CHAPTER II

LITERATURE REVIEW

This chapter was divided into seven parts as follows:

I. The use of magnets in orthodontics

II. The corrosion products released from the magnets

III. The cytotoxic effect of corrosion products from the magnets

IV. *In vitro* cytotoxicity tests

V. Apoptosis or programmed cell death

VI. Apoptosis detection systems

VII. Detection of apoptotic cells with the exposed phosphatidylserine (PS) on cell membrane

I. The use of magnets in orthodontics

The interest in using magnets for generating orthodontic forces began with the widespread availability of rare earth magnetic alloys, such as samarium cobalt and neodymium-iron-boron magnets (Tsutsui, 1979; Sandler, 1989; 1991). These rare earth magnets are categorized into hard or permanent magnets due to their high coercivities (the ability of the magnet to resist demagnetization) and high magnetizations when removed from the applied magnetic field. The ideal magnets used in orthodontics should be small, powerful, permanent, and resistant to corrosion. In addition, magnets should produce optimal physiological force. The most important property of intraoral magnets is biocompatibility with oral environment.

The significant advantages for using magnets as an alternative orthodontic forces generating system are their abilities to produce a measurable force continuously over a long period of time. They can be made to attract (to pull) or to repel (to push) the teeth. The force they deliver can be directed, and they can exert their forces through mucosa and bone as there is no need for the magnets to directly

contact oral tissues. Several reports revealed the successful use of these permanent magnetic devices generating both intra and intermaxillary forces (Blechman and Smiley, 1978; Kawata and Matsuka, 1979; Kawata *et al*., 1987; Muller, 1984; Blechman, 1985; Gainelly *et al*., 1989; Bondemark and Kurol, 1992; Sandler *et al.,* 1989; Sandler and Springate, 1991; Darendelier and Friedli, 1994; Dellinger, 1986; Wood and Nanda, 1988; Darendelier *et al.,* 1992; 1993; 1995; Bondemark *et al.,* 1997; Vardimon *et al*., 1987). Although magnets are very useful, there are a number of drawbacks that severely affect their performances, such as 1) dramatic reduction of force generated between two magnets with increasing distance, 2) significant and irreversible loss in force if the magnets are heated, 3) dramatic reduction in force if the magnets are not aligned to the others, and 4) severe corrosion of magnets in the mouth. In addition, their prices are high. Therefore, the use of magnets for orthodontic treatment is currently limited.

Watanakit and Jotikasthira (2001) investigated available low cost commercial magnets, being potentially useful for future clinical application. Their findings indicated that these magnets were categorized in the neodymium-iron-boron group. The compositions were comparable with those of the orthodontic magnets, and they could generate the attracting force to orthodontic brackets greater than that generated by orthodontic magnets with an equal volume.

II. The corrosion products released from the magnets

When the metals are attacked by natural agents, such as air and water, they are known to be corroded via the degrading process known as corrosion, resulting in dissolution or formation of respective dissolved metal ions or corrosion products. The corrosion products are chemical compounds which are produced by metal undergoing chemical or electrochemical reaction with nonmetallic elements, such as oxygen and chloride in the environment. The corrosion products may accelerate, retard, or have no influence on the subsequent deterioration of the metal surface.

The most common example of corrosion is rusting of iron. The iron is combined with oxygen in air and water to form hydrated oxide of iron. This oxide

compound is porous. The iron-oxide is bulkier, weaker, and more brittle than the non-oxide form. Another example is the use of magnetic alloys in the oral environment. Intraoral magnets can produce general mechanisms of corrosion and subsequently release corrosion products. Water, oxygen, and chloride ions that are present in saliva contribute to corrosion. Various acids such as phosphoric, acetic, and lactic can also lead to corrosion (Marek, 1996).

In the oral cavity, numerous variables facilitating the corrosion of metal are temperature, quantity and quality of saliva, plaque, pH, protein, physical and chemical properties of food and liquids, and oral health conditions (Park and Shearer, 1983). Chemical compositions of alloys (Geis-Gerstorfer and Weber, 1987) and masticatory habits (Obatake *et al*., 1991) also influence corrosion.

III. The cytotoxic effect of corrosion products released from the magnets

The intraoral magnets may be used for a long period of time, and then be corroded. Subsequently, corrosion products may be released. Various methods have been suggested to eliminate the corrosion with varying degrees of success (Riley *et al*., 1999; Bonemark *et al.,* 1994a; 1994b; Vardimon and Mueller, 1985). Noble metal, i.e. aluminum or titanium, is coated on the magnet surface to prevent corrosion.

There have been some experiments reported in the literature pertaining to the biocompatibility of either leachable or corrosion products of magnets. Sandler *et al.* (1989) demonstrated that the leacheable products from neodymium-iron-boron magnets had no short-term cytotoxic effect on UMR-106 cells (cloned hormone-respond osteosarcoma cell line). Evan and McDonald (1995) showed that corrosion products released from these magnets consisted mainly of iron compounds, and that these corrosion products did not adversely affect cell proliferation. The findings from these studies were in agreement with the results of Panichakul and Jotikasthira (2003). They reported that corrosion products released from commercial magnets did not have short-term effect on the viability and the growth of cultured human gingival fibroblasts. The quantities of corrosion products released from the commercial magnets were usually greater than those from the orthodontic magnets. The compositions of

corrosion products released from these magnets consisted mainly of boron and silicon.

Only a few studies have focused on the possible cytotoxic effects of corrosion products released from magnets. The *In vitro* study of Kawahara *et al*. (1968) revealed that the iron showed cytotoxic effect by preventing cellular outgrowth and multiplication, and that the degree of cytotoxicity did not necessarily correspond with the degree of corrosion. Linder-Aronson (1992) made the assumption that either the inhomogeneous static magnetic field or the magnetic material was able to influence vital process in biologic tissue. The subsequent experimental results confirmed the adversely biological responses (Camilleri and McDonald, 1993; Linder-Aronson *et al*., 1992; 1995; 1996).

IV. *In vitro* cytotoxicity tests

In general, the *in vitro* cytotoxicity test could be performed by the growth, viability, and the apoptosis assays. The pre-clinical tests (level 1 biocompatibility test) may be considered the screening phase for evaluating materials to be used in a clinical application (level 3 biocompatibility test). These easily controlled test situations can save lives of animals while simulating specific *in vitro* condition. These methods are based on cell culture techniques which are more economic than performing experiments with animals (level 2 biocompatibility test). Cell culture technique can provide a continuous supply of homogenous cellular materials for biochemical experiments. Furthermore, the statistical analysis can be performed with high accuracy.

The gingival epithelial cells are one of the most appropriate cells for *in vitro* studies. The epithelium could be easily separated from their connective tissue with the specific enzyme. Furthermore, the culture procedures are not too complicated. The most important reason for selecting these cells in this *in vitro* study is that the leachable cytotoxic elements released from the intraoral magnets directly contact these cells.

Epithelia

1) Development of the epithelia

Epithelia are derived from all three germ layers of the embryo (ectoderm, mesoderm and endoderm). The relative degree of development of these three germ layers varies considerably in different epithelia. Ectoderm, for example, gives rise to the covering of the skin and body openings, while endoderm generates the lining and glands of the digestive tract, and the lining of the respiratory tract. Epithelia of mesodermal origin are classified separately as mesothelium (the serous lining of the pericardium, pleural, and peritoneal cavities) and endothelium (the lining of the blood and lymphatic vessels) (Shaw, 1996).

2) Structure of the epithelia

Epithelia are essentially surface tissue. They usually form a covering for connective tissue from which they are separated by a basement membrane. These tissues consist of close-knit polyhedral cells bound laterally to each other by cell junction. They are invariably avascular tissue, and are nourished by the capillaries of the underlying connective tissue. The classification of lining and covering epithelia is based on their morphologies and characteristics.

The gingival epithelia are one of best known protective epithelia which are composed of the oral epithelium, the sulcular epithelium, and the junctional epithelium, all of which are stratified squamous epithelia. The various layers of stratified squamous epithelia as seen by electron microscopy consist of a stratum basale, stratum spinosum, stratum graulosum, and stratum corneum, also referred to as basal, prickle-cell, granular, and cornified layers, respectively. About 90 percent of the gingival epithelial cells are keratin-forming cells, so-called keratinocytes. The remainders are referred to as nonkeratinocytes. They are replaced every 1 to 2 weeks. New cells are produced by cell division in the basal layer, while superficial layers are lost by desquamation into the oral cavity (Ramfjord and Major, 1979).

3) Role of the epithelia

The functions of epithelia are those that are clearly related to a superficial tissue, namely protection, absorption, and secretion. (Cole and Eastoe, 1988). The nature of epithelia is a front-line defense against physical, chemical, and microbial insults; therefore, the epithelial cells are responsible quickly to any sudden cell loss (for example injury).

V. Apoptosis or programmed cell death.

Most cells from higher eukaryotes have the ability to be self-destroyed by activation of an intrinsic cellular suicide program when they are no longer needed or have become seriously damaged (Kerr *et al.,* 1972). The term apoptosis, which is often equated with programmed cell death (PCD), is used in Greek to describe dropping off or falling off petals from flowers or leaves from trees.

Apoptosis is a well-documented phenomenon that has been observed in many cell systems. It plays an important role in a variety of physiologic and pathologic conditions, in tissue and organ development during embryogenesis as well as in adult tissue during cell turn over (Cohen, 1992). Some reports have demonstrated that various types of cell death including apoptosis occur in epithelial cells of the dental lamina, fibroblasts, bone-related cells, and amaeloblasts during tooth development (Khaejornbut *et al.,* 1991; Ten Cate and Anderson, 1986; Bronckers, 1996a; 1996b; Nishikawa and Sasaki, 1995). The process varies with cell types and stimuli. Although accumulating data imply that apoptosis is a gene-regulated phenomenon, little is known about the mechanisms of apoptosis at the molecular level (Ueda and Shah, 1994).

Apoptosis refers to morphological alterations exhibited by dying cells that include cell shrinkage, membrane blebbing, increased cytoplasmic density, chromatin condensation, and segregation into sharply circumscribed masses that abut the nuclear membrane and form blister-like protrusions (“budding”). Cells undergoing apoptosis often fragment into membrane-bound apoptotic bodies that are readily phagocytosed and digested by macrophages or neighboring cells without generating

an inflammatory response. These changes distinguish apoptosis from cell death by necrosis.

. Necrosis refers to the morphology most often seen when cells die from severe and sudden injury, such as ischemia, sustained hyperthermia, or physical and chemical trauma. In necrosis, there are early changes in mitochondrial shape and function, and the cell loses its ability to regulate osmotic pressure, swells, and ruptures. The contents of the cell are spilled into the surrounding tissue, resulting in generation of a local inflammatory response.

VI. Apoptosis detection systems

A variety of techniques have been proposed for detection of apoptotic cells. The most widely used methods include the study of morphology, the analysis of DNA degradation, the DNA end-labeling techniques, the flow cytometric analysis, and the nuclease assays. Features of the different methods are as follows; 1) morphological analysis by microscopy, 2) analysis of DNA degradation, 3) DNA end-labeling technique, 4) analysis by flow and laser-scanning cytometry, 5) analysis of endonucleases (Sgonc and Gruber, 1998).

1) Morphological analysis by microscopy.

The traditional visualization method is used to measure apoptosis. It is characterized by morphological alterations exhibited by dying cells that are already explained above. These morphological changes can be detected with the **electron microscope** (Kerr *et al*., 1972; Gorman *et al*., 1996). The application of quantitative digital imaging techniques to electron microscopy to visualize fine degradations of gray levels enables the detection of even subtle changes in nuclear densities (Payne and Cromey, 1992).

Budding and formation of apoptotic bodies can also be seen by **phase contrast microscopy**. Another versatile tool is the **confocal laser scanning microscopy**, powerful for both morphological analysis and macromolecular localization (Smith *et al*., 1991). In principle, morphological assessment has been the most reliable method for the

identification of individual apoptotic cell. However, quantification of apoptosis by microscopy is cumbersome and not an enough powerful technique. Morphological evidence of apoptosis is not consistent in all cell types and is not always followed by DNA fragmentation (Cohen *et al*,. 1992). Moreover, this method is subjective, offers only a numerical impression of the occurrence of apoptosis in cytological preparation, and is not suitable for kinetic studies or statistical analysis.

2) Analysis of DNA degradation

During early apoptosis, a Ca^{2+}- dependent endonuclease cleaves cellular DNA into histone-associated DNA fragments (mono- and oligo-nucleosomes) prior to the appearance of morphological changes. Initially, the DNA fragments are large (50-300 kb), but are later digested to oligonucleosomal size (multimers of 180-200 bp). Internucleosomal cleavage by an endonuclease can be visualized after electrophoresis as a typical DNA “ladder” that is considered to be a biochemical hallmark of apoptosis. Nevertheless, this method is time consuming, lacks cell specificity, is qualitative rather than quantitative, and does not offer kinetic information about the apoptosis process.

3) DNA end-labeling technique

This DNA based assay is designed for the specific detection of apoptotic cells within a cell population consisting of both apoptotic and nonapoptotic cells .With the ***in situ*** **nick translation assay**, TUNEL (TdT-mediated dUTP Nick-End Labeling) assay, the fragmented DNA of apoptotic cells is labeled by catalytically incorporating fluorescent dyes at the 3’-hydroxyl ends of the fragmented DNA using enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail. The fluorescein-labeled DNA then can be visualized directly by fluorescence microscopy or quantified by flow cytometry.

4) Analysis by flow and laser-scanning cytometry

Flow cytometry (FCM), by providing the possibility of rapid, accurate, and unbiased measurement of a variety of cell components on a cell by cell basis, has become an indispensable methodology in the analysis of cell death. Several applications of flow cytometry include the studies of cell death mechanism, the studies

of cell function, the identification and quantification of apoptotic or necrotic cells, which is generally based on the presence of a particular biochemical or molecular marker that is a characteristic for either apoptosis or necrosis (Daezynkiewicz and Bender, 2001).

The common principles of flow cytometric method are that primary or cultured cell lines are treated with various apoptotic agents, after a period of time, numerous fluorescent dyes or markers can be added to the cells, and the cells are immediately examined. During flow cytometry, the cells individually pass through a laser that excites the dye or marker. The fluorescent emission then passes through various filters and is detected by photomultiplier tubes. Several different cellular characteristics, **such as cellular viability, cell size, DNA content, changes in the mitochondrial membrane potential, phosphatidylserine exposure, caspase activity**, can be determined using flow cytometry (Ormerod *et al.,* 1993; Dive *et al*., 1986; van Engeland *et al*., 1996; Koopman *et al*., 1994; Sgonc and Gruber, 1998; Bortner and Cidlowski, 2001).

A variety of flow cytometric methods, developed for the identification of apoptotic and necrotic cells, have been modified and adapted for laser-scanning cytometry (LSC). The laser-scaning cytometer (CompuCyte, Cambridge, MA) is a microscope-based cytofluorometer that offers advantages of both flow cytometry and image analysis. Thus, fluorescence of individual cells is measured rapidly and accurately by LSC similar to that of FCM.

5) Analysis of endonucleases

Because DNA fragmentation is a primary event in apoptosis and may be the actual cause of cell death, the identification of the endonuclease (s), which is (are) responsible for this cleavage, is also of interest in studies of apoptosis. Nuclease assays have been described that the detection of DNA-degrading activity in SDS-polyacrylamide gels in which a nonradioactive or ^{32}P-labeled nucleic acid substrate is incorporated into the gel matrix, and the nuclease activity is ascertained by loss of the substrate from the gel. This assay is simple to perform, but lacks the specificity to detect internucleosome DNA degradation.

Another nuclease activity assay, specifically developed to study nucleases cleaving DNA at internucleosomal sites (Schwartzman and Cidllowski, 1991; Compton, 1991), utilizes nuclei of cells not susceptible to glucocorticoid-induced lysis (HeLa or chicken red blood cells) as a substrate to measure nuclease activity of glucocorticoid-treated thymocyte nuclear protein extracts. This assay allows differentiation between random DNA degradation and the internucleosomal cleavage that is typical during apoptosis.

VII. Detection of apoptotic cells with the exposed phosphatidylserine (PS) on cell membrane

Both prokaryotic and eukaryotic cells are surrounded by a plasma membrane or cellular membrane, which defines the boundary of the cell and separates its internal contents from the environment. The plasma membranes are composed of both lipids and proteins. The basic structure of the plasma membrane is the phospholipid bilayer, which is impermeable to most of water-soluble molecules. Proteins embedded within the phospholipid bilayer carry out the specific functions of the plasma membrane, including selective transport of molecule and cell-cell recognition. The plasma membrane thus plays a dual role, i.e. isolates the cytoplasm and mediates interactions between the cell and its environment.

The plasma membranes of animal cells contain four major phospholipids. The outer leaflet consists predominantly of phosphatidylcholine, sphingomyelin, and glycolipids, whereas the inner leaflet contains phosphatidylserine (PS) and phosphoinositol. In addition to the phospholipids, cholesterol and glycolipids are distributed in both leaflets.

In recent years, a novel assay for the detection of apoptotic cells by flow cytometry, using fluorescein isothiocyanate (FITC) labeled Annexin V to phosphatidylserine had been developed (Vermes *et al*., 1995). Quantification of the frequency of cells exposing phosphatidylserine (PS) by measuring a number of cells that bind to Annexin V is one of the simplest methods with high sensitivity of the assays available for early detection of apoptosis. This novel Annexin V binding assay, in combination with a dye

exclusion test, permits the detection of the early phase of apoptosis before the loss of membrane integrity. Moreover, it appears to be sensitive, easily performed, and routinely tested using primary cells or cell lines undergoing an apoptotic death (Overbeeke *et al*., 1998).

In the early stages of apoptosis, changes occur at the cell surface. One of these plasma membrane alterations is the translocation of PS from the inner to the outer leaflet (Fadok *et al*., 1992). Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS, and binds to cells with exposed PS (Raynal and Pollard, 1994). Hence, Annexin V can be used as a marker of early detection of apoptotic cells. Apoptotic cells become annexin V-positive after nuclear condensation has started, but before the cell has become permeable to the fluorescent dyes such as propidium iodide (PI).

Translocation of PS to the external surface is not unique to apoptosis, but occurs also during necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs; the cell membrane loses its integrity and becomes leaky. Therefore, staining cells with FITC-conjugated annexin V and propidium iodide (PI) can identify subpopulation of cells with membrane changes and associated loss of membrane integrity. PI is used for a standard flow cytometric viability assay to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damage cells are permeable to PI. In necrotic cells with damaged cell membrane, PI induces a red fluorescence of the DNA, while it is excluded by a preserved cytoplasmic membrane. Hence, during the initial phase of apoptosis, the cells are still able to exclude PI, and therefore do not show any red fluorescence signal similar to that of living cells.

This two-bivariate flow cytometry assay for apoptosis allows not only discrimination among **viable cells** (PI and Annexin V-FITC negative), **apoptotic cells** (PI negative, Annexin V-FITC positive), and **necrotic cells** (PI and Annexin V-FITC positive), but also quantification of those cell subpopulations (Lawrence and Jonathan, 2001; Studzinski, 1999).