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Indirect Analysis of the Biocompatibility of Root-End Filling Materials In Vitro

Robert Ray Haglund Jr

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INDIRECT ANALYSIS OF THE BIOCOMPATIBILITY
OF ROOT-END FILLING MATERIALS IN VITRO

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B.S., Providence College, 1995
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INDIRECT ANALYSIS OF THE BIOCOMPATIBILITY OF ROOT-END FILLING MATERIALS IN VITRO

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**REVIEW OF LITERATURE**

*Periapical pathogenesis*

Pulpal and periapical pathosis develop as a result of bacterial infection. Kakehashi *et al.*, in 1965 demonstrated the importance of microbial infection in the development of periapical inflammation. Germ-free rats were subjected to surgical pulp exposure left open to the oral cavity. Control rats with normal bacteriologic flora underwent the same procedure. Germ-free rats exhibited no periapical destruction and formed reparative dentin bridge over exposure site whereas control rats developed pulpal necrosis and periapical inflammation. Subsequent studies have confirmed this direct relationship of bacteria to periapical inflammation (Sundqvist, 1976). In studies in which pulps in monkeys were aseptically rendered necrotic and sealed within the root canal, no inflammatory reaction was observed periapically (Moller *et al.*, 1981). In contrast, teeth with root canals infected by indigenous oral flora had inflammatory reactions in their tissues. The severity of periapical/pulpal inflammation is directly related to the bacterial load contaminating the tissue (Korzen *et al.*, 1974). A known amount of *Streptococcus mutans* was exposed into rats for a controlled length of time. A direct correlation...
was found between total microbial content within root canals and length of time the periapical tissues were exposed to the infecting microorganisms.

The infected root canal represents an ecological environment favorable for continued bacterial growth due to the low oxygen tension and availability of host tissue fluids for nutrients (Sundqvist, 1992). The microbial population is overwhelmingly anaerobic and gram negative (Sundqvist, 1976; Farber and Seltzer, 1988; Sundqvist, 1994). Prominent isolates in both human and animal studies have included *Prevotella, Porphyromonas, Peptostreptococci, Streptococci, Enterococci, Camphylobacter, Fusobacteria, Eubacteria, and Propionibacteria* (Farber and Seltzer, 1988). Longitudinal studies indicate that the relative proportion of obligate anaerobes increases over time (Fabricius *et al.*, 1982; Tani-Ishii *et al.*, 1994). Black pigmented bacteria, such as *Porphyromonas* and *Prevotella* species, as well as other microorganisms produce an array of virulence factors that may contribute to their pathogenicity. In particular, bacterial lipopolysaccharides (LPS) from cell walls of gram-negative bacteria have been shown to stimulate bone resorption *in vitro* (Haussman *et al.*, 1970) and *in vivo* (Umezu *et al.*,
Studies have shown a correlation between the levels of LPS in root canals and the presence of periapical lesions (Dahlen and Bergenholtz, 1980). Recognition of the need for strict aseptic technique during root canal treatment was identified well before adequate culturing techniques were available (Coolidge E, 1919). Future studies have supported obtaining a negative culture prior to obturation (Ingle and Zedlow, 1958). Some clinicians still adhere to this philosophy today.

**Disinfection of the root canal**

Various antimicrobials and disinfectants have been used in an attempt to remove bacteria and fungi from root canals. In Great Britain, a study was done comparing the antiseptic properties of phenol, salicylic acid, hydrogen peroxide, iodine, mercuric chloride, silver nitrate, sodium hypochlorite, benzene sodium sulphochloramine, paratoluene sodium sulphochloramine, and acetylchloramino-dichlorbenzene (Dakin HD, 1915). Materials were exposed to one drop of *Staphylococcus aureus*. Dakin found sodium hypochlorite to be an effective antiseptic that was irritating when applied to wounds (empirical evidence from application on wounded soldiers in World War 1). He suggested a diluted
preparation to maximize antiseptic properties and minimize harmful side effects. Dakin arrived at 0.5% sodium hypochlorite solution. “Dakin’s solution” was soon put to use as an endodontic irrigant. However, more specific studies were needed to discern which properties were best suited for root canal therapy. More sophisticated techniques were used to evaluate efficacy of root canal irrigants and medicaments in subsequent studies. A five part study investigating the toxicity and efficacy of irrigation solutions and canal dressings was done involving the application of each antimicrobial to known endodontic pathogens, specifically *Staphylococcus aureus, Streptococcus faecalis, Psuedomonous aeruginosa*, and *Candida albicans* (Spangberg and Engstrom, 1968). It was found that 0.5% sodium hypochlorite, Iodopax 0.04%, Biopsept 0.1%, and Biosept 1.0% exhibited an adequate ratio between antimicrobial effect and cytotoxicity for irrigants. The authors recommend 0.5% sodium hypochlorite or Iodopax 0.04%. When cell death was evaluated by release of radioactively-labeled chromium, all irrigants studied were found to be markedly cytotoxic compared to their antimicrobial effects (Spangberg *et al.*, 1973). The appropriate dilution of sodium hypochlorite also came into question. Serial dilutions of
Clorox (5.25% sodium hypochlorite) were exposed to bacterial preparations of *Streptococcus faecalis* and *Staphylococcus aureus* (Shih *et al.*, 1970). Bacteria were killed within 30 seconds with full strength, 1:10 dilution, 1:100 dilution, and 1:1000 dilution of Clorox. However, seven days post-irrigation revealed that at least 50% of all samples showed growth with both bacterial specimens. The authors recommend 5.25% sodium hypochlorite, the use of antimicrobial medicament between appointments, and caution regarding negative cultures (although the microbial population in the canal may be reduced, the canal is not sterile). However, a study evaluating the effect of 0.5% sodium hypochlorite in the treatment of teeth with necrotic pulps and apical periodontitis compared with saline concluded differently (Bystrom and Sundqvist, 1983). Bacterial samples were taken at the beginning of treatment, after instrumentation and irrigation, and after each subsequent appointment of instrumentation and irrigation. No canal dressing was used between appointments. It was found that 0.5% sodium hypochlorite was more effective than saline as a root canal irrigant in necrotic teeth.

Another mechanism of reducing bacterial contamination within the root canal system involves the use of intracanal medicament between
appointments. The toxicity and efficacy of irrigation solutions and canal dressings has been investigated (Spangberg and Engstrom, 1968). The following medicaments were studied: 2% iodide potassium iodine, 5% iodide potassium iodine, Biosept 0.1%, Biosept 1.0%, Iodopax (0.4% iodoform), Grossman’s solution, chloramine, Dakin’s solution (0.5% sodium hypochlorite), Chlumsky’s solution (camphorated phenol), tricresol formalin, eugenol, aetanolum, chloroform, resin chloroform, EDTA, and sulfuric acid. Formulations of 9.9ml of each medicament were shaken in a test tube with 0.1ml of bacterial suspension (organisms included Streptococcus feacalis, Staphylococcus aureus, Psuedomonas aeruginosa, and Candida). The results showed numerous differences between medicaments and effect on organisms. 5% iodine potassium iodide, Dakin’s solution, Iodopax, and Biosept 0.1% had bactericidal effects on all test organisms, however there were no definitive recommendations made by the authors regarding choice of interappointment medicament.

Other studies have also addressed the issue of antimicrobial efficacy relative to host cytotoxicity. A study investigating root canal medicaments used corneal irritation of the eyes of white male rabbits as a
model for toxicity (Harrison and Madonia, 1971). The materials investigated included 1% aqueous parachlorophenol, camphorated parachlorophenol, 2% parachlorophenol, crestatin, and eugenol. A dermal connective tissue response was also observed. Eugenol and camphorated parachlorophenol exhibited severe inflammation and tissue necrosis while all others showed very mild inflammation. Another method of evaluating material toxicity in vitro involves the use of radiolabeled cells as described by Spangberg in 1973. HeLa cells and L cells were labeled with $^{51}$Cr (sodium chromate). For testing fluid or soluble materials, labeled cell suspension was mixed with the solution to be tested and incubated at 37°C for 1, 2, 3, 4, and 24 hours. For testing solids or semisolids, the materials were spread over the culture chamber and incubated with labeled cells for various time periods. At the end of the culture, media was collected and centrifuged. The supernatent was measured for $^{51}$Cr. The percentage of $^{51}$Cr release was calculated according to the total amount of label incorporated into the target cells. This method was used to study and correlate the toxicity and antimicrobial effect of root canal antiseptics (Spangberg et al., 1973). Materials tested included chlorhexidine, camphorated monochlorophenol,
formocresol, Iodopax, iodine potassium iodide, paramonochlorophenol, sodium hypochlorite, and Wescodyne. The results showed that all materials are markedly toxic compared to their antimicrobial effect. A recommendation for the use of an adequate irrigation solution and a medicament for dressing between appointments was made.

**Biocompatibility**

Although removal of bacteria from the root canal is of primary importance, the biocompatibility of endodontic materials is necessary for adequate healing. The materials used in surgical endodontics are placed in intimate contact with the hard and soft tissues of the periodontium. Therefore, it is essential that a potential root-end filling material be non-toxic and biocompatible with its surrounding host tissue (Torabinejad and Pitt Ford, 1998). One widely accepted definition of biocompatibility is “the ability of a material to elicit an appropriate response in a given application” (Wataha, 2001). This definition implies that the interaction between the host, the material, and the expected function of the material is congruous and not pathologic. All three factors must be considered and be in harmony before a material can be
considered biocompatible. No biomaterial is truly inert. When a material is placed into living tissue, interactions with the complex biological systems will occur and will result in some sort of biologic response (Mjor et al., 1977). The environment in which a root-end filling is placed is dynamic and changing. Following endodontic surgery, the periapical tissues recruit a variety of cells that change temporally as healing occurs (Harrison and Jurosky, 1992). Furthermore, the function of the tooth will alter the periapical environment from pressure caused by mastication. These factors can affect the timeliness of post-operative healing.

Biocompatibility is not only a property of a material, but of a material interacting with its environment. Consider an example comparing dental and orthopedic implants (Wataha and Hanks, 1997). A properly placed titanium implant will osseointegrate with mandibular bone over time. However, a chromium-cobalt alloy placed in the same dental implant situation (same host, same placement technique, same load) will not achieve successful osseointegration. Conversely, when titanium is used as an orthopedic implant for hip athroplasty, the femoral head (ball) portion of the implant causes wear of the acetabulum (socket).
The small particles created cause irritation locally and at remote sites due to movement of debris within blood vessels, ultimately leading to the failure of the implant. Yet, the much harder chromium-cobalt alloy will perform well in this orthopedic application. Thus, it is not always appropriate to label a material “biocompatible” or “incompatible” because the classification depends upon the material and its interaction within a certain environment.

Recently, public and various government agencies have required some assurance that materials used in medicine and dentistry are safe and effective (Federation Dentaire Internationale, 1980). These regulatory pressures have resulted from many sources, including the movement towards ethical treatment of patients, an increased awareness of patients about the risks involved in health care, and concerns of health practitioners about litigation by patients. Biocompatibility is measured using *in vitro* tests and *in vivo* tests.

*In vitro* biocompatibility tests are performed outside of a living organism, usually in a test tube or culture dish. These tests are quite diverse, but generally place cells or bacteria in contact with an experimental material. The three most commonly used cytotoxicity tests
in dentistry include agar overlay technique, Millipore filter method, and radiochromium release test (Torabinejad and Pitt Ford, 1996). Agar overlay method involves test cell monolayers separated from experimental materials by agar (Guess et al., 1965). Following 24-hour incubation, the cell monolayers are examined under microscope and the plates are visually inspected for cellular lysis. Wennberg et al. (1979) introduced the “Millipore filter method” in which Millipore filters are placed in tissue culture plates and covered with suspensions of test cells, usually HeLa cells or mouse L929 fibroblasts. An experimental material can be exposed to cells indirectly using an agar medium or aqueous culture medium. The relative degree of cytotoxicity of the material can be registered by the extent of cellular death or inhibited enzyme activity. Spangberg (1973) introduced the radiochromium release test. This involved the use of prelabeled test cells and the release and subsequent measurement of radiochromium following incubation with test materials. The amount of radiochromium reflects cell death and estimates material biocompatibility relative to other experimental materials.

*In vivo* biocompatibility tests, such as implantation of a material into animals, allows for the observation of the many complex interactions
between the biological environment and the material. The biological response is generally more comprehensive and relevant than *in vitro* tests. However, *in vivo* testing is time-consuming and expensive. Furthermore, the question of relevance remains because of the appropriateness of using an animal species to represent the human response. Usage tests are clinical trials of the material on human volunteers and represent the final measure of biocompatibility in the most relevant environment (Wataha, 2001). Biocompatibility testing has been used to evaluate all materials used in medicine and dentistry. Both *in vitro* and *in vivo* evaluation of materials are relevant and necessary and will continue to be used to evaluate new materials introduced in the United States as enforced by the Food and Drug Administration.

**Immunology of the periapical lesion**

The periapical lesion represents a local immune response to infection of the root canal, the purpose of which is to localize the infection within the root canal system. Many studies have described the inflammatory cell infiltrate in chronic periapical lesions in both human and non-human models (Pulver *et al.*, 1978; Stern *et al.*, 1981; Dahlen *et
al., 1982; Bergenholtz et al., 1983; Torabinejad et al., 1985a; Kopp and Schwarting, 1989; Akamine et al., 1994; Kawashima et al., 1996; Stashenko et al., 1998). These reports demonstrate that a mixed infiltrate is recruited to the periapical tissues in response to an insult. Investigators have found varying ratios of T and B lymphocytes, polymorphonuclear leukocytes (PMNs), macrophages, plasma cells, natural killer cells, eosinophils, and mast cells (Stern et al., 1981; Bergenholtz et al., 1983; Torabinjad et al., 1985a; Stashenko and Yu, 1989; Kopp and Schwarting, 1989; Kawishima et al., 1996). The inflammatory infiltrate constitutes approximately 50% of the cellular response in periapical lesions; non-inflammatory connective tissue cells, including fibroblasts, vascular endothelium, proliferating epithelium, osteoblasts, and osteoclasts, comprise the balance of cells (Langeland et al., 1977). Many studies agree that macrophages are the most numerous immune cell type responding to periapical infection (Stern et al., 1981; Kopp and Schwarting, 1989; Kawashima et al., 1996).

The rat model has been used to study the genesis of periapical disease (Kakehashi et al., 1965; Yu and Stashenko, 1987; Kawashima et al., 1996). In this model, pulpal necrosis was initiated on day 2 followed
by periapical bone destruction beginning about day 7. A period of rapid
destruction occurs between days 7 and 20 (active phase), with slowed
resorption thereafter (chronic phase) (Yu and Stashenko, 1987;
Stashenko and Yu, 1989; Kawashima et al., 1996).

Tissue destruction in periapical tissues involves the resorption of
bone and its replacement by granulomatous and/or cystic tissue
extensively infiltrated with leukocytes. Communication between
leukocytes as well as other non-inflammatory cells involves cytokines.
Interleukin-1α (IL-1α), IL-1β, tumor necrosis factor-α (TNFα), TNFβ,
IL-6, and IL-11 have been found to possess bone-resorptive activity, and
collectively comprise the entity formerly termed osteoclast-activating
factor (OAF) (Dewhirst et al., 1985; Ishimi et al., 1990; Girasole et al.,
1994). In humans, IL-1β constitutes most OAF activity (Dewhirst et al.,
1985; Lorenzo et al., 1987). Prostaglandins have been implicated in bone
resorption as well. Resorption stimulated by all these cytokines is
partially indomethicin-inhibitable, indicating that they act through both
prostaglandin dependent and independent pathways of osteoclast
activation (Stashenko et al., 1987). Most bone resorptive activity can be
inhibited by blocking the expression of IL-1α, with approximately 10-
15% of bone-resorption activity attributed to PGE IL-1β and IL-1α are 500- and 25-fold more potent in stimulating bone resorption, respectively, than TNFs (Stashenko et al., 1987).

Although IL-1α plays a central part in the pathogenesis of bone resorption in the periapical lesion, its production and activity are likely to be regulated by other, primarily T-cell derived, cytokines (Stashenko et al., 1998). T-helper 1 cells (Th1) are generally proinflammatory, expressing IFNγ and IL-2 as well as IL-12 (Seitz et al., 1996). IFNγ is the key mediator of macrophage activation and has been shown, in combination with other stimuli, to upregulate IL-1 and TNF expression (Collart et al., 1986; Gerrard et al., 1987; Seiling et al., 1994). TNFα induces production of IL-1 which stimulates its own synthesis in a positive feedback loop (Kaushansky et al., 1988; Dinarello et al., 1987). These proinflammatory pathways may accelerate bacterial infection-induced bone destruction. In contrast, cytokines produced by T-helper 2 (Th2) lymphocytes including IL-4, IL-6, IL-10, and IL-13, downregulate the production of IL-1 and inhibit the expression and activity of the Th1-type mediators (Fiorentino et al., 1991; Seitz et al., 1996; Mosmann and Coffman, 1989). These IL-1 inhibitory functions may serve to protect
periapical bone from destruction. The balance between Th1 produced cytokines and Th2 cytokines may determine the extent of bone resorption in the periapical tissue of a tooth with pulpal necrosis.

Several aspects of the inflammatory response may be modulated by the secretion of neuropeptides, a process referred to as neurogenic inflammation (Jancso et al., 1967). Neurogenic responses include increases in blood flow, plasma extravasation, and leukocyte accumulation. Neuropeptides, particularly calcitonin gene-related peptide (CGRP), have been shown to promote wound healing in non-dental systems and have been shown to be closely associated with reparative dentin formation (Taylor and Byers, 1990). Although neurogenic inflammation has been most extensively studied in the pulp, pulpal necrosis as a result of infection or injury also leads to a local proliferation of sensory nerve fibers in the periapical region (Lin and Langeland, 1981; Holland, 1988; Byers et al., 1990). Other neuropeptides that contribute to neurogenic inflammation include substance P (Olgart et al., 1977), vasoactive intestinal peptide (Uddman et al., 1980), neuropeptide Y (Uddman et al., 1984), and neurokinin A (Wakisaka et al., 1988). Neuropeptides are synthesized in the nerve cell
bodies of the trigeminal ganglion and are transported to the periphery by
axonial streaming to be stored in the terminal nerve branches.
Neuropeptides are subsequently released into the pulp and periapical
tissues, via the axonal reflex, in response to electrical, mechanical,
chemical, or immunologic stimuli (Olgart et al., 1977).

**Non-surgical root canal therapy**

Complete cleaning and shaping of root canals followed by
adequate sealing often result in resolution of periradicular lesions in
patients who have undergone non-surgical root canal treatment. The
degree of success following root canal therapy has been reported as high
as 98.7% (Hession, 1981) and as low as 45% (Meeuwissen and Eschen,
1983). Many studies have reported good healing of periapical lesions
following root canal treatment (Strindberg, 1956; Bergenholtz et al.,
1979; Sjörgren et al., 1990). Ingle and Glick in 1965 reported a success
rate of 95% of all treated endodontic cases.

Many investigators have attempted to identify individual factors
affecting the prognosis of non-surgical endodontic treatment. Ingle in
1956 found a statistical association with improper root canal fillings and
a lack of apical seal with more than 75% of root canal failures. Sjorgren et al., in 1990 have shown that the level of root filling in relation to the root apex has a significant influence on the outcome of treatment of teeth with apical periodontitis. In a long-term follow up study, non-vital teeth with radiographic periapical lesions that were instrumented and filled within 0-2mm of the radiographic apex had a success rate of 94%. Teeth with the same preoperative diagnosis, but were filled short (>2mm) of the apex during treatment had a success rate of just 68%. Overfilled teeth had a 76% success rate. Most follow-up studies on endodontic therapy report overall success rates between 85-90% (Strindberg, 1956; Grahnen and Hansson, 1961; Kerekes and Tronstad, 1979; Sjorgren et al., 1990).

If conventional root canal treatment fails, the tooth can undergo orthograde retreatment or endodontic surgery to achieve adequate healing periapically. Decision making is usually based on the reason for failure. Procedural problems such as inadequate aseptic control, poor access cavity design, missed canals, ledged canals, inadequate instrumentation, leaking temporary fillings, and lack of permanent restoration are all examples of problems that can eventually lead to endodontic failure. Bergenholztz et al. (1979) evaluated the effects of endodontic retreatment
on quality of apical seal and periapical healing on 660 previously filled
teeth. The results showed that root-fillings with technical shortcomings
could be markedly improved as regards to effectiveness of seal and
distance to the apex. A large number of lumina discernable apical to root-fillings could also be treated and filled. 78% of the cases with pathologic lesion present periapically prior to retreatment either completely healed or displayed an obvious size reduction. Retreatments carried out because of technical difficulties alone were successful in 94% of the cases. The authors recommend root canal retreatment prior to endodontic surgery. Sjorgren et al., in 1990 showed that the preoperative diagnosis has a strong correlation with overall prognosis. When strict aseptic technique was applied, they found that teeth with irreversible pulpitis were clinically and radiographically successful in 96% of the cases, whereas necrotic teeth with apical periodontitis had a reduced success rate of 86%. In the same study, Sjorgren et al. found that root filled teeth with periapical lesions which then underwent retreatment had an even lower success rate of 62% (follow-up period of 8-10 years).
Surgical endodontics

Periapical surgery was first described in the literature by Farrar in 1880. He commented on a radical new approach to the treatment of periapical abscesses involving heroic amputation of the apical segment of roots of teeth. Periapical surgery and apicoectomy of teeth with persistent infections has since become a common treatment modality among endodontists and oral surgeons. Coolidge in 1930 was the first to apply histologic evidence to healing in the periapical region following apicoectomy. The goal of such surgery is to adequately seal the apical foramen. In some instances, this is the only direction available because of large post/core, fractured silver point filling, or a narrow canal space that is obstructing the clinician’s ability to clean the apical canal segment. In these cases that were unable to be instrumented and present with endodontic failure and/or periapical symptoms, endodontic surgery is often indicated (Luebke et al., 1964). Historically, other indications for surgery described in the literature include presence of periapical pathology, blunderbust apices, instrument fracture, lateral or apical perforations, dilacerated roots, and dens-in-dente (Maxmen, 1959). Currently, techniques for achieving high quality orthograde treatment and
retreatment have warranted reconsideration of the indications for periapical surgery. Apical periodontitis is a prerequisite for all endodontic surgery, diagnosed with radiography and clinical exam. Provided that the root canal treatment or retreatment is of good quality, the occurrence, persistence, or exacerbation of a periapical radiolucency is indication for root canal retreatment or periapical surgery. Extraradicular infection of the root canal has also been documented (Nair et al., 1999). The authors described six clinical cases that demonstrated persistent radioluscent lesions after conventional root canal treatment. Surgical biopsies were analyzed with correlative light and transmission electron microscopy. Factors associated with failure included not only persistent intraradicular infection, but also extraradicular infection that remained following non-surgical endodontics. Cholesterol crystals have been identified in therapy resistant endodontic cases (Nair, 1999).

Depending on the etiology of apical pathology, the tooth may or may not be responsive to apical surgery. Excess filling material apically may result in apical periodontitis (Sjogren et al., 1995). Perforation of the root canal laterally or apically can sometimes be repaired with endodontic surgery. Fractured instruments can harbor bacteria and
become an indication for apical surgery. Inaccessible root canals, particularly those with a preoperative diagnosis of necrosis, can also be treated surgically. Exploratory surgery is sometimes required to investigate the presence of a vertical root fracture or perforation. Occasionally, an apical lesion that is not responding to conventional endodontics may be of malignant origin and in need of biopsy.

The most frequently performed endodontic surgical procedure is the apicoectomy. This involves the resection of the root-end to provide access to the infection remaining in the root canal. Resection of the root-end can also result in the elimination of certain etiologic factors contributing to the pathology such as accessory apical foramen that continue to harbor bacteria, apical perforations, and some conservative vertical root fractures. Traditionally the root-end has been beveled to improve visual and mechanical access to the root canals (Rud J and Andreasen, 1972; Gutmann and Harrison, 1985). More recent literature reveals that bevels may have a deleterious effect on healing because more dentinal tubules are opened increasing the amount of apical leakage (Gilheany et al., 1994). Other investigations have shown no difference when comparing beveled and perpendicular root-end preparations.
(O’Connor et al., 1995). Currently, it is recommended to make as little bevel as possible without compromising access (Pitt Ford, 1998).

Following root-end resection, the canal space is prepared by removing 2-3mm of apical gutta percha. It is recommended that the apical segment of the root canal be prepared adequately to remove irritants from within the canal (Lin et al., 1983). Traditionally this was accomplished with a rotary handpiece and burs, however a well-centered preparation was often difficult to achieve. Wuchenich et al. (1994) investigated the apical preparation using a rotary handpiece compared with ultrasonic root-end preparation in cadaver teeth with regards to retention, cleanliness, and root canal parallelism. Using SEM analysis, it was shown that ultrasonic instruments produced more parallel walls and more depth for retention. Preparation with burs has been found to interfere with proper angulation resulting in bacteria remaining on the buccal aspect of the root-end cavity and increased risk of palatal perforation (Chong et al., 1997). The advent of specially designed tips for use with ultrasonic handpieces has allowed the root-end preparation to be accomplished along the long-axis of the root canal. Furthermore, the preparation is well centered and more conservative (Gutmann et al.,
1994; Sultan and Pitt Ford, 1995). Ultrasonic preparation has been shown to clean the root-end of bacteria more effectively than burs (Gutmann et al., 1994). It has also been shown to be more effective than retrograde hand filing the apical preparation (Sultan and Pitt Ford, 1995). Comparison of root-end cavity preparation using either a 008 rosehead bur or ultrasonic preparation with Super EBA root-end filling was investigated (Chailertvanitkul et al., 1998). Results showed leakage of bacteria in 59% of the cases prepared with burs compared with 22% prepared with ultrasonics after 90 days.

The type of root-end preparation often will be dictated by the selection of root-end filling material. Traditionally, root-end cavity preparation has been designed for amalgam, involving a retentive internal configuration to compensate for the inability of amalgam to bond to tooth structure (Ross, 1935). This type of single surface preparation is often difficult to achieve in vivo with limited access (Moorehead, 1927; Taylor and Doku, 1961). An alternative single surface cavity prepared at right angles to the beveled root face has also been recommended (Gutmann and Harrison, 1985). A slot preparation has been indicated where surgical access is very limited (Matsura, 1962; Block and Bushell, 1982;
Gutmann and Harrison, 1985). A slot preparation can also be helpful when preparing two canals in close proximity to each other, commonly found in maxillary molars and premolars. It can be accomplished with ultrasonics or burs, and has the advantage over single surface cavity of allowing better cleaning of the root canal below the pulpal ends of the dentinal tubules of the resected root face (Gutmann and Harrison, 1985). The preparation for adhesive materials involves a saucer preparation accomplished with a round bur (Rud et al., 1991; Chong et al., 1993). A saucer preparation is conducive to covering a large surface area with composite resin or glass ionomer allowing for dentin bonding and sealing of dentin tubules (Rud et al., 1991). Both ultrasonics and handpiece preparation will create a smear layer on the exposed root-end. Better apical healing and cementum deposition was reported when the smear layer was removed with citric acid demineralization (Craig and Harrison, 1993). Other investigations concluded that no difference in cell adhesion and attachment was observed following smear layer removal (Zhu et al., 2000).

Once the apical root segment is adequately prepared, the root-end is obturated. The purpose of the root-end filling material is to fill the
apical canal space and achieve a seal, preventing access of microbial 
products contained within the root canal system to periapical tissues, thus 
allowing tissue repair to occur (Pitt Ford, 1998). The need for a root-end 
filling has been investigated. Apical resection alone, with no root-end 
filling and with or without burnishing of the exposed gutta percha, has 
been supported by some authors (Bramwell and Hicks, 1986; Rapp et al., 
1991). Other investigators maintain that the removal of apical gutta 
percha and dentin along with use of a root-end filling will improve the 
prognosis of endodontic surgery. In a retrospective study evaluating the 
prognosis of 572 teeth treated with periapical surgery, placement of a 
root-end filling was found to be the factor most associated with 
successful healing (Hirsch et al., 1979). Ideally, a root-end filling 
material should be biocompatible, easy to use, radiopaque, adhesive, 
antibacterial, insoluble, and unaffected by moisture. A wide variety of 
materials have been investigated, but very few possess these properties.

**Root-end filling materials**

Farrar in 1880 described the orthograde placement of gold foil in 
the apical third of root canals during obturation to prevent abscess
formation in teeth undergoing future surgical treatment. Placement of filling material from a retrograde approach at the time of surgical removal of the apex was not described until many years later. Von Hippel (1914) published a case report detailing the placement of amalgam to seal the root canal following apicoectomy. Luks (1956) described the preparation of a small retentive cavity at the root-end following removal of the apical root segment. Luks used amalgam as a root-end filling material and observed an increase in prognosis. Messing (1958) developed an amalgam carrier for use in periapical surgery to improve the technique of delivering small amounts of material to sites with limited access. Amalgam was considered to possess many properties which rendered it ideal for sealing root canals, such as adequate biocompatibility, easy handling, antiseptic properties, and expansion on setting (Messing, 1958; Trice FB, 1959). The suggestion of non-alloy materials for root-end filling was made by Nicholls (1962). He described the selection of material for root-end filling was confined to (1) amalgam, (2) fast-setting zinc oxide-eugenol compound, and (3) zinc phosphate cement. Nicholls states the advantages of amalgam as being easy to condense and, thus, an “effective apical seal is assured”. For zinc
oxide-eugenol (ZOE), he identified easier handling as the primary advantage, and states that post-operative results have been just as satisfactory. Zinc phosphate cement, according to Nicholls, did not seal as well as ZOE, as proven by Grossman in 1939, and was no longer recommended. An in vitro dye leakage study comparing various polycarboxylate cements with amalgam showed a significantly inferior seal with polycarboxylate cements when used for root-end filling (Barry et al., 1976). An in vivo study involving cyanoacrylate cement (Biobond) showed better healing than no root-end filling after two years when evaluated radiographically and clinically (Nordenram, 1970). Some investigators continued to advocate gold foil stating less dimensional change, ability to be autoclaved, and ease of use over amalgam (Kopp and Kresberg, 1973). The disadvantages of amalgam included the presence of mercury, corrosion, lack of sterility, scatter of particles during placement, staining of soft tissues, and slow setting time. Kopp & Kresberg (1973) performed 440 surgical cases using gold foil as a root-end filling. They reported 398 (90.5%) successful outcomes after two years based on radiographic and clinical examination. Amalgam root-end fillings in Rhesus monkeys were evaluated histologically (Marcotte
et al., 1975). Adequate healing of periodontal ligament and osseous tissue was shown with both amalgam and gutta-percha when compared with no root-end filling. Cunningham (1975) compared the apical appearance of eight teeth obturated with silver cones and sealer, gutta-percha and sealer alone, and gutta percha and sealer with root resection, with and without placement of root-end amalgam. The results showed breakdown of the apical seal in roots apicected after gutta percha obturation. Retrograde amalgam fillings appeared satisfactory under SEM.

In 1970, Nord used Cavit for root-end filling. Cavit is a zinc-oxide based material that does not contain eugenol (Widerman et al., 1971). Subsequent studies involving apical seal of Cavit have shown it to be less effective than amalgam in surgical cases in vivo followed for three years (Finne et al., 1977). The authors report dissolution of Cavit from the root-end preparation in several cases that could lead to microleakage and ultimate failure. It was found that Cavit did not provide complete canal obliteration. Delivanis (1978) compared the apical seal of polycarboxylate cement, Cavit, and amalgam in vitro and in vivo. He placed root-end fillings in dogs and evaluated the apical seal after six
months using radioisotopes. A second part of the study included the placement of root-end filling in extracted single rooted teeth and evaluation of radioisotope leakage after two days. It was concluded that the seal provided by amalgam improved over time and was significantly more effective than Cavit or polycarboxylate cement.

The scanning electron microscope (SEM) has been used in endodontic research to study the marginal adaptation of root-end filling materials to the adjacent tooth structure. The interface between the restoration and tooth structure has been implicated in the breakdown of the apical seal. Moodnik et al. (1975) found defects of 6-150μm around amalgam root-end fillings using SEM. The authors made no clinical correlation to their findings. Other SEM publications investigating the marginal integrity of root-end fillings would follow.

Pashley et al. (1977) described a simple in vitro method of quantitatively measuring the rate at which isotopically labeled substances permeate human dentin using fluid filtration. This technique has evolved and is currently used to measure microleakage of dental materials. The quality of the apical seal has been assessed through leakage studies. Investigators have attempted to quantify the integrity of the apical seal
using the penetration of dye, radioisotopes, bacteria, fluid filtration, and electrochemical means. Some researchers showed that microorganism penetration might be more appropriate than dye or isotope penetration for studying leakage in vivo (Mortensen et al., 1965; Krakow et al., 1977).

Oynick and Oynick (1978) published a case report documenting successful root-end obturation using a material called Stailine, now known as Super EBA. The material consists of powder and liquid mixed together in equal parts. The powder is composed of 60% zinc oxide, 34% silicone dioxide, and 6% natural resin; the liquid contains 62.5% ethoxybenzoic acid (EBA) and 37.5% eugenol. The authors state the advantage of Super EBA over IRM being its ease of manipulation and adhesiveness to dentin walls.

Although the literature has shown that placement of a root-end filling is paramount in prognosis of apical surgery (Hirsch et al., 1979), some researchers disagreed. Tanzilli (1980) evaluated the marginal adaptation of retrograde amalgam, heat-sealed gutta-percha, and cold-burnished gutta-percha prepared in extracted teeth. SEM analysis reported significantly smaller marginal defects with cold-burnished gutta-percha (average defect 1.8 microns) than with amalgam or heat-sealed
gutta-percha (average defects ranging from 22-104 microns). They concluded that cold-burnished gutta-percha was 90% better than any other technique investigated. A subsequent dye leakage study reported less leakage with cold-burnished gutta-percha than amalgam or heat-sealed gutta-percha (Kaplan et al., 1982).

Kimura (1982) underwent a two-part study to evaluate the effect of zinc in amalgam root-end fillings. He evaluated the periradicular tissue reaction of teeth in dogs with root-ends obturated with zinc- or zinc-free alloys in vivo. Histologic examination of four specimens at various time intervals (1-22 months) showed presence of some inflammation in the 1- and 7-month specimens and severe inflammation adjacent to both materials after 12- and 22-months. No statistical difference was found between zinc-containing and zinc-free amalgams. In part two of Kimura’s study, he determined the concentration of elements by optical emission spectrographic analysis in eight specimens at each time interval (1-22 months) and found no zinc precipitate in periapical bone adjacent to root-ends filled with zinc-containing or zinc-free alloys. Zinc-free amalgam was compared with poly-hydroxyethylmethacrylate (polyHEMA) and gutta-percha using bacterial microleakage (Kos et al.,
PolyHEMA showed better sealing capabilities against bacterial microleakage than zinc-free amalgam or gutta-percha in extracted teeth. The sealing ability of dental amalgam root-end fillings was evaluated by Tronstad *et al.* (1983). In this study, different types of amalgams were evaluated: (1) zinc-containing silver amalgam, (2) zinc-free silver amalgam, (3) dispersed phase alloy with 19% copper, and (4) spherical alloy with 27% copper. The results showed that all specimens leaked. The authors advocate the use of cavity varnish prior to placement of amalgam as a root-end filling. Stabholz *et al.* (1985) found poor marginal adaptation of amalgam root-end fillings when analyzed with scanning electron microscope.

The quality of the apical seal has been investigated using titanium screws as root-end fillings (Luomanen and Tuompo, 1985). The tightness of titanium screw fillings and retrograde amalgam fillings was compared in 17 human, single-rooted teeth using the leakage of bacteria *in vitro*. The authors report a tighter apical seal with titanium screw fillings than with amalgam.

Smee *et al.*, in 1987 used passive dye leakage to evaluate the apical seal of P-30 resin bonded ceramic, Teflon, amalgam, and IRM as root-
end fillings in extracted teeth. The results showed that P-30 resin bonded ceramic, IRM, and Teflon had less leakage than amalgam after 48-hours. The authors suggest that composite resin bonded to dentin is adequate for root-end filling.

Zetterqvist (1987) investigated glass ionomer cement in monkeys as a root-end filling. Glass ionomer and amalgam was used at random as root-end fillings in monkey central incisors. Tissue reaction and eventual healing were similar in both amalgam and glass ionomer.

Cyanoacrylate was also evaluated as a root-end filling material, and was shown to leak less than amalgam, with and without varnish, heat-sealed gutta-percha, and cold-burnished gutta-percha when observed for passive dye leakage (Barkhorder et al., 1988).

Safavi et al. (1988) developed a model for the evaluation root-end fillings using attachment of fibroblasts as a measure of biocompatibility. The quantity and the quality of cell attachment were examined using scanning electron microscopy. The apices of extracted teeth were prepared and obturated with root-end fillings. Two different amalgams, a composite resin, and a root canal sealer were used as experimental materials. The model showed differences among the materials. The
density of cells on the surface of composite resin was markedly less than that of amalgam. Gaps were observed between amalgam and dentin walls ranging from 2μm to 8μm. In composite resin fillings, good marginal adaptations were observed in the majority of specimens.

Andreasen et al. (1989) introduced a new technique of root-end filling involving the use of composite resin with a dentin bonding (Gluma) agent. The histologic response to this material was examined in two monkeys. The authors report reformation of Sharpey's fibers and regeneration of new cementum directly upon the composite resin root-end filling. A subsequent paper by Munksgaard et al. (1989) evaluated composite resin root-end fillings with Gluma dentin bonding agent in vitro. They described a concave root-end preparation to allow the maximum amount of surface area at the root-end for dentin bonding. It was found that the bond strength between composite and apical dentin to be 18Mpa and is unaffected by eugenol containing sealers. Contamination of Gluma with saliva or serum decreased the bond strength significantly. Microscopy of the composite-dentin border as well as SEM of the composite surface adjacent to dentin revealed good adaptation. A passive dye penetration study was used to compare the
sealing ability of composite resin, with and without dentin bonding agent, amalgam with varnish, EBA cement, glass ionomer cement, and absence of root-end filling (Thirawat and Edmunds, 1989). It was concluded that none of the materials provided an impenetrable seal but that glass ionomer cement, composite resin with dentin bonding agent, and composite resin without dentin bonding agent produced a better seal than amalgam with varnish, EBA cement, and resection alone.

Dorn and Gartner (1990) published a retrospective study documenting the success rates of 488 apical surgery cases completed with Super EBA, IRM, and amalgam. Radiographs were taken at a minimum of 6 months to a maximum of 10 years. The results revealed that both Super EBA and IRM demonstrated statistically significant improvements in success rates when compared with amalgam. The success rates were 75% for amalgam, 91% for IRM, 95% for Super EBA. The difference between IRM and Super EBA was not statistically significant.

Injectable thermoplasticized gutta-percha was compared to glass ionomer and amalgam when used as root-end filling materials (Olson et al., 1990). Statistical analysis of the results indicated that injectable
gutta-percha without sealer demonstrated significantly more leakage than other materials.

Yoshimura et al. (1990) evaluated apical seal of amalgam root-end fillings over time using microleakage of dye from both directions (coronally and apically) under pressurized fluid filtration. It was found that coronal leakage was greater than apical leakage at each time period, but the difference was not statistically significant. This experimental system was shown to reliably measure microleakage in retrograde amalgam fillings. King et al. (1990) also used the fluid filtration technique to evaluate the seal of root-end fillings. Materials evaluated were cold-burnished gutta-percha, amalgam, amalgam with varnish, Super EBA, and glass ionomer over 24-hours, 1-, 2-, 3-weeks, and 1-, 2-, 3-month intervals. Multiple range analysis indicated that glass ionomer produced a significantly inferior seal when compared with the other materials at all time periods. There was no significant difference among Super EBA, amalgam, and amalgam with varnish. Inoue et al. (1991) published a 24-week study of the microleakage of amalgam, glass ionomer, and IRM when evaluated with fluid filtration. They concluded
at the 1.5-hour interval, that glass ionomer and IRM leaked significantly less than amalgam.

Friedman et al., in 1991, attempted to correlate dye leakage with radiographic healing. Amalgam, glass ionomer, and composite resin root-end fillings placed in beagle dogs were evaluated for radiographic healing after six months. Teeth were then extracted and suspended in methylene blue dye for 8 hours. The authors found no correlation between radiographic healing and dye leakage. There was no statistically significant difference among the materials.

Rud et al. (1991) elaborated on the technique of using composite resin as a root-end filling. In this article, they introduced “Retroplast” as a redesigned composite resin better suited for use in surgical endodontics. Retroplast is a chemically curable composite resin containing silver for radiopacity and aerosil to obtain a suitable consistency. The desired cavity design is reinforced to be one that is slightly concave followed by smear layer removal with ethylenediaminetetraacetic acid (EDTA). A subsequent study reported enhanced healing with removal of the smear layer from resected root-ends during periapical surgery (Craig and Harrison, 1993). Adhesion of Retroplast to prepared dentin is achieved
with the dentin bonding agent Gluma. Gluma is a water-based solution of 5% glutaraldehyde and 35% 2-hydroxyethyl methacrylate (HEMA). This technique was evaluated in vivo in monkeys and humans. Retroplast used as a root-end filling in monkeys showed a tight seal between the composite and the cavity surface when evaluated with SEM. Histologic specimens of tissue surrounding root-end fillings revealed absence of inflammatory cells and intimate contact between Retroplast and fibroblasts. In some cases, cementum and Sharpey’s fibers formed in contact with the filling. Retroplast used as root-end filling in humans performed successfully in most cases, the main cause of failure being inadequate hemostasis during obturation or undiagnosed root fracture. Histologic analysis of two failed endodontic surgical cases treated with Retroplast were sectioned and examined under scanning electron microscope (Andreasen et al., 1993). Two anterior teeth were extracted following apical surgery due to root fracture. Teeth were removed with adjacent periapical bone for analysis. Examination of tissue showed reformation of periodontium adjacent to the composite, including reformation of a lamina dura, Sharpey’s fibers extending into the material, and cementum deposition.
A clinically based article exposed amalgam root-end fillings as having an unreliable long-term prognosis (Frank et al., 1992). The coauthors contacted as many patients as possible that had undergone surgical endodontics obturated with amalgam. Excluded from this study were cases for which failure could be attributed to any reason other than failure of the apically placed amalgam. Therefore, the authors state, “all cases included had to demonstrate periapical healing prior to ultimate breakdown”. Based on this criteria, 60 of 104 teeth (57.7%) were considered to be successful and 44 teeth (42.3%) were determined to be failures.

The biocompatibility of IRM and amalgam root-end filling materials was tested in ferrets (Maher et al., 1992). Ferret canines were instrumented, obturated, and treated surgically with root-end fillings of IRM, amalgam, or no root-end filling. Teeth with surrounding bone (block sections) were examined clinically, radiographically, and prepared for histologic examination. Clinical and radiographic examination indicated both materials to be well tolerated by periapical tissues. Microscopic evaluation of histologic sections of amalgam revealed a decrease in inflammation and the formation of a fibrous capsule over the
15-week period. IRM specimens showed persistent inflammation and slower healing potential.

The biocompatibility of root-end filling materials towards cells was evaluated in vitro (Peltola et al., 1992). Gingival fibroblasts and rat sarcoma cells were incubated with freshly mixed amalgam, glass ionomer, composite resin, and titanium to evaluate cytotoxicity. Cultures were photographed through a light microscope after 7 days incubation and counted after 14 days. It was shown that the proliferation of gingival fibroblasts was less disturbed by titanium, being approximately 96% of the control value (cell cultures without test materials), followed by composite, amalgam, and glass ionomer (61%, 49%, and 35% of the control value respectively). In rat sarcoma cell cultures, 76% of the control value was observed with titanium, followed by 12% with composite, and 5% with both amalgam and glass ionomer.

Torabinejad et al. (1993) introduced mineral trioxide aggregate (MTA) as a root-end filling material. This in vitro study used rhodamine B fluorescent dye and a confocal microscope to evaluate the sealing ability of amalgam, Super EBA, and MTA in thirty extracted single-rooted teeth. Statistical analysis showed that MTA leaked significantly
less than amalgam and Super EBA. MTA consists of calcium oxide and calcium phosphate. Calcium oxide is a crystal containing 87% calcium and 12.4% silica. Calcium phosphate contains 49% phosphate, 35% calcium, 6% silica, 3% chloride, and 2% carbon (Torabinejad et al., 1995).

Chong et al. (1994a) investigated the antibacterial activity of various root-end filling materials. The experimental materials included a light-cured glass ionomer cement (Vitrebond), three zinc-oxide eugenol based cements (Kalzinol, IRM, Super EBA), and amalgam. Using the agar diffusion inhibitory test with Streptococcus anginosus and Enterococcus faecalis, they found that glass ionomer had the most antibacterial efficacy, followed by the ZOE-based cements. Amalgam showed no antibacterial activity. A follow-up study evaluated the cytotoxicity of these materials (Chong et al., 1994b). Fresh and aged specimens of each material were assessed for cytotoxic effects using a Millipore filter and a cell monolayer. They used the cytochemical demonstration of succinate dehydrogenase activity expressed in Swiss mouse 3T3 fibroblasts as a measure of cytotoxicity. Fresh IRM exhibited the most pronounced cytotoxic effect and the difference was statistically
significant compared with Vitrebond, Kalzinol, EBA cement, and amalgam. When aged, Kalzinol and IRM had a significantly greater cytotoxic effect than the other materials. Torabinejad et al. (1995) investigated the antibacterial effects of amalgam, IRM, Super EBA, and MTA on nine facultative bacteria (*Enterococcus fecalis*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus subtilis*, and *Escherichia coli*) and seven strict anaerobic bacteria (*Prevotella buccae*, *Bacteroides fragilis*, *Prevotella intermedia*, *Prevotella melaninogenica*, *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, and *Peptostreptococcus anaerobius*). After growing these bacteria on solid media, freshly mixed materials were placed on the surfaces and the zone of inhibition was measured. IRM and Super EBA had some antibacterial effects on both types of bacteria. MTA was found to have effects only against facultative organisms. Amalgam had no antibacterial effect against any of the bacteria tested in this study.

The effect of contamination on apical seal of root-end fillings was evaluated (Torabinejad et al., 1994). Ninety extracted human teeth were instrumented, obturated, and filled apically with 2-3mm of amalgam,
Super EBA, IRM, or MTA. For each material, half of the root-end cavities were dried prior to placing the filling materials. The remaining half was obturated after being contaminated with blood. The results showed that contamination with blood had no significant effect on the amount of dye leakage. MTA was shown to leak significantly less than other materials tested with or without contamination of the root-end cavities. Miles et al. (1994) evaluated the contamination of dentin bonding agents on apical radicular dentin. Results indicated that the bond strengths of all groups contaminated with blood were significantly less than control groups, except with Amalgambond.

The effect of storage time on the degree of dye leakage of root-end filling materials was investigated (Higa et al., 1994). Sixty root-end cavities were filled with either amalgam, super EBA, or IRM. Ten roots in each group were placed immediately into India ink and ten were allowed to set for 24 hours before placement into ink. Results showed that storage time had no significant influence on the amount of dye leakage. Super EBA and IRM leaked significantly less than amalgam.

McDonald et al. (1994) introduced a self-setting apatite cement for use in root-end filling. The study involved fluid filtration evaluation of
amalgam, EBA cement, and apatite cement in single-rooted human teeth over time. Results indicated that apatite cement provided a comparable seal to amalgam and EBA cement. The authors suggest that apatite cement, in conjunction with its excellent biocompatibility, is an acceptable alternative to present-day root-end filling materials.

The ratio of powder to liquid in IRM root-end fillings was evaluated using fluid filtration (Crooks et al., 1994). Various ratios were investigated at 1, 2, 3, 4, 8, and 12 weeks after insertion. There were no significant differences in the microleakage of the experimental groups after week 3. However, the handling characteristics of IRM improved with an increase in powder to liquid ratio and the sealing ability remains unaffected.

Torabinejad et al. (1995b) investigated MTA for root-end filling in dogs. Periapical lesions were induced in the periradicular tissues of six beagle dogs. Orthograde root canal treatment was performed followed by endodontic surgery and placement of either amalgam or MTA root-end fillings. The periapical tissue response was observed histologically 2-5 weeks and 10-18 weeks post-operatively. Statistical analysis of the results showed less periapical inflammation and more fibrous capsules
adjacent to MTA compared with amalgam. In addition, the presence of
cementum on the surface of MTA was a frequent finding in the 10-18
week specimens.

Torabinejad et al. (1995c) investigated the physical and chemical
properties of MTA, IRM, Super EBA, and amalgam. The results showed
that MTA had a pH of 10.2 initially, which rises to 12.5 three hours after
mixing. MTA has a radiopacity that is equal to aluminum. MTA is more
radiopaque than Super EBA and IRM, but not amalgam. Amalgam had
the shortest setting time (4 minutes) and MTA the longest (2 hours and
45 minutes). At 24-hours MTA had the lowest compressive strength (40
Mpa) among the materials, but increased to 67 Mpa after three weeks.
Only IRM showed any solubility under the conditions of this study.
Another study involved the marginal adaptation of these material
investigated under scanning electron microscope (Torabinejad et al.,
1995d). Due to artifacts in the specimens, resin replicas were created and
evaluated under SEM. Data comparing gap sizes between root-end
filling materials and their surrounding dentin showed that MTA had
better adaptation compared with amalgam, Super EBA, and IRM.
Bacterial leakage of root-end filling materials was also investigated
(Torabinejad et al., 1995e). The number of days for *Staphylococcus epidermis* to penetrate root-end fillings was used to evaluate apical seal. Most samples whose apical 3mm were filled with amalgam, Super EBA, and IRM began leaking at 6 to 57 days. The majority of samples whose root-ends were filled with MTA did not show any leakage throughout the experimental period (90 days). Statistical analysis of the data showed that MTA leaked significantly less than other root-end filling materials. No significant difference was found between the leakage of amalgam, Super EBA, and IRM.

Johnson *et al.* (1995) investigated the effect of different formulations of amalgam on sealing ability. These included zinc-free spherical amalgam, zinc-free admixture amalgam, two zinc-containing admixture amalgams, and a zinc-containing lathe-cut amalgam. Fluid filtration was used to measure microleakage at 1, 2, 4, 8, 12, and 24 weeks after placement. Results showed that zinc-free spherical amalgam had significantly greater leakage than all the other products evaluated.

The radiopacity of root-end fillings was investigated by Shah *et al.* (1996). Radiographs of 1mm thick specimens of eight different materials (amalgam, Kalzinol, IRM, Super EBA, Vitrebond, Fuji II LC, Chemfil,
and gutta-percha) were assessed densitometrically relative to 1mm of aluminum. Results showed that glass ionomers (Vitrebond, Fugi II LC, Chemfil) had radiopacities below the international standard for root canal sealers (<3mm aluminum); zinc-oxide eugenol cements (Kalzinol, Super EBA, IRM) had radiopacities equivalent to 5-8mm aluminum; and gutta-percha had a radiopacity equivalent to 6.1mm aluminum.

Rud et al. (1996a) published a long-term evaluation of dentin-bonded resin composite (Retroplast) root-end fillings. Patients were examined clinically and radiographically. Thirty-three of the first 34 resin composite root-end fillings were examined; all cases were at least 8 years post-operatively. Only one patient showed recurrence of periapical inflammation, likely due to root fracture. The remaining cases showed complete bone healing periapically. The authors conclude that the bond established between apical dentin and resin composite was stable during the observation period and that the filling material had not been harmful to the surrounding tissues. Another study evaluated the effect of replacement of silver, used for radiopacity, with ytterbium trifluoride (Rud et al., 1996b). Silver was found to lower the working time and storage stability of the composite and might cause discolorations. Apical
fillings with the new formulation of Retroplast were performed and evaluated one year post-operatively. Of 351 surgical cases, 80% showed complete healing, 2% scar tissue, 12% uncertain healing, and 6% failure. No significant difference in healing pattern was found compared with that obtained using silver-containing Retroplast.

Trope et al. (1996) investigated the healing of various root-end filling materials in dogs. Mandibular premolars in seven beagle dogs were infected and periapical lesions were induced. Apical surgery was performed without disinfection of the root canal system. Super EBA, glass ionomer, amalgam, IRM, and light-cured composite resin were used for root-end obturation. After six months, the dogs were sacrificed and the apical tissues were prepared for histologic examination. Relative percentages of bone and inflammation were calculated against controls (no treatment was performed following root canal inoculation, resulting in full progression of periapical lesions). The results showed that Super EBA had less inflammation and less bone loss than other materials. In overall periapical inflammation, Super EBA was statistically better than all other materials except IRM. IRM was superior to glass ionomer, but not statistically different from the other materials. Regarding bone loss,
Super EBA was superior to glass ionomer, composite resin, and the positive control, but not statistically different from amalgam or IRM.

Williams and Gutmann (1996) evaluated the periapical healing response to a polyvinyl resin (Diaket) root-end filling with and without tricalcium phosphate. Non-surgical root canal treatment was performed on 56 premolar roots in mongrel dogs. Roots were then treated with apicoectomy and root-end filling with Diaket alone or Diaket with tricalcium phosphate. Post-surgical histologic evaluation was completed after 30 to 60 days. No difference was found between the two groups. Cementogenesis was documented to have occurred in both Diaket groups, with and without tricalcium phosphate.

The sealing ability of MTA, amalgam, and Super EBA was compared using fluid filtration (Bates et al., 1996). Seventy-six single rooted extracted teeth were instrumented and obturated prior to apicoectomy and root-end filling with either amalgam, with and without varnish, MTA, or Super EBA. Microleakage was assessed at 24-hours, 72-hours, 2-weeks, 4-weeks, 8-weeks, and 12-weeks using fluid filtration. Results showed that MTA demonstrated excellent sealing ability throughout the 12-week experimental period. Microleakage of the MTA
group and the Super EBA group was significantly less than amalgam, with and without varnish, at 24-hours, 72-hours, and 2-weeks. At subsequent periods, there were no significant differences among the three materials.

Torabinejad _et al._ (1997) evaluated the periapical tissue response in monkeys following apicoectomy and root-end filling with either MTA or amalgam. After five months, maxillary incisors of three monkeys, treated with both orthograde root canal treatment and surgical endodontics, were extracted and evaluated histologically. The authors observed no periapical inflammation adjacent to five of six root-ends filled with MTA. A complete layer of cementum was observed over MTA in all five cases. All root-ends filled with amalgam showed periapical inflammation and no new cementum formation.

Rud _et al._ (1997) compared the effect of root canal contents on periapical healing of teeth with surgically placed dentin-bonded composite resin root-end fillings. Healing results were compared among 551 infected roots apically sealed with Retroplast composite resin combined with the dentin bonding agent Gluma. These roots contained either (1) root filling to the apex following resection, (2) insufficient root
filling, or (3) empty root canals with necrotic pulp remnants. At 2-4 year follow-up, complete bone healing was observed (clinically and radiographically) in 92% of adequately obturated teeth, 85% of insufficiently obturated teeth, and 81% with no orthograde treatment. The authors also report that, of 21 roots classified as “failures”, healing was achieved by a second surgery using Retroplast and Gluma in 76% of the cases.

Wu et al. (1998) used standard bovine root sections to evaluate the sealing ability of amalgam, Super EBA, MTA, and glass ionomer. At 24-hours, 3-months, 6-months, and 1-year after filling, leakage along these filling materials was determined under low pressure using a fluid transport model. During the first three months, the percentage of leakage increased for amalgam and Super EBA, whereas it decreased for MTA. Leakage of amalgam and Super EBA decreased over time, whereas the seal of MTA was maintained throughout the experiment (one year). Another longitudinal leakage study comparing amalgam and MTA in a fluid filtration model showed amalgam to have significantly higher leakage at week-4 than MTA (Yatsushiro et al., 1998).
The cytotoxicity of endodontic sealers and root-end filling materials was evaluated using cell death of L929 mouse fibroblasts (Osorio et al., 1998). The authors used Millipore filter insert (3.0μm pore size) containing root-end filling materials (amalgam, Gallium GF2 [metal alloy], Ketac silver, MTA, and Super EBA) incubated in culture wells with L929 mouse fibroblasts. Cytotoxic effects were assessed using the MTT assay for mitochondrial enzyme activity and the CV assay for cell numbers. MTA was shown to have no cytotoxic effects on L929 fibroblasts. Gallium GF2 showed little cytotoxicity. Ketac silver, Super EBA, and amalgam showed higher levels of cytotoxicity.

Zhu et al., in 1999 evaluated the cytotoxicity of amalgam, IRM, and Super EBA using human periodontal ligament cells and human osteoblast-like cells. Ten-millimeter long plastic tubes were filled with 3mm of freshly mixed root-end filling material and cultured with PDL and osteoblast-like cells. The size of the cell-free zones around the materials and the total cell number per dish were calculated after 3 and 7 days. Amalgam produced a larger cell-free zone compared with IRM and Super EBA. Amalgam also showed a statistically significant reduction in total cell number for both cell types tested. This study concluded that
amalgam had greater cell toxicity to human periodontal ligament cells and human osteoblast-like cells than IRM and Super EBA.

Bacterial microleakage of root-end filling materials was investigated (Adamo et al., 1999). Sixty extracted single-rooted human teeth were treated with conventional root canal therapy and prepared for root-end filling. Root-end filling materials included MTA, Super EBA, composite resin with ProBond dentin-bonding agent, and amalgam. The coronal access of each tooth was inoculated with Streptococcus salivarius every 48 hours and a chamber containing indicating solution was evaluated for turbidity. The authors found no statistical difference in the rate of bacterial microleakage among the groups tested at either 4, 8, or 12 weeks. A similar experiment using the penetration of Prevotella nigrescens against Geristore, MTA, and Super EBA root-end fillings also showed no statistically significant difference in microleakage among the experimental groups (Scheerer et al., 2001).

In 2000, Zhu et al., evaluated the adhesion of human osteoblasts to root-end filling materials using scanning electron microscopy (SEM). Approximately 1mm thick disks of MTA, IRM, composite, and amalgam were condensed into 96-well flat-bottom plates and cultured with human
osteoblasts for one day. Results showed that osteoblasts attached and spread on MTA and composite forming a monolayer. Osteoblasts also attached on amalgam, but with few cells spreading. In the presence of IRM, osteoblasts appeared rounded with no spreading. Results indicate that osteoblasts have a favorable short-term response to MTA and composite resin compared with amalgam and IRM.

Keiser et al., in 2000, investigated the cytotoxicity of root-end filling materials in vitro using human periodontal ligament cells. The cytotoxicity of MTA, Super EBA, and amalgam was evaluated using a cell viability assay for mitochondrial dehydrogenase activity in human PDL cells. In the freshly mixed states, results showed amalgam to be most toxic followed by Super EBA, and then MTA. After 24 hours, Super EBA was the most toxic relative to amalgam and MTA. Overall, the authors conclude that the cytotoxicity of MTA was minimal relative to amalgam and Super EBA.

**Prognosis of endodontic surgery**

The prognosis of endodontic surgery has been reported by several investigators. Mattila and Altonen (1968) evaluated the prognosis of
periapical surgery. They used radiographic and clinical examination to determine success or failure in 143 teeth that underwent apicoectomy. Radiographically, 78 out of 143 (55%) were regarded as successful based on the authors interpretation and 98 cases were symptom-free (65%). Rud et al. (1972a) published results of a study investigating factors affecting the prognosis of 1,000 cases treated with endodontic surgery. A subsequent study involved the multivariate analysis of the influence of various factors upon healing after these documented periapical surgery cases (Rud et al., 1972b). Some factors affecting the prognosis of cases in this study include presence or absence of root-end filling, presence or absence of orthograde root canal treatment, and the retreatment to failing root canal therapy prior to surgery. A retrospective study of 572 teeth treated with periapical surgery showed that 46.7% of the cases healed completely, 26.2% showed a decrease in bone destruction, 22% were unchanged, and 5.1% presented with increased bone destruction (Hirsch et al., 1979). They found the most significant variable for success to be the presence or absence of a root-end filling. Grung et al. (1990) followed 477 surgical cases for five years. Complete healing was observed in 78% of the cases and incomplete healing (scar tissue) in 9%;
13% were regarded as failures. In this study, 85% of the teeth that underwent orthograde retreatment had success following apical surgery, whereas 65% of cases had success without retreatment. Dorn and Gartner (1990) published results of a retrospective study comparing success rates of surgical cases performed with three different root-end filling materials. The success rates were 75% for amalgam, 91% for IRM, and 95% for Super EBA. The overall prognosis of periapical surgery in this study was 82.0%. Frank et al. (1992) evaluated the long-term prognosis of surgically placed amalgam fillings. Over a ten-year observation period, all cases included in this study demonstrated periapical healing prior to ultimate breakdown. On this basis, 60 of 104 teeth (57.7%) were considered to be successful and 44 teeth (42.3%) were determined to be failures.

Few studies have attempted to examine the correlation between clinical success of root canal therapy and in vitro examination. Torabinejad et al. (1994) examined the apical adaptation of three orthograde and four retrograde amalgam root-end fillings of extracted human teeth using dye leakage and scanning electron microscopic methods. Despite the appearance of successful root canal therapy by
clinical and radiographic standards, these examinations showed dye
penetration through the apical foramen to the level of the root canal
filling and the presence of small gaps between root canal filling materials
and dentinal walls. SEM evaluation revealed gaps of various sizes in
clinically successful root-end amalgam fillings. In unsuccessful cases,
similar examinations showed the presence of gaps between the root-end
cavity preparation and the amalgam filling as well as complete
penetration of dye between amalgam, root canal filling materials, and
dentinal walls.

In 2000, Zuolo et al., reported 91.2% success out of 102 surgical
cases prepared with ultrasonics and treated with IRM root-end fillings 1-
4 years post-operatively.

Periapical wound healing

Few studies have investigated wound healing following periapical
surgery. A two-part study published by Harrison and Jurosky (1991)
details findings of soft and hard tissue healing in the rhesus monkeys
(Macaca mulatta) following endodontic surgery. Periapical surgery was
completed on six monkeys. The monkeys were killed and histologic
sections were evaluated under light microscope. The healing of both the incisional wound and the dissectional wound involves the migration of immunologic and non-immunologic cells. In soft tissue, polymorphonuclear leukocytes were the predominant inflammatory cell at 24 hours, but were reduced in numbers by 48 hours as the macrophage cell number became the predominant cell in the wound site during the 48-to 72-hour period. Epithelial cells formed a cohesive layer of stratified squamous epithelium throughout the wound by 96 hours. Connective tissue healing by fibroblasts appeared to be directly related to the speed with which epithelial healing occurred. Regarding the dissectional wound, polymorphonuclear leukocytes and macrophages were recruited early during clot formation. At day 2, an increase in macrophage cell number was observed and continued to be the predominant inflammatory cell in dissectional wound healing. Also by day 2, fibroblasts and ectomesenchymal cells were evident along the fibrin strands. Fibroblasts were found in the healing periapical tissues until day 14. Granulation tissue replaced the fibrin clot in the wound site as early as 4 days after surgery, and is replaced by fibrous connective tissue by 14 days. Harrison and Jurosky concluded that the healing of the dissectional
wound is very advanced at the 4th post-surgical day and essentially complete by the 14th day, with remodeling and maturation of epithelial and osseous tissues continuing through the 28th day.

A biocompatible root-end filling is necessary for adequate and timely healing in the periapical tissues. Although many studies have investigated the effect of root-end filling materials upon cells in vitro, no published literature reports observations of the morphology of cells. Furthermore, recovery and vitality of cells reacted with root-end filling materials have seldom been reported.
AIM OF STUDY

The purpose of this investigation was to determine the cellular response to four root-end filling materials (Amalgam, IRM, Retroplast, and MTA) based on cell morphology and cell growth. Specifically to:

A. Quantify the biocompatibility of root-end filling materials by counting the total cell number of fibroblasts.

B. Quantify the biocompatibility of root-end filling materials by counting the total cell number of macrophages.

C. Assess the biocompatibility of root-end filling materials by observing morphology of fibroblasts using phase-contrast microscopy.

D. Assess the biocompatibility of root-end filling materials by observing morphology of macrophages using phase-contrast microscopy.

E. Compare differences in cell growth and morphology patterns in response following exposure to amalgam, IRM, MTA, and Retroplast root-end filling materials.
The hypothesis to be tested is as follows: There is no difference between the *in vitro* effects of various root-end fillings on the growth and morphology of fibroblasts and macrophages. There is no difference between the response of fibroblasts and the response of macrophages following incubation with various root-end filling materials.
MATERIALS AND METHODS

Experimental root-end filling materials

Root-end filling materials used in this study were MTA (ProRoot\textsuperscript{TM}, Dentsply, Tulsa, OH), IRM (Caulk/Dentsply, Milford, DE), Amalgam (Valiant Ph.D., Ivoclar Norht America Inc., Amherst, NY) and Retroplast composite resin (Retroplast\textsuperscript{TM}, Retroplast Trading, Ronne, Denmark). Root-end filling materials were mixed according to manufacturer’s instructions. In one set of experiments, fresh pellets (1x1x3 mm\textsuperscript{3}) of root-end filling material were used. In another set of experiments, the pellets of root-end filling material were allowed to set in cell culture medium at 37\degree C and 100% humidity. The medium was changed every day for 2 weeks. Then the set pellets of root-end filling material were used.

Cell culture

L929 mouse fibroblasts and the mouse macrophage cell line RAW 264.7 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Eagle’s minimum essential medium (ATCC) supplemented with 10% fetal bovine serum (Hyclone
Laboratories Inc. Logan UT) and 1% antibiotic/antimycotic cocktail (300 units/ml penicillin, 300 μg/ml streptomycin, 5 μg/ml amphotericin B; Gibco BRL, Gaithersburg, MD) under standard cell culture conditions (37 °C, 100% humidity, 95% air and 5% CO₂).

Cells were seeded into 6-well cell culture plates (Sigma, St. Louis, MO) at an initial density of 2 x 10⁵ cells/well for fibroblasts and 4 x 10⁵ cells/well for macrophages with 2 ml medium. Cells were incubated for 24 hr to allow adhesion. Then culture plate inserts (30 mm diameter, 0.4 μm pore size; Millipore Corp. Bedford, MA) with pellets (one pellet/insert) of fresh or set root-end filling material were placed into the cell culture plates. Cells cultured with only culture plate insert served as control. After 3-day incubation, cell morphology was examined under phase-contrast microscope (CK40 Culture Microscope, Olympus American Inc. Melville NY) and photographed. Cells were detached with 0.25% trypsin and 1 mM EDTA in Hanks’ balanced salt solution (Gibco BRL, Gaithersburg, MD), and the total cell number per well was electronically counted (Coulter Electronics, Hialeah, FL).
The experiments were done in triplicate. Data were statistically analyzed using one-way ANOVA by comparing total cell numbers per well within the fresh or the set material groups.

Cytokine analysis

For cytokine assay, root-end filling materials were inserted into the wells of 24-well flat-bottom plates (Becton Dickinson, Oxnard, CA), and condensed to disks of ~1mm thickness and the same diameter as the wells. Cell culture medium (500 µl/per well) was put into the wells and incubated at 37°C and 100% humidity. The medium was changed every day for 2 weeks. This allowed the materials to set. Then mouse macrophages were seeded into the wells (10^6 cells/1 ml medium/per well) with the material disks in the bottom. The plates were incubated for 24 hours. After incubation, culture media were collected and analyzed for IL-1β and IL-6 content by ELISA (R&D Systems, Inc. Minneapolis, MN). Cells cultured without root-end filling materials served as a negative control, and cells treated with 1 µg/ml LPS served as a positive control.
RESULTS

*Effect of root-end filling materials on fibroblasts:*

CELL PROLIFERATION

Quantitative evaluation of total number of fibroblasts revealed differences between the materials when freshly mixed. Table 1 shows the total cell number after 3-days incubation with root-end filling materials. The mean of the three experiments quantifying total cell number was used to compare materials. In the fresh material experiment, the total cell number in the control group was significantly greater ($p < 0.01$) than the group exposed to the experimental materials. Total cell number cultured with fresh IRM was significantly less ($p < 0.01$) than that cultured with other fresh materials. IRM was not statistically different from Retroplast. Fibroblasts cultured with Retroplast had a total cell number significantly less than controls ($p < 0.01$). Freshly mixed amalgam and freshly prepared MTA showed a calculated number of fibroblasts similar to control. Both materials were not significantly different from controls. Fresh amalgam and fresh MTA were not statistically different from each other, however, both were higher in fibroblast number than IRM and Retroplast. This difference was
statistically significant. The total cell number in the fresh Retroplast group was significantly less (p<0.01) than that in the fresh MTA group or fresh amalgam group.

In the set materials experiment, the total fibroblast cell number in the control group was significantly greater (p < 0.01) than that in the set root-end filling material groups. Set materials showed similar results with fibroblasts, however Retroplast exhibited a fibroblast cell number similar to control. No significant differences were seen between the set MTA group and the set amalgam group, or between the set amalgam group and the set Retroplast groups. However, the total cell number in the set MTA group was greater (p < 0.01) than that in the set Retroplast groups. The total cell number cultured with set IRM was significantly less (p < 0.01) than that cultured with other root-end filling materials. IRM that had been set for 2 weeks showed a statistically significant decrease in fibroblast number compared with controls.

CELL MORPHOLOGY

Phase-contrast microscopy revealed differences in fibroblast morphology. Morphologically, mouse fibroblasts grew to confluence and
formed a monolayer in control groups (Figure 1). With fresh amalgam, an area of cell death was observed directly beneath the material (Figure 2.a), however normal cell morphology was found from the periphery of the material to the outer edge of the culture well (Figure 2.b). With set amalgam, normal cell morphology was found throughout the culture well (Figure 2.c). With both fresh (Figure 3.a) and set IRM (Figure 3.b), cell death occurred both beneath the material and in the periphery. With fresh Retroplast, cell death occurred throughout the culture well (Figure 4.a). With Retroplast that had been set for two-weeks, cell death was found directly beneath the material (Figure 4.b), but normal morphology of L929 fibroblasts was found in the peripheral region (Figure 4.c). Cell detachment and floating were also seen in the fresh IRM and fresh Retroplast groups. In wells with fibroblast exposed to freshly mixed MTA we observed three zones of cellular response (Figure 5.a). Direct exposure of cells to MTA, placed in culture wells without filter inserts, resulted in an area around the MTA that appeared whitish in color surrounded by a clear zone (Figure 6.a). Under microscopy, the morphological changes were the same as that observed with the presence of cell culture inserts which displayed three distinct zones of cellular
response (Figure 6.b). In the first zone, directly beneath the material, we observed altered cell morphology consistent with cell death along with the presence of a multitude of yellow granules (Figure 5.b). The granules were round and symmetrical and were consistent throughout all specimens examined. In the second zone, an area of cell death was observed distinct from the area containing the yellow granules (Figure 5.c). And in the third zone, fibroblasts grew to confluence and appeared morphologically normal (Figure 5.d). Fibroblasts exposed to set MTA had normal morphology throughout the culture well (Figure 5.e).

**Effect of root-end filling materials on macrophages:**

**CELL PROFILERATION**

Table 2 shows the total cell numbers after 3-day incubation with pellets of root-end filling materials. In the fresh material experiments, the total cell number in the control group is significantly greater (p < 0.01) than that in the fresh root-end filling material groups. The total cell number cultured with fresh IRM was significantly less (p < 0.01) than that cultured with other fresh materials. There was no statistically cell number difference between the fresh MTA group and the fresh amalgam
group. The total cell number in the fresh Retroplast group was significantly less ($p < 0.01$) than that in the fresh MTA group or in the fresh amalgam group.

In the set material experiments, the total cell number in the control group is significantly greater ($p < 0.01$) than that in the set root-end filling material groups. The total cell number cultured with the set IRM was significantly less ($p < 0.01$) than that cultured with other root-end filling materials. The total cell number in the set Retroplast group was significantly less ($p < 0.01$) than that in the set MTA group or in the set of amalgam group. There were no statistically cell number differences between the set MTA group and the set amalgam group.

CELL MORPHOLOGY

The morphology of the macrophages in the presence of the various materials was similar to that of fibroblasts. Cells cultured without root-end filling material formed a monolayer (Figure 7). With fresh amalgam, cell death was seen directly underneath the material (Figure 8.a). However, cells grew normally beyond this area (Figure 8.b). With set amalgam, we observed a confluent mass of macrophages with
morphology similar to cells in controls (Figure 8.c). IRM demonstrated cell death throughout the culture well in both freshly mixed and set experiments (Figures 9.a and 9.b). Specimens with fresh Retroplast revealed cell death throughout the culture well (Figure 10.a). Set Retroplast, however, resulted in cell death only directly beneath the material (Figure 10.b) and normal macrophage morphology from the area surrounding the material to the edge of the culture well (Figure 10.c). With fresh MTA, the three zones observed with fibroblasts were also present with macrophages (Figure 11.a). We observed an area of altered cell morphology and yellow granules (Figure 11.b) surrounded by an area of cell death (Figure 11.c) encompassed by cells of normal morphology (Figure 11.d). Macrophages with set MTA grew to confluence and had normal morphology (Figure 11.e), similar to controls.

**Cytokine production:**

There was no detected IL-1β or IL-6 production in any of the root-end filling material groups or in the negative control group. In the positive control group, LPS stimulated IL-1β and IL-6 production at 68.5 pg/ml and 230.8 pg/ml separately.
DISCUSSION

The biocompatibility of root-end filling materials to the cells involved in wound healing is an important aspect of post-surgical healing. However, the removal of bacteria from the periapical tissues and confinement of bacteria remaining in the root canal system remains the most important factor in surgical prognosis. Improved techniques for eliminating bacteria from the apical segment of teeth with failing root canals as well as the development of superior root-end filling materials is necessary for increasing the number of successful outcomes. Researchers search for a root-end filling to adequately seal the apical portion of the root canal without compromising the host’s ability to repair and regenerate periapical tissues. We found that freshly mixed and set IRM had a cytotoxic effect against fibroblasts and macrophages, whereas amalgam seemed more biocompatible. MTA appeared to be biocompatible when exposed to both fibroblasts and macrophages, but affected the cells in a rather unique manner. This finding would not have been observed if conventional techniques for evaluating biocompatibility, such as agar overlay or radiolabelled cells, had been implemented. The examination of the morphology of the cells following incubation with
MTA was vital for this observation. Freshly mixed Retroplast showed marked cytotoxicity when reacted with both fibroblasts and macrophages. However, Retroplast that had been set for 2-weeks did not differ from controls. Although these findings were consistent in two cell types, conclusions regarding which root-end filling should be used is still very debatable.

Harrison and Jurosky (1991) found fibroblasts to be the most numerous cells type in the healing periapical tissues following endodontic surgery. In the same study, they identified macrophages to be the most numerous immunologic cell involved in periapical wound healing. We chose to use L929 mouse fibroblasts and RAW 264.7 mouse macrophages based on this study. Many studies have used these cell lines when evaluating the biocompatibility of dental and non-dental materials. Kawahara et al. (1960) used fibroblasts from heart muscle of chicken embryos to analyze the cytotoxicity of methylmethacrylate. He used fibroblasts cell death as a marker of biocompatibility. Guess and Autian (1964) used mouse L929 fibroblasts to evaluate the biocompatibility of plastics in the United States. Guess et al. (1965) developed the agar diffusion test using the indirect effect of experimental
materials upon a cultured monolayer of cells, usually L929 mouse fibroblasts. This has become a standard test of biocompatibility. Engstrom and Spangberg (1969) used cell death as a marker of cytotoxicity when evaluating dental irrigants and medicaments. Quantification of total cell number will approximate the effect of materials upon test cells allowing for some conclusion regarding cytotoxicity. Wennberg et al. (1979) developed the Millipore filter method to evaluate the soluble factors present in materials and their effect on cells. The cells involved in periapical wound healing do not contact root-end filling materials immediately following surgery, but would be affected by chemicals that are eluted from biomaterials. Thus, indirect analysis of these materials is an important aspect of biocompatibility in endodontic surgery. In this study, root-end filling materials were separated from the test cells with a filter similar to that described by Wennberg et al. The presence of the filtered inserts between the root-end filling material and the experimental cells eliminated material particles from interfering with accurate electronic cell counting.
Both quantitative and qualitative analysis of the effect of root-end filling materials upon cells provided information regarding their cytotoxicity. Total cell number of fibroblasts and macrophages allowed for direct comparison of materials to each other. Although no material had the same cell number as control specimens, some materials proved to be better than others in this regard. Amalgam and MTA in both freshly mixed and set experiments showed cell numbers that were not statistically different from controls. However, all materials showed some level of cytotoxicity. It has been shown in previous publications that a root-end filling is necessary for healing following periapical surgery (Rud et al., 1972; Hirsch et al., 1979). Relative cytotoxicity is one factor that should be considered when choosing a root-end filling material. Freshly mixed IRM and fresh Retroplast showed significantly lower total cell number when compared to amalgam and MTA with both fibroblasts and macrophages. However, other factors are also involved when choosing a root-end filling material. Pitt Ford (1998) defined the ideal properties of a root-end filling to be not only biocompatible, but also easy to use, radiopaque, adhesive, antibacterial, insoluble, and unaffected by
moisture. According to this criteria, the ideal root-end filling has yet to be discovered.

Analysis of the morphology of cells following exposure to root-end filling materials has not been previously published. This aspect of biocompatibility, although subjective, can provide important information about the physical response of cells when exposed to materials. In general, freshly mixed materials showed greater cytotoxicity to both cell types than materials that had been set for two weeks. Cytotoxicity was defined by the appearance of cells under phase-contrast microscope at approximately 200 times magnification. Non-vital cells were round in shape and appeared to have lost all internal cell components. They did not grow to confluence and were very distinct from one another spatially. This finding was compared to controls which exhibited cells that grew to confluence, showing very little space between adjacent cells, and appeared to have intact cell membranes and cell components. In many cases, cells directly beneath the material reacted differently than cells on the periphery of the culture well. This can be attributed to the solubility of cytotoxic substances leaching from the experimental materials through the culture medium. The exposure to harmful substances seemed to
effect cells in the periphery of the well much less than cells in close proximity to the materials. This gradient observed may be present in vivo as well.

Dental amalgam is an alloy that results when mercury is combined with silver, tin, copper, and sometimes zinc. Mercury is a dense liquid metal that is highly toxic. Amalgam releases free mercury during setting (Kawahara et al., 1962). In this study, freshly mixed amalgam showed a region of cell death directly below the material when examined microscopically. We hypothesize that free mercury released during the setting of the amalgam was the etiology for this finding. Both L929 fibroblasts and RAW 264.7 macrophages responded similarly when exposed to fresh amalgam. However, we observed cells of normal morphology, grown to confluence in the periphery of the culture well. These observations concur with the findings of Kawahara et al. (1962). They exposed L929 fibroblasts to amalgam and observed a “reaction ring” of cell death around the material. This zone of cytotoxicity was attributed to the release of mercury from the setting amalgam. In this study, the free mercury release was probably not soluble through the culture medium. It is likely that the concentration of mercury was not
high enough to cause cell death in the outer region of the culture well. This finding would not have been available with cell counting alone. Total cell number with freshly mixed amalgam was not statistically different from control specimens. This finding conflicts with the results found by Osorio et al. (1998) evaluating the indirect effect of amalgam on fibroblasts in vitro. Our findings regarding the indirect effect of amalgam differ from those found with direct exposure of cells to amalgam (Zhu et al., 1999; Keiser et al., 2000). A significant decrease in total cell number was observed in previous studies with amalgam upon test cells in vitro. Amalgam set for two weeks showed excellent morphology and cell confluence. Total cell number of set amalgam was not significantly different from freshly mixed amalgam for both fibroblasts and macrophages. The difference was seen only with microscopic examination.

Amalgam has been used for root-end filling for almost a century, and many studies report post-operative healing with amalgam placed apically. Adequate biocompatibility of amalgam has been shown in vivo (Marcotte et al., 1975; Cunningham, 1975). However, other studies report that the prognosis of amalgam is significantly worse than other
root-end filling materials that are available today and should no longer be used by clinicians (Dorn and Gartner, 1990; Frank et al., 1992). Some researchers state that if amalgam were introduced as a new root-end filling material today, questions regarding its toxicity, delayed expansion and corrosion, potential for tissue staining, and marginal leakage would probably argue against its clinical testing and use (Johnson B, 1999). Our findings indicate that amalgam is relatively biocompatible when exposed to mouse fibroblasts and macrophages in vitro.

It is well documented that IRM releases eugenol initially during setting and continues its release for a considerable time thereafter (Markowitz et al., 1992). Eugenol release, although cytotoxic, is useful in certain clinical scenarios. Practitioners have used this side effect as an obtundant to treat potentially symptomatic teeth in deep caries lesions and pulpal exposures. Eugenol released from IRM is also antibacterial, which may be an indication for its use in certain situations. Using the methods present in this study, IRM exhibited marked cytotoxicity towards fibroblasts and macrophages. This concurs with the findings of Chong et al. (1994) whom performed a similar experiment with indirect analysis of materials upon mouse 3T3 fibroblasts. The morphology
observed in culture wells following exposure to freshly mixed as well as
set IRM were consistently poor. Cells from both cell lines exhibited
round morphology of the few remaining cells and extensive cells death
from beneath the material to the outer edge of the culture well. This
finding confirms that eugenol is relatively soluble through the culture
medium and was able to effect cells at a distance from the material. The
qualitative findings with IRM were reflected in the quantitative analysis.
The total cell number found with IRM in both fibroblasts and
macrophages were significantly lower than controls. The total cell
number in both cell lines was also significantly lower than any other
experimental root-end filling material except freshly mixed Retroplast.
The mechanism of cytotoxicity involving eugenol involves the
coagulation of cell proteins ultimately leading to cell death.

Rud et al. (1991) introduced Retroplast composite resin for use in
root-end filling, suggesting that the seal with dentin bonding would result
in improved surgical prognosis. Studies evaluating the effect of
composite resin upon experimental cells have been reported, but none
specifically on Retroplast. Although Retroplast is not yet available for
use in the United States, its promising results reported in prognosis
studies performed in Europe warranted its evaluation. The findings observed with freshly mixed Retroplast were consistent when examined microscopically. Cells appeared rounded and were scattered throughout the culture well. Neither fibroblasts nor macrophages grew to confluence. This cell death observed with freshly mixed Retroplast may be attributed to the release of free monomer during setting. It has been reported that composite resins leach substances or a combination of substances into the surrounding medium that can cause a decrease in cell attachment, growth, and proliferation (Huang et al., 2002). The setting reaction of composite resins has been investigated (Ferracane and Condon, 1990). Of concern was the effect that the unpolymerized material might have on the biocompatibility of the composite with oral tissues. Composite has been shown to exert significant cytotoxicity in cell culture (Rathburn et al., 1987; Anderson et al., 1988). The cytotoxicity has been attributed to the release of uncured monomer and oligomer. Ferracane and Condon (1990) found that 50% of the leachable species were eluted from the composite within three hours of soaking in water. Elution of nearly all of the leachable components was complete within 24-hour period. In this study, when Retroplast had been allowed to set for two weeks prior to
incubation with experimental cells, cells death occurred only directly beneath the material. The peripheral fibroblasts and macrophages were normal morphologically and grew to confluence. From this finding we conclude that the level of monomer released from Retroplast after two weeks does not reach the concentration required to affect cells distant from the material. The quantitative results seemed to reflect our microscopic findings. Total cell number of fibroblasts showed a statistically significant increase when exposed to set Retroplast compared with freshly mixed specimens. Similar findings were observed with macrophages. This was the only root-end filling material in this study for which a significant difference was found in total cell number between fresh and set experiments. According to Harrison and Jurosky (1991), connective tissue cells are not found in the healing periapical tissues until 48-hours post-operatively. The amount of monomer released from Retroplast at 48-hours and its effect upon the cells involved in wound healing was not evaluated in this study, but may provide further information about its biocompatibility. An in vivo investigation by Rud et al. (1989) using composite resin root-end fillings in monkeys showed excellent periapical healing. Retrospective studies of clinical cases
performed with Retroplast composite resin have also shown excellent post-operative healing (Andreasen et al., 1993; Rud et al., 1996a). Our findings with Retroplast revealed an initial cytotoxicity with freshly mixed material that resulted in altered cell morphology and decreased cell numbers relative to controls and other experimental materials (MTA and amalgam). Retroplast that had been set for two weeks showed an area of dead cells beneath the material and normal cell morphology in the periphery. Total cell number with set Retroplast was not significantly different from MTA, amalgam, or controls with both fibroblasts and macrophages. If these results can be extrapolated to a clinical setting, the rapid leaching of cytotoxic monomer from Retroplast would negate the possibility of the composite’s unbound components providing a chronic source of irritation to the periapical tissues.

The findings with MTA were unique relative to the other materials. Since its introduction by Torabinejad et al. (1993), MTA has been used for root-end filling, perforation repair, pulp capping, apexification, and root canal obturation. Numerous studies have been done evaluating the physical and chemical characteristics of MTA. MTA has been found to be equal or superior to other root-end filling materials in studies...
evaluating bacterial leakage (Adamo et al., 1999), cytotoxicity (Keiser et al., 2000), fluid-filtration microleakage (Fogel and Peikoff, 2001), dye leakage (Higa et al., 1994), marginal adaptation (Torabinejad et al., 1994b; 1995d), antibacterial efficacy (Torabinejad et al., 1995), and in vivo studies (Torabinejad et al., 1995b; Torabinejad et al., 1997). In this study, MTA affected cells differently than any other material evaluated. Three distinct zones of cell reaction were found when exposed to freshly mixed MTA. Both fibroblasts and macrophages directly beneath freshly mixed material exhibited flattened, irregular cell morphology consistent with the loss of cell vitality. The yellow granules found throughout this zone of cell reaction were present with both cell types. The second zone adjacent to the MTA involved a layer of dead cells. In third zone of cell reaction, further away from the material, we observed normal, healthy cells grown to confluence. Furthermore, a pilot study done in conjunction with the current project analyzed the reaction of cells to root-end filling materials following direct contact of materials on cells. With freshly mixed MTA, we again observed the three zones of cell reaction found with indirect examination. We hypothesize that the yellow granules represent the dissolution of cell component and the coagulation of serum
proteins in the culture medium. This reaction was likely the result of an increase on pH cause by the setting reaction of MTA. The pH of MTA has been reported to be approximately 12.6 (Torabinejad et al., 1995c). Calcium hydroxide has a pH that has been reported to be approximately 12 and has been shown to effect cells similarly (Holland et al., 1999). MTA has the same effect as calcium hydroxide on pulp capping and apexification (Torabinejad and Chivian, 1999). MTA elicits calcium hydroxide upon setting and may function similarly in vivo (Holland et al., 1999). The yellow granules were observed only beneath the MTA, the area likely to have the highest pH. The area of cell death surrounding the MTA, but with the absence of granules, may have had a pH high enough to be cytotoxic to cells without causing the coagulation of cell components and serum proteins. Although the morphologic evaluation suggested areas of cells death with freshly mixed MTA, total cell counts were not significantly different from controls. The majority of the culture well contained healthy fibroblasts and macrophages following the incubation period. Only cells in close proximity to the material were affected. Cells exposed to MTA that had been set for two weeks appeared normal and grew to confluence throughout the culture well. We
conclude that MTA is relatively biocompatible compared with other root-end filling materials. Our results regarding the biocompatibility of MTA agree with those found by Osorio *et al.*, 1998). Furthermore, it is possible that the increase in pH that caused the coagulation of serum proteins *in vitro* may have a positive effect of the healing of periapical tissues *in vivo*. Further studies are needed for more definitive conclusions regarding the unique reaction MTA to healing periapical cells and tissues.

Some shortcomings of this study involve the interaction of the material with the experimental cells. The microscopic observations with the various materials reveals differences in cells in close proximity or distant to the material. The presence of dead cells was not always reflected in the quantitative analysis. In contrast, cells that grew to confluence in the periphery of the culture well could increase the value during cell counting independent of the cytotoxicity observed beneath the material.

The size of root-end filling material pellets was chosen based on the speculated clinical amount. In this experiment, we try to determine the effect of the whole material on cell growth. Clinically, the material
would be placed into the prepared apical root canal segment resulting in the exposure of less material to the cells involved in periapical healing. Thus, the effect of the material on the surrounding tissue may be less compared with the effect observed in this study.

Cytokines elicited from cells following exposure to root-end filling materials was not produced at a detectable level. Stashenko et al. (1987) were able to measure the level of cytokine elicited from test cells in response to root-end filling materials. Other studies have been able to quantify cytokine production from test cells in vitro (Koh et al., 1998; Stashenko et al., 1998). However, this was unable to be accomplished in our study. One possible reason for the lack of cytokine quantification is the time between the incubation of cells and measurement of cytokines. Three days following incubation, the vital cells remaining may not have been able to elicit a detectable amount of cytokine. For accurate cytokine analysis, future studies should consider an earlier time-point for cytokine measurement following incubation. Another possible reason concerns the sensitivity of this test. The lack of production of cytokines may be because the small number of cells had an inhibitory feedback on the production of cytokines. There are many possible interactions between
the material, the medium, and the cytokines that could result in a level not detectable by our methods. Cytokines released by test cells in response to the materials may have been broken down by enzymes present in the culture medium prior to being quantified.

Biocompatibility is one characteristic of an ideal root-end filling. However, decision making can rely on many factors, most notably, the clinical scenario of a specific endodontic surgery. Biocompatibility involves the interaction of a biomaterial, the host, and the expected function of the material. In this in vitro study, some materials exhibited better biocompatibility than others. However, it has been shown that all materials tested are able to provide an environment conducive to healing following periapical surgery. This study concurs with previous publications documenting the favorable outcome between MTA and test cells in vitro. Further research is needed regarding the effect of root-end filling materials upon the cells involved in periapical healing, the importance of biocompatibility, and the influence of root-end filling materials on the long-term prognosis of periapical surgery.
CONCLUSIONS

The indirect analysis of the biocompatibility of root-end filling materials revealed the following conclusions:

A. There was no statistically significant difference between the effect of amalgam and MTA on cell growth.

B. Cell growth was greatly inhibited in the presence of IRM and freshly mixed Retroplast.

C. Cells exhibited altered morphology when examined microscopically following incubation with IRM and freshly mixed Retroplast. Normal morphology was observed following incubation with amalgam, MTA, and set Retroplast.

D. Morphologic analysis of cells exposed to freshly mixed MTA revealed a unique dissolution of cell components and denaturation of serum proteins that resulted in three distinct zones of cellular response.
E. Few differences between fibroblasts and macrophages were found in cell growth and cell morphology following exposure to various root-end filling materials.
Table 1. Total cell number per well of mouse fibroblasts after 3-day incubation with pellets of root-end filling materials

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MTA</th>
<th>Amalgam</th>
<th>IRM</th>
<th>Retroplast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>1195.74</td>
<td>965.01</td>
<td>972.87</td>
<td>95.96</td>
<td>229.38</td>
</tr>
<tr>
<td></td>
<td>(144.44)</td>
<td>(25.28)</td>
<td>(23.79)</td>
<td>(8.37)</td>
<td>(62.12)</td>
</tr>
<tr>
<td>Set</td>
<td>1195.74</td>
<td>1071.31</td>
<td>1042.63</td>
<td>105.53</td>
<td>1004.86</td>
</tr>
<tr>
<td></td>
<td>(144.44)</td>
<td>(53.25)</td>
<td>(50.46)</td>
<td>(26.56)</td>
<td>(27.93)</td>
</tr>
</tbody>
</table>

Values are means, with SD in parentheses.

Table 2. Total cell number per well of mouse macrophages after 3-day incubation with pellets of root-end filling materials

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MTA</th>
<th>Amalgam</th>
<th>IRM</th>
<th>Retroplast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>1395.32</td>
<td>921.48</td>
<td>836.53</td>
<td>139.63</td>
<td>442.70</td>
</tr>
<tr>
<td></td>
<td>(214.55)</td>
<td>(92.14)</td>
<td>(44.96)</td>
<td>(17.61)</td>
<td>(115.63)</td>
</tr>
<tr>
<td>Set</td>
<td>1395.32</td>
<td>1136.67</td>
<td>1136.97</td>
<td>299.43</td>
<td>667.07</td>
</tr>
<tr>
<td></td>
<td>(214.55)</td>
<td>(129.53)</td>
<td>(19.57)</td>
<td>(74.74)</td>
<td>(153.65)</td>
</tr>
</tbody>
</table>

Values are means, with SD in parentheses.
Fig. 1.  Cell morphology of normal mouse L929 fibroblasts. (200X)
Fig. 2. Cell morphology of mouse fibroblasts cultured with fresh amalgam directly beneath the material (a) and from the periphery of the culture well (b). Cell morphology of mouse fibroblasts with set amalgam (c). (200X)
Fig. 3. Cell morphology of mouse fibroblasts cultured with fresh (a) and set (b) IRM. (200X)
Fig. 4. Cell morphology of mouse fibroblasts cultured with fresh Retroplast (a). Cell morphology with set Retroplast from the area directly beneath the material (b) and from the periphery of the culture well (c). (200X)
**Fig. 5.** Cell morphology of mouse fibroblasts cultured with fresh (a, b, c, d) and set MTA (e). Direct observation of fibroblasts without magnification following incubation with fresh MTA (a). Three zones of cell reaction observed surrounding fresh MTA: from directly beneath MTA (b), from the area surrounding MTA (c), and from the periphery (d). (200X)
Fig. 6. Cell morphology of mouse fibroblasts cultured with fresh MTA without cell culture insert. Direct contact of fresh MTA also results in three zones of cell reaction viewed without magnification (a) and microscopically (b). (200X)
**Fig. 7.** Cell morphology of normal mouse RAW 264.7 macrophages. (200X)
Fig. 8. Cell morphology of mouse macrophages cultured with fresh (a, b) and set (c) amalgam. Macrophages observed from directly beneath fresh amalgam (a) and from the periphery of the culture well (b). (200X)
Fig. 9. Cell morphology of mouse macrophages cultured with fresh (a) and set (b) IRM. (200X)
Fig. 10. Cell morphology of mouse macrophages cultured with fresh (a) and set (b, c) Retroplast. Cell reaction from directly beneath set Retroplast (b) and from the periphery of the culture well (c). (200X)
Fig. 11. Cell morphology of mouse macrophages cultured with fresh (a,b,c,d) and set (e) MTA. Low magnification view of three zones of cell reaction (a). High magnification view of area directly beneath the MTA (b), from the zone surrounding the MTA (c), and from the periphery (d). (200X)
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