The Role of Collagen Scaffold and Stromal Vascular Fraction on Healing Process in Growth Plate Injury (SOX9 and Histological Examination)

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Abstract

BACKGROUND: Pediatric skeletal trauma presents a distinct approach to its treatment because of its unique auses and possibility of its troublesome complications. One of its major complications is growth plate injury which may disturb the longitudinal growth of child’s bone.

AIM: In this study, the author combined stromal vascular fraction (SVF) and collagen scaffold as biomaterial for future treatment of physeal injury in skeletally immature patients using SOX9 and histological examination as a marker.

METHODS: The study was conducted experimentally on Rattus norvegicus growth plate based on Erickson study, and the SVF was from R. norvegicus fat tissue with ZUK method. Histological examination was evaluated with 8 times magnification and SOX9 from the growth plate was measured with enzyme-linked immunosorbent assay.

RESULTS: Histological examination showed that the best result was obtained in SVF + collagen group judging by the bony bridge diameter. The result was differed significantly from the positive control group with p < 0.05 in Tukey test. The result from SOX9 level measurement shows that all of the treatment groups SOX9 level almost the same as normal value (negative control group) and it is significantly differ from the positive control group with p < 0.05 in Mann–Whitney U-test.

CONCLUSIONS: This study showed that the combination of collagen and SVF had a great effect on healing process in growth plate injury.

Introduction

Injuries incurred by skeletally immature patients are unique both in their causes and gravity of their consequences. Physis, or growth plates, are cartilaginous regions at the ends of children’s long bones which act as primary sites of bone elongation. Growth plate injury may result from trauma, infection, metabolic abnormalities, or malignancy. The major concern with growth plate injury is the damaged cartilage in growth plate that could be replaced by bony repair tissue, forming a “bony bridge.” Depending on the size and location of the injury within the growth plate, the bony bridge may cause asymmetric growth arrest with subsequent angular deformity or complete cessation of longitudinal growth. The latter is a devastating outcome for children that have not yet reached their full height. The current treatment involves surgical resection of the bony bridge and replacement with an interpositional material to preserve normal growth in the remaining growth plate. Bony bridge reformation and additional growth effects, however, remain major complications of bar excision.

The current management, surgical or non-surgical, has significant limitations and may result in further morbidity in the form of additional surgeries, further development of growth arrest, or progression of angular limb deformities. As such, there is a dire need to develop new treatment strategies for growth plate injury that not only prevents bony bridge formation but also leads to regeneration of healthy growth plate cartilage.
thus restoring normal bone elongation. Methods under investigation include modulating intrinsic injury pathways to prevent osteogenesis as well as recruiting or adding stem cells for regenerating damaged growth plate [1].

The morbidity and unpredictable nature of growth plate injuries combined with current therapeutic limitations establish a critical need to develop effective treatments for affected children. Successful treatments should prevent bony bridge formation and simultaneously regenerate growth plate and restoring normal bone elongation. Tissue engineering approaches utilizing stem cells, growth factors, and biomaterials have the potential to overcome the shortcomings of current approaches by restoring growth plate and, thus, may play an important role in the treatment of growth plate injuries. In this study, we used mesenchymal stem cell from stromal vascular fraction (SVF) and collagen scaffold as biomaterial to make a regeneration of growth plate injury with SOX9 and histological examination as a marker [2].

**Methods**

This study was an experimental *in vivo* on *Rattus norvegicus* growth plate, with post-test only control group design. Ethical permission was granted from the Ethical Committee of Research in Medical Science, Universitas Brawijaya. This study was conducted at Biomedical Laboratory of Faculty of Medicine, Universitas Brawijaya, Malang, from January to May 2019. There were two groups of treatment which received SVF only and combination of SVF and collagen. The growth plate sample was taken from the proximal tibia of male Wistar strain of *R. norvegicus* which aged 6 weeks. The number of sample is nine rats in each group. The inclusion criteria were a healthy male Wistar strain *R. norvegicus* rat which aged 6 weeks while the exclusion criteria were deformity in rat’s extremity, infection, and deceased rat during the treatment. Growth plate injury model of *R. norvegicus* is based on Erickson study [3]. SVF was originated from *R. norvegicus* fat tissue with ZUK method [4].

The rats were sacrificed with decapitation method. The adipose tissue was obtained from epidymal and perirenal fat. The rats were positioned in supination. Skin incision was done longitudinally and widely until it opens up the abdominal region. The testicle was obtained and cleared from the surrounding fatty tissue. The fatty tissue was then compiled. The perirenal fat was obtained from innervation of retroperitoneal fat pad. The fatty tissue was then washed with phosphate buffer saline which contains 10% of antibiotic and antimycotic. It was then cut with knife into small fragments. The fragments were then immersed at collagenase solution type IA at 0.075% concentration for 30 min at 37°C. The digested tissue was then filtered with 100 µm of mesh filter and centrifuged at 1200 rpm speed for 10 min at 20°C. The supernatant was tossed out. The final results were a suspension containing heterogeneous cells with $2 \times 10^6$ of cells in every gram of fatty tissue.

The next step is surgical technique to make growth plate injury model in rats. Rat’s lower extremity from medial malleolus up to the hip was cleaned and prepared. Skin incision was made about 1 cm along the anteromedial proximal tibia, started from the distal end of the medial femoral condyle. The skin was stretched to the bone deep to it and the foot was held steady. A 0.5 cm incision was made through the fascia and soft tissue at the anteromedial aspect of the proximal tibia from the growth plate until the inferior part of the skin incision. Gently dissect the fascia and soft tissue from the tibia with a surgical knife. The tibia cortical bone was drilled at the diaphysis with a 2 mm Kirschner wire to make a cortical window. The same cortical window was made again in parallel with the insertion of the distal semitendinosus. The drill was held perpendicularly to the tibia diaphysis and slowly pushed for 2 mm. Hemorrhage control was done by wound pressure. The growth plate injury was done by drilling the growth plate using 1,8 mm Kirschner wire. The wound was cleaned using 3 mL of sterile saline. The wound was closed with suture. Moist dressing was then applied and phenylbutazone at the dose of 10 mg/day was given as analgesic.

The bone bridge diameter was counted using histological examination with hematoxylin-eosin (HE) staining with 8 times magnification. The examiner was pathologist from Pathology Anatomy Department of Universitas Brawijaya. SOX9 concentration was measured from growth plate injury tissue using enzyme-linked immunosorbent assay (ELISA).

**Results**

From histological examination in Figure 1, it was clear that in the positive control group, a bony bridge was formed which was distinguished from the normal growth plate surrounding it. In the treatment group, the growth plate tissue regeneration was obtained where the best results were in the SVF + collagen group. This regeneration process can be distinguished from normal growth plate tissue, namely, the presence of immature chondrocytes characterized by the absorption of basophilic color from HE staining, with a round nucleus and outside the lacuna, accompanied by irregular arrangement of zones on the growth plate.
Bony bridge diameter measurements were carried out on histological examination with the positive control group being the largest, while the smallest bony bridge diameter in the treatment group was found in the SVF + collagen group which is shown from Figure 2. The data were then tested for normality and homogeneity. The results obtained are normal and homogeneous distribution and thus it can be continued with the parametric test, namely, Tukey which is shown in Table 1. The Tukey test showed that the positive control group differed significantly when compared with each treatment group. In this examination, it was found that the average value of SOX9 levels from the tissue that experienced growth plate injury is shown in Figure 3.

Table 1: Tukey test of the positive control group and treatment group

<table>
<thead>
<tr>
<th>Group comparison</th>
<th>p-value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>0.002</td>
<td>Significant</td>
</tr>
<tr>
<td>SVF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVF + collagen</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>SVF</td>
<td>0.214</td>
<td>Insignificant</td>
</tr>
</tbody>
</table>

SOF: Stromal vascular fraction.

From Figure 3, it can be seen that in the positive control group, the value is very low when compared to other groups, whereas in the treatment group, all values approached normal values (negative control).

Discussion

Tissue engineering to solve problems in orthopedic field has been widely applied including in growth plate injury. The basis of tissue engineering is the presence of three components, namely, cells, signal, and scaffold. In this study, tissue engineering was carried out using mesenchymal stem cells (cell components) and growth factors (signal) contained in SVF and collagen as a scaffold [5].

In histological examination, it was clearly seen qualitatively the difference between the treatment group and the positive control group where the treatment group showed the regeneration process of the growth plate injury. The best results are shown in the SVF + collagen group that shows the regeneration process.
which almost like normal tissue with bony bridge diameter of 365.5 µm compared to SVF only and positive control group (654.67 µm and 1256.83 µm). The regeneration process can be distinguished from normal growth plate tissue, namely, the presence of immature chondrocytes which is characterized by the absorption of basophilic color from HE staining, with a round nucleus and outside the lacuna, accompanied by irregular arrangement of zones on the growth plate. This finding gives great hope that tissue engineering can create a very important condition in the process of regeneration of the growth plate injury. As is known from the literature, the healing process of growth plate injury still has a lot of complications such as the formation of a bony bridge which clinically will cause growth disturbance in the extremities that experienced growth plate injury. Histological examination can prove that tissue engineering has an enormous potential in the regeneration process of the growth plate and prevents the formation of a bony bridge in the growth plate injury. Bony bridge is a cause of complications in growth plate injury. Therefore, if it can prevent its formation, it can also prevent complications which may arise in growth plate injury. This research can prove that tissue engineering can inhibit the formation of a bony bridge in growth plate injury [6].

Other variables examined in this study aim to explain and prove how tissue engineering has the ability to regenerate from growth plate injury and also inhibit the formation of a bony bridge. Research variable was the SOX9 level in growth plate tissue as measured by the ELISA method. From the results of the study, it was found that SOX9 levels in the positive control group were significantly different (p < 0.05) with a total measurement of 2754 ng/mL when compared to the treatment group with SOX9 level of 554 ng/mL in SVF only group and 5626 ng/mL in the SVF and collagen group. In accordance with SOX9 theory, a transcription factor plays a very large role in the process of chondrogenesis, so it is often referred to as the master of chondrogenesis. If the level of SOX9 decreases, the chondrogenesis process does not occur properly. From the results of the study, it was found that SOX9 levels in the positive control group decreased more than twice the normal value, and with the treatment with SVF and collagen scaffold, it increases the level of SOX9 almost the same as normal tissue. SOX9 is a chondrogenesis marker that is very important. Therefore, this study uses the marker as one of the research variables [7]. According to Veronique Lefebvre, SOX9 is an important transcription factor for cartilage and plays a role in chondrocyte differentiation. The molecular mechanism of SOX9 action in chondrocyte cells is that SOX9 directly targets genes which contribute to the formation of extracellular matrices such as type II, IX, and XI collagen as well as aggrecan and its regulators such as chondroitin 4-sulfotransferase. This extracellular matrix is very important in providing microenvironment for cells in growth plate tissue which was injured for regeneration [8].

Conclusions

This study can prove that the combination of collagen and SVF had a great effect on healing process in growth plate injury, with SOX9 and histological examination as a marker.

Acknowledgment

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