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Original Article

Characterization and Stability Study of Amniotic Membrane Stem Cell Metabolite Product (AMSC-MP)

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ABSTRACT

The purpose of this study was to determine the characteristic and stability of Amniotic Membrane Stem Cell Metabolite Product (AMSC-MP) in fluid and freeze dried form. Conducted a qualitative test of the liquid and freeze dried AMSC-MP form using the SDS-PAGE method, also determined the quantitative TGF-β levels, stability stored testing both materials at room and cold temperature during 28 days. Characterization of the freeze dried form was also carried out including FTIR profile, DTA, SEM and XRD.SDS-PAGE results obtained that qualitatively the liquid and freeze dried forms have the same protein component a molecule weight (MW) of 75.33 kDa. Quantitatively, the form of fluid has higher TGF-β levels compared to freeze dried on day 0. However, the results of the stability test showed that the form of freeze dried has better stability than the fluid, as indicated by a decrease in TGF-β levels greater on the day 21st. Liquid form that is stored at room temperature changes the color after 7days. Furthermore characterization of freeze dried shows has a crystalline structure based on its XRD profile with an endothermic peak at 163.8 this is supported by SEM a tetragonal crystal. FTIR profile showed the maximum absorption at wave numbers 1674-1640 and 3350-3200 which indicates the presence of C=O and N-H bonds which are functional groups of protein compounds.Conclusion: The freeze dried AMSC-MP form has better stability than the fluid form in cold temperature storage and AMSC-MP is a protein.

Keywords:AMSC-MP,Characterization, Freeze dried,Stability,TGF-β.

1. INTRODUCTION

Aging is a multifactorial process that occurs in humans due to factors based on time and external factors due to sun exposure and pollution, indicated by color change, wrinkles, fine lines and relief [1]. The development of antiaging cosmetics is currently reaching a rapid stage, the technology used is modern and the active ingredients derived from

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chemicals to the body can be used as a natural antiaging. But there are limitations to natural antiaging, one of which is storage instability due to the many components of water in it. So the problem must be developed using a new method. One of the natural ingredients that can used as antiaging cosmetics comes from the body is an amniotic membrane stem cell metabolite product (AMSC-MP)[2,3]. AMSC-MP is a transparent, thin and strong fetal membrane sac with a thickness of 0.02-0.05 mm[4] containing growth hormones that function as antiaging including Transforming Growth Factor Beta (TGF-β), Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF)and Keratinocytes Growth Factor (KGF)[5]. TGF-β acts as a multifunctional growth factor that has an important role in modulating cell behavior in tissues, so that it has the potential to be one of the antiaging components. However, there is no literature stating the standardization and characterization of AMSC-MP as a raw material for biological products and its stability. The importance of AMSC-MP characterization and standardization is closely related to the reproducibility and stability that affect the handling of materials which can have an impact on the effectiveness of antiaging cosmetics products.Standardization parameters including stability and characterization are important for pharmaceutical products, especially cosmetics to ensure the effectiveness and efficacy of the use and storage of products in a long time. In this study AMSC-MP stability and characterization tests were carried out on the form of fluid and freeze dried. The data that has been obtained from this research can be used for information on the use of antiaging cosmetics raw materials, especially those made from amnion.

2. MATERIALS AND METHODS

Materials

AMSC-MP was obtained from the Stem Cell Bank Laboratory at the DR Soetomo General Hospital, in Surabaya, Indonesia. The procedures for obtaining amniotic membrane from the donor, and separation of AMSC-MP from AMSCs were performed legally in accordance (Clinical Research Unit DR Soetomo general hospital) with the international standards for tissue donor and stem cell culture. TGF-βColor Burst TM Electrophoresis Marker C 1992-1VL Sigma Aldrich from Singapore. Human TGF-β ELISA Kit Bioassay Technology Laboratory.

Freeze drying AMSC-MP

The first step of the process using VirTis 4 K, BenchTop K, Chinaat DR Soetomo General Hospital, was freezing the compound, followed by putting it under vacuum which allowed the solvent to adhere and solidify. Finally, it was frozen at -40ºC to allow the solvent in the material to sublimate. Condensation in low temperature has the ability to remove the solvent which evaporates in the vacuum for it to return to the solid form. This final result of this process formed a yellowish-white powder.

Qualitative test of AMSC-MP withsodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE)

The AMSC-MP fluid and freeze dried were analyzed using (Mini-PROTEAN Tetra CELL-BIO-RAD) at the Institute of Tropical Disease, in Surabaya, Indonesia. A total of 5 mg of sample was diluted using 300 µl H_2O, with 15% separating gel (pH 8.8), and 4% stacking gel (pH 6.8) with silver stained staining.

Quantitative TGF-β levels with enzyme linked immuno sorbent assay (ELISA)

The AMSC-MP fluid and freeze dried were analyzed using an iMark microplate reader (Bio Rad), and ELISA kit (human TGF-β, Bioassay Tech, Lab) at Infection Specific Hospital, in Surabaya, Indonesia. In each, 50 µl of standard, 40 µl of sample and add 10 µl of anti TGF-β were added to the wells containing the samples. It was further incubated for 60 min (37ºC), with the plates washed 5 times. An approximate amount of 50 µl of the substrate solution A and B were added to the solution and incubated for 10 min (37ºC), till it turns yellow. The sap content determination is carried out at a wavenumber of 450 nm.

Study stability test ofAMSC-MP

AMSC-MP samples of the form of fluid and freeze dried were stored at cold temperature (7ºC) and room temperature (25ºC) for 28 days, the parameters observed were changes in color and pH observed on days 0, 7, 14, 21, and 28. TGF-β levels The AMSC-MP fluid and freeze dried forms are carried out on days 0 and 21.

X-Ray Diffraction (XRD)

X-ray diffraction using Philips X'Pert Jeol JDX-3530, Netherlands, at Department Materials and Metallurgic Engineering Faculty of Industrial Technology, Institute Teknologi Sepuluh November, Surabaya, Indonesia. Test was carried out on dried AMSC-MP with the powder placed into a glass sample holder and uniformly seared onto a glass slide, with a flat upper surface. The glass holder contained the sample was inserted into X-ray diffractometer and observed with an angle of 2θ from 5º to 40º. X-ray diffractor was set at 40 kV and 40 mA .

Scanning Electron Microscope (SEM)

Freeze dried AMSCM was analyzed using Jeol, JSM-6360, Japan at Department Materials and Metallurgic Engineering Faculty of Industrial Technology, Institute Teknologi Sepuluh November, Surabaya, Indonesia. Approximately 10 mg of sample was placed in a specimen tube with double-sided adhesive. The sample was inserted into the room holder and coated with 10 mm gold aluminum. In addition, the morphology was observed with a voltage of 20 kV and 12 mA using appropriate magnification.

Differential Thermal Analysis (DTA)

In the Thermal analyzer DTA using Mettler Toledo FP 85 TA Cell, US, approximately 3.1 mg of dried AMSC-MP was transferred to the aluminum crucible pan, which was inserted to the DTA. The instrument was then set with a heating

speed of 10ºC /min, with the observations carried out in a range of 30 – 300ºC.

Fourier Transform Infra Red (FTIR)

FTIR using Cary 630 with Diamond ATR, UK, test was approximately 10 mg of dried AMSC-MP and placed on the glass between the holder which was lowered till it sounded and waited for 15s to obtain the result. The spectra were observed at a wavelength number of 650 - 4000 cm^{-1}

Statistical Analysis

Mann Whitney test was performed to evaluate the pH parameter on days 0, 7, 14, 21, and 28. In addition, the paired t-test was used to evaluate the TGF-β level on days 0 and 21. Data were analyzed using ANOVA one way method, with a significant level of $p < 0.05$.

3. RESULTS AND DISCUSSION

The purpose of this study was to determine the characteristic of raw material freeze dried Amniotic Membrane Stem Cell Metabolite Product (AMSC-MP) in addition, TGF-β which is one of the AMSC-MP components has a low stability against temperature and water so this study compares its liquid and freeze dried form.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Both liquid and freeze dried indicate protein markers at RF (0.76) which are same as MW 75.33 kDa. SDS-PAGE results is seen in (Figure 1).

Enzyme Linked Immunosorbent Assay (ELISA)

The results of the determination of TGF-β levels indicate that the fluid has decreased significantly after storage at cold temperatures (7^{0}C) for 21 days. TGF-β levels using the ELISA method obtained fluid levels on day 0 765.7 ng/L and freeze dried for 595.5 ng/L. Whereas AMSC-MP freeze dried did not experience a decrease in TGF-β levels and was stable during storage for 21 days in cold temperatures.Based on paired t-test, it is known that the two treatment groups showed no differences ($p = 0.053 > 0.05$). TGF-β levels results is seen in (Table 1).

Table 1: TGF-β levels of AMSC-MP fluid and freeze dried on days 0 and 21 stored at cold temperature (7^{0}C)

AMSC-MP	Day- 0ng/L Mean ± SD	Day-21ng/L Mean ± SD
Fluid	765.7± 1.27	568.7± 30.19
Freeze dried	595.5± 6.22	563.5± 25.73

Stability Study

The results of AMSC-MP fluid and freeze dried on visual tests at cold and room temperatures for 28 days showed a change in color in the fluid starting on day 7th of storage at room temperature. While in cold temperatures there is no change. The form of freeze dried at room temperature and cold storage does not change the smell and color. Proteins such as AMSC-MP generally stable in water for a short period of time, while Pharmaceutical dosage consists of adequate storage stability for months[6]. Color and odor changes occur on the 7th day of AMSC-MP fluid day at room temperature due to the large number of microorganisms that grow in the water component. Water component is a good medium for the growth of microorganisms. Cold temperatures will prevent the growth of microorganisms and stop all enzyme activity, so there are no significant changes [6, 7]. Stability study result is seen in (Figure 2).The results of the pH test did not change significantly either fluid or freeze dried at room temperature or cold storage.The measurement of pH value had no statistically significant difference ($p= 0.878 > 0.05$). pH results is seen in (Figure 3).The results of the pH fluid and freeze dried do not differ because while freeze drying no ingredients added.

X-Ray Diffraction (XRD)

There were three peaks at angles 31.59º, 45.35º and 56.39ºas shown by the XRD result in (Figure 4). Protein crystals usually have a lower symmetry system due to the activity of its molecules which is between the polar groups. It also has larger cell units than crystals with small molecular sizes, therefore, they are more diffracted [8]. Clare said that the peaks that appear in XRD indicate the nature of the crystals in the sample which is a representative of the existing growth hormone.Each peak that appears in the XRD pattern represents a crystal field consisting a certain orientation. Protein crystals are sensitive to X-ray irradiation, especially the fresh ones, therefore,the crystal peaks tends to be increasingly obtained [9]. Peak positions at an angle of 31.59º, 45.35º and 56.39º with the highest intensity from AMSC-MP indicate that this area contains growth factors. The plot formed represents the number of amorphous and crystalline elements in the sample, while the two highest peaks represents growth factors in AMSC-MP [10].

Scanning Electron Microscope (SEM)

Freeze dried AMSC-MP had tetragonal crystal. The SEM results is seen in (Figure 5).The flat shape indicates its flexibility with the morphological structure of AMSC-MP characterized using scanning electron microscopy (SEM). According to Chai, J and Hink, tetragonal cystals is a type of growth hormone [9, 11].

Differential Thermal Analysis (DTA)

Thermal analysis with DTA produced thermograms in the form of endothermic peaks with melting temperatures of 163.8ºC and enthalpy 305 J/g. The DTA result is seen in (Figure 6).

Fourier Transform InfraRed (FTIR)

The functional group in AMSC-MP was examined using Fourier Transform Infrared (FTIR) with a wavenumber of 4000 cm^{-1}-500 cm^{-1}. Spectra FTIR results is seen in (Figure 7). The strong intensity was seen in the functional group of C = O at 1675-1640 cm^{-1} with a C=O and N-H bond at 3200 cm^{-1}. The type of spectra which is not sharp, with a peak C=O in the range of 1640 cm^{-1}, has an NH group of 3200 cm^{-1}. AMSC-MP data consists of amides and alkyl groups containing growth factor content [12].

From the results of this study it was found that the molecular size of the sample was 75.33 kDa, the level of TGF-β fluid was higher than that of freeze dried but during 21 days of storage the fluid sample was unstable. The storage stability of freeze dried is better than fluid, while the pH does not differ significantly between the two. The characterization of freeze dried AMSC-MP has high crystallinity properties of XRD results having three specific peaks, the results are supported by SEM parameters in the form of tetragonal crystals, the nature of crystals possessed by AMSC-MP results in melting temperatures reaching 163.8ºC. One of the characteristics of protein functional groups is the presence of C=O bonds in wave number 1675-1640 cm^{-1} and N-H 3350-3200 cm^{-1}. TGF-β growth hormone is not the only one that can be used as an anti-aging in AMSC-MP. Other examples are EGF, bFGF, and KGF, further research is needed so that stability and efficacy can be compared.

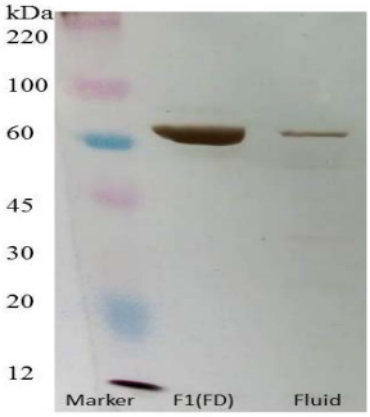

Fig1:The result of qualitative determination of protein marker in fluid and freeze dried (FD) AMSC-MP using SDS-PAGE

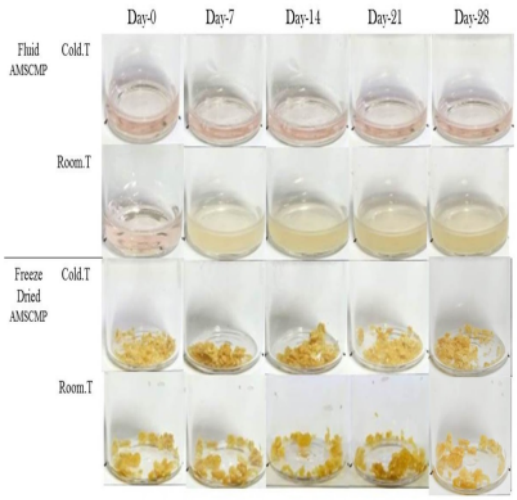

Fig 2:Visual observation of fluid and freeze dried AMSC-MP stored at cold and room temperatutes for 28 days

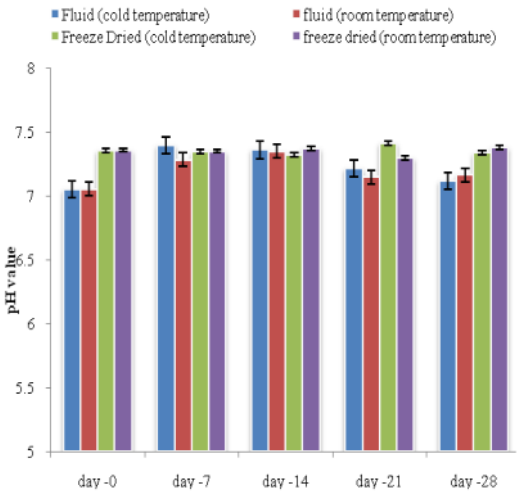

Fig 3: pH evaluation of fluid and freeze dried are stored at room and cold temperatures during study period for 28 days

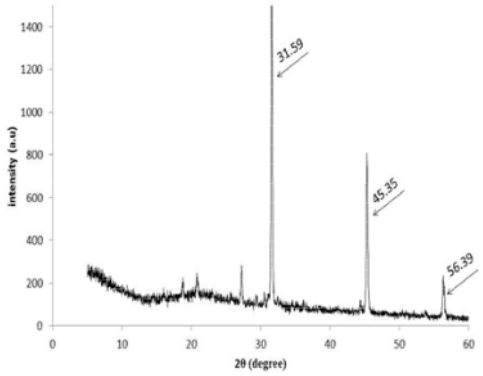

Fig 4:Profil of powder x-ray diffraction of freeze dried AMSC-MP

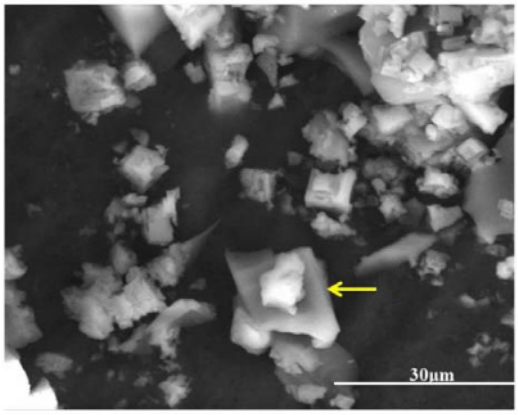

Fig 5: Scanning electron microscope (SEM) image of freeze dried Amniotic Membrane Stem Cell Metabolite Product (AMSCMP) (Scale bar : 30 µm)

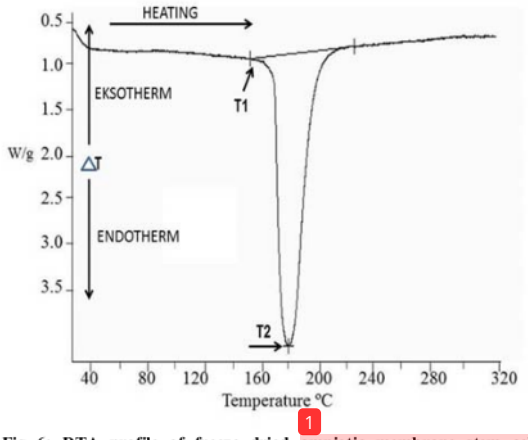

Fig 6: DTA profile of freeze dried amniotic membrane stem cell metabolite product(AMSC-MP)

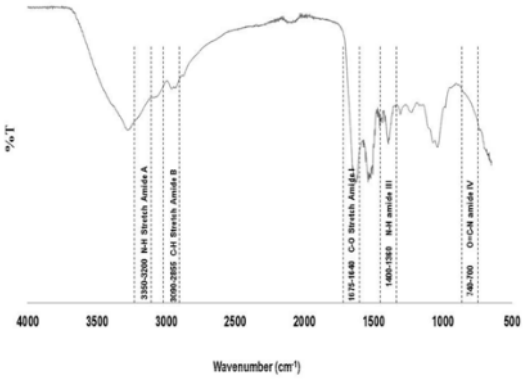

Fig 7: The FTIR Spectra of Freeze Dried AMSC-MP

4. CONCLUSIONS

The AMSC-MP freeze dried form has better stability than its fluid form at both room and cold temperature storage and from further test results AMSC-MP is a protein.

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