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Evaluation of Secretome Tenogenic Potential from Adipose Stem Cells (ACS) in Hypoxic Condition with Fresh Frozen Tendon Scaffold Using Scleraxis (Scx), Insulin-Like Growth Factor 1 (IGF-1) and Collagen Type 1

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Abstract. Various studies have been conducted to see the scaffold that supports the regeneration of tendon. This study aims to analyze the *in vitro* secretome tenogenic potential produced by ASCs culture with fresh frozen tendon scaffold in hypoxic conditions. ELISA tests for Scx and IGF-1 levels in secretome were obtained from ASC culture with fresh frozen tendon scaffold under normoxic (21%) and hypoxia (2%) conditions. The immunohistochemical examination of COL-1 was also carried out on the 2nd and 6th days of cell culture. The secretion of Scx and IGF-1 was increased in secretome from ASC cultures using a fresh frozen tendon scaffold compared with those which did not ($p < 0.05$). In the normoxia condition, Scx and IGF-1 in secretome with fresh frozen tendons had better results than hypoxic conditions ($p < 0.05$). The highest Scx levels were obtained in culture on the 6th day ($p < 0.05$), while the highest IGF-1 levels were obtained in the culture on the 2nd day ($p < 0.05$). There was an increase in the secretion of Scx and IGF-1 from ASC cultures with fresh frozen tendon scaffold under the hypoxic condition of 2%.

Introduction

The management of therapy and regeneration of musculoskeletal tissue keeps promoting a challenge in the modern era of health. In our body, musculoskeletal tissue plays a significant role in providing support, stability, and body movements. Because of its essential role in our daily routine, it can be easily imagined if musculoskeletal injury is a significant cause of disability [1].

More than 30 million of tendons and ligaments injuries occur annually throughout the world. This injury causes a reduced ability to do sports and daily activities [2]. There are two main types of therapy in tendon injuries, namely, conservative and operative. The conservative treatment includes steroid injection, ultrasound, shockwave, and physiotherapy [3]. Operations include direct sewing, autograft, allograft, xenograft, or tendon prosthesis [4]. However, there has not been any therapy that is considered quite satisfactory. The problems that arise include a long recovery period, morbidity from the donor site, immunological reactions, and tendon tissue necrosis [5].

Mesenchymal Stem Cells (MSCs) were introduced 25 years ago for the designation of cells from the bone marrow or human and mammalian periosteum that can be cultured and isolated *in vitro* and developed into various kinds of phenotypes and mesodermal tissue [6]. *In vitro* tests, MSCs can become bone, cartilage, fat, and other tissues [7].

At present, various types of growth factors have been reported to affect the ethnogenesis of ASCs. The growth factor is the main modulator of collagen production. During the tendon healing process, various types of growth factors such as Transforming growth factor-β (TGF-β), insulin-like growth factors I and II (IGF-I and II), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) work synergistically in the process [8,9].

The scaffold is an important element of tendon tissue engineering. Scaffolds provide significant progress in terms of structural integrity and biological compatibility for tendons [10,11]. It is known that most growth factors are regulated in various stem cells under hypoxic conditions [12]. Besides, hypoxia makes it possible to maintain the undifferentiated phenotype - MSC for self-renewal [13].

In diseased or damaged tissue, MSCs rarely or never immediately differentiate according to the tissue [14]. At this time, there is an increase in research to study the characterization and potential of secretors by various cells. The main challenge in this research is the difficulty in collecting samples due to a few numbers of secretors in nanogram units [15-17]. Based on the explanation above, it can be assumed that the administration of scaffold decellularized extracellular matrix (ECM) or fresh frozen tendons seeded with secretory ASCs in hypoxic conditions stimulates tenogenesis.

Methods

Study Design

This is an *in vitro* exploratory laboratory study with Randomized Control Group Post-Test Only design. The study was conducted at the Cell & Tissue Bank of Dr. Soetomo General Academic Hospital/ Faculty of Medicine Universitas Airlangga. Study time was scheduled from July to September 2019. The study protocol was approved by the Animal Care and Use Committee of our institution (certificate number: 1386/KEPK/VIII/2019).

Sample

The research materials are adipose stem cells and fresh frozen tendons. The subjects were divided into four groups consisting of 1) Secretome without Scaffold in Normoxic Condition; 2) Secretome with Scaffold in Normoxic Condition; 3) Secretome without Scaffold in Hypoxic Condition, and 4) Secretome with Scaffold in Hypoxic Condition.

Isolation of Mesenchymal Stem Cells from Adipose Tissue

The fat tissue is rinsed with phosphate-buffered saline (PBS) solution until it is clean (red blood cells detached from the fat tissue). Adipose tissue is cut until smooth and mixed with the collagenase enzyme then poured in bottles containing magnetic stirrer. The tissue in the bottle is then incubated on a hot plate at 370°C for 30 minutes until the adipose tissue is dissolved. A medium stopper is added and incubated again for 10 minutes until it becomes a homogeneous solution. This solution is then poured into a 50 ml cone that has been given a filter consisting of sterile gauze until the rest of the insoluble fat tissue is separated. The filter results are then centrifuged at a speed of 3000 rpm for up to 5 minutes until a pellet is formed. The pellets are then resuspended with an alpha Minimum Essential Medium (MEM) until it becomes homogeneous solution. After being planted in a 10 cm petri dish and incubated in a CO_2 incubator for 24 hours until the cells are attached to the base of the petri dish. The media of cells that have been attached are then replaced every two days until the cells that form colonies and grow to reach 80% confluent.

Culture of Mesenchymal Stem Cells

Mesenchymal stem cells that have successfully grown to form colonies can then be reproduced until they reach the dose needed for clinical application. Cells that have formed a monolayer layer with 80% confluency need to be rejuvenated by passage.

The passage is performed by removing the medium of a petri dish and then rinsing the monolayer with a PBS solution. After that express, a triple enzyme is added, and incubation is performed for 5 minutes until the monolayer separated regardless of the basic Petri. After a monolayer separated, it needs to be added with a stopper and do resuspension medium until it turns into a single cell. The solution containing the single cell is poured in a conical tube and centrifuge to form pellets. The pellet is then given in α-MEM medium and resuspended until it becomes a homogeneous solution and then implanted in a new petri dish.

Induction of Hypoxic and Fresh Frozen Tendon Scaffold Preparation Culture

The provision of hypoxic conditions in stem cell culture with 1% oxygen level was carried out for several days of cultivation time to reach passage four and passage 8. The cells from MSC obtained from phase 1 research are grown to passage four and passage 8. The cells took aseptically are then placed on a culture dish with a density of $2x10^7$ cells/cm^2 on ten dishes, 5 cm with an IMDM medium containing 15% FBS, an MSC simulator supplement and antibiotics (100 U penicillin/100µg/mL Streptomycin) at 37°C, 5% CO_2 and 95% air. Maintenance of quiescence of BM-MSC cells is done by administering hypoxic conditions with a dose of 1% O_2 concentration by inserting a culture flask into a special incubator for hypoxic conditions (Modular Incubator Chamber) that is cultured until early passage (4^{th} passage) and late passage (8^{th} passage).

The mesenchymal stem cells that have been successfully isolated from fat tissue need to be characterized using CD-105, i.e., a specific marker for mesenchymal stem cells. After that, the mesenchymal stem cells that have been labeled are examined using flow cytometry and immunocytochemical techniques. A positive test on CD-105 is shown by the presence of color fading on the surface of the mesenchymal stem cell membrane. While the use of CD-45, a specific marker of stem cell hemopoietic, needs to be done to ensure that the results of isolation from fat tissue are pure mesenchymal stem cells. The negative test indicates this in the absence of color fading on the surface of the mesenchymal stem cell membrane.

Secretome Preparation

The collected conditioning medium is then put into the 50 ml dialysis tubing membrane. Furthermore, connective dialysis tubing at both end to meeting and enter it in a 500 ml beaker already containing PBS solution in cold conditions. After that, enter the magnetic bar and place the beaker on a magnetic stirrer hot plate. Then, rotate at a speed of 500 rpm and leave it overnight until the color conditioning of the medium in the dialysis tube is faded. After being faded, remove PBS solution and cut into dialysis tubing using sterile scissors and then pour in 250 ml glass beaker to further filter metabolite products with 0.22 microns size and pack in conical 50 ml. Medipack enters the sterile and sealing as well as the store at a temperature of -20°C.

Data Collection

Data collection of this research uses adipose stem cells and fresh frozen tendons. The independent variables in this study are secretome and fresh frozen tendons. The dependent variables in this study are Scleraxis (Scx), IGF-1, and COL-1. The control variable in this study is hypoxia condition 2%. The Scleraxis (Scx) and IGF-1 were assessed using ELISA examination. The collagen type 1 was assessed using microscopic examination after immunohistochemistry staining.

Statistical Analysis

The data retrieved from the evaluation were compiled and analyzed using IBM SPSS Statistics version 26. Normality test using the Kolmogorov Smirnov with Lilliefors significance correction test was then done, normal data groups were then tested using One-Way ANOVA, and not normal data groups were tested using the Kruskal-Wallis Test.

Results and Discussion

This study assessed the secretion of scleraxis and IGF-1, as well as the expression of COL-1 in four treatment groups. The comparison of the mean Scx levels of each group is shown in Table 1, with the highest mean value in the Secretome with Scaffold in Normoxic condition was on the 6^{th} day. A trend of declination of mean IGF-1 concentration was shown in each group, in which the lowest level was on the 6^{th} day. The comparison of mean Scx and IGF-1 based on groups and days was shown in Figure 1.

Table 1. Scleraxis and IGF-1 secretion based on treatment group on the 2nd day, 4th day, and 6th day

		Secretome without Scaffold in Normoxic Condition (n=6)	Secretome with Scaffold in Normoxic Condition (n=6)	Secretome without Scaffold in Hypoxic Condition (n=6)	Secretome with Scaffold in Hypoxic Condition (n=6)	P Value
Scx	Day 2	11.998 ± 0.166	11.575 ± 0.075	11.091 ± 0.106	11.558 ± 0.141	p≤0.001
	Day 4	12.090 ± 0.143	12.992 ± 0.722	11.355 ± 0.210	11.353 ± 0.147	p≤0.001
	Day 6	11.951 ± 0.321	14.025 ± 0.300	10.775 ± 0.099	13.116 ± 0.115	p≤0.001
IGF-1	Day 2	24.029 ± 0.321	27.737 ± 0.302	23.442 ± 0.463	25.637 ± 0.391	p≤0.001
	Day 4	23.026 ± 0.509	25.067 ± 0.415	23.774 ± 0.490	24.448 ± 0.425	p≤0.001
	Day 6	21.620 ± 0.420	23.152 ± 0.237	22.239 ± 0.528	23.159 ± 0.516	p≤0.001

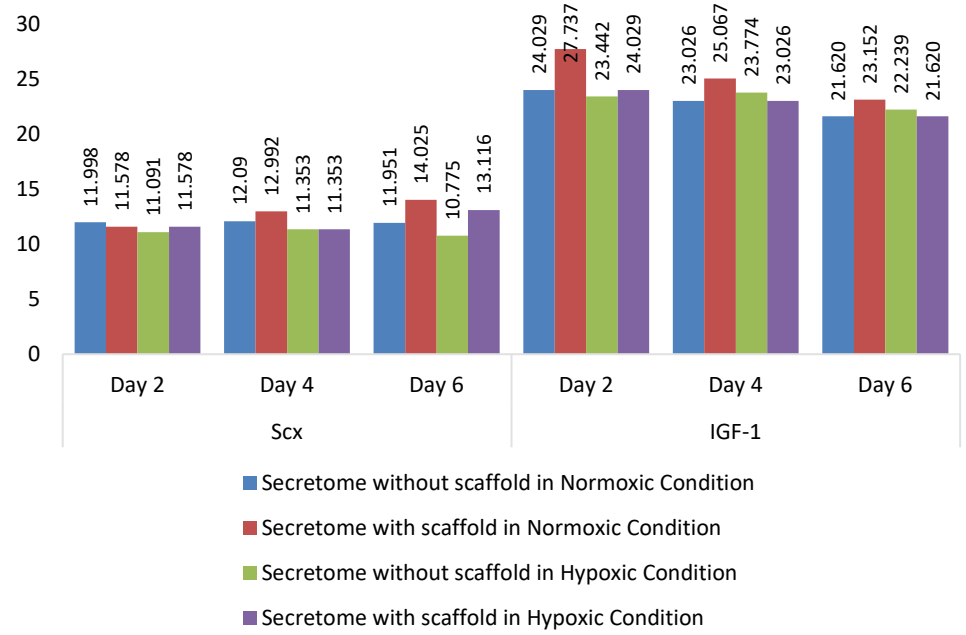


Figure 1. Comparison of Scx and IGF-1 based on groups and days

The result of the study showed that both secretion of Scx and IGF-1 in four treatment groups were different significantly (p≤0.001). This result was also further confirmed using Least Significance Difference and Dunnett T3 post hoc test. Among the treatment groups, on the 2nd day, Scx secretion on Secretome without Scaffold in Normoxic condition showed more significant, while on either the 4th day or 6th day, Secretome with Scaffold in Normoxic condition showed more significant. IGF-1 secretion on Secretome with Scaffold in Normoxic condition showed more significant on the 2nd and 4th day. Interestingly, on the 6th day, both Secretome with Scaffold in Normoxic condition or Secretome with Scaffold in Hypoxic condition showed the same high significantly.

Figure 2 shows the comparison of collagen type 1 in immunohistochemical staining based on each group. The results show a decreased number of collagen type 1 in the 6th day compared to the second day in each group.

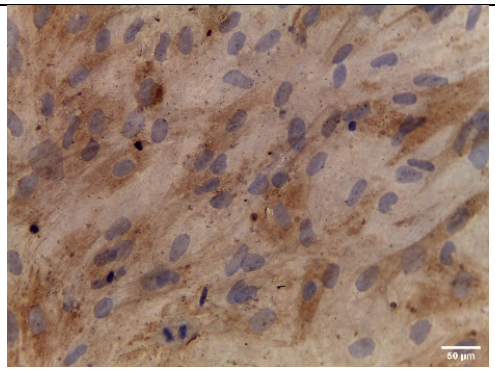
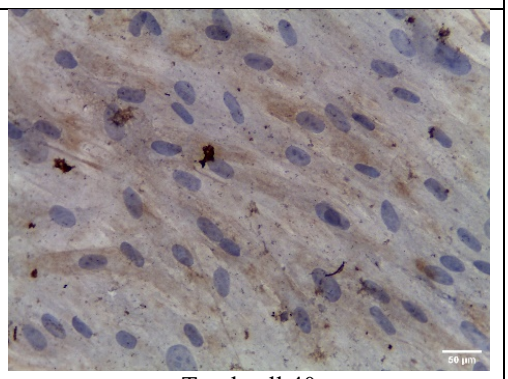
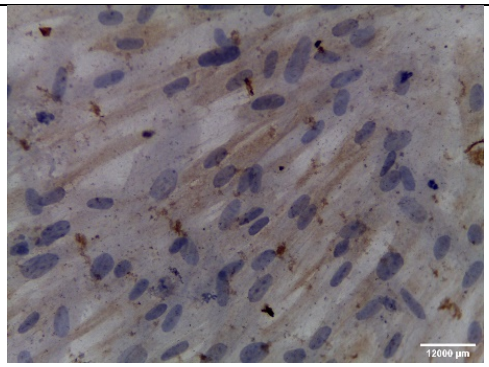
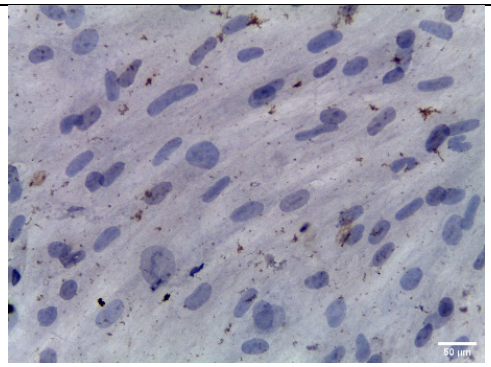
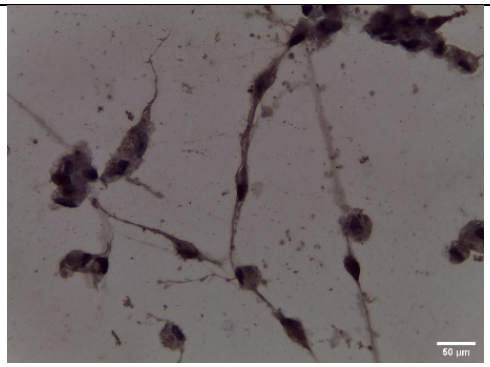
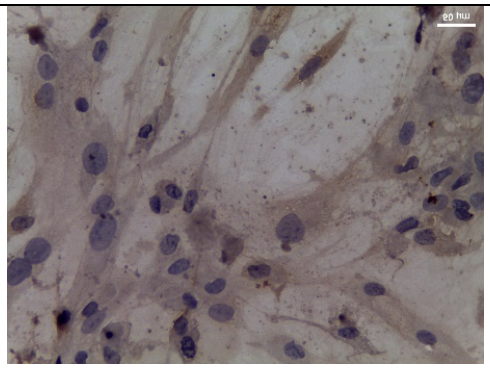
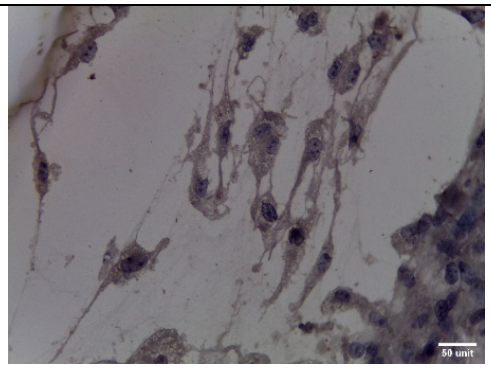
	Day 2	Day 6
Secretome without scaffold in normoxic condition	 Total cell 65	 Total cell 40
Secretome without scaffold in hypoxic condition	 Total cell 51	 Total cell 53
Secretome with scaffold in normoxic condition	 Total cell 16	None
Secretome with scaffold in hypoxic condition	 Total cell 48	 Total cell 26

Figure 2. Comparison of collagen type 1 immunohistochemical staining based in each group (400x power microscope magnification)

This study analyzed the secretion of Scx and IGF-1 secretory proteins contained in tenogenesis. Secretome is an alternative in the field of tissue engineering, which is designated as cell-free therapy. In this study, secretions were obtained from a human adipose-derived mesenchymal stem cells (ASCs). The marker proteins used in this study were Scx and IGF-1 using ELISA on the

2nd, 4th, and 6th day. Besides, examining the immunohistochemistry of COL-1 in the sample was also carried out to see differences in the culture medium.

There are four groups with opposing culture, namely: the ASC group is cultured without scaffolding, and in normoxic conditions, the ASC group is cultured with scaffolding, and in normoxic conditions, the ASC group is cultured without scaffolding under hypoxic conditions (2%), and the ASC group is cultured with scaffolding in hypoxic conditions (2%).

The results of this study show a significant increase in Scx secretion in the group using scaffold fresh frozen tendons compared to those which did not ($p < 0.05$). Sex expression was seen in the early stages of tendon development and encouraged the expression of tenogenic markers. It is thought that early scleraxis-expressing progenitor cells led to the formation of tendon tissue and other muscle attachments [18]. Likewise, a study by Yin *et al.* obtained tendon-derived stem/progenitor cells (TSPCs) implanted in ECM decellularized tendons/ligaments increased proliferation and differentiation of phenotype of tendon cells [19].

A study by Sakabe *et al.* in 2018 stated that the transcription of Scx contributed linearly in wound healing in adult rat test samples [20]. There are also other studies showing that intratendinous injection of tendon derived stem cells transduced with Scx in the patellar tendon window defect in mice significantly improved the histological and biomechanical properties of repair tissue compared to tendons treated with tendon derived stem cells alone [21]. Scleraxis (Scx) is a member of the basic-helix-loop-helix (bHLH) superfamily transcription factor, having direct regulation in increasing the transcription of COL-1, which makes up the largest ECM component in the tendon. Besides, Scx also increases the expression of protein and mRNA from Tenomodulin (Tnmd), which plays a role in tenogenesis [18].

The same thing was found in the increase in IGF-1 secretion, which was significantly more pronounced in the group using scaffold fresh frozen tendons than those which did not ($p < 0.05$). IGF-1 stands out in the early stages of tendon healing. The main effect of IGF on tendon healing is mitogenesis, stimulating fibroblast proliferation, and tenocytes at the site of injury [22]. In a study by Dahlgren *et al.* about the model of flexor tendinitis in horses, intralesional IGF-I injection increased cell proliferation and collagen synthesis, reduced overall lesion size, and tended toward increasing mechanical strength in the treated tendon rather than the control tendon [23].

Higher levels of Scx and IGF-1 were obtained in the secretory group of ASC, which was seeded with fresh frozen tendons in normoxia ($p < 0.05$) compared to the hypoxia group. This is different from research conducted by circumcision that most of the growth factors are regulated in various stem cells under hypoxic conditions [12]. Some studies showed that hypoxic conditions increase the proliferation of cells but do not increase the differentiation of these cells [24]. However, several studies have shown the negative effect of ambient O_2 concentrations on MSC, inducing premature aging at longer population doubling times and DNA damage [25].

The interesting thing in this study is the increased secretion of Scx in the ASCs culture group with fresh frozen tendon scaffold in day 2 to day 6. There was a significant difference ($p < 0.05$) in the group in both normoxic and hypoxic conditions. This was different in IGF-1 secretion, which experienced a significant decrease in all groups from day 2 to day 6 ($p < 0.05$). These data indicate that cultural time influences differences in secretion levels. Kawasaki et al., in their study, said that the expression of Scx genes under low oxygen conditions and even anoxia increased significantly after 24 hours [26].

At the COL-1 immunohistochemical examination in the four groups of samples on the 2nd and 6th day of culture, the number of cells with COL-1 expression was lower in the scaffold culture group both in hypoxia and normoxia conditions. These results are inversely proportional to the ELISA test, which shows a significant increase in levels of Scx and IGF-1 in secretors. A study by Tan *et al.* showed that the expression of COL-1 only increased on the 2nd to 4th weeks and decreased again after that [21].

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Conclusion

This study showed that the application of secretome combined with fresh frozen tendon scaffold in normoxic conditions has the highest significant tenogenic healing potential through increased modulation of scleraxis (Scx), insulin-like growth factor 1 (IGF-1) and collagen type 1 (COL-1).

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