行政院國家科學委員會專題研究計畫　成果報告

不同成分根尖充填劑植入老鼠之生物效應比較

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-040-018-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學牙醫學系

計畫主持人：黃翠賢
共同主持人：高嘉澤

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行政院國家科學委員會補助專題研究計畫成果報告

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一、中英文摘要

摘要

於根尖手術治療中所填補之材料對於組織之修復扮演重要之角色，所使用之材料必須是對於生物體具有生物相容性。本研究比較三種不同之根尖充填劑氫氧化鈣 (calcium hydroxide-based)，丁香由酚 (eugenol-based) 和三氧礦化物 (mineral trioxide aggregate)植入於老鼠體內後之生物學上反應。根尖充填材料經硬化後，將之植入到老鼠體內，分別經過六週與八週後，將老鼠犧牲作 X 光檢查與組織切片觀察分析。結果發現第八週氫氧化鈣組之 X 光片上出現有鈣化環之影像，其他組則不見。氧化鋅丁香油酚組則於組織切片上出現發炎性細胞堆積，三氧礦化物組則呈現與組織有良好之生物相容性。

關鍵字：根尖充填劑　植入　生物相容性

Abstract

The use of root-end filling materials designed to stimulate hard and soft tissue repair in periradicular tissues is highly recommended. The materials should demonstrate good cell and tissue compatibility. The aim of the present study was to compare in vitro biocompatibility and in vivo tissue reaction with calcium hydroxide-based, eugenol-based and mineral trioxide aggregate root-end filling materials. The human osteosarcoma cell line was treated with immersed root end filling materials. The test materials were implanted in rats and the results observed at 6 and 8 weeks. In vitro, the highest survival rate was demonstrated for the mineral trioxide aggregate ($p<0.05$). In vivo, a radiopaque ring was evident in the calcium hydroxide implants on the eight-week radiograph. Histopathology revealed eugenol-based material with the inflammatory cells around the implant, with fibrous connective tissue forming around the calcium hydroxide-based analog. The mineral trioxide aggregate appears to be well tolerated by the tissue.

Key words: root apex filling material, implantation, biocompatiblitliy

二、報告內容

Introduction

The primary objective of root-end surgery is appropriate placement of a seal between the root canal system and the periodontium [1]. An ideal root-end filling material should not only hermetically seal the root-end cavity, but it should also be biocompatible,

non-toxic, insoluble in tissue fluids, non-resorbable, dimensionally stable, capable of inducing osteogenesis and cementogenesis, easy to prepare and use, sterilizable, radio-opaque, inexpensive, and not susceptible to denaturing in the presence of moisture [1,2]. A number of research and clinical studies have attempted to identify the ideal root-end filling material [3,4]. These investigations have focused on preservation of the requisite physical properties and material usage in the surgical site, as well as radiographic assessment of their periadicular tissue response.

Historically, materials that have been advocated for root-end fill include amalgam, a zinc oxide eugenol-based cement (Super-EBA, Bosworth Co., Durham, England), composite resin, glassionomer cement, intermediate restorative materials, mineral trioxide aggregate (MTA, ProRoot, Tlusa Dental, Tulsa, OK, USA) together with many others [5,6]. As these materials are in direct and prolonged contact with the periodontal tissue, biocompatibility is of primary importance. Previous studies have determined that the root end filling materials are cytotoxic to many cell lines in vitro [7-9]. These materials also inhibit cell growth and the viability of gingival fibroblasts and PDL cells derived from the human periodontium [10]. Biocompatibility studies have shown that MTA is superior to other commonly used root-end filling materials[9,11,12].

Outcome varies where root-end filling material is implanted into different animals, as described below. In vivo study of tissue reactions after subcutaneous and intraosseous implantation of MTA and ethoxybenzoic acid cement has shown that these materials are not osteoinductive upon subcutaneous implantation, but rather osteoconductive upon intraosseous implantation. Reactions to intraosseous implants are less intense with both materials in comparison to subcutaneous implantation [13]. Another study of rat connective-tissue reaction to implanted MTA has shown a layer of granulation in the dentin wall tubules [14]. Implantation of MTA or Portland cement into adult guinea pigs produced bone healing and was associated with minimal inflammatory response at the implant sites [15]. Further, in vitro biocompatibility study has demonstrated that MTA has a favorable bone reaction when implanted in the tibia and mandible of guinea pigs [16].

The satisfactory experimental results suggest that outcomes should be comparing in vitro and in vivo study. There are no studies that have simultaneously compared biocompatibility of the various root-end filling materials cultured in vitro and implanted in vivo. The aim of the present study was to compare in vitro biocompatibility and in vivo tissue reactions of calcium hydroxide-based,

eugenol-based and mineral trioxide aggregate root-end filling materials.

Material and methods

Material preparation

Three kinds of root-end filling materials were compared: calcium hydroxide (Life; Kerr Co. Romulus, MI, USA) and eugenol-based cements (Super EBA; Bosworth Co., Durham, England), and mineral trioxide aggregate (ProRoot; Tulsa Dental, Tulsa, OK, USA). (Table I)

The cylindrical acrylate applicators were 2 mm in diamter and 2 mm in length, and were sterilized before use. The root-end filling materials were mixed and inserted in the applicator hole. After material setting, 30 pieces of the material were sealed in polyethylene tube without adding medium and stored in an incubator at 37 ℃ before the in vivo test. For the in vitro test, three pieces of the set end-filling material were immersed in McCoy's medium for one day and one week. The immersed solutions were then used to detect the cell survival rate.

In vitro testing

Cell suspensions of the human osteogenic sarcoma cell (U_2OS, BCRC no 60187, Food Industry Research and Development Institute, Taiwan) line were seeded into 96-well flat-bottomed plates at 5×10^3 cells/well, as determined using a hemocytometer, in complete McCoy's medium (SIGMA; Sigma Chemical Co., St Louis, MO, USA), and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 hours. The culture medium was then replaced with 200-μL aliquots of the test extracts or control media (DMSO 5% prepared as positive control and complete culture medium as negative control), and the cells were then incubated for 24 hours at 37 °C in humidified air with 5% CO_2. Test samples were then divided into two groups consisting of cells exposed to the one-day or one-week test extracts. Each well was tested in triplicate.

After the exposure, cell viability was determined from the ability of the cells to cleave the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphen yl tetrazolium bromide; MTT, SIGMA) to a formazan dye. The medium was removed with a sterile pipette, and 200 μL of phosphate-buffered saline was added to each well, swirled gently for 1 min and then replaced with 100 μL of complete medium and 10 μL of a 5-mg/L solution of MTT. The cells were incubated in the MTT/medium solution for 4 hours at 37°C in an atmosphere of 5% CO_2. Then, 100 μL of a 6.25% solution (vol/vol) of 0.1 mol/L NaOH in DMSO was added to each well, and the plates were incubated overnight to solubilize any formazan crystals that had formed. Plates were shaken for 60 min at room temperature on a plate shaker to achieve uniform color. Optical densities were then measured at 550 nm using a multiwell spectrophotometer. The survival rates are presented as mean ± standard deviation (%; mean ± SD). The

results were compared using one-way analysis of variance (ANOVA). Differences in treatment means were analyzed using the Student-Newman-Keul test, and considered significant at probabilities of less than 0.05.

Animal implantation test

The experimental protocol was ethically approved by the Animal Care Committee at the Chung Shan Medical University of Taiwan. Forty rats weighing between 150-200 g were quarantined for 2 weeks before commencement of the experimental procedure. Each animal was anesthetized by an intramuscular injection of ketamine and xylazine [17].

Surgery was performed on the leg of each animal, with the site shaved and disinfected with 5% tincture of iodine. Local anesthesia was administered by infiltration with 0.25 mL of 3% lidocaine. After a skin incision in the hip area, the materials were placed inside the muscle proximate to the bone. In the experimental group, each animal received one implant of root-end filler. After secure placement of the implants, the muscle layer and skin were repositioned and sutured with cotton. The control group animals received a water injection to create comparable stress.

Twenty rats were euthanized after 6 weeks using CO_2 overdose, and the remaining 20 animals after 8 weeks. The legs were dissected free and prefixed in 10% formalin. Digitalized X-rays were taken of the implant site of the rat leg. Within an hour, the leg was trimmed back to a final specimen size of approximately 10 mm and fixed in formalin at 4 ℃ for 24 hours. The samples were dehydrated in alcohol, embedded in paraffin and serially sectioned with a microtome set at 5 μm. Sections were stained with hematoxylin and eosin. Histological slides of the tissue adjacent to the implanted materials were taken through a light microscope.

Results

U_2OS compatibility

The U_2OS cells treated with one-day or one-week immersed root-end filling materials showed the MTA group had the highest survival rate (135.13 ± 21.68 in one-day group, 106.04 ± 4.30 in one-week group) ($p<0.05$). The survival rates of U_2OS cells treated with one-day or one-week for the calcium hydroxide and eugenol-based materials were not statistically different ($p>0.05$; Figure I).

X-ray observation

X-ray examination of the implant site revealed that root-end filling materials were properly placed in the tissue. The structure of the root-end filling material was still intact after six weeks of observation (Fig. II). At eight weeks, the structure of the root-end fillers was still intact, except for the Life material (Fig. III). A radiopaque ring was visible around the implant for the Life group (Fig. III d).

Histological observation

Control group

Tissue sections from the rat legs showed muscle layer and some adipose tissue. No inflammatory cells were noted (Figure. IV a).

Life calcium hydroxide base

The implant material was surrounded by fibrous tissue with ingrowth of the connective tissue observed at 6 and 8 weeks. No evidence of inflammatory cells, collagen deposition or mineral deposition was detected in the experiment (Figure IV b, c and Figure V a, b).

ProRoot mineral trioxide aggregate base

Muscle structure was evident in the tissue surrounding the root-end filling material by week 6, and a fibrous layer by week 8. There was no evidence of inflammation, collagen deposition or mineral deposition in the experiment (Figure IV d, e and Figure. V c, d)**.**

Eugenol-based Super EBA

The muscle structure surrounding the implant consisted of fibrous connective tissue and inflammatory cells at weeks 6 and 8 of observation respectively (Figure IV f, g and Figure V e, f). There was no evidence of mineral deposition.

Discussion

Extracts of root-end filling materials are useful for toxicity screening *in vitro*. This offers the advantages of easy filtration sterilization, and affords examination of the effect of these materials on cells that are both distant to, and in contact with root end filling material [18]. This *in vitro* extract testing simulates the immediate post-surgical periradicular environment, where toxic elements of the root-end filler may leach into the surrounding fluids in the bony crypt as the filling material is in contact with the osseous tissue. Thus, a human osteogenic sarcoma cell line culture system was employed in our study. These cells closely resemble human osteoblasts in their ability to express high levels of bone markers [19]. The present results show that MTA has the highest biocompatibility in vitro (Figure. I), confirming the findings of other reports [5,7,9,18]. The low survival rate with calcium hydroxide root-end fillings may be associated with excessive pH of the extracts. Eugenol, which is potentially damaging to cells, is the main component of eugenol-based materials. When freshly mixed zinc oxide-eugenol cements contact fluid, an immediate and initially high release of eugenol occurs [20]. Eugenol is toxic to Chinese hamster lung fibroblast V79 cells, and can also cause chromosome damage [21]. Thus, when the original extracts of root-end filling materials were added to the cultures, most of the cells did not survive.

In the present investigation, the longest study period was 8 weeks. This may not be sufficient for adequate assessment of long-term response to

materials that are intended to be in contact with living tissues in humans for many years, however. Moretton et al. showed that, in terms of life-span, 1 month in rats is equivalent to approximately 30 months in humans [13]. Further, with respect to observed biological response, if material reactions are favorable at 8 weeks, it is unlikely that a subsequent inflammatory reaction will develop barring physiochemical deterioration of the material or its colonization by bacteria, which may form a superficial biofilm with subsequent adverse effects. Thus, time periods of 6 and 8 weeks were used in the present study.

From X-ray observation, only the calcium hydroxide-based implant showed a ring-like radiopaque morphology at 8 weeks relative to the initial radiograph. The rest of the implants showed no change. We suggest that the radiopaque ring probably comes from dissolved implant material. From histopathology observation of the calcium hydroxide implant, there was no evidence of either bone formation or induction. There was ingrowth of connective tissue into the calcium hydroxide implant, however. Dissolution of the periphery of the calcium hydroxide implant may account for the radiopaque ring. The chronic pulp inflammation and complete dentin bridge resulted from calcium hydroxide cement capping on the dog pulp was found [22]. Normally, areas of coagulation necrosis and dystrophic calcification are found in calcium hydroxide implants [23]. The present study demonstrated the existence of a fibrous connective tissue-like structure without necrosis around the calcium hydroxide implant. Although cytotoxicity has been noted for the calcium hydroxide based root-end filling material in vitro, it does not appear to produce any tissue necrosis in present findings.

From in vitro study, the highest survival rate was demonstrated for MTA in Figure 1, indicating its biocompatibility. In vivo, MTA-toxicity study over 7, 15, 30, 60 and 90 days showed moderate inflammatory response developed in subcutaneous connective tissues in rats at 7 days [24]. Inflammation had reduced by day 60 and, by day 90, the implant material was surrounded by an increasingly thick fibrous connective tissue [24]. The present study showed that fibrous tissue surrounding the MTA muscle implant at weeks 6 and 8. No inflammatory cells were found. The fibrous connective tissue appears to indicate that the material was well tolerated by the tissue. In the present study, there were different outcomes with respect to tissue as comparing the calcium hydroxide-based and MTA implant sites. It has been demonstrated that MTA causes hard-tissue deposition in rat subcutaneous connective tissue [14]. However, this was not replicated in the

present study.

The zinc oxide eugenol cements have been recommended for root-end fillings by clinicians for many decades [25-27]. The eugenol based cement was promoted as root end fillings [27]. A significantly higher success rate has been demonstrated for root-end fillings using two versions of zinc oxide eugenol (IRM, super EBA) in comparison to amalgam [28]. Pitt Ford et al. have shown that tissue response to eugenol-based cement involves toleration rather than bioacceptability [28]. The results of our in vitro study showed low survival rate for Super EBA. Implant assay revealed that inflammatory cells surrounded the material at weeks 6 and 8. The structure of the tissue change is dystrophic. A frequent finding on histological examination is the presence of giant cells on the surface of the root-end filling material [29,30]. The cause of this inflammatory reaction may be either the predominantly moderate inflammatory response initially observed with subcutaneous implantation of Super EBA, which is probably attributed to ortho-ethoxybenzoic acid [31]or eugenol irritation [32]. Thus, it is concluded that based on the in vitro and in vivo results of the present study, that Super EBA is not biocompatible.

Conclusion

This study demonstrates that in vivo tissue reaction and in vitro cell reaction results can differ. High survival rates were demonstrated from in vitro testing of cultured cells exposed to MTA root-end fillings. In vitro testing of the calcium hydroxide-based root-end materials indicates good biocompatibility. By contrast, good cell or tissue reactions were not demonstrated for the eugenol-based root-end filling materials in the present study.

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計畫成果自評

本研究計畫為一系列之研究之一，乃比較根尖充填材料對於活生物體之相容性，由結果發現，三氧礦化物類對組織之相容性最優，此可提供臨床醫師作為使用此材料時之選擇參考。由本計劃之延伸，日後可再加以研究是否有其他類材料也可以得到相同之結果，並且可由切片部份做免疫反應測試，觀察材料之免疫學上變化。

Table 1. The composition of the root end filling materials.

Product		Composition	Reference no.
Super EBA (Bosworth Co., Durham, England)	powder	Zinc oxide 60% Alumina 34% Natural Resin 6%	0921007
	liquid	Ortho Ethoxy Benzoic acid 62.5% Eugenol 37.5%	
Life (Kerr Co. Romulus, MI, USA)	base	Calcium hydroxide 6.01g Zinc Oxide 1.65g	2-1032
	catalyst	Barium Sulphate 4.32g Polymethylensalicylate Resin 3.98g Methylsalicyate 1.81g Barium Sulphate 4.32g Polymethylensalicylate Resin 3.98g Methylsalicyate 1.81g	
MTA (ProRoot, Tlusa Dental, Tulsa, OK, USA)	powder	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, calcium sulfate, bismuth oxide	A04050000 0100
	liquid	Distilled water	

Figure I. Survival rates for various immersion-time root-end fillings on U_2OS cells.

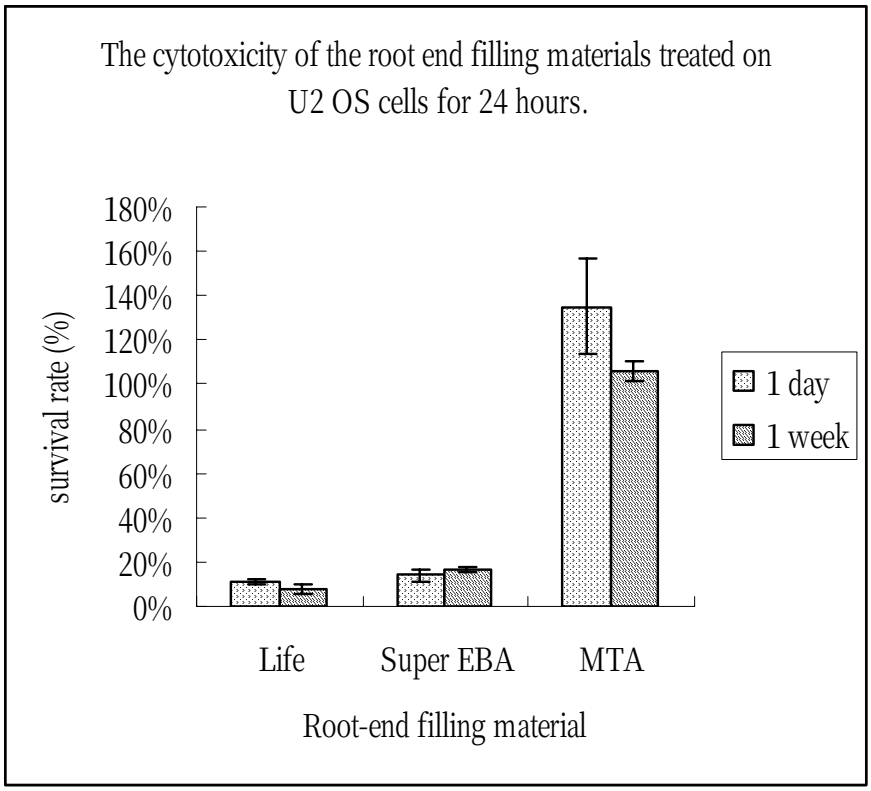
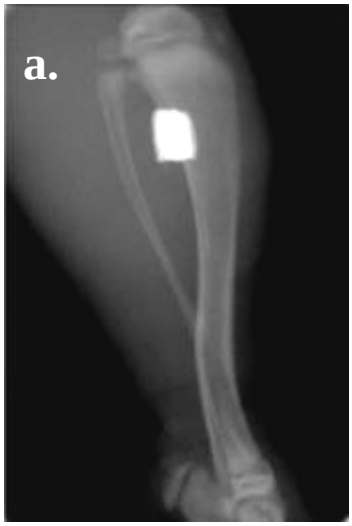
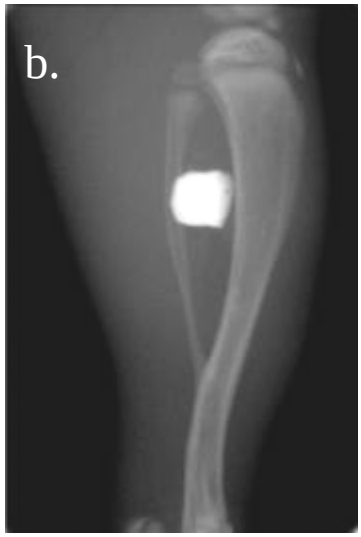
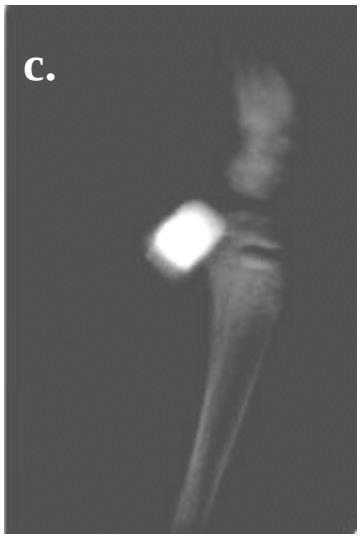


Fig II. Radiology examination of root-end filling-material implantation at day 0 for: a)

MTA implant; b) Super EBA implant; c) Life implant; d) control group.







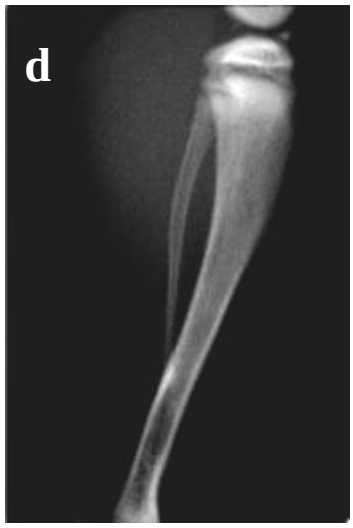


Fig III. Radiology examination of root-end implantation for: MTA at weeks 6 (a) and 8 (b), Super EBA at weeks 6 (c) and 8 (d); Life implant at weeks 6 (e) and 8 (f).

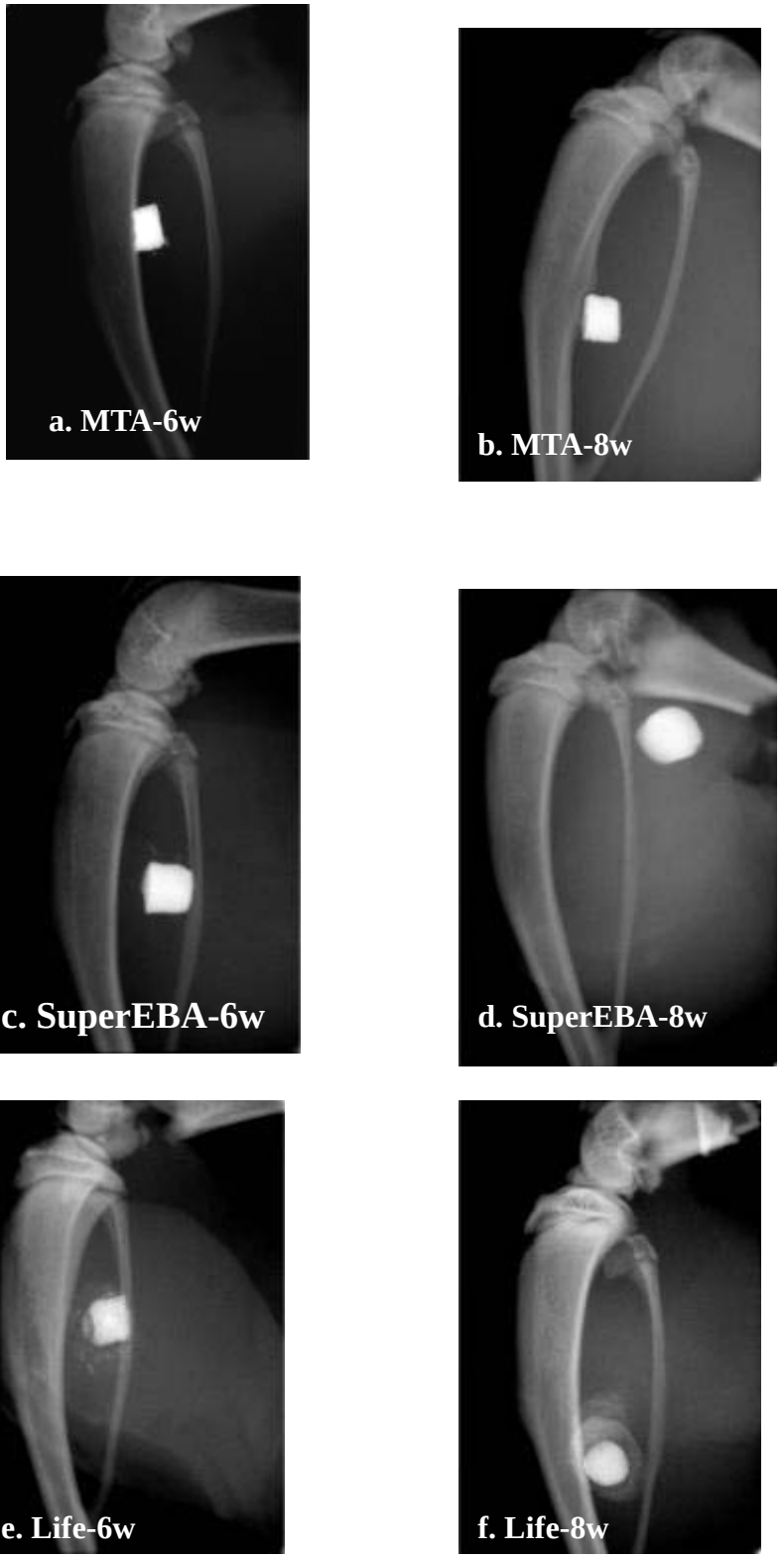


Fig IV. Histological observation of root-end fillers implanted on the muscle layer of the rat leg (phase contrast microscope, original magnifications x40 and x100, hematoxylin and eosin stain): control group (a), Life implant at weeks 6 at 40x (b) and 100x (c), MTA implant at week 6 at 40x (d) and 100x (e), Super EBA implant at week 6 at 40x (f) and 100x (g).

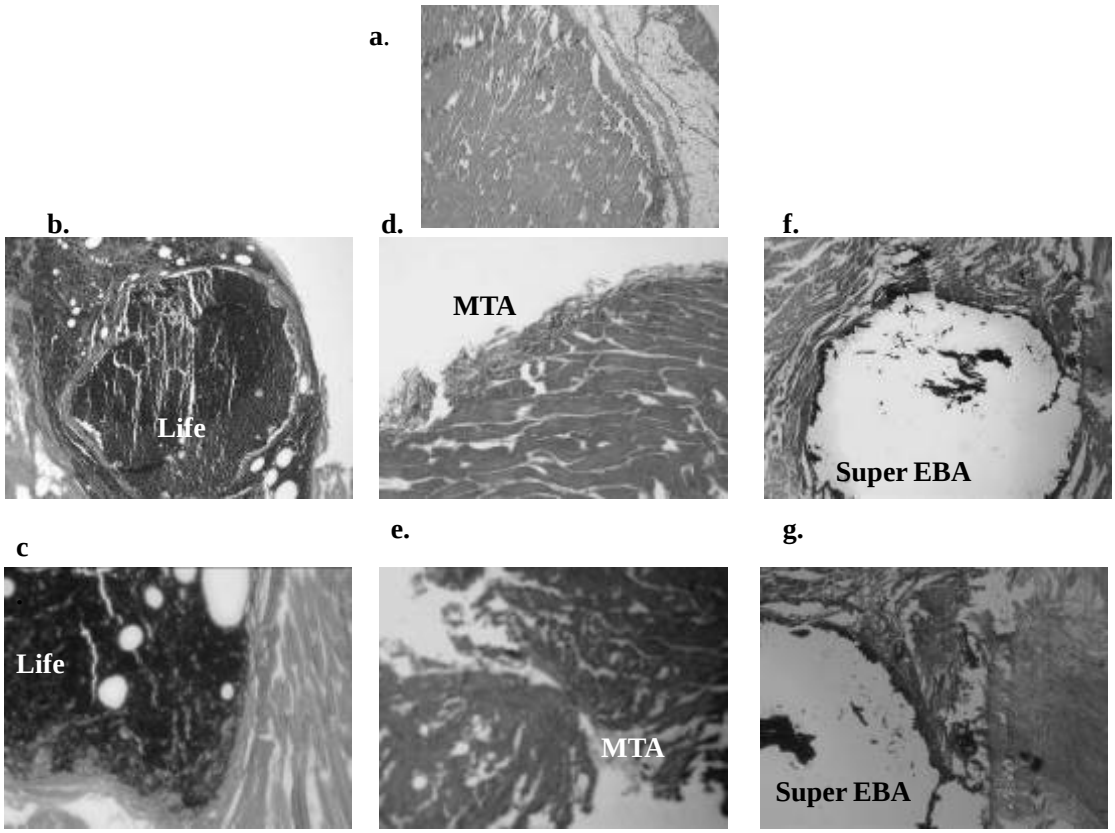


Fig V. Histological observation of root-end filling materials implanted on muscle layer of rat leg (phase contrast microscope at x40 and x100; hematoxylin and eosin stain): Life implant at weeks 8 at 40x (a) and 100x (b), MTA implant at week 8 at 40x (c) and 100x (d); Super EBA implant at week 8 at 40x (e) and 100x (f).