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ORIGINAL ARTICLE

An MTT-based method for quantification of periodontal ligament cell viability

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OBJECTIVE: Survival of periodontal ligament cells is crucial issue after tooth replantation. To understand this matter, we introduced MTT assay, which can be used as a tool for measuring the viability of periodontal ligament cells from extracted rat molars.

MATERIALS AND METHODS: The maxillary molars of 4-week-old Sprague–Dawley white female rats were used. Ten teeth of each immediate, 1 h Viaspan and 1 h dry were processed for MTT evaluation. Another 10 teeth from each group were treated in the same manner as above, but were replanted into their original socket. After 2 weeks, the animals were killed and the prevalence of resorption pits was evaluated.

RESULTS: *In vivo* MTT assay corresponded with the histological results of the resorption pits ($P \leq 0.05$). The polarizing and optical microscopic findings were consistent with the *in vivo* MTT assay results.

CONCLUSIONS: *In vivo* MTT measurements were consistent with the histologic observations and suggest that the *in vivo* MTT assay could be a tool for evaluating the viability of periodontal ligament cells directly from the extracted root surface. The advantages are shorter analysis time compared with animal or cell culture experiments, easy manipulation, clear quantification and immediate identification of the vital cells.

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Keywords: MTT; periodontal cell; viability; formazan crystal; polarizing microscope

Introduction

The existence of the viable periodontal ligament fibroblasts is most important for the success of tooth replantation. Various methods have been used to evaluate the periodontal ligament cells viability after different preservation methods of the extracted teeth. Among those, the mitotic activity using radioactive DNA precursors (Hupp *et al*, 1997), clonogenic ability (Lekic *et al*, 1998) and direct cell counting stained with neutral red, trypan blue (Ashkenazi *et al*, 1999) or fluorescein diacetate (Patel *et al*, 1994) are the most frequent. However, these *in vitro* methods have some drawbacks in that the collected periodontal cells may not reflect the real condition of the periodontal ligament *in situ*. This is because the characteristics of the collected cells can change during the harvesting and sub-culturing procedure. Another study reported that the clinical results differed from the biological state of cultured periodontal cells when the viability, mitogenicity and clonogenic capacity were evaluated (Ashkenazi *et al*, 1999, 2000).

A tetrazolium-based colorimetric (MTT) assay is widely used as a cytotoxicity test in cultured cells on account of its speed of analysis and relative objectivity. The principle behind this technique depends on the capacity of living cells to reduce tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a formazan crystal in their metabolizing mitochondria. This purple-colored formazan crystal is soluble in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and can be measured using an ELISA reader at a wavelength of 570 nm. The value of the optic density is related to the number of viable cells in each sample. The MTT method is reported to have several advantages with respect to the speed of analysis, quantification and the management of many samples (Shimoyama *et al*, 1989). The predictability of the MTT assay was compared with other conventional techniques (Shimoyama *et al*, 1989; Basha *et al*, 1996) and it was found that the MTT assay showed excellent reproducibility and was equivalent to the 90% predictable rate obtained from the clonogenic assay. Even with these advantages, there are few reports related to the *in vivo* application of this method. Pig skin was examined after giving it an experimental burn using the MTT staining (Henze *et al*, 1997). The depth of the burn was determined

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morphometrically after coloring it with a modified MTT-staining on frozen sections of the skin biopsies. When examined under the optical microscope, they observed a well-defined demarcation line between the vital (MTT positive) and necrotic tissues (MTT negative). Moreover, the created formazan crystals were visualized under the polarized light microscope for the purpose of pixel analysis in the intact mice tissue (Colangelo *et al*, 1992).

Therefore, if the *in vivo* MTT assay can be applied to the periodontal ligament cells *in situ*, many of the experimental variables can be eliminated by reducing the observation time and experimental trauma. Accordingly, the aim of this study was to determine whether the *in vivo* MTT assay in periodontal ligament cells is comparable with a histological examination.

Materials and methods

Pretreatment of experimental animals

A total of 30 Sprague–Dawley female rats with an average body weight of approximately 100 g (Daehan Biolink, Seoul, Korea) were used. The rats were fed powdered Purina rat chow diet containing 0.4% beta-aminoproprionitrile (β-APN; Sigma, St Louis, MO, USA) for 3 days. β-APN is known to reduce the cross-linkage of collagen, and lower the tensile strength of the collagen molecules, which allows the gentle extraction of teeth with minimal trauma to the surrounding tissues (Bornstein, 1970). Under ketamine anesthesia (0.1 cc per animal), the first and second maxillary molars were extracted using gentle luxation. The extracted molars were examined for the integrity of all roots and the absence of any fracture lines.

MTT assay

Two hundred microliters of a MTT solution (0.05 mg ml^{-1}; Sigma Chemical Co.) was placed in each well of the 96-well plate. Each treated tooth was immersed in a well. Immediate and dry groups were established in order to compare the two extremes. 1-h Viaspan? was chosen to determine the intermediate response of the two extremes. The classifications were as follows:

Immediate Group ($n = 10$); 10 teeth were immersed in a MTT solution immediately after extraction.

Dry Group ($n = 10$); 10 teeth were immersed in a MTT solution after drying with warm air for an hour.

One-hour Viaspan Group ($n = 10$); 10 teeth were treated with Viaspan? (Dupont Pharma, Willmington, DE, USA) for 1 h, and then immersed in a MTT solution.

After incubating the wells for 3 h at 37°C, the supernatant of each well was removed by pipetting and 150 μl of DMSO (Merck) was then added. The plate was further incubated for 30 min. After removing the teeth, the optical density (OD) of each well was measured using an ELISA (Benchmark microplate reader; BIO-RAD, Hercules, CA, USA) at a 570 nm wavelength. Each OD value was divided by the relative tissue volume of each tooth in order to eliminate the measurement errors caused by the different tissue volumes. To obtain the relative tissue volume, each tooth, which had already been used for the MTT assay, was again stained with hematoxylin–eosin for 10 min. The teeth were treated with ethanol and 1% acidified alcohol for color extraction. Each of the extract was then measured in the manner same as above at 450 nm. Each MTT value was divided by the relative tissue volume to obtain the final value.

Histological observation

Quantification of the resorption pits

Ten teeth from each group were treated in the same manner as in the MTT assay but were replanted into the original socket in order to evaluate the histological features. The animals were killed at 14 days after replantation by a heart infusion of 40 ml of 10% formalin. The maxillae were removed and fixed in Karnovsky's fixative for additional 3–4 h, then demineralized at room temperature in 5% nitric acid. After completing the demineralization, the specimens were washed with a 0.1 M sodium cacodylate buffer, dehydrated in a graded series of ethanol, and embedded with paraffin. For the light microscopic observation, 4 μm-thick sections perpendicular to the long axis were cut and stained with hematoxylin–eosin. The histological images were stored using Matrox Intellicam (Matrox Graphics Inc., Quebec, Canada) and transferred to Microsoft Powerpoint 2002 (Microsoft Co., Seattle, WA, USA). The resorbed root surface was evaluated according to Andreason's study (Andersson *et al*, 1987). Briefly, a point was marked at the center of the root so that four lines could be established to meet at a marked center point to make eight cross-sections with the root outline (Figure 1). Any cross-section lines that met with the resorption pit were counted and recorded. The data were analyzed using a one-way ANOVA. A P-value ≤ 0.05 was considered significant. The Student–Newman–Keuls method was used to determine the differences among the groups.

Qualitative evaluation of MTT granules

Ten teeth from each group were treated in the same manner as in the MTT assay. After incubation, all the teeth were decalcified for 1 week and sectioned at 10 μm to observe the patterns of the MTT granules.

The created formazan crystals were observed under normal optical and polarizing microscopes. The histological images were stored using Matrox Intellicam (Matrox Graphics Inc.) and transferred to Microsoft Powerpoint 2002 (Microsoft Co.) and evaluated qualitatively.

Results

MTT Assay

The mean measurement of the immediate group was 2.81 while that of the dry group was 0.98 ($P < 0.05$). The mean measurement of the Viaspan group was 2.65, which was significantly different from the dry group

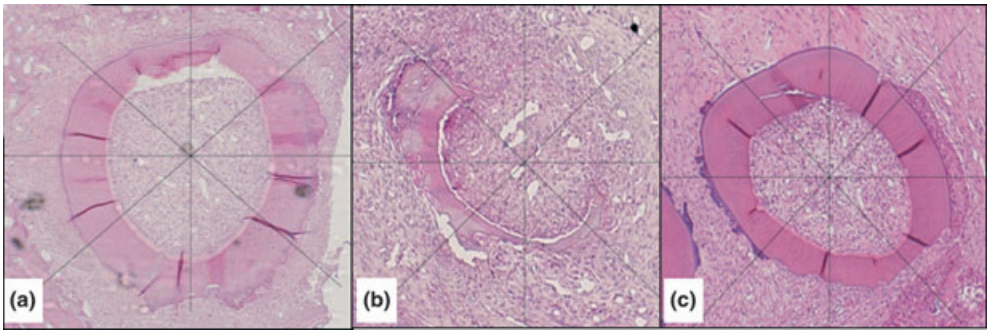

Figure 1 Quantification of resorption pits. (**a**) The immediate treated group showing root resorption at 2 points, (**b**) 1 h dried group showing root resorption at 7 points, (**c**) 1 h Viaspan® treated group showing root resorption at 2 points

Table 1 Average of the optical density (OD) and mean number of resorption pits

Group	*OD/tooth weight*	*No. of resorption pits*
Immediate	2.81 ± 0.45	3.03
Dry	0.98 ± 0.35[a]	6.50[a]
1 h Viaspan?	2.65 ± 0.49	2.75

[a]Statistically significant at the 95% level of confidence.

($P < 0.05$) but not from the immediate group ($P > 0.05$) (Table 1).

Microscopic observation

Quantification of resorption pits

In the immediate group, an average of 3.03 resorption pits was observed (Table 1). The pulp and periodontal cells appeared to be within the normal limit without showing any signs of inflammation (Figure 1a). In the dry group, an average of 6.5 resorption pits was observed with abundant inflammatory cells. Some specimens showed extensive root resorption (Figure 1b). The Viaspan group showed an average 2.75 resorption points (Figure 1c), which was significantly different from the dry group ($P < 0.05$) but not from the immediate group ($P > 0.05$). These findings were consistent with the results from the MTT assay.

Qualitative evaluation of MTT granules

In the immediate and 1-h Viaspan groups, densely stained blue crystals were observed all over the tissues except in dentin. The density of the formazan crystals was the highest in the cementum. The MTT granules appeared as a bright orange-purple color while the dentin and cementum appeared as white-grey. The granules appeared as blue spots at lower magnification, which was in contrast to the needle-like appearance observed at higher magnification (Figure 2).

Discussion

'How deep the MTT staining can penetrate in to the deep layered tissue is of concern in this study. Similar study has been shown that MTT staining was used on the scalded pig skin to evaluate the extent of the burn (Henze *et al*, 1997). After scalding, the full-thickness skin of the scalded area and the normal area were taken and treated with MTT solution for 1 h. The sections were then frozen at −70°C for sectioning. Healthy skin showed a blue coloration in all cell types of the dermis, particularly in the basal cell layer. MTT staining of the cross-sections of scald pig skin showed a clear demarcation line between the viable and necrotic tissues, allowing morphometric analysis. The observed average depth of the demarcation was as thick as 1.94 ± 0.07 mm.

In this experiment, serial frozen-sections were obtained from the root end in the immediate group tooth to determine the penetration pattern of the formazan crystals. MTT staining was observed in the deep layer of the periodontal ligament as well as in the cemental layers. In some specimens, MTT staining could be observed in the pulp tissue as deep as 1 mm away from the root tip (Figure 3). Therefore, we believe that MTT staining can be achieved at the whole thickness of the periodontal ligament, which was approximately 0.5 mm in thickness.

In this experiment, many of the pulpal cells as well as the periodontal cells were stained together. However, it is not clear whether this mixed staining caused any problem in evaluating the viability of the periodontal ligament cells only. Considering that the major cell types of the pulp and periodontal ligament have the same ectomesenchymal origin, and that the main purpose of this study was to assess the damaging effect of any living cells, we presumed that this question would not pose a serious problem.

Two separate evaluations were performed to determine whether the *in vivo* MTT assay of this study was reliable. The first was a histological examination, in which the total number of the resorbed root surface was calculated according to the Andreason's method (Andersson *et al*, 1987). The results of these histological findings were in consistent with the ELISA assay. Although the number of the resorption pits in the 1-h Viaspan group was slightly lower (2.75) than that in the immediate group (3.03), there was no statistical difference in the MTT and histological evaluations between the two groups (Table 1). The dry group showed significantly more resorption pits (6.50) than the immediate or 1-h Viaspan group. This was comparable with the MTT assay, which showed the lowest OD value in the dry group (0.98 *vs* 2.81 and 2.65, Table 1).

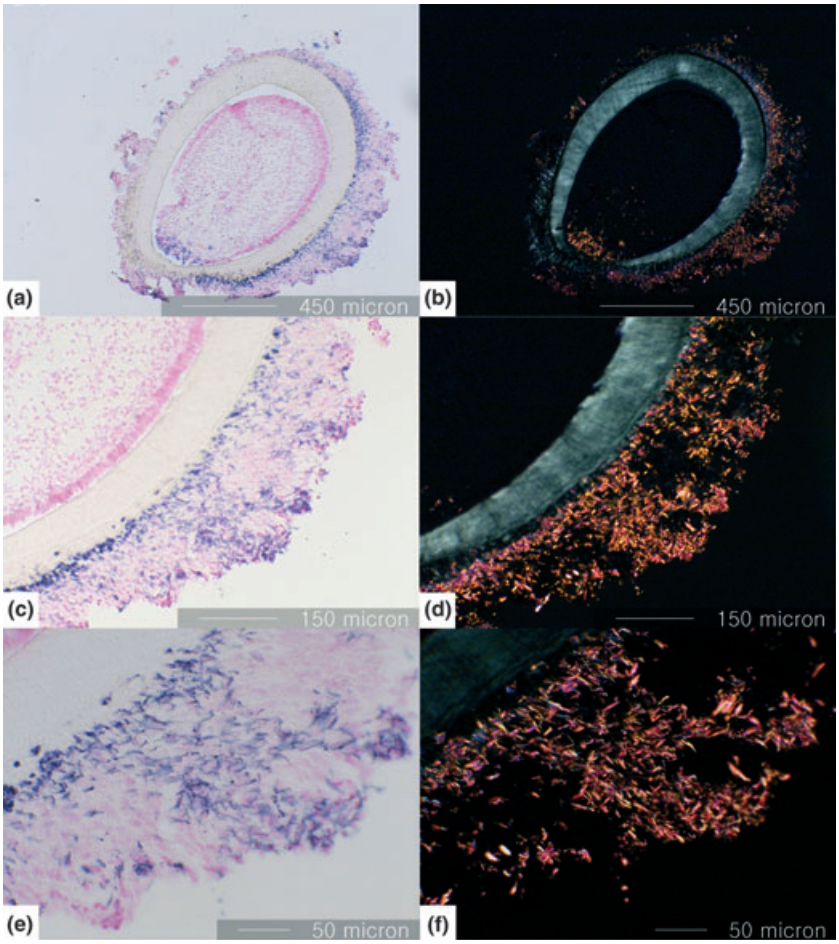

Figure 2 Qualitative evaluation of MTT granules. H&E in the light microscope (**a**,**c**,**e**) and the polarizing microscope (**b**,**d**,**f**)

The second trial involved a qualitative evaluation of the created formazan crystals *in situ*. In the immediate and 1-h Viaspan groups, densely stained blue crystals were observed all over the tissues except in the dentin. The density of the formazan crystals was the highest in the pulp, which was followed by cementum and periodontal ligament. This was again observed by polarizing microscopy. Initially, we tried to count the number of MTT granules using optical microscopy. Previous studies demonstrated that the formazan crystals formed by the reduction of MTT reflected polarized light and could be measured using pixel analysis in the intact tissue (Colangelo *et al*, 1992; Furukawa *et al*, 1992). However, this was not possible because many of the MTT granules overlapped (Figures 1 and 2). In higher magnification of the specimens (Figure 2f), long needle-like crystal threads of the stained cells were observed, indicating the intracytoplasmic location of the blue formazan crystal.

The different tissue volume of each tooth was another concern in this experiment. In an *in vivo* MTT experiment of the venom injected muscle tissue, the MTT value was divided with the corresponding muscle weight to compensate for any minor variations during the tissue dissection (Lomonte *et al*, 1993).

In this experiment, each measured OD value was divided by the relative tissue volume of each tooth. To obtain the relative tissue volume, each tooth, which had

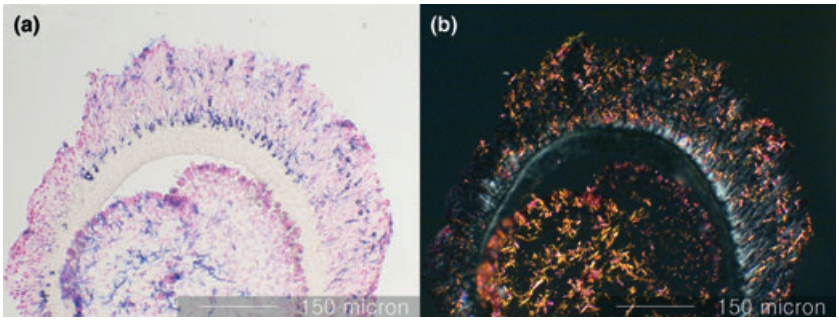

Figure 3 MTT staining was observed in the pulp as well as cemental-periodontal layers at 1 mm position from the root apex. light microscopy (**a**) and polarizing microscopy (**b**)

already been used for the MTT assay, was again stained with hematoxylin–eosin for 10 min. The teeth were treated with ethanol and 1% acid alcohol for color extraction, and each of the extracts was measured by ELISA at 450 nm. Each MTT value was divided by the relative tissue volume to obtain the final value.

Minimal injury to the periodontal apparatus during extraction is another important factor for replantation studies. The rats used in this study were fed a powdered Purina rat diet containing β-APN for 3 days. β-APN interferes with the action of the amine oxides, and prevents the formation of lysyl-derived aldehyde groups on the collagen alpha-chain (Bornstein, 1970). Because of the consequent reduction of the reactive aldehyde groups, the collagen molecules fail to become cross-linked and the tensile strength of the collagen fibers is greatly reduced (Fry *et al*, 1962). In this study, most of the teeth could be extracted with all five roots intact using the β-APN pretreatment. For the biological effect of β-APN, the periodontal ligaments return to their normal state at 3 days after the cessation of the β-APN treatment and that there were no irreversible changes in collagen fiber or periodontal ligament cells (Barrington and Meyer, 1966).

This study would be clinically useful to evaluate the long-term storage media of the teeth for the transplantation and replantation. Future study should examine whether or not the MTT staining in the periodontium *in situ* can show the cellular activities of the different zones of periodontal healing. This should increase the understanding of the healing dynamics of the replanted or transplanted tooth periodontium.

To sum it up, the *in vivo* MTT measurements were consistent with the histologic observations and suggest that the *in vivo* MTT assay can be a reasonable tool for evaluating the viability of periodontal ligament cells directly from the extracted root surface. The advantages of this technique are shorter analysis time compared with animal or cell culture experiments, easy manipulation, clear quantification and immediate identification of the vital cells.

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References

Andersson L, Jonsson BG, Hammarstrom L, Blomlof L, Andreasen JO, Lindskog S (1987). Evaluation of statistics and desirable experimental design of a histomorphometrical method for studies of root resorption. *Endod Dent Traumatol* **3:** 288–295.

Ashkenazi M, Sarnat H, Keila S (1999). In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in six different media. *Endod Dent Traumatol* **15:** 149–156.

Ashkenazi M, Marouni M, Sarnat H (2000). In vitro viability, mitogenicity and clonogenic capacity of periodontal ligament cells after storage in four media at room temperature. *Endod Dent Traumatol* **16:** 63–70.

Barrington EP, Meyer J (1966). Recovery of the rat dental organ from experimental lathyrism. *J Periodontol* **37:** 453–467.

Basha G, Yap P, Penninckx F (1996). Comparative study of classical, colorimetric and immunologic staining methods for the assessment of tumor cell viability. *Tumour Biol* **17:** 354–361.

Bornstein P (1970). The cross-linking of collagen and elastin and its inhibition in osteolathyrism. Is there a relation to the aging process? *Am J Med* **49:** 429–435.

Colangelo D, Guo HY, Connors KM, Kubota T, Silvestro L, Hoffman RM (1992). Correlation of drug response in human tumors histocultured in vitro with an image-analysis MTT end point and in vivo xenografted in nude mice. *Anticancer Res* **12:** 1373–1376.

Fry P, Harkness ML, Harkness RD, Nightingale M (1962). Mechanical properties of tissues of lathyritic animals. *J Physiol* **164:** 77–89.

Furukawa T, Kubota T, Watanabe M, Takahara T, Yamaguchi H, Takeuchi T *et al*. (1992). High in vitro–in vivo correlation of drug response using sponge-gel-supported three-dimensional histoculture and the MTT end point. *Int J Cancer* **51:** 489–498.

Henze U, Lennartz A, Hafemann B, Goldmann C, Kirkpatrick CJ, Klosterhalfen B (1997). The influence of the C1-inhibitor BERINERT and the protein-free haemodialysate ACTIHAEMYL20% on the evolution of the depth of scald burns in a porcine model. *Burns* **23:** 473–477.

Hupp JG, Trope M, Mesaros SV, Aukhil I (1997). Tritiated thymidine uptake in periodontal ligament cells of dogs' teeth stored in various media for extended time periods. *Endod Dent Traumatol* **13:** 223–227.

Lekic PC, Kenny DJ, Barrett EJ (1998). The influence of storage conditions on the clonogenic capacity of periodontal ligament cells: implications for tooth replantation. *Int Endod J* **31:** 137–140.

Lomonte B, Gutierrez JM, Romero M, Nunez J, Tarkowski A, Hanson LA (1993). An MTT-based method for the in vivo quantification of myotoxic activity of snake venoms and its neutralization by antibodies. *J Immunol Methods* **161:** 231–237.

Patel S, Dumsha TC, Sydiskis RJ (1994). Determining periodontal ligament (PDL) cell vitality from exarticulated teeth stored in saline or milk using fluorescein diacetate. *Int Endod J* **27:** 1–5.

Shimoyama Y, Kubota T, Watanabe M, Ishibiki K, Abe O (1989). Predictability of in vivo chemosensitivity by in vitro MTT assay with reference to the clonogenic assay. *J Surg Oncol* **41:** 12–18.