CHAPTER V

RESULTS AND DISCUSSION

5.1 Fat removal

5.1.1 Effects of freeze thaw on fat removal

The percentages of fat removal of porcine skin treated in different groups (FR1-FR5) were shown in Figure 5.1. It was noticed that, fat in the skin treated in hexane for 12 hr without pretreatment (FR1) was poorly removed (50% fat removal). When freeze thaw pretreatment was applied prior to a treatment in hexane for 12 hr, as represented in FR2, the percentage of fat removal was increased to 82%. These results revealed that, pretreatment using freeze thaw technique can improve the performance of fat removal from porcine skin. In general, freeze thaw technique is used to disrupt cells in tissues [26]. The denaturation of macromolecules occurs due to the fact that at such low temperatures, the molecule's conformation changes. In the other words, process of freezing tissues will damage cells and whole tissues irreversibly because an osmotic shock together with formation of ice crystals disrupts cell membranes. As a result, lipid combined in tissue matrix can be easily removed by solvent.

Comparing the influence of freeze thaw cycles on fat removal (FR2-FR4), it was noticed that when the numbers of freeze thaw cycles were increased from 5 cycles (FR2) to 10 cycles (FR3) and 15 cycles (FR4), the percentage of fat removal was increased from 82% to 91% and 92%, respectively. These results revealed that, pretreatment porcine skin with 10 cycles of freeze thaw was suitable to enhance the performance of fat removal from porcine skin by hexane treatment for 12 hr.

5.1.2 Effects of type of solvent on fat removal

Various types of solvent have been long known to remove fat from tissues, for example hexane and chloroform/methanol. The effects of hexane and chloroform/methanol (2/1 v/v) on the fat removal from porcine skin were compared. As presented in FR3 (use of hexane) and FR5 samples (use of chloroform/methanol) in Figure 5.1, it could be noticed that the mixture of chloroform/methanol (2/1 v/v) solvent can be used to remove fat more effectively than hexane. 99.5% and 92% of fat removal were achieved when porcine skin was treated with chloroform/methanol (2/1 v/v) and hexane, respectively. In order to extract lipids from tissues, it is necessary to use solvents that not only dissolve the lipids readily but overcome the interactions between lipid and tissue matrix, and it is essential to perturb both the hydrophobic and polar interaction at the same time [27]. No single pure solvent appears to be suitable for a purpose of lipid extraction. These results confirmed that, pure hexane was insufficient to achieve a complete removal of fat. Most lipid analysts have accepted that a mixture of chloroform and methanol in the ratio of 2/1 (v/v) can extract lipids more thoroughly from animals, plants, and bacterial tissues than other simple solvent combinations. These probably result from the capacity of chloroform to associate with water molecules, presumably by week hydrogen bonds [28].

5.1.3 Effects of treatment time on fat removal

As evident in Figure 5.1 that chloroform/methanol (2/1 v/v) can be effectively used to remove fat from porcine skin. This experiment was carried out in order to confirm the optimum time necessary for fat removal using chloroform/methanol. All samples were pretreated with 10 freeze thaw cycles. In Figure 5.2, the result from FR5 was replotted and compared to FR6-FR9, of which the treatment time in chloroform/methanol was reduced to the range of 3 hr to 20 min. It was noticed that the percentages of fat removal in FR5 (treatment time = 12 hr), FR6 (treatment time = 3 hr), and FR7 (treatment time = 2 hr) were as high as 98-99.5%, implying a complete removal of fat from porcine skin. When further reducing the treatment time to 1 hr (FR8) and 20 min (FR9), the percentages of fat removal was significantly decreased

The results suggested that, after pretreatment of porcine skin by 10 cycles of freeze thaw, a treatment by chloroform/methanol (2/1 v/v) at room temperature for 2 hr was beneficial to effectively remove fat in porcine skin.

5.2 Cell removal

5.2.1 Effects of enzyme refreshment on cell removal

The efficiency of acellular dermal matrix (ADM) in the treatment of full thickness injuries as a dermal substitute depends on its low antigenicity. This directly relates to the performance of cell removal from skin. Most previous studies have evaluated the performance of cell removal qualitatively, preferably by histological examination [4,7,11]. This was, however, insufficient to ensure that cells were completely removed from skin. In this study, the performance of cell removal was investigated both qualitatively and quantitatively by H&E staining and DNA assay, respectively. Since DNA, a macromolecule presented in every living cell, is the basis for all biological processes, the amount of other molecules produced by cells can be measured in relation to the DNA content of the cells [29]. The amount of DNA can then represent the number of cells in porcine skin. Therefore, the percentage of cell removal form porcine skin can be easily determined by DNA assay.

In this experiment, the decellularization by enzymatic treatment was carried out using 1% (w/v) trypsin solution of which the activity was 56 unit/g. The percentages of cell removal from porcine skin determined by DNA assay for various groups of treatment were depicted in Figure 5.3. It was noticed that, cells in ADM treated by trypsin using continuous stirring (CR1) were poorly removed (33% of cell removal). Since porcine skin has a tightly compacted network of thick collagen bundles [7], the access of enzyme solution into the dermis is difficult. In addition, at such a long incubating time as 24 hr, the activities of trypsin greatly dropped by 58%, as shown in Figure 5.4. This is due to the fact that the activity of enzyme was dependent on the incubation time [28]. The results from Figure 5.4 showed that, after trypsin was incubated at room temperature and pH 7.4 for 6 hr, the activity was

sharply decreased by 43%. During the decellularized process, a number of protease inhibitor can be released from disrupted cells, resulting in the inhibition of enzyme-substrate interaction [8]. From this information, the decellularized process using trypsin was carried out by refreshing trypsin solution at every 6 hr (CR2). The influence of enzyme refreshment on cell removal observed in CR2 group (Figure 5.3), showed that the percentage of cell removal was slightly increased compared to the process without enzyme refreshment in CR1. This obviously proved that enzyme activity was not the main reason of the poor performance of cell removal. The alternative reason, therefore, was likely to be the difficulty for enzyme to access into active sites in such a dense porcine skin.

5.2.2 Influence of periodic pressurized technique on cell removal

To overcome such a problem, periodic pressurized technique was introduced into the decellularized process using trypsin. In CR3 group, trypsin incubation time was shorten from 24 hr (CR2) to 12 hr and 24 pressurized periods were applied. The DNA assay results in CR2 and CR3 groups (Figure 5.3) shown that, percentage of cell removal in a short-time treatment under pressurized period (12 hr for CR3) was much greater than a long-time treatment with continuous stirring (24 hr for CR2). This indicated that the periodic pressurized process was able to reduce the incubation time in trypsin solution and progressively improved the efficiency of cell removal for 34%. Under pressurized condition, enzyme solution was able to penetrate into porcine skin more thoroughly. A greater increase in the percentage of cell removal probably resulted from an increasing of enzyme-substrate interaction.

However, previous studies reported that lengthy enzyme incubation could disrupt ECM structure, remove fibronectin, elastin, and GAG [30,31]. In the present study, short-time process was alternatively selected to prevent the problem mentioned above. However, short-time process may reduce the performance of cell removal. In order to improve the performance of cell removal treatment in a short-time process, increasing of a number of pressurized periods was employed in the decellularized process. The experiment on CR4 group was then performed by shortening trypsin

incubation time to 3 hr and increasing a number of pressurized periods to 36. Enzyme refreshment was also performed more often, i.e. every 1.5 hr. Comparing the result on CR4 to CR3 treatments in Figure 5.3, one could notice that when the incubation time of enzyme was decreased from 12 hr to 3 hr and pressurized periods was increased from 24 periods to 36 periods, the percentage of cell removal was increased from 72% to 77%. These results suggested that, the percentage of cell removal under short-time trypsin treatment of de-fat skin with short pressurized period time (greater number of pressurized periods) was comparable to, or even slightly higher than, the one under long-time treatment with long pressurized period time. However, as cells and other components were quickly removed from porcine skin within a short-time, it was suspected that high concentration of cells in enzyme solution might inhibit enzyme-substrate interactions.

The experiment on CR5 group was therefore carried out same as on CR4 group, except the schedule of enzyme refreshment which was at every 45 min instead of 1.5 hr. The results on CR4 and CR5 groups in Figure 5.3 found that the percentage of cell removal in CR5 was significantly higher than that in CR4. Additional results on the activity of trypsin assayed at the same condition as in CR4 and CR5 treatments, shown in Figure 5.4, ensured that the activities of trypsin during the first 45 min of treatment were relatively high. In other words, trypsin was still active during the refreshment. This proved the hypothesis mentioned earlier that cells and other cellular components removed from skin inhibited enzyme accession into active sites of skin. These results corresponded to histological examination depicted in Figure 5.5. Histological photograph of porcine skin without any treatments used as a control was shown in Figure 5.5a. It exhibited a dense structure of collagen fiber (pink color) with a lot of bound fibroblast cells (bluish purple color indicated by arrows). Histological photograph of the samples of CR4 and CR5 groups shown in Figure 5.5b and 5.5c, respectively, revealed that fibroblasts found in CR4 and CR5 treatments were much fewer than in fresh porcine skin (Figure 5.5a). In addition, there were fewer fibroblasts found in CR5 sample comparing to CR4 sample.

From the results on CR1-CR5 treatments, it could be confirmed that periodic pressurized technique could be effectively utilized in the decellularized process, providing an advantage over typical stirring method. With an appropriated number of pressurized periods applied and enzyme refreshment in a short-time enzymatic treatment, ADM from porcine skin could be achieved.

5.2.3 Comparison of the type of enzyme

Two types of enzyme including trypsin and dispase II have been employed in the decellularized process of porcine skin. In this study, dispase II was used in comparison with trypsin at the same total activity (115 unit), which was determined by enzyme activity assay [37], and same pressurized period condition. From the results in CR4 (trypsin) and CR6 (dispase II) in Figure 5.3, dispase II could be used to remove cells better than trypsin (92% and 77% cell removal for CR6 and CR4, respectively). These results on DNA assay corresponded with the results on histological examinations, shown in Figure 5.5. A small amount of fibroblasts could be found in the histological photograph of CR4 sample (Figure 5.5b) while fibroblast cells were not observed in dermal matrix from CR6 treatment. The qualitative results from histological examination of CR6 group implied a complete removal of cells from porcine skin. Though, the quantitative results on DNA assay showed the highest percentage of cell removal, but not 100% removal. The discrepancy in DNA assay was possibly due to the use of L929 mouse fibroblast for the calibration. Nevertheless, the results from DNA assay and histological revealed that, dispase II was effectively used to remove cell better than trypsin. This might be because trypsin acts on lysine and arginine residues, while dispase II cleaved fibronectin, laminin, and collagen type IV (basement membrane), which is a major constituent of the anchoring fibroblast [11].

5.3 Morphology observation

SEM micrographs of ADM samples from each cell removal treatment were shown in Figure 5.6. SEM micrographs of fresh porcine skin, elucidated in Figure

5.6a as a reference, shown a dense and slightly porous structure. After treatment with trypsin for 24 hr (CR1 in Figure 5.6b and CR2 in Figure 5.6c), the dermal structures looked much denser than the original porcine skin. This indicated that a lengthy treatment time with trypsin induced collagen degradation in dermal structure [8]. Decreasing incubation time in trypsin to 12 hr resulted in a slightly porous structure of ADM as observed in Figure 5.6d. Apparently, the structural pattern of collagen fiber remained in ADM samples treated for 3 hr in trypsin and dispase II solutions as shown in Figure 5.6e and Figure 5.6g, respectively. In comparison of Figure 5.6e and 5.6g to Figure 5.6a, structure of ADM samples looked to be more fibrous than that of fresh porcine skin.

The results elucidated that, to prevent collagen degradation affected by treatment under high enzyme concentration, the treatment time in enzyme solution must be short. Short-time treatment with periodic pressurized technique not only effectively removed cells from porcine skin, but also preserved the natural dermal structure and the characteristics of collagen fiber in ADM.

5.4 Fourier transforms raman (FT-Raman) spectrophotometric analysis

In this study, characteristics of collagen were observed using FT-Raman spectroscopy. FT-Raman spectra obtained from fresh porcine skin, de-fat skin, and ADM derived from various treatments (CR4-CR6) were shown in Figure 5.7. Six characteristics of vibration bands at the wavenumbers of 1665, 1270, 1245, 1004, 884, and 855 cm^{-1} were observed. Generally, amide I (1665 cm^{-1}) originate from C=O stretching vibration. Amide III vibration band (1270 and 1245 cm^{-1}) is a combination peak between N–H deformation and C–N stretching vibrations [32]. Amino acid residues were observed in the region of 1000-800 cm^{-1} where amino acid such as phenylanaline (Phe), proline (Pro), and hydroxyproline (Hyp) shown strong raman scattering due to aromatic or saturated side chain ring [33]. A band corresponded to phenylanaline appeared at 1004 cm^{-1}, whereas two bands for proline and hydroxyproline residue were observed at 855 and 884 cm^{-1}, respectively. The specific vibration band obtained form porcine skin under various treatment corresponded to

specific vibration band of type I collagen. Significant differences between porcine skin, de-fat skin, and ADM derived from various treatment (CR4-CR6) were not observed. This indicated that, the structure of type I collagen remained in ADM. In other words, type I collagen in porcine dermis was not affected by decellularized process using periodic pressurized technique.

5.5 Animal study

5.5.1 Cell infiltration into ADM

ADM samples produced by using periodic pressurized technique were shown in Figure 5.8. Cell infiltration into ADM produced from CR5 (trypsin model) and CR6 (dispase II model) was investigated by SEM. Figure 5.9 showed photographs of ADM samples from CR5 (trypsin model) and CR6 (dispase II model) retrieved at 1-, 2-, and 4-week postoperatively. After 1-week of implantation (Figure 5.9a and 5.9d), capillaries were observed in both models. It was noted that no significant degradation of implanted ADM was noticed at 1-week postoperatively. After 2-week implantation (Figure 5.9b and 5.9e), significant difference was not observed in both models compared to 1-week implantation. After 4-week implantation (Figure 5.9c and 5.9f), ADM prepared from both models markedly degraded. In addition, ADM samples from both models were filled with numerous capillaries from the host tissue. Infiltration of cells was accompanied by degradation of acellular tissue [34]. The implants provoke cellular responses which led to physical invasion by various cells such as polymorphonuclear leukocytes, macrophages, and fibroblasts [35].

Scanning electron microscopy was employed to visualize cell migration into the implanted ADM. The cross section of samples was defined by C1, C2, and C3. The C1 section represented region of tissue at the sample edge, C2 was at the depth of 0.25 cm from the edge, and C3 was at the center of implanted samples. Figure 5.10 showed the SEM photographs of the retrieved samples produced from CR5 treatment at 1-, 2- and 4-week. Cells only adhered at the edge of ADM during the first week. The absence of cells in implanted ADM probably results from tightly compacted

network of collagen bundles, blocking migration of cells [7]. After 2-week implantation, there were some cells observed at C2 section, indicating that cells could migrate into ADM sample. However, no cells appeared at the center of ADM (C3 section). After 4-week implantation, a number of cells could be found at the center of the ADM samples. Similar phenomena of cell infiltration was observed in the case of CR6 samples, as illustrated in Figure 5.11. These results indicated that a number of cells could infiltrate into ADM samples prepared from both trypsin and dispase II models.

Figure 5.12 presented histological micrographs of implanted ADM samples from CR5 (trypsin model) and CR6 (dispase model) treatments at 2- and 4-week postoperatively. After 2-week of implantation (Figure 5.12a and 5.12b), inflammatory and fibroblast cells were able to infiltrate into open spaces of ADM samples. The depth of cell infiltration into both ADM samples was similar. In addition, neo-collagen fibril and neo-capillaries were observed in both ADM samples. After 4-week of implantation (Figure 5.12c and 5.12d), the results showed that ADM samples degraded and were filled with cells. There were still some inflammatory cells presented in acellular tissue. Instead, fibroblasts (migrating from the host tissue), neo-collagen fibril, neo-capillaries, and red blood cell were observed. The depth of cell infiltration into ADM after 4 weeks was significantly greater than that observed after 2 weeks. In addition, cell penetrated into CR6 sample (dispase model) deeper than CR5 sample (trypsin model). Penetration of cells into the ADM may be caused by low antigenicity of ADM. In addition, once inflammatory cells infiltrated into ADM scaffolds, proteolytic enzymes such as collagenase secreted by macrophages started to degrade the original porcine tissue fibrils [36]. This allowed fibroblasts from the host tissue (rat) to migrate into the ADM. Consequently, more neo-collagen fibrils were produced.

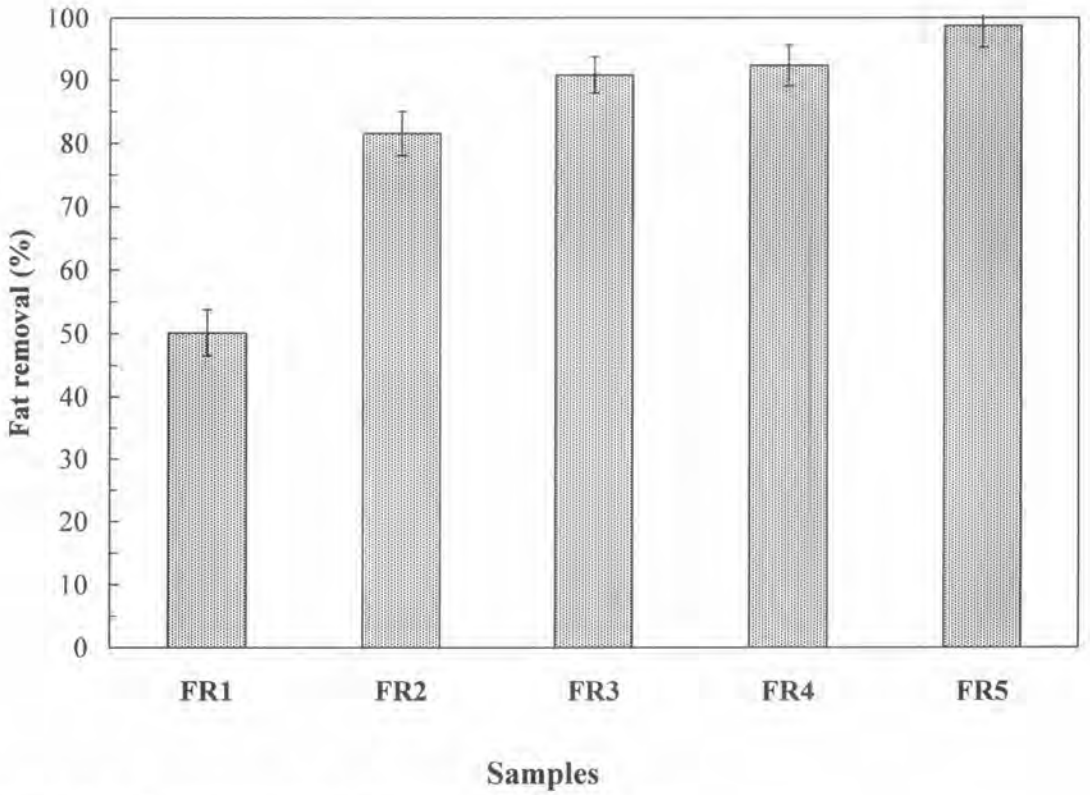


Figure 5.1 Percentage of fat removal from porcine skin;

FR1 = hexane treatment for 12 hr

FR2 = 5 freeze thaw cycles and hexane treatment for 12 hr

FR3 = 10 freeze thaw cycles and hexane treatment for 12 hr

FR4 = 15 freeze thaw cycles and hexane treatment for 12 hr

FR5 = 10 freeze thaw cycles and chloroform/methanol treatment for 12 hr

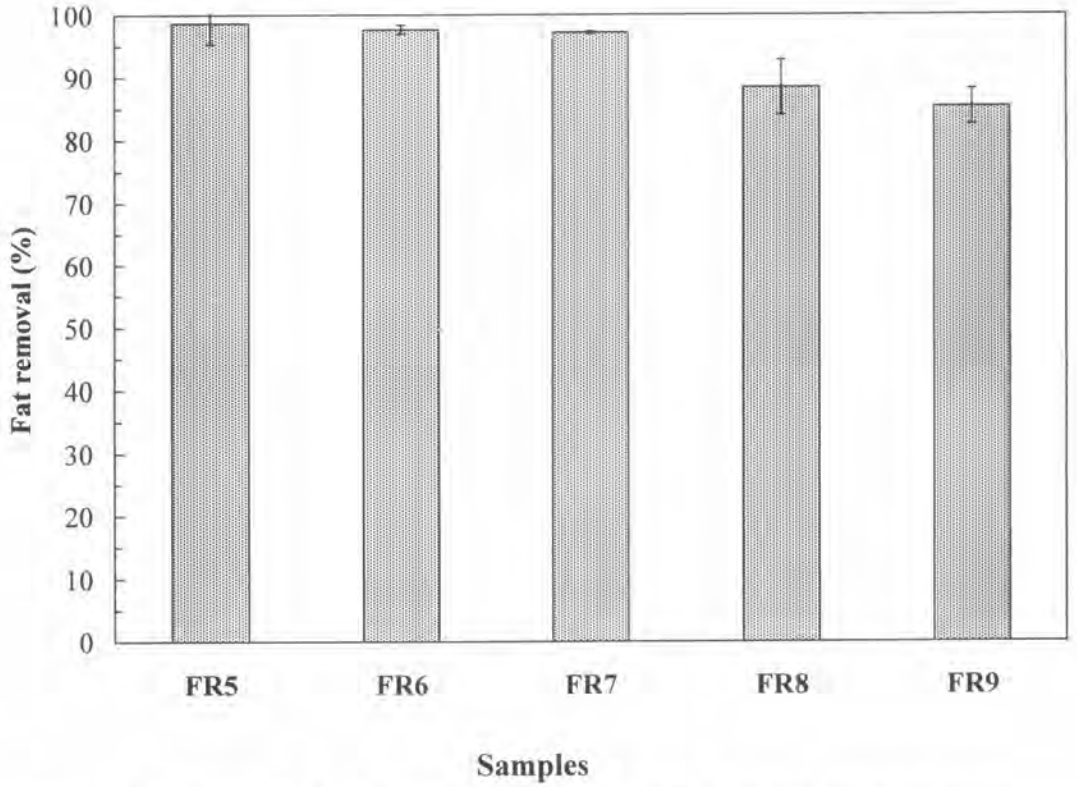


Figure 5.2 Percentage of fat removal from porcine skin by chloroform/methanol treatment;

FR5 = 10 freeze thaw cycles and chloroform/methanol treatment for 12 hr

FR6 = 10 freeze thaw cycles and chloroform/methanol treatment for 3 hr

FR7 = 10 freeze thaw cycles and chloroform/methanol treatment for 2 hr

FR8 = 10 freeze thaw cycles and chloroform/methanol treatment for 1 hr

FR9 = 10 freeze thaw cycles and chloroform/methanol treatment for 20 min

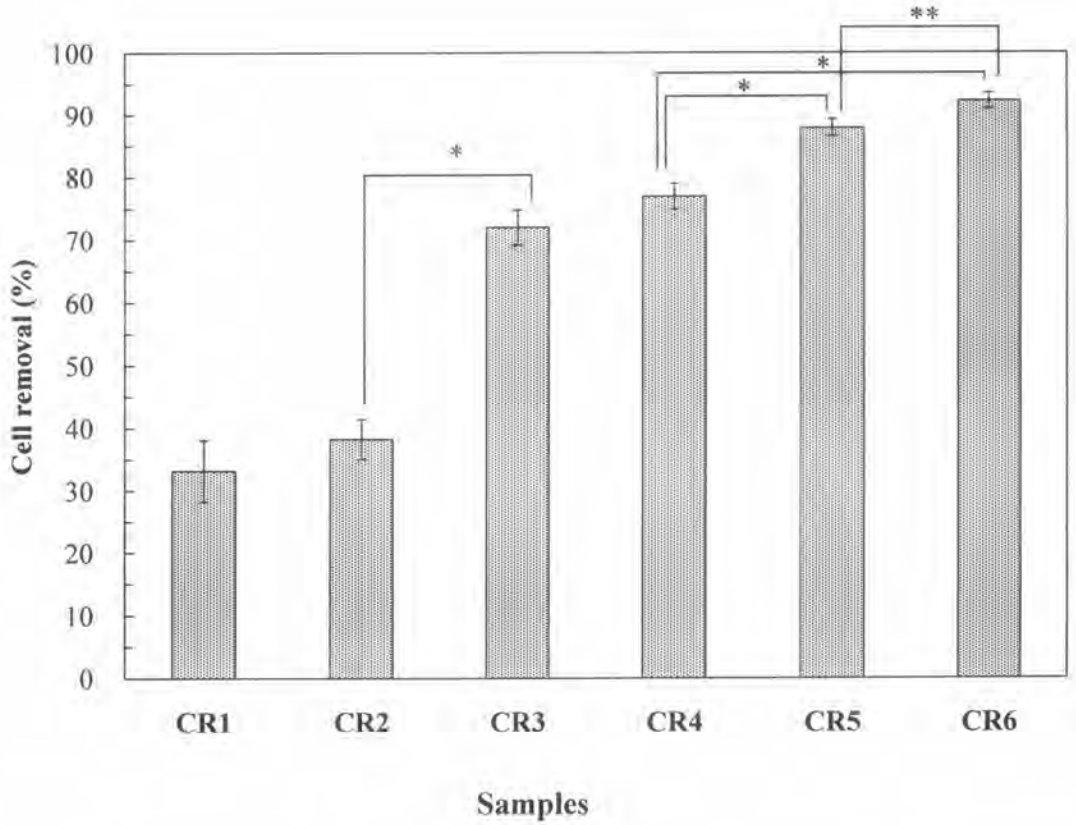


Figure 5.3 Percentage of cell removal from porcine skin determined by DNA assay.

CR1 = trypsin, continuous stirring, treatment time 24 hr

CR2 = trypsin, enzyme refreshment 6 hr, continuous stirring, treatment time 24 hr

CR3 = trypsin, enzyme refreshment 6 hr, 24 pressurized periods, treatment time 12 hr

CR4 = trypsin, enzyme refreshment 1.5 hr, 36 pressurized periods, treatment time 3 hr

CR5 = trypsin, enzyme refreshment 45 min, 36 pressurized periods, treatment time 3 hr

CR6 = dispase II, enzyme refreshment 1.5 hr, 36 pressurized periods, treatment time 3 hr

* represented a significant difference at $P<0.01$, ** represented a significant difference at $P<0.05$

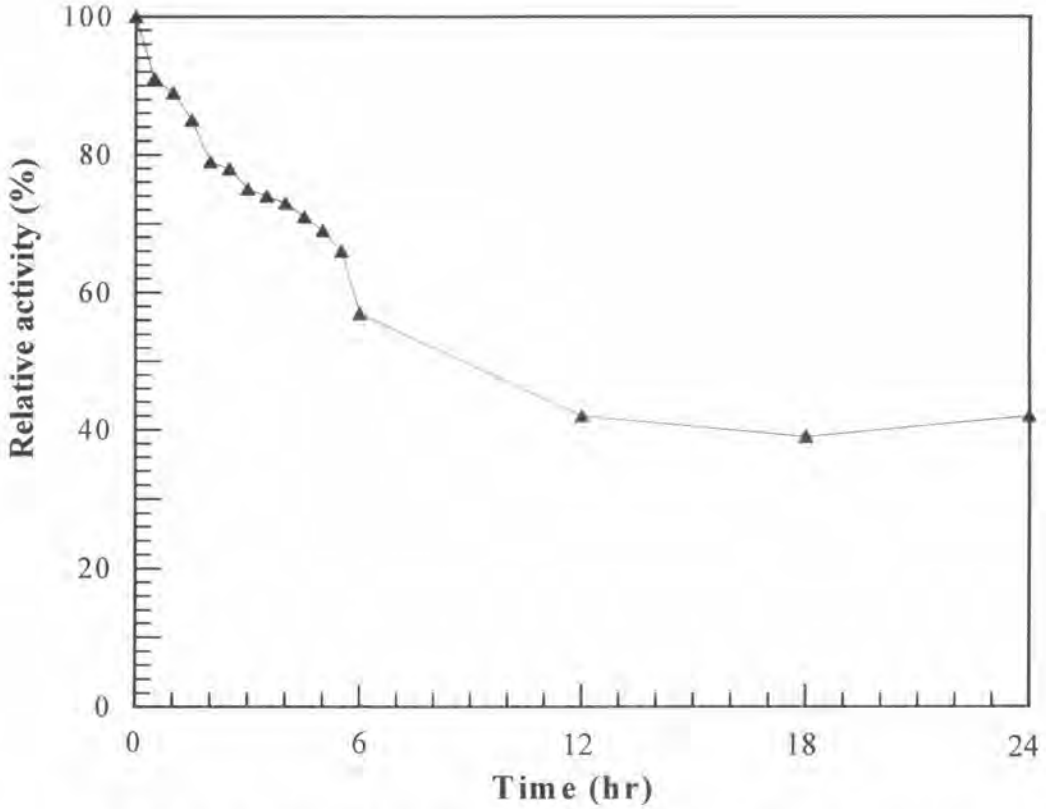


Figure 5.4. Relative activity of trypsin as a function of time at room temperature and pH 7.4.

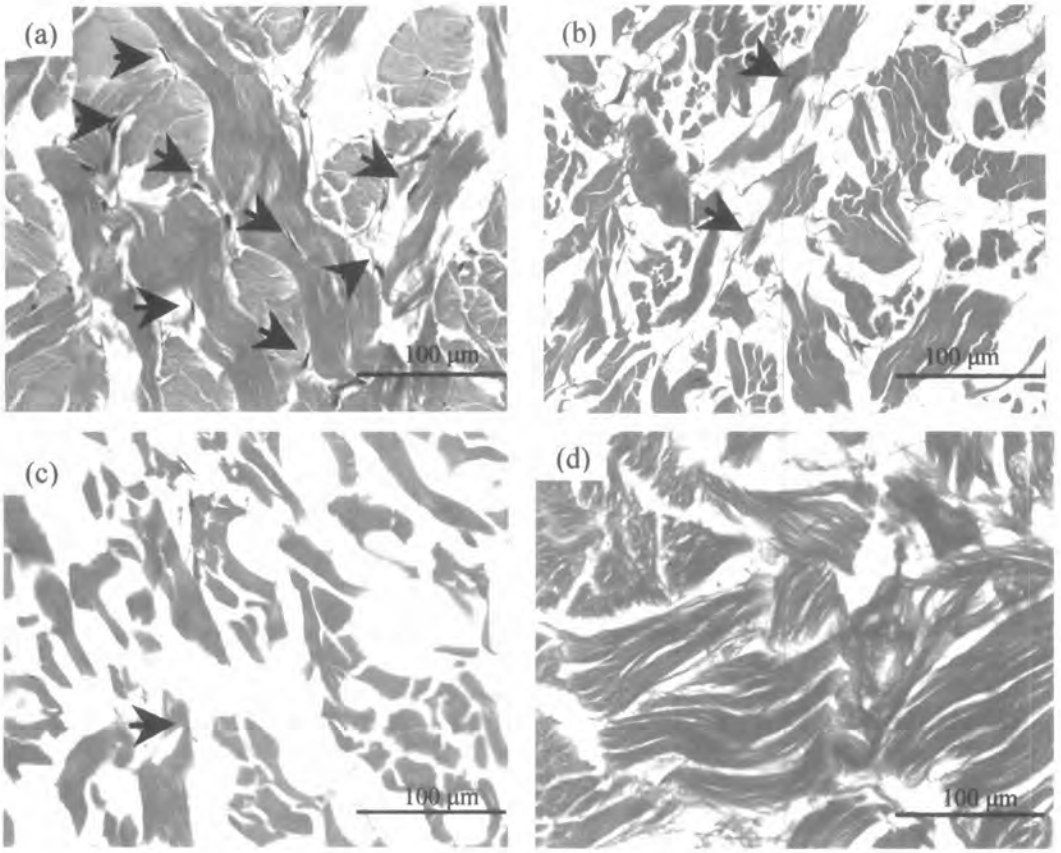


Figure 5.5 Histological photographs of fresh porcine skin and ADM samples stained with H&E (400 x magnification): (a) fresh porcine skin, (b) CR4, (c) CR5, (d) CR6 (arrows indicated fibroblast cells).

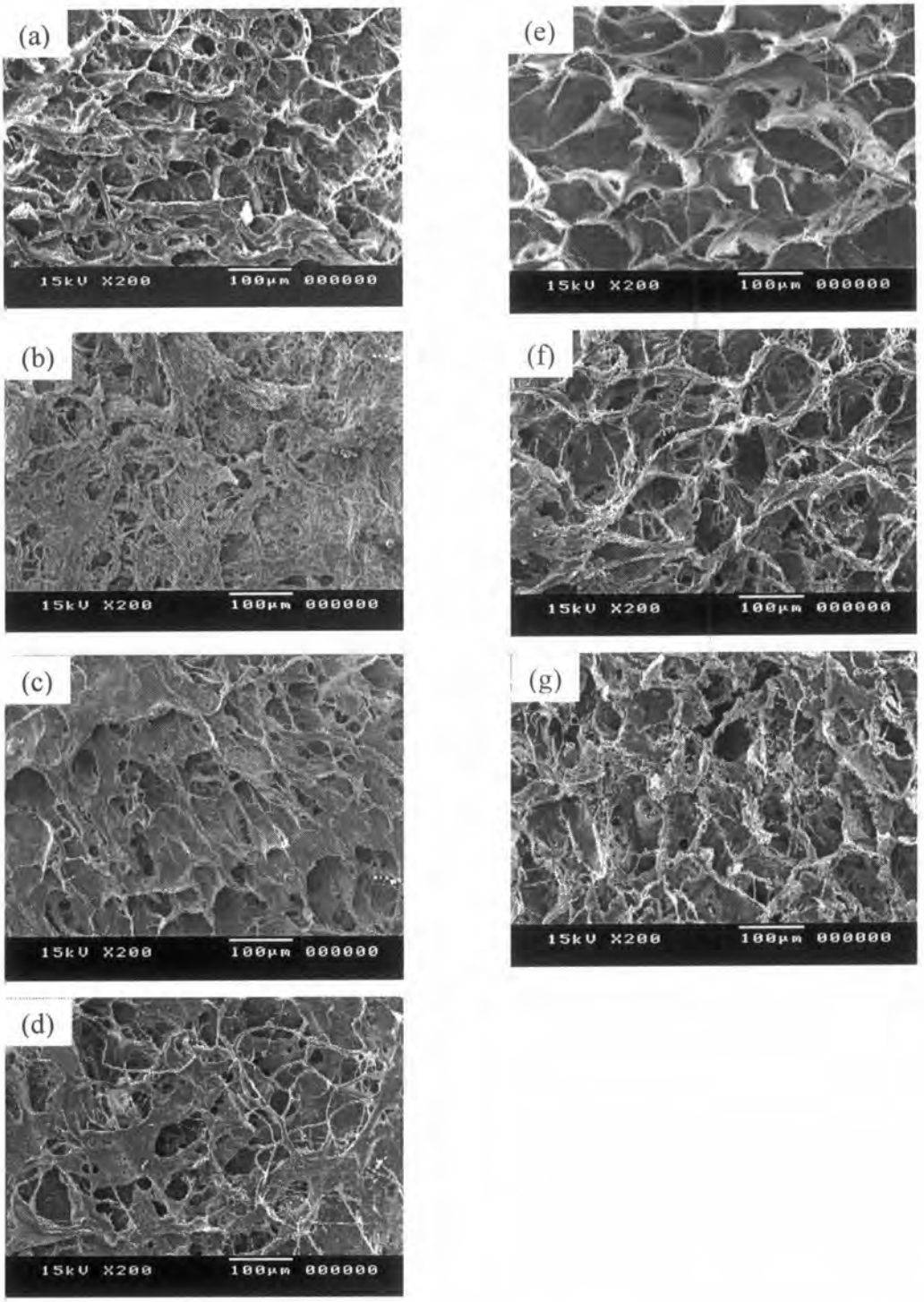


Figure 5.6 SEM micrographs of fresh porcine skin and ADM samples from different treatments: (a) fresh porcine skin, (b) CR1, (c) CR2, (d) CR3, (e) CR4, (f) CR5, and (g) CR6.

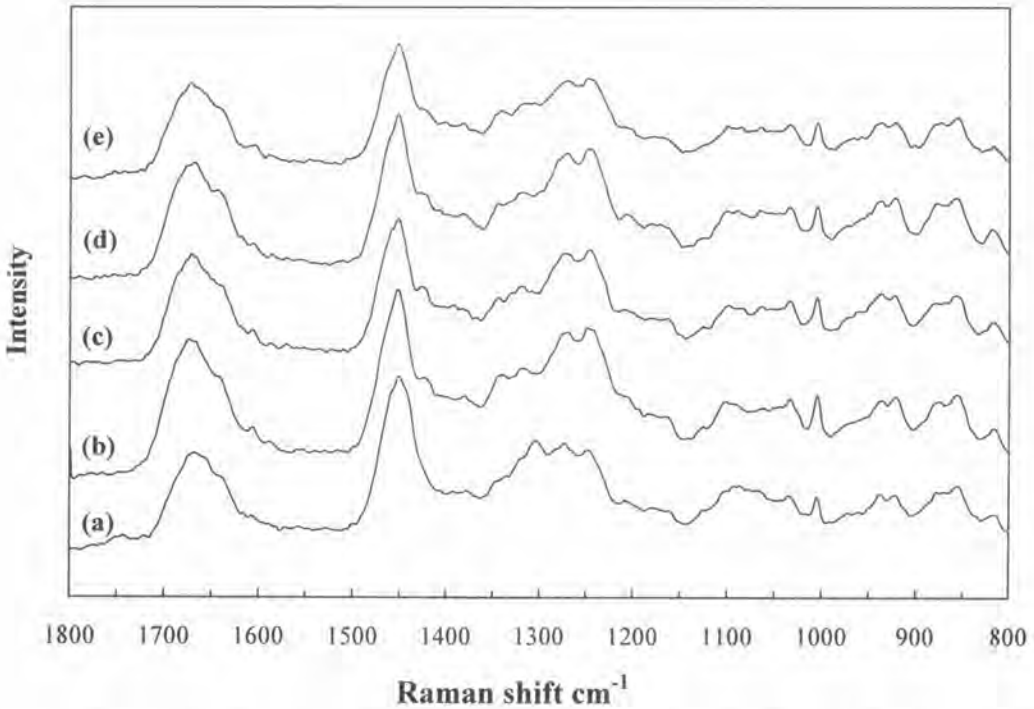
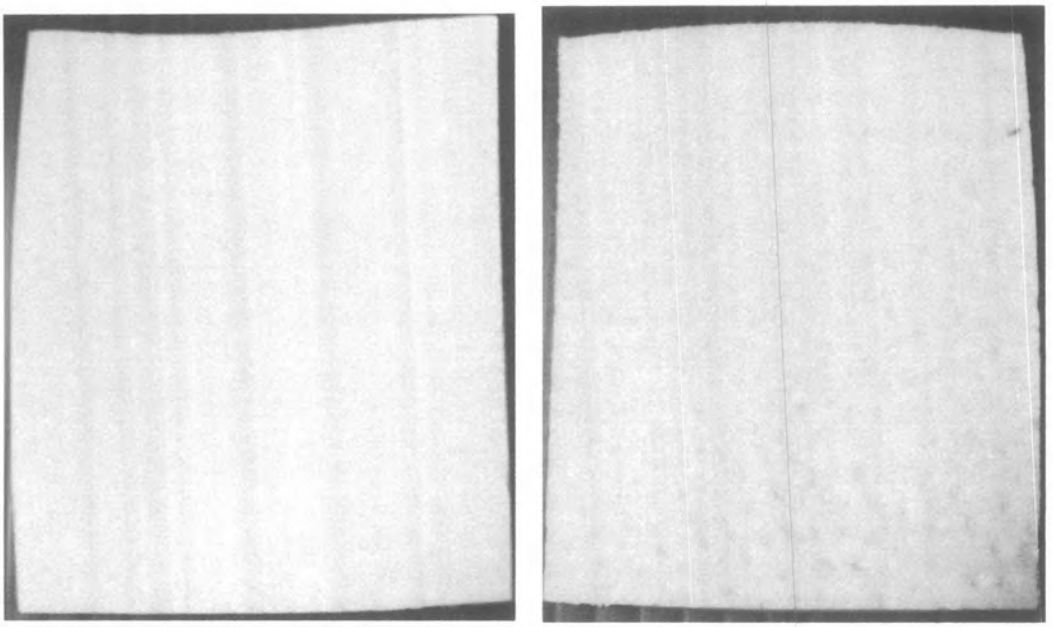


Figure 5.7 FT-Raman spectra of porcine skin; (a) fresh porcine skin, (b) de-fat skin, (c) ADM from CR4 treatment, (d) ADM from CR5 treatment, and (e) ADM from CR6 treatment



(a) **(b)**

Figure 5.8 Acellular porcine dermis; (a) top surface, (b) bottom surface

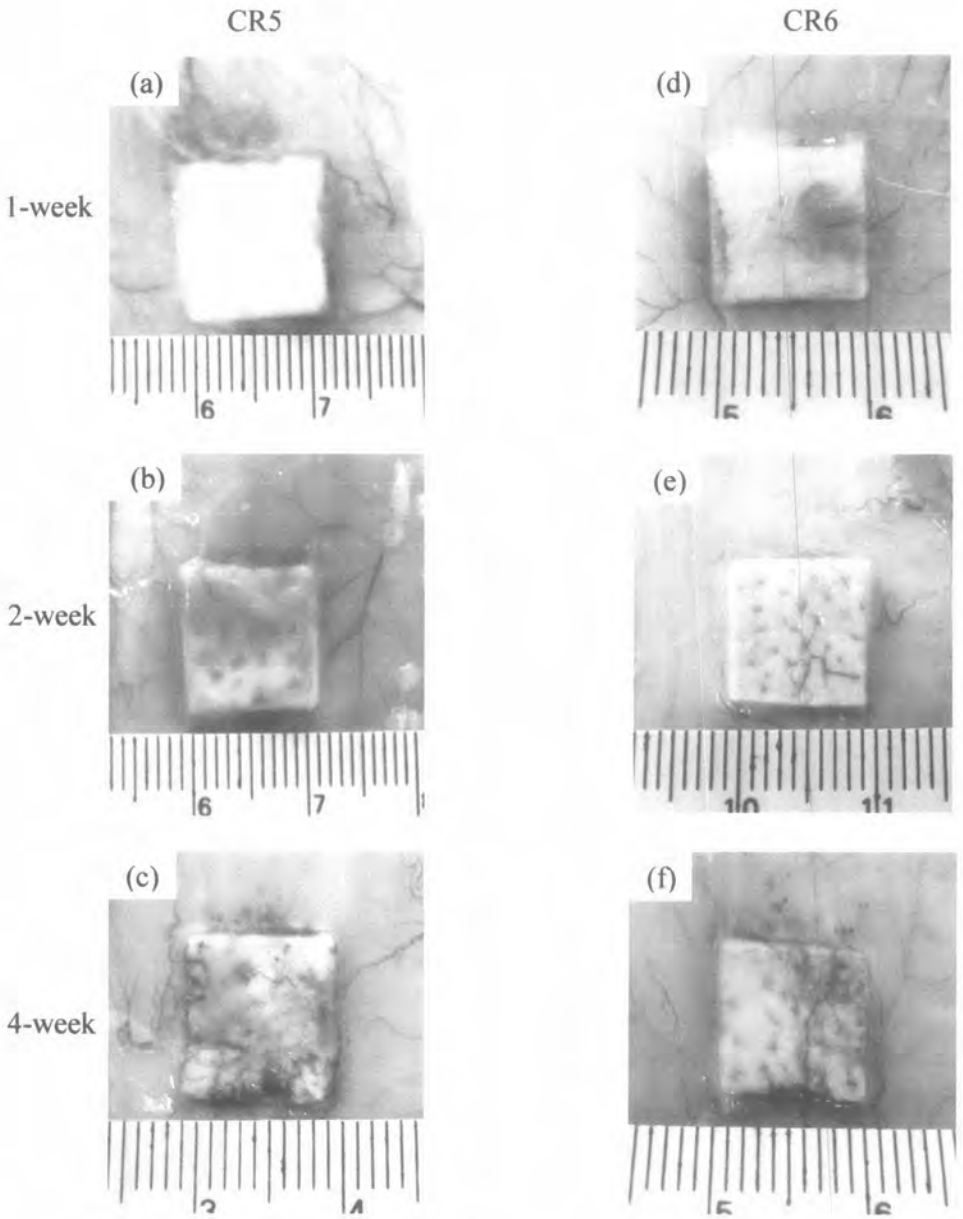


Figure 5.9 Photographs of retrieved ADM samples produced from CR5 (trypsin model) and CR6 (dispase II model) treatment at 1-, 2- and 4-week postoperatively: (a) CR5 after 1-week, (b) CR5 after 2-week, (c) CR5 after 4-week, (d) CR6 after 1-week, (e) CR6 after 2- week, and (f) CR6 after 4-week.

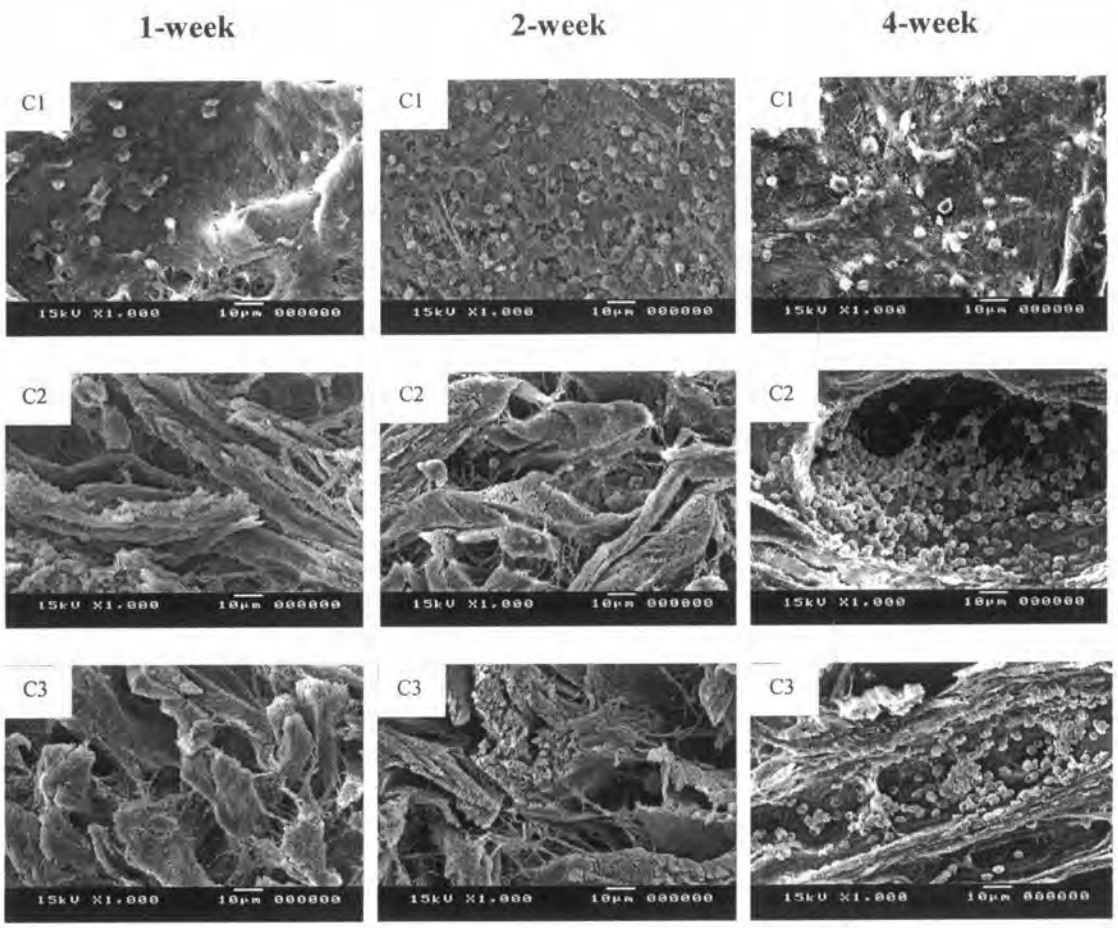


Figure 5.10 SEM micrographs of CR5 (trypsin model) implanted samples retrieved at 1-, 2- and 4-week postoperatively: C1 represents region at the sample edge, C2 is at the depth of 0.25 cm from the edge, and C3 is at the center of implanted samples.

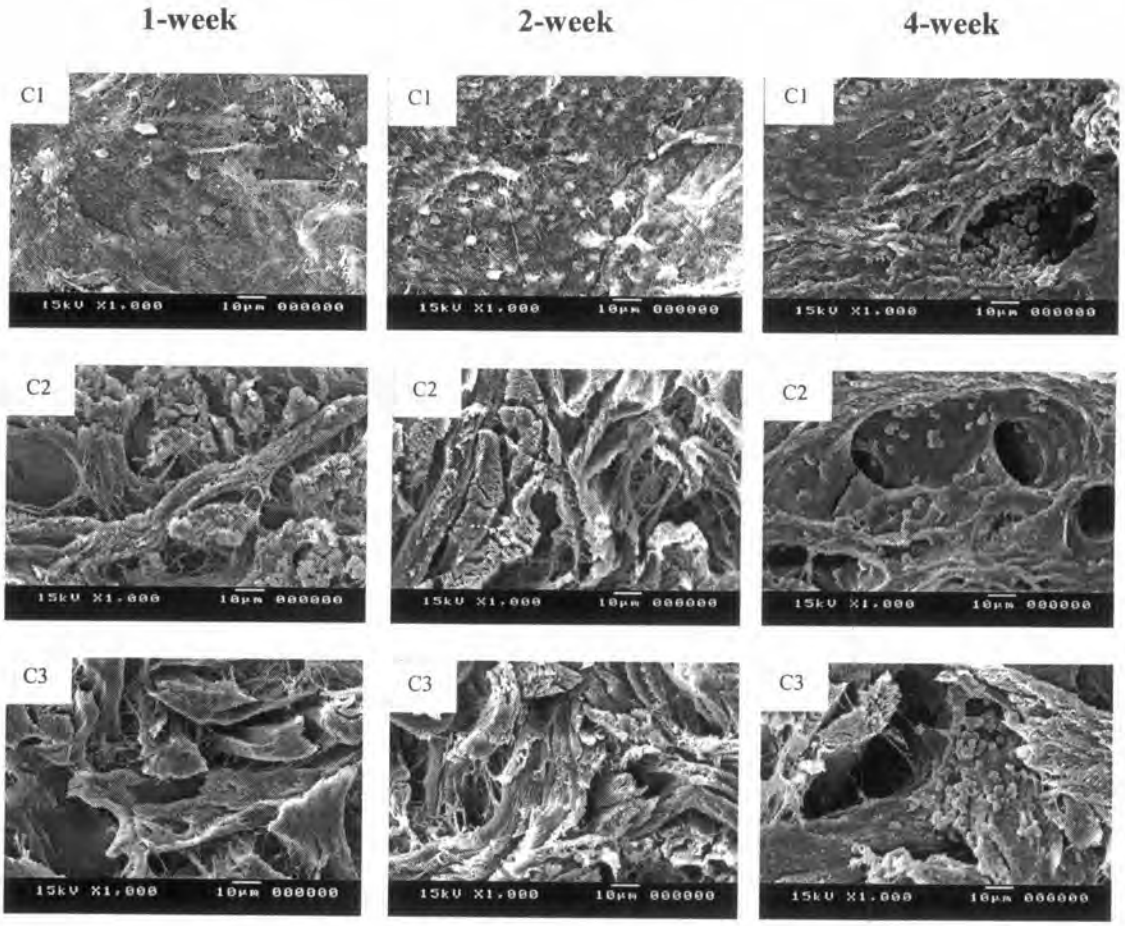


Figure 5.11 SEM micrographs of CR6 (dispase II model) implanted samples retrieved at 1-, 2- and 4-week postoperatively: C1 represents region at the sample edge, C2 is at the depth of 0.25 cm from the edge, and C3 is at the center of implanted samples.

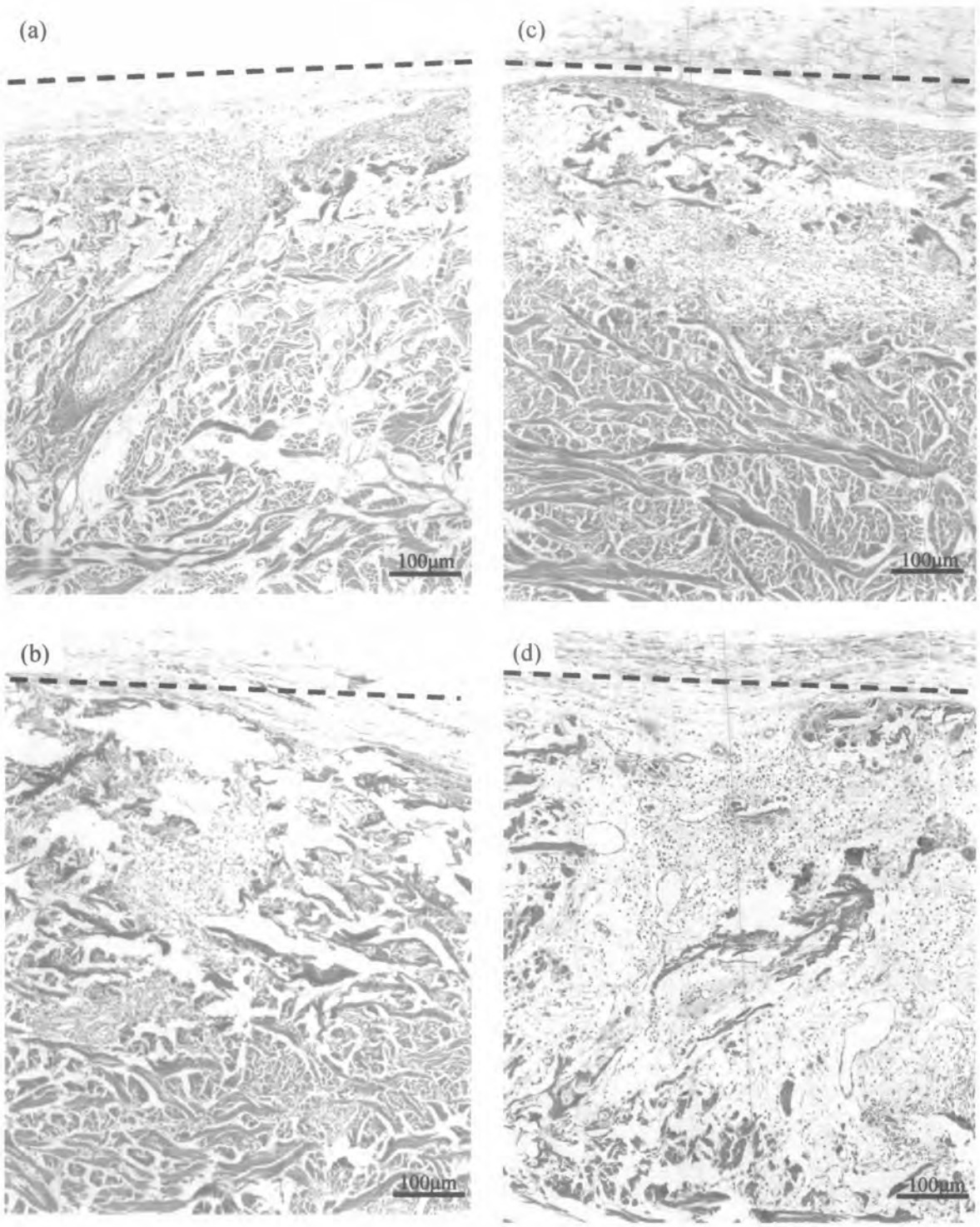


Figure 5.12 Histological photographs of CR5 (trypsin model) and CR6 (dispase II model) implanted ADM at 2- and 4-week postoperatively (100 x magnification): (a) CR5 at 2-week, (b) CR6 at 2-week, (c) CR5 at 4-week, (d) CR6 at 4-week. Dash line represented an interface between host tissue (rat) and implanted samples.