UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

SUELEN PAINI

F1 protein fraction obtained from latex incorporated into CaP-materials improve critical-size defect bone repair in a concentration-dependent manner

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A fração proteica F1 obtida do látex incorporado à biomateriais a base de CaP melhora o reparo defeitos ósseos de tamanho crítico de maneira dependente da concentração

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Orientador: Prof. Dr. Gerson Francisco de Assis.

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DEDICATÓRIA

Dedico essa dissertação...

Aos meus pais, Rosa e Roberto

Ao meu irmão Bruno

E aos mestres Dra. Tania, Dr. Gerson e Dr.Rumio.

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Só os que não fazem nada nunca erram.

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ABSTRACT

One strategy for bone regenerative engineering is to use matrices associated to osteogenic and angiogenic molecules to increase bone formation. The aim of the present study was to evaluate the efficacy of treatment of extensive cranial bone defects with the F1 protein obtained from latex adsorbed at different concentrations (0,01%, 0,025%, 0,05% e 1%) to two different bone-substitutes biomaterials, deproteinized bovine bone (DBB) and biphasic calcium phosphate ceramics (pBCP) using a preclinical model in rats. Defects of 8-mm diameter were created in parietal bones of 72 rats filled with the pure biomaterial or carried with the different concentrations of F1 protein, in the microtomographic images a visual analysis of the microtomographic reconstructions of the skull through transverse, coronal and sagittal sections. Subsequently, the segmentation of the defect in the reconstructions will be done through an image processing algorithm to quantify the parameters. Analyzing, in the BP-G group, the total volume of bone (TV), in the CSBD-CG group, the total volume of new bone (TV-NB), and in the treated groups, the total volume of the grafted region (TV/GR), the total volume of new bone (TV-NB) and biomaterials (TV/DBB and TV/pBCP). In tissue sections stained with Hematoxylin and Eosin a descriptive histological analysis was performed to verify the tissue response to treatment with F1 protein and its association with osteoconductive biomaterial and correlate it with the histomorphometric determination to obtain percent values and volume of neoformed bone tissue, biomaterial, bone marrow and soft tissue. In the characterization of DBB and pBCP biomaterials, it was performed through the combined analytical methodology by SEM and SDD-EDS, to analyze external morphology and elemental chemical composition. All the results were compared between the groups by the ANOVA variance analysis and the Tukey tests at the 5% level of significance (Statistica v.5.1, StatSoft). After 12 weeks, defects treated with biomaterials without F1 presented greater bone formation in relation to the control group. The association of 0.025% and 0.05% of F1 plus DBB showed higher bone formation (32.6% and 25.1%, respectively) when compared to pBCP, being 19.3% and 15.1%, respectively.We conclude that the stimulation of angiogenesis and osteogenesis depends on its concentration of F1e and the physicochemical properties of the carrier material.

Key words: Bone Regeneration, Angiogenesis Inducers Agents, Biocompatible Materials, Rats.

RESUMO

Uma estratégia da engenharia regenerativa óssea é usar matrizes associadas a moléculas osteogênicas e angiogênicas para aumentar a formação óssea. O objetivo do presente estudo foi avaliar a eficácia do tratamento de defeitos ósseos cranianos extensos com a proteína F1 obtida do látex adsorvido em diferentes concentrações (0,01%, 0,025%, 0,05% e 1%) a dois diferentes biomateriais ósseo-substitutos, osso bovino desproteinizado (DBB) cerâmica de fosfato de cálcio bifásica (pBCP) utilizando um modelo pré-clínico em ratos. Defeitos de 8 mm de diâmetro foram criados nos ossos parietais de 72 ratos preenchidos com o biomaterial puro ou carregados com as diferentes concentrações da proteína F1, nas imagens microtomográficas uma análise visual das reconstruções microtomográficas do crânio através de cortes transversais, coronais e sagitais. Subsequentemente, foi feito segmentação do defeito nas reconstruções através de algoritmo de processamento de imagem para quantificação dos parâmetros. Analisando, no grupo BP-G, o volume total de osso (TV), no grupo CSBD-CG o volume total de osso novo (TV-NB), e nos grupos tratados, o volume total da região enxertada (TV-GR), volume total de osso novo (TV-NB) e biomaterial (TV-DBB e TV-pBCP). Nos cortes teciduais corados pela Hematoxilina e Eosina foi realizado uma análise histológica descritiva para verificar a resposta tecidual frente ao tratamento com a proteína F1 e a sua associação com biomaterial osteocondutor e correlaciona-la com a determinação histomorfométrica para a obtenção dos valores percentuais e de volume de tecido ósseo neoformado, biomaterial, medula óssea e tecido conjuntivo. Na caracterização dos biomaterias DBB e pBCP, foi realizado através da metodologia analítica combinada por SEM e SDD-EDS, para analisar morfologia externa e composição química elementar. Todos os resultados foram comparados entre os grupos pela análise de variância ANOVA e o tests de Tukey ao nível de significância de 5% (Statistica v.5.1, StatSoft). Após 12 semanas, defeitos tratados com biomateriais sem F1 apresentaram maior formação óssea em relação ao grupo controle. A associação de 0,025% e 0,05% de F1 mais DBB mostraram maior formação óssea (32,6% e 25,1%, respectivamente) quando comparados com pBCP, sendo 19,3% e 15,1%, respectivamente.Nós concluímos que, a estimulação da angiogênese e osteogênese depende de sua concentração de F1e das propriedades físico-químicas do material carreador.

Palavras-chave: Regeneração Óssea, Indutores da Angiogênese, Materiais Biocompatíveis, Ratos.

LIST OF ABREVIATIONS AND ACRONYMS

β-TCP	Beta-tricalcium phosphate
μA	Microampère
μm	Micrometer
®	Trademark
2D	Two-Dimensional
3D	Three-Dimensional
ºC	Degrees Celsius
™	Trademark
μA	Microamp
ANOVA	Analisys of variance
B	Border
BCP	Biphasic calcium phosphate
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein 2
BP-G	Bone-plug group
BV	Bone volume
BV/TV	Bone volume/Total Volume
C	Carbon
Ca	Calcium
cc	Cubic centimeter
CEEPA	Ethics Committee on Animal Education and Research at FOB-USP
CEVAP	Center for the study of venomous and venomous animals
CG	Control group
cm	Centimeter
CSBD	Critical size bone defects
CT	Connective tissue
DBB	Deproteinized bovine bone
DEAE-cellulose	Diethylaminoethyl cellulose
EDS	Energy dispersive spectroscopy
EDTA	Ethylenediamine tetraacetic acid

E.G.	Example
F1	Fraction 1
FBGCs	Large multinucleated foreign body giant cells
FGFs	Fibroblast growth factors
Fig.	Figure
FOB	Bauru school of dentistry
G	Group
GR	Grafted region
HA	Hydroxyapatite
HE	Hematoxylin and eosin
I.E.	In other words
IGFs	Insulin-like growth factor
IL-10	Interleukin-10
Inc.	Incorporated
kg	Kilogram
k	Kilovolt
LB	Left border
Ltda.	Limited
M	Molar
MB	Marrow bone
Mg	Magnesium
mg	Milligram
Micro-CT	Microcomputed tomography
Min	Minutes
mL	Milliliter
mm	Millimeter
mm^3	Cubic millimeter
n°	Number
NaCl	Sodium chloride
NB	New bone
Nm	Nanometers
O	Oxygen
P	Phosphorus

p	Statistical p-value; descriptive level
pBCP70/30	Biphasic calcium phosphate 70/30
PDEM	Probability density evolution method
PDGF	Platelet-derived growth factor
pH	Hydrogenation potential
RB	Right border
rhBMP-2	Recombinant human bone morphogenetic protein 2
ROI	Region of interest
S	Section
SDD	Silicon drift detector
SEM	Scanning Electron Microscopy
SP	São Paulo
ST	Soft tissue
t	Test
TA	Total area
TAi	Total area of each structure
TC	Connective tissue
TCP	Tricalcium phosphate
TG	Treatment group
TV	Total volume
UNESP	Paulista state university “Júlio Mesquita Filho”
USA	United States of America
USP	University of São Paulo
V	Vessel
v	Volume
Vv	Volume density
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
vs.	Versus
VT	Total volume
X	Volume density belonging to a given constituent

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

The reconstruction of large bone defects or critical size bone defects caused by trauma, infection, fracture nonunion, bone tumor resection and spinal deformities are a major challenge in regenerative medicine, especially for oral and maxillofacial surgeons (Cancedda *et al*., 2007).

The autogenous bone remains as the gold standard for grafting because it has the three classic properties for bone regeneration and engineering. Osteogenesis is the capacity of synthesizing new bone by its bone cells. Osteoinductivity occurs due to the morphogens contained in the bone matrix which are capable of inducing the pluripotent mesenchymal cells to differentiate into osteoblasts. Osteoconductiveness is possible by the 3D structure of bone, it serves as a scaffold for migration, adhesion, osteoblasts differentiation and bone matrix synthesis on the surface (Tadic and Epple, 2004). However, a second surgical intervention for graft harvesting is necessary and this results in an increased patient's morbidity in addition to the limited amount of disponible tissue in the donor site (Laurencin *et al*., 2006). Consequently, to these limitations numerous bone substitutes have been or are being developed as an alternative to the autogenous graft (Campana *et al*., 2014) and currently, has been associated with factors of growth and / or osteoinducers (Chin *et al*., 2005; Liu *et al*., 2009; Li *et al*., 2011) in the intention to potentiate the repair.

Vascularization is extremely important during the bone repair process, which can provide a bone source (Zimmerer *et al*., 2017), the blood vessels are responsible for the distribution of oxygen, in addition to the transport of nutrients, cells (osteoblasts and osteoclast precursors) growth factors and cytokines. Among the growth factors, we have VEGF that has a direct stimulating effect on angiogenesis and also on the differentiation of osteoblasts (Marenzana and Arnett, 2013; Liu *et al*., 2017) highlighting a link between the two cell types (endothelial cells and osteoblasts) and morphogenetic protein (BMP) that is capable of recruiting and inducing differentiation of undifferentiated mesenchymal cells into osteoblasts (Santos *et al*., 2005).

In Brazil, products obtained from the rubber tree Hevea brasiliensis, a tree native to the Amazon River basin, has shown positive effects in several clinical situations due to its biocompatibility and angiogenic activity accelerating the cicatricial process without producing hypersensitivity response (Mrue *et al*., 2004). The latex membrane was used in humans in the

reconstruction of tympani destroyed by chronic infections (Araujo *et al.,* 2012). Possessing bioactive and angiogenesis-inducing properties, the membrane was successfully used in treatment of chronic wounds in patients with diabetes. In all cases they presented a vigorous and healthy formation of new blood vessels, although healing has varied person to person according to age or other factors (Frade *et al.,* 2004).

At dissertation of Mauricio (2006) evaluated by DEAE chromatography cellulose obtained 3 fractions of the latex which denominated F1, F2 and F3. The angiogenic activity of the isolated fractions was evaluated in the chorio-allantoic membrane of chicken eggs Gallus domesticus. The F1 also showed greater activity on vascular permeability during the repair of dermal ulcers performed on rabbit ears. In another study, was assessed whether the F1 was able to stimulate human umbilical vein endothelial cells. In this case, Although F1 did not directly stimulated endothelial cells from human umbilical cord, it acted in angiogenesis indirectly through stimulation of growth factors by monocytes / macrophages stimulated, or other mechanisms not yet clear-cut. Already, Lamounier (2004) evaluated the production of inflammatory cytokines *in vitro* in culture of peripheral blood mononuclear cells induced by Membrane. Cells did not proliferate in the presence of membrane due to changes during processing, total serum and F2 did not change in lymphoproliferative response and cytokine production, whereas F1 led to an increase in IL-10.

Regarding to concentration of the protein to be used, the F1 was incorporated in 4% carboxymethylcellulose gel (Bayer - São Paulo, SP) in 3 different concentrations: 0.01%, 0.1% and 1% and applied on the ulcers on the ears of rabbits, showing that the concentration 0.01% of F1 was the most efficient to stimulate the healing of the lesion in a shorter time (Mendonça, 2004 and Mendonça et al., 2010). Recently, the company Boticario has proposed a new anti-aging gel containing F1, able of restoring collagen production and skin elasticity. In a preclinical study with 60 women aged approximately 50 years, the use of F1 gel for 1 month led to a reduction of 80% in eye wrinkles and also the wrinkles in the forehead region, a study later confirmed with a larger group of 300 women.

In relation the application of F1 to bone repair, few studies have evaluated its potential, a preclinical study using latex in rat alveolar bone repair has shown that the latex was biocompatible and able of leading to a progressive osseointegration stimulating angiogenesis and accelerating the process of new bone formation in the first days of alveolar bone repair (Balabanian *et al.,* 2006). In another study, compared the osseointegration of the dental implant

(3.3 diameter x 10.0 mm length) in circumferential defects in dog jaws (5.0 diameter x 6.3 mm depth) treated with latex angiogenic proteins (2.5% collagen + 2.5% hyaluronic acid and 0.01% of the latex protein) versus autogenous bone, study did not show significant differences regarding bone formation and implant bone contact (Manfrin Arnez *et al.,* 2012).

Machado and collaborators, evaluated the repair of bone defects in the tibia of rats with fibrin sealant derived from snake venom (CEVAP) associated or not with BMP-2 and/or F1 at concentrations of 5 or 8μg, the results showed higher bone formation in the groups that used the BMP-2 and F1 in relation to the control (without treatment), being more favorable to BMP2. However, the use of fibrin showed better results in relation to defects without fibrin, but the association of fibrin with BMP-2 was the one that presented better results in the treatment of the tibial defect (Machado *et al.,* 2015). Was also evaluated the use of two different concentrations of BMP-2 (5μg/10μg) and (5μg/10μg) of F1 associated with or without the monoolein gel vehicle in the treatment of cranial bone defects of 6mm in diameter. After 4 weeks, they showed greater bone formation in the defects treated with the vehicle associated with higher protein concentration (10μg), being more favorable to BMP-2 (Issa *et al.,* 2012). In this sense, is important to emphasize that although rhBMP-2 is a potent growth factor for bone repair (Carreira *et al.,* 2014), has been reported to have collateral effects such as bloating, seroma and an increased risk of cancer when used at high dosages (Carragee *et al.,* 2013).

Another essential factor that determines success or failure during inductive bone grafts is the competence of the carrier material. A good carrier material must be biodegradable, biocompatible, and the measure that carrier is absorbed, there must be replacement with new bone tissue without allowing the bone site to be exceeded (Toriumi and Robertson, 1993). Several carriers are being used, they are made from metals, ceramics, polymers and composites. Natural or synthetic ceramics based on calcium phosphate have been used in various medical applications due to their mechanical resistance, biocompatibility, but with low biodegradability (Agrawal and Sinha, 2016) and should have also properties of osteointegration, osteoinduction (Moore *et al.,* 2001). In a recent study, biphasic ceramics of porous calcium phosphate in the ratio HA/TCPp 7:30, showed high osteoinduction power and osteoconduction in the bone repair process, promoting bone neoformation similar to autogenous bone (Santos *et al.,* 2018). In addition to the inorganic bone, studies show that they promote osteoconduction and have a slower resorption, being an alternative in bone repair therapy in the craniomaxillofacial region (Cestari *et al.,* 2009).

The products proposed in this project have as objective to evaluate and demonstrate the level of safety and efficacy of medical products for health, clarifying the type of interaction that different materials may present in front of the association with a protein of vegetable origin with potential osteo stimulating activity, becoming more an alternative to bone substitutes in current use in procedures to fill bone defects caused by trauma or pathologies.

2 PROPOSITION

2 PROPOSITION

The objective of this study was to evaluate whether F1 protein obtained from natural latex interferes positively or negatively in the vascularization and bone formation of critical-size bone defects and, if positive, the best concentrations of F1 and the type of carrier biomaterial have potential for future clinical applications.

3 ARTICLE

3 ARTICLE

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F1 protein fraction obtained from latex incorporated into CaP-materials improve critical-size defect bone repair in a concentration-dependent manner

Suelen Paini[1], Rumio Taga[1] and Gerson Francisco de Assis[1]

[1] *Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, São Paulo, Brazil*

* To whom correspondence should be addressed Suelen Paini, Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Av. Dr. Octavio Pinheiro Brisolla, 9-75, Vila Universitária, Bauru SP, Brazil. Tel: +55 1498833-8090; Email: suelenpaini@usp.br

Keywords:. Bone Regeneration, Angiogenesis Inducers, Biocompatible Materials, Rats

Running title: biphasic ceramic

ABSTRACT

One strategy of bone regenerative engineering is to use matrices associated to osteogenic and angiogenic molecules to increase bone formation. This study evaluated the bone formation in the rat critical-size defects (CSBD) treated with deproteinized bovine bone (DBB-TG) and biphasic calcium-phosphate (pBCP-TG) incorporated with different concentrations of the angiogenic F1 protein fraction obtained of Hevea brasiliensis latex (without, 0.01%, 0.025%, 0.05% and 0.01%) after 12 weeks. The defects of control group (CSBD-CG) did not received treatment. 2D and 3D μCT analyses were used to quantify the density and total volume of new bone, biomaterial and soft tissue. 2D-histomorphometric analysis was performed for qualitative and quantitative analysis. All treatments of DBB-TG and pBCP without F1 and with 0.01% and 0.025% of F1 showed higher new bone volume compared to CSBD-CG. In DBB-TG new bone volume was significantly higher when incorporate with 0.025% and 0.05% of F1 than others concentration and pBCP with and without F1. In pBCP-TG the incorporation of the F1 did not promote bone tissue gain compared to pBCP without F1 ($p>0.05$) and a significant bone reduction was observed with 0.1% of F1. Overall, the F1 incorporated to DBB significantly improve the bone regeneration dependent on its concentration in a standardized rat CSBD model.

KEYWORDS

Bone Regeneration, Angiogenesis Inducers, Biocompatible Materials, Rats

4 DISCUSSION

4 DISCUSSION

Bone regeneration is a complex and dynamic biological process involving cellular and molecular elements during the new bone formation (Ai-Aql *et al*., 2008). However, large bone defects remain a clinical challenge, pathological situations or trauma, may compromise the healing process of the repair (Gómez-Barrena *et al.,* 2015; Panteli *et al.,* 2015). Another crucial factor for a good healing is that the damaged bone location needs to be highly vascularized (Portal-Núñez *et al.,* 2012; Hu and Olsen, 2016; 2017). Additionally, current strategies for tissue engineering developed therapies with growth factors associated to bone grafts attempting to induce new vessels formation then they could be good alternatives because they can provide oxygen, nutrients, and promote direct migration to bone defects (Gorustovich *et al*., 2010). In this context angiogenesis is closely linked to a better bone formation and we studied latex F1 protein performance versus 2 different bone biomaterials. Recently, the experimental model most commonly used to evaluate bone replacement strategies has been in calvaria of rats (Vajgel *et al.*, 2014), based on the critical size defect (Cooper *et al.*, 2010). Schmitz & Hollinger (1986), gave rise to the term "Critical Size Defect" (CSBD), defined as a defect that lacks the natural repair capability during the life of the animal. In our study, was performed in calvaria of the rat a creating a unilateral defect of 8-mm of diameter. A other pilot study carried out in our laboratory showed spontaneous healing of a 5-mm bone defect in adult rats leading to closed of defect (47.5 ± 9.8% at 12 weeks and 83.3 ± 13.1% at 24 weeks, data not included) considered as a subcritical size defect that can heal without intervention. Other study, also showed that bilateral defects of 5mm in association with the application of factors of growth, the control site may be contaminated due to defects being near (Vajgel *et al.*, 2014).

The methods used to verify bone repair in these experimental models include histological and histomorphometric evaluations (Park *et al.*, 2009), because this technique is considered gold standard (Vidal *et al.*, 2012) and allows to evaluate elements present in the tissue samples by means of two-dimensional cuts, qualitatively and quantitatively. Therefore, it is not possible to evaluate the sample volume, requiring three-dimensional samples (Li *et al.*, 2010). In order to complement the method of histological quantification, we associate to the morphometric method the computerized microtomography, allowing the obtaining of the three-dimensional samples (Efeoglu *et al.*, 2007; Parkinson *et al.*, 2008), being a new technique with many advantages, among them: the ability to evaluate several parameters depending on the

software and hardware it has, does not damage the sample, ensuring the integrity of the sample for other techniques used later (Ho and Hutmacher, 2006) and considered the safest method in quantitative analysis (Gundersen *et al.*, 1988). Although, it has the disadvantage of the microtomographic analysis of bone tissue, the program makes it impossible to change the gray levels during quantification of bone volume (Parkinson *et al.*, 2008). Many studies have used two techniques and have done compared microtomographic and histomofometric data (Yeom *et al.*, 2008; Particelli *et al.*, 2012).

In the present study, some difficulties were found during quantifications correlation between 2D-micro-CT morphometry and histomorphometry measures. The values obtained were discordant between both analyzes and similar difficulties are also found in the work of Chappard et al., (2005). Most of the methodologies only evaluate a single region of the graft, in this regard, taking into account that the bone repair does not occur simultaneously in the whole defect, we had the idea of creating a new methodology of stratified analysis considering the whole region and also performing comparisons between similar micro-CT and histological cuts. Since there are no studies that use these methodology thresholds were standardized and after segmentation we had the following analyzes: volume of density (Vv), new bone (Vv-NB), biomaterials (Vv-DBB e Vv-pBCP), bone marrow (Vv-MB) and soft tissue (Vv -ST) in the CTAn program for the microCT samples, and in the AxionVision program, in the histological samples.

The findings showed that, there was no statistical difference between the mean values of volumetric density of the bone tissue, biomaterial and soft tissue, obtained in micro-CT and histological images (Figs. 5D2 e 6D2) e DBB (Fig. 6D1). Regarding histomorphometry the CSBD group, a small bone formation occurred, being 20.7 ± 8.2%, considered a critical-size defect. Regarding the treatment groups, the implanted volume remained stable over the period of 120 days in both groups. However, a volume loss of the pBCP (Table of the Fig. 8B), associated with the appearance of multinucleate foreign body giant cells (FBGCs, see Fig. 8A) at concentrations of 0.05% and 0.1% F1. Recent studies have shown that the presence of FBGCs found on the surface of calcium phosphate ceramics is correlated with increased vascularity in tissue repair, considering important the vascular endothelial growth factor (VEGF) during bone formation (Ghanaati *et al.*, 2010) (Yang *et al.*, 2012). However, in our work the presence of FBGCs was associated with the presence of anomalous microspheres, verified in concentrations above 0.05% F1. Experimental data have shown that the application of excessive amounts of growth

factors causes irregular blood vessels to appear (Zisch *et al.*, 2003; Davies *et al.*, 2008). Moreover, pathologies of angiogenesis may occur (Moldovan and Moldovan, 2002), as seen, the dosage of VEGF should be strictly controlled (Davies *et al.*, 2008). Considering the importance of the dosage of these growth factors when associated with biomaterials in tissue engineering, other studies have investigated the release of these drugs (Liu *et al.*, 2013; Li *et al.*, 2015).

Highlighting a link, the characteristics of the biomaterials to be proposed in therapy, such as pore sizes, chemical and physical properties as some studies have done, should be taken into account. (Klein *et al.*, 1984; Hamada *et al.*, 2010; Otsuka *et al.*, 2013; Ebrahimi and Botelho, 2017; Ebrahimi *et al.*, 2017). For this reason, we performed the characterization of the biomaterials carried to F1 (Fig. 2), verifying the properties of the ceramics pBCP and DBB. The analysis showed morphology, porosity of the different granules between one material and another. And there was a relationship between the Ca / P elements 1.66 and 1.67 in both materials. On the other hand, the solubility of the material may also interfere with the regeneration process. In this context, (Ghanaati *et al.*, 2012), used in its study pure HA and TCP granules and associated HA / TCP, in the proportion 60:40, analyzing action when used pure and associated, after the experiment period being of up to 30 days, the results showed that, when using pure ceramics, the TCP was absorbed faster to the connective tissue, presence of multinucleated giant cells and an intense vascularization, already HA, presented the opposite, a slow degradation, few multinucleated giant cell cells. That is, when used in conjunction, HA / TCP 60/30, the ceramic presents an equilibrium between the vascularization and the degradation of the material, being an excellent combination to restore the bone tissue. In relation to HA / TCP in the ratio 70:30, used in our work, in the study of (Lomelino *et al.*, 2012), showed that 70:30 biphasic pottery ceramics an increase in bone repair, and presented similar characteristics to the autogenous bone, such as bone tissue cells. These synthetic ceramics are largely research and studies show to be biocompatible, osteoconductive and allow the formation of a new bone on its surface (Damien and Parsons, 1991; Hak, 2007)

Concerning DBB ceramics, in our study pottery promoted good osseointegration of DBB as well as pBCP (Details in the Fig. 7B), the total volume of region grafted on DBB-TG and pBCP-TG (Table in the Fig. 9C) was 99,8% bigger (average of 85 mm^3) than surgically removed, with an average of 42,6 mm^3. It was also observed direct communication of the new bone and the surface of the material without interposition of soft tissue. In study of Cestari et al 2009, we can see the similarity to our results, study was carried out with the sintered bovine-derived anorganic bone material (sBDAB) as in form of block in the repair of a bone defect of

critical size and observed that it promoted excellent osseointegration from the edges of the defects towards the center, bone volume was 3.8 times greater compared to surgically removed bone, whereas in the control group, filled only with the blood clot of the animal itself, bone formation was limited. They concluded that the DBB block possesses osteocondudore property, block providing osteocondudore, a slow reabsorption and osteoconductive. In the study of Rocha et al. (2011), evaluated the Gen-Ox®inorg associated with and without the platelet-rich plasma (PRP), after the 4-week experimental period, observed the presence of a small bone formation in edge region and in some defects was observed in central region, was seen the presence of giant cells in the group Gen-Ox®inorg without and with the PRP, where they concluded that in addition to being osteoinductive, it maintains the new bone volume.

Given the evidences found here, we can say that biomaterials are well accepted in the literature, and are closely linked to growth factors. In this way, the results presented in this study showed the potential of F1 carried to the biomaterials, being dose dependent and the choice of biomaterial can also be influenced. in addition, new methodologies proposed in this work using the combination 2D/3D micro-CT and histomorphometric analysis to evaluate bone regeneration could help future studies in a calvary model of critical size in rats.

5 CONCLUSIONS

5 CONCLUSIONS

Our study demonstrates that the potential of F1 in stimulating angiogenesis and osteogenesis is dependent on the concentration and carrier biomaterial. In the other hand, we present a new methodology standardized in this study using the combination 2D/3D micro-CT and histomorphometric analysis for future preclinical studies in critical size defect as an experimental model in rat calvaria, in bone regeneration.

REFERENCES

REFERENCES

AGRAWAL, V.; SINHA, M. A review on carrier systems for bone morphogenetic protein-2. **J Biomed Mater Res B Appl Biomater**, Jan 2016. ISSN 1552-4981 (Eletronic)

AI-AQL, Z. S. et al. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. **J Dent Res,** v. 87, n. 2, p. 107-18, Feb 2008. ISSN 0022-0345 (Eletronic)

ARAUJO, M. M.; MASSUDA, E. T.; HYPPOLITO, M. A. Anatomical and functional evaluation of tympanoplasty using a transitory natural latex biomembrane implant from the rubber tree Hevea brasiliensis. **Acta Cir Bras,** v. 27, n. 8, p. 566-71, Aug 2012. ISSN 1678-2674 (Eletronic)

BALABANIAN, C. A. et al. Biocompatibility of natural latex implanted into dental alveolus of rats. **J Oral Sci,** v. 48, n. 4, p. 201-5, Dec 2006. ISSN 1343-4934 (Eletronic)

CAMPANA, V. et al. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. **J Mater Sci Mater Med,** v. 25, n. 10, p. 2445-61, Oct 2014. ISSN 1573-4838 (Eletronic)

CANCEDDA, R.; GIANNONI, P.; MASTROGIACOMO, M. A tissue engineering approach to bone repair in large animal models and in clinical practice. **Biomaterials,** v. 28, n. 29, p. 4240-50, Oct 2007. ISSN 0142-9612 (Eletronic)

CARRAGEE, E. J. et al. Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. **J Bone Joint Surg Am,** v. 95, n. 17, p. 1537-45, Sep 2013. ISSN 1535-1386 (Eletronic)

CARREIRA, A. C. et al. Bone morphogenetic proteins: facts, challenges, and future perspectives. **J Dent Res,** v. 93, n. 4, p. 335-45, Apr 2014. ISSN 1544-0591 (Eletronic)

CESTARI, T. M. et al. Bone repair and augmentation using block of sintered bovine-derived anorganic bone graft in cranial bone defect model. **Clin Oral Implants Res,** v. 20, n. 4, p. 340-50, Apr 2009. ISSN 1600-0501 (Eletronic)

CHIN, M. et al. Repair of alveolar clefts with recombinant human bone morphogenetic protein (rhBMP-2) in patients with clefts. **J Craniofac Surg,** v. 16, n. 5, p. 778-89, Sep 2005. ISSN 1049-2275 (Eletronic)

COOPER, G. M. et al. Testing the critical size in calvarial bone defects: revisiting the concept of a critical-size defect. Plast Reconstr Surg, v. 125, n. 6, p. 1685-92, Jun 2010. ISSN 1529-4242 (Eletronic)

DAMIEN, C. J.; PARSONS, J. R. Bone graft and bone graft substitutes: a review of current technology and applications. **J Appl Biomater,** v. 2, n. 3, p. 187-208, 1991. ISSN 1045-4861 (Eletronic)

DAVIES, N. et al. The dosage dependence of VEGF stimulation on scaffold neovascularisation. **Biomaterials,** v. 29, n. 26, p. 3531-8, Sep 2008. ISSN 0142-9612 (Eletronic)

EBRAHIMI, M.; BOTELHO, M. Biphasic calcium phosphates (BCP) of hydroxyapatite (HA) and tricalcium phosphate (TCP) as bone substitutes: Importance of physicochemical characterizations in biomaterials studies. Data Brief, v. 10, p. 93-97, Feb 2017. ISSN 2352-3409 (Eletronic)

EBRAHIMI, M.; BOTELHO, M. G.; DOROZHKIN, S. V. Biphasic calcium phosphates bioceramics (HA/TCP): Concept, physicochemical properties and the impact of standardization of study protocols in biomaterials research. Mater Sci Eng C Mater Biol Appl, v. 71, p. 1293-1312, Feb 2017. ISSN 1873-0191 (Eletronic)

EFEOGLU, C. et al. Quantitative morphometric evaluation of critical size experimental bone defects by microcomputed tomography. **Br J Oral Maxillofac Surg,** v. 45, n. 3, p. 203-7, Apr 2007. ISSN 0266-4356 (Eletronic)

FRADE, M. C. et al. Management of diabetic skin wounds with a natural latex biomembrane. **Med Cutan Iber Lat Am,** v. 32, n. 4, p. 157-62, 2004 (Eletronic)

GHANAATI, S. et al. The chemical composition of synthetic bone substitutes influences tissue reactions in vivo: histological and histomorphometrical analysis of the cellular inflammatory response to hydroxyapatite, beta-tricalcium phosphate and biphasic calcium phosphate ceramics. Biomed Mater, v. 7, n. 1, p. 015005, Feb 2012. ISSN 1748-605X (Eletronic)

_____. Influence of β-tricalcium phosphate granule size and morphology on tissue reaction in vivo. **Acta Biomater,** v. 6, n. 12, p. 4476-87, Dec 2010. ISSN 1878-7568 (Eletronic)

GORUSTOVICH, A. A.; ROETHER, J. A.; BOCCACCINI, A. R. Effect of bioactive glasses on angiogenesis: a review of in vitro and in vivo evidences. Tissue Eng Part B Rev, v. 16, n. 2, p. 199-207, Apr 2010. ISSN 1937-3376 (Eletronic)

GUNDERSEN, H. J. et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS, v. 96, n. 5, p. 379-94, May 1988. ISSN 0903-4641(Eletronic)

GÓMEZ-BARRENA, E. et al. Bone fracture healing: cell therapy in delayed unions and nonunions. Bone, v. 70, p. 93-101, Jan 2015. ISSN 1873-2763(Eletronic)

HAK, D. J. The use of osteoconductive bone graft substitutes in orthopaedic trauma. J Am Acad Orthop Surg, v. 15, n. 9, p. 525-36, Sep 2007. ISSN 1067-151X (Eletronic)

HAMADA, H. et al. Effect of geometrical structure on the biodegradation of a three-dimensionally perforated porous apatite/collagen composite bone cell scaffold. Biol Pharm Bull, v. 33, n. 7, p. 1228-32, 2010. ISSN 1347-5215 (Eletronic)

HO, S. T.; HUTMACHER, D. W. A comparison of micro CT with other techniques used in the characterization of scaffolds. Biomaterials, v. 27, n. 8, p. 1362-76, Mar 2006. ISSN 0142-9612 (Eletronic)

HU, K.; OLSEN, B. R. The roles of vascular endothelial growth factor in bone repair and regeneration. Bone, v. 91, p. 30-8, 10 2016. ISSN 1873-2763 (Eletronic)

_____. Vascular endothelial growth factor control mechanisms in skeletal growth and repair. Dev Dyn, v. 246, n. 4, p. 227-234, 04 2017. ISSN 1097-0177 (Eletronic)

ISSA, J. P. et al. Biological evaluation of the bone healing process after application of two potentially osteogenic proteins: an animal experimental model. **Gerodontology,** v. 29, n. 4, p. 258-64, Dec 2012. ISSN 1741-2358 (Eletronic)

KLEIN, C. P.; DRIESSEN, A. A.; DE GROOT, K. Relationship between the degradation behaviour of calcium phosphate ceramics and their physical-chemical characteristics and ultrastructural geometry. Biomaterials, v. 5, n. 3, p. 157-60, May 1984. ISSN 0142-9612 (Eletronic)

Lamounier, F. Avaliação da produção de citocinas inflamatórias "in vitro", induzidas pela membrana de látex natural seringueira Havea Brasiliensis, 115. Mestrado. Departamento de Clínica Médica, Divisão de Dermatologia, Faculdade de Medicina de Ribeirão Preto, Univerdade de São Paulo, Ribeirão Preto, 2004.

LAURENCIN, C.; KHAN, Y.; EL-AMIN, S. F. Bone graft substitutes. **Expert Rev Med Devices,** v. 3, n. 1, p. 49-57, Jan 2006. ISSN 1743-4440 (Eletronic)

LI, L. et al. Controlled dual delivery of BMP-2 and dexamethasone by nanoparticle-embedded electrospun nanofibers for the efficient repair of critical-sized rat calvarial defect. Biomaterials, v. 37, p. 218-29, Jan 2015. ISSN 1878-5905 (Eletronic)

LI, W. et al. Nell-1 enhances bone regeneration in a rat critical-sized femoral segmental defect model. **Plast Reconstr Surg,** v. 127, n. 2, p. 580-7, Feb 2011. ISSN 1529-4242 (Eletronic)

LI, X. et al. Osteogenic induction of adipose-derived stromal cells: not a requirement for bone formation in vivo. **Artif Organs,** v. 34, n. 1, p. 46-54, Jan 2010. ISSN 1525-1594 (Eletronic)

LIU, T. et al. Deproteinized bovine bone functionalized with the slow delivery of BMP-2 for the repair of critical-sized bone defects in sheep. Bone, v. 56, n. 1, p. 110-8, Sep 2013. ISSN 1873-2763 (Eletronic)

LIU, W. C. et al. Angiogenesis Assays for the Evaluation of Angiogenic Properties of Orthopaedic Biomaterials - A General Review. **Adv Healthc Mater,** v. 6, n. 5, Mar 2017 (Eletronic)

LIU, Y. et al. Segmental bone regeneration using an rhBMP-2-loaded gelatin/nanohydroxyapatite/fibrin scaffold in a rabbit model. **Biomaterials,** v. 30, n. 31, p. 6276-85, Oct 2009. ISSN 1878-5905 (Eletronic)

LOMELINO, R. E. O. et al. The association of human primary bone cells with biphasic calcium phosphate (βTCP/HA 70:30) granules increases bone repair. J Mater Sci Mater Med, v. 23, n. 3, p. 781-8, Mar 2012. ISSN 1573-4838 (Eletronic)

MACHADO, E. G. et al. A new heterologous fibrin sealant as scaffold to recombinant human bone morphogenetic protein-2 (rhBMP-2) and natural latex proteins for the repair of tibial bone defects. **Acta Histochem,** v. 117, n. 3, p. 288-96, Apr 2015. ISSN 1618-0372 (Eletronic)

MANFRIN ARNEZ, M. F. et al. Implant osseointegration in circumferential bone defects treated with latex-derived proteins or autogenous bone in dog's mandible. **Clin Implant Dent Relat Res,** v. 14, n. 1, p. 135-43, Mar 2012. ISSN 1708-8208 (Eletronic)

MARENZANA, M.; ARNETT, T. R. The Key Role of the Blood Supply to Bone. **Bone Res,** v. 1, n. 3, p. 203-15, Sep 2013. ISSN 2095-4700 (Eletronic)

MAURÍCIO, V. Aceleração do reparo tissular induzido por uma fração angiogênica purificada do látex natural da seringueira Hevea brasiliensis. 2006. 111 p. Dissertação

(Mestrado em Medicina) – Faculdade de Medicina, Universidade de São Paulo, Ribeirão Preto

MENDONÇA, R.J. Caracterização biológica de uma fração angiogênica do latex natural da seringueira Hevea brasiliensis. Dissertação de Mestrado. FMRP-USP, Ribeirão Preto, 2004.

MENDONÇA, R. J. et al. Increased vascular permeability, angiogenesis and wound healing induced by the serum of natural latex of the rubber tree Hevea brasiliensis. **Phytother Res,** v. 24, n. 5, p. 764-8, May 2010. ISSN 1099-1573 (Eletronic)

MOLDOVAN, L.; MOLDOVAN, N. I. Trends in genomic analysis of the cardiovascular system. Arch Pathol Lab Med, v. 126, n. 3, p. 310-6, Mar 2002. ISSN 0003-9985(Eletronic)

MOORE, W. R.; GRAVES, S. E.; BAIN, G. I. Synthetic bone graft substitutes. **ANZ J Surg,** v. 71, n. 6, p. 354-61, Jun 2001. ISSN 1445-1433 (Eletronic)

MRUE, F. et al. Evaluation of the biocompatibility of a new biomembrane. **Materials Research,** v. 7, n. 2, p. 277-283, 2004. ISSN 1516-1439.

OTSUKA, M. et al. Effect of geometrical structure on the in vivo quality change of a three-dimensionally perforated porous bone cell scaffold made of apatite/collagen composite. J Biomed Mater Res B Appl Biomater, v. 101, n. 2, p. 338-45, Feb 2013. ISSN 1552-4981 (Eletronic)

PANTELI, M. et al. Biological and molecular profile of fracture non-union tissue: current insights. J Cell Mol Med, v. 19, n. 4, p. 685-713, Apr 2015. ISSN 1582-4934 (Eletronic)

PARK, J. W. et al. Bone formation with various bone graft substitutes in critical-sized rat calvarial defect. Clin Oral Implants Res, v. 20, n. 4, p. 372-8, Apr 2009. ISSN 1600-0501 (Eletronic)

PARKINSON, I. H.; BADIEI, A.; FAZZALARI, N. L. Variation in segmentation of bone from micro-CT imaging: implications for quantitative morphometric analysis. Australas Phys Eng Sci Med, v. 31, n. 2, p. 160-4, Jun 2008. ISSN 0158-9938 (Eletronic)

PARTICELLI, F. et al. A comparison between micro-CT and histology for the evaluation of cortical bone: effect of polymethylmethacrylate embedding on structural parameters. J Microsc, v. 245, n. 3, p. 302-10, Mar 2012. ISSN 1365-2818 (Eletronic)

PORTAL-NÚÑEZ, S.; LOZANO, D.; ESBRIT, P. Role of angiogenesis on bone formation. Histol Histopathol, v. 27, n. 5, p. 559-66, 05 2012. ISSN 1699-5848 (Eletronic)

ROCHA, C. A. Avaliação comparativa da neoformação óssea após enxertia de HA/TCPp, Bio-Oss e osso autógeno associados ou não ao PRP em cirurgias de levantamento de seio maxilar de coelhos 2015. 158 Universidade de São Paulo. Faculdade de Odontologia de Bauru

SANTOS, A. A. D. et al. The role of bone morphogenetic protein on bone tissue repair. **Acta Ortopédica Brasileira,** v. 13, n. 4, p. 194-195, 2005. ISSN 1413-7852.

SANTOS, P. S. et al. Osteoinductive porous biphasic calcium phosphate ceramic as an alternative to autogenous bone grafting in the treatment of mandibular bone critical-size defects. **J Biomed Mater Res B Appl Biomater,** v. 106, n. 4, p. 1546-1557, May 2018. ISSN 1552-4981 (Eletronic)

SCHMITZ, J. P.; HOLLINGER, J. O. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res, n. 205, p. 299-308, Apr 1986. ISSN 0009-921X (Eletronic)

TADIC, D.; EPPLE, M. A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone. **Biomaterials,** v. 25, n. 6, p. 987-94, Mar 2004. ISSN 0142-9612 (Eletronic)

TORIUMI, D. M.; ROBERTSON, K. Bone inductive biomaterials in facial plastic and reconstructive surgery. **Facial Plast Surg,** v. 9, n. 1, p. 29-36, Jan 1993. ISSN 0736-6825 (Eletronic)

VAJGEL, A. et al. A systematic review on the critical size defect model. Clin Oral Implants Res, v. 25, n. 8, p. 879-93, Aug 2014. ISSN 1600-0501 (Eletronic)

VIDAL, B. et al. Bone histomorphometry revisited. Acta Reumatol Port, v. 37, n. 4, p. 294-300, 2012 Oct-Dec 2012. ISSN 0303-464X (Eletronic)

YANG, Y. Q. et al. The role of vascular endothelial growth factor in ossification. Int J Oral Sci, v. 4, n. 2, p. 64-8, Jun 2012. ISSN 1674-2818 (Eletronic)

YEOM, H. et al. Correlation between micro-computed tomography and histomorphometry for assessment of new bone formation in a calvarial experimental model. J Craniofac Surg, v. 19, n. 2, p. 446-52, Mar 2008. ISSN 1049-2275 (Eletronic)

ZIMMERER, R. M. et al. In vivo tissue engineered bone versus autologous bone: stability and structure. **Int J Oral Maxillofac Surg,** v. 46, n. 3, p. 385-393, Mar 2017. ISSN 1399-0020 (Eletronic)

ZISCH, A. H.; LUTOLF, M. P.; HUBBELL, J. A. Biopolymeric delivery matrices for angiogenic growth factors. Cardiovasc Pathol, v. 12, n. 6, p. 295-310, 2003 Nov-Dec 2003. ISSN 1054-8807 (Eletronic)

APPENDIX

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION

We hereby declare that we are aware of the article "*F1 protein fraction obtained from latex incorporated into CaP-materials improve critical-size defect bone repair in a concentration-dependent manner*" will be included in Dissertation of the student **Suelen Paini** and can not be used in other works of the Graduate Programs of the Faculty of Dentistry of Bauru of the University of São Paulo.

Bauru, 7 de Agosto de 2018.

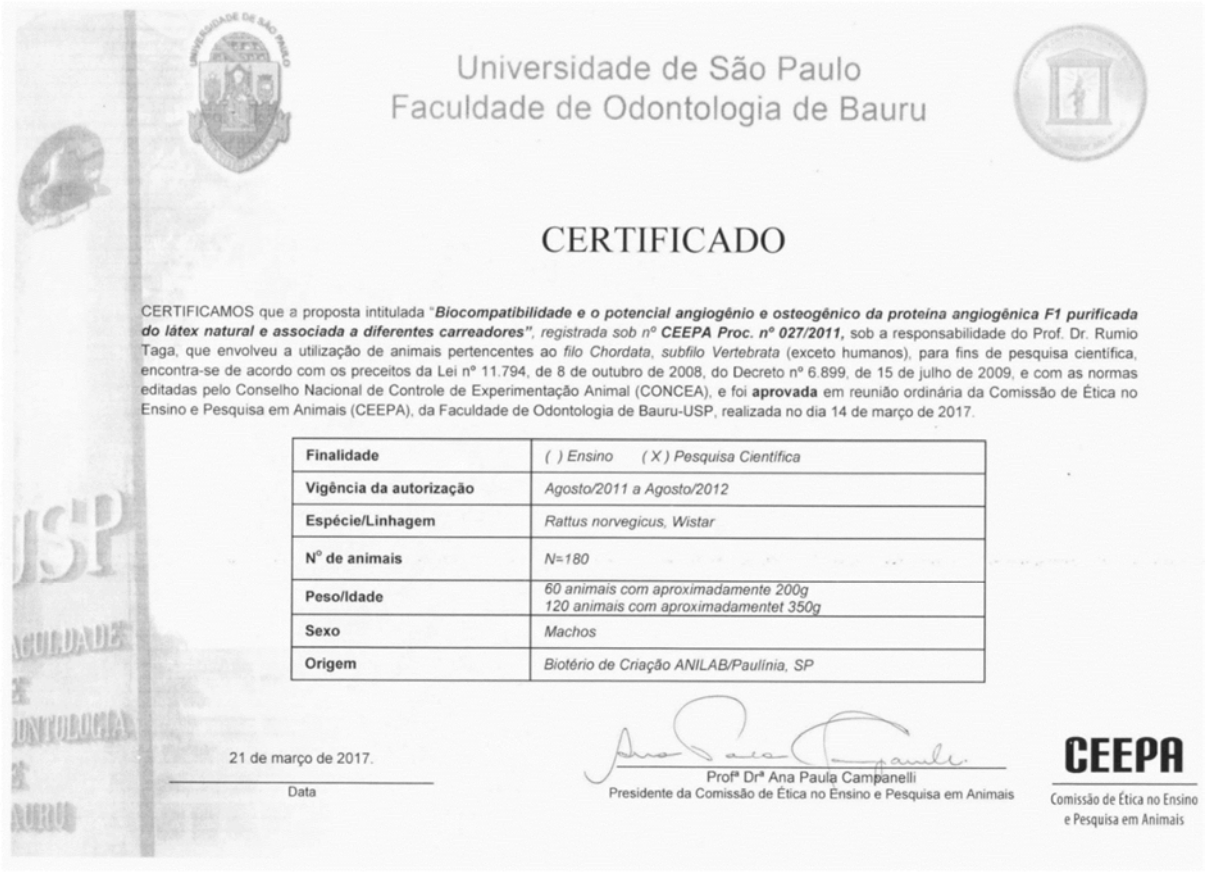
Suelen Paini

Gerson Francisco de Assis

Rumio Taga

ANNEXS

ANNEXS



Universidade de São Paulo
Faculdade de Odontologia de Bauru

CERTIFICADO

CERTIFICAMOS que a proposta intitulada "***Biocompatibilidade e o potencial angiogênio e osteogênico da proteína angiogênica F1 purificada do látex natural e associada a diferentes carreadores***", *registrada sob nº* ***CEEPA Proc. nº 027/2011,*** sob a responsabilidade do Prof. Dr. Rumio Taga, que envolveu a utilização de animais pertencentes ao *filo Chordata, subfilo Vertebrata* (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **aprovada** em reunião ordinária da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), da Faculdade de Odontologia de Bauru-USP, realizada no dia 14 de março de 2017.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*Agosto/2011 a Agosto/2012*
Espécie/Linhagem	*Rattus norvegicus, Wistar*
Nº de animais	*N=180*
Peso/Idade	*60 animais com aproximadamente 200g* *120 animais com aproximadamentet 350g*
Sexo	*Machos*
Origem	*Biotério de Criação ANILAB/Paulínia, SP*

21 de março de 2017.
Data

Profª Drª Ana Paula Campanelli
Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA
Comissão de Ética no Ensino e Pesquisa em Animais

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 004/2017.

Bauru, 5 de maio de 2017.

Senhor Professor,

Informamos que Projeto de Pesquisa denominado ***"Influência da concentração da proteína F1 purificada do látex natural (Heve brasiliensis) e do biomaterial carreador na vascularização e formação ósse em defeitos ósseos cranianos de tamanho crítico. Estudo microtomgráfico, histomofométrico e imunistoquímico"*** tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais (roedores), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi analisado e considerado APROVADO em reunião ordinária da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), realizada nesta data.

Vigência do projeto:	*Maio/2017 a Abril/2019*
Espécie/Linhagem:	*Amostras biológicas (calvária de ratos incluídos em Histosec-Merck) provenientes do protocolo CEEPA 027/2011*
Nº de animais:	*Não se aplica*
Peso/Idade	*Não se aplica*
Sexo:	*Não se aplica*
Origem:	*Não se aplica*

Esta CEEPA solicita que ao final da pesquisa seja enviado um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Profª Drª Ana Paula Campanelli

Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Gerson Francisco de Assis
Docente do Departamento de Ciências Biológicas

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
e-mail: ceepa@fob.usp.br – Fone/FAX (0xx14) 3235-8356
http://www.fob.usp.br