CHAPTER III

MATERIALS AND METHODS

MATERIALS

The following materials were used in this study:

1) Specimens

1.1 The orthodontic magnets (Ormco, USA, Part No. 671-0001)

The orthodontic magnets were cylindrical samarium-cobalt magnets. They were coated with a biocompatible polymer to avoid leach out products. The size of orthodontic magnets was 3.75 mm in diameter and 2.0 mm in thickness with the central pole (0.5 mm in diameter). Nine specimens of orthodontic magnets were used in this experiment (Figure 3.1).

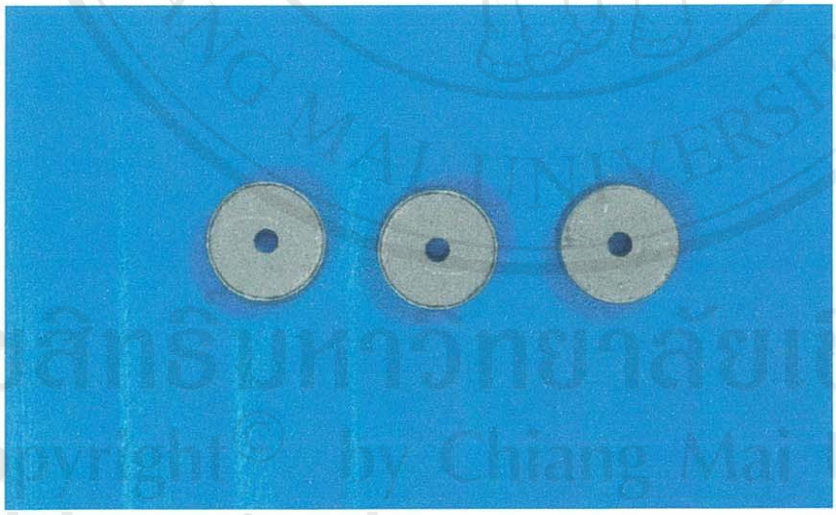

Figure 3.1 The orthodontic magnets

1.2 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). Nine rectangular specimens were prepared with the same figure and dimension so that their surface area (48.41 mm^2) was equal to that of the orthodontic magnets. All commercial magnets were mechanically grounded and polished using wet abrasive papers in different grades (320, 600, and 1000), respectively (Figure 3.2).

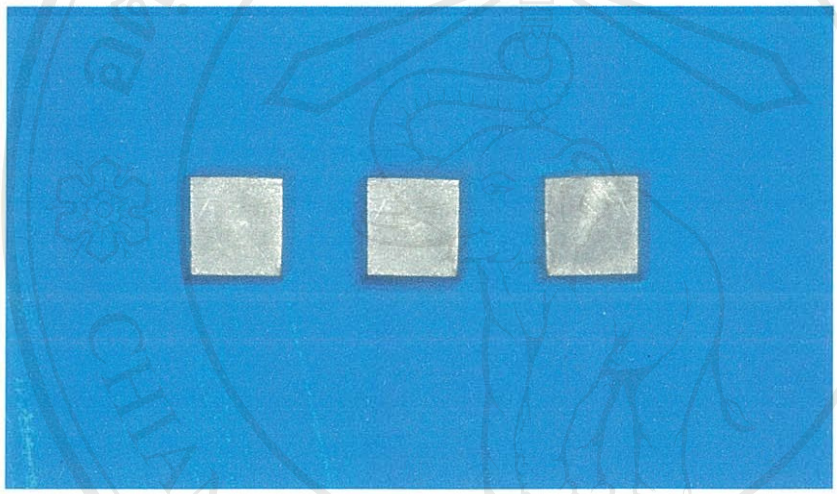

Figure 3.2 The commercial magnets

2) Chemical reagents

2.1 Cell culture medium: Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) contains L-glutamine and antibiotic-antimycotic solution (Figure 3.3).

2.2 Trypsinizing solution (Trypsin – EDTA) (Figure 3.3)

2.3 Fetal bovine serum (Figure 3.3)

2.4 0.1% Trypan blue solution (Figure 3.13)

2.5 Bromodeoxyuridine (BrdU) flow kit (Figure 3.4)

2.6 Tumor Promoting agent (TPA): Phorbol 12-myristate 13 acetate 1 mg/ml

2.7 Corrosive media, follows in ISO 10993-5,15 (1999, 2000):

2.7.1 Cell culture medium (mentioned above)

2.7.2 0.9% Sodium chloride (NaCl) aqueous solution. This solution is very corrosive without inhibitory action.

2.7.3 Artificial saliva. This simulates natural saliva in the oral cavity.

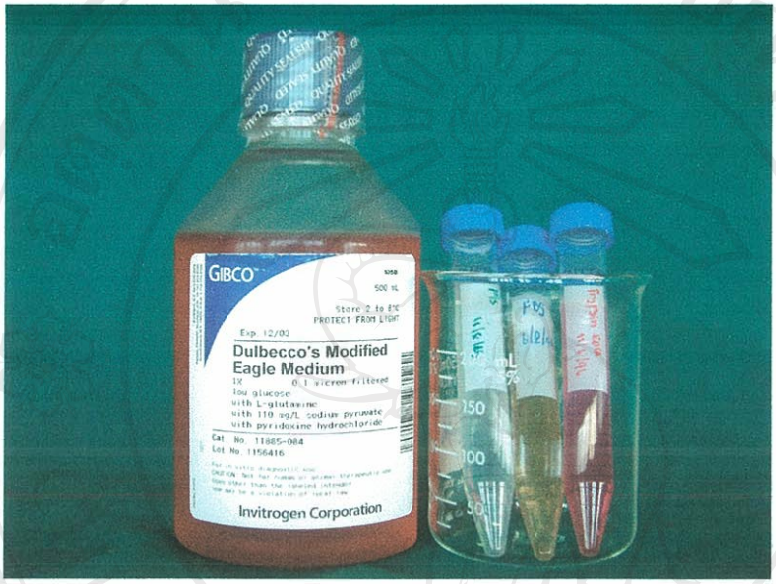

Figure 3.3 Chemical reagents for cell culture

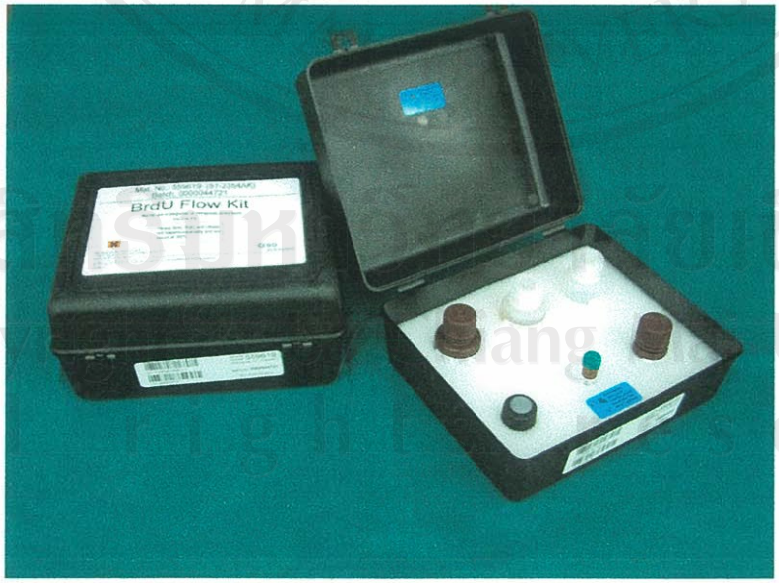

Figure 3.4 The bromodeoxyuridine (BrdU) flow kit

3) Supplies

3.1 Plastic culture flasks (25 cm^3) (Figure 3.5)

3.2 6-well culture plates (Figure 3.5)

3.3 Test tubes (50 ml)

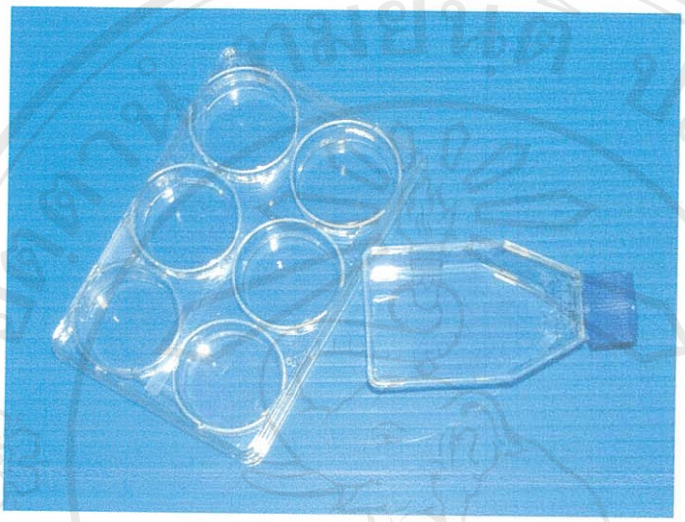

Figure 3.5 A 6-well culture plate and a plastic culture flask (25 cm^3)

4) Instruments

1. Atomic Absorption Spectrophotometer (AAS), which makes possible to assess the quantity and differentiate various types of dissolved metal ions (Figure 3.6).

2. Microscope

3. Flow cytometer with a 488 nm argon laser (Figure 3.7)

4. Incubator, maintained at 37 °C in 5% CO_2/ 95% air atmosphere with 100% humidity (Figure 3.8).

5. Laminar flow biological cabinet, which minimizes contamination of cultures (Figure 3.9).

6. Autoclave

7. Desk-top centrifuge

8. Haemocytometer for cell counting. This instrument is a double chamber with Neubauer rulings. There are nine squares on each chamber side. Each large square represents an area of 1 mm^2 and a depth of 0.1 mm, i.e., a volume of 0.1 mm^3.

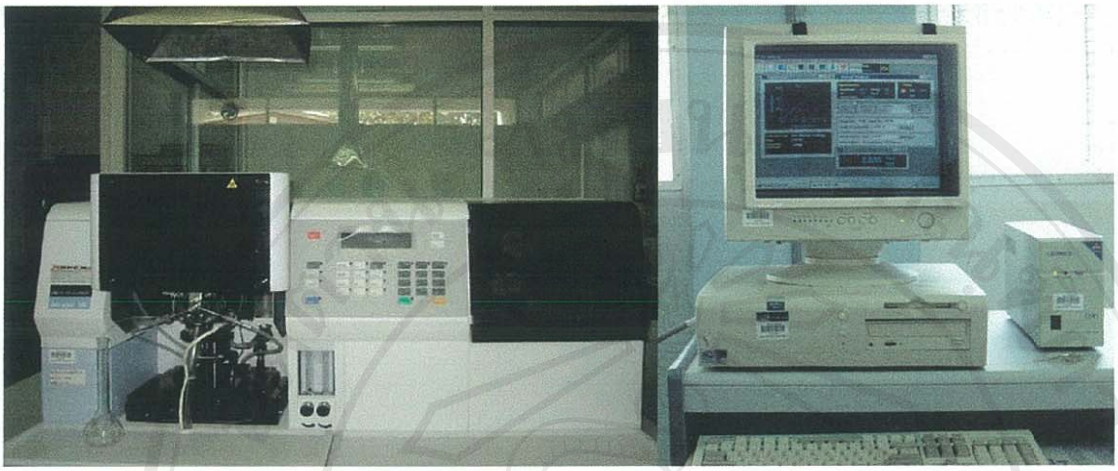

Figure 3.6 The Atomic Absorption Spectrophotometer

Figure 3.7 The flow cytometer

Figure 3.8 The incubator

Figure 3.9 The laminar flow biological cabinet

METHODS

The methods of this study were divided into two parts.

Part I: The composition and quantities of corrosion products released from orthodontic magnets and commercial magnets

Part II: Biocompatibility test of corrosion products released from orthodontic magnets and commercial magnets

Part I: The composition and quantities of corrosion products released from orthodontic magnets and commercial magnets

Specimen preparation

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

Extraction method

Three specimens of commercial magnet were separately immersed in 10 ml of cell culture medium, 0.9% NaCl, or artificial saliva. Also, three specimens of orthodontic magnets were immersed in each solution as described above. Duplication of this experiment with two different magnets and three different solutions were performed.

The cell culture medium, 0.9% NaCl, and artificial saliva without any magnet in equivalent volume were used as a negative control for each corrosive medium.

The magnets were incubated in various types of solution at 37 °C for 7 days. Subsequently, the extracts of corrosion products were collected, and then specimens were removed from corrosive medium. Two aliquots of each extract were used as stock solution for further chemical and biological analyses.

Chemical analysis

Each extract of corrosion products was analyzed to detect the composition and quantities of corrosion products released from specimens. Test media were analyzed for boron (B), copper (Cu), cobalt (Co), iron (Fe), nickel (Ni), and silicon (Si) ions by Atomic Absorption Spectrophotometer (Perkin-Elmer AAnalyst 100). The recommended atomization flame temperature was selected according to the Perkin-Elmer manual to give maximum sensitivity and precision. The specific parameters (wavelength, flame type and temperature, slit width) used for detection of each element were listed in Table 3.1. Standard solution was prepared by dilution of purchased 1,000 ppm stock solution with deionized water. The standard linear curve was generated for each element. The presence of element in control medium without corrosion products was calibrated for blank setting (0 ppm) before evaluating that element in each medium. Three absorbance readings per tested element were made for each corrosive medium of both magnets. The means and standard deviations of element concentration in ppm (mg/L) were then determined. Each test was repeated twice in both test and control groups.

Table 3.1 Atomic absorption parameters used for elements

Element	Wavelength (nm)	Flame	Flame temperature (°C)	Slit width (nm)
Boron (B)	249.7	Nitrous oxide-acetylene	2,900	0.70
Cobalt (Co)	240.7	Air-acetylene	2,300	0.20
Cupper (Cu)	324.8	Air-acetylene	2,300	0.70
Iron (Fe)	248.3	Air-acetylene	2,300	0.20
Nickel (Ni)	232.0	Air-acetylene	2,300	0.20
Silicon (Si)	251.6	Nitrous oxide-acetylene	2,900	0.20

Part II: Biocompatibility test of corrosion products released from orthodontic magnets and commercial magnets

The experimental process was divided into two parts as follows:

I. Preparation of the cultured human gingival fibroblasts

II. Experimental process to evaluate the viability and growth of the cultured human gingival fibroblasts in the presence of corrosion products

I. Preparation of the cultured human gingival fibroblasts

The normal gingival biopsies, overlying an impacted third molar, obtained from the Oral Surgery Department 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 removed from the gingival biopsy using 0.5 mg/ml thermolysin, collagenase type X (Sigma; St. Louis MO), and 1.125 mM Ca^{2+} in HEPES-buffered saline overnight at 4 °C.

Primary gingival fibroblasts were isolated from connective tissue after the epithelium was removed. Connective tissue was incubated in DMEM supplemented with 1% penicillin/streptomycin (GIBCO BRL) and 10% FBS until a sufficient number of fibroblasts spread from the tissue. Trypsin-EDTA was used to remove fibroblasts from tissue culture dishes. To expand the number of fibroblasts, fibroblasts were further cultured in several flasks determined by the number of experimental conditions. Otherwise, primary fibroblasts can be kept frozen in 8.33% DMSO (Sigma), 20% FBS in DMEM at $1x10^6$ cells/cryotube (Nunc).

When the biological tests were performed, the frozen cells were thawed. The fibroblasts were diluted with serum-containing medium to dilute out the DMSO, and seeded in a culture flask at 37 °C.

II. Experimental process to evaluate the viability and growth of cultured human gingival fibroblasts in the presence of corrosion products

When human gingival fibroblasts in culture flasks reach proper confluence (80%), they were harvested by trypsinization. These fibroblasts were seeded in 6-well culture plates for conducting the experiment. The experimental process in this study was divided into 2 sections as follows:

1) Pre-experimental process

This study used trypan blue dye exclusion assay and flow cytometry for determining cell viability and cell growth, respectively.

The cell growth in terms of newly synthesized DNA can be determined by the immunofluorescent staining of bromodeoxyuridine and flow cytometric analysis. This application allows for enumeration and identification position in cell-cycle kinetics. The BrdU Flow Kit was used to assess cell's new DNA synthetic activity and a flow cytometer was used to analyze fluorescent activity. Cells were incorporated with fluorescent dyes, i.e., bromodeoxyuridine (BrdU: an analog of thymidine) that enters into DNA of cell in S phase. Subsequently, labeled fibroblasts were treated according to the BD PharMingen BrdU staining protocol. The major steps of this protocol consist of cell fixation and permeabilization, treatment with DNase, staining with specific anti-BrdU fluorescent antibodies (Anti-BrdU conjugated with FITC), couple treatment with 7-amino-actinomycin D (7-AAD) that binds to total DNA, and acquisitive analysis with flow cytometer. The assessment methods require relatively sophisticated computer techniques using the BD CellQuest flow cytometry analysis software. However, the flow cytometer should be initially set up for a particular cell type before analysis by adjusting voltage and compensation of fluorescence emission. The Figure 3.10 showed values of voltage and compensation after the instrument was set to eliminate the spectral overlap by 7-AAD and FITC anti-BrdU cells.

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Cytometer Type:   FACSort

Detectors/Amps:
Param   Detector  Voltage   AmpGain     Mode
P1        FSC       E-1       2.70      Lin
P2        SSC       340       1.00      Lin
P3        FL1       476       1.00      Log
P4        FL2       150       1.00      Log
P5        FL3       689       1.00      Lin
P6        FL2-A               1.00      Lin
P7        FL2-W               1.00      Lin
Threshold:
Parameter:   FL1
Value:  35

Compensation:
FL1 - 5.5 % FL2
FL2 - 1.5 % FL1
FL2 - 0.9 % FL3
FL3 - 0.9 % FL2
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Figure 3.10 The flow cytometer setup guidelines for the cultured human gingival fibroblasts

This flow cytometric analysis related to the cell cycle is the determination of the fraction of cells in the G_0/G_1, S, and G_2/M phases. The information obtained from flow cytometry is shown as Figure 3.11. The "horseshoe" fluorescence staining profile of 7-AAD versus BrdU dot plot was created for acquisition and subsequent analysis of data. Each fraction was produced by variation of fluorescent level and DNA content.

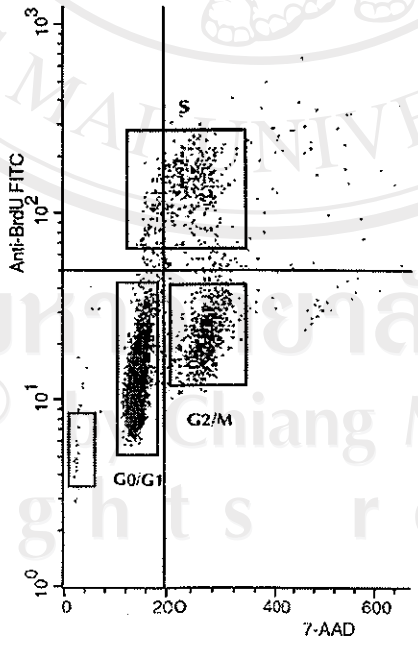

Figure 3.11 The 7-AAD versus BrdU dot plot diagram

The length of time for labeling with BrdU depends on the tested cell population rate of cell cycle entry and progression. It should be recognized that the cellular properties and nature in cell-cycle activity of each cell type are different. Prior to this procedure, the choice of optimal labeling time point should be considered for each particular cell population by a time-course analysis experiment. Therefore, the cultured human gingival fibroblasts were labeled with BrdU and incubated for various time intervals (30, 60, 90, 120 min). Subsequently, these groups were processed by the flow cytometry as mentioned above. The results of all groups in a dot plot diagram of this experiment were shown in Figure 3.12.

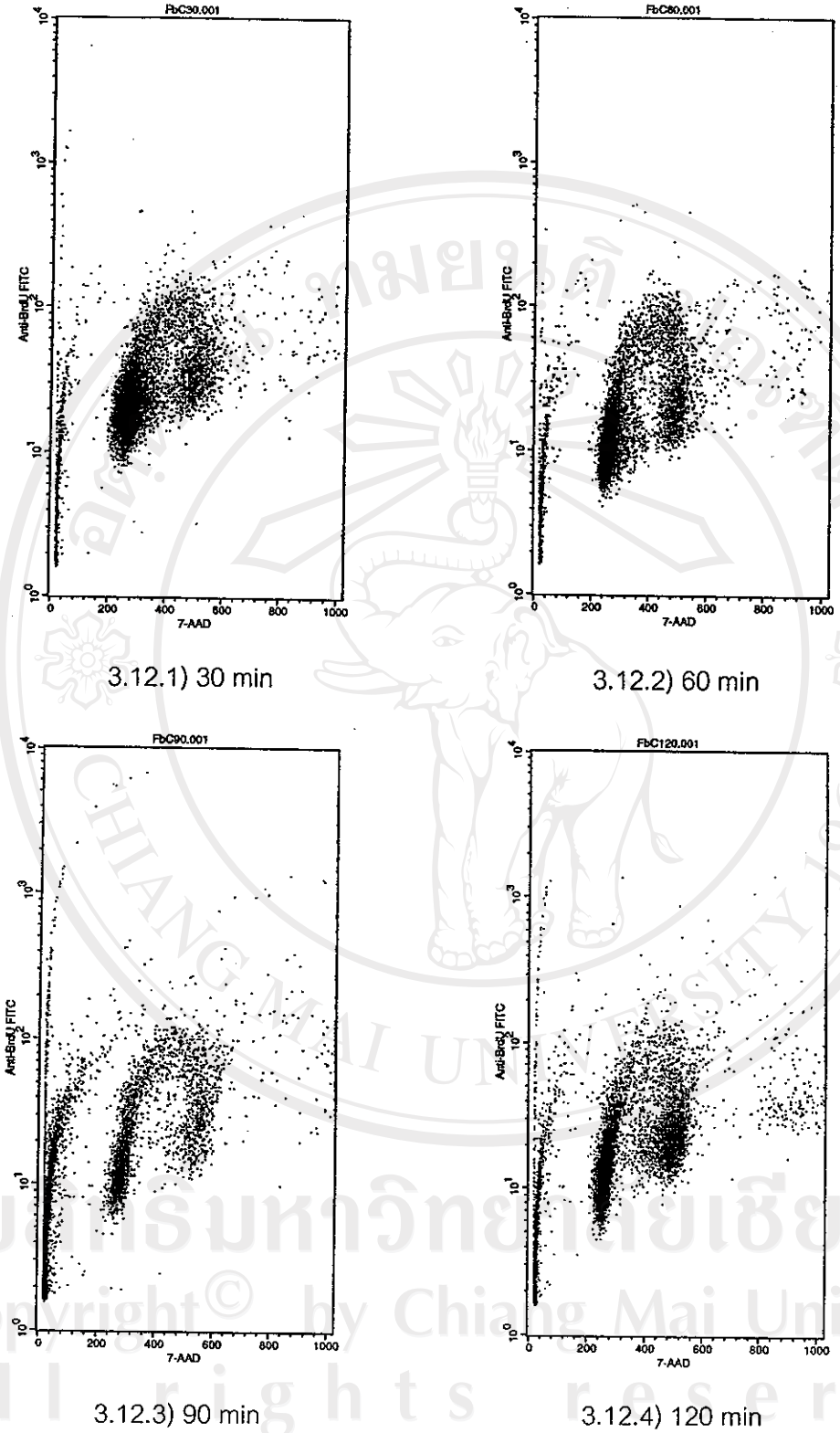

Figure 3.12 The 7-AAD versus BrdU dot plot diagrams of the time-course analysis experiment to determine the effective period of labeling time for the cultured human gingival fibroblasts

The dot plot diagram should demonstrate the most excitation level and amount of fluorescent signal from S phase marker (BrdU) for the effective length of time. In addition, a population of cells among each region of DNA distribution were much more discernible. So, the effective length of labeling time, that is 60 min, was selected for the whole experiments of this study.

Moreover, it is necessary to evaluate the efficacy of flow cytometer to detect an alteration of cell population in S phase. So, the other experiment was conducted by setting into two groups, i.e., a control group and a positive control group. The control group was the human gingival fibroblast cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C for 3 and 7 days. The positive control was Phorbol12-myristate13-acetate, i.e., Tumor Promoting Agent (TPA). The TPA (1 mg/ml) was diluted ten fold to 100 µg/ml or 100 ng/µl with DMSO. To stimulate cells, 100 ng of diluted TPA was added directly to each ml of culture medium and incubated overnight at 37 °C for both 3- and 7-day experiments. The immunofluorescent staining of incorporated BrdU and flow cytometry were used to measure the state of stimulated cell proliferation.

Table 3.2 The comparison of mean ranks of the percentages of the growth (the rate of new DNA synthesis) of the cultured human gingival fibroblasts that was induced by a Tumor Promoting Agent (TPA)

Cultured time	Group	n	Median	P_{25}	P_{75}	Mean rank	P-value*
3 days	Control	3	29.69	28.31	29.75	2.00	0.034*
	TPA	4	32.65	30.86	35.14	5.50	
7 days	Control	4	15.35	14.64	19.06	2.50	0.021*
	TPA	4	21.86	21.03	33.14	6.50	

* Mann-Whitney U Test

* $P < 0.05$

n = Number of repeated experiments

The Mann-Whitney U test indicated that there were human gingival fibroblasts in S phase of cell cycle in TPA stimulation group significantly greater than the control unstimulated group (P= 0.034 for 3 days, P= 0.021 for 7 days) (Table 3.2).

Therefore, TPA could induce cell proliferation of the cultured human gingival fibroblasts, and the flow cytometry was efficient to detect an alteration in new DNA synthesis.

2) Experimental process

The human gingival fibroblasts were seeded in a 6-well culture plate at a density of 2,000 cells and 20,000 cells in 2 ml medium per well for 3-and 7-day experiment, respectively. They were cultured in 5% CO_2 incubator at 37 °C for overnight. On the following day, 200 µl of either extract solution containing corrosion products released from the commercial magnet or the orthodontic magnet was added in each well. The control solution without corrosion products was added in the remaining wells served as a negative control.

Two types of solution, containing corrosion products of commercial magnets or orthodontic magnets selected for this experiment, were 0.9% NaCl and DMEM, except artificial saliva. This was because the inhibitory effect on cell proliferation was observed while artificial saliva was added in cell culture. Therefore, the types of magnet and corrosive medium were selected for the experiment as follows.

1) Control group: Cell culture medium (DMEM)

2) Experimental group: Corrosion products of the orthodontic magnet in DMEM

3) Experimental group: Corrosion products of the commercial magnet in DMEM

4) Experimental group: 0.9% sodium chloride

5) Experimental group: Corrosion products of the orthodontic magnet in 0.9% NaCl

6) Experimental group: Corrosion products of the commercial magnet in 0.9% NaCl

All of the experiments were conducted in a 37 °C incubator for 3 and 7 days.

The effects of corrosion products released from magnets on the cultured human gingival fibroblasts were evaluated by:

1) Measurement of viability

The trypan blue dye exclusion assay was used to analyze the cell viability. The cultured human gingival fibroblasts were trypsinized with Trypsin-EDTA. This enzymatic reaction was stopped with DMEM and cells were resuspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. An aliquot of 10 µl of cell suspension was mixed with 5 µl of trypan blue (GIBCO BRL) for staining. After 5 min, 10 µl was transferred to each chamber side of the haemocytometer for cell counting. The unstained cells were live cells, while the blue stained cells were considered nonviable. The unstained and stained cells on the four corners and the center square of each chamber side, that is 10 of 0.1 mm^3, were visually counted (Fig 3.13). Cells that overlapped the border on two sides of the square were included in cell count and then cells on the border of the other two sides were not counted. The percentage of viability was calculated from the number of cells that excluded the dye and the total number of cells. These values were compared among different groups.

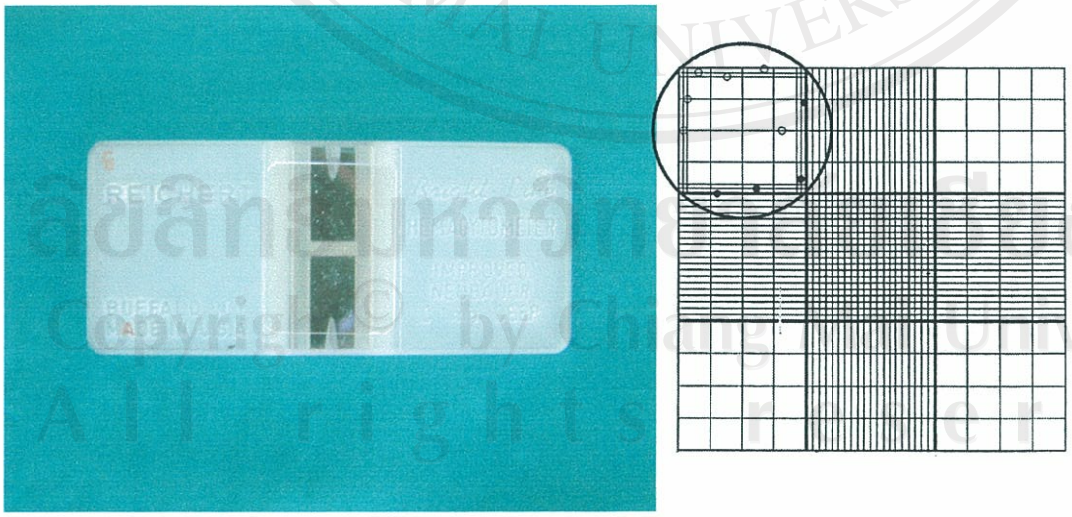

Figure 3.13 A haemocytometer for the trypan blue dye exclusion assay

2) Measurement of growth

After incubated for 3 and 7 days, the cultured human gingival fibroblasts in each group were labeled with BrdU (10 µl of 1 mM BrdU / each ml of culture medium). Then, these labeled cells were incubated at 37 °C for 60 min that was the effective period of labeling time for these cells (see Figure 3.12). The labeling step was important to avoid disturbing the cells in any way that might disrupt their normal cell cycling patterns.

Subsequently, labeled fibroblasts were treated according to the BD PharMingen BrdU staining protocol. Briefly, the major steps of this protocol consisted of cell fixation and permeabilization, treatment with DNase, staining with FITC anti-BrdU, staining of total DNA with 7-AAD, and cell cycle analysis by the flow cytometer. Fibroblasts, which were not labeled with BrdU, were a negative cell staining control. This allowed the determination of background staining levels for the FITC anti-BrdU.

The cell cycle positions and actively new DNA synthesis of cells can be determined by analyzing the correlated expression of total DNA and incorporated BrdU levels as shown in the 7-AAD versus BrdU dot plot diagram. The region gates of dot plot were discriminated in G_0/G_1, S, and G_2/M phase for quantitative cell cycle analysis. The cell counts in each region of DNA distribution are proportional to the fractions of cells in the corresponding cell cycle phase. And, the percentage of the rate of new DNA synthesis (S phase) was calculated to determine the cell growth. Each experiment was performed at least three times.

STATISTICAL ANALYSES

1. Descriptive analysis as mean and standard deviation was used to determine the quantity of corrosion products released from orthodontic magnets and commercial magnets in cell culture medium, 0.9% NaCl, and artificial saliva.

2. Descriptive analysis as median and quartiles was used to show the percentage of the viability and growth (the rate of new DNA synthesis). These values indicated the effects of corrosion products released from orthodontic magnets and commercial magnets on the viability and growth of the cultured human gingival fibroblasts after incubated in solution containing corrosion products for 3 and 7 days.

3. The non-parametric tests, i.e. the Kruskal Wallis test and the Mann-Whitney U test, were used to compare the effects of corrosion products released from orthodontic magnets and commercial magnets on the viability and growth of the cultured human gingival fibroblasts at each time point at the significance level of 0.05.