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Characterization of human dermal solutions: New raw material for tissue engineering

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Background : *Tissue engineering requires scaffold for supporting tissue construction. The scaffold materials affect the scaffold properties and the achievement of tissue construction. Human skin, which is abundant of extracellular matrix, is a promising source for fabricating tissue engineering scaffolds.*

Objective : *To determine extracellular matrix components in human dermal solutions, and characterize fundamental physical and biological properties of the scaffolds made from the human dermal solutions.*

Methods : *Cadaveric human skin was prepared to be 3 fractions of dermal solutions, denoted as DS-1, DS-2 and DS-3. These dermal solutions were determined for the content of collagen and sulfated glycosaminoglycans (sulfated GAGs). Tissue engineering scaffolds were prepared form these dermal solutions and their properties were characterized comparing with scaffolds from type I collagen, commercially provided by Sigma-Aldrich corporation. The characterized physical properties included pore structure and mechanical strength and the characterized biological properties included degradation by collagenase, cell attachment and cell proliferation of human bone marrow-derived stem cells (human BMSCs).*

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Results : *The components in each fraction of dermal solutions are obviously different. The dermal solutions contained collagen 92.23, 79.07 and 161.68 μg/mg dry weight and contained sulfated GAGs 3.09 ± 0.51, 1.36 ± 0.39 and 6.91 ± 0.87 μg/mg dry weight of DS-1, DS-2 and DS-3, respectively. The scaffolds from DS-3 had the smallest average pore size, the highest elastic modulus and the longest degradation time with significant difference (p <0.05) while those of the scaffolds from DS-1 and DS-2 are insignificantly different. The scaffolds from type I collagen (Sigma®) provided the most favorable cell attachment with significant difference (p <0.05) whereas no significant difference were found among the scaffolds from all types of human dermal solutions. The lowest cell proliferation was found in the scaffolds from DS-3 with significant difference (p <0.05). The highest cell proliferation was found in the scaffolds from DS-2, the second was the scaffolds from type I collagen (Sigma®), and the third was the scaffolds from DS-1 but no significant difference was found.*

Conclusion : *Human dermal solutions can be prepared from human dermis and used as raw materials for scaffold fabrication. The contents of collagen and sulfated GAGs in each type of the human dermal solutions are different, as a result, the properties of the scaffolds fabricated from each type of the human dermal solutions are also different. The scaffolds from the human dermal solutions support the proliferation of human BMSCs without any sign of cytotoxicity. Even though the scaffolds from type I collagen (Sigma®)) provided better cell attachment than all types of the scaffolds from human dermal solutions, the scaffolds from DS-2 provide better cell proliferation.*

Keywords : *Scaffold, tissue engineering, human dermal solution.*

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เหตุผลของการทำวิจัย : *วิศวกรรมเนื้อเยื่อต้องอาศัยโครงเนื้อเยื่อสังเคราะห์ในกระบวนการสร้างเนื้อเยื่อ วัสดุของโครงเนื้อเยื่อสังเคราะห์มีผลต่อคุณสมบัติของโครงเนื้อเยื่อสังเคราะห์และผลสำเร็จของการสร้างเนื้อเยื่อผิวหนังมนุษย์ซึ่งอุดมไปด้วย extracellular matrix เป็นแหล่งวัตถุดิบหนึ่งที่เป็นไปได้สำหรับใช้เตรียมโครงเนื้อเยื่อสังเคราะห์ในวิศวกรรมเนื้อเยื่อ*

วัตถุประสงค์ : *เพื่อวิเคราะห์องค์ประกอบในสารละลายจากผิวหนังมนุษย์ และวิเคราะห์คุณสมบัติขั้นต้นทางกายภาพและทางชีวภาพของโครงเนื้อเยื่อสังเคราะห์ที่เตรียมจากสารละลายจากผิวหนังมนุษย์*

วิธีการศึกษา : *ผิวหนังจากผู้เสียชีวิตถูกนำมาเตรียมเป็นสารละลายจากผิวหนัง 3 ชนิด ตั้งชื่อว่า DS-1 DS-2 และ DS-3 และ นำมาวิเคราะห์ปริมาณคอลลาเจนและซัลเฟตไกลโคสะมิโนไกลแคน จากนั้นจึงนำมาเตรียมเป็นโครงเนื้อเยื่อสังเคราะห์สำหรับวิศวกรรมเนื้อเยื่อ คุณสมบัติของโครงเนื้อเยื่อสังเคราะห์ที่เตรียมได้จะถูกนำมาวิเคราะห์คุณสมบัติเปรียบเทียบกับโครงเนื้อเยื่อสังเคราะห์ที่เตรียมจากคอลลาเจนชนิดที่ 1 คุณสมบัติที่วิเคราะห์ได้แก่ โครงสร้างรูพรุน ความแข็งแรงเชิงกล การย่อยสลายโดยเอนไซม์คอลลาเจเนส การยึดเกาะและการเจริญเติบโตของเซลล์ต้นกำเนิดจากไขกระดูก*

ผลการศึกษา : *องค์ประกอบในสารละลายจากผิวหนังมนุษย์แต่ละชนิดมีความแตกต่างกัน โดยDS-1 DS-2 และ DS-3 มีปริมาณคอลลาเจนเป็น 92.23 79.07 และ 161.68 ไมโครกรัม/มก.น้ำหนักแห้ง ตามลำดับ และมีปริมาณซัลเฟตไกลโคสะมิโนไกลแคนเป็น 3.09 $\pm$ 0.51, 1.36 $\pm$ 0.39 และ 6.91 $\pm$ 0.87 ไมโครกรัม/มก.น้ำหนักแห้ง ตามลำดับ โครงเนื้อเยื่อสังเคราะห์จาก DS-3 มีขนาดรูพรุนเล็กที่สุด ค่าอีลาสติกโมดูลัสสูงที่สุด และ ใช้เวลาในการย่อยสลายนานที่สุดอย่างมีนัยสำคัญ ในขณะที่ความแตกต่างระหว่างโครงเนื้อเยื่อสังเคราะห์จาก DS-1 และ DS-2 ไม่มีนัยสำคัญ เซลล์สามารถยึดเกาะในโครงเนื้อเยื่อสังเคราะห์จากคอลลาเจนชนิดที่ 1 ได้ดีที่สุดอย่างมีนัยสำคัญ ในขณะที่ความแตกต่างระหว่างโครงเนื้อเยื่อสังเคราะห์จากสารละลายผิวหนังแต่ละชนิดไม่มีนัยสำคัญ เซลล์มีการเจริญเติบโตต่ำที่สุดอย่างมีนัยสำคัญในโครงเนื้อเยื่อจาก DS-3 แต่มีการเจริญเติบโตสูงสุดในโครงเนื้อเยื่อจาก DS-2 รองลงมาคือ DS-1 และคอลลาเจนชนิดที่ 1โดยไม่มีนัยสำคัญ*

สรุป : *สารละลายจากผิวหนังมนุษย์สามารถเตรียมขึ้นมาและใช้เป็นวัตถุดิบสำหรับทำโครงเนื้อเยื่อสังเคราะห์ได้ โดยองค์ประกอบในสารละลายจากผิวหนังแต่ละชนิดมีความแตกต่างกันซึ่งส่งผลให้โครงเนื้อเยื่อสังเคราะห์ที่เตรียมจากสารละลายจากผิวหนังแต่ละชนิดมีคุณสมบัติแตกต่างกัน โครงเนื้อเยื่อสังเคราะห์ที่เตรียมจากสารละลายจากผิวหนังมนุษย์ไม่มีความเป็นพิษต่อเซลล์โดยเซลล์สามารถเจริญเติบโตได้ตามปกติ ถึงแม้ว่าเซลล์สามารถยึดเกาะในโครงเนื้อเยื่อสังเคราะห์จากคอลลาเจนชนิดที่ 1 ได้ดีที่สุด แต่เซลล์มีการเจริญเติบโตสูงสุดในโครงเนื้อเยื่อสังเคราะห์ที่เตรียมจากสารละลายจากผิวหนัง DS-2*

คำสำคัญ : *โครงเนื้อเยื่อสังเคราะห์, วิศวกรรมเนื้อเยื่อ, สารละลายจากผิวหนังมนุษย์.*

Tissue engineering is useful for the treatment of damaged organs when natural healing is insufficient effective. Tissue engineering constructs artificial tissues to replace the lost tissues or provides scaffolds to facilitate the natural healing mechanism. Scaffolds play an important role by supporting cell growth and 3 dimensional tissue constructs. Effective scaffold is a critical factor that affects the success of tissue construction in tissue engineering. The property of scaffolds mainly depends on the raw materials from which they are made. Diverse biocompatible materials have been investigated. Among the materials, extracellular matrix is interesting because it is the natural scaffold of the tissues in the body and possesses biological properties involving in healing mechanism.

Skin is the large-volume organ in the human body with abundant of extracellular matrix. The main matrix in the human dermis is protein (90% dry weight) and 95% of which is collagen. (1) Most collagens in the human dermis are type I and type III collagen. The ratio of type I collagen/type III collagen is around 4.0 - 4.8.(2) The major components of glycosaminoglycans in the human skin are hyaluronic acid and dermatan sulfate (3) while chondroitin-4-sulfate and chondroitin-6-sulfate are found in small amount. (4) Hyaluronic acid is approximately 75% of the total dermal GAGs. (5) Uronic acid in the human dermis is in the range 800 – 1,100 μg/g dried dermis in which 3.9% is heparin. (6) The major proteoglycans detected in the human skin are decorin and versican. (7) High content of extracellular matrix in the human dermis has a potentiality to be used as a raw material for scaffold preparation in tissue engineering.

This research is aimed to prepare human dermal solutions from human skin to be used as raw materials for preparation of tissue engineering scaffolds. The human dermal solutions are characterized by their extracellular matrix components and the scaffolds made from the dermal solutions are analyzed for fundamental properties.

Methods

Human dermal solution preparation

Human cadaveric skin was immersed in 1 Normal NaCl overnight. The epidermis was peeled off. The dermis was blended in a meat blender. The blended dermis was dissolved in 0.5 M acetic acid solution (pH 2) and was homogenized. Non-dissolved matrix was removed by centrifugation at 1,500 rpm for 2 minutes. The solution was denoted as the dermal solution-1 (DS-1). After DS-1 was centrifuged at 12,000 rpm for 10 minutes, the supernatant was denoted as the dermal solution-2 (DS-2), and the sediment was denoted as the dermal solution-3 (DS-3).

Collagen-Sulfated Glycosaminoglycans analysis

Each type of dermal solutions was analyzed for collagen and sulfated GAGs contents by colorimetric methods. Collagen content was assayed by determination of hydroxyproline via chloramine-T reagent according to AOAC's official method of 990.26 (8) and sulfated GAGs was assayed by dimethylmethylene blue method according to Richard W Farndale, et al. (9) The percentage of the matrix mass content was determined by weighting the human dermal solutions before and after freeze-drying.

Scaffold preparation

Scaffolds from human dermal solution and 0.8% type I collagen (Sigma®, Sigma-Aldrich Corp., St. Louis, USA) were fabricated by freeze-drying. The material solutions were frozen at -40°C for 24 hrs, warm up 0°C for 4 hrs and drying was performed at 0°C for 2 days under vacuum condition. Scaffolds were punched by skin biopsy punch to attain circular-shaped scaffolds with 6 mm diameter and 2 mm thickness.

Scaffold characterization

Scaffolds were characterized physical properties including pore imaging by SEM (JEOL, JSM-5410LV), average pore size calculation by ImageJ software and compressive modulus by universal testing machine (Instron No.5567, USA) with compression rate 0.5 mm/min. Enzymatic degradation was assessed by weighting dry weight of scaffold after immersion in 0.5 U/ml Collagenase type I solution (Sigma-Aldrich Corp., St. Louis, USA).

Human BMSCs culture

Bone marrow was harvested from a patient, after having his informed consent. This protocol has been approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (COA No.134/2009 and IRB No.459/51). Human BMSCs were isolated and cultured according to Donald P Lennon and Arnold I Caplan. [10] The culture medium was DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin.

Human BMSCs characterized by flow cytometry

Human BMSCs were analyzed for stem cell surface antigen markers by flow cytometry. The stem cell markers (CD29, CD44, CD90 and CD105) were detected by specific fluorochrome-conjugated antibodies (CD29 was conjugated with PE-Cy5, CD44 and CD105 were conjugated with PE, CD-90 was conjugated with FITC). All fluorochrome-conjugated antibodies were purchased from Biolegend (San Diego, USA).

Attachment and proliferation of h-BMSCs in scaffolds

Human BMSCs were seeded into scaffolds at 25,000 cells/scaffold by dynamic seeding technique. Briefly, scaffolds were placed in 48-well plate with cell suspension and were shaken by horizontal shaker (Innova 2000, New Brunswick Scientific Co., Inc., USA). Cell attachment and proliferation was determined by MTT method according to Dai-Chian Chen, et al. [11] after culture for 1, 7, 14, 21 days.

Statistical analysis

Throughout the experiment, the sample size in all samples groups was performed in triplicate and the obtained data were expressed in means ± standard deviation. Student's t-test was used for significance testing within individual pair considered at $p < 0.05$.

Results

Apparently, the prepared human dermal solutions were viscous whitish solutions. The matrix contents and extracellular matrix components in the human dermal solutions were determined and the

results are shown in Table 1. The matrix content in DS-3 (30.80 ± 0.24 mg/ml) was highest and significantly higher than DS-1 (9.20 ± 0.42 mg/ml) and DS-2 (8.92 ± 0.24 mg/ml) ($p < 0.05$). No significant difference was found between the matrix contents of DS-1 and DS-2. Collagen content was also found highest in DS-3 (161.68 μg/mg) while collagen contents in DS-1 and DS-2 were 92.23 and 79.07 μg/mg, respectively. Sulfated GAGs contents in DS-1, DS-2 and DS-3 were 3.10 ± 0.51, 1.36 ± 0.39 and 6.91 ± 0.87 μg/mg, respectively. Sulfated GAGs contents in each type of the human dermal solutions were significantly different ($p < 0.05$) when compared to others.

Scaffolds were prepared from the dermal solutions and 0.8% type I collagen (Sigma®). Pore structures of the scaffolds were visualized and analyzed by SEM. All types of scaffolds had interconnected pores, as shown in Figure 1. Average pore diameter of type I collagen (Sigma®) scaffolds (188.84 ± 35.41 μm) was significantly higher than the others ($p < 0.05$). Scaffolds from DS-3 had the lowest average pore diameter (97.81 ± 21.99 μm) with statistically different ($p < 0.05$). No significant difference was found between the average pore diameter of scaffolds from DS-1 (145.68 ± 46.86 μm) and DS-2 (142.54 ± 45.39 μm).

The stiffness of the scaffolds were analyzed by compression test and expressed in the value of compressive modulus. The stiffest scaffolds was DS-3 scaffolds which their average compressive modulus (147.54 ± 11.07 KPa) was significantly higher than the others ($p < 0.05$). The average compressive moduli for scaffolds from DS-1, DS-2 and type I collagen (Sigma®) were 88.72 ± 27.61, 84.30 ± 21.62 and 56.32 ± 16.98 KPa, respectively, with insignificant difference. Degradation time for 50% weight loss was also found highest in DS-3 scaffolds (6.30 hr). Degradation time for 50% weight loss of the scaffolds from DS-1 and DS-2 were the same (3.30 hr) and degradation time of the scaffolds from type I collagen (Sigma®) was shortest (2.57 hr). All characterized properties of the scaffolds are concluded in the Table 2.

Their characteristics were analyzed for surface antigen markers for stem cells which included CD29, CD44, CD90 and CD105 by flow cytometry. More than 95% of cells were expressed stem cell antigen markers in each analyzed marker. This result confirms that the stem cell characteristic of the isolated cells before they are submitted to following experiments. Human BMSCs of passage 2 were seeded into scaffolds and assessed for cell attachment and proliferation.

Table 1. Matrix contents and extracellular matrix contents in each type of dermal solutions.

Dermal solutions	Matrix content(mg/ml)	Extracellular matrix content (μg/mg dry weight)	
		Collagen	Sulfated GAGs
DS-1	9.20 ± 0.42	92.23	3.10 ± 0.51
DS-2	8.92 ± 0.24	79.07	1.36 ± 0.39
DS-3	30.80 ± 0.24	161.68	6.91 ± 0.87

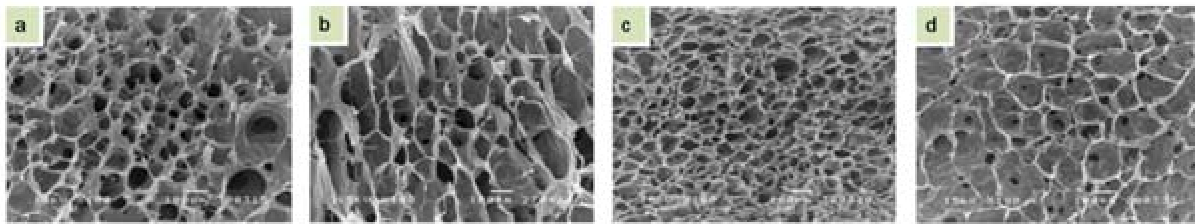

Figure 1. Pore structures of scaffolds from: (a) DS-1, (b) DS-2, (c) DS-3, and (d) type I collagen (Sigma®) visualized by scanning electron microscope.

Table 2. Physical properties of scaffolds from: DS-1, DS-2, DS-3 and type I collagen (Sigma®). Data are represented as means ± SD

Scaffolds	Average pore diameter (mm)	Compressive modulus (KPa)	Degradation time for 50% weight loss (hours)
DS-1	145.68 ± 46.86	88.72 ± 27.61	3.30
DS-2	142.54 ± 45.39	84.30 ± 21.62	3.30
DS-3	97.81 ± 21.99	147.54 ± 11.07	6.30
Type I collagen (Sigma®)	188.84 ± 35.41	56.32 ± 16.98	2.57

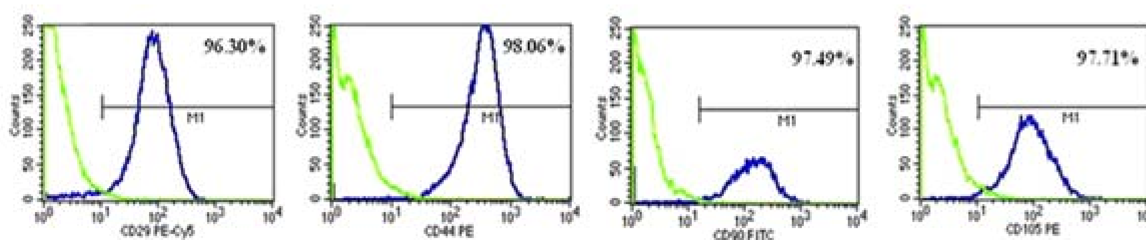

Figure 2. Flow cytometry analysis of surface antigen markers of h-BMSCs. The pale line represents non-stained cells used as negative control and the dark line represents fluorescent intensity of each CD expression on the cell surfaces.

After dynamic cell seeding, amount of cell attachment on the scaffolds was determined at the first day and the result is shown in Figure 3. Human BMSCs greatest attached on the type I collagen (Sigma®) scaffolds (22,448.46 ± 1,643.32 cells/scaffold) with significant difference ($p < 0.05$). Cell attachment for scaffolds from DS-1 (18,288.69 ± 1,680.46 cells/scaffold), DS-2 (18,288.69 ± 1,138.53 cells/scaffold) and DS-3 (18,001.80 ± 1,829.92 cells/scaffold) were insignificant different. Percentages of cell attachment for scaffolds from type I collagen (Sigma®), DS-1, DS-2 and DS-3 were 89.79 ± 6.57%, 73.15 ± 6.72%, 73.15 ± 4.55% and 72.01 ± 7.31%, respectively. Proliferation of human BMSCs in each

type of scaffolds is shown in Figure 4. The amount of human BMSCs in scaffolds from DS-3 was lowest both in day 7 (43,175.64 ± 2,566.95 cells/scaffold) and day 14 (51,064.88 ± 2,857.14 cells/scaffold). At day 7, it was lower with significant different (p <0.05) compared to the scaffolds from DS-2 (57,232.83 ± 2,581.93 cells/scaffold) and type I collagen (Sigma®) (54,292.29 ± 4,196.10 cells/scaffold) but insignificant different compared to the scaffolds from DS-1 (47,909.18 ± 3,458.23 cells/scaffold). At day 14, it was lower with significant different (p <0.05) compare to all other scaffolds (58,452.07 ± 2,446.91 cells/ DS-1 scaffold, 65,624.11 ± 4,962.72 cells/ DS-2 scaffold, and 65,050.34 ± 1,643.32 cells/ type I collagen scaffold). The highest amount of human BMSCs was found in the scaffolds from DS-2 and the second was the scaffolds from type I collagen (Sigma®) both in day 7 and day 14 with insignificant difference among them.

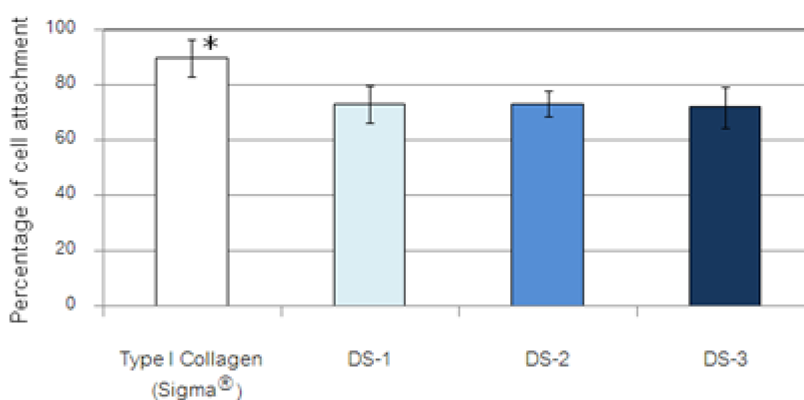

Figure 3. Percentage of cell attachment on the test scaffolds (* presented significant difference of type I collagen (Sigma®) scaffolds at p <0.05 relative to all scaffolds from dermal solution).

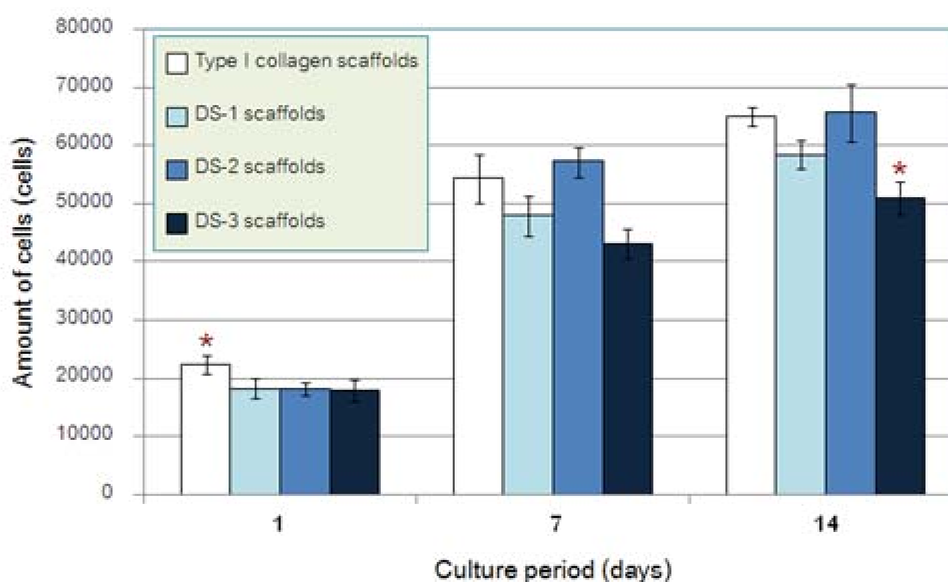

Figure 4. Proliferation of BMSCs in the scaffolds shown in the amount of cells at the determined days (* presented significant difference of the scaffolds at p <0.05 relative to all other scaffolds).

Discussion

The dermal solutions were a mixture of extracellular matrices. They contained at least collagen and sulfated GAGs which were the two components analyzed herein. The dermal solution DS-1, the initial solution obtained from the preparation process and has not been fractionated to other types of dermal solutions, contained the content of collagen close to the collagen content in the intact human skin analyzed by Shingo Tajima, et al. [1] and likewise for the content of sulfated GAGs. The centrifugation step caused the matrices deposit denser in the lower part (DS-3) while the upper part (DS-2) is lighter and caused the difference in the content of the extracellular matrices in the human dermal solutions.

The matrix content in DS-3, which was significantly higher than DS-1 and DS-2, causes its scaffolds possess the smallest average pore size, the highest stiffness and longest digestion time with significant difference compared to the scaffolds from DS-1 and DS-2 as well as the scaffolds from type I collagen (Sigma®). Despite the average pore size of the scaffolds from type I collagen (Sigma®) was significant larger than those of the scaffolds from all dermal solutions, the interconnected pores of the scaffolds from type I collagen (Sigma®) was appearently smaller.

Eventhough the scaffolds from DS-3 possess average pore size significantly smaller than the scaffolds from DS-1 and DS-3, no significant difference in the percentage of cell attachment was found among all scaffolds from the human dermal solutions. The highest cell proliferation was found in the scaffolds from DS-2, the second was the scaffolds from type I collagen (Sigma®), the third was in the scaffolds from DS-1, and the worst cell proliferation was found in the scaffolds from DS-3. The scaffolds from DS-2 provided greater cell proliferation than the scaffolds from other dermal solutions and also better than the scaffolds from type I collagen (Sigma®). It may be because of the sulfated GAGs to collagen ratio in DS-2 which is lower than those in the other human dermal solutions.

Conclusion

The proposed process for preparation of the dermal solutions is incomplicate and only common laboratory equipments are required. Each fraction of the attained dermal solutions composes of different portions of the collagen and sulfated GAGs. The contents of collagen and sulfated GAGs in DS-1 are similar to the contents found in the intact human dermis. By comparison to DS-1, the contents are denser in DS-3 and lighter in DS-2. Many physical and biological properties of the scaffolds from DS-1 and DS-2 are quite similar except for the cell proliferation. The results show that the scaffolds from DS-2 provide the best cell proliferation compare to all other types of scaffolds in the experiment.

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