CHAPTER IV

RESULTS

The results from the cytotoxic study of corrosion products released from commercial magnets on human gingival epithelial cells were sequentially 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

From the electron microscopic study, *necrotic cells* show cytoplasmic vacuolization, nuclear swelling, rupture of both nuclear and plasma membranes, and appear as faintly stained cells with nuclear ghosts. In contrast, apoptotic cells show shrinking, chromatin clumping, and nuclear fragmentation. In this case the cell membrane and cell organelles stay intact although the plasma membrane often shows some blebbing. Nuclear collapse is the main indicator that the cell has undergone apoptosis (Vermes *et al*., 1995). PI-stained normal nuclei have a uniform diffuse stain and brightly staining bodies, whereas apoptotic nuclei demonstrate a reduction of their diameter and an evident condensation of chromatin. Necrotic nuclei are different from apoptotic nuclei by PI, which stained leaked DNA, presented nuclei fragmentation and emitted red fluorescence (Studzinski, 1999). The nuclear morphological characteristics of PI-stained cultured human gingival epithelial cells under fluorescence microscopic investigation of this study were shown in Figure 4.1-4.5.

The result from fluorescence microscopic investigation at the magnification of 40 fold revealed that there was no obvious morphological difference in nuclear shape between untreated control gingival epithelial cells and cells treated with 0.9% NaCl

without any corrosion products (Figure 4.1 and 4.5, respectively). However, the appearance of the nuclei from gingival epithelial cells treated with 0.5 mM H_2O_2 for 10 h showed a characteristic of apoptosis (Figure 4.2), i.e. the majority of these treated nuclei showed a marked reduction in their diameter and an evident condensation of chromatin (small arrows in Figure 4.2). Moreover, there was a difference in the nuclear morphology between untreated control cells and cells treated with two different volumes, i.e. 50 and 500 μl, of corrosion products released from commercial magnets (Figure 4.3 and 4.4, respectively). The epithelial cells treated with corrosion products showed a slight reduction in their nuclear diameter and condensation of their chromatin (Figure 4.3 and 4.4).

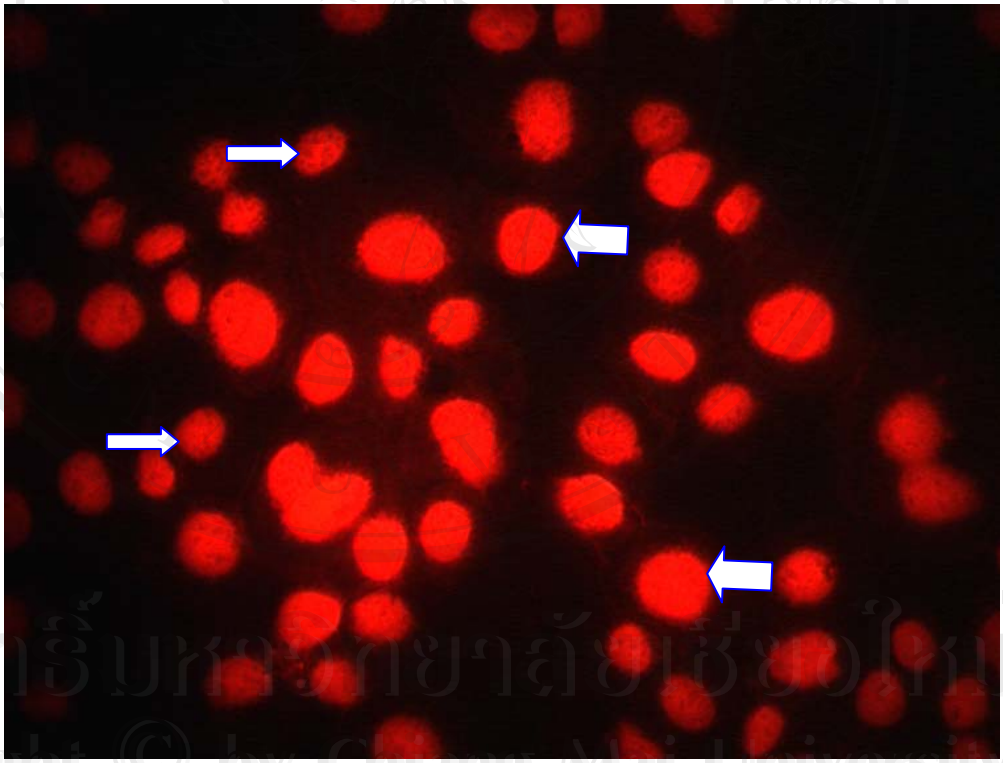

Figure 4.1 Fluorescence microscopic appearance of methanol-fixed and PI-stained nuclei of cultured human gingival epithelial cells after 5 days incubation in KGM alone (control group 1). The large arrows indicated the nuclei of normal cell, while the small arrows displayed the nuclei that were characteristic of apoptosis. The nuclei of apoptotic cells were smaller than those of normal cells, i.e. their smaller nuclear diameter and an evident condensation of chromatin. However, the characteristic of necrosis was not clearly found in this field. Original magnification x40.

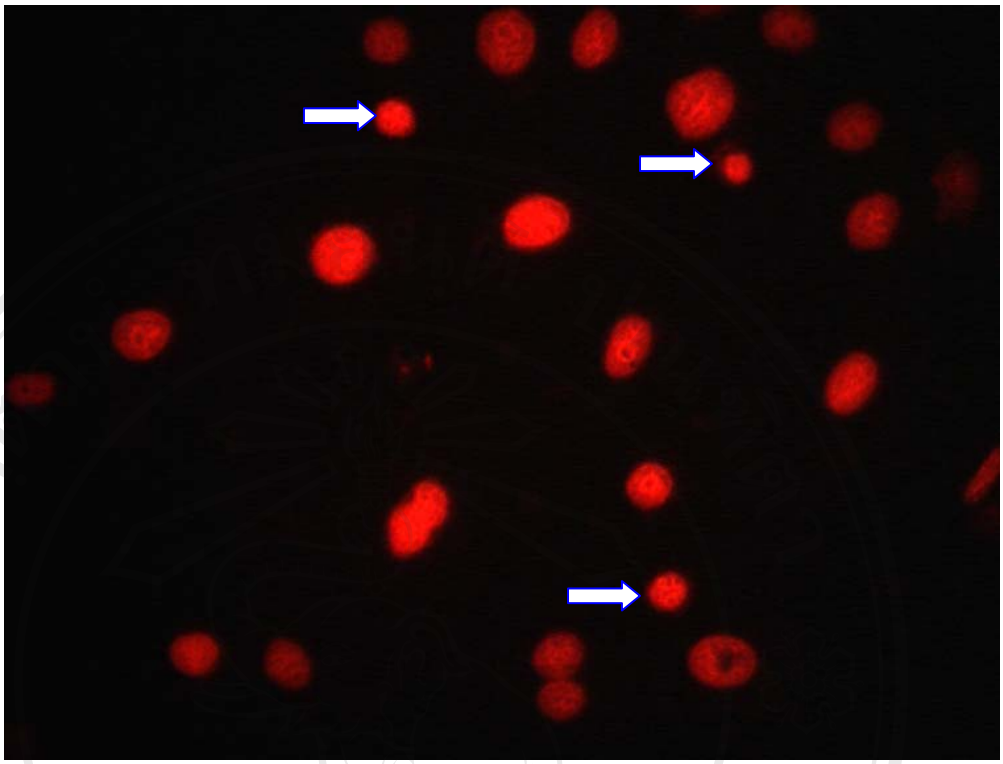

Figure 4.2 Fluorescence microscopic appearance of methanol-fixed and PI-stained nuclei of cultured human gingival epithelial cells after being induced to undergo apoptosis by 0.5 mM H_2O_2 for 10 h (positive control group 2). The apoptotic nuclei, which were indicated by the small arrows, showed a marked reduction in their diameter and evident condensation of chromatin. However, the appearance of normal nuclei was not observed in this field. Original magnification x40.

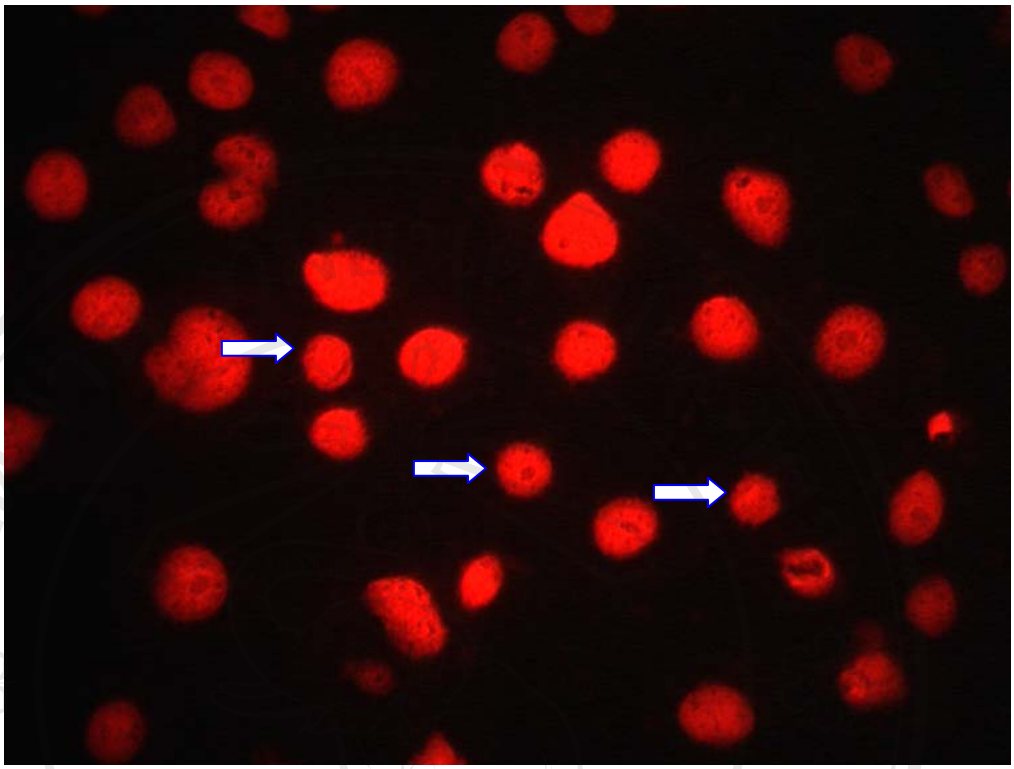

Figure 4.3 Fluorescence microscopic appearance of methanol-fixed and PI-stained nuclei of cultured human gingival epithelial cells after being incubated in 50 µl of 0.9% NaCl containing maximum corrosion products released from commercial magnets for 5 days (experimental group 3). Note the apoptotic cells with an evident chromatin condensation and reduction in nuclear size, as indicated by the small arrows. Overall, the diameter of these nuclei was smaller than that of control untreated cells. Original magnification x40.

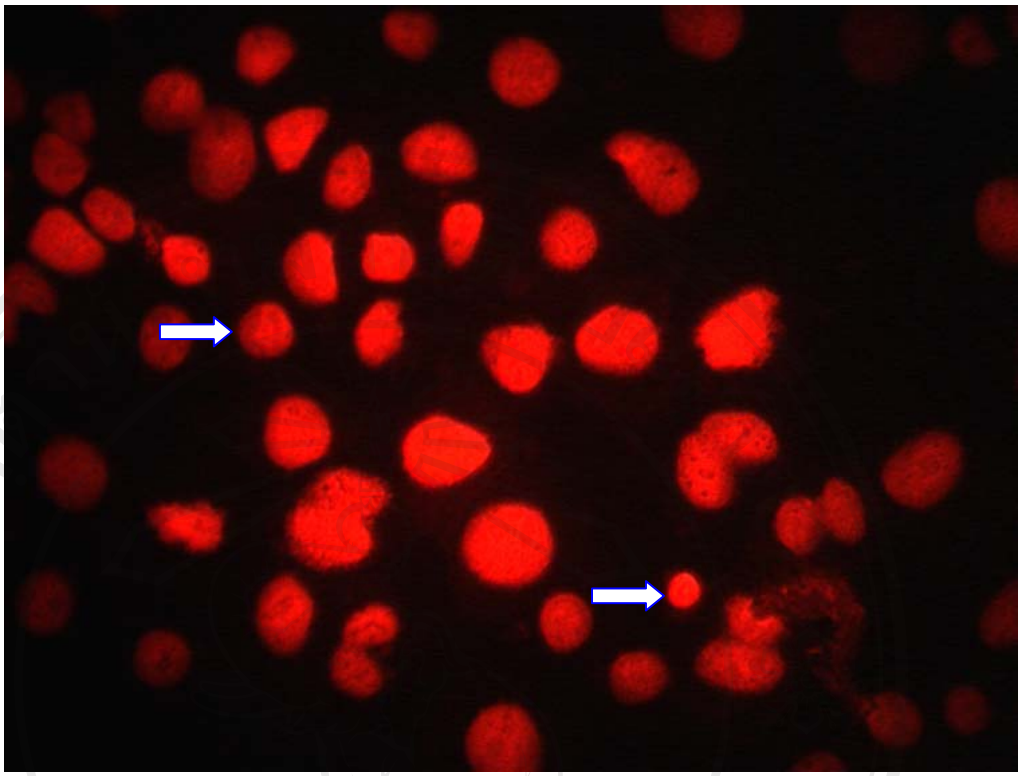

Figure 4.4 Fluorescence microscopic appearance of methanol-fixed and PI-stained nuclei of cultured human gingival epithelial cells after being incubated in 500 µl of 0.9% NaCl containing maximum corrosion products released from commercial magnets for 5 days (experimental group 4). Note the apoptotic cells, as indicated by the small arrows. Interestingly, the nuclear morphology of cultured human gingival epithelial cells treated with 50 µl of corrosion product solution was not generally different from that treated with 500 µl of corrosion product solution. Original magnification x40.

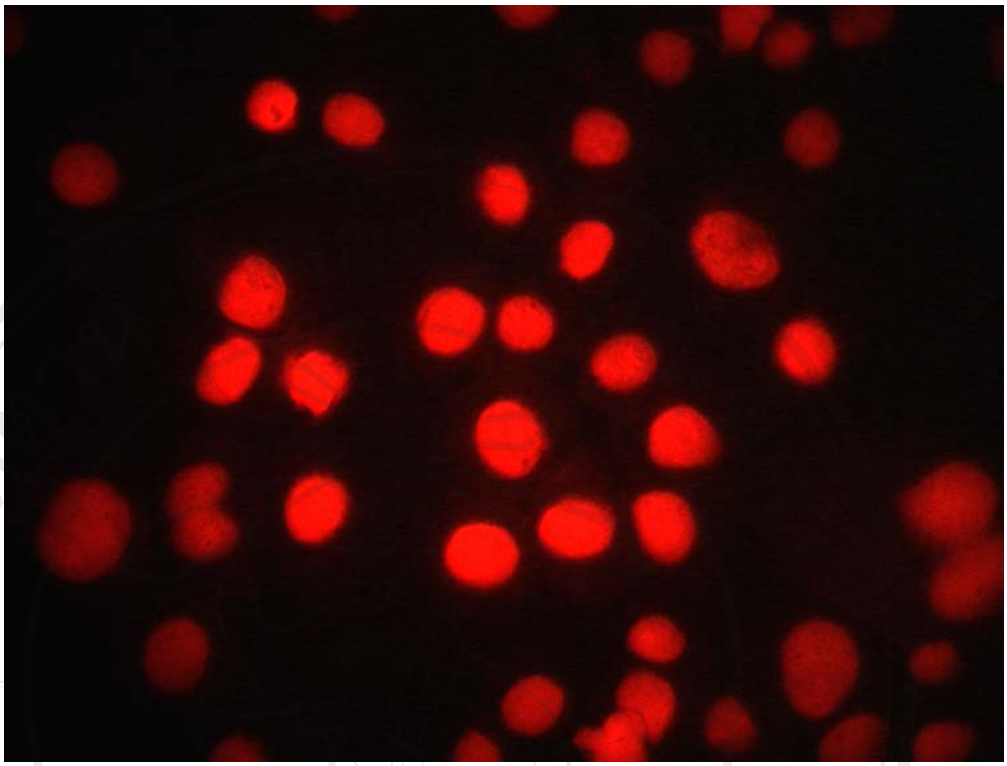

Figure 4.5 Fluorescence microscopic appearance of methanol-fixed and PI-stained nuclei of cultured human gingival epithelial cells after being incubated in 0.9% NaCl alone for 5 days (control solution group 5). In general, the nuclear appearance of epithelial cells after being exposed to 0.9% NaCl solution without any corrosion products showed no obvious difference from that of the control untreated cells. Original magnification x40.

In conclusion, both normal and apoptotic nuclei of cultured human gingival epithelial cells could be recognized under the fluorescence microscope by a PI-nuclear counterstained technique. The size of apoptotic nuclei was smaller than that of normal nuclei with an evident chromatin condensation. However, the appearance of necrotic nuclei of these cells was not found in any groups. It could be concluded that corrosion products released from commercial magnets had the cytotoxic effect on cultured human gingival epithelial cells by inducing cells to undergo apoptosis.

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

This study investigated the cytotoxic effect of corrosion products released from commercial magnets on cultured human gingival epithelial cells. The study included five groups (a negative control, a positive control, and three of experimental groups as described previously). Gingival epithelial cells were incubated in 5% CO_2-95% air, fully humidified atmosphere at 37^0C for 5 days. The percentage of normal viable, apoptotic, and necrotic cells were measured by flow cytometry (FMC), using FITC-conjugated annexin V and propidium iodide assay. The experiments were repeated at least three times for each group. The FITC-Annexin V versus PI dot plot diagram demonstrated the green fluorescence emission signal on X-axis (FITC-Annexin V) and the red fluorescence emission signal (PI) on Y- axis.

The apoptosis and the necrosis of human gingival epithelial cells were determined by the percentage of lower right quadrant (FIITC-Annexin V positive/ PI negative) and upper right quadrant (FITC-Annexin V positive/ PI positive), respectively. The findings from the dot plot diagrams of bivariate FITC-Annexin V/PI FCM of positive control cells, untreated control cells, and cells in all three experimental groups were shown in Figure 4.6.

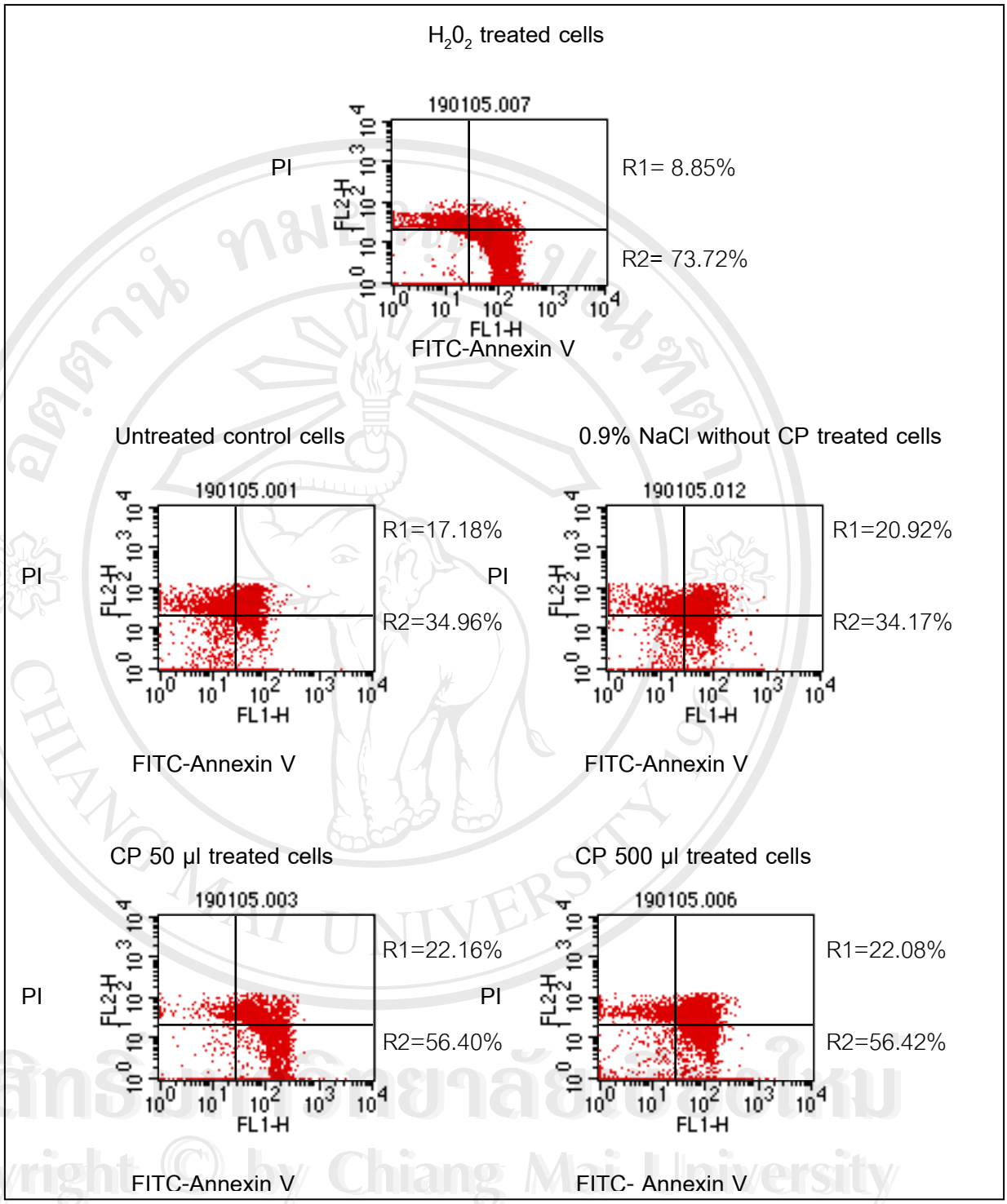

Figure 4.6 A contour diagram of FITC-Annexin V/PI flow cytometry of cultured human gingival epithelial cells treated with H_2O_2 (upper), untreated control cells (middle left), cells treated with 0.9% NaCl alone (middle right), and cells treated with 50 or 500 µl of 0.9% NaCl containing maximum corrosion products released from commercial magnets (lower left or lower right, respectively). This was representative of five experiments.

The lower left quadrant of each dot plot diagram showed the percentage of *viable cells*, which excluded PI and were negative for FITC-Annexin V binding. The upper right quadrant (R1) indicated the percentage of non-viable *necrotic cells* (positive for FITC-Annexin V binding and for PI uptake). The lower right quadrant (R2) indicated the percentage of *apoptotic cells* that excluded PI but were positive for FITC-Annexin V.

The results showed that the percentages of the apoptotic cells (R2) increased in the experimental group 3 and 4, i.e. 0.9% NaCl solution containing maximum corrosion products released from commercial magnets when compared with control untreated cells (group 1) and cells treated with 0.9% NaCl alone (group 5) (Figure 4.6). Furthermore, the percentage of the apoptotic cells (R2) in the positive control group (group 2) dramatically increased from that of control untreated cells (group 1). Interestingly, the percentages of the necrotic (R1) and apoptotic (R2) cells between untreated control cells and cells treated with 0.9% NaCl alone were not evidently different. The raw data of the percentages of the necrotic and apoptotic cells from all experiments performed in this study were presented in an Appendix section.

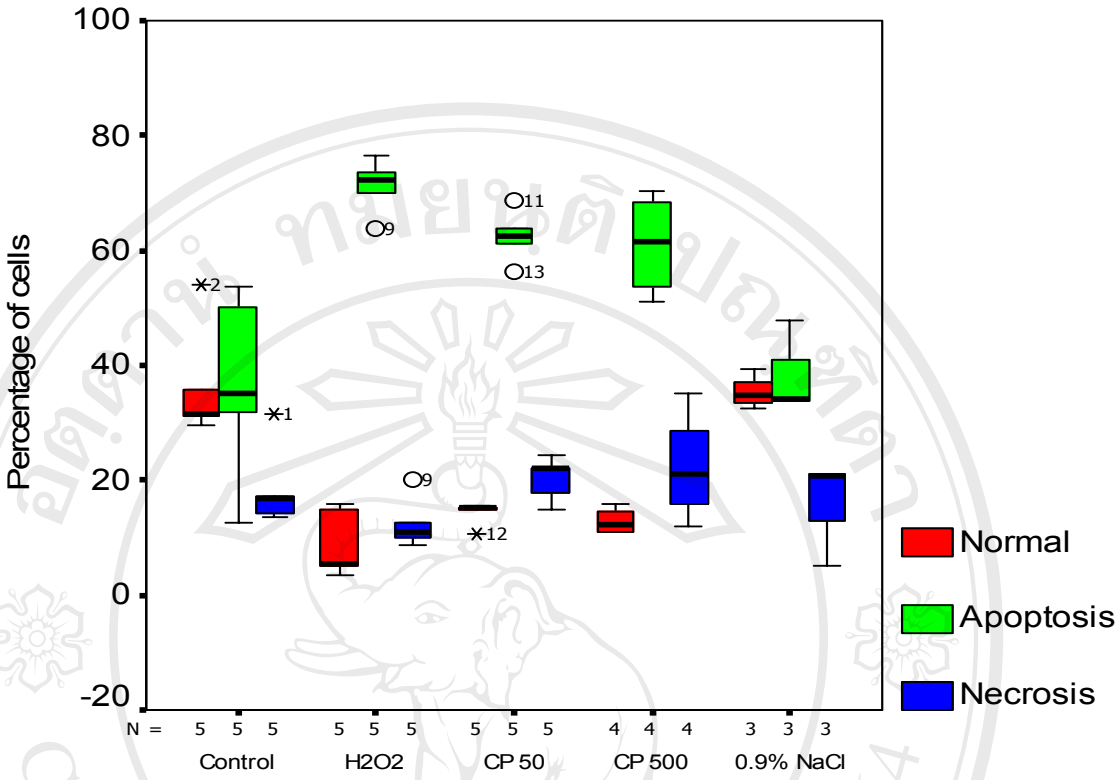

Figure 4.7 A box plot graph of the percentages of normal viable, apoptotic, and necrotic gingival epithelial cells in all five groups.

The medians as well as their 25th and 75th percentiles of the percentages from *normal viable*, *apoptotic*, and *necrotic* gingival epithelial cells in all five groups were shown in Figure 4.7. The box plot graph demonstrated that the percentages of *normal viable populations* of untreated control cells and cells treated with 0.9% NaCl alone (group 1 and 5, respectively) were higher than those of cells treated with H_2O_2 and 0.9% NaCl containing maximum corrosion product released from commercial magnets (group 2, 3,and 4). On the other hand, the percentages of apoptotic cells of control untreated cells and cells treated with 0.9% NaCl alone (group 1 and 5, respectively) were lower than those of cells treated with H_2O_2 and 0.9% NaCl containing maximum corrosion products released from commercial magnets (group 2, 3, and 4).

However, the percentages of the *necrotic cells* of control untreated cells and cells treated 0.9% NaCl alone (group1 and 5, respectively) did not clearly differ from those of the cells treated with H_2O_2 or corrosion products (group 2, 3, and 4). Furthermore, there was no difference in terms of the median percentages of the *normal viable*, *apoptotic,* and *necrotic* cells between cultured human gingival epithelial cells treated with 50 and 500 µl of 0.9% NaCl containing maximum corrosion products released from commercial magnets (group 3 and 4, respectively).

The mean ranks of the percentages of the apoptotic and necrotic cells in all five groups were presented in Table 4.1 and 4.2, respectively.

Table 4.1 The comparison of mean ranks of the percentages of the *apoptosis* (FITC-Annexin V positive/ PI negative) of the cultured human gingival epithelial cells by groups

Groups	N	Median	P 25	P 75	Mean rank	P-value *
1. Control	5	34.96	31.83	50.22	4.80	
2. H_2O_2	5	72.22	69.92	73.72	19.10	
3. 0.9% NaCl + CP 50 µl	5	62.40	61.27	63.90	13.30	0.002*
4. 0.9% NaCl + CP 500 µl	4	61.48	55.12	67.46	13.50	
5. 0.9% NaCl	3	34.17	33.94	41.06	4.33	

* Kruskal Wallis test =16.599 (P=0.002*)

N = Number of repeated experiments

Table 4.2 The comparison of mean ranks of the percentages of the *necrosis* (FITC-Annexin V positive/ PI positive) of the cultured human gingival epithelial cells by groups

Groups	N	Median	P 25	P 75	Mean rank	P-value *
1. Control	5	16.90	14.10	17.18	11.40	
2. H_2O_2	5	11.03	10.03	12.63	5.80	
3. 0.9% NaCl + CP 50 µl	5	22.15	17.90	22.16	15.60	0.158
4. 0.9% NaCl + CP 500 µl	4	21.09	18.05	25.35	14.25	
5. 0.9% NaCl	3	20.92	12.97	20.97	10.67	

* Kruskal Wallis test =6.614 (P=0.158)

N = Number of repeated experiments

When comparing the percentages of *apoptotic cells* between all groups (Table 4.1), the Kruskal Wallis test indicated that the percentages of apoptotic cells in the control group 1 and the experimental groups (groups 3 and 4) in the presence of corrosion products released from commercial magnets were statistically significant different (P= 0.002). However, the percentages of *necrotic cells* between control untreated cells (group 1) and the experimental groups (groups 3 and 4) in the presence of corrosion products released from commercial magnets were not statistically significant different (P= 0.158).

The Mann-Whitney U test was used to determine the statistical difference of the apoptosis of the cultured human gingival epithelial cells between the control group (group 1) and the other experimental groups (groups 2, 3, 4, and 5) at $P<0.05$.

Table 4.3 The comparisons of P-values from the percentages of *apoptotic* cells between the control group (group 1) and the remaining experimental groups (groups 2, 3, 4, and 5).

		Mann-Whitney U test	P-value
Group 1 control	Group 2 H_2O_2	0.000	P=0.009*
	Group 3 0.9% NaCl + CP 50 µl	0.000	P=0.009*
	Group 4 0.9% NaCl + CP 500 µl	1.000	P=0.027*
	Group 5 0.9% NaCl	7.000	P=0.881

A significant difference was found between the percentages of *apoptotic cells* in the control untreated cells (group 1) and cells treated with 0.5 mM H_2O_2 , 50 µl and 500 µl of 0.9% NaCl containing maximum corrosion products released from commercial magnets (P= 0.009*, P=0.009*, and P=0.027*, respectively) (Table 4.3). However, the percentages of apoptotic cells between the control untreated cells (group 1) and cells treated with 0.9% NaCl alone (group 5) were not significantly different (P=0.881).

Table 4.4 The comparison of mean ranks of the percentages of the apoptotic cells (FITC-Annexin V positive/ PI negative) between two different volumes of corrosion products.

Groups	N	Median	P 25	P 75	Mean rank	P-value *
3. 0.9% NaCl + CP 50 µl	5	62.40	61.27	63.90	5.00	1.00
4. 0.9% NaCl + CP 500 µl	4	61.48	55.12	67.46	5.00	

* Mann- Whitney U test = 10.000 (P=1.00)

N = Number of repeated experiments

When comparing the percentages of *apoptotic cells* between two different volumes of corrosion products, the Mann-Whitney U test showed that the percentages of apoptotic cells in groups 3 and 4 (cells treated with 50 µl and 500 µl, respectively, of 0.9 % NaCl containing maximum corrosion products released from commercial magnets) were not statistically significant different (P= 1.00) (Table 4.4).

Table 4.5 The comparisons of mean ranks of the percentages of *dead cells* (apoptosis and necrosis) of cultured human gingival epithelial cells by groups

Groups	N	Median	P 25	P 75	Mean rank	P-value *
1. Control	5	63.38	52.14	64.32	4.80	
2. H_2O_2	5	82.57	82.25	84.07	16.00	
3. 0.9% NaCl + CP 50 µl	5	83.30	81.80	83.42	15.20	0.0049*
4. 0.9% NaCl + CP 500 µl	4	84.27	81.25	86.44	15.25	
5. 0.9% NaCl	3	54.72	53.84	54.91	4.00	

* Kruskal Wallis test =14.683 (P=0.0049*)

N = Number of repeated experiments

When comparing the percentages of *dead cells* (apoptotic and necrotic cells) between all groups (Table 4.5), the Kruskal Wallis test indicated that the *dead cells* in the control group and the 0.9% NaCl alone group (groups 1 and 5, respectively) were significantly different from groups 2, 3, and 4 (P= 0.0049*) (Table 4.5).

The Mann-Whitney U test was used to determine the statistical difference of the *dead cells* between the control group (group 1) and the remaining experimental groups (groups 2, 3, 4, and 5) at $P<0.05$.

Table 4.6 The comparisons of P-values of the percentages of *dea*d *cells* (apoptosis and necrosis) of the cultured human gingival epithelial cells between the control group and the remaining experimental groups.

		Mann-Whitney U test	P-value
Group 1 control	Group 2 H_2O_2	0.000	P=0.009*
	Group 3 0.9% NaCl + CP 50 µl	0.000	P=0.009*
	Group 4 0.9% NaCl + CP 500 µl	0.000	P=0.014*
	Group 5 0.9% NaCl	6.000	P=0.655

Similar to the significant difference found in the percentages of apoptotic cells (Table 4.3), a significant difference was found in terms of the percentages of the *dead cells* between the untreated control cells (group 1) and cells treated with 0.5 mM H_2O_2 (group 2), 50 µl (group 3), and 500 µl (group 4) of 0.9% NaCl containing maximum corrosion products released from commercial magnets (P = 0.009*, P=0.009*, and P=0.014*, respectively). However, the percentages of the *dead cells* in the untreated control cells (group 1) did not significantly differ from those in cells treated with 0.9% NaCl alone (group 5) (P=0.665).

Table 4.7 The comparison of mean ranks of the percentages of the *dead cells* (apoptosis and necrosis) between two different volumes of corrosion products.

Groups	N	Median	P 25	P 75	Mean rank	P-value *
3. 0.9% NaCl + CP 50 µl	5	83.30	81.80	83.42	5.00	1.00
4. 0.9% NaCl + CP 500 µl	4	84.27	81.25	86.44	5.00	

* Mann- Whitney U test = 10.000 (P=1.00)

N = Number of repeated experiments

When comparing the percentages of *dead cells* (apoptosis and necrosis) of cultured human gingival epithelial cells between two different volumes of corrosion products, the Mann-Whitney U test showed that the percentages of the *dead cells* in groups 3 and 4, i.e. cells treated with 50 µl and 500 µl of 0.9% NaCl containing maximum corrosion products released from commercial magnets, respectively, were not significantly different (P=1.00) (Table 4.7).

In conclusion, the percentages of the apoptotic cells in the control group 1 and groups 2, 3, and 4, i.e. cells treated with H_2O_2, 50 µl and 500 µl of 0.9% NaCl containing corrosion products for 5 days, respectively, were significantly different. In contrast, the percentages of the necrotic cells between the control untreated and treated cells were not. Consequently, the corrosion products released from commercial magnets had cytotoxic effect on the cultured human gingival epithelial cells via an apoptotic cell death.