CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Magnet specimens

A total of fourteen disk-shaped commercial magnets with the diameter of 20 mm and the thickness of 2 mm were used in this experiment (Figure 3.1). Four pieces of magnets were used as single magnet, another ten magnets were used in pairs as attractive magnets.

2. Chemical reagents

2.1. Cell culture reagents

2.2. Trypan blue dye

2.3. Bromodeoxyuridine Flow Kits (Figure 3.2)

2.4. Phorbol12-myristate13-acetate, a tumor promoting agent (TPA) at the stock concentration of 1mg/ml

3. Supplies

3.1. Six well flat bottom microtiter culture plates (Figure 3.3)

3.2. Culture flasks (Figure 3.3)

4. Instruments

4.1. A tesla meter 2000 with a Hall probe (Figure 3.4)

4.2. A haemocytometer (Figure 2.6)

4.3. A laminar flow biological cabinet (Figure 3.5)

4.4. A carbondioxide incubator (Figure 3.6)

4.5. A phase contrast microscope (Figure 3.7)

4.6. A desk top centrifuge

4.7. A flow cytometer (Figure 2.8)

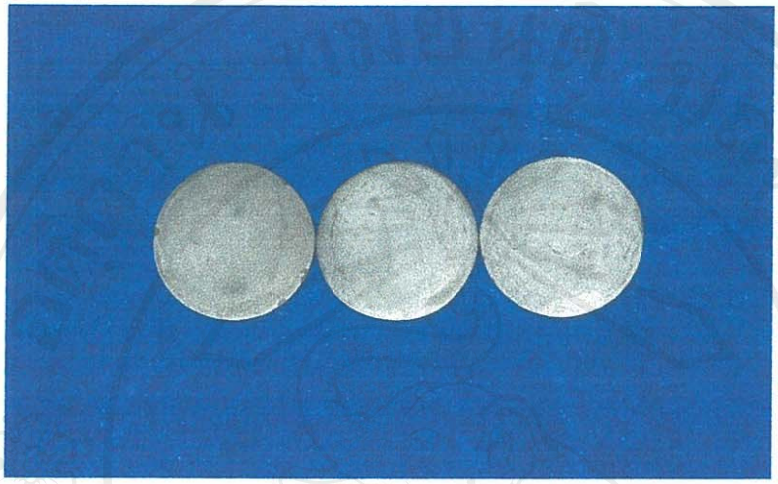


Figure 3.1 Magnet specimens in the dimension of 20 mm in diameter and 2 mm in thickness

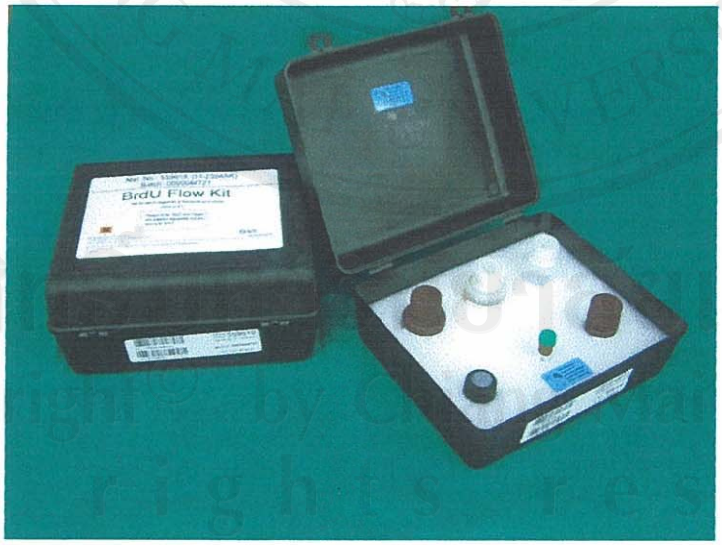


Figure 3.2 Bromodeoxyuridine Flow Kits

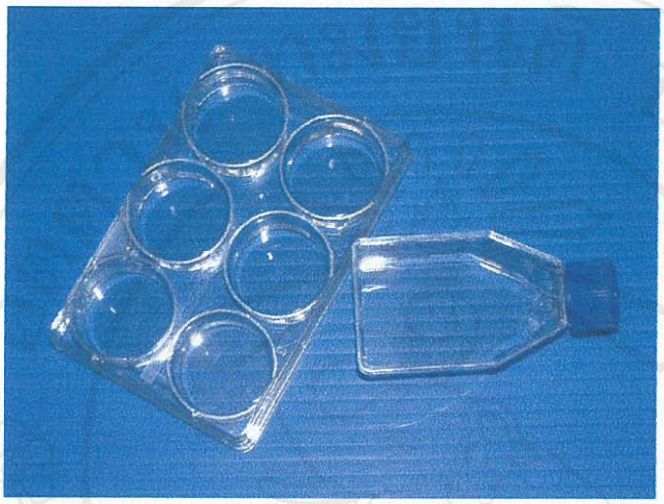


Figure 3.3 A six-well flat bottom microtiter culture plate and a culture flask

Figure 3.4 A tesla meter 2000 with a Hall probe

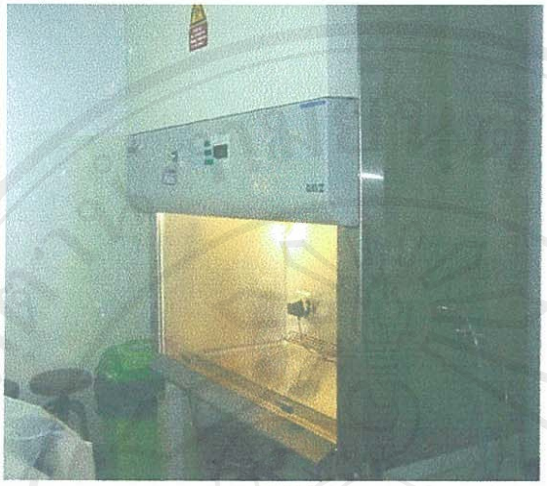


Figure 3.5 A laminar flow biological cabinet

Figure 3.6 A carbondioxide incubator

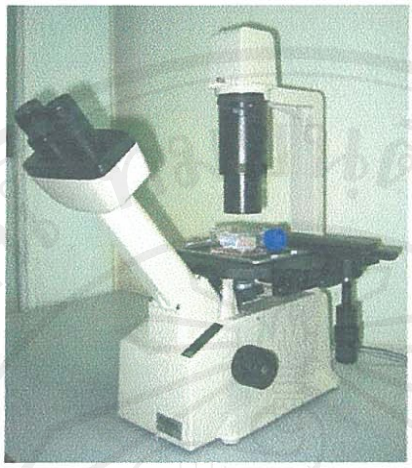


Figure 3.7 A phase contrast microscope

Methods

In this study, the evaluation of cytotoxicity of magnetic field was done by *in vitro* methods with the cultured human gingival fibroblasts grown in Dulbecco's Modified Eagle Medium (DMEM) under the static magnetic field. The growth and viability of cells were determined after the human gingival fibroblasts were cultured under the static magnetic field for 3 and 7 days.

The experimental process was divided into four parts as follows:

I. The measurement of magnetic field intensity
II. The preparation of the cultured human gingival fibroblasts
III. The pre-experimental processes
IV. The experimental processes to evaluate the viability and growth of the cultured human gingival fibroblasts under the static magnetic field

I. The measurement of magnetic field intensity

A culture plate without magnetic field was set as a control group. Another two culture plates with magnetic field were set as experimental groups, which were divided into 2 patterns according to the placement of magnets.

Pattern 1: The single position

The magnet specimen was placed under the first well of culture plate, which was defined as the position 1. The superior surface of magnet contacted with the outer surface of the floor of the plate, and the center of magnet was always placed in the center of the well with the north pole up.

Pattern 2: The attractive position

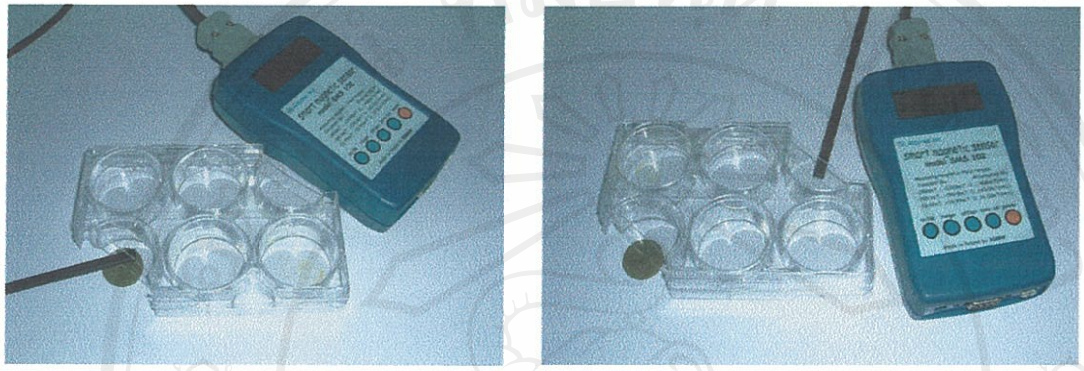
Two magnet specimens were placed on the opposite side of the well to give attractive force to each other. One specimen was placed under the first well of culture plate in the same position, while the other was placed on the culture plate's cover in the parallel position to the lower one. The pole faces of these two magnets, the upper and the lower, were set to have opposite pole type to each others (always placed the north pole up).

Prior to measuring the magnetic field intensity with the Hall probe, the 6-well culture plates were cut at the 2 corners opposite to each other so that it would be accessible for the Hall probe to measure the magnetic field intensity within the well. Subsequently, the magnetic field of the position 1 for pattern 1 was measured at the center of well, which was assumed to have the highest magnetic field intensity. Additionally, the magnetic field of the position 2 was measured at the center of farthest well from the magnet, which was assumed to have the lowest magnetic field intensity (Figure 3.8).

Similarly, the magnetic fields for pattern 2 were measured at two positions, the position 1 and 2, between the upper and the lower magnets in an attractive position (Figure 3.9).

The extent of flux density of magnetic field at the end of hall probe was measured by the Teslameter, which could measure as low as 0.01 millitesla (mT). The magnetic field intensity of each magnet in both positions of 2 patterns was recorded and

calculated to determine the average extent of static magnetic field generated by the commercial magnets, and expressed as mean and standard deviation, the values of the magnetic field intensity were shown in Table 3.1.



A) B)

Figure 3.8 The measurement of single magnetic field at two positions of culture plate: A) the position 1 was the closest to the magnet, B) the position 2 was the farthest from the magnet.

A) B)

Figure 3.9 The measurement of attractive magnetic field at two positions of culture plate: A) the position 1 was the closest to the magnets, B) the position 2 was the farthest from the magnets.

After the magnets had been used in the cell culture experiment several times, the flux density of these magnets was measured again. It was found that the flux densities remained unchanged throughout the experimental period, although some corrosion products appeared on the surface of magnets. This finding was in accordance with the result from a previous *in vitro* experiment (Linder-Aronson and Linskog, 1995).

Table 3.1 The distribution and the amount of magnetic field intensity of the single magnet and the attractive magnets at the position 1 and the position 2

pattern	position	number of specimens	magnetic field intensity (mT)	
			mean	SD
single magnet	1	4	74.05	14.98
	2	4	0.06	0.01
attractive magnets	1	10	79.52	18.24
	2	10	0.11	0.01

II. Preparation of the cultured human gingival fibroblasts

Normal gingival biopsies overlying an impacted third molar, obtained from the Oral Surgery Department, were kept in cold HEPES-buffered saline containing fungisone (GIBCO BRL) and penicillin/streptomycin (GIBCO BRL). Each 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 fibroblasts were isolated from connective tissue after the epithelium had been removed. Connective tissue was incubated in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL) supplemented with 1% penicillin/streptomycin and 10% FBS

(GIBCO BRL). When a sufficient number of fibroblasts spread from the tissue, the trypsin-EDTA (GIBCO BRL) was used to remove fibroblasts from tissue culture dishes. To expand the number of fibroblasts, determined by the number of experimental conditions, fibroblasts were further cultured in several flasks. Otherwise, primary fibroblasts could be kept frozen in DMEM containing 8.33% DMSO (Sigma) and 20% FBS at $1x10^6$ cells per cryotube (Nunc).

III. The pre-experimental processes

In this *in vitro* study, the immunofluorescent staining of bromodeoxyuridine together with the flow cytometric analysis was a method of choice to evaluate the growth of the cultured human gingival fibroblasts.

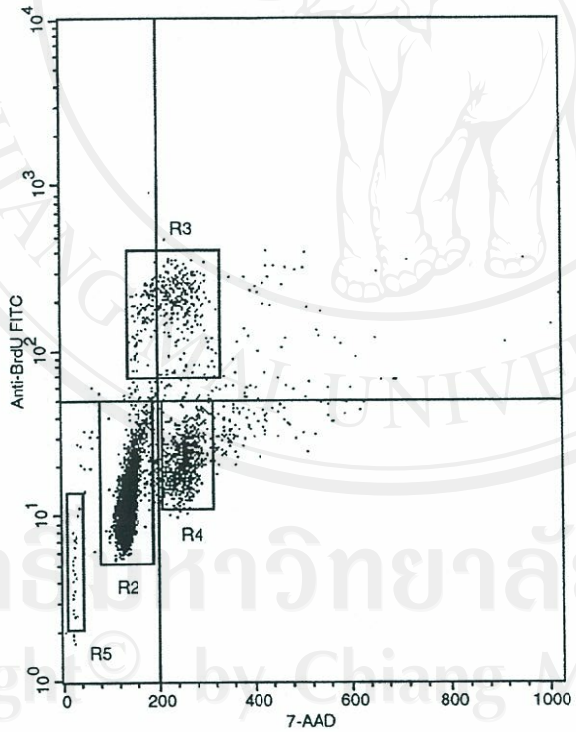


Figure 3.10 A 7-AAD versus FITC anti-BrU dot plot diagram and the percentages of cell population in each phase of cell cycle: R_2 is G_0+G_1-phase, R_3 is S-phase, R_4 is G_2+M phase, and R_5 is apoptotic phase.

The process of BrdU staining was done according to the manufacturer's instructions (BD Pharmingen). BrdU (an analog of the DNA precursor, thymidine) was incorporated into the cultured human gingival fibroblasts to determine the newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. The incorporated BrdU was stained with anti-BrdU conjugated with FITC, specific fluorescent dye. Subsequently, the cultured human gingival fibroblasts were stained with 7-amino-actinomycin D (7-AAD) in order to determine the total DNA synthesis. Finally, the levels of incorporated BrdU were measured by the flow cytometry. The amount of incorporated BrdU detected by a the flow cytometer was determined as the fluorescent levels, and demonstrated as a dot plot diagram on the connected computer monitor, as shown in Figure 3.10.

Although the BrdU staining protocols have been generally used to determine the growth of several types of eukaryotic cells, each cell type differs from one another in its nature of cell cycle, particularly the intervals of cell division. Therefore, before conducting the experiments, it was necessary to set up two pre-experimental runs in order to determine the labeling time with BrdU and the efficiency of the BrdU staining protocol to detect new DNA synthesis.

1) The labeling time set-up for the cultured human gingival fibroblasts

The cultured human gingival fibroblasts at approximate 80% confluence were divided into 4 groups, including 30, 60, 90, and 120 min for labeling time. The staining protocol and the flow cytometry were similarly performed for these four groups according to the protocol of the BrdU Flow Kit.

The result showed that the S phase of cell cycle was most discernable at 60 min of labeling time (Figure 3.11). Therefore, the labeling time of 60 min was selected for the whole experiments.

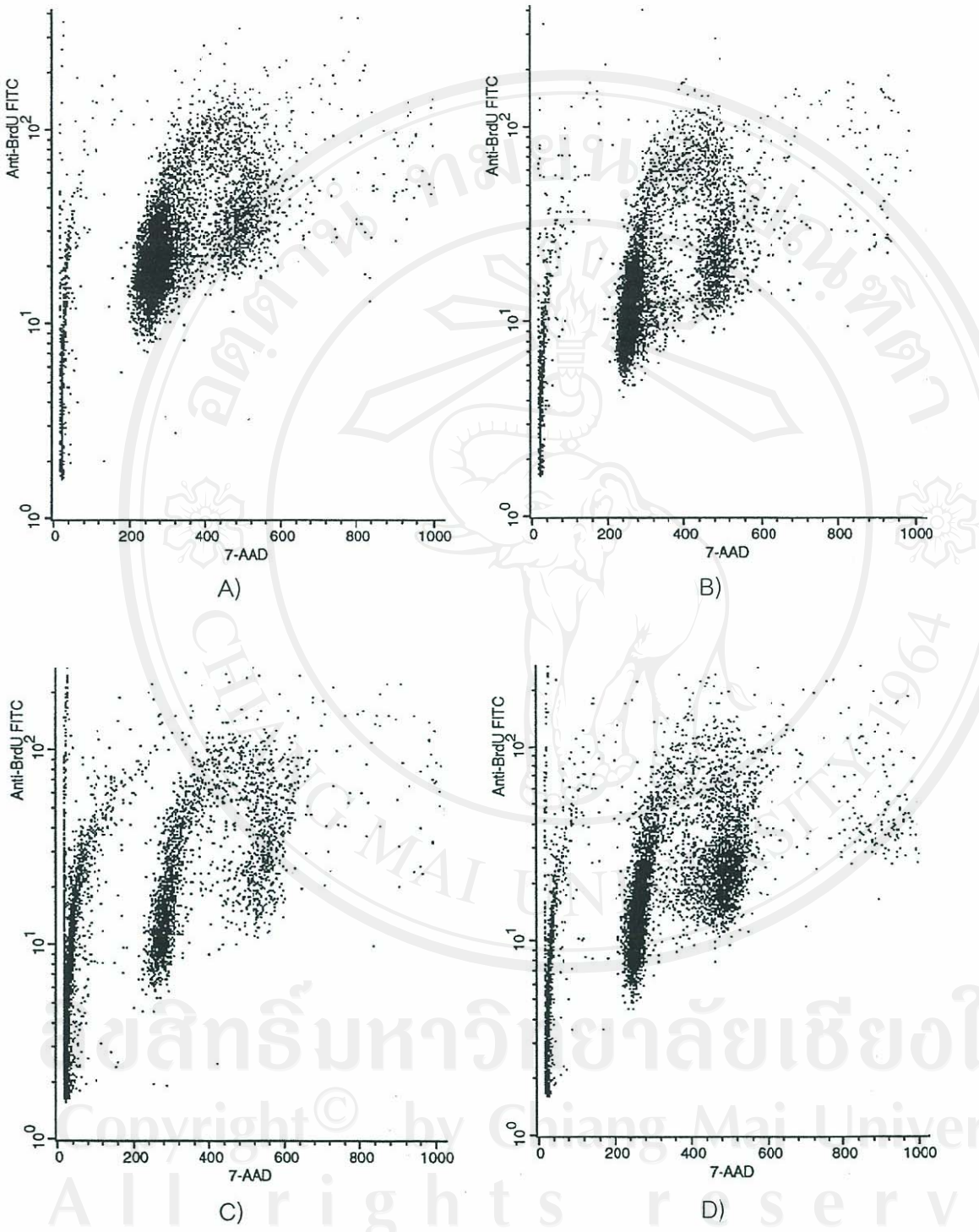


Figure 3.11 The dot plot diagrams of the BrdU-labeled cultured human gingival fibroblasts at different duration for BrdU uptake, A) 30 min B) 60 min C) 90 min D) 120 min

2) The verification of the ability of flow cytometer to detect new DNA synthesis in the cultured human gingival fibroblasts

In addition to the labeling time, it is important to assess the ability of flow cytometer to detect an alteration of new DNA synthesis in the experimental groups. Therefore, phorbol12-myristate13-acetate, a tumor promoting agent (TPA) that can induce cell proliferation, was added into the cultured human gingival fibroblasts as a positive control for new DNA synthesis.

The result using Mann-Whitney U test statistical analysis showed that that there was a significant change of the growth, the percentage of S phase, in the cultured human gingival fibroblasts stimulated with this tumor promoting agent compared to the control group (Table 3.2). This indicated that the BrdU protocol and the flow cytometric analysis were efficient to detect new DNA synthesis.

Table 3.2 The mean ranks of growth (the rate of new DNA synthesis) of the cultured human gingival fibroblasts between the positive control group (TPA stimulation for 1 day) and the control group at days 3 and 7.

culture time	group	number of repeated experiment	Median (P25,P75)	mean rank	P value*
3 days	Control	3	29.69 (28.31, 29.75)	2.00	
	Positive control	4	32.65 (30.86, 35.14)	5.50	0.03*
7 days	Control	4	15.35 (14.64, 19.06)	2.50	
	Positive control	4	21.86 (21.03, 33.14)	6.50	0.02*

* Mann-Whitney U test

IV. The experimental processes to evaluate the viability and growth of the cultured human gingival fibroblasts under the static magnetic field

To study the effect of static magnetic field on the viability and growth of the cultured human gingival fibroblasts, those cells were seeded in 6-well culture plates and

grown under static magnetic field for short period of 3 day and for longer period of 7 days

The cultured human gingival fibroblasts were seeded equally in each well at the constant density, approximate 20,000 cells per well for the 3-day culture time and 2,000 cells per well for the 7-day culture time. The newly seeded cells were incubated for 24 hours to allow cell attachment before being exposed to the magnetic field. The patterns of magnet placement were described in Table 3 (*see in The measurement of magnetic field intensity*).

Table 3.3 Five experimental groups under different conditions of magnetic field

Culture plate number	Magnet pattern	Position in culture plate	Magnetic field intensity (Mean ± SD)	Experimental Group
1	Without magnet	1	0.00 ± 0.00	1
2	Single magnet	1	74.05 ± 14.98	2
		2	0.06 ± 0.01	3
3	Attractive magnets	1	79.52 ± 18.24	4
		2	0.11 ± 0.01	5

Therefore, the experimental conditions were divided into 5 groups as follows:

Group 1: Without magnetic field (The control group)

Group 2: High magnetic field from single magnet (the position 1)

Group 3: Low magnetic field from single magnet (the position 2)

Group 4: High magnetic field from attractive magnets (the position 1)

Group 5: Low magnetic field from attractive magnets (the position 2)

All culture plates, both control and experimental groups, were incubated at 37°C in a humidified chamber with 5% CO_2. The cultured fibroblasts were harvested at day 3 and day 7. Their viability and growth under each condition were evaluated.

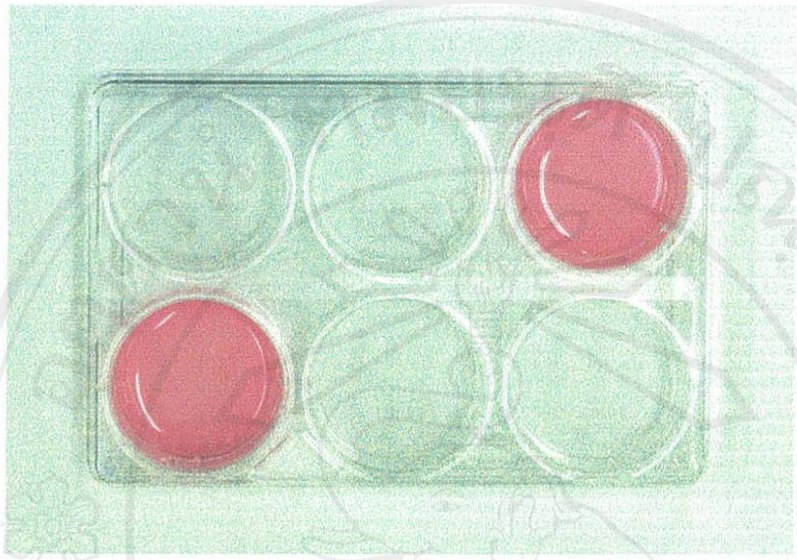


Figure 3.12 The cultured human gingival fibroblasts grown in DMEM with 10% FBS and 1% penicillin / streptomycin without exposure to the static magnetic field (Group 1)

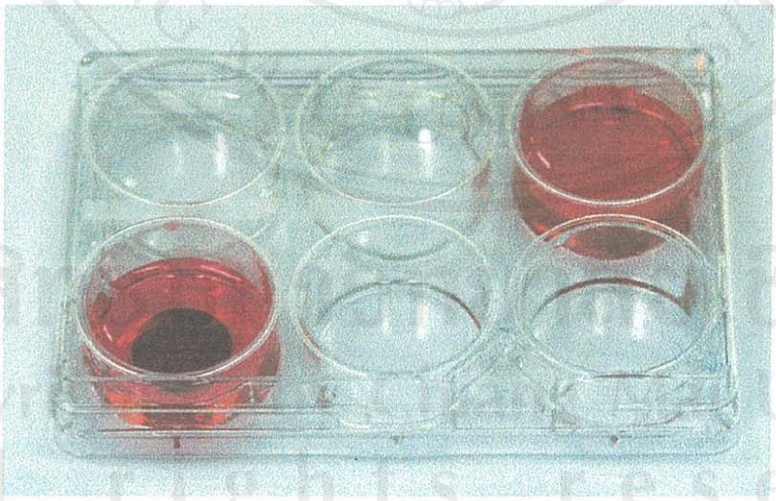


Figure 3.13 The cultured human gingival fibroblasts grown in DMEM with 10% FBS and 1% penicillin / streptomycin under high and low single static magnetic field (Group 2 and 3, respectively)

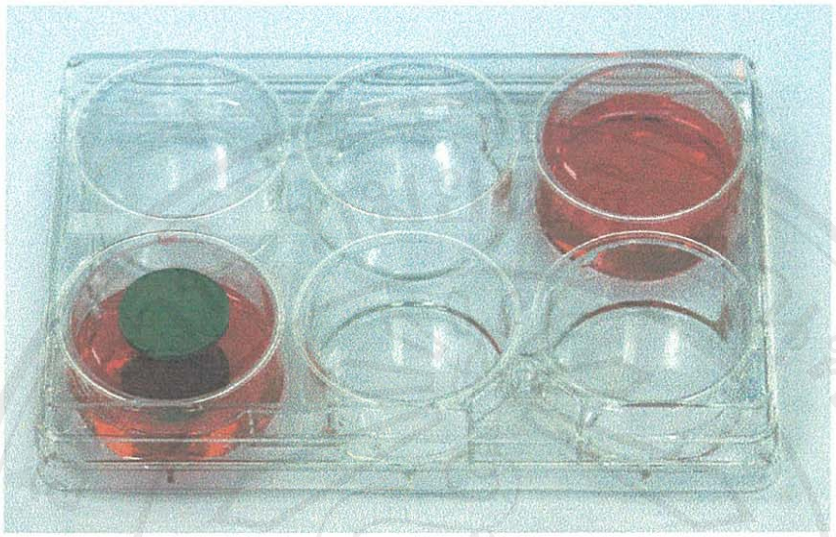


Figure 3.14 The cultured human gingival fibroblasts grown in DMEM with 10% FBS and 1% penicillin / streptomycin under high and low attractive static magnetic field (Group 4 and 5 respectively)

In this study, two methods were used to investigate the *in vitro* effects of magnetic field. The first method was trypan blue dye exclusion assay with cell count to evaluate the cell viability of the cultured human gingival fibroblast. The second was the flow cytometric analysis to evaluate the cell proliferation.

Trypan blue dye exclusion assay

The number of fibroblasts from both control and experimental groups was estimated by counting viable and nonviable cells under a microscope. The fibroblast cells were first detached from the well plates and the cell numbers were counted in the chamber of haemocytometer. The stained cells were non-viable cells, while the unstained cells were viable cells. The percentage of viability was determined by the ratio of the number of viable cells to the total number of cells. The details of this method were presented as follows.

1) Cell staining

After the culture human gingival fibroblasts were trypsinized, a 10 microlitre (µl) of the total volume of cell suspension was transferred to a separate tube containing 5 µl of trypan blue dye solution. Cells were left in the dye for 5 min before the 10 µl of cell suspension in dye were transferred to the chamber of a haemocytometer.

2) Cell count using a haemocytometer

A haemocytometer contains two chambers, as shown in Figure 3.12 and 3.13, each of which when filled and coverslipped contains a total volume of 0.9 µl. Each chamber is ruled into nine large squares. Thus, when coverslipped, the volume of each square is 0.1 mm^3 or 0.1 µl.

Cell count was performed under a microscope using a 10x objective lens. The total number of cells and the number of viable cells were counted in five of the nine large squares in each chamber of the haemocytometer for a total of ten squares. The total number of cells was calculated from the number of cells in a total of ten squares.

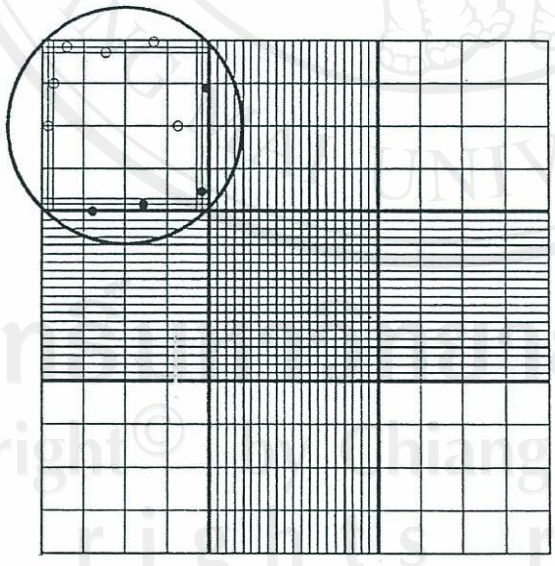


Figure 3.15 A standard haemocytometer chamber which are ruled into nine large squares. The circle represents an area of a large square.

3) Calculation of the percentages of viability

The percentage of viability was calculated from the total number of viable cells.

Example:

Total cell count in ten fields of haemocytometer (N) = 540

The number of stained cells (n) in ten fields = 62

The percent viability [(N – n) / N x 100] = (540 – 62) / 540 x 100 = 88.5%

Immunofluorescent staining of bromodeoxyuridine and flow cytometric analysis

The percentages of growth of the culture human gingival fibroblasts from both control and experimental groups were determined by the process of BrdU staining and flow cytometric analysis, previously explained (*see in The pre-experimental processes*).

To confirm the results, the experimental processes to evaluate the viability and growth of the cultured human gingival fibroblasts under static magnetic field were repeated for several times. The number of repeated experiments with applicable results of each group are shown in Table 3.4 and Table 3.5.

Table 3.4 The number of repeated experiments for evaluating cell viability

Culture time	Number of repeated experiments in evaluation of cell viability				
	Group 1	Group 2	Group 3	Group 4	Group 5
3 days	5	5	5	5	5
7 days	4	4	4	4	4

Table 3.5 The number of repeated experiments for evaluating cell growth

Culture time	Number of repeated experiments in evaluation of cell growth				
	Group 1	Group 2	Group 3	Group 4	Group 5
3 days	5	5	4	4	5
7 days	4	3	4	4	3

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

1. The descriptive analysis was used to determine medians and quartiles of the viability and growth (rate of new DNA synthesis) of the cultured human gingival fibroblasts in each experimental group at day 3 and day 7.

2. The non-parametric analyses (The Kruskal-Wallis test and Mann-Whitney U test) were used to compare the viability and growth (rate of new DNA synthesis) of the cultured human gingival fibroblasts in each experimental group at day 3 and day 7, $P<0.05$ significance level.

3. The correlation analysis (Spearman's rho correlation) was used to analyze the correlation between the viability and growth (rate of new DNA synthesis) of the cultured human gingival fibroblasts and the extent of magnetic field intensity.