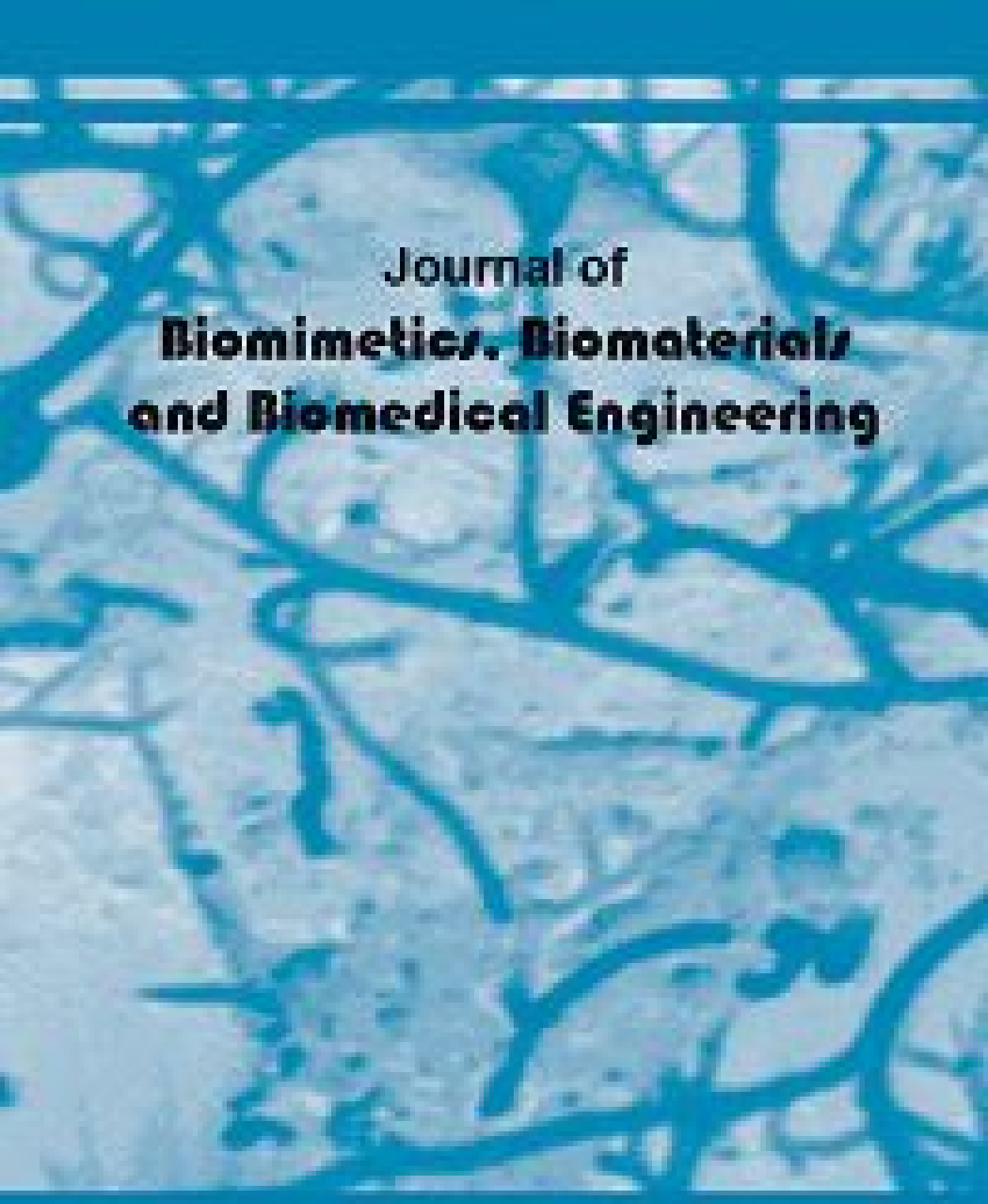

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Characterization of Scleraxis and SRY-Box 9 from Adipose-Derived Stem Cells Culture Seeded with Enthesis Scaffold in Hypoxic Condition

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Abstract. The use of mesenchymal stem cells can add local improvements potential to enthesis tissue regeneration based on tropical activity through secretions of growth factors, cytokines, and vesicles (e.g. exosomes), collectively known as secretomes. This study aims to analyze secretomes characterization from adipose-derived mesenchymal stem cells seeded with enthesis tissue scaffold in hypoxic conditions and to analyze the influence of hypoxic environment to the characterization of secretomes. This is an in-vitro study using a Randomized Control Group Post-Test Only design. This study using Adipose Stem Cells (ASCs) were cultured in hypoxia (Oxygen 5%) and Normoxia (21%) condition. The scaffolds are fresh-frozen enthesis tissue and was seeded in the treatment group and compared to control. The evaluation of Scleraxis (Scx) and SRY-box (Sox9) was measured using ELISA on the 2nd, 4th, and 6th days. Comparison of Scx levels between each evaluation time showed a positive trend in a group with scaffold in hypoxia condition although it has no significant differences (p=0.085), with the highest level on day 6, that is 13,568 ng/ml. Conversely, the comparison of Sox9 showed significant differences (p=0.02) in a group with scaffold in hypoxia condition, with the highest level on day 4, that is 28,250 ng/ml. The use of enthesis scaffold seeded in adipose-derived mesenchymal stem cells in hypoxic conditions shows a positive trend as regenerative effort of injured enthesis tissue through Scleraxis and Sox9 secretomes induction.

Introduction

Rotator cuff tendon tear is one of the injuries to the tendon-bone interface (enthesis) tissue that accounts for almost 20% of all injuries and 80% of those injuries suffer repeated tears [1]. Although almost all total tear of the rotator cuff tendon can be repaired through arthroscopy surgical techniques and with the latest material, the failure rate in the healing of the tendon-bone interface tissue area can reach 94% in the long-term follow-ups that can significantly decrease strength and clinical-end results. Biological failure of healing in tendon-bone interface tissue area can be related to the muscle and tendon degeneration, decreased cellular activity, and decreased healing potential of tendons [1]. A total tear of the rotator cuff tendon can be found in 13% of individuals around the age of 50, 25% in individuals at age 60, and 50% in individuals at age 80. The etiology of rotator cuff tendon tear is multifactorial and is a combination of degenerative changes and the presence of micro/macro trauma. In addition to these factors, other factors such as age, smoking, hypercholesterolemia, and family history are also predisposing factors for an individual to experience a tear in the rotator cuff. The anatomical location of the flexor and rotator cuff being intrasynovial, makes it unable to undergo spontaneous healing in case of injury [2].

There are two standard therapy options to treat the injury to the tendon mentioned above, such as nonoperative (pharmacological) and operative management. In addition to surgical and pharmacological interventions, the use of therapy with stem cells has been gaining popularity in recent years as a promising therapeutic tool for the repair and regeneration of tendons and muscles. Mesenchymal stem cells (MSC) become one of the therapeutic tools that can add local repair potential and promote the regeneration of tendons and muscles [3]. Recently there was a study that stated the positive effects of stem cells on adipose tissue in the healing of rotator cuff tendon tear in rabbits [4].

Stem cells from adipose tissue are progenitor cells that can proliferate and differentiate into several different types of mesenchymal cells, such as tenocytes, myocytes, and also could affect the production of growth factors and cytokines. This ability can be beneficial in accelerating the healing of the tendon-bone interface tissue area. In addition to the multi differentiation capacity of stem cells, it has now begun to be widely researched that most of the therapeutic effects obtained from mesenchymal stem cells are based on tropical activity through secretions of growth factors, cytokines, and vesicles (e.g. exosomes), collectively referred to as secretomes [4]. Secretomes are produced from stem cell cultures in conditioned-media that have been implanted with scaffolds [5]. MSC secretomes contain many cell signaling molecules, including growth factors and cytokines that modulate cell behavior, such as proliferation, differentiation, and production of extracellular matrices or provide pro and anti-inflammatory effects [6]. In addition, to engage in intercellular communication, secretomes mimic the genetic and proteomic content of secreted MSCs and are therefore assumed to reproduce the therapeutic effects of stem cells [7]. Recently, there is research showing that tendon-bone tissue from mice is derived from SRY-box9 (Sox9) and Scleraxis (Scx) set that are important for chondrogenesis and tenogenesis, respectively [8]. Although Sox9 and Scx role in enthesis development is still not clear, research in adult meniscal fibrochondrocyte showed that Sox9 is important to maintain fibrochondrocyte phenotype. Therefore, cartilage and tendon/ligament marker seems important to enthesis characterization. Scleraxis (Scx) is helix-loop-helix base factor transcription that implicated in tendon development and regeneration [9].

In conditioned-media for stem cell culture that can produce secretomes, the term hypoxia condition in the context of cell culture is a condition where the oxygen levels ranging from 0-10%. In general, hypoxic conditions strengthen MSC regeneration and cytoprotective effects [10]. Conversely, there is study that showed a negative influence of ambient O_2 concentrations on MSC, inducing premature aging, longer population multiplication times, and DNA damage [11].

Hypothesis of this study is the increasing level of Scx and Sox9 from Adipose Stem Cells (ASCs) metabolite that were implanted with tendon-bone interface tissue scaffold in hypoxic condition. This study will investigate how hypoxic condition influence the characterization of scleraxis (Scx) and SRY-Box9 (Sox 9) from metabolites of mesenchymal stem cell culture that were implanted with tendon-bone interface tissue scaffold.

Method

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 January to June 2020. The study protocol was approved by the Health Research Committee of our institution (certificate number: 0082/LOE/301.4.2/VIII/2020).

Sample

The research materials are adipose stem cells and fresh frozen enthesis tissue. The control groups defined as adipose-derived mesenchymal stem cells seeded without enthesis tissue scaffold in either hypoxic or normoxic conditions. The intervention groups defined as adipose-derived mesenchymal stem cells seeded with enthesis tissue scaffold in either hypoxic or normoxic conditions. The subjects were divided into four groups consisting of 1) Secretome without Scaffold in Normoxic Condition (Control-Normoxic); 2) Secretome with Scaffold in Normoxic Condition (Scaffold-Normoxic); 3) Secretome without Scaffold in Hypoxic Condition (Control-Hypoxic), and 4) Secretome with Scaffold in Hypoxic Condition (Scaffold-Hypoxic). In this study, according to Federer's formula, the sample size in each group were 6 with the total sample were 24 of each observation day.

Isolation of Mesenchymal Stem Cells from Adipose Tissue

The adipose tissue is rinsed with phosphate-buffered saline (PBS) solution until it is clean (red blood cells detached from the adipose tissue). Adipose tissue is cut until smooth and mixed with the collagenase enzyme then poured into bottles containing magnetic stirrer. The tissue in the bottle is then incubated on a hot plate at 37°C for 30 minutes until the adipose tissue is dissolved to optimize collagenase enzyme so that stem cells from adipose tissue can be dissociated. 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 adipose 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 a 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 is 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 Enthesis Scaffold Preparation Culture

The provision of hypoxic conditions in stem cell culture with 5% 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 because we need time to obtain cells that enough for treatment dose, so we maintain it within that time. 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 Iscove's Modified Dulbecco's Medium (IMDM) containing 15% Fetal bovine serum (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 MSC cells is done by administering hypoxic conditions with a dose of 5% 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 adipose 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 adipose 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, tie dialysis tubing at both ends and enter it in a 500 ml beaker that 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 the 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 enthesis. The independent variables in this study are secretome without scaffold in normoxic condition (Control-Normoxic), secretome with scaffold in normoxic condition (Scaffold-Normoxic), secretome without scaffold in hypoxic condition (Control-Hypoxic), and secretome with scaffold in hypoxic condition (Scaffold-Hypoxic). The dependent variables in this study are Scleraxis (Scx) and SRY-box9 (Sox9). The control variable in this study is oxygenation condition (hypoxic and normoxic). The Scx and Sox9 were assessed using ELISA examination.

Statistical Analysis

The data retrieved from the evaluation were compiled and analyzed using IBM SPSS Statistics version 26. A normality test using the Shapiro-Wilk was done, normal data groups were then tested using One-Way ANOVA, and not normal data groups were tested using the Kruskal-Wallis Test. The post-hoc analysis for intragroup comparisons was tested using Least Significant Difference (LSD) test.

Result and Discussion

In vitro research has been conducted to analyze scleraxis and SRY-box 9 (Sox9) secretions in 4 groups of samples as shown in Table 1, namely secretome without scaffold in normoxic condition, secretome in normoxic condition with fresh frozen enthesis tissue scaffold, secretome without scaffold in hypoxic condition, and secretome in hypoxic condition with fresh frozen enthesis tissue scaffold. The examination was carried out with six samples for each group and carried out on the 2nd day, the 4th day, and the 6th day. The comparison of mean Scx and Sox9 based on groups and days were shown in Figure 1.

Table 1. Scleraxis and Sox9 secretion based on treatment group on the 2nd day, 4th day, and 6th day

		Secretome without scaffold in Normoxic Condition	Secretome with scaffold in Normoxic Condition	Secretome without scaffold in Hypoxic Condition	Secretome with scaffold in Hypoxic Condition	P Value
Scx	Day 2	12.318±0.837	13.551±1.492	12.679±1.595	11.806±0.882	P=0.136
	Day 4	14.550±1.515	15.318±1.384	12.580±1.895	12.875±1.205	P=0.014
	Day 6	14.102±2.430	14.087±2.211	10.273±0.789	13.568±1.617	P=0.005
SOX9	Day 2	24.663±2.892	20.074±2.039	24.173±1.811	21.559±1.612	P=0.004
	Day 4	23.677±2.008	21.172±2.236	28.250±2.048	27.565±2.931	P=0.000
	Day 6	22.923±0.847	24.837±1.231	25.873±2.204	24.820±2.392	P=0.064

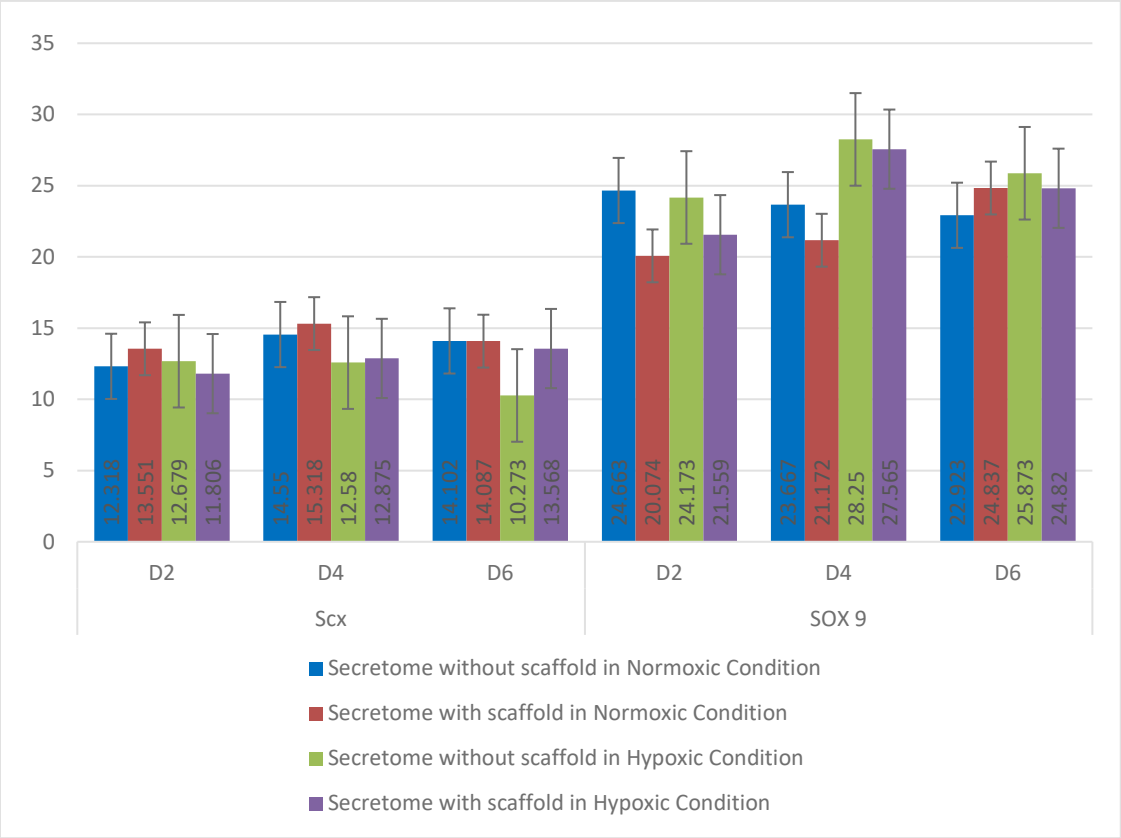

Figure 1. Comparison of Scx and Sox9 based on groups and days

The secretome group that received the treatment with scaffold and in normoxic condition had the highest Scx levels compared to the other group with an average score of 13,551 on the 2nd day. Comparative tests showed no significant difference in average Scx levels on day 2 between each group (p=0.136).

The secretome group that received the treatment with scaffold and in normoxic condition had the highest Scx levels compared to the other group with an average score of 15,318 on the 4th day. Comparative tests showed significant differences in average Scx levels on day 4 between each group (p=0.014).

Secretome group without scaffold and in normoxic condition obtained the highest Scx levels compared to the other group with an average score of 14,102, followed by the group with scaffold in normoxic condition with an average value of 14,087 on the 6th day. Comparative tests showed significant differences in average Scx levels on day 6 between each group (p=0.005).

From the statistical analysis that has been done, the Scx value obtained on the 4th and 6th days are significant, and then this result is followed by post hoc analysis. From post hoc analysis on day 4, there were significant differences between the hypoxic control group and both normoxic control group and normoxic scaffold group. In addition, on day 4 there were also significant differences between the hypoxic scaffold group and normoxic scaffold.

The scaffold-free in normoxic condition group had the highest Sox9 levels compared to the rest of the group with an average score of 24,663 on day 2. Comparative tests showed significant differences in average Sox9 levels on day 2 between each group (p=0.004).

The scaffold-free in hypoxic condition group had the highest Sox9 levels compared to the rest of the group with an average score of 28,250 on day 4. Comparative tests showed significant differences in average Sox9 levels on day 4 between each group (p=0.000).

The scaffold-free in hypoxic condition group had the highest Sox9 levels compared to the rest of the group with an average score of 25,873 on day 6. Comparative tests showed no significant difference in average Sox9 levels on day 6 between each group (p=0.064).

From the statistical analysis that has been done, the value of Sox9 in the 2nd and 4th day is significant, then this result is followed by post hoc analysis. From post hoc analysis on day 2, there were significant differences between the hypoxic control group and both hypoxic scaffold group and normoxic scaffold group. Significant differences were also found between the hypoxic scaffold group and the normoxic control group, as well as between the normoxic scaffold group and normoxic control group. On day 4, there was a significant difference in Sox9 levels between the hypoxic control group and both normoxic control group and normoxic scaffold group. Significant differences were also found between the hypoxic scaffold group and both normoxic control group and normoxia scaffold group.

This study also compared the results of the ELISA Scleraksis (Scx) and SRY-box9 (Sox9) in the 2nd, 4th, and 6th day based on the group as shown in Figure 2 and Figure 3.

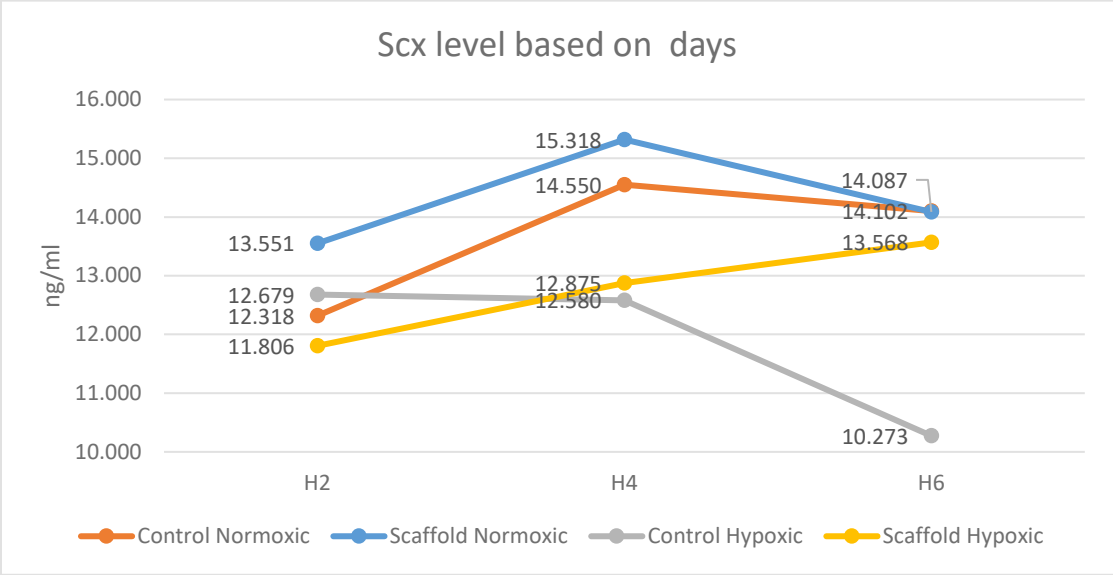

Figure 2. Comparison of Scx based on days

Based on Figure 2, the normoxic control group improved on day 4 but dropped on day 6 with an insignificant increase difference (p=0.091). Normoxic scaffold group has the highest levels especially on day 2 and 4, where on the 6th day the levels of scleraxis decrease (p=0.227). The hypoxic control group continued to decline until day 6, which became the peak of significant decline (p=0.023). The hypoxic scaffold group has an increasing trend even though it is not significant (p=0.085).

From the results of statistical analysis that has been done on the comparison of Scx levels based on the evaluation time, there were significant differences in the hypoxia control group, then this result is followed by post hoc analysis. From post hoc analysis, there were significant differences between the hypoxic control group between day 2 and day 6, and between day 4 and day 6.

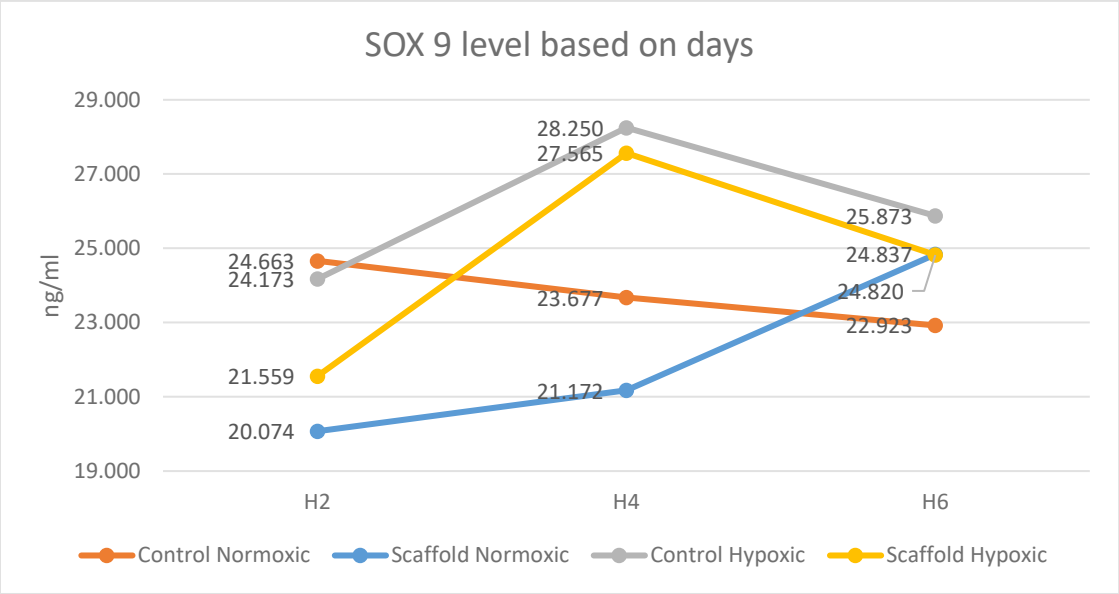

Figure 3. Comparison of Sox9 based on days

Based on Figure 3, the normoxic control group has the highest levels on day 2 but has a downward trend. The normoxic scaffold group has an upward trend, especially on day 6 which has a significant increase ($p=0.01$). The hypoxic control group had the highest levels compared to other groups especially on days 4 and 6 with significant average differences from this group on the 2nd, 4th, and 6th days ($p=0.011$). The hypoxic scaffold group also has the same trend as the hypoxia scaffold-free group, which is rising on the 4th day meaningfully ($p=0.002$) and then down on the 6th day.

From the statistical analysis that has been done on sox9 level comparison based on evaluation time, there are significant differences in hypoxic control group, hypoxic scaffold group, and normoxic scaffold group, then this result is followed by post hoc analysis. From post hoc analysis, there were significant differences in the hypoxic control group between day 2 and day 4. Significant differences were also found in the hypoxic scaffold group between day 2 and both day 4 and 6. In the normoxic scaffold group, there is a significant difference between day 6 and both day 2 and 4.

This study analyzed the secretions of scx that play a role in tenogenesis and Sox9 secretions that play a role in chondrogenesis. Secretome is an alternative way in the field of tissue engineering that has the potential as cell-free therapy. In this study, the secretome was obtained from human adipose-derived mesenchymal stem cells (ASCs) culture.

This study results showed an increase in Scx secretion in the group using fresh frozen enthesis scaffold in normoxic condition, where this group experienced a significant increase on day 4. Scx expression is seen in the early stages of tendon development and also encourages the expression of tenogenic markers. It is thought that early scleraxis-expressing progenitor cells lead to the formation of tendon tissue and other muscle attachments [12]. Likewise, Yin et al in their research found that tendon-derived stem/progenitor cells (TSPCs) implanted in decellularized tendons/ECM ligaments increasing the proliferation and differentiation of tendon cell phenotypes [13]. Sakabe et al, in 2018 in their research stated that transcription of Scx contributed linearly to wound healing in adult mouse animal test samples [14]. There is also another study in which intratendinous injection of tendon derived stem cell transduced with Scx on window defects of mouse patellar tendons significantly improved the histological and biomechanic properties of tissue repair compared to tendons treated only with derived stem cells tendons [15].

Higher Scx levels were obtained in the ASC secretome group seeded with fresh frozen enthesis scaffold in normoxic conditions compared to the hypoxic group on day 2, 4, and 6. This is different from the research conducted by Pawitan in which most growth factors are regulated in various stem

cells in hypoxc conditions [16]. Yamamoto, et al do mention in his research that hypoxic conditions increase the proliferation of cells but do not increase the differentiation of these cells [17]. Nevertheless, several studies have shown a negative influence of ambient O_2 concentrations on MSC, inducing premature aging, longer population multiplication times, and DNA damage [11,18,19]. Another study said that hypoxic conditions can improve Scx expression within 14-21 days after cultured with tenocytes compared to groups in normoxic conditions [20]. In this study, the scleraxis group with scaffold in hypoxic condition showed a positive trend, where the levels continued to increase from day 2, 4, and 6 even though the statistical analysis was not significant.

The same was not found in Sox9 secretions, where the group using fresh frozen enthesis scaffold had a lower number than the group without fresh frozen scaffold although it was not significant, the group given hypoxia conditions had higher sox9 levels, especially on day 4. This is supported by research stating that chondrogenesis can be triggered by hypoxic conditions [21]. Other studies have also said that hypoxic conditions can increase the proliferation and differentiation of chondrogenesis, which can be triggered by an increase in Hypoxia-inducible factor-1α (HIF-1α) that occurs in hypoxic conditions [22,23]. This hypoxic condition resembles an in vivo condition in cartilage, where the hyaline cartilage tissue is avascular, has no nerves and blood vessels, so oxygen can only diffuse through the synovial fluid. Physiological oxygen pressure in articular chondrocytes is 5-10% and maybe only 1% in the deeper layers. Chondrocytes from joint cartilage can survive by maintaining their phenotypes under low oxygen pressure. This could a piece of evidence that low oxygen pressure can be an important regulatory factor in chondrogenic proliferation and differentiation [24]. Another research also said that moderate hypoxia (5%) can increase chondrogenic potency in adipose-derived mesenchymal stem cells [25].

Mesenchymal stem cells are taken from adipose tissue secrete IGF-1 and or IGF-2 in vitro [26]. Chondrogenic differentiation of mesenchymal stem cells, along with increased glycosaminoglycan expression, is triggered by the presence of Parathyroid Hormone-related Peptide (PTHrP), TGF-β, Insulin Growth Factor (IGF)-1 or Bone Morphogenetic Protein (BMP)-2. The same study also said there was a dramatic increase in VEGF expression in the proliferation of in vitro chondrocytes in hypoxic conditions, which also depend on HIF-1α levels. In the process of chondrogenesis, several growth factors strongly influence it, such as TGF-β1, BMP2/-4/-6, IGF-1, and FGF. The increase in hepatocyte growth factor (HGF) secretions by ASCs is potentially involved in the role of chondroprotection on chondrocytes. All of these growth factors were increasing in the culture of ASCs given hypoxia conditions. In normoxic condition, mesenchymal stem cells based on its origin (bone, adipose, or synovial), showed no hypertrophic path when the cells were implanted to cartilage intraarticularly [27].

The comparison between Sox9 level using scaffold and scaffold-free is not significant. This can be caused by storage factor of the scaffold. There is research showing that cells in tissue still can be grown in vitro from allograft fresh frozen after at least 6 months storage in tissue bank. Cells from frozen graft were not different morphologically from cells derived from fresh tissue and still have the same mRNA profile to that in fresh tissue [28]. The longer the storage, the less viable cells can be found inside the frozen graft and it can also change the morphology of the cells. The limitation of this study is that we used scaffold that kept in tissue bank's storage for about 8 months.

Conclusion

There is no significant increasing in scleraxis secretion from ASC implanted with enthesis fresh frozen scaffold in 5% hypoxic condition, but there is positive trend showed in scleraxis secretion with the same condition. Conversely, there is significant increasing in Sox-9 secretion from ASC implanted with enthesis fresh frozen scaffold in 5% hypoxic condition. The use of enthesis scaffold seeded in adipose-derived mesenchymal stem cells in hypoxic conditions showed a positive trend as regenerative effort of injured enthesis tissue through Scleraxis and Sox9 secretomes induction.

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