Doxycycline and Autogenous Bone in Repair of Critical-Size Defects

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DOI:
10.1097/ID.0000000000000783

Doxycycline and autogenous bone in repair of critical-size defects

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ABSTRACT (175 words)

PURPOSE The association of doxycycline and autogenous bone on repair of critical-size defects was evaluated.

MATERIALS AND METHODS Fifty albino rats were divided in 5 groups (n=10). A 5-mm diameter defect was treated with: CO – Blood clot (control); DOX – 10% Doxycycline in natrosol gel; NAT – Natrosol gel; PAB – Particulate autogenous bone; PAB + DOX – Particulate autogenous bone associated with 10% doxycycline in natrosol gel. The animals were euthanized at 4 and 8 weeks postoperatively. Histomorphometric analysis was performed to assess the percentage of new bone in the defect area. Statistical analysis of the results was performed using ANOVA and Tukey's tests ($p < 0.05$).

RESULTS The results showed that new bone formation was limited to the margins of the defect. At 4 and 8 weeks, the group PAB + DOX showed the higher bone formation (38.59% and 47.86%, respectively), with statistical difference in comparison with the CO (19.52%) at 4 weeks and CO (18.80%), DOX (22.05%) and NAT (15.89%) at 8 weeks ($p < 0.05$).

CONCLUSIONS The association of 10% doxycycline with autogenous bone improved significantly bone healing in critical-size defects.

KEYWORDS: Bone regeneration, bone graft, osseous defects.

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The repair of extensive bone defects due to trauma, infections, pathologies or congenital malformation was always considered a challenge for reconstructive surgery.[1] Bone defects are considered of critical size when the extension is large enough to jeopardize complete healing during a lifetime.[2] The critical-size defects are not able to repair spontaneously and thus, depend on the use of bone substitutes to complete the regeneration.[3]

Tetracyclines have been investigated extensively for decades for their antibiotic activity[4], anti-inflammatory effect[5] and inhibition of collagenolytic enzymes responsible for degradation of connective tissue and bone resorption.[6] Minocycline and doxycycline have been used as a possible therapy for bone regeneration.[7] The semi-synthetic derivative of tetracycline, the doxycycline is reported to have positive effects on bone regeneration. Doxycycline stimulates osteogenesis and apoptosis of osteoclasts, inhibits inflammatory bone resorption and osteoclastogenesis.[8,9] The effect of 10% doxycycline in the repair of critical-size defects in rat calvaria was previously evaluated.[10] The addition of 10% doxycycline in natrosol gel in the bone defects enhanced repair and thus this antibiotic could be used with graft materials and as a scaffold for bone regeneration.[10]

Despite several graft biomaterials developed in the last decades, autogenous bone is still highly recommended for treatment of critical-size defects.[11,12] Autogenous bone is considered the gold standard graft for bone regeneration.[13] The autogenous bone has several advantages including favorable bone quality, no risk of disease transmission or antigenicity, and predictability in the repair of larger defects or greater atrophy.[13] The high rate of success on reconstruction surgery with autogenous bone seems that this graft is ideal to test the combination with doxycycline for repair of extensive defects.[12,13] The aim of the study was to evaluate the use of 10% doxycycline in gel form and particulated autogenous bone on repair of simulated critical-size defects in rat calvaria. Qualitative and quantitative histological analyses were performed to analyze the percentage of new bone formation.

MATERIALS AND METHODS

Experimental design

Fifty adult male albino rats (*Rattus norvegicus*), weighing approximately 500 g were selected (Ethical approval in the Animal Ethics Committee - 590/2014). The animals were kept in an environment with temperature ranging 22-24ºC, with a controlled light cycle and consumption of solid food and water *ad libitum* throughout the experimental period.

For the surgical procedures, the rats were anaesthetized by intra-muscular injection of a combination of ketamine (Dopalen, Sespo, São Paulo, Brazil) (70 mg/kg) and xilasin (Rompum, Bayer S. A., Brazil) (6 mg/kg). The frontal-parietal region was shaved and disinfected with 10% povidone-iodine. A U-shaped incision was made through the skin using a no. 15 scalpel blade, with the caudal base in the rat calvaria and a full-thickness flap was folded posteriorly. A trephine drill of 5-mm diameter in a contra-angle was used to prepare a critical size defect in the rat calvaria under constant cooling with sterile saline solution. The defect included a portion of the sagittal suture. Then, "L"-shaped markings were performed using a carbide conical drill, 2 mm anterior and 2 mm posterior to the surgical defect margins. The markings were performed to allow identifying the center of the original defect during laboratorial processing. The major axis of each "L" was located on an imaginary longitudinal cranial-caudal line that divided the surgical defect in half. The markings were filled with amalgam as previously described.[14]

The animals were randomly divided into five groups (n = 10) according to the treatment applied:

- CO – Untreated. The defect was filled by a blood clot (control);
- DOX – 10% Doxycycline in natrosol gel;
- NAT – Natrosol gel;
- PAB – Particulated autogenous bone;
- PAB + DOX – Particulated autogenous bone associated with 10% doxycycline in natrosol gel.

In DOX and NAT groups, 0.5 mL of the substances were applied directly in the defect using a spatula. In PAB group, the skullcap removed with trephine was crushed using a bone crusher and inserted into the defect.

In PAB group, the particulated skullcap was mixed with 10% doxycycline in natrosol gel and inserted into the defect.

After filling the defects with the respective material, the tissues were placed in their original position and sutured with silk 5-0.

After 4 and 8 weeks, the animals were euthanized by excessive inhalation of carbon dioxide (CO_2). The frontal-parietal skin was shaved, disinfected and the calvaria was removed. The specimens were kept in a 10% formalin solution for 2 weeks. Then, the samples were washed in water, decalcified in 18% etilenediaminetetracetic solution (EDTA) and embedded in paraffin. Sections of 6-µm thickness were performed from the center of the original defect. The sections were stained with hematoxylin and eosin.

Microscopic analysis

The microscopic analysis was performed qualitatively and quantitatively using a binocular optical microscope by a trained technician. The histological slices of the specimens were evaluated qualitatively according to:

- Quality and intensity of the inflammatory reaction;
- Presence of fibroblasts and collagen fibers;
- Presence of granulation tissue and bone trabecula formation;
- Type and quality of tissue formed within the surgical defect.

The histological sections were evaluated histomorphometrically. Images of the sections were captured with a digital camera (Olympus DP71, Tóquio, Japão) connected to a binocular optical microscope (Olympus BX50, Tóquio, Japão) with an original magnification of 4X and saved on a computer. Histomorphometric analysis was performed using Image J 1.48 software. The software allowed calculating the area of bone formation. The total area of the surgical defect was measured in mm^2, based in the right and left margins, following the "L" markings. A 5-mm length was determined for the surgical defect and 1-mm was added considering the trephine oscillation, completing a total of 6-mm length. The height of total area of the defect was established

according to the thickness of the skullcap. The area of new bone present in the delimited total area was measured in mm^2. The values were converted in percentage in relation to the total area of the defect.[10]

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (Graph Pad software Inc., La Jolla, California, USA). Data were submitted to normality test of D'Agostino & Pearson. Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's test ($p < 0.05$).

RESULTS

Representative microscopic sections of specimens of each group at 4 and 8 weeks are present in Figures 1 and 2, respectively. The mean, standard deviation and statistical differences of the percentage of bone formation at 4 and 8 weeks are represented in Table 1.

4 weeks

The qualitative microscopic analysis of the calvaria of the specimens of each group showed bone formation restricted to the margins of the defect. In all specimens the bone formation occurred in a centripetal way. The new-formed bone was less dense and more vascularized than the native bone, allowing the differentiation of the interface (Fig. 1 - B, D, F, H and J). For the groups CO, DOX and NAT, the bone formation in the margins was slight and the thickness of the tissue in the center of the defect was thinner (Fig. 1 - A, C and E). In the groups PAB and PAB + DOX, the bone formation was more evident and particles of autogenous bone was found in the center of the defect involved by connective tissue (Fig. 1 - G and I).

The histomorphometric analysis of the specimens at 4 weeks showed that the group PAB + DOX had the high percentage of bone formation (38.59 ± 3.09). Statistical differences were found between this group and CO and NAT, which presented the low values (19.52 ± 4.36 and 15.94 ± 6.67, respectively) ($p < 0.05$). In the other comparisons, no statistical differences were verified ($p > 0.05$).

8 weeks

At this period, the qualitative analysis of each group showed more intense bone formation, still restricted to the margins of the defect. The groups CO, DOX and NAT presented the less intense bone formation with thin thickness in the center of the defect (Fig. 2 - A, C and E). The groups PAB and PAB + DOX had a high percentage of new bone formation in relation to the anterior period of analysis. In this period the particles of autogenous bone could be still identified in the center of the defect (Fig. 2 - G and I).

The histomorphometric analysis showed that the group PAB + DOX had the high percentages of new bone formation (47.86 ± 13.96). The lower values were found for NAT group (15.89 ± 4.91). Statistical differences were verified between PAB + DOX and CO, NAT and DOX groups ($p < 0.05$). Statistical differences were also verified in the comparison between the groups PAB and NAT ($p < 0.05$). In the analysis of the groups in relation to the periods (4 to 8 weeks), no statistical differences were found ($p > 0.05$).

DISCUSSION

The study evaluated the effect of the addition of 10% doxycycline in gel form and particulated autogenous bone in the repair of critical-size defects created in rat calvaria. Evaluation of bone repair, is essential and the conditions need to be ideal to allow the new formation of cortical and medullar bone when a critical sized defect is present.[3,15] Several models of study have been proposed to evaluate bone repair.[16] The calvaria provide a site with considerable area of cortical and medullar bone, easy surgical access, low action of muscular contraction and mechanical loads, minimizing the risk of fracture and interference in the repair.[14,15,17]

In the current study, the diameter of the defects was 5-mm. Divergences are found in the literature regarding the diameter for critical-size defects.[10,14] Independently of the diameter, the defect created should have a diameter sufficient to not allow spontaneous repair during the lifetime of the animal[2] or the entire laboratorial experiment.[3] In the study, no complete repair was observed for any group, indicating that the diameter of 5 mm was sufficient to create a defect to be considered as critical-size.[17]

The ability of doxycycline to inhibit osteoclastogenesis and collagenolytic enzymes (responsible for degradation of connective tissues and bone resorption) and induces apoptosis of osteoclasts, encouraged several authors to evaluate the effect of this antibiotic on bone repair.[8,10,18] In the study, the doxycycline was applied directly in the defect with a concentration of 10% as previously reported[10] or added to particulated autogenous bone. Doxycycline alone showed lower bone forming ability (26.65% and 22.05% at 4 and 8 weeks, respectively) in comparison with the group in which doxycycline was associated with autogenous bone (38.59% and 47.86% at 4 and 8 weeks, respectively). These results indicate that the use of bone particles together with doxycycline enhanced the healing ability.[1] The presence of particles of bone in the center of the defect has two main functions: the physical action as a scaffold to allow osteoconduction and the biological action of providing osteogenic cells. When the autogenous bone acts as scaffold, it prevents invagination of connective tissue from the surgical flap into the center of the defect[1,10] as evidenced by the thickness of the calvaria in the defects. The thickness is markedly thinner in the center of the defect in the groups in which autogenous bone was not used. Conversely, in the groups in which the particles of bone were applied, the thickness of the calvaria was maintained in all extension, suggesting that the bone particles acted as a scaffold.[10] This is in accordance to a previous report, where doxycycline applied directly in the defect presented higher values than the defects left untreated (filled by blood clot).[10] In the current study, doxycycline alone (26.65% and 22.05% at 4 and 8 weeks, respectively) showed high percentages of bone formation in comparison to the control group (19.52% and 18.80% at 4 and 8 weeks, respectively). These results suggest that the doxycycline had a positive effect on bone repair of the critical-size defects.[10]

Natrosol gel was used as a vehicle for doxycycline.[10] This substance is an inert agent, soluble in water.[19-21] As an inert substance, no effect on the repair was expected. The results showed that the specimens of the natrosol group (15.94% and 15.89% at 4 and 8 weeks, respectively) had low percentage of bone formation than that of doxycycline group (26.65% and 22.05% at 4 and 8 weeks, respectively). The values found suggest that the natrosol really acted as an inert vehicle and the doxycycline was the component inductor of bone formation, corroborating with the previous findings.[10]

The addition of 10% doxycycline in natrosol gel with autogenous bone was effective in increasing the percentage of bone formation in the critical-size defects at 4 and 8 weeks of analysis. Statistical differences were found among this group (38.59%) and control (19.52%) and natrosol (15.94%) at 4 weeks and among this group (47.86%) and control (18.80%), doxycycline (22.05%) and natrosol (15.89%) at 8 weeks. When autogenous bone was applied without doxycycline, the percentage was lower than with doxycycline, suggesting that the doxycycline increased the ability of bone repair of the autogenous bone.[10] The results are in agreement with those reported in literature.[7,22-24] The specific processes associated with this finding are probably related with the properties of doxycycline in inhibiting collagenolytic enzymes and osteoclastogenesis and induce the apoptosis of osteoclasts.[7,10,18,25]

The perspectives of therapeutic use of doxycycline to improve the process of bone repair are wide.[10,23,24] In critical-size defects, the complete repair requires the aid of a graft material[2] and the activity of 10% doxycycline in association with autogenous bone was very effective for bone healing of this type of defect, corroborating with previous finding.[10] Nevertheless, other graft materials still have to be tested in association with doxycycline and in other surgical sites to ensure the efficacy of this therapy.

CONCLUSION

The use of 10% doxycycline in gel form with particulate autogenous bone significantly improved bone healing in critical-size defects in rat calvaria.

ACKNOWLEDGEMENTS: The State of São Paulo University (UNESP – Araçatuba) for support laboratorial process.

DISCLOSURE: The authors claim to have no financial interest, either directly or indirectly, in the products or information listed in the paper.

APPROVAL: Ethical approval in the Animal Ethics Committee of Federal University of Alfenas - 590/2014

ROLE/CONTRIBUTION OF CO-AUTHORS:

Ribamar Lazanha Lucateli - Contributed to conception and design, contributed to analysis, drafted the manuscript, critically revised the manuscript.

Marina Angélica Marciano - Contributed to analysis, drafted the manuscript, critically revised the manuscript.

Sabrina Ferreira - Contributed to analysis, drafted the manuscript, critically revised the manuscript.

Idelmo Rangel Garcia Júnior - Contributed to analysis, drafted the manuscript, critically revised the manuscript.

Josette Camilleri - Contributed to analysis, drafted the manuscript, critically revised the manuscript.

Ronaldo Célio Mariano - Contributed to conception and design, contributed to analysis, drafted the manuscript, critically revised the manuscript.

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LEGENDS

Fig. 1 – Representative sections of the studied groups at 4 weeks of analysis. It is possible to notice a slight new bone formation limited to the margins of the defect for CO (A), DOX (C) and NAT (E) groups. In the magnification images (B, D, F and J), the interface between native bone (red asterisk) and new-formed bone (black asterisk) is evident. High degree of new formed bone can be observed in PAB (G) and PAB + DOX (I) groups. The particles of autogenous bone (H – black asterisk) can be seen in the center of the defect in these groups. In the high magnification images (B, D, F and J), it is evident the aspect less dense of new-formed bone (black asterisk) in comparison with native bone (red asterisk). (A, C, E, G and I – 4x mag; B, D, F, H and J – 40x mag).

Fig. 2 – Representative sections of the studied groups at 8 weeks of analysis. In the low magnification images, it is possible to observe more evident new bone formation in comparison with the period of 4 weeks. In CO (A), DOX (C) and NAT (E) groups, it is noticeable that the bone formation was not so intense as that presented by PAB (G) and PAB + DOX (I) groups. Moreover, the thickness of the calvaria was thinner for the groups in which autogenous bone was not applied (A, C and E). In the high magnification images (B, D, F and H) the interface between native bone (red asterisk) and new-formed bone (black asterisk) can be seen. (A, C, E, G and I – 4x mag; B, D, F, H and J – 40x mag).

Table 1 – Mean and standard deviation of the percentage of the corresponding area of new bone formed after 4 and 8 weeks for each studied group. The lower case in each column represents statistical differences among groups in each period ($p < 0.05$). The capital letters in each line indicate the statistical differences in a same group between 4 and 8 weeks ($p < 0.05$).