liquid intake within the ideal limits not characterizing protein malnutrition or dehydration. The animals of the CT and OZ group, according to table 1, presented adequate feed and liquid intake, data that corroborate the confirmation that during the experiment the animals did not present malnutrition or dehydration. This could leave doubt as to whether the results found were due to the use of ozonio.

A secure and correct ozone dose represents a non-deleterious acute oxidative stress that induces an antioxidant cellular response which is able to revert a chronic oxidative stress, i.e. helps normalizing the unstable redox balance in many diseases. This act can help improving circulation (local vasodilatation and angiogenesis) and oxygen support, favoring metabolism and cytokines liberation, autacoids and growth factors, that, alongside antimicrobial activity, represent crucial elements in the treatment of metabolic, inflammatory, infectious and neoplastic diseases. The action of ozone therapy can be interpreted as a nontoxic “therapeutic shock”, capable of restoring homeostasis from being a modifier of the physiologic response (Buliés JCE, 1996) (Bocci VA, 2006).

Bucci, 1997, points in his study that the oxidative capacity of ozone reflects directly in the healing process. A study conducted by Kan et al. concluded using histological and radiological analysis that the use of hyperbaric oxygen and ozone in osseous defects of 5 mm in skulls of Winstar rats, separately and combined, efficient in increasing osseous consolidation. Alpan, Toker and Ozer (Alpan AL & Toker H & Ozer H, 2016) investigated the effects of systemic and topic applications of ozone in alveolar osseous consolidation after dental extraction and detected that a systemic postoperative application of ozone can accelerate the process of alveolar osseous cicatrization after extraction on the long run. The osseous defects produced in animals from the CT and OZ groups were totally closed, demonstrating macroscopical tissue repair.

The analyzes of morphometric properties did not reveal differences among experimental groups.

Table 2. The average comparison among the experimental groups for morphometric properties of the right tibias, 8 animals per group. Results expressed in averages ± standard error. Alfenas, Minas Gerais, Brazil, 2014.

Variables	CT	OZ
Tibia length (mm)	24,43±0,40[a]	24,31±0,40[a]
Proximal width of tibia (mm)	9,81±0,50[a]	9,56±0,50[a]
Diaphesys width of tibia (mm)	7,21±0,25[a]	6,71±0,25[a]

Averages followed by the same minor letter in line are statistically the same by the Tukey test ($P \leq 0,05$). Source: Authors.

As illustrated in Table 2, we can observe that the variables in question (Tibia length, proximal width of tibia and diaphesys width of tibia) did not show significant differences when comparing the groups of animals studied.

Results of the biomechanical properties (structural and material) showed significant interactions mediant the exposure to ozone, with osseous resistence decrease in animals from the control group, observed by the decrease of the necessary maximum force (N) to rupture the bone compared to the needed value to break the bones of the animals from the ozone group (Table 3).

Table 3. Tibia structural and material properties in rats of groups CT and OZ. Results expressed in average ± standard error.

Mechanical properties	CT	OZ
Structural properties		
Maximum load (N)	140,16±5,86[a]	192,46±5,86[b]
Displacement (mm)	201,00±5,86[a]	193,27±5,86[a]
Stiffness (N/mm)	0,35±0,04[a]	0,75±0,04[a]
Material properties		
Maximum stress (mpa)	142,57±4,35[a]	15,13±0,71[a]
Elastic modulus (mpa)	89,85±1,94[a]	84,68±1,94[a]
Strain (mm/mm)	0,35±0,01[a]	0,06±0,01[a]

Source: Authors.

In the results expressed in Table 3, the lines indicate the results referring to the biomechanics test. The only variable that presented significant alteration was the Maximum load of the OZ group 192.46±5.86b compared to the CT group 140.16±5.86

Although there is literature that associates the use of ozone and osseous repair, the work developed by Kim et al. revealed in histomorphological analysis that the use of ozonized water can alter the osseous repair process, improving and expanding vascular neoformation and the number of osteoclasts next to the injured region, but is not able to stimulate the neoformation of osseous trabeculae.

4. Conclusion

Analysis of the maximum force of rupture demonstrated itself different between the groups, as the force needed to rupture the tibias of the group treated with ozone was greater. Biomechanical analysis evaluated diverse parameters and even though the force parameter was the only one that presented significant different results, it indicates that the bone tissue is more resistant. We believe these data reveal the need of future studies that histologically evaluate the tissue in terms of calcium and collagen deposition.

As a proposal of a new study on the effects of ozõnio we will seek the analysis of the neoformed bone and the use of immunohistochemistry to corroborate the beneficial findings of biomechanics presented in this study. Furthermore the study with ozone for biomechanics in repair in other types of tissues such as ligaments and tendons can be expanded.

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