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Effects of Amnion Lyophilization Sterile Radiation against the Number of Osteoblasts and Osteocytes in Nonunion Fractures: An Experimental Research Study

Ahmad Fauzi[1], Alvarino Alvarino[2], Yanwirasti Yanwirasti[3], Roni Eka Sahputra[2], Suharmanto Suharmanto[4]*

[1]Department of Surgery, Faculty of Medicine, Universitas Lampung, Bandar Lampung, Indonesia; [2]Department of Surgery, Faculty of Medicine, Universitas Andalas, Padang, West Sumatera, Indonesia; [3]Department of Anatomy, Faculty of Medicine, Universitas Andalas, Padang, West Sumatera, Indonesia; 4Department of Public Health, Faculty of Medicine, Universitas Lampung, Bandar Lampung, Indonesia

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***Correspondence:** Suharmanto Suharmanto, Faculty of Medicine, Universitas Lampung, Bandar Lampung, Indonesia. E-mail: ahmadfauzi_dr@yahoo.co.id
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Abstract

BACKGROUND: The amnion lyophilization sterile radiation helps the process of bone regeneration. Bone density with amnion lyophilization sterile radiation intervention was seen from histomorphometry and tomography results which showed a density similar or higher than the research group that was administered collagen membranes.

AIM: The aim of this study was to determine the effects of amnion lyophilization of sterile radiation on the number of osteoblasts and osteocytes in nonunion fractures.

METHODS: This study used an experimental post-test-only control group design that involved white rats of the Sprague–Dawley strain. The samples used were 8-week-old rats, weighing 250–350 g of the male sex. The study was conducted on two groups (control and intervention) of rats with a total of seven rats per group. The osteotomy procedures, periosteal stripping, cauterization, and internal fixations were conducted. Independent T-test was performed to assess number of osteoblasts and osteocytes.

RESULTS: The mean value of osteocytes in the control group was 00.00 ± 00.00 and in the amnion lyophilization sterile radiation group it was 87.14 ± 44.85 with a p = 0.002. The mean value of osteoblasts in the control group was 50.06 ± 5.76 and in the amnion lyophilization sterile radiation group it was 283.63 ± 22.86. This study showed that there were differences in the number of osteocytes and osteoblasts in the two groups with a p = 0,000.

CONCLUSION: Amnion lyophilization sterile radiation is effective in increasing the number of osteoblasts and osteocytes in nonunion fractures.

Introduction

In general, the fracture healing process is affected by the number of osteoblasts and osteocytes. During the bone formation process, osteoblasts (the main bone-forming cells) synthesize bone matrix proteins, including alkaline phosphatase, osteocalcin, type I collagen, calcium, and phosphate-based minerals. These proteins and minerals would then be deposited into the extracellular matrix to form strong and mineralized tissues that would be differentiated into osteocyte cells [1]. This differentiation is tightly controlled by specific extracellular regulatory proteins such as growth factors, hormones, vitamins, and cytokines [2].

Osteoblasts are surrounded by extracellular matrix proteins including type I collagen and fibronectin primarily through integrin-β1. Osteoblasts can express integrin-β1, and the involvement of integrin-β1 in osteoblasts by specific antibodies or ligand matrix, induces proliferation, differentiation, and the synthesis of the osteoblast bone matrix through tyrosine kinase, especially focal adhesion kinase. This suggests that bone formation is also tightly regulated by adhesion in the bone matrix [3].

Osteocytes are the result of osteoblast differentiation. In immature bones, osteocytes make up about 95% of total bone cells. Osteocytes are the only cells embedded in the extracellular matrix of bones. The physiological function of osteocytes is not well understood yet, but some scientists have stated that osteocytes work as lining cells and regulate the exchange of mineral ions between interstitial and extracellular fluids. Thus, osteocytes help to maintain the local ion environment to be suitable for the mineralization of the bone matrix. Based on its unique location in this bone matrix, osteocytes are responsible for detecting micro-damage and for initiating the repair process [4]. Moreover, osteocytes play an important role in the formation and remodeling of bones through the sclerotin mechanism.

The amnion lyophilization sterile radiation (ALSR) approach has been developed by the National

Nuclear Power Agency and stored in the Batan Research Network Bank. It consists of amnion that has been processed by the lyophilization method that is then dried and sterilized with γ beam radiation (gamma rays) to inhibit the activity of microorganisms. This method can minimize tissue damage and preserve organic compounds such as the growth factors contained in amnion. In fracture healing, the scaffold factor depends on the type and source of living cells and components of the extracellular matrix of the scaffold. One of the scaffold biomaterial used is the amniotic membrane. Matrix extracellular membranes in the amniotic membrane and its components, such as factor growth, suggesting that the amniotic membrane is a very good candidate for good to use as a scaffold in the healing process. Besides that, amniotic membrane is a biomaterial that can be easily obtained, processed and transported [5].

The growth factors contained in ALSR include vascular endothelial growth factor (VEGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), bone morphogenetic protein-2 (BMP-2), bone morphogenetic protein-4 (BMP-4), and scaffolding cells such as collagen and laminin. With this method, ALSR is believed to function as an osteoconductive/scaffold, osteogenic, and growth factor in the fracture area. Importance of this study is radiation sterilized lyophilized amnion (ALSR) and is expected to help overcome problems in the fracture healing process, namely, nonunion along with its complications. Evidence on the effect of ALSR in assisting the process fracture healing and increased expression of osteoblasts, osteocytes, VEGF, and cells CD34, especially in the nonunion condition, has not been found. Therefore, research is needed to prove this [6].

Preliminary study was conducted to determine the non-union fracture model. The results showed that osteotomy with cauterization at the tip of the fracture fragments can lead to failure of new bone tissue formation. Based on research Winanto *et al.* (2013), on bone density defects in rabbit femurs, it has been proven that ALSR helps the process of bone regeneration. Bone density with ALSR intervention was seen from histomorphometry and tomography results which showed a density similar or higher than the research group that was administered collagen membranes. Higher densities compared to the results were obtained from the control group (without membranes). Moreover, currently, ALSR is cheap and easy to obtain [7].

The goal of this study was to determine the effects of amnion lyophilization of sterile radiation (ALSR) on the number of osteoblasts and osteocytes in nonunion fractures.

Methodology

Study design

This study used an experimental post-test-only control group design that involved white rats of the Sprague–Dawley strain. The research began with a pilot study to determine the nonunion model. The samples used were 8-week-old rats, weighing 250–350 g of the male sex. The study was conducted on five groups of rats with a total of seven rats per group, and thus the total number of animals needed for this study was 35, 21 of which were used for the preliminary study (pilot study) and 14 were for the research with ALSR.

Research subjects

After the preliminary research, a nonunion model was obtained for further research with ALSR. In the study with ALSR, the rats were divided into two groups. Group 1 was experienced nonunion in a preliminary study and Group 2 was given amnion lyophilization sterile radiation. The two groups were euthanized in the 4^{th} and 8^{th} weeks, respectively, for histological examination for VEGF and CD-34 expression.

Surgical procedure

The osteotomy procedures, periosteal stripping, cauterization, and internal fixations were conducted according to the following procedures: (1) The rats that were selected for this study were divided into five groups. In the preliminary study, there were three groups, while in the main study there were two groups. Each group contained seven rats; (2) each rat underwent procedures according to their respective groups; (3) the anesthesia used in this study were ketamine (Ketamil (R), Troy laboratories PTY limited Australia) that was 80 mg/kg of the subject's body weight, and xylazine (Seton 2%$^{(R),}$ Laboratorios Calier S.A. Spain), that was 10 mg/kg of the subject's body weight. Anesthesia was administered intraperitoneally; and (4) after the right thigh region of the rat's fur was shaved, in a tilted position of the right side at the top, under aseptic conditions using 10% Povidone-iodine and 70% alcohol and covered with a sterile duct cloth, a 20 mm anterolateral approach was taken on the right thigh. The vastus lateralis muscle was separated from the biceps femoris muscle, and the patellar was dislocated towards the medial. Then, the lateral vastus muscle and biceps femoris were elevated by keeping the periosteum fixed intact along the surface of the femur bone; (5) osteotomy was performed in the middle of femur diaphysis using a saw in the shape of an A-semicircular letter with a saw eye size of 1 mm [8], [9]; (6) in the preliminary research, cauterization and periosteal stripping were performed using a sharp knife, the incision was as far as 8 mm

each from the fracture line to proximal and distal; (7) in the main study, periosteal stripping corresponded to the results in the preliminary studies that led to non-union; (8) internal fixation was performed by using intramedullary K-wires size 1.4 mm retrogradely through the intercondylar femur; (9) soft tissue was sewn with catgut 3.0 and the skin was sewn with silk 3.0 [10]; and (10) all rats were administered antibiotics (Ampicillin 50 mg/kg of the subject's body weight/day) and analgesics (paracetamol 50 mg/kg of the subject's body weight/day) for a week.

Euthanasia, network retrieval, and preparation

Euthanasia was done by intraperitoneally administering ketamine-xylazine cocktails at doses of 10−20 mg/kg of the subject's body weight [11]. The femur diaphysis segment was then released from the surrounding tissue for preparation. Each segment of the femur bone was fixated in 10% neutral buffered formalin for 48 h at room temperature. The volume of the fixation fluid is at least 5–10 times the volume of the tissue. Then, decalcification of the femur pieces was conducted for 24 h with a commercial solution Plank Rychlo containing $AlCl(H_20)_6$ 2.1728 g, HCl 3% 30 mL, 30 mL of 5% formic acid, and 30 mL of dH_2O. The next process was dehydration, the samples were soaked in alcohol with concentrations of 70% and 95% for 30 min each, then they were soaked in alcohol 100% for 3 h, and then Xylol for 30 min. The next step would be the clearing process; the preparations were soaked with xylol solution twice, 1st for 1 h (Xylol 1), and then 2 h (Xylol 2). The next step is the infiltration and embedding process, the samples were placed in liquid paraffin at a temperature of 45°C twice for every 3 h, then they were placed in an oven or incubator with a temperature of 55−57°C for 12 h (18.00−06.00 am) [8], [9], [12]. After that, the hard paraffin blocks were cut using a transverse microtome. Next, each material from the paraffin block was made into a slide and given H&E staining (three slides).

Assessment of osteoblast and osteocyte expression

The formation of new bone can be detected by all histological staining methods at the proximal and distal ends of the fracture zone. Bones appear as compact structures in dark red. Connective tissue is displayed as cell tissue and collagen fibers are structured in bright pink. Cartilage appears in a gray-pink mixture, but all tissues must be morphologically separated. Scaffold material has also dissolved during the depolymerization process. Therefore, the white spots on the histological section present the original implant material. The number of new calcified bones in the fracture area as well as inside the porous implant can be easily estimated by H&E staining [13]. The examination of histopathology in this study was conducted by using a standard Zeiss Primo Starlight microscope with an Infinity-1 camera conducted by veterinary pathologists of the Faculty of Veterinary Medicine, Bogor Agricultural Institute.

Analysis data

The research data are normally distributed using Kolmogorov–Smirnov test. Independent T-test was performed to assess number of osteoblasts and osteocytes. The characteristics of the research subjects' average size are exhibited in a table. The results of the analysis are displayed with their mean ranks between control groups and treatment groups [13].

Results

The number of osteocytes and osteoblasts was more in the ALSR group than the control group. Tables 1 and 2 show that the ALSR group had significantly different numbers of osteoblasts and osteocytes than the control group.

Table 1: Analysis of osteocyte in the amnion lyophilization sterile radiation study

Group	n	Osteocyte (mean ± SD)	p	CI 95%
Control	7	00.00 ± 00.00	0.002*	87.14 (45.66–128.63)
ALSR	7	87.14 ± 44.85		

*Independent t-test. SD: Standard deviation, ALSR: Amnion lyophilization sterile radiation, CI: Confidence interval.

The study showed that the group administered with ALSR had a higher number of osteoblasts than the control group (283.63 ± 22.86 vs. 50.06 ± 5.76) with a p = 0.000. Statistically, ALSR administration in nonunion fracture cases can affect the number of osteoblasts. This was evidenced by the clinical assessment of bone tissue between the ALSR group and the control group. More than 50% of the samples in the ALSR group showed union conditions, while in the control group all bone samples remained in a nonunion condition (mean rank was 10.71 vs. 4.29).

Table 2: Analysis of osteoblast in the amnion lyophilization sterile radiation study

Group	n	Osteoblast (mean ± SD)	p	CI 95%
Control	7	50.06 ± 5.76	<0.001 *	233.57 (212.35–254.80)
ALSR	7	283.63 ± 22.86		

*Independent t-test. SD: Standard deviation, ALSR: Amnion lyophilization sterile radiation, CI: Confidence interval.

Discussion

The number of osteocytes and osteoblasts was more in the ALSR group than the control group. Tables 1 and 2 show that the ALSR group had significantly

different numbers of osteoblasts and osteocytes than the control group.

Osteoblasts are derived from bone marrow stromal osteoprogenitor cells and play a role in the process of matrix synthesis and bone mineralization [14]. Osteoblasts are stimulated through increased osteoid secretion and the inhibition of the ability of osteoclasts to break down bone tissue. Osteoblasts are one of the materials contained in ALSR and are products of the differentiation of chondrocytes.

The study showed that the group administered with ALSR had a higher number of osteoblasts than the control group (283.63 ± 22.86 vs. 50.06 ± 5.76) with p = 0.000 (Table 2). Statistically, ALSR administration in nonunion fracture cases can affect the number of osteoblasts. This was evidenced by the clinical assessment of bone tissue between the ALSR group and the control group. More than 50% of the samples in the ALSR group showed union conditions, while in the control group all bone samples remained in a nonunion condition (mean rank was 10.71 vs. 4.29). This is in line with the previous research which showed that an increase in the number of osteoblasts has an effect on the speed of bone healing in fractures [15]. Osteoblasts that differentiate from chondrocytes can regulate the activity of osteoclasts and the deposition of the bone matrix. Moreover, osteoblasts trapped in the bone matrix are rich in alkali phosphatase, an organic phosphate-breaking enzyme, and have parathyroid hormone receptors and estrogens that can trigger bone compaction with the help of growth, thyroid, and sex hormones [16].

Furthermore, the osteoblast findings on the H&E stains suggest that the administration of ALSR in fracture conditions may increase the number of osteoblasts so that the formation process of new bone tissue and bone compaction can occur. Moreover, this suggests that the bone condition in the ALSR group will be denser compared to the control group.

In addition to osteoblasts, osteocytes are also a part contained in ALSR. Osteocytes and osteoblasts are the results of differentiation from the chondrocytes in the ALSR tissue. Osteocytes are interrelated through plasma membrane extension. The role of osteocytes as mechanoreceptors can instruct osteoblasts to form bones and osteoclasts to absorb parts of bone [17].

This study showed that the group administered with ALSR had a higher number of osteocytes than the control group (87.14 ± 44.85 vs. 00.00 ± 00.00; CI 95% 87.14 (45.66−128.63) with a p = 0.002 (Table 1). Therefore, this statistically suggests that ALSR administration in nonunion fracture cases can affect the number of osteocytes. This is supported by the microscopic findings of bone tissue between the ALSR group and the control group which found that during this study, 100% of the samples in the ALSR group experienced faster looping maturation than in the control group with a mean rank of 11 versus 4.

Furthermore, these findings are supported by previous study which found that osteocytes play a role in the healing process of fractures in rats [18]. A decrease in the number of osteocytes in fracture conditions can extend the healing time of fractures and increase the incidence of bone growth failure. Moreover, osteocytes can regulate the exchange of mineral ions between the interstitial and extracellular fluids so that the appropriate local ion environment for the mineralization of the bone matrix can be formed. The location of osteocytes in the extracellular matrix can detect microdamage, which results in the occurrence of IGF-1 expression and the stimulation of both resting osteoblasts to become active, thereby inducing the formation of new osteoblasts [13]. This study showed ALSR administration in nonunion fracture cases can affect the number of osteocytes. The limitation of this study is the use of rats that have not involved other animals and in larger numbers. This study showed that ALSR with different experimental animals and on a larger scale can strengthen the results of this study so that ALSR can be used as a therapy in the healing process of nonunion fractures.

Statement of Ethics

This study was ethically approved by the research ethics board of Medical Faculty of Lampung University and obtained approval number 187/UN26.18/PP.05.02.00/2022.

Author Contributions

Ahmad Fauzi conceived of the presented idea. Ahmad Fauzi and Alvarino developed the theory and performed the computations. Yanwirasti and Suharmanto verified the analytical methods. Ahmad Fauzi and Roni Eka Sahputra encouraged to investigate intervention and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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