Brazilian Dental Journal (2016) 27(2): 187-191
http://dx.doi.org/10.1590/0103-6440201600447
ISSN 0103-6440

In Vitro Cytotoxicity Evaluation of Three Root-End Filling Materials in Human Periodontal Ligament Fibroblasts

Hernán Coaguila-Llerena[1], Abraham Vaisberg[2], Zulema Velásquez-Huamán[1]

[1]Department of Endodontics, Dental School, Universidad Peruana Cayetano Heredia, Lima, Peru
[2]Laboratories for Research and Development, School of Sciences and Philosophy, Universidad Peruana Cayetano Heredia, Lima, Peru

Correspondence: Hernán Coaguila Llerena, Urb. Tahuaycani K-19, Sachaca, Arequipa, Peru. Tel:+51-96-800-1084. e-mail: eric.coaguila.l@upch.pe

The aim of this study was to evaluate *in vitro* the cytotoxicity on human periodontal ligament fibroblasts of three root-end filling materials: MTA Angelus®, EndoSequence Root Repair Material Putty® and Super EBA®. A primary culture of human periodontal ligament fibroblasts was previously obtained in order to evaluate the cytotoxicity of the three extracts from the root-end filling materials after 2 and 7 days of setting. Serial dilutions of these extracts (1:1, 1:2, 1:4 and 1:8) were evaluated at 1, 3 and 7 days using the methyl-thiazol-tetrazolium (MTT) colorimetric assay. Cell viability was evaluated as percentage of the negative control group, which represented 100% cell viability. Statistical analyses were done with t-test, ANOVA and Kruskal-Wallis test at a significance level of 5%. It was found that the main difference among root-end filling materials was in the higher dilutions ($p<0.05$), but there was a similar behavior in lower dilutions ($p>0.05$). Cell viability of MTA Angelus® was superior for 2-day setting ($p<0.05$), compared with the other two root-end fillings. There were no statistically significant differences between 7-day set MTA Angelus® and EndoSequence Root Repair Material Putty®. Super EBA® showed the lowest percentage of cell viability at higher dilutions ($p<0.05$). Therefore, MTA Angelus® and EndoSequence Root Repair Material Putty® were less cytotoxic in the highest dilution (1:1) compared with Super EBA®.

Key Words: apicoectomy, EndoSequence root repair material, materials testing, mineral trioxide aggregate, super EBA.

Introduction

Apical surgery is the last resort to maintain endodontically treated teeth with persistent periapical pathology. The objective is to create optimal conditions in order to allow healing by tissue regeneration, including formation of a new periodontal attachment (1).

A hermetic seal prevents permanently the leakage of the biological periapical tissue toxins. Therefore, a retrograde preparation followed by placement of a root-end filling must provide a long-term sealing in a dynamic environment that responds to the possibility of dentin and cementum resorption, and to changes in the defense mechanisms of the patient (2).

Periodontal ligament is an important element in apical healing (3-5). Furthermore, human periodontal ligament fibroblasts (PDLF) participate in bone remodeling by stimulating the formation of osteoclast-like cells by producing various active cytokines and enzymes that are essential for osteoclastic differentiation (6).

A cell culture may be defined as an *in vitro* growth of previously isolated cells in a suitable medium. The goal is to obtain an original group of cells, which can maintain the identity and characteristics of the primary cells over time. Cell viability could be measured by the methyl-thiazol-tetrazolium assay (MTT) (7).

Root-end fillings play an important role in retrograde obturation, since they can potentially damage periapical tissues, promote inflammation or destruction, or hamper apical regeneration instead of fulfilling the goal of promoting generation of the periodontal attachment, including cementum to cover the resected root surface and alveolar bone (8).

Mineral trioxide aggregate (MTA) was developed by Torabinejad, initially for use in root perforations, and it has also been widely used as root-end filling. MTA releases calcium ions and has the ability to form hydroxyapatite. Furthermore, its physicochemical reaction with distilled water affords MTA its superior sealing ability, tissue compatibility and osteogenic potential. (9-12).

Recently, a bioceramic-based material, the EndoSequence Root Repair Material Putty® (ERRM) was introduced on the market and is available ready-to-use in a premixed putty form. According to the manufacturer, this material consists of calcium silicates, zirconium oxide, tantalum oxide, monobasic calcium phosphate and fillers. It has many desirable properties, which include hydrophilicity and insolubility, adequate radiopacity, free of aluminum and a high pH. Studies have also reported its high *in-vitro* tissue compatibility (13-17).

Super EBA® (SEBA) was developed in the 1960's as a substitute for zinc oxide-eugenol cements, which has high strength due to the presence of ethoxybenzoic acid, but unpredictable setting times. SEBA has a neutral pH, low solubility and a suitable radiopacity. However, tissue

compatibility studies reported that SEBA causes mild to moderate reactions due to the presence of eugenol (18-21). It has been proposed as root-end filling because of its good sealing properties and its excellent clinical results (22-24).

This research is relevant to apical healing because it is directly related to the establishment of a favorable environment in the periapical area. This is provided in part by the materials used on the root-end at the completion of an endodontic surgical procedure.

The purpose of this study was to evaluate *in vitro* the cytotoxicity of MTA Angelus®, EndoSequence Root Repair Material Putty® and Super EBA® in human periodontal ligament fibroblasts.

Material and Methods

Preparation of PDLF

PDLF were obtained from erupted premolars, which were extracted for non-pathological reasons as well as for orthodontic purposes. An informed consent was provided prior to these extractions.

The remaining gingival tissue tags were removed by thorough curetting of the cervical area of the root surface to avoid contamination of the periodontal tissues by the gingival connective tissue. All laboratory procedures were performed in a laminar flow cabinet to ensure aseptic conditions. The samples were placed in a called "biopsy medium" [Dulbecco modified Eagle medium (DMEM) with 50 µg/mL gentamicin sulfate, 5 µg/mL amphotericin B and fetal bovine serum 10% (FBS 10%)] and were subsequently washed three times with this medium before processing. Only tissues from the middle third of the root surface were used.

Small pieces of tissue were placed in culture plates. After that, they were placed in an incubator with humidity control at 37 °C, 100% relative humidity and 5% CO_2 atmosphere in "biopsy medium". On the following day, the medium was replaced with "culture medium" (DMEM supplemented with 50 µg/mL gentamicin sulfate, 5 µg/mL amphotericin B, 1.16 g/L glutamine and FBS 10 %).

After confluency, PDLF cells were transferred to 75 cm^2 tissue culture flasks using 0.08% trypsin and 0.04% ethylenediaminetetraacetic acid, and were designated as "first transfer cells". Then, the cells were subcultured and used between passages 4 and 5.

Root-End Filling Materials

MTA Angelus® (Angelus, Londrina, PR, Brazil), EndoSequence Root Repair Material Putty® (Brasseler, Savannah, GA, USA) and Super EBA® (Bosworth, Skokie, IL, USA) were obtained from commercial sources to avoid conflicts of interest. Two discs 10 mm diameter and 3 mm thick of each material were prepared and incubated for 2 and 7 days under aseptic conditions at 37 °C and 95% relative humidity.

To ensure sterility, the discs were exposed to ultraviolet light for 20 min on each surface after setting transferred to 24-well tissue culture plates containing 1 mL DMEM per well and incubated at 37 °C, 95% relative humidity for 24 h, thus obtaining extracts of the root-end filling materials.

Cell Viability Assays

PDLF suspension (100 µL/well) were seeded in 96-well culture plates at a density of $5x10^3$ cells/100 µL and incubated for 24 h to allow cell attachment to the plates before the addition of the extracts.

Subsequently, each sample was divided into 6 experimental groups corresponding to 2- and 7-day setting time: MTA 2, ERRM 2, SEBA 2 and MTA 7, ERRM 7, SEBA 7. In addition, there was a negative control (which contained the PDLF suspension with DMEM). Afterwards, a two-fold serial dilution was carried out using DMEM in order to obtain four dilutions of each extract (1:1, 1:2, 1:4 and 1:8), which were incubated for 1, 3 and 7 days.

Cytotoxicity was determined by the "methyl-thiazol-tetrazolium assay" (MTT) according to the manufacturer's instructions. Cell viability was obtained as percentage of the negative control group, which represented 100% cell viability. Statistical differences between groups were analyzed with t-test, ANOVA and Kruskal-Wallis test. Statistical software SPSS 20.0, was utilized at a significance level of 5%.

Results

Cytotoxicity was significantly correlated with material type and setting time (Fig. 1). The percentage of cell viability was higher for the 2-day set MTA in the highest dilution (1:1) and in all periods of evaluation compared with the other groups ($p<0.05$). There was no statistically significant difference with the 7-day set MTA and ERRM.

An interesting finding was that ERRM showed a decreased proportion of tissue compatibility in all serial dilutions at 2 days of setting and inversely displayed an increased proportion of tissue compatibility at 7 days of setting.

Statistically significant differences were found at higher dilutions between SEBA and the other root-end fillings, SEBA showing decreased cell viability ($p<0.05$). The behavior of all root-end fillings was similar at the lower dilutions ($p>0.05$).

Discussion

At the conclusion of an apical surgery with the placement of a root-end filling, "programmed events" should allow for apical healing, which may have the

form of repair or regeneration, depending on extrinsic and intrinsic factors (5). In this study, PDLF were used to simulate a clinical environment since these cells are required for specific sensitivity testing (11,15) and play an essential role in wound healing (5). Additionally, human cells can be conveniently cultured with a low number of passages, resulting in minimal cell changes due to cell culture manipulation (9). To increase the reliability of the sample collection, PDLF were harvested only from the middle third of the root to avoid any contamination with gingival and apical tissues (3).

Evaluation periods of 1, 3 and 7 days were primarily chosen due to the fact that they correspond to the exponential increase and posterior decrease participation of fibroblasts after an injury, such as a complementary apical surgery (4). PDLF are important because they participate in bone remodeling (6).

With regard to calibration of the setting time of the root-end fillings, according to Damas et al. (14), 48 h is not enough time; only after 168 h (7 days) a complete setting of all three cement samples can be obtained, particularly for ERRM. Ma et al. (15) showed that ERRM requires 2 days in order to be considered "relatively fresh" and 7 days to be considered "completely set".

Cytotoxicity has been widely investigated to determine the tissue compatibility of endodontic materials before testing in clinical trials (17). It is especially interesting for root-end fillings, since cytotoxicity may cause the degeneration of the periapical tissues and delay wound healing. Cytotoxicity tests are simple and may be carried out under controlled *in vitro* conditions. In this investigation, extracts at different dilutions were used in order to observe which are cytotoxic to the PDLF, as well as to simulate the postsurgical apical zone, where toxic elements of root-end fillings could leak into fluid surrounding the bony crypt (11).

MTA, SEBA and ERRM release ionic components that may affect cell viability and for that reason the latter parameter was evaluated with the MTT assay. This assay measures mitochondrial dehydrogenase activity of cells by MTT reduction , which forms dark blue formazan crystals. The resulting absorbance is correlated with the amount of cells and shows cytotoxicity levels. The advantages of this method are simplicity, rapidity, accuracy and no use of radioisotopes (7,14).

MTA cell viability on PDLF was very high with 2 days setting time, which is consistent with the study performed by Osorio et al. (10), who demonstrated in mouse and human gingival fibroblasts that 2-day set MTA, compared to other materials, including SEBA, did not cause cytotoxic reactions after 72 h.

The present results also agree with the study by Keiser et al. (11), who demonstrated that fresh MTA and 1-day

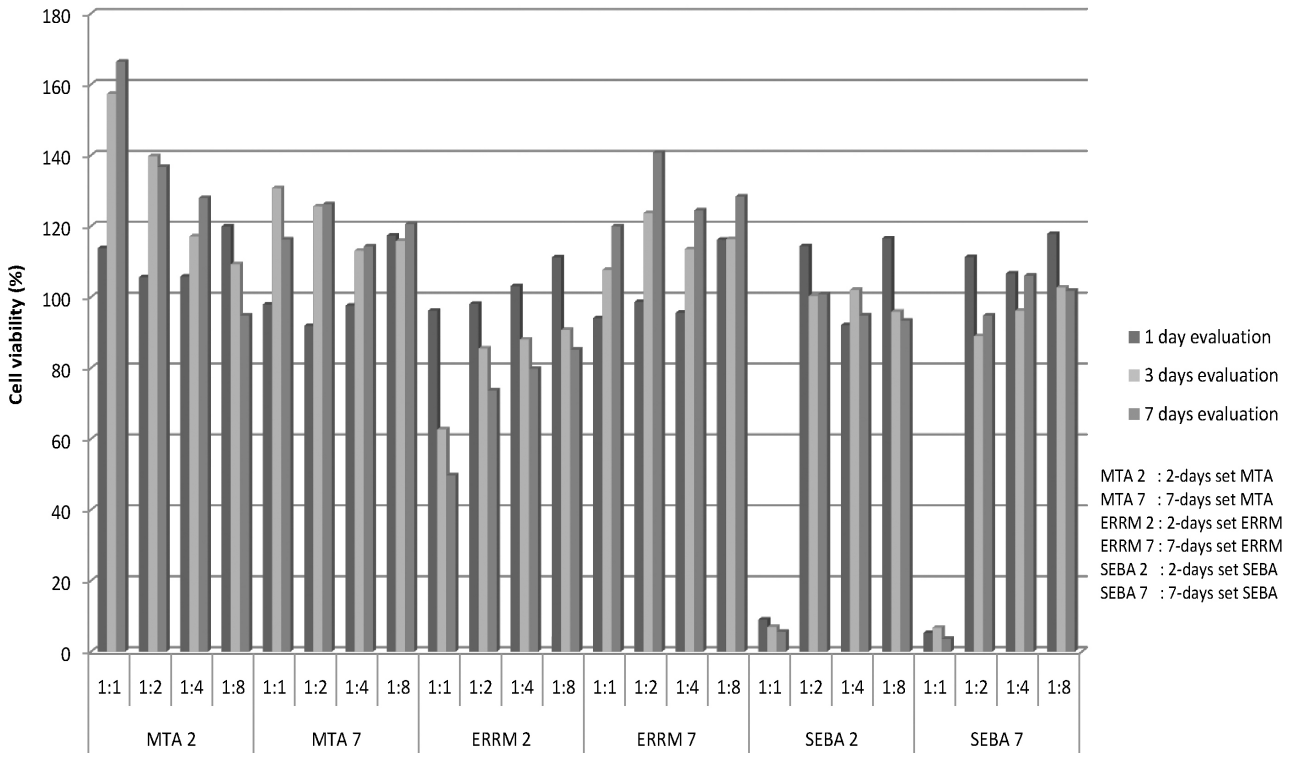

Figure 1. Percentage of PDLF cell viability of MTA Angelus®, EndoSequence Root Repair Material® and Super EBA®.

set MTA are less cytotoxic than amalgam and SEBA in all serial dilutions. These results are also in agreement with those of Gorduysus et al. (12) who compared MTA with other endodontic sealers using MTT assay with 1-day set samples and evaluation periods of 24, 48 and 72 h, and showed that the MTA is the most biocompatible material and thus less cytotoxic. Yoshino et al. (9) also observed that 1-day set MTA and Portland cement displayed better tissue compatibility on PDLF in comparison to MTA Fillapex.

However, it was found that cell viability in 7-days set MTA was slightly lower than in 2-days set. This finding partially agrees with the study of Ma et al. (15) who observed that 2 and 7-days set MTA showed high cell viability. Similar methodology was used in the present study, but the values were higher. Differences could be explained by the lower iron levels of the white MTA-Angelus composition used in this study, which is consistent with Willershausen et al. (17). The role of setting time (2 and 7 days) on cell viability could be explained by toxic leaching out from "relatively fresh" and "completely set" root-end fillings, which is in accordance with Damas et al. (14) and Ma et al. (15).

Regarding ERRM, this study was performed with putty formulation and it exhibited a behavior similar to 7-day set MTA , which is also consistent with Ma et al. (15), who evaluated the cytotoxicity of ERRM, MTA, IRM and Cavit on gingival fibroblasts and found that MTA and ERRM showed similar behavior.

Al Anezi et al. (13) demonstrated in mouse fibroblasts that ERRM viability is comparable to white and grey MTA. The present results on PDLF had a similar tendency, although higher values were obtained. The authors also agree with Damas et al. (14), who showed that 7-day set ERRM and MTA had a similar behavior at 24 h of evaluation in epithelial fibroblasts.

Regarding evaluation periods, Ma et al. (15) showed that ERRM had higher cell viability in the 3rd day of evaluation versus the 7th day. This study had the same evaluation period, but on one hand it showed a proportional decrease in cell viability with 2-day set, and on the other hand an increased cell viability with 7-day set. Differences with the study of Ma et al. may be explained by the fact that although here was used similar methodology with ERRM, the cell samples were PDLF instead of gingival fibroblasts. The authors partially agree with Hirschmann et al. (16) who observed that 7-days set ERRM was more cytotoxic than MTA at 2 days of evaluation, was similar at 5 days and was less cytotoxic after 8 days. A study by Willershausen et al. (17) evaluated PDLF proliferation with white MTA, grey MTA and ERRM set for 1 day, and were evaluated at 6, 24, 72 and 96 h. They observed a decrease of PDLF proliferation with MTA in the evaluations at 24 to 96 h, a similar tendency observed in the present study, although they used the Alamar Blue test and disks instead of extracts. The role of evaluation periods is to demonstrate changes in cell viability when incubated for a short or extended time with extracts from root-end fillings.

This study revealed that SEBA showed a higher cytotoxicity at highest dilution (1:1)than other root-end fillings. This was confirmed by the low survival rate of PDLF. An increase in cell death was observed from the first day of evaluation and up to the 7th day when minimal cells remained. Samara et al. (20) indicated that although SEBA has been widely used in clinical practice, the cytotoxicity, induced mainly by the release of eugenol, remains problematic.

The aforementioned findings from the present study are consistent those of Osorio et al. (10) and Song et al. (21), who compared on PDLF the cytotoxicity of SEBA with two MTA presentations and Endocem. They found that SEBA is significantly more cytotoxic than the two MTA presentations. It should be mentioned that although the findings of their study were in agreement with the present ones, different methodologies were used. While MTA and ERRM had a similar behavior at higher dilutions, the presence of eugenol in SEBA induced a more cytotoxic effect.

On the basis of the present *in-vitro* study, it may be concluded that MTA Angelus® and EndoSequence Root Repair Material Putty® were less cytotoxic compared with Super EBA® in the highest dilution. However, the behavior of all root-end fillings was similar at the lower dilutions.

Acknowledgements

The authors gratefully acknowledge the clinical skills, expertise and assistance of Drs Martha Somerman, Alexis Evangelista, Clovis Bramante, Ashley Millstein, Gisele Faria and Rensso Vértiz.

Resumo

O objetivo deste trabalho foi avaliar *in vitro* a citotoxicidade em fibroblastos do ligamento periodontal humano de três cimentos de retrobturação: MTA Angelus®, EndoSequence Root Repair Material Putty® e Super EBA®. Uma cultura de fibroblastos primários do ligamento periodontal humano foi obtida anteriormente a fim de avaliar a citotoxicidade dos três extratos dos cimentos de retrobturação após 2 e 7 dias de endurecimento. As diluições em série destes extratos (1:1, 1:2, 1:4 e 1:8) foram avaliados em 1, 3 e 7 dias empregando o ensaio colorimétrico metil-tiazol-tetrazólio (MTT). A viabilidade celular foi calculada em base da porcentagem do grupo de controle negativo, que representou 100% de viabilidade de células. As análises estatísticas foram realizadas com o teste t, ANOVA e teste de Kruskal-Wallis a um nível de significância de 5%. Verificou-se que a principal diferença entre os cimentos de retrobturação estava nas diluições mais elevadas ($p<0,05$) e houve um comportamento semelhante nas diluições mais baixas ($p>0,05$). A viabilidade celular dos fibroblastos do ligamento periodontal humano foi superior para MTA Angelus® de 2 dias de endurecimento ($p<0,05$), em comparação com os outros materiais de retrobturação. Não houve diferença significante entre MTA Angelus® e EndoSequence Root Repair Material Putty® de 7 dias de endurecimento.

Super EBA® mostrou a menor percentagem da viabilidade celular nas diluições mais altas (p<0,05). Portanto, os cimentos de retrobturação MTA Angelus® e EndoSequence Root Repair Material Putty® foram menos citotóxicos na diluição mais alta (1:1) em comparação com Super EBA®.

References

1. Kim S, Kratchman S. Modern endodontic surgery concepts and practice: A review. J Endod 2006;32:601-623.
2. Carr GB. Ultrasonic root end preparation. Dent Clin North Am 1997;41:541-554.
3. Somerman MJ, Archer SY, Imm GR, Foster FA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. J Dent Res 1998;67:66-70.
4. Witte MB, Barbul A. General principle of wound healing. Dent Clin North Am 1997;77:509-528.
5. Lin L, Chen MY, Ricucci D, Rosenberg PA. Guided tissue regeneration in periapical surgery. J Endod 2010;36:618-625.
6. Kook SH, Son YO, Hwang JM, Kim EM, Lee CB, Jeon YM, et al.. Mechanical force inhibits osteoclastogenic potential of human periodontal ligament fibroblasts through OPG production and ERK-mediated signaling. J Cell Biochem.2009;106:1010-1019.
7. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;16;65:55-63.
8. Shahi S, Rahimi S, Lotfi M, Reza Yavari H, Reza Gaderian A. A comparative study of the biocompatibility of three root-end filling materials in rat connective tissue. J Endod 2006;32:776-780.
9. Yoshino P, Nishiyama CK, Modena KC, Santos CF, Sipert CR. In vitro cytotoxicity of white MTA, MTA Fillapex® and Portland cement on human periodontal ligament fibroblasts. Braz Dent J 2013;24:111-116.
10. Osorio RM, Hefti A, Vertucci FJ, Shawley AL. Cytotoxicity of endodontic materials. J Endod 1998;24:91-96.
11. Keiser K, Johnson CC, Tipton DA. Cytotoxicity of mineral trioxide aggregate using human periodontal ligament fibroblasts. J Endod 2000;26:288-291.
12. Gorduysus M, Avcu N, Gorduysus O, Pekel A, Baran Y, Avcu F, et al.. Cytotoxic effects of four different endodontic materials in human periodontal ligament fibroblasts. J Endod 2007;33:1450-1454.
13. Al Anezi AZ, Jiang J, Safavi KE, Spangberg LSW, Zhu Q. Cytotoxicity evaluation of EndoSequence root repair material. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:e122-125.
14. Damas BA, Wheater MA, Bringas JS, Hoen MM. Cytotoxicity comparison of mineral trioxide aggregates and EndoSequence bioceramic root repair materials. J Endod 2011;37:372-375.
15. Ma J, Shen Y, Stojicic S, Haapasalo M. Biocompatibility of two novel root repair materials. J Endod 2011;37:793-798.
16. Hirschman WR, Wheater MA, Bringas JS, Hoen MM. Cytotoxicity comparison of three current direct pulp-capping agents with a new bioceramic root repair putty. J Endod 2012;38:385-388.
17. Willershausen I, Wolf T, Kasaj A, Weyer V, Willershausen B, Marroquin BB. Influence of a bioceramic root-end material and mineral trioxide aggregates on fibroblasts and osteoblasts. Arch Oral Biol 2013;58:1232-1237.
18. Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD. Cytotoxicity of four root end filling materials. J Endod 1995;21:489-492.
19. Zhu Q, Safavi KE, Spangberg LS. Cytotoxic evaluation of root-end filling materials in cultures of human osteoblast-like cells and periodontal ligament cells. J Endod 1999;25:410-412.
20. Samara A, Sarri Y, Stravopodis D, Tzanetakis GN, Kontakiotis EG, Anastasiadou E. A comparative study of the effects of three root-end filling materials on proliferation and adherence of human periodontal ligament fibroblasts. J Endod 2011;37:865-870.
21. Song M, Yoon TS, Kim SY, Kim E. Cytotoxicity of newly developed pozzolan cement and other root-end filling materials on human periodontal ligament cell. Restor Dent Endod 2014;39:39-44.
22. Rubinstein RA, Kim S. Short-term observation of the results of endodontic surgery with the use of a surgical operation microscope and Super-EBA as root-end filling material. J Endod 1999;25:43–48.
23. Marques JH, Silva-Sousa YT, Rached-Júnior FJ, Mazzi-Chaves JF, Miranda CE, da Silva SR, et al.. New methodology to evaluate bond strength of root-end filling materials. Braz Dent J 2015;26:288-291.
24. Li H, Zhai F, Zhang R, Hou B. Evaluation of microsurgery with SuperEBA as root-end filling material for treating post-treatment endodontic disease: A 2-year retrospective study. J Endod 2014;40:345-350.

Received August 18, 2015
Accepted February 26, 2016