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Early activation of macrophage-2 with IL-4 in stromal vascular fraction increases VEGF levels and adipocyte count and maintains volume of fat graft in Wistar rats (*Rattus norvegicus*)

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Abstract

Several previous studies have demonstrated the benefits of early macrophage 2 activation fat grafts supplemented with macrophage culture. However, this approach is considered impractical in clinical settings because of intraperitoneal induction use. The aim of this study was to investigate the effect of early stromal vascular fraction (SVF) macrophage-2 activation with IL-4 on fat graft survival compared to SVF alone using an animal model for better fat graft viability. This experimental study included inguinal fat harvesting, isolated with collagenases to retrieve the SVF, and then injected with a combination of fat graft and SVF (0.3 mL) into the scalp region. The intervention group received an IL-4 intralesional injection on the third day, and the fat grafts were biopsied on days 7, 14, and 30. The primary outcomes were the final volume of the fat graft, vascular endothelial growth factor (VEGF) expression, and the adipocyte cell count using perilipin staining on immunohistochemistry examination. The group receiving IL-4 exhibited significantly higher VEGF on days 7, 14, and 30 (p=0.009, 0.009, and 0.021, respectively). Similarly, the IL-4 treatment significantly increased the perilipin concentration on days 7, 14, and 30 (p=0.008, 0.008, and 0.029, respectively). In this group, VEGF concentration was significantly increased on day 14 as compared to day 7 (p=0.009), while no significant difference was observed in the control group (p=0.090). Additionally, the IL-4 group displayed significantly less reduction of fat graft volume than the control group, as observed on days 7, 14, and 30 (p=0.009, 0.009, and 0.021, respectively). Overall, the study underscores the potential benefits of early M2 polarization in fat grafting, as well as providing practical advantages for improving fat graft volume retention.

Keywords: Fat graft, adipocytes, macrophages, regeneration, interleukin-4

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Introduction

Autologous fat grafting is widely used in reconstructive surgery. However, its high resorption rate, ranging from 20–90%, makes it difficult to predict final volume [1,2]. Adipose stem cells (ASCs) are prone to death in the regenerative zone within the first 72 hours after the fat graft is transferred [1]. To address this problem, researchers have explored various strategies, including macrophage polarization into the anti-inflammatory macrophage-2 (M2) phenotype induced by

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interleukin-4 (IL-4) [3,4]. Unlike pro-inflammatory macrophage-1 (M1), which initiates inflammation and cell death, M2 promotes tissue growth and suppresses inflammation, which is crucial for balanced wound healing [5]. The wound healing process relies on a balance between M1 and M2, and their proportions can be regulated to increase fat survival without compromising macrophage homeostasis [6].

Early M2 polarization has proven to be effective in promoting angiogenesis and preserving fat graft volume [7]. Previous studies have supported the hypothesis that M2 infiltration within the stromal vascular fraction (SVF) increases long-term fat graft survival by supporting neovascularization and adipocyte maintenance [8,9]. However, previous studies did not conduct M2 polarization in vivo, instead using cultured M2 to the fat graft that was received by intraperitoneal Brewer's thioglycollate injection and in vitro culture, which is impractical in human clinical settings [4,10,11]. However, there are studies focused on M2 polarization that implied the effectiveness without the need for precultured M2 [7,12].

While numerous modifications in fat grafting stages and components, such as the administration of growth factors, platelet-rich plasma, and the addition of adipose stem cell cultures, have shown statistical significance in enhancing cell regeneration and reducing necrosis, the understanding of macrophage dynamics remains limited [13-18]. Limited studies using IL-4 to activate the macrophage showed an increase in CD34+ and the expression of the adipogenic proteins PPAR-γ, C/EBP and AP2 [4]. This study innovatively focuses on activating M2 within SVF using IL-4 on the third day to enhance angiogenesis and early adipogenesis, which are critical for fat graft viability. The angiogenesis is assessed by measuring the vascular endothelial growth factor (VEGF) level, a key angiogenic factor in the revascularization process [19]. The aim of this study was to address this gap by investigating early IL-4-induced macrophage activation in SVF, potentially offering insights into optimizing fat graft outcomes through improved tissue integration and volume retention.

Methods

Animals and fat grafting model

The Faculty of Medicine Udayana University Ethics Committee approved this study. This research was conducted in the Integrated Laboratory Unit, Sub Animal Laboratory Unit, Department of Pharmacology and Therapeutics, Faculty of Medicine, Udayana University. The sample size was estimated using unpaired comparative numerical analysis [20]. Twenty-eight healthy female Wistar rats (*Rattus norvegicus*) weighed 250–300 grams each were used in this study. The rats were housed under standard laboratory conditions with a 12:12 hour light-dark cycle. Each rat was placed in a cage measuring 50 cm (length) × 40 cm (width) × 20 cm (height). The humidity of the cage was maintained at 50–70%, and the temperature was kept between 25–30°C, which was monitored with a thermometer. Tap water and high-protein pelletized food were provided as needed.

Experimental design

This research employed a true experimental design using an animal model. Twenty-eight female rats were randomly divided into two groups, which were labeled as control and IL-4 groups by non-blinding simple random sampling. The control group (n=14) underwent fat grafting and SVF isolation, where the fat graft and SVF were injected into the scalp region. As for the IL-4 group (n=14), the same grafting procedure was applied but with the addition of intralesional IL-4 injection on day 3. Each group of rats was divided into three sample collection days: on days 7, 14, and 30. The rats were maintained until the day the biopsy was performed and were terminated immediately after the biopsy was completed. All animals showed neither significant weight reduction nor sudden aggressive behavior.

Inguinal fat pad collection

To collect inguinal fat, all rats underwent intramuscular anesthesia using ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg), following the recommendation from a previous study [21] The experimental animals were placed in a supine position, where the

bilateral inguinal region was shaved and disinfected. Additionally, the inter-ear and nape areas were shaved. A skin incision was made 1 cm oblique to the midline of the pubic-sternum, parallel to the inguinal ligament. After skin excision, a skin flap was created using blunt dissection with Metzenbaum scissors, preserving the inguinal veins until the adipofascial inguinal fat pad was reached (**Figure 1A**). Approximately 1 gram of fat was harvested and transferred directly to a 4°C wash buffer. The donor site was then closed with primary closure.

SVF isolation

SVF isolation was performed according to the Star Protocols [22]. The collected inguinal fat pad samples were thoroughly washed in a 50 mL Falcon tube containing 20 mL of wash buffer to remove the residual blood. The adipose tissue was minced using sterile forceps and scissors. Using a liquid overflow method, 0.25 mL of the tissue was set aside for the fat graft. The remaining sample was digested with 200 mL of Collagenase II (2844 U, Product number: C6885-500MG, Sigma Aldrich, St Louis, MO, USA) and 2.5 mL of DNase (27.75 U, Product number: D4527-10KU, Sigma Aldrich, St Louis, MO, USA) per mL of wash buffer. The samples were placed in a shaker-incubator at 37°C and 225 rpm for 25–30 minutes until homogeneous and pinkish white in color. The solution was then transferred to a Falcon tube containing 20 mL of cold wash buffer and centrifuged at 800× g for 10 minutes at 4°C. The supernatant was discarded, leaving a pellet that contained the stromal vascular fraction cells. This pellet was mixed with the reserved fat graft until a final volume of 0.3 mL was reached.

Fat injection and macrophage polarization

A small incision was made in the head region and the middle of the ear, followed by the insertion of a 14-G blunt cannula. A supragaleal dissection was performed in a fan-shaped pattern to create a pocket for the fat graft. Subsequently, 0.3 mL of the prepared mixture of fat graft and SVF was injected into each rostral region of the rat's head using a 1 cc syringe and a 14-G abocath (**Figure 1B**). The wound was then closed with 6–0 nylon sutures and treated with gentamicin eye ointment. On day 3, rats in the IL-4 group were injected with IL-4 (Product number; I 1020-5UG, Sigma Aldrich, St Louis, MO, USA) intralesionally into the fat graft in the head region at a dose of 2 ng (5 µg diluted in 50 mL of double-distilled water, 2 mL taken).

Figure 1. Harvesting inguinal fat pad (A) and injecting fat + stromal vascular fraction (SVF) to the scalp (B).

Fat harvesting

On day 7, five rats from each group were sampled and terminated. An incision was made in the midline between the two eyes for 1 cm long using tissue scissors, a dissection was carried out in the scalp pocket, and the fat graft was taken. Fat graft biopsy volume was measured using the liquid overflow method. On day 14, another 5 rats from each group underwent graft sampling and terminated. On day 30, the remaining 4 rats from each group were harvested and terminated. Each group of mice was anesthetized intracardially with ketamine until they died. Each sampled tissue was photographed, and its volume was measured using the liquid overflow method. The tissue was halved, with one portion used for biopsy and the other for VEGF analysis via enzyme-linked immunosorbent assay (ELISA).

Perilipin examination

The tissue was prepared by using 10% phosphate-buffered formalin fixation for 24 hours and was dehydrated through a graded alcohol series (30%, 40%, 50%, 70%, 80%, and 96%) for three cycles of 25 minutes each. The tissue was then cleared in xylene for three cycles of 1 hour until it became transparent and was infiltrated in pure paraffin three times, each for 1 hour. Subsequently, it was solidified into blocks for slicing. Tissue sections of 5 µm sections were cut using a microtome, mounted on Poly-Lysine-coated slides, and incubated at 60°C for 2 hours. For Perilipin staining (Dako EnVision®+ Dual Link System-HRP (DAB+)), the slides were deparaffinized, rehydrated, and subjected to antigen retrieval in a citrate buffer with microwave heating. Endogenous peroxidase was blocked, followed by the application of 5% FBS before incubating with primary antibody overnight. After washing, labeled polymer-HRP and DAB substrate for color development was applied, followed by Hematoxylin Meyer counterstaining, dehydration, and mounting. Finally, the slides were imaged with an Olympus CX41 microscope, capturing images at 400× magnification, and Perilipin expression was quantified by counting the brown-stained cells.

VEGF examination

On the harvesting day, the fat graft was collected from the scalp. The specimen was placed in a 1.5 mL microtube with phosphate-buffered saline (PBS) and stored at -20°C. VEGF levels were measured using the Rat VEGF ELISA kit (BT Laboratory, Shanghai, China), and the result was expressed in ng/L. The tissue was rinsed with cold PBS (pH 7.4) to remove blood residues, minced, and homogenized in PBS at a ratio of 1:9 (tissue weight in grams to PBS volume in mL). The homogenate was centrifuged at 12,000 RPM for 15 minutes at 4°C, and the supernatant was used for ELISA analysis. Reagents were prepared according to ELISA kit protocol. Standard wells were filled with 50 µL of standard solution and sample wells were filled with 40 µL of supernatant, followed by the addition of 10 µL of anti-VEGF antibody and 50 µL of streptavidin-HRP to each well. The plate was incubated with a plastic sealer for 60 minutes at 37°C. After incubation, the plate was washed five times with wash buffer, using 0.35 mL buffer per well for each wash in 30 seconds and inverted to remove excess liquid. Substrate solution A was added, followed by substrate solution B to each well, and the plate was incubated in the dark for 10 minutes at 37°C. Finally, 50 µL of stop solution was added to each well, and VEGF levels were measured by reading optical density at 450 nm using a microplate reader.

Statistical analysis

For the analysis of quantitative data, the SPSS Version 25.0 (SPSS Inc., Chicago, IL, USA) software program was used to test the predetermined hypotheses. A statistical significance level of 0.05 was established along with 95% confidence interval (CI) for this study. The normality of the data was evaluated using the Shapiro-Wilk test. Comparative analysis was conducted using an unpaired t-test for normally distributed data, while the Mann-Whitney test was used for data with a non-normal distribution.

Vania et al. Narra J 2024; 4 (3): e1080 - http://doi.org/10.52225/narra.v4i3.1080

Results

Macroscopic observation

Until the end of observation, none of the subjects experienced significant complications such as abscesses, necrosis, infection, hematoma, or seroma in the scalp area. Photographed images of fat grafts resulted in this study are presented in **Figure 2**. In the treatment group, the tissue appeared larger, more reddish in color, and had a softer consistency compared to the control group. In contrast, the fat in the control group was smaller, the tissue was pale, and had a firmer consistency. On day 7, the biopsied tissue appeared more gel-like compared to days 14 and 30. On day 30, the biopsy showed a greater size reduction in the control group compared to the treatment group.

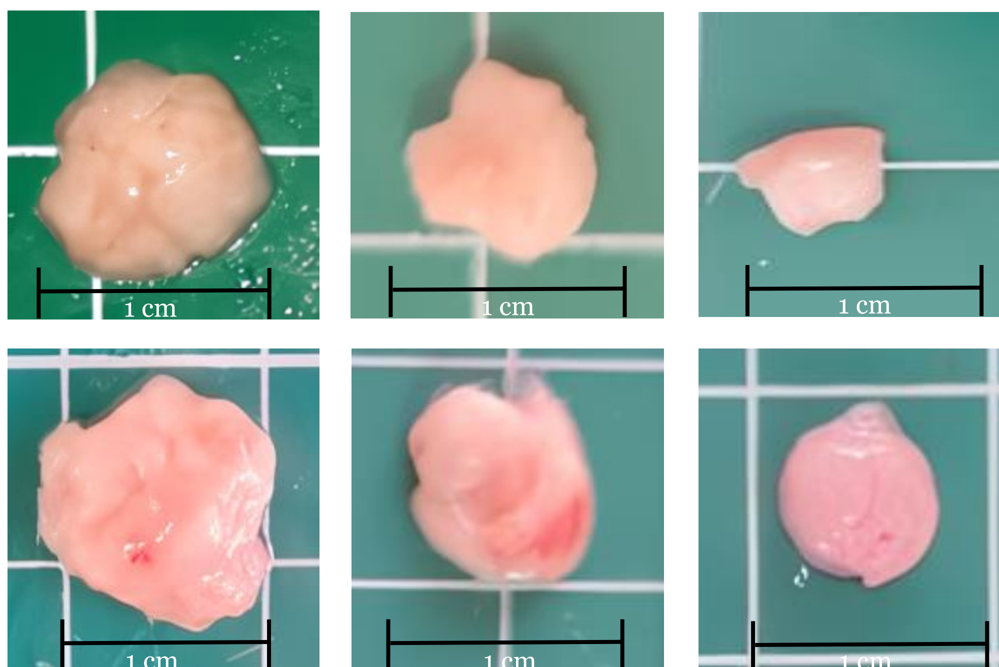

Figure 2. Harvested fat on the control group on days 7 (A), 14 (B), and 30 (C), and on the intervention group on days 7 (D), 14 (E), and 30 (F).

VEGF level

VEGF levels measured in both control and IL-4 groups are presented in **Table 1**. There was a significant increase in VEGF levels on days 7, 14, and 30 in both control (p=0.003) and intervention (p=0.006) groups. Significant differences in VEGF levels between the groups were observed on days 7, 14, and 30 (p=0.009, 0.009, and 0.021, respectively). In the control group, VEGF levels increased significantly on day 14 compared to day 7 (p=0.009). However, in the intervention group, the level did not change significantly from day 7 to day 14 (p=0.090). Additionally, VEGF levels in the control group increased significantly on day 30 compared to day 14 (p=0.014), while no significant change was observed in the IL-4 group (p=0.086). The trend for VEGF levels is presented in **Figure 3**.

Adipocyte count

Perilipin-positive cell counts in control and IL-4 groups that were observed on days 7, 14, and 30 are presented in **Table 1**. There was a significant increase in perilipin-positive cell counts on days 7, 14, and 30 in both the control (p=0.003) and intervention (p=0.003) groups. The mean perilipin results indicated differences in the number of perilipin-positive cells per visual field between the control and treatment groups on days 7, 14, and 30 (p=0.009, 0.009 and 0.019, respectively).

Vania et al. Narra J 2024; 4 (3): e1080 - http://doi.org/10.52225/narra.v4i3.1080

Table 1. Effects of M2 activation using IL-4 on VEGF, perilipin, and fat graft volume

Variable	Control			IL-4			Between groups
	Median/mean difference (min-max)	Day 7 vs 14	Day 14 vs 30	Median (min-max)	Day 7 vs 14	Day 14 vs 30	
		p-value [a]	p-value [a]		p-value [a]	p-value [a]	p-value [a]
Vascular endothelial growth factor (VEGF) (ng/mL) (n=14)							
Day 7 (n=5)	51 (44–57)	0.009*		217 (193–233)	0.090		0.009*
Day 14 (n=5)	226 (211–242)		0.014*	287 (257–345)		0.086	0.009*
Day 30 (n=4)	278 (263–297)			318 (306–347)			0.021*
Perilipin-positive cell count cells/visual field (n=14)							
Day 7 (n=5)	15.67 (12.33–16.33)	0.009*		35.33 (30.33–42.67)	0.009*		0.008*
Day 14 (n=5)	19.33 (17.33–22.33)		0.014*	51.67 (46.67–61.67)		0.019*	0.008*
Day 30 (n=4)	26.00 (25.67–26.33)			68.67 (61.67–73.67)			0.029*
Preoperative volume (mL) (n=14)							
Day 7 (n=5)	0.30 (0.29–0.30)	n/a	n/a	0.30 (0.30–0.30)	n/a	n/a	0.317
Day 14 (n=5)	0.30 (0.29–0.30)			0.30 (0.29–0.30)			1
Day 30 (n=4)	0.30 (0.30–0.30)			0.30 (0.30–0.30)			1
Postoperative volume (mL) (n=14)							
Day 7 (n=5)	0.134 (0.112–0.182)	n/a	n/a	0.284 (0.271–0.287)	n/a	n/a	0.009*
Day 14 (n=5)	0.102 (0.092–0.125)			0.262 (0.255–0.279)			0.009*
Day 30 (n=4)	0.083 (0.06–0.092)			0.254 (0.24–0.261)			0.021*
Volume differences (mL) (n=14)							
Day 7 (n=5)	0.156 (0.12–1.19)#	0.028*		0.016 (0.01–0.03)#	0.036*		0.009*
Day 14 (n=5)	0.193 (0.18–0.21)#		0.019*	0.038 (0.01–0.05)#		0.065	0.009*
Day 30 (n=4)	0.218 (0.21–0.24)#			0.045 (0.04–0.06)#			0.021*

[a] The data were analyzed using the Mann-Whitney test
#Presented as mean difference (95%CI)
*Statistically significant at $p<0.05$

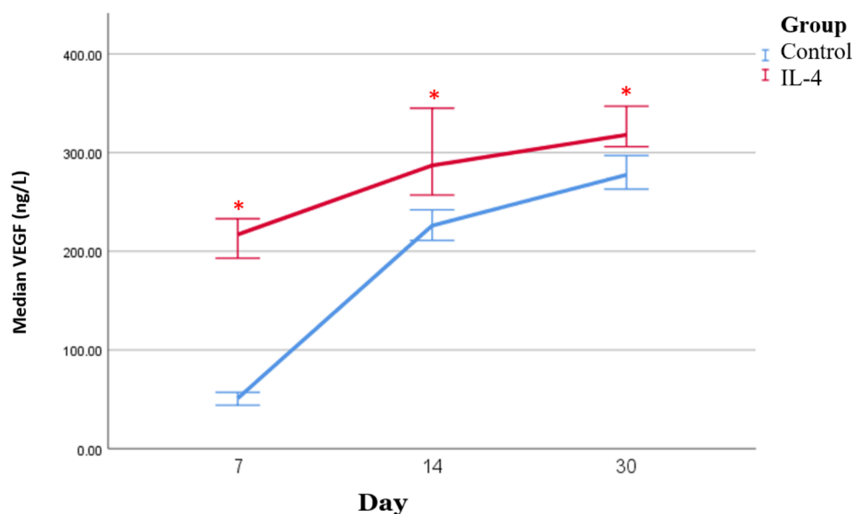

Figure 3. Changes of VEGF levels in the control and IL-4 groups across the observation days. *Statistically significant at $p<0.05$ as compared to the previous day of observation.

There was a significant difference in perilipin numbers per visual field in both the control and treatment groups on day 14 compared to day 7 (p=0.009 and 0.009, respectively) and on day 30 compared to day 14 (p=0.014 and 0.019, respectively). The histological images depicting fat tissues of control and IL-4 groups are presented in **Figure 4**.

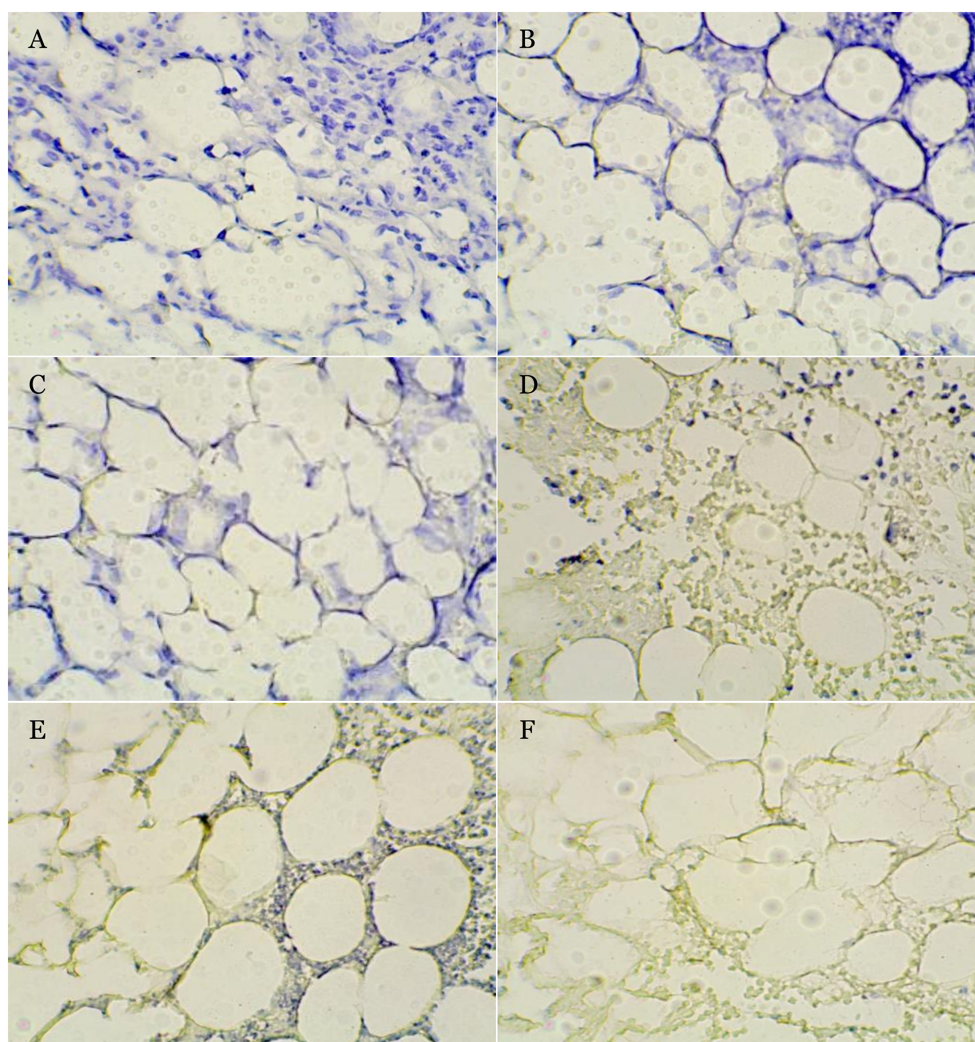

Figure 4. Histological images of fat tissue in the control group on day 7 (A), day 14 (B), and day 30 (C). Histological images of fat tissue in the IL-4 group on day 7 (D), day 14 (E) and day 30 (F). Observed with perilipin immunohistochemistry under 400× magnification.

Original Article

Volume difference

There was a significant increase in preoperative volume on days 7, 14, and 30 in both control (p=0.006) and intervention (p=0.011) groups (**Table 1**). There was no difference in preoperative volume between the control and treatment groups on days 7, 14, and 30 (p=0.690, 1.00, and 1.00, respectively). When the postoperative volume measurements were deducted from the preoperative volume, a greater reduction was observed in the control group than in the treatment group on days 7, 14, and 30 (p=0.043, p=0.043, and 0.68, respectively). The volume reduction in the treatment group was less than in the control group on days 7, 14, and 30 (p=0.009, 0.009, and 0.029, respectively). Among rats receiving IL-4, the postoperative volume was significantly different when observed on day 14 as compared to day 7 (p=0.036), but the volume change was no longer significant on day 30 (p=0.065) (**Table 1**). The trend for volume differences is presented in **Figure 5**.

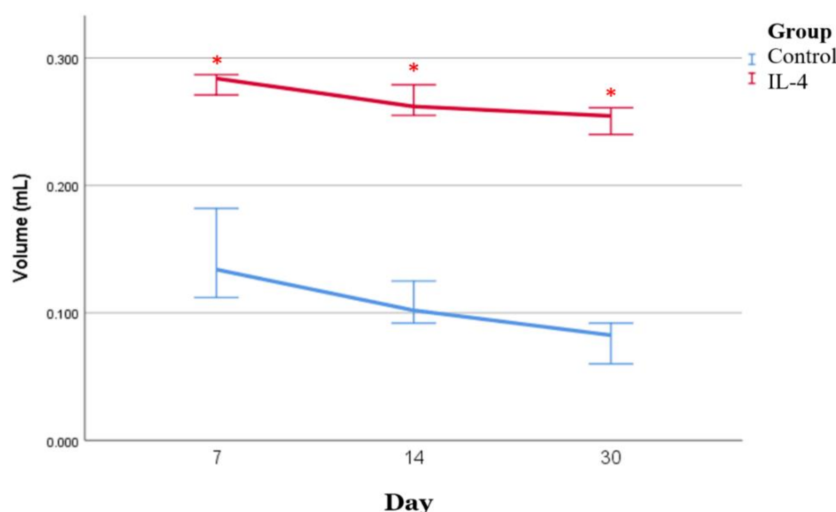

Figure 5. Trend of delta volume (mL) observed in control and IL-4 group. *Statistically significant at p<0.05 as compared to the previous day of observation.

Discussion

This present study suggested that IL-4 administration resulted in significantly higher concentrations of VEGF and perilipin. Additionally, the IL-4 group showed significantly less volume reduction compared to the control group at all time points. This study emphasized the beneficial effects of early IL-4-induced M2 polarization on Fat Graft outcomes. This is in line with the study by Li *et al.*, which performed M2 polarization at the early stage and resulted in promoted vascularization and regeneration, thereby improving the fat graft retention rate [4]. The early activation of macrophage by IL-4 was first studied by Phipps *et al.*, who observed 3 months after injection and resulted of M2 macrophage-supplemented grafts remained stable, whereas controls experienced further volume loss by 2.5× smaller [10].

In this study, although there was a volume reduction in the IL-4 group, it was only 0.045 mL (16.7%) on day 30, which is better compared to the 0.218 mL (72.7%) reduction in the non-IL-4 group on day 30. Thus, the volume reduction with IL-4 is 22% smaller compared to non-IL-4. The final volume on day 30 in the treatment group was three times larger compared to the fat graft volume in the control group (median 0.254 mL vs 0.08 mL). These results align with Li *et al.* (2022), which found that early polarization with IL-4 resulted in 1.3 times higher volume in fat grafts compared to the no IL-4 group (52% ± 6.5% vs 40% ± 3.5%) [4]. Similarly, Cai *et al.* (2017) found that fat graft retention weight was 1.39 times higher in the treatment group compared to the control group (117±12 mg vs 84±15 mg, p=0.043) [11].

This present research highlights that adipocyte stem cells can survive up to 72 hours post-graft, differentiating into mature adipocytes and determining the final Fat Graft volume [1]. Early intervention within this time frame is critical because it induces earlier vascularization for survival and saves more dying Adipocyte Stem Cells [23]. The more ASCs that remain viable, the more they proliferate and mature into adipocytes.

Within the IL-4 group, the VEGF concentration was significantly higher on day 14 compared to day 7, while no significant difference was observed in the control group. Neovascularization is crucial for tissue survival, with Macrophage-2 contributing to the angiogenesis process [11]. In hypoxic conditions, such as newly grafted fat, M2 releases pro-angiogenic factors like VEGF to promote angiogenesis. Increased VEGF levels stimulate endothelial cell proliferation, prevent apoptosis, and mediate endothelial cell mitosis and migration within the graft. Following fat grafting, the low-oxygen environment triggers ASCs to release HIF and VEGF, which activate macrophages. M2 directly interacts with endothelial cells through sprouting mechanisms, where donor blood vessels extend and eventually connect with recipient vessels (anastomosis)[6]. VEGF works synergistically with M2, significantly increasing endothelial cell proliferation and enhancing surface integrin expression on endothelial cells by 2–7 times [24]. Once blood vessels are connected, the recipient tissue oxygenates the donor tissue and facilitates progenitor cell migration within 4–14 days post-grafting [25]. Neovascularization, which was indicated by VEGF expression, was significantly higher in the treatment group compared to the control group on days 7 (p=0.009), 14 (p=0.009), and 30 (p=0.019). Notably, while the control group showed significant increases in VEGF levels between days 7 and 14, and days 30 and 14, the treatment group did not exhibit significant changes during these periods (p=0.090 and p=0.086, respectively). This suggested that IL-4-induced M2 polarization had already increased earlier and sustained the level so that the increase was not as significant as the control group later on days 14 and 30. The early neovascularization potentially aids in graft integration and survival [6,11,24-26].

Based on the statistical analysis, the adipocyte numbers also demonstrated significant differences between the treatment and control groups. The IL-4 group showed substantial increases from the first to the second week (p=0.009) and from the second to the fourth week (p=0.019), indicating enhanced adipogenesis facilitated by IL-4-induced M2 polarization. Additionally, perilipin levels, a marker of mature adipocytes, were greater in the IL-4 group throughout the study period (p=0.009 on days 7 and 14, p=0.019 on day 30), underscoring improved adipocyte maturation and retention in the treated Fat Grafts.

This study also found more inflammatory cells in the control group compared to the treatment group. This aligns with Keskin's research, where the control group exhibited acute inflammation, lipogranuloma, and cyst formation as the M2 is known to mark the anti-inflammatory process, thus reducing the number of neutrophils infiltrating the tissue [26]. Inflammatory response analysis revealed fewer inflammatory cells in the treatment group compared to the control, aligning with previous research indicating that early M2 polarization promotes an anti-inflammatory environment [24,27].

The limitation of this study is described in the following sentences. First, although this study supports the notion that the early presence of M2 macrophages in fat grafts increases volume retention, it does not explore how long M2 macrophages should remain at the wound site. If M2 macrophages are present for too long, they may trigger fibrosis processes, ultimately reducing the final volume in the long term (Cai *et al.*, 2017). This study does not address the stability of final fat graft volume but focuses on vascular regeneration and adipogenesis growth. Another limitation is the minuscule amount of fat available from the rat samples. Rats have limited fat pockets, resulting in restricted tissue harvesting. Even though the number of macrophages and adipose stem cells added to the wound decreases after stromal vascular fraction extraction, the ratio, body weight, and extracted cells do not affect the results beyond the research hypothesis.

This study induced macrophage-2 polarization on day three, manipulating macrophage-1 and -2 dynamics at the end of the inflammatory phase. Jetten *et al.*'s research, which stimulates M2 polarization on days 1 and 3, is expected to affect wound closure and healing in diabetic rats. However, there is no similar research reporting on wound closure and healing complications in non-diabetic rats. Another limitation is that M2 cells are immune cells often associated with tumor growth. Although they have proven benefits, macrophage-2 therapy is not without contraindications. The effects of interleukin-4 on macrophage polarization-2, which may lead to faster cell proliferation and tissue growth in tumors, are not yet fully understood. Future research could be conducted on subjects with malignancy risks. However, in this study, a small dose was used and applied locally through intralesional injection in fat, aiming to minimize the risk of

unwanted cell growth. The limited research duration also prevented further observation of systemic effects.

Conclusion

Overall, administering IL-4 on the third day of Fat graft and SVF helps its viability by increasing the VEGF level and regeneration process of adipocytes. This study investigated the impact of IL-4-induced early Macrophage-2 polarization on Fat Graft and SVF viability over a period of 30 days and suggested that optimizing macrophage polarization through IL-4 administration in SVF could represent a promising strategy to improve the clinical efficacy by using the precultured macrophage obtained by intraperitoneal induction. The more ASCs that remain viable, the more they proliferate and mature into adipocytes. Further research should be conducted with a longer duration and should be performed with subjects that have a larger amount of fat tissue. Further investigation of the safety profile and optimal dosage of interleukin-4 in experimental subjects should be detailed.

Ethics approval

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Udayana, Denpasar, Indonesia (08/05/2023/No: 2023.02.1.0493).

Acknowledgments

The authors have nothing to declare.

Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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