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IN VIVO EVALUATION OF THE INFLUENCE OF POSSIBLE CHANGES IN THE OSTEOCLASTOGENESIS PROCESS CAUSED BY THE ACTION OF INFLAMMATORY DRUGS IN RATS WITH INDUCED DIABETES MELLITUS

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ABSTRACT

The insertion of potentially inflammation-controllingmedications common ground among health professionals since their performance provides postoperative comfort to patients; they can be divided into two types, steroidal anti-inflammatory drugs, and non-steroidal anti-inflammatory drugs, each having its particularity regarding the site of action. Steroidal anti-inflammatories have their action initiated at the top of the inflammatory process, limiting the appearance of isoformsfrom local trauma, apart from the therapeutic advantages, they also present significant disadvantages such as the action on proteins responsible for bone formation and resorption. Otherwise, non-steroidal anti-inflammatory drugs have a lower level of action on the inflammatory process, which limits their therapeutic advantage to only one pathway, the cyclooxygenase, and it can cause a delay in tissue repair.In patients with Diabetes Mellitus their therapeutic effects are significant regarding possible changes in cutaneous and bone repair.The aim of this study was to evaluate in vivo the influence of 3 different types of anti-inflammatory drugs. The results showed that the action of anti-inflammatory drugs did not influence the osteogenesis process, therefore the systemic factor is the main responsible for the increase in the time of the beginning of bone neoformation.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of chronic hyperglycemia caused by metabolic endocrine failures that can be followed to a greater or lesser degree by changes in the conversion of carbohydrates, proteins, and lipids (Mauri *et al*., 2017 and Paz, 2017). The World Health Organization, in 2019, classified DM into 6 categories according to its diagnosis: type I, type II, hyperglycemia first detected during pregnancy, hybrid type (slow-evolving DM and immunological in adults and with a tendency to ketosis), specific types (monogenic diabetes, exocrine pancreas diseases, etc.) and unclassified DM that do not fit into other categories (WHO, 2019 and Sociedade Brasileira De Diabetes, 2016). DM compromises various parts of the human body, and may cause visual disturbances, nephropathies, neuropathies, vascular and microcirculation impairment, reduction of immune capacity, oral manifestations, among others, can also cause osteopenia with direct alteration in the osteogenesis process thus affecting bone production, maturation and consolidation process (Silva *et al*., 2017; Mainardes *et al*., 2007; Oliveira, 2016 and Khouja, 2019).

According to researches one of the ways to induce diabetes mellitus in rats is through streptozocin (STZ) injections, an antibiotic with diabetogenic characteristics due to its potential for destruction and disjunction of beta cells of Langerhans islets in the pancreas (Negri, 2005 and Lenzen, 2008). Thus, giving a single dose in the proportion of 50 to 60 mg.kg which can be administered intravenously by opting for the caudal and sublingual veins as forms of puncture, or introduced via intraperitoneal. High doses of STZ leads to severe damage to pancreatic β cells in this way, making the availability of systemic insulin unfeasible, favoring hyperglycemia. This condition and the way the pathology is induced can be compared to the mechanism that occurs in DM type 1 because it does not present insulin resistance (Srinivasan, 2005; Mu, 2006; Correia-Santos, 2011). Inflammation is a necessary process for tissue and bone repair, however in diabetes they are directly affected;[12] within this limitation scope, these are incorporated: the phases of hemostasis, inflammation, proliferation, maturation and remodeling occurred due to the deficit of nutrients caused by the reduction of vascular microcirculation, favoring the reduction of osteogenesis that depends on biomechanical and biological factors, as well as the potential delay in tissue repair, thus causing the patient damage that may be

mild, moderate or severe, depending on the degree of tissue hypoxia (Bagheri *et al*., 2013 and Athyros, 2018). Therefore, the control of inflammation through drugs in these patients should be analyzed and reconsidered regarding the chosen type of anti-inflammatory treatment (Pelissoni, 2003). Anti-inflammatory drugs can be administered to control inflammation in cases where there is prolonged exacerbation, or in cases major tissue injury, adjusting the action of inflammatory control with their analgesic potential. These drugs are usually classified as steroidal and non-steroidal (Mateus, 2002). The steroidal type has its inhibition factor concentrated in the neutralization of the action in the initial stage of the cascade, acting directly on the phospholipase A2 enzyme, thus it can compromise osteogenesis because it interferes in the production of collagen matrix and potential osteoproliferative zones after lesions through direct action in osteoblasts by interfering with phosphatidysmnase (Cornell, 1992 and Li, 2012). Non-steroidal anti-inflammatory drugs, on the other hand, act by neutralizing the cycloxygenase (COX) enzyme, which has isoforms 1 and 2. (Andrade, 2013) Cox isoform 1, after the direct action of NSAIDs, inhibits the production and action of prostaglandins, and as a result, the entire bone repair phase done by the process of osteoclastogenesis, and tissue reconstitution are altered due to the reduction of fibroblasts present at the site of injury (Andrade, 2013 and Lisowska, 2020) Evidently, both classes of anti-inflammatory drugs can interfere in the osteogenesis process, a fact that has been a cause for concern, since these drugs are often prescribed in the postoperative period of oral surgeries (Cavagni, 2005).

Proposition

General proposition: The aim of this study was to evaluate in vivo the influence of 3 different types of anti-inflammatory drugs: dexamethasone, ketoprofen and parecoxib on bone repair and inflammatory process, through histological and histomorphometric studies, after semi-critical defects in the calvaria of rats with high blood glucose rates caused by the addition of streptozocin inducing diabetes.

MATERIAL AND METHOD

This study began after approval by the Ethics Committee for the Use of Animals (CEUA) of The São Leopoldo Mandic College, under protocol 2021/26. The research was carried out in the Laboratory of Anatomo-Pathology and Bioterium of The São Leopoldo Mandic College, Campinas/SP campus (Brazil). For the execution of the research, male Wistar rats were used (N=60) of the *Rattus Norvegicus Albinus* species, obtained from the Bioterium Anilab Ltda. (Paulínia, São Paulo, Brazil), aged between 2 to 4 months of life and body mass between 200 and 300g. (Park *et al*. 2009). The statistical planning of the experimental groups was done by adopting a critical value for the 95% confidence interval: 1.96 and acceptable maximum deviation of 0.19 (19%) (Ekaterina *et al*., 2012), minimum standard error of 5% of the mean (50%) and significance level of 5%. The choice of rat was convenient due to low cost, ease of accommodation and handling, and physiologically it is considered that their healing process is similar to human's (Cohen, 1990). Cohen & Mast studies (1990) show that first-intention tissue healing present better analyses when compared to the second and third intentions. The animals were put in a bay with controlled lighting conditions with light-dark cycle of 12 hours, room temperature (21°C), balanced feeding, controlled humidity, and water at will (*ad libitum*), until the time of surgery (Feijó *et al*., 2010).

Experimental groups

The animals were divided into 4 experimental groups (G1- Control, G2- Dexamethasone, G3- Parecoxib and G4- Ketoprofen), in all groups an injection was previously performed in the intraperitoneal region of streptozocin (STZ) 50mg/kg diluted in citrate buffer 50mM with pH 4.5, in a single dose after 4 hours of fasting, and 03 days of induction of DM were waited, after that, the blood glucose level was measured using Baker's glucometer. Once induction was verified according to the procedure, a semi-critical defect was made in the calvary region and after suturing was performed to favor first-intention healing (Delfino, 2002). Three analysis times (3, 7 and 21 days) were performed, evaluating 05 animals per subgroup, totaling 15 animals per group, according to the medications used. In G1 (n=15) group with DM, the defect was performed but without the use of postoperative therapeutic AI. In G2 (n=15) dexamethasone (0.52 mg/kg/day) was administered for injection and the first dose was performed 1 hour before surgery and the second dose 24 hours after.[29] In G3 (n=15) a defect was performed and administered Parecoxib (6.4 mg/kg/day) injectable one dose 1 hour before the procedure and the same dose every 12 hours for 5 days (Meunier, 2006). In G4 (n=15) a defect was made and Ketoprofen was administered (12.5 mg/kg/day) for injection 1 hour before the procedure and the same dose every 12 hours for 5 days (Martins, 2005 and Silva, 2012).

Surgical procedures: The animals were previously weighed, submitted to preoperative medication, and intramuscularly anesthetized with anesthetic solution of Xylazine and Ketamine, in the proportion of 75 mg/kg of ketamine and 10 mg/kg of xylazine. After the certification of the anesthetic effect, trichotomy was performed with the aid of a hair trimmer in the frontonasal region until the occipital bulge. The animals were positioned in a stabilizer developed for the experiment, where they were maintained and immobilized at 2 places, anterior to the auditory canal; previous antisepsis of the region with 2% chlorhexidine in gel was performed and the position of fenestrated sterile surgical fields was performed (Figure 1). A rectilinear incision of approximately 15 mm paramedian with a 15c scalpel blade was performed in the fronto-occipital region of the rats with posterior limit until occipital protuberance, using the superior sagittal line as anatomical guide (Figure 2).

Source: Own authorship

Figure 1. Initial photo, with field position after antisepsis

Source: Own authorship

Figure 2. Parasagittal linear incision with periosteum disruption

It was followed by a wide lateral divulsion with the aid of a Molt 2-4 elevator, in which the skin, muscles and periosteum were removed (Figure 3), followed by the exposure of the parietal region contralateral to the incision site for calvary access, keeping the tissues folded with the use of a retractor (Figure 4).

Source: Own authorship.

Figure 3. Tissue detachment with periosteum exposure

Source: Own authorship

Figure 4. Total divulsion with parietal exposure contralateral and field clearance

With the help of a trephine drill (Neodent®, JJGC Ind. and Com. de Mat. Dental S.A., Brazil) of 3.3 mm of diameter, internal to a low-speed handpiece/contra-angle system, to the 20:1 a semi-critical circular bone defect of 4.0 mm in diameter and depth of no more than 2.0 mm was performed with dura mater exposure, under constant irrigation with 0.9% sterile saline, with motor programming for low-speed rotation aiming careful handling of the bone surface (Figure 5), the drill enabled cortical perforation to make a bone cap, which was removed resulting in dura mater exposure (Figure 6).

Source: Own authorship.

Figure 5. Osteotomy for semi-critical defect in parietal

Source: Own authorship

Figure 6. Dura mater exposure and parietal bone cap removal

The following steps were tissue repositioning and wound synthesis with suture by planes initiating at the periosteum (Figure 7), skin was sutured with a 5-0 absorbable polygalactin 910 thread (Vycril®, Ethicon, Johnson & Johnson) in continuous suture. After tissue synthesis and aiming at wound care, topic rifocin was administered in the operated region respecting all the necessary biosafety principles (Figure 8).

Source: Own authorship

Figure 7. Synthesis of the periosteum

Source: Own authorship.

Figure 8. Epithelium synthesis and rifocin application in surgical wound

All rats received analgesic subcutaneously (Tramadol Hydrochloride, Cristália Prod. Quím. Farm. Ltda, Itapira/SP, Brazil; 5 mg/kg, once a day for 3 days) and were put in cages until they returned from anesthetic deepening without dietary restrictions. Five animals were placed per cage (divided by subgroups), totaling 12 cages. Anti-inflammatory medications were administered according to the subgroups and the animals were euthanized, as proposed, at 3, 7 and 21 days, and 5 animals of each subgroup were evaluated totaling the amount of 20 animals per analysis.

Euthanasia: The animals were euthanized with deepening anesthetic with barbiturate (sodium thiopental 150 mg/kg and Lidocaine 10 mg/ml), intraperitoneally, after 3, 7 and 21 days of performing the cranial defects.

Material Collection and analysis: Immediately after euthanasia, the skulcaps were removed, sectioned in blocks, and fixed in a solution containing 10% buffered formaldehyde (pH 7.4) for histomorphometric analysis, according to Spicer *et al*. (2012)[33] and Strauss *et al*. (2020)[34], remaining for a maximum time of 7 days, when then the caps were processed for making the blades.

Qualitative analysis

Processing of blades: Each skullcap after being removed was placed in 10% buffered formaldehyde in identified vials. Then the samples were placed on also identified cassettes. After that, the descaling process began, with 20% formic acid solution [Merck (Merck®, Darmastadt, Germany)] for 6 days. After the descaling period, the samples were washed under running water for 24 hours and immersed in distilled water. Dehydration was the subsequent stage, performed to remove water from the tissue. This step was performed in the tissue processor for a total time of 12 h in the equipment. Successively, the washing process occurred for 1 hour with the solutions: alcohol 70%, alcohol 80%, alcohol 90%, alcohol 95%, absolute alcohol I, absolute alcohol II, absolute alcohol III, alcohol/xylol. The next step was diaphanization. The samples then were treated with xylol and were involved in histological paraffin (Synth®, Diadema, Sao Paulo, Brazil) was performed in the paraffin equipament. At this moment, the molds that were used were made. The next stage was microtomy, using the microtome (RM 2245®, Leica Biosystems, Buffalo Grove, Ilinois, United States) to obtain thin 4 μm sections and distended in glass slides.

Quantitative analysis

Inflammatory score: For the evaluation of inflammatory infiltrate and histomorphometric analysis, the sections were stained with hematoxylin-eosin (HE). The histological sections were examined and punctuated by optical microscope analysis, according to the intensity of the inflammatory infiltrate by a blind examiner, about the experimental conditions. The protocol used for the analysis of the inflammatory score was adapted according to a previous study.[35] The inflammatory infiltrate score was classified according to the following qualitative scale from 0 to 5, as described: 0 (absence of inflammatory cells); 1 (1-10% of inflammatory cells); 2 (11-25% of inflammatory cells); 3 (26-50% of inflammatory cells); 4 (51-75% of inflammatory cells) and 5 (>75% of inflammatory cells).

Masson's Trichrome staining: For the analysis of bone neoformation, the color recommended in the present study was Masson's Trichrome, which allows identification of tissues by different colors, shades, and morphological identification. This staining has fundamental importance in the identification of soft and hard tissue components distinctly, differentiating it from other histochemical staining techniques because it also distinguishes cell types and tissue maturation, according to Gruber (1992)[36] and Tandon *et al*. (2019)[37]. This analysis was performed on the group of animals 21 days after the defect was made in the skull defect.

Histomorphometric analysis: The slides were histomorphometrically analyzed, evaluating the total amount of new formed bone. The analyses used the micrometer (μm) or square micrometer (μm2) as a measurement parameter. For the analyses, the areas of bone neoformation were used. The data were analyzed using the ImageJ software (ImageJ®, National Institute of Health, Bethesda, MD, USA). The ImageJ area counting tool was used, calibration was performed with the bar in μm and then this measure was converted

into mm, to facilitate counting and statistical procedures. All procedures were performed by the same calibrated pathologist, in a blind procedure.

Analysis of Results

Statistical analysis

The data found were submitted to a normal curve adherence analysis, verifying its non-normal distribution. Therefore, the Kruskal-Wallis nonparametric test was used, followed by Dunn's test for multiple comparison between groups. In all tests, the level of significance adopted was 5% ($p<0.05$). GraphPad Prism software (Graph Pad Prism 6.0®, San Diego, CA, USA) was used for statistical calculation and graphics making.

RESULTS

In the analysis of the group euthanized 3 days after of the preparation of the bone defect, the control group presented a higher score of inflammatory infiltrate ($p<0.05$), predominantly composed of neutrophils, besides the presence of a greater number of bleeding areas when compared to the treated groups. The group treated with dexamethasone and the group treated with ketoprofen presented lower inflammatory infiltrate when compared to the control group ($p<0.05$), with about 5% of inflammatory cells, absence or small hemorrhagic areas, in addition to the presence of connective tissue in the bone defect. The group treated with parecoxib showed a reduction in the inflammatory score, about 15% of inflammatory cells when compared to the control group ($p<0.05$). The groups that received the treatments showed no statistical difference between them, as shown in Figure 9.

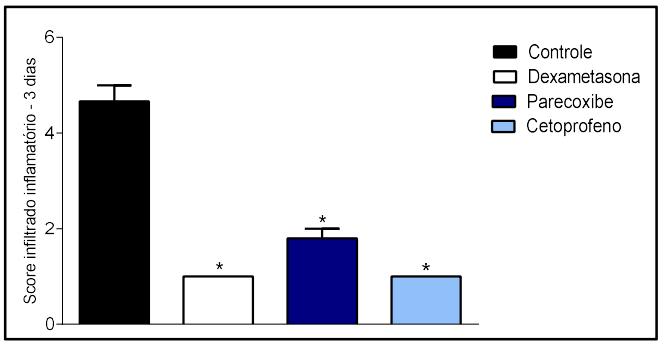

Figure 9. Evaluation of inflammatory score in the group of euthanized animals after 3 days of critical defect in the calvaria

The control group presented higher inflammatory score when compared to the groups that received the treatments. The groups treated with dexamethasone, parecoxib and ketoprofen showed a significant reduction in inflammatory score when compared to the control group. The symbol "*" represents a statistically significant decrease when compared to the control group. The obtained data was submitted to the Kruskal-Wallis test, followed by Dunn's multiple comparison post-test. The level of significance established was 5%. $P<0.0001$.

In the analysis of the group euthanized 7 days after the bone defect was made, the control group presented a higher score of inflammatory infiltrate ($p<0.05$), predominantly composed of lymphocytes, when compared to the treated groups, in addition to the presence of a greater amount of hemorrhagic areas when compared to the treated groups.

The groups treated with dexamethasone, parecoxib and ketoprofen showed lower inflammatory infiltrate when compared to the control group ($p<0.05$), about 10% of inflammatory cells, in addition to a larger layer of connective tissue coating the bone defect. The groups that received the treatments showed no statistical difference between them, as shown in Figure 10.

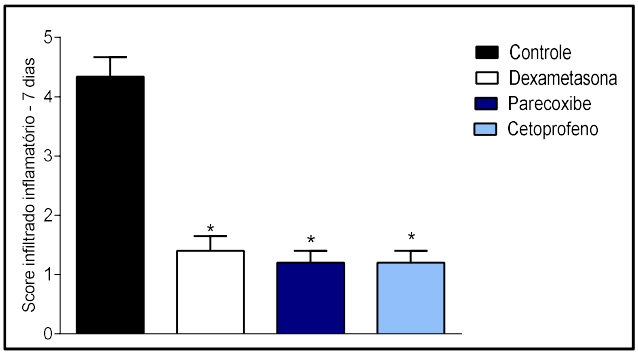

Figure 10. Evaluation of inflammatory score in the group of euthanized animals after 7 days of critical defect in the calvaria

The control group presented higher inflammatory score when compared to the groups that received the treatments. The groups treated with dexamethasone, parecoxib and ketoprofen showed a significant reduction in inflammatory score when compared to the control group. The symbol "*" represents a statistically significant decrease when compared to the control group. The obtained data were submitted to the Kruskal-Wallis's test, followed by Dunn's multiple comparison post-test. The level of significance established was 5%. $p=0.0090$.

In the analysis of the group euthanized after 21 days of the preparation of the bone defect, the control group presented a higher score of inflammatory infiltrate ($p<0.05$), when compared to the treated groups. The group treated with parecoxib and the group treated with ketoprofen presented lower inflammatory infiltrate when compared to the control group ($p<0.05$), about 5% of inflammatory cells, in addition to the presence of connective tissue in the bone defect. The group treated with dexamethasone showed no statistical difference when compared to the control group ($p<0.05$), as shown in Figure 11.

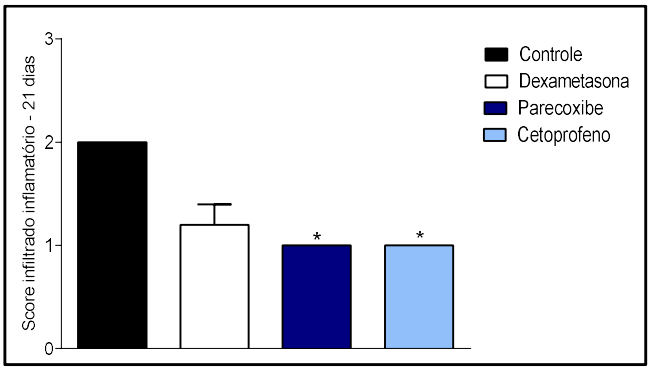

Figure 11. Evaluation of inflammatory score in the group of euthanized animals after 21 days of critical defect in the calvaria

The control group presented higher inflammatory score when compared to the groups that received the treatments with parecoxib and ketoprofen. The groups treated with parecoxib and ketoprofen showed a significant reduction in inflammatory score when compared to the control group. The symbol "*" represents a statistically significant decrease when compared to the control group. The data obtained were submitted to the Kruskal-Wallis's test, followed by Dunn's multiple comparison post-test. The level of significance established was 5%. $p=0.0098$.

Histomorphometric analysis

Regarding bone new formation, although the parecoxib group presented a tendency with a greater area of bone neoformation, the analysis of the results showed no statistical difference between the groups evaluated ($p>0.05$), as shown in Figure 12.

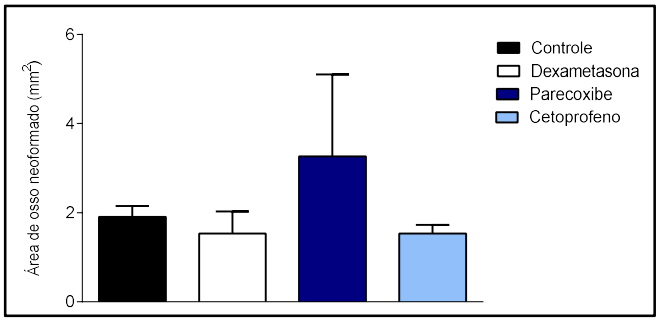

Figure 12. Evaluation of the area of new formed bone in the group of euthanized animals after 21 days of critical defect in the skull cap

In the groups evaluated, there was no statistical difference between the groups evaluated. The data obtained were submitted to the Kruskal-Wallis' test, followed by Dunn's multiple comparison post-test. The level of significance established was 5%. P<0.9067.

DISCUSSION

The idea of the use of AIs to control the inflammatory phase is based on the prerogatives that the phenomenon lasts, on average, 7 to 10 days on the process of cellular diapedesis to homeostasis, coming to an end when there are no evident clinical manifestations at the site of injury, however patients with local microvascular circulation alterations may not be within these parameters.[14,38,39] Complications are systematized in microvascular and macrovascular disorders and are also considered responsible for diseases in the digestive, musculoskeletal and even cognitive systems.[40,41] Corroborating the present study, it was observed greater inflammatory infiltrate in groups not submitted to therapy in the periods of 3 to 7 days, but the inflammatory rate remained high. When compared the period of 21 days there was no significant difference between the groups, evidencing that the alterations caused by DM can delay the regenerative potential. The quantitative analysis of the newformed bone in this study when using steroidal anti-inflammatory, for example Dexamethasone in G2, was evidenced as follows: the 3-days and 7-days groups did not present formed bone, meaning that the therapeutic factor associated with the comorbidity may propitiate the delay of osteogenic formation. This disagrees with the work of Lima *et al*. (2020)[29] that claimed that on the seventh day there are indications of bone neoformation, even though steroidal anti-inflammatory drugs are used. Corroborating with the proposal of Mergoni *et al*. (2019)[42], Li *et al*. (2012)[19], and Byers *et al*. (2009)[43] whose studies highlighted that the action of dexamethasone may cause reduction of the osteoclastogenesis process due to the inhibition of fibroblast differentiation and thus direct interruption in collagen production and activation of matrix cells such as osteoblasts. Only in the period of 21 days, bone formation was observed, but showed no distinction when compared to the group without the use of steroidal anti-inflammatories, however it is an understanding that the increase in glycemic rates evidences the contraindication of this anti-inflammatory in cases of DM.

In studies of non-selective NSAIDs such as Ketoprofen, the medication of group G4, Silva *et al*. (2012)[30], Martins *et al*. (2005)[31], and Zeng *et al*. (1993)[44] share the resolution that non-selective AI may interfere in the process of prostaglandin activation, which consequently affects the bone and tissue repair process even if doses are used in the proportion of 12.5 mg/kg/day or greater. This statement do not agree with the present study because regarding inflammatory control with tissue formation, group G4 presented a promising response when compared to the group not submitted to therapeutic medication at periods of 3, 7, and 21 days, and no interference was observed in the osteoclastogenesis process, since in the period of 21 days there were no significant differences in the processes evidencing the presence of bone matrix in all histological sections taking into account that the rats had hyperglycemia.

The link of NSAIDs on osteogenesis process interference is still controversial[45]; invalidating this argument, the analysis of COX 2 expression is necessary to activate the growth factor of the vascular endothelium (VEGF), necessary for the nutritional reconstitution of the area under repair and therefore its absence would condemn the entire homeostasis process.[46] The present study however corroborates Carvalho's(2007)[45] statement, because there was no significant interference of the action of drugs in the osteoformation process when these were compared with the group without their action, on periods of 3, 7 and 21 days, even if there was the presence of a disease; and thus covering the theoretical basis found in the assumptions of Padoins (2012)[39] and Tiseo *et al*. (2006)[47] regarding the therapeutic choice of NSAIDs, being non-selective or selective of COX 2, which according to the results have no influence on the healing process of bone in formation. In the present study for the period of 21 days, a greater bone distribution was evidenced in the animals of group G3, when compared to the other drugs and the group with DM without AI administration, however in the studies conducted by Xie *et al*. (2019)[48], Simon *et al*. (2002)[49], Cai *et al*. (2014)[50], and Ghalayani (2014)[51] all reached the consensus that COX 2 selective AI, as is the case of parecoxib (same dose as administrated in this study or higher), did not present a relevant influence on the bone formation rate rather short or long term, denoting and complementing that the systemic factor conducted in the experiment was the major responsible for the delay in the beginning of the osteogenesis process when compared to studies in healthy rats.[52,53]

CONCLUSION

Therefore, according to qualitative analysis, the study revealed that there was a control over the inflammatory infiltrate on the 3 groups in all periods of analysis when compared to the group without the use of anti-inflammatories. As for the bone quantitative visualized in analysis by Masson Trichrome, there was no significant difference in the final process of osteogenesis when using anti-inflammatory drugs or not; however, the factor of induced systemic comorbidity (DM) was significant for the delay of the bone new formation phase, and this was observed on the twenty-first day only. Among the groups that used drugs, it was also evidenced that the group using selective NSAIDs, Parecoxib, had a greater area of bone formation when compared to other drugs, however their bone forming rates were equivalent.

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