

## **Microscopic analysis of the repair of critical bone defects in rabbits calvaria after the use of particulated autogenous bone or particulate autogenous bone associated with inorganic biomaterial**

*Análise microscópica do reparo de defeitos ósseos críticos em calvária de coelhos após a utilização de osso autógeno particulado ou osso autógeno particulado associado a biomaterial inorgânico*

*Análisis microscópico de la reparación de defectos óseos críticos en conejos de calvaria tras el uso de hueso autógeno particulado o hueso autógeno particulado asociado con biomaterial inorgánico*

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### **Abstract**

The aim of this study was to evaluate, through microscopic analysis, the repair of critical defects in rabbit calvaria after the use of particulate autogenous bone or particulate autogenous bone associated with inorganic biomaterial. Six male albino rabbits, New Zealand, were used, in which 4 defects were performed in each calvaria and randomly divided into 4 equitable groups: Group I – performed bone defect; Group II – performed bone defect and filled with particulate autogenous bone; Group III – performed bone defect and filled with particulate inorganic material (Bio-Oss®, Geistlich from Brazil); Group IV – performed bone defect and filled with particulate autogenous bone

associated with particulate inorganic material (Bio-Oss® - Geistlich from Brazil) with proportion being 20:80. The analyze time of the procedure was 60 days. The microscopic analyze allowed to conclude that the autogenous, the Bio-Oss® and the Bio-Oss® associated with autogenous bone groups showed a bone neoformation on the defects, characterizing the material osteoconductive. However, the complete defect closure occurred on the autogenous bone and the autogenous bone associated with the inorganic bone, showing that the presence of the autogenous bone improves the characteristics of the biomaterial. The use of inorganic bone associated with the autogenous bone allowed a complete bone neoformation of the critic defect in the calvaria of rabbits.

**Keywords:** Biocompatible Materials; Bone Regeneration; Bone Substitutes.

### Resumo

Este estudo teve por objetivo avaliar por meio das análises microscópicas o reparo de defeitos ósseos críticos de calvária de coelhos após a utilização de osso autógeno particulado ou osso autógeno particulado associado a biomaterial inorgânico. Foram utilizados seis coelhos albinos machos, Nova Zelândia, nos quais foram realizados 4 defeitos em cada calvaria e divididos aleatoriamente em 4 grupos equitativos, sendo Grupo I – realizado defeito ósseo e preenchido por coágulo, Grupo II – realizado defeito ósseo e preenchimento com osso autógeno particulado, Grupo III – realizado defeito ósseo e seu preenchimento com (BioOss®, Geistlich do Brasil), Grupo IV – realizado defeito ósseo e seu preenchimento com osso autógeno particulado associado ao material inorgânico particulado (BioOss®-Geistlich do Brasil) na proporção de 20:80. O tempo de análise desse processo de reparo foi de 60 dias. A análise microscópica permitiu verificar que tanto o grupo autógeno quanto o grupo do Bio-Oss® e o grupo do Bio-Oss® associado ao osso autógeno tiveram uma neoformação óssea nos defeitos, caracterizando a osteocondução dos materiais. Contudo o fechamento completo dos defeitos ocorreu no grupo do osso autógeno e do osso autógeno associado ao osso inorgânico, demonstrando que a presença do osso autógeno melhora as características do biomaterial. O uso do osso inorgânico associado ao osso autógeno permitiu uma neoformação óssea completa do defeito crítico em calvária de coelhos.

**Palavras-chave:** Materiais Biocompatíveis; Regeneração Óssea; Substitutos Ósseos.

### Resumen

Este estudio tuvo como objetivo evaluar mediante análisis microscópico la reparación de defectos óseos críticos en la calva de conejo después de utilizar hueso autógeno particulado o hueso autógeno particulado asociado con biomaterial inorgánico. Se utilizaron seis conejos machos albinos, Nueva Zelanda, en los cuales se realizaron 4 defectos en cada calvaria y se dividieron aleatoriamente en 4 grupos equitativos: Grupo I - defecto óseo y relleno de coágulo, Grupo II - defecto óseo y relleno óseo de partículas autógenas, Grupo III - defecto óseo realizado y su relleno con (BioOss®, Geistlich do Brasil), Grupo IV - defecto óseo y su relleno con hueso autógeno particulado asociado con material inorgánico particulado (BioOss®-Geistlich do Brasil) en una proporción de 20:80. El tiempo de análisis para este proceso de reparación fue de 60 días. El análisis microscópico mostró que tanto el grupo autógeno como el grupo Bio-Oss® y el grupo Bio-Oss® asociado a hueso autógeno presentaron una neoformación ósea en los defectos que caracterizan la osteoconducción de los materiales. Sin embargo, el cierre completo de los defectos se produjo en el grupo de hueso autógeno y hueso autógeno asociado a hueso inorgánico, demostrando que la presencia de hueso autógeno mejora las características del biomaterial. El uso de hueso inorgánico asociado con hueso autógeno permitió una neoformación ósea completa del defecto crítico en la calva del conejo.

**Palabras clave:** Materiales Biocompatibles; Regeneración Ósea; Sustitutos de Huesos.

## 1. Introduction

The repair of bone defects represents a major surgical challenge, especially in cases of large defects, where their physiological regenerative capacity is exceeded (Vajgel et al., 2014; Carlisle et al., 2019; Götz et al., 2015).

Bone is a connective tissue with a high potential to repair its damaged structure, however, it directly depends on adequate vascularization, mechanical stability and competition with tissues with high proliferative activity (Sohn et al., 2019). Some bone tissue damage may take a longer time to complete the repair or even not be repaired normally by the body, requiring the use of surgical strategies to try to optimize the repair of bone failure, as in cases of critical bone defects, which do not have the ability to repair themselves spontaneously, and may cause a temporary or permanent sequelae for the patient (Li et al., 2015; Porto et al., 2012).

Defects resulting from aging, trauma, osteotomy sites, pathological resections, infections and developmental anomalies are clinical situations in which faults need to be reconstructed (García-Gareta et al., 2015, Kim et al., 2022). It is known that the loss of dental elements causes a series of biological events in the supporting tissues, which must be carefully

analyzed to assess the need and possibilities for treatments in each case, providing the patient with a comfortable rehabilitation, with adequate function and aesthetics. Tooth extraction causes a decrease in masticatory function, thus decreasing bone stimulation, which consequently undergoes an irreversible resorption process and volume reduction of the alveolar ridge (Noelken et al., 2020). Depending on this degree of resorption, it can make it difficult or even contraindicate the placement of implants in the proper position for a correct treatment (Boyne et al., 1997). Therefore, sometimes it is necessary to use grafts before to implant placement in cases where there is not enough bone thickness or height for implant placement (Norton et al., 2003).

Reconstruction of large defects in hard tissue has always been a difficult situation for surgeons, and a variety of bone grafts have been suggested to aid repair, such as autogenous, homogeneous, lyophilized, demineralized, and also artificial or synthetic materials (Saulacic et al., 2021). Among these options, autogenous bone is still considered the “gold standard” due to its osteoinductive, osteoconductive and osteogenic characteristics. However, obtaining enough bone tissue from the donor area to fill the defect in its entirety becomes a challenge in some complex clinical conditions that require a greater quantity of the substitute and its use increases the morbidity of the surgical procedure, which drives the constant search for alternative materials with equivalent biological nobility. (Porto et al., 2012; Noelken et al., 2020; Saulacic et al., 2021, Kim et al., 2022).

Nowadays, bone graft is frequently used in oral surgery, facial maxilla for alveolar reconstruction for future rehabilitation with osseointegrated implants and orthopedic surgeries. It is also indicated for the treatment of congenital and traumatic defects, as well as the reconstruction of the mandible or maxilla after tumor resection or removal of a cyst that often involves these bones (Pallesen et al., 2002, Kim et al., 2022).

Therefore, the search for materials that can replace autogenous bone is great. Among the available biomaterials, Bio-Oss® has been the most used clinically. It is a natural bone substitute obtained from the mineral part of bovine bone, its structural and morphological characteristics favor the proliferation of blood vessels and the migration of bone cells (Açil et al., 2000). Literature demonstrates its biocompatibility and osteoconduction capacity (Norton et al., 2003; Gokhale et al., 2012), as well as well-established and documented clinical outcomes (Handschel et al., 2009). Such as in bone regeneration, for the vertical increase in the posterior region of the maxilla through the elevation of the maxillary sinus (Traini et al., 2008), in peri-implant defects (Gokhale et al., 2012), and in other post-tooth extraction defects. Thus, understanding the relationship of this biomaterial and its association with autogenous bone can improve the characteristics of bone reconstructions.

The critical size defect has been used as an experimental model to evaluate the effectiveness of newly developed biomaterials. There are several models of bone defects used for research on bone regeneration in different anatomical regions, including those of the tibia, radius, mandible and skull of animals (Li et al., 2015; Carlisle et al., 2019; Liu et al., 2020).

Faced with the need for complete bone repair and the great challenge that the treatment of critical defects represents, both in dentistry and in the medical fields, the search for mechanisms that modulate the reparative process is extremely valuable for the therapeutic success of reconstructive surgeries. In the case of bone regeneration, the study with biomaterials and surgical techniques that help in the principles of bone repair, makes possible the constant studies in search of more efficient alternatives, with lower cost and greater availability and accessibility to surgeons.

The aim of this study was to evaluate the repair of critical bone defects in rabbit calvaria after using particulate autogenous bone or particulate autogenous bone associated with inorganic biomaterial through microscopic analysis.

## 2. Methodology

This research was approved by the Animal Research Ethics Committee of the Faculty of Dentistry, São Paulo State University (UNESP), Araçatuba– SP (COBEA) processo: 00999/2011.

## Experimental Groups

For this study, 6 male New Zealand albino rabbits were used, weighing approximately 3.0 kilograms (kg) each, and aged between 5 and 6 months, provided by the Animal Faculty of Medicine of Botucatu, Universidade Estadual Paulista - UNESP which were randomly divided into 4 equal groups that made up the study sample.

Group I: Control – 6 defects: After the 7 mm bone defect had been performed, there was protection with a bovine cortical membrane (GenDerm®- Baumer) (Figure 1A).

Group II: Autogenous bone – 6 defects: After the 7 mm bone defect had been made, it was filled with autogenous particulate bone obtained on the contralateral side (Figure 1B). Then, it was covered with GenDerm® membrane (Baumer).

Group III: Inorganic bone (Bio-Oss®, Geistlich) – 6 defects: After the 7 mm bone defect was performed, it was filled with particulate inorganic material (Bio-Oss®, Geistlich) (Figure 1C). Then, it was covered with a bovine cortical membrane (GenDerm®-Baumer).

Group IV: Autogenous bone associated with Inorganic bone (Bio-Oss®, Geistlich) – 6 defects: After the 7 mm bone defect had been performed, it was filled with particulate autogenous bone associated with Bio-Oss® (Geistlich) in a proportion of 20:80. The autogenous bone was obtained on the contralateral side. Subsequently, protection with a bovine cortical membrane was performed (GenDerm®- Baumer).

**Figure 1** - Grafts used to fill the defects. A: Bovine cortical membrane (GenDerm®- Baumer); B: Autogenous particulate bone; C: Particulate inorganic material (Bio-Oss®, Geistlich).



Source: Authors.

## Surgical Procedure

After fasting for 12 hours, the animals underwent general anesthesia via intramuscular with a mixture of 2% xylazine hydrochloride (4mg/Kg) and 10% ketamine hydrochloride (10mg/Kg) followed by trichotomy in the fronto-parietal region, positioning the animal in prone position, antisepsis of the area with topical povidone-iodine (PVPI) and placement of sterile field (Figure 2A).

Then, surgical access was performed through a straight median incision from the occipital protuberance to the eyes with a 15 scalpel (Figure 2B). The total flap was detached and folded with the detachers to expose the parietal bone on both sides (Figure 2C).

**Figure 2** – Antisepsis and approach. A: antisepsis of the area with topical povidone-iodine (PVPI); B: median incision in the calvaria; C: surgical site exposure.

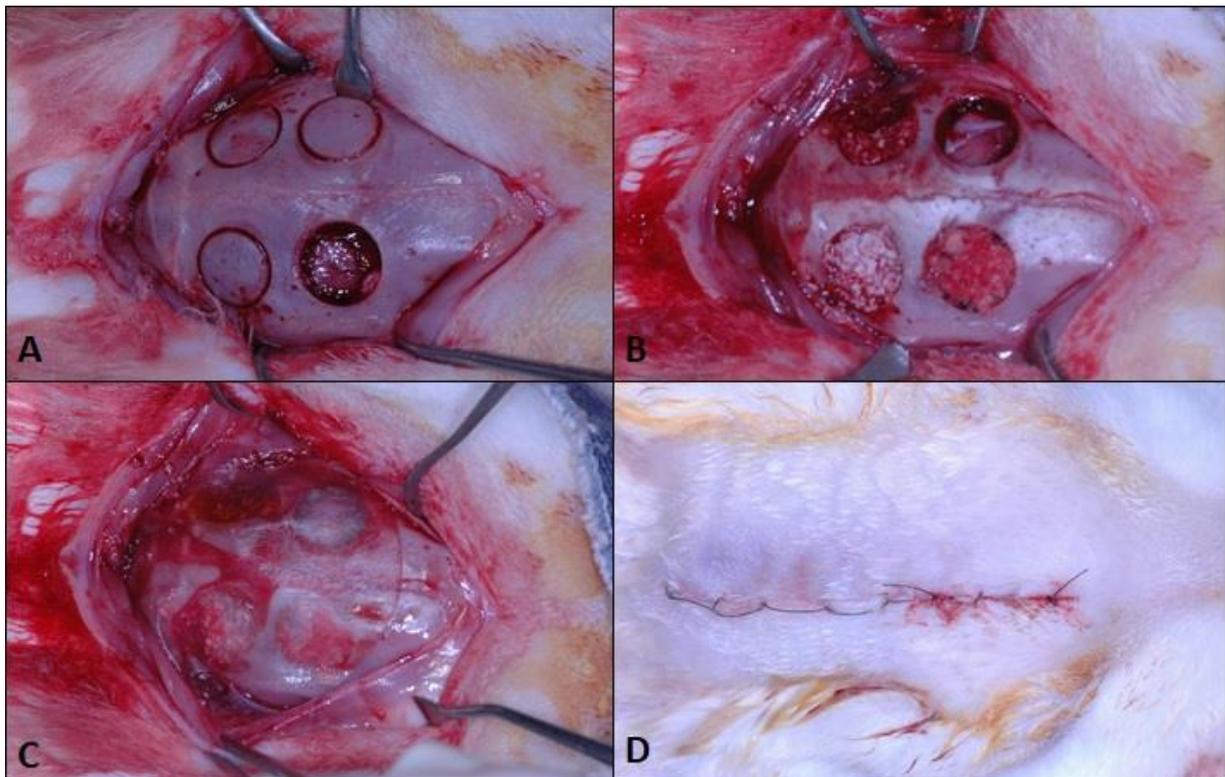


Source: Authors.

Using a trephine drill (7 mm) and using a low-speed motor, two osteotomies were performed in the right parietal bone and two osteotomies in the left parietal bone with saline irrigation, creating four critical defects in the same animal. The osteotomized parietal bone was removed and the dura mater maintained intact (Figure 3A). Bone defects were performed in 6 animals, and each animal received procedures relating to four experimental groups as reported in the item of division of groups (Figure 3B). Covering was performed with a bovine cortical membrane (GenDerm® Baumer) (Figure 3C). After placing the membrane and closing the surgical wound, the skin tissue was sutured using 5-0 nylon (Figure 3D). The animals received a dose of pentabiotic to avoid infection and a dose of dipyrone for postoperative analgesia.

For these animals, euthanasia were performed 60 days after surgery and the material was fixed in 10% neutral buffered formalin. Then the pieces were decalcified and submitted to processing for staining with Hematoxylin-Eosin.

**Figure 3** – Surgical Procedure. A: four critical defects made in calvaria with trephine drills keeping the dura mater intact; B: Filling critical defects with the respective biomaterials; C: covering the defects with bovine cortical membrane; D: Suture.



Source: Authors.

### Obtaining slides for microscopic analysis

The samples obtained were immediately stored in individual containers, properly identified, in 10% neutral buffered formalin for fixation for 7 days. After fixation, the samples were washed in running water for twenty-four hours. Subsequently, the samples were demineralized in a formic acid solution at pH 7.2, which was renewed weekly until total demineralization was verified by radiographic evaluation. The samples were dehydrated in increasing concentrations of alcohol, diaphanized in xylene and embedded in paraffin blocks, to be cut (thickness of 5 $\mu$ m) in a microtome, producing 5 slides per piece, with 3 tissue cuts each. The slides produced were stained with Hematoxylin and Eosin for study in an optical microscope.

Microscopic analysis was performed using an optical microscope to evaluate morphological aspects of the bone repair of the cavity, the evolution of the closure and the presence or absence of biomaterial and bone, trying to compare these events between the four experimental groups.

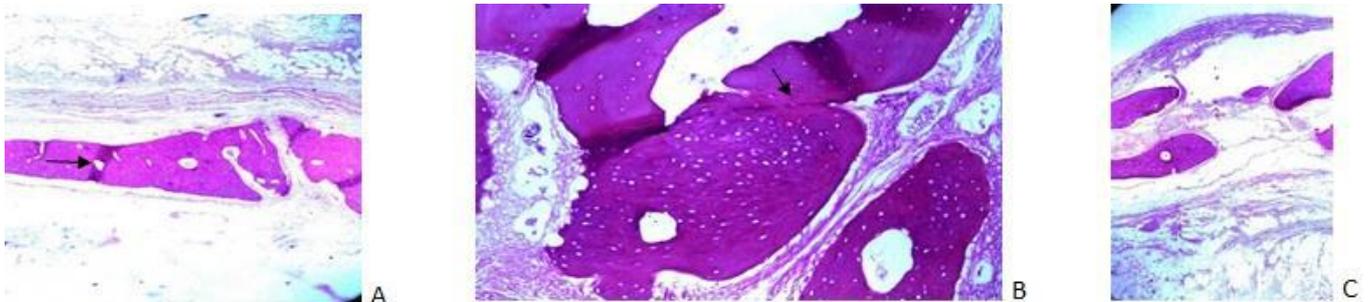
## 3. Results and Discussion

### Control Group

In the evaluation of the control group, it was possible to observe the osteotomy line and bone neoformation occurring near the margins of the bone defect (Figure 4A). At the highest magnification, it is possible to observe bone neoformation close to the stump (Figure 4B).

In the center of the bone defect, no total closure was observed, which was mostly filled with fibrous connective tissue, demonstrating that the defect performed was critical. (Figure 4C).

**Figure 4** - Control Group – A: Control Group, 40x: Left stump, osteotomy line (arrow); Bone neoformation. B: Control Group, 100x: Left stump, osteotomy line (arrow). Bone neoformation; C: Control group, 40x: Center of the defect without bone formation.

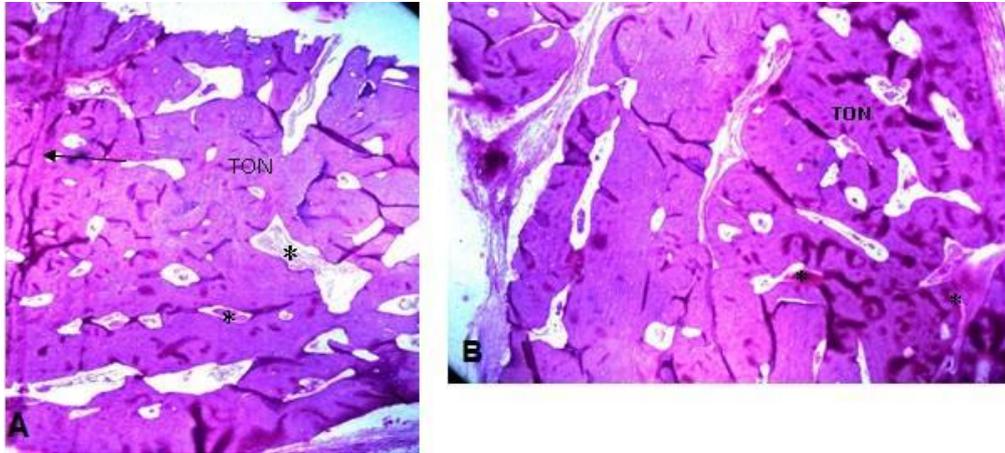


Source: Authors.

### Autogenous Group

In the group where the autogenous bone was used as filling material, a bone neoformation was observed both in the regions close to the stump (Figure 5A) and in the center of the defect (Figure 5B).

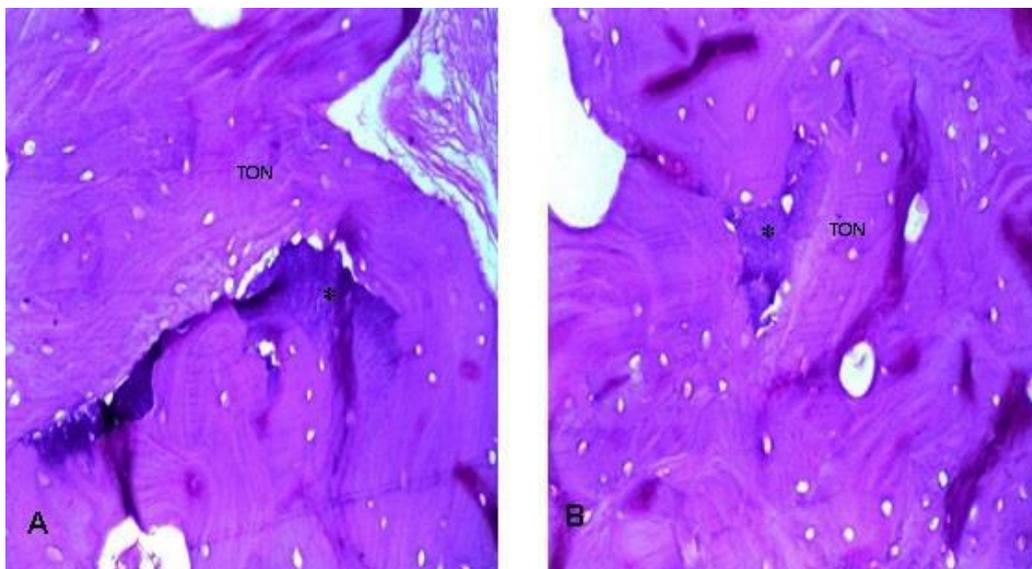
**Figure 5** – Results of Autogenous Group. A: Autogenous Group 40x. Bone neoformation starting from the stump. Arrow indicates osteotomy line. Fragments of bone tissue (\*) incorporated into the neoformed formed bone tissue (TON); B: Autogenous Group 40x. Defect center. Neoformed bone tissue (TON) completely filling the cavity. Presence of autogenous graft particles (\*).



Source: Authors.

At 200x magnification, it is noted that the autogenous bone particles are incorporated into the neoformed bone tissue (Figure 6A e 6B).

**Figure 6** - Autogenous Group 200x. Defect center. Figures A and B show in detail the autogenous bone particle (\*) that was incorporated into the neoformed bone tissue (TON).

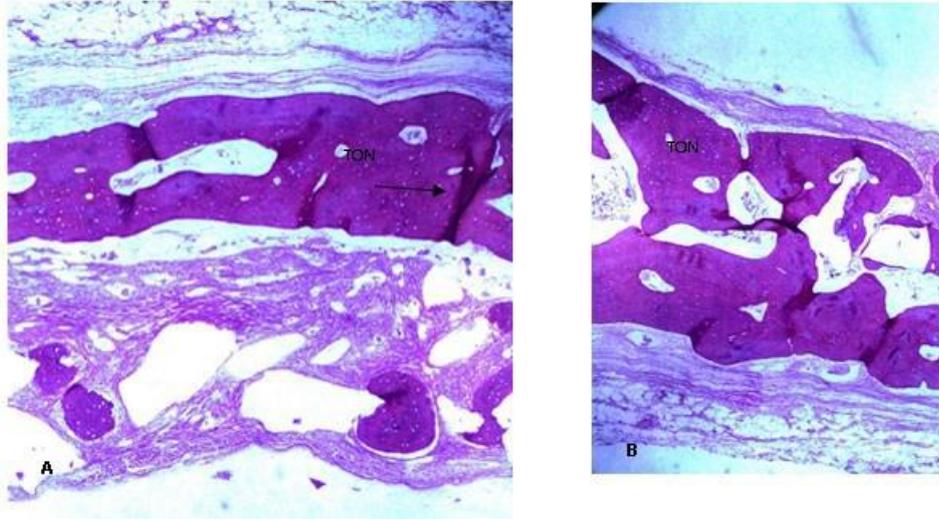


Source: Authors.

### **Inorganic Bovine Bone Group (Bio-Oss®)**

In the group where the inorganic bovine bone biomaterial (Bio-Oss®) was used, there is bone neoformation of the stump towards the center of the defect (Figure 7A, 7B), however it became thinner as it develops towards the center of the defect.

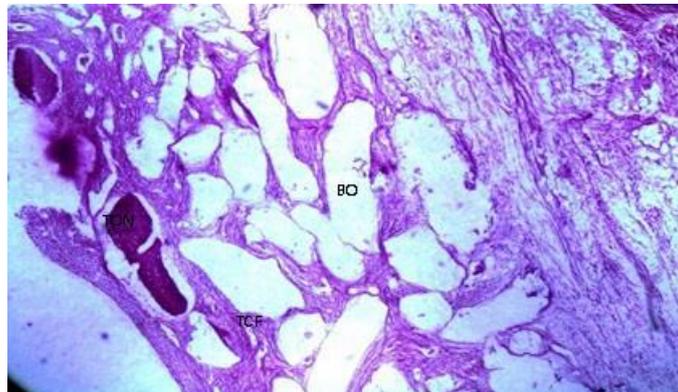
**Figure 7** – Bio-Oss Group. 40x. Close to the stump. Neofomed bone tissue (TON) from the stump (arrow osteotomy line). In the center of the bone defect, the TON becomes thinner.



Source: Authors.

It was possible to observe that the complete closure of the critical defect in the center of the defect did not occur. In some specimens, particles of biomaterial surrounded by neofomed bone tissue were observed, and others by fibrous connective tissue. (Figure 8).

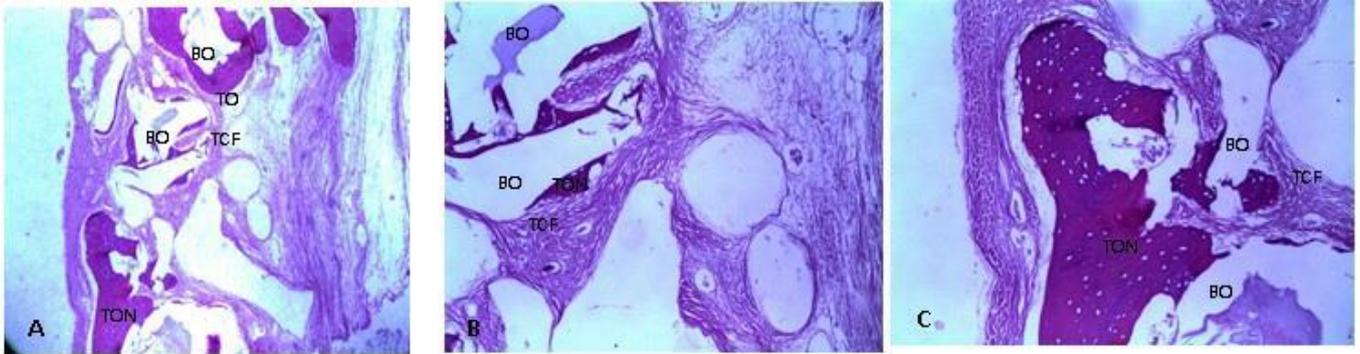
**Figure 8** – 40x. Defect center. Bio-Oss® (BO) particle surrounded by fibrous connective tissue (TCF) and neofomed bone tissue (TON).



Source: Authors.

At 100x magnification, it was observed in more detail the formation, sometimes of fibrous connective tissue, sometimes of neofomed bone tissue, in the same specimen, characterizing osteoconduction (Figure 9A, 9B e 9C).

**Figure 9** – A: 40x. Neoformed bone tissue (TON) close to the Bio-Oss® granules (BO), and fibrous connective tissue (TCF); B and C: 100x magnification. Detail of fibrous connective tissue (TCF) formation close to the Bio-Oss® particle (BO) and osteoconduction (TON) in the same specimen.

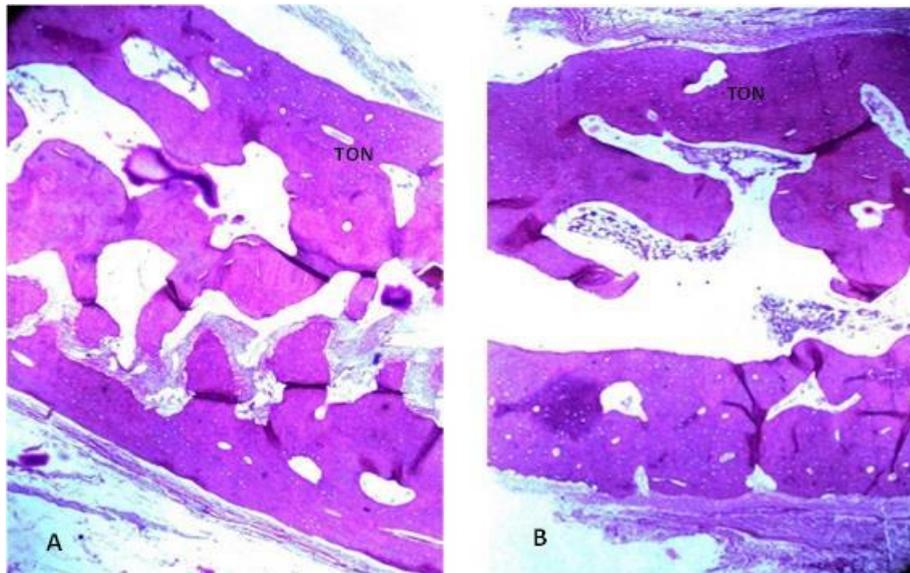


Source: Authors.

### Inorganic Bovine Bone Group (Bio-Oss®) and Autogenous

In the group of the association of inorganic bovine bone biomaterial (Bio-Oss®) with particulate autogenous bone in the proportion of 80:20, bone formation is noted from the stumps towards the center of the defect (Figure 10A, 10B). This bone neoformation was more constant, different from the Bio-Oss® group which was thinner when it was in a more central portion.

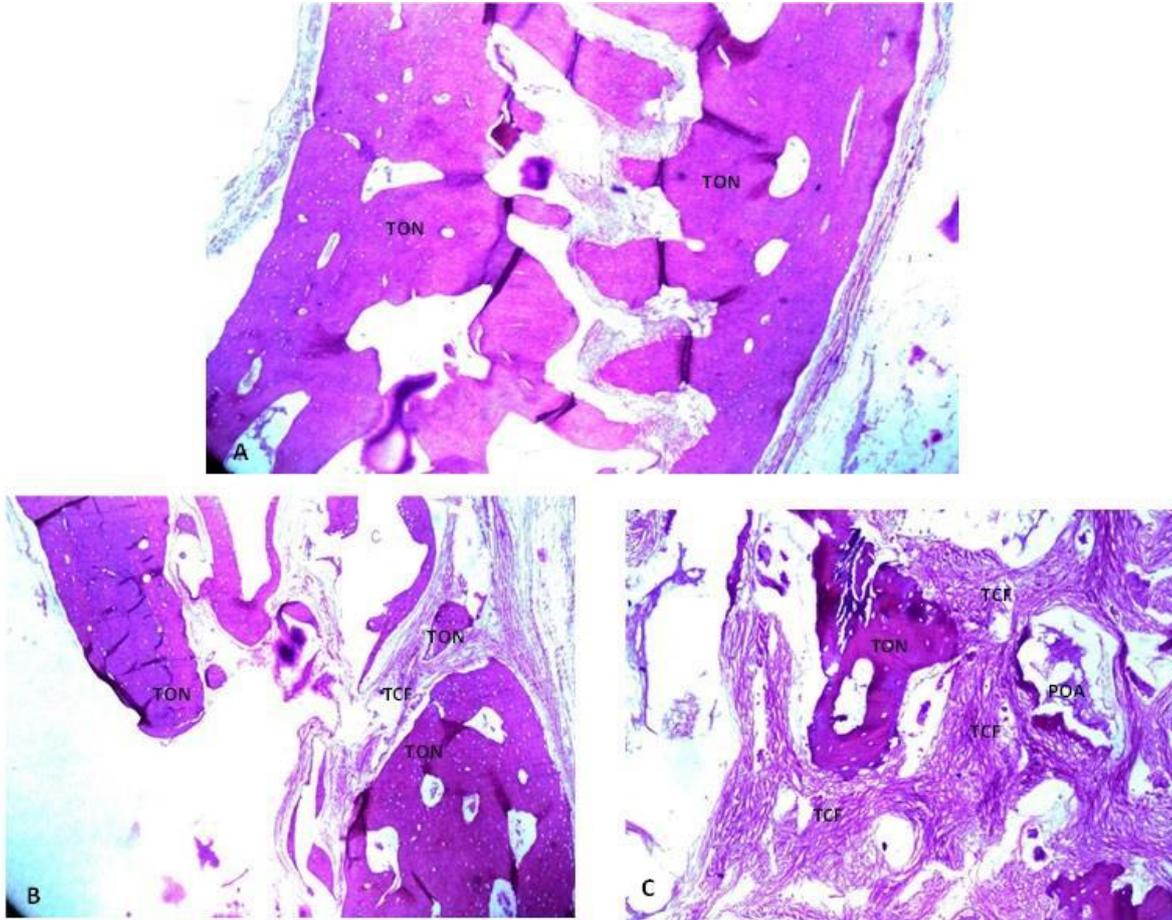
**Figure 10** – A: 40x. Right stump. TON from bone stump; B: 40x. Left stump. TON from bone stump.



Source: Authors.

In the central region, it is noted that in some specimens there was complete closure of the bone defect (Figure 11A), but in one specimen a poor result was observed with a large formation of fibrous connective tissue and without cavity closure (Figure 11B, 11C).

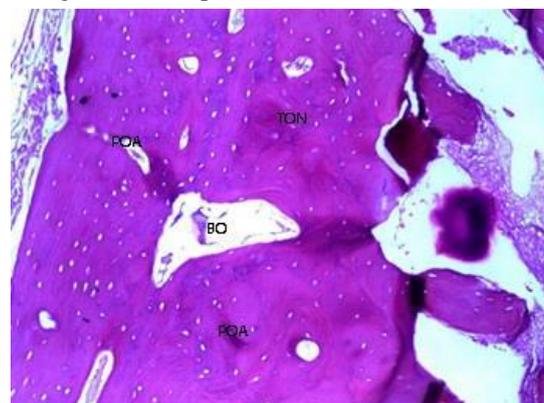
**Figure 11** – A: 40x. Defect center. Neofomed bone tissue (TON) in the center of the defect demonstrating osteoconduction. B: 40x Defect Center. Center with little neofomed bone tissue (TON) being practically filled by fibrous connective tissue (TCF); C: 100x. Defect center. Detail in the central region where a large amount of fibrous connective tissue (TCF) is observed, little neofomed bone tissue (TON) and remnant of the autogenous bone particle (POA).



Source: Authors.

At 100x magnification, both particles of autogenous bone and biomaterial enveloped by neofomed bone tissue are observed, characterizing the osteoconduction process. (Figure 12)

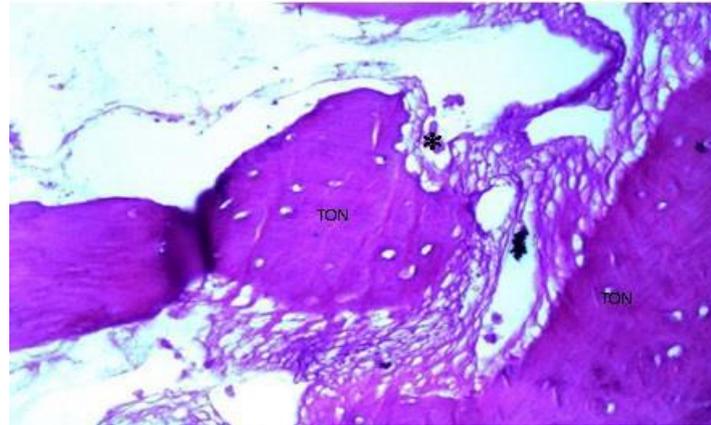
**Figure 12** - Defect center. 100x. Neofomed bone tissue (TON) in the center of the defect demonstrating osteoconduction around Bio-Oss® particles (BO) and autogenous bone particles (POA).



Source: Authors.

It was also possible to observe areas that suggest resorption of autogenous bone promoted by macrophages called Howship Gaps. (Figure 13)

**Figure 13** - Bio-Oss®/Autogeno Group. 200x magnification. Howship gaps (\*).



Source: Authors.

At an increase of 100, 200 and 400x, the presence of autogenous bone particles in the replacement phase was verified. (Figure 14A, 14B e 14C).

**Figure 14** - Bio-Oss®/Autogeno Group. A: 100x magnification. Autogenous bone particle (POA) suggesting that it is in the replacement phase. B: 200x magnification and C: 400x magnification.



Source: Authors.

#### 4. Discussion

Advances in the field of reconstructive surgery have boosted the search for biomaterials that enhance bone repair, replace autogenous bone, are efficient, economically accessible and with the advantages of unlimited supply without the need for a donor area, which would reduce patient morbidities (Li et al., 2015).

Autogenous intra and extra-oral grafts are tools frequently used to provide physical and biological demands in the reconstruction of bone defects caused by trauma, pathologies and physiological factors in the medical field and with various indications. However, in addition to autogenous bone graft, other materials can also be used for these procedures, including allogeneic, xenogeneic and alloplastic biomaterials, with or without the use of membranes for guided bone regeneration. In implant dentistry, there is a constant search for the best material in the use of these reconstructions, therefore, many researches and studies are carried out so that there is a minimum of failure. (Dahlin et al., 1994).

In this study, to analyze the biological behavior of the inorganic bovine bone and its association with the autogenous bone graft, the rabbit calvaria was used as an experimental model for presenting a reduced period of bone repair. For this purpose, clot was used as a negative control group to confirm that the 7mm cavities created were really critical. This fact was confirmed because defects where there was only a blood clot, complete repair in the center of the defect was not observed, with a fibrous connective tissue in the region.

At 60 days, histologically, a good bone repair process was observed in both materials studied (autogenous and Bio-Oss®-Geistlich), a fact that was characterized by the presence of osteoblasts in large numbers in the groups during this period of analysis. Autogenous bone showed bone neoformation around all of its particles, confirming its excellent osteoconductivity (Crespi et al., 2007; Scarano et al., 2006). No inflammatory infiltrate was found in specimens grafted with autogenous bone (Hallman et al., 2002; Barone et al., 2005; Crespi et al., 2007).

There are other properties of autogenous grafts that are considered important in different surgical situations, such as biocompatibility, the ability to act as a matrix for bone neoformation, and mechanical stability. Considering that few techniques contain all these requirements in a proportional way, the surgeon needs to have selection criteria for each specific situation (Marzola & Pastori, 2006; Porto et al., 2012; Noelken et al., 2020; Saulacic et al., 2021, Kim et al., 2022). The biocompatibility of autogenous grafts is well defined among the available materials and, therefore, it was considered our positive control in the analysis of the groups.

Due to the superior tissue compatibility, the best material for bone defects reconstruction is the autogenous bone graft because it has a minimal inflammatory response, faster angiogenesis and osteogenesis, in addition to having osteoinductive properties. (Porto et al., 2012; Saulacic et al., 2021).

Inorganic bovine bone has more than 25 years of clinical and scientific results as a bone substitute with osteoconductive action. It is indicated for defects, fenestrations and gaps in the peri-implant region (Araujo et al, 2008), as well as for vertical increases in the posterior region of the maxilla, through the technique of elevation of the maxillary sinus membrane, associated or not with particulate autogenous bone (Lundgren s et al., 1996; Traini T et al., 2008; Xuan F et al., 2013).

In this work, the group that received inorganic bovine bone (Bio-oss®- Geistlich) also demonstrated good bone neoformation in the defects created, and these results are consistent with the findings in the literature (Barone et al., 2005; Jensen et al., 2006). However, it can be observed that at 60 days in this group we did not obtain complete closure of the defects created, with moments when the material particle was surrounded by bone and in others it was surrounded by fibrous connective tissue, a result also observed in other studies (Tovar et al., 2014). Inorganic bovine bone (Bio-oss®- Geistlich) proved to be an osteoconductive material serving as a scaffold for bone matrix deposition and new bone formation (Iezzi et at., 2008). No inflammatory reaction was observed at 60 days in the studied groups, a fact that was also observed in other studies. (Scarano et al., 2006).

In the group where the association of autogenous bone and inorganic bovine bone (Bio-Oss®-Geistlich) was performed, the results were quite satisfactory, with most specimens being able to observe the closure of the created defects. The autogenous bone particles were in processes of incorporation by the neoformed bone tissue and the biomaterial particles were enveloped by the neoformed bone, characterizing the osteoconduction process. This better stability and quality of bone formation was also observed in others studies (Hallman et al., 2002).

Inorganic bovine bone also has very important characteristics, which a biomaterial needs, such as free availability, safe (preventing disease transmission), biocompatible, reduce surgical time, and present less morbidity is necessary for the evolution of implant dentistry (Iezzi et al., 2008).

The critical size defect has been used as an experimental model to evaluate the efficacy of newly developed

biomaterials. Skull defects are widely used in studies with biomaterials, and the rabbit is commonly used in animal experiments in health research. Healing of the critical size defect should be evaluated by correlating the size of the defect with the repair period, as well as the quantity and quality of healing tissue. (Li et al., 2015; Carlisle et al., 2019; Liu et al., 2020). In this study, the use of rabbits in the experimental model was of great value because it was possible to perform 4 defects in the same animal, reducing operating time, costs and observation errors among individuals; in addition to being easily handled and having a high rate of bone turnover.

The autogenous bone, in this work, presented better results when comparing new bone neoformation, as a group where only the biomaterial was used. However, its association with inorganic bovine bone (Bio-oss®- Geistlich) allowed this neoformed area to have the advantages attributed by the presence of autogenous bone, plus the stability of the gain in volume due to the presence of the biomaterial, as it will not be reabsorbed in the same way as autogenous bone particles.

#### 4. Conclusion

Within the limitations of the work, the results obtained and the parameters proposed and used in this research, the microscopic analysis allowed to conclude that the use of both the particulate autogenous bone and the particulate autogenous bone associated with inorganic bovine bone (Bio-Oss®-Geistlich) allowed a complete repair of critical defects in rabbit calvaria.

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