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Physiobiochemical and microbiologic stability characteristics of freeze-dried cartilage secretome Adipose Mesenchymal Stem Cell (AdMSC)

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Abstract---Cartilage is an avascular, alymphatic, and aneurysmal structure, so it is difficult to heal properly if an injury occurs. Using Chondrogenic Adipose Mesenchymal Stem Cell (AdMSC) secretome as an adjuvant is one of the therapeutic options to overcome the problem of injury to the cartilage. Storage and distribution problems must be solved if we use this method. This study aims to characterize the Freeze-dried chondrogenic AdMSC secretome. Secretome's physical, biochemistry, and microbiology stability are derived from the chondrogenic AdMSC secretome. This study's design is prospective observational analytics research. Freeze-dried cartilage secretome AdMSC was analyzed for physical (organoleptic changes (shape, color, and odor), pH, biochemistry (TGF- β 3), and microbiology (microbe contamination) stability. The difference was observed in 0, 4, 8, and 12 weeks. Data normality and homogeneity were tested using the Shapiro-Wilk test and followed up with the Friedman and Wilcoxon tests to analyze the data further. Organoleptics change showed no color change until four weeks with no change of shape and smell until

12 weeks. No significant difference was found in porosity and pH change—TGF- β 3, which has a role in chondrogenesis, has no considerable decrease until 12 weeks. No contamination was found until 12 weeks.

Keywords---Adipose-Derived Mesenchymal Stem Cell, Freeze-Dried, Organoleptic Changes, Secretome.

1 Introduction

Joint cartilage is a structure that is avascular, alymphatic, and aneural. When joint cartilage is injured, it is difficult for it to heal and returns to normal. If it does not recover, it will quickly deteriorate and develop into osteoarthritis (OA) (Merkely, Ackermann, & Lattermann, 2018). Autologous Chondrocyte Implantation (ACI), Osteochondral autograft/allograft transplantation, and microfracture are the treatment options often used to treat cartilage injuries today. Cell-based therapies such as Mesenchymal Stem Cells (MSC) also have great potential as adjuvant therapy to help overcome this problem (Mancuso, Raman, Glynn, Barry, & Murphy, 2019).

Still, cell-based therapy has various drawbacks, including complicated storage and distribution protocols, the risk of immunological reactions, and limited retention of target tissues (Damous et al., 2018). This leads us to a new paradigm of cell-free therapy, namely secretomes (Mancuso et al., 2019; Perbowo, Utomo, Widhiyanto, Airlangga, & Purwati, 2020).

Secretomes are proteins secreted by cells, tissues, or organisms into the extracellular space at certain times and under certain conditions (Merivaara et al., 2021). The secretome also contains many cell signaling molecules (Lee, Chang, Park, & Park, 2011), growth factors (Ping-Wing, Chung-Hsi, & Ya-Churn, 1996), and cytokines that act like paracrine glands to regulate cell behavior such as proliferation, differentiation, and extracellular matrix production (Daneshmandi et al., 2020; Utomo et al., 2019). Secretomes derived from adipose tissue have great potential to be used as adjuvant therapy in cartilage injuries. In vitro studies have shown that MSCs from synovial adipose tissue (DiLorenzo & Schell, 2014), or infrapatellar fat pad (IFP), have better proliferative ability and chondrogenic potential than other types (Koh & Choi, 2012; Utomo et al., 2019).

Previous studies have shown that the combination of DFLP (Dwikora-Ferdiansyah-Lesmono-Purwati) and AdMSC scaffolds has good potential for cartilage regeneration by increasing the expression of type 2 collagen such as hyaline cartilage (Bari et al., 2019), which is abundant in joint cartilage (Perbowo et al., 2020; Sumarwoto et al., 2021). Besides its great potential for cartilage healing, the combination of scaffold and Adipose-Derived Mesenchymal Cell Secretome (AdMSC) poses its challenges in storage and distribution. The secretomes produced from cell cultures are carried in media containing fetal bovine serum (FBS) or human serum and preserved by cryopreservation (Damous et al., 2018), making it difficult for distribution and storage.

The freeze-drying method is expected to be an answer to this problem. The cryopreservation and freeze drying methods are fundamentally different; when using the cryopreservation method, the sample is cooled to a cryogen temperature (-80°C to -196°C). In contrast, the freeze drying or lyophilization method removes water content through the sublimation process (Theodoridis et al., 2019). The freeze-drying method is currently one of the promising methods because this storage method has been proven to be effective for preserving biomaterials at room temperature for up to several months (Thorp et al., 2021). The freeze-drying method has several advantages, including sample stability at room temperature, maintained structural stability (Mocchi et al., 2021), re-application (Nadesh et al., 2021), distribution, and easy storage (Bari et al., 2021). It is expected to be a future solution for the mass production and distribution of secretome products (Zhang et al., 2010).

Organoleptic is a test based on the sensory process. The uses of organoleptic tests are shelf life assessment, product matching, product mapping, product specifications, quality control, product reformulation, testing for potential odor deviations, and the appearance of foreign odors determining product acceptance (Susiwi S, 2009). The TGF- β superfamily includes a variety of multifunctional growth factors for cellular processes such as proliferation, cell differentiation, and cell apoptosis. TGF-1 and TGF-2 were identified as cartilage driving factors-A (CIF-A) and CIF-B, respectively, in bone extracts in the chondrogenesis assay. TGF- β 3 has emerged as one of the relatively new isoforms to be discovered and studied. TGF- β 3 is believed to regulate molecules involved in cellular adhesion and extracellular matrix (ECM) formation during cartilage development, especially in the palate. Without TGF- β 3, mammals develop a deformity known as cleft lip (Bari et al., 2019). It is caused by the failure of the epithelial cells on both sides of the developing palate to fuse (Mirabdollahi, Haghjooyjavanmard, & Sadeghi-Aliabadi, 2019). TGF- β 3 also plays a vital role in controlling the development of mammalian lung cartilage (El Baradie et al., 2020); it also regulates cell adhesion and ECM formation in tissues (Bari et al., 2021).

However, the molecular biology of the effects of TGF- β during chondrogenesis remains unclear. One pathway currently being investigated is the involvement of transcriptional activation by SRY-type high mobility group box 9 (SOX9) and its co-activator cAMP, which responds to element-binding protein (CBP) and the p300 paralogue (Quen et al., 2009). All types of microorganisms, including fungi, viruses, bacteria, and protozoa, can contaminate cells in culture. Airborne microorganisms can easily enter and grow in cell culture if good manufacturing practices are not followed. The presence of serum in cell culture media primarily makes it susceptible to contamination by microbial species. Contamination in research can jeopardize the quality of research and affect the results that interfere with conclusions. In contrast, when applied to products, contamination can affect normal cell growth, causing cell death within a few days after contamination (Ali, 2017).

Considering the above advantages, the combination of cartilage scaffold and Adipose-Derived Mesenchymal Stem Cell (AdMSC) preserved by the freeze-drying method is a combination that has great potential to be mass-produced and used. However, this production process requires Good Manufacturing Practice (GMP) in

its production, storage, and distribution processes. To achieve this, it is necessary to physically, biochemically, and microbiologically evaluate the stability characteristics. This study aimed to observe and analyze the physical, biochemical, and microbiological stability characteristics of the freeze-dried secretome of chondrogenic AdMSC.

2 Materials and Methods

We conducted a character analysis of the freeze-dried secretome biomaterial of chondrogenic AdMSC by evaluating its physical, biochemical, and microbiological stability. Physical stability was measured by assessing organoleptic changes (shape, color, and odor) with clinical observation and pH with litmus paper at weeks 0, 4, 8, and 12. Biochemical stability was assessed by evaluating TGF beta three levels using ELISA at weeks 0, 4, 8, and 12. Meanwhile, aerobic culture and sensitivity testing was carried out for microbiological stability by observing the presence or absence of bacterial contamination.

The sample size in this study was calculated using the sample size formula from Lemeshow. From the existing procedure, it is found that a minimum of 25 samples is required. The freeze-dried secretome product from chondrogenic AdMSC is produced at the Cell and Tissue Bank of Dr. Soetomo General Hospital. The research was conducted for three months starting in July 2021 at the Cell and Tissue Bank of Dr. Soetomo General Hospital and the Department of Clinical Microbiology at Dr. Soetomo General Hospital, Airlangga University Surabaya.

Secretome Processing

The research material was Adipose Mesenchymal Stem Cells obtained from the infrapatellar pat pad. Before sampling, it was necessary to prepare alpha MEM transport media to carry samples from 1 cm³ adipose tissue without connective tissue or blood clots. The collected samples were immediately put into the transport medium and taken to the laboratory to isolate mesenchymal stem cells. The fatty tissue from the transport medium was then removed and rinsed with PBS solution until clean until the red blood cells attached to the fatty tissue were lost. Adipose tissue was chopped and mixed with collagenase, then poured into bottles equipped with a magnetic stirrer. The tissue in the bottle was then incubated on a hot plate at 37°C for 30 minutes until the fat tissue was completely dissolved. After dissolving, the stopper medium was added and set again for 10 minutes until it became a homogeneous solution. The solution is then poured into 50 ml conical and filtered in the form of sterile gauze until the remaining insoluble fat tissue is separated. The filter results were centrifuged at 3000 rpm for 5 minutes to form pellets. The pellets were resuspended with the alpha MEM medium to become a homogeneous solution. After that, it was planted in a 10 cm petri dish and incubated in a CO₂ incubator for 24 hours until the cells were attached to the bottom of the petri dish. The cells that had been bound were then replaced with the medium every two days until the cells formed colonies and grew to 80% confluent. The obtained ADMSCs were then prepared to culture the secretome, followed by a seeding process with frozen tendon scaffolds to extract the chondrogenic secretome from these cells. The culture and seeding process takes about five days. MSCs that have successfully grown to form

colonies can be propagated to reach the required dose for clinical application. Cells forming a monolayer with confluency of up to 80% need to be rejuvenated by passage. The passage was carried out by removing the medium from the petri dish and then rinsing the monolayer with PBS solution. Subsequently, add the triplex expression enzyme and incubate for 5 minutes until the monolayer is separated from the cup. After the monolayer is removed, it needs to be resuspended by adding a stopping medium until it becomes one cell. The solution containing single cells was poured into a conical tube and centrifuged to form pellets. The pellets were added to Alfa MEM media and resuspended in a homogeneous solution before sowing in new Petri dishes. The secretome produced in this process is one cc per tube. The secretome obtained was then freeze-dried and then packaged in sterile cones and stored at room temperature with a weight of 0.0179 [grams per tube](#).

Statistical Analysis

All data obtained from this study are presented in tables, graphs, and figures to determine the distribution of data characteristics. The distribution of the data will be tested using the Shapiro-Wilk test. The hypothesis test will be analyzed using a repeated ANOVA test if the data distribution is normal. If the data distribution is abnormal, the Friedman test will test the hypothesis analysis. Furthermore, the study was carried out using the Wilcoxon method. The value of $p < 0.05$ was determined as a significant value. Tables and graphs are processed using the Microsoft Excel program. Hypothesis testing was processed using the SPSS version 24 program.

3 Results and Discussions

Physical Stability

The results of organoleptic observations of freeze-dried secretome samples from chondrogenic Ad-MSCs are described in Table 1, and the documentation of the samples is summarized in Figure 1. The shape and odor of the samples did not change until week 12, but the color changed from pink to light yellow. Looking at the acidity level, the pH of the sample also did not show a change from week 0 to week 12 and was at pH 4-5.

Table 1
Results of organoleptic observations and sample pH

Observation result	Week 0	Week 4	Week 8	Week 12
Color	Pink	Pink	Dark Yellow	Light Yellow
Shape	Porous Solid	Porous Solid	Porous Solid	Porous Solid
Odor	Odorless	Odorless	Odorless	Odorless
pH	4-5	4-5	4-5	4-5



Figure 1. *Changes in color and shape of the freeze-dried secretome of chondrogenic AdMSC*

Biochemical Stability

Biochemically the concentration of TGF- β 3 in Freeze-Dried Chondrogenic Secretome AdMSC had a downward trend from the initial 675.67 ± 44.1 at week 0 to 610.4 ± 14.61 at week 12 (Table 3).

Table 2
The concentration of TGF- β 3 in Freeze-Dried Chondrogenic Secretome AdMSC (ng/l)

TGF- β 3 Concentration	Mean	Std. Deviation	Minimum	Maximum
Week 0	675.67	44.08	630.17	718.17
Week 4	753.07	25.27	735.22	782
Week 8	507.85	99.30	397.67	590.44
Week 12	610.40	14.61	597.6	626.33

Statistically, the normality test of the data using the Shapiro – Wilk, and Kolmogorov – Smirnov methods found that the data distribution was not normal and then analyzed using the Friedman method. The data analysis results did not show a significant difference in TGF- β 3 levels between weeks 0, 4, 8, and 12 (table 4).

Table 3
Comparison of TGF- β 3 concentration from week to week using Wilcoxon test

	Week 0	Week 4	Week 8	Week 12
Week 0		0.25	0.25	0.25
Week 4			0.25	0.25
Week 8				0.25
Week 12				

Microbiologic Stability

Microbiologically, Freeze-Dried Chondrogenic Secretome AdMSC showed no bacterial contamination at weeks 0, 4, 8, and 12 (table 4).

Table 4
Microbiological stability evaluation of Freeze-Dried Chondrogenic Secretome AdMSC

Sample	Week 0	Week 4	Week 8	Week 12
1	No Bacterial Growth	No Bacterial Growth	No Bacterial Growth	No Bacterial Growth
2	No Bacterial Growth	No Bacterial Growth	No Bacterial Growth	No Bacterial Growth
3	No Bacterial Growth	No Bacterial Growth	No Bacterial Growth	No Bacterial Growth

We hope this study could pave the way for the mass development of Freeze-Dried Chondrogenic Secretome AdMSC production and further studies in the secretome production field. Freeze Drying was chosen because of its ease of improvement in preparation for large-scale production. It has several advantages over other techniques commonly used to isolate MSC, including its relatively low cost and shorter time required for the entire process. This process also ensures aseptic conditions when changing from liquid to powder or vice versa. In addition, the final product of this process is easy to store; a shorter reconstitution time also provides long-term stability. Apart from its advantages, the freeze-drying method also has disadvantages; the process of freezing and drying can cause pressure that can disrupt the stability of this secretome product.

Ice formation during the freezing process produces a reaction, one of which is protein degradation. When a material undergoes a freezing and drying process, the material receives stress that can change the pH before and after the freeze-drying process. This process can potentially cause biomaterial proteins to denatured, osmotic balance disturbances, and mechanical stress ([Merivaara et al., 2021](#)). The results of the evaluation of pH for 12 weeks showed no change, but it is necessary to further study the optimal pH for freeze-dried chondrogenic secretome AdMSC. The pH analysis in this study was in the range of 4-5. This acidity level is too acidic for human physiological conditions (pH 7-7.4), so further research is needed on alkaline substances that can reconstitute freeze-dried secretomes to suit human physiological requirements ([Li et al., 2006](#)).

Chondrocyte differentiation is regulated by members of the growth factor superfamily TGF- β . TGF- β plays a role in all stages of chondrogenesis, mesenchymal condensation, chondrocyte proliferation, extracellular matrix deposition, and finally, terminal differentiation. TGF- β is a crucial initiator of chondrogenesis in mesenchymal precursor cells. TGF- β also stimulates chondroblast proliferation and the deposition of cartilage-specific extracellular matrix molecules such as aggrecan and type II collagen. TGF- β 3 can enhance stem cell chondrogenesis in culture, and its application in chondrocytes and a suitable scaffold may provide new biotherapy at the forefront of cartilage tissue engineering (Li et al., 2006; Quen et al., 2009). From the observations, it was found that there were no significant differences in the levels of growth factor TGF- β 3 at weeks 0, 4, 8, and 12. More extended observations may be needed to observe a substantial decrease in growth factor. Previous research conducted by Pan showed that freeze-dried MSC preparations could preserve growth factors for more than four weeks (Pan et al., 2016).

The results of the evaluation of microbiological stability showed that no bacterial growth was detected for 0, 4, 8, and 12 weeks, so it is expected that the freeze-dried secretome material from chondrogenic adipose mesenchymal stem cells (AdMSC) meets good GMP and is safe to be stored for long term.

4 Conclusion

In this study, there was a change in color after the fourth week, while there was no change in shape and smell until the twelfth week. In addition, there was no significant change in pH and decreased levels of TGF-3 growth factor and contamination until the twelfth week.

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