Demineralized Human Dentin Matrix as an Osteoinductor in the Dental Socket: An Experimental Study in Wistar Rats

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Purpose: This study evaluated the bone-forming potential of the demineralized human dentin matrix by performing histologic and morphometric analyses. The immunolabeling of osteopontin, a determinant protein for bone repair, was also evaluated. Materials and Methods: Wistar rats were selected and submitted to the extraction of the right and left second molars. Tooth sockets were separated into two groups: the control group (right), which was filled with the blood clot, and the experimental group (left), which was filled with demineralized human dentin matrix. Animals were sacrificed at 5, 10, and 21 days. Histologic and histoquantitative analyses (analyses of variance [ANOVA] and Tukey’s test) were performed, as well as immunostaining for osteopontin as an osteogenesis indicator. Results: After 5 days, demineralized human dentin matrix was incorporated by new trabeculae. After 10 days, connective tissue organization and new trabeculae were observed in the experimental group, and intense staining for osteopontin close to demineralized human dentin matrix was observed in the experimental group. After 21 days, the experimental group was showing mature trabeculae. A statistical difference was observed (P < .05). There was a higher number of trabeculae in the experimental groups in all periods of analysis. The presence of osteopontin was observed more intensely at 10 days close to demineralized human dentin matrix. Conclusion: This study indicates that demineralized human dentin matrix implanted in tooth sockets induces the acceleration of osteogenesis. Int J Oral Maxillofac Implants 2020;35:910–916. doi: 10.11607/jomi.8279

Keywords: bone repair, demineralized human dentin matrix, tooth sockets, osteopontin

Factors that may contribute to the stability of dental implants are the quality of the implant, metabolism, and bone density after a healthy and efficient process of osseointegration is achieved. Bone regeneration at the appropriate width and height is particularly important in posterior implant sites where more volume support is required to withstand occlusal forces, as well as at anterior maxillary sites where increased bone contour is sought in order to reach acceptable aesthetic demands.1 After tooth loss, the absence of the periodontal ligament activates a physiologic and irreversible process of reabsorption of the alveolar bone, resulting in an alteration in both volume and bone height.2 These dimensional changes cannot be avoided but may be attenuated by means of surgical grafting techniques for the preservation of the alveolar bone with the use of different types of biomaterials or autogenous tissues.3 In such cases, the implementation of strategies for tissue reconstruction, accelerated osteogenesis, or both can be necessary.4–6

It is generally accepted that osteogenesis demands the combination of osteoinduction with osteoconduction and that one process does not work without the other.7 However, a main limiting factor of current therapies for stimulating osteogenesis using growth factors is the expensive costs of these substances.8 The techniques based on autogenous bone grafts have disadvantages, such as morbidity and the need of a second surgical site. Moreover, the sterilization and purification processes used in biomaterials can eliminate the osteoinductive capacity of the cells or tissues, suppressing their viability.8,9 These disadvantages increased the need for studies in search of better alternatives for osteogenic therapies.

The embryologic and histologic similarities between dentin and bone have guided studies that evaluated the use of the dentin matrix as an osteoinductive and osteoconductive element in osteogenic therapies. Studies have also demonstrated that human demineralized dentin matrix, or demineralized dentin matrix cryopreserved for up to 6 months, maintained the same properties of biocompatibility and osteoinduction.9,10

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However, there is insufficient data to support the effects of demineralized human dentin matrix in the alveolar bone repair process. The aim of this study was to conduct histologic and histomorphometric analyses of demineralized human dentin matrix during bone repair in rat dental alveoli after extraction.

**MATERIALS AND METHODS**

The present study was approved by the Animal Ethics Committee from the University of Uberaba (UNIUBE - Protocol No 022/2009) in compliance with the ARRIVE guidelines.11

**Experimental Design**

Based on a priori power analysis,11,12 an experimental in vivo study on dental alveoli of Wistar rats was designed. In each rat, the alveoli were separated into two groups: the control group (maxillary right side), which did not receive demineralized human dentin matrix and was naturally filled only by a blood clot, and the experimental group (maxillary left side), which was filled with demineralized human dentin matrix.

**Animals**

Twenty-four male Wistar rats, approximately 2 months old, weighing between 150 and 200 g, were selected for this study. Animals were kept in a room with controlled climatic conditions, in 12-hour cycles of light/darkness and ad libitum access to food and water.

**Demineralized Human Dentin Matrix**

Teeth were collected from the tooth bank of the Dentistry Faculty at the University of Uberaba. Demineralized human dentin matrix was obtained from teeth without caries or other disease. The roots were cut out, cleaned through the removal of the dental pulp and periodontal ligaments, and demineralized with diethylamine tetraacetic acid (EDTA) 10%, with a pH of 7.3 at 25°C for approximately 3 months, and cut in a cryostat. Slices of demineralized human dentin matrix were washed in sterilized deionized water for 10 minutes, then stored in phosphate-buffered saline (PBS) containing 100 units/mL of penicillin (Gibco BRL) and 100 mg/mL of streptomycin (Gibco BRL) for decontamination.

**Surgical Procedures**

The surgical protocol followed the method established by the present study group.5,7 Animals were randomly grouped and subjected to extraction of the left and right maxillary second molars after anesthesia, which consisted of a solution of ketamine 10%/xylazine 2%, 1:1, in a volume of 0.1 mL per 100 g of intramuscularly injected body weight. The alveoli were separated into two groups: the control group (right), which did not receive demineralized human dentin matrix and was naturally filled only by a blood clot, and the experimental group (left), which was filled with demineralized human dentin matrix and was gently placed with an amalgam carrier.

The animals were kept in a supine position throughout the duration of anesthesia (approximately 4 hours) to prevent the displacement of demineralized human dentin matrix in the tooth socket. Animals received pasty food for 2 days after surgery. The animals were sacrificed by cervical displacement under anesthesia consisting of ketamine 10% and xylazine 2% (1:1 in a volume of 0.1 mL per 100 g body weight intramuscularly). Euthanasia was performed 5, 10, or 21 days after surgery. Histologic, morphometric, and immunohistochemical analyses were carried out on eight animals per group.

**Histologic and Histoquantitative Analyses**

The maxillae were dissected and fixed in a solution of 10% neutral buffered formalin for 48 hours at room temperature. After fixation, the maxillae were demineralized in 10% EDTA (pH 7.3), dehydrated using graded ethanol solutions, embedded in paraffin, and cut on a microtome in the sagittal plane, producing sections of 5 µm. Sections were stained with hematoxylin-eosin for histologic and morphometric analyses and processed for an immunohistochemistry evaluation of the osteopontin expression. Images were observed with an optic Olympus BX50 microscope and captured using the QCapture 6.0 software. The analysis was concentrated on the apical area of the sockets (bottom) of the distal root, as the location of initial bone formation. The images were obtained using QCapture 6.0 software and a microscope (Olympus BX50), coupled with a personal computer and image processing software. The new bone trabeculae were counted using a grid with vertical and horizontal lines placed on the photomicrograph, with the aid of the confocal assistant. A calibrated person counted the number of bone trabeculae located on the grid line intersections.

**Immunohistochemistry Analyses**

This study analyzed the osteogenic potential of demineralized human dentin matrix through the biologic effects evaluated by the osteopontin expression during bone repair in sockets of distal roots of the second molars of rats after extraction.

Osteopontin expression in each time period was assessed in at least three different animals. Sections were deparaffinized with xylene, rehydrated in a graded series of ethanol, diluted in phosphate-buffered saline, incubated in 10% H2O2, and diluted in methanol for neutralization of endogenous peroxidase activity.
Nonspecific binding sites were blocked with bovine serum albumin and diluted in 2% phosphate-buffered saline for 1 hour. Sections were incubated overnight at 4°C with the anti-mouse osteopontin antibody (Santa Cruz Biotechnology, Osteopontin: code sc-21742), with a 1:50 dilution factor. After three washes in phosphate-buffered saline, the sections were incubated with the biotinylated secondary antibody for 30 minutes at room temperature (Dako LSAB Kit - K0690). Sections were then washed again in PBS and incubated with streptavidin-conjugated peroxidase complex for 30 minutes at room temperature. Sections were washed again in phosphate-buffered saline, and the peroxidase-binding activity was detected using the substrate 3,3-diaminobenzidine tetrahydrochloride and H₂O₂ 0.01% in phosphate-buffered saline, then counterstained with hematoxylin. Controls were subjected to the same procedure, omitting the incubation with the primary antibody. To determine the amount, location, and intensity of stained structures, 12 randomly selected sections of the apical area of the socket were evaluated in both control and experimental sides. The marked structures were described and compared between groups and tooth socket sides.

**Statistical Analyses**

The differences between stages of the tissue repair process were tested in each group through an analysis of variance (ANOVA) and Tukey test using the software GraphPad Prism 6.0. The following parameters were analyzed: the formation and organization of collagen fibers and fibroblasts, connective tissue, blood vessels, and the formation of newly formed trabeculae in three different stages of postsurgical procedures (days 5, 10, and 21). Data from all groups were checked for normality by the Kolmogorov-Smirnov test. All experiments were performed in triplicate, and the significance was set at \( P \leq .05 \).

**RESULTS**

**Histologic and Histoquantitative Analysis**

Histologic analysis showed the demineralized human dentin matrix fragments immersed in dental sockets surrounded by connective tissue in different stages of resorption. On day 5, the apical portion of the sockets in the experimental group (left side) exhibited fewer inflammation sites than the control side. The implanted material (demineralized human dentin matrix) was in contact with organized connective tissue, increasing the formation and organization of collagen fibers and promoting the initial tissue repair process (Fig 1a). A detailed observation of the interaction of demineralized human dentin matrix with connective tissue showed multiple resorption areas of dentin in contact with sites of organized tissue. Blood vessels were also present in these areas (Fig 1b).

On day 10, the differences between the control and experimental sides regarding the stages of tissue repair became more relevant. On the control side, the most prominent areas were collagen fibers and fibroblasts, organizing the connective tissue preceding the formation of newly formed trabeculae (Fig 2a). The experimental socket showed the tissue repair in a later stage. Newly formed trabeculae were observed close to highly organized connective tissue, and new vessels were also present. Organized connective tissue surrounded by the new trabeculae formed the medullary spaces that characterize the architecture of a final conformation of medullary bone (Fig 2b).

Day 21 of the control side matches with approximately day 10 of the experimental socket side. Newly formed trabeculae, still very cellular and less mineralized, were observed close to the organized connective tissue (Fig 3a). On the experimental side, the maturation was characterized by continuous incremental deposition layers of lamellar bone, and by the substitution of cellular bone for a more mineralized tissue (Fig 3b). The histoquantitative results showing the number of trabeculae are expressed in Fig. 4, where a statistically significant difference \( (P < .05) \) between the number of
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Trabeculae of control and treated sockets could be observed in all periods of analysis.

**Immunohistochemistry Analyses**

The immunohistochemical staining for osteopontin in the experimental group on day 5 can be observed in two predominant regions: in the blood vessel walls and the area of connective tissue close to demineralized human dentin matrix (Fig 5b). This marker characterized the final stages of osteoblast differentiation and the initiation of bone matrix production (Figs 5a and 5b). The immunostaining for osteopontin in the experimental group (10 days) was very intense in the regions close to demineralized human dentin matrix and less intense in areas of mineralized trabecular bone (Figs 6 and 7).
DISCUSSION

Osteopontin is one of the most abundant non-collagenous proteins of bone tissue. It performs several functions, including control of mineralization, organization of osteogenesis, adhesion of osteogenic cells to the bone matrix, and resorption.\textsuperscript{12} Osteopontin and osteocalcin have been considered important indicators of osteogenesis, detected in late stages of osteoblast differentiation and early stages of osteogenesis.\textsuperscript{13}

The correct choice of an osteoinductive and osteoconductive agent is critical to the success of osteogenic therapies for tissue regeneration after trauma or surgery. The major advantages of using demineralized dentin matrix as an osteoconductive and osteoinductive agent include the ease of obtaining it, the low cost, and the simplicity of preparation and placement at the treatment site. Surgical procedures involved in this process are minimally invasive, especially compared with treatments using autogenous bone implants.

Embryologically, both dentin and alveolar bone have the same origin: cells of the neural crest and ectomesenchyme. Alveolar bone and dentin have very similar chemical compositions; both constitute up to approximately 65% inorganic components and 35% organic matter. The organic composition in both dentin and alveolar bone are also very similar. Clinically, when a root is left in the socket after an incomplete extraction, it usually does not generate an inflammatory response. Usually, the root fully integrates to the adjacent bone, confirming the biocompatibility between these two tissues.\textsuperscript{14} Thus, the use of dentin matrix as an osteoinductive and osteoconductive component of osteogenic therapy is justified, and has obtained very favorable results.

In the present study, the first analysis was chosen after only 5 days due to the results obtained in a previous study,\textsuperscript{4} when the inflammatory process observed after 3 days negatively influenced the immunohistochemical markers and the main histologic events. Thus, in the present study, an improved observation of tissue repair was possible. The other evaluation periods (10 and 21 days) were also based on milestones\textsuperscript{2,7,15,16} of the socket repair after tooth extraction. The present study evaluated the basal portion of the socket. This choice, which was based on results of previous work,\textsuperscript{4,10} occurred because this region is the first to produce bone tissue after tooth extraction during alveolar repair. Thus, as the present study was evaluating an early marker of osteogenesis (osteopontin), the observation of an early bone synthesis region was shown to be adequate.

The results of the present study showed a higher amount of trabecular bone in the experimental alveoli compared with the control. This statistically confirmed result is probably due to the presence of demineralized human dentin matrix in experimental sockets. It was observed that the demineralized

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\caption{The immunostaining for osteopontin in the experimental group (10 days) is very intense in the regions close to the demineralized human dentin matrix and less intense in areas of mineralized trabeculae bone. Bar = 100 μm. DHDM = demineralized human dentin matrix. T = trabeculae.}
\end{figure}
human dentin matrix after 5 days in the alveoli has an uneven surface and is covered with cells, indicating clear resorption. This gradual resorption of demineralized human dentin matrix is shown in reports from other studies. One of the biggest advantages of biodegradation is the slow and gradual release of growth factors present in dentin matrix: bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). In areas of dentin or bone resorption, resorbed tissues work as a source of growth factors and other molecules, increasing the viability of these substances in the medium. Demineralized human dentin matrix acts as a dynamic zone of action, capable of influencing the cell phenotype. Extracellular matrix proteins can directly interact with cell surface receptors, thereby initiating a signal transduction pathway and/or modulating the cellular response to growth factors. Thus, the growth and differentiation factors released slowly can induce adjacent mesenchymal cells to differentiate into chondrocytes and osteoblasts. The presence of demineralized human dentin matrix was probably also responsible for the faster repair of the socket compared with control alveoli.

One of the growth factors in the complex network of cytokines present in demineralized human dentin matrix, gradually released during resorption, is TGF-β. It is a potent stimulator of bone cell growth, consistently implicated in osteogenesis in vivo. Direct contact between demineralized human dentin matrix and the newly formed bone tissue found in sections during this period is in accordance with these statements. Regarding connective tissue organization, TGF-β has been directly related to both the synthesis and contraction of the collagen fibers in the connective tissue cells. This contraction of the connective fibers that characterizes the organization of the connective tissue can be considered crucial for tissue repair and regeneration.

Concurrently with the degradation or incorporation of demineralized human dentin matrix to bone, intense osteopontin staining close to demineralized human dentin matrix was found. The presence of this biomolecule as an indicator of repair and regeneration of mineralized tissue has been reported. Osteopontin is a multifunctional cytokine that is considered to be an initial marker of osteogenesis as one of the indicators of the final stage of osteoblast differentiation. In fact, osteogenesis starts shortly after the differentiation of osteoblasts. Thus, the presence of osteopontin detected more intensely in immunohistochemical sections of the alveoli treated with demineralized human dentin matrix denotes osteogenesis at a faster pace than that observed in the alveoli control in the same period.

**CONCLUSIONS**

Based on the results of this study, the presence of demineralized human dentin matrix in rat alveoli induced a satisfactory and faster bone repair process.

**ACKNOWLEDGMENTS**

This study received research funding/grants from the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq]), the coordination group for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento do Pessoal de Nível Superior [CAPES]). The authors have no direct financial interests. Dr A.M. Fernandes received research funding/grants from CNPq, the coordination group for CAPES.

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