ORIGINAL ARTICLE

Adding collagen to adipose tissue transplant increases engraftment by promoting cell proliferation, neovascularisation and macrophage activity in a rat model

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Abstract

To clarify the effect of collagen addition to transplanted adipose tissue on angiogenesis, cell proliferation and tissue remodelling process and reveal whether collagen addition contributes to improving transplanted adipose tissue engraftment in rats. Adipose tissue was harvested from the inguinal and injected into the back of the rat, in addition to collagen. Engraftment tissue was harvested, semi-quantitatively evaluated and underwent haematoxylin and eosin or Perilipin staining. Moreover, we evaluated viable adipocyte counts and neovascularisation. Macrophages were evaluated using flow cytometry, and the adiponectin or vascular endothelial growth factor (VEGF) mRNA was detected using real-time polymerase chain reaction. By collagen addition to transplanted adipose tissue, higher engraftment rate semi-quantitatively and a greater number of new blood vessels histologically were identified. Perilipin staining revealed a higher adipocyte number. The total cell, M1 macrophage and M2 macrophage count were higher. There was increased adiponectin mRNA significantly at week 4 compared to that at week 1 after transplantation. Note that the expression levels of VEGF mRNA increased. In rats, adding collagen enhanced cell proliferation, induced M2 macrophages, which are involved in wound healing, and promoted adipocytes and neovascularisation. Therefore, collagen addition to transplanted adipose tissue could increase the engraftment rate of adipose tissue.

KEYWORDS
adipose tissue transplantation, cell proliferation, collagen addition, M1 macrophage, M2 macrophage

Key Messages

- adipose tissue transplantation is a relatively minimal invasive procedure
- however, as per the current method, the amount per transplant is limited
- the purpose of this study is finally to contribute to establishing a more effective fat transplantation method
- by collagen addition, a greater number of new blood vessels histologically were identified and M1 macrophage and M2 macrophage count were higher
- collagen addition to transplanted adipose tissue could increase the engraftment rate of adipose tissue

1 | INTRODUCTION

Adipose tissue transplantation is a relatively minimal invasive procedure; because it can be performed relatively easily and does not require advanced techniques such as for vascular anastomosis, it is widely performed in various settings such as in reconstruction after craniofacial surgery and following mastectomy.[1-6] However, as per the current method, the amount per transplant is limited; because there is no blood flow in transplanted adipose tissue, the tissue might become ischemic. If this happens, the majority of cases develop necrosis, thus making graft survival unstable and making multiple transplants necessary in multiple cases.[2,7-9] Many studies have indicated that there is considerable discrepancy with regard to the related effects and complications of adipose tissue transplantation, which vary as per the level of skill of the surgeon. Thus, the current method still has room for improvement.[8,10] Transplanted adipose tissue is engrafted through the penetration of capillaries from surrounding tissue. However, this process requires time, and when blood flow cannot be maintained, it becomes fat necrosis and scar tissue.[2] Therefore, to stabilise and achieve engraftment of adipose tissue transplants, the reperfusion of blood flow to the adipose tissue needs to be achieved more quickly. Furthermore, the tolerance to ischemia of the actual transplanted adipose tissue needs to be improved. Previously, we reported that adding lipid fraction obtained from adipose tissue to artificial dermis induces early neovascularisation in the artificial dermis.[11] When the artificial dermis contains collagen, this collagen induces neovascularisation. Therefore, based on this empirical data, it is possible that while the mechanism is unclear, the interaction between collagen and adipocytes strongly enhances neovascularisation. The purpose of this study is to clarify the effect of collagen addition to transplanted adipose tissue on angiogenesis, cell proliferation and tissue remodelling process, as well as to reveal whether the addition of collagen contributes to the improvement of transplanted adipose tissue in rats and finally to contribute to establishing a more effective fat transplantation method.

2 | METHODS

2.1 | Animals

Approval was obtained from the animal care and use committee of the Tokyo Medical University Animal Experiment Ethics Committee (Approval Number: H31-0056) for the protocol of all animal experiments, and all experiments were conducted in accordance with relevant guidelines. Female Sprague–Dawley (SD) rats (Clea Japan, Inc.) of 15-20 weeks of age were utilised. The rats were maintained in a breeding facility at a room temperature of 23°C, with 24-hour air conditioning, and a cycle of 12 hours light and 12 hours dark. Stainless steel cages that enabled individual feeding were utilised because each animal was maintained in a separate cage after injection.

2.2 | Harvesting adipose tissue and modification of adipose specimens for transplantation

The rats were anaesthetised by isoflurane inhalation with an intra-abdominal injection of a combination of three anaesthetic agents, including medetomidine at 0.75 mg/kg, midazolam at 4.0 mg/kg and butorphanol at 5.0 mg/kg. Note that adipose tissue harvested from the inguinal region of the rats was utilised for autologous tissue transplantation. Harvested fat was thinly sliced using surgical scissors; after washing with physiological saline, centrifugal separation was performed (1800 rpm for 3 minutes at 12°C). As the adipose specimen for transplant, we harvested the portion after removing the oil component in the supernatant and the fluid in the underlay, which was defined as the control group (Figure 1). Bovine collagen suspension adjusted to a collagen concentration of 0.25% (Olympus Terumo Biomaterials) was added to the harvested adipose specimen for transplantation and utilised as the collagen group. Collagen derived from the dermis of young Australian cows

FIGURE 1 Adipose tissue for transplantation. Adipose tissue is harvested from the inguinal region. Harvested adipose tissue was thinly sliced using surgical scissors; after washing with physiological saline, centrifugal separation was performed (1800 rpm for 3 minutes at 12°C). As the adipose specimen for transplant, we harvested the portion after removing the oil component in the supernatant and the fluid in the underlay
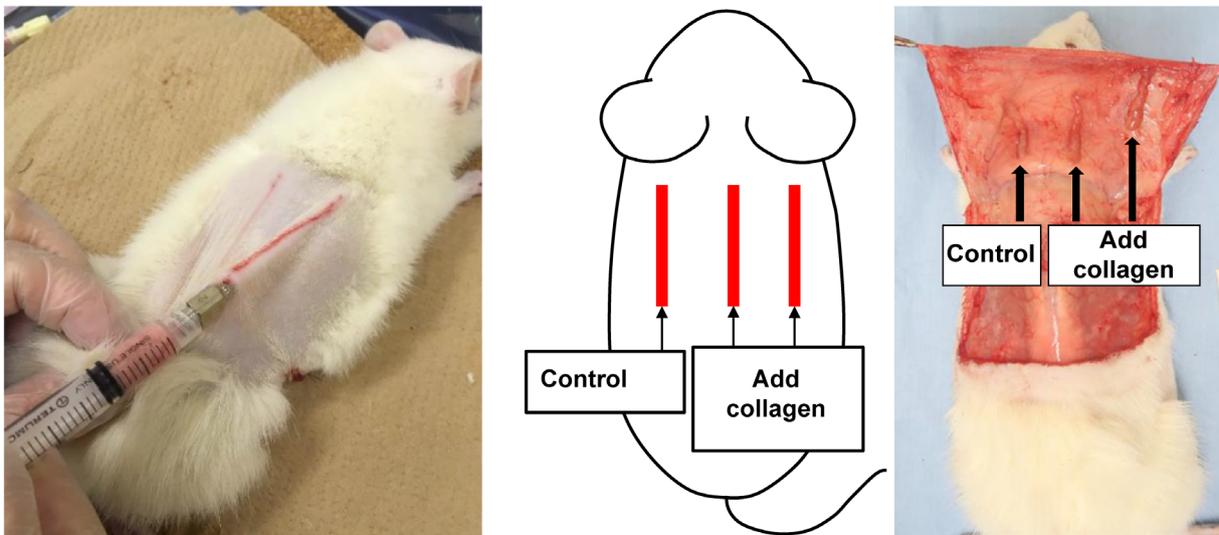
FIGURE 2 Transplantation of the adipose specimen. The adipose specimen for transplantation was injected beneath the fibrous membrane on the back of the same individual rat, in addition to an adipose specimen for transplantation containing collagen using an 18 G cannula to infuse 0.1 cc/1 cm

was treated with protease, the telopeptide part was digested and cleaved and atelocollagen, which has almost no antigenicity, was utilised as a raw material. When injecting 0.5 mL of fat, the collected fat sample was placed for transplantation in a 2.5 mL syringe. The 0.25% collagen turbid solution was taken with tweezers, added to the fat of the syringe and mixed well with tweezers.

2.3 | Tissue transplant

The adipose specimen for transplantation was then injected beneath the fibrous membrane on the back of the same individual rat, in addition to an adipose specimen for transplantation containing collagenusing an 18 G cannula to infuse 0.1 cc/1 cm (Figure 2).

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2.4 | Immunohistochemistry

Perilipin (GP29, PROGEN, Heidelberg) was adopted for immunohistochemical analyses. Heat-induced epitope retrieval with antigen retrieval solution (pH 9, Nichirei Biosciences, Tokyo) was performed. Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxidase and 0.1% sodium azide containing 0.01 M phosphate-buffered saline (PBS), and a Histofine® simple staining system (Nichirei Biosciences) was utilised for secondary detection. The final product was visualised using 3,3′-diaminobenzidine.

2.5 | Isolation of mononuclear cells infiltrating to implanted fat tissue

Implanted fat tissue was removed, washed with cold PBS, sliced with scissors into small pieces and digested in PBS containing 1 mg/mL collagenase (Sigma-Aldrich) with gentle shaking at 37°C for 30 minutes. After filtering through a 100-μm-sized nylon cell strainer, the single cells were collected and centrifuged, and then erythrocytes were lysed in the ammonium-chloride-potassium lysing buffer. After centrifugation, the cells were collected for flow cytometry analysis.

2.6 | Flow cytometry

Cells were washed and incubated for 20 minutes at 4°C with staining buffer (2% FCS in PBS containing 0.02% NaN$_3$). Subsequently, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD163 (ED2, Bio-Rad), phycoerythrin (PE)-conjugated anti-CD86 (24F, BioLegend) and pacific blue-conjugated anti-CD45 (OX-1, BioLegend) or PE-conjugated anti-CD3 (1F4, BioLegend) and pacific blue-conjugated anti-CD45. Then, the cells were fixed and permeabilised with a fixation/permeabilisation solution kit (BD Biosciences) for 1 hour at 4°C in the dark, stained intracellularly with Alexa Fluor 647-conjugated anti-CD68 (ED1, Bio-Rad) and analysed on a FACSCanto II Flow Cytometer (BD Biosciences), followed by analysis with FlowJo (Tree Star, Inc.; OR, US).

2.7 | Quantitative real-time polymerase chain reaction

Total RNA of fat grafts was extracted as previously described.[12] High-capacity RNA to cDNA kit (Thermo Fisher Scientific, Waltham, MA) was utilised for reverse transcriptase reactions. The expression levels of adiponectin and VEGF were determined using quantitative real-time polymerase chain reaction (PCR) using ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). The expression level of the housekeeping gene *GAPDH* was utilised as an internal control. Primers and probes utilised for target genes were TaqMan Gene Expression Assays (Rn01775763_g1 for GAPDH; Rn00595250_m1 for adiponectin; Rn01511602_m1 for VEGF) purchased from Thermo Fisher Scientific (Waltham, MA).

2.8 | Statistics

Differences between multiple groups were analysed using the Mann–Whitney *U*-test. Statistical significance was set as $P < .05$ and analysed using StatMate V (ATMS Co., Ltd., Tokyo, Japan).

3 | RESULTS

3.1 | Semiquantitative evaluation of the engraftment rate of the transplanted adipose tissue

At 1 week and at 4 weeks after lipoinjection, the engraftment rate of the transplanted adipose tissue expressed by engrafted tissue length/transplanted tissue length was higher in the group with added collagen (control group vs collagen group: 1 week $[0.27 \pm 0.35$ vs $0.52 \pm 0.37]$, 4 weeks $[0.27 \pm 0.41$ vs $0.60 \pm 0.39])$ (Table 1).
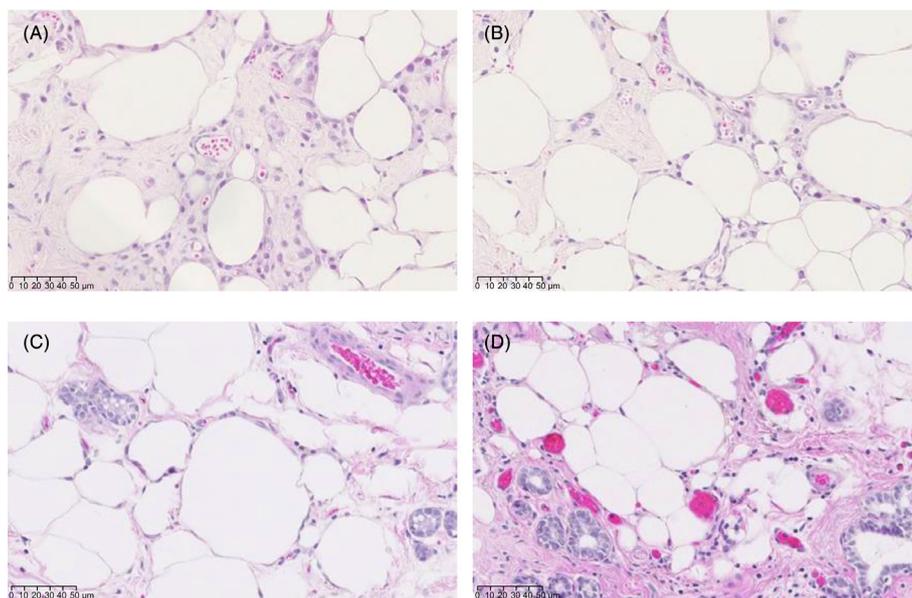
3.2 | Histological evaluation

As histological evaluations after adipose tissue transplantation, we performed haematoxylin and eosin (HE) staining

TABLE 1 Engraftment rate of the transplanted adipose tissue

	Control	Collagen
1 wk	0.27 ± 0.35	0.52 ± 0.37
4 wk	0.27 ± 0.41	0.60 ± 0.39

Note: At 1 wk and at 4 wk after lipoinjection, the engraftment rate of the transplanted adipose tissue expressed by engrafted tissue length/transplanted tissue length. The result was expressed as the engrafted rate ± SD.

FIGURE 3 Histological evaluations of the engraftment tissue. Haematoxylin and eosin staining were performed for (A) the control group at 1 week, (B) the collagen group at 1 week, (C) the control group at 4 weeks and (D) the collagen group at 4 weeks. The number of new blood vessels in the engrafted tissue was then evaluated
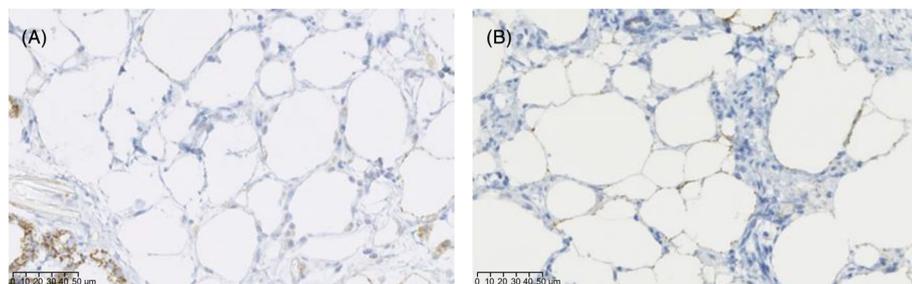
FIGURE 4 Immunohistochemistry of the engraftment tissue. Perilipin staining was performed for (A) control group and (B) collagen group. Perilipin-positive cells were then counted as viable adipocytes

and Perilipin staining. By evaluating the number of new blood vessels in the engrafted tissue on HE staining at 1 week and at 4 weeks, a greater number of new blood vessels was observed in the collagen group (control group vs collagen group: 1 week [3.33 ± 0.58/visual field vs 14.33 ± 0.58/visual field] and 4 weeks [3.33 ± 1.15/visual field vs 23.33 ± 4.93/visual field]) (n = 3, $P < .05$) (Figure 3). Furthermore, on Perilipin staining of viable adipocytes, additional Perilipin-positive cells were reported in the collagen group (Figure 4). These results suggested that in the group with collagen, greater angiogenesis and viable adipocytes were induced.

3.3 | Changes in cell count and macrophage count caused by adding collagen

Sliced control group and collagen group adipose tissue for transplantation were both injected into the back of an individual rat, and then engrafted tissue in the animal's back was extracted 1 week later (n = 12). In the extracted tissue, the total cell count, M1 macrophage count and M2 macrophage count were analysed using flow cytometry by fluorescence-activated cell sorting. Assuming the control group was 1.0, the collagen group had a significantly higher total cell count of 1.89 ($P < .05$), M1 macrophage count of 1.69 ($P < .05$) and M2 macrophage count of 2.19 ($P < .05$). These results suggest that collagen addition induces additional M1 and M2 macrophages (Figure 5).

3.4 | Gene expression of adiponectin and VEGF in grafted adipose tissue

To investigate whether collagen treatment improves the survival of transplanted adipose tissue, the expression of adiponectin mRNA, as a marker for functional adipocytes, was compared (Figure 6A). At 1 week after transplantation, there was no significant difference between control- and collagen-treated grafts. The expression of adiponectin mRNA was similar at 1 week and 4 weeks after transplantation in control conditions. However, collagen treatment significantly increased adiponectin mRNA at 4 weeks compared to that at 1 weeks after transplantation ($P < .05$). To evaluate the effects of
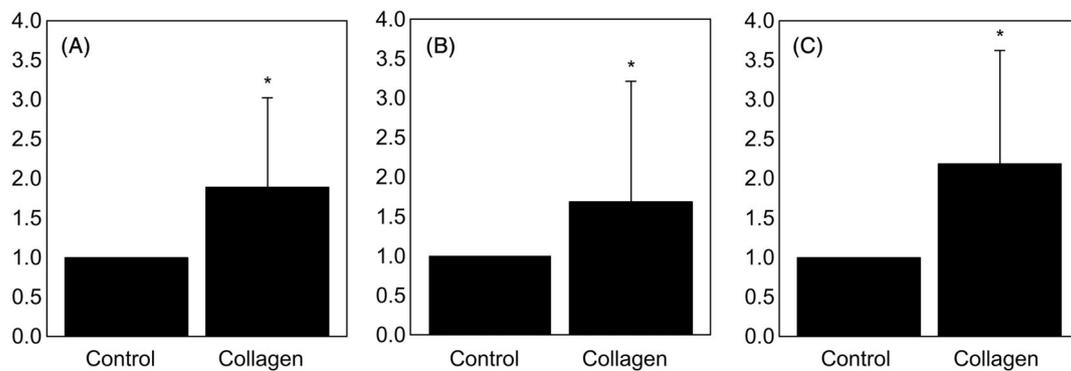
FIGURE 5 Cell proliferation and macrophage activity in the engraftment tissue. Engrafted tissues of control group and collagen group were evaluated (A) total cell number, (B) M1 macrophage number and (C) M2 macrophage number by flow cytometry (n = 12, *$P < .05$)
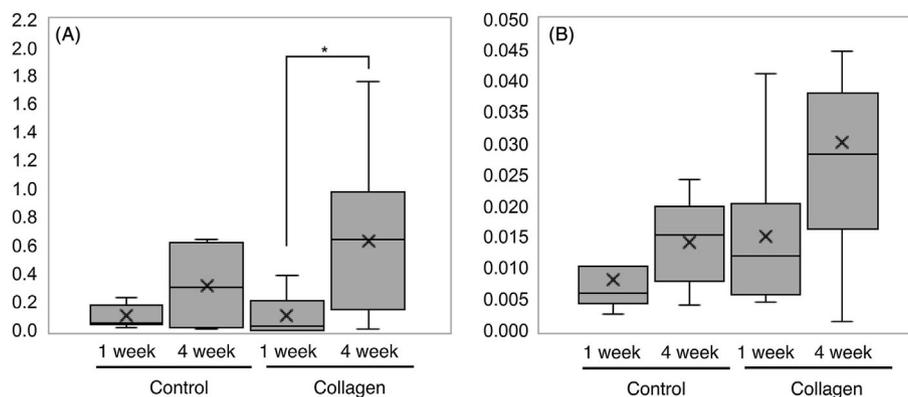
FIGURE 6 Gene expression in the engraftment tissue. Engrafted tissues of control group and collagen group were evaluated adiponectin mRNA and vascular endothelial growth factor (VEGF) mRNA expression by quantitative real-time polymerase chain reaction. (A) Adiponectin (control group 1 week: 0.11 ± 0.087 [n = 7], 4 weeks: 0.32 ± 0.30 [n = 5]; collagen group 1 weeks: 0.10 ± 0.14 [n = 8], 4 weeks: 0.63 ± 0.55 [n = 10]); (B) VEGF (control group 1 week: 0.0081 ± 0.0059 [n = 7], 4 weeks: 0.014 ± 0.0073 [n = 5]; collagen group 1 week: 0.015 ± 0.012 [n = 8], 4 weeks: 0.030 ± 0.023 [n = 10]) (*$P < .05$)

collagen on angiogenesis in grafts, the expression levels of VEGF mRNA were investigated (Figure 6B). There was no significant difference between control and collagen-treated grafts, although the expression levels of VEGF mRNA seemed to be increased by collagen treatment at 4 weeks after transplantation. These results suggested that collagen treatment improves the functional adipocytes in the grafts at 4 weeks after transplantation, possibly via angiogenesis.

4 | DISCUSSION

Fat transplantation is extensively employed as a postoperative reconstruction method such as craniofacial surgery and mastectomy; however, most of the transplanted adipose tissue is necrotic, engraftment is unstable and developing more stable fat transplantation method is awaited. The engraftment instability of adipose tissue transplantation

arises when the adipose tissue for transplantation is placed in an ischemic environment, and the transplanted adipose tissue developed necrosis.[2,7-9,13] To improve adipose tissue engraftment, we believe that it is important to reduce the cell count with necrosis arising from such ischemic environment, that is, neovascularisation and cell proliferation within the transplanted adipose tissue are required.[14-16] This study's results suggest that in rat adipose tissue, the addition of collagen to the transplanted adipose tissue increases angiogenesis and improves the fat engraftment rate via the number of adipocytes and the proliferation of macrophages. In this study, adipose tissue transplanted into rats histologically promoted angiogenesis by adding collagen and therefore improving the engraftment rate. The tissue remodelling mechanism appeared to be involved in the increase in engraftment rate. Histological evaluation after fat transplantation revealed the production of macrophages around adipocytes when HE staining, Perilipin staining and CD31 staining colour were performed. As similar

findings were observed in many samples, additional experiments were conducted on the type of macrophages produced. After transplantation, the adipose tissue is placed in an ischemic environment, which causes tissue necrosis and tissue remodelling.[16] Macrophage migration is strongly involved in the process of such remodelling.[17,18] Macrophages are broadly divided into M1 and M2 macrophages. M1 macrophages induce the inflammatory reaction required for tissue engraftment, and they are involved in the scavenging of necrotic adipose tissue, whereas M2 macrophages are involved in tissue repair, and both M1 and M2 macrophages carry out an important role in the process of adipose tissue remodelling and engraftment.[18-23] However, the mechanism and the appropriate amount of load have not yet been elucidated. If an increase in M2 macrophages is associated with improved liveability after adipose tissue transplantation, M2 macrophage induction is likely to increase fat growth.[18]

Furthermore, macrophages improve the survival of adipose tissue grafts by inducing neovascularisation and activated stem cells.[18,24] In this study, we reported that adding collagen to adipose tissue transplants significantly increased M1 and M2 macrophages in the transplanted tissue. In particular, M2 macrophages considerably increased, and we believe that this result reflects a state whereby adding collagen to adipose tissue for transplantation induces the inflammatory reaction required for tissue remodelling and induces a potent tissue repair action.[19,24,25]

In this study, no significant difference was observed in the expression level of vascular endothelial growth factor (VEGF) mRNA, which is a factor that promotes neovascularisation at 1 week and 4 weeks after transplantation; moreover, the level tended to be higher in the collagen group. This result might reflect the fact that adding collagen serves as a platform for neovascularisation and helps give rise to abundant neovascularisation in the transplanted tissue. It is possible that the ligation and clustering of integrin receptors α1β1/α2 β1 on the surface of endothelial cells (EC) via the GFPGER (502-507) sequence of the collagen fibres is important for neovascularisation activity.[26] While there is still room for debate, to summarise the results of the histological evaluation, we can draw the conclusion that collagen addition to transplanted adipose tissue produced additional neovascularisation.[25-27] Recently, attention has been drawn to the effectiveness of adipose-derived stem cells (ASC) as a means to promote neovascularisation in transplanted adipose tissue. ASC induce differentiation to adipocytes by co-culturing with mature adipocytes,[28] and they contribute to neovascularisation by differentiating into vascular EC.[14,29-32] Furthermore, it has been reported that hypoxia results in the ASC secretion of neovascularisation growth factors such as VEGF and

therefore, induces neovascularisation in an ischemic environment after transplantation.[33] ASC presents in subcutaneous adipose tissue can differentiate into adipocytes[34]; it has been reported that when ASC are cultured from collagen, more lipids are produced, which is a marker of adipose differentiation.[35] Therefore, adding collagen could support ASC differentiation into adipocytes. In this study, on comparing the level of adiponectin mRNA expression at 1 and 4 weeks after transplantation, the expression level tended to be higher, although not significantly higher, in the control group. In the collagen group, a significant increase in the level of adiponectin mRNA expression was observed at 4 weeks. Furthermore, while there was no significant difference observed, at 4 weeks, the level of adiponectin mRNA expression tended to be higher in the collagen group than in the control group. Adiponectin is an adipocytokine produced particularly in adipose tissue and that produces and secretes various bioactive factors.[36] In humans, it is the gene with the most abundant expression in adipose tissue.[37] This study's results show that when collagen was added; there was a greater viable adipocyte count at 4 weeks after transplantation, which supports the higher engraftment rate in the collagen group indicated by semiquantitative evaluation. Moreover, in the collagen group, there was a greater change over time from 1 to 4 weeks after transplantation. This suggests that adding collagen leads to greater viable adipocyte proliferation. Our results suggested that adding collagen increases the adipocyte count and increases neovascularisation in the transplanted adipose tissue. It is possible that a greater effect can be anticipated by combining ASC with collagen, and therefore, this needs to be studied further in future.

In conclusion, on adding collagen to the transplanted adipose tissue, we report that the level of adiponectin mRNA increased, and the histological viable adipocyte count increased in the rats. Furthermore, the level of VEGF mRNA tended to increase, and histologically, an increase in neovascularisation was observed, which suggested that adding collagen might contribute to increased neovascularisation in transplanted adipose tissue in the rats. Moreover, we reported that adding collagen increased M1 and M2 macrophages in transplanted adipose tissue in the rats and, therefore, might contribute to tissue remodelling. It was suggested that adding collagen to transplanted tissue increases the engraftment rate of transplanted adipose tissue by increasing the viable adipocyte count, promoting neovascularisation and inducing macrophages.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Chika Suzuki designed the research, conducted experiments and wrote the manuscript. Hana Inoue contributed to perform the quantitative real-time PCR. Takayuki Yoshimoto contributed to perform flow cytometry. Takako Komiya has made substantial contribution to study design, interpretation of data and made critical comments on the study. Hajime Matsumura organised this research as project managers. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Approval was obtained from the animal care and use committee of Tokyo Medical University (Approval Number - H31-0056) for the protocol of all animal experiments, and all experiments were conducted in accordance with relevant guidelines.

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