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Mouse Subcutaneous Tissue Reaction to Calcium Hydroxide-based Root Canal Filling Material

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Abstract: Mouse subcutaneous tissue reaction to an embedded calcium hydroxide-based root canal filling material was analyzed histopathologically. After the material was placed within the mouse dorsal subcutaneous tissues, we performed examinations using histopathological, histochemical and immunohistochemical techniques. Two weeks after embedment, the proliferation of granulation tissue had already begun to surround the calcification. Most of the cells observed were macrophages. Likewise, multinucleated giant cells increased significantly. The multinucleated giant cells were observed as two types. In one, the centers of the giant cells were vacuoles, while in the others there were deeply stained calcifications with hematoxylin. Twelve weeks after embedment of the materials, further growth of multinucleated giants cells were sighted. Histochemically, von Kossa-stain-positive granules were observed within the macrophages and multinucleated giant cells as black fine granules. According to the TRAP stained specimens, the multinucleated giant cells especially reacted strongly at 4 weeks. However, the reaction became very weak at 12 weeks. CD68 immunohistochemical staining showed positive reactions in the cytoplasm of the proliferating macrophages and multinucleated giant cells. These results suggest that multinucleated giant cells are present in the surrounding tissues due to implantation of the calcium hydroxide-based root canal filling material, and that the presence of ACP in the cells is due to ingested calcium during active phagocytosis, which would disappear later on.

Key words: Calcium hydroxide, Tissue reaction, macrophage, multi nucleated giant cells, TRAP

Introduction

Calcium hydroxide-based root canal filling material is widely used in clinical practice. Consequently, in vivo studies and experiments are actively being carried out. Histological studies of tissue reaction have also been reported [1-6]. However, most of the studies focused on the formation of localized calcification associated with the components of the material[7-9]. In this study, the mouse subcutaneous tissue reaction to the embedded calcium hydroxide-based root canal filling material was analyzed histologically.

Materials and Methods

The examined material in the study are Vitapex®(Neo Dental Chemical Products Co., Tokyo, Japan). A total of 6 mice purchased from Japan SLC (Shizuoka, Japan) were used as experimental animals. The mice were 5-week-old, male and from ddY strain. After purchase, the animals were bred for 3 weeks and no abnormality in health was confirmed (At the age of 8 week-old). During breeding, the animals were housed in exclusive breeding plastic container and fed with mouse/rat solid feed a Picolab Rodent Diet 20® pursed from Japan SLC Co., Shizuoka, Japan. Also, the animals had free access to food and drinking water. Embedment of Vitapex® was done in the following manner. Briefly, after being subjected to general anesthesia by inhalation of isoflurane, the mouse was fixed on the surgical table. The dorsal site where the surgical field would be done was wiped with cotton with alcohol and shaved with electric razor. An incision was made near the midline using a scalpel and the subcutaneous tissue was peeled off using a blunt ophthalmic blade. Then after, approximately 50 mg of Vitapex® was injected into the subcutaneous tissue. The incision was closed using single thread

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suture. After 2, 4 and 12 weeks, the mice were subjected to the same general anesthesia and the area where Vitapex® was embedded including the surrounding tissues were excised as one lump of tissue. The harvested tissues were fixed in 10 % neutral buffered formalin solution, embedded in paraffin following routine histopathological manner and examined histopathologically. In addition, some sections were stained with tartrate resistant acid phosphatase (TRAP) and von Kossa stains. Furthermore, in order to mark the macrophages, immunohistochemistry (ICH) with CD68 (Anti-CD68 antibody, ab125047, Abcam, Cambridge, UK; dilusion: 1/100) was used. Concisely, after deparaffinization, the slides were pre-treated with Dako Protease K for about 5 min. For staining, Dako Chem Mate Envision Kit (Dako Japan, Tokyo, Japan) was used and antigenic sites were revealed using DAB. The experiment was carried out in accordance with the Animal Experiment Guidelines of Matsumoto Dental University.

Results

Two weeks after embedment, small and large irregular calcifications and silicone oil in small vacuoles were observed in the surrounding tissues. The proliferation of granulation tissue had already begun to surround the calcification. In addition, the proliferated granulation tissue was filled with spindle-shaped fibroblasts and macrophages with rounded nucleus pushed on one side. Several capillaries were also noticed in between cells. Also, few multinucleated giant cells were detected. Four weeks after embedment, an increased in cellular components within the growing granulation tissue was apparent. Most of the cells observed were macrophages (Fig. 1). Likewise, multinucleated giant cells increased significantly. The multinucleated giant cells were observed as two types. One the center of the giant cells were vacuoles (Fig. 1), the other deeply stained calcifications with hematoxylin (Fig. 2). Twelve weeks after the embedement, further growth of multinucleated giants cells were sighted.

Histochemically, von Kossa-stain-positive granules were observed within the macrophages and multinucleated giant cells, as black fine granules. The proliferating granulation tissue were around the material was embedded (Fig. 3). The stained granules increased over time revealing the shape of the cells within the macrophages and multinucleated giant cells (Fig. 4). According to the TRAP stained specimens, the multinucleated giant cells especially reacted strongly at 4 weeks (Fig. 5). However, the reaction became very weak at 12 weeks. CD68 immunohistochemical staining showed positive reactions in the cytoplasms of the proliferating macrophages and multinucleated giant cells (Fig. 6).

Discussion

Calcium hydroxide-based root canal filling material has been widely used in the clinical practice on a daily basis, frequently used in children in particular[10]. The material offers convenient and simple root canal treatment in a short period of time providing excellent results. Histopathological studies reported no damage or tissue reaction from calcium hydroxide-based root canal filling materials[4-6]. Kawakami et al did histopathological studies regarding tissue reactions to calcium hydroxide-based root canal filling material[5-8]. We also previously reported and discussed in detail the tissue damage and its fate in vivo[9-12]. The data presented by Kawakami et al in 2012 were published as a monographs[12]. Moreover, the experimental purpose in previous reports did not include detailed pathological analysis. In this study, the authors went beyond histopathological investigation, in which von Kossa and TRAP stains were used together with immunohistochemical staining using CD68 to specifically examine the pathological response of the surrounding tissue to calcium hydroxide-based root canal filling material.

The results of this experiment were similar to those previously described[5,6]. Particularly, necrotic layer was observed in the surrounding tissues where Vitapex® was embedded. Macrophages and multinucleated giant cells proliferated in the granulation tissues. The role of those cells in active phagocytosis having significant impact on the surrounding tissues has been confirmed. Furthermore, the presence of those cells was established by the positive reaction to CD68. Although the results have already been confirmed, innovations in the medical field are constantly arising and so it justifiable to perform more experiments that would further verify the results for safety purposes since of calcium hydroxide-based root canal filling materials is very commonly used among children.

Von Kossa stain revealed the presence of multinucleated giant cells 4 and 12 weeks after the embedment. It was inferred that the multinucleated giant cells ingested the calcifications brought about by the calcium component of Vitapex®. This tissue reaction to conventional calcium hydroxide-based root canal filling material [5,11], is considered to be an important finding.

Although TRAP is used as an osteoclast marker in general, its expression are also detected in other foreign materials[14]. Multinucleated giant cells were not observed 2 weeks after embedement. However, at 4 weeks, multinucleated giants cells showed positive reaction (reddish purple) to TRAP. But then again, at 12 weeks, the reaction became weak. This might be due to the absence of acid phosphatase (ACP) in multinucleated giants cells at 2 and 12 weeks compared to those at 4 weeks. The multinucleated giants cells, which ingested calcium contained high amount of ACP, which can be seen in osetoclasts during advanced phagocytosis and then disappeared during the inactive state. The presence of multinucleated giant cells can be explained in the same phenomenon[15]. Multinucleated giant cells ingested mucus thereby altering its cellular morphology. The same mechanism occurred during the decline in the activity of TRAP in

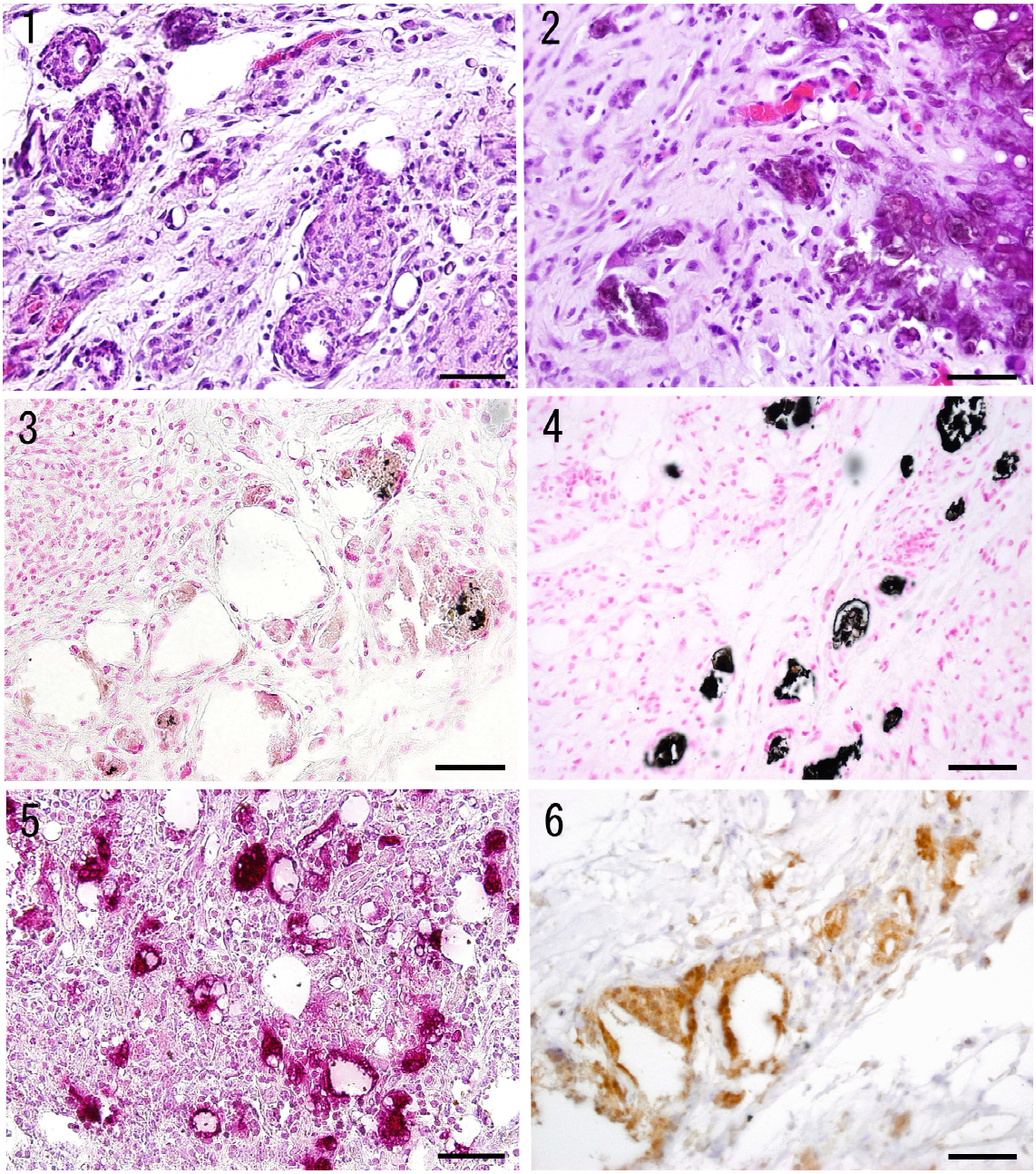

Figure 1. Histopathological feature of the embedded area showing proliferation of granulation tissues (4-week-specimen; HE; scale bar=100μm).
Figure 2. Histopathological feature showing calcified portions in the embedded area (4-week-specimen; HE; scale bar=100μm).
Figure 3. Some von Kossa-positive black small granules within the multinucleated giant cells (4-week-specinen; von-Kossa; scale bar=100 μm).
Figure 4. A large amount of von Kossa-positive black small granules within the multinucleated giant cells showing the out-line of the cells (12-week-specinen; von-Kossa; scale bar=100μm).
Figure 5. TRAP-positive products appearing within the multinucleated giant cells (4-week-specinen; TRAP; scale bar=100μm).
Figure 6. Positive products showing in the multinucleated giant cells (12-week-specinen; ICH-CD68; scale bar=100μm).

multinucleated giant cells. Nevertheless, further investigation is needed to verify the hypothesis. The results suggest that multinucleated giant cells are present in the surrounding tissues due to Vitapex® implantation and the presence of ACP in the cells is due to ingested calcium during active phagocytosis which would disappear later on.

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