CHAPTER III

MATERIALS AND METHODS

MATERIALS

The following materials were used in this study:

1) Specimens

The commercial magnets

The commercial magnets used in this study contain mainly iron (Fe), Neodymium (Nd) with trace of cobalt (Co), Copper (Cu), and Gadolinium (Gd) (Panichakul and Jotikasthira, 2003). The disk-shaped commercial magnets with the diameter of 20 mm and the thickness of 2 mm were used in this study (Figure 3.1). All commercial magnets are mechanically ground and polished using wet abrasive papers in different grades (320, 600, and 1000), respectively.

Figure 3.1 The commercial magnets

2) Chemical reagents

2.1) Cell culture medium (Figure 3.2):

2.1.1) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), containing L-glutamine and antibiotic-antimycotic solution.

2.1.2) Keratinocyte Growth Medium (KGM) (Clonetics) supplemented with epidermal growth factor (rh EGF), bovine insulin, gentamycin A, hydrocortisone and bovine pituitary extract (BPE).

2.2) Trypsinizing solution (trypsin – EDTA)

2.3) Phosphate-buffered saline (PBS)

2.4) Dimethylsulfoxide (DMSO)

2.5) HEPES-buffered saline

2.6) Thermolysin (Collagenase type X) (Sigma)

2.7) Annexin V-FITC apoptosis detection Kit (BD Pharmingen) (Figure 3.3)

2.7.1) Annexin V-FITC

2.7.2) Propidium Iodide

2.7.3) Annexin V Binding Buffer

2.8) Corrosive media (ISO 10993-5; 15, 1999; 2000)

: 0.9% sodium chloride (NaCl) aqueous solution. This solution is very corrosive without inhibitory action.

2.9) Apoptosis inducers

2.9.1) Hydrogen peroxide (H_2O_2)

2.9.2) Thapsigargin, a SERCA pump inhibitor

2.9.3) Cell wall extract of *Fusobacterium nucleatum*, a Gram-negative periodontal bacterium

2.10) Absolute methanol

2.11) Glycerol

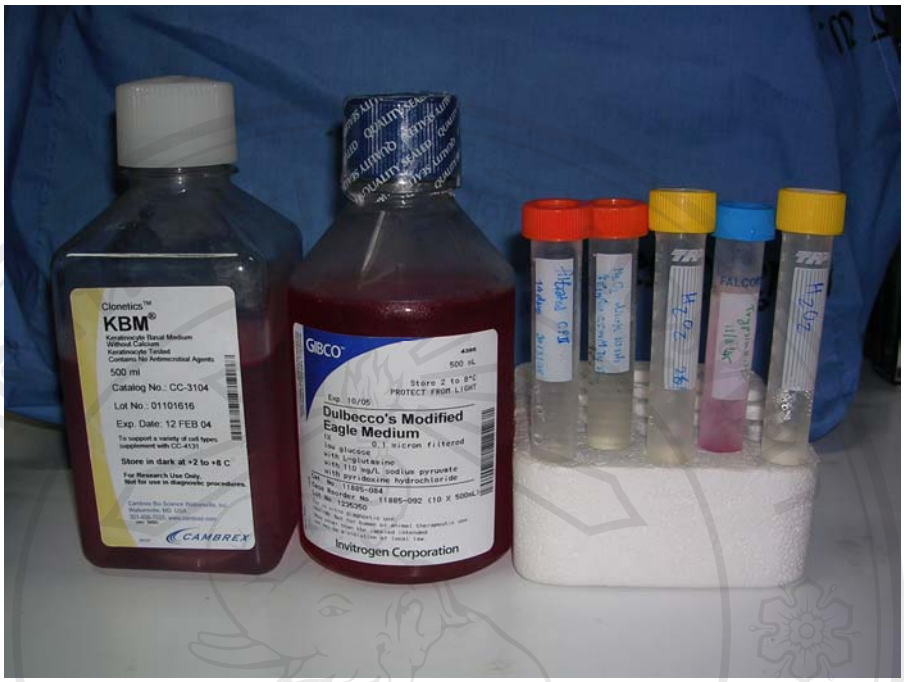

Figure 3.2 Chemical reagents for cell culture

Figure 3.3 The annexin V-FITC apoptosis detection Kit

3) Supplies

3.1) Plastic culture flasks (25 cm)^2
and plastic culture dishes (6 cm in diameter) (Figure 3.4)

3.2) Pasteur pipettes and automatic pipettes

3.3) Centrifuge tubes (50 ml, 15 ml)

3.4) Disposable syringe (10 ml) with filtration papers (0.22 µm)

3.5) Rubber policeman (Figure 3.5)

3.6) Glass slides

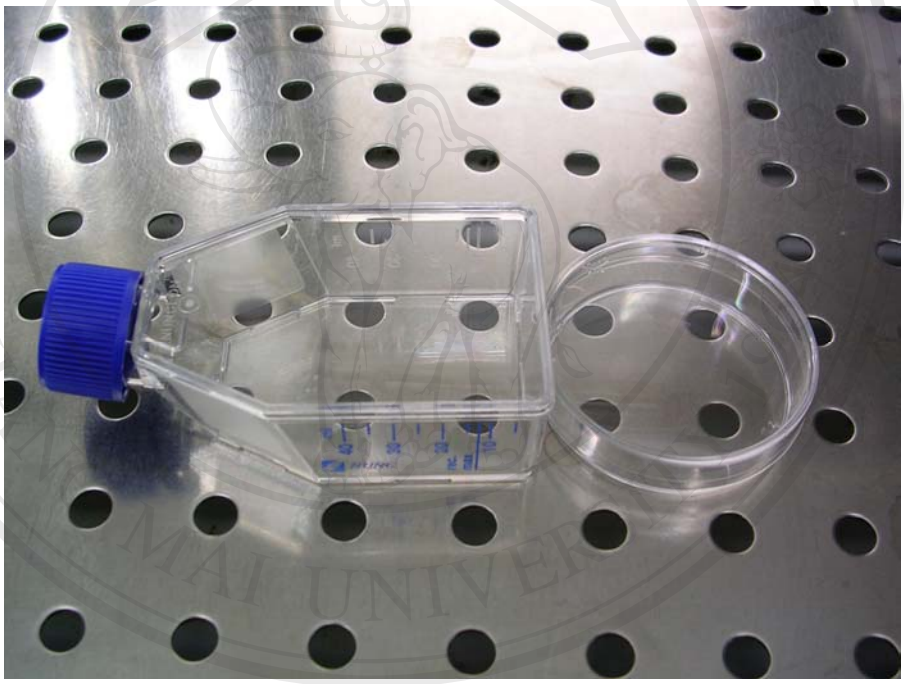

Figure 3.4 A plastic culture flask (25 cm^2) and a culture dish (6 cm in diameter)

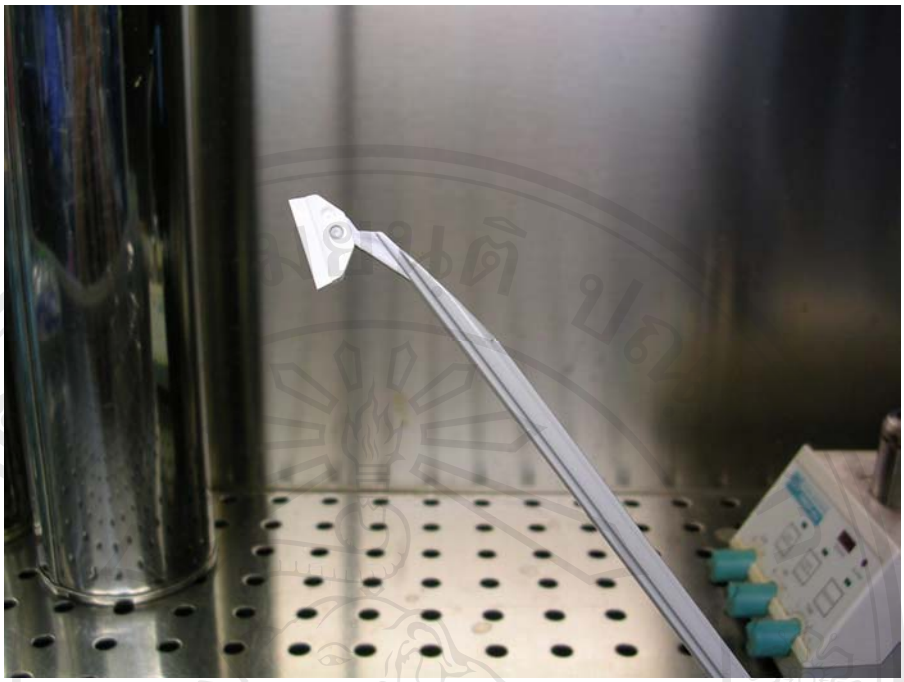

Figure 3.5 A rubber policeman

4) Instruments

4.1) A flow cytometer with a 488 nm argon laser (Figure 3.6)

4.2) A simple phase contrast microscope (Figure 3.7)

4.2) A fluorescence microscope (Figure 3.8)

4.3) An incubator, maintained at 37°C in 5% CO_2/ 95% air atmosphere with 100% humidity (Figure 3.9)

4.4) A lamina flow biological cabinet, which minimizes contamination of cultures (Figure 3.10)

4.5) An autoclave

4.6) A desk-top high speed refrigerated centrifuge (Figure 3.11)

4.7) A cytospin (Figure 3.12)

Figure 3.6 The flow cytometer

Figure 3.7 The phase contrast microscope

Figure 3.8 The fluorescence microscope

Figure 3.9 The CO_2 incubator

Figure 3.10 The lamina flow biological cabinet

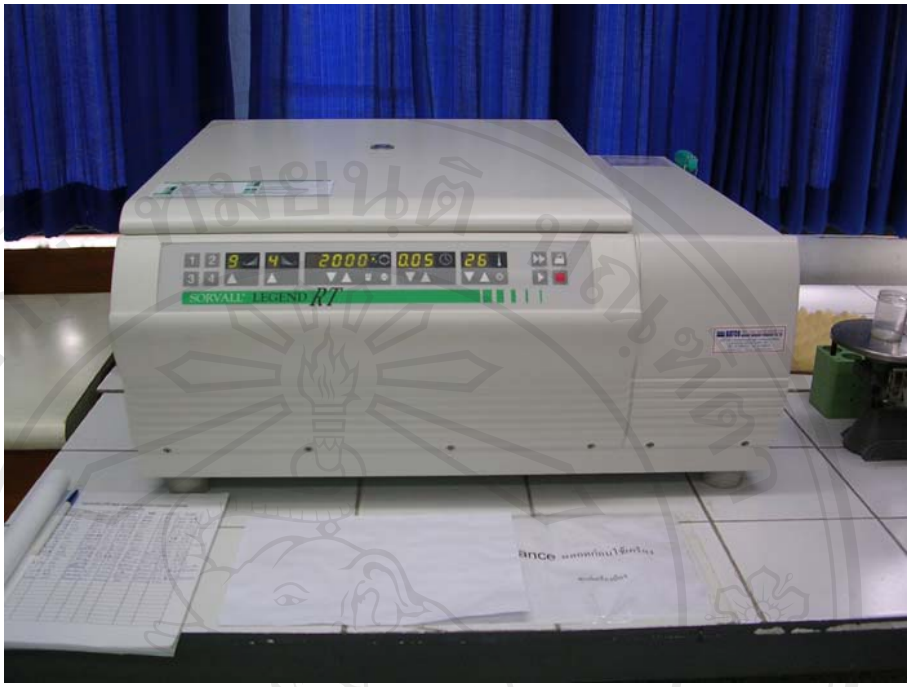

Figure 3.11 The desk-top high speed centrifuge

Figure 3.12 The cytospin

METHODS

The methods of the cytotoxicity test of corrosion products released from commercial magnets were divided into three parts as follows:

Part I: Preparation of corrosion products

Part II: Preparation of cultured human gingival epithelial cells

Part III. Experimental process to assess the apoptosis of cultured human gingival epithelial cells in the presence of corrosion products

Part I: Preparation of corrosion products

Specimen preparation

The sterilization process of specimens was performed before being used in the experiment, according to current clinical practice. All specimens were scrubbed by soft brush, immersed in 70% ethanol for 5 minutes, and then thoroughly rinsed twice with 10 ml sterile distilled water for 5 minutes. Finally, they were autoclaved at 121°C for 20 minutes.

Extraction method

The specimens of commercial magnet were carefully immersed in 0.9% NaCl.

The solution of 0.9% NaCl without any magnet byproducts in equal concentration was used as a negative control for the corrosive solution.

The magnets were incubated in 0.9% NaCl solution for 14 days at 37°C. After that, the corrosion products were collected, and then the specimens were removed from corrosive solution, which was later filtered by 0.22 μm filtration papers. The corrosion products were used as a stock solution for further apoptosis analyses.

Part II. Preparation of cultured human gingival epithelial cells

The normal gingival biopsies, overlying an impacted third molar and obtained from the Oral Surgery Department, Faculty of Dentistry, Chiang Mai University, were kept in cold HEPES-buffered saline containing fungizone and penicillin/streptomycin to kill microorganisms. The tissue was rinsed and vigorously shaked twice to remove any

blood or tissue debris. An epithelial sheet was separated from the gingival biopsy using 0.5 mg/ml thermolysin (collagenase type X) (Sigma; St. Louis MO) in HEPES-buffered saline containing 1.125 mM Ca^{2+} overnight at 4°C.

Single epithelial cells were mechanically removed from the epithelial sheet after incubating with trypsin-EDTA (0.05% trypsin, 0.53 nM EDTA) (GIBCO BRL). Primary epithelial cells were then grown in 0.03 mM Ca^{2+} serum-free keratinocyte growth medium (KGM) (Biowhittaker) at 37^0C in a 5% CO_2 humidified incubator until they reached 80 % confluence. Then, primary epithelial cells were removed from tissue culture dishes by incubation with trypsin-EDTA (GIBCO BRL) for 5 min. The trysinization was stopped by addition of an equal volume of Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL) supplemented with 1% penicillin/streptomycin (GIBCO BRL) and 10% FBS. To expand the number of cells, epithelial cells were further cultured in several tissue culture dishes determined by the number of experimental conditions. Otherwise, primary cultured cells could be kept frozen in 8.33% DMSO (Sigma), 20% FBS in DMEM at $1x10^6$ cells/cryotube (Nunc).

Part III. Experimental process to assess the apoptosis of cultured human gingival epithelial cells in the presence of corrosion products

When the biological tests were performed, the frozen cells were thawed. The cultured cells were diluted in KGM and seeded in culture dishes at a density of 10^5 cells in 5 ml medium per dish for conducting the experiment. The experimental process in this study was divided into 2 sections as follows:

1) Pre-experimental process

The cytotoxicity study of corrosion products released from commercial magnets on cultured human gingival epithelial cells was assessed in terms of cell apoptosis and cell necrosis (two forms of cell death). The percentage of normal viable, apoptotic, and necrotic cells was measured by two-bivariate FITC-conjugated annexin V and propidium iodide staining assay using flow cytometric analysis (FMC).

As mentioned previously, the measurement of Annexin V binding apoptosis assay, fluorescein isothiocyanate (FITC)-labeled Annexin V binding protein can be

used as a sensitive probe for PS exposure on the cell membrane. In viable cells with PS located at the inner surface of the plasma membrane, they are not reactive with green fluorescence (FITC-Annexin V negative), whilst in translocated PS to the external surface of the plasma membrane, apoptotic and necrotic cells were reactive with green fluorescence (FITC-Annexin V positive). However, apoptotic cells exclude PI which is used for this cell bivariate FCM assay, while necrotic cells do not. In cells with a damaged cell membrane, PI induced a red fluorescence of the DNA (PI positive), while it is excluded by cells with a preserved cell 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 (PI negative). Therefore, by staining cells with a combination of FITC-Annexin V and PI, it is possible to detect unaffected viable cells (Annexin V negative/ PI negative), early apoptotic cells (Annexin V positive/ PI negative), and late apoptotic cells ("necrotic stage" of apoptosis) as well as necrotic cells (Annexin V positive/ PI positive).

The FITC- Annexin V versus PI dot plot diagram demonstrates the green fluorescence emission signal on X-axis (FITC-Annexin V) and the red fluorescence emission signal (PI) on Y- axis. The information obtained from our preliminary study by flow cytometric analysis was shown in Figure 3.13.

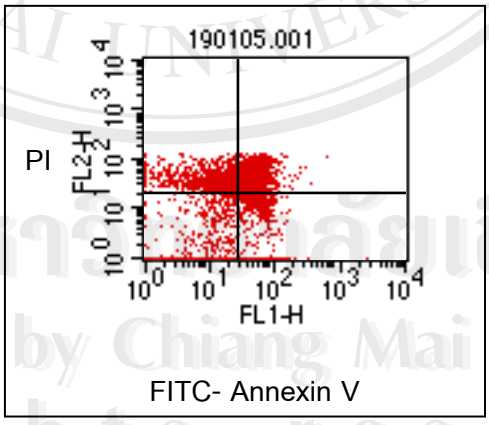

Figure 3.13 The dot plot diagram of FITC-Annexin V / PI flow cytometry of untreated control cultured human gingival epithelial cells after incubated in a 5% CO_2 – 95% air, fully humidified atmosphere at 37 ^{0} C for 5 days.

The lower left quadrant of the diagram showed the viable cells, which excluded PI and were negative for FITC-Annexin V binding. The upper right quadrant contained the non-viable necrotic cells, positive for FITC-Annexin V binding and for PI uptake. The lower right quadrant represented the apoptotic cells, FITC-Annexin V positive and PI negative, demonstrating cytoplasmic membrane integrity.

However, the flow cytometer should be initially set up for the epithelial cells before analysis by adjusting voltage and compensation of fluorescence emission. The values of voltage and compensation of fluorescence after the instrument was set to eliminate the spectral overlap by Annexin V-FITC and propidium iodide intensity were summarized in Table 3.1. All measurements were done under this same instrument setting.

Table 3.1 The flow cytometer setup conditions for cultured human gingival epithelial cells

Cytometer Type: FACSCalibur

Detectors/ Amps:

Parameter	Detector	Voltage	AmpGain Mode
P1	FSC	E00	1.00 Lin
P2	SSC	350	1.00 Lin
P3	FL1	350	1.00 Log
P4	Fl2	280	1.00 Log
P5	FL3	150	1.00 Lin
P6	FL1-A		1.00 Lin
P7	FL4	100	Lin

Threshold: Primary Parameter: FSC

Value: 52 Secondary Parameter: None

Compensation:

FL1 - 40.8% FL2 FL2 - 35.5% FL1

FL2 - 0.0% FL3 FL3 - 0.0% FL2

In a pilot experimental study, the positive control groups should be initially investigated for selection of the appropriate apoptosis inducing agent. In general, cells are treated with a stimulus like chemical or chemotherapeutic agents, serum deprivation, irradiation, low oxygen, or proapoptotic genes, to induce apoptosis (Mila and Scott, 2001).

Three different stimuli, i.e., hydrogen peroxide, thapsigargin and cell wall extract of *Fusobacterium nucleatum*, were used for this pilot study. It was found that 0.5 mM hydrogen peroxide was the most appropriate stimulus to induce cultured human gingival epithelial cells to undergo apoptosis since the percentage of apoptotic cells was highest with hydrogen peroxide, followed by thapsigargin and *Fusobacterium nucleatum* cell wall extract, respectively (Table 3.2).

Table 3.2 Apoptosis inducing agent

Agent	Dose	Incubation time (h)	Percentage of apoptotic cells		
			Exp 1	Exp 2	Exp 3
Hydrogen peroxide	0.5 mM	10	76.40	63.90	72.22
Thapsigargin	1 μM	10	49.41	47.25	41.43
Fusobacterium nucleatum	20 μl	10	31.35	29.33	28.11

Kruskal Wallis test= 8.727(P=0.013)

2) Experimental process

The human gingival epithelial cells were seeded in the culture dishes at a density of 10^5 cells in 5 ml medium per dish. They were cultured in 5% CO_2 incubator at 37^0C for a night. On the following day, in the positive control, cells were induced by 0.5 mM H_20_2 for 10 h (group 2). In the experimental groups, cells were treated with either 50 or 500 μl of the extract solution containing maximum corrosion products

released from the commercial magnets, or 0.9% NaCl, the corrosive solution, without any magnet byproducts for 5 days (group 3, 4, or 5 respectively). The untreated control cells were served as a negative control group (group1). Therefore, the measurement of apoptosis was divided into 5 groups as follows:

1) Negative control group: untreated control cells
2) Positive control group: cells treated with 0.5 mM H_2O_2
3) Experimental group: cells treated with 50 µl of 0.9% NaCl solution containing maximum corrosion products released from commercial magnets
4) Experimental group: cells treated with 500 µl of 0.9% NaCl solution containing maximum corrosion products released from commercial magnets
5) Experimental group: cells treated with 0.9% NaCl solution without any magnet byproducts

All of the experiments were conducted in a 37^0C incubator for 5 days.

The cytotoxic effects of corrosion products released from commercial magnets on human gingival epithelial cells were evaluated by:

1) The morphological analysis with propidium iodide under fluorescence microscopy.
2) The flow cytometric analysis with FITC-conjugated annexin V and propidium iodide.

1) The morphological analysis by propidium iodide (PI) staining and fluorescence microscopy

Propidium iodide (PI) is the most common red fluorescent nuclear stain for identifying dead cells in a population and used as a counterstain in multicolor fluorescent techniques. Propidium iodide is suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, and fluorometry. Propidium iodide, which binds to nucleic acid, is an excellent counterstain for DNA and

chromatin. Because of membrane impermeable property, it is thus generally excluded from viable cells and can only penetrate the plasma membrane when membrane integrity is breached, as occurred in the later stages of apoptosis or in necrosis.

After incubating cells with corrosion products, they were harvested by gentle mechanical scrape with a rubber policeman and centrifuged at 1200 rpm using a high speed cytocentrifuge. Then, the cells were cytospun onto glass slides for 10 min, air dried, fixed with absolute methanol for 10 min at -20^{0}C, washed twice with PBS ,air dried, stained with PI (final concentration 200 µg/ml) for 10 min at room temperature, and washed again with PBS. After drying, the slides were mounted with 90% glycerol and finally examined for any nuclear changes that were consistent with apoptosis under fluorescence microscope (Nicolletti, 1991).

2) The flow cytometric analysis by FITC-conjugated annexin V and propidium iodide assay.

Koopman *et al*. were the first to describe detection of apoptotic cells *in vitro* using FITC-labeled annexin V (Koopman *et al,* 1994). Hapten-labeled annexin V can bind to externalized PS in the outer plasma membrane leaflet of apoptotic cells. It will not bind to normal cells because the molecule cannot trespass the phospholipids bilayer. In necrotic cells, however, the inner leaflet of the plasma membrane is available for binding, since the integrity of the plasma membrane is lost. To discriminate between necrotic and apoptotic cells, a membrane impermeable DNA dye, for example propidium iodide (PI), can be included in the assay. In this way vital, apoptotic, and necrotic cells can be discriminated on the basis of double-labeling for annexin V and PI and analyzed by flow cytometry and fluorescence microscopy.

When treated and untreated cells reach almost confluence (80%), they were harvested by gentle mechanical scrape with the rubber policeman, and then transferred to the 15 ml culture tube. Some amount of KGM was added to balance the volume in each tube prior to being centrifuged immediately for 15 min.

The assessment of apoptotic cells by using the following modified protocol of Annexin V/PI kit from BD Biosciences Pharmingen (BD Pharmingen) was perfomed. The cells were washed with cold PBS, resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/ml, and transferred 100 µl of the solution (1x 10^5 cells) to a 5 ml flow cytometer tube. Then 5 µl of Annexin V-FITC and 5 µl of PI were added for cell staining. The cells were later gently vortexed and incubated for 15 min at room temperature in the dark. Finally, 400 µl of 1X Annexin V binding buffer was added to each tube before flow cytometer detection. The flow cytometric analyses were performed within one hour with a laser excitation at 488 nm. FITC could be detected at 530 ± 20 nm and PI at > 600 nm. Apoptotic cells shrink substantially during the cell death process; therefore, it was important not to “gate out” the small cells that were often considered debris. This apoptotic assay was performed at least three times.

STATISTICAL ANALYSES

The data collection and the data analysis were performed by using statistical analyses as follows:

1. Descriptive analysis such as median and percentile was used to describe the cytotoxic effects of corrosion products released from commercial magnets on cultured human gingival epithelial cells among each experimental group. This would allow us to assess the cytotoxicity of corrosion products released from commercial magnets in terms of apoptosis and necrosis in cultured human gingival epithelia cells after being incubated in solution containing corrosion products for 5 days.

2. The nonparametric Kruskal Wallis test was used to compare the effects of corrosion products on **apoptosis** in cultured human gingival epithelia cells after being incubated in solution containing corrosion products for 5 days at the significance level of 0.05. If there was significant difference, each pair of groups would be tested with the Mann-Whitney U test.

3. The nonparametric Kruskal Wallis test was used to compare the effects of corrosion products on **necrosis** in cultured human gingival epithelial cells after being incubated in solution containing corrosion products for 5 days at the significance level of 0.05. If there was significant difference, each pair of groups would be tested with the Mann-Whitney U test.