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Histological evaluation of the osteoinductive potential of demineralized dentin matrix

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Abstract

Objective: The aim of this study was to verify the osteoinductivity of the xenogeneic and ectopic implantation of demineralized dentin matrix (DDM) in muscle tissue of rats in short-term.

Methods: Ten human teeth were utilized to obtain the DDM. Surgical access was made by incisions on two pockets created in the dorsal musculature of 10 Wistar rats, where the implants were inserted. The animals were divided into 2 groups: in the first group the implants remained in place for one month and in the second group for two months. Biopsies were taken at the end of the experimental times.

Results: Histological examination showed the presence of an intense chronic inflammatory infiltrate, and in some areas, osteoclasts were observed surrounding the material, and no mineralization foci were found.

Conclusion: It can be concluded that DDM implanted for periods of 1 or 2 months in the dorsal musculature of rats did not result in osteoinduction.

Keywords: Biocompatible materials; dental implantation; dentin

Avaliação histológica do potencial osteoindutivo da matriz dentinária desmineralizada

Resumo

Objetivo: A proposta deste estudo foi verificar a osteoindução da implantação xenogênica e ectópica de matriz dentinária desmineralizada (DDM) em tecido muscular de ratos em curto prazo.

Métodos: Dez dentes humanos foram utilizados para obtenção da DDM. Acesso cirúrgico foi feito através de incisões resultando em duas bolsas na musculatura dorsal de 10 ratos Wistar, onde os implantes foram inseridos. Os animais foram divididos em 2 grupos: no primeiro grupo os implantes foram mantidos por um mês e o segundo grupo foram mantidos por dois meses. As biópsias foram realizadas no final do tempo experimental.

Resultados: O exame histológico apresentou a presença de um intenso infiltrado inflamatório crônico, e em algumas áreas, osteoclastos foram observados em torno do material, nenhum foco de mineralização foi encontrado.

Conclusão: Pode-se concluir que DDM implantado na musculatura dorsal de ratos no período de 1 ou 2 meses não resulta em osteoindução.

Palavras-chave: Materiais biocompatíveis; implantação dentária; dentina

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Introduction

Biomaterials can have possible clinical applications, since they are capable of providing adequate tissue repair and inducing new tissue formation with biochemical characteristics of the original tissue.

Bone autogenous graft has showed excellent results to repair of critical-size defects (ANDERSON), but there are disadvantages such as the creation of a second surgical site. Dentin is a tissue that comes from the dental papilla and has been utilized in various studies investigating its possible osteo-inducing potential [2-4]. Thus, intramuscular allogeneic implants of demineralized dentin matrix (DDM) from the teeth of rabbits showed osteo-inducing potential 28 days after the surgical procedure [3]. Homogeneous DDM implanted for 30 days in the mandibles of rabbits with surgically created defects induced bone neoformation [2]. Similar results were seen with autogenous implants of DDM in surgically created defects in parietal bone of rabbits, where fragments of the material underwent resorption concomitant with the process of osteoinduction [4].

The organic fraction of mineralized tissues is the main constituent with osteoinducing potential, thus, it should attempt to establish what proteins are directly involved in this process. Bone morphogenetic proteins (BMPs) are probably the most important growth factors associated with bone tissue [5]. Futhermore, it was demonstrated that dentin-derived BMP-2 is an important required factor to induce differentiation of stem cells into odontoblasts [6].

Studies demonstrated osteoinductivity of bioimplants containing recombinant human bone morphogenetic protein-2 (rhBMP-2) [7] and recombinant human bone morphogenetic protein-7 (rhBMP-7) [8], where the clinical outcome of treatment using rhBMP-2 has shown success equivalent to autologous graft [9]. However, the effects of BMP-2 and BMP-7 in the regeneration of bone tissue using different methods and sites of application were seen by De Biase and Capanna [10]. The authors concluded that many variables are involved in these investigations and that the above mentioned growth factors are not the only ones that influence the process of bone tissue repair.

Non-collagen proteins (NCPs) are found in the dentin and are directly involved in tissue mineralization processes. These proteins have been characterized and analyzed biochemically and physiologically, demonstrating the great potential of biomaterials [11-13]. Dentin matrix protein-1 (DMP-1), dentin matrix protein-2 (DMP-2) and dentin sialoprotein (DSP) are some examples. The expression of DMP-1 in *E. coli* was studied by Srinivasan et al. [2] for the purpose of determining their functional, structural and post-secretory properties. The authors determined the amino acid sequence of DMP-1, which could reveal important roles in the activity of this protein, and among them the processes of induction of mineralization. In other experiment, Chaussain et al. [13] observed that DMP-1 was able to promote reparative dentin formation in pathological situations by differentiatons of mesenchymal cells. The DMP-1, DMP-2 and DSP are expressed in a varied manner according to the stage of development of the formation of mineralized tissues. This fact should be considered because the processing of these proteins would allow their use in future studies [11].

Furthermore, in relation about experimental period, studies on allogeneic/homogeneous implants of DDM resulted in osteoinduction in periods of up to 30 days [1,3], and they contradicted the findings of Sanada et al. [14] who did not observe osteoinduction with xenogeneic implants, or of Machado et al. [15] who demonstrated osteoinduction with xenogeneic implants only after 180 days.

In view of the above findings and the evidence that demineralized dentin tissue displays osteoinductive properties. The aim of this study was verify if xenogeneic and ectopic implantation of DDM in muscle tissue of rats would induces the formation of bone tissue in a short-term.

Materials and Methods

After approval by ethics committee in accordance with the Declaration of Helsinki, the DDM was obtained according to the method used by Conover and Urist [3]. Ten extracted human teeth were utilized, regardless of type. The crowns were removed, along with the pulp tissue, periodontal tissue and cement. The remaining tooth root tissue was immersed in 0.6 N hydrochloric acid (HCl) (1 g/20 mL) at 2ºC for 48 hours. Afterward, the demineralized dentin was removed and placed in a solution of chloroform-methanol (1:1) at 25ºC for 1 hour. The material was then placed again in 0.6 NHCl at 2ºC for 24 hours, followed by incubation in 2 M calcium chloride ($CaCl_2$) at 2ºC for 1 hour. Then, the material was placed in ethylenediaminetetraacetic acid (EDTA), pH 7.4, at 2ºC for 1 hour, and finally in water at 55ºC for 1 hour. This process yielded DDM, which was then fragmented into small round pieces of approximately 1 mm thickness and 4 mm in diameter. This material was stored dry at 2ºC. Before the surgical procedure, DDM was sterilized in ethylene oxide, since this method has been previously shown to be efficacious and suitable for this work [16,17].

Ten adult male Wistar rats (*Rattus norvegicus albinus*) weighing an average of 250 to 350 g were anesthetized using a mixture of 2% Xylazin (Roncum, Cristália, São Paulo, Brazil) and 10% Ketamine (Ketalar, Cristália, São Paulo, Brazil) (0.1 mL per 100 g of rat bodyweight). Then, surgical access for the implantation of DDM was made by incisions lateral to the vertebral column, on the right and left sides of each animal. After tearing away the subcutaneous tissue, two pockets were created (one on each side) in the dorsal musculature, where the implants were placed. After suture of the musculature and then the skin, the animals remained in separate cages under observation until the end of sedation.

The animals were divided into 2 groups of 5, where in the first group the implants remained in place for one month and in the second group for two months. After the end of the experimental times, biopsies were taken of the muscle tissues containing the implants and kept in 10%

buffered formalin solution for 24 hours until histological processing. After 7 days of decalcification in 20 % formic acid, the specimens were subjected to routine processing. The paraffin-embedded specimens were sectioned at 5 µm and stained with hematoxylin and eosin, since there are reports in the literature [18,19] that show the efficacy of this method in the analysis of processes related to osteoinduction.

Results

Histological examination of the materials collected at both sampling times, showed the presence of an intense chronic inflammatory infiltrate in the regions adjacent to the implants. In some areas, giant multinucleated cells of the osteoclast type were observed surrounding the material. No mineralization foci were found concomitant with the resorption process 1 and 2 months after implantation. Figure 1A shows a representative photograph of a DDM implant at 1 month after being inserted. An intense chronic inflammatory infiltrate can be seen, as well as sites of resorption. Figure 1B gives a magnified view, which shows multinucleated cells reabsorbing the material. Figure 2A shows a representative photograph of a DDM implant at 2 months after intramuscular insertion, indicating a more intense inflammatory process and greater extent of implant resorption. Figure 2B gives a magnified view, which shows multinucleated cells continuing to reabsorb the material.

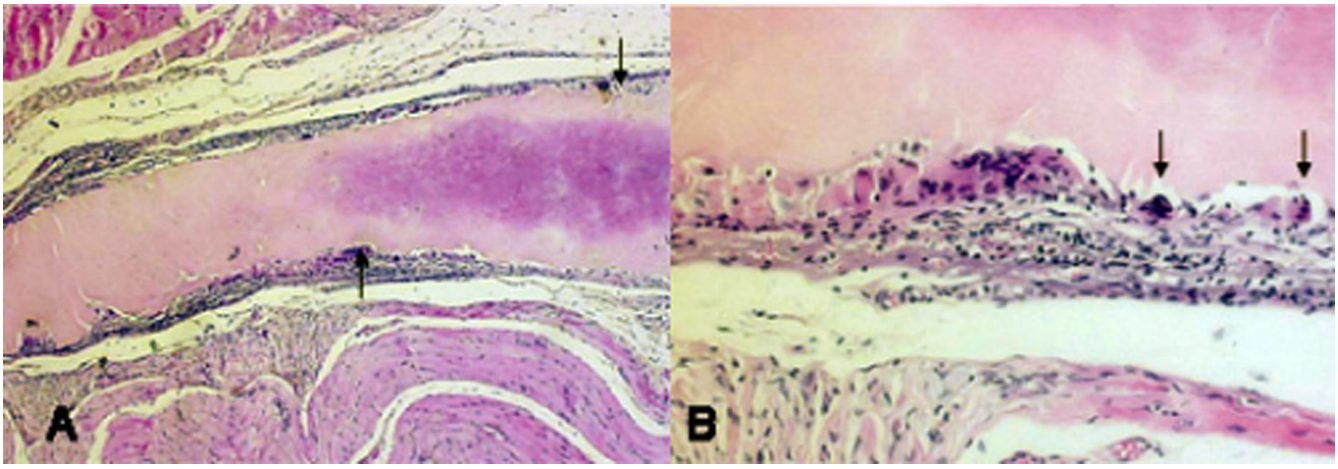

Fig. 1. Experimental period of 1 month. **A.** Sites of resorption around DDM are indicated by narrows (at 40x the original magnification). **B.** Narrows show multinucleated cells reabsorbing the DDM (at 400x the original magnification).

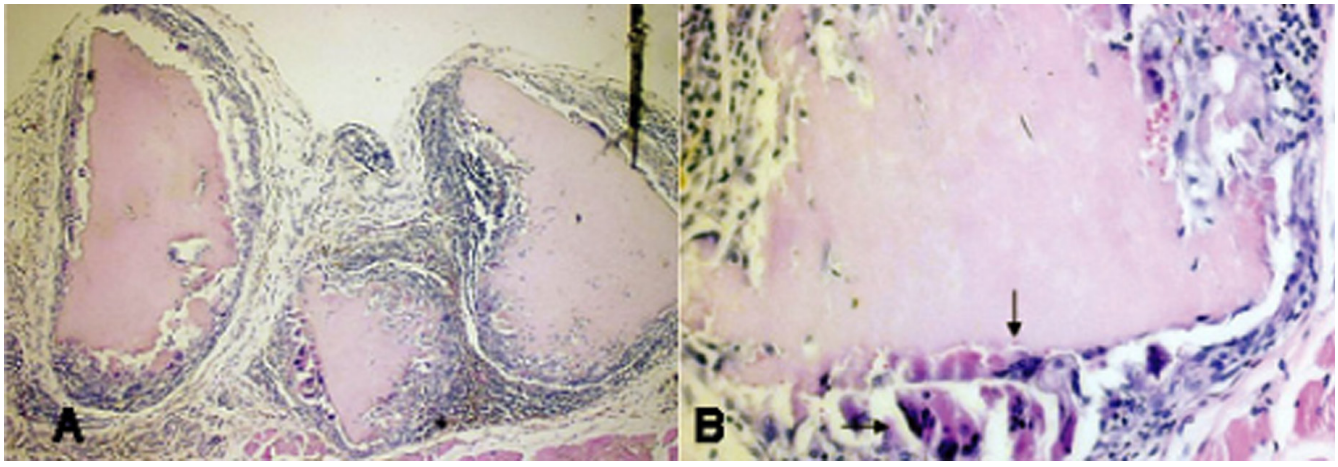

Fig. 2. Experimental period of 2 months. **A.** Greater extent of implant resorption (at 40x the original magnification). **B.** Multinucleated cells reabsorbing the DDM are indicated by narrow (at 400x the original magnification).

Discussion

Many studies have been conducted in search of a material capable of favoring osteoinduction in tissues, with the aim of repairing bone structures lost due to trauma, infectious processes, and diseases, among others. Dentistry is one of medical areas that have a great interest in this subject, because in various clinical situations, it is necessary the therapeutic use of some biocompatible material, capable of favoring suitable tissue repair. The Aggregate Trioxide Mineral and calcium hydroxide are examples of materials of mineral nature, widely utilized in dentistry, which show biocompatibility characteristics and also provide favorable conditions for the repair of tissue damaged by complications or pulp disease [20,21]. However, there are still doubts as to whether these materials have some osteoinductive property, since there is evidence that high alkalinity, as well as mineral composition, favors only the healing process of the injured tissue, by impeding microbial growth and providing a good supply of mineral at the site. Moreover, it is the organic fraction and not the mineral one of biomaterials that is responsible for osteoinduction [25]. Bone tissue has also been studied to determine its osteoinductive properties. There are reported works that bone refrigerated dry [22] and demineralized bone tissue as well [23] are materials that have this potential. BMPs, which are growth factors involved in processes of bone tissue formation, are also studied for their role in osteoinduction [5-10].

In relation to the main focus of this present study, namely demineralized dentin, many investigations have been conducted with the objective of determining its possible osteoinductive activity [2-4]. This work aimed at determining if xenogenous and ectopic implants of DDM in muscle tissue of rats induce the formation of bone tissue in a short-term.

In evaluating the osteoinductive potential of any materials, it is necessary to determine bone formation in an area of the body where it does not naturally exist. That is why the fragment was inserted in muscle tissue, since it is a tissue that is naturally distinct from bone tissue; in case there is some evidence of osteoinduction in this location, the chance of this occurring in bone would be even greater. It has also been established that increased blood supply to the tissue, in which the fragment is placed, improves the osteoinductive process [15].

All the organic components, including collagens and non-collagen proteins, would remain in the dentin matrix, because it can be seen that the concomitant action of the whole mixture could influence the osteoinductive process. The mineral fraction was removed by demineralization using EDTA, because according to Cooper et al. [24] the dentin has an extra cellular matrix which comprises growth factors, phosphorylated proteins termed Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGS) as dentine sialoprotein, dentine phosphoprotein, bone sialoprotein, dentin matrix protein-1 and osteopontin, and non-phosphorylated proteins, such as osteocalcin and the small leucine-rich proteoglycans. These bioactive molecules need to be released to regulation of dental tissue regenerative process, such release occurs after demineralization process. The dental tissue regenerative process occurs because these bioactive molecules act on stem/progenitor cell resulting odontoblast-like cells differentiation and dentine secretion. According to Simon et al. [25], the odontoblast regulation is very similar to osteoblast, because It appears that several markers of dental cells are also up-regulated in bone cells.

Osteoinduction can be influenced by the presence of infection, which may damage the repair process. The dentin was sterilized beforehand with ethylene oxide because this method does not interfere with the inductive process [15].

The results for 1 and 2 months after the implantation of this material did not show the presence of mineralization centers. The implants embedded in muscle tissue were surrounded by an intense inflammatory infiltrate, and multinucleated cells of the osteoclast type were shown to be reabsorbing this material, which is in accordance with Machado et al. who did not observe the formation of mineralization in a period of 2 months, where fibroblasts were observed starting only at 180 days [15]. According to Cooper et al. [24], the activiation of pro-inflammatory signaling cascade can result in inhibition and obstruction of the differentiation processes that induces bone resorption around periapical periodontitis; however the inflammation is an important role and prerequisite for regeneration.

There is evidence that fragment resorption may play a major role in the calcification process since resorption, in the absence of contamination, is the first stage of hard tissue formation [15].

The size and shape of the dentin may have influenced the time of the repair process because larger sizes need longer times to be reabsorbed, which should be considered in these results. Compared to studies conducted with autogenous and allogeneic implants [2-4], the present study also indicates that the type of implant could influence the time required for osteoinduction.

Thus, further studies are needed with longer experimental times and smaller-sized implants, in order to better understanding of the DDM osteo-inducing potential.

Conclusion

Based on our results, the implants of human DDM with dimensions of approximately 1 mm thickness and 4 mm in diameter, remained for periods of 1 to 2 months in the dorsal musculature of rats, did not result in osteoinduction.

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