PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
MESTRADO EM ESTOMATOLOGIA

RAFAEL DE ARAÚJO NORONHA

EFEITO DO CANABIDIOL NO PROCESSO CICATRICIAL DE DEFEITOS ÓSSEOS CRÍTICOS MECANICAMENTE INDUZIDOS EM CALOTA CRANIANA DE RATOS: AVALIAÇÃO CLÍNICA E HISTOLÓGICA

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Mestrado - Área de Concentração: Estomatologia Clínica

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Linha de Pesquisa

Enfermidades da Região Bucomaxilofacial: estudos clínicos, imunológicos e anatomopatológicos

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Orientadora: Profª. Dra. Maria Antonia Zancanaro de Figueiredo

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RESUMO

As fraturas ósseas bucofaciais de origem traumática possuem uma elevada incidência na população mundial. Estas podem ser tratadas de distintas formas, que variam desde métodos conservadores até procedimentos amplamente invasivos. Dentre as múltiplas possibilidades de tratamento deste tipo de enfermidade, está a utilização de fitoterápicos, onde insere-se o canabidiol (CBD). Esse é o principal componente não-psicomimético da *Cannabis sativa (Can-ns)* e desempenha potentes efeitos anti-inflamatórios, antioxidantes e analgésicos em diversas condições patológicas. A presente dissertação está estruturada na forma de 2 artigos científicos. No primeiro foi realizada uma revisão de literatura, cujo objetivo foi avaliar os mecanismos de ação do CBD que possam estar envolvidos no reparo ósseo. Os resultados deste estudo sugerem que o CBD é uma substância capaz de interferir no processo cicatricial de defeitos ósseos a partir do seu potencial anti-inflamatório. O segundo trata de um experimento desenvolvido em modelo animal, utilizando 64 ratos *Wistar,* divididos randomicamente em 2 grupos (teste com CBD e controle). Avaliou-se clínica e histologicamente o efeito da administração intraperitoneal do CBD, na dose de 10 mg/kg/dia, por duas, 4, 6 e 8 semanas, no processo cicatricial de defeitos ósseos mecanicamente induzidos em calota craniana de ratos. Constatou-se que o CDB exerceu um efeito benéfico na quarta semana pós tratamento onde observou-se um aumento na média de osso neoformado no interior do defeito. Contudo não foi observada diferença estatisticamente significativa no processo cicatricial dos defeitos ósseos nos tempos analisados. Estes resultados sugerem a necessidade de aprofundar o tema, visando uma melhor compreensão sobre possíveis efeitos do uso do CBD no metabolismo e reparo ósseo.

Palavras-chave: canabidiol, canabinoides, Cannabis sativa, consolidação da fratura, regeneração óssea, patologia óssea

ABSTRACT

Maxillofacial fractures of traumatic origin have a high incidence in the world population. These can be treated in different ways, ranging from conservative methods to widely invasive procedures. Among the multiple possibilities of treatment of this type of disease is the use of herbal medicines, where cannabidiol (CBD) is inserted. This is the main non-psychomimetic component of *Cannabis sativa* (Can-ns) and plays potent anti-inflammatory, antioxidant and analgesic effects in a variety of pathological conditions. The present dissertation is structured in the form of 2 scientific papers. The first one consists of a literature review, whose objective was to evaluate the mechanisms of action of CBD that may be involved in bone repair. The results of this study suggest that CBD is unable substance of interfering in the healing process of bone defects from its anti-inflammatory and antioxidant potential. The second is an experiment in an animal model, using 64 *Wistar* rats, divided into two groups (test with CBD and control). At the periods of two, four, six and eight weeks, they were evaluated clinically and histologically in the cicatricial process of mechanically induced bone defects in the skull cap of rats. It was found that the CBD exerted a beneficial effect in the fourth week after treatment where an increase in the mean of neoformed bone within the defect was observed, however, no statistically significant difference was observed in the healing process of the bone defects at the times analyzed. These results suggest the need to deepen the theme, aiming at a better understanding of the possible effects of CBD on bone metabolism and repair.

Keywords: cannabidiol, cannabinoids, Cannabis sativa, bone regeneration, fracture healing, bone pathology

SUMÁRIO

1. INTRODUÇÃO

As propriedades terapêuticas da *Cannabis sativa* (popularmente conhecida como maconha) são conhecidas há milhares de anos. Na década de 80, foram descobertos receptores específicos, capazes de interagir com propriedades químicas da planta no organismo de mamíferos, despertando assim, o interesse científico das propriedades farmacológicas da mesma (Iannotti; Di Marzo; Petrosino, 2016).

Os receptores canabinóides tipo 1 (CB1) e tipo 2 (CB2) estão presentes no organismo humano, mais precisamente na membrana celular, acoplados à proteína G, de células do sistema imune e do sistema nervoso, respectivamente (Iannotti; Di Marzo; Petrosino, 2016). A partir desses conhecimentos alguns autores direcionaram seus estudos visando detectar seus ligantes endógenos. Com isso foram identificadas duas principais substâncias endocanabinoides, sendo elas a Anandamida (AEA) e 2-araquidonil glicerol (2AG). O sistema endocanabinoide (sEC) é constituído pelos receptores, ligantes, bem como por enzimas de degradação e de síntese (Lu; Mackie, 2016; Russo, 2016).

O sEC é considerado um regulador fisiológico homeostático único e difundido (Russo, 2016). Sua principal função é a neuromodulação, capaz de gerar efeitos que alteram a percepção da dor, fome, esquecimento, ansiedade, aprendizado e memória. Além disso, influencia no controle motor, na imunidade, proliferação de células tumorais e, inclusive, no processo inflamatório (Fasinu Phillips, Elsohly, et al., 2016).

No sEC foram identificadas mais de 400 substâncias, das quais, acima de 70 são canabinoides. As duas mais abundantes são o delta-9-tetra-hidrocanabinol (THC) e o canabidiol (CBD).

Em 1964, Mechoulam e Gaoni caracterizaram a estrutura química do principal componente psicoativo da *Cannabis spp*., o THC. Este canabinoide possui propriedades lipofílicas, o que facilita a sua absorção no corpo e a consequente rapidez no aparecimento de seus efeitos. É um agonista parcial do CB1 e do CB2 (Katchan, David, Shoenfeld, 2016; Pisanti**,** Malfitano AM, Ciaglia E**,** et al., 2017), utilizado em medicações para o tratamento de náusea e vômitos induzidos, por exemplo, em decorrência da quimioterapia e da anorexia associada à AIDS (Burstein; Zurier, 2009).

O CBD é um composto que não possui propriedades psicoativas, por isso seus possíveis efeitos clínicos ainda estão sendo investigados. Sabe-se que o tratamento com CBD atenua algumas das alterações psicológicas induzidas por altas doses de THC (0,5 mg / kg), tais como ansiedade e pânico (Zuardi Shirakawa, Finkelfarb E, et al., 1982). Da mesma forma, é atribuído ao CBD um papel neuroprotetor, uma vez que atua como um antioxidante contra os efeitos oxidativos produzidos nos neurônios pela liberação excessiva de glutamato. A partir deste efeito explica-se a capacidade de imunomodulação atribuída aos canabinoides (Netzahualcoyotzi-Piedra, Muñoz-Arenas G, García IM, et al., 2009). Quando comparado ao THC, o CBD demonstra menor afinidade pelos receptores canabinoides (Mcpartland, Duncan M, Di Marzo et al., 2015; Pisanti, et al., 2017). A revisão de literatura conduzida por Bergamaschi e colaboradores em 2011, e atualizada por Iffland e Grotenhermen em 2017, sugere que a utilização do CBD é segura e bem tolerada, tanto em animais quanto em humanos. Sua administração não influencia a função motora, memória e tampouco a temperatura corporal (Bergamaschi, Queiroz, Zuardi, et al., 2011; Iffland; Grotenhermen, 2017).

Na área odontológica, são escassos os estudos relacionados ao uso do CBD. Um experimento desenvolvido por Napimoga et al. em 2009, testou o efeito de 5mg/kg/dia dessa substância em modelo animal de doença periodontal em ratos. Os resultados obtidos demonstraram que o CBD reduziu a reabsorção óssea, modulando o processo inflamatório através da diminuição da produção de interleucina 1beta (IL1β) e fator de necrose tumoral alfa (TNF-α).

Uma revisão de literatura recentemente publicada pelo nosso grupo de pesquisa sugere que o CBD possa ser testado como uma alternativa para o tratamento de mucosites orais, considerando seu potencial anti-inflamatório e antioxidante (Cuba, Salum, Cherubini et al., 2017).

Sabe-se que o desenvolvimento da inflamação representa a primeira etapa no processo de cicatrização óssea. O recrutamento celular estimulado pelo dano ao tecido ósseo, juntamente com a produção de citocinas inflamatórias e fatores de crescimento, é responsável por estimular a formação do tecido ósseo imaturo (Bab, Zimmer, 2009; Napimoga, Benatti, Lima, et al., 2009)

Considerando-se os estudos que evidenciam o efeito anti-inflamatório, antioxidante e a fisiologia do reparo ósseo, propõe-se que esta substância possa ser analisada, testando seu uso no favorecimento da cicatrização óssea. Assim

sendo, este experimento teve como objetivo avaliar o efeito do CBD, na dose de 10 mg/kg/dia, como uma alternativa capaz de auxiliar no tratamento desta enfermidade.

A presente dissertação foi estruturada sob a forma de 2 artigos científicos. Apresenta-se, inicialmente, uma revisão de literatura, destacando o potencial uso do CBD como agente estimulante da cicatrização óssea. No segundo artigo, foi feito um estudo experimental, analisando-se clínica e histologicamente o processo cicatricial de defeitos ósseos mecanicamente induzidos em calota craniana de ratos tratados com CBD.

2. OBJETIVOS

2.1 Objetivo geral

- Avaliar clinica e histológicamente o efeito da administração intraperitoneal de 10 mg/kg/dia de canabidiol no processo cicatricial de defeitos ósseos críticos mecanicamente induzidos em calota craniana de ratos.

3. ARTIGO CIENTÍFICO 1

O artigo a seguir intitula-se *"*Cannabidiol: a therapeutic alternative in the bone healing process?*"* e foi formatado de acordo com as normas estabelecidas pelo periódico *Quintessence International*.

Cannabidiol: a therapeutic alternative in the bone healing process?

Running title: Cannabidiol in bone healing process

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Keywords: cannabidiol, cannabinoids, Cannabis sativa, bone regeneration, fracture healing, bone pathology

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ABSTRACT

Bone fractures show a high incidence in the general population, and can be treated conservatively or through invasive procedures. Among the possible treatments are the phytotherapeutic agents, where there is a possibility to use cannabidiol. *Cannabis sativa* is a plant of Asian origin used for many years for medicinal purposes. Cannabidiol is one of the best known cannabinoids. The therapeutic properties of *Cannabis sativa* result from the interaction of its compounds with receptors located in the central nervous system and cells of the immune system. We reviewed the available literature in Pubmed between 1994 and 2017 on the bone repair process and the activity of cannabidiol in bone, considering the possibility of its use in the treatment of fractures. Studies have shown that cannabidiol is an alternative capable of interfering with the RANK/ RANKL/OPG system, which is directly linked to the bone healing process. It also has antiinflammatory properties, where it acts to control the release of inflammatory cytokines. The literature on the use of cannabidiol in the area of dentistry is still scarce, and therefore, studies on the role of cannabidiol in the repair of bone tissue are encouraged.

SUMMARY

1. INTRODUCTION

Cannabis sativa (*Can-ns*) or marijuana is a plant of Asian origin used for thousands of years for medicinal purposes. Its therapeutic properties result from the interaction of its compounds with so-called cannabinoid systems in cells of the human central nervous system (CNS) and other organs. [1,2] Since its discovery nearly a decade ago, the cannabinoid system consisting of CB1 and CB2 receptors has attracted great attention. CB1 receptors are most commonly found in the CNS, whereas CB2 receptors are found in other human organs, mainly linked to cells of the immune system. [1,2]

There are more than 70 types of cannabinoids reported in the literature, but the two most known are cannabidiol (CBD) and tetrahydrocannabinol (THC). THC has a high affinity for CB1 and CB2 receptors, which makes it the main constituent responsible for the psychoactive effect of *Cs.* CBD, however, has a low affinity for these receptors and thus does not display this characteristic. [3,4,5,6]

CBD has been tested in the treatment of various pathologies, such as epilepsy, diabetes, Alzheimer's, sepsis, nephrotoxicity, cardiotoxicity and even cancer. Studies have shown that CBD is well tolerated and nontoxic and exerts beneficial effects in various neuropsychiatric diseases and inflammatory disorders through its antioxidant, antiinflammatory, immunomodulatory and analgesic actions. [3,4,5,6]

Recently, several components of the endocannabinoid system were detected in bone tissue. [7,8,9] The main ones were anandamide (AEA) and 2-arachidonoylglycerol (2AG), present at levels similar to those found in the brain. There is a tendency for the two components to be synthesized locally in bone tissue. Although there have been few studies so far, some authors suggest that CBD has favorable effects on the bone healing process. [9,10,11,12,13]

Bone fractures have a high incidence in the general population and can be treated in a more conservative way, with immobilization and/or reduction, or even with surgery, physical therapy and medication. The last can be done using phytotherapeutics, where CBD can be used. On the basis of its antiinflammatory potential and ability to inhibit the expression of receptor activator of nuclear factor kappa B ligand (RANKL), it is believed that CBD can be an alternative in the repair process of bone fractures. [9,10,11,12,13]

The objective of this review was to evaluate the therapeutic viability of CBD on the basis of scientific publications that discuss its mechanism of action, suggesting possibilities for future studies on the use of this compound in the repair of fractures of the maxillofacial complex.

2. DATA SOURCES

A research literature review was performed in the PubMed database. The keywords "bone repair," "cannabidiol," "healing bone" and "cannabis" were used to search for complete articles published between 1994 and 2017. Additional papers were obtained from the articles' reference lists. Recent studies addressed the mechanisms of action and possible uses of CBD.

3. REVIEW

3.1 Bone healing

In addition to excellent mechanical properties, bone has the potential for repair as a result of its regenerative capacity in response to local fractures or defects, forming new tissue with the same previous structure but without the formation of a scar. [14]

After a bone injury, a sequence of events occurs to restore bone shape and function, and many of these are still not completely elucidated. It is known that this repair process occurs through the local release of cytokines and growth factors. [15]

When a bone fracture occurs, other structures are ruptured, such as the periosteum, vessels in the cortical-medullary portion and surrounding tissues. Immediately, there is blood leakage from the edge of the injured bone or adjacent soft tissues, forming between the ends of the fracture and the periosteum a clot or hematoma within the medullary canal. [16]

The fracture clot consists of the extravasation of erythrocytes, fibrin and platelets, these structures release platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor (TGF)-alpha, and chemotactic and regulators of cellular activity as well. [15,16]

For the repair process to begin, the newly formed clot and the cell debris and matrix need to be phagocytosed by macrophages. Simultaneously with clot formation, tissue necrosis adjacent to the fracture occurs, promoting an intense inflammatory response, characterized by vasodilation, plasma exudation, and presence of leukocytes and mesenchymal cells. [16]

As of the third day after fracture, connective tissue rich in osteogenic cells forms, constituting a collar around this area, which penetrates between the ruptured bone ends. Osteoblasts synthesize collagen and matrix, creating an immature mineralized tissue. This process evolves, generating after some time, the bone callus, which forms in a disordered way, characterized by an irregular arrangement of osteocytes and collagen fibers. While the repair process continues, concomitant bone remodeling occurs. Callus becomes unnecessary and is then resorbed at this repair stage. Bone trabeculae are formed, oriented along stress lines, becoming functional. [16,17]

With the synthesis of new collagen and other matrix proteins, mineral deposition begins, which lasts for several days, long enough for collagen to form its binding bridges. Few osteoblasts remain attached to the matrix in mineralization, being transformed into osteocytes, while most undergo apoptosis [17]. Reduction of osteoblastic activity may result from inhibition by negative feedback or induction of osteoblast apoptosis by tumor necrosis factor (TNF) released by adjacent medullary cells. [18,19,20,21]

3.2 Cannabidiol

C. sativa, is a commonly used drug, being illegal in several countries and can be associated with various health problems. This plant has more than 500 substances, which trigger different effects on the human body. [1,2] Most of them are classified as cannabinoids, and since the 19th century, marijuana has been extensively studied for medicinal purposes. Cannabinoids are heterogeneous molecules that act by binding to receptors present in the body. These can be produced endogenously, where AEA and 2AG are the best-known endocannabinoids studied so far. There are also other plant derivatives known as phytocannabinoids, where the most popular and best known ones are THC and CBD. [1,2,9]

THC has major limitations in its therapeutic use due to its psychoactive effects mediated by receptors located in the CNS. In contrast, CBD does not have this effect due to the low affinity of these receptors for this phytocannabinoid, thus offering greater safety in its medicinal use. [1,2]

The cannabinoid receptors were discovered in the 1980s and their nomenclature established according to chronological order of identification, i.e., CB1 (mostly located in the CNS) and CB2 (located in other organs of the human

body and the immune system) [2]. CB1 is associated with the perception of pain and memory, while CB2 plays an important role in mediating inflammatory processes, acting to reduce proinflammatory cytokines. Accordingly, it is known that the type of receptor with greater affinity for each compound is determinant for the pharmacological effects to be achieved. [9,10,12,13]

In the last decade there has been a marked growth in medical studies on the use of CBD, mainly prompted by the discovery of its antiinflammatory, antioxidant and neuroprotective action. Research has shown that CBD has the ability to decrease proinflammatory mediators by suppressing the cellular response of the immune system, which may be important in the treatment of diseases of this nature. Decreased adenosine uptake and the production of some inflammatory mediators, such as interferon (IFN), tumor necrosis factor - alpha (TNF), proinflammatory citokines (IL-1) and antiinflammatory (IL-10), appear to be important in the antiinflammatory action of CBD. [1,2,22,23]

Recent studies have shown that two of the main components of the endocannabinoid system occur bone tissue, namely AEA and 2AG (mentioned above). Accordingly, CBD has been found to interfere with the RANK/RANKL/OPG system, which is directly related to the process of bone healing. This occurs due to the link of CBD with GPR55 receptors, coupled to G protein. Therefore CBD would inhibit the expression of RANKL, decreasing the activity and maturation of osteoclasts through the interaction with transient receptor potential cation channels (TRPV 1, 2, 4 and 5) and, consequently, resulting in a lower bone resorption. [10,11,12]

4. DISCUSSION

Bone fractures of all types are among the most prevalent group of injuries in the general population. The repair process of these fractures occurs especially through enzymes that catalyze the synthesis of collagen.[14,15,16] It is known that bone cells possess cannabinoid receptors and endocannabinoid metabolic enzymes, which are expressed at skeletal sympathetic nerve endings. Cannabinoids play an important role in the remodeling and structuring of bone mass.[9,10,11,12] With the current approval of the use of *Cannabis* for medicinal purposes it is important to evaluate its effects on the healing of this tissue. Many fractures are repaired by a process called endochondral ossification. In this process, the fracture line is initially filled by a mineralized, cartilaginous callus, which will be resorbed and replaced by a bone callus, which in will be gradually remodeled, forming the mature bone. [14,15]

Kogan et al. [24] produced bone fractures in the femur of rats and evaluated the effect of CBD on the healing phase. They found that the stimulation of the bone repair process induced by the phytocannabinoid occurred during the later stages of healing (about 6 weeks). In contrast, Napimoga et al. e Nogueira et al. [12,25] demonstrated that inhalation of *Cs* smoke inhibited the early stages of bone healing around implants in rats. The possible explanation for such disagreement is based on the pattern of the intramembranous healing that occurs around implants, in which early stages are the formation of a blood clot and/or its organization through primary bone formation. This process may be susceptible to the deleterious effects of *Cannabis*, through the expression of CB1 receptors in collagen fibers, which release norepinephrine thus restricting bone formation and stimulating the resorption of this tissue. [12,25]

After some components of the endocannabinoid system (AEA and 2AG) were found in the bone in amounts similar to those found in the CNS, it was suggested that they could be synthesized locally in bone tissue. It is known that the cannabinoid receptors (CB1 and CB2) are also present in bone, with CB2 showing the highest expression. This is found in osteoblasts, pre-osteoblasts, stromal cells derived from bone marrow and osteocytes. CB2 is of potential importance in bone metabolism, where its expression is low in undifferentiated osteoblast precursors but increases progressively along with the expression of osteoblastic marker genes. [9,11]

The discovery of the RANK/RANKL/OPG system led a better understanding of the mechanisms regulating osteoclastic differentiation and activation by the immune system. Ofek et al. [10] demonstrated that CB2 receptors are expressed in both osteoblasts and osteoclasts and that exposure of these cells to a specific CB2 agonist (GPR55) results in a distinct response. GPR55 is a newly discovered G protein coupled receptor that is expressed in human and rat osteoclasts and osteoblasts. This suggests that this receptor contributes significantly to the maintenance as well as the formation of bone mass through two different mechanisms, 1) direct stimulation of osteoblasts and 2) direct inhibition of osteoclasts and RANKL expression, therenby repressing osteoclastogenesis.

Napimoga et al. [12] reinforced these findings when they tested the effect of CBD on experimental periodontitis in rats. The authors observed that CBD was able to inhibit the expression of RANK and RANKL. Few studies have reported the direct effect of CBD on the RANK/RANKL/OPG system. However, treatment of ovariectomized animals with a specific CB2 agonist has been reported to suppress osteoclastogenesis, apparently by a mechanism involving CB2-mediated

mitogenic inhibition of osteoclast precursors and inhibition of RANKL expression in stromal cells and osteoblasts. [10,26]

5. CONCLUSION

In analyzing the results obtained in studies so far, it is believed that CBD has the potential to contribute the consolidation of fractures. Several preclinical and clinical studies have demonstrated that CBD is a viable and safe agent, which encourages new studies investigating its true contribution in the bone healing process.

6. CONFLICTS OF INTEREST

The authors declare not having any conflict of interest.

6. REFERENCES

[1] - Burstein S. Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. Bioorg Med Chem 2015;23(7):1377-1385

[2] - Cuba LF, Salum FG, Cherubini K, Figueiredo MAZ. Cannabidiol: an alternative therapeutic agent for oral mucositis?. J Clin Pharm Ther 2017;42(3): 245-250

[3] - Pan H, Mukhopadhyay P, Rajesh M, Patel V, Mukhopadhyay B, Gao B, et al., Cannabidiol attenuates cisplatin-induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation, and cell death. J Pharmacol Exp Ther 2009;328(3):708-714

[4] - Cassol-Jr OJ, Comim CM, Silva BR, Hermani FV, Constantino LS, Felisberto F, et al., Treatment with cannabidiol reverses oxidative stress parameters, cognitive impairment and mortality in rats submitted to sepsis by cecal ligation and punctura. Brain Res 2010;1348:128-138

[5] - Rajesh M, Mukhopadhyay P, Batkai S, Patel V, Saito K, Matsumoto S, et al., Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. J Am Coll Cardiol 2010;56(25):2115-2125

[6] - Booz GW, Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress. Free Radic Biol Med 2011;51(5):1054-1061

[7] - Yang L, Rozenfeld R, Wu D, Devi LA, Zhang Z, Cederbaum A, Cannabidiol protects liver from binge alcohol-induced steatosis by mechanisms including inhibition of oxidative stress and increase in autophagy. Free Radic Biol Med 2014;68:260-267

[8] - Elbaz M, Nasser MW, Ravi J, Wani NA, Ahirwar DK, Zhao H, et al., Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: novel anti-tumor mechanisms of cannabidiol in breast cancer. Mol Onco 2015;9(4): 906-919

[9] - Bab I, Zimmer A, Melamed E. Cannabinoids and the skeleton: from marijuana to reversal of bone loss. Ann Med 2009;41(8):560-567

[10] - Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, et al., Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc Natl Acad Sci U.S.A. 2006;103(3):696-701

[11] - Bab I, Zimmer A. Cannabinoid receptors and the regulation of bone mass. Brit J Pharmacol 2008;153:182-188

[12] - Napimoga MH, Benatti BB, Lima FO, Alves PM, Campos AC, Pena-dos-Santos DR, et al., Cannabidiol decreases bone resorption by inhibiting RANK/ RANKL expression and pro-inflammatory cytokines during experimental periodontitis in rat. Int immunepharmacol 2009;9:216-222

[13] - Rettori E, De Laurentiis A, Zubilete MZ , Rettori V, Elverdin JC. Anti-inflammatory effect of the endocannabinoid anandamide in experimental periodontitis and stress in the rat. Neuroimmunomodulat 2012;19:293-303

[14] - Schenk RK. Bone regeneration: biologic basis. In: D. Buser, C. DahlIn, R.K. Scherk, Guided bone regeneration in implant dentistry. Quintessense Books 1994. Chicago: Quintessence p. 49-100

[15] - Hollinger J, Wong MEK. The integrated processes of hard tissue regeneration with special emphasis on fracture healing. Oral Surg Oral Med Oral Pathol 1996;82(6):594-606

[16] - Trowbridge HO, Emling RC. Regeneração e cicatrização. In: Inflamação: uma revisão do processo. (ed. 7) (1996) Quintessence p.146-147

[17] - Marssel R , Einhorn TA. The biology of fracture healing injury. Bristol 2011;42(6):551-555

[18] - Hill PA, Orth M. Bone remodeling. Br J Orthod 1998;25(2):101-107

[19] - Amadei SU, Silveira VAS, Pereira AC, Carvalho YR, Rocha RF. J Bras Patol Med Lab. Rio de Janeiro 2006;42(1):5-12

[20] - Kwan TS, Padrines M, Théoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev 2004;15:49-60

[21] - Burkiti HG, Young B, John W. Histologia funcional. Guanabara Koogan, Rio de Janeiro, 1994 p.409

[22] - Baron EP. Comprehensive review of medicinal marijuana, cannabinoids, and therapeutic implications in medicine and headache: what a long strange trip it's been. Headache 2015;55:885-916

[23] - Ibeas BC, Chen T, Nunn AV, Bazelot M, Dallas M, Whalley BJ, Molecular targets of cannabidiol in neurological disorders. Neurotherapeutics 2015;12:699-730

[24] - Kogan NM, Melamed E, Wasserman E, Raphael B, Breuer A, Stok KS, et al., Cannabidiol, a major non-Psychotropic Cannabis constituent enhances fracture healing and stimulates lysyl hydroxylase activity in osteoblasts. J Bone Miner Res 2015;30(10):1905-1913.DOI: 10.1002/jbmr.2513

[25] - Nogueira-Filho GR, Cadide T, Rosa BT, Neiva TG, Tunes R, Peruzzo D, et al., Cannabis sativa smoke inhalation decreases bone filling around titanium implants: a histomorphometric study in rat. Implant Dent 2008;17(4):461-470

[26] - Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ, Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999;20:345-357

2. ARTIGO CIENTÍFICO 2

Este artigo intitula-se “**Cannabidiol as an alternative in bone healing of critical size defect in rat calvarium”,** foi formatado de acordo com as normas estabelecidas pelo periódico *Phytotherapy Research.*

CANNABIDIOL AS AN ALTERNATIVE IN BONE HEALING OF CRITICAL SIZE DEFECT IN RAT CALVARIUM

Running title: Cannabidiol in bone healing process

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STATEMENT OF CLINICAL RELEVANCE:

Treatment with cannabidiol at the concentration and times previously established did not significantly affect the healing process of bone defects created in the calvarium of rats. The results point to the need for new studies using different methods that can make it possible to gain a better understanding of the effect of this substance on bone metabolism, to determine possible alternatives in fracture management and repair of mineralized tissue.

ABSTRACT

Objective. To evaluate the effect of cannabidiol on bone healing in the rat calvarium.

Study design. Two standardized bone defects, 5 mm in diameter, were created bilaterally in the calvarium of 64 male *Wistar* rats with a trephine drill. The rats were divided randomly into 2 groups of 32 animals each, and they were weighed daily to adjust the doses of the substances to be administered. Each rat was given an intraperitoneal (IP) injection of 10 mg/kg/day cannabidiol (CBD) or vehicle in the test and control groups, respectively. Afterwards, they were evaluated clinically and histologically at two, 4, 6 and 8 weeks of treatment.

Results. CBD did not significantly affect the healing of the bone defects produced at any of the observation times. However, in the 4-week test group, there was a greater formation of bone tissue, resulting in almost complete closure of the critical defect, which was not observed in the other experimental groups.

Conclusions. Daily administration of 10 mg/kg CBD for the different experimental times did not have a beneficial effect on the repair of the bone defects produced, suggesting whitin this methodology the inability of CBD to accelerate bone healing.

Keywords: cannabidiol, Cannabis sativa, bone healing, bone repair, cannabinoids, bone disease

1. INTRODUCTION

Bone is a specialized form of connective tissue composed of cells and extracellular matrix, which has the peculiar characteristic of mineralizing. The maturation of the matrix results in an extreme hardness of this tissue, allowing it to provide support, protection and resistance. In turn, the collagen matrix offers a certain degree of malleability to the bone tissue, reducing the risk of fractures. (Burkiti, Young & John, 1994; Junqueira & Carneiro, 1995).

Bone healing is a complex biological process that follows specific patterns of regeneration and involves changes in the expression of thousands of genes. The renewal of the mineralized bone matrix in the repair of a fracture or bone defect occurs initially with the resorption phase, through specialized cells called osteoclasts, followed by the neoformation process, through the osteoblasts. The balance of this process (resorption/neoformation) occurs through a system called RANK/RANKL/OPG (Amadei, Silveira, Pereira, Carvalho & Rocha, 2006; Putnam, Scutt, Bicknell, Priestley & Williamson, 2007; Marssel & Einhorn, 2011).

Inhibition of receptor activator of nuclear factor kappa-B ligand (RANKL) prevents the binding between the receptor (RANK) and the ligand (RANKL), thus inhibiting the activity of osteoclasts and consequently the bone resorption process (Kwan, Padrines, Théoleyre, Heymann & Fortun, 2004; Marssel, et al., 2011).

Cannabis sativa (*Cs*) is a plant of Asian origin used for thousands of years for medicinal purposes. Its properties result from the interaction of its compounds that are part of the so-called cannabinoid systems with cells of the central nervous system (CNS) and various organs (Iannotti; Di Marzo & Petrosino, 2016). Since their discovery almost a decade ago, the cannabinoid receptors, CB1 and CB2 receptors, have aroused great attention in the scientific community.

CB1 receptors are most commonly found in the CNS, while CB2 receptors are found in different organs, mainly linked to cells of the immune system (Lu & Mackie, 2016; Russo, 2016).

There are more than 70 types of cannabinoids reported in the literature, but the two most known are cannabidiol (CBD) and tetrahydrocannabinol (THC). CBD is a component of *Cs*, which has low affinity for CB1 and CB2 receptors and thus has no psychoactive effect. However, THC has a great affinity for these receptors, which gives it the characteristic of being the main compound responsible for the psychoactive effect of the plant (Mechoulam, Ben-Shabat, Hanus, Ligumsky, Kaminski, Schatz, *et al.*, 1995; Rajesh, Mukhopadhyay, Bátkai, Patel, Saito, Matsumoto, *et al.*, 2010; Booz, 2011).

CBD has been tested in the treatment of various pathologies such as epilepsy, diabetes, Alzheimer's, sepsis, nephro- and cardiotoxicity and cancer as well (Pan, Mukhopadhyay, Rajesh, Patel, Mukhopadhyay, Gao, *et al.*, 2009; Cuba, Salum, Cherubini & Figueiredo, 2017). Studies have shown that CBD is well tolerated, shows no toxicity and has beneficial effects in the treatment of several neuropsychiatric diseases and inflammatory disorders, through its antioxidant, antiinflammatory, immunomodulatory and analgesic actions (Mechoulam, *et al.*, 1995; Booz, 2011; Burstein, 2015).

Recently, several components of the endocannabinoid system (ES) have been detected in bone tissue (Ofek, Karsak, Leclerc, Fogel, Frenkel, Wright, *et al.*, 2006), especially anandamide and 2-arachidonoylglycerol, which are considered the main components, appearing at levels similar to those found in the brain. These compounds tend to be synthesized locally in the bone tissue. Although this has been addressed little, some authors suggest that CBD promotes the bone healing process of bone (Ofek *et al.,* 2006; Bab & Zimmer, 2008).

In view of the growing benefits of the therapeutic use of CBD, the National Agency for Health Surveillance (ANVISA) in Brazil recently reported its use. The compound is no longer on the list of prohibited substances and changes status to category C1, therapeutic use allowed but subject to control (ANVISA, 2016, 2017).

Based on its antiinflammatory potential and ability to inhibit the expression of RANKL, through antiinflamatory cytokine production, it is believed that CBD

may be an alternative in the healing process of bone fractures (Ofek *et al.*, 2006; Bab *et al.*, 2009)

Bone fractures have a high incidence in the general population and can be treated a more conservative way by immobilization and the reduction of the fracture to surgery, physiotherapy and drug therapy. The last option can be done through herbal products, where CBD can be included.

Accordingly, the objective of this study was to evaluate the clinical and histological effect of CBD on the repair of standardized bone defects created in the calvarium of rats.

2. MATERIALS AND METHODS

This study was approved by the Research Cinentific Committee and the Committee on Animal Use (protocol No. 7694) of the Pontifical Catholic University of Rio Grande do Sul. All procedures were conducted in accordance with the ethical principles applied in the use laboratory animals established by the National Board of Animal Experimentation Control.

2.1. Selection of sample

The sample consisted of 64 male, heterogenic *Wistar* rats, *Rattus norvegicus*, with a mean age of 10 weeks, weighing 250 ± 25 g and clinically healthy. The rats were obtained from the Center for Experimental Biological Models of the Pontifical Catholic University of Rio Grande do Sul (CeMBE/ PUCRS). During the experiment, the animals were given irradiated pelleted Nuvilab-Cr1 chow (Nuvilab, Colombo, PR, Brazil) and filtered water *ad libitum*.

The rats were kept in plastic boxes lined with autoclaved wood chips and labeled according to the respective groups. They were arranged in micro-isolators with maintenance of the temperature at 23 ± 1°C, relative humidity at 50 ± 5% and light-dark cycle at 12 h.

The animals were randomly divided into 2 groups of 32 rats each (test and control) and subdivided according to the previously established analysis times as listed below:

Test Group (TG) CBD: Intraperitoneal (IP) administration of CBD 10 mg/ kg/day (in 2% *Tween* 80)

- TG - CBD 1: Experimental time (2 weeks) n = 8
- TG - CBD 2: Experimental time (4 weeks) n = 8
- TG - CBD 3: Experimental time (6 weeks) n = 8
- TG - CBD 4: Experimental time (8 weeks) n = 8

Control Group (CG): IP administration of saline + 2% *Tween* 80

- CG 1: Experimental time (2 weeks) n = 8
- CG 2: Experimental time (4 weeks) n = 8
- CG 3: Experimental time (6 weeks) n = 8
- CG 4: Experimental time (8 weeks) n = 8

After weighing and anesthesia, 2 standardized critical defects were mechanically created in the center of the parietal bones (right and left) of the calvarium of each rat. Immediately, IP injection of the products used in the respective study groups was started. These injections were performed daily, in the morning, always at the same time.

The treatment described above proceeded until euthanasia, which occurred on the 15th, 29th, 43rd and 57th day after the surgery, based on a study where the same time intervals were used (Kogan, Melamed, Wasserman, Raphael, Breuer, Stok, *et al.,* 2015).

2.2. Creation of critical bone defect in calvarium

Initially, each animal was weighed on a Uranus scale (model IDU 2500/0.5) to adjust the analgesic and anesthetic doses. Subsequently, the skin on the skull was shaven, followed by antisepsis with 2% chlorhexidine. The animals received 5% tramadol hydrochloride (IP, 10 mg/kg) and were anesthetized with 10% ketamine hydrochloride (IP, 100 mg/kg) and 2% xylazine hydrochloride (IP, 5mg / kg). Only after complete anesthesia, with loss of protective reflexes, a linear incision of approximately 1.5 cm was made, following the midline of the animal, in the region of the skull, incising skin and periosteum. The skullcap was exposed and with a 5-mm trephine drill mounted on a straight surgical handpiece, using a speed of 20,000 rpm, 2 critical bone defects were made in the central portion of each parietal bone. The procedure was done under constant irrigation with saline,

avoiding damage to the brain tissue. The osteotomized portion of the skull carefully removed, and the surgical wound was irrigated with saline to remove the generated residues. Afterwards, the incision was sutured using Vicryl 4-0 resorbable suture (Spicer, Kretlow, Young, Jansen, Kasper & Mikos, 2012).

2.3. Treatment of animals

The IP injection of 10 mg/kg CBD (in 2% *Tween* 80) (Test Group) and saline (in 2% *Tween* 80) (Control Group) was done immediately after the surgery. On all subsequent days, according to the groups and analysis times established in the study, the test substance and vehicle were always injected in the morning.

2.4. Procurement of surgical fragments for histological analysis

Immediately after euthanasia, performed by anesthetic overdose with isoflurane, an incision was made in the rat skullcap region. The segment containing the critical bone defect was removed after incision of the soft tissues between the medial canthi of the eyes, respecting skull circumference. The bony segment of the calvarium was separated from the remainder of the animal's head with the aid of a 701 frustoconical drill mounted on a straight handpiece, using low speed and under irrigation with saline at a distance of 4 mm from the edges of defects.

The samples obtained were clinically evaluated and then placed in labeled flasks and fixed in 10% buffered formalin for at least 24 h.

2.5. Clinical evaluation

All animals were evaluated macroscopically to determine the presence of bone neoformation in the critical defect. In both groups (test and control), a No. 5 exploratory probe was used to evaluate the consistency of the neoformed bone to see if it was rigid, fibrous or mixed. The critical defects with total or partial bone neoformation over the defect area produced were evaluated as a percentage.

2.6. Processing of histological sections and microscopic analysis

Histological processing and analysis of the samples were done in the Oral Pathology Laboratory of PUCRS.

All samples, after being fixed, were decalcified using 5% HNO_3 for the required time (10 to 15 days), until the material could be cut and examined. The decalcified pieces were cut longitudinally and embedded in paraffin with the cut surface faced down and sliced with a microtome (3 semi-serial sections of each sample, 100 µm apart). The slides were stained with hematoxylin and eosin (HE).

Microscopic analysis was performed by locating the area of the critical defect with the objective of quantitatively evaluating bone neoformation. The histological images at 40x magnification were taken from the microscope by a computer and analyzed with the help of Image J software. This program was used to measure the initial size of the bone defect (5 mm standard) and later the mean smallest (d) and largest (D) distance between the margins of the neoformed bone. A mean value per specimen was calculated based on the measurements of the 3 central sections. The specimen largest distance was taken into account. We opted for this type of analysis, since repair did not occur uniformly within the defect (Figure 1).

2.7. Calibration of examiner

The researcher was trained by an experienced pathologist to standardize the analysis criteria. In the intra-examiner calibration, 20 microscopic fields were used and blinded evaluations were performed in duplicate at intervals of 7 days, without predetermined order. The analysis of pairs of measures occurred when there was concordance by the Kappa test. The examiner was considered calibrated when these measurements showed values greater than 0.7.

2.8. Statistical analysis

The analyses were performed with SPSS version 22.0. The qualitative variables were expressed as absolute frequencies, percentage and the quantitative ones as mean and standard deviation or median and interquartile range. The time-stratified Mann-Whitney test was performed to determine the p value. The graph was prepared by plotting medians. $p<0.05$ was considered statistically significant.

3. Results

The surgical procedures were performed without anesthetic intercurrence. Intraoperative complications occurred due to the high precision required to make the critical defect in an extremely fragile anatomical area. During the study, 12 animals were accidentally killed, 5 in the control group and 7 in the test group. This occurred when the drill penetrated the animal's brain.

3.1. Clinical evaluation

In both groups (test and control), there was bone neoformation. In the table below we show the percentage of animals that had defects partially or totally closed. The results obtained through this evaluation were confirmed by histological analysis (Table 1)

3.2. Histological evaluation

Two weeks after performing the procedures a slow healing in the CBD group was observed compared to the control. At this experimental time, histological analysis showed that the CBD group had less neoformed bone along the defect than the control group. The formed tissue showed inflammatory infiltrate on the margins of the defect and formation of a dense connective tissue in its most central portion.

After 4 weeks, an improvement in the healing of the CBD group was observed in relation to the control. At this time, although there was no statistically significant change, a favorable, more consistent trend was detected in the group treated with CBD.

The critical defects of some animals in different groups were completely filled with mature and organized bone tissue. However, this trend was not maintained at 6 and 8 weeks, with similar results for both groups (Figure 2).

In most animals, in both groups, the neoformed bone was surrounded by a substantial amount of osteoblasts, filling the area of the bone defect. It was possible to differentiate, in all sections, the neoformed bone in the critical defect (Figure 3).

DISCUSSION

The healing of bone defects occurs through a complex process which is regulated by a system called RANK/RANKL/OPG (Bab *et al.*, 2009; Ofek *et al.*, 2006). Although CBD acts on this system, there was no significant influence on the repair of critical defects in the animals, although it showed a beneficial effect at the experimental time of 4 weeks.

The present study suggests that the CBD negatively influenced the bone healing process at the experimental time of 2 weeks, with marked improvement at other experimental times, especially the fourth postoperative week. These data can be explained by the antioxidant and antiinflammatory effects of CBD, causing it to attenuate the inflammatory process during the healing period (Cuba *et al.*, 2017; Pan *et al*., 2009).

The effect of CBD on the healing process of bone defects has been previously described in some studies such as that of Koogan *et al.* 2015, where, through a tomographic analysis, the authors demonstrated that, in the sixth and eighth week, there was a substantial gain in defect repair compared to the control group. These findings are in agreement with the results obtained in the present study, where the CBD showed a gain in the fourth week and a balance in the eighth week.

It is known that bone cells have receptors and enzymes of the endocannabinoid system (Ofek *et al.*, 2006). The cannabinoid receptors (CB1 and CB2) are also expressed in skeletal sympathetic nerve endings, which play an important role in the bone remodeling process (Bab *et al*., 2008, 2009). Studies have reported that, based on the antiinflammatory potential of CBD, inhibition of RANKL expression, preventing binding to the receptor (RANK) and thus blocking osteoclast activity and consequently bone resorption (Ofek *et al.*, 2006; Bab *et al.*, 2008, 2009; Rettori, De Laurentiis, Zubilete, Rettori & Elverdin, 2012)

The critical defect has been widely used as a test model for bone regeneration for testing the effects of biomaterials on this process (Donos, Dereka & Mardas, 2015; Stavropoulos, Sculean, Bosshardt, Buser & Klinge, 2015). According to a recent systematic review, 5-mm diameter defects in the calvarium

of rats can be considered critical defects in these animals (Vajgel, Mardas, Farias, Petrie, Cimões & Donos, 2014). This was confirmed in our study where complete closure was not observed in any of the defects of the control group at any experimental time. Thus, the procedure performed with the 5-mm trephine drill met the criteria for making a critical defect and was also in agreement with other studies where this calvarium defect model was used in rats (Mardas, Kostopoulos & Earring, 2002; Mardas, Stavropoulos & Earring, 2008).

The present study showed that CBD did not have a significant effect on the bone healing process, but studies report that CB2 receptors are expressed in bone cells exerting an anabolic and antiresorptive action in osteoporosis. These studies report a polymorphism in the gene encoding CB2 (CNR2) as important genetic risk factors for osteoporosis. Activation of CB2 attenuates bone loss induced by ovariectomy in mice, limiting bone resorption and increasing the formation of this tissue (Bab *et al.*, 2008).

Regarding body weight, there was a decrease in the first days after the defects were made in all the experimental groups. However, there was no difference in weight loss in the groups treated with CBD compared to control, at the established experimental times.

Critical defects are invasive and painful procedures, leaving the animals prostrate in the first 2 days. Analgesic properties were reported with the use of CBD (Pisanti, Malfitano, Ciaglia, Lamberti, Ranieri, Cuomo, *et al.*, 2017). The administration resulted in an antihyperalgesic effect in a model of chronic (Costa, Trovato, Comelli, Giagnoni & Colleoni, 2007) and acute (Costa, Colleoni, Conti, Parolaro, Franke, Trovato & Giagnoni, 2004) inflammation. It was proposed that this effect was mediated by the modulation of TRPV1 (transient receptor potential vanilloid 1), also known as capsaicin receptor. In this study, no weight difference was found between the CBD and control groups. These findings also agree with the results of Jamontt, Molleman, Pertwee and Parsons (2010) who concluded that the administration of CBD for the treatment of colitis did not influence the body weight of the rats.

Corroborating with the findings described above, an experimental study with male *Wistar* rats demonstrated that the administration of CBD at doses of 2.5 and

5 mg/kg for 13 days caused a significant weight loss (Ignatowska-Jankowska, Jankowski & Swiergiel, 2011). Considering that weight loss/gain as a side effect of CBD is still controversial, a study by Iffland and Grotenhermen (2017) proposed that the influence on weight is discrete, since changes in appetite and weight results are considered multifactorial and complex (Bergamaschi, Queiroz, Zuardi & Crippa, 2011; Iffland *et al.*, 2017).

Regarding the dose of CBD used in this study, it is important to emphasize that it was chosen based on the scarce results obtained on the subject in the available literature. Previous studies in rats used CBD at IP doses ranging from 1 to 480 mg/kg (Bergamaschi *et al.* 2011). Other authors suggest that the properties of CBD have an inverted U-shaped response (Malfait, Gallil, Sumariwalla, Malik, Andreakos, Mechoulam *et al.*, 2000; Zuardi, 2008; Jamontt *et al.,* 2010). Treatment with CBD (2.5 to 10 mg/kg, IP, daily for 3 days) was used by Pan *et al.* (2009). The authors concluded that this substance caused a dose-dependent attenuation of renal injury induced by cisplatin, decreasing the expression of TNF-α and IL-1β. In the present study, CBD did not have a significant effect on the healing of bone defects.

Adverse effects were not observed in the administration of CBD, and it can be used safely at the chosen dose. In two literature reviews by Iffland *et al.* 2017 and Bergamaschi *et al.* 2011, the authors suggested that this drug has a low undesirable effects profile if given in a controlled manner. It is considered safe and well tolerated, both in animal and human models, and does not promote cellular toxicity. This reinforces the results obtained in a randomized clinical study conducted by Naftali, Mechulam, Marii, Gabay, Stein, Bronshtain, *et al.,* 2017, where 20 patients with Crohn's disease were treated with CBD at a daily dose of 20 mg for 8 weeks. The patients showed high tolerability and no adverse effects. These data encourage further studies seeking to extend our knowledge of the benefits and harmful effects of CBD.

FINAL CONSIDERATIONS

The present study demonstrated that the use of CDB for the repair of a mechanically created bone defect showed a beneficial effect only in the fourth week after treatment. No statistically significant difference was seen in the healing of bone defects at the times analyzed. These results suggested that new studies are needed to determine the influence of CBD on bone metabolism.

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REFERENCES

Amadei, SU, Silveira, VAS, Pereira, AC, Carvalho, YR & Rocha, RF (2006). The influence of estrogen deficiency on the bone remodeling and repair process. *J Bras Patol Med Lab* 42(1):5-12

Bab, I & Zimmer, A (2008). Cannabinoid receptors and the regulation of bone mass. *Brit J Pharmacol* 153:182-188

Bab, I, Zimmer, A & Melamed, E (2009). Cannabinoids and the skeleton: from marijuana to reversal of bone loss. *Ann Med* 41(8):560-567

Bergamaschi, MM, Queiroz, RHC, Zuardi, AW & Crippa, JAS (2011). Safety and side effects of cannabidiol, a Cannabis sativa constituent. *Curr Drug Saf* 6(4): 237-249 doi:10.2174/157488611798280924.

Booz, GW (2011). Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress. *Free Radic Biol Med* 51(5):1054-1061

Burkiti, HG, Young, B & John, W. *Histologia funcional.* (3 ed.) (1994) Rio de Janeiro: Guanabara Koogan p.409

Burstein, S (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg Med Chem* 23(7):1377-1385

COBEA. *Princípios Éticos de Experimentação Animal*. Sociedade Brasileira de Ciência em Animais de Laboratório (2015) - Colégio Brasileiro de Experimentação Animal.

Costa, B, Colleoni, M, Conti, S, Parolaro, D, Franke, C, Trovato, AE & Giagnoni, G (2004). Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation

in the rat paw. *Naunyn Schmiedebergs Arch Pharmacol* 369(3):294-299 doi: 10.1007/s00210-004-0871-3

Costa, B, Trovato, AE, Comelli, F, Giagnoni, G & Colleoni, M (2007) The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *Eur J Pharmacol* 556(1-3):75-83 doi:10.1016/j.ejphar.2006.11.006

Cuba, LF, Salum, FG, Cherubini, K & Figueiredo, MAZ (2017) Cannabidiol: an alternative therapeutic agent for oral mucositis?. *J Clin Pharm Ther* 42(3): 245-250

Diretrizes da Prática de Eutanásia do CONCEA. Ministério da Ciência, Tecnologia e Inovação Conselho Nacional de Controle de Experimentação Animal – CONCEA. (2015) Disponível em URL:http://www.mct.gov.br/upd_blob/ 0234/234055.pdf.

Donos, N, Dereka, X & Mardas, N (2015) Experimental models for guided bone regeneration in healthy and medically compromised conditions. *Periodontol* 2000 68: 99-121

Figueiredo JA, Pesce HF, Gioso MA & Figueiredo MA (2001) The histological effects of four endodontic sealers implanted in the oral mucosa: submucous injection versus implant in polyethylene tubes. *Int Endod J* 34(5): 377-385

Fletcher, RH & Fletcher, SW. *Epidemiologia Clínica* - Elementos Essenciais. (4 ed.) (2006) Porto Alegre: Artmed

Iffland, K & Grotenhermen, F (2017) An update on safety and side effects of Cannabidiol: A review of clinical data and relevant animal studies. *Cannabis Cannabinoid Res* 2(1):139-154. doi:10.1089/can.2016.0034

Ignatowska-Jankowska, B, Jankowski, MM & Swiergiel, AH (2011) Cannabidiol decreases body weight gain in rats: Involvement of CB2 receptors. *Neurosci Lett* 490(1):82-84. doi:10.1016/j.neulet.2010.12.031.

Jamontt, JM, Molleman, A, Pertwee, RG & Parsons, ME (2010). The effects of Delta- tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis. *Br J Pharmacol* 160 (3):712-723

Junqueira, LC & Carneiro, I. *Histologia básica*. (8 ed.) (1995) Rio de Janeiro: Guanabara Koogan p.108-126

Kogan, NM, Melamed, E, Wasserman, E, Raphael, B, Breuer, A, Stok, KS, Sondergaard, R, Escudero, AV, Baraghithy, S, Attar-Namdar, M, Friedlander-Barenboim, S, Mathavan, N, Isaksson, H, Mechoulam, R, Mueller, R, Bajayo, A, Gabet, Y & Bab, I (2015) Cannabidiol, a major non-psychotropic Cannabis constituent enhances fracture healing and stimulates lysyl hydroxylase activity in osteoblasts. *J Bone Miner Res* 30(10):1905-1913.DOI: 10.1002/jbmr.2513

Kwan, TS, Padrines, M, Théoleyre, S, Heymann, D & Fortun, Y (2004) IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev* 15:49-60

Lu, HC & Makie, K (2016) An introduction to the endogenous cannabinoid system. *Biol Psychiatry* 79(7):516-525

Malfait, AM, Gallily, R, Sumariwalla, PF, Malik, AS, Andreakos, E, Mechoulam, R & Feldmann, M (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci* 97(17):9561-9566. doi:10.1073/pnas.160105897.

Manual de cuidados e procedimentos com animais de laboratório do biotério de produção e experimentação da FCF-IQ/USP (2013). Instituto de Química da Faculdade de Ciências Farmacêuticas da USP. FCF-IQ/USP, São

Paulo. Disponível em URL:http://www.fo.usp.br/wp-content/uploads/Manual-Cuidados-com Animais.pdf.

Mardas, N, Busetti, J, de Figueiredo, JAP, Mezzomo, LA, Scarparo, RK & Donos, N (2016) Guided bone regeneration in osteoporotic conditions following treatment with zoledronic acid. *Clin Oral Impl Res* 1-10 doi:10.1111/clr.12810

Mardas, N, Kostopoulos, L & Karring, T (2002) Bone and suture regeneration in calvarial defects by e-PTFE-membranes and demineralised bone matrix and the impact on calvarial growth: an experimental study in the rat. *J Cranio-facial Surg* 13: 453-462

Mardas, N, Stavropoulos, A & Karring, T (2008) Anorganic bovine-derived hydroxyapatite matrix coupled with a synthetic cell-binding peptide (PepGen P-15) for bone regeneration. an experimental study in rats. *Clin Oral Implants Res* 19: 1010-1015

Marssel, R & Einhorn, TA (2011) The biology of fracture healing injury. *Bristol* 42(6):551-555

Mechoulam, R, Ben-Shabat, S, Hanus, L, Ligumsky, M, Kaminski, NE, Schatz, AR, Gopher, A, Almog, S, Martin, BR, Compton, R, Pertwee, RG, Griffin, G, Bayewitch, M, Barg, J & Vogel, Z (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 50: 83-90

Naftali, T, Mechoulam, R, Marii, A, Gabay, G, Stein, A, Bronshtain M, Laish, I, Benjaminov, F & Konikoff, FM (2017) Low-dose Cannabidiol is safe but not effective in the treatment for Crohn's disease, a randomized controlled trial. *Dig Dis Sci* 62(6):1615-1620 doi:10.1007/s10620-017-4540-z

Napimoga, MH, Benatti, BB, Lima, FO, Alves, PM, Campos, AC, Pena-dos-Santos, DR, Severino, FP, Cunha, FQ & Guimarães, FS (2009) Cannabidiol decreases bone resorption by inhibiting RANK/RANKL expression and pro-

inflammatory cytokines during experimental periodontitis in rats. *Int immunepharmacol* 9:216-222

Ofek, O, Karsak, M, Leclerc, N, Fogel, M, Frenkel, B, Wright, K, Tam, J, Attar-Namdar, M, Kram, V, Shohami, E, Mechoulam, R , Zimmer, A & Bab, I (2006) Peripheral cannabinoid receptor, CB2, regulates bone mass. *Proc Natl Acad Sci U.S.A.* 103(3):696-701

Pan, H, Mukhopadhyay, P, Rajesh, M, Patel, V, Mukhopadhyay, B, Gao, B, Haskó, G & Pacher, P (2009) Cannabidiol attenuates cisplatin-induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation, and cell death. *J Pharmacol Exp Ther* 328(3):708-714

Pisanti, S, Malfitano, AM, Ciaglia, E, Lamberti, A, Ranieri, R, Cuomo, G, Abate, M, Faggiana, G, Proto, MC, Fiore, D, Laezza, C & Bifulco, M (2017) Cannabidiol: State of the art and new challenges for therapeutic applications. *Pharmacol Ther* 175:133-150. doi:10.1016/j.pharmthera.2017.02.041

Putnam, SE, Scutt, AM , Bicknell, K, Priestley, CM & Williamson, EM (2007) Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytother Res* 21:99-112

Rajesh, M, Mukhopadhyay, P, Bátkai, S, Patel, V, Saito, K, Matsumoto, S, Kashiwaya, Y, Horváth, B, Mukhopadhyay, B, Becker, L, Haskó, G, Liaudet, L, Wink, DA, Veves, A, Mechoulam, R & Pacher, P (2010) Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. *J Am Coll Cardiol* 56(25): 2115-2125

Rettori, E, De Laurentiis, A, Zubilete, MZ, Rettori, V & Elverdin, JC (2012) Anti-inflammatory effect of the endocannabinoid anandamide in experimental periodontitis and stress in the rat. *Neuroimmunomodulat* 19:293-303

Russo, EB (2016) Beyond Cannabis: Plants and the Endocannabinoid System. *Trends Pharmacol Sci* 37(7):594-605

Spicer, PP, Kretlow, JD, Young, S, Jansen, SY, Kasper, FK & Mikos, AG (2012) Evaluation of bone regeneration using the rat critical size calvarial defect. *Nat Protoc* 7(10):1918-1929.

Stavropoulos, A, Sculean, A, Bosshardt, D, Buser, D & Klinge, B (2015) Pre-clinical in vivo models for the screening of bone biomaterials for oral/ craniofacial indications: focus on small-animal models. *Periodontol* 68: 55-65

Vajgel, A, Mardas, N, Farias, BC, Petrie, A, Cimões, R & Donos, N (2014) A systematic review on the critical size defect model. *Clin Oral Implants Res* 25: 879-893

Yang, L, Rozenfeld, R, Wu, D, Devi, LA, Zhang, Z & Cederbaum, A (2014) Cannabidiol protects liver from binge alcohol-induced steatosis by mechanisms including inhibition of oxidative stress and increase in autophagy. *Free Radic Biol Med* 68:260-267

Zhang, J, Zhou, H, Yang, K, Yuan, Y & Liu, C (2013) RhBMP-2-loaded calcium silicate/calcium phosphate cement scaffold with hierarchically porous structure for enhanced bone tissue regeneration. *Biomaterials* 34:9381-9392

Zuardi, AW (2008) Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action. *Rev Bras Psiquiatr* 30(3):271-280. doi:10.1590/S1516-44462008000300015.

FIGURE LEGENDS

FIGURE 1 - Photomicrografy ilustrating the measurement was made of the largest and smallest distance between the margins of the neorformed bone in the critical defect. The formula (D+d)/2 was used to measure the formed bone

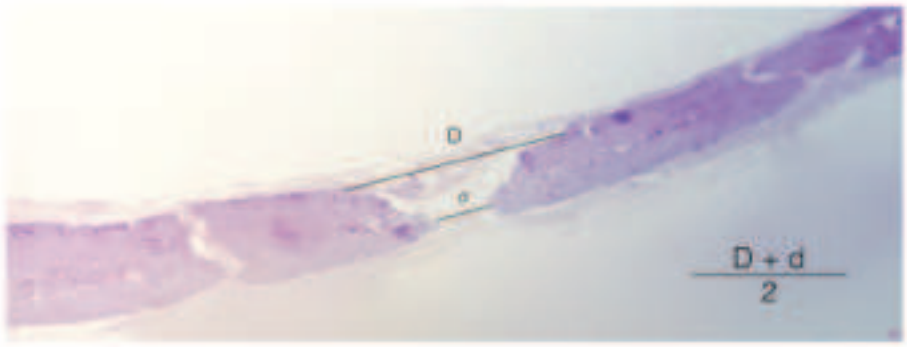


TABLE 1 - Clinical analysis in percentages in the test (T) and control (C) groups at different experimental times

Time (weeks) / Repair %	2	4	6	8
Parcial bone defect closure	T - 100 % (n = 6) C - 88.33 % (n = 6)	T - 57.14 % (n = 7) C - 88.33 % (n = 6)	T - 57.14 % (n = 6) C - 88.33 % (n = 7)	T and C - 100% (n = 6 for both groups)
Total bone defect closure	C - 11.67% (n = 6)	T - 42.86 % (n = 7) C - 11.67% (n=6)	T - 42.86% (n = 6) C - 11.67 % (n = 7)	——

FIGURE 2 - The effect of cannabidiol on the healing process of critical defects according to the times of 2, 4, 6, and 8 weeks post-surgery (40X).

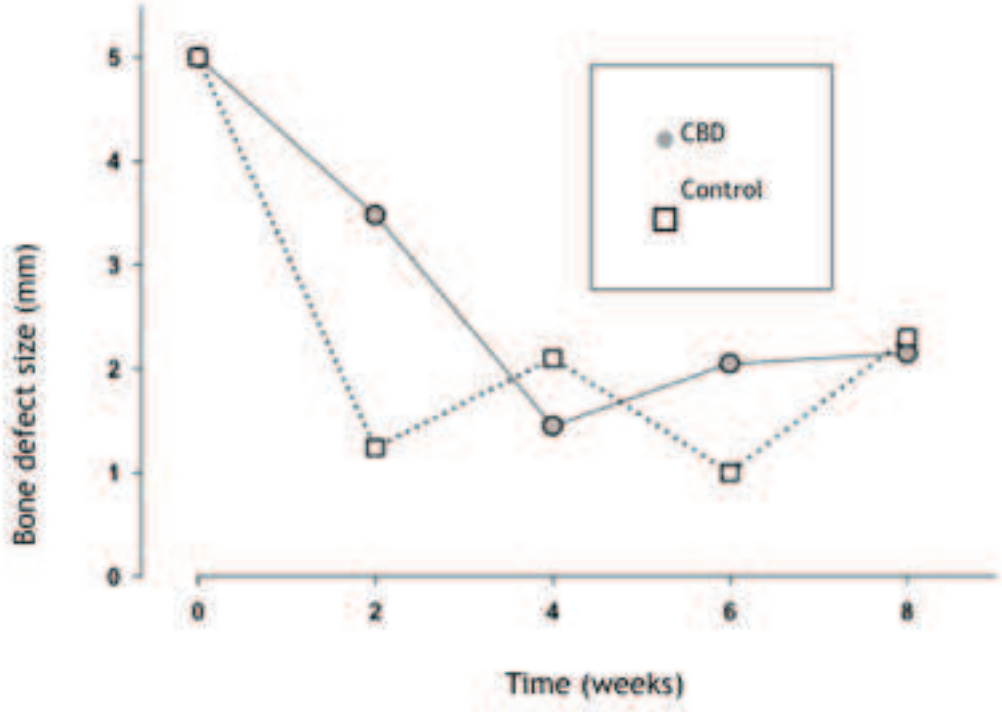
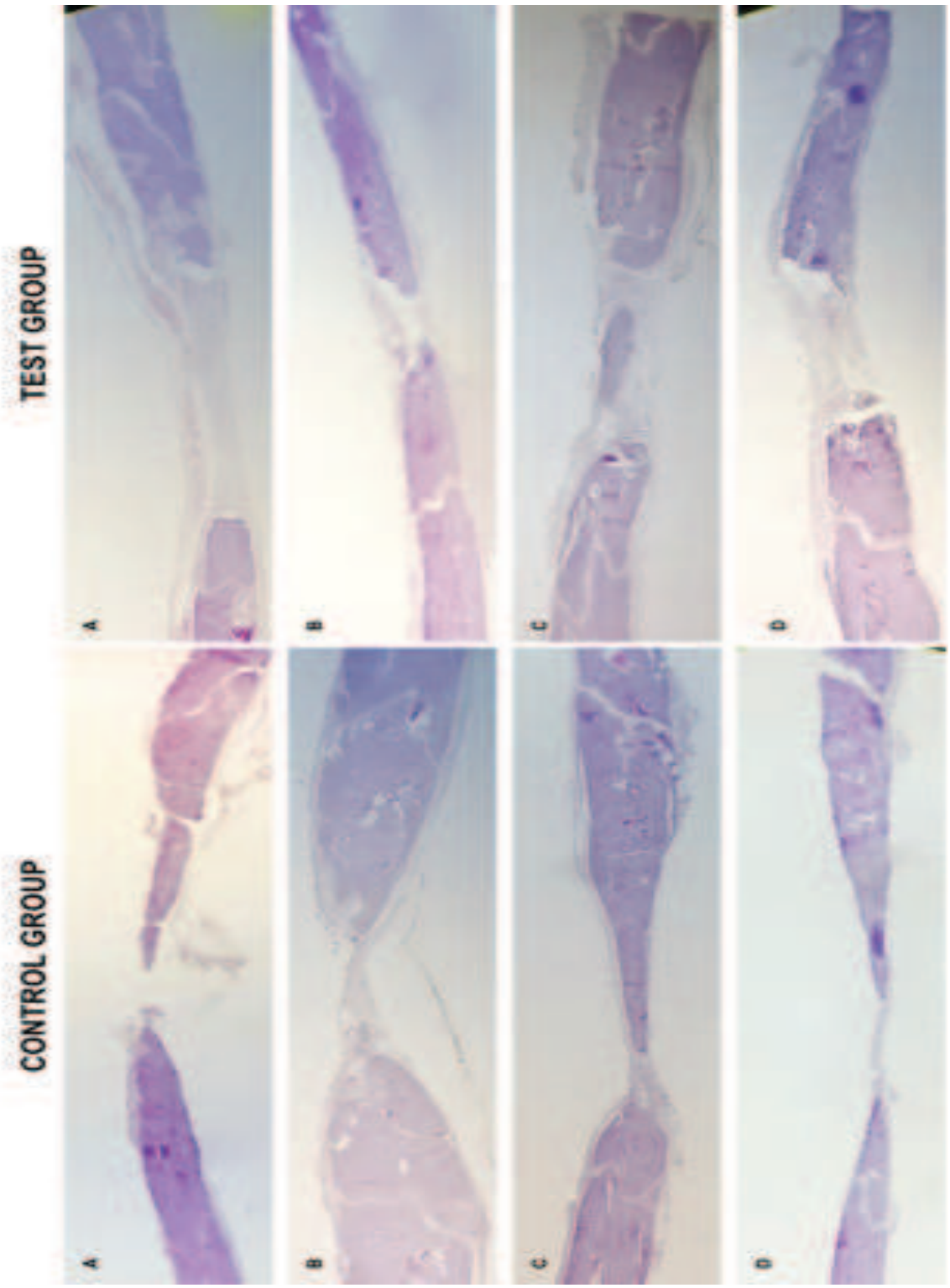


FIGURE 3 - Histological image of the neoformed bone at 4 experimental times: A) 2 weeks; B) 4 weeks; C) 6 weeks; D) 8 weeks. Staining (HE) and magnification.



5. DISCUSSÃO COMPLEMENTAR

A utilização da maconha para fins medicinais tem sido um assunto muito polêmico no mundo inteiro acarretando inúmeras discussões. No Brasil, o primeiro medicamento a base de *Cannabis sativa* registrado foi o Mevatyl, conhecido mundialmente como Sativex. A administração deste produto se dá sob a forma de *spray* e contém em sua formulação os fitocanabinóides THC e CBD. Este medicamento é utilizado para o tratamento de algumas doenças como por exemplo, a esclerose múltipla, naqueles pacientes que não respondem de forma favorável a terapia convencional (ANVISA, 2017a).

Em 2015 a utilização desses produtos era autorizada exclusivamente sob prescrição profissional por médico habilitado, além de uma criteriosa avaliação e aprovação da Agência Nacional de Vigilância Sanitária. Em 2016, essas mesmas substâncias foram excluídas da lista de compostos proibidos e passaram a ser incluídos como medicamentos psicotrópicos sujeitos a controle especial (ANVISA, 2016).

Na década de 60 o cultivo da planta foi proibido pela Organização das Nações Unidas (ONU), exceto para fins médicos e científicos, através da convenção de Substâncias Entorpecentes. A utilização desses compostos também foi regulamentada nos anos de 1971 e 1988 nas convenções da ONU sobre Substâncias Psicotrópicas e Contra o Tráfico Ilícito de Entorpecentes. (ANVISA, 2017b). Entretanto, diversos estudos desenvolvidos, evidenciando os efeitos benéficos da maconha no tratamento de inúmeras doenças, pressionaram os órgãos públicos mundiais para a sua liberação com finalidades medicinais (Pisanti et al.,2017).

Devido ao insucesso no tratamento convencional de doenças incuráveis, várias famílias ao redor do mundo passaram a testar e utilizar de forma clandestina os efeitos dos medicamentos a base de CBD e THC. A falta de controle no uso e processamento dessas substâncias é considerada perigosa e prejudicial aos pacientes, por desconhecerem a pureza do produto gerado, bem como a dosagem adequada para cada situação (Sabóia, 2017). Esse fato tornou inadiável a regulamentação dos compostos para fins terapêuticos, visando avaliar cientificamente a segurança, qualidade e eficácia desses medicamentos.

Apesar do crescimento no número de estudos na área médica e odontológica, ainda pode-se considerar tímida a discussão em torno dos

benefícios terapêuticos da droga (Napimoga et al., 2009; Cuba et al.,2017). O presente estudo visa um maior conhecimento da área na odontologia nesse debate. Mesmo com os entraves burocráticos existentes no país, é expressiva a participação de pesquisadores brasileiros nos estudos científicos com os canabinóides. Os investimentos científicos nesta temática contemplam inclusive a criação de um Centro de Pesquisa em Canabinóides vinculado à Faculdade de Medicina de Ribeirão Preto (Stella, 2017).

O projeto executado nesta dissertação apresenta alguns resultados e tópicos passíveis de serem discutidos. O desenho do estudo assim como o procedimento empregado, foram realizados de acordo com os achados da literatura atual (Mardas, Busetti, de Figueiredo, et al., 2016).

A diluição do CBD com *Tween* 80 e a solução salina foi utilizada em diversos estudos prévios (Napimoga et al.,2009; Vuolo, Petronilho, Sonai, et al., 2015). O processo de diluição do CBD é bastante delicado, uma vez que esta substância apresenta-se na forma de um pó de baixa solubilidade aquosa. Para evitar formação de grânulos, que poderiam obstruir o lúmen da seringa durante a administração intraperitoneal, incorporou-se lentamente o pó ao líquido, com posterior auxílio de uma cuba ultrassônica (Apêndice 3).

A via de administração intraperitoneal (IP) é a mais comumente utilizada e citada na literatura para avaliação do efeito do CBD em roedores. Ocorre devido a conveniência do seu uso e das características da substância (Iffland, et al., 2017). Optou-se então, pela consagrada técnica de administração via IP afim de facilitar a análise dos resultados e comparar aos achados já existentes.

Estudos que avaliam o processo cicatricial ósseo com frequência utilizam ratos, por apresentarem este tecido estruturalmente semelhante ao dos humanos bem como uma fisiologia óssea similar. Outras vantagens incluem o baixo custo e a facilidade na manipulação desses animais. Os ratos machos são preferidos nos experimentos, possivelmente pelo preço mais acessível, além da menor influência de alterações hormonais o que, nas fêmeas, poderia interferir na cicatrização de lesões com perda de integridade epitelial (Peplow; Chung; Baxter, 2010).

No presente estudo optou-se por realizar o procedimento em calvária de ratos visto que este método é amplamente difundido na literatura (Mardas et al., 2002, 2008; Stravopoulos, Sculean, Bosshardt et al., 2015). Outras localizações também foram utilizadas conforme descrito no estudo de Kogan e colaboradores

em 2015, que realizaram fratura em fêmur de ratos. De acordo com uma revisão sistemática recente, os defeitos em calvária com diâmetro de 5 mm podem ser considerados como defeitos críticos em ratos (Vajgel, Mardas, Farias et al., 2014). Isso foi confirmado em nosso estudo onde não observou-se o fechamento completo dos defeitos não tratados (grupo controle), nos respectivos tempos de cicatrização analisados. Isto indica que o defeito parietal com a broca trefina de 5 mm, preencheu os critérios necessários para a formação de um defeito crítico, estando em concordância com estudos anteriores usando defeitos similares na calvária de ratos (Mardas, Kostopoulos, Karring et al., 2002: Mardas, Stavropoulos, Karring, 2008; Donos, Dereka, Mardas et al., 2015). A broca utilizada para confecção dos defeitos garantiu a padronização dos mesmos. O manejo delicado da técnica cirúrgica utilizada neste estudo não impediu que, acidentalmente, fosse transfixada na calota craniana de alguns animais, os quais, foram excluídos do estudo e eutanasiados para evitar o distresse e sofrimento.

O tamanho dos defeitos, bem como os tempos experimentais para avaliar a cicatrização óssea, são amplamente variáveis na literatura. Os resultados obtidos nos 4 tempos experimentais do presente estudo (duas, 4, 6 e 8 semanas) justifica-se por pesquisas previamente desenvolvidas como a de Mardas, Busetti, de Figueiredo et al. em 2017, onde os autores utilizaram os tempos de 30 e 60 dias para observar a cicatrização óssea de defeitos críticos tratados com biomateriais. Kogan et al. em 2015 utilizou os mesmos tempos experimentais utilizados neste estudo para avaliar o reparo ósseo em fratura de fêmur de ratos, visto que as alterações neste processo cicatricial são significativamente identificadas nesses intervalos de tempo.

A avaliação clínica do reparo ósseo foi realizada através da presença ou não do calo ósseo e/ou osso neoformado, bem como a consistência do mesmo. Para uma melhor análise optou-se por realizar mensurações do osso recém formado através do uso de um *software* denominado *Image J* como utilizado no estudo de Mardas et al. em 2017. Realizou-se o cálculo de porcentagem de cicatrização o que permitiu calcular a média entre o tamanho total do defeito e da quantidade de osso neoformado.

De acordo com o que foi exposto nesta dissertação, acredita-se que esta pesquisa possa subsidiar estudos que envolvam e favoreçam o uso do CBD em diferentes temáticas da área médica e odontológica.

REFERÊNCIAS

Amadei SU, Silveira VAS, Pereira AC, Carvalho YR, Rocha RF (2006) J Bras Patol Med Lab. Rio de Janeiro: 42(1):5-12.

ANVISA. Canabidiol e THC: norma permitirá registro de produto. 2016. Disponível em: <http://portal.anvisa.gov.br/noticias/-/asset_publisher/FXrpx9qY7FbU/content/canabidiol-e-thc-norma-permitira-registro-de- produto/219201>.

ANVISA. Esclarecimentos a respeito do registro do medicamento Mevatyl. 2017ª. Nota Técnica no 01/2017/GMESP/GGMED/ANVISA. p. 1-4.

ANVISA. Maconha: Anvisa não é contra uso para fins medicinais. 2017b. Disponível em: <http://portal.anvisa.gov.br/noticias/-/asset_publisher/FXrpx9qY7FbU/content/anvisa-nao-e-contra-uso-para-fins- medicinais/219201>.

Bab I and A. Zimmer (2008) Cannabinoid receptors and the regulation of bone mass. Brit J Pharmacol. 153:182-188

Bab I and A. Zimmer, E. Melamed (2009) Cannabinoids and the skeleton: from marijuana to reversal of bone loss. Ann Med. 41(8):560-567

Baron EP (2015) Comprehensive review of medicinal marijuana, cannabinoids, and therapeutic implications in medicine and headache: what a long strange trip it's been. Headache 55:885-916

Bergamaschi MM, Queiroz RHC, Zuardi AW, Crippa JAS (2011). Safety and side effects of cannabidiol, a Cannabis sativa constituent. Curr Drug Saf 6(4):237-249. doi:10.2174/157488611798280924

Booz GW (2011) Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress. Free Radic Biol Med 51(5):1054-1061.

Burkiti HG, Young B, John W. Histologia funcional. (3 ed.) (1994) Rio de Janeiro: Guanabara Koogan p.409.

Burstein S (2015) Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. Bioorg Med Chem 23(7):1377-1385.

Cassol-Jr OJ, Comim CM, Silva BR, Hermani FV, Constantino LS, Felisberto F, et al., (2010) Treatment with cannabidiol reverses oxidative stress parameters, cognitive impairment and mortality in rats submitted to sepsis by cecal ligation and punctura, Brain Res 1348:128-138

COBEA. Princípios Éticos de Experimentação Animal. Sociedade Brasileira de Ciência em Animais de laboratório - Colégio Brasileiro de Experimentação Animal 2015.

Costa B, Colleoni M, Conti S, Parolaro D, Franke C, Trovato AE, et al., (2004) Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw. Naunyn Schmiedebergs Arch Pharmacol 369(3):294-299. doi:10.1007/s00210-004-0871-3.

Costa B, Trovato AE, Comelli F, Giagnoni G, Colleoni M (2007) The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. Eur J Pharmacol 556(1-3):75-83. doi:10.1016/j.ejphar.2006.11.006.

Cuba LF, Salum FG, Cherubini K, Figueiredo MAZ (2017) Cannabidiol: an alternative therapeutic agent for oral mucositis?. J Clin Pharm Ther 42(3):245-250

Diretrizes da Prática de Eutanásia do CONCEA. Ministério da Ciência, Tecnologia e Inovação Conselho Nacional de Controle de Experimentação Animal – CONCEA. (2015) Disponível em URL:http://www.mct.gov.br/upd_blob/0234/234055.pdf.

Donos N, Dereka X, Mardas N (2015) Experimental models for guided bone regeneration in healthy and medically compromised conditions. Periodontol 2000 68: 99-121.

Elbaz M, Nasser MW, Ravi J, Wani NA, Ahirwar DK, Zhao H, et al., (2015) Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: novel anti-tumor mechanisms of cannabidiol in breast cancer. Mol Oncol 9(4): 906-919

Fasinu PS, Phillips S, Elsohly MA, Walker LA (2016) Current status and prospects for cannabidiol preparations as new therapeutic agents. JACCP 36(7):781-796

Figueiredo JA, Pesce HF, Gioso MA, Figueiredo MA (2001). The histological effects of four endodontic sealers implanted in the oral mucosa: submucous injection versus implant in polyethylene tubes. Int Endod J 34(5):377-385

Fletcher RH, Fletcher SW. Epidemiologia Clínica - Elementos Essenciais. (4 ed.) (2006) Porto Alegre: Artmed

Fundação Oswaldo Cruz – Fiocruz. Manual de utilização de animais. Fiocruz Rio de Janeiro, 2008. Disponível em: http://www.bioterio.net/Files/Manual%20de%20utilizacao%20de%20animais_FIOCRUZ.pdf > Acesso em: 03/07/2016.

Hill PA, Orth M (1998) Bone remodeling. Br J Orthod 25 (2):101-107

Hollinger J, Wong MEK (1996) The integrated processes of hard tissue regeneration with special emphasis on fracture healing. Oral Surg Oral Med Oral Pathol 82(6): 594-606

Iannotti, FA, Di Marzo V, Petrosino S (2016) Endocannabinoids and endocannabinoid-related mediators: Targets, metabolism and role in neurological disorders. Prog Lipid Res 62:107-128

Ibeas BC, Chen T, Nunn AV, Bazelot M, Dallas M, Whalley BJ (2015) Molecular targets of cannabidiol in neurological discords. Neurotherapeutics 12:699-730

Iffland K, Grotenhermen F (2017). An update on safety and side effects of Cannabidiol: A review of clinical data and relevant animal studies. Cannabis Cannabinoid Res 2(1):139-154. doi:10.1089/can.2016.0034.

Ignatowska-Jankowska B, Jankowski MM, Swiergiel AH (2011) Cannabidiol decreases body weight gain in rats: Involvement of CB2 receptors. Neurosci Lett 490(1):82-84. doi:10.1016/j.neulet.2010.12.031.

Jamontt JM, Molleman A, Pertwee RG, Parsons ME (2010) The effects of Delta-tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis. Br J Pharmacol. 160 (3): 712-723

Junqueira LC, Carneiro I. Histologia básica. (8 ed.) (1995) Rio de Janeiro: Guanabara Koogan p.108-26.

Katchan V, David P, Shoenfeld Y (2016) Cannabinoids and autoimmune diseases: A systematic review. Autoimmun Rev 15(6)513-528

Kogan NM, Melamed E, Wasserman E, Raphael B, Breuer A, Stok KS, et al., (2015) Cannabidiol, a major non-psychotropic Cannabis constituent enhances fracture healing and stimulates lysyl hydroxylase activity in osteoblasts. J Bone Miner Res. 30(10):1905-1913.DOI: 10.1002/jbmr.2513

Kwan TS, Padrines M, Théoleyre S, Heymann D, Fortun Y (2004) IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev 15:49-60.

Lee K, Weir MD, Lippens E, Mehta M, Wang P, Duda GN, et al., (2014) Bone regeneration via novel macroporous CPC scaffolds in critical-sized cranial defects in rats. Dent Mater 30:199-207.

Lu, HC, Mackie, K (2016). An introduction to the endogenous cannabinoid system. Biol Psychiatry. 79(7):516-525

Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreakos E, Mechoulam R, et al. (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen- induced arthritis. Proc Natl Acad Sci U S A. 97(17): 9561-9566. doi:10.1073/pnas.160105897.

Manual de cuidados e procedimentos com animais de laboratório do biotério de produção e experimentação da FCF-IQ/USP. Instituto de Química da Faculdade de Ciências Farmacêuticas da USP. FCF-IQ/USP, São Paulo; 2013. Disponível em URL:http://www.fo.usp.br/wp-content/uploads/Manual-Cuidados-com Animais.pdf.

Mardas N, Busetti J, de Figueiredo JAP, Mezzomo LA, Scarparo RK, Donos N (2017) Guided bone regeneration in osteoporotic conditions following treatment with zoledronic acid. Clin Oral Impl 1-10 doi:10.1111/clr.12810

Mardas, N., Kostopoulos, L, Karring, T. (2002) Bone and suture regeneration in calvarial defects by e-PTFE-membranes and demineralised bone matrix and the impact on calvarial growth: an experimental study in the rat. J Craniofac Surg 13: 453-462.

Mardas, N, Stavropoulos A, Karring T (2008) Anorganic bovine-derived hydroxyapatite matrix coupled with a synthetic cell-binding peptide (PepGen P-15) for bone regeneration. an experi- mental study in rats. Clin Oral Implants 19:1010-1015.

Marssel R & Einhorn TA (2011) The biology of fracture healing injury. Bristol 42(6): 551-555.

Mcpartland JM, Duncan M, Di Marzo V, Pertwee RG (2015) Are cannabidiol and Δ9-tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. Br J Pharmacol 172(3)737-753

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, et al., (1995) Identification of an endogenous 2- monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50: 83-90.

Naftali T, Mechulam R, Marii A, Gabay G, Stein A, Bronshtain M (2017) Low-dose Cannabidiol is safe but not effective in the treatment for Crohn's disease, a randomized controlled trial. Dig Dis Sci 62(6):1615-1620. doi:10.1007/s10620-017-4540-z.

Napimoga MH, Benatti BB, Lima FO, Alves PM, Campos AC, Pena-dos-Santos DR, et al., (2009). Cannabidiol decreases bone resorption by inhibiting RANK/RANKL expression and pro-inflammatory cytokines during experimental periodontitis in rats. Int immunepharmacol 9:216-222.

Netzahualcoyotzi-Piedra C, Muñoz-Arenas G, García IM , Garduño BF, de León DLP (2009) La marihuana y el sistema endocanabinoide: De sus efectos recreativos a la terapéutica. Rev Biomed 20:128-153

Nogueira-Filho GR, Cadide T, Rosa BT, Neiva TG, Tunes R, Peruzzo D, et al., (2008) Cannabis sativa smoke inhalation decreases bone filling around titanium implants: a histomorphometric study in rat. Implant Dent 17(4):461-470

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, et al., (2006). Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc Natl Acad Sci U.S.A. 103(3): 696-701.

Pan H, Mukhopadhyay P, Rajesh M, Patel V, Mukhopadhyay B, Gao B, et al., (2009) Cannabidiol attenuates cisplatin-induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation, and cell death. J Pharmacol Exp Ther 328(3): 708-714.

Peplow PV, Chung T, Baxter GD (2010) Laser photobiomodulation of wound healing: a review of experimental studies in mouse and rat animal models. Lasers Surg Med 28(3)291-325

Pisanti S, Malfitano AM, Ciaglia E, Lamberti A, Ranieri R, Cuomo G, et al., (2017). Cannabidiol: State of the art and new challenges for therapeutic applications. Pharmacol Ther. 175:133-150. doi:10.1016/j.pharmthera.2017.02.041.

Putnam SE, Scutt AM , Bicknell K, Priestley CM, Williamson EM (2007) Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytother Res 21:99-112

Rajesh M, Mukhopadhyay P, Bátkai S, Patel V, Saito K, Matsumoto S, et al., (2010) Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. J Am Coll Cardiol 56(25):2115-2125.

Rettori E, De Laurentiis A, Zubilete MZ, Rettori V, Elverdin JC (2012) Anti-inflammatory effect of the endocannabinoid anandamide in experimental periodontitis and stress in the rat. Neuroimmunomodulat 19:293-303.

Russo, EB (2016). Beyond Cannabis: Plants and the Endocannabinoid System. Trends Pharmacol Sci 37(7):594-605

Sabóia, G. Famílias criam redes clandestinas de distribuição de remédios à base de cannabis. 2017. Disponível em: <http://cbn.globoradio.globo.com/especiais/ maconha-alem-do- tabu/2017/10/09/FAMILIAS-CRIAM-REDES-CLANDESTINAS-DE-DISTRIBUICAO-DE- REMEDIOS-A-BASE-DE-CANNABIS.htm>

Schenk RK, Bone regeneration: biologic basis. In: D. Buser, C. DahlIn, R.K. Scherk, Guided bone regeneration in implant dentistry. Quintessense Books, Chicago 3 (1994), pp.49-100

Spicer PP, Kretlow JD, Young S, Jansen SY, Kasper FK, Mikos AG (2012). Evaluation of bone regeneration using the rat critical size calvarial defect. Nat Protoc 7(10): 1918-1929.

Stavropoulos A, Sculean A, Bosshardt D, Buser D, Klinge B (2015) Pre-clinical in vivo models for the screening of bone biomaterials for oral/craniofacial indications: focus on small-animal models. Periodontol 68:55-65.

Stella R. USP Ribeirão terá centro de pesquisa em canabidiol - Universidade de São Paulo - Serviço de Comunicação Social - Campus Ribeirão Preto. 2017. Disponível em: <http://ribeirao.usp.br/?p=11169>.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345-357

Tavares CO, Bottcher DE, Assmann E, Kopper PMP, Figueiredo JAP, Grecca FS, et al., (2013) Tissue reactions to a new mineral trioxide aggregate-containing endodontic sealer. J Endod 39(5):653-657.

Trowbridge HO, Emling RC. Regeneração e cicatrização. In: Inflamação: uma revisão do processo. (7 ed.) (1996) Quintessence p.146-147

Vajgel A, Mardas A, Farias N, Petrie BC, Cimões A, Donos RN (2014) A systematic review on the critical size defect model. Clin Oral Implants Res 25: 879-893.

Vuolo, F, Petronilho F, Sonai B, Ritter C, Hallak JE, Zuardi AW, et al., (2015) Evaluation of serum cytokines levels and the role of cannabidiol treatment in animal model of asthma. Mediators of Inflammation 2015:1-5

Yang L, Rozenfeld R, Wu D, Devi LA, Zhang Z, Cederbaum A (2014) Cannabidiol protects liver from binge alcohol-induced steatosis by mechanisms including inhibition of oxidative stress and increase in autophagy. Free Radic Biol Med 68:260-267

Zhang J, Zhou H, Yang K, Yuan Y, Liu C (2013) RhBMP-2-loaded calcium silicate/ calcium phosphate cement scaffold with hierarchically porous structure for enhanced bone tissue regeneration. Biomaterials 34:9381-9392.

Zou D, Zhang Z, He J, Zhu S, Wang S, Zhang W, et al., (2011) Repairing critical-sized calvarial defects with BMSCs modified by a constitutively active form of

hypoxia-inducible factor-1α and a phosphate cement scaffold. Biomaterials 32:9707-9718.

Zuardi AW (2008) Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action. Rev Bras Psiquiatr 30(3):271-280. doi:10.1590/S1516-44462008000300015.

Zuardi AW, Shirakawa I, Finkelfarb E, Karniol IG (1982) Action of cannabidiol on the anxiety and other effects produced by delta-9-THC in normal subjects. Psychopharmacology 76:245-250.

ANEXO 1 - Aprovação do Projeto de Pesquisa pela Comissão Científica da Faculdade de Odontologia da PUCRS

De: Sistema de Pesquisas - SIPESQ <noreply@pucrs.br>
Enviado: quarta-feira, 25 de janeiro de 2017 05:00
Para: Maria Antonia Z de Figueiredo
Assunto: [SIPESQ] Resultado da Análise do Projeto

Esta mensagem foi emitida automaticamente pelo SIPESQ - Sistema de Pesquisas da PUC
Prezado(a) Coordenador(a) de Projeto de Pesquisa,
A CEUA considerou que o projeto **7694 - EFEITO DO CANABIDIOL NO PROCESSO CICATRICIAL DE DEFEITO ÓSSEO CRÍTICO INDUZIDO MECANICAMENTE EM CALOTA CRANIANA DE RATOS: AVALIAÇÃO CLÍNICA E HISTOLÓGICA** atende aos requisitos por ela definidos.
Desta forma, o projeto já pode ser iniciado.

Atenciosamente,
Pró-Reitoria de Pesquisa, Inovação e Desenvolvimento

Apêndice 1 - Ficha de avaliação clínica

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA/FO-PUCRS
ÁREA DE CONCENTRAÇÃO EM ESTOMATOLOGIA CLÍNICA
EFEITO DO CANABIDIOL NO PROCESSO CICATRICIAL DE DEFEITOS ÓSSEOS CRÍTICOS MECANICAMENTE INDUZIDOS EM CALOTA CRANIANA DE RATOS: AVALIAÇÃO CLÍNICA E HISTOLÓGICA
FICHA DE AVALIAÇÃO CLÍNICA

IDENTIFICAÇÃO

Rato nº: _____ **Peso inicial:** _____Kg **Peso final:** _____Kg

Tratamento: **Tempo:**

[ ] Defeito + CBD + *Tween* 80 – 2% [ ] 2 semanas [ ] 6 semanas

[ ] Defeito + Soro + *Tween* 80 – 2% [ ] 4 semanas [ ] 8 semanas

AVALIAÇÃO CLÍNICA LOCAL

Sinais inflamatórios:

[ ] Eritema [ ] Edema [Nenhum]

Áreas de supuração:

[ ] Sim [ ] Não Localização:_____

Presença de calo ósseo:

[ ] Sim [ ] Não

Consistência:_____

Sinais secundários:

[ ] Sim Quais?:_____ [ ] Não

Outras informações:
_____
_____
_____
_____

Fotos: _____

Data da avaliação:___/___/____

Apêndice 2 - Ficha de avaliação histológica

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO EM ESTOMATOLOGIA CLÍNICA
EFEITO DO CANABIDIOL NO PROCESSO CICATRICIAL DE DEFEITOS ÓSSEOS CRÍTICOS MECANICAMENTE INDUZIDOS EM CALOTA CRANIANA DE RATOS: AVALIAÇÃO CLÍNICA E HISTOLÓGICA
FICHA DE AVALIAÇÃO HISTOLÓGICA

IDENTIFICAÇÃO

Rato nº: _____ Lâmina nº:_____

Tratamento:

[ ] Defeito + CBD + *Tween* 80

[ ] Defeito + Soro + *Tween* 80 – 2%

Tempo:

[ ] 2 semanas [ ] 6 semanas

[ ] 4 semanas [ ] 8 semanas

Escore:

[ ] - / - : Ausência de osso neoformado, sem fechamento do defeito
[ ] + / - : Pouco osso neoformado, sem fechamento do defeito
[ ] ++ / - : Neoformação óssea significativa, sem fechamento do defeito
[ ] +++ / + : Neoformação óssea significativa, defeito fechado
[ ] +++ / + : Neoformação óssea fora da área do defeito, fechamento do defeito

Observações:_____

Fotos:_____

Data da avaliação:__/__/____

Apêndice 3 - Manipulação das soluções utilizadas no experimento

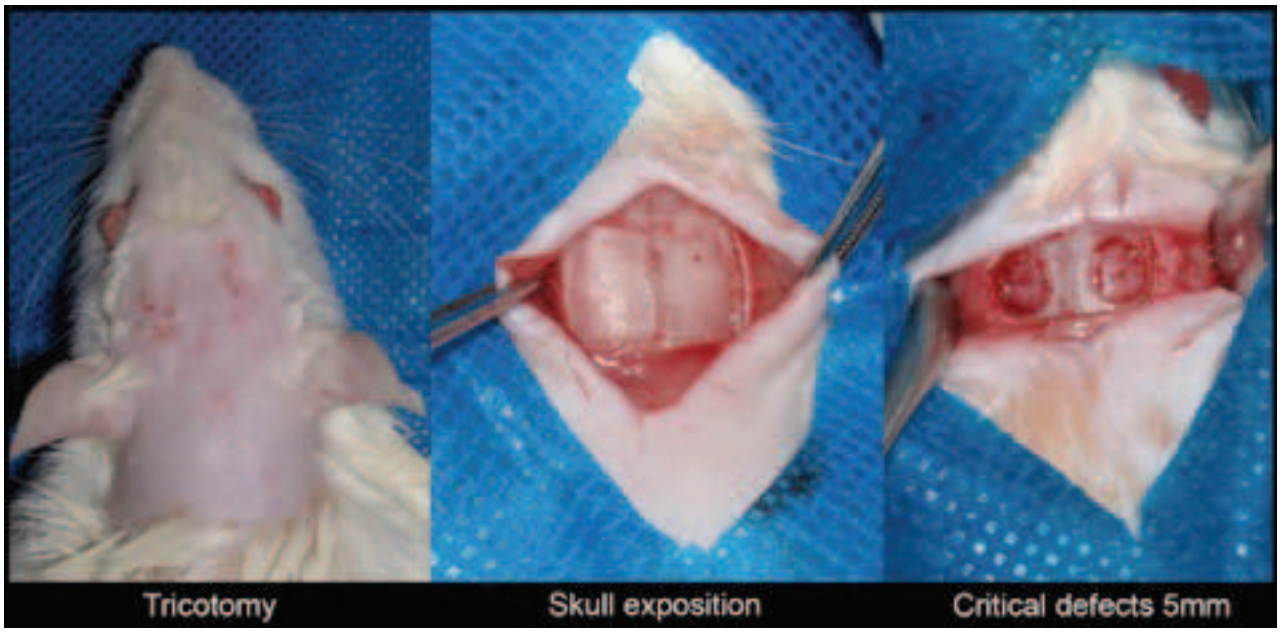
4. As soluções foram preparadas imediatamente antes da injeção IP nos animais, no volume de 1 mg/kg.

5. Após a incorporação das substâncias o produto final foi levado a uma incubadora ultra-sônica para dissolução de grânulos insolúveis.

6. Aspecto final da solução de CBD após seu preparo.

7. As soluções foram protegidas da luz solar e ambiental até o momento exato de sua administração.

Apêndice 4 - Sequência do procedimento cirúrgico executado em calota craniana de ratos



Broca trefina de 5 mm de diâmetro usada para realização dos defeitos ósseos