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ORIGINAL ARTICLE

Biological response to lyophilized demineralized dentin matrix implanted in the subcutaneous tissues of rats

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Abstract *Aim:* Lyophilized demineralized dentin matrix (LDDM) consists of a type 1 collagen complex matrix containing growth factors and no mineral crystals. Although the efficacy of LDDM for bone grafting is well known, there is limited evidence on the biological response to human lyophilized DDM. Therefore, the aim of this study was to evaluate the biological response of subcutaneous tissues in rats to powdered LDDM, mineral trioxide aggregate (MTA), and Biodentine implanted using polyethylene tubes.

Methods: Forty Wistar rats were divided into four groups (n = 10 each) depending on the experimental time intervals and were placed in polyethylene tubes containing LDDM, MTA, biodentine, and one empty control. After 3, 7, 15, and 30 observation days, the animals were sacrificed and quantitative and qualitative analysis of the subcutaneous tissue samples was carried out. The intensity of the inflammatory response was scored from 0 (no response) to 3 (severe response), and the data were statistically analyzed using ANOVA and Bonferroni tests ($p < 0.05$).

Results: All groups exhibited moderate inflammation after 3 and 7 days of observation, with presence of inflammatory infiltrate predominantly consisting of macrophages and angioblastic proliferation being observed. After 15 observation days, the control group exhibited mild inflammation and a predominance of fibroblasts, and this differed significantly from the remaining cement groups

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that exhibited moderate inflammation. After 30 days of observation, all groups exhibited a mild inflammatory response, predominance of fibroblasts, and a greater amount of collagen fibers.

Conclusion: Within the limitations of this study, it can be concluded that LDDM exhibited an acceptable biological response similar to MTA and Biodentine in the subcutaneous tissues of rats.

1. Introduction

Lyophilized demineralized dentin matrix (LDDM) typically consists of a type-1 collagen complex containing growth factors and no mineral crystals (Liu et al., 2016). Dentin is a source of bioactive molecules that have significant potential for use in the repair of damaged or restored dentinal structure (Smith et al., 2012). Previous studies have used dentin as a scaffold to examine cell binding, growth, and differentiation, and to identify sources of stem cells and bioactive molecules for tissue and organ regeneration (Albuquerque et al., 2014). A variety of techniques for preparation of this material have also been reported, with the majority of them being used to evaluate the efficacy of this material for bone grafting (Urist, 1965; Bang, 1972; Chalmers et al., 1975). The demineralization process is essential for release of various growth factors and proteins (Kim et al., 2013), and previous evidence has shown that demineralized bone matrix stimulates osteogenesis (Urist, 1965; Chalmers et al., 1975) and dentinogenesis (Nakashima, 1994; Zhang et al., 2012).

Currently, mineral trioxide aggregate (MTA) and Biodentine are the materials most commonly used for pulp capping, pulpotomy, sealing of root perforations, resorption, apexification, and retrograde surgical filling (Parirokh & Torabinejad, 2010). MTA has the advantages of good biocompatibility, low solubility (Cavenago et al. 2014), excellent sealing capacity (Torabinejad et al., 1993), high alkalinity (Duarte et al., 2012), and release of calcium ions, while Biodentine is known to be a biocompatible (Mori et al., 2014) and bioactive (Zanini et al., 2012) cement. However, despite the advantages of these cements, they sometimes release small amounts of arsenic and lead upon coming in contact with bodily fluids (Camilleri et al., 2012). Additionally, previous studies have shown that radiopacifiers used in MTA can cause pigmentation of dental crowns when in contact with sodium hypochlorite (Marciano et al., 2015).

Although LDDM is available in a variety of particle sizes (Nam et al., 2016), most studies tend to use larger particles for bone grafting (Kim et al., 2013; Nam et al., 2016). Yun et al. (2016) carried out pulp capping in dogs using powdered demineralized dentin obtained from bovine teeth (xenogeneic), and compared the results to those obtained with MTA. The powdered forms of these materials are suitable for various endodontic procedures such as restoration of perforation areas, pulp capping, and resorption, as the small particle size allows good adaptation.

Although the efficacy of LDDM for bone grafting is well known, there is limited evidence on the biological response to human lyophilized DDM. Therefore, the aim of this study was to evaluate the biological response of subcutaneous rat tissue to powdered LDDM, MTA, and Biodentine implanted using polyethylene tubes.

2. Materials and methods

2.1. Ethical committee approval

This study was approved by the Ethics Committee on the Use of Animals in the Biological Sector at the Federal University of Paraná (CEUA/BIO-UFPR process 23075167918/2016–35; Approval 1050).

2.2. Surgical procedures

This study used 40 healthy adult female Wistar rats (*Rathus norvegicus*) kept in plastic cages containing five animals each. The animals were given water and fed using commercially balanced feed ad libitum, and the cages were stored in a room with controlled temperature (23 °C ± 1 °C) before and after completion of the surgical procedures. A combination of Xylazine hydrochloride (0.04 mL/100 g Xilazin® - Syntec do Brasil Ltda, Santana de Parnaíba, Brazil) and Ketamine hydrochloride (0.08 mL/100 g Cetamin® - Syntec do Brasil Ltda, Santana de Parnaíba, Brazil) was administered via intramuscular injection to achieve anesthesia.

Additionally, 160 polyethylene tubes (Embramed Ind. Com. Ltda. São Paulo, Brazil) with an internal diameter of 1.5 mm, external diameter of 2 mm, and length of 10 mm were sterilized using ethylene oxide and divided into the following four groups:

1. MTA (Angelus, Londrina, Brazil) filled (n = 40);
2. LDDM filled (n = 40);
3. Biodentine (Septodont, St-Maur-desFosses, France) filled (n = 40);
4. Negative control group made up of empty tubes (n = 40).

Healthy third molar teeth with clinical indications for extraction were selected from the UFPR (Federal University of Paraná) tooth bank. A powdered (particle size < 75 μm) (Kim et al., 2010) form of the matrix was obtained by removing the periodontal ligament and pulp from the extracted tooth, sterilizing it using an autoclave, and then pulverizing and sieving it using graduated sieves. Chemical separation of the enamel, dentin, and cementum was achieved by fractioning liquids of adequate density, and the separated dentin was then demineralized using a sequence of EDTA solutions at the following concentrations (17%, 10%, and 5%), in accordance with the protocol suggested by Li et al. (2011). After drying, the matrix was placed in a lyophilizer, and the resultant LDDM was sterilized using gamma rays and cryopreserved at − 80 °C.

The MTA and Biodentine cements were prepared as per the manufacturer's recommendations. LDDM was manipulated using distilled water on a sterile glass plate until a paste-like consistency was obtained.

After manipulation, the materials were inserted into the polyethylene tubes with the help of an insertion spatula and endodontic condensers (Maillefer, Ballaigues, Switzerland) to allow complete, homogeneous filling.

After anesthetizing the rats, a trichotomy was performed on their backs and the area was sterilized using povidone iodine solution (Rioquímica Indústria Farmacêutica Ltda., São José do Rio Preto, Brazil). Thereafter, a longitudinal incision extending approximately 15 mm into the median region of the back of each animal was made using a no.15 scalpel blade (Embramac, Itajaí, Brazil). The polyethylene tubes were positioned inside a Transofix device (B.Braun, São Gonçalo, Brazil), together with a arthroscope, TM Action for TMJ (Traumec, Rio Claro, Brazil) used as a trocar. The instrument was introduced using a catheterization movement with slight pressure, and tissue divulsion was carried out up to a depth of 18 mm where the tubes were deposited.

The implants were inserted perpendicular to the incision line, with each animal receiving four implants (two in the upper part of the incision and two in the lower part). The edges of the incisions were sutured using 4.0 silk thread (Ethicon, Johnson & Johnson, São José dos Campos, Brazil), and the animals were divided into four experimental groups based on observation period (3, 7, 15, and 30 days; n = 10, respectively).

After completion of the observation period, the animals were sacrificed using an overdose of anesthetic solution and trichotomy of the dorsal region was performed to allow location of the implanted tubes using palpation. The implant site was dissected such that a margin of tissue was retained around the tube, and the tissues were fixed in 10% formalin solution.

2.3. Histologic processing and analysis

Following removal, the tubes were placed in paraffin blocks and longitudinal sections of 5 μm thickness were made. The slides were stained with hematoxylin and eosin, and descriptive, histomorphometric, and histological analyses were carried out using light microscopy.

Histological analyses were carried out at 100 × and 400 × magnification, and the biological response of the subcutaneous tissues to the tested materials was examined. The tissues located at the mouth of the polyethylene tube and those in contact with the materials tested were analyzed for inflammatory infiltrate and other cellular phenomena indicative of a tissue reaction. (Fig. 1)

Histomorphometric analyses were performed using light microscopy at 400 × magnification, and the tissues in contact with the material at the end of the tube were examined. Three adjacent fields were captured and the images were analyzed using ImageJ software (ImageJ, Bethesda, USA). In accordance with previous studies using similar methodology, the biological responses were scored as follows: 0 (no reaction) - none or few inflammatory cells; 1 (mild reaction) - < 25 inflammatory cells seen; 2 (moderate reaction) – 25 to 125 inflammatory cells seen; and 3 (severe reaction) - > 125 inflammatory cells seen (Gomes-Filho et al., 2012; Cintra et al., 2017).

2.4. Statistical analysis

The distribution of scores was calculated as proportions, and the median value for each group and each experimental period was examined. The data were analyzed as ranks using the ANOVA test with Bonferroni correction, and the statistical significance level was set at 5% ($p < 0.05$).

3. Results

3.1. Descriptive histological analysis

All examined groups exhibited formation of connective tissue around the tube implantation area, and traces of the tested

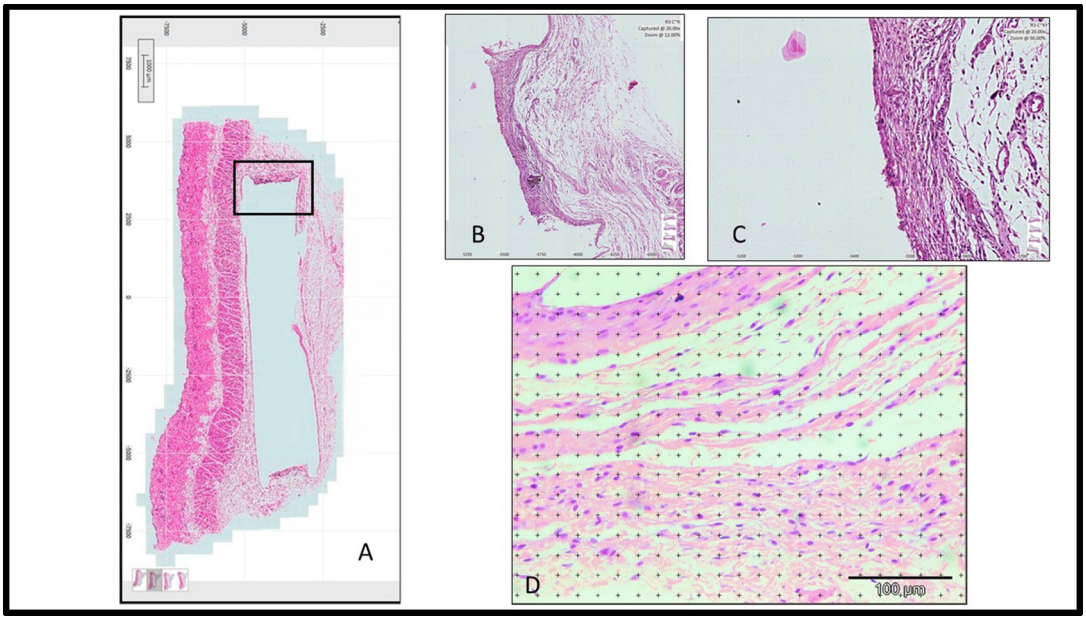

Fig. 1 (A) Image obtained from a microscope slide scanner. Longitudinal section showing the tube placed in the subcutaneous tissue. The square represents the analyzed area. (B) and (C) Enlarged image of the analyzed area. (D) Counting grid used for determining the inflammatory score.

materials were found to be present in the tissues. Additionally, a reduction of inflammatory infiltrate as a function of time was observed in all groups.

3.1.1. Three day observation period

Moderate inflammatory infiltrate with a predominance of macrophages and evidence of some fibroblasts, blood vessels, and edema was observed in all groups after a period of three days (Fig. 2).

3.1.2. Seven day observation period

Moderate inflammatory infiltrate with a predominance of macrophages and angioblastic proliferation was observed in all groups after 7 days. Additionally, the cement groups showed evidence of phagocytosis, apparent from positioning and interaction of the macrophages with the extravasated material (Fig. 2).

3.1.3. Fifteen day observation period

The control group exhibited a greater reduction in inflammatory infiltrate (mild intensity), while the remaining groups exhibited moderate inflammatory infiltrate with greater fibro-angioblastic proliferation after 15 days (Fig. 3).

3.1.4. Thirty day observation period

All groups exhibited mild inflammatory infiltrate, thin capsules, large amounts of collagen fibers, and decreased population of inflammatory cells and blood vessels after 30 days. Additionally, a predominance of fibroblasts was also observed (Fig. 4).

3.2. Histomorphometric analysis

Table 1 shows the distribution of inflammatory response scores by group and observational period. No differences were observed between the groups over a period of 3 and 7 days, and the specimens exhibited cell counts ranging between 25 and 125 (score 2). A greater inflammatory response (score 2) was observed in the cement groups after 15 days, with a statistically significant difference ($p < 0.05$) being observed in comparison to the control group which exhibited a reduction of the inflammatory infiltrate (score 1). Within 30 days, all groups exhibited a mild inflammatory response (score 1).

4. Discussion

The results of the current study showed that LDDM elicited a biological response similar to that of MTA and Biodentine.

Some characteristics of subcutaneous tissue studies make this method a reference by FDI (FDI, 1980) and ISO (ISO, 2016). The method used in this study had several advantages, as follows: a) implantation of empty polyethylene tubes in the subcutaneous tissues on the backs of rats induced repair, apparent from the formation of fibrous tissue, and non-persistent inflammation; b) it allowed implantation of readily manipulated materials, thus decreasing diffusion into the surrounding tissues and standardizing material/tissue contact; and c) the external diameter selected for this study minimized material leakage while providing an adequate and standardized area for histological analysis. Although material overflow can be limited by carrying out implantation after the material has set, this limits the ability to examine the effects of the setting reaction itself (Olsson et al., 1981).

Lyophilization of DDM is a preparation technique commonly used for graft biomaterials as it decreases the risk of transmission of infections such as HIV, reduces antigenicity, and induces only a slight immune response in the patient (Zasacki, 1991; Urist, 2002). The process of lyophilization promotes dehydration of the demineralized dentin matrix by mobilizing the water molecules from a solution of protein macromolecules. This promotes hydrolysis of the macro-

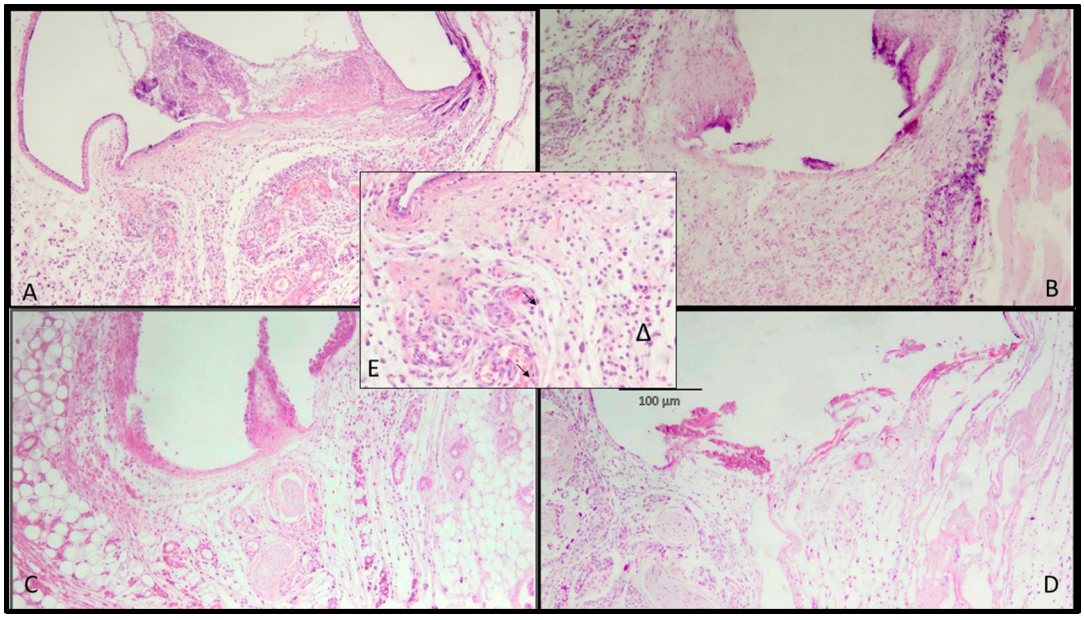

Fig. 2 3 and 7 day observation periods - (A) Control (B) LDDM (C) MTA (D) Biodentine. Biodentine [100 × magnification]. (E) 400 × magnification. Triangles indicate inflammatory infiltration. Arrows indicate blood vessels.

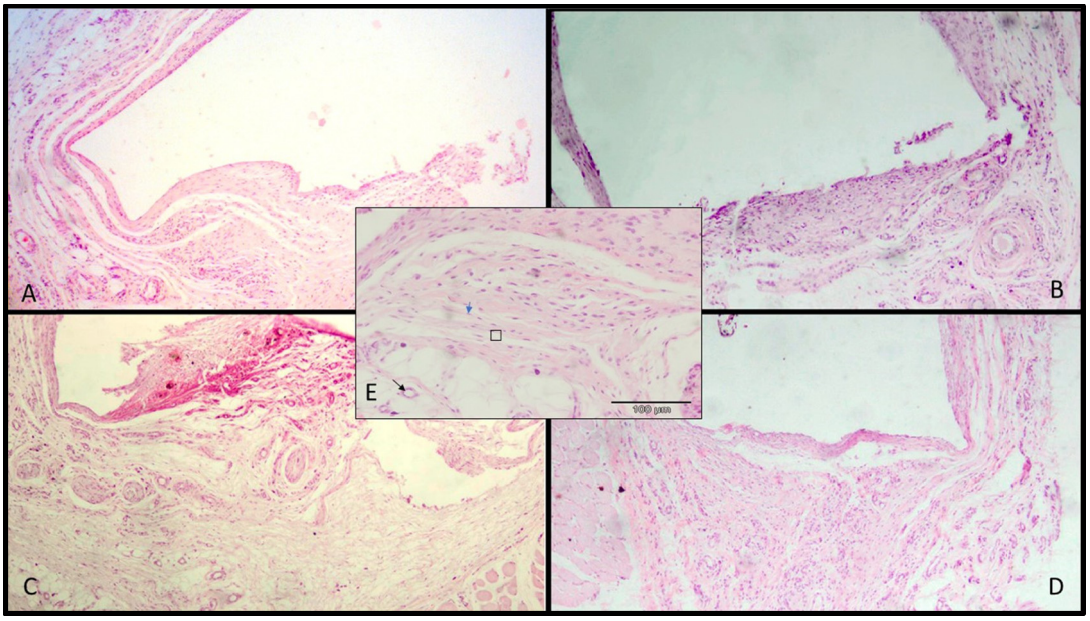

Fig. 3 15 day observation period - (A) Control (B) LDDM (C) MTA (D) Biodentine [100 × magnification]. (E) 400 × magnification. Black arrows indicate blood vessels. Blue arrows indicate fibroblasts. Squares indicate collagen fibers.

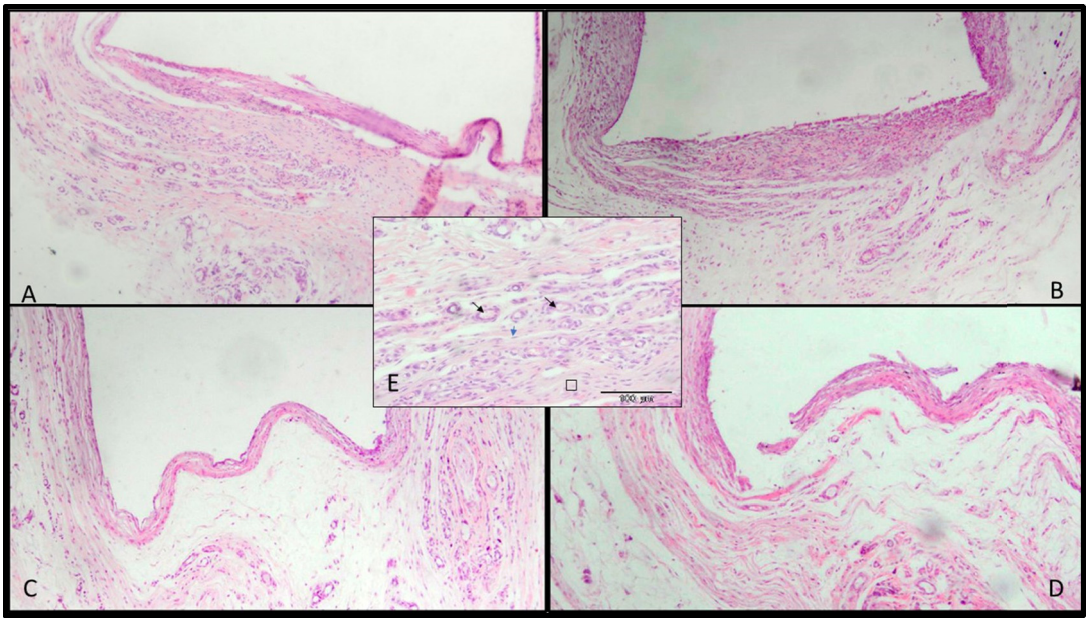

Fig. 4 30 day observation period - (A) Control (B) LDDM (C) MTA (D) Biodentine [100 × magnification]. (E) 400 × magnification. Blue arrows indicate fibroblasts. Squares indicate collagen fibers.

molecules while keeping the polypeptides that constitute the collagenous or non-collagenous protein segments and are biologically active in the process of tissue regeneration intact. This could, in turn, explain the decrease in antigenicity without loss of osteo-promotion characteristics exhibited by LDDM. The biocompatibility observed in this study could be attributed to lyophilization of the xenogeneic graft used.

Particle size and pore interconnection of DDM play an important role in bone formation. Nam et al. (2016) histologically evaluated two sizes of DDM bone grafts and concluded that smaller particles with less space between them were more effective in promoting osteogenesis. However, powdered materials were recommended for use in endodontics as the dimensions of the treated structures were very small, making application of granular material difficult.

MTA was selected for comparison as it has been commercially available for several years and is extensively studied (Parirokh & Torabinejad, 2010; Marciano et al., 2015; Cavenago et al., 2017). It is the gold standard for treatment of resorption, perforation, pulpotomies and *retro*-blocking due to its physico-chemical characteristics and biocompatibility (Torabinejad & Parirokh 2010). Clinical studies have also

Table 1 Percentage of scores that categorize the inflammatory response among the groups evaluated in different experimental periods.

	Period/Group	Scores				Result
		0	1	2	3	
3 days	Control	0	0	100	0	2
	MTA	0	0	100	0	2
	Biodentine	0	0	100	0	2
	LDDM	0	0	100	0	2
7 days	Control	0	0	100	0	2
	MTA	0	0	100	0	2
	Biodentine	0	0	100	0	2
	LDDM	0	0	100	0	2
15 days*	Control	0	100	0	0	1[a]
	MTA	0	0	100	0	2[b]
	Biodentine	0	6.66	93.34	0	2[b]
	LDDM	0	13.33	86.67	0	2[b]
30 days	Control	0	100	0	0	1
	MTA	0	100	0	0	1
	Biodentine	0	93.34	6.66	0	1
	LDDM	0	100	0	0	1

Scores – 0 (no inflammatory reaction): none or few; 1 (mild inflammatory reaction): < 25 cells; 2 (moderate inflammatory reaction): between 25 and 125 cells; 3 (severe inflammatory reaction): 125 or more cells.
*In the period of 15 days, different letters indicate statistically significant difference between the groups (ANOVA, Bonferroni, $p < 0.05$).

demonstrated the biological superiority of this material compared to other cements when used for *retro*-blocking (von Arx et al., 2010; Tsesis et al., 2013). The results of this study showed that MTA resulted in a moderate inflammatory reaction over the initial observation periods of 3, 7, and 15 days, with inflammation reduction occurring over time. A mild inflammatory response and formation of new organized tissue with a predominance of collagen fibers and fibroblasts was observed after 30 days, and this was in agreement with previous findings (Moretton et al., 2000; Cavenago et al., 2017). Mori et al. (Mori et al. 2014) also reported a mild inflammatory response in the initial observation periods, but this was in contrast to the findings reported by Moretton et al. (Moretton et al. 2000) who observed severe inflammation and necrosis after 15 and 30 days of observation.

Despite the clinical suitability of MTA, its long setting time and difficult manipulation have led to the search for a better substitute. Biodentine was included in this study as it is the most commonly used alternative to MTA and has similar clinical indications. In their clinical study, Nowicka et al. (2013) compared the pulpal response of human teeth to Biodentine and MTA after direct pulp capping, and found that Biodentine exhibited efficacy similar to MTA. Additionally, it also exhibited simpler management and manipulation compared to MTA. This was in accordance with other studies that also reported good pulpal response (Laurent et al., 2012; Tran et al., 2012; Zanini et al., 2012) and stimulation of dentin formation (Laurent et al., 2012; Tran et al., 2012). In an in vitro study comparing the occurrence of crown discoloration, the authors found a significant difference between Biodentine and MTA, with the former exhibiting less color change in the teeth. In this study, a moderate and mild inflammatory response were observed in the initial and final observation periods, respectively, and this was in agreement with previous evidence (Mori et al., 2014; da Fonseca et al., 2016).

The main limitation of LDDM is that it is a non-setting material with limited sealing ability, a property essential for some clinical treatments such as root canal perforations and root-end fillings, in comparison to bioceramic cements. However, this was a preliminary study evaluating the biological characteristics of this material, with the aim of eventually improving and further developing it for use as an endodontic material.

The presence of inflammation in the first two observation periods of this study could be attributed to surgical trauma, while the inflammatory response observed after 7 days was most likely caused by the materials themselves. The adequate biological response exhibited by LDDM in this study suggests that it could be considered as a possible treatment alternative for MTA. However, further studies examining the biological and physico-chemical properties of LDDM are necessary before it can be considered suitable for use in humans.

5. Conclusions

No differences were observed between the groups over a period of 3 and 7 days (score 2). A greater inflammatory response (score 2) was observed in the cement groups after 15 days, with a statistically significant difference ($p < 0.05$) being observed in comparison to the control group which exhibited a reduction of the inflammatory infiltrate (score 1). Based on the findings of this study, it can be concluded that LDDM exhibits an acceptable biological response similar to MTA and Biodentine in the subcutaneous tissues of rats.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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