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Morphological Behavior and Attachment of p19 Neural Cells to Root-End Filling Materials

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Summary: Some techniques and instruments like stereomicroscopy and confocal microscopy used for observing neural cells are too complicated and dependent on preparation and cell fixation methods. This may question the results of these methods. Though, we have used scanning electron microscopy on replicated specimens to observe p19 neural cells and their cellular extensions. This manuscript has shown the feasibility of using replica (indirect) method instead of direct methods for observing morphological characteristics of this high sensitive cell line. As neural cells are very sensitive to fixation solutions and processes, we have used replica mode and observed neural cells with a novel indirect method. We have used replica mode in this study to indirectly and noninvasively evaluate the state of p19 neural cells and their cellular extensions. SCANNING 32: 369–374, 2010. © 2010 Wiley Periodicals, Inc.

Key words: mineral trioxide aggregate, p19 neural stem cells, replica, scanning electron microscope

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Introduction

An ideal root-end filling material should have various properties such as easy manipulation, conductivity to create an apical seal, ability to promote periapical healing, no toxicity for the periradicular tissues, noncorrosiveness or electrochemical activity, and the ability to adhere to the root canal system in three dimensions (Gartner and Dorn 1992). These materials should be biocompatible because of their direct and prolonged contact with the periodontium and should permit healing of periradicular tissues via regeneration of cementum, periodontal ligament, and alveolar bone across the resected root-end surface (Fouad *et al.* 1993). Amalgam, composite resin, intermediate restorative material, Super EBA, glass-ionomer, polycarboxylate cements, Cavit, gutta-percha (GP) (Friedman 1991), and mineral trioxide aggregate (MTA) all have been used as root-end filling materials. Many studies have shown that MTA is one of the most tissue friendly retrofilling materials (Torabinejad *et al.* 1997; Koh *et al.* 1997; Camilleri *et al.* 2004).

The cytotoxicity of root-end filling materials has been tested on different cell lines such as fibroblasts, osteoblasts, kidney-cell line, and L-929 fibrosarcoma cells (Keiser *et al.* 2000). Determining the mitochondrial function (Keiser *et al.* 2000) and measuring the inhibition zone of cell growth (Bruce *et al.* 1993) are common methods of evaluating the toxicity of endodontic materials. These assays mostly measure the inhibition of cell replication or activity, and they do not measure the cell death directly. Some toxicity assays have measured actual cell death (Lobner 2000).

There are few studies on neurotoxicity of endodontic materials. It seems that the use of neural cells

is more sensitive than other methods of biocompatibility evaluation (Asrari and Lobner 2003). Neurons, much more than fibroblasts, can provide a highly sensitive system which is susceptible to free-radical-mediated injury, metabolic insults, and environmental toxins (Mattson *et al.* 1995).

Root-end filling materials are in close contact with nerve endings and local nerves, which lack cell bodies found in cortical cultures. Death of neurons can result from damage to the neuronal processes in cortical cultures (Tecoma *et al.* 1989). In addition, one of the components of tissue regeneration that has sometimes been neglected is the neural supplement regeneration. There are limited studies on the effect(s) of white mineral trioxide aggregate (WMTA) on neural cells. The aim of this study was to evaluate the neurotoxicity of WMTA on p19 stem cells when exposed to different biological fluids.

Materials and Methods

P19 stem cells were obtained from Pasteur Institute (Pasteur Institute, Tehran, Iran). P19 cultures started from a frozen stock by thawing the cells rapidly at 37°C and were cultured in a standard medium consisting of α-MEM (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 7.5% calf serum (GIBCO BRL, Life Technologies), 5% fetal serum (FCS) (GIBCO BRL, Life Technologies), and 100U/mL penicillin-streptomycin. The cells were maintained at sub-confluency by being sub-cultured into tissue culture-grade dishes (Nunc, Roskilde, Denmark) every 48 h and then removed from the culture dishes by being treated with trypsin (GIBCO BRL, Life Technologies) to induce neurally differentiated p19 stem cells. The cells were plated at a density of 5×10^4 cell/mL in the medium supplemented with 0.3 mM of retinoic acid (RA) (Sigma, St. Louis, MO) into 100 mm bacteriological-grade Petri dishes (Nunc) initially and grew as aggregates for 4 days. The medium was refreshed every 48 h during this period.

Twenty WMTA (Tooth-colored Formula, Dentsply, Tulsa Dental, Tulsa, OK) sachets were mixed separately with distilled water under aseptic conditions according to manufacturer's instructions and packed into 40 cylindrical polycarbonate tubes (Falcon Plastics, Div. of BioQuest, Oxnard, CA). A suitable micro-condenser (Hu-Friedy, Chicago, IL) was chosen and checked to ensure that the tip would fit the diameter and length of the cylindrical polycarbonate tubes with an inner diameter of 8 mm and a height of 10 mm.

Four groups with ten specimens each were prepared. Each specimen was placed in a separate vial and stored at 37°C and 100% relative humidity for 24 h to ensure WMTA setting. Three wet pieces of gauze soaked in blood, deionized water (DW), and synthetic tissue fluid (STF), respectively, covered all the specimens in each group for 24 h. Gauze pieces were replaced every 6 h with fresh ones to ensure a sufficient environment. In the fourth group, WMTA sachets were mixed with STF at a STF-to-powders ratio of 0.33 and prepared according to a previous study (Ratner *et al.* 2004). Ten tubes were filled with GP (Bifill System, VDW, Munich, Germany), and ten tubes were filled with zinc oxide-eugenol (ZOE) (Kemdent; Associated Dental Products Ltd, Wiltshire, U.K.). Finally, all the specimens were taken out of the polycarbonate tubes and stored separately under aseptic conditions.

After 4 days of RA treatment, the aggregates were plated onto tissue culture-grade dishes containing WMTA and GP tablets in a medium without RA and the culture continued for 10 days. GP tablets were fixed into culture dishes by Nickel Titanium (Ni-Ti) needles to ensure that GP was placed at the bottom of culture plates.

Immediately after the tablets were taken out of the tissue media, each tablet was placed in a Petri dish and cooled down to −70°C at a rate of 10°C/min (MDF-C8V1, Sanyo Electric Co., Japan). Petri dishes were placed on a tray and stored in a freezer at −70°C for 24 h.

Immediately after the tablets were retrieved from the freezer, a replica of each tablet was prepared using a resin (RepliSet-F1 50 mL System, Struers, Denmark). The prepolymer was mixed with the curing agent, and placed without delay on each tablet. The tablets were kept for 20 min at room temperature so that the replicas would become cured. The peeled-off replicas were observed under a scanning electron microscope (SEM, Cambridge Instrument S360, Cambridge, England). Figure 1 shows the design of the experiment.

Each replica surface was sputter coated with gold and visualized under SEM at 15 kV by a secondary detector. A grading system was developed with respect to the amount of cell attachment and their traces on the replica surface in each sample according to the following criteria:

1. None to slight (0–25%).
2. Mild (26–50%).
3. Moderate (51–75%).
4. High (76–100%).

Two investigators scored the amount of cell attachments in a blind manner according to the grading criteria. In case of discrepancies in the scores determined by the two investigators, a third investigator was asked to score the samples.

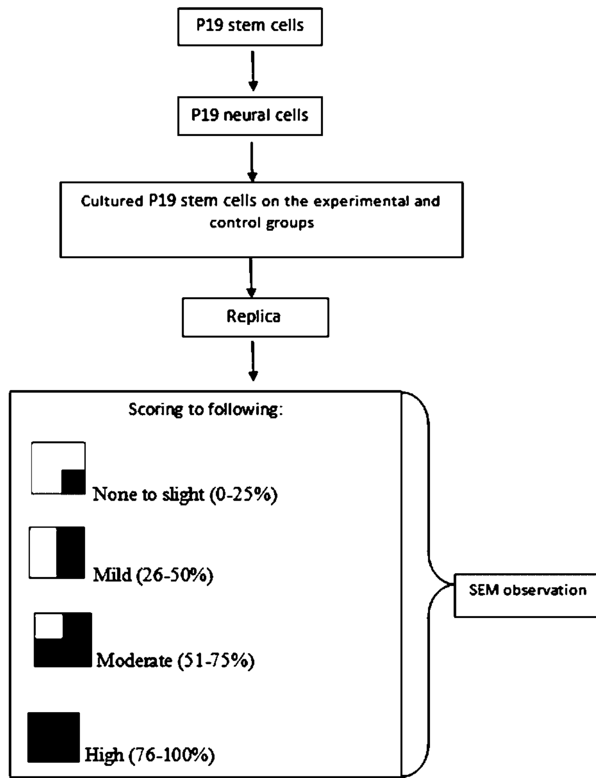

Fig 1. Flowchart of the experiment.

Finally, two similar scorings were considered as the final score.

ANOVA and a post hoc Tukey test were used for statistical analysis.

Results

The mean ± standard deviations of the amount of cell attachment for WMTA exposed to blood, DW, and STF, and WMTA mixed with STF, ZOE, and GP were 3.5 ± 0.52, 1.0 ± 0.00, 2.7 ± 0.67, 1.5 ± 0.52, 1.0 ± 0.00, and 3.5 ± 0.52, respectively. ANOVA revealed significant differences among the groups ($p < 0.0001$) (Fig. 2). Post hoc Tukey test did not demonstrate any significant differences between WMTA exposed to blood and GP ($p = 1.00$), but there were significant differences between WMTA exposed to blood and other groups (Fig. 2).

SEM micrographs are displayed in Figure 3. The secondary detector (SE) detected a neuron structure, including the nucleus, cell body, axon, dendrite, and synapses in WMTA/B, and GP specimens. In addition, p19 cells in WMTA/B and GP groups grew normally and their morphology was comparable to that of the parent cell (Tanaka *et al.* 2003). Trace of NiTi needle is shown by (▶) in Figure 3(e). SEM micrographs revealed that uniformly differentiated p19 cells predominantly covered the surface of WMTA/B and GP groups. Consistent with the morphological observation, dendrite fibers which emanate from the cell body and provide the receptive zones that receive activation from other neurons were clearly observed (Fig. 3(a) and (b)). In the WMTA/DW group the cells had disappeared. Differentiated p19 stem cells showed similar morphology to those observed in previous studies (Tanaka *et al.* 2003).

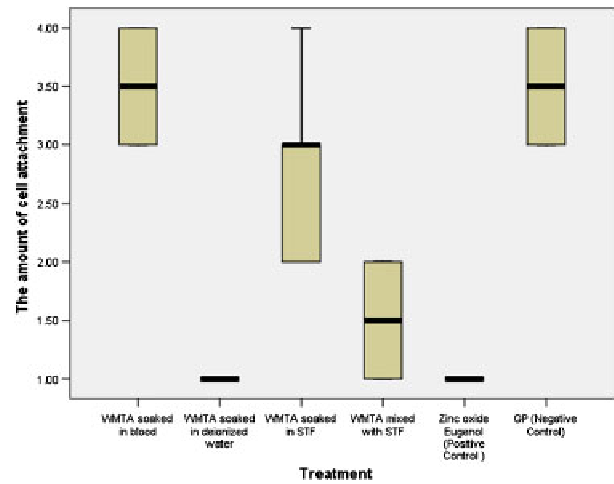

Fig 2. Mean amount of cells grade for materials.

Discussion

Root-end filling materials may inadvertently be overextended beyond the root canal space and invade the surrounding periodontium and bone. The material introduced within such a sensitive anatomical space may mechanically or chemically affect the peripheral nerve fibers, such as the inferior alveolar nerve and neural cells (Gallas-Torreira *et al.* 2003), resulting in a series of complications such as paresthesia (Orstavik *et al.* 1983).

The quality and quantity of cell attachment, adhesion, and spreading on the root-end filling materials can be used as a criterion for evaluation and as an indicator of the materials' biocompatibility (Zhu *et al.* 2000). Cell attachment is one of the most sensitive methods for measuring toxicity. Different cells, such as MG-63 osteosarcoma cells (Koh *et al.* 1998), Saos-2 osteosarcoma cells (Zhu *et al.* 2000), human gingival fibroblasts, periodontal ligament fibroblasts (Bonson *et al.* 2004), human alveolar bone cells (Perinpanayagam *et al.* 2006), and cementoblast cell lines (Thomson *et al.* 2003), have been used for the evaluation of MTA biocompatibility.

P19 stem cells are the major cell lines available and can be used for evaluating neurotoxicity of different materials. A previous study (Khorasani *et al.* 2009) has shown the suitability and reliability of cultured p19 stem cell test for the evaluation of

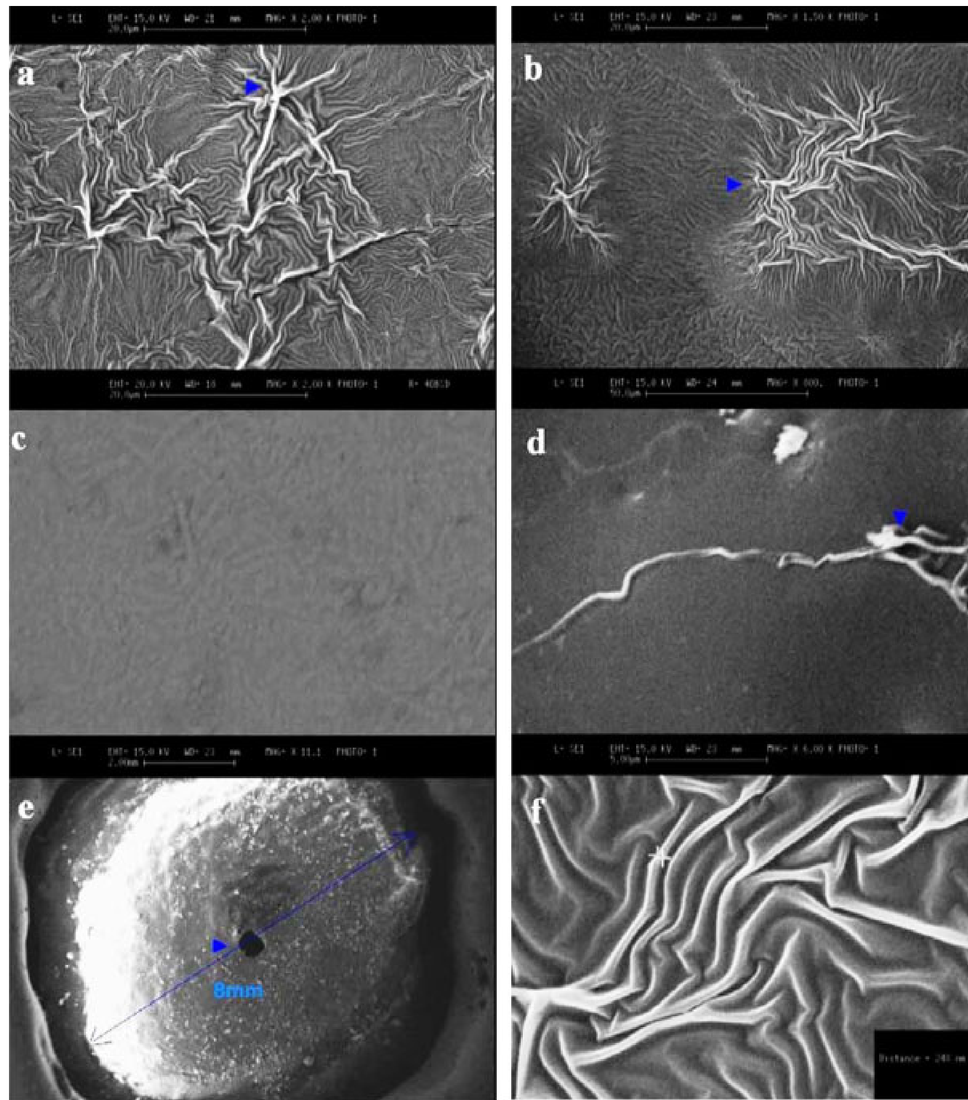

Fig 3. Scanning electron microscopy images of specimens which were exposed to WMTA/Blood (**a**) (× 2000), GP (**b**) (× 1500), and, WMTA/DW (**c**) (× 2000), WMTA/STF (**d**) (× 800), respectively, More Cells (▶) can be seen on the surface of WMTA exposed to blood; in addition, overview of replica impression on WMTA tablet, inner diameter (8 mm) and trace of NiTi (▶)can be seen on the surface (**e**) (× 11). Synapse formation (+) in great detail (**f**) (× 6000) and Abnormal cells can be seen in WMTA with STF (d).

neurocompatibility, which is more sensitive than biocompatibility evaluation (Asrari and Lobner 2003). Like other neural cells, viable differentiated p19 stem cells exhibit dendritic and axonal parts, while loss of cell compartments reveals cell death, indicating neurotoxicity (Finley *et al*. 1996).

WMTA comes in contact with blood or tissue fluids when placed as root-end filling material; therefore, blood was selected to simulate clinical conditions, whereas STF was selected to simulate dentinal and tissue fluids. WMTA mixed with STF was selected to mimic placement of dry MTA as confirmed by some investigators (Budig and Eleazer 2008) that dry MTA can be adequately hydrated by moisture penetration into the root body in clinical settings.

In this study, the superior neurocompatibility of WMTA, when exposed to blood rather than STF, may be attributed to the production of a unique and more neurocompatible surface, resulting from the presence of different bio-active elements in blood rather than in STF, which affect WMTA surface. In addition, it has been reported that when MTA is placed upon tissues, some bio-active molecules may be produced (Tomson *et al*. 2007). WMTA itself may not be neurocompatible because the exposure of WMTA to DW produced the least neuro-compatibility. DW was selected because it has no proteins, enzymes, or saturated ions.

The potential of blood to react with WMTA surface might produce a mechanical barrier against the solution's penetration, which limits the ability

for exchanging any materials with surrounding tissues. The composition and surface texture of the root-end filling material have an influence on the morphology and cell attachments, reflecting the biocompatibility of the substratum (Balto and Al-Nazhan 2003). It seems that provision of bioactive elements by blood is an important factor involved in the differentiation of p19 stem cells in WMTA/Blood group.

GP and ZOE were selected as controls because they are generally considered biocompatible and nonbiocompatible root canal filling materials, respectively (Tani-Ishii and Teranaka 2003).

Anti-nociceptive effects of WMTA were evaluated. WMTA did not irritate nerve tissues and was more effective than eugenol in relieving orofacial nociceptive pain of formalin injection (Abbasipour *et al.* 2009). An investigation with osteoblast-like cells on MTA has found that MTA has low toxicity and does not inhibit cell growth but can suppress the differentiation of osteoblast-like cells (Nakayama *et al.* 2005). In contrast to our study, the experimental time interval in that study (Nakayama *et al.* 2005) was up to 3 days, which has been reported to be too short for rat bone marrow cells to be divided and converted to functional osteoblasts. Furthermore, the use of gray or white MTA has not been mentioned in that study. It is an established fact that some of the ingredients of white and gray MTA are not similar (Song *et al.* 2006).

Despite the significant differences between WMTA/STF, WMTA/DW and GP, the comparison of WMTA/B and GP showed no significant differences. These findings might be attributed to the creation of a layer on the surface of WMTA after being exposed to blood, which may prepare the surface to meet specific biocompatibility requirements such as morphology, wettability, and solubility (Ratner *et al.* 2004). MTA produces calcium hydroxide when mixed with water; therefore, when a mixture of MTA and water is exposed to tissue fluid, hydroxyapatite crystals (the major component of bone) are produced that cover the surface of MTA (Sarkar *et al.* 2005). Therefore, low neurotoxicity of WMTA in the presence of blood might be attributed to the formation of hydroxyapatite crystals on WMTA surface. Comparison between WMTA/STF and WMTA mixed with STF showed significant differences which can be explained by the ability of STF to release all of its major cationic components (Ratner *et al.* 2004), leading to an influence on neural cells.

Neural cells are very sensitive to fixation solutions and processes. Some techniques and instruments such as stereomicroscopy and confocal microscopy (Ugel *et al.* 2004) used for observing neural cells are dependent on preparation and cell fixation methods which are too complicated (Fedoroff and Richardson 2001). A novel indirect method by scanning electron microscopy on replicated specimens was used to observe p19 neural cells and their cellular extensions. In addition, this study demonstrated feasibility and suitability of replica technique to observe p19 neural cells morphology and behavior.

Conclusion

It can be concluded that replica technique is suitable for studying morphological behavior and attachment of p19 neural stem cells. Further studies may prove the suitable of this technique for studying other cell lines morphological behaviors. Also, biological systems react to the surface characteristics of the materials in contact with it; therefore, exposure of WMTA to blood may alter its surface properties, making it more neurocompatible. In addition, the exposure of WMTA to blood during reparative usage of WMTA may enhance its neurocompatibility.

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