Characterization physical-chemical and biological the bovine bone graft, Bonefill in biogical assays – Part 1

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ABSTRACT
The use of biomaterials for the repair of bone defects has received great attention from researchers and dentists that due to good results in last years. Moreover, the market has become very promising for companies that dominate the technology. In the case of products derived from bovine tissues, the concern with the traceability of raw materials, control of manufacturing processes and ensuring the biosecurity of the material produced should be key points in the choice of occupation. The purpose of this was assessed and laboratory tests to characterize the material produced by BoneFill® Biomedical Bionnovation SA from bovine bone cortical. Evaluation hypersensitivity, test for in vitro cytotoxicity, test for systemic toxicity, test for irritation an delayed-type hypersensitivity, test implant, gene reverse mutation test and histological evaluation of bone defects were performed to characterize the material given the requirement of ISO 10993. The results demonstrated the effectiveness of bovine bone Bonefi ll® as material to fi ll bone defects in animals. Within the limitations of this study, it is the biocompatibility and biosafety of the product as bone substitutes in treatments for regenerating tissue with a technique of bone grafts.
Key Words - Bone transplantation; Biocompatible materials; Biological Assay, Graft vs host reaction.

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Introduction and Literature Review

The possibility to selectively influence bone formation, controlling quality and quantity became a reality since the technological development of biomaterials, and the important evolution of methods and knowledge of cellular and molecular biology present in these events. However, researches on the implant material ideal for autogenic bone graft replacement still persists as one of the greatest challenges of modern Dentistry[1].

It is known that the implant best material is the autogenic bone, due to its biological properties, along with the rejection absence. However, there are some inconveniences for its frequent use, such as the need, in most cases, of a second surgical bed, increased infection risk, longer convalescence period, and higher cust[2-3]. These factors hinder or make the procedure impracticable, contributing to the development and production of bone substitutes, synthetic or biological. A great variety of biomaterials can be found on the market, with different sizes, morphological shapes and composition, and application procedures, and various authors report that such materials are exhaustively studied[4-5]. Among them we highlight the lyophilized bovine bone that has been widely studied for bone graft[6]. Bovine bone matrix is easy to obtain, widely available, with long-term storage, and physical-chemical properties similar to those of human bone[6-7].

With that, several studies have been published with regarding the biological quality and biocompatibility concerns of the bovine bone matrix used as implantable material. In a study, the citotoxity, systemic toxicity, irritating potential, progenic reaction, and Bioburden were determined in bovine bone samples processed, in which it was concluded that the lyophilized bovine bone production is possible with excellent biocompatibility[6].

The authors evaluated the bone graft material of bovine origin in implantation testing, in rats, in order to assess the tissue response, in which they concluded that it is biocompatible[8].

The histological response of bovine bone matrix graft in human was evaluated and it was concluded that the combination of graft material with barrier is favorable for the periodontal bone defect healing[9].

Other authors proved the osteoconduction properties of demineralized bovine bone, through a comparative study between different grafting materials in bone defect and the control group, with bone clot[10]. The evaluation of xenogeneic graft osteoconduction in alveolar defect repair in humans, was also proposed in the study, which concluded that it is suitable for the treatment before tooth implants installation[11].

For some authors, the bovine bone graft provides uniform and homogeneous degradation. And regarding the tested products, autogenous graft and bovine xenogeneic materials, both were considered appropriate for bone grafting procedures[12]. Also, in another study, it was demonstrated that the bone grafting material of inorganic bovine matrix supports the anchoring and proliferation of osteoblasts[13].

In view of the need for more evidences that prove, by means of laboratory tests, the effectiveness of bone graft material of bovine origin, the purpose of this study was to evaluate the responses of the living beings organism compared to the grafted material.

Proposition

In order to evaluate the possible sensitizing and cytotoxic effects, reversible damaging or not on the skin, biocompatibility, histological responses, and mutagenic potential of Bonefill bovine bone graft material, produced by Bionnovation Biomedical Products S/A, Anvisa registration 10392710012, tests for hypersensitivity, potential for cytotoxicity, primary irritation, implant, histological, and Ames test were performed.

Material and Methods

Hypersensitivity Test in Guinea Pigs – Maximized method

A pre-test was conducted with two guinea pigs, and test substance composed of Bonefill and saline solution, at a ratio of 1:5, in which no dermal irritation or systemic reactions were observed. Therefore, for the hypersensitivity test 15 albino guinea-pigs (Cavia porcellus), young, female, weighing between 287 g and 513 g of live weight were used. The animals were healthy, with no external injuries and had not been used in other essays. Then, they were divided into two groups (five animals in the control group and 10 in the test group) and kept in polypropylene boxes, covered with wood shavings and covered with metallic grid. The diet was composed of commercial feed and filtered water supplementation, both provided at will. They were prepared, with hair removal in the application area, with shearing equipment and minimum aggression to the region. Then, they were subjected to the application of three pairs of intradermal injections, of a volume of 0.1 ml of each solution. The solutions to the test group were: A - ratio of 1:1 of ACF/saline solution; B - test substance diluted at a ratio of 1:5 in saline solution; and

C -50% of solution B in 50%of the solution; and solutions for the control group were: A -ratio of 1:1 of ACF/saline solution; B - only saline solution, and C - 50% the solution B in 50% of the solution. Injections 1 and 2 were administered in the shoulder region, close to the head, one on each side; and the third near the tail, test area for the induction period (first day). On the seventh day, the animals of both groups were treated with topical application of 0.5 ml of 10% solution of sodium lauryl sulphate in liquid vaseline, in the test area and on the eighth day, the test group received the product topical application through occlusive gauze maintained for 48 hours, and the control group treated with gauze moistened with saline solution. In the challenge period, on the 22nd day, one of the animals' sides of the test and control groups was given occlusive gauze with test substances and saline solutions, respectively, for 24 hours. After 48 hours and 72 hours the test guinea pigs were examined and dermal reactions were clinically observed, determined according to Magnusson and Kligman classification, described as:

0 – No visible changes.
1 – Discreet and small erythema.
2 – Moderate and confluent erythema.
3 - Intense erythema and edema.

Evaluation for cytotoxicity potential in vitro

NCTC clone 929 cell lines and conjunctive tissue cells of mice (ATCC CCL1) were sown in Petri dishes and incubated for 48 hours for cell monolayer formation. Then, samples, Bonefill, were placed in solid culture medium (composed of equal parts of middle twice concentrated and neutral red agar) and incubated again for 24 hours. The positive and negative controls, respectively, were made with non-toxic paper filter discs with 0.5 cm in diameter and 0.5 cm fragment x 0.5 cm of toxic latex. Samples were tested in quadruplicated. Macro and microscopic observations were carried out and classified with the zone index (IZ), to identify non-stained areas by vital dye according to ASTM F895-84[14]:

0 - No area under and around the sample - None
1 - Few changes or cellular degeneration in the sample – Weak
2- Limited area in the sample – Slight
3- Area between 0.5-1.0 cm around the sample – Moderate
4- Area greater than 1.0 cm around the sample – Severe

Systemic Acute Toxicity Test

Twenty Swiss albino mice, weight between 19.800 g and 21.70 g, properly prepared for the test were also distributed in four experimental groups, tests (oleic extract and saline extract) and controls (cottonseed oil and saline solution). Each animal received an injection with 1.0 ml/20 g of body weight of each solution according to each group. The oleic extract test group animals (250 mg of Bonefill solution and 10 ml of sterile cottonseed oil, prepared in autoclave, 121°C for 60 minutes) were compared with the cottonseed oil control group, and injected via intraperiotonial. The group injected with saline solution was the saline extract test control group (250 mg of Bonefill solution and 10 ml of despyrogenized sterile saline solution) intravenously. Macroscopic abnormalities were analyzed during the test period and deaths cases.

Primary skin irritation test in rabbits

Three albino rabbits (Oryctolagus cuniculus), of New Zealand breed, white, adults, both genders, with 2290 g and 2410 g of live weight, healthy and without detectable pathological changes in the skin were used. They were maintained acclimated to controlled laboratory conditions and diet consisting of pelletized commercial feed and filtered water supplementation, both provided at will. For the test, hair was carefully removed from region immediately posterior to the neck, with shear device, without irritating or hurting the region. 0.5 ml of the test solution, dilution, in ultrasound, out of Bonefill into deionized water at a ratio of 1:5, was applied on the skin of the exposed region, approximately 6 cm2 and protected with occlusive gauze for four hours. For negative control, adjacent untreated areas were analyzed. After the exposure period, the area was cleaned by washing with water and cotton aid. Animals were clinically observed in periods of 60 minutes, 24 hours, 48 hours, and 72 hours after application and reactions were analyzed regarding the presence of erythema, edema, scabs formation, and local and systemic changes.

Implant test in rabbits

Two albino rabbits, New Zealand, females, adult, healthy (2.5 kg to 3.5 kg) were selected for this test. The procedure was initiated with the depilation of the animals' dorsal region to perform the samples implanting. The animals were sedated before surgery and samples measuring 10 mm x 1 mm were implanted in paravertebral muscle, in six incisions made 2.5 cm from the spine and 2.5 cm from each other, being four incisions on the left and two on the right sides, containing the controls. After implantation, the animals were observed for a period of 30 days.

Reverse gene mutation test in Salmonella typhimurium (Ames test)

A preliminary test was performed with the TA 100 strain and concentrations of 10 µl, 25 µl, 50 µl, 100 µl, and 200 µl of extract/plate were determined for the ultimate test that was performed according to the methodology described in Cetesb Technical Standard L5.621[15]. Glucosic minimal medium and surface agar were used, top agar. For tests with metabolic activation, 0.5 ml/plate of S9 fraction were added to the top agar, with protein concentration of 36.2 mg/ml. Positive and negative controls were included in all tests. For negative control, performed in triplicate, deionized water was used, 100 µl/plate in order to obtain the number of colonies per plate and compare to the test group. For positive control, in duplicate, admittedly mutagenic substances were used to ensure the responsiveness capacity of each strain to mutagen, and the metabolic activation system effectiveness. All concentrations were tested in triplicate in the absence and presence of metabolic activation. The strains TA98; TA100; Ta 102; TA1535 and TA1537 of Salmonella typhimurium were incubated for 72 hours. Mutagenicity reasons (RM) were assessed, and also the presence or absence of metabolic activation system that corresponds to the microsomal fraction of rat liver induced with Aroclor 1254.

Histological evaluation of bone defects made in rabbit tibiae

Six adult albino rabbits, New Zealand, of both genders, weighing between 3.5 kg and 4.0 kg were used, supplied by the Vivarium of the Federal University Santa Maria (UFSM). The study was approved by the Animal Ethics Committee under the No. 23081.003273/2007-33. They were divided into two groups (three animals in each group) being kept in individual cages fed with balanced feed once a day and water ad libitum. The animals underwent intravenous anesthesia with acepromazine 0.2% (0.1 mg/kg dose), after 15 minutes they were administered 0.5 mL/kg by deep intramuscular via, or the mixture in equal parts of ketamine (vetanarcol 5%) and xylazine hydrochloride (kensol 2%). After tibiae trichotomy in inner portion, the skin antisepsis was performed by friction with topic iodopovidine. Bone defects were made with trephines of 4 mm diameter (Meisinger) under intense cooling by saline solution, being two in each tibia (Figure 1). All animals received the same anti-inflammatory – subcutaneous dose of ketoprofen 2 mg/kg, once daily for three days. The entire procedure was performed at the Department of Veterinary Medicine of UFSM. The animals were divided into two experimental groups, the control group (GC), defects filled only with clot (c and d beds); and test group (GT), defects filled with Bonefill (a and b beds). The animals sacrifice was accomplished by intraperitoneal injection of an overdose of the chloral hydrate anesthetic substance, with three animals being sacrificed with 30 days, and three other 60 days after surgery. The tibiae were removed and placed for fixation in buffered formaldehyde solution of Lillie 10%, pH 7.4, for 48 hours. Then they were washed for 24 hours in running water and demineralized in Morse solution, for a period of 30 days. After that, the pieces were washed in running water for 24 hours, neutralized in sodium sulphate solution for 24 hours, washed again in running water for another 24 hours, reduced and placed in 70% alcohol. In the lab they were dehydrated in increasing concentration alcohols (alcohol 80%, 90% alcohol and absolute alcohol), diaphanized in xylene, and included in paraffin for the realization of the cuts in rotational microtome. Longitudinal serial cuts were then made, 6 µm thick, which were stained by the hematoxylin and eosin (HE) method, which allows an overview of cell morphology and the presence of collagen fibers. Histologic evaluation of events was made in a descriptive manner, using a semi-quantitative method based on normality-related aspects knowledge. For this analysis, a light optical microscope Nikon E200, of Biotecnos microscopy laboratory, was used. For determining Bonefill effectiveness, during the bone neoformation, some parameters have been evaluated, based on the presence of cells characteristics of the inflammatory process, the formation of fibrous capsule around the implanted material, the type of neoformed tissue in the region, and in the presence of the implanted material amid the receptor site during periods observed. The plates were analyzed by a single professional experienced in bone tissue histology, who conducted a descriptive and semi-quantitative analysis considering representative parameters of the repair process, such as: cortical repair, collagen fibers, inflammatory infiltrate, and bone neoformation.

Results

Table 1 shows the results obtained in the hypersensitivity test for 48 and 72 hours, and the results observed for the control group.

The data shown in Table 1 when compared with the classification data indicate that analyzed samples did not cause any type of dermal or systemic reaction in guinea pigs. This result indicates that the substance is considered non-sensitizing in these animals. control.

Table 2 shows the results found in the test for evaluating the cytotoxicity potential in vitro to BoneFill, negative control and positivecontrol.

***TABLE 1** – RESULTS OBTAINED IN THE NYPERSENSITIVITY TEST FOR 48 AND 72 HOURS OF TEST AND CONTROL GROUPS*

Test Group						Control Group		
Guinea Pigs	48 h	72 h	Guinea Pigs	48 h	72 h	Guinea Pigs	48 h	72 h
1	0	0	6	0	0	1	0	0
2	0	0	7	0	0	2	0	0
3	0	0	8	0	0	3	0	0
4	0	0	9	0	0	4	0	0
5	0	0	10	0	0	5	0	0

TABLE 2 – RESULTS OBTAINED IN THE CYTOTOXICITY POTENTIAL ASSESSMENT TEST IN VITRO

Sample	1	2	3	4
	IZ	IZ	IZ	IZ
BoneFill	0	0	0	0
Negative control	0	0	0	0
Positive control	4	4	4	4

***TABLE 3** – ACUTE SYSTEMIC TOXICITY RESULTS*

Readings	Time (h)				Number of deaths
	4	24	48	72	
Test group (oleic extract)	0	0	0	0	0/5
Control group (cottonseed oil)	0	0	0	0	0/5
Test group (saline extract)	0	0	0	0	0/5
Control group (saline solution)	0	0	0	0	0/5

***TABLE 4** - DEGREE OF ANIMAL SKIN LESIONS FOR THE PRODUCT BONEFILL*

Rabbit 1		Rabbit 2		Rabbit 3		Time
Erythema	Edema	Erythema	Edema	Erythema	Edema	
1	0	1	0	0	0	60 minutes
0	0	2	0	0	0	24 hours
0	0	0	0	0	0	48 hours
0	0	0	0	0	0	72 hours

The data presented in Table 2 compared with the classification, indicate that the product BoneFill showed no toxic effect to the NCTC Clone 929 cell line (ATCC CCL-1).

The results obtained in the acute systemic toxicity test by intravenous and intraperitoneal administration via are shown in Table 3 and indicate that there were no deaths in the analyzed periods and, therefore, the material was considered approved. No macroscopic abnormalities were observed in animals during the test period.

For the primary skin irritation test in rabbits the lesions degree on the skin of the animals treated with the product Bonefill, is expressed in Table 4.

The histological analysis of samples from each animal in the control group showed that the bone area solely regenerated by clot featured structure with plenty of bone tissue formation; the samples were collected in 30 days with ample trabeculae (green arrows), however, with a few incremental growth lines and discrete presence of cells (osteoclasts and osteoblasts) inside it in all samples evaluated. The existence of collagen fibers with smaller number of osteocytes present are also noticed in images (Figure 1). After 60 days, the studied areas, in turn, showed normalcy aspect without inflammatory infiltrate (Figure 2), when compared to the test group, however, with small amounts of osteocytes present (blue arrows). In addition, inside the trabeculae formed, the aspect was of viable bone cells absence, showing a feature more similar with the adipose tissue formation (yellow arrows). For the test group, grafted with Bonefill, in the area grafted with bovine bone, in 30 days, large areas of cellular activity (yellow arrows) and areas of intense formation of collagen fibers (green arrows) were noticed, characteristic of bone neoformation, however, with a trabecular quantity and density lower than that shown in the control

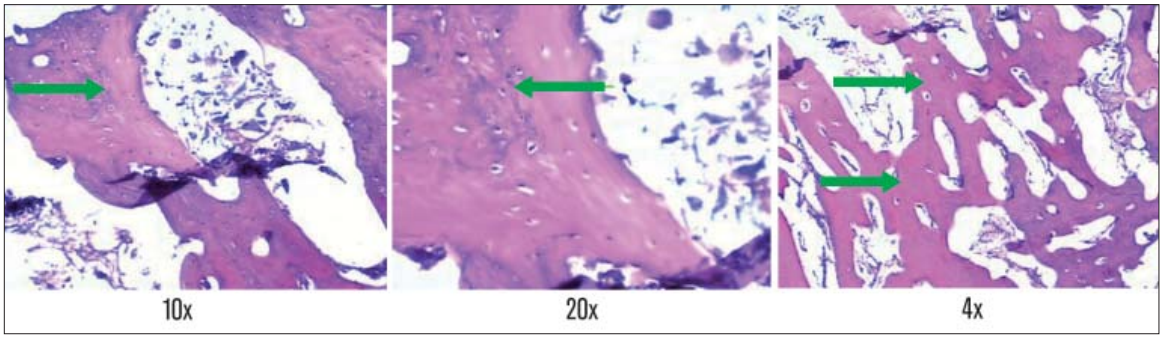

Figure 1
Control group-30 days. The green arrows indicate the bone tissue formation.

(Figure 3). The spaces where the formation (calcification) was not complete showed large amounts of blood vessels and collagen fibers with intense presence of cellular activity.

In all the studied plates a discreet presence of residual material (Bonefill) not reabsorbed (black arrows) was detected. After 60 days, on grafted, an an intense bone neoformation, with good

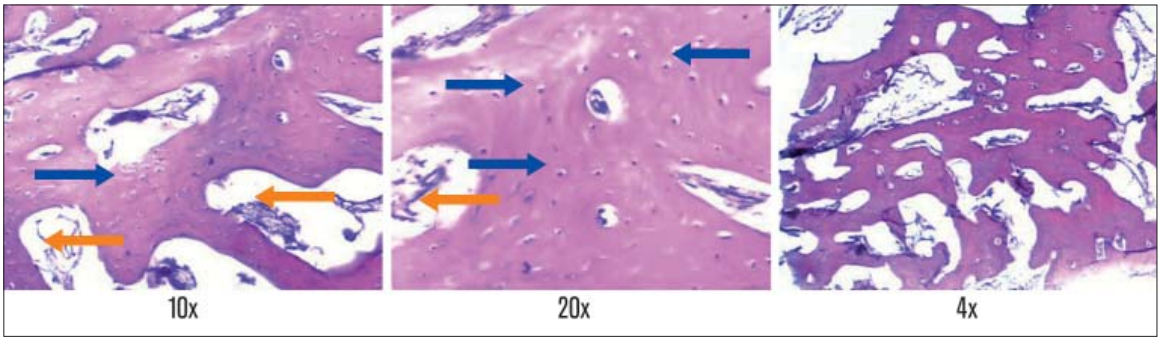

Figure 2
Control group - 60 days. Yellow arrows show adipose tissue formation and blue arrows small amounts of osteocytes.

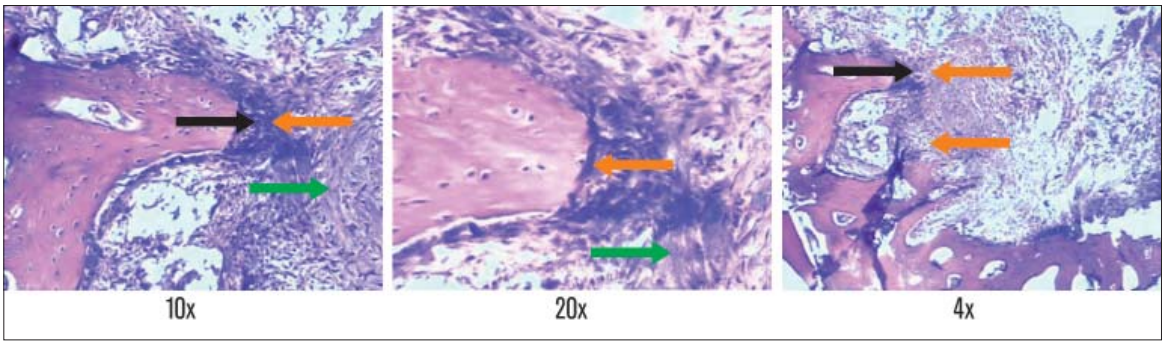

Figure 3
Test group with Bonefill-30 days. Arrows indicate great cellular activity (yellow), intense formation of collagen fibers (green) and residual material, Bonefill, not reabsorbed (black).

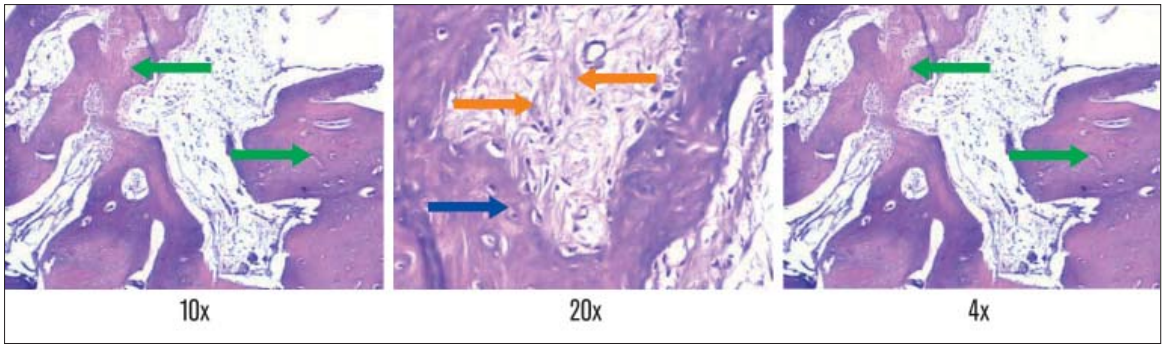

Figure 4
Test group with Bonefill-60 days. Arrows indicate intense bone neoformation (green), osteocytes (blue) and collagen fibers with intense cellular activity (yellow).

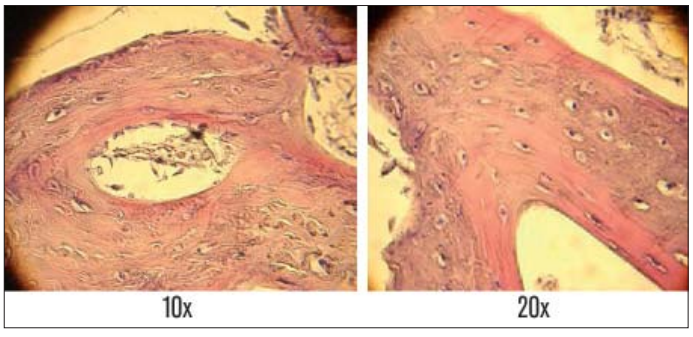

Figure 5
Test Group with Bonefill - 60 days – presence of blood s vessels.

trabecular density and large areas of bone neoformation (green arrows) was observed. Neoformation zones presented viable cells inside it (osteocytes - blue arrows), besides several incremental growth lines. In non-calcified spaces of the area grafted with Bonefill it was noted, also, in all cases, the presence of collagen fibers with intense cellular activity (yellow arrows), when compared with the control group (Figure 4). Another fact always noticed was the presence of dilated blood vessels, denoting intense vascularity in the repair area (Figure 5). In these plates, the presence of the grafted material particles was not detected.

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In order that medical products can be marketed in Brazil its approval by the National Agency of Sanitary Surveillance (Anvisa) is required, which grants the product registration. This registration, controlled by a number, is granted to the manufacturer after the submission of the relevant documentation. Therefore, in order that the regulatory agency grants registration and authorization for sale it is necessary to comply with requirements established, among which are the physical, chemical and biological analyses, in clinical and laboratory aspects, which prove and guarantee the quality and effectiveness of each product. To do so, evaluations and laboratory tests that follow the standards established by the International Organization for Standardization (ISO) are required. The tests for health and medical use products are described in the standard ISO 10993[16], which sets the products biological assessments. Hypersensitivity test[17], evaluation of citotoxity potential[18], acute systemic toxicity test[19], skin irritation test[19], implant test[20], reverse gene mutation test[21], and histological evaluation of bone defects were performed to characterize the material in view of ISO 10993[16] requirements.

Therefore, on the basis of the data obtained in this study, the efficiency and quality of the product examined, on the basis of tests performed was evidenced. It was noted that no test results were unfavorable. The uses of living organisms close to human organism, with reactions that can resemble, allow extrapolating the results for use in humans without causing problems or unpleasant reactions. The product use did not cause death in any of the guinea pigs of any test.

Clinically analyzing the animals it can be seen that Bonefill bovine bone use for the bone defects repair developed satisfactorily, since all bone grafting surgeries were performed without complications. During the grafting surgery it was observed that the material allows an easy handling for the installation in the surgical bed despite the abundant bleeding. Histologically, the consistent new bone

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formation and with active remodeling of Bonefill particle was confirmed. The photomicrographs of the plates showed viable bone tissue in grafted areas and, often, it was not possible to accurately distinguish the neoformed trabeculae of the material particle (Figure 3), even after a period of 30 days. Inside the trabeculae, signs of viable cells within the cellular spaces (figures 3 and 4) were found. Another strong presence was the clearly detectable growth lines in bone trabeculae (figures 3 to 5), with 30 and 60 days. In the trabeculae, suggestive images of an active bone remodeling process in progress were evidenced (Figure 5). A frequent feature in Medullary spaces was the presence of low-to-moderate mononuclear inflammatory infiltrate (Figure 3), probably associated with the very process of bone repair in progress on site. In smaller increases, a greater presence of viable bone (with higher amounts of cellular activity) in areas grafted with Bonefill were noticed when compared with the areas of control groups (figures 1 to 4)[22]. With these data we can conclude that the intensity of the created defects filling, thorough the bone neoformation and remodeling that were in progress in those areas filled with Bonefill, is far superior to the control group. A dynamic process can be observed due to the good osteoconduction quality of Bonefill, allowing that newly formed bone is deposited involving and uniting the particles, which are being reabsorbed while new bone is formed.

Among the reasons that stimulate the use of Bonefill bovine bone, we can describe: it does not require a surgical area donor due to its laboratory origin; its required properties as biomaterial were demonstrated by the tests presented and the good response when used in filling bone cavities.

Conclusion

It can be concluded that the bovine bone Bonefill used proved to be effective as material for filling bone defects in animals, histologically showing effective for propitiating neoformation and bone growth, resulting in a neoformed tissue of firm clinical consistency, similar to natural bone tissue. Also, the distinguished features in the areas treated with the proposed material could be evidenced when compared with the control sites, showing an increased cellular activity. The results showed that the studied material use for filling bone defects provides, due to the possibility of a more intense cellular activity, the foreseeability (not studied in this work) of greater response towards the installation of implants in these sites.

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