Effect of anti-inflammatory agents on the integration of autogenous bone graft and bovine bone devitalized matrix in rats

Efeito de antiinflamatórios na integração de enxerto ósseo autógeno e de matriz óssea bovina desvitalizada em ratos

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ABSTRACT

Objective: To study the repair of bone defect filled with autograft or bovine bone devitalized matrix in rats under anti-inflammatory action. Methods: Two hundred and forty Wistar rats were distributed to two groups of 120 animals each. A 2mm-diameter defect was created in the femoral diaphysis. Animals of Group I had the bone defect filled with autograft and those of Group II, with bovine bone devitalized matrix. Animals of each group were redistributed to four subgroups according to the intramuscular administration of anti-inflammatory drug or saline solution: A – diclofenac sodium; B - dexamethasone; C - meloxicam; D - saline solution. Evaluation periods were 7, 14 and 30 days. Histological evaluation consisted of quantifying the inflammatory process, the bone neoformation, the collagen formation and the presence of macrophages. Results: Animals of Group I did not show significant difference considering inflammatory reaction. Significant and progressive increase of bone neoformation was observed in both groups. The animals that received meloxicam and autograft showed less collagen formation at 14 and 30 days. The number of macrophages was higher in Group II than in Group I. The animals treated with dexamethasone and saline solution did not show statistically significant difference. Conclusions: Diclofenac sodium and meloxicam delayed bone graft repair and dexamethasone did not interfere in it.

Key words: Bone Transplantation. Anti-Inflammatory Agents. Rats, Wistar.

RESUMO


Introduction

In orthopedics, repair of bone defects is frequently difficult because a fast growth of connective tissue inside them impedes the formation of bone tissue, making bone graft the classical method of treatment in anatomofunctional reconstruction\(^1\). However, the use of autogenous bone graft presents as limitation an inadequate amount when the donor source is scarce.

Studies on bone graft and reparative osteogenesis have been focused on the search for substitutes for autogenous bone or pharmacological factors and agents that interfere in such processes. Bovine devitalized matrix is one of the options; when used to fill bone defects, it acts as a mold for fibroblast cells of the host bone to invade and differentiate in cartilaginous and bone tissues\(^5\).

A comparative study on implants of devitalized bovine bone, autogenous bone, coral hydroxyapatite and castor-oil polyurethane in standardized bone defects of femoral condyles in rabbits demonstrated that although bovine devitalized matrix implant has osteoconductive property, it causes more intense inflammatory reaction and presents minor osteogenic potential as well as slower bone tissue recovery, when compared to autogenous bone graft, which demonstrated greater osteogenic potential and less intense inflammatory reaction\(^1\).

The action of non-hormonal anti-inflammatory (NHAI) agents is based on the inhibition of prostaglandin synthesis which, besides producing analgesic and anti-inflammatory effect, decreases the number of macrophages, fibroblasts and collagen fibers\(^8\), as previously demonstrated in studies about the healing of several tissues such as abdominal wall, alimentary canal, muscles, cartilages and bones\(^7\).

The daily use of diclofenac sodium may impair bone integration. Experiments with mice demonstrated that the use of diclofenac sodium interfered in the fractures remodeling, leading to instability of the bone callus\(^6\). Meloxicam, another NHAI agent, increased neutrophils flow and decreased osteoclasts on the seventh day of observation\(^11\). Tenoxicam presented inhibitory effect on osteogenesis. In osteogenesis, steroidal anti-inflammatory agents have been considered inhibitors of phosphatase-kinase\(^1\), an essential enzyme for the triggering of osteoblasts agents have been considered inhibitors of phosphatase-osteogenesis. In osteogenesis, steroidal anti-inflammatory agents have been considered inhibitors of phosphatase-kinase\(^1\), an essential enzyme for the triggering of osteoblasts differentiation in cartilaginous and bone tissues\(^5\).

The use of anti-inflammatory drugs in orthopedics as well as in traumatology during immediate post-operative period, the absence of papers in literature about the effect of such medicines on bone repair using graft, and the result of research indicating that bovine bone devitalized matrix in spite of presenting osteoconductive potential leads to slower tissue repair and more intense inflammation stimulated the present investigation, which used a standard experimental model and aimed at histologically studying the repair of a bone defect that was created in rat femoral diaphysis and filled with autogenous bone graft or bovine bone devitalized matrix under the action of anti-inflammatory agents.

Methods

The present experiment used 240 adult male Wistar- EPM rats (weighed 250–300g, aged 78–90 days old), which were randomly distributed to two groups: Group I, in which the standardized bone defect was filled with autogenous bone graft, and Group II, in which the standardized bone defect was filled with bovine bone devitalized matrix.

Both groups were redistributed to four subgroups of 30 animals each, according to the anti-inflammatory agent used: A - diclofenac sodium (Voltaren®), 2mg.Kg\(^{-1}\) a day; B - dexamethasone (Decadron®), 0.5 mg.Kg\(^{-1}\) a day; C - meloxicam (Movatec®), 0.5 mg.Kg\(^{-1}\) a day; and D - isotonic saline solution as control. The anti-inflammatory drugs and the saline solution were intramuscularly (IM) administered at the immediate post-operative period and for six consecutive days.

Intraperitoneal injection of pentobarbital sodium (Tiopental®), at the dose of 50mg.Kg\(^{-1}\), was used as anesthetic procedure. The animal was positioned in left lateral decubitus in order to let its right inferior member exposed for the operation. For antisepsis, 2% iodized alcohol and sterile operative cloth were used; then, longitudinal incision was made in the lateral surface of the right thigh (30mm length), from the saliency of the right lateral femoral condyle, aimed at reaching the skin and subcutaneous cells.

After rupture of the muscle and exposure of the right femoral diaphysis, an incision was made and the peristomeum was delicately removed by using a bistoury and a metallic trephine (2mm diameter) coupled to a low-rotation electric perforator; the lateral cortical layer was crossed, reaching the marrow layer, thus creating the standardized defect.

As it is a cortico-spongy tissue, the bone content removed was fragmented during the operation. It was collected to immediately fill the defects in animals from Group I. Bone defects of animals from Group II were filled with bovine bone devitalized matrix.

The muscular plan was closed by using 4-0 catgut interrupted simple stitches and the skin by using 4-0 polyamide monofilament thread and continuous suture; then the anti-inflammatory drug or the saline solution was IM administered at the immediate post-operative period and for six consecutive days, comprising seven days of treatment.

After daily clinical observation for 7, 14 and 30 days, ten animals of each subgroup received anesthesia similar to that received during operation, had the right posterior limb disarticulated and the femur dissected. Euthanasia was obtained by the continuous inhalation of ethyl ether under fume hood.

The pieces were immersed in individual flasks containing 10% buffered formaldehyde and sent to the Department of Pathological Anatomy, São Paulo State University (UNESP), Botucatu, for histological processing. From each block, three slides were prepared: the first was stained with Hematoxylin and Eosin (HE) for quantitative analysis of the inflammatory reaction and bone neof ormation; the second was stained with Picrosirius Red for analysis of the presence of collagen; and the third was prepared for macrophages identification through immunohistochemical test.

In the qualitative analysis under optical
microscopy, the presence of inflammatory cells like neutrophils, fibroblasts and macrophages in the area of the defect filled with graft was considered as inflammatory reaction; bone tissue surrounded by osteoblasts evidencing osteoblastic activity was considered as bone neoformation.

The quantitative analysis was carried out using a computerized program of image analysis (ImageLab 2.3), based on spectrophotometry principles.

Two sites were analyzed in each slide, allowing the calculation of the arithmetic mean of two parameters: the percentage of the area occupied by the parameter to be analyzed and the light optical density reflected by that parameter. Quantitative measures for the statistical analysis were obtained using Kruskal-Wallis analysis of variance to compare the variables’ values at 7, 14 and 30 days for each anti-inflammatory drug in each group separately. The same analysis was used to compare data among the anti-inflammatory agents at each experiment day in each group. The Mann-Whitney test was used to compare the values between Group I and Group II for each anti-inflammatory drug at each day of the experiment. Nullity hypothesis rejection level was set at 5%.

**Results**

More intense inflammatory reaction was observed at all periods in the animals of Group II, in which bone defect was filled with bovine bone devitalized matrix (Figures 1 and 2). Statistical analysis showed there was no significant difference regarding inflammatory reaction among subgroups whose defects were filled with autogenous graft. However, among subgroups whose defects were filled with bovine devitalized matrix, there was less intense inflammatory reaction in animals that received dexamethasone and saline solution (Figure 3).

A significant and progressive increase of bone neoformation was observed in both Groups I and II at all periods analyzed, indicating bone neoformation had a time-dependent aspect, regardless the use of anti-inflammatory agents. In Group II, the animals that received diclofenac sodium and meloxicam presented less neoformation, but the animals treated with dexamethasone and saline solution showed no significant difference at 14 and 30 days, when comparing Groups I and II (Figure 4).
Collagen formation was time-dependent and was significantly high at all periods studied (Figures 5 and 6). The animals that received meloxicam and autogenous graft showed less collagen formation than the other subgroups at 14 and 30 days. Those that received diclofenac and bovine devitalized matrix presented less collagen concentration at all periods analyzed. The group that received dexamethasone and autogenous graft had less collagen formation than the group that received dexamethasone and bovine bone devitalized matrix at 7 days (Figure 7).

FIGURE 4 - Quantification of bone neoformation in Groups I and II, and their respective subgroups A, B, C and D (values are expressed as percentages)

FIGURE 5 - Photomicrograph showing areas of collagen, marked in red by Picrosirius Red, zoom 100 X, in animals of Group I, at 7 days (first column), at 14 days (second column) and at 30 days (third column)

FIGURE 6 - Photomicrograph showing areas of collagen, marked in red by Picrosirius Red, zoom 100 X, in animals of Group II, at 7 days (first column), at 14 days (second column) and at 30 days (third column)
The immunohistochemical study for macrophages identification (Figure 8) showed a higher amount of macrophages in Group II (in animals treated with diclofenac and meloxicam) than in Group I at 7 and 14 days. There was no statistically significant difference between Groups I and II for animals treated with dexamethasone and saline solution. At 30 days, no group presented macrophages (Figure 9).

Discussion

Rats were used in the present experiment because they can be easily obtained and handled, and show anatomical and biological similarities to humans, such as the pharmacokinetics of NHAI agents and the binding of medicines to plasma proteins, which makes rat the appropriate animal model for comparison of NHAI drugs effects as well as for clinical prognostics. Also, comparative studies among several animal species have established that the pharmacokinetic profile of male rats is the most similar to that of humans.

For homogeneity, only male rats were used, avoiding the factor of estrous cycle inherent to females.

The correction of bone defects with substance loss has in the autogenous bone graft the ideal pattern (gold standard). Its use, however, has some limiting factors such as the amount of material available in patients with low reserve of autogenous bone (children and elderly) or previously subjected to operative procedures using such reserve.

To fill bone defects as well as to induce bone formation, several synthetic products have been used such as morphogenetic protein, bone devitalized matrix, castor oil polymer, and coralline hydroxyapatite. These synthetic materials present varied degree of integration according to the implanted site and the animal studied. However, when compared to autogenous graft, those products have not been efficient enough as bone substitutes.

In an attempt to solve such problem, materials of biological origin, known as biomaterials, have been increasingly used to fill bone cavities due to their osteoinduction and/or osteotransport properties in a procedure denominated guided bone regeneration.

Among grafts derived from biological sources are coral hydroxyapatite, castor oil polyurethane, and inorganic bovine matrix. Figueiredo compared the osteointegration of autogenous bone graft, coral porous hydroxyapatite, castor oil polyurethane and inorganic bovine matrix in femoral bone defects in rabbits, and observed more intense inflammatory reaction and more reabsorption of the graft in defects filled with inorganic bovine matrix.

The use of bovine bone devitalized matrix is advantageous due to its easy obtaining, great amount of material available for graft, and high osteogenic capacity, serving as a mold for bone neoformation. It is a xenogenic, microporous, natural hydroxyapatite with physical properties appropriate for filling cavities; it is adaptable to the irregularities of the implant site, allowing good bone juxtaposition in the repair of defects and facilitating bone growth. Besides, it has low production cost and can be stored at room temperature. At bone implantation, it triggers intense inflammatory reaction and can be partially or completely removed from the receptor site through a slow process of surface reabsorption.

Reparative osteogenesis of bone defects, with or without the use of bone graft, can be influenced by biological
The administration period of the NHAI agents was established as 7 days based on previous studies using experimental animals involving healing20. In biomedical literature, there is not any paper about its application in the study of organic bone matrix19. For its quantification, the direct visualization principle and the staining method with Picrossirius Red F3BA derived from picric acid were used. This method is easy, efficient for material collection, subsequent filling 3, 47, 48, 50 in control animals of Group I. The technique was easy, efficient for material collection, and adequate for filling the standardized defects. Besides, it could be easily replicated.

The inorganic bovine bone matrix easily filled the standardized defects. It was obtained from lyophilized bovine bone, which is marketed as bars composed of small, medium or big sterilized particles. In the present study, the bars used were composed of small particles and were fragmented to allow appropriate filling of the defect.

All groups had weight gain, which indicated that there was no compromising of the animals’ health in spite of the operation and the use of implants and drugs.

To evaluate the action of the anti-inflammatory agents on the bone healing process, representative histological parameters were obtained at 7, 14 and 30 days. For data analysis, a histological study was carried out and included slides stained with hematoxylin-eosin for quantification of the inflammatory process and the neoformed bone. Another parameter analyzed was the formation of collagen, which is the main component of the bone organic matrix19. For its quantification, the direct visualization principle and the staining method with Picrossirius Red F3BA derived from picric acid were used. This method is easy to use, selective, allows ready interpretation, and is frequently employed in works involving healing20. In biomedical literature, there is not any paper about its application in the study of organic bone matrix, which is an important component of the bone and, therefore, a relevant parameter to be analyzed when studying bone regeneration.

Macrophages quantification was considered relevant due to the importance these cells have in a certain moment of the healing process, when they generate a stimulus and regulate the proliferation process, modulating stimuli that result in the collagen synthesis23. An immunohistochemical method was adopted for macrophage identification and consisted of antigen-antibody reaction using a standard technique. The antigen CD68, found in the macrophage cytoplasm, was detected by the
monoclonal antibody HAM56; to identify the reaction, a
discloser that stains the cellular bodies dark-brown was
used. Experiments involving healing had already used
such method for macrophage identification; however, all of
them involved healing of the abdominal23 wall. A study was
carried out in mice to explain the pathogenesis of bone
reabsorption associated with periapical lesion, and could
quantify macrophages and verify their prevalence until the
fifth day of observation as well as the prevalence of
osteoblasts after that period. However, a different method
was used to identify those cells19.

All those parameters were quantified by using a
computer program developed to allow identification,
selection and subtraction of structures of the same image
through spectrum of 32 x 1,000 shades of colors, defining
the morphologic patterns in the area and the perimeter for
the calculation of the form factor, which was obtained from
the relationship between those two variables25. Such method
is already well established and has been used in experimental
studies involving abdominal wall healing and bone
regeneration1.

More intense inflammatory reaction was observed
at all periods in animals whose bone defects were filled with
bovine bone devitalized matrix. Such data corroborated
those from literature1 which demonstrated intense
inflammatory reaction triggered by the presence of bovine
bone devitalized matrix.

There was a significant and progressive increase
in bone neoformation in both Groups I and II at all periods
analyzed, suggesting it was time-dependent, regardless the
use of anti-inflammatory drugs.

Collagen formation was also time-dependent and
significantly high at all periods studied, suggesting that
NHAI agents interfered in such process both in the group
that received autogenous graft and in the group that received
bovine bone devitalized matrix.

At 7 days, the group that received dexamethasone
and autogenous graft showed less collagen formation than
the group that received dexamethasone and bovine bone
devitalized matrix, which demonstrated that the corticoid
interfered in the collagen synthesis only in the initial phase
of regeneration.

Studies have shown that corticoid impairs the
formation of bone collagen20, 26, indicating the need for
additional studies aimed at identifying the type of collagen
formed. The organic bone matrix consists of collagen Type
I (95%), and the cartilage is mainly composed by collagen
Type II, and as the corticoid does not interfere in the
formation of cartilaginous collagen, further investigation
on the type of collagen formed could contribute for a better
understanding of that result.

The immunohistochemical study for macrophage
identification showed no statistically significant difference
between animals of Groups I and II that were treated with
dexamethasone and saline solution. At 30 days of evaluation,
no group presented macrophages because these cells are
part of the healing initial phase. Similar results were obtained
in an experimental study on bone repair carried out by Lin
et al34, in which there was macrophage prevalence at 5 days
and osteoblast prevalence at 15 and 20 days, a later stage
of bone repair.

Higher macrophage concentration was observed
in animals of Group II that were treated with diclofenac and
meloxicam probably because the bovine bone devitalized
matrix caused significant inflammatory reaction, delaying
the bone repair process.

Some works in literature suggested that NHAI
agents interfere in the bone repair10, 21. Sell et al18 reported
that the use of diclofenac sodium, at a daily dose of 50mg,
impaired bone integration of non-cemented prostheses in
humans, since it interfered in the proliferation and
metabolism of fibroblasts and osteoblasts, accelerating the
differentiation of the former and the inhibition of the latter.

Akman et al19 studied the effect of diclofenac
sodium, IM administered at daily doses of 1mg and 2mg, on
the repair process of tibia closed fracture in 55 Wistar rats
that were sacrificed after 2, 4 and 6 weeks for clinical-
radiological and histological analyses. After two weeks,
bone callus in control group animals was more stable than
that in animals treated with diclofenac sodium. Radiological
and histological analyses did not show any difference
among groups in the subsequent periods. Yugoshi et al25
investigated the interference of diclofenac administration
in the alveolar repair process. They used 42 Wistar mice,
out of which 21 received diclofenac sodium, at a daily dose of 10mg.Kg⁻¹ a day) and
the remaining received saline solution. The animals were
sacrificed at 7, 14 and 21 days. The progressive formation
of bone and the reduction of clot volume and connective
tissue from 1 to 3 weeks after dental extraction were
quantified through histometric method using the point
counting technique. Treatment with diclofenac caused
significant delay of bone neoformation, associated with the
delay in the clot organization and remission.

Giordano Neto21 studied the effect of tenoxicam, a
non-hormonal anti-inflammatory inhibitor of COX-2, and
observed that such drug presented inhibitory effect on the
osteogenesis, which could be related to the treatment
precocity and to the dose used. However, studies have
demonstrated that meloxicam can decrease bone
reabsorption by 43% in periapical lesions since they occur
due to COX-2 production.

In biomedical literature, there are no studies about
the effect of corticoids used for a short period on bone
regeneration. The harmful effects of long-lasting use of
corticoids on bone are well known and documented.
Researchers carried out a biomechanical study of bone graft
repair in rabbits with metilpredinisolone during six
months and observed significant deterioration of the graft
mechanical properties, when compared to the control group.
The main role of corticoids in osteoporosis induction and
bone formation decrease is to inhibit the enzyme
phosphatase-kinase128, which is essential to induce
osteoblast proliferation and is time-dependent.

Control groups presented better results than the
groups treated with NHAI drugs, which corroborate data
from previous works that demonstrated the interference of those drugs in reparative osteogenesis.

The animals treated with dexamethasone and saline solution presented no statistically significant difference, which suggested that dexamethasone did not interfere in the physiological process of bone repair because such result was similar to that observed in the subgroup that was not treated with anti-inflammatory drugs.

The methodology used in the present experimental model (such as collagen and macrophage quantification) to study the effect of anti-inflammatory agents on bone repair was not found in literature. Even studies on inflammatory process and bone neoformation did not use the quantification of those parameters but employed a qualitative analysis, which hindered the confrontation of the findings of the present research with the ones from literature; however, the present results suggest many hypotheses for future investigation on bone repair under drug action.

The present study showed that NHA1 agents (diclofenac sodium and meloxicam), despite their anti-inflammatory action, were not effective in reducing inflammatory reaction triggered by the implantation of devitalized bone matrix and, thus, did not contribute to a better integration of such graft. Dexamethasone did not interfere in the process as it led to results similar to those obtained in the control group.

Further studies are needed, therefore, to better explain the integration of inorganic bone matrix, which produces intense inflammatory reaction at the implantation site. It opens up the perspective of using other types of anti-inflammatory, chelant or cytostatic drugs.

Conclusions

The non-hormonal anti-inflammatory agents diclofenac sodium and meloxicam delayed the repair of autogenous bone graft and bovine bone devitalized matrix.

The anti-inflammatory steroid dexamethasone did not interfere in the repair of autogenous bone graft and bovine bone devitalized matrix.

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