CHAPTER VII

GALLIC ACID GRAFTED MACROPOROUSE SCAFFOLD TO SUPPORTTISSUE REGENERATIONWITH A TARGET APPLICATION IN BONE DEFECT

7.1 Abstract

Three dimensional model of cell seeded on Gallic acid grafted macroporouse scaffold was successfully investigated for their behavior both before and after treated with pyocyanin (seeding HDFa from 2 patients and MSCs from 6 patients). The grafted macroporouse scaffold showed excellent and stable free radical scavenging activity using DPPH assay. After treated with pyocanin, the cell seeded on GA-grafted collagen-I scaffolds showed the impressive oxidant-antioxidant balance which provided higher cell differentiation in comparable to the neat one. All of the three dimensional cell studies can be concluded that GA-grafted collagen-I scaffold at optimum concentration had potential to regenerate cell performance after the cell damages.

7.2 Introduction

Reactive oxygen species (ROS) was generated by the regulated cell metabolism and have beneficial on cell signaling and homeostasis (Devasagayam, 2004) but can be cumulated to oxidative stress under the stress of environment. ROS can cause many diseases and damages including aging, cancer, metabolic disorders, cardiovascular disease, ischemic injuries, and inflammation (Datta, Sinha, & Chattopadhyay, 2000; Halliwell, 1997; Kehrer & Smith, 1994). Pyocyanin was evaluated to be a toxic substance to the cells due to their ability to reduce by nonenzymatic NAD(P)H resulting in the generation of oxidative stress. However, the oxidative stress can be depleted using free radical scavenger (antioxidant). There are many antioxidant substances which were natural (Chen, Zhang, Qu, & Xie, 2008; Qiao et al., 2009; Tseng, Yang, &Mau, 2008; Yuan, Zhang, Fan, & Yang, 2008) and synthetic substance.

Gallic acid (GA, 3,4,5-trihydroxy benzoic acid) which was derived from natural is the well known antioxidant due to their ability to provide H-atom to deplete oxygen free radical. Since their excellent activity of gallic acid, there were many studies interested on modified structure of gallic acid or combining with other molecules to improve antioxidant activity, solubility, biocompatibility and bioactivity (Curcio et al., 2009; Pasanphan & Chirachanchai, 2008; Spizzirri et al., 2009; Zuo et al., 2003).Gallic acid were generally applied to use as additive in food, pharmaceuticals and cosmetic due to their beneficial.

The aim of this study was interested in synthesis grafted gallic acid on collagen scaffold through amine reactive group in collagen scaffold structure led to forming of ester bonds between gallic acid and collagen chains. All of GA-grafted collagen sample were used to be a three dimensional substrate for cell seeding including normal human fibroblast (cells from 2 patients) and Mesenchymal stem cells (cells from 6 patients) and then studied on cell proliferation and cell behavior before and after treating with pyocyanin.

7.3 Materials and methods

7.3.1 Materials

Gallic acid, MES monohydrate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Pyocyanin from *Pseudomonas aeruginosa*, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Germany). Ethanol was purchased from Merck (Germany). All the chemicals and reagents were used as received without further purification.

7.3.2 Preparation of MES Solution

To prepare 1000 ml of MES buffer (pH 5.6), 9.76 g MES (M_w = 195.2) was dissolved in 900 ml of distilled water. HCl or NaOH were added to the solution to get pH 5.7. Finally, the distilled water was added to the solution to make up the volume.

7.3.3 Effect of Oxidative Stress Concentrations and Times on Human Dermal Fibroblasts (HDFa) and Mesenchymal Stem Cells (MSCs)

Pyocyanin was selected to be a substance providing oxidative stress to the cells. Human dermal fibroblast, HDFa (23m-pat.273 and 71m-pat.361) were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Germany), supplemented with 10% fetal calf serum (FCS; BIOCHROM, Germany), 1% L-glutamine (Invitrogen Corp.), and a 1% penicillin/streptomycin (Biochrom, Germany). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h and measured for their viability using alamar blue assay. After 48 h, the cells were treated with pyocyanin 0, 50, 100, 250, 500 and 1000 μM for 30 min and 2 h. Finally, the pyocyanin solutions were removed from the cells and then measured for their viability using alamar blue assay. Same procedure also did with MSCs (40m-pat.357 and 80- pat.374).

7.3.4 Synthesis of Gallic Acid-Grafted Collagen Scaffolds

The collagen-I scaffolds were obtained from Optamaix-3D™ scaffolds (Matricel, Germany) and were cut into circular shape of 2 mm diameters and the thickness was 3 mm to use in the experiments. The collagen scaffolds were grafted with gallic acid 5 different concentrations (5, 10, 20, 40 and 80 mM of GA) by using *N*-(3-dimethylaminopropyl)-*N'* ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Fig.1 showed the chemical pathway of gallic acid-grafted collagen scaffolds in detail. Gallic acid with different weight corresponding to each concentration were dissolved in 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.6) 10 ml. For each GA concentration, *N*-(3-dimethylaminopropyl)-*N'* ethylcarbodiimide hydrochloride (2 mole equivalent to GA) was added in the solution to obtained amine-reactive-*O*-acylisourea intermediate and *N*-hydroxysuccinimide (2 mole equivalent to EDC) was further added to obtained amine-reactive-NHS ester. Carboxylic acid groups of gallic acid were converted to amine-reactive-NHS ester using EDC and NHS. The reaction was stirred in 4 °C room for 1h.

The collagen-I scaffolds were obtained from Optumaix-3D^{TM} scaffolds (diameter 2 mm, thickness 3 mm) were equilibrated with MES buffer (0.05 M, pH 5.6) for at least 30 min. And then the specimens were immersed in the amine-reactive-NHS ester solution (20 pieces of specimens per each 10 mL of the amine-reactive-NHS ester solution) and stirred in 4 °C room for 3 h. The reaction was carried out heterogeneously for 24 h. After 24 h, the sample was washed with phosphate buffer solutions (PBS) to obtained gallic acid-grafted collagen-I scaffolds and immersed all samples in PBS solution in order to maintain the shape of the sample at 4°C.

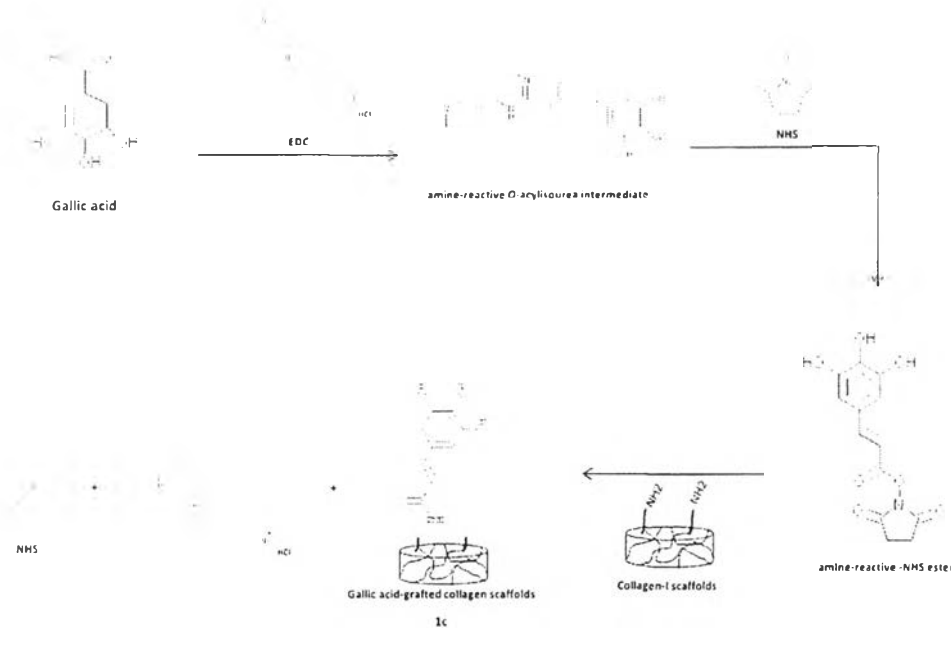


Fig. 7.1 The chemical pathway of gallic acid-grafted collagen-I scaffolds

7.3.5 Characterization

A Nicolet Fourier transform infrared (FT-110 IR) 360 spectrophotometer was used to confirm chemical structure of the obtained GA-grafted collagen scaffold with 5 different GA concentrations (5, 10, 20, 40 and 80 mM). FT-IR spectra of neat collagen scaffold were also recorded for comparison using the KBr pellet method.

7.3.6 Measurement of Antioxidant Activity of GA-Grafted Collagen Scaffolds with DPPH Free Radical Scavenging Assay

The antioxidant activity of insoluble materials was developed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) solution. The GA-grafted collagen scaffolds with 5 different GA concentrations were put separately into eppendorf with 300 µL DPPH solution (0.1 mM of DPPH in 50% ethanol) was added to start the reactions. The mixture was vortexed in order to help the reaction between insoluble materials and DPPH reagent. The solution was incubated at room temperature for 30 min in darkness. The absorbance of the final solution was recorded using spectrophotometer at the wavelength of 517 nm. The antioxidant activity was showed as the percentage of DPPH that decrease compared to the blank DPPH, according to following equations (Yu et al., 2011),

$$\text{Free radical scavenging activity (\%)} = [1 - \frac{Abs_{sample}}{Abs_{control}}] \times 100,$$

Where Abs_{sample} and $Abs_{control}$ are the absorption of sample in DPPH solution and blank DPPH, respectively.

7.3.7 Cell Proliferation of GA-Grafted Collagen-I Scaffolds

HDFa (from 2 patients) and MSCs (from 6 patients ; 40m-pat.357, 42m-pat.275, 49m-pat.392, 85m-pat.336, 80m-pat.355 and 80- pat.374) from the culture were trypsinized and counted by a cell counter (CASY system, AG Innovatis AG). The GA-grafted collagen scaffolds and neat collagen scaffold were dropped into the mixed cell suspension. The culture was maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 for 1 hour to adhere the cells into the scaffolds. After that, the additional medium was added into the wells. The cells on the scaffold were cultured for 1, 2, 3 and 7 days for HDFa and 1, 2, 3 and 5 days for MSCs. At the different time points, the cells on the scaffolds were measured for their viability using alamar blue assay. After testing cell viability, the cells on the scaffolds were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature. The fixed cells were washed twice with 10 % PBS buffer solutions for 5 min and once with distilled water for 5 min.

The sample were stained with DNA-specified fluorochrome 4,6-diamidine-2-phenylindole dihydrochloride DAPI for 5 min, and stored at 4 °C. The staining images of the cells were examined by using fluorescence microscopy.

7.3.8 Effect of oxidative stress on Human dermal fibroblasts (HDFa) and Mesenchymal stem cells (MSCs) seeded on GA-grafted collagen-I scaffolds

HDFa (from 2 patients) and MSCs (from 6 patients) from the culture were seeded on GA-grafted collagen-I scaffolds with different grafted GA concentrations using the same procedure as cell proliferation experiment. All of the samples were treated with corresponding pyocyanin concentration as 2D experiment for 30 min. Afterward, the viability of the cells was tested using alamar blue assay and DNA-specified using cyquant experiment.

7.4 Results and discussion

7.4.1 Effect of oxidative stress concentrations and times on Human dermal fibroblasts (HDFa) and Mesenchymal stem cells (MSCs)

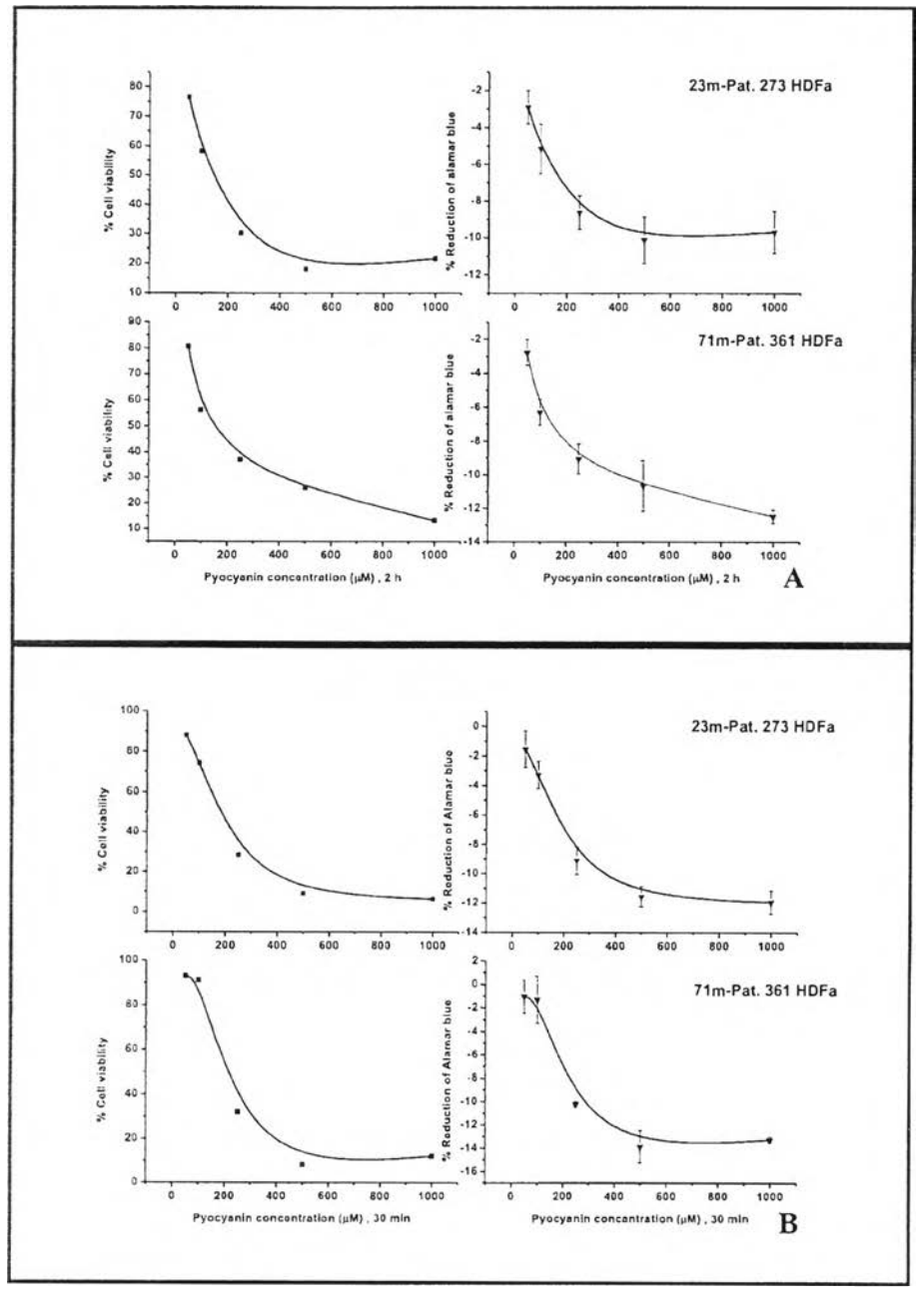


Figure 7.2 Representative of cell viability of HDFa from 2 patients after treated with pyocyanin different concentrations including 50, 100, 250, 500 and 1000, respectively, using 30 min treated time (A) and 2 h treated time (B).

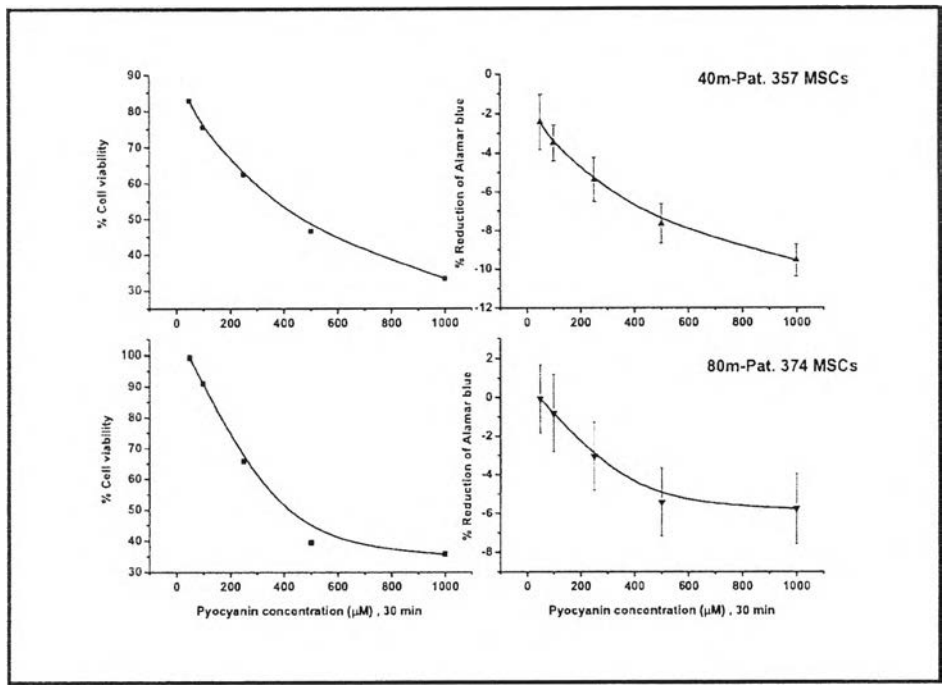
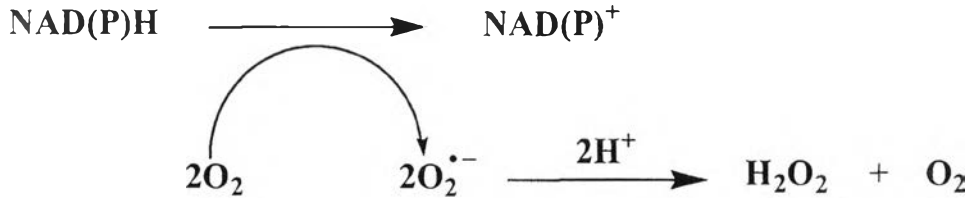


Figure 7.3 Representative of cell viability of MSCs from 2 patients after treated with pyocyanin different concentrations including 50, 100, 250, 500 and 1000, respectively, using 30 min treated time.

HDFa was isolated from 2 patients which were selected based on different age of patient (23m-pat.273 and 71m-pat.361). Cell viability of both cell after treated with 50, 100, 250, 500 and 1000 μM decreased when concentration increased. From figure 7.2a showed 60% cell viability of both cell when treated with 100 μM for 30 min which meant to be at that concentration not extremely harmed to the cell and the cell can be recovered for their activity. At 2 h treated time, cell responding showed much lower activity with the same treated pyocyanin concentrations (figure 7.2b). Therefore, pyocyanin at 100 μM and 30 min were satisfied concentration and time to further applied to the cell in 3 dimensional (cell seeded in scaffold). Figure 7.3 showed cell responding of MSCs (40m-pat.357 and 80- pat.374) after treated with corresponding pyocyanin concentrations, the result showed that 300 μM pyocyanin was satisfied concentration for further study due to expressed 60% cell viability of MSCs.

Pyocyanin was reduced by intracellular reducing agent (e.g. NAD(P)H) created oxidative stress in the cells. The intracellular reaction pathway of the presented pyocyanin was directly generated superoxide radical ($O_2^{\bullet-}$) and hydrogenperoxide (H_2O_2) by following reaction (*) (Malley, Reszka, Spitz, Denning & Britigan, 2004; Hassan & Fridovich, 1980).



(*) The intracellular reaction of pyocyanin reduction by NAD(P)H

Additionally, there was reported that pyocyanin had effect on the cell in complex manner. Pyocyanin increased the level of glutathione reductase in a cell-free system which reduced GSH level. While the redox reaction, the formation of hydrogenperoxide also reduced the GSH by conjugation (Chang, Shi& Forman, 1992; Muller, 2002).

7.4.2 Characterization

Figure 7.4 showed FTIR spectra of collagen-I scaffold (NEAT), 5 mM GA-grafted collagen-I scaffold (GA 5), 10 mM GA-grafted collagen-I scaffold (GA 10), 20 mM GA-grafted collagen-I scaffold (GA 20), 40 mM GA-grafted collagen-I scaffold (GA 40) and 80 mM GA-grafted collagen-I scaffold (GA 80). Collagen-I scaffold spectrum exhibited band at 1690, 1520 and 1310 cm^{-1} (amide I, amide II and amide III, respectively) which were indicated C=O stretching vibration (amide I) and amide N-H bending vibration and C-N stretching vibration (amide II). The amide III consisted of the stretching of C-N and N-H bending from amide linkage (Ligia et al, 2011). [1] The stretching of –OH and $-CH_3$ bands were shown at 3500 and 2850 cm^{-1}, respectively. All of the result clearly indicated the consisting of amino acids on the network of the collagen-I scaffold.

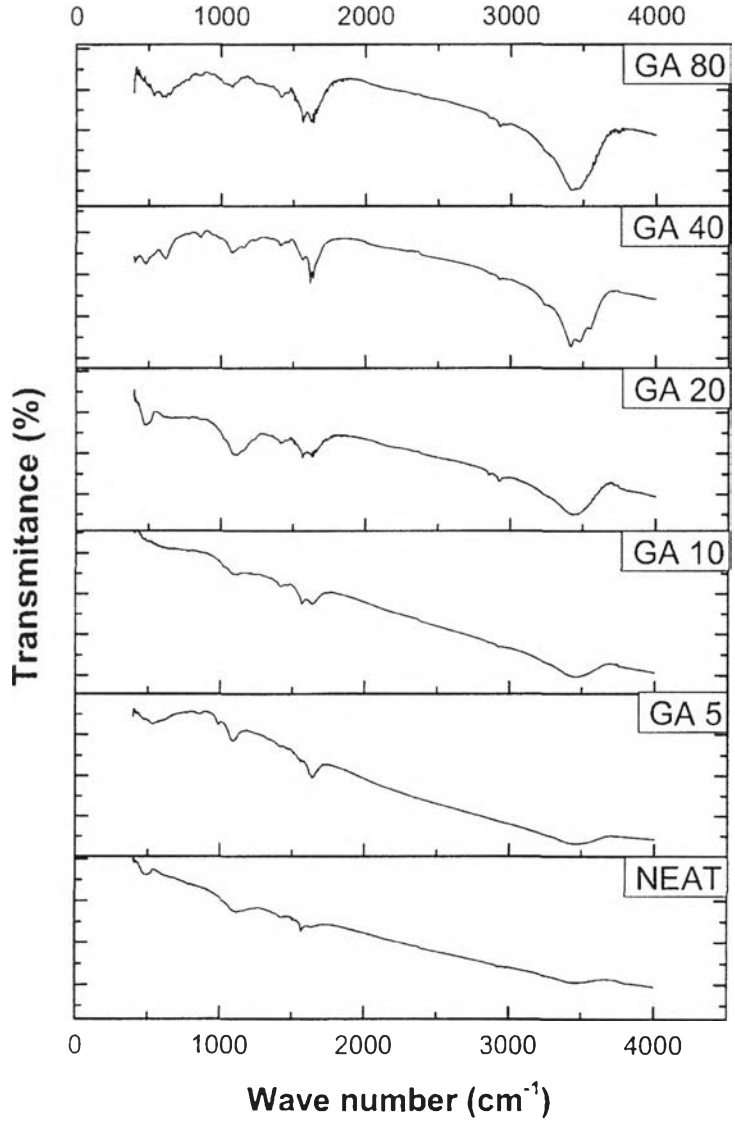


Figure 7.4 Representative of FT-IR spectrum of GA-grafted collagen-I scaffolds ; neat collagen scaffold, 5 mM GA-grafted collagen scaffold, 10 mM GA-grafted collagen scaffold, 20 mM GA-grafted collagen scaffold, 40 mM GA-grafted collagen scaffold and 80 mM GA-grafted collagen scaffold (hereafter; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively).

The grafted of GA on collagen backbone generated amide linkage from amine groups which existed in the scaffold (Bo˘zi˘c, Gorgieva & Vanja Kokol, 2012) (fig.7.1). The pattern of absorption spectra of GA-grafted collagen scaffold at all concentrations showed similar pattern to parent molecule (collagen-I scaffold). Furthermore, the introducing GA molecules into collagen scaffold led to aromatic C-H stretching and -OH stretching of phenolic derivatives indicated by absorption band at 3030 and 3400 cm^{-1}, respectively.

7.4.3 Free Radical Scavenging Antioxidant Activity of GA-Grafted Collagen Scaffolds

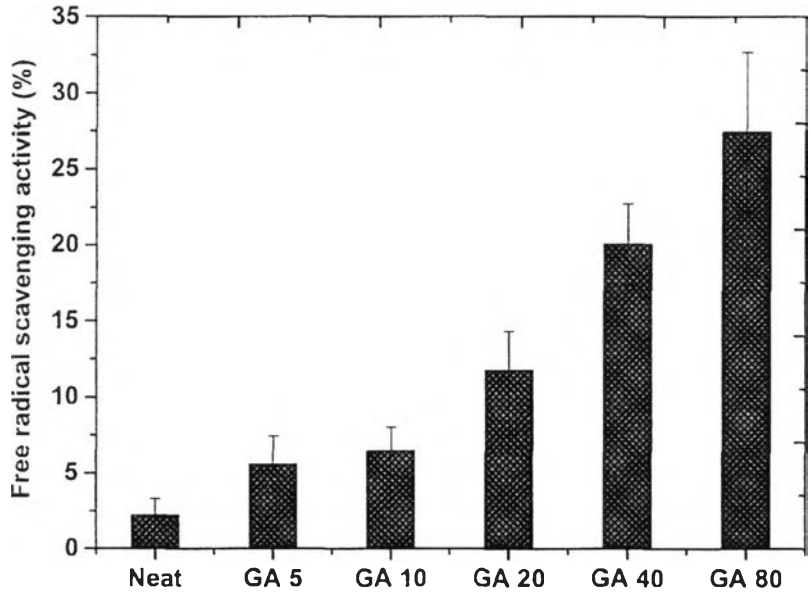


Figure 7.5 Representative of free radical scavenging activity (%) of GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80.

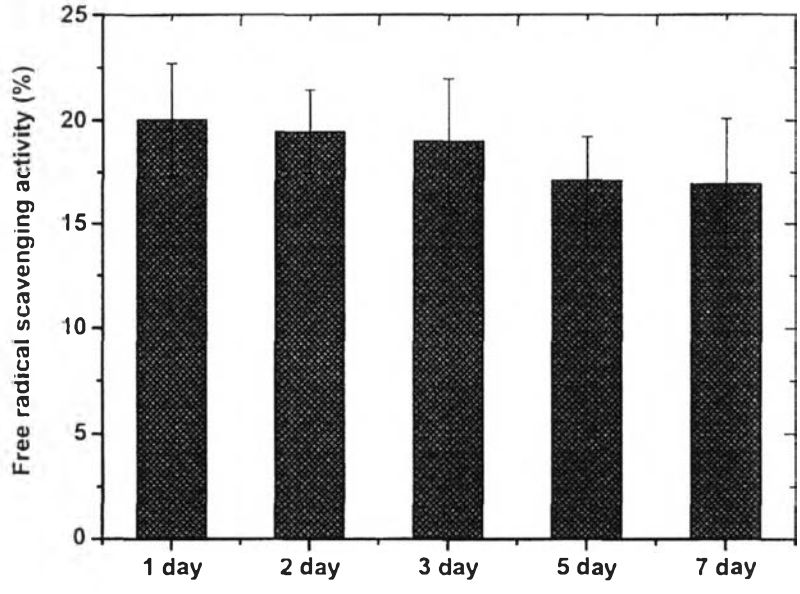


Figure 7.6 Representative of free radical scavenging activity (%) of GA-grafted collagen

Antioxidant of GA was identified as scavenger of DPPH radical in polar solvent. Free radical scavenging activity of GA-grafted collagen-I scaffold was directly proportional to GA concentrations, GA 80 showed the highest free radical scavenging activity (27.42 ± 5.24) following by GA 40 (20.03 ± 2.68), GA 20 (11.72 ± 2.59), GA 10 (6.45 ± 1.57), GA 5 (5.55 ± 1.86), NEAT (2.91 ± 1.10), respectively.

H-atom-donating ability of GA (ArOH) was the main process of phenolic antioxidant activity, there were 2 main pathways including H-atom transfer from phenolic compound (1) and electron-transfer/proton-transfer consequently (2) (Ji, Zhang & Shen, 2006). Additionally, in second step required polar environment due to charge separation processes.

$$RO^{\bullet} + ArOH \rightarrow ROH + ArO^{\bullet} \quad (1)$$

$$RO^{\bullet} + ArOH \rightarrow RO^{-} + ArOH^{\bullet +} \rightarrow ROH + ArO^{\bullet} \quad (2)$$

In order to increase GA concentration directly led to higher scavenging activity of the scaffolds because of more transferred H-atom.

The stability of antioxidant activity was evaluated after finished grafting procedure, the 40 mM GA-grafted collagen-I scaffolds (GA 40) were selected to evaluate for their antioxidant activity at different storage time (1, 2, 3, 5 and 7 days). The result showed no significantly different of scavenger ability of the scaffold at each storage time selected which can be expressed that amide linkage was stable and dissoluble in water (fig. 7.6)

7.4.4 Cell Proliferation of GA-Grafted Collagen-I Scaffolds

Cell proliferation of HDFa showed the dose-dependent manner of GA concentrations which were used to grafted the collagen-I scaffold (fig.7.7). The dose-dependent manner was showed the highest at GA 10 following by GA 5 and Neat, respectively which was showed similarly trend between both cells. When using higher concentration of GA (GA 20, GA 40 and GA 80), expressed the lower proliferation of the cells which meant to be the excess of GA that the cells required to get rid of oxidative stress in the cells. The proliferated result at the different day indicated the same pattern of the cell growth.

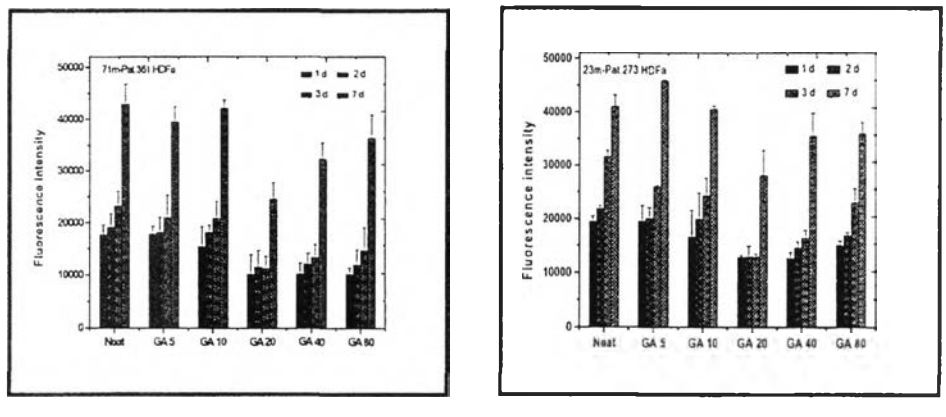


Figure 7.7 Representative of cell proliferation of HDFa from 2 patients seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively, at 1, 2, 3 and 7 days

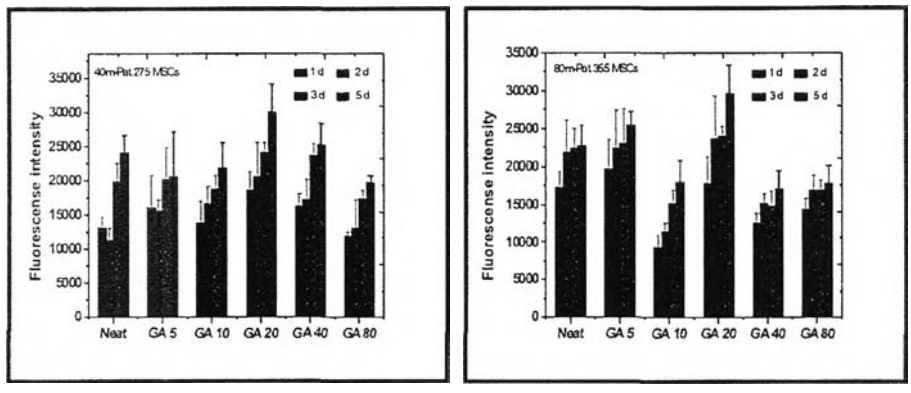


Figure 7.8 Representative of cell proliferation of MSCs from 2 patients seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively, at 1, 2, 3 and 5 days.

Figure 7.8 showed the cell proliferation of MSCs which similarly showed their dose-dependent manner when grafted GA concentration increased until GA 20. The proliferated results when using higher GA concentration slightly decreased at GA 40 and much lower at GA 80.

The study on cell proliferation of this three dimensional experiment quite clearly understood the behavior of the cell which incorporated in the matrix structure. This can be related to the cellular microenvironment which facilitated the study of tissue regeneration (Dhaliwal, 2012). Aerobic cells can be generated ROS mainly from the mitochondrial electron transport chain (ETC) and subsequently transformed into superoxide anion (Alfadda & Sallam 2012). In order to undergo cell activities regularity required oxidant-antioxidant balance which facilitated cell function including maintaining integrity of organelles and expression of immune system. The proliferated resulted of both cells clearly showed the oxidant-antioxidant balance between cell proliferation and GA concentration, there were a certain optimal concentration of antioxidant to be used resulting in providing better cell regeneration.

7.4.5 Effect of Oxidative Stress on Human Dermal Fibroblasts (HDFa) and Mesenchymal Stem Cells (MSCs) Seeded on GA-Grafted Collagen-I Scaffolds

The decided experiment for three dimensional cell seeded on GA-grafted collagen-I scaffolds behavior slightly complicated. The model was set to evaluate the activity of the cells both before and after treated with corresponding pyocyanin concentration (100 μM for HDFa and 300 μM for MSCs) for 30 min. The behavior of the HDFa before and after treated with pyocyanin was shown in Figure 7.9, the 24h cell proliferation was not expressed different between the set of samples. After treating with stress to the cells was surprisingly showed faster growth rate of the cells when using GA 20 for 23m-273 HDFa and GA 10 for 71m-361 HDFa. The behavior of both before and after cell response can be interpreted to cell differentiation (fig. 10) which provided the valuable data leading to facilitate study of three dimensional cell behaviors in form of predicable curve and directly provided the possibly concentration of oxidation-antioxidant balance of the specified cells.

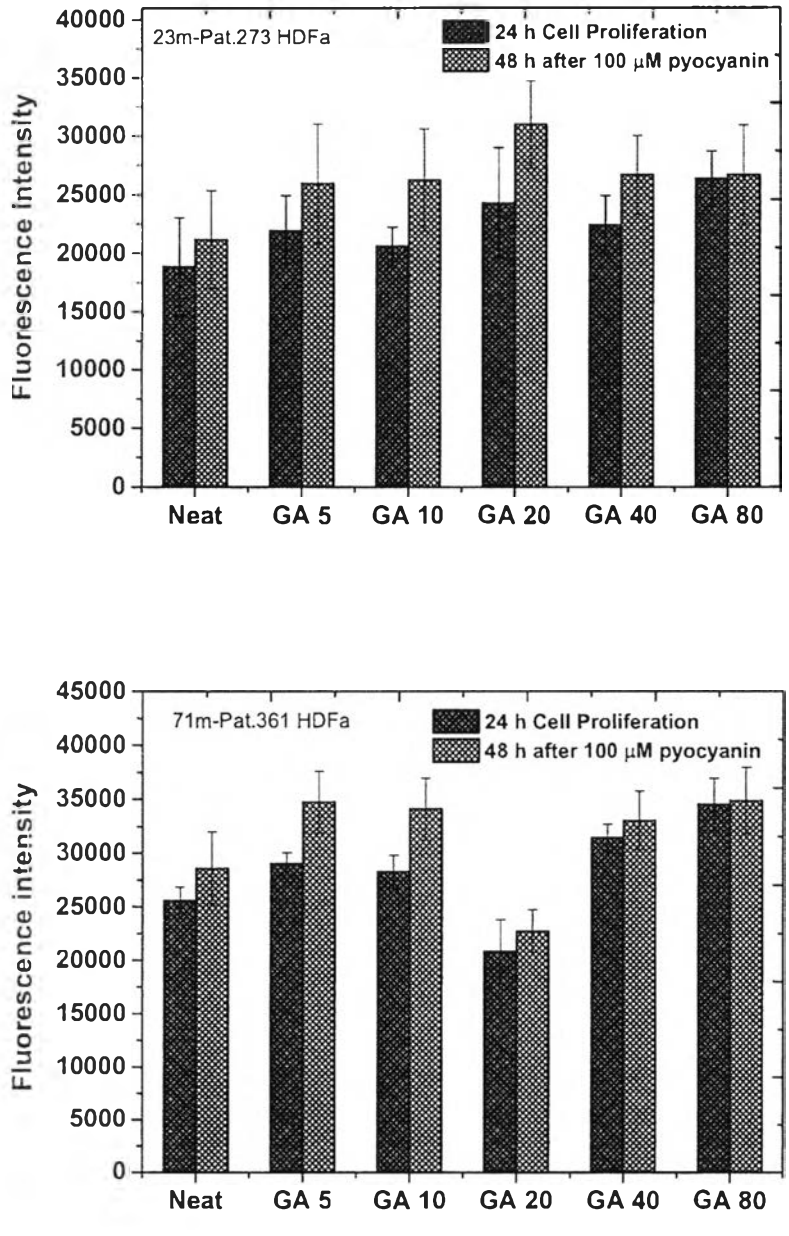


Figure 7.9 Representative of cell viability of HDFa from 2 patients seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively, at 24 h cell growth and 48 h after treated with 100 μM pyocyanin for 30 min.

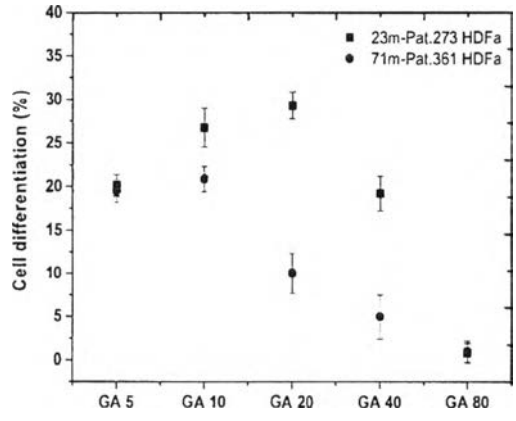


Figure 7.10 Representative of cell differentiation of HDFa from 2 patients seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively, calculated from number of the cells at 24 h cell growth relative to number of cells at 48 h after treated with 100 μM pyocyanin for 30 min.

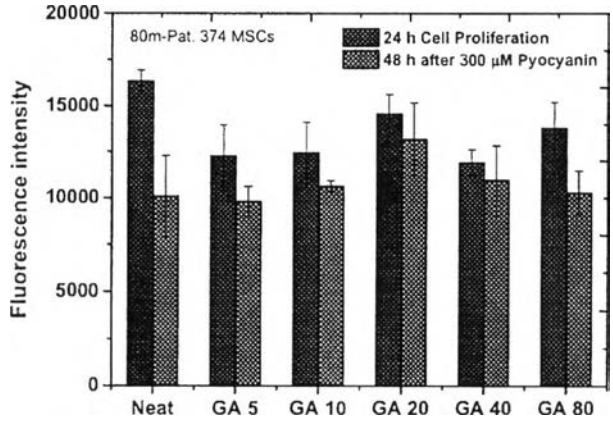


Figure 7.11 Representative of cell viability of MSCs from 6 patients seeded on GA-grafted collagen-I scaffolds with 100 μM pyocyanin for 30min.

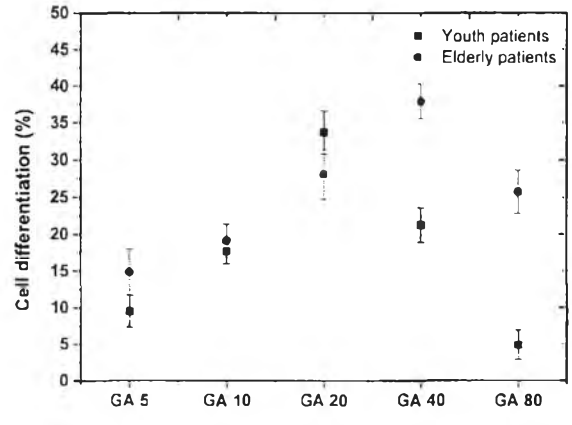


Figure 7.12 Representative of average cell differentiation of MSCs (derived from 3 youth patients and 3 elderly patients) were seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively, calculated from number of the cells at 24 h cell growth relative to number of cells at 48 h after treated with 100 μM pyocyanin for 30 min.

The MSCs were isolated from 6 different patients which aimed to study on how the cells in different age respond after applied stress. Figure 7.11 showed cell viability of MSCs from 6 patients before and after treated with pyocyanin, the result indicated that 24 h cell proliferation was not different in each batch. The cell viability data was also calculated to cell differentiation (fig. 7.12), the cell growth after treated with pyocyanin was impressively high when using GA 40 for aged patients and GA 20 for young patients.

The cell responding after treated with stress expressed an understandable result which depended on GA concentration due to their scavenging ability. Although, aerobic cells were generated ROS by themselves regularly but the amount of free radical generation was depended on age of person. It is well known that aged person (> 65 y of age) trended to generate more free radical and lipid peroxidation contributes in the cell than young person (Meydani, Wu, Santos & Havek, 1995). Glutathione is an intracellular thiol group that protected the cell from reactive oxygen species including oxygen intermediate and free radical.

There was reported that glutathione level inversely proportional to aging, the lower glutathione concentration blood occurred in person > 60 year of age (Lang et al., 1992). From all of the factors possibly explained that why MSC cell from aged person required more GA concentration than the young MSC cell.

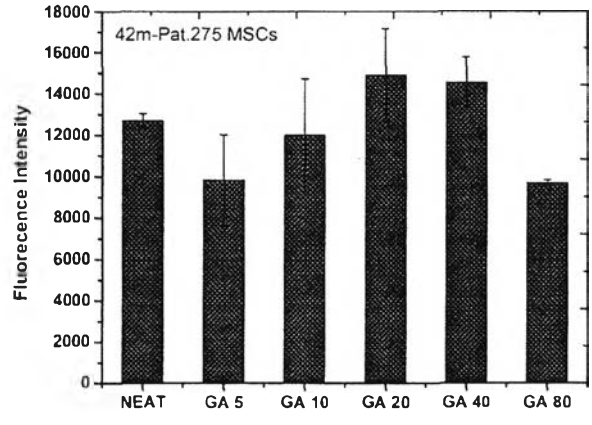


Figure 7.13 Representative of cyquant cell proliferation of MSCs which were seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively.

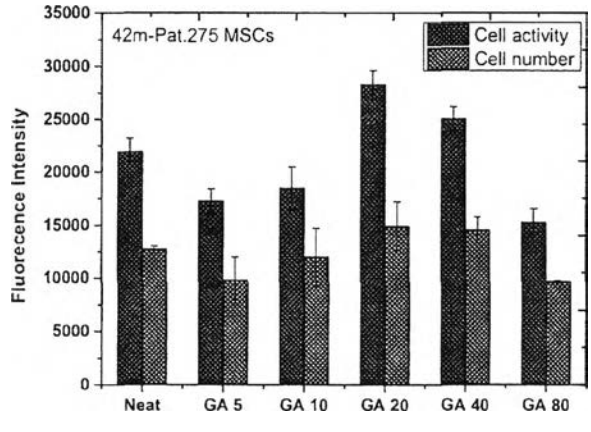


Figure 7.14 Representative of cell activity and cell number of MSCs which were seeded on GA-grafted collagen-I scaffolds; Neat, GA5, GA10, GA20, GA40 and GA 80, respectively.

Figure 7.13 represented cell number after treated with pyocyanin from cyquant experiment. The trend of the obtained cell number from all patients was the same, therefore only cell number data of 42m-275 MSCs was selected to report. The pattern of cell number from DNA count was similar to cell performance in viability from alamar blue experiment (fig. 7.14). Although, the cell number of all samples (with and without GA grafting) was slightly fluctuated but the cellular health showed much different when using scavenging agent which meant to be the cell in the antioxidant environment can be recovered for their performance. From this phenomenon can be possibly concluded that the generation of intra/inter oxidative stress directly led to the reduction of cell performance as well as induced cell damages but not directly killed the cell (Bloknina, Virolainen & Fagerstedt, 2003). Additionally, the damaged cell can be regenerated by electron transfer from antioxidant agent.

7.5 Conclusion

The GA-grafted collagen-I scaffolds were successfully prepared using the amine-reactive-NHS ester solution which provided excellent and stable scavenging activity depending on the usage of GA concentration. The modified scaffolds were used to be the 3D substrate to proliferate the cells including HDFa (2 patients) and MSCs (6 patients).The cell proliferation of all cells indicated the dose-dependent manner of the cell growth as well as suggested the cell responding of oxidant-antioxidant balance when using too high antioxidant substances. The study model of cell activities after applied stress was showed the impressively high cell growth when using GA 20 for HDFa and young MSCs and GA 40 for aged MSCs. Another factor that was affected on the responding of 6 different MSCs patients after exposed to the pyocyanin was the aging of patient. The generation of free radical of aged person was higher than young person that required more scavenging agent to regeneration. Additionally, the cell number of all batches was obtained from DNA count after expose to pyocyanin which directly indicated the same trend as cell proliferation in the less sensitivity manner. All of the three dimensional cell study can be concluded that GA-grafted collagen-I scaffold at optimum concentration had potential to regenerate cell performance after the cell damages.

7.6 Acknowledgement

The authors acknowledge partial support received from the Center of Excellence on Petrochemical and Materials Technology, Chulalongkorn University, and The Petroleum and Petrochemical College (PPC), Chulalongkorn University.

7.7 Refferences

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