



Comparison of TGF- β 3, FGF-2, and BMP-7 Levels Between Freeze Dried Cartilage-Cultured Secretome and Freeze-Dried Crude Secretome: An Experimental Study

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ABSTRACT

Background: The secretome from Mesenchymal Stem Cells (MSCs) has recently emerged as a promising cell-free therapeutic for cartilage defects. The cartilage-cultured secretome is expected to encourage better cell chondrogenesis process. TGF- β 3, FGF-2, and BMP-7 are several growth factors that influence the process of cartilage regeneration. The freeze-drying process itself functions to maintain the stability of growth factors and minimize contamination of the secretome. As a promising type of tissue engineering, this research compares the cartilage-cultured secretome with the crude secretome.

Methods: This study compared TGF- β 3, FGF-2, and BMP-7 levels between Freeze Dried Cartilage-cultured secretome and Crude Secretome. This research is a true experimental with randomized post-test-only design. A comparison of these level was carried out on days 4, 10, 17, and 21.

Results: This study showed that not all of the growth factor parameter levels between the two groups showed significant differences. The BMP-7 parameter showed the highest levels in the freeze-dried cartilage secretome group on day 10, while the FGF-2 parameter showed the highest levels in the freeze-dried crude secretome group on day 4, and the TGF- β 3 parameter showed the highest levels in the freeze-dried cartilage secretome group on day 17.

Conclusions: This research shows that the freeze-dried cartilage secretome group had higher levels of growth factors BMP-7 and TGF- β 3 than the freeze-dried crude secretome group with the highest results for BMP-7 on the 10th day and TGF- β 3 on the 17th day. Meanwhile, the FGF-2 parameter showed that the freeze-dried crude secretome group was higher, especially on day 4.

Keywords: Freeze dried cartilage-cultured secretome, Crude secretome, TGF- β 3, FGF-2, BMP-7

INTRODUCTION

The body has three types of cartilage, and one of them is hyaline cartilage, which is a type of cartilage on joint surfaces that is avascular, alymphatic and anisotropic. Damage to joint cartilage caused by trauma, degenerative diseases, or congenital abnormalities can cause joint pain and reduce quality of life. Secondary fibrocartilage tissue is often formed by osteochondral damage as a result of the inflammatory response; it is biomechanically worse than hyaline cartilage tissue and can break down and separate more quickly. Damage to the cartilage that is not treated will cause pain, joint dysfunction and osteoarthritis (OA) which greatly affect the quality of life. Due to hypocellularity and avascularity, articular cartilage has limited regeneration capacity upon injury (Lee and Wang, 2017).

Mesenchymal stem cells (MSCs) have attracted tremendous research interest due to their ability to repair tissue and reduce inflammation when implanted to damaged or diseased organs. These therapeutic effects have been largely attributed to reactions of secreted biomolecules (i.e., the secretome) (Phelps, *et al.*, 2018). MSC are unspecified cells that can be isolated from various tissues in the body including bone marrow, adipose, dermal, umbilical cord blood, and synovial fluid. Recent studies show that the biological activity of MSCs can be achieved with MSC conditioned medium containing bioactive secretory factors, indicating the potential for developing cell-free therapy strategies for tissue repair (Lee and Wang, 2017). The use of the MSC secretome, or its components, has advantages compared to implantation of the MSC itself, including: the signal can be engineered and scaled to a certain dose, and the nonliving nature of the secretome allows it to be stored and transported efficiently (Jones, *et al.*, 2004; Hsiao, *et al.*, 2012; Pires, *et al.*, 2016)

Secretome defined as a set of factors/molecules secreted into the extracellular space. These factors include soluble proteins, free nucleic acids, lipids, growth factors and extracellular vesicles. The secretome of individual cells and tissues is specific, and changes in response to fluctuations in physiological states or pathological conditions (Beer, *et al.*, 2017; Vizoso, *et al.*, 2017). The MSC secretome contains many cells signaling molecules, including growth factors and cytokines that modulate cell behavior such as proliferation, differentiation, and extracellular matrix production or exert pro- and anti-inflammatory effects (Phelps, *et al.*, 2018).

Chondrogenic differentiation of MSCs must be induced by various intrinsic and extrinsic factors. Growth factors play the most important role in this process. Growth factors are a group of biologically active polypeptides produced by the body, which can stimulate cell proliferation and differentiation. In hyaline cartilage, growth factors regulate homeostasis and integrity, as well as development. Several growth factors that play a role in chondrogenic differentiation include Transforming Growth Factor- β 3 (TGF- β 3), Bone Morphogenetic Protein-7 (BMP-7), and Fibroblast Growth Factor-2 (FGF-2). TGF- β 3 functions to increase the production of cartilage extracellular matrix (ECM), BMP-7 functions to inhibit cell proliferation and induce chondrogenic differentiation, while FGF-2 upregulates Sox9 during chondroblast cellular expansion and early activation of chondrogenesis and increases ECM synthesis. Danišovič *et al* in their research in 2012 have shown that growth factors, especially from the TGF- β and FGF superfamilies, can promote chondrogenic differentiation of MSCs in *in vitro* and *in vivo* conditions. Therefore, growth factors and MSCs are promising tools for tissue engineering and regenerative medicine (Danišovič, *et al.*, 2012; Fortier, *et al.*, 2011; Wang, *et al.*, 2019).

Growth factors in liquid form it will have a direct effect on storage stability because it is a good medium for microbial growth, so special handling is required to maintain the stability of existing growth factors using the freeze dry method. Freeze dry is a drying process where the solvent (generally water) or suspension medium crystallizes at a low temperature and then undergoes sublimation from the solid phase to the gas phase directly. Freeze dry is used to ensure long-term stability and ease of product storage and reconstitution.

This process produces various freezing and drying stresses that can alter the stability of biological samples. Stability studies comparing the fluid form and freeze-dried form show that freeze dried is more stable at room temperature or cold storage, while the fluid form changes at room temperature, this is due to the large amount of water in the medium which causes the growth factor components to be unstable and grow by microbes (Bari, *et al.*, 2018).

Tissue engineering has developed rapidly in the last few decades. One of the most important factors in cartilage tissue engineering is scaffold preparation (Chen, *et al.*, 2011; Tavassoli, *et al.*, 2012). To support cell growth, nutrient transport, and removal of metabolic waste products, scaffolds must have highly porous properties and pores that are connected in three dimensions. By using scaffolds that have good biodegradable and biocompatible properties, replacement of new tissue can be facilitated without inducing capsular fibrous tissue. Scaffolds that are not biocompatible damage body tissue and can cause rejection and death of surrounding tissue. Biocompatibility was assessed based on acute systemic toxicity and pyrogenicity. ECM-derived scaffolds can support cell growth due to their natural components and biocompatibility. Acellular scaffolds have been successfully applied in both preclinical and human applications (Gilbert, *et al.*, 2006).

Until now, evaluation of freeze-dried cartilage cultured secretome mesenchymal stem cells using cartilage scaffold seeding is not yet available. This is important to know so that in the future it can be used as a therapeutic option for OA patients which functions as cartilage regeneration. In addition, the production of cartilage cultured secretome in freeze dried form is expected to be more stable compared to the previous product in liquid form, so that it can be used for a longer period of time.

METHODS

This research is a true experimental with a randomized post-test-only design with the aim of analyzing the growth factor content in the cartilage cultured secretome that underwent cartilage scaffold seeding with the group that did not undergo cartilage scaffold seeding. This research was conducted at the Tissue Bank of Academic General Hospital Dr. Soetomo Surabaya. Ethical approval for this study was obtained from Academic General Hospital Dr. Soetomo Surabaya health research ethics committee. The population of this study was healthy adult humans, both men and women. This study used samples in the form of mesenchymal stem cells taken from human adipose mesenchymal stem cells originating from the infrapatellar fat pad. First, an adipose biopsy was performed from the infrapatellar fat pad of patients who met the inclusion criteria. The adipose obtained was subjected to isolation, cell expansion and cell culture. After obtaining cells that are 80% confluent, the process of seeding the cells onto the scaffold is carried out by inserting the scaffold into the culture container. Then the cells that were 80% confluent were carried out by the process of detaching the cells and turning them into single cells, then the Alpha MEM culture medium was added. Single cells with Alpha MEM are inserted into the scaffold, incubated for a minimum of 2x24 hours. After that, the media was changed every 3-4x24 hours. When changing the media, the secretome production process is carried out. The secretome production technique is carried out by harvesting cells that have grown to 80% confluence, centrifuging for 10 minutes until pellets are formed, and the supernatant obtained is collected. The supernatant was dialyzed with phosphate buffer saline for 2x24 hours until the color of the secretome faded, then centrifuged again, after which the secretome was ready for use. For the freeze-dried technique, the secretome that has been collected is stored in a cryo tube and stored at -80°C for 24 hours. Then the cryo tube is put into the freeze-dried machine for 24 hours and can be stored as needed.

Evaluation of growth factors in this study included levels of TGF- β 3, FGF-2, and BMP-7. ELISA (enzyme-linked immunosorbent assay) is a tool that is widely used to detect and measure proteins and antigens from various samples. The secretome produced by MSCs, then measured the levels of TGF- β 3, FGF-2, and BMP-7 using an ELISA kit that corresponds to these growth factors. The collected data was analyzed statistically using the SPSS 26 program. In this research, the data was obtained in the form of quantitative data. The normality test was carried out using Shapiro – Wilk. Comparison of each growth factor in the two sample groups with descriptive and analytical analysis in the form of an independent sample T-test with the alternative Mann Whitney test.

RESULTS

The data in this study were analyzed statistically using the SPSS 26 program. Comparison of BMP-7 in two sample groups with descriptive and analytical analysis in the form of an independent sample T-test with the alternative Mann Whitney test. An overview of the descriptive analysis can be seen in Table 1, where the mean values in the freeze-dried crude secretome group sequentially on days 4, 10, 17, and 21 were 5.25, 10.25, 5.17, and 13.08. Meanwhile, the mean values of freeze-dried cartilage secretome on days 4, 10, 17, and 21 were 5.75, 14.75, 5.57, and 11.92, respectively. These results show that the mean BMP-7 in the freeze-dried cartilage secretome group on days 4, 10, 17 is higher than the freeze-dried crude secretome group, but on day 21 the mean value of the freeze-dried crude secretome group is higher than the freeze-dried cartilage secretome group (**Fig. 1**).

Table 1. Comparative evaluation of BMP-7

BMP-7	Mean Freeze-Dried Crude Secretome	Mean Freeze-Dried Cartilage Secretome	P value
Day 4	5.25	5.75	0.08
Day 10	10.25	14.75	0.12
Day 17	5.17	5.57	0.19
Day 21	13.08	11.92	0.69

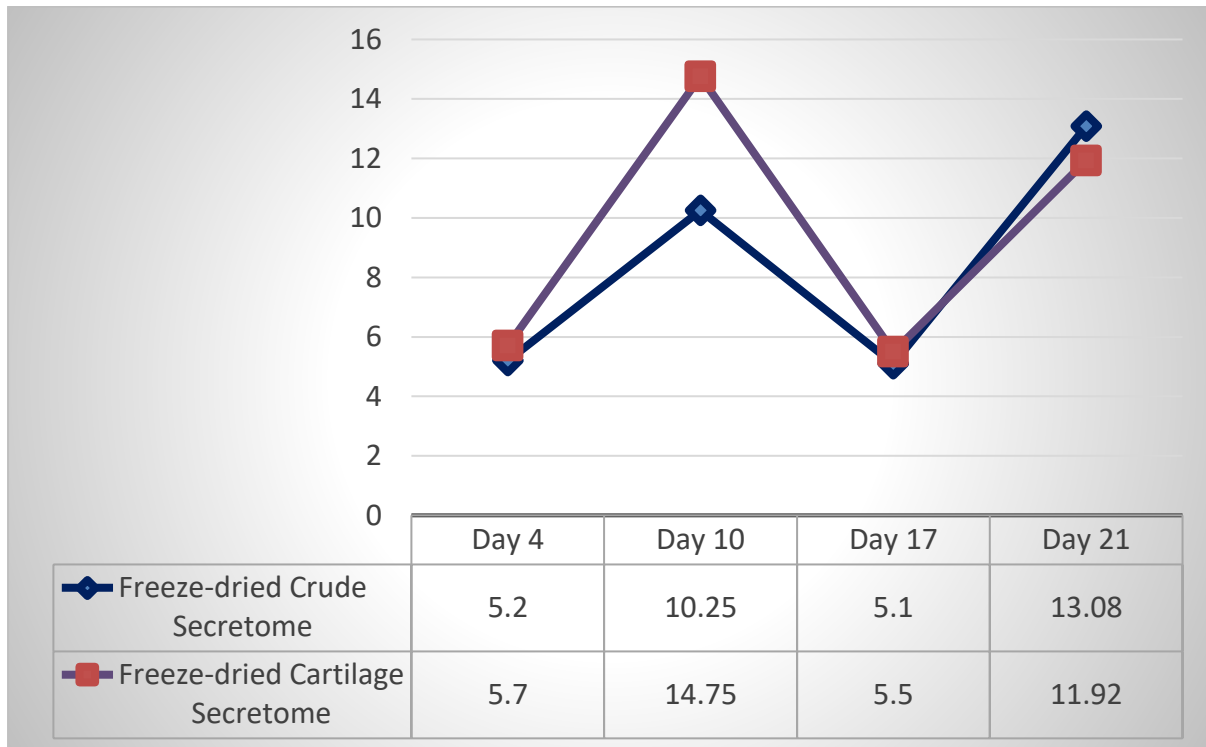


Fig. 1. Comparison of BMP-7 Levels Between Freeze-Dried Cartilage-Cultured Secretome and Freeze-Dried Crude Secretome

Comparison of FGF-2 in two sample groups with descriptive and analytical analysis in the form of an independent sample T-test with the alternative Mann Whitney test. An overview of the descriptive analysis can be seen in Table 2, where the mean values in the freeze-dried crude secretome group sequentially on days 4, 10, 17, and 21 were 13.5, 0.42, 0.44, and 0.39. Meanwhile, the mean values of freeze-dried cartilage secretome on days 4, 10, 17, and 21 were 11.5, 0.38, 0.41, and 0.35, respectively. These results show that the mean FGF-2 in the freeze-dried crude secretome group on days 4, 10, 17 and 21 is higher than in the freeze-dried cartilage secretome group (**Fig. 2**). The results of the comparison of FGF 2 between the freeze-dried crude secretome and freeze-dried cartilage secretome groups on days 4 and 10 showed no significant differences, while on days 17 and 21 there were significant differences.

Table 2. Comparative evaluation of FGF-2

FGF-2	Mean Freeze-Dried Crude Secretome	Mean Freeze-Dried Cartilage Secretome	P value
Day 4	13.5	11.5	0.49
Day 10	0.42	0.38	0.13
Day 17	0.44	0.41	0.00
Day 21	0.39	0.35	0.03

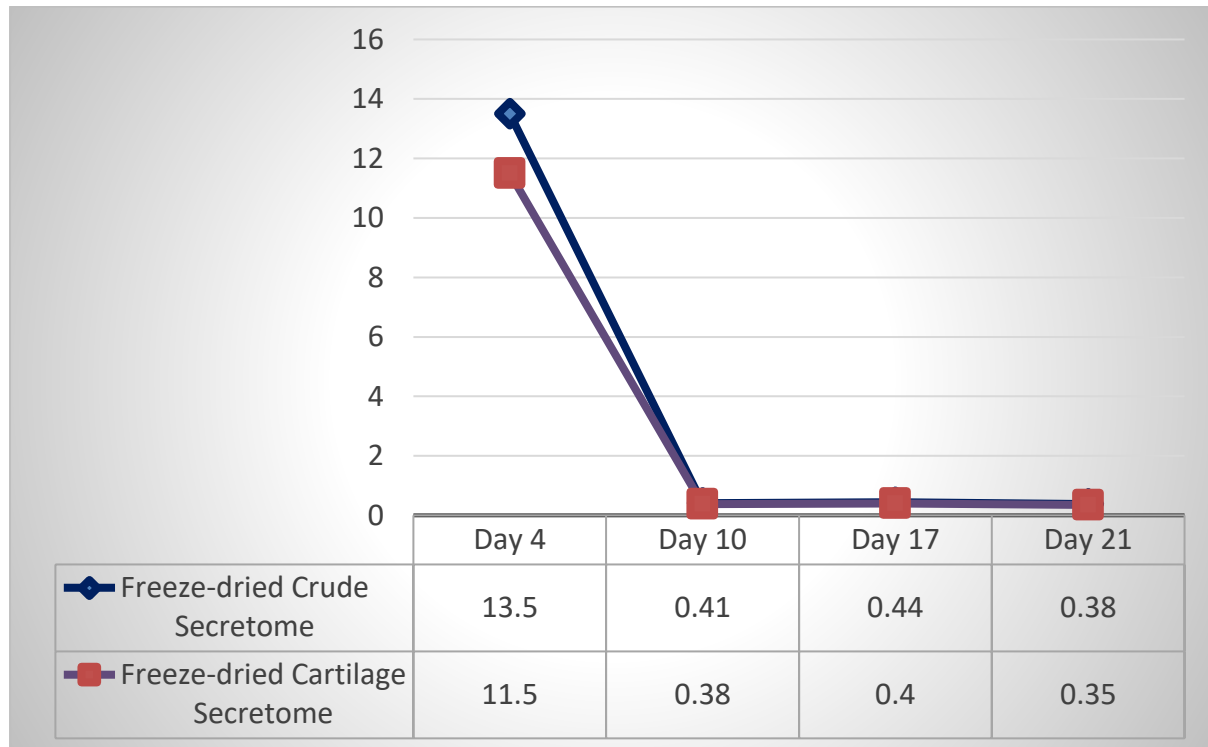
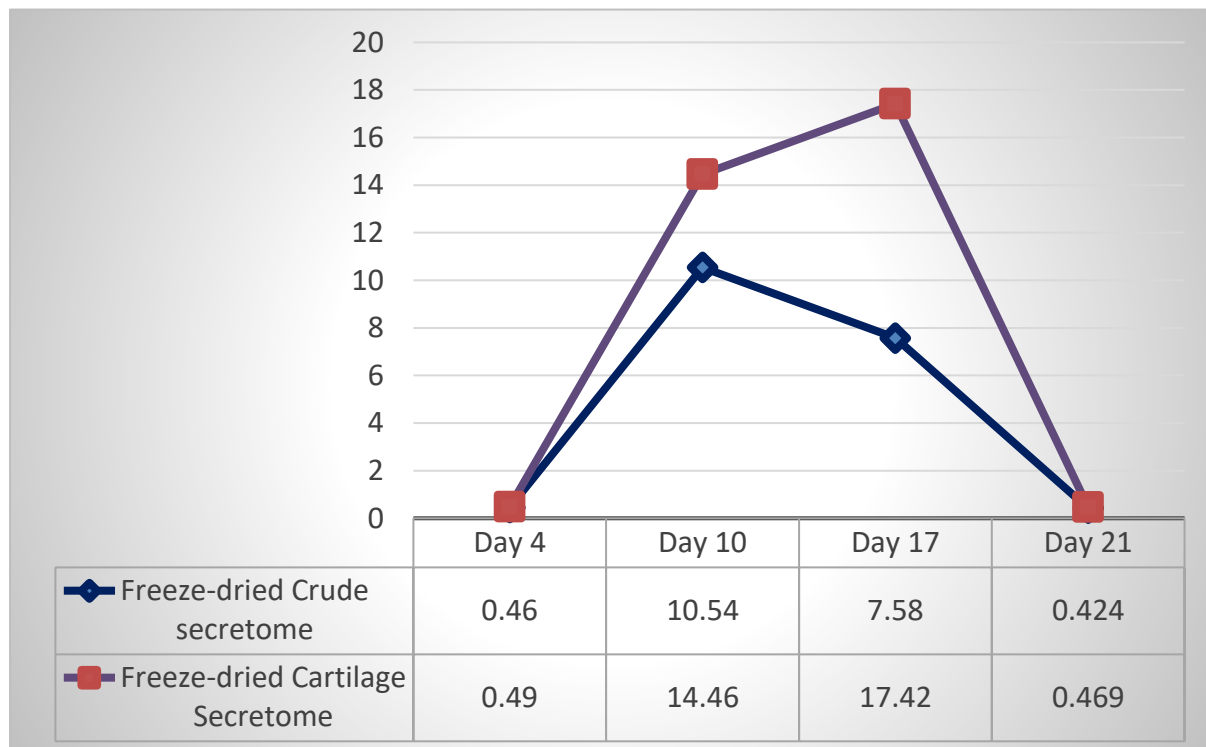


Fig. 2. Comparison of FGF-2 Levels Between Freeze-Dried Cartilage-Cultured Secretome and Freeze-Dried Crude Secretome

Comparison against TGF-β3 in two sample groups with descriptive and analytical analysis in the form of an independent sample T-test with the alternative Mann Whitney test. An overview of the descriptive analysis can be seen in Table 3, where the mean values in the freeze-dried crude secretome group sequentially on days 4, 10, 17, and 21 were 0.46, 10.54, 7.58, and 0.42. Meanwhile, the average value of freeze-dried cartilage secretome respectively on days 4, 10, 17, and 21 were 0.5, 14.46, 17.42, and 0.47. These results show the average TGF-β3 in the freeze-dried cartilage secretome group on days 4, 10, 17 and 21 was higher than the freeze-dried crude secretome group (**Fig. 3**). The results of the comparison of TGF-β3 between the freeze-dried crude secretome and freeze-dried cartilage secretome groups on days 4, 17 and 21 showed a significant difference, while on day 10 there was no significant difference.

Table 3. Comparative evaluation of TGF- β 3

TGF- β 3	Mean Freeze-Dried Crude Secretome	Mean Freeze-Dried Cartilage Secretome	P value
Day 4	0.46	0.5	0.03
Day 10	10.54	14.46	0.18
Day 17	7.58	17.42	0.00
Day 21	0.42	0.47	0.04

**Fig. 3.** Comparison of TGF- β 3 Levels Between Freeze-Dried Cartilage-Cultured Secretome and Freeze-Dried Crude Secretome

DISCUSSION

BMP-7 is a growth factor that influences the process of cartilage regeneration. BMP-7 (also known as osteogenic protein-1) is synthesized by chondrocytes and plays an important role in articular cartilage regeneration. BMP-7 has significant anabolic activity where BMP-7 protects cartilage from damage. BMP-7 functions to inhibit cell proliferation and induce chondrogenic differentiation.³ Experiments with MSCs showed that BMP-7 decreased their proliferative activity but stimulated cartilaginous ECM expression. BMP-7 provided statistically significant results in the evaluations studied on day 4, day 10, day 17 and day 21. In a study by Fu, *et al.* (2017), a significant increase in BMP-7 was found on day 4 and day 6 (Fu, *et al.*, 2017).

In other research by Kumar *et al.* (2018), it shows osteogenic potential due to the content of proteins that are important in the osteogenic process, namely BMP-7, which is higher in Dental Stem Cells compared to Bone Marrow Stem Cells (Kumar, *et al.*, 2018). Other studies explain the differences in growth factor levels in various different culture techniques. In a study by Edward *et al.* show that BMP-2 levels were found to be significantly higher in the cultured group hypoxic compared to normoxic (Edward, *et al.*, 2022). This research shows that there were no significant differences in BMP-7 levels between the Freeze-Dried Crude Secretome and

Freeze-Dried Cartilage Secretome groups on days 4, 10, 17, and 21. This is an additional interesting insight into the influence of the freeze-dried process on the secretome.

Fibroblast growth factor 2 (FGF-2) is reported to have various functions in organisms. FGF-2 was reported to protect cells from various forms of death, such as apoptosis or necrosis through inhibiting autophagy (Tang, *et al.*, 2017) and is involved in cell self-renewal and differentiation (Zhao, *et al.*, 2018). FGF-2 is a key mitogen for mesenchymal stem cells in humans, so it is often added to increase the growth rate of mesenchymal stem cells in humans. The pro-survival effect of FGF-2 can be seen from cell apoptosis. Suppression of FGF-2 expression inhibits neural stem cell proliferation and preconditioning with FGF-2 significantly increases the production of angiogenic secretions of periodontal stem cells.

In addition, there have been several studies examining the role of FGF-2 in cell differentiation. FGF-2 is associated with faster differentiation in stem cell culture expansion (Lee and Wang, 2017). In addition, it was also reported that FGF-2 is an important neurotrophic factor that can stimulate neurogenesis and angiogenesis has been shown to have neuroprotective effects after brain injury. Various previous reports have shown that FGF-2 induces cell proliferation, migration and differentiation of various types of cells and tissues. Additionally, FGF-2 has been applied for clinical use in the regeneration of damaged tissue (Lee and Wang, 2017). FGF-2 plays an important role in promoting wound healing and reducing the risk of scarring and is suggested to be a potential therapeutic agent to improve stem cell-based approaches for the treatment of diabetes mellitus and its complications (Nawrocka, *et al.*, 2017). In addition, a synergistic action of FGF-2 and bone marrow transplantation can reduce radiation-induced intestinal injury (Kim, *et al.*, 2018).

In the evaluation results of FGF-2 in the Freeze-Dried Crude Secretome and Freeze-Dried Cartilage Secretome groups on day 4 and day 10 there were no significant differences. On days 17 and 21 in the Freeze-Dried Crude secretome and Freeze-dried cartilage secretome groups showed significant differences. In another study by Gromolak *et al.* (2020), FGF-2 has the potential to increase osteogenic differentiation ability much better when combined with BMP-2 with confirmation of Alizarin Red S staining which assesses osteocalcin and type I collagen in the secretome prepared from ovine bone marrow (Gromolak, *et al.*, 2020). In another study by Konala *et al.* (2020), FGF-2 had the highest increase in the secretome from the dentine pulp compared to the secretome made from Wharthon's jelly and bone marrow with an increase of 1.6 in the secretome of the denticular pulp, 1.5 in the secretome of Warthon's jelly and 1.0 in the secretome from bone marrow (Konala, *et al.*, 2020).

MSCs form a host of stem cells in almost all tissues and organs that regulate organ homeostasis by regenerating lost cells under physiological and pathological conditions. This can be achieved either by physical migration to the site of tissue injury and/or through paracrine secretion of MSCs or both. This in-depth understanding of the properties of MSCs will help us in selecting appropriate tissue types for MSCs for the treatment of certain diseases. TGF- β 3 is a multifunctional cytokine required for embryonic tissue development and adult tissue homeostasis. TGF- β 3 stimulates autocrine and paracrine structures that are important in maintaining and developing MSCs. Bone tissue and cartilage contain trace amounts of TGF- β 3. TGF- β 3 stimulates proliferation, differentiation, and osteoblast formation osteoprogenitors (Edward, *et al.*, 2022).

In this study, the results of the TGF- β 3 evaluation on day 4, day 10, day 17 and day 20 showed significant differences according to statistical results on each day of the evaluation carried out. There are various other studies that have studied the levels of TGF- β 3 in the secretome using various culture techniques and conditions. TGF- β 3 showed higher yields in the secretome prepared from derivatives bone marrow of 1.0 compared to the secretome from Warthon's jelly with a value of 0.73 and the secretome from the dental pulp with an increase of 0.62 (Konala, *et al.*, 2020). In a study conducted by Edward *et al.*, it was found that the mean levels of TGF- β 3 were higher in the secretome group with hypoxic culture conditions compared to those in normoxia conditions. Hypoxic conditions are believed to approach the physiological conditions of a fracture (Edward, *et al.*, 2022). In addition, in a study conducted by Mingyuan *et al.*, it was found that TGF- β 3 levels in intracellular and secreted conditions were significantly higher in hypoxic conditions compared to normoxic conditions. The results of this study provide additional data regarding the best culture conditions and techniques to achieve optimal TGF- β 3 levels.

This study has several limitations, namely the number of samples can still be increased to produce a more specific evaluation. Sample storage in this study was also carried out in tropical areas, so it could affect sample quality. Apart from that, there are still few studies that discuss the comparison of the expression of growth factors in the Freeze-Dried Crude Secretome and Freeze-Dried Cartilage Secretome, so the author cannot provide examples of comparisons from this research to previous studies.

CONCLUSION

This research shows that the freeze-dried cartilage secretome group had higher levels of growth factors BMP-7 and TGF- β 3 than the freeze-dried crude secretome group with the highest results for BMP-7 on the 10th day and TGF- β 3 on the 17th day. Meanwhile, the FGF-2 parameter showed that the freeze-dried crude secretome group was higher, especially on day 4.

ABBREVIATIONS

Alpha MEM: Alpha Minimum Essential Medium

BMP-7: Bone Morphogenetic Protein-7

ECM: Extracellular Matrix

ELISA: Enzym-Linked Immunosorbent Assay

FGF-2: Fibroblast Growth Factor-2

MSC: Mesenchymal Stem Cell

OA: Osteoarthritis

TGF- β 3: Transforming Growth Factor- β 3

AVAILABILITY OF DATA AND MATERIALS

Data will be made available on reasonable request

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AUTHORS CONTRIBUTIONS

R.E.S.: Data curation, Investigation, and Writing

D.N.U.: Conceptualization, Methodology, and Supervision

H.S.: Conceptualization, Methodology, and Supervision

M.E.: Conceptualization, Supervision, and Writing

K.D.H.: Conceptualization, Supervision, Resources, and Writing

B.U.: Data curation, Supervision, and Writing

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None

DECLARATIONS

Ethics approval and consent to participate

This study has received approval from ethical committee of Academic General Hospital Dr. Soetomo Surabaya health research ethics committee (Ethical Clearance No. 0076/ KEPK/ IX/ 2020)

Consent for publication

Not applicable

Competing interests

The authors declare no conflict of interest.

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