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Effect of Selected Accelerants on pH, Setting Time and Biocompatibility of MTA

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Effect of selected accelerants on pH, setting time and biocompatibility of MTA

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Effect of selected accelerants on pH, setting time and biocompatibility of MTA

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To my father, my mother, my brother Hamdan, and all my family. I hope I make you proud.

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Introduction:

The primary goal of root canal treatment is the prevention and treatment of apical periodontitis. There is overwhelming evidence for the essential role of microbial agents in dental pulp infection and the pathogenesis of apical periodontitis. The presence of bacteria in the necrotic dental pulp of teeth with associated pathologic conditions was reported more than a century ago when Miller hypothesized in 1894 that microorganisms are the causative agents of endodontic disease (Miller 1894). However, it was not until more than a half century later, when the causal relationship between periapical inflammation and bacterial infection was convincingly demonstrated. In the classic study by Kakehashi and coworkers in 1965, it was shown that pulpal necrosis and periapical inflammation developed in rats subjected to mechanical pulp exposure and kept exposed to the conventional microbial environment of the oral cavity. Whereas germ-free animals demonstrated minimal pulpal inflammation and no periapical destruction (Kakehashi et al 1965)

In the US, it is estimated that more than 24 million endodontic procedures are performed annually, and up to 5.5% of these procedures are apical surgery, perforation repair, and apexification procedures (Roberts et al 2008, Nash et al 2002). Recent meta-analysis reports estimated endodontic treatment success rate to be between 68% and 85% (Iqbal et al 2008, Ng et al 2007). Surgical placement of a root end filling has been increasingly used in the management of endodontic failure. Mineral trioxide aggregate (MTA) is the most widely used root end filling material in surgical endodontics. MTA is shown to be biocompatible. The following literature review aims to discuss apical

periodontitis and its causative factors. Surgical endodontics and wound healing, as well as bone cells and regulation of bone metabolism. MTA composition and biocompatibility with periapical tissue will be reviewed.

Apical periodontitis:

Apical periodontitis is an inflammatory disease of periradicular tissues caused by persistent microbial infection within the root canal system of the affected tooth (Kakehashi et al 1965, Sundqvist 1976, Nair 2006). The infected and necrotic pulp offers a selective habitat for the organisms (Fabricius et al 1982, Nair 2006).The microbes grow in sessile biofilms, aggregates, coaggregates, and as planktonic cells suspended in fluid phase (Nair 2006). Planktonic bacteria have easy access to nutrients, multiply rapidly and often are highly motile (Olson et al 2002). They are more susceptible to antibiotics, environmental and host factors. Sessile bacteria grow in colonies encased by an extracellular matrix of carbohydrates or exopolysaccharides (Olson et al 2002). These colonies often are called a bacterial biofilm. Sessile bacteria have limited nutrient, grow more slowly and have restricted mobility (Olson et al 2002).

A biofilm (Costerton et al 2003) is a community of microorganisms embedded in an exopolysaccharide matrix that adheres onto a moist surface (Nair 2006). Apical periodontitis persisting after root canal treatment is believed to be harder to eradicate compared to apical periodontitis affecting teeth without previous endodontic treatment. Endodontically treated teeth with apical periodontitis present the clinician with a resistant microbiological etiology, and limited treatment options compared to apical periodontitis in non-root canal treated teeth (Nair 2006).

A clear understanding of the microbiology and pathology the two types of apical periodontitis is essential to deliver optimum treatment. Intra-radicular microorganisms are the main etiological agents of apical periodontitis (Kakehashi et al 1965, Sundqvist 1976), hence the treatment consists of eradicating or reducing the microbial population and preventing re-infection by root canal filling (Nair et al 2005).

Once this is achieved, hard tissue healing and reduction of periapical radiolucency is expected (Strindberg 1956, Grahnen & Hansson 1961, Seltzer & Bender 1963, Storms 1969, Molven 1976, Kerekes & Tronstad 1979, Molven & Halse 1988, Sjogren et al 1990 & 1997, Sundqvist et al 1998). However, some periapical tissues will not heal. Inadequate aseptic control, poor access cavity design, untreated root canals, insufficient instrumentation, and leaking temporary or permanent restorations are common problems that may lead to persistent apical periodontitis (Sundqvist & Figdor 1998, Nair 2006). Complex apical third root canal anatomy and factors in periapical tissue that interfere with post-treatment healing are the main reasons for persistent apical lesions despite the clinician's careful instrumentation and meticulous microbiological control (Nair 2006).

Microbial Factors:

Intra-radicular infection

Several histological studies have shown bacteria present in the root canal system of teeth with asymptomatic periapical periodontitis. Bacteria were mostly located in the uninstrumented recesses of the main canals, isthmuses, and accessory canals (Nair 1990, Nair 2006). The bacteria existed as biofilms and were not removed by instrumentation and irrigation with NaOCl. Considering the complex root canal anatomy and difficulty to

eradicate bacterial biofilm, it is unlikely that clinicians will be able to get a bacteria free root canal system.

The endodontic microbiology of treated teeth is less understood than that of untreated infected necrotic dental pulps (Nair 2006). *Enterococcus faecalis* is the most recovered organism from the canals of failed root filled teeth, with a prevalence ranging from 22% to 77% of cases analyzed (Moller 1966, Molander et al 1998, Sundqvist et al 1998, Peciuliene et al 2000, Hancock et al 2001, Pinheiro et al 2003, Siqueira & Rocas 2004, Fouad et al 2005, Nair 2006). The organism is resistant to most of the intra-canal medicaments (Bystrom et al 1985), and can tolerate a pH up to 11.5, which may be one reason why this organism sometimes survives antimicrobial treatment with calcium hydroxide dressings. This resistance occurs because of its ability to regulate internal pH with an efficient proton pump (Evans et al 2002). *Enterococcus faecalis* can survive prolonged starvation (Figdor et al 2003). It can grow as a mono-infection in treated canals in the absence of synergistic support from other bacteria (Fabricius et al 1982). Therefore, *E. faecalis* is regarded as being a very recalcitrant microbe among the potential etiological agents of persistent apical periodontitis (Nair 2006).

Chen et al (2009) reported that Herpes-viruses are present but not required for the development of acute apical abscesses and cellulitis of endodontic origin. Li et al (2009) reported that Epstien-Barr virus may be associated with irreversible pulpitis and apical periodontitis. Although not reported in teeth with previous root canal treatment, further research is required to exclude viruses as a potential factor in persistent apical periodontitis.

Extra-radicular infection

Actinomycosis is a chronic, granulomatous, infectious disease in humans and animals caused by the genera *Actinomyces* and *Propionibacterium* (McGhee et al 1982, Nair 2006). Actinomycotic organisms are able to establish extraradicular infections and can perpetuate the inflammation at the periapex even after proper root canal treatment (Nair 2006). Therefore, periapical actinomycosis is important in endodontics (Sundqvist & Reuterving 1980, Nair & Schroeder 1984, Happonen et al. 1985, Happonen 1986, Sjogren et al 1988, Nair et al 1999, Nair 2006). *Actinomyces israelii* and *P. proprionicum* are consistently isolated and characterized from the periapical tissue of teeth which did not respond to proper non-surgical endodontic treatment (Happonen 1986, Sjogren et al 1988, Ricucci & Siqueira 2008).

In his review (Nair 2006) summarized all the possible causative agents of persistent apical periodontitis. They are: (i) intraradicular infection persisting in the complex apical root canal system,(ii) extraradicular infection, generally in the form of periapical actinomycosis, (iii) extruded root canal filling or other exogenous materials that cause a foreign body reaction,(iv) accumulation of endogenous cholesterol crystals that irritate periapical tissues, (v) true cystic lesions, and (vi) scar tissue healing of the periapex. In both poorly and properly treated teeth, residual microbes in the apical portion of the root canal system are the most important etiological factor, while the other remaining factors occur rarely. Treatment options for such cases are retreatment, surgery or extraction.

Surgical Endodontic Treatment:

In the US, it is estimated that more than 24 million endodontic procedures are performed annually, and up to 5.5% of these procedures are apical surgery, perforation repair, and apexification procedures (Roberts et al 2008, Nash et al 2002). Recent reviews estimated the cumulative endodontic success rates, with and without apical periodontitis, to be between 68% and 85% (Iqbal et al 2008, Ng et al 2007).Surgery is performed on failed root canals due to complex root canal anatomy, perforations, instrument fractures, ledges, and restorative reasons where crowns and posts cannot be removed. Apical surgery involves accessing the root apical third, removal of all granulation tissue, root end resection, root end cavity preparation and placement of root end filling material. Following the surgical procedure the tissue undergoes changes to restore its structure and function. The ultimate goal of every surgical procedure is regeneration of all lost tissues. The next section will discuss healing after surgical procedures in soft tissue and bone.

Healing:

A wound is defined as any injury that causes disruption of the anatomical continuity and/or function of living tissues and results in cellular injury and death (Krawczyk 1978). All surgical procedures involve intentional wounding of specific tissues and are dependent upon the wound healing responses of those tissues for success (Harrison 1991). Wound healing is, in turn, dependent upon the type of tissues wounded and the type of wound inflicted (Harrison 1991).

Wound healing is dependent upon: (a) the type of tissue wounded and (b) the type of wound the tissue receives (Krawczyk 1978, Harrison 1991). It involves a complex

series of biological events. It can be divided into; (a) clotting and inflammation, (b) epithelial healing, (c) connective tissue healing, and (d) maturation and remodeling.

Clotting and Inflammation:

Surgical wounding causes disruption of the microvasculature supplying the mucoperiosteal tissues. The first step of healing is to achieve hemostasis. Vascular injury releases plasma fractions (eg. albumin, fibrinogen, globulins, fibronectin, and plasminogin) and formed elements (erythrocytes, platelets, and leukocytes) into the surrounding tissues (Harrison 1991). A plethora of humoral and cellular biochemical mediators are activated, causing vasodilatation and increased permeability in intact microvessels, as well as intravascular platelet aggregation (platelet plugs) in severed vessels. The activated biochemical mediators influence both the clotting and inflammatory mechanisms (Harrison 1991).

Clotting:

Extrinsic and intrinsic clotting mechanisms are triggered, each giving rise to a cascading sequence of events leading to clot formation (Boucek 1984, Harrison 1991). The crucial step in both clotting mechanisms is the conversion of fibrinogen to fibrin and formation of fibrin clot.

The fibrin clot form strengthens the initial platelet plug (Harrison 1991). A few hours after clot formation, the fibrin strands undergo contraction this provides a tenuous attachment between opposing wound edges and forms pathways of migration, first for inflammatory cells and, subsequently, for cells which will affect repair or regeneration (Ruben et al 1960, Harrison 1991).

Inflammation:

The response of all living tissues to all forms of injury is inflammation, which involves vascular, humoral, and cellular reactions at the injury site and prepares the site for healing (Boucek 1984, Harrison 1991). Healing is dependent upon the inflammatory process to create a favorable environment for cellular metabolism by removing microorganisms, necrotic or damaged tissue, and particulate matter (Harrison 1991). Inflammation dominates the early stages of wound healing. The more rapidly the inflammatory process can create an environment conducive to rapid wound healing (Harrison 1991).

The events of the inflammatory response are mediated by the production, activation, and release of inflammatory mediators. The interactions between inflammatory mediators are extremely complex (Harrison 1991). Histamine and Serotonin cause hemodynamic changes in the microvasculature, resulting in vasodilation, an increased blood volume, and a decreased rate of flow (Harrison 1991). Leukocytes become displaced to the periphery of the blood stream and actively pass through the vessel wall and proceed to the wound site (Harrison 1991).

The first wave of leukocytes is largely comprised of polymorphonuclear neutrophils (PMN's) which are the most active cells in the early inflammatory response, phagocytizing microorganisms, particulate matter, and cellular debris (Boucek 1984, Harrison 1991). Rapid destruction of microorganisms quickly reduces the need for continued migration of these cells and the chemotactic mechanisms responsible for their early migration are soon deactivated (Boucek 1984, Harrison 1991). Under ideal wound-healing conditions, PMN activity subsides and their numbers decrease rapidly between 24 and 48 h after wounding (Boucek 1984, Harrison 1991).

After the initial influx of PMN's, plasma-derived monocytes appear in increasing numbers, undergoing rapid morphological and functional changes under the influence of inflammatory mediators to become activated macrophages. Macrophages become the predominant cell in the wound site (Krawczyk 1978, Trowbridge 1983, Harrison 1991). Macrophages release biochemical mediators which stimulate fibroblast and migration, collagen and ground substance synthesis, and angiogenesis (Harrison 1991). The macrophages are control the extent and degree of the immediate inflammatory response, creating an environment in which connective tissue healing can occur, and directing the ingress of cells (fibroblasts, undifferentiated ectomesenchymal cells, endothelial and smooth muscle cells) that will affect repair or regeneration of the wound site (Krawczyk 1978, Hunt et al 1984, Harrison 1991).

Epithelial Healing:

Epithelial cells in the wound edges undergo specific changes within hours after surgical wounding. The basal and suprabasal prickle cells undergo dedifferentiation, acquire the potential for amoeboid movement, and develop phagocytic capabilities (Krawczyk 1978, Melcher 1969, Rubben 1980, Harrison 1991). Spaces appear between these cells as desmosomal attachments are loosened. The cells become elongated and begin to migrate across the fibrin scaffolding of the clot (Harrison 1991). The basal and suprabasal ceils adjacent to these migrating cells of the wound edge subsequently begin to undergo mitosis at a high rate. The migrating cells move as a monolayer or sheet of cells, which may be several cells in thickness, toward the center of the surface of the wound until cellular contact is made with epithelial cells migrating from the opposing wound edge (Melcher 1969, Harrison 1991).

Connective Tissue Healing:

The connective tissue healing phase is the most complex of the wound healing phases. It begins with the formation of the epithelial seal and progresses rapidly after epithelial barrier formation (Harrison 1991). The primary connective tissue cell involved in this phase is the fibroblast which synthesizes ground substance and collagen, the essential elements of regeneration or repair (Rubben 1980, Harrison 1991). Fibroblasts are mobilized primarily from undifferentiated ectomesenchymal cells in the perivascular tissues surrounding the wound site (Boucek 1984, Harrison 1991). Fibroblasts begin the synthesis of collagen, glycosaminoglycans, glycoproteins, and fibrous proteins (Harrison 1991). The first-formed collagen appears as a fine, branching network of delicate type III (reticulin) fibers formed by young fibroblasts. Mature fibroblasts soon initiate synthesis of type I collagen. As collagen molecules enter the extracellular environment, angiogenesis factors are released from macrophages and other sources, stimulating the migration of endothelial and smooth muscle cell chords from the microvasculature of the wound edges into the wound site (Krawczyk 1978, Trowbridge 1983, Harrison 1991).

Maturation and Remodeling:

After sufficient collagen is produced, the number of fibroblasts in the former wound site decreases and there is a concomitant reduction in the vascular channels. This signals the end of the connective tissue healing phase and the beginning of the maturation and remodeling phase (Harrison 1991). Under ideal healing conditions the later phase may begin as early as a few days after simple incisional wounding in oral mucoperiosteal tissues (Harrison 1991). Collagen fibers undergo pronounced changes in form, bulk, strength, and orientation, during the maturation phase (Harrison 1991). Maturation involves simultaneous collagen deaggregation and reaggregation which results in a

change in the architecture of the fibers to an organized pattern and density similar to those of normal adjacent tissues (Kline&Weiss1966, Harrison 1991). As the fiber pattern and density take on a normal appearance, the fibrohlast population decreases to the same level as adjacent tissues (Harrison 1991). This step in healing process is followed closely by the formation and remodeling of bone. The next section provides a detailed overview of the bone structure and metabolism.

Bone:

Bone is a specialized connective tissue. It is highly mineralized with calcium phosphate in the form of hydroxyapatite ($[Ca_3(PO_4)_2]\ Ca(OH)_2$) (Datta et al 2008). Bone provides the body with rigidity, shape, protection, and support. Bone undergoes continuous remodeling for repair and adaptation to body needs. In childhood bone formation exceeds resorption, while both processes are balanced in adult life. Bone strength is regulated thorough a carefully regulated process involving multiple cells and mediators (Dattaet al 2008).

Bone structure:

There are two types of mature bone: (1) cortical or compact bone which has a dense, ordered structure; (2) cancellous or trabecular bone which is lighter, less compact and has an irregular structure (Datta et al 2008). Cortical or compact bone is found mainly in the shaft of long bones and the surfaces of flat bones. It is composed of bone laid down concentrically around central canals known as Haversian systems. These contain blood vessels, lymphatics, nerves and connective tissue. A concentric layer of rings or lamellae of bone matrix surround each Haversian canal. Within the lamellae are

tiny spaces called lacunae containing osteocytes (Datta et al 2008). Trabecular or cancellous bone forms the ends of long bones and the inner parts of flat bones. It contains interconnecting plates and bars called trabeculae, and have a honeycomb appearance. The trabeculae are aligned along lines of stress; this connectivity adds considerably to its strength.

Trabecular bone is ideally suited to withstanding compressive stress. Another important element in the structure of bone is the collagen network. In adults, collagen fibers adopt a preferential orientation resulting in the formation of lamellar bone. In cortical bone the lamellae are concentrically arranged, and in trabecular bone they are parallel to one another (Datta et al 2008). Woven bone is formed in the growing skeleton and in various pathological conditions in the adult. This type of bone has a random arrangement of collagen fibers, with non-uniformly sized and distributed osteocytes.

In general each bone has an outer layer of cortical bone overlying trabecular bone and the medullary cavity. The cortical bone has an outer membrane called the periosteum. The periosteum has two layers: an outer and an inner layer. The inner layer has osteogenic potential and lays down new bone allowing the bone to enlarge, a process known as periosteal apposition. The inner surface of the cortex has another lining called the endosteum. Bone tends to undergo resorption from the endosteal surface. Both the periosteum and endosteum contain osteoblasts and osteoclasts and their progenitors. Osteoblasts and osteoclasts function in a coordinated manner, to carry out remodeling, growth and repair (Datta et al 2008).

Bone cells:

Osteoclasts and osteoblasts carry out bone metabolism. Osteoclasts function to resorb existing bone and are active early in the bone remodeling cycle. They are derived from fusion of cells of monocyte lineage. Osteoclasts are multinucleated, they have apical and basolateral poles that differ both morphologically and functionally. The osteoclast's apical pole has a ruffled membrane that is oriented toward the bone matrix and is the site for secretion of enzymes and protons that are essential for resoprtion of bone. The osteoclast's basolateral pole faces toward the local environment and it has receptors for hormones and other substances such as, fibroblasts growth factor, transforming growth factor and platelet derived growth factor (Christenson 1997).

The osteoclast functions by attaching the outside edge of its apical membrane to the mineralized matrix on the bone's surface, creating a microenvironment termed a "sealing zone." This zone provides a bone-resorbing compartment where the pH is lowered when protons and potent enzymes such as acid phosphatase, aryl-sulfatases, and metalloproteinases are released. This microenvironment functions to resorb bone and form a depression termed a lacuna (Christenson 1997).

Osteoblasts are responsible for bone formation. After formation of lacunae by the osteoclasts, osteoblasts cells lay down osteoid, a replacement bone matrix. Osteoblasts derive from mesenchymal progenitors that can differentiate into chondrocytes or osteoblasts (Fig 1), adapted from Maes et al (2007). The transcription factors involved in driving the cell to a chondrocyte fate are Sox9, L-Sox5, and Sox6, while osteoblast differentiation requires Runx2 and later Osx and β-catenin. Progressive osteoblast differentiation from committed osteoblast progenitor cells is characterized by changes in

gene expression, providing markers that typify specific stages. Runx2 is the earliest marker of preosteoblasts, followed by Osx. Preosteoblasts further differentiate into osteoblasts that produce large amounts of type I collagen (Collagen 1) and later mineralize the osteoid matrix as mature osteoblasts expressing osteocalcin. Eventually, the cells can become entrapped within the bone matrix as osteocytes, remain quiescently at the surface of the bone as lining cells, or die by apoptosis, Maes et al (2007). Osteoblasts have one nucleus and an extensive network of rough endoplasmic reticulum (ER), which is responsible for synthesis of bone matrix proteins. After osteoblasts have filled the lacunae with osteoid, the process of bone formation is halted (Christenson 1997).

Some of the osteoblasts are entrapped in the bone matrix where they become osteocytes. Despite their relative inactivity compared with osteoblasts, osteocytes play a central role in the determination and maintenance of bone structure (Mikuni-Takagaki1999, Han et al 2004, Holmbeck et al 2005, Kamioka et al 2001, Zhao et al 2002, Datta et al 2008). Osteocytes embedded in bone interact with other osteocytes and bone cells by an elaborate network of dendritic processes, which run inside lacunar cannaliculi. There is emerging evidence that osteocytes function as mechanosensory cells, and dendritic processes carry the signaling molecules responsible for bone resorption process. (Duncan& Turner 1995, Pavalko et al 2004, Tarbell et al 2005, Datta et al 2008)

Bone Metabolism Cycle:

The bone cycle proceeds in one direction only, in a well-coordinated, process controlled by hormones and other factors via mechanisms which are incompletely

understood at present (Christenson 1997). Bone remodeling always begins in the quiescent phase. After activation is initiated, osteoclasts are attracted to the new bone forming unit or bone mineralization unit (BMU). Osteoclasts erode the bone matrix, forming lacunae. This process takes about 10 days and osteoclasts normally resorb bone until the lacunae are approximately 100 μm in diameter and 50 μm deep. Resorption is then halted and osteoblasts are recruited to the BMU site. Osteoblasts begin at the bottom of lacunae and lay down the osteoid matrix, comprised mainly of type 1 collagen. It takes about 80 days to fill the lacunae. The newly formed matrix is then mineralized with hydroxyapatite, giving the BMU tensile strength. The remodeled area then passes into the quiescent phase to complete the bone cycle (Christenson 1997).

Regulation of bone metabolism:

Bone metabolism and regulation is an area under extensive research, however the exact mechanisms are not yet completely understood. Fibroblast growth factors (FGF) increase both osteoblast proliferation and collagen synthesis within bone (Canalis et al 1987). The FGFs precise mechanism of action is unknown (Abraham et al 1986). Insulin-like growth factors (IGF, type I and II) increase the protein content of osteoid by promoting preosteoblastic proliferation and by decreasing collagen degradation as well as increasing protein synthesis (Forlik et al 1988, Canalis et al 1989).

Transforming growth factors (TGF) are important in the maturation process. They stimulate the precursor cells committed to becoming osteoblasts (Centrella et al 1988). TGFs appear to stimulate the synthesis of alkaline phosphatase. TGF is also related to synthesis of type I collagen (Bortell et al 1990). Platelet derived growth factor (PDGF)

found in the bone matrix also stimulates osteoprogenitor cells and protein synthesis (Canalis 1981).

Both osteoblasts and osteoclasts are responsive to various prostaglandins, which may mediate their response to other growth factors. Tissue necrosis factors (TNF) increase collagen synthesis in preosteoblasts, but appear to decrease collagen synthesis in more mature cells (Raisz & Martin 1984). Colony stimulating factors (CSF) are implicated in osteoclast proliferation and may be involved in the signaling mechanism between osteoclasts and osteoblasts (Raisz & Martin 1984, Chyun & Raisz 1984). The effects of parathyroid hormone (PTH) are modulated by IGF-I and CSF; the effects on bone metabolism are dependent on circulating PTH concentrations as well as on the time of exposure to excess PTH concentrations (McCarthy et al 1997, Horowitz et al 1988). Vitamin D is comprised of two substances, calcifediol (25-dihydroxyvitamin D) and calcitriol (1,25trihydroxyvitamin D). PTH is important for conversion of calcifediol to calcitriol (Schmidt-Gayk et al 1997). Calcifediol and calcitriol are involved in maturation of osteoblasts as well as normal growth and inhibition of bone (Canalis 1990, Civitelli et al 1990). Also, decreased concentrations of calcifediol and calcitriol are associated with increased BMU activation.

Estrogen reportedly decreases production of the osteoid matrix, and appears to enhance the formation of trabecular bone (Takano-Yamamoto & Rodan 1990). The decrease in estrogen that occurs with menopause results in a decline in bone mass. Calcitonin is a calcitropic hormone that is an effective inhibitor of bone resorption (Gruber et al 1984, Avioli 1982). Calcitonin inhibits osteoclast activity. Increased concentrations of thyroid hormone, either due to hyperthyroidism or from therapeutic

administration of this hormone, cause increased bone turnover (Canalis 1993, Martin et al 1989).

Bone resorption and formation require interaction between osteoblasts and osteoclasts. This interaction is regulated by hormones cytokines, especially IL-1 alpha and beta. Osteoblasts have receptors for IL-alpha, when activated they will stimulate osteoclasts. Osteoblasts presence is essential for osteoclasts IL-1 mediated bone breakdown. At low levels IL-1 beta stimulate bone formation, while at high concentrations it inhibits it (Canalis 1986, Mitchell et al 1998).

Markers of bone formation:

Alkaline phosphatase (ALP)

The exact function of this enzyme is unknown. It has broad tissue distribution and appears to be involved in the transport of substances from the intracellular compartment across the membrane to extracellular region (Christenson 1997). In bone, ALP may also be involved in the breakdown of pyrophosphate, a potent inhibitor of calcium phosphate deposition at the extracellular level (Risteli & Risteli 1993, Christenson 1997)

Four ALP isoenzymes are commonly present in blood (Datta et al 2008). These isoenzymes are relatively specific for the organs they represent which are; liver, bone, placenta, and intestine. The bone specific ALP isoenzyme (B-ALP) predominates during childhood and adolescence (Christenson 1997).

B-ALP is produced in extremely high amounts during the bone cycle's formation phase and is therefore, an excellent indicator of bone formation activity (Whyte 1983, Vanstraalen et al 1991, Christenson 1997).Evidence for B-ALP involvement in bone formation can be seen in patients with hypophosphatasia, a rare inherited autosomal

recessive disorder characterized by defective mineralization of bone and teeth. The disease is diagnosed by deficient levels of total ALP (Gundberg 2000).

Osteocalcin

Osteocalcin is a vitamin K-dependent protein. It is only found in bone tissue and dentin. Osteocalcin is a peptide of 49 amino acids. It is assumed that much of the newly synthesized protein is incorporated into the bone matrix. Osteoclacin functions in binding calcium (Christenson 1997). The synthesis of osteocalcin in osteoblasts is controlled by calcitriol, 25-dihydroxyvitamin D, which activates its synthesis. Some of the newly synthesized osteocalcin is released into the circulation. The serum level of osteocalcin is a very specific marker for the rate of bone formation when formation and resorption are uncoupled. When resorption and formation are coupled, serum osteocalcin levels reflect bone turnover (Cormier 1995).

Root End Filling Materials:

The purpose of root end filling procedure is to seal the root canal system. The root end filling material must be stable and biocompatible. Root end filling material need to adhere to root structure, maintain hermetic seal, be insoluble in tissue fluids, dimensionally stable, non-resorbable, radiopaque, non-toxic, and be biocompatible with tooth and surrounding structures (Roberts et al 2008). A variety of root end filling materials, GP, amalgam, cavit and zinc oxide eugenol based cement such as super EBA and IRM. A relatively recent root end filling material, Mineral trioxide aggregate (MTA) is now believed to be most biocompatible root end filling material and it is widely used in apical surgery, as well as perforation repair, direct pulp capping and apexification (Roberts et al 2008).

MTA:

MTA is a cement advocated for the dental use in peri-radicular surgeries, perforation repairs and as a pulp capping material. It is commercially available as ProRoot MTA (Tulsa Dental Products,Tulsa, OK, USA). MTA received FDA approval in 1998 (Camilleri et al 2006). MTA is available in the USA in two forms, grey MTA (GMTA) and white MTA (WMTA). In Brazil, MTA Angelus is also available in grey and white forms (Angelus Solucoes Odontologicas, Londrina, Brazil).

MTA consists of 50–75% (wt) of calcium oxide and 15–25% silicon dioxide. When mixed they produce tricalcium silicate, dicalcium silicate, tricalcium aluminate and tetracalcium aluminoferrite (Torabinejad & White 1995, Torabinejad & Hong 1995, Camilleri et al 2006). However, in a recent review by Roberts et al (2008), several studies reported MTA to consist of, dicalcium silicate, tricalcium silicate, tricalcium aluminate, tricalcium aluminoferrite, gypsum and bismuth oxide. MTA also contains trace amounts of silicon oxide (SiO_2), calcium oxide (CaO), magnesium oxide (MgO), potassium sulfate (K_2SO_4), and sodium sulfate (Na_2SO_4).

Compared to Portland cement, MTA has less gypsum than portland cement. Bismuth oxide is added to MTA for radio-opacity. MTA also has a smaller mean particle size, fewer heavy metals and longer setting time, when compared to Portland cement (Roberts et al 2008).

GMTA is a grey powder which is mixed with sterile water in a 3:1 (w/v) ratio according to manufacturer's instruction. Setting time of GMTA is between 3-4 hours (Roberts et al 2008, Dammaschke et al 2005, Torabinejad et al 1995). It sets in a hydration reaction of tricalcium silicate ($3CaO{\cdot}SiO_2$) and dicalcium silicate ($2CaO{\cdot}SiO_2$).

Upon mixing it forms a colloidal gel, which solidifies into a hard structure in the presence of moisture (Roberts et al 2008). In apical surgery applications moisture is provided from surrounding tissues. The pH of setting MTA rises from 10.2 to 12.5 three hours after mixing (Torabinejad & Pitt Ford 1995, Camilleri et al 2006).

In 2002, white MTA (WMTA) was introduced by Dentsply Endodontics, Tulsa, OK, USA. Asgary et al (2005) characterized the differences between GMTA and WMTA using SEM and probe microanalysis. WMTA was found to have 54.9% less $Al_2 O_3$, 56.5% less MgO, and 90.8% less FeO than GMTA. The author concluded that reduced ferrous oxide concentration is most likely the cause for the white color of WMTA.

Song et al (2006) compared the chemical and crystalline composition of Portland cement, ProRoot GMTA, ProRoot WMTA, and gray MTA-Angelus. The author used X-ray diffraction analysis, and energy dispersive x-ray spectrometry of both freshly mixed and set materials. The author found the crystalline structure and chemical composition of GMTA and WMTA were similar except for the presence of iron in GMTA, which was different from findings of Asgary et al (2005). Both were composed mainly of bismuth oxide and calcium silicate oxide. Portland cement was composed mainly of calcium silicate oxide and had no bismuth oxide. Gray MTA-Angelus had a lower content of bismuth oxide than ProRoot MTA. The author found no noticeable differences in the chemical composition and crystalline structures between the powder and set forms of any of the material tested.

Biocompatibility:

The biocompatibility of new dental materials is established *in vivo* and *in vitro* methods to evaluate interactions between host and material. MTA has undergone different types of investigation, usage tests, implantation, cells attachment and cytotoxicity tests. MTA was found to be biocompatible; it also induces osteogenesis and cementogenesis. MTA effectively seal root and furcal perforations and apical foramina.

Studies *In Vivo*:

Torabinejad et al (1995) examined the periradicular tissue response of dog's teeth with apical periodontitis to GMTA and amalgam. Forty six roots in six beagle dogs were used and periradicular tissue lesions were developed. The canals on half of the roots were instrumented and obturated with gutta-percha and sealer, and their access cavities were sealed with GMTA. The remaining root canals were instrumented and obturated with gutta-percha without root canal sealer, and their access was left open. After 2 weeks, roots were resected; half of the root-end cavities were filled with amalgam and the rest with GMTA. The periradicular tissue response of the dogs was evaluated histologically at 2 to 5 and 10 to 18 wks following periradicular surgery. The results showed less periradicular inflammation and more fibrous capsules adjacent to GMTA, compared with amalgam. In addition, the presence of cementum on the surface of GMTA was a frequent finding.

In a similar study, Bernabe et al (2007) compared GMTA to zinc oxide eugenol cement (ZoE). Histological examination was done after 180 days. Results showed cementum deposited only over GMTA. ZoE showed severe inflammation compared to GMTA. No bacteria were found at the interface between both materials and dentin. The

author proposes that MTA antibacterial properties are due to formation of calcium hydroxide from reaction of calcium oxide with water. In another dog study, Baek et al (2005) using 24 roots compared the periapical tissue reaction, as well as cementum regeneration in contact with amalgam, Super EBA, and GMTA using undecalcified ground sections. Five months after periapical surgery, GMTA showed minor inflammatory reaction with plasma cells, lymphocytes and some macrophages. Cementum deposition occurred on the GMTA surface. The author speculated that new cementum formation could have originated from periodontal ligament and bone cells.

To observe the healing process with GMTA repaired root canal perforation: Holland et al (2001) created lateral root perforation in 48 root canals of dog`s teeth after complete instrumentation. The perforations were repaired with GMTA, and Sealapex was used as a control. At 30 and 180 days histological analysis was done. Results showed no inflammation and cementum deposition around MTA, while Sealapex was surrounded with chronic inflammation and slight deposition of cementum.

Furcation repair was studied by Yildirm et al (2005). Furcal perforations were created in 90 mandibular premolars and molars in 9 mongrel dogs. Seventy two teeth were repaired with GMTA or Super EBA and 18 without repair served as negative controls. Histological examination was done at 1, 3, and 6 months. GMTA showed mild inflammation at 1 month, which decreased at 3 months and diminished at 6 months. Cementum deposition was found on all GMTA specimens at 6 months.

Regan et al (2002) compared periapical tissue reaction to GMTA and Diaket. The experiment was similar to Torabinejad et al (1995), however no lesions were developed in the periapical tissues. Two months after surgery, histological sections were compared

according to detailed criteria. The results showed no difference between GMTA and Diaket in the presence of inflammation and bone formation. New periodontal ligament formation was greater on the Diaket surface compared to GMTA. Cementum coverage was not complete but, variable and unpredictable on both materials.

In a retrospective human study, Main et al (2004) looked at all repaired root perforations completed in the University of Loma Linda endodontic residency program. Only GMTA repaired perforations with radiographs and a minimum of 1 year recall were included. Only 16 patients met the criteria, 5 were strip perforations, 5 laterals, 3 furcal, and 3 apical perforations. All cases had preoperative radiolucency that decreased in size at 1 year recall.

In conclusion, the reviewed studies support the biocompatibility of GMTA when it's in contact with periapical tissue. Also these studies show GMTA to be more biocompatible when compared with other commonly used dental materials.

Studies *In Vitro*:

Periodontal ligament cells:

Several studies looked at the cytotoxicity, attachment and cytokines production of periodontal ligament cells when in contact with MTA. Thomson et al (2003) investigated the effects of GMTA, IRM, and amalgam on murine cementoblast growth, in tissue culture. Cells and materials were incubated for 48 h then fixed for scanning electron microscopic analysis. Gene expression was evaluated on GMTA and IRM using reverse transcriptase polymerase chain reaction (RT-PCR) with primer sets for glyceraldehyde-3-phosphate dehydrogenase, type I collagen, alkaline phosphatase, osteocalcin, and bone sialoprotein after 3 and 5 days. The presence of osteocalcin on GMTA was evaluated

using conofocal microscopy after 7 and 12 days of incubation. Images were compared with controls to assess qualitative differences. Results showed better cell attachment on GMTA compared to IRM and amalgam. RT-PCR results were not conclusive; IRM did not yield enough products for PCR use. However, GMTA results were similar to control. Osteocalcin production increased from 5 to 12 days in GMTA and control. The author concluded that GMTA is cemento-inductive by allowing the expression of genes and proteins involved in the cementogenesis process.

The attachment and morphology of human periodontal ligament fibroblasts on GMTA was evaluated using a scanning electron microscope (Balto et al 2004). GMTA was placed in an apical cavity of 30 single-rooted slices of extracted human teeth, which were divided in 2 groups freshly prepared and set, as well as 2 control slices. A suspension of human periodontal ligament fibroblast cells was placed over the MTA filling and control. In the set-MTA group, cells were round and flattened, displayed smooth surfaces, and appeared to be tightly attached to MTA. In the freshly mixed state cells did not show attachment.

Vajrabhaya et al (2006) evaluated the cytotoxicity of furcal perforation repair materials, Ketac molar, glass ionomer and GMTA, using cell culture technique. The extract of GMTA and Ketac Molar were added on PDL cells in tissue-culture plates. Cell proliferation after an incubation period of 3 days was determined by using the MTT assay. The growth of cultured human periodontal fibroblast cells were suppressed by both materials, However the GMTA group showed more viable cells.

The cytotoxicity of amalgam, glass-ionomer, SuperEBA, N-Rickert, GMTA and gutta-percha cytotoxicity was compared against permanent V79 fibroblasts and murine

granulocyte-macrophage progenitor cells (Souza et al 2006). Eluates from the materials, using extraction medium were added to the culture medium for 72 h and their cytotoxicities were assessed over 24 h and 7 days. Cytotoxicity was judged using the total nucleic acid content (NAC), neutral red uptake (NRU) and reduction of the tetrazolium salt (MTT). GMTA was ranked as the least cytotoxic cement in both cell systems.

Gorduysus et al (2007) compared the cytotoxicity, induced apoptosis and/or necrosis, and apoptotic mechanisms in human periodontal ligament (PDL) fibroblasts treated with four different endodontic materials: ProRoot (WMTA), Diaket, Endion, and CYMED 8410. The effects of these four materials on the viability of PDL fibroblasts were determined by MTT (3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide) assay. Apoptotic pathways were evaluated via Annexin-V/PI double-staining Assay, and cell cycle analysis. Exposure to WMTA for 24, 48, and 72 hours resulted in no significant differences in MTT reduction and viable cell numbers comparable to controls. However, treatment of PDL fibroblasts with Diaket, Endion, and CYMED 8410 resulted in a reduction of viable cell number compared with controls. Annexin V/PI staining and cell cycle analysis showed that Diaket, Endion, and CYMED 8410 induced higher percentages of apoptosis and/or necrosis than in controls. The results suggest that WMTA is a less damaging material.

BMP-2 induces bone, cartilage, cementum and dentin formation, while TGF beta-1 has been shown to inhibit or stimulate bone formation and thought to block the effect of BMP-2 on bone cells. Guven et al (2007) compared the effects of ProRoot GMTA and grey MTA Angelus on transforming growth factor (TGF)-beta1 and bone morphogenetic protein (BMP)-2 levels produced by cultured human gingival fibroblasts (HGFs). After

24 and 72 hours of exposure to the MTA products, HGF viability was determined by using the MTT assay. TGF-beta1 and BMP-2 levels in cell-free culture media were determined by enzyme-linked immunosorbent assay. Cell viability of the test groups was significantly lower than that of control at 24 and 72 hours but showed an increase at 72 hours. MTA Angelus group displayed higher TGF beta-1 levels than control and ProRoot MTA groups at 24 and 72 hours. BMP-2 levels of both test materials were similar at 24 and 72 hours. These results suggest that both MTA products are capable of stimulating HGF to produce BMP-2, whereas the stimulatory effect for TGF beta-1 is material dependent. Although both BMP-2 and TGF beta-1 were increased, the inhibitory effect could not be detected.

Osteoblast cells:

The reaction of osteoblasts to MTA was first tested by Koh et al (1997). The materials and methods were repeated by many researchers. In his study Koh et al (1997) used osteoblasts (MG-63) derived from human osteosarcoma. GMTA (Loma Linda) and polymethylmethacrylate commonly used orthopedic cement, were tested in both the freshly prepared and set state. Cells were grown in presence of materials and tested over 1-144hrs period. Standard ELISA assays were used for assessment of interleukin (IL)-1 alpha, IL-1 beta, IL-6, macrophage colony stimulating factor (M-CSF), and osteocalcin. In brief, the assays employ a ''sandwich'' enzyme immunoassay technique which was used per the manufacturer's instructions. A color develops, the intensity of which is in proportion to the amount of cytokine present, and it then is measured and compared with known standards prepared from doubling dilutions. The samples were read at 450 nm

with a correction at 570 nm using a spectrophotometer. The levels of alkaline phosphatase were measured to establish the level of differentiation of the cells.

Cells were collected and assayed using the Sigma protocol for alkaline phosphatase activity. Following the periods of growth, the cells were washed and three times with phosphate-buffered saline and by three cycles of freezing and thawing. The basis the assay was to utilize a 2-amino-2-methyl-1-propanol buffer with an incubation period of 15 min (Sigma, Procedure 104). The reaction was stopped with 10 mL of 0.05 NaOH, and the color, generated by the presence of p-nitrophenol, was measured by absorbance at 400–420 nm. In all dishes cells were seen adhering to the base and GMTA at 6 h and had increased to confluence at 144 h. IL-1 alpha, IL-1 beta, and IL-6 were raised when the cells were grown in the presence of GMTA at 144 h. M-CSF appeared to be unaffected although the overall value was high. Cells grown with PMA produced negligible levels of cytokines. Osteocalcin production increased when cells were grown on GMTA. No osteocalcin could be detected with PMA. Cells in contact with GMTA also appeared to have higher levels of alkaline phosphatase. No cells could be found attached to PMA and so no alkaline phosphatase activity could be measured.

In another study involving osteoblasts, Koh et al (1998) used the same materials and methods to test the morphology of osteoblasts (MG-63) when grown with GMTA and IRM. SEM analysis revealed healthy cells in contact with GMTA at 1 and 3 days; in contrast, cells in the presence of IRM appeared rounded. The ELISA assays revealed raised levels of all tested cytokines at all periods when cells were grown in the presence of GMTA; in contrast, cells grown alone or with IRM produced undetectable amounts.

The M-CSF was produced by cells irrespective of the group. The author concluded that GMTA offers a biologically active substrate for bone cells and stimulates IL production.

Mitchell & Pitt ford (1999) carried out the same experiment using a variant of GMTA. The authors did not specify the changes they made to GMTA; however their results were in agreement with Koh et al (1997 and 1998). Nakayama et al (2005) used bone cells from rat femur without specifying the cell line he used. Materials used were GMTA and IRM. Cells were then seeded on three dishes of each material, and cultured for 3 days, and then evaluated using SEM and transmission (TEM) electron microscopy. The calcium released from hydrated material, the cell proliferation ratio and alkaline phosphatase (ALP) activity were analyzed, and the expression of type I collagen and bone-related protein mRNAs were evaluated using RT-PCR. In the GMTA group, SEM and TEM showed that the cells attached in the same manner as the control group. The calcium released and ALP activity was similar to the control group. Cell proliferation and expression of type I collagen mRNA was significantly lower, while the expression of osteopontin mRNA was significantly higher than the control group at the third day of culture. In IRM groups, a few rounded cells were observed on the material but no living cells were seen. The author concluded that GMTA is a material of low toxicity which does not inhibit cell growth, but does suppress the differentiation of osteoblast-like cells.

Zhu et al (2000) compared the attachment of human osteoblast –like-Saos-2 cells to GMTA, IRM, composite and amalgam. Materials were condensed into bottoms of a 96 –well culture dish using an amalgam to make 1 mm thick disks, then cells and culture medium were added and incubated for 2 weeks. SEM observation showed that osteoblasts attached and spread on GMTA and composite by forming a monolayer.

Osteoblasts also attached on amalgam, but with few cells spreading. In the presence of IRM, osteoblasts appeared rounded with no spreading.

Tani-Ishii et al (2007) grew MC3T3-E1 cells with GMTA, amalgam and Dycal. RT-PCR with primer sets for type I collagen, osteocalcin, and bone sialoprotein was used to measure the gene-expression response of the cells with the tested materials. GMTA caused an up-regulation of type I collagen and osteocalcin messenger RNA expression after 24 hours. In a similar study using the same cell line, Yoshimine et al (2007) compared the cell response to GMTA, IRM and 4-META/MMA-TBB resin. The results showed better attachment of cells to GMTA compared to other materials.

Al-Rabeah et al (2007) analyzed the response of human alveolar bone cells to GMTA and WMTA. Cells were grown from human alveolar bone explants. The author did not specify the cell line obtained. Cells were seeded onto set ProRoot GMTA, WMTA, and MTA prepared with local anesthetic solution, instead of water. SEM showed cells were attached and spread out on all materials within 24 hours, and proliferated to form a matrix-like layer within 7 days. Cell attachment and cell-surface interactions with all materials were similar.

Camilleri et al (2004) examined the biocompatibility of Pro Root GMTA and WMTA when grown with Saos-2 osteosarcoma cells. Both materials were placed on glass cover slips and allowed to set for 1 or 28 days. Saos-2 osteosarcoma cells were seeded and placed in medium over the material-coated coverslips for 1, 5 and 7 days. Cell morphological changes were evaluated by SEM. The response to both materials was similar. The 1-day cured samples of two commercial forms of MTA showed good biocompatibility. However, the 28-day cured samples were less biocompatible after 1 and

5 days. The author believes the biocompatibility of the 1 day mixed material due to calcium hydroxide production. The setting reaction of MTA yields Ca $(OH)_2$ from tricalcium silicate. Free tricalcium silicate was not available once all material was fully set after 28 days.

In a similar study, Perez et al (2003) tested the same materials against primary osteoblasts and MG-63 osteosarcoma cells. The cells were exposed to beta-glycerophosphate and dexamethasone to assess mineralized nodule formation as a function of osteogenic behavior. The results showed the number of cells on the surface of the materials increased in all samples throughout the experiment, except for WMTA where no primary osteoblasts were visible on top of the material by the end of 13 days. After exposing cells to differentiation medium, nodules were observed in cultures of primary osteoblasts, but not of MG-63 osteosarcoma cells. MG-63 cells do not behave osteogenically by forming mineralized nodules, and primary osteoblasts are more sensitive than MG-63 osteosarcoma cells to WMTA in cell culture. The author recommended primary osteoblasts for testing endodontic materials in cell culture.

MTA mixed with water has a short working time, delayed setting, and poor consistency. Recently, research was focused on obtaining an alternative delivery vehicle for MTA, to improve its setting time and handling characteristics.

Improving MTA Properties:

Many clinicians subjectively report that the handling properties of MTA are less than ideal. The consistency is difficult to maintain due to the setting reaction that results in desiccation of the MTA/water mixture .Long setting time of MTA makes it less than ideal for apexification and perforation repair. Several researchers worked on improving the handling characteristics of MTA.

Wiltbank et al (2007) added Portland cement (PC) accelerators (calcium chloride $CaCl_2$, calcium nitrite/nitrate, and calcium formate) to GMTA, WMTA and PC. Initial setting time, dimensional stability, temperature during set, and pH were measured. It was found that all 3 additives significantly accelerated the setting of GMTA and PC; only calcium chloride and calcium formate significantly accelerated WMTA. Dimensional stability was not significantly different between control and experimental groups. Calcium chloride decreased the pH of GMTA and PC, while pH increased with calcium nitrite/nitrate and calcium formate. The author concluded that further studies are needed to evaluate the biocompatibility of these additives to MTA.

In a similar study, Ber et al (2007) added the following accelerants 2% $CaCl_2$ to 1%, 2%, and 3% methylcellulose (MC), to GMTA powder. Those three different mixtures were then added to GMTA and PC. One percent of MC and 2% of $CaCl_2$ added to GMTA resulted in a mixture that handled similarly to a reinforced zinc oxide-eugenol cement. The mixture gave an approximately equal compressive strength, and set three times faster (57 +/- 3 minutes) compared to 3-4 hours for regular GMTA.

Karimjee et al (2006) compared the biocompatibility of WMTA mixed with water or with KY Jelly (Johnson & Johnson, New Brunswick, NJ) to that of Fuji II LC (GC America, Alsip, IL), cement and amalgam. Periodontal ligament (PDL) cells were cultured. Eluates from mixed materials were placed in contact with the PDL cells for 24, 48, and 72 hours. Cell viability was determined by measuring mitochondrial enzyme activity using the MTT assay. Cytotoxicity was also measured in terms of cell lysis using the lactate dehydrogenase assay. One mL α–minimum essential medium (α-MEM)

(Gibco, Grand Island, NY) supplemented with 100 μg/mL penicillin G (Sigma Chemical Co., St. Louis, MO), 50 μg/mL of streptomycin (Sigma), and 0.2% fetal bovine serum (FBS; Hyclone, Logan, UT) was added to test material, and then eluates were extracted. Eluate extractions from all materials caused significantly less cell viability and more cell death than control at all times. However, at 72 h the WMTA/water, WMTA/KY, and amalgam eluate extractions led to significantly better cell viability than the Fuji II eluates. The author concluded that WMTA/KY, WMTA/water, and amalgam have similar biocompatibility regarding effects of their eluates on human PDL cells.

Kogan et al (2006) added several additives to MTA to enhance its setting properties. Additives tested included saline, 2% lidocaine, 3.0% NaOCl gel, Chlorhexidine gulconate gel, KY Jelly, 3% and 5% $CaCl_2$. The setting times were evaluated using Vicat apparatus. Compressive strength of the set materials was evaluated using an Instron machine. NaOCl , KY jelly and $CaCl_2$ decreased the setting time to 20-25 minutes. Compressive strengths of these materials were lower than MTA mixed with water. The author found NaOCl, KY jelly, and $CaCl_2$ had improved handling properties. The author also recommended these materials to be mixed with MTA, when root canal system obturation and MTA repair are to be done in one visit.

There are numerous studies of the biological properties of GMTA and WMTA (Roberts et al 2008). Studies *In vitro* of cell attachments and morphology, cytokine production, and cytotoxicity have been well documented (Roberts et al 2008, Beranbe et al 2007, Baek et al 2005, Thomson et al 2003, Balto et al 2004, Souza et al 2006, Gorduysus et al 2007, Guven et al 2007, Koh et al 1997 and 1998, Zhu et al 2000). These materials are also osteoconductive. However, substantially no information is available on

how these new chemical additives changes the biological properties of the grey and white.MTA formulations. This appears especially important as the biological effects of MTA are related to biochemical interactions between tissues and the surface of the material.

Therefore, this study was designed to add KY, NaOCl and 5% $CaCl_2$ to grey MTA and investigate the biological effects of adding several additives to GMTA.

Aims of this study:

The aims of this study were to:

1) Compare the pH, temperature, and setting time of the following additives; KY liquid, Calcium Chloride (CaCl2), and Sodium Hypochloride gel to GMTA mixed with water (Part 1).

2) Compare the cell attachment to test materials using fluorescent microscope (Part 2).

3) Compare the cell attachment to dentin and test materials using fluorescent microscope (Part 3).

4) Measure levels of the following bone formation markers: TNF-Alpha (TNF-α), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), and macrophage colony stimulating factor (M-CSF), (Part 4).

Materials and Methods:

All experiments were performed in triplicates. Materials were divided into the following groups. Group 1(control): GMTA mixed with sterile water in 3:1 (w/v) ratio. Group 2 (test): GMTA mixed with 5% calcium chloride $CaCl_2$ (Sigma-Aldrich, St. Louis, MO). Group 3 (test): GMTA mixed with KY liquid (Johnson & Johnson, Langhorne, PA). Group 4 (test): GMTA mixed with sodium hypochloride ($NaOCl_2$) gel (ChlorCid V, Ultradent Products. Inc, South Jordan, UT).

Part 1:

Temperature and pH measurement:
Test and control materials were mixed using 0.5 g of grey MTA in 3:1 ratio (w/w)

- Group 1: Grey MTA with water (positive control)
- Group 2:KY liquid
- Group 3: Calcium Chloride ($CaCl_2$)
- Group 4: Sodium Hypochloride gel (NaOCl)

Calibrated pH probe (HANNAA pH21, pH/mV; HANNA instruments, Woonsocket, RI),Figure 1, was introduced to each group. The probe was calibrated after each reading. Readings were taken immediately after mixing and every 5 minutes for 15 minutes.

Setting time:
Test and control materials were mixed; 1 full GMTA pack was mixed for each group and was placed in one well of a 24 well culture plate (Costar, Corning, NY). During the experiment, samples were covered with deionized water moistened gauze and placed in a 37°C incubator. The samples were tested just before their anticipated setting time and at 5 minutes interval until fully set. A Gilmore apparatus, Fig 2, was used with a stainless steel indenter with 100-g mass applied at a right angle to the surface of the sample for 5

seconds. Setting time was defined as the length of time the indenter failed to leave a definite mark at the surface of the sample.

Part 2:

Osteoblasts:

Mouse MC3T3-E1 osteoblasts were grown in Dulbecco's Alpha Modified Eagle Medium (DMEM-Alpha; Gibco BRL, Gaithersburg, MD) supplemented with L-glutamine ,ribonucleosides, deoxyribonucleosides, 10% fetal bovine serum (FBS; Hyclone laboratories, Logan ,UT) sodium pyruvate, glucose , and 1% of antibiotic/ antimycotic cocktail (300 U/mL penicillin, 300 μg/mL streptomycin, 5 μg/mL amphotericin B). Cells were incubated at 37°C, 100% humidity, 95% air and 5% CO_2.

Material disks:

Part 1 control and test materials were used to make uniform disks, using orthodontic elastic bands (6mm wide x 1.5mm deep). Disks were covered with deionized water moistened gauze until fully set. Amalgam Valiant PH.D.(Ivovlar Vivadent, Amherst, NY) IRM caps (Dentsply Caulk, Milford, DE), and composite 340 A4 Tetric Flow Ivovlar Vivadent, Amherst, NY), served as negative controls. Three disks of each were made. After setting all disks were smoothed and polished using carbide bur (Alpine, FG 7664, Coltene/Whaledent. Germany).

Cell attachment:

Six disks of each group were placed in the bottom of 12-well cell culture plates (Costar, Corning, NY) as shown in Fig 3. Cells with medium were added to the wells and incubated for 4 days under 37°C, 100% humidity, 95% air and 5% CO_2. At day 1and 4

days, 3 samples of each group were removed and washed twice with phosphate- buffered saline.

Fluorescent Microscope:

The protocol was adapted from Jiang et al (2003).Specimens were fixed for 15 minutes at room temperature with 2% paraformaldehyde, washed with phosphate buffered saline, and treated for 20 minutes with 0.2% Triton X-100 solution (Sigma, St. Louis, MO) to permeabilize cell membranes. For the identification of F-actin, the cells were incubated with 1μg/mL of flourescein isothiocyanate-conjugated phalloidin (Sigma) for 20 minutes at room temperature. For the identification of nuclei, the cells were stained with 10μg /ml of Hoechst 33342 for 20 minutes. All specimens were washed with phosphate- buffered saline, and the distribution of the actin ring was determined using fluorescence microscopy (Ziess observer Z1), shown in figure 4.

Part 3:

Dentin disks:

Caries free freshly extracted single rooted teeth were collected. Roots were scraped with surgical blade to remove periodontal ligament cells. Crown and apical 3 mm were removed using high speed hand piece with straight fissure carbide bur (Midwest, FG #57, Dentsply, York, PA) under water spray. Longitudinal buccal and lingual grooves were created and teeth were split into 2 halves using straight fissure carbide bur (Midwest, FG #57, Dentsply, York, PA). Each half was cut in the middle to create roughly 6x6mm dentin disks using the same bur. Class I cavity measuring roughly 3x3x3mm were created using high speed hand piece with water and straight fissure carbide bur (Midwest, FG #57, Dentsply, York, PA). Disks were stored in saline

supplemented with 1% antibiotic/antimycotic cocktail (300 units/ml penicillin, 300 μg/ml streptomycin, 5 μg/ml amphotericin B (Gibco BRL, Gaithersburg, MD).

Test materials:

Negative, positive controls, and test materials in part 1 were mixed and placed in cavities using amalgam carrier and condenser. Disks were placed in 12-well cell culture plates (Costar, Corning, NY) with storage medium for 24 hrs until set (*Fig 6*). Disk surfaces were smoothed and polished using a high speed hand piece with carbide bur (Alpine, FG 7664, Coltene/Whaledent. Germany) under water spray.

Cell attachment:

Two cell lines were used. Mouse MC3T3-E1 osteoblasts were grown in Dulbecco`s Alpha Modified Eagle Medium (DMEM-Alpha; Gibco BRL, Gaithersburg, MD) supplemented with L-glutamine ,ribonucleosides, deoxyribonucleosides, 10% fetal bovine serum (FBS; Hyclone laboratories, Logan ,UT) sodium pyruvate, glucose , and 1% of antibiotic/ antimycotic cocktail (300 U/mL penicillin, 300 μg/mL streptomycin, 5 μg/mL amphotericin B). Cells were incubated at 37°C, 100% humidity, 95% air and 5% CO_2.

L929 mouse fibroblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Eagle's minimum essential medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT) and 1% antibiotic/antimycotic cocktail (300 units/mL penicillin, 300 mg/mL streptomycin, 5 mg/mL amphotericin B; Gibco BRL, Gaithersburg, MD) under standard cell culture conditions (37°C, 100% humidity, 95% air and 5% CO_2).

Disks placed in 12-well cell culture plates (Costar, Corning, NY). Cells and culture mediums were added to disks at 10^5/ well and incubated for 24 hrs under standard cell culture conditions (37 ^{0}C, 100% humidity, 95% air and 5% CO_2). Disks were prepared for fluorescent microscope as previously mentioned.

Part4:

10 additional disks of the test materials and controls in part 2 were made.

Mouse Macrophages:

The mouse macrophage cell line RAW 264.7 was 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 ^{0}C, 100% humidity, 95% air and 5% CO_2).

After MTA disks were placed into the 12-well cell culture plates (Costar, Corning, NY), cells were seeded at a density of 10^5/well in DMEM with 10% fetal bovine serum. After 3-day incubation, culture medium was collected. The following bioactive molecules were measured by use of a commercially available sandwich ELISA kit (R&D System, Minneapolis, MN, USA):

- Macrophage colony stimulating factor (M-CSF):
- Interleukin-1 alpha (IL-1α)
- Interleukin-1 beta (IL-1β)
- Tumor necrosis factor alpha (TNF-α)

Interleukin 1 Alpha (IL-1α), Interlukin-1-Beta (IL-1β), Tumor necrosis factor-Alpha (TNF-α), and Macrophage colony stimulating factor (M-CSF):

The Koh (1998) protocol was followed. One mL of media was removed from wells of each group at day 3; and stored at -20°C until required. The assay used a quantitative "sandwich" technique (Quantikine R&D Systems, Oxford, UK). The microtitre plate had a monoclonal antibody specific to the interleukin (IL) being investigated that was coated in each well. Control and samples were placed in wells and any cytokine present bound to the immobilized antibody. After several washes, polyclonal enzyme-linked antibodies were then placed in the wells. Unbound polyclonal antibodies were then washed off and a substrate added. A yellow color developed, and the intensity, which was proportional to the amount of cytokine present, was then measured. A spectrophotometer set at 450 nm was used to measure the optical density of each well.

Statistical Analysis:

Results from cytokine tests were analyzed via one-way ANOVA and student t-test.

Results:

Part 1:

The pH and setting time of each group are shown in Tables 1, 2 and 3.This table shows a gradual increase in pH in the control and KY group over the 15 minute period. While all other groups showed gradual decrease in pH over the same period. KY had lower pH values at 0, 10, and15 minutes which was statistically significant when compared to control. $CaCl_2$ at 9 minutes had pH of 10.49 which was statistically

significant when compared to control. NaOCl had lower pH than control which was statistically significant at 5 and 15 minutes.

Temperature in all four groups remained relatively constant in all test groups, while control showed gradual decrease between 0-15 minutes. KY and NaOCl were lower than control at 0 minutes, which was statistically significant.

This table shows the KY group with the shortest setting time of 70 minutes. The Control group had the longest time. NaOCl and $CaCl_2$ setting time was on average 60 minutes less than control. Both KY and $CaCl_2$ showed statistically significant difference when compared to control group.

Part 2:
Cell attachment:

All groups showed cell attachment after 1 day (*Image 1*).Group 3 ($CaCl_2$) had fewer cells attaching to the surface compared to the remaining groups. At day 4 the growth and attachment of cells in all groups was extensive (*Image 2*). No differences in cell attachment were noted.

Image 3 shows the control disks at day 1. Cells attached and spread on GMTA with water as well as composite. Limited number of cells attached to amalgam and IRM. Cells were rounded and showed no spreading. Similar results were obtained at day 4.

Part 3:

Mouse osteoblasts attached to dentin and cement in all groups. Cells spread from dentin into the cement surface. Image 4, shows spread of cells at the junction of cement and dentine in all four groups. There was no difference in the quantity of cells attached in all four groups. Similar results were obtained when mouse fibroblasts were seeded on

cement surface (*Image 5*). Both image 4 and 5 show nuclei and cytoplasm of cells. Due to different thickness between dentine and cement images could not be digitally enhanced to show cytoplasm in some of the test groups.

Image 6 shows osteoblasts cells attachment to GMTA and water as well as composite. Cells did not attach to IRM. Few cells attached to amalgam. Cells were rounded and showed no spreading. Fibroblasts showed similar results (*Image 7*).

Part 4:
IL-1 Alpha (IL-1α), IL-1-Beta (IL-1β), TNF-Alpha (TNF-α), and Macrophage colony stimulating factor (M-CSF):

TNF-alpha was released when cells were cultured with all groups. Compared with control group, there were significantly increased release of TNF- from NaOCl group ($p<0.05$) and significantly less release from KY group ($p<0.05$) (Fig. 7).

There were no detectable changes for Interleukin-1 alpha (IL-1α), Interleukin-1 beta (IL-1β), and Macrophage colony stimulating factor (M-CSF) when cells were cultured with the various MTA disks.

Discussion:

The pH of the test groups was different from the control group. The control group pH on average increased from 9.88 immediately after mixing to 10.35 fifteen minutes after mixing. The NaOCl group showed reduction from 9.42 at 0 minutes to 8.87 at 15 minutes, which was statistically significant at 5 and 15 minutes. This can be explained by the low pH of NaOCl itself. The $CaCl_2$ group started with 10.49 at 0 minutes, which was statistically significant when compared to control, and the pH decreased to 10.22 at 15 minutes, the final pH was similar to control.

For the KY group the pH showed the greatest change from 7.68 at 0 minutes to 9.89 at 10 minutes, then dropping back to 9.15 at 15 minutes. KY groups showed statistical significance at all time expect for 5 minutes period. Torabinejad et al (1999) showed that commercial MTA has a pH of 12.5. It has also been assumed that the high pH of MTA is responsible for its biological activity and the formation of a calcium hydroxide layer (Roberts et al 2008, Camilleri et al 2004).

Wiltbank et al (2007) measured pH using the same protocol. pH was measured from 1-10 minutes. In his study, KY and NaOCl gels were not used. The pH of control and $CaCl_2$ were higher than our results, measuring 11.75 and 11.50, respectively. Measurements were presented on a graph instead of tables. In our results a drop in pH was noted toward the 15 minute period with all test groups. The pH of all groups did change with time. Increasing the time interval might yield different results.

We have noted great difficulty in measuring the pH for all the test groups. Separate probes were used for each group and they were calibrated with a standard solution before each measurement was undertaken. However, it took longer for the pH reader to get a stable reading once the probe was placed in the cement. This can be due to the small amount of cement used. Increasing the amount of MTA and the time interval might give more consistent results similar to Torabinejad et al (1999). Due to the high cost of GMTA large amounts could not be experimented. However, a pilot can be done using Portland cement to specify the amount and time needed before carrying the final experiment.

Temperature also changed slightly between 0 minutes and 15 minutes in all groups. Only KY and NaOCl at 0 minutes had lower temperatures than control , which

were of statistical significance. Temperature readings were almost similar with 1-3^0 C change between the groups, which was similar to Wiltbank's (2007) results. The pH and temperature probes were connected and measurements were taken simultaneously. It is important to remember that this experiment was carried out at room temperature; when these materials are used *in vivo* different readings are expected.

The setting time test showed the great differences between all groups. The control group took 195±18.027 minutes to set which is consistent with Torabinejad et al (1995 &1999). The KY group had the shortest setting time 70±13.22 minutes, which have shown statistical significance. The $CaCl_2$ and NaOCl setting times were 123.33±2.88 and 130±13.22, respectively. $CaCl_2$ setting time was significantly shorter than control. Our results were different from previous research. Wiltbank et al (2007) had a setting time of 35 minutes for the $CaCl_2$. However, the author used the Vickers hardness test and recorded the initial time to set. In this experiment the setting time was recorded when the stainless steel arm of the Gilmore apparatus failed to make an indentation on the cement surface. In a similar study Ber et al (2007) added to 2% $CaCl_2$ to either1%, 2%, or 3% methylcellulose (MC). Those 3 new additives were then mixed with either GMTA or PC to improve. These mixtures gave an approximately equal compressive strength, and sat three times faster (57 +/- 3 minutes) compared to 3-4 hours of regular GMTA.

In our experiment mixing GMTA with the test materials resulted in a thick cement that handled similarly to IRM. It was easier to remove and handle compared to control group. However, these were experiments *in vitro*; the setting time might vary when the conditions of the experiment change to human or animal models.

Mouse osteoblast cell attachment to the new cements surface was tested at 1 and 4 days, using fluorescent microscopy. All groups showed cell attachment after 1 day. Group 3 ($CaCl_2$) had fewer cells attaching to the surface compared to the remaining groups. At day 4 the growth and attachment of cells in all groups was extensive. No differences in cell attachment were noted.

In control disks, cells attached and spread on GMTA with water as well as composite. Limited number of cells attached to amalgam and IRM. Cells were rounded and showed no spreading. Similar results were obtained at day 4. Our results are consistent with Zhu et al 2000.

Torabinejad et al (1995) examined the periradicular tissue response of dogs to GMTA and amalgam. The periradicular tissue response of the dogs was evaluated histologically at 2 to 5 and 10 to 18 wks following periradicular surgery. The results showed less periradicular inflammation and more fibrous capsules adjacent to GMTA, compared with amalgam. In addition, the presence of cementum on the surface of GMTA was a frequent finding.

In a similar study, Bernabe et al (2007) compared GMTA to Zinc Oxide Eugenol (ZoE). Histological examination was done after 180 days. Results showed cementum deposited only over GMTA. ZoE showed severe inflammation compared to GMTA. To observe the healing process with GMTA repaired root canal perforation, Holland et al (2001) created lateral root perforation in 48 root canals of dog's teeth after complete instrumentation. The perforations were repaired with GMTA, and Sealapex was used as a control. At 30 and 180 days histological analysis was done. Results showed no

inflammation and cementum deposition around MTA, while Sealapex was surrounded with chronic inflammation and slight deposition of cementum.

In a retrospective human study Main et al (2004) looked at all repaired root perforations completed in the University of Loma Linda endodontic residency program. Only GMTA repaired perforation with radiographs and a minimum of 1 year recall were included. Only 16 patients met the criteria, 5 were strip perforations, 5 laterals, 3 furcal, and 3 apical perforations. All cases had preoperative radiolucency that decreased in size at 1 year recall.

Karimjee et al (2006) compared the biocompatibility of WMTA mixed with water or with KY Jelly to that of Fuji II cement and amalgam. Periodontal ligament (PDL) cells were cultured. Eluates from mixed materials were placed in contact with the PDL cells for 24, 48, and 72 hours. Cell viability was determined by measuring mitochondrial enzyme activity using the MTT assay. Eluate extractions from all materials caused significantly less cell viability and more cell death than control at all times. However, at 72 h the WMTA/water, MTA/KY, and amalgam eluate extractions led to significantly better cell viability than the Fuji II eluates. The author concluded that WMTA/KY, WMTA/water, and amalgam have similar biocompatibility regarding effects of their eluates on human PDL cells.

The reviewed studies support the biocompatibility of GMTA when it's in contact with periapical tissue. Also these studies show MTA to be more biocompatible when compared with other commonly used root end filling materials.

Hakki et al (2009) used different concentrations of MTA and tested cementoblast cell survival and gene expression. The results showed that 20mg/mL concentration of MTA was toxic for cementoblasts, while using 0.02 mg/mL supported the survival of cells at 72 hrs period. Mineralization ability of the cells was also decreased with the increase in MTA concentration. However when smaller amount of cement was used with dentin disks, both mouse osteoblasts and fibroblasts showed continuous attachment to dentin and test cement surface. When compared to control disks mouse osteoblasts attached to GMTA and water as well as composite. Cells did not attach to IRM. Few cells attached to amalgam. Cells were rounded and showed no spreading. Fibroblasts showed similar results. This proves the biocompatibility of our test materials with the cells used.

The reaction of osteoblasts to MTA was first tested by Koh et al (1997). In his study Koh used osteoblasts (MG-63) derived from human osteosarcoma. In all dishes cells were seen adhering to the base and GMTA at 6 h and had increased to confluence at 144 h. IL-1 alpha, IL-1 beta, and IL-6 were raised when the cells were grown in the presence of GMTA at 144 h. M-CSF appeared to be unaffected although the overall value was high. Cells in contact with GMTA also appeared to have higher levels of alkaline phosphatase.

In another study, Koh et al (1998) used the same materials and methods to test the morphology of osteoblasts (MG-63) when grown with GMTA and IRM. SEM revealed healthy cells in contact with GMTA at 1 and 3 days; in contrast, cells in the presence of IRM appeared rounded. The ELISA assays revealed raised levels of all tested cytokines at all periods when cells were grown in the presence of GMTA; in contrast, cells grown

alone or with IRM produced undetectable amounts. The author concluded that GMTA offers a biologically active substrate for bone cells and stimulates IL production.

Mitchell and Pitt Ford (1999) carried out the same experiment using variant of GMTA. The authors did not specify the changes they made to GMTA; however their results were in agreement with Koh (1997 and 1998). Nakayama et al (2005) used bone cells from rat femur without specifying the cell line used. Materials used were GMTA and IRM. In the GMTA group, SEM showed that the cells attached in the same manner as the control group. The calcium released and ALP activity was similar to the control group.

Tani-Ishii et al (2007) grew MC3T3-E1 cells with GMTA, amalgam and Dycal. RT-PCR with primer sets for type I collagen, osteocalcin, and bone sialoprotein was used to measure the gene-expression response of the cells with the tested materials. GMTA caused an up-regulation of type I collagen and osteocalcin messenger RNA expression after 24 hours.

Cytokines levels were evaluated in this study using mouse macrophage cell line RAW 264.7. Macrophages were chosen because of their essential role in healing and inflammation. They produce variable cytokines the regulate bone formation. The results showed significant increase in TNF-α in the NaOCl group compared to control, which was consistent with Jafarnia et al (2009) results. KY group showed the least amount of TNF-α produced. $CaCl_2$ groups produced similar levels when compared to control. There were no detectable changes for Interleukin-1 alpha (IL-1α), Interleukin-1 beta (IL-1β), and Macrophage colony stimulating factor (M-CSF) when cells were cultured with the various MTA disks.

Jafarnia et al (2009) used the same additives in our study. These additives were mixed with GMTA and WMTA to test the viability of mouse fibroblasts using MTT assay. The test and control cements were tested in freshly prepared and set state in the form of pellets measuring 1mm in width x 1mm in height. The author found when the material was set the additives had no effect on cell viability. While in freshly prepared NaOCl gel had the lowest cell viability ranging between 29% -50%. Both WMTA and GMTA had similar results.

In our experiment all groups did not significantly stimulate cells to produce cytokines. This can be explained either by; materials used did not initiate a significant inflammatory reaction, or the number of live and active cells was too low to produce detectable amounts of cytokines.

In a recent study by Huang et al (2008) 15% sodium phosphate dibasic (Na_2HPO_4) was used as an accelerant with White MTA. The author found setting time reduced to 26 minutes, pH increased to 13.2 and the tensile strength to be 4.9 MPa after 6 hours. In a follow-up study by Ding et al (2008) the setting time was 38 minutes and fibroblasts showed attachment to both control and test group under SEM observation. This new material has not been fully tested for cytokines and it might provide a good alternative to MTA mixed with water.

Conclusion:

Under the conditions of this study, mouse MC3T3-E1 osteoblasts and L929 mouse fibroblasts attached to the surface of GMTA and composite. Mouse MC3T3-E1 osteoblasts and L929 mouse fibroblasts did not attach to the surface of amalgam and IRM. Adding KY, $CaCl_2$, and NaOCl to grey MTA improved the handling properties and decreased setting time. Mouse osteoblasts and fibroblasts attached and spread on GMTA mixed with additives in a similar manner to GMTA mixed with water. These test cements could provide a possible alternative to GMTA mixed with water. Further *in vitro* investigations are required to prove the clinical applications of these materials.

Index (tables and figures):

Figure 1:

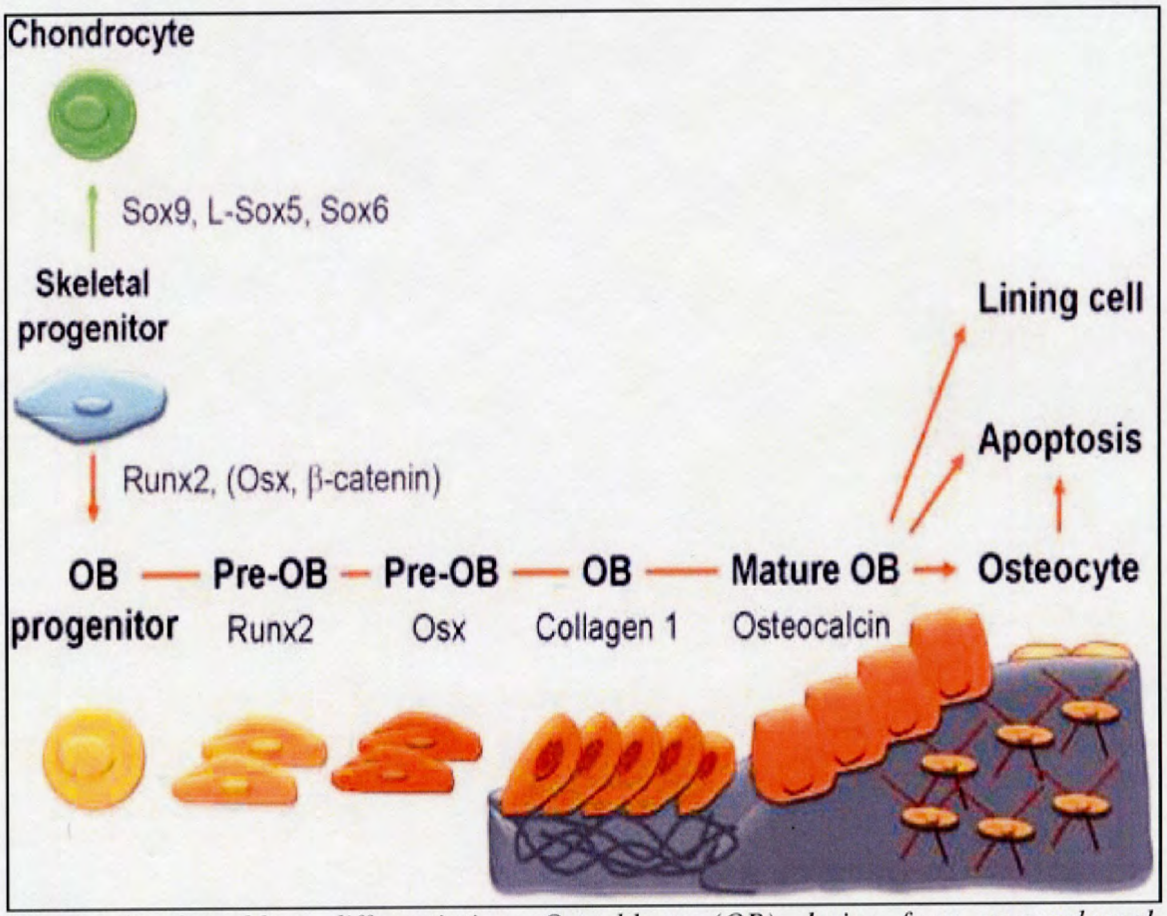

Figure 1: *Osteoblast differentiation. Osteoblasts (OB) derive from mesenchymal progenitors that can differentiate into chondrocytes or osteoblasts. The transcription factors involved in driving the cell to a chondrocyte fate are Sox9, L-Sox5, and Sox6, while osteoblast differentiation requires Runx2 and later Osx and -catenin. Progressive osteoblast differentiation from committed osteoblast progenitor cells is characterized by changes in gene expression, providing markers that typify specific stages. Runx2 is the earliest marker of preosteoblasts, followed by Osx. Preosteoblasts further differentiate into osteoblasts that produce large amounts of type I collagen (Collagen 1) and later mineralize the osteoid matrix as mature osteoblasts expressing osteocalcin. Eventually, the cells can become entrapped within the bone matrix as osteocytes, adapted from Maes et al (2007).*

Figure 2*:*

Figure 2*: HANNAA pH 21.*

Figure 3:

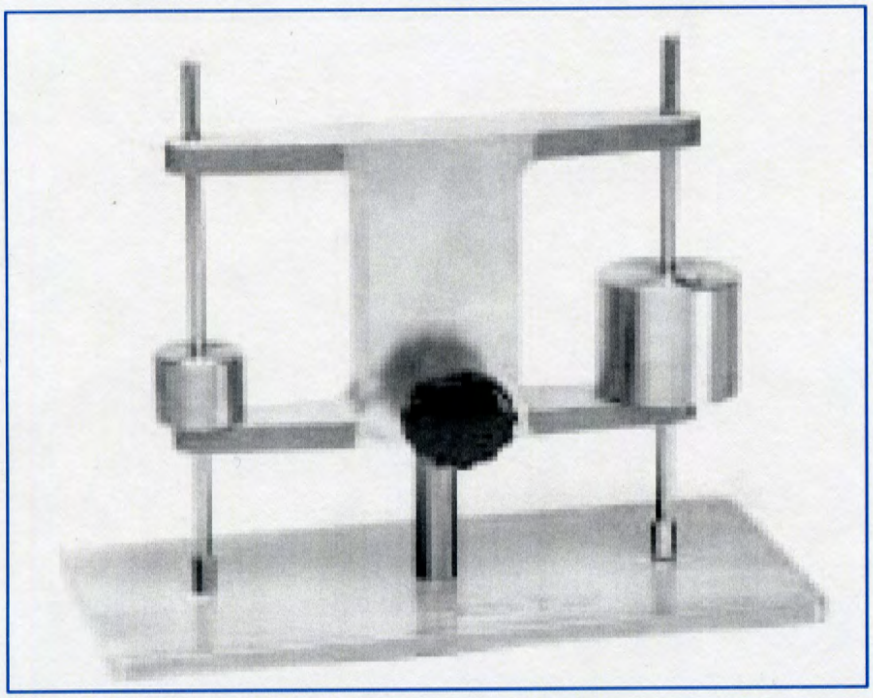

Figure 3*: Gilmore apparatus.*

Figure 4:

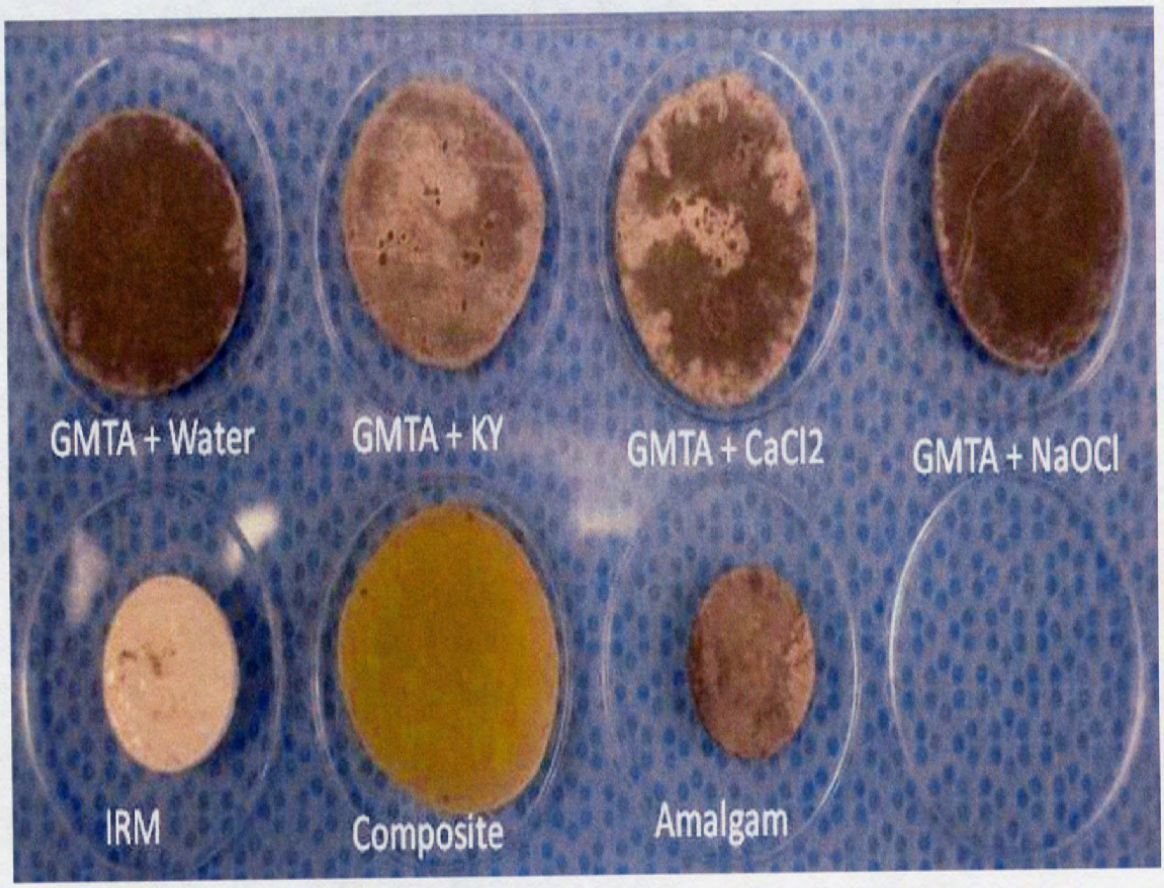

Figure 4: *MTA disks, polished and placed in culture wells.(A): GMTA with water,(B): GMTA with KY ,(C): GMTA with* $CaCl_2$*,(D):GMTA with NaOCl, (E): Amalgam, (F):Composite, (G): IRM.*

Figure 5*:*

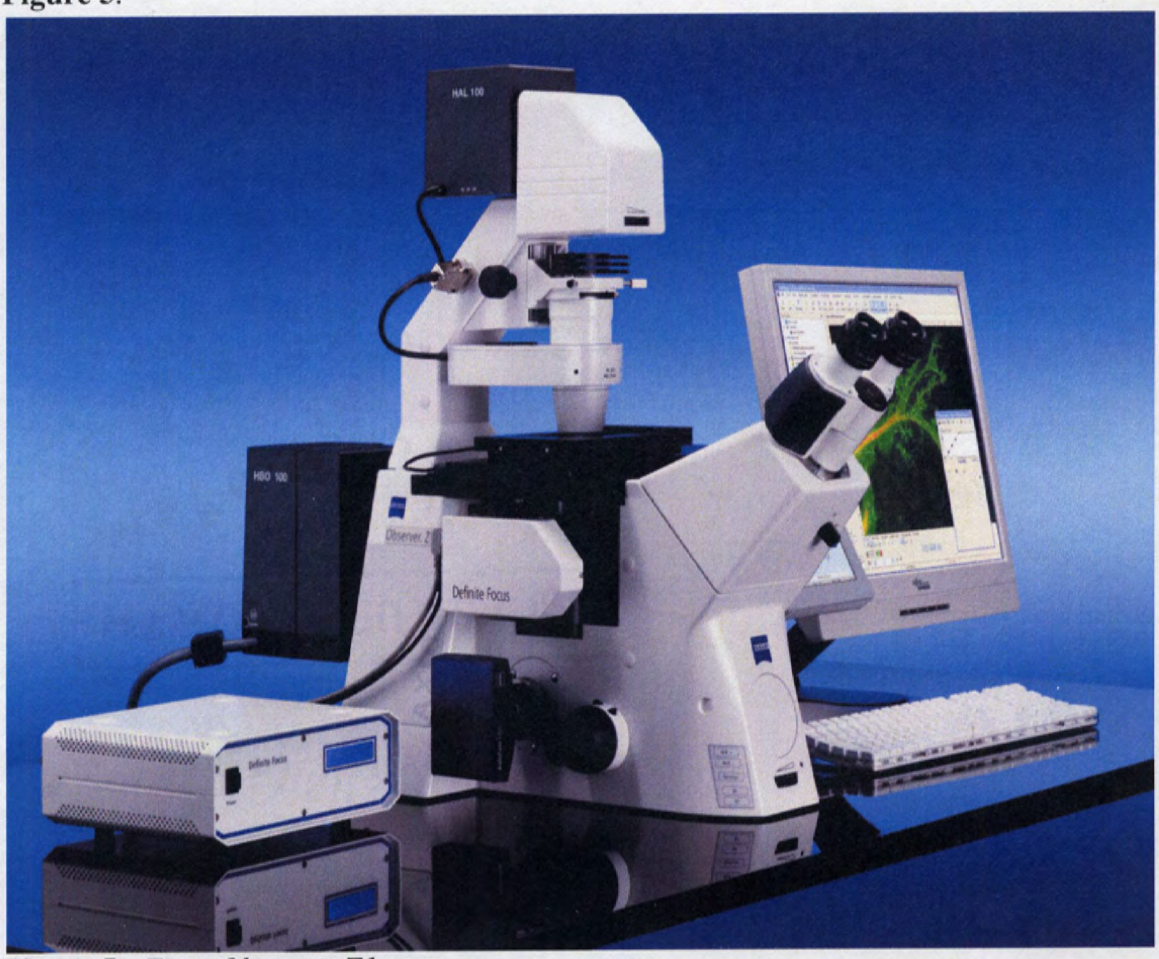

Figure 5*: Ziess Observer Z1*

Figure 6:

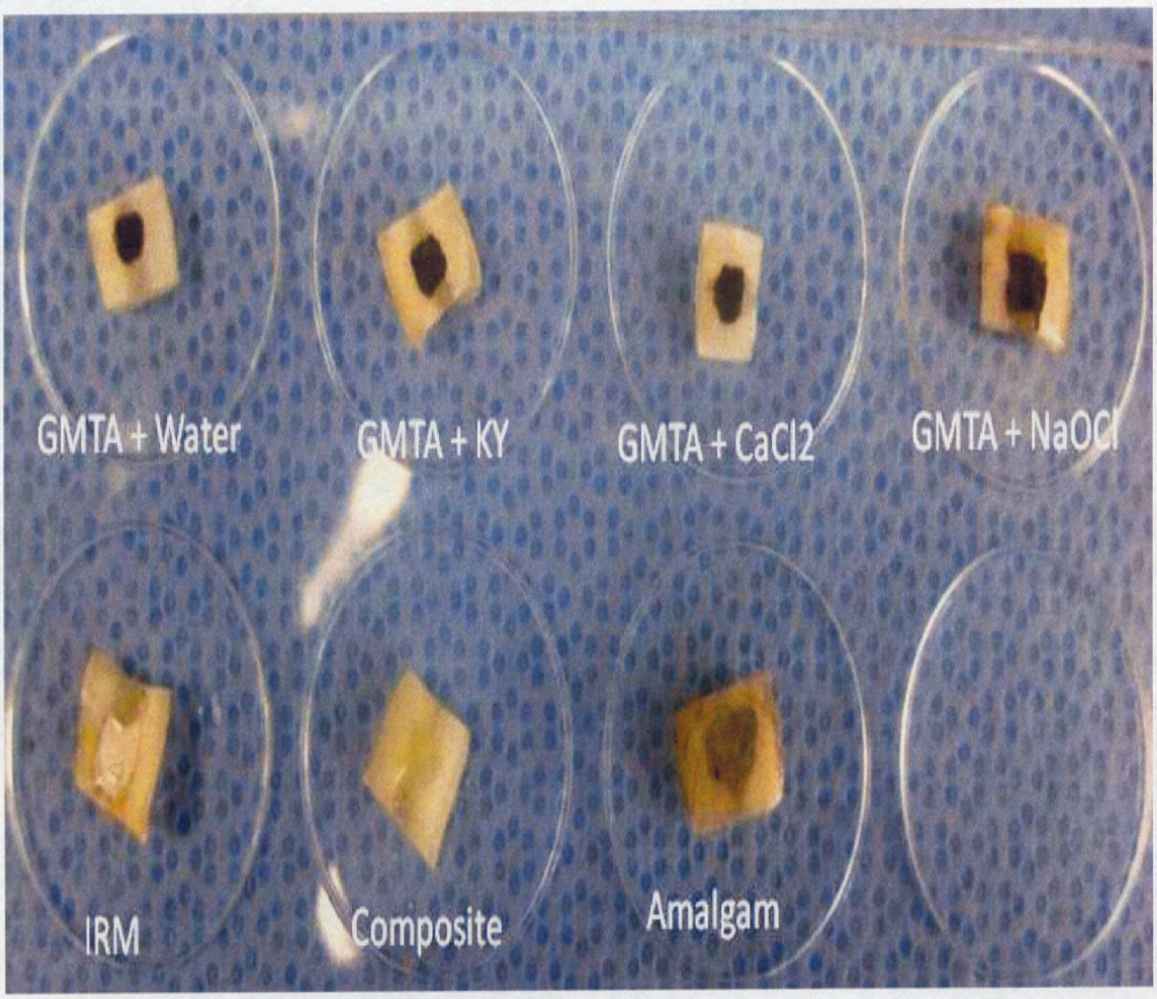

Figure 6: *MTA and dentin disks, polished and placed in culture wells.(A): GMTA with water,(B): GMTA with KY ,(C): GMTA with $CaCl_2$,(D):GMTA with NaOCl, (E): Amalgam, (F):Composite, (G): IRM.*

Table 1:

Group	0 minutes	5 minutes	10 minutes	15 minutes
MTA with water (control)	9.88±0.089	10.5±0.390	10.46±0.19	10.35±0.0416
MTA with KY	7.68±0.531 ▲	9.29±0.7161	9.89±0.090 ▲	9.15±0.312 ▲
MTA with $CaCl_2$	10.49±0.040 ▲	10.25±0.236	10.24±0.135	10.22±0.330
MTA with NaOCl	9.42±0.843	9.25±0.194▲	9.70±1.010	8.87±0.243 ▲

Table 1: *Means and standard deviation of the pH in four different groups. (▲= P<0.05)*

Table 2:

Group	0 minutes	5 minutes	10 minutes	15 minutes
MTA with water(control)	25.36±0.416	24.2±0.655	23.16±0.642	22.9±0.655
MTA with KY	23.5±0.1527▲	23.5±0.608	23.23±0.404	23.1±0.288
MTA with $CaCl_2$	24.13±1.184	23.93±0.923	23.4±1.3	23±1.212
MTA with NaOCl	22.1±0.871 ▲	22.7±0.838	22.7±1.53	22.1±1.607

Table 2: *Means and standard deviations of the Temperature in four different groups. (▲= P<0.05)*

Table 3:

Group	Setting time (minutes)
MTA with water (control)	195±18.027
MTA with KY	70±13.22 ▲
MTA with $CaCl_2$	123.33±2.88 ▲
MTA with NaOCl	130±13.22

Table 3: *Means and standard deviation of the setting time. (▲= P<0.05)*

Image 1:

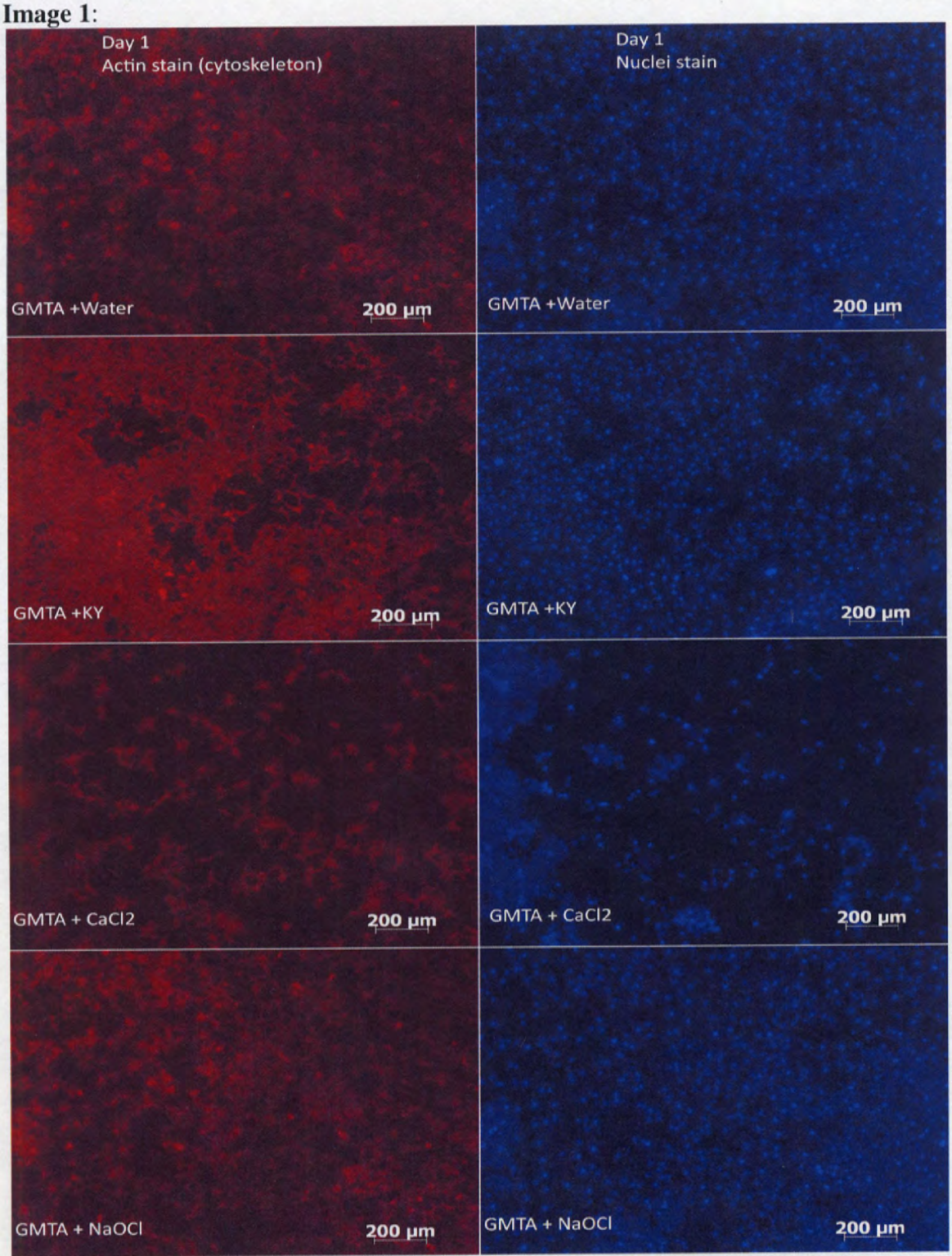

Image 1: *Shows all groups at day 1. Group 3 had fewer cell attaching to the surface compared to the remaining groups. The cytoskeleton was stained red and nuclei were stained blue. Fluorescent microscope image at 5x magnification.*

Image 2:

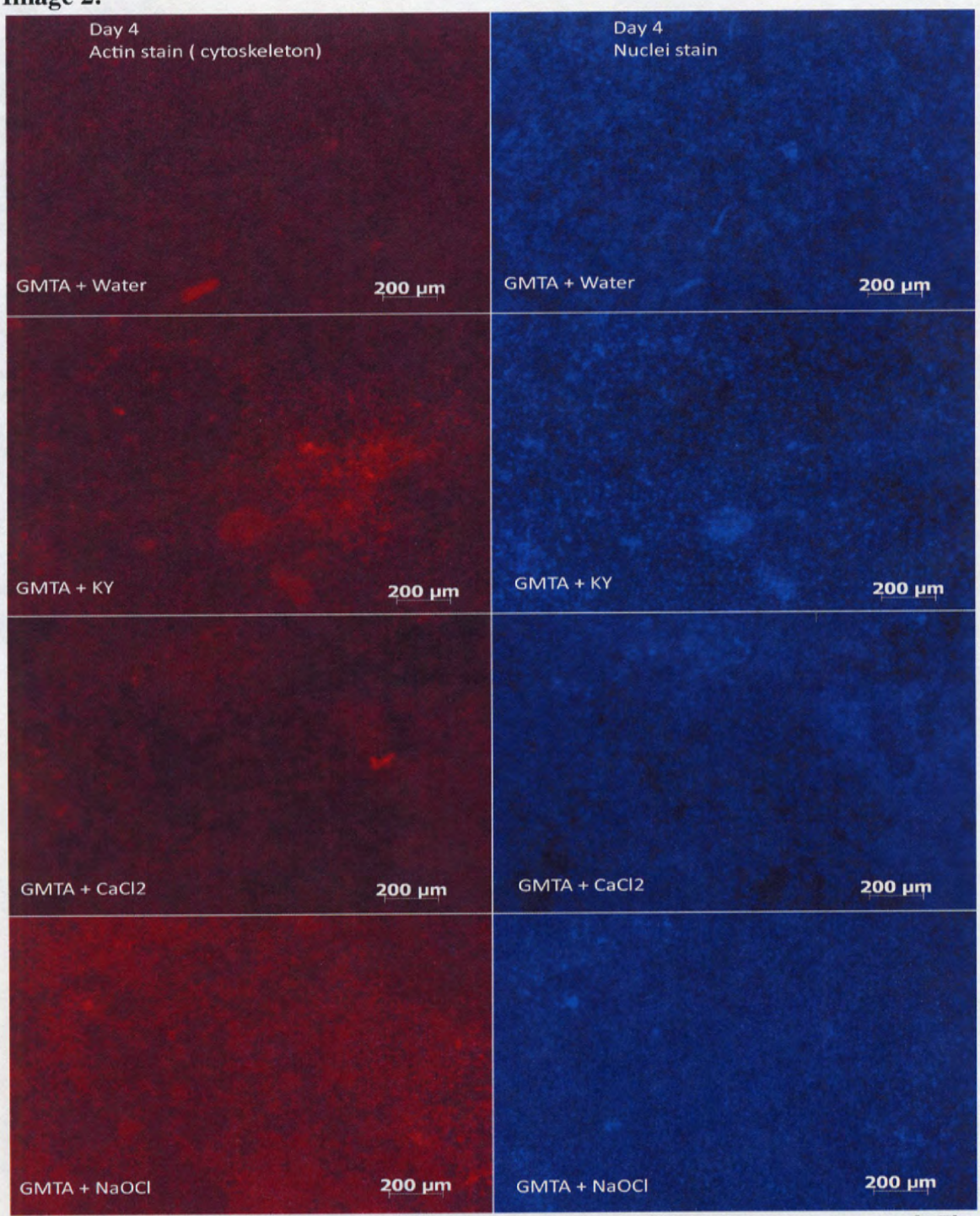

Image 2: *Shows all groups at day 4. No differences in cell attachment were noted. The cytoskeleton was stained red and nuclei were stained blue. Fluorescent microscope image at 5x magnification.*

Image 3:

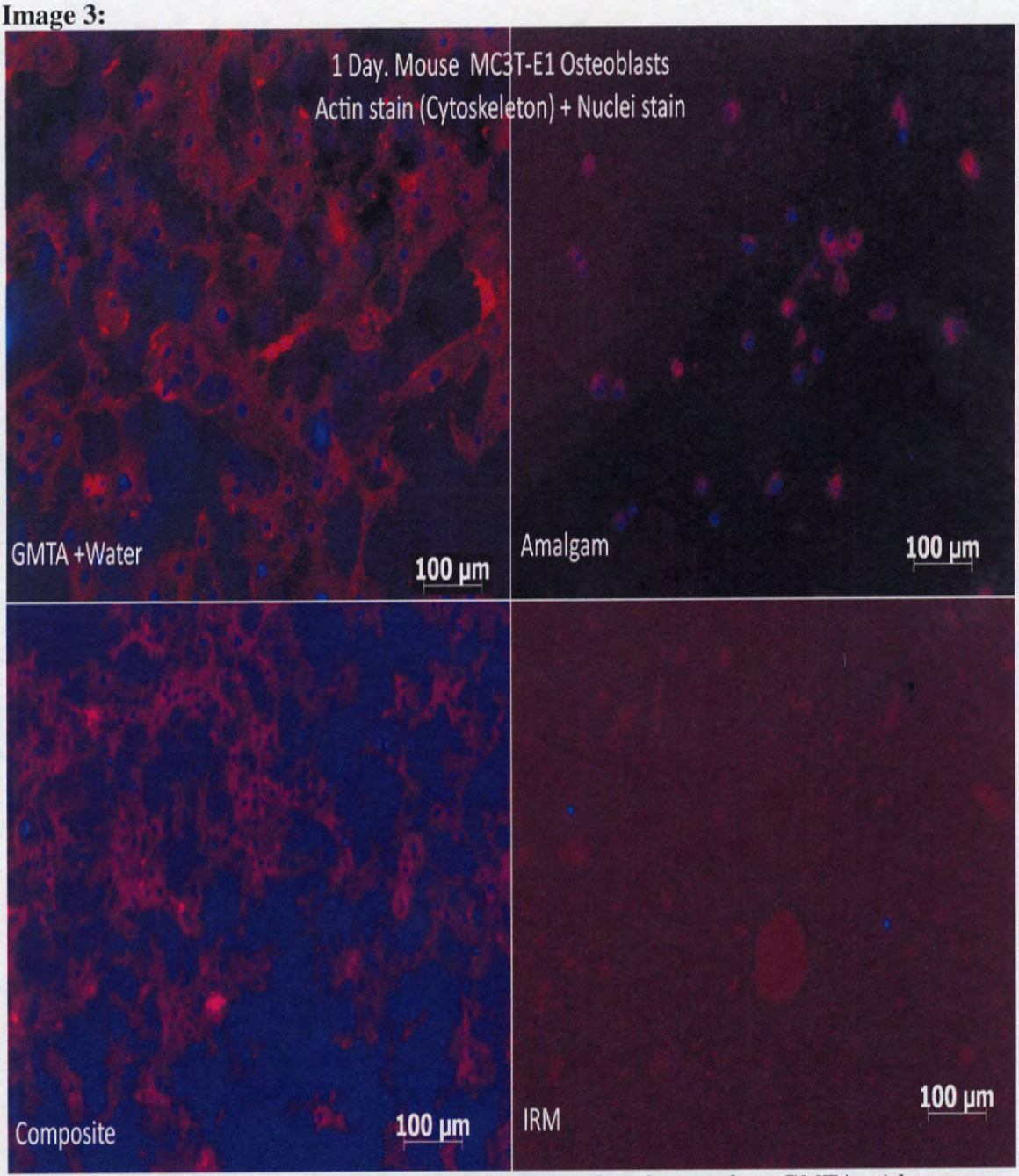

Image 3: *Shows all controls at day 1. Cells attached and spread on GMTA with water as well as composite. Limited number of cells attached to amalgam and IRM. Cells were rounded and showed no spreading. The cytoskeleton was stained red and nuclei were stained blue. Fluorescent microscope image at 10x magnification.*

Image 4:

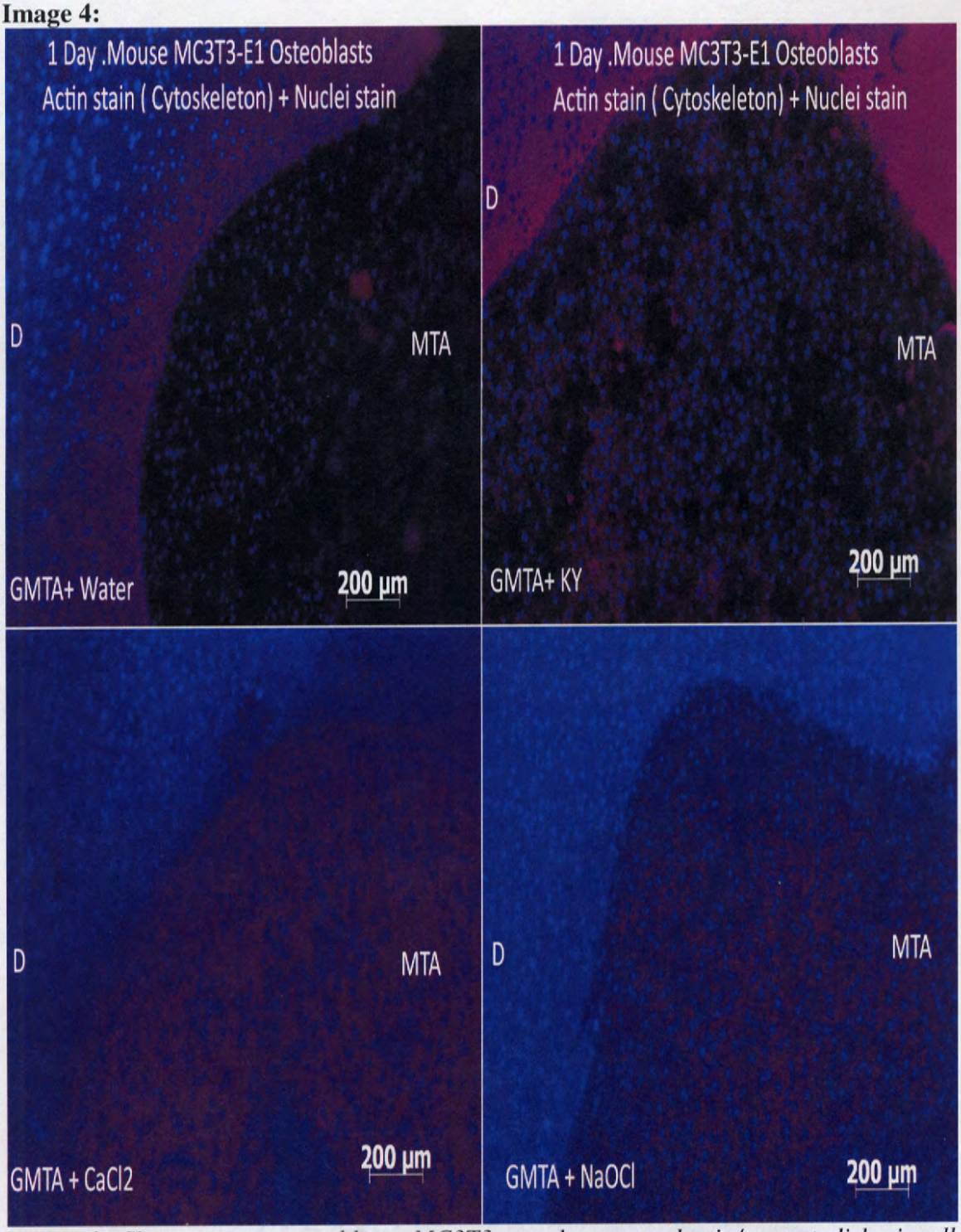

Image 4: *Shows mouse osteoblasts MC3T3 attachment to dentin/cement disks in all groups after 24 hrs. No differences in cell attachment were noted .(D): dentin. The cytoskeleton was stained red and nuclei were stained blue. Fluorescent microscope image at 5x magnification.*

Image 5:

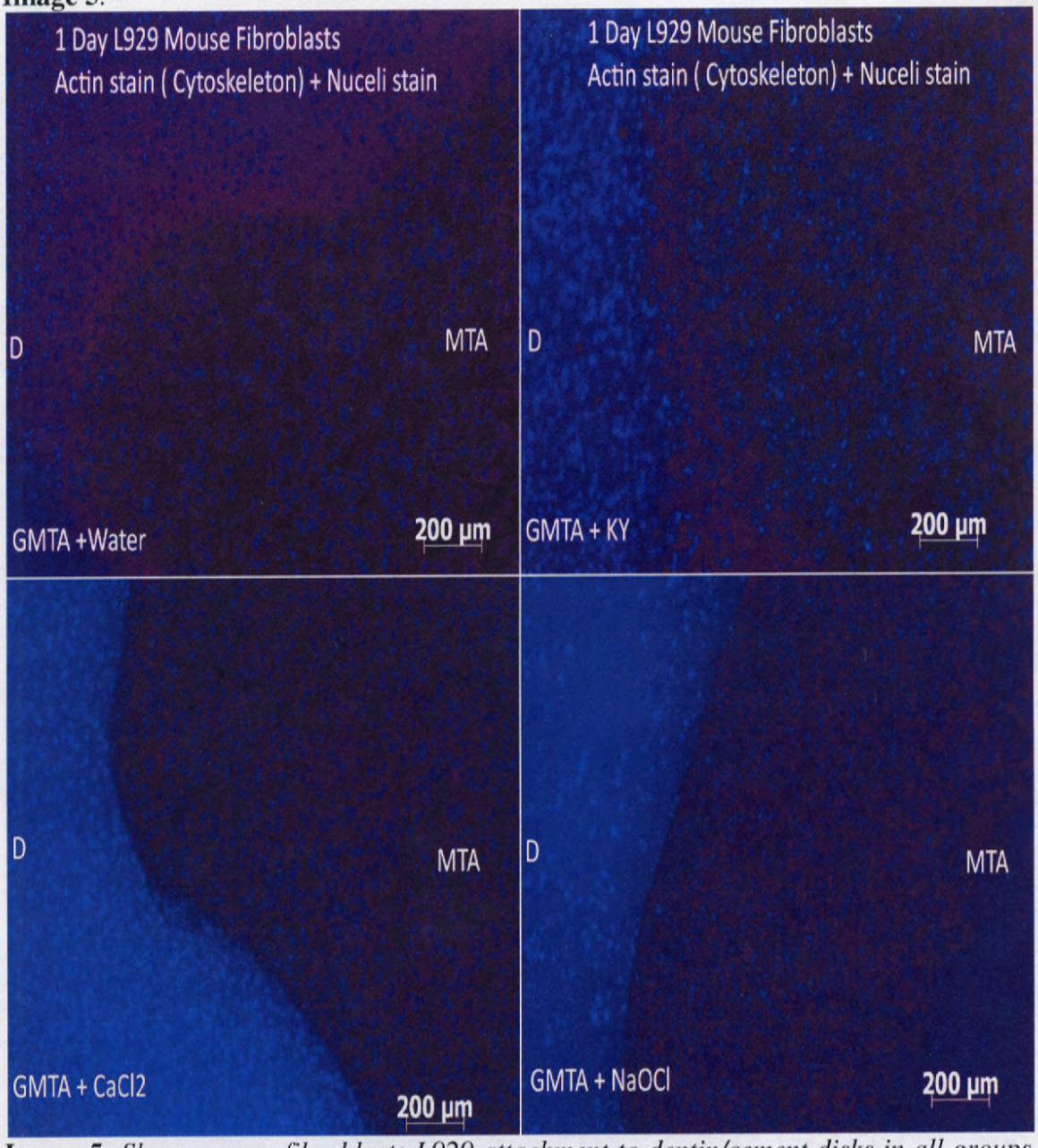

Image 5: *Shows mouse fibroblasts L929 attachment to dentin/cement disks in all groups after 24 hrs. No differences in cell attachment were noted.(D): dentin. The cytoplasm was stained red and nuclei were stained blue. Fluorescent microscope image at 5x magnification.*

Image 6:

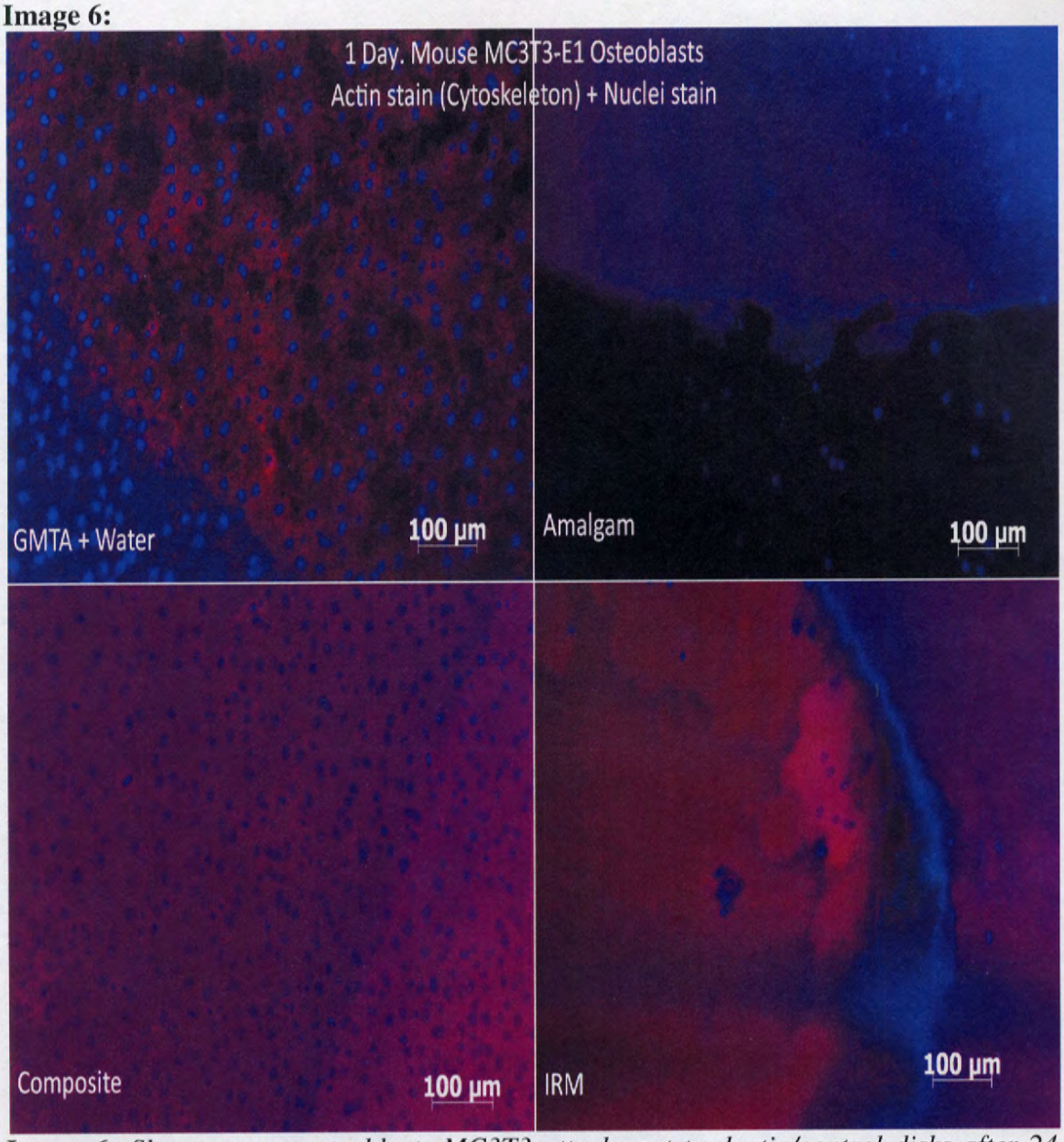

Image 6: *Shows mouse osteoblasts MC3T3 attachment to dentin/control disks after 24 hrs. Osteoblasts attached to GMTA and water as well as composite. Cells did not attach to IRM. Few cells attached to amalgam. Cells were rounded and showed no spreading. The cytoskeleton was stained red and nuclei were stained blue. Fluorescent microscope image at 10x magnification.*

Image 7:

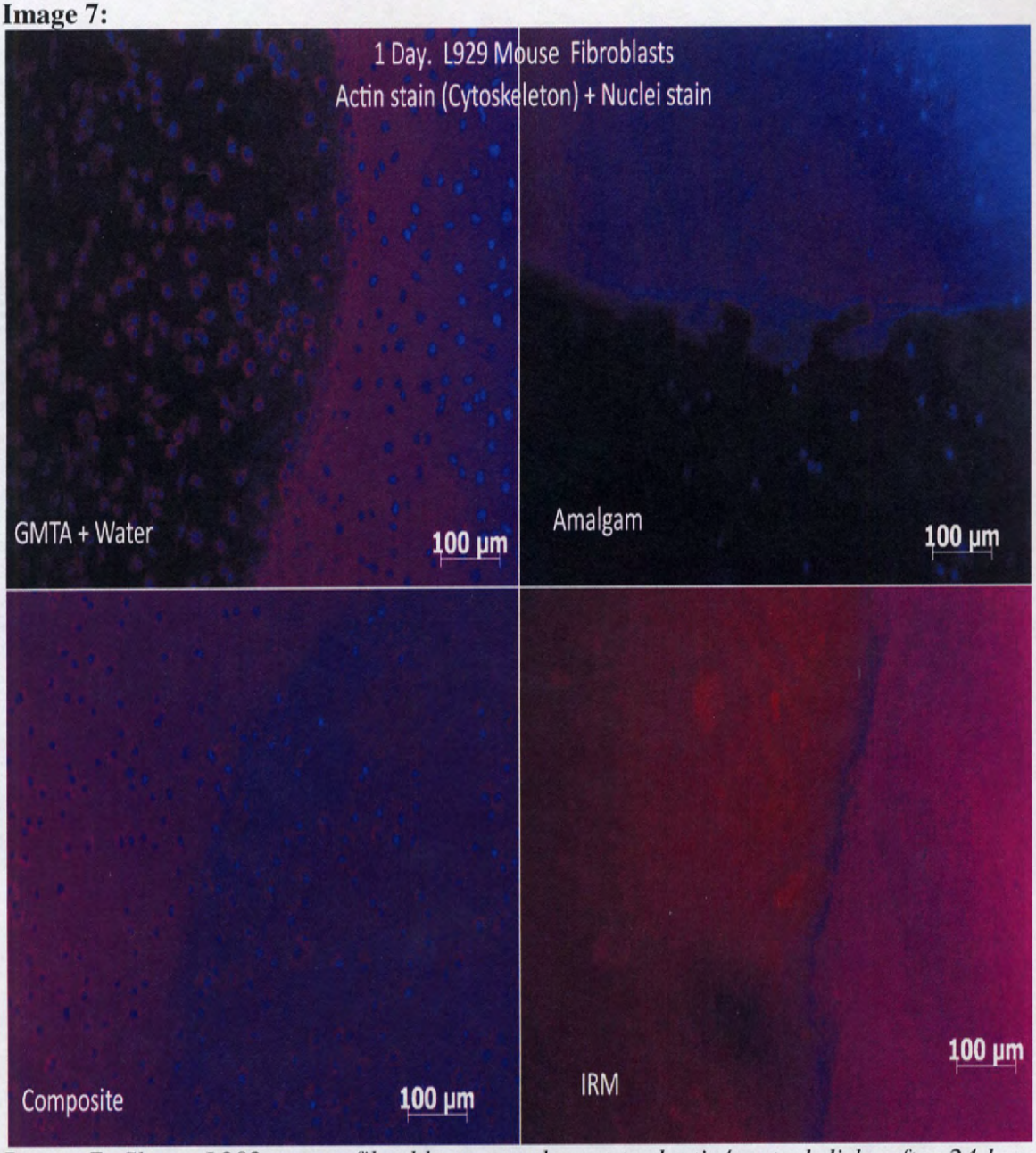

Image 7: *Shows L292 mouse fibroblasts attachment to dentin/control disks after 24 hrs. Osteoblasts attached to GMTA and water as well as composite. Cells did not attach to IRM. Few cells attached to amalgam. Cells were rounded and showed no spreading. The cytoskeleton was stained red and nuclei were stained blue. Fluorescent microscope image at 10x magnification.*

Figure 7:

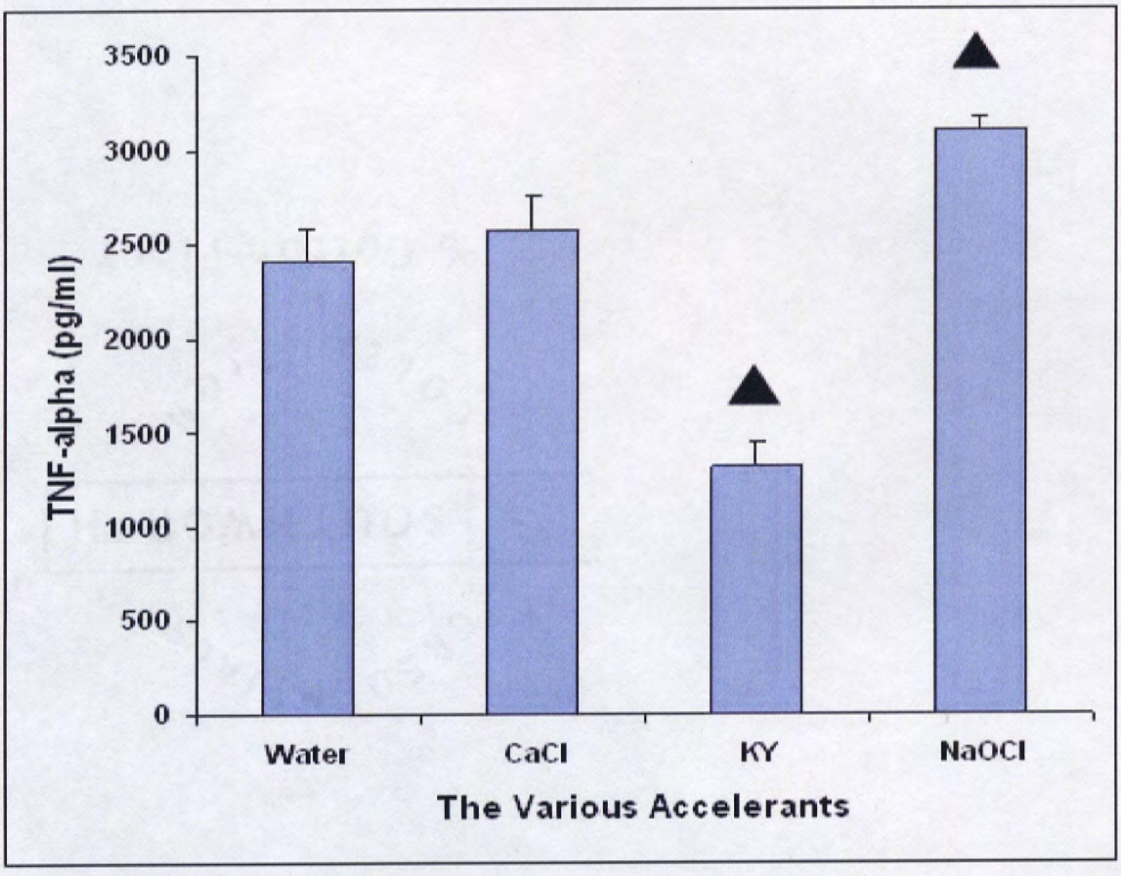

Figure 7: *TNF-alpha release when cells were cultured with the various MTA disks. (▲= P<0.05)*

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