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Research Article

Evaluation of TNF-A Expression After Adipose-Derived Mesenchymal Stem Cells (ASCs) Treatment Combined with Freeze-Dried Amniotic Membrane Wrapping in Rat Sciatic Nerve Defect Model

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ABSTRACT

Nerve regeneration presents a clinical challenge to biomedical engineering. Recently, adipose-derived stem cells (ASCs) are known as one of the strategies in tissue regeneration. ASC has good potential to differentiate into neurogenic cells with capability in producing neuronal markers. This study aimed to evaluate the role of application of ASCs on sciatic nerve healing in association with the expression of TNF-α. Thirty-six Spraque-Dawley rats weighing approximately 250 g were divided into 6 control group and 6 experimental groups (n:3). The 6 experimental group was divided into evaluation on the 1st,3rd,5th,7 th 14 th and 21 st day after surgery. ASCs was applied to the injury site (dose 1 x 10^6 cells/nerve) with the freeze-dried amniotic membrane. Histopathology was evaluated with Hematoxylin-eosin and Masson's Trichrome staining. Application of ASCs could down-regulate the TNF-α expression, decrease inflammation and fibrous collagen formation during nerve healing. Moreover, it can conclude that ASCs with freeze-dried amniotic membrane wrapping can decrease the inflammation and perineural fibrous collagen formation during Sciatic nerve healing.

Keyword: amnion, healing, neuron marker, regeneration.

INTRODUCTION

Injury to the peripheral nerve is common problems, associated with long-term disability and have a devastating impact on quality of life[1]. Nerve regeneration presents a clinical challenge to biomedical engineering. It is known that formation of fibrotic tissue is considered as one of the problems that interfere the process of nerve regeneration[2]. It has been suggested that the fibrous tissue inhibits nerve regeneration by acting as a physical barrier that neurons cannot penetrate. Fibrocytes secrete a variety of cytokines including TNF-α that promote the proliferation, migration, and extracellular matrix production by the local fibroblasts. TNF- α known as one of the cytokines that promote fibrosis formation during tissue regeneration including skin, muscle and cornea[3,4]. However, the role of this cytokine in fibrosis formation during nerve regeneration is not well understood.

Recently, adipose-derived stem cells (ASCs) are known as one of the strategies in tissue regeneration[5]. ASCs are an abundant population of multipotent progenitor cells that reside in adipose tissue[6]. Application of ASC has recently been suggested as a possible novel therapy in peripheral nerve regeneration[7]. ASC has good potential to differentiate into neurogenic cells with capability in producing neuronal markers[8,9]. This study is aimed to evaluate the role of TNF-α in inflammation and fibrous collagen formation during nerve regeneration after application of ASCs.

MATERIALS AND METHODS

Study design and animals

Thirty-six Spraque-Dawley rats weighing approximately 250 g were divided into 6 control group and 6 experimental groups (n:3). The 6 experimental group was divided into evaluation on the 1st,3rd,5th,7th 14th and 21st day after surgery. The rats had free access to standard rodent laboratory food and tap water.

Cell isolation and culture

Adipose tissue samples were obtained from intraabdominal fat tissue of 3-month-old Spraque – Dawley rat under anesthesia. The adipose tissue was extensively washed with PBS to remove blood and fibrous material and vessels were carefully dissected and discarded.The remaining tissue was finely minced and digested with 0.1% of Collagenase Type I (Gibco, California, USA) for 60 min with gentle agitation. Enzyme activity was neutralized with a twofold volume of standard medium containing Dulbecco's modified Eagle medium (DMEM, Gibco) with 20% of fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and centrifuged for 12 min at 400×g. The supernatant containing the lipid droplets was discarded. The stromal vascular fraction settled at the bottom was resuspended in standard medium and seeded in culture dishes . Stromal vascular fraction cultures were incubated at 37 °C in a 5% CO_2 atmosphere. After 48 h, no adherent

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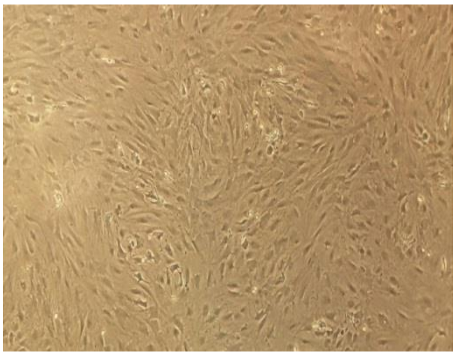

Figure 1: ASC after 80% confluence under inverted microscope.

cells were removed. When they reached 80% of confluence, adherent cells were trypsinized (0.25% at 37 °C for 5 min,Sigma), harvested, and washed with standard medium to remove trypsin and then expanded in larger dishes. A homogenous cell population of ASCs was obtained after 3-4 weeks of culture. Cells at early passages (4) in culture were used for the experiments. Confirmation of mesenchymal stem cells was performed by immunocytochemistry with positive marker (CD 44,CD 90,CD 105) and negative marker (CD 14, CD 19, CD 54).

Freeze-dried amniotic membrane

The freeze-dried amniotic membrane was produced by Tissue Bank unit of Dr. Soetomo General Hospital, Surabaya, Indonesia. We performed histology evaluation for the acellularity status of this membrane with Hematoxylin-eosin staining.

Surgical procedure

Animals were anesthetized by intramuscular administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Animal Ethics Committee of the Gadjah Mada University. The University Research Council approved all experiments. Following surgical preparation, the right sciatic nerve was exposed through a gluteal muscle incision and sciatic nerve was injured with a knife and repaired with 7/0 monofilament non-absorbable suture. After careful homeostasis, the muscle was sutured with resorbable 4/0 sutures, and the skin was closed with 3/0 nylon. In the control group, the sciatic nerve only repaired without any specific treatment. In the experiment group, after being repaired the nerve was wrapped with freeze-dried amniotic membrane and ASC was applied into the repaired nerve. After the expected day of evaluation has been reached, all animal were evaluated for clinical outcome with walking track analysis. Afterward, the animals were anesthetized and euthanized with cervical dislocation technique for further histopathology evaluation.

Histological studies

The nerve in the area of repair of the control and experiment group were harvested. They were fixed in 10% formaldehyde, dehydrated through an ethanol series and embedded in paraffin. The nerves were sectioned in 4 µm sections in the longitudinal plane then stained with Hematoxylin-eosin for general evaluation and Masson's Trichrome staining for fibrous collagen tissue evaluation.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, blocked to prevent nonspecific antibody binding and incubated with primary antibodies at 4 °C overnight. Following a PBS washing, the plates were incubated with avidin/biotin blocking kit. The primary antibodies used were anti TNF-α for nerve specimens and anti CD-14, anti CD-19, anti CD-44,anti CD-54,anti CD-90, and anti CD-105 for ASCs culture (®Bioss). Dishes were examined under the fluorescence microscope (NIKON ECLIPSE E400).

RESULTS

ASC isolation and culture

Adipose-derived Stem Cells culture was performed until 4th passage (3-4 weeks) and reach 80% of confluence. Afterward, the cells then ready for evaluation of Mesenchymal stem cells marker on immunocytochemistry.

Amniotic membrane

The freeze-dried amniotic membrane that produced by Tissue Bank unit of Dr.Soetomo General Hospital was then evaluated for the status of acellularity with HE staining.

Histopathology evaluation

Under microscopic evaluation, all specimens showed higher fibrosis in repair area of the control group, compared to experiment group with Masson's Trichrome staining. Evaluation of collagen formation was guided by Ersoy et al[10] scoring system: Score 0: small scattered areas of green/blue staining, Score 1 :for thin bands of green/blue staining, score 2: for thicker, connected bands of green/blue staining, score 3: for thick and dense areas of green/blue staining. Collagen formation was significantly reduced in experiment group compared to control group.

Immunohistochemistry of TNF-α

Expression of TNF-α categorized into Score 0 (negative), Score 1 (Sporadic), 2 (Focal), 3 (General)[11]. In control group showed mean score 2,67 in day 1st,3rd,5th after surgery and decrease to mean score 2,33 in day 7th and 14th, after that decrease to mean score 0,33 at day 21st after surgery. In experiment group, the result showed mean score 2,67 at day 1st, decrease gradually to mean score 0,33 at day 7th. However, re-increase of expression occur at day 14th and 21st after injury.

DISCUSSION

Tumor necrosis factor alpha (TNF-α) is a cytokine that plays an important role in numerous physiological and pathological processes including immunity and inflammation. Elevated TNF-α is documented in various neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and also after injury, in which it is thought to exhibit a pro-inflammatory function[12].

A study performed by Nadeau et al., demonstrates that sciatic nerve injury induces a rapid production and release of IL-1 and TNF and causes infiltration of neutrophils

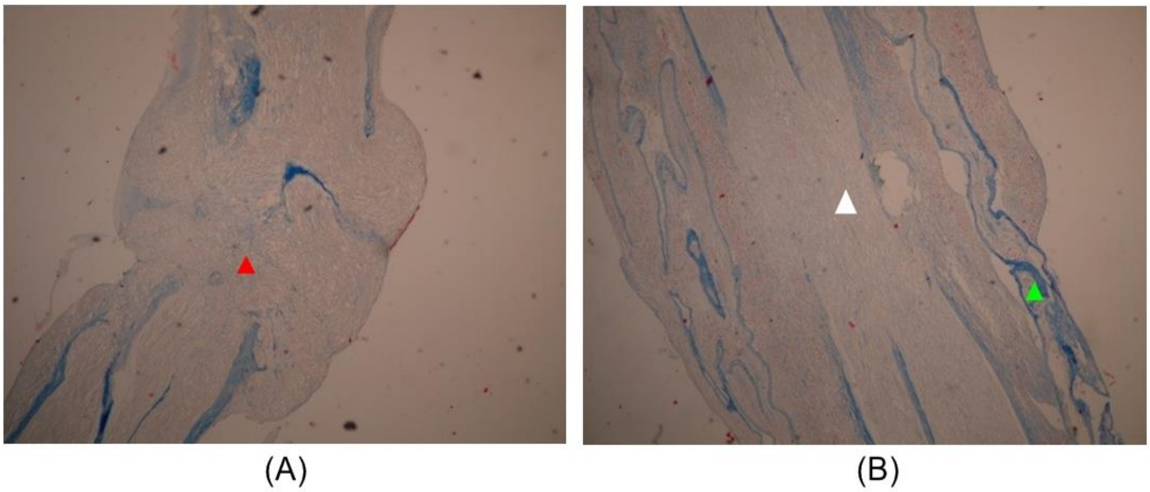

Figure 2: Histopathology (Masson's Trichrome) after nerve repair: (A) Control group; (B) experiment group. Note: a blue color sign of fibrous tissue, red triangle is fibrous collagen at nerve anastomosis site with poor axon continuation, white triangle is good axon continuation without fibrous collagen formation, green triangle is Collagen of amniotic membrane.

Table 1: Scoring after nerve repair for collagen formation evaluation[10].

Control Group	Score	Treatment Group	Score	p
Day-1	1.33 ± 0.57	Day-1	2.67 ± 0.57	0.0478*
Day -3	3.00 ± 0.00	Day -3	2.67 ± 0.57	0.37
Day -5	3.00 ± 0.00	Day -5	1.33 ± 0.57	0.007*
Day -7	3.00 ± 0.00	Day -7	1.67 ± 0.57	0.016*
Day -14	3.00 ± 0.00	Day -14	0.67 ± 0.57	0.002*
Day -21	3.00 ± 0.00	Day -21	0.67 ± 0.57	0.002*

Table 2: Result of inflammation scoring according to Ersoy et al[10].

Control Group	Mean ± SD	Treatment Group	Mean ± SD	p
Day-1	2.33 ± 0.57	Day-1	2.67 ± 0.57	0.519
Day -3	3.00 ± 0.00	Day -3	2.33 ± 0.57	0.116
Day -5	3.00 ± 0.00	Day -5	2.33 ± 0.57	0.116
Day -7	3.00 ± 0.00	Day -7	1.67 ± 0.57	0.016*
Day -14	3.00 ± 0.00	Day -14	1.67 ± 0.57	0.016*
Day -21	3.00 ± 0.00	Day -21	1.67 ± 0.57	0.016*

and proinflammatory monocytes/macrophages into the distal stump of the nerve[13]. Tumor necrosis factor-a (TNF-α) is produced by a wide variety of cells, particularly monocytes, activated macrophages, and fibroblasts[14,15]. TNF-α directly upregulates p-selectin on endovascular cells, which then interact with integrins leading to leukocyte infiltration[16].

Following injury, TNF-α expression and release are induced within minutes up to a few hours and persists during the following days in damaged tissue[17]. This condition is consistent with the result of this study in the control group which showed increased expression since day 1st until day 5th after surgery, and gradually decrease from day 7th until day 21st after injury. Expression of TNF-α was down-regulated in experiment group especially until day 7th after surgery. This result indicates that after application of ASCs and amniotic membrane at the injury site can decrease the inflammatory process significantly at day 21 which showed the result of Ersoy et al scoring system.

The previous study performed by Lei et al., showed that antagonism of TNF-α result in a reduction of neuroinflammation and improve neurological recovery after intracerebral hemorrhage[16]. Although its exact role remains unclear, TNF-α does not serve as a simple 'biomarker' of inflammation, but rather plays a central role in mediating and extending neuronal injury after insult. TNF blockade can inhibit NF-kB activation, promoting increased apoptosis of inflammatory cells, and reducing the inflammatory response[18].

A study performed by Khan et al., Neutralization of endogenous TNF-a reduces glomerular inflammation, and tubulointerstitial scarring, with preservation of renal function, in experimental glomerulonephritis[14]. The mechanism of TNF-α on promoting scar tissue had been investigated[19]. They found that fibroblasts stimulated by TNF-α secrete the small leucine-rich proteoglycan called

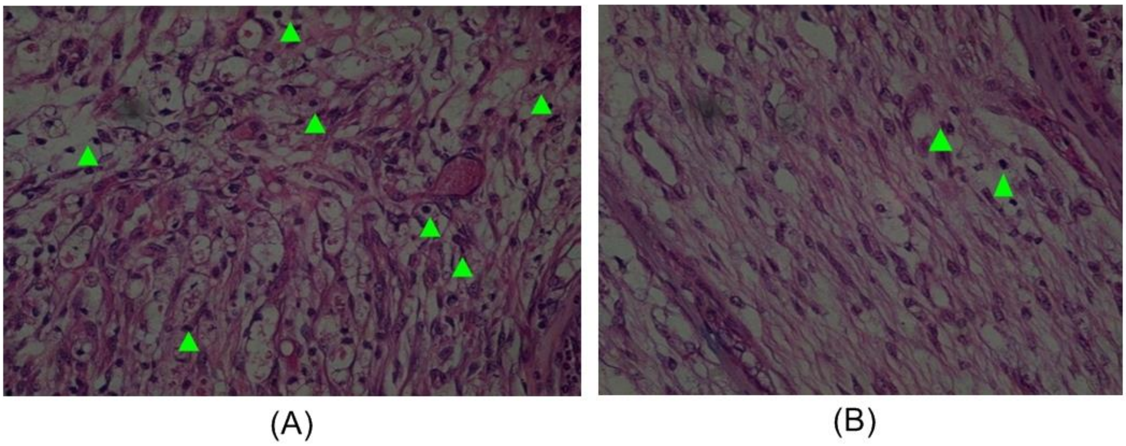

Figure 3: Comparison of inflammation at day 7 after injury between the control group (A) and experiment group (B). Note abundant of inflammatory cells (Green triangle) on the control group.

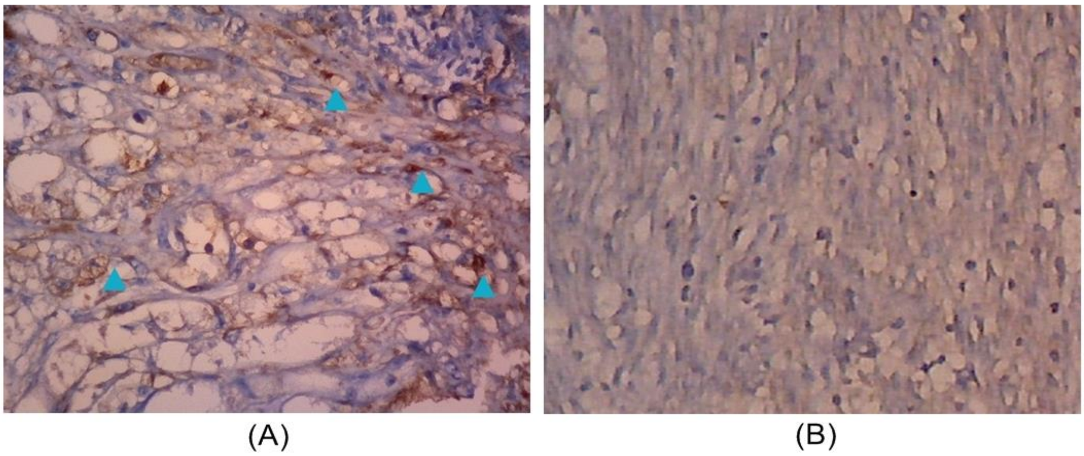

Figure 4: Expression of TNF-α at day 7 after injury in the control group (A) and experiment group (B). Note Blue triangle: Positive expression of TNF-α.

Table 3: Result of TNF-α expression scoring by Nooijen et al[11].

Control group	Score	Treatment group	Score	p
Day-1	2,67 ± 0,57	Day-1	2,67 ± 0,57	1,0
Day-3	2,67 ± 0,57	Day-3	1,33 ± 0,57	0,047*
Day-5	2,67 ± 0,57	Day-5	0,67± 0,57	0,013*
Day-7	2,33 ± 0,57	Day-7	0,33 ± 0,57	0,013*
Day-14	2,33 ± 0,57	Day-14	1,00 ± 1,00	0,116
Day-21	0,33 ± 0,57	Day-21	2,67 ± 0,57	0,08

lumican and that lumican promotes fibrocyte differentiation and fibrous formation. Several studies stated that TNF- α has some early protective effect on nerve tissue after injury. A study by Mac Nair et al., found that long-term exposure to TNF-α is toxic to retinal root ganglion cells (RGCs)[20]. However, TNF-α appears to initiate protective pathways that improve RGC survival immediately following optic nerve injury. The mechanism of protection may be occurring through TNF-α activation of Müller cells. This result is similar to the present study,which TNF-α decreased significantly at day 3,5 and 7 after injury. However, at day 1 after injury, TNF-α still expressed with mean score 2,67. Thus, the role of TNF-α as early protective cytokine was still present after ASCs treatment on peripheral nerve injury.

CONCLUSION

Application of ASCs with freeze-dried amniotic membrane wrapping can decrease the inflammation and perineural fibrous collagen formation during Sciatic nerve healing.

CONFLICT OF INTEREST

Author declare there is no conflict of interest

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