CHAPTER V
DISCUSSION

The discussion was presented as follows.

Part I: The morphological analysis by propidium iodide and fluorescence microscopy

Part II: The flow cytometric analysis by FITC-conjugated annexin V and propidium iodide assay

Part I: The morphological analysis by propidium iodide and fluorescence microscopy

In order to obtain a more precise picture of the potential cytotoxicity from the corrosion products, the morphological study was initially conducted. It was found that the apoptosis was rather easily detected by the characteristic nuclear morphology using propidium iodide and fluorescence microscopy, which allowed apoptotic cells to be distinguished from healthy and necrotic cells. Surprisingly, the features of necrotic cells could not be found in any control and experimental groups, even when the cells were exposed to corrosion products for 5 days. In general, cell death caused by toxic agents had a morphological characteristic of necrosis. Therefore, it could be postulated from this study that the corrosion products released from commercial magnets have mild cytotoxic effect on cultured human gingival epithelial cells, or an apoptosis is a major pathway for cell death by corrosion products. Therefore, it would be interesting to further elucidate a molecular mechanism concerning the activation of the apoptotic cell death pathway, such as activation of a cascade of caspase enzymes, endonucleases responsible for DNA cleavage, etc., by corrosion products in the future study.

It was interesting to note that the results from this study showed no marked difference in terms of the number of apoptotic nuclei by fluorescence microscopy between the untreated control and corrosion product-treated cells. The reason might

be because these treated cells were detected during the initial phase (latent phase) of apoptotic cell death and the cells might therefore look morphologically normal. However, these cells might be actively preparing for death at the molecular levels (Pollard and Earnshaw, 2002). Otherwise, the morphological analysis is solely a qualitative assessment of nuclear shrinkage and chromatin condensation, so we were not able to really quantify the exact number of apoptotic cells in both control and treated groups. This limitation could then be overcome by another analysis for the presence of phosphatidylserine on the outer cell membrane, another hallmark of apoptosis, by the flow cytometry in the next section.

Although different cell types do not necessarily display all the hallmarks of apoptosis, there are similar features shared by cells undergoing apoptosis, which include shrinkage, blebbing, and chromatin condensation. Therefore, a detection technique based on apoptosis-specific biochemical changes, such as DNA fragmentation, exposure of phosphatidylserine (PS) on the outer cell membrane, and proteolysis of protein should be simultaneously conducted to ensure that the damage phenomenon of the cells has certainly occurred (Studzinski, 1999). This was why we had conducted two different apoptotic analyses, i.e. the morphological study of apoptotic nuclei by staining with a fluorescent dye and the flow cytometric analysis of phosphatidylserine exposure on the outer cell membrane, which would be discussed in the next section.

Part II: The flow cytometric analysis by FITC-conjugated annexin V and propidium iodide assay

The results from this study showed that the corrosion products released from commercial magnets significantly affect cells by inducing apoptotic cell death, whereas they did not significantly induce necrotic cell death *in vitro*. An increase in the number of apoptotic cells, when exposed to the solution of commercial magnet byproducts, may probably be due to the breakdown metal compounds from the magnet, such as neodymium chlorides and iron oxides. Rare earth magnets and, in particular, those containing neodymium, are susceptible to corrosion (Vardimon and

Muller, 1985) with a release of potential harmful products. Several studies have confirmed that rare earth magnets are more likely to be corroded and their susceptibility to corrosion depends on the types of alloys in the magnet and their relative position in the electrochemical series. The study by Vardimon and Muller (1985) showed that uncoated neodymium rare earth magnets were highly susceptible to corrosion because they contained high amounts of iron (72 % by weight). Furthermore, it was previously shown that irons could induce a cytotoxic response by preventing cellular outgrowth and multiplication (Kawata et al, 1968). In addition, while corrosion preferentially takes place by oxidizing the neodymium-rich phase that resides between the $Nd_2Fe_{14}B$ grains, the identification of $NdCl_3$ particles in the rare earth magnet is another concern, since this compound is known for its cytotoxic potential having a cited LD_{50} of 600 mg/kg body weight from the intraperitoneal route in mice, and 5.25 g/kg body weight when administered orally to guinea pigs (Haley, 1965). Its existence can lead to the possibility of organ destruction and toxicity (Evan and McDonald, 1995).

The biological safety of commercial magnets containing rare earth elements had been previously evaluated for the possible cytotoxic effect of their corrosion products on both cultured human gingival fibroblasts and epithelial cells (Panichakul and Jotikasthira, 2003; Jotikasthira *et al*., 2005). The outcomes from their study had demonstrated that corrosion products released from commercial magnets had no short-term adverse effect on the viability and growth of both cultured human gingival fibroblasts and epithelial cells. The possible explanations of the contradictory results between their study and ours may be due to the difference in the analyses of targeted mechanisms used between their study and ours. They studied the effects of corrosion products on the cellular growth by measuring newly DNA synthesis, while we measured the percentages of apoptotic and necrotic cells treated with corrosion products. Consequently, although the growth of cultured cells is not affected by corrosion products according to the results from their *in vitro* study, it does not mean that cultured oral cells will not be damaged and ultimately dead by corrosion products.

Indeed, we have shown in this study that cultured oral epithelial cells were damaged by corrosion products until they had to commit apoptotic cell death. Moreover, the change in PS externalization occurs in the very early stage of apoptosis prior to any changes that might occur in the nucleus. Therefore, it is possible that the detection of apoptotic cell death by examining the translocation of phospholipid molecules on the cell membrane should be quicker and earlier than a study of DNA changes done in their study.

With regard to the types of solvent used to corrode the magnet, either culture medium or 0.9% NaCl solution could be used in accordance with ISO 10993-12:1996. However, 0.9% NaCl solution was selected in this study because the quantities of corrosion products released in 0.9% NaCl were greater than those in culture medium (Panichakul and Jotikasthira, 2003). Furthermore, we have proven in this study that 0.9% NaCl solution did not cause any further cytotoxic effect in terms of an increase in the percentages of apoptotic and necrotic cells when compared with those in control untreated group.

A flow cytometric analysis with FITC-conjugated annexin V and propidium iodide was conducted in this study to measure the percentages of two different forms of cell death, i.e. apoptosis and necrosis. This two-parameter FCM method had more ability to detect apoptotic cells than other three traditional methods (microscopy, DNA flow cytometry, DNA electrophoresis). It appears to be more sensitive and correlated well with other tests. It also permits the detection of the early phase of apoptosis before the loss of cell membrane integrity and is relatively easy to perform. Moreover, it is an appropriate method for quantitative analysis of apoptotic and necrotic cells, which makes possible to measure the kinetics of the cell death process over time and in relation to the cell cycle (Vermes et al., 1995). The finding from Koopman *et al* (1994) demonstrated that this assay was likely to be applicable to all cell types. In contrast, the morphological assessment, which has been the most reliable method for the identification of individual apoptotic cells, is subjective and offers only a numerical impression of the occurrence of apoptosis in cytological preparation. In particular, it is

not suitable for a kinetics study or statistical analysis. The method, described by Nicoletti *et al* (1991) for quantitative measurement of hypo-diploid cells using DNA-FCM, is easy to perform and gives a reliable estimation of the number of apoptotic cells. However, it measures only the cell, which has already reached the stage where a substantial amount of the DNA has been fragmented and leaked from the cell or has been altered and lost staining capacity. Therefore, this method provides no information regarding the number of apoptotic cells in the initial phase of the process. Measurement of DNA fragments provides a sensitive assay for the detection of DNA fragmentation, but this method is time consuming, lacks cell specificity, and is qualitative rather than quantitative; therefore, it does not offer kinetics information about the apoptotic process (Facchinetti *et al*., 1991; Olive *et al*., 1993).

For cells cultured in suspension, detection of PS exposure is relatively easy. For adherent cells, however, the situation is more complicated. When cells are grown on glass slides, they can be analyzed immediately after annexin V labeling using a fluorescence microscope. To obtain a single cell suspension of adherent cells for flow cytometric analysis, cells have to be detached from culture vessels. It has been shown that harvesting adherent cells by enzymatic digestion interferes with a reliable detection of PS exposure. In addition, digestion with trypsin and ethylenediaminotetraacetic acid (EDTA) before annexin V labeling can induce changes in the cell membrane, which lead to a false positive result. Trypsin or EDTA treatment after annexin V labeling interferes with the detection of bound annexin V because trypsin removes bound annexin V by proteolysis and EDTA chelates calcium ions, which are necessary for binding between annexin V and PS. Consequently, the method of cell scraping, used in this study, to harvest the cells from culture vessels was suggested prior to labeling adherent monolayer cells with annexin V (van Engeland, 1996). Nevertheless, extremely good care should be taken while scraping the adherent cells to prevent physical trauma, since cell death can happen with cell scraping like the results in this study, in which the percentages of apoptotic cells were relatively high even in the control untreated group.

The commercial magnets used in this study were uncoated and therefore not protected at all from high corrosive 0.9% NaCl solution. The study by Vardimon and Muller (1985) demonstrated that the uncoated neodymium containing magnets were susceptible to corrosion with the release of potentially harmful products. Therefore, coating the magnets with any protective biocompatible substances, such as noble metals (titanium, gold, etc), epoxy resin, stainless steel, and a thin layer of parylene will generate an effective barrier against corrosion (Bleachman, 1985, Vardimon et al., 1991, Cerny, 1978). Accordingly, several reports have demonstrated the acceptable biological compatibility of coated magnets with no adverse effects on oral tissue (Sandler et al., 1989; Bonemark et al., 1994a, 1994b, 1994c; Donohue et al., 1994).

According to the biological evaluation for *in vitro* cytotoxicity tests of ISO 10993-5; 1996, numerous methods are recommended to determine the cytotoxicity. These include 4 categories of evaluation: 1) the assessments of cell damage by a morphological means, 2) the measurements of cell damage, 3) the measurements of cell growth, and 4) the measurements of specific aspects of cellular metabolism. The pioneer work, done by Panichakul and Jotikasthira (2003) and Jotikasthira *et al.* (2003), of biocompatibility test of the corrosion products on cell viability and growth of cultured human gingival fibroblasts and epithelial cells, *(in vitro* evaluation category 1 and 3) had supported the safety of the commercial magnets for a use in Clinical Dentistry, in particular Orthodontics. However, the assessments of cytotoxic effect of these corrosion products on apoptosis and necrosis of cultured human gingival epithelial cells (in *vitro* evaluation category 1 and 2) have shown an opposite result, i.e. the commercial magnet is not safe for a use in the oral cavity with a moisture environment unless it is properly coated by any protective biocompatible substances or noble metals. Therefore, it is suggested that the cytotoxicity test on the oral cells be performed with the coated commercial magnets in the future study. In addition, the specific aspects of cellular metabolisms, i.e. an apoptotic pathway (in *vitro* evaluation category 4), should be further investigated for a complete evaluation of the biological testing of the commercial magnets.

LIMITATION OF THIS INVESTIGATION

1. The limited use of flow cytometer is allowed. Therefore, this study could only investigate the cytotoxic effect of CP solution on cultured human gingival epithelial cells, but not on other cell types, such as fibroblasts.

2. The *in vitro* study with cell culture requires technical experiences. There are several factors influencing cell culture procedures, and the control of these variables is essential for experimental condition.

3. The duration of exposure time to corrosion products was too short because the cells took only a few days to become confluent.

4. The price of materials, e.g. Annexin V-FITC apoptosis detection kit and cell culture medium, is high; therefore, fewer experiments could be repeated in this study.

SUGGESTIONS FOR FURTHER STUDY

1. The intraoral magnets directly contact with oral gingival fibroblasts when used for guiding eruption of impacted tooth. Therefore, the *in vitro* cytotoxicity evaluation of corrosion products released from coated commercial magnets should also be conducted in oral fibroblasts as well.

2. The cytotoxic effect of corrosion products released from commercial magnets on apoptosis should be studied in a dilution manner of CP solution to evaluate a lethal dose of CP in oral cells.

3. The *in vitro* study of cytotoxic effect of corrosion products released from commercial magnets on apoptosis of epithelial cells should be investigated at different time intervals to evaluate a possible time response.

4. Intraoral magnets are used for several months during orthodontic therapy. It would therefore be of particular interest to investigate possible cytotoxic effect of long-term exposure to corrosion products released from coated commercial magnets on oral tissue and cells.

5. The results of this *in vitro* study showed that the number of apoptotic cells in the presence of corrosion products was significantly higher than that of the control untreated cells (α<0.05). To determine the possible deleterious effect of corrosion products released from coated commercial magnets, an additional test, i.e. *in vivo* and/or a clinical trial, should be performed for a complete evaluation of the cytotoxic effect at the significant difference level of 0.01 (α<0.01).

6. The measurement of specific aspects of cellular metabolism (*in vitro* cytotoxicity evaluation category 4) associated with apoptosis, e.g. caspase enzymes, catalase enzymes, endonucleases, the mitochondrial transmembrane potential, intracellular reactive oxygen intermediates (ROI), and glutathione, should be further determined for a complete evaluation of the biological testing of the commercial magnets.