J Oral Res
oral and craniofacial sciences

Article

Osteogenic potential of different chalcones in an *in vivo* model: A preliminary study.

Xana Raquel Ortolan,[1] Telmo José Mezadri,[1]
David Rivero Tames,[1]Rogério Corrêa[2] & Fátima Campos-Buzzi.[2]

Affiliations: [1]Laboratory of Histology, University of Vale do Itajaí (UNIVALI), Itajaí, Santa Catarina, Brazil. [2]Department of Chemistry, Chemical-Pharmaceutical Investigations Center (NIQFAR)/CCS, University of Vale do Itajaí (UNIVALI), Itajaí, Santa Catarina, Brazil.

Corresponding author: Telmo Mezadri. Universidade do Vale do Itajaí, Rua Uruguai, 458, Bloco 27, CEP: 88302-202. Phone: (55-47) 33417901. E-mail: t.mezadri@univali.br

Receipt: 07/17/2017 **Revised:** 08/11/2017
Acceptance: 08/24/2017 **Online:** 08/24/2017

Abstract: Aim. To evaluate the osteogenic potential of chalcones using the rat critical size calvarial defect. Methods. The chalcones were synthesized from acetophenone following the Claisen-Schmidt aldol condensation method by varying the substituted benzaldehydes (3,4-Cl; 4-Cl; 4-CH_3; 4-OCH_3, H). The five chalcone molecules were evaluated in three concentrations (1%, 5% and 10%) in comparison to control and vehicle (Vaseline) groups. The results of the remaining wound areas were calculated statistically by the ANOVA method followed by the Student-Newman-Keuls test and the histological sections were analyzed qualitatively by light microscopy. Results. All molecules at 10% concentration showed significant bone closure compared to the control, vehicle and chalcone groups at 1% concentration (p<0.01). Active osteoblasts were observed on the repair surfaces in all groups treated with chalcones. Treatment with the C5 molecule at concentration of a 10% resulted in greater bone neoformation compared to the other molecules, with features of secondary bone observed. Conclusion. The chalcones evidenced a dose-dependent osteogenic potential and C5 was more effective in bone repair.

Keywords: Chalcone; osteogenesis; organic synthesis.

INTRODUCTION.

Bones are tough and rigid structures that, despite their inert appearance, grow, remold, and remain active throughout the life of organisms. When bone is injured, as occurs in fractures, they are capable of repair, a phenomenon that demonstrates their permanent vitality. At normal conditions, most of the fractures do not present problems of consolidation, however there are situations in which the repair process may be accelerated, ensuring a faster return of musculoskeletal function.[1] Despite the great regenerative potential of bone tissue, in some cases the wound can be filled by fibrous connective tissue and the repair may be inadequate.[2,3]

Bone reconstructions are frequent in the routine of oral and maxillofacial surgeries, and may be indicated in face traumas, pathologies and orthognathic surgeries.[4] Therefore, some mechanisms used in the treatment of bone defects correspond to autologous, allogeneic or xenotransplantation transplants. However, these methods require strict control associated to the transmission of infectious agents.[5,6]

Further, within a variety of bioactive factors, bone morphogenetic proteins (BMPs) have been widely studied and show satisfactory results regarding osteogenesis in tissue engineering areas. However, these proteins isolated from allogeneic or xenogenic bone have associated health risks,

Conflict of interests: None.

Ethics approval: The present study was approved by the Ethical Committee on the Use of Animals of University of Vale do Itajaí (UNIVALI) (041/2011).

Funding: The present study is supported by the University of Vale do Itajaí (UNIVALI).

Authors' contributions: This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Acknowledgements: None.

Cite as: Ortolan XR, Mezadri TJ, Tames DR, Corrêa R & Campos-Buzzi F. Osteogenic potential of different chalcones in an *in vivo* model: A preliminary study. J Oral Res 2017; 6(8):209-215.
doi:10.17126/joralres.2017.059

such as the transmission of infection and the induction of comorbities. In addition, the clinical use of BMPs has been substantially reduced due to the high cost of obtaining these substances.[7] Limitations associated with bone repair and the materials that can promote the osteogenesis have motivated the development of alternative strategies to enhance the repair of large bone defects.

Through organic synthesis, many classes of organic compounds have shown promising biological effects.[8] Chalcones represent a group of intermediate compounds or end products in flavonoid biosynthesis. Considering that different substituents on the chalcone rings may result in compounds with different biological activities, many pharmacological studies of several synthetic chalcones show antioxidant, antibacterial, anti-fungal, antiviral, antiparasitic, analgesic, anti-inflammatory, cytotoxic and chemopreventive potential for cancer cells.[9-11] Plant-isolated molecules have been shown to play a role in the repair of non-mineralized tissues.[12-14] However the potential of these molecules in the repair of bone wounds is not known.

Kim *et al.,*[15] showed that licochalcone A has osteogenic activity, increasing alkaline phosphatase and calcium levels, interfering with cell differentiation, with a possible induction of the expression levels of BMPs. Moreover, Ortolan *et al.,*[16] suggested induction of bone repair by chalcone 1-phenyl-3-(4-chlorophenyl)-2-propen-1-one in the rat critical size calvarial defect, as the wounds treated with this molecule showed complete closure after 45 days of treatment.

The aim of this study was to evaluate the osteogenic potential of five synthetic chalcone molecules with different substituents (3,4-Cl; 4-Cl; 4-CH_3; 4-OCH_3, H), in three concentrations (1%, 5% and 10%) on critical rat calvarial defects using Vaseline as a vehicle.

MATERIALS AND METHODS.

Synthesis of chalcones

The 1,3-diphenylprop-2-en-1-ones were obtained by the Claisen Schimdt aldol condensation method of. The method consisted in dissolving an equimolar mixture of different substituted benzaldehydes (0.05mol) and acetophenone (0.05mol) in 25ml of ethanol in basic medium (5g of sodium hydroxide).

Surgical procedure

After approval of the Ethical Committee on the Use of Animals of University of Vale do Itajaí (UNIVALI) (041/2011), 170 Wistar female rats (45 days old) were used. Under sterile conditions of the surgical field the animals were anesthetized with a solution of 3.75ml ketamine hydrochloride Cetamin® (10%), 2.5ml of Dopasen® xylazine (2%) and 3.35ml of distilled water; (0.1ml/kg IM) and manually trichotomized in the right parietal bone region.

The cutaneous and periosteal planes were folded and a disk of bone tissue was completely removed with a 5mm diameter surgical trephine mounted on a low-rotation handpiece, and under constant irrigation with saline, exposing the dura mater (Figure 1).

The wounds of the control group (n=10) were irrigated with saline solution; in the vehicle group (n=10) Vaseline was applied (single application). In the each of the other groups one of the five molecules-types was applied (Figure 2) with vehicle at concentrations of 1%, 5% and 10% (n=10 animals/concentration).

Vaseline, used as a drug-carrier, was used to provide the drug with a semisolid form and to carry the chalcone (powder), facilitating the single administration onto the calvaria of the animal.

Then, the periosteum and soft tissues were sutured. For analgesia, ketoprofen Ketofen® 1% (0.25ml/100g) was applied subcutaneously within the first 72 hours.

After 30 days, the animals were euthanized with anesthetic overdose and the repair tissue was removed for analysis of the critical wound area. The collected material was fixed by immersion in 4% paraformaldehyde in phosphate buffer, pH 7.4 for 72 hours. After this period, for quantitative analysis the areas of the remaining wounds were calculated (in mm^2) using Image J software (NIH, USA).

Following the histological technique, the samples were demineralized in 10% ethylenediamine tetraacetic acid (EDTA) in phosphate buffer (0.1M, pH 7.4), dehydrated in alcohol at increasing concentrations (70, 90 and 100%), bleached in xylol and embedded in paraffin. Five semi-seriate cuts (1:10) were obtained in a 7µm rotatory microtome and stained with hematoxylin and eosin.

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Macro and microscopic analysis and statistical treatment

The Image J software (NIH, USA) was used to measure the area of critical wounds for comparison between groups. Quantitative data were statistically treated using variance analysis (ANOVA) followed by the Student-Newman-Keuls multiple comparisons test using the GraphPad InStat (GraphPad, USA). The histologic sections were analyzed qualitatively by transmitted light microscopy, evaluating the cellular and tissue types in wound repair. The specimens of the repair areas were documented with a photomicroscope (BX50, Olympus®, Tokyo, Japan).

Figure 1. Critical wound and chalcone application.

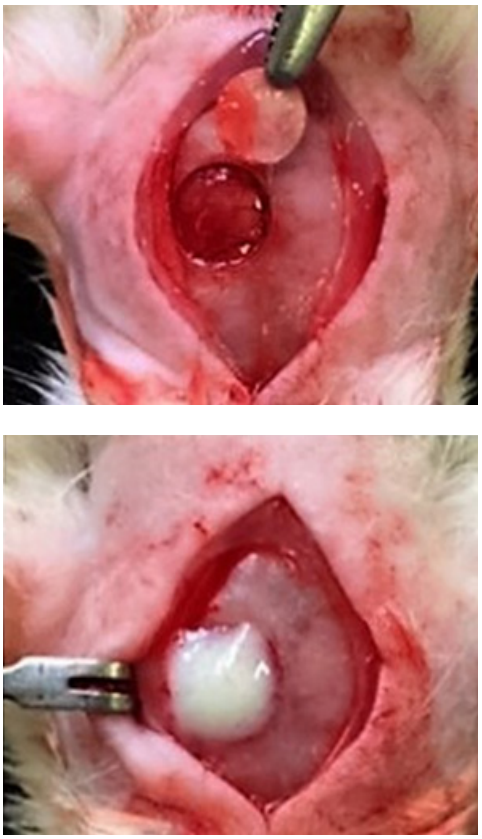
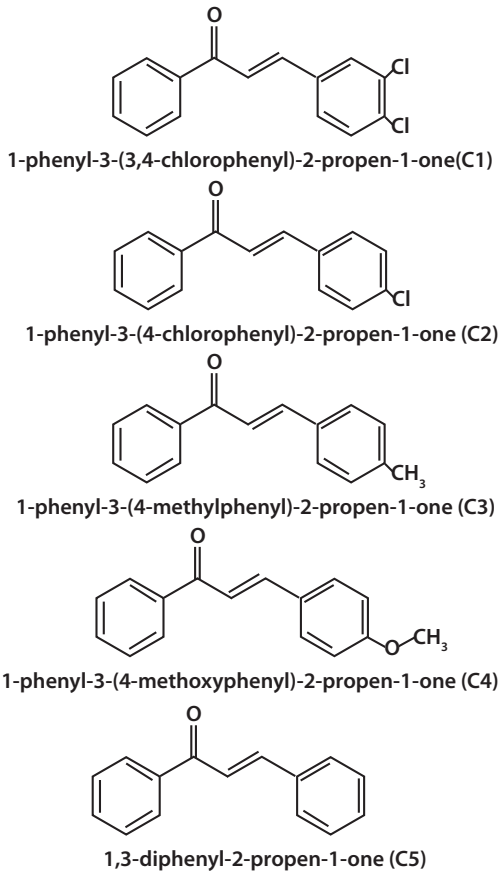


Figure 2. Chemical structure of chalcones.



RESULTS.

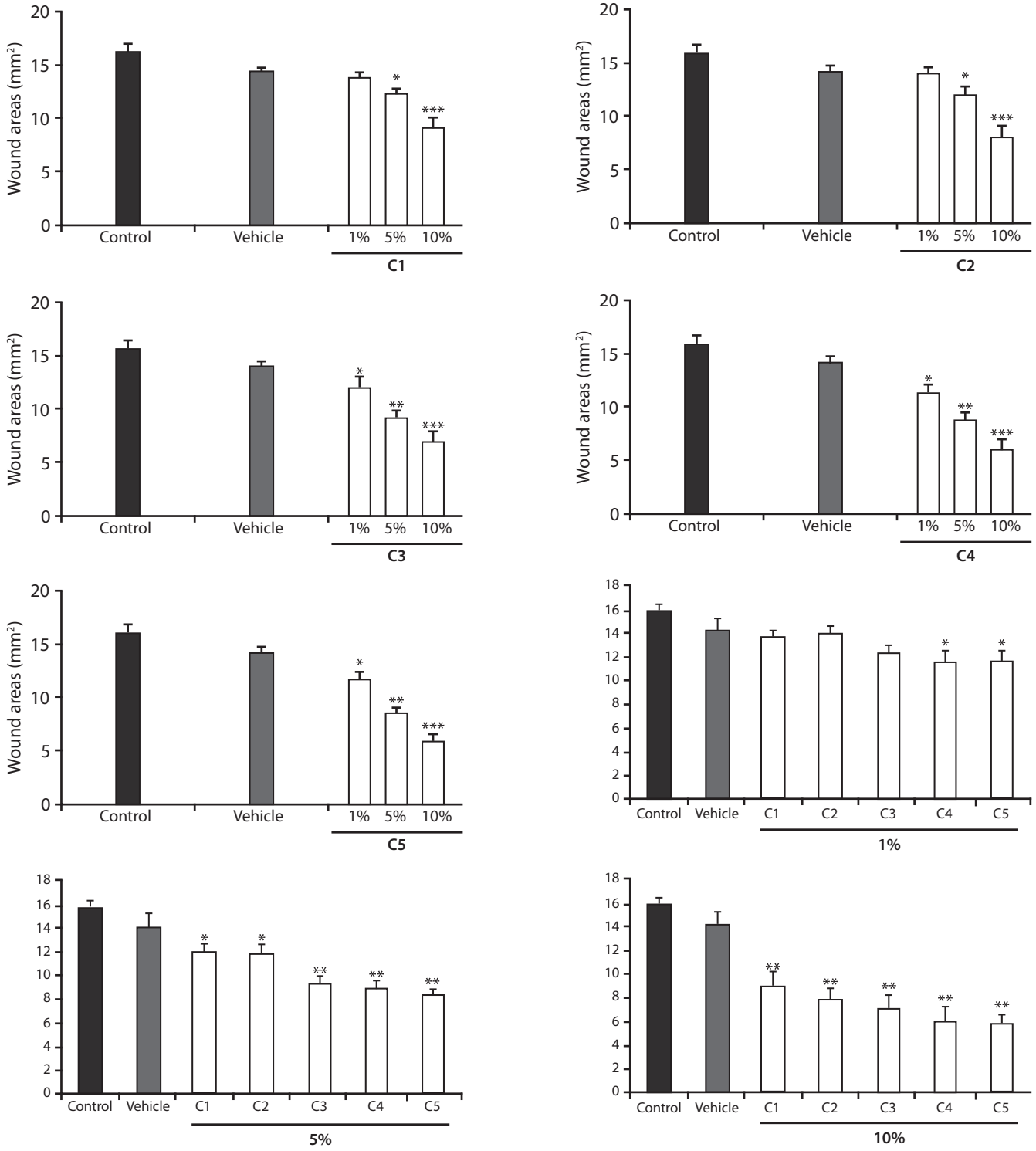
Table 1 and Figure 3 show the mean values for the remaining wound areas from the critical wound, analyzed quantitatively in each of the groups and the doses used. Statistical analysis did not show significant differences between the control and vehicle groups regarding the mean area values of the remaining wounds.

The groups treated with C4 and C5 molecules at 1% concentration showed significant differences compared to the control group (p<0.01). At 5% concentration, the C1 and C2 chalcones showed a significant increase in bone wound closure compared to the control group (p<0.01) and the C3, C4 and C5 molecules, in this same concentration, show statistically significant differences in comparison to the control and vehicle group (p<0.01).

At 10% concentration, all the molecules showed a significant increase in bone closure compared to the control, vehicle and chalcone at 1% concentration groups (p<0.01). No statistical differences were observed between the 5% and 10% concentrations. No significant differences were observed between the molecules at the same concentrations (Figure 3).

Thirty days after the surgical procedure, microscopic analysis showed disorganized connective tissue with no signs of inflammation in the control group; no significant bone neoformation from the edges of the critical defect coated by flattened cells (inactive osteoblasts) (Figure 4-A). Regarding the vehicle group, the wounds showed

Figure 3. Areas of remaining wounds of the animals treated with chalcones (C1, C2, C3, C4 and C5) at 1%, 5% and 10% concentration and the control and vehicle groups after 30 days of treatment.



inflammatory infiltrate characterized by mononuclear cells and wound edges coated by inactive osteoblasts (Figure 4-B).

The group treated with concentrations of 1%, 5% and 10% of chalcone displayed connective tissue and absence of inflammatory infiltrate at central wound areas, osteogenesis at the edges of the critical defect coated with active osteoblasts was observed (Figure 4-C, D and E).

In the groups treated with C4 and C5 chalcones at 10%, the repair tissue displayed a lamellar aspect with observed osteocytes oriented in the same direction, featuring secondary bone (Figure 4-F).

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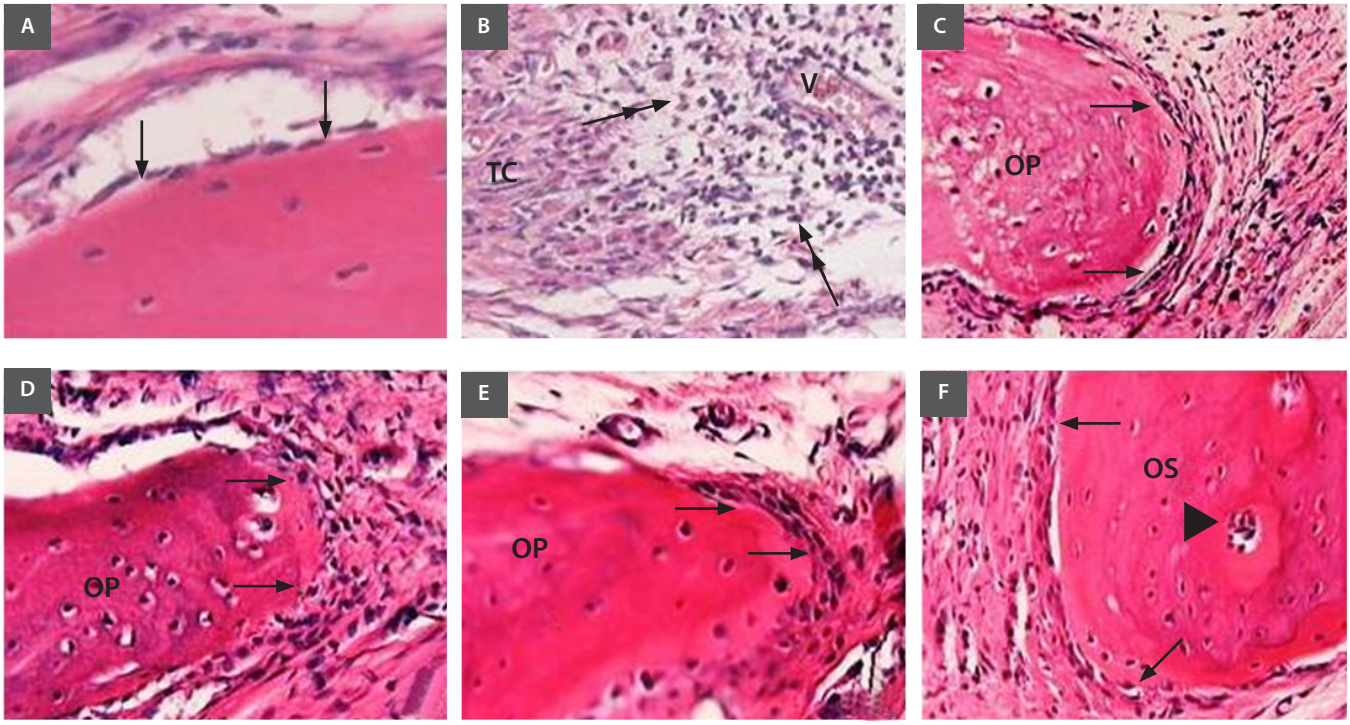
Table 1. Remaining areas of wounds 30 days after treatment.

Groups	Mean±standard error		
Control	16.28±0.48		
Vehicle	14.97±1.20		
	Concentrations		
Molecules	1%	5%	10%
C1	13.83±0.53	12.24±0.63*	9.11±1.19**
C2	14.06±0.61	11.96±0.91*	7.93±1.05**
C3	12.36±0.60*	9.53±0.62**	7.34±1.05**
C4	11.59±0.95*	9.18±0.64**	6.25±1.05**
C5	11.78±0.61*	8.58±0.53**	5.94±0.72**

Areas of remaining wounds of experimental groups.
Data are presented as mean ± standard error. [*]=p<0.01; Significant for the control group; [**]=p<0.01
Significant for the control and vehicle groups.
ANOVA followed by the Student-Newman-Keuls test, n=10 animals per group.

Figure 4. Photomicrographs of experimental wounds.



A. Control group: inactive osteoblasts (arrows) in the bone surfaces.
B. Vehicle group: severe inflammation in the connective tissue (CT), presence of blood vessels (V) and mononuclear cells (double arrow).
C. C2 group-1% concentration; active osteoblasts (arrow) at the borders of the newly formed bone tissue and primary bone (OP).
D. C4 group-5% concentration; active osteoblasts (arrows) in the bone surfaces and primary bone (OP).
E. C3 group-10% concentration; active osteoblasts (arrows).
F. C5 group-10% concentration; bone surfaces coated by active osteoblasts (arrows), lamellar bone with concentric structures (arrowhead) featuring secondary bone (OS).

DISCUSSION.

This study aimed to evaluate the osteogenic potential of different concentrations (1%, 5% and 10%) of chalcone molecules with distinct substituents as proposed by Topliss, using Vaseline as vehicle, in the rat critical size calvarial bone defect. The calvaria has been chosen as a model to simulate bone loss because it is an area anatomically free of mechanical stress, with bone defects structures stably surrounded, and with a limited blood supply resulting in a low regenerative potential.[17]

In addition, a critical defect should be large enough to avoid spontaneous repair during the animal life, making the study of the osteogenic potential of a substance possible and to evaluate the interactions between the implanted material and the bone *in situ*.[18] These considerations validate the experimental model used in our study.

The period of 30 days of treatment with chalcone allows for the measuring of the diameters of the remaining bone wounds, without total repair of the defect, since previous studies have shown that 45 days after treatment with chalcone a complete repair of the critical wound has occurred, limiting comparisons among the groups.[16]

The results show that both groups, control and vehicle, present a small area of bone neoformation and none of the wounds evolved to significant regeneration during the 30 days of the experiment, characterizing the critical wound model. In both groups, the osteoblasts present in the borders of bone wounds show a reduced ability of bone synthesis, suggesting limited bone formation. Oliveira *et al.,*[19] obtained similar results after evaluating the critical wound repair promoted by a demineralized bovine bone implant. The authors showed that biomaterial has been replaced by fibrous tissue at 21 days after treatment, and after 90 days the repair was still incomplete. On the other hand, the chalcone molecules showed osteogenic potential in our study. The vehicle-treated wounds showed inflammatory reaction with engorged blood vessels and mononuclear cells. Although primarily inflammation represents one of the defense mechanisms of the body, the vehicle used in this study (Vaseline) may have compromised the bone regeneration, because even after 30 days this inflammatory repair phase remains evident.

Huang *et al.,*[20] show that a suitable initial inflammatory response is required to initiate and guide subsequent bone regeneration processes. In contrast, excessive or prolonged inflammation may inhibit or delay the bone tissue repair. Thus, the anti-inflammatory property of chalcones as reported by Chen *et al.,*[21] may have contributed to the improvement of the bone repair stimulus observed in our study, as in animals treated with chalcones no inflammation signs were observed.

Previous studies[16] have shown that a single application of chalcone in rat calvarial defects reduces the critical defect size after 30 days of treatment and promotes complete wound closure after 45 days. In our study, we observed predominantly active osteoblasts on the repair surfaces from neoformed bone in animals treated with chalcones. Therefore, it is possible that chalcones induce complete repair of critical defects in longer periods, especially at 10% concentration, which was the most effective dose in reducing critical defect size.

Although Kim *et al.,*[15] have demonstrated that licochalcone A is more effective in bone repair at low concentrations (2.5µM and 5µM) compared to higher doses (10µM), our results show that five different chalcone molecules presented osteogenesis activity in a dose-dependent manner. In addition, chalcones used in higher concentration (10%) did not present toxicity, as observed by the absence of necrosis processes and already reported by Pingaew *et al.*[22]

The degree of bone repair observed with treatment with the different chalcone molecules at the same concentrations, as measured in remaining wound area at the experimental endpoint, did not differ, suggesting that all the assayed molecules have osteogenic potential.

Although the result was not statistically significant regarding the amount of bone formed, the 10% concentration appeared to be more effective, and treatment with C4 and C5 molecules resulted in higher bone formation compared to the other molecules, and showed some features of secondary bone in histological analysis.

Therefore, the repair promoted by C4 and C5 chalcones seems to be similar to that obtained by the autogenous bone graft, considered a gold standard among the materials used in the osteogenesis process. Trotta *et al.,*[23] showed that this biomaterial was able to promote significant bone repair with the presence of Haversian canals in the newly formed bone after 30 days.

The chalcone structure formed by two aromatic rings and substituents proposed by Topliss, confer to these molecules lipophilic characteristics,[24,25] enabling them to interact with membrane or cytoplasmic receptors of bone cells during some of the phases of the repair, or with transcription factors and secreted non-collagenous proteins such as BMPs, responsible for the elaboration of extracellular matrices that mineralize. This data are found in a study by Kim *et al.,*[15] regarding the possible induction of the expression of BMP, an important factor in the osteogenesis mechanism, by licochalcona A.

In contrast, studies by Sung *et al.,*[26] Yadav *et al.,*[27] and Suh *et al.,*[28] showed that different chalcones may also suppress osteoclastogenesis by inhibiting osteoclast receptors such as RANKL. Considering the osteogenic potential presented by chalcones in the present study, these molecules may present a pharmacological approach for the acceleration of bone repair. However, it is important to continue studies evaluating other molecules and carriers, employing other

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methodologies, in order to determine the factors involved in the process of osteogenesis promoted by these substances, considering the cost-benefit aspect involved in the process of chalcone synthesis.

CONCLUSION.

The chalcones displayed a dose-dependent osteogenic potential and C5 was the most effective at promoting bone repair.

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