Histomorphometric Analysis of Homogenous Demineralized Dentin Matrix as Osteopromotive Material in Rabbit Mandibles

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Purpose: *The aim of this work was to evaluate the effectiveness of homogenous demineralized dentin matrix (HDDM) slices in surgical bone defects created in the mandibles of rabbits and occluded with a polytetrafluoroethylene (PTFE) membrane in the promotion of bone growth.* ***Materials and Methods:*** *Surgical bone defects were created in 36 adult rabbits and divided into 4 groups: bone defect (control), bone defect with PTFE membrane, bone defect with HDDM, and bone defect with both HDDM and a PTFE membrane (HDDM + PTFE). The rabbits were sacrificed after 30, 60, and 90 days, and the bone defects were examined histologically and by histomorphometric analysis (analysis of variance and the Tukey test).* ***Results:*** *The volume of newly formed bone matrix was significantly greater in the HDDM and HDDM + PTFE groups than in the control and PTFE groups. The discrete inflammatory reaction found in the HDDM and HDDM + PTFE groups did not prevent the osteopromotive activity of the dentin matrix.* ***Discussion:*** *HDDM slices were biocompatible and were resorbed during the bone remodeling process. They stimulated the newly formed bone until 30 days after implantation.* ***Conclusion:*** *Bone repair was accelerated in the bone defects treated with HDDM in comparison to the control group.* INT J ORAL MAXILLOFAC IMPLANTS 2004;19:679–686

Key words: *bone repair, guided tissue regeneration, homogenous demineralized dentin matrix, osteopromotion, tissue engineering*

Research has been conducted on acceleration of the bone repair response in reconstructive surgeries, using biologic materials of both animal and vegetal origin.[1–6] Osteoinduction is based on the increase of osteoblastic proliferation and bone matrix synthesis. This process might be controlled by complex molecular interactions, such as cellular messages of short and long extension, that influence the speed and duration of the osteoblastic and osteoclastic cellular work as well as proliferation, differentiation, and chemotaxis.[4,7] Some authors have stated that cellular proliferation begins with a local stimulating factor, ie, with bone morphogenetic proteins.[2,8,9] The structural proteins, such as type I collagen and its fragments, might also induce proliferation.[10] According to Bruder and associates,[11] undesired soft tissue invasion within the bone defect inhibits the presence of potentially osteogenic tissue, hindering the occurrence of bone repair. Alper and colleagues[1] and Rabie and coworkers[12] believe that this phenomenon could be eliminated with bone graft implantation on the defect area. The bone graft would act as a physical barrier.

Autogenous demineralized dentin matrix (ADDM) implanted in the subcutaneous connective and muscular tissues of the abdomen, dental pulp, and bone defects has induced the formation of bone tissue in experimental animals.[3–6,13] In bone defects the dentin

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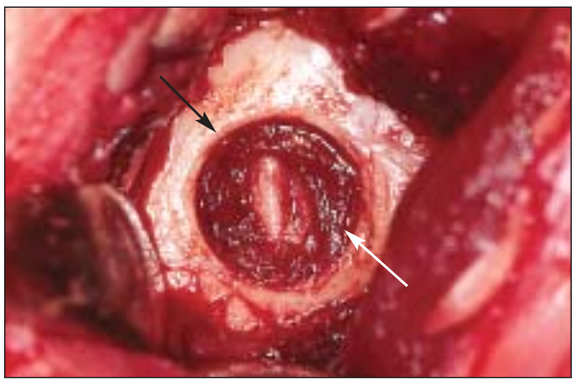

Fig 1 The spherical bone defect was 5.0 mm in diameter. HDDM slices *(arrows)* were placed around the edges of the bone defect.

Fig 2 The HDDM slices, which were approximately 8 µm thick, were whitish, with translucent aspects and a soft consistency.

matrix is reabsorbed during the bone remodeling process and the newly formed bone tissue grows by extension and cellular proliferation of the osteogenic tissue derived from the periosteum, bone marrow, and adjoining mesenchymal cell differentiation.[3–6,13]

Although ADDM has been widely used experimentally as graft material, few studies on homogenous demineralized dentin matrix (HDDM) in bone defects were found in a search of the literature. Bang[14] compared the degree of antigenicity of xenogenic demineralized dentin matrix (XDDM) with that of HDDM. Bang showed that XDDM presented a high degree of antigenicity and lacked osteoinductive properties, while HDDM caused a discrete immune reaction and had potential as an osteoinductor.

The aim of this work was to evaluate the effectiveness of the osteopromotive and osteoconductive properties of HDDM in surgical bone defects created in the mandibles of rabbits and occluded with a polytetrafluorethylene (PTFE) membrane.

MATERIALS AND METHODS

Thirty-six young adult rabbits with an average weight of 3.5 kg were divided into 4 groups: *(1)* bone defect filled with blood clot (control), *(2)* bone defect implanted with HDDM slices, *(3)* bone defect covered with a PTFE membrane, and *(4)* bone defect implanted with HDDM slices and covered with a PTFE membrane (HDDM + PTFE). The dentin was placed around the edges of the created bone defect (Fig 1).

To make the HDDM, the central incisors of the control-group rabbits were extracted and prepared in slices according to the technique of Catanzaro-Guimarães and associates[3] and Gomes and coworkers.[5] The pulp tissue was totally removed by the retrograde technique, and the root was planed. After being washed with sterile physiologic serum at 2°C, the teeth were immersed in a 0.6 N hydrochloric acid solution at 2°C until complete demineralization. The specimens were then washed for 3 hours in distilled water under constant agitation to completely remove the acid. After this process, the HDDM was cut into slices approximately 8 µm thick with the aid of freezing microtomy (Model CTD International-Harris Cryostat; International Equipment Company, Needham Heights, MA). These slices were immersed in a sterile glass container filled with a solution of 70% ethyl alcohol and gentamicin (5 mL of alcohol per 0.2 mL of gentamicin) and stored at 2°C until the time of implantation (Fig 2).

The animals were anesthetized intramuscularly with Rompum (Bayer, São Paulo, Brazil), a preanesthetic, and Ketalar (Holliday-Scott, São Paulo, Brazil). The preanesthetic agent was applied 5 minutes before administration of the anesthetic. An incision was made in the molar regions of the mandibles, followed by muscular dissection, plane to plane. An incision was made in the periosteum, and a surgical bone defect was created with a 5.0-mm trephine constantly irrigated with sterile saline solution. The round bone defect was 5.0 mm wide and a maximum of 2.0 mm deep (the depth was equal to the thickness of the removed cortical bone). After the bone defect had been created and the HDDM and/or the occlusive barrier placed, the periosteum, masticatory muscle, and skin were sutured.

The animals were treated with an antibiotic (Pentabiótico; Fort Dodge Saúde Animal, São Paulo, Brazil) 24 hours before and 1 hour after the surgery. For 3 days after the surgery, an analgesic and anti-inflammatory agent (Voltaren; Parker-Davis, São Paulo, Brazil) was also administered. These drugs were injected intramuscularly in all animals. Three

animals from each group were sacrificed at 30, 60, and 90 days after surgery. The bone containing the surgical defect was removed en bloc, fixed in 10% formalin for 72 hours, demineralized in a 0.2 mmol/L solution of ethylenediaminetetraacetate acid (EDTA) (Merck S.A. Industrias Químicas, São Paulo, Brazil) and embedded in paraffin. The sections were obtained in the longitudinal direction and showed about 6 μm of thickness. They were stained using hematoxylin-eosin and picrosirius (sirius red).[15]

Histomorphometric Analysis

Histomorphometric analysis was accomplished using the experimental principle of stereology, which consists of observing the 3-dimensional quantitative parameters of the anatomic structures in the histologic sections using geometry and statistical analysis.[6,16] This method was applied to study a sample of the arbitrary and isotropic histologic sections.

Nine sections from each animal were selected arbitrarily. Three fields from the surgical bone defect region of each section were analyzed. The KS400 program (Kontron Elektronik, Munich, Germany) was connected to a 70-square reticule, with each point corresponding to 10×10^4 μm^2. This reticule was placed over histologic fields, which were analyzed with the aid of an 10× objective and an ocular KPL 10× optical microscope (Zeiss 1.3; Kontron Elektronik). Bone tissue area was measured. The images obtained were transferred to a high-resolution monitor using a video camera.

The morphometric count was done through quantification of the number of points that incurred on the newly formed bone matrix. The values obtained were converted into volume density and presented in terms of percentage using the equation $BD = N_{Bone}/N_{Total}$, where BD = bone density, N_{Bone} = the number of points in bone trabeculae, and N_{Total} = the total number of points in the bone defect.

RESULTS

Microscopic Features

The bone defect was divided into peripheral and central regions to describe microscopic aspects of the specimens (Fig 3).

30 Days. Control Group. The histologic sections showed a fine layer of immature bone tissue superficially covering the entire bone defect. Beneath this layer, in the deeper portions of the defects, there were a large quantity of osteogenic connective tissue and a few immature bone trabeculae. The connective tissue showed a discrete inflammatory infiltrate (Fig 4a).

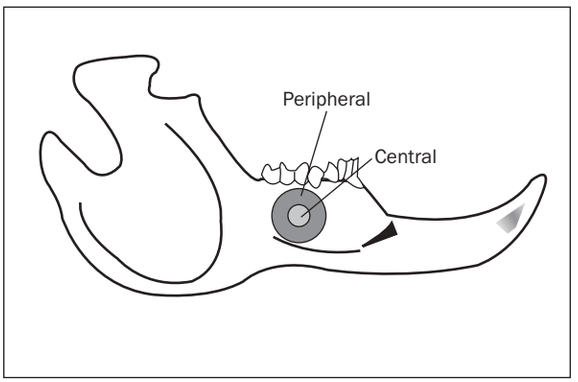

Fig 3 Schematic drawing of the rabbit mandible with the surgical defect.

HDDM Group. The bone defects were almost totally filled by bone tissue; however, osteogenic connective tissue was seen in other regions. Bone trabeculae were distributed along the entire defect, but mainly at its borders (Fig 4b). In the peripheral regions of the bone defects, HDDM slices were observed incorporated in the newly formed bone matrix along the whole extent of the histologic section. Large osteon haversian canals and osteoclasts were seen in the newly formed bone tissue. In the central regions of the bone defect, numerous osteoprogenitor cells were seen, and a large quantity of osteoid tissue near the HDDM slices and discrete mononuclear inflammatory cells had infiltrated beside the HDDM slices (Figs 4c and 4d).

PTFE Group. The bone defects were filled by a large quantity of osteogenic connective tissue and by a few irregular, immature bone trabeculae, which were found mainly at the defect borders. The bone trabeculae found in the central portions were finer and less numerous than those at the periphery of the surgical defect. The bone surface showed immature bone trabeculae irregularly disposed (Fig 5).

HDDM + PTFE Group. The bone defect was filled by bone tissue. Thick and regular immature bone trabeculae were observed (Fig 6). In some areas, numerous osteoprogenitor cells and osteoid tissue were observed near the HDDM. Large medullary channels, osteoblasts, osteoclasts, and discrete inflammatory cells were observed surrounding the HDDM slices. In some cases, HDDM slices were incorporated in the newly formed bone trabeculae (Fig 7).

60 Days. Control Group. The defects were not completely filled by bone trabeculae. A continuous bone layer covered their superficial portion. This fine bone layer was formed by highly cellularized immature bone tissue. Its growth began at the basal and lateral portions of the defects, toward the central

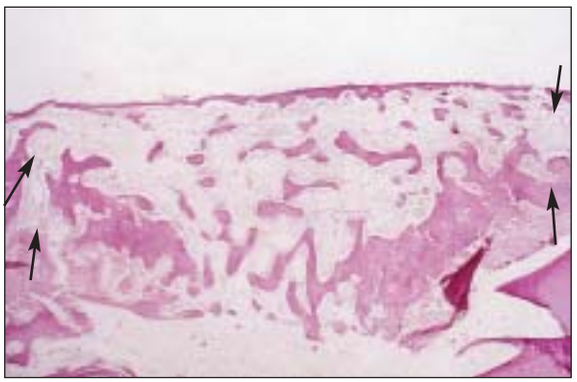

Fig 4a Control group (30 days). The bone defect region *(arrows)* is filled by bone tissue with disperse and thin immature bone trabeculae and osteogenic connective tissue (hematoxylin-eosin; original magnification ×25).

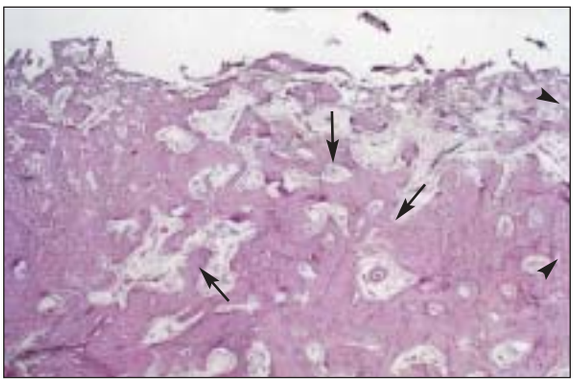

Fig 4b HDDM group (30 days). The bone defect region *(arrowheads)* is totally filled by irregular and thick newly formed bone trabeculae *(black arrows).* A small amount of osteogenic connective tissue can also be seen (hematoxylin-eosin; original magnification ×25).

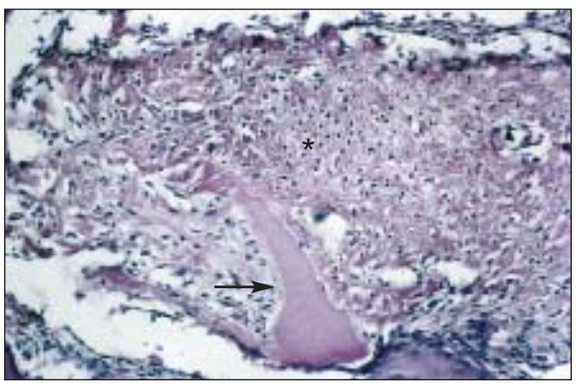

Fig 4c HDDM group (30 days). Osteoid tissue *(asterisk)* and numerous osteoprogenitor cells were observed near the HDDM *(arrow)* (hematoxylin-eosin; original magnification ×400).

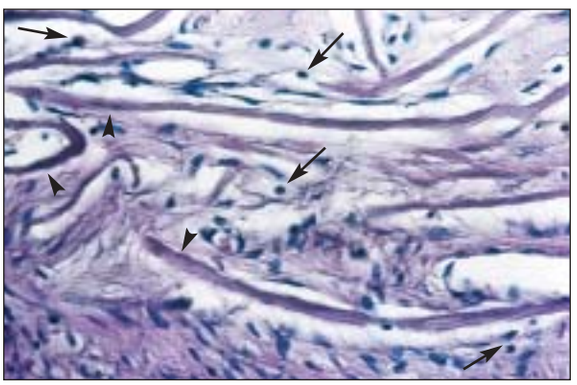

Fig 4d HDDM group (30 days). Discrete inflammatory cells *(black arrows)* infiltrated were observed beside the HDDM slices *(arrowheads)* (hematoxylin-eosin; original magnification ×400).

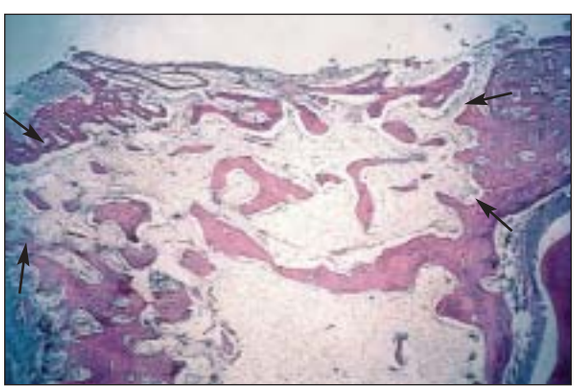

Fig 5 PTFE group (30 days). The bone defect region *(arrows)* is filled by a large quantity of osteogenic connective tissue and by a few irregular immature bone trabeculae (hematoxylin-eosin; original magnification ×25).

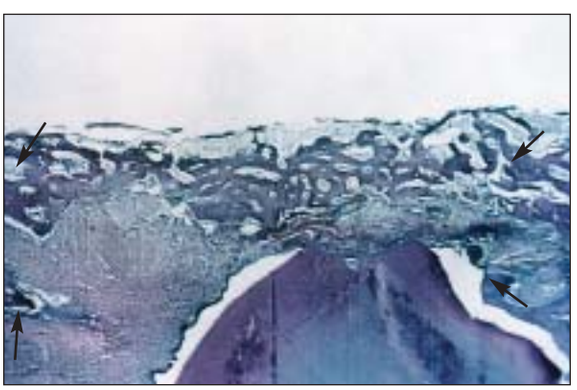

Fig 6 HDDM + PTFE group (30 days). The bone defect region *(arrows)* is filled with newly formed bone trabeculae (hematoxylin-eosin; original magnification ×25).

regions of the defect, forming a bone bridge in the central region of the surgical locus. Below the continuous bone layer, loose connective tissue containing osteogenic cells was found. The bases of the bone defects were almost completely filled by highly cellularized immature bone tissue.

HDDM Group. The bone defects contained a large quantity of bone tissue formed by highly cellularized immature trabeculae, with large medullary spaces. Bone growth occurred at the lateral limits of the defects, toward the defect center. In the central portions of the defects, osteogenic connective tissue was observed, with moderate inflammatory cell infiltrate. The HDDM slices were incorporated in the immature bone trabeculae, which showed large osteon haversian canals.

PTFE Group. The bone defects were filled by immature bone tissue in the peripheral portions only. In the central region, large quantities of loose connective tissue were observed, including osteogenic cells and a small quantity of inflammatory cells, in addition to a few bone trabeculae. The bone tissue was highly cellularized, showing large haversian canals and ample medullary spaces.

HDDM + PTFE Group. The bone defects were almost completely filled by immature bone trabeculae, with large medullary spaces filled by osteogenic connective tissue. In the peripheral regions of the bone defects, the trabeculae showed more tissue organization and formed characteristic haversian systems. In the central portion, immature bone trabeculae showed large medullary channels. In the histologic sections, no HDDM slices were seen incorporated in the bone tissue or the osteogenic connective tissue.

90 Days. Control Group. The bone defects were not completely filled by bone tissue. A continuous bone tissue layer covered the superficial portions of the defects. This bone layer was formed by an organized tissue. Below this, loose connective tissue with osteogenic cells was observed. The bases of the bone defects were completely filled by bone tissue.

HDDM Group. In this group, the bone defects showed a larger quantity of bone tissue than in the control group, with bone growth at the defect borders. The newly formed tissue was comprised of highly cellularized immature trabeculae with large haversian canals and medullary spaces. No HDDM slices were incorporated in the neoformed bone tissue.

PTFE Group. The surgical defects were filled by bone tissue in the peripheral region, showing highly cellularized immature bone trabeculae and large haversian canals and medullary spaces. In the central regions of the surgical defects, rare bone trabeculae and loose connective tissue were seen.

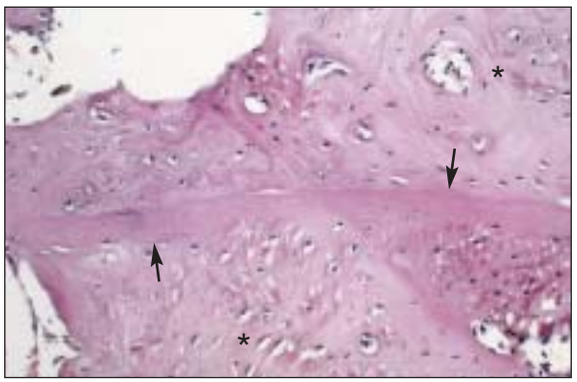

Fig 7 HDDM + PTFE group (30 days). HDDM slices *(arrows)* are incorporated into the immature bone tissue *(asterisks)* (hematoxylin-eosin; original magnification ×400).

HDDM + PTFE Group. The defects were almost totally filled by immature bone tissue, with large medullary spaces filled by osteogenic connective tissue. The trabeculae showed good tissue organization; characteristic haversian systems had been formed. Some HDDM slices were observed incorporated in the bone tissue and the connective tissue.

Histomorphometric Analysis

The purpose of the histomorphometric analysis was to measure the density of newly formed bone matrix in the bone defects, providing the necessary data for statistical analysis.

The statistical difference between the densities of newly formed bone matrix of the experimental groups was significant at all given observation times. Differences between sacrifice groups within the same experimental group were not significant in most cases; a balanced bone neoformation process was seen from 30 days of observation onward in all experimental groups except the PTFE group.

After the analysis of variance, the means and standard deviations of the densities of the newly formed bone matrix were calculated for each experimental group. Based on these values, a statistically significant difference among the experimental groups related to density averages of the neoformed bone matrix was evidenced. The Tukey test was applied with a 5% significance level. In the control, HDDM, and HDDM + PTFE groups, there was no significant difference in the quantity of neoformed bone matrix found in animals sacrificed at 30, 60, or 90 days. However, in the PTFE group, the difference between the quantities of neoformed bone matrix at 30 and 60 days was significant.

At 30 days, the control and PTFE groups presented a similar degree of new bone formation, as

Table 1 New Bone Formation (Mean ± SD) in μm³ at Sacrifice

Days after surgery	Control	HDDM	PTFE	HDDM + PTFE
30	0.359 ± 0.215^{Aa}	0.688 ± 0.216^{Ab}	0.331 ± 0.209^{Aa}	0.675 ± 0.160^{Ab}
60	0.311 ± 0.191^{Aa}	0.632 ± 0.180^{Abc}	0.507 ± 0.228^{Bb}	0.686 ± 0.161^{Ac}
90	0.462 + 0.184^{Aab}	0.610 ± 0.170^{Aac}	0.418 ± 0.240^{ABb}	0.652 ± 0.155^{Ac}

Means followed by different letters (uppercase in the vertical and lowercase in the horizontal) are not similar (Tukey test; $P \leq .05$).

did the HDDM and HDDM + PTFE groups (Table 1). Mean newly formed bone matrix was greater in the HDDM and HDDM + PTFE groups than in the control and PTFE groups. At 60 days, the Tukey test showed that the control group presented a statistically significant amount of new bone formation; however, mean new bone formation was lower in this group compared to the HDDM, PTFE, and HDDM + PTFE groups. The HDDM group had a quantity of newly formed bone matrix similar to that of the HDDM + PTFE group. The PTFE and HDDM + PTFE groups did not present the same quantity of neoformed bone matrix. The HDDM and HDDM + PTFE groups showed the greatest mean new bone formation at 60 days. At 90 days, the control and PTFE groups presented the lowest mean new bone formation, while the HDDM and HDDM + PTFE groups had the highest mean new bone formation (Table 1). Bone density was greater in the experimental groups HDDM and HDDM + PTFE during all observation periods.

DISCUSSION

Many investigators have been studying osteopromotive materials that may aid the bone repair process. Compared to several materials being studied, ADDM in bone defects has been used with excellent results, since it contains bone morphogenetic protein and is reabsorbed.[3,5,6] Several studies have shown that bone repair occurs with greater intensity when purified bone morphogenetic protein, bone, and/or dentin demineralized matrix are used.[2–6,8,17–19] In the present study, formation of a statistically significant quantity of newly formed bone matrix in the HDDM and HDDM-PM groups could be explained by the presence of this protein in its organic structure.

The chemotactic and osteogenic potential of dentin matrix has been reported by several investigators.[1,3,5,6] The results of the present study showed that the slices of HDDM induced the migration and deposition of osteogenic cells and bone matrix over the dentin matrix surface.

Some authors believe that the grafted dentin matrix might promote an adequate surface for undifferentiated mesenchymal cell fixation and aid in cell orientation, which would explain their osteoconductibility.[6,7,20–22] The presence of a great quantity of osteoblasts located over the HDDM slices at 30 days in the HDDM and HDDM + PTFE groups is in agreement with the results of Nakashima,[20–22] Catanzaro-Guimarães,[7] and Gomes and colleagues.[6] These investigators used ADDM in the form of gel, particles, and/or slices. They reported that the quantity of newly formed bone tissue in the ADDM slices was greater than when ADDM was used in powder or gel form. The present authors believe the use of slices increased the amount of surface contact between the HDDM and the osteogenic connective tissue, providing a greater substratum for bone matrix deposition.

In this study, neovascularization within the defect was seen after 30 days in the HDDM and HDDM + PTFE groups. Large vascular channels permeating the newly formed bone tissue were visible, and there was intense osteoprecursory cell proliferation in the osteogenic connective tissue.

The microscopic analysis of samples from the HDDM and HDDM + PTFE groups showed the presence of moderate mononuclear inflammatory cell infiltrate during all periods studied. However, HDDM showed excellent biocompatibility, with a high level of biologic tolerance by the animals in which it was implanted, as observed in the studies of Bang,[14] Nordenram and Bang,[23] and Knudsen and coworkers.[24]

The microscopic analysis showed newly formed bone tissue filling the bone defects in the HDDM and HDDM + PTFE groups in all observation periods, which confirms the results of Gould and associates,[25] who used HDDM gelatin in critical-size bone defects in the parietal bones of rats and concluded that the defects were completely repaired in all groups treated.

According to Mundy and associates[10] and Catanzaro-Guimarães,[7] the complexes of molecular interactions present in the bone and dentin matrices might influence the proliferation, migration, anchorage, speed, and lifespan of the osteoblastic and osteoclastic cells. The present authors hypothesize that the intense process of bone remodeling that occurred in the bone defects of the HDDM and HDDM + PTFE groups during the first 60 days after surgery might be explained by this phenomenon, since bone remodeling consists of a sequence of events, such as reabsorption and deposition of bone matrix.

Certain characteristics of biologic materials have been considered ideal, including biocompatibility, storage without loss of feasibility, ease of material obtainment, and cost-effectiveness.[1,5,6,14,22–29] In the present study, HDDM was found to have some characteristics that could be considered ideal for use as a bone grafting material, such as a significant potential for osteopromotion, excellent biological tolerance, discreet inflammatory reaction, and easy obtainment, handling, and storage. HDDM evidenced positive results and considerable potential for clinical applicability in the medical and dental areas; perhaps the creation of a tooth bank will be deemed desirable in the future.

Supplementary studies with specific methodologies for the identification of the activating substances responsible for HDDM's osteopromotion would complement the results obtained in this research.

CONCLUSIONS

The results obtained suggest that HDDM was biocompatible and stimulated newly formed bone until 30 days after its implantation, which demonstrates that HDDM is osteopromotive. The volume of newly formed bone matrix was statistically significant in the HDDM and HDDM + PTFE groups. The inflammatory reaction found in the HDDM and HDDM + PTFE groups did not prevent the osteopromotive activity of the dentin matrix. The HDDM was resorbed during the bone remodeling process.

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