ORIGINAL ARTICLE

TGF-1 biomarker level evaluation on fracture healing in a murine model with a bone defect after stromal vascular fraction application

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ABSTRACT

Aim Bone defect is a challenge even for experienced orthopaedic surgeons and it is a significant cause of morbidity in patients and a source of high economic burden in health care. A severe bone defect is a condition whereby the bone tissue cannot undergo natural healing despite surgical stabilization and requires further surgical intervention. Stromal vascular fraction (SVF) is a heterogeneous cell population derived from adipose tissue that results from minimal manipulation of the adipose tissue itself. TGF is essential in maintaining and expanding mesenchymal stem cells or progenitors of osteoblasts. Furthermore, TGF-β signalling also triggers osteoprogenitor cell proliferation, early differentiation, and maintenance of osteoblasts in the bone healing process. The aim of this study was to determine the effect of administering SVF on bone defects' healing process assessed based on the TGF- β1.

Methods This was an animal study involving twelve Wistar strain *Rattus norvegicus*. They were divided into three groups: negative group (normal rats), positive group (rats with bone defect without SVF application), and SVF group (rats with bone defect with SVF application). After 30 days, the rats were sacrificed, the TGF- β1 biomarker was evaluated (quantified using ELISA).

Results TGF- β1 biomarker expressions were higher in the group with SVF application than in the group without SVF application. All comparisons of the SVF group and positive control group showed significant differences (p=0,000), respectively.

Conclusion Giving SVF application could aid the healing process in a murine model with bone defect, marked by an increased level of TGF- β1.

Key words: animal experimentation, biomarkers, mesenchymal stem cell

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INTRODUCTION

Fractures and segmental bone defects are a significant cause of morbidity in patients and are a source of a high economic burden in health care. A severe bone defect is a devastating condition in which the bone cannot heal naturally despite surgical stabilization and usually requires further surgical intervention, such as stem cell or stromal vascular fraction (SVF) application (1). In humans, it is called a bone defect when there is a loss of bone components reaching 2-2.5 times the diameter of the bone involved (2), while other researchers define when the distance of the defect is more than 1 cm plus more than 50% of the bone circumference (2). In animal research objects such as rats, it is called a bone defect when there is a loss of bone components up to 3 mm (2).

These conditions often complicate fractures and require additional reconstructive procedures, such as bone transport, acute limb shortening and lengthening, massive allograft or vascularized fibular allograft, masque let technique (induced membrane), and bone grafting with BMP and extensive and segmental bone defects requiring no salvage action (3,4). Despite these efforts, there is an excellent long-term functional outcome but is limited by high complications and reoperation (3,4).

Stromal Vascular Fraction (SVF) was first described in 1960 by Hollenberg, and it was found in 2001 that SVF contains a large number of MSCs (5,6). The SVF is a heterogeneous cell population that arises from adipose tissue that is minimally manipulated. It has been reported that SVF contains various cells like adipose-derived mesenchymal stem cells (ADMSC), hematopoietic stem cells (HSC), T regulatory cells (T-reg), and progenitor cells (4,7,8). The SVF also contains growth factors like insulin-like growth factor-1 (IGF-1), transforming growth factor β (TGF β), and fibroblast growth factor (FGF), which plays a role in cell proliferation and differentiation (4,7).

The healing process in bone defect goes through several phases: hematoma formation, inflammation, soft callus formation in cartilage, neovascularization, soft callus mineralization, hard callus formation, and remodelling of the osteoclastic hard callus to make flat bones (9,10). This process, however, is not sufficient to cover large defects in bones. Under such circumstances, an autograft is a preferred method to replace bone loss (11). During the healing process of bone defect, several biomarkers fluctuate dynamically to signify an ongoing bone formation, such as BMP-2, osteocalcin, and TGF-β1 (11). During the bone formation process, the levels of these biomarkers mentioned above will elevate, thus making them a suitable parameter for fracture healing with bone defect (11).

TGF-β1 is the most abundant isoform, the most prominent sources are platelets (20 mg/kg) and bone (200 g/kg). The TGF-β1 is a multifunctional, ubiquitous growth factor, first discovered as a synergistic factor with TNF-α which stimulates the formation of fibroblast colonies, cartilage (11). TGF-β1 is found abundantly in the tissues that make up the skeleton, where it helps regulate bone growth and the intricate lattice forms in the spaces between cells (the extracellular matrix) (11).

The benefits of SVF application in the medical and orthopaedic fields have been widely observed. SVF has been used in cases of burnt trauma, nerve injury, osteoarthritis, osteonecrosis, rheumatoid arthritis, rupture of Achilles tendon, and growth plate defect (12-14). An SVF application in bone defect therapy has been done, but up until now, there has been no study that measures the effect of SVF application for bone healing from TGF-β1 biomarker.

The aim of this study was to determine the effect of SVF from adipose tissue in the process of bone defect healing, measured by TGF-β1 biomarker.

MATERIALS AND METHODS

Study design and animal model

The research design used was an experimental laboratory method with a randomized post-test only control group design. This study aimed to determine the effect of SVF in increasing osteogenic and osteo inductive activity of fracture regeneration with bone defects with an indicator of TGF-β1.

The study started by selecting Wistar strain *Rattus norvegicus* rats that met the inclusion and exclusion criteria acclimatized for seven days. Twelve rats were randomly divided into three groups, four rats in each group: the negative group included four normal rats without fracture and large-sized bone defect and SVF application, P (-); the positive group was the murine model with fracture and large-sized bone defect, but without

SVF application, P (+). The SVF group was the murine model with fracture and large-sized bone defect and SVF application, SVF. These three groups were observed for 30 days and tested for biomarker level of TGF-β1 (Table 1).

The study was conducted in 2021 and in the period of three months. Maintenance and treatment of rats in the form of making fracture models with bone defects accompanied by the provision of SVF according to treatment groups carried out in the Animal lab of Universitas Brawijaya Faculty of Medicine. The making of SVF and the application of SVF were carried out at the Physiology Laboratory of Universitas Brawijaya Faculty of Medicine. TGF-β1 biomarker was examined in the Biomedical Laboratory of Universitas Brawijaya Faculty of Medicine.

The Ethics Committee of Universitas Brawijaya Faculty of Medicine has approved all animal protocols, and all subsequent experiments were carried out according to the ARRIVE guidelines and regulations (15). Animals were kept in standard conditions of accredited vivarium with gentle handling, daily cage cleaning and close monitoring were done to minimize animal suffering.

Methods

The making of stromal vascular fraction from adipose tissue. Five 12-week-old male Wistar strain rats were sacrificed by dislocating their cervical. Adipose tissue was harvested from the epididymal and perirenal fat. The rats were in the supine position, then a skin incision was made longitudinal to expose the abdomen. The testicles were removed, and the fat surrounding them was harvested. Perirenal fat was collected by cutting off the innervation from the retroperitoneal fat pad.

The harvested adipose tissue was then washed with a solution of PBS (phosphate-buffered saline; Sigma-Aldrich, Germany) which contains a mixture of 10% antibiotic-antimycotic, then mashed with a knife. It was then immersed in a 0.075% type IA collagenase mixture (Sigma-Aldrich) and PBS for 30 minutes at 37 °C. The processed tissue was then strained with a 100 μm mesh (Sigma-Aldrich) and centrifuged om 1200 rpm for 10 minutes at 20 °C. The supernatant was discarded, and the resulting suspension yielded a heterogeneous cell mixture with an estimate of $2x10^6$ cells for 1 gram of adipose tissue (16).

Preparation procedure for animal fracture model with bone defect and plaster of Paris application. Murine models were acclimatized for seven days before a bone defect was made on rats in positive and intervention groups. Rats were anesthetized with 100 mg/kg ketamine injection and intraperitoneal 10mg/kg xylazine hydrochloride before the procedure. To ensure the rats were under anaesthesia the pedal reflex technique by extending the extremities and pinching the web between the toes was applied. If murine shielded away or twitched a muscle and made a sound, the anaesthesia was not enough. After that, an antibiotic injection of 20 mg/kg cefazolin was administered on the right leg. The operating area was then shaved and cleaned with chlorohezadine. The murine were placed in a prone position on the operating area and incised for 3-4 cm. The incision was made layer by layer until the bone was exposed. Osteotomy was done using a 3 mm Kerrison, so the bone defect made was 3 mm wide. The intervention was then done according to the assigned groups. After that, plaster of paris was applied from the proximal femur to the ankle with a 90° knee flexion. Analgesia was given every 8 hours (using IM 5mg/kg ketorolac), and an antibiotic was administered 24 hours post-surgery using intramuscular 20 mg/kg cefazoline. Monitoring was done periodically for 30 days.

Laboratory analysis with ELISA method. After 30 days, murine models were harvested. The area of bone defect with callus formation was collected and then extracted. The levels of TGF-β1) were assessed using the ELISA method.

Statistical analysis

The hypothetical comparative test steps were as follows: data normality test, variant homogeneity test, and comparative student T-test or One-way ANOVA test or Kruskal-Walli's test according to the normality and homogeneity test result. After the ANOVA or Kruskal-Walli's test result had been received and the test was significant ($p<0.05$), the next was the post hoc test. If the data collected was not homogenous by ANOVA, a non-parametric test with the Kruskal-Walli's method done.

RESULTS

The TGF-β1 level was measured using ELISA reader and resulted with the highest TGF-β1 in SVF group and the lowest one in the negative group (the normal rat's group) (Table 1).

Table 1. Serum TGF-ß1 level in each of 12 rats according to the groups

Rat group	Ordinal number of the rat in the group	TGF-β1 (ng/mL)
Negative	1	0.497
	2	0.627
	3	0.554
	4	0.619
Positive	1	0.795
	2	0.614
	3	0.708
	4	0.708
SVF	1	1.899
	2	1.932
	3	1.995
	4	1.875

Negative, normal rat group without fracture, bone defect and SVF application; positive, rat group with fracture and large-sized bone defect, but without SVF application; SVF, rat group with fracture and large-sized bone defect and SVF application

The TGF-β1 comparative hypothesis test in each three groups was carried out by ANOVA test because the data were normally distributed and homogenous. A significant difference in TGF-β1 levels due to the application of SVF was found (p=0.000) (Table 2).

Table 2. ANOVA comparative hypothesis test results

Group	Mean±SD	p
Negative	0.57425±0.061	
Positive	0.70625±0.07392	0.00
SVF	1.92525±0.05204	

Negative, normal rat group without fracture, bone defect and SVF (Stromal Vascular Fraction) application; positive, rat group with fracture and large-sized bone defect, but without SVF application; SVF, rat group with fracture and large-sized bone defect and SVF application

Based on the post hoc test results (Table 3), the comparison between the groups showed that the groups had a significantly different TGF-β1 mean values in case of $p<0.05$; conversely, in case of $p>0.05$, the TGF-β1 average values were not significantly different. Accordingly, the application of SVF had a significant effect on osteogenic and osteo inductive activity increase of fracture regeneration with bone defects (with an indicator of TGF-β1SVF) comparing to groups without SVF application (Figure 1).

Table 3. Post-hoc analysis on Stromal Vascular Fraction (SVF) application related serum TGF-ß1

Groups comparison		p
Negative	**Positive**	0.038
	SVF	0.000
Positive	**Negative**	0.038
	SVF	0.000
SVF	**Negative**	0.000
	Positive	0.000

Negative, normal rat group without fracture, bone defect and SVF (Stromal Vascular Fraction) application; positive, rat group with fracture and large-sized bone defect, but without SVF application; SVF, rat group with fracture and large-sized bone defect and SVF application

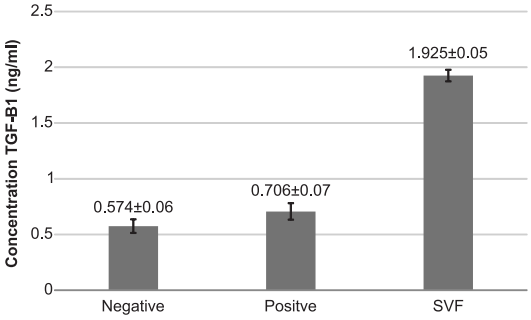

Figure 1. Average TGF-ß1 in each of the three groups
Negative, normal rat group without fracture, bone defect and SVF application; positive, rat group with fracture and large-sized bone defect, but without SVF application; SVF, rat group with fracture and large-sized bone defect and SVF application

DISCUSSION

In this study, there was a significant difference in the levels of TGF-β1 after the application of SVF, respectively (p=0.000), compared to the control (positive) group, and it was confirmed with post hoc test too. It implies that the SVF application will increase the level of TGF-β1 in fractures with bone defects because with TGF-β1, a greater number of osteoblasts will play a role in bone formation so that the process of fracture healing would be more significant.

The stromal vascular fraction has several advantages over other biologically active materials: the patient's discomfort can be minimized when fat tissue is taken, SVF contains a high ratio of stem cell volume, tissue extraction is relatively easy and can be adjusted to the needs, processing fat tissue into ADMSCs which then becomes SVF with high mesenchymal cells can be done quickly, and the multipotent cells contained in SVF can bind quickly to the scaffold material, increase quickly, and can differentiate into osteogenic elements (17,18).

With the application of SVF (stem cells), the bone healing process becomes more active and efficient because SVF contains progenitor cells, namely mesenchymal cells (mainly ADMSc), and also several growth factors such as TGFβ (transforming growth factor), IGF1 (insulin), growth factor 1), FGF2 (fibroblast growth factor 2), and PDGF (platelet-derived growth factor). It is known that bone and cartilage contain large amounts of TGF-β, which is essential in the maintenance and expansion of mesenchymal stem cells or progenitors of osteoblasts and triggering

osteoprogenitor cell proliferation, early differentiation, and maintenance of osteoblasts (11).

A limitation of this study is group size that was rather small, which is a common limitation with the animal studies. Future research is highly recommended to use a larger group size, or randomized clinical trial with humans. We recommend that future research incorporate a combination of SVF and scaffold and a different fixation method, such as external or internal fixation, to broaden the scope of the study to include a variety of orthopaedic modalities.

In conclusion, the application of SVF could aid healing processes in a murine model with bone defect, marked by increased levels of TGF-β1 as a bone formation marker.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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