Adipose Tissue Formation Utilizing Fat Flap Distraction Technique

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Adipose Tissue Formation Utilizing Fat Flap Distraction Technique

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Tissue engineering and regenerative medicine have been regarded as an area of research and experiment in the past. Nonetheless, medical doctors and scientists concentrated on it, to derive a promising outcome. From now on, significant and practical methods are necessary, enabling organs to overcome problems for themselves. The whole procedures on preparing this doctoral thesis were cautious and fruitful; quoting the phrase, "*Effort is more important than talent.*„

I sincerely appreciate of academic advisors; professor Yong Oock Kim, Won Jai Lee, Byung Il Lee, Kee Yang Chung, and Jae Woo Kim. The academic teaching and advices were more than the experimental process, and besides provided answers on troubles. The research scientist Eun Hye Kang has contributed to the researching process thoroughly. In addition, I would like to give thanks to colleagues, who have shared moments overcoming daily challenges in our lives.

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ABSTRACT

Adipose Tissue Formation Utilizing Fat Flap Distraction Technique

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Co-regulation between adipocytes and supporting vasculature is considered a significant process in adipose tissue generation. The objective of this study was to evaluate the mechanical and biological effects of a distraction technique on adipose tissue formation and maintenance. Based on the hypothesis that fat flaps gradually receding from each other can develop an adipose tissue construct, perforated polycarbonate syringe-shaped chambers were implanted in rabbit model. Latency (1 week) and distraction (3 weeks) periods were followed by a consolidation period in different

experimental groups (4, 8, and 12 week). In the distraction group, the volume of fat pad gradually increased up to 16 weeks. A transition zone was observed at 8 weeks, indicating the initiation of tissue generation. Histomorphologic analysis showed adipose and collagen connective tissue at 8 weeks. At 16 weeks, the relative composition has altered significantly. Adipose components occupied most of the tissue, and connective tissue was reduced. Blood vessels with endothelial lining were noted adjacent to adipocyte clusters, as well as in inter-adipocyte areas. The vessels had increased in number and were evenly distributed by 16 weeks. The distraction technique produced more balanced adipose tissue generation than a non-distraction method, demonstrating the co-development of adipose and vascular tissues.

Key words: Distraction, Adipocyte, Adipose tissue, Angiogenesis, Vascular tissue

I. INTRODUCTION

Adipose tissue engineering methods have recently progressed to address the generation and development of fatty tissue to restore lost or defective sites. To treat large tissue defects, a tissue engineering strategy is necessary to provide promising results[1]. Autologous fat graft has advantages in reconstruction, enabling augmentation of soft-tissue volume and contour defect correction. Autologous fat is biocompatible, available in sufficient amount in most patients, and naturally integrates with host tissues. However, adipose tissue transfer success is frequently limited due to low and unpredictable graft survival rate[2]. Vascularization is the major limitation affecting the survival of grafted or engineered adipose tissue constructs[3,4]. Viable vessels adjacent to the adipose tissue are crucial for nutrient diffusion and volume maintenance[5]. In addition, extracellular matrix elements secreted by endothelial cells greatly affect preadipocyte proliferation and differentiation[6]. There is significant evidence of co-regulation and inter-dependence between adipose tissue and the vasculature that supports it[7].

Distraction techniques are utilized in various clinical fields, including distraction osteogenesis on craniosynostosis or hemifacial microsomia and soft-tissue distraction to improve chronic flexion contractures of digits[8,9]. The procedure induces gradual histomorphogenesis and effective tissue generation based on distraction angiogenesis[10]. We considered that the advantages of this technique could improve adipose tissue generation.

The objective of this experiment was to evaluate the mechanical and biological effects of distraction technique on adipose tissue generation. With the hypothesis that fat flaps gradually receding from each other can develop an adipose tissue construct, perforated

polycarbonate syringe-shaped chambers were implanted in a rabbit model. The hypothesis was based on studies about a fat flap enclosed in perforated chamber induces adipose tissue formation and expansion[1,5]. Two fat flaps elevated from the dorsal cervical area were enclosed in the chamber. After different distraction periods, the generated tissue was harvested for morphological and histological analyses.

II. MATERIALS AND METHODS

1. Experimental Animals

This study protocol was conducted under the regulations of the Review Board of Experimental Ethics, College of Medicine, Yonsei University, Seoul, Korea. The Institutional Animal Care and Use Committee (IACUC) approval number was 2014-0326. All animals were maintained, fed, and euthanized under standard protocols of Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute. Thirty male New Zealand albino rabbits (2500–3500 g) were selected for assignment to three experimental and three control groups.

2. Experimental Study

New Zealand albino rabbits were used as an adipose tissue distraction model. Surgery was performed after an overnight fast. Animals were anesthetized intramuscularly with

Zoletil 50 (Virbac, France) and 2% Rompun solution (Bayer, Leverkusen, Germany) (1:2 ratio, 1 ml/kg). Dorsal surfaces of the cervical area were shaved and disinfected with povidone-iodine solution. A 7-cm vertical incision was made on the cutaneous layer of prepared area. For the experimental group, two fat flaps measuring approximately 1 × 1 × 5 cm^3 were elevated after subcutaneous dissection around the cervical area. The researcher-manufactured perforated polycarbonate syringe-shaped chambers were implanted within the subcutaneous layer, and fat flaps were fixed at (a) and (b) points with #4-0 nylon sutures (Fig. 1 and 2). Subcutaneous and skin layers were repaired with #3-0 vicryl and #4-0 nylon sutures, respectively. Afterwards, 5 mg/kg enrofloxacin (Baytril®, Bayer Healthcare, Shawnee Mission, KS, USA) was administered intramuscularly for 3 days to prevent infection. After 7 days of rest, distraction in the distraction group was conducted at the rate of 0.7 ml / 7 days × 3 times (Total 2.1 ml / 21 days) (Fig. 3). Consolidation periods were for 4, 8, and 12 weeks (5 rabbits × 3sub-groups). For non-distraction groups, a perforated polycarbonate chamber was implanted in the state of distraction being completed, and cervical fat tissues were fixed at each end of implant (Fig. 2). Ten rabbits were euthanized at postoperative 8, 12, 16 weeks respectively, and generated tissue between (a) and (b) points in addition to adjacent fat lumps were harvested for analysis (Fig. 4).

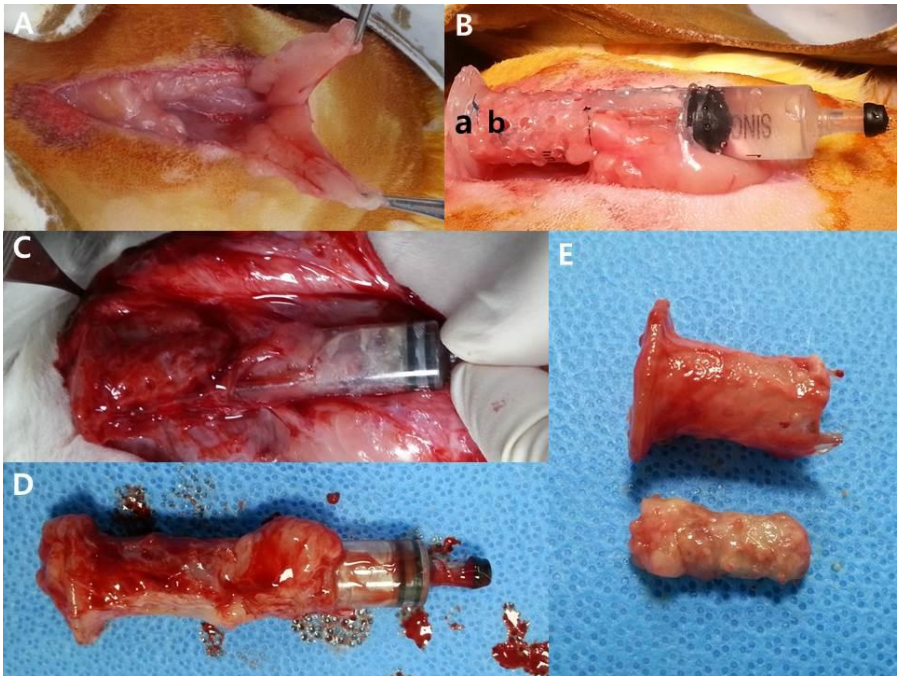

Figure 1. Adipose tissue formation using a distraction technique. Two fat flaps are elevated on the dorsum of cervical area (A). They are inserted into the chamber, and the tips of flaps are placed at point (b), dynamic distraction part. A counter fat tissue is sutured at point (a), fixed part of the chamber (B). Distraction is performed for assigned period, and chamber including adjacent tissue is harvested (C, D). Newly generated adipose tissue is separated from the chamber carefully (E).

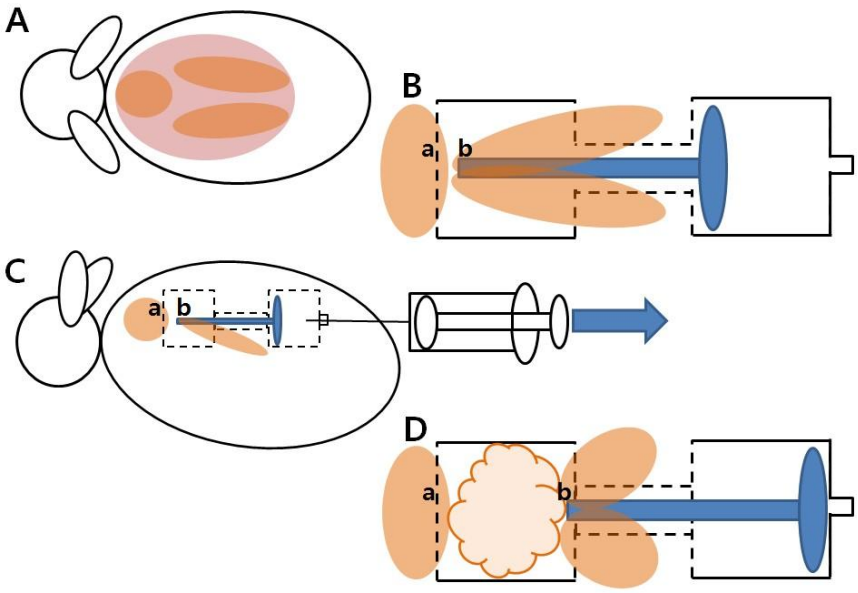

Figure 2. Schematic figure showing distraction procedures. Two fat flaps are elevated following subcutaneous dissection (A). The flaps are inserted into the chamber, and the tips of flaps are placed at point (b), dynamic distraction part. A counter fat tissue is sutured at point (a), fixed part of the chamber (B). Whole distraction chamber is inset into subcutaneous layer, and distraction is performed using a syringe (C). Newly generated tissue can be observed between points (a) and (b) after consolidation period (D). On the other hand, control group chambers were implanted in the state of distraction being completed.

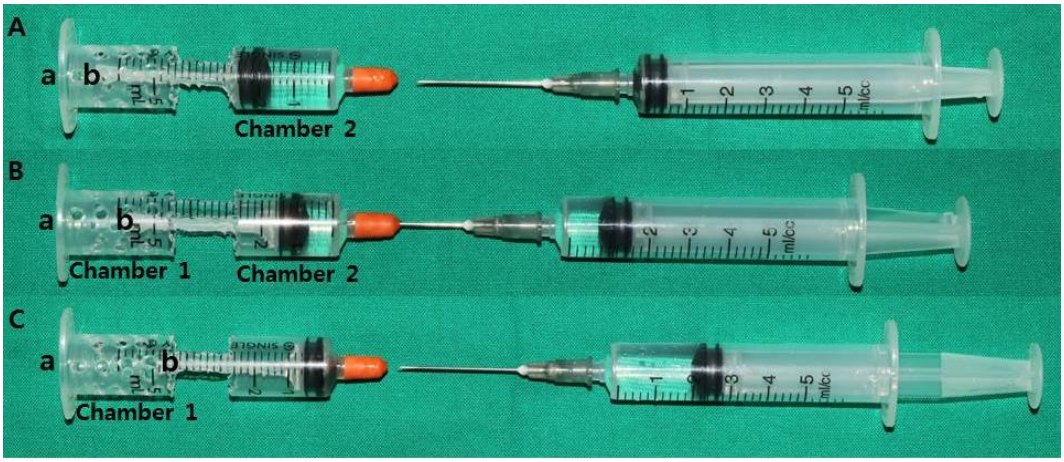

Figure 3. Distraction chamber model. Before distraction is initiated, fat tissues fixed at points (a) and (b) points are contact with each other (A). Distraction is undergone through gradual extraction of saline solution from chamber 2 (B). As point (b) recedes from point (a), expansion of chamber 1 is achieved (C).

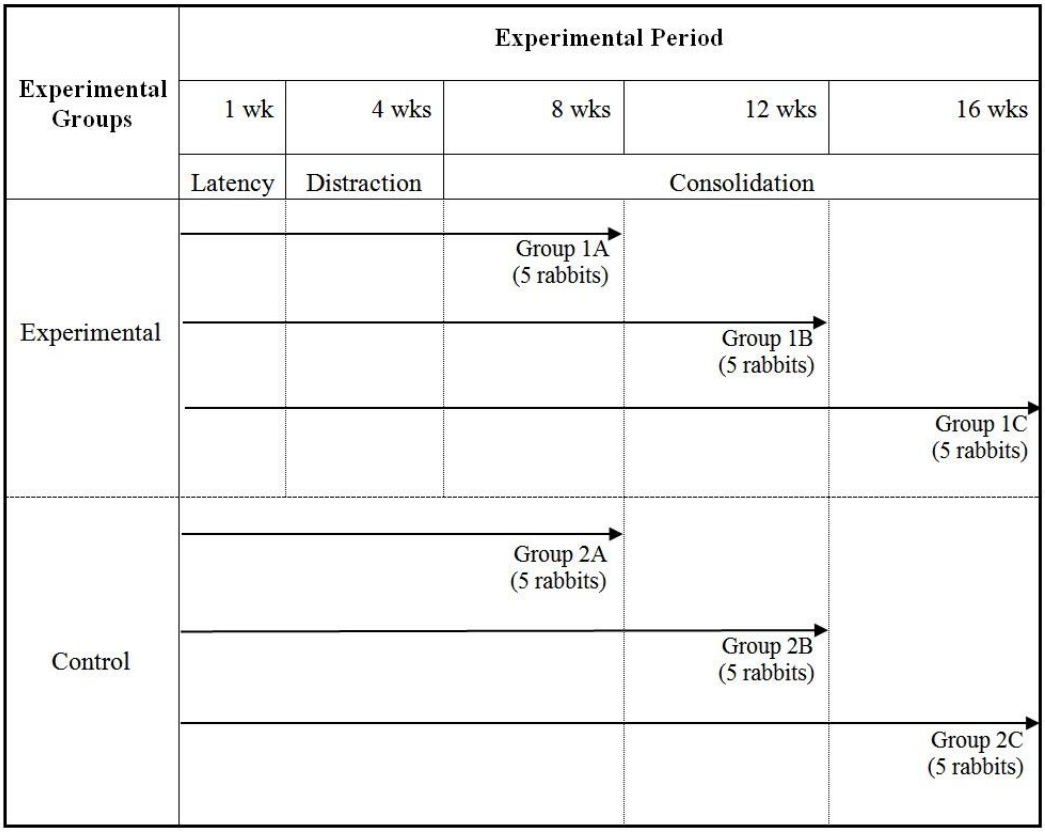

Figure 4. Experimental time table. After 7 days of latent period, distraction is conducted in the rate of 0.7 ml / 7 days x 3 times (Total 2.1 ml / 21 days) on experimental group. Ten rabbits (5 in experimental, and 5 in control groups) are euthanized on postoperative 8, 12, 16 weeks respectively. Experimental (Group 1A, 1B and 1C) and control (Group 2A, 2B and 2C) groups are assigned with regard to the consolidation periods.

3. Quantitative and Histologic Analysis

Noninvasive quantification of adipose tissue was performed using micro-computerized tomography (micro-CT) on the day of tissue harvest. The raw file was reconstructed into an ordered sequence of two-dimensional (2D) sections of the scan region. The final grayscale file reflected the apparent density of each voxel, with denser and less dense tissues appearing brighter and darker, respectively. In vivo micro-CT scans have been validated over a broad range of body weights and adiposity[11].

Newly generated and adjacent normal fat tissues were subsequently harvested. The fat pad was dissected, and its volume was measured using the liquid overflow method. Following gross observation, tissue section was performed in the middle of points (a) and (b), namely the farthest site from previously existing adipose tissues. Sections of the fat pad were stained with (1) hematoxylin and eosin (H&E), (2) Masson's trichrome, and (3) CD31 immunohistochemistry labeling methods prior to examination with light microscopy (BX61VS, Olympus Corp., Tokyo, Japan).

Masson's trichrome staining was performed to analyze connective tissues including collagen and fibrin. The staining solution was formulated using Bouin's solution (picric acid solution, 75 ml; 37% formalin, 25 ml; glacial acetic acid, 5 ml), Weigert's iron hematoxylin solution (hematoxylin, 1 g; 95% ethanol, 100 ml; ferric chloride, 2 g; concentrated HCl, 1 ml; distilled water, 95 ml), Biebrich scarlet-acid fuchsin solution (1% Biebrich scarlet, 90 ml; 1% acid fuchsin, 10 ml; Glacial acetic acid, 1 ml), phosphomolybdic–phosphotungstic acid (phosphomolybdic acid, 2.5 g; phosphotungstic acid, 2.5 g) and an aniline blue solution (aniline blue, 2.5 g; distilled water, 100 ml; glacial acetic acid, 2 ml).

A semi-quantitative analysis of adipose and connective tissue densities was carried out using MetaMorph® image analysis software (Universal Image Corporation, Buckinghamshire, UK). Results were demonstrated as the average optical density (OD) on five different digital images, and the average was calculated. OD can quantify the opacity of slides exposed to transmitted light, and outcomes could be considered inversely proportional to the grayscale values, which are related to the amount of spectral light.

CD31 immunohistochemistry staining was carried out to measure the quantitative extent of angiogenesis within adipose tissue. Sections were pretreated with 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase and processed with protein block serum-free reagent (X0909; DAKO, Carpinteria, CA, USA) for 30 min to prevent non-specific binding. The sections were incubated at 4°C overnight with primary antibodies (rabbit anti-vascular endothelial growth factor, RB-222-P, Laboratory Vision, Fremont, CA; anti-mouse platelet endothelial cell adhesion molecule-1 [PECAM/CD31] polyclonal antibody, M20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated additionally at room temperature for 20 min with DAKO Envision Kit (DAKO) secondary antibodies.

To evaluate neovascularization, we counted the numbers of CD31-positive vessels enclosed with a single layer of endothelial cells and without a muscular layer. A comparative analysis of the number of blood vessels was performed in each high-power field (×200). For each slide, the numbers of vessels were measured on five different sites at the junction between adipose and connective tissues, where vessels are abundant. Vascular density was presented with regard to the time period in each group.

4. Statistical Analysis

Unpaired t-tests were performed to compare fat pad volumes, quantities of generated adipose and connective tissues, and numbers of new blood vessels between experimental and control groups. One-way analysis of variance was also used to test whether there was any difference between time periods on those variables in each group. Significant analysis of variance results were followed by the post hoc test for pairwise comparisons, adjusted by Bonferroni correction. Statistical significance was evaluated with a 95% confidence interval.

III. RESULTS

1. Gross Morphology and Volume Analysis

Each group started with 15 animals (n=15, distraction group; n=15, non-distraction group). One rabbit in distraction group showed delayed wound healing, and postoperative oozing necessitated additional conservative management. The other implantation sites exhibited no gross indication of acute inflammation or abnormality in the chamber tissues. Gross observation of each group demonstrated gradual adipose tissue expansion. In the distraction group, adipose tissue showed irregular contours with a constricted transition zone at 8 weeks. The transition zone represented a core area where tissue was gradually

generated. After 12 weeks of consolidation, adipose tissue exhibited regular contours and filled the perforated chamber. In the non-distraction group, generated tissue showed irregular contours with pliable characteristics and was fragile at 8 weeks. After maturation for 16 weeks, the adipose tissue had gathered volume, and the surface fitted to the chamber. The non-distraction group tissue had a coarse capsular layer with heterogeneous color compared to the distraction group (Fig. 5).

Harvested adipose tissue was subjected to volume measurement using the saline solution overflow method. In the distraction group, the fat pad volume gradually increased up to 16 weeks (final volume 1.92 ± 0.06 ml). Nonetheless, significant increases were noted at 8 weeks (1.14 ± 0.13 ml) and 12 weeks (1.65 ± 0.16 ml). The non-distraction group also exhibited a volumetric increment at 16 weeks (final volume 1.31 ± 0.14 ml), and significant growth was noted at 8 weeks (0.74 ± 0.07 ml) and 12 weeks (1.14 ± 0.18 ml). In comparative inter-group analysis, the fat pad volume of the distraction group was larger than that of the non-distraction group, indicating significant discrepancies in each period (8, 12, and 16 weeks; $p < 0.05$) (Fig. 6).

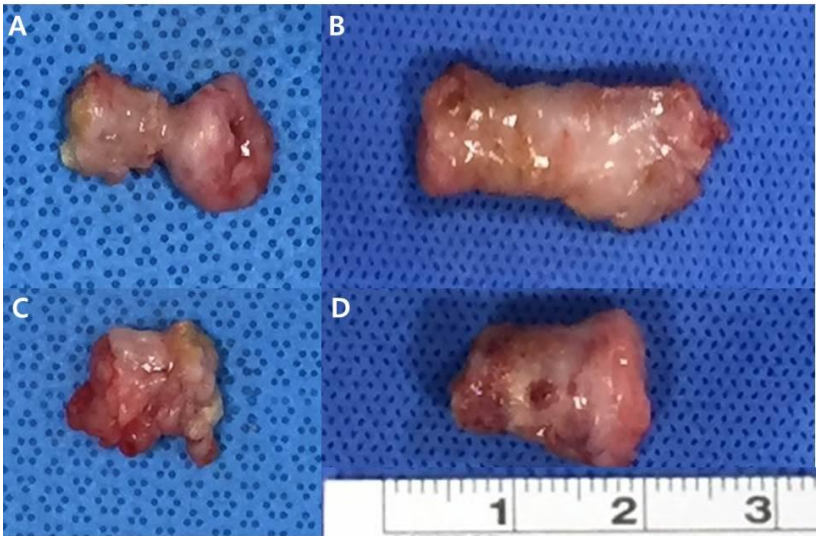

Figure 5. Gross observation of generated tissue in each group. In experimental group, adipose tissue showed irregular contour with constricted transition zone at 8 weeks (A). After 12 weeks of consolidation period, adipose tissue construct was noted following volume expansion (B). In control group, generated tissue exhibited pliable characteristic with irregular contour at 8 weeks (C). After maturation for 16 weeks, adipose tissue has gathered volume and fitted to the chamber (D).

Table 1. Volume measurement of the generated tissue in distraction chamber (ml ± SD)

	8 weeks	12 weeks	16 weeks
Distraction group	1.14 ± 0.13	1.65 ± 0.16	1.92 ± 0.06
Non-distraction group	0.74 ± 0.07	1.14 ± 0.18	1.31 ± 0.14
P value	<0.05	<0.05	<0.05

In distraction group, the volume of generated tissue showed gradual increase up to 16 weeks. Nonetheless, significant expansion was noted at 8 and 12 weeks. Non-distraction group also exhibited volumetric increment at 16 weeks, and significance alteration was noted at 8 and 12 weeks. In comparative inter-group analysis, the volume of distraction group resulted in larger amounts than non-distraction group, indicating significant differences in each period (8, 12 and 16 weeks; $p < 0.05$).

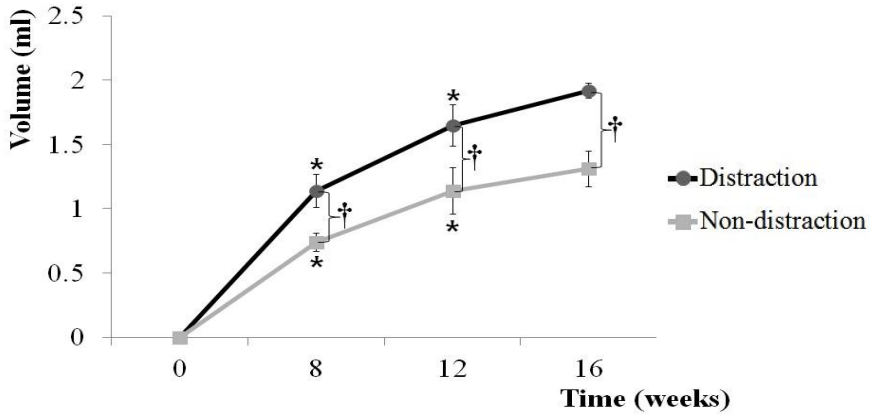

Figure 6. Comparative volume measurements with regard to the time period. In experimental group, the volume of generated tissue showed gradual increase up to 16 weeks (final volume 1.92 ± 0.06, Group 1C). Nonetheless, significant expansion was noted at 8 and 12 weeks (Group 1A, 1.14 ± 0.13 ml; Group 1B, 1.65 ± 0.16 ml; $p<0.05$). Control group also exhibited volumetric increment at 16 weeks (final volume 1.31 ± 0.14, Group 2C), and significance alteration was noted at 8 and 12 weeks (Group 2A, 0.74 ± 0.07 ml; Group 2B, 1.14 ± 0.18 ml; $p<0.05$). In comparative inter-group analysis, the volume of experimental group resulted in larger amounts than control group, indicating significant differences in each period (8, 12 and 16 weeks; $p<0.05$).

* $p<0.05$, compared to measurement on previous experimental period

† $p<0.05$, compared to control group

2. Micro-CT Imaging

Micro-CT of samples including the whole chamber was performed immediately after tissue harvest. The method enabled adequate evaluation before separating the tissue from the chamber. Images demonstrated generated tissues composed of adipose and connective tissues with distinguishable Hounsfield units (fat, -100 to -50; connective tissue, +100 to +300).

The distraction group showed adipose tissue formation on the central area of chamber at 8 weeks, when consolidation had been allowed for 4 weeks. At 16 weeks, more adipose tissue was noted, occupying most of the chamber space. In addition, there was minimal connective tissue. In the control group, mixed characteristic tissue with loose adipose and dense connective tissue components had developed at 8 weeks. The size was smaller compared with the experimental group. Tissue generation had progressed at 16 weeks; however, the connective tissue was dispersed mimicking lipodystrophy (Fig. 7).

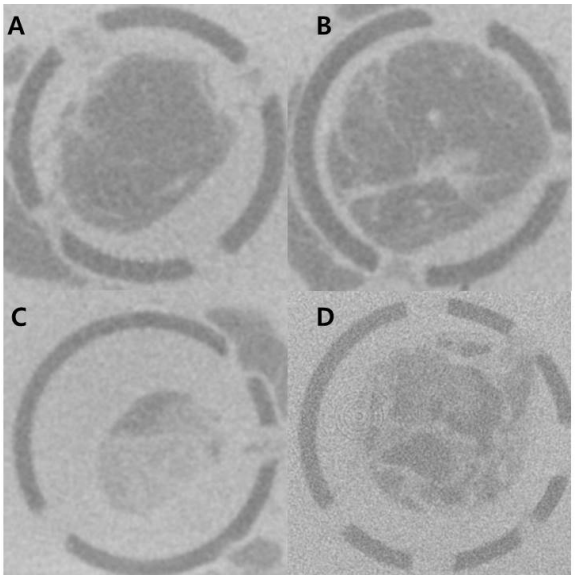

Figure 7. Micro-CT images in each group. Experimental group exhibited adipose tissue formation on the central core at 8 weeks (A). At 16 weeks, enlarged adipose tissue occupied the chamber, showing minimal fibrous tissue (B). In control group, tissue generation has been noted, although smaller in size compared to experimental group (C). At 16 weeks, tissue expansion has progressed, however adipose and fibrous tissues existed in mixed characteristic (D).

3. Histomorphologic Assessment

Histomorphologic analysis was performed using H&E and Masson's trichrome staining methods. Serial tissue section at three different sites; namely cephalic, transition zone, caudal areas was followed by microscopic observation under low magnification (x 10.25). The cephalic area results consisted of cervical fat tissue originally, and did not present significant difference between 8 and 16 week observations. The transition zone, composed of newly generated tissue has noted small diameter with inflammatory reaction at 8 weeks. Nonetheless, the diameter has enlarged and inflammation has subsided at 16 weeks. The caudal area, which was close to the caudally based fat flaps showed connective tissue core at 8 weeks. Later observation at 16 weeks, however, exhibited adipose tissue components with septa; which had undergone alteration in tissue characteristic (Fig. 8). On the other hand non-distraction group did not show transition zone and cephalo-caudal discrepancy.

Under high magnification (x 200), Generated tissue in the distraction group consisted of uniform adipocytes at 8 weeks, although connective tissues including collagen and fibrin were also observed. At 16 weeks, the relative composition was significantly altered. Adipose component occupied most of the tissue, and connective tissue was reduced. Each adipocyte showed mature and viable characteristics without signs of atrophy, hypertrophy, or necrosis. In the control group, connective tissue was more prevalent than adipose tissue at 8 weeks, and inflammatory cell infiltration was noted. Subsequent results showed more regular adipocyte distribution at 16 weeks, but fibrotic connective tissue was still present (Fig. 9, 10).

Masson's trichrome stain enabled tissue identification, and adipose and adjacent

connective tissue were quantitatively evaluated. In the distraction group, adipose tissue increased significantly throughout 16 weeks (8 week: 50030 ± 4237 OD and 16 week: 92104 ± 3636 OD). Adjacent connective tissue, however, showed a trend curve with a gradual decrease (8 week: 46738 ± 3252 OD and 16 week: 7208 ± 2219). In the non-distraction group, adipose tissue increased up to 12 weeks (8 week: 44939 ± 2817 OD and 12 week: 58894 ± 3841 OD), while adjacent connective tissue decreased (8 week: 51786 ± 4641 OD and 12 week: 33929 ± 3312). At 16 weeks, the control group showed similar tendencies; however, the value was not distinguishable from the 12-week measurement. With regard to inter-group analysis, the experimental group showed higher adipose tissue quantity than the control group at 12 and 16 weeks, and an inverse relationship was noted in connective tissue density ($p < 0.05$) (Fig. 10, 11).

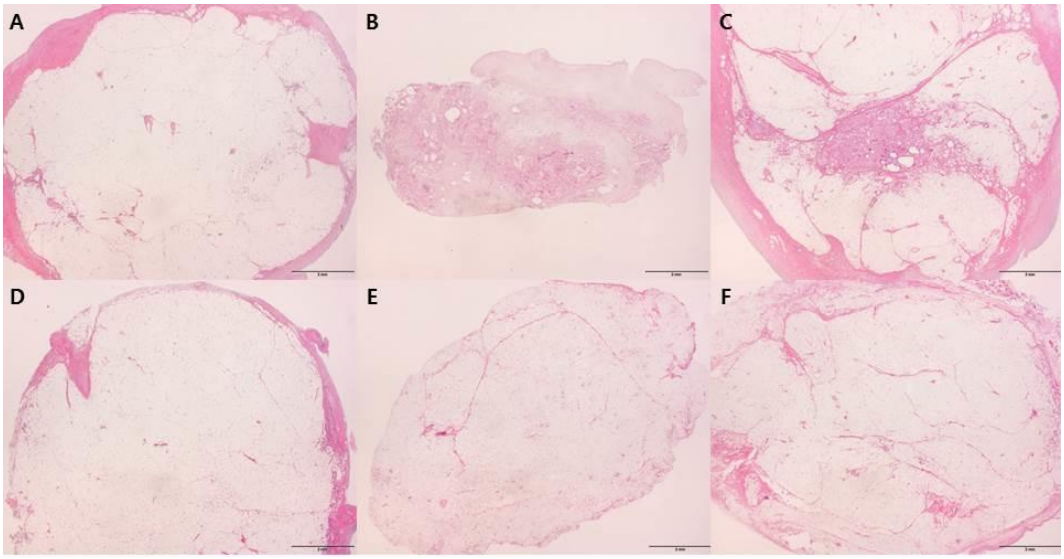

Figure 8. Serial tissue section at three different sites; namely cephalic, transition zone, caudal areas was followed by microscopic observation under low magnification (x 10.25). The cephalic area results consisted of cervical fat tissue originally, and did not present significant difference between 8 week (A) and 16 week (D) observations. The transition zone, composed of newly generated tissue has noted small diameter with inflammatory reaction at 8 weeks (B). Nonetheless, the diameter has enlarged and inflammation has subsided at 16 weeks (E). The caudal area, which was close to the caudally based fat flaps showed connective tissue core at 8 weeks (C). Later observation at 16 weeks, however, exhibited adipose tissue components with septa; which had undergone transition in tissue characteristic (F).

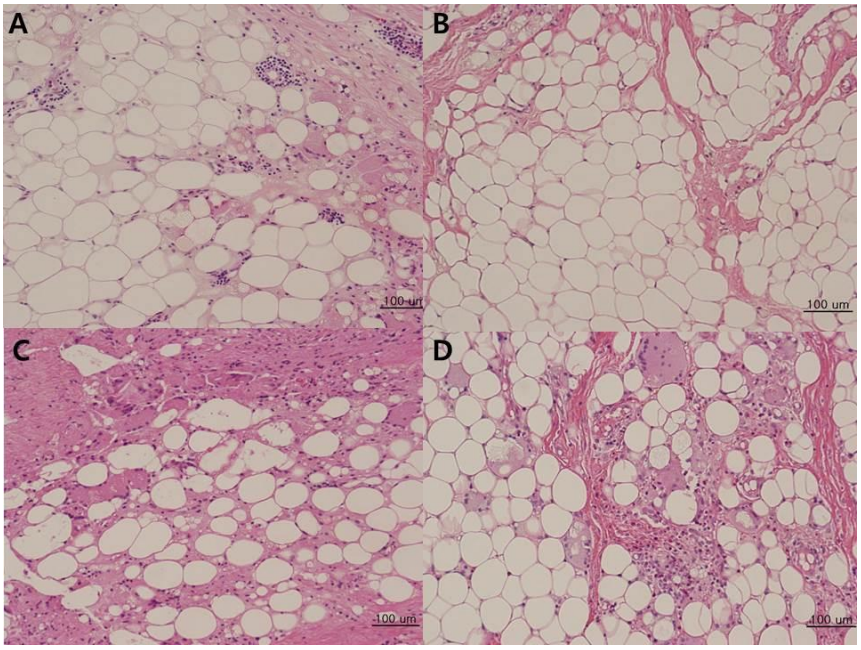

Figure 9. Experimental group demonstrated uniform adipocytes at 8 weeks, and fibrous connective tissue existed in part (A). At 16 weeks, each adipocyte exhibited mature and viable characteristic with consistency (B). In control group, fibrous connective tissue existed prominently with inflammatory cell infiltration at 8 weeks (C). The later result, at 16 weeks, showed fibrous tissue notably (D).

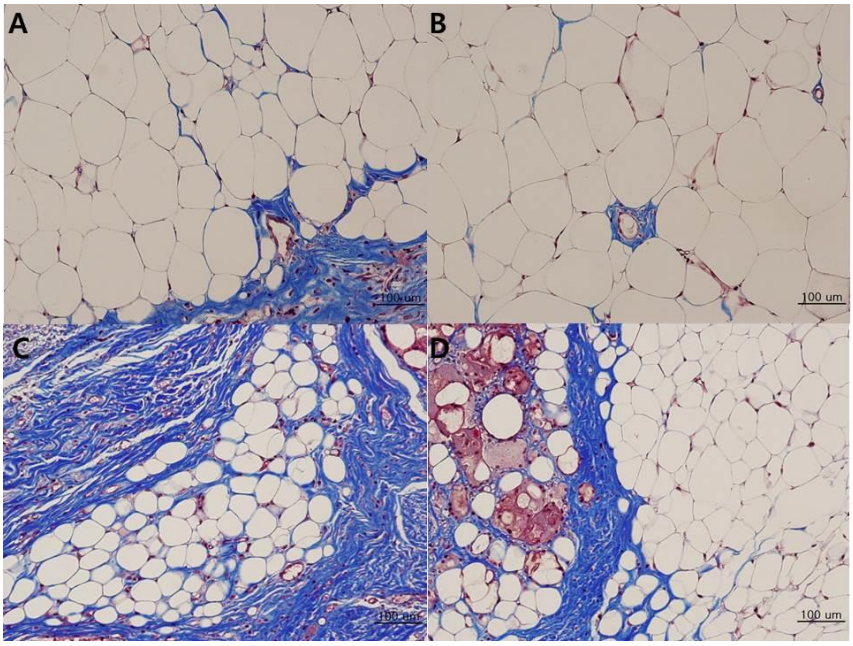

Figure 10. Masson's trichrome stain facilitated adequate distinction between tissues. Experimental group consisted of adipose tissue with little fibrous connective tissues, such as collagen and fibrin at 8 weeks (A). At 16 weeks, Adipose components occupied most of the tissue; meanwhile fibrous tissue has reduced (B). In control group, fibrous connective tissue exhibited a higher prevalence than adipose tissue at 8 weeks (C). Subsequent observation revealed more adipose tissue at 16 weeks; however fibrotic tissue and inflammation were still evident (D).

Table 2-1. Measurement of adipose tissue area using Metamorph® image analyzing software. (Pixels ± SD)

	8 weeks	12 weeks	16 weeks
Distraction group	50030 ± 4237	78117 ± 2078	92104 ± 3636
Non-distraction group	44939 ± 2817	58894 ± 3841	62773 ± 2332
P value	>0.05	<0.05	<0.05

Table 2-2. Measurement of fibrous connective tissue area using Metamorph® image analyzing software. (Pixels ± SD)

	8 weeks	12 weeks	16 weeks
Distraction group	46738 ± 3252	18239 ± 3473	7208 ± 2219
Non-distraction group	51786 ± 4641	33929 ± 3311	30001 ± 2300
P value	>0.05	<0.05	<0.05

In distraction group, adipose tissue showed a gradual increase; meanwhile adjacent connective tissue has decreased during 16 weeks. In non-distraction group, adipose tissue noted increment, and fibrous tissue exhibited decrement up to 12 weeks. With regard to inter-group analysis, experimental group demonstrated higher adipose tissue quantity than control group at 12 and 16 weeks, and connective tissue density showed an inverse relationship ($p < 0.05$).

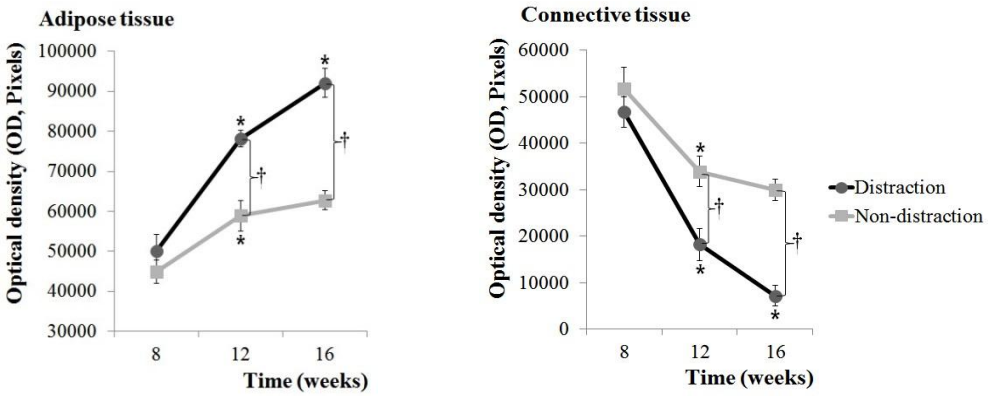

Figure 11. Quantitative analysis on adipose tissue (A) and fibrous connective tissue (B). In experimental group, adipose tissue showed a gradual increase; meanwhile adjacent connective tissue has decreased during 16 weeks. In control group, adipose tissue noted increment, and fibrous tissue exhibited decrement up to 12 weeks. With regard to inter-group analysis, experimental group demonstrated higher adipose tissue quantity than control group at 12 and 16 weeks, and connective tissue density showed an inverse relationship ($p < 0.05$).

* $p<0.05$, compared to measurement on previous experimental period

† $p<0.05$, compared to control group

4. Angiogenesis

CD31 immunohistochemistry was performed to assess angiogenesis. Blood vessels with endothelial lining were noted at the junction between adipose and connective tissues in the distraction group. The vessels had increased in number and were evenly distributed at 16 weeks compared to earlier time points. Evidence of angiogenesis was less prominent in the non-distraction group (Fig. 12).

New blood vessels increased throughout 16 weeks in the distraction group. Significant alterations were noted at 8, 12, and 16 weeks (8 week: 2.92 ± 0.98 and 16 week: 12.3 ± 1.62). The control group also had more blood vessels up to 12 weeks, showing significance at 8 and 12 weeks (8 week: 1.96 ± 0.67 and 12 week: 4.48 ± 0.91). In inter-group comparative analysis, the numbers of blood vessels were significantly different between groups at 12 and 16 weeks ($p < 0.05$) (Fig. 13). Furthermore, new blood vessels were noted among adipocytes at high magnification (×200) in the distraction group, and evidence of angiogenesis in adipocyte clusters was more prominent than in the non-distraction group. Inter-adipocyte angiogenesis was a significant finding in the distraction group (Fig. 14).

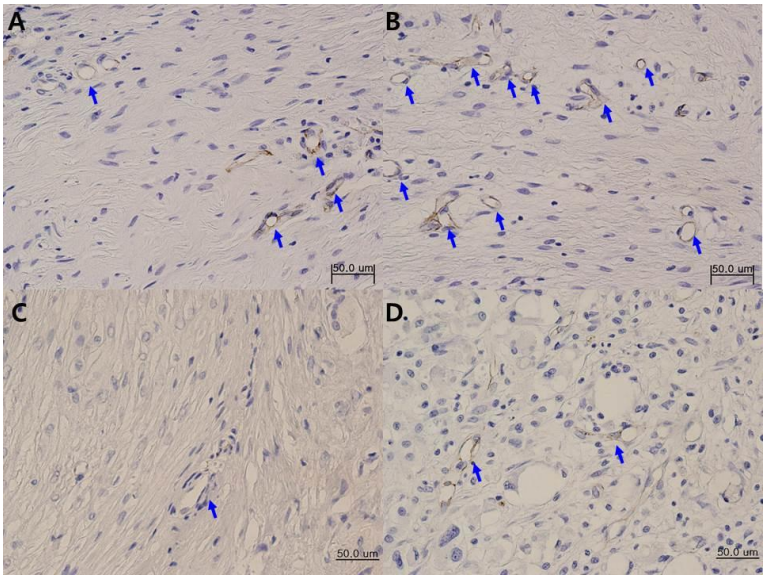

Figure 12. CD 31 immunohistochemistry stain results showing the evidence of angiogenesis. The arrows indicate endothelial lining with CD 31 positive cells. Blood vessels were noted adjacent to adipocyte clusters at 8 weeks in experimental group (A). The vessels has been increased and distributed evenly at 16 weeks (B). In control group, labeled cells were exhibited infrequently at 8 weeks (C). At 16 weeks, vessels were apparent than earlier observation; nonetheless the evidence of angiogenesis was less prominent compared to experimental group (D).

Table 3. Number of blood vessels in the generated tissue (number ± SD)

	8 weeks	12 weeks	16 weeks
Distraction group	2.92 ± 0.98	6.92 ± 0.87	12.3 ± 1.62
Non-distraction group	1.96 ± 0.67	4.48 ± 0.91	5.92 ± 1.25
P value	>0.05	<0.05	<0.05

In distraction group, new blood vessels increased throughout 16 weeks, and significant alteration was noted at each period; 8, 12 and 16 weeks. Non-distraction group has exhibited increment up to 12 weeks. In comparative analysis between experimental and control groups, significant discrepancy was noted at 12 and 16 weeks ($p < 0.05$).

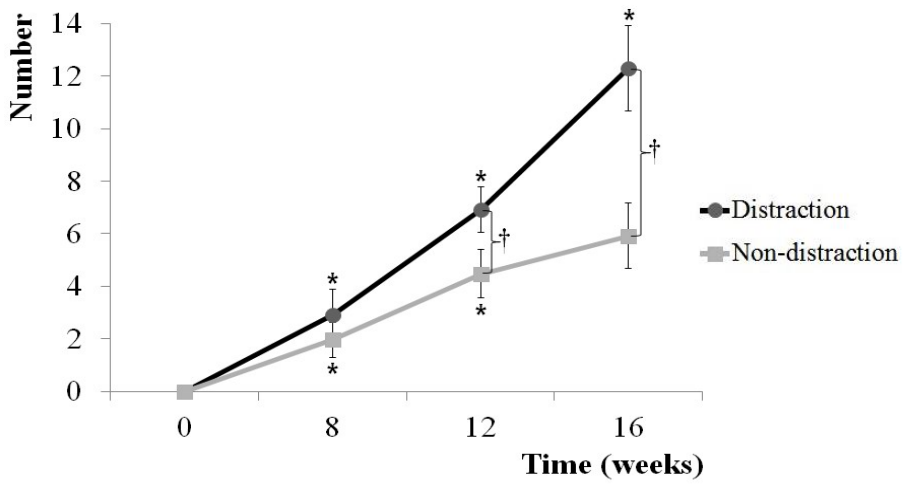

Figure 13. In experimental group, new blood vessels increased throughout 16 weeks, and significant alteration was noted at each period; 8, 12 and 16 weeks. Control group has exhibited increment up to 12 weeks. In comparative analysis between experimental and control groups, significant discrepancy was noted at 12 and 16 weeks ($p < 0.05$).

* $p<0.05$, compared to measurement on previous experimental period

† $p<0.05$, compared to control group

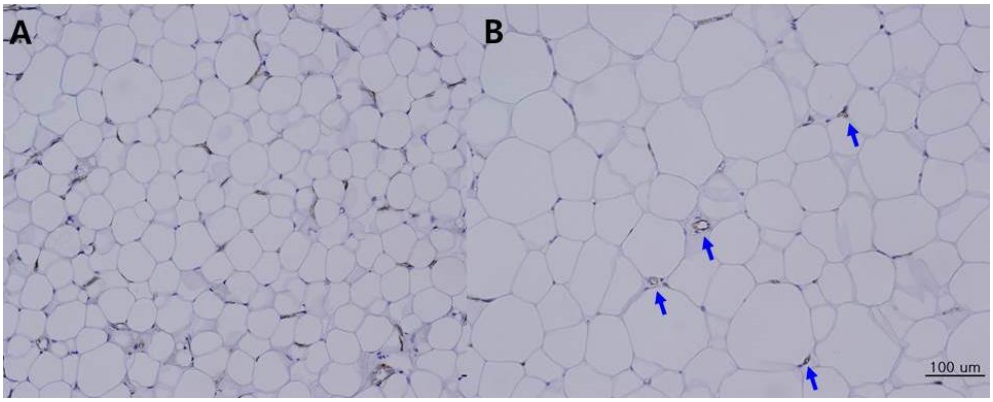

Figure 14. Inter-adipocyte angiogenesis in distraction model. Labelled capillaries were distributed throughout uniform adipocytes (A). New blood vessels were noted among adipocytes at high magnification (x200) image in experimental group, and the evidence of angiogenesis in adipocyte clusters was prominent (B).

IV. DISCUSSION

Adipose tissue is a metabolically active and highly vascularized organ. It was proposed that every adipocyte has one or more supportive capillaries[3]. Considering oxygen and nutrient supplies, the vascular system is essential for transporting cytokines, hormones, and growth factors. The vasculature therefore has a significant role in adipocyte survival and maintenance[4]. An adipose tissue construct exceeding a critical size may suffer from poor supply and subsequent cell necrosis[7]. In the clinical setting, transferred adipose tissue is at risk of volume loss up to 60 %[1]. Adipocyte necrosis results from an inadequate initial blood supply and low tolerance against ischemia. A number of strategic approaches have been developed to improve adipose tissue constructs, but the desired characteristics of cellular components in tissue constructs has remained unclear.

Findlay et al. developed a perforated chamber that was subcutaneously inserted into the swine groin enclosing a fat flap based on the superficial circumflex iliac pedicle[5]. They reported new vascularized tissue that filled a 78.5 ml chamber in 22 weeks. Histologic analysis exhibited increased adipose tissue volume associated with adipocyte hyperplasia rather than hypertrophy.

Tissue engineering chambers (TECs) have been utilized to generate adipose tissue constructs[1,5,12-14]. Peng et al. noted that collagen tissue was dominant at early observation at 30 days, but it was replaced by newly formed adipocytes over time[12]. They suggested four stages of TEC adipose tissue generation: the inflammation period (0–15 days), the angiogenesis stage (15–30 days), the adipogenesis stage (30–45 days), and the maturation stage (45–60 days). In the inflammation stage, surgical procedures and chamber implantation prompted macrophage and mesenchymal stem cell (MSC) infiltration,

followed by the release of angiogenic and growth factors. The inflammatory reaction remarkably enhanced angiogenesis, extracellular matrix (ECM) expansion, and perivascular cell proliferation. In the adipogenesis stage, perivascular cells have switched into adipose precursor cells, and adipogenic differentiation occurs if there is an adequate blood supply. Finally, in the maturation stage, adipose tissue regeneration is completed following the expansion of newly generated adipocyte tissue and ECM remodeling.

We hypothesized that TEC using the fat flap distraction technique could induce the gradual generation and maintenance of adipose tissue with natural histologic characteristics. Early observation at 8 weeks demonstrated a constricted transition zone, which confirmed gradual tissue generation from the core area. At 16 weeks, the tissue exhibited regular contours and fitted to the chamber. The distraction method achieved more efficient volume expansion than in the non-distraction group, where the fat tissues had been separated since chamber implantation (Fig. 5).

The distraction technique has been utilized in reconstructive procedures for which 1) gradual tissue generation is advantageous; 2) a considerable amount of tissue is required; or 3) adjacent tissue circumstance is relatively poor due to hypoxia, irradiation, or chronic inflammation[8,15-18]. Tissue generation using distraction involves mechanical traction, transient ischemia, and subsequent angiogenesis[10]. The generated tissues have demonstrated stability and resistance against poorly vascularized condition[19,20]. Notably, our distraction group showed mature adipocytes with significant vascularity in histomorphological and quantitative analyses. In addition, the generated tissue at 8 weeks consisted of connective tissue in the core area, but this had been replaced with mature adipose tissue at 16 weeks, which is in accordance with previous findings (Fig. 8) [1,12,13].

The synergy between angiogenesis and adipogenesis has been verified through the incorporation of preexisting vascularized adipose tissue[4,6]. Furthermore, changes in the mechanical force within the chamber provide additional mitogenic stimulus in accordance with mechano-transduction[21]. A lack of adequate cell-to-cell contact in fat flap has been associated with a poor adipogenesis rate[22]. Our histomorphologic and quantitative findings demonstrate comparable results in terms of vascular and adipose tissue co-development (Fig. 9-13). In addition, vascular growth among adipocytes supported the viability of the adipose tissue construct (Fig. 14). On the other hand, our non-distraction group had fibrous connective tissue construct without adequate tissue contact and mechano-transduction.

Nie et al. suggested the delivery of adipose-derived stem cells (ASCs) through an acellular dermal matrix, and several authors have successfully developed the model in different scaffold types[23]. In our distraction model, connective tissue initially generated at the transition zone expanded into adipose tissue with fullness and natural contours. The primary tissue could promote adipose tissue formation by serving as a natural adipogenic scaffold.

Aseptic inflammation has been reported to promote adipose tissue regeneration[12]. Lilija et al. found that adipogenesis was induced by macrophage-derived factors such as monocyte chemotactic protein-1 (MCP-1), which recruited macrophages, and bone marrow-derived precursor cells[13]. Adipogenesis in TECs is thought to ensure the relationship between controllable inflammation and adequate adipogenesis. Inflammation is usually considered as the connector showing different comorbidities, but a recent study noted that hypoxia could be a potential risk factor for chronic inflammation[24]. Hypoxia can lead to inflammation in adipose tissue and induce gene expression in macrophages

and adipocytes. It is believed that local inflammation related to hypoxia serves as a physiological promoter for angiogenesis and ECM remodeling in adipose tissue; however, when inflammatory reactions persist, chronic inflammation occurs[25]. During chronic inflammation, vascular networks cannot provide adequate oxygen to adipocytes, and local hypoxia arises, which could trigger adipose tissue dysfunction[26]. In that instance, hypoxia occurs in a group of adipocytes distal from the vascular network as adipose tissues expand[27]. The researches on impaired adipogenic capacity elucidate distinguishable results in distraction and non-distraction groups. When hypoxic and inflammatory states were overcome by sufficient vascularity with an adequate matrix, mature adipose tissue could be generated in the distraction group. On the other hand, the void in non-distraction group was filled with fibrous connective tissue infiltrated with inflammatory cells. Although there was an adipose component, the evidence of chronic inflammation still existed.

Lilja et al. suggested that gene expression and various cytokines are involved in the early development of engineered adipose tissue[13]. They utilized TECs in mouse model and reported macrophages entering the TEC at 12 hours post-insertion. This caused prompt increments in MCP-1 and macrophage inflammatory protein-1 alpha (MIP-1α), providing a signal for further macrophage recruitment. The cytokines attracted precursor cells including MSCs to the chamber to facilitate tissue generation. The authors reported that cytokine levels decreased in the chamber since 2 days post-insertion, while growth factors such as tumor necrosis factor-α, lipocalin-2, and interleukin-1β increased. These factors served as additional stimuli for de novo blood vessel formation. In addition, different stages of angiogenesis are modulated by ASCs and adipocytes. A recent study reported that vascular endothelial growth factor (VEGF) expression by ASCs and

adipocytes could destabilize existing vessels for vascular sprouting[7]. Following destabilization, endothelial cell migration and proliferation are induced by angiotensin II and basic fibroblast growth factor (b-FGF) from cellular components of adipose tissue cluster[28,29]. Adipocyte-derived factors including leptin, monobutyrin, and adenosine enhance the process and promote vascular fenestration[30,31]. Several important mediators also play roles in adipogenesis-angiogenesis crosstalk. PPAR-γ and the human CCAAT/enhancer binding protein alpha (C/EBP-α), the main modulators of adipogenesis, are activated through various endocrine modes, and VEGF expression in adipocytes utilizes common mediators[7,32,33]. The linkage between adipocyte and endothelial cell-derived mediators suggests inter-dependence between the two components.

Previous publications have utilized various expressions including adipose tissue generation[1,34], regeneration[12,14], development[13,35], and expansion[36,37]. To accurately define tissue regeneration, it is important to ensure regenerative processes of the tissue injured or undergoing degenerative changes. In addition, the origin of adipocytes needs to be clarified, with de novo adipogenesis of particular interest. We utilized the terms adipose tissue generation and formation. Nonetheless, tracking cell sources is important in future studies and it is critical to state whether they have been migrated, recruited, or generated.

Our results revealed a significant correlation between adipose tissue formation and angiogenesis induced by a distraction technique. An adjacent connective tissue network supported tissue generation, acting as a natural scaffold. The non-distraction model resulted in adipose and fibrous tissue formation in the perforated chambers, while the distraction method generated well-vascularized adipose tissue with a more balanced adipocyte-endothelial interaction. Further research utilizing larger distraction chambers with long-term observation should precede clinical applications.

V. CONCLUSION

Tissue generation using a distraction chamber was introduced to validate the mechanical and biological effects of distraction technique on adipose tissue formation and maintenance. We hypothesized that fat flaps receding gradually from each other could develop adipose tissue construct.

Distraction group demonstrated gradual expansion of adipose tissue up to 16 weeks. A transition zone was observed at 8 weeks, clarifying the initiation of tissue generation. Distraction group has achieved well-vascularized adipose tissue formation, in comparison with non-distraction group. The crosstalk between endothelial cells and adipocytes has been known to play a key role in both adipogenesis and angiogenesis. Distraction technique resulted in more balanced adipose tissue formation than non-distraction method.

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ABSTRACT (IN KOREAN)

지방 피판 신연술을 이용한 지방 조직 형성

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이명철

지방 조직 생성에 있어 지방 세포와 혈관 형성 간 상호 작용은 중요한 과정으로 여겨진다. 본 실험의 목적은 지방 조직 신연술이 지방 조직 형성 및 유지에 미치는 물리적 생물학적 효과를 확인 하는 것이다. 지방 조직 피판을 거상하여, 적절한 시간 간격과 양을 정해 조직간 간격을 점차 멀어지게 하면 지방 조직 구조물이 생성된다는 가설하에 다공성 폴리카보네이트 주사기형 신연기를 가토 모델에 적용하였다.

30 마리의 가토를 본 실험을 위해 사용하였으며 15 마리에 지방 조직 신연 모델을 적용 (실험군: 1A, 1B, 1C군), 15 마리는 비신연 모델을 적용 (대조군: 2A, 2B, 2C군) 하였다. 두개의 지방 조직 피판을 가토 경부의 배측에서 거상한 후 실험 계획에 따라 신연기에 고정하였다. 1주간 휴지기 (latency) 와 3주간 신연기 (distraction) 이후 실험군에 따라 경화기 (consolidation) 를 정하

였다. (1A군-4주, 1B군-8주, 1C군-12주) 이후 신연기 내에 생성된 조직을 채취하여, 육안 검사와 부피 측정을 하였다. 현미경 조직 검사를 위해 지방 조직 절편을 (1) 헤마톡실린 에오신 염색, (2) 마손 삼색 염색, (3) CD31 면역조직화학염색으로 처리하였다.

실험군에서 지방 조직의 부피는 16주간의 실험기간동안 점차 증가 하였다 (최종 부피 1.92 ± 0.06 ml). 통계적으로 의미있는 증가는 8주, 12주에 관찰되었다 (1A군: 1.14 ± 0.13 ml, 1B군: 1.65 ± 0.16 ml, $p < 0.05$). 현미경 조직 검사상 8주째 풍부한 지방 조직이 관찰 되었으며, 부분적으로 콜라겐과 피브린을 포함한 결합조직이 확인 되었다. 실험군 16주째 조직내 상대적인 구성 성분이 의미있는 변화를 보였다. 지방 세포 비율이 증가한 반면 결합조직 비율은 감소하여, 조직 대부분이 지방 세포 조직으로 구성 되었다. 또한, 내피세포로 구성된 혈관이 지방조직군 주변과 지방세포 사이구역에서 관찰 되었다. 혈관 개수는 16주 동안 의미있는 증가를 보였으며, 지방 조직내에 균일하게 분포 하였다.

지방 조직 신연 모델을 통해 16주 동안 지방조직의 점진적인 증가를 확인하였다. 실험군 8주째 조직 형성 개시를 증명하는 이행 구역 (transition zone)이 관찰 되었다. 신연 모델에서 비신연 모델 보다 혈행이 풍부한 지방 조직 형성을 관찰 하였다. 대조군과 비교하였을 때, 지방 조직 신연술을 통해 더 균형있는 지방 세포 및 혈관 조직 형성을 확인할 수 있었다.

핵심되는 말: 신연술, 지방 세포, 지방 조직, 혈관 형성, 혈관 조직