ISSN: 03875547
Volume 45, Issue 09, November, 2022

The Effect of Stromal Vascular Fraction to Inhibit Bony Bridge Formation in Growth Plate Injury (Evaluation Using Alizarin Level and Scanning Electron Microscope) in Rattus Norvegicus Rats

Panji Sananta[1], Respati S Dradjat[1], Ganang Dwi Cahyono[1]

Orthopaedic and Traumatology Department, Faculty of Medicine University of Brawijaya - Saiful Anwar General Hospital, Malang, East Java, Indonesia[1]

Keywords:
Stromal Vascular Fraction, Bony bridge, COL2A1, Aggrecan

ABSTRACT

The growth plate is important in bone growth. Injury to the growth plate may develop premature fusion or asymmetric growth at the ends of the bones caused by the interaction of bone formation and remodeling in growth plate injuries leading to adverse bone tissue growth, a bony bridge. This study applied Alizarin and scanning electron microscope (SEM) method to measure and compare the bone bridge area in the bones between injured mice had received stromal vascular fraction (SVF) and mice that had not received SFV treatment. An in vitro study using laboratory experimental methods with post-test only control group design. Sample of this research is stored biological material in the form of bone tissue from Rattus norvegicus strain Wistar. Samples were divided into two research groups; the control group was consisted of tissue with growth plate injury minus tissue engineering treatment, and the treatment group received tissue engineering treatment in form of SVF administration. The examination of research samples was carried out in the radiology and biomedical laboratories. Average area of the bony bridge was smaller in the growth plates of rats given SVF compared to those not given SVF either by the Alizarin method or by the SEM method. This has shown that SVF administration can reduce the size of the bony bridge in growth plate injuries. SVF administration can reduce the size of the bony bridge in growth plate injuries on evaluation using an Alizarin staining and scanning electron microscope (SEM).

1. INTRODUCTION

Growth plate is the area of tissue near the ends of long bones in children and adolescents that determines bone growth process through endochondral ossification, therefore it is crucial for rapid and integrated longitudinal bone growth [1]. Growth plate injuries occur in patients with immature bone [2]. A growth plate injury can cause a portion of the growth plate to undergo premature fusion or asymmetric growth at both ends of bones. This complication is caused by the interaction of bone formation and remodeling in growth plate injuries

leading to adverse bone tissue growth, also known as a bony bridge [3].

In orthopaedics, tissue engineering has been generally applied to cases of osteonecrosis, non-union fractures, and chondral-osteochondral defects. Tissue engineering involves cells, scaffolds, and signaling processes where all these components are arranged in such way resembling natural regeneration that occurs in cells, tissues, and organs. Tissue engineering technique is also believed to prevent the formation of bony bridge in growth plate injuries. The use of chondrocyte transplantation or mesenchymal stem cells (MSCs) – based therapy in the healing process of growth plate injuries has been investigated yet the results have not been satisfactory [3]. In addition, the use of mesenchymal stem cells (MSCs) as a treatment for growth plate injuries has also been investigated. However, MSC presents with following limitations; considerable time for MSC harvest, in vitro expansion and implantation [3], [4]. Therefore, in this study, researchers wished to seek alternative biological therapy in the form of stromal vascular fraction (SVF) conducive to management of growth plate injuries.

Stromal vascular fraction (SVF) is a heterogeneous cell population derived from adipose tissue. SVF contains variety of cells consisting of adipose-derived mesenchymal stem cells (AD-MSC), hematopoietic stem cells (HSC), T-regulatory cells (T-Reg), and progenitor cells. SVF also contains variety growth factors, such as insulin-like growth factor-I (IGF-1), transforming growth factor (TGFβ) and basic fibroblast growth factor (bFGF) which are significant in the process of cell proliferation and differentiation [5]. The use of SVF in the medical field has proven its benefits, in cases of rheumatoid arthritis, burns, neurotrauma, osteoarthritis, osteonecrosis, and rupture of Achilles tendon [6- 8].

Alizarin is a method to determine bone formation that also able to detect bone calcification process. Alizarin stains the bone with dark red in color, indicating that the bone has undergone calcification. The dark red color is produced from the bond between the dye and calcium in the bone matrix, therefore facilitate application and observation in laboratories setting [9]. Furthermore, scanning electron microscope (SEM) can produce very accurate images of various materials by analyzing organic and inorganic materials at the scale of nanometer to micrometer (μm). SEM is often used to analyze bone structure, in vivo and in vitro bone resorption, as well as to examine the surface structure and cell-matrix interactions of bone cells with various substrates.

Given the benefits of Alizarin and SEM in measuring the hypertrophic differentiation process of chondrocytes for which is the basic evaluation for the growth plate healing process, researchers applied Alizarin and SEM to determine the effect of SVF administration from fat tissue on healing growth plate injuries. The main focus is the content of T-reg, AD-MSCs, and growth factor SVF which are expected to regenerate growth plates and inhibit bone bridge formation in growth plate injuries.

2. Methods and Material

2.1 Research Design

This research is an in vitro study using laboratory experimental methods with post-test only control group design. The aim of this study is to determine the effect of tissue engineering in inhibiting the formation of bony bridges on injury growth plates using the Alizarin test and scanning electron microscope (SEM). The research was conducted at the biomedical laboratory of the Faculty of Medicine, Universitas Brawijaya Malang starting in April 2020 until April 2021.

2.2 Research Samples

The sample of this research is stored biological material in the form of bone tissue from Rattus norvegicus strain Wistar mice. The amount of biological material stored in the form of bone for each group (replication) was obtained using the Federer formula [10] and the number found for stored biological material in the form of bone used in each group was 16. Inclusion criteria for the samples were male Rattus norvegicus strain Wistar, with weight ranging from 230-280 grams, age ± 3 months (12 weeks) [11], healthy and active condition mice with no limb defects, and had not experienced any treatment or received any chemical intake. Whereas the exclusion criteria were infection in extremity, mice that had undergone treatment, and the death of mice before completing the research.

Samples were divided into two research groups; the control group was consisted of tissue with growth plate injury minus tissue engineering treatment, and the treatment group was consisted of tissue with growth plate injury that had received tissue engineering treatment in form of SVF administration. Each group was examined for the formation of a bony bridge. The examination of research samples was carried out in the radiology and biomedical laboratories of Brawijaya University.

2.3 Stromal Vascular Fraction

Stromal vascular fraction of adipose tissue is a heterogeneous cell population that has been purified by the Zuk method. The heterogeneous cells consist of Adiposed Derived Stem Cells (AD-MSCs), Hematopoietic Stem Cells (HSCs), and T-Regulatory Cells (T-Reg).

2.4 Growth Plate Injury

Growth plate injury is an injury that occurs on the growth plate of Rattus Norvegicus strain Wistar rats made by drilling the proximal tibia using a 2 mm drill bit. Drilling was performed from distal to proximal to the growth plate [3].

2.5 Alizarin

Alizarin is a method to determine bone formation that also able to detect bone calcification process. Alizarin stains the bone with dark red in color, indicating that the bone has undergone calcification. The dark red color is produced from the bond between the dye and calcium in the bone matrix.

2.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM), an effective tool in the analysis of organic and inorganic materials at the nanometer to micrometer (μm) scale. SEM operates up to 1,000,000 times magnifications thereby can produce very precise images for wide range of materials, in addition it has often been used to analyze bone structure.

2.7 Rattus novergicus wistar strain

The type of experimental animal was Rattus norvegicus wistar strain according to the inclusion criteria. Wistar strain of Rattus norvegicus; white rat with red eyes, wide head, long ears and has a long tail shorter than its body length, about 6 weeks old, male and physically healthy (active movement and responding to its surroundings). The selection of experimental animals which were all male was based on the considerations that the sample was more homogeneous. Body weight is determined by weighing of the mice with the aim that the sample is homogeneous. Healthy sample is characterized by its active movement, with no defects and infections of the extremities.

2.8 Bony Bridge Evaluation

Bony bridge measurements were conducted using Alizarin staining and scanning electron microscope (SEM).

Bony bridge area measurements were done out using image raster 4.1 software (OptiLab). The preparations were stained using the Hematoxylen-Eosin technique and photographed at 0.63x microscope magnification (Leica stereo microscope). The photos are stored on a memory card, in JPEG format and then opened using raster images. The measurement results were then analyzed using a different test (average) using SPSS ver. software 21. The results are presented in the form of images, tables of mean and ± SD data and graphs.

2.9 Data Analysis

The data obtained were tabulated and compared between the control group and the treatment group. This study applied Shapiro-Wilk method for normality test, subsequently the result of Alizarin and SEM level data held significance value ($p = 0.028$) > 0.05. In conclusion, the research data did not spread following a normal distribution. Data homogeneity was tested using Levene test and the result held significance value ($p = 0.967$) > 0.05, therefore the research sample has a homogeneous data variance. Given the result, normality test showed that data were not normally distributed and necessarily analyzed using Mann-Whitney test to compare the difference between mean data from the two groups.

3. Result

In the study the sample amounted to 32 mice. Alizarin levels were measured to observe the formation of bony bridge on the injury growth plate (Table 1). In this study, it was found that the average Alizarin level in the control group was higher, i.e. 150.59 micrometers compared to the treatment group, that had received stromal vascular fraction.

Table 1. The average area of the bony bridge in the control and treatment group

Control Group		**Treatment Group**	
Number	**Measurements (Micrometer)**	**Number**	**Measurements (Micrometer)**
1	115.58	1	95.25
2	135.62	2	47.59
3	105.95	3	75.32
4	158.35	4	78.59
5	114.36	5	78.32
6	175	6	96.58
7	182.34	7	102.25
8	157.11	8	103.56
9	169.14	9	95.65
10	157.26	10	85.65
11	108.25	11	115.36
12	186.21	12	102
13	143.58	13	115.26
14	112.54	14	96.56
15	185.14	15	97.36
16	168.34	16	105.65
Control Group Mean	150.59±29.71	**Treatment Group Mean**	94.95±18

Examination using a Scanning Electron Microscope (SEM) was conducted to determine the formation of bony bridge in the injured growth plate (Figure 1). It was later discovered the average difference between alpha (total bone cross section) and beta (bony bridge formation) in the treatment group was greater than the control (+), which was 1,350.

ISSN: 03875547
Volume 45, Issue 09, November, 2022

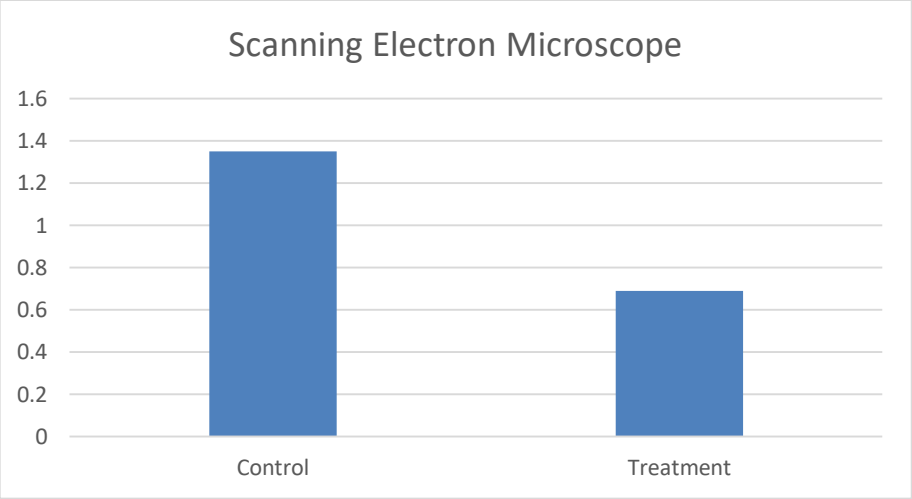

Figure 1 Bony bridge area graph using SEM method

The decrease in the average bony bridge in the control group compared to the treatment group was 55.64 micrometers (Figure 2).

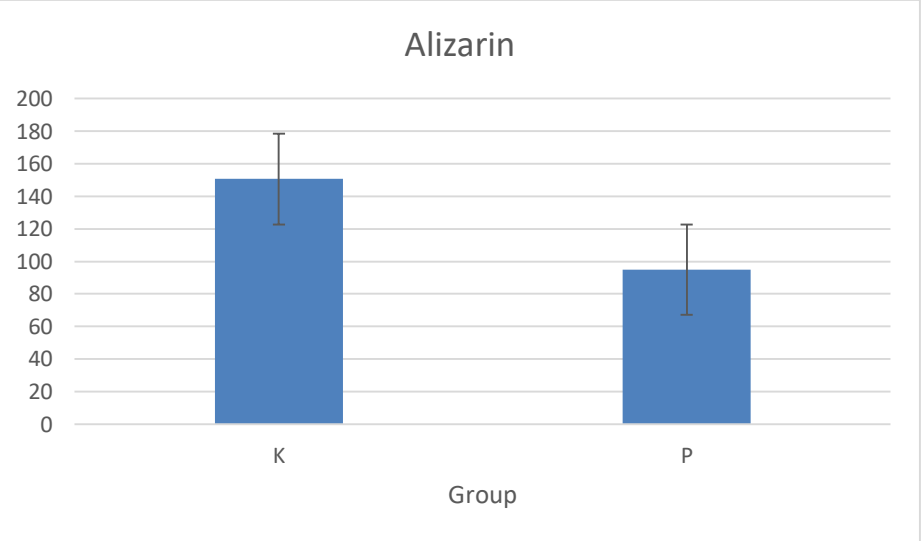

Figure 2 Bony bridge area graph using the Alizarin method. K for Control and P for Treatment Group

From Mann-Whitney test, the average Alizarin and SEM levels obtained a significance value ($p = 0.001$) <0.05 hence pointing a significant difference between average Alizarin and SEM levels from both research groups (Table 2 & 3).

Table 2. Alizarin Evaluation

Groups	Bony Bridge Area	p-value
Control Group	150.59±29.71	
Treatment Group	94.95±18	<0,05

Controls: Samples had growth plate injury without tissue engineering treatment; Treatment group: Samples suffered growth plate injury with tissue engineering treatment in the form of administration of SVF;

Table 3. Scanning Electron Microscope (SEM) Examination

Groups	Bony Bridge Area	p-value
Control Group	1,350	

Treatment Group	0,690	<0,05

Controls: Samples had growth plate injury without tissue engineering treatment; Treatment group: Samples suffered growth plate injury with tissue engineering treatment in the form of administration of SVF;

4. Discussion

In this study, SVF was administered to the growth plates of injured mice and compared with samples that were not receiving SVF. Afterwards, bony bridge measurements were performed using Alizarin and SEM method. The aim of this study was to determine whether there would be effect towards the formation of bony bridge in the injured growth plates following SVF administration.

In the measurement using Alizarin staining, it was discovered that bony bridge formation level in the growth plate of the control sample (150.59±29.71) was higher than that in the growth plate of the sample treated with SVF (94.95±18). Mann-Whitney test result had proven the bony bridge in the control sample and the treatment group had a significant difference ($p<0.05$). Whereas measurements using scanning electron microscope (SEM) method, showed bony bridge formation level in the growth plate of the control sample (1.350) was more than the growth plate of the sample receiving SVF (0.690). From the Mann-Whitney test had figured the bony bridge in the control sample and the treatment group had a significant difference ($p<0.05$).

This study has concluded that mice with growth plate injury receiving stromal vascular fraction (SVF) treatment had a smaller mean bone bridge area than those without SVF treatment. It is possibly correlated with the properties of SVF contents; adipose derived mesenchymal stem cells (AD-MSC), growth factors, and T regulatory cells (T-reg) [5].

The content of AD-MSC in SVF will likely increase the number of AD-MSC in the growth plate injured area. The presence of growth factors and T-reg triggers increase in the proliferation and differentiation of AD-MSC through the chondrogenesis pathway, instead of osteogenesis pathway. Activation of the chondrogenesis pathway through transcription factor SoX9 converts AD-MSCs into chondrocytes, thereby preventing growth disorders in growth plate injuries. Meanwhile, the presence of growth factors in SVF can cause an increase in Insulin-like Growth Factor-I (IGF-1), Transforming Growth Factor-β (TGF-β), and Fibroblast Growth Factor-2 (FGF-2) in the plate injury area. The following growth is expected to stimulate the proliferation and differentiation of cells, in this case AD-MSC [5].

In addition, another role of T-reg as a cell in the immunological system is the regulation of monocytes. The injured growth plate will release a biomolecule called damage associated molecular pattern (DAMP) which will affect the T-reg to regulate monocytes to turn into type 2 macrophages, consequently there will be exceeding number of type 2 macrophages in the injured area. This correlating type 2 macrophage promotes the process of repairing growth plate injuries, as well as being anti-inflammatory that can prevent chronic inflammation which is very detrimental in the ideal healing process [5].

5. Conclusion

Based on the results and discussion in this study, it was found that administration of SVF can reduce the size of the bony bridge in growth plate injuries on evaluation using a Scanning Electron Microscope (SEM) and Alizarin staining. To support the conclusions of this study, further research is needed on fracture identification using microCT to improve accuracy in measuring epiphyseal growth plate.

6. References

[1] Saladin KS. Anatomy & Physiology: The Unity of Form and Function. 5th Editio. McGraw-Hill; 2018.

[2] Levine RH, Foris LA, Nezwek TA, Waseem M. Salter Harris Fractures. Adv Emerg Nurs J. 2022;29(1):10-19. doi:10.1097/01261775-200701000-00003

[3] Chung R, Xian CJ. Mechanisms for growth plate injury repair and potential cell-based therapies for regeneration. J Mol Endocrinol. 2014;53(1):T45-T61. doi:10.1530/JME-14-0062

[4] Shukrimi AB, Afizah MH, Schmitt JF, Hui JHP. Mesenchymal stem cell therapy for injured growth plate. Front Biosci. 2013;S5(2):S407. doi:10.2741/S407

[5] Alexander RW. Understanding Adipose-derived Stromal Vascular Fraction (AD-SVF) Cell Biology and use on the Basis of Cellular, Chemical, Structural and Paracrine Components: A Concise Review. J Prolotherapy. 2012;4(March):855-869.

[6] Pak J. Regeneration of human bones in hip osteonecrosis and human cartilage in knee osteoarthritis with autologous adipose-tissue-derived stem cells: A case series. J Med Case Rep. 2011;5(1):296. doi:10.1186/1752-1947-5-296

[7] Rodriguez JP, Murphy MP, Hong S, et al. Autologous stromal vascular fraction therapy for rheumatoid arthritis: Rationale and clinical safety. Int Arch Med. 2012;5(1):5. doi:10.1186/1755-7682-5-522313603

[8] Gentile P, Orlandi A, Scioli MG, Di Pasquali C, Bocchini I, Cervelli V. Concise Review: Adipose-Derived Stromal Vascular Fraction Cells and Platelet-Rich Plasma: Basic and Clinical Implications for Tissue Engineering Therapies in Regenerative Surgery. Stem Cells Transl Med. 2012;1(3):230-236. doi:10.5966/sctm.2011-0054

[9] Shabani I, Haddadi-Asl V, Soleimani M, Seyedjafari E, Hashemi SM. Ion-Exchange Polymer Nanofibers for Enhanced Osteogenic Differentiation of Stem Cells and Ectopic Bone Formation. ACS Appl Mater Interfaces. 2014;6(1):72-82. doi:10.1021/am404500c

[10] Ihwah A, Deoranto P, Wijana S, Dewi IA. Comparative study between Federer and Gomez method for number of replication in complete randomized design using simulation: Study of Areca Palm (Areca catechu) as organic waste for producing handicraft paper. In: IOP Conference Series: Earth and Environmental Science; 2018. doi:10.1088/1755-1315/131/1/012049

[11] Sengupta P. The laboratory rat: Relating its age with human's. Int J Prev Med. Published online 2013.