ORIGINAL ARTICLE

EFFECTS OF CHEMICAL PROCESSING AND OXIDE ETHYLENE STERILIZATION ON CORTICAL AND CANCELLOUS RAT BONE: A LIGHT AND ELECTRON SCANNING MICROSCOPY STUDY

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ABSTRACT

Objective: To evaluate, under microscopic examination, the structural changes displayed by the trabecular and cortical bones after being processed chemically and sterilized by ethylene oxide. Methods: Samples of cancellous and cortical bones obtained from young female albinus rats (Wistar) were assigned to four groups according to the type of treatment: Group I- drying; Group II- drying and ethylene oxide sterilization; III- chemical treatment; IV- chemical treatment and ethylene oxide sterilization. Half of this material was analyzed under ordinary light microscope and the other half using scanning electron microscopy. Results: In all the samples, regardless the group, there was good preservation of the general morphology. For samples submitted to the chemical processing there was better preservation of the cellular content, whereas there was amalgamation of the fibres when ethylene oxide was used. Conclusion: Treatment with ethylene oxide caused amalgamation of the fibers, possibly because of heating and the chemical treatment contributed to a better cellular preservation of the osseous structure.

Keywords – Bone transplantation; Ethylene oxide; Microscopy, electron; Sterilization; Rats

INTRODUCTION

Modern orthopedics has increasingly been using bone grafts or its substitutes for the treatment of complex conditions such as severe trauma with bone loss, arthroplasty revision, hip dysplasia surgery, and orthopedic oncology, among others[1,2].

There are many options available for several types of bone grafts, with grafts of autologous origin being the ideal[3], but sometimes there is insufficient bone for use in large substitutions, when the donor area has been depleted by previous crops, when the donor bone or the patient are small (pediatric patients), or if there are any constitutional illnesses that affect the integrity of the donor bone. In this context, frozen cadaver bone banks have appeared, and are already common in large cities, but require expensive infrastructure and labor, which often limits the access of smaller centers and/or those with fewer resources, besides not providing absolute assurance against the transmission of infectious diseases[4,5].

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However, the bone can undergo chemical and/or physical processes in order to ensure sterilization and to allow for cheaper methods of preservation in order for it to be used as a graft while preserving its osteogenic capacity[6].

Irradiation has been the most common form of sterilization and one of the most acceptable methods of decontaminating human tissue[7]. Another option is autoclaving, recommended as an alternative process. The influence of preservation and sterilization in the biological performance of bone grafts, as well as their mechanical and immunological properties, have been investigated in previous studies[8].

The chemical processing with dehydration in absolute alcohol, ethyl ether degreasing, and bleaching in hydrogen peroxide, followed by sterilization with ethylene oxide, developed by Volpon[9,10], has been shown in experimental studies to constitute grafts with osteoconductive capacity, and is therefore an alternative to autologous bone graft, although with lower performance. This type of graft has the advantage of being of low cost, since it can be prepared in a basic laboratory routine, sterilized with ethylene oxide, stored at room temperature, and can be transported without any special equipment.

The potentiality of this graft has been tested on animals, both in terms of biological performance and for mechanical strength[10,11]. This last feature is interesting to study because it may be desirable that the grafted bone has the strength to allow for the insertion of implants, or to resist local mechanical stresses[10]. Voggenreiter et al.[12] showed that bone preparation methods can affect the mechanical strength by altering the collagen or through the production of cracks. Haje et al.[9] studied the microstructural changes caused by the processing of the graft described here, finding amalgamation and the production of microcracks. However, they did not identify which of the steps in the processes employed were responsible for the changes. In addition, these authors investigated only the cortical bone and, in practice, cancellous grafts and cortico-cancellous grafts are the ones most often used.

Thus, the study of changes in the microstructure of the bone caused by the processing methods is of interest not only as a way to increase knowledge about the bone to be implanted, but also from the practical point of view in the sense of anticipating any limitation imposed by the preparation of the graft.

The present study aims to investigate, from the image point of view, the possible changes in bone caused by a sequence of chemical treatment (alcohol, hydrogen peroxide, and ethyl ether) with or without ethylene oxide sterilization. It is our hypothesis that the two treatment processes produce different effects on bone.

METHODS

Eight young albino female rats from routine disposal of the Ribeirão Preto School of Medicine Bioengineering Laboratory, USP, were used, with a mean weight of 250 g. These animals were killed according to the recommendations established by the American Veterinary Medical Association[13] and, after euthanasia, the femurs were removed and cleaned of soft tissue. With the aid of metallographic cutter (Isomet Buehler, USA), three cross-sectional rings were obtained of the mediodiaphyseal area of the femurs, and a 1.2-mm thick frontal section was obtained from the metaphysioepiphyseal region. Specimens of the diaphysis served as a sample of cortical bone and fragments of the metaphysioepiphyseal region obtained were used to study the cancellous bone. These fragments were separated into four groups, each of which contained the two types of bone. The groups were established based on the type of treatment to which the bones were submitted:

Group I – Bone fragments simply dried in an oven (40°C);

Group II – Bone fragments dried in an oven (40°C) and sterilized with ethylene oxide;

Group III – Bone fragments chemically treated according to Volpon et al.[14].

Group IV – Bone fragments chemically treated in the same manner as in Group III, but sterilized with ethylene oxide, in the same manner as in Group II.

The bone fragments were preserved and subjected to processing, using a technique already described by Volpon et al.[14], whose basic steps include: removal of remnants of attached soft tissue, immersion in absolute alcohol for 48 hours, treatment with hydrogen peroxide 40 volumes until bleached and ethyl ether bath, with successive changes until supernatant fat droplets disappear. Again, the material was placed in absolute alcohol for residual dehydration and finally dried in an oven (40°C). The samples were then separated into their respective groups in equal portions of cortical and cancellous samples and packaged individually.

A routine hospital ethylene oxide sterilization

technique was used (2.0 kg of gas per cycle for three hours, followed by two hours each of aeration and hyperventilation at a temperature of 55°C, 0.5 kgf/cm^2 pressure, with -0.5 kgf/cm^2 vacuum in a SERCON® unit).

HISTOLOGICAL PROCESSING

The bone fragments from each group were separated into two halves. The first was reserved for study with a conventional light microscope, stained with toluidine blue and pyronin, and the second was allocated for study with a scanning electron microscope.

The processing of the material to make the slides was initiated by fixation in 10% buffered formaldehyde solution for 10 days, changed every two days. Next, there was dehydration in alkaline solution following a sequence of increasing alcohol concentration while stirring for four hours a day. Every two days the solution was replaced by a higher alcohol concentration. After dehydration, the specimens were embedded in acrylic resin (LR White, USA) stabilized with benzoyl peroxide, agitated for one hour, and refrigerated overnight. On subsequent days the samples were subjected to a cycle of agitation and placement in a vacuum oven (Shell Lab) to remove any bubbles, returning to refrigeration after the cycle. This procedure was performed for nine days, with a change of resin every 48 hours. On the tenth day, they were placed in Teflon molds and placed in an oven (60°C). When dried, the pieces went through a process of manual sanding, followed by polishing. The acrylic blocks with the bone fragments were cut into microtome cross-sections, glued to slides with resin, hand sanded and polished until the thickness reached 10 to 20 micrometers.

After polishing, the samples were stained with toluidine blue and pyronin. The material was examined and microphotographed in segments under a conventional light microscope (Leica Microsystems Nussloch GmbH, Germany), with an original magnification of 25x. Portions of the images were reconstituted into a single image using Adobe Photoshop®. The details of the regions of interest were examined under magnification of up to 100x.

Bone fragments were prepared specifically for examination under electron microscopy: 1 - dehydration in increasing concentrations of ethanol; 2 - drying in liquid carbon dioxide in a CPD 030 (Critical Point Dryer, Bal-Tec®, Germany); 3 - fixation with silver- and graphite-based conductive glue onto aluminum stubs to improve the flow of electrons; 4 - covered with a thin layer of 24 k gold in high vacuum using an SCD 050 (Super Cool Sputtering System, Bal -Tec®, Germany), making the samples reflective to the electron beam.

The specimens were examined and eletronmicrographed in 120-mm Fuji ACROS® ISO 100 black and white film under a scanning electron microscope (JSM-5200, Jeol®, Japan), and cortical and cancellous bone were analyzed in magnifications of 75x, 150x, and 350x. The overall look of the piece, the preservation of the global morphology, the trabecular bone, and surface features were observed at the lower magnifications. In the larger magnifications, details of the surface and trabecular bone were studied. The following comparisons were made:

a. Group I versus Group II (bone dried in an oven vs. bone dried and sterilized with ethylene oxide)

b. Group III versus Group IV (chemically treated bone vs. chemically treated bone sterilized with ethylene oxide)

c. Group I versus Group III (bone dried in an oven vs. chemically treated bone)

d. Group II versus Group IV (bone dried in an oven and sterilized with ethylene oxide vs. chemically treated bone sterilized with ethylene oxide).

RESULTS

Optical microscopy with conventional light

In terms of overall comparison, the results were quite similar among the specimens regardless of the type of treatment.

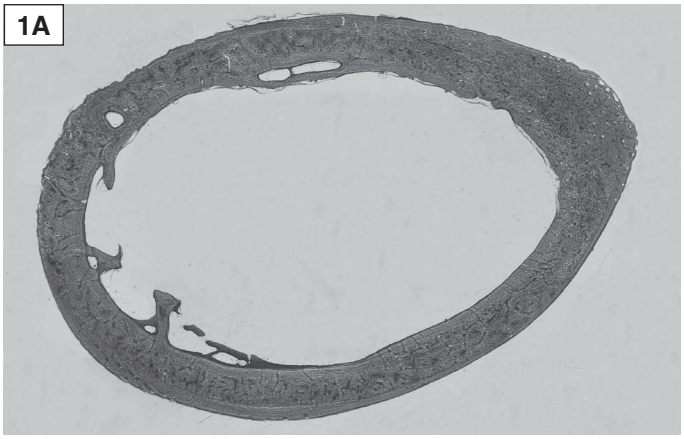
Transverse sections in the diaphyseal region produced bony rings with preserved morphology (Figure 1A). Under higher magnification, three layers were identified forming the bone cortex. The outer (subperiosteal) layer was composed of overlapping lamellae without any trabecular space and placed transversely to the longitudinal axis of the bone. The wider middle layer was composed of compact bone, with the lamellae arranged longitudinally, so that osteons formed by the central vessel, and concentric lamellae connected by a system of canaliculi between the osteocytic lacunae (Haversian system), which were seen both empty and with cellular content (Figure 1B), were clearly visible and typical in the cross section. In some regions, Volkmann system vessels appeared, and in others, the outer and inner layers were separated by cementing lines.

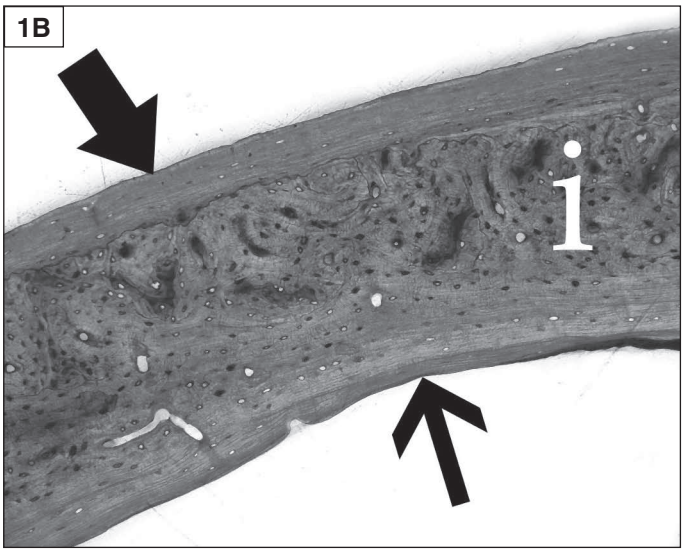
The inner layer (endosteal) was formed with the same distribution as that of the external layer, only more slender (Figure 1B).

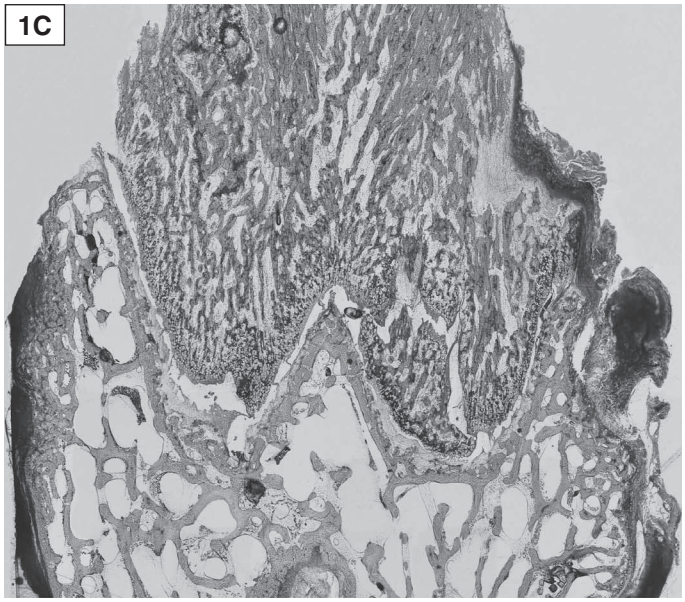
In the region corresponding to the linea aspera of the femur, the separation of three layers was less typical, predominantly bone with characteristics that were more similar to the intermediate layer.

Cancellous bone from the epiphysiometaphyseal segment had maintained the anatomy of the region, preserving the format and general structure of trabecular bone that, in the epiphysis, favored the longitudinal direction and, in the metaphysis, trabeculae were thicker and had a more random distribution (Figure 1C).

In the comparison between groups, what drew attention was that in groups III and IV there was preservation of cellular content, most typically illustrated in the trabecular bone (Figure 1D). Indeed, in groups I and II, there were virtually no cells or remnants of bone marrow, whereas in the first, osteoblasts arranged on the lamellae were abundant and residues of bone marrow were present.







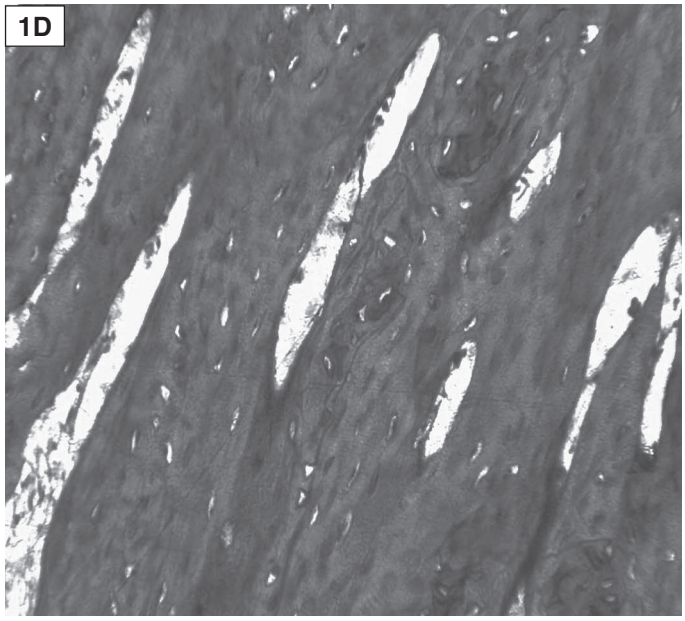


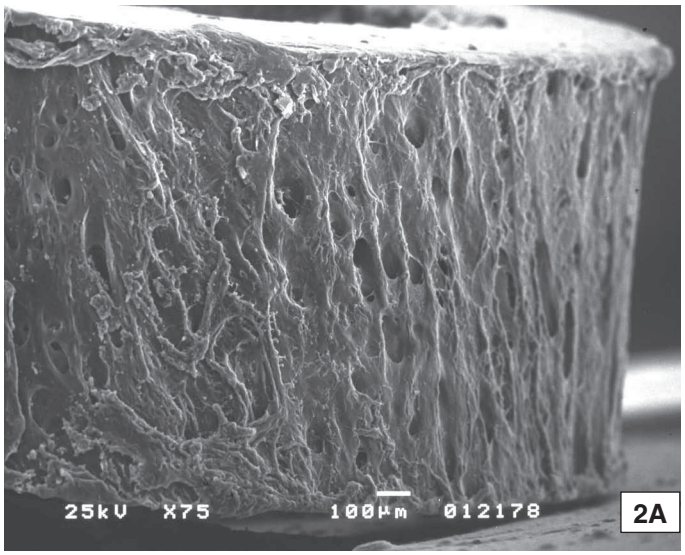
Figure 1 – Histological aspects of diaphyseal and epiphysiometaphyseal bone samples from chemical processing (Group III) examined under conventional light microscopy. The general morphology of the bone is maintained. A – sample of dry bone (Group I). B – detail of the previous sample illustrating the three layers that form the cortex: the outer layer presents compact bone with fibers arranged circularly (filled arrow). In the intermediate layer (i), lamellae are arranged longitudinally, which is evidenced by the numerous visible Haversian systems. The inner layer is narrower and presents the lamellae arranged circularly, as in the outer layer (narrow arrow). C – frontal view of the distal femur illustrating the general morphology. D – detail of same specimen illustrating the preservation of cellular content, seen as numerous osteoblasts on the lamellae. (A and C: 25x magnification; B and D: 100x magnification. Staining: toluidine blue and pyronin).

Scanning electron microscopy

In the lower magnifications, there was preservation of microarchitectural space in segments of both compact bone and cancellous bone (Figure 2).

In diaphyseal segments at 75x magnification, the outer surface of the cortex showed the typical fibrillar aspect perforated by vascular holes, whereas lamellar matrices, intertrabecular spaces, and vascular holes were predominant in cancellous bone (Figure 2).

In compact bone, especially at higher magnifications, all groups had microfractures on the surface. The greatest difference occurred in groups II and IV, which had amalgamation of the microfibers (Figure 3 A, B). This was not found in groups I and III.



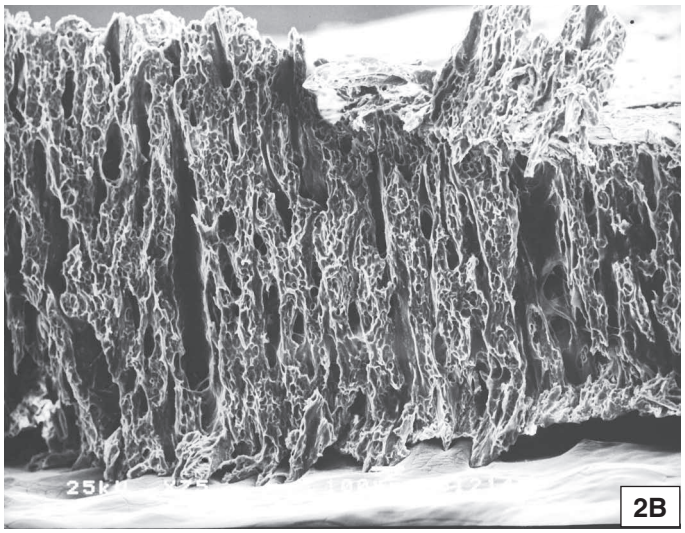
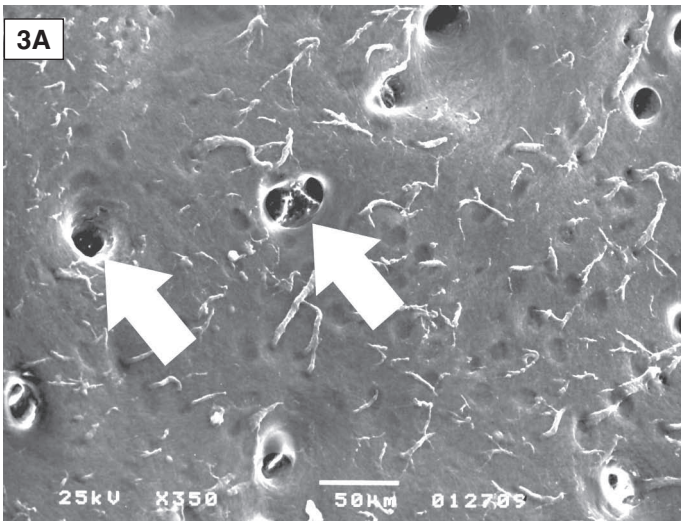
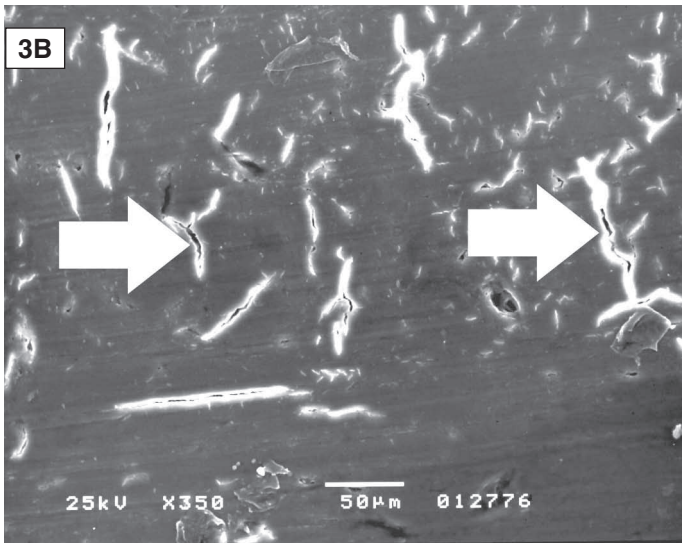
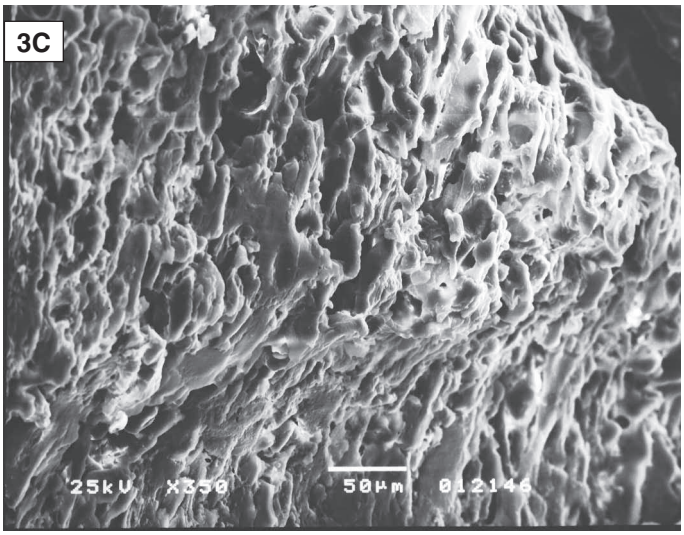


Figure 2 – Eletronmicrographs of diaphyseal (A) and metaphyseal (B) bone samples at 75x magnification. Both samples come from bones that were simply dried (Group I). Microarchitectural space is maintained for both types of samples.

With respect to cancellous bone, findings were similar to those of cortical bone in the samples of groups II and IV (Figure 3 C, D).







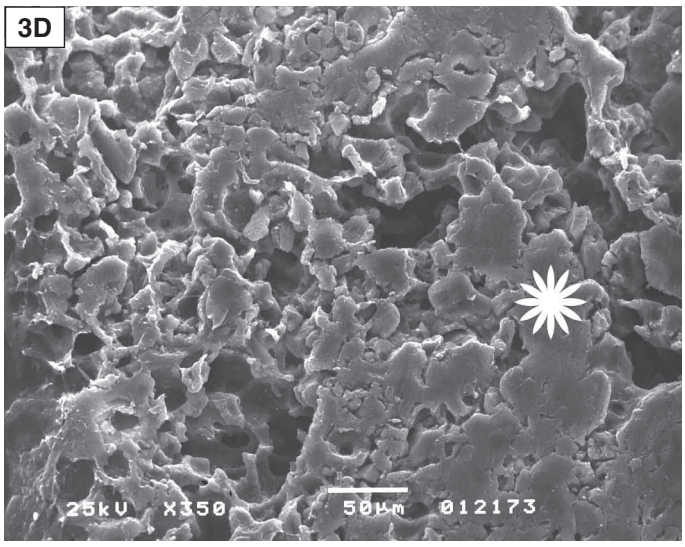


Figure 3 – Scanning eletronmicrographs of cortical diaphyseal bone and cancellous bone. A – In the dried cortical bone with no chemical treatment (Group I) the bone surface is preserved and has numerous vascular holes (arrows, 350x), while in the bone in Group II (dried and sterilized with ethylene oxide, Figure B) the surface is less preserved and holes are less clear (amalgamation). The microfractures are evident (arrows, 350x). C – Dried cancellous bone (Group I; 350x) D – Chemically treated cancellous bone sterilized with ethylene oxide (Group IV, 350x). The trabecular bone is less visible; there is amalgamation of fibers that lose their individuality and which appear swollen (asterisk).

DISCUSSION

The evolution of orthopedic surgery has made possible the preservation of severely affected limbs. Its contributions include greater damage control, a deeper understanding of the pathophysiological mechanisms of trauma, the development of microsurgical techniques, and infection control. Thus, more patients are surviving many of whom have bone disorders presenting consolidation that needs to be repaired.

More recently, there has been research to stimulate osteogenesis with bone substitutes[15,16], growth factors[17], and through the use of electromagnetic fields and ultrasonic stimulation[18,19]. However, bone grafts persist as the most commonly used option.

Among the grafts, autologous grafts are the most efficient[20] because there is no immunoincompatibility, but there may be limitations related to the amount available. The fact that additional surgery is required to harvest bone also causes increased morbidity[9].

Thus, new sources of grafting have been sought, the oldest of these being xenogenous bone, in which bone of a different species is used. Although there are some isolated favorable reports in the literature[21], this type of graft has the immune response as a limitation, which compromises its performance.

The homogenous (or homologous) graft is an intermediate option between the two previous options, since it involves two individuals of the same species that are more genetically similar. However, a disadvantage is the potential risk of disease transmission[14].

The chemical and physical processing of bone for grafting purposes reduces the possibility of transmitting infections and allows for easier storage and handling[6,21], but diminishes its osteogenic capacity.

Investigations with the graft prepared according to the technique described in this study showed good osteogenic performance by osteoconductive mechanism[14] and the possibility of production in block or even bone screws[22]. We seek to further our study, since the type of preparation used to treat bone has been shown to change its mechanical properties[11,22,23]. Other authors have reported that structural changes may occur on the surfaces of bones depending on the duration of exposure to ethylene oxide, or when another type of processing is used for sterilization[12,24,25].

In the present study, we sought to investigate which step of the process of graft preparation (chemical treatment or exposure to ethylene oxide) could be causing damage to the bone by changing its microstructure and mechanical properties, as has already been demonstrated[11,22,23], damage basically formed by amalgamation, cracks, and weakening to torsion. These findings may be clinically relevant to making the best graft selection and in machining bone in order to obtain implants with specific shapes.

Our results show that when only chemical processing was used, there was greater preservation of bone microstructure, including cellular content, which can be explained by the use of alcohol at the beginning of the preparation, which acts as a fixative. However, from an osteogenic standpoint, these cells are inactive because they are dead and such a graft acts only as an osteoconductor in the stimulation of bone formation[10]. This processing is important, because in addition to fixing and dehydrating, it promotes the removal of all fatty tissue. Kakiuchi et al.[6] showed that for ethylene oxide to actually sterilize the bone tissue, it is necessary that the water and fat tissue have been removed, so that gas can effectively penetrate

the bone tissue. In addition, dissolution in organic solvents such as ethanol reduces the antigenicity of the allograft[6].

Another point is that ethylene oxide gas is toxic and must be completely removed after bone sterilization, which is efficiently accomplished by appropriate ventilation, with residues of up to 100 parts per million for medium-sized samples (10-100g)[6]. However, caution should be taken when using large bone fragments, since, in these cases, sterility is less efficient and the technical parameters of the process should be adjusted[6].

The scanning electron microscopy findings showed that application of ethylene oxide caused the amalgamation of fibers, especially in cortical bone. This change probably also occurred in the cancellous bone, however, the presence of much fewer trabeculae with varied orientation may have hindered the observation of this fact. This finding has been encountered by others[6,22]. Haje et al.[22] found that when bone was subjected to the processing described in this study, the maximum strength in bending tests increased, but became more fragile under torsion stress. Amalgamation may also account for these changes.

Moreover, it is likely that the warming that occurs during sterilization is the causal factor of the amalgamation and not the gas itself, since it is inert, but only toxic to and active in living structures. This reasoning is suggested by the fact that the amalgamation was found only in heated groups and reinforced by the findings of Voggenreiter et al.[12], who studied the effects of temperature and irradiation on the microstructure of cortical bone and found that autoclaving produces denaturation of the organic matrix, with amalgamation and swelling of the fibrillar matrix.

Finally, in terms of the methodological limitations of our study, more samples could have been used, although the number of samples in this type of research is usually small[6, 12], and through the methodology the results also suggest, but do not demonstrate fully, that heat has been the causative agent of amalgamation. This can be determined in another study designed specifically for this purpose.

CONCLUSION

The chemical processing used in this research, as well as sterilization with ethylene oxide, caused minimal changes when examined by conventional light microscopy. However, when the material was analyzed microstructurally, evidence of microcracks was also common in the control group, as well as the fibrillar amalgamation that seems to be the result of heating and can explain the changes in bone mechanical parameters found by other authors.

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