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Original Article

FORMULATION OF MESENCHYMAL STEM CELL SECRETOME AS ANTIAGING CREAM

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ABSTRACT

Objective: Skin aging occurs not only due to age but is influenced by various environmental factors such as lifestyle, pollution, and excessive exposure to UV rays. Secretomes can act as antiaging agents that stimulate collagen biosynthesis naturally in the skin. This study aims to create a cream containing 5% secretome with essential oil, dermatological examination, and Fibroblast Growth Factor (FGF) checking.

Methods: Antiaging efficacy testing involves examination by an expert dermatologist for assessment before and after using the cream. The antiaging impact test criteria include moisture, elasticity, and collagen values. Consequently, the cream shows an antiaging activity.

Results: The antiaging efficacy test revealed increased moisture from 24.375±11.97 to 25.125±7.1; the skin elasticity also increased from 40.375±8.39 to 48.5±9.09 and the collagen value increased from 48.25±13.54 to 56.5±8.63. The result shows that the cream contains a 61.143 pg/ml concentration of FGF using an ELISA kit.

Conclusion: The formula for cream preparations containing 5% secretome with essential oil meets the requirements of cosmetics through several evaluations of the trial. It has an antiaging effect, proven through several tests.

Keywords: Secretome, Antiaging, Cream, Fibroblast growth factor

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INTRODUCTION

Skin aging is a biological process that everyone cannot avoid due to age and environmental factors. The process of skin aging is caused by environmental factors such as ultraviolet radiation (UV), long-term use of cosmetics, pollution, and lifestyle [1–3]. These factors increase the production of reactive oxygen species (ROS), which damage the skin collagen matrix, such as decreased collagen synthesis and increased collagen degradation. Extrinsic skin aging is characterized by black spots, wrinkles, epidermal atrophy, and dry and rough skin [4, 5].

Progress in biotechnology increases expectations of cosmetics trends. The industries focus on product development using the latest technology, such as stem cell culture and exosome extraction [6]. Demand for cosmetics has become a global trend with extraordinary progress in developing cosmetics with additional pharmacological properties, such as slowing premature aging or regenerating damaged skin. A conditioned medium is a medium from cultured cells. The secretome contains metabolites secreted by cells, growth factors, and extracellular matrix proteins. The secretome of the mesenchymal stem cell has growth factors such as epidermal growth factors (EGF), fibroblast growth factor (FGF), vascular endothelial growth factors (VEGF), and many more. These three factors play a role in wound healing and fibroblast activity, providing antioxidant effects by increasing collagen biosynthesis, reducing wrinkles, and reducing oxidative stress on the skin [7].

The secretome acts as an antiaging agent by stimulating natural collagen biosynthesis on the skin. Secretomes also do not contain cells, and no need to match the donor and recipient. This condition can avoid rejection when providing conditioned-medium therapy to patients. Moreover, the secretome can be more easily produced, frozen, dried, packaged, and delivered [8].

At present, many cosmetic innovations have appeared through scientific research. In cosmetics, many ingredients are used commercially and claimed to have a particular effect when applied. The cosmetics industry is competing to produce cosmetic preparations that claim the best ingredients with the most antiaging benefits based on stem cells and the products they release. However, this related research is still not much, so the results are questionable, especially because the dosage forms tested are different. Previously, Kim *et al.*, had tested AD-MSC in liquid form [9]. Therefore, this study will examine the effect of cream preparations containing conditioned media mesenchymal stem cells.

MATERIALS AND METHODS

Tools and materials

Pipette Gun CV10 (Cleaver®, England), Pippete Controller 94700 (TPP®, Switzerland), Biosafety Cabinet 1300 (Thermofisher Scientific®, United States), CO2 AMUS080 incubator (Hanil®, Korea), Inverted AMEX1000 (TPP Union), Waterbath Swb10L1 (Cleaver®, England), Flask T25 Vent Cap 90026 (TPP®, Switzerland), Centrifuge Tube 15 ml (SPL®, Korea), Centrifuge Tube 50 ml (TPP®, Swiss), Obups Plastic (Parafilm ®, United States), 2 ml serology pipette (TPP®, Switzerland), 5 ml serology pipette (SPL®, Korea), Standard Tip (SSI®, US), Disposable Syringe 5 ml (Onemed®, Indonesia), Alcohol Swab (Onemed®, Indonesia), Syringe Filter 0.22 µm (TPP®, Switzerland), Analytical Scales (Shimadzu®, Japan), Heating Magnetic Stirrer (Arec®, Italy), PH Meter (Hanna Instruments® Hi98107, United States), viscometer Brookfield (Broekfield Synchrrorctic, United States), Centrifuge (PLC Series-03, China), Microscopes and Opttilab Viewer (Zeiss Primo Star, Germany), Microplate Reader (Thermo Scientific®, United States), Nanoparticle Size (Shimadzu Sald 2300, Japan), Sony A6000 Camera, EH-900U Skin Analyzer and the other laboratory equipment. Adipose mesenchymal stem cells, medium DMEM (CR20210567014, Invitrogen®, United States), FBS (Fetal Bovine Serum) (FO20126140079 Invitrogen®, United States), penicillin-streptomycin (CR20515240062 Invitrogen®, United States), Trypsin-Edta 0,25% (CR20512563029 Invitrogen®, United States, PBS (Phosphate Buffer Saline) (FO20126140087 Invitrogen®, United States), Aquabidest (Double Distillated Water) (Bratacho, Indonesia), Olivem® 1000 (Hallstar® (Daarjeling, Indonesia), patchouli oil (Pelangi Harum Nusantara, Indonesia), Methyl Paraben (Bratacho, Indonesia), Aquadest (Bratacho, Indonesia).

Preparation of culture medium

DMEM, FBS, and antibiotics were made into aliquots with 20% FBS+1% penicillin-streptomycin antibiotics in the DMEM medium. All procedures were performed under sterile conditions. This culture medium can be stored in a refrigerator (-20 ℃) when not used and warmed in a water bath to 37 °C if reused.

Cell culture multiplication

In passage two, 70% of confluent cells were removed from the growth medium and washed with sterile PBS (Phosphate Buffer Saline) 2 times. The trypsinization process was carried out to remove cells from the surface of the flask by adding 1 ml of trypsin-EDTA 0.5% into the flask, then incubated in a 5% CO2 incubator at 37 °C for 5 min. 1 ml of culture medium was added to inactivate the trypsin. Cells were put into a microtube and centrifuged at 2000 rpm for 5 min. The supernatant had discarded, and the pellet was resuspended with 4 ml of culture medium. The cell suspension was put into flask T25.

Collecting secretome

The medium is taken from cells passage four which is 70% confluent and then filtered with a 0.22 µm filter to remove the remaining cell debris. The filtrate was taken and stored at-20 ℃.

Preparation of cream

The concentration of the efficacious ingredients and the composition of the cream base is based on previous research, which is 5% [9].

Table 1: Cream formulation

Ingredients	Function	Formula I (%)	Formula II (%)	Formula III (%)
CM-MSC	Active ingredients	5	5	5
Olivem® 1000	Emulsifier/cream base	5	5	5
Orange oil	Enhancer	-	4	-
Patchouli oil	Enhancer	-	-	1
DMDM Hydantoin	Preservative	0.1	0.1	0.1
Aquadest	Solvent	Ad 100	Ad 100	Ad 100

The formulation of the cream is based on the previous research [10–12]

The oil and water phases were each heated at the same temperature (70 ^{0}C) for 10 min. The temperature was lowered to 45 ^{0}C. Orange oil and CM-MSC were added to a magnetic stirrer with a speed of 1200 rpm and a stirring time of 10 min. The finished cream is cool to room temperature and put in a container. Preparations made as much as 100 grams [13].

Evaluation of cream

- Organoleptic observations can be seen visually from the cream's shape, texture, color, and homogeneity [14]
- The homogeneity test is carried out by applying±0.1 g of the preparation on a piece of transparent glass, it must show a Homogeneities arrangement, and there should be no particle specks [15]
- pH test. It was carried out with a pH meter calibrated with a buffer solution of 4.01 and a buffer solution of 7.01. The position of the needle indicates the pH value.
- Test preference for cream-based aroma. For the preference test, 1 gram of bases 1, 2, and 3 in a separate container was given to 10 volunteers. To identify the most suitable aroma in the formulation by volunteer, it used the Nine-Point Hedonic Scale, where one is the lowest score and nine is the highest [16].
- To see the emulsion types, one drop of methylene blue was dropped in 100 g of samples and mixed. If the methylene blue color is evenly distributed, it indicates the kind of oil-in-water emulsion (o/w). Meanwhile, if it doesn't, it is a type of water-in-oil (w/o) emulsion [18].
- Determination of cream viscosity using a Brookfield viscometer with spindle no. 6. Viscosity results are obtained from the multiplication of dial reading with the correction factor for each spindle [18].
- Determination of stability: by mechanical tests and freeze and thaw methods.

Clinical trial on human skin

- The skin irritation test was carried out directly on humans with a patch test. The test preparation of approximately 0.1 g was applied to the inner arm with a diameter of 2 cm, then covered with gauze. After 24 h, observed symptoms that arise. This examination was conducted on ten volunteers [19].
- Examining skin conditions: An antiaging cream effect test was conducted on eight human volunteers aged 30 to 50. Volunteers were asked to use cream preparations on the corners of the eyes or the part recommended by a dermatologist and genital specialist for eight weeks at night. A dermatologist and gynecologist measured several parameters, such as the value of moisture, elasticity, and collagen fibers in the skin, using a skin analyzer [20].

Characterization and measurement of FGF concentration by ELISA test and microplate reader

Samples with a cream supernatant containing CM-MSC were added with 100 µl of dilution sample and standard, then incubated for 90 min at 37℃. This characterization work is carried out according to the stated criteria. A well-plate containing standard solution, secretome, and cream samples containing FGF growth factor was used to determine the concentration, which was carried out with the sandwich ELISA test procedure. Then the absorbance was measured on a Microplate Reader with a wavelength of 450 nm.

RESULTS

Preparation of secretome

The stem cells used are cells that have entered the fourth life phase or called passage 4 (P4). P4 cells are cells that have been subcultured three times. *Subculture* is the process of multiplying cells and can make cells more Homogeneous and affect cell growth kinetics which can increase the potential of cells to treat degenerative diseases. After two days, cells that had entered the P4 were observed using an inverted microscope. When it was confluent, the medium was taken and filtered with a 0.22 µm filter to remove any remaining cell debris. This medium is used as an active ingredient in cream preparations.

Organoleptic

The evaluation of the cream preparations showed no changes before and during the storage of cream preparations (5 w). The organoleptic properties, including physical appearance, color, texture, and homogeneity, are displayed in table 2. Results showed that all formulations had a cosmetically appealing appearance, ivory color, smooth texture, and homogenous with no sign of phase separation.

pH evaluation

The pH measurement was carried out for five weeks to see how much the pH changed during storage. The pH value of the product indicates the degree of acidity of a product. The pH of cosmetic

preparations must follow the pH of skin acceptance. Based on the results, it can be seen that the formulas from Base 1 and 3 have the closest pH value to the normal pH of the skin. According to SNI 16-4399-1996, the ideal pH for cream preparations is 4.5-8 [21]. If the cream has a pH that is too alkaline, it will cause dry skin; if the cream's pH is too acidic, it will cause skin irritation [22].

Table 2: Organoleptic evaluation results

Formulas	Description	Week					
		0	1	2	3	4	5
Base 1	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
	Color	White	White	White	White	White	White
	Smell	Odorless	Odorless	Odorless	Odorless	Odorless	Odorless
	Homogeneity	Homogen	Homogen	Homogen	Homogen	Homogen	Homogen
Base 2	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
	Color	Ivory	Ivory	Ivory	Ivory	Ivory	Ivory
	Smell	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic
	Homogeneity	Homogen	Homogen	Homogen	Homogen	Homogen	Homogen
Base 3	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
	Color	White-yellowish	White-yellowish	White-yellowish	White-yellowish	White-yellowish	White-yellowish
	Smell	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic
	Homogeneity	Homogen	Homogen	Homogen	Homogen	Homogen	Homogen

Table 3: pH evaluation results during storage for five weeks

Formulas	An Average of pH in-weeks						Average±SD
	Week-0	Week-1	Week-2	Week-3	Week-4	Week-5	
Base 1	6.27	7.03	6.84	6.55	6.43	6.14	6.54±0.34
Base 2	6.98	6.98	7.04	7.09	7.11	7.13	7.06±0.07
Base 3	5.62	5.96	5.79	5.10	5.07	5.08	5.44±0.40

Base scent preference test

The three bases were tested on ten volunteers without explaining the composition of the bases. Volunteers filled out a questionnaire to provide an assessment of the three bases. The evaluation was carried out according to the olfactory perception of the volunteers. To assess the level of volunteer acceptance of the formulation using the Nine-Point Hedonic Scale, where 1 is the lowest score and nine is the highest. The mean assessment of the three bases by ten volunteers against bases 1, 2, and 3 was 3.4±0.84, 7.±0.53, and 4.6±1.17. Based on the Shapiro-Wilk test, the data distribution was not expected at the base two scores ($p<0.05$). Therefore, the Kruskal Wallis non-parametric test was carried out. There was a statistically significant difference between the three bases ($p=0.000$).

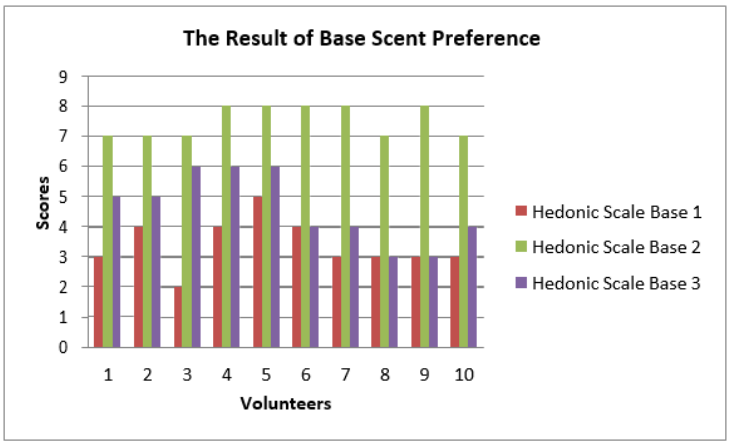

Fig. 1: Graph for the base scent preference test results using nine-point hedonic scale [15]

Type of cream

The tests found that the base formula cream were oil-in-water (o/w) types. The o/w type is more comfortable when applied to the skin because it is not too oily, easily washed off with water, and quickly absorbed [10].

Viscosity

The viscosity values of CM-MSC cream bases and preparations ranged from 3500-23250 cPs and were still within the cream viscosity range that met the standard (SNI 16-4399-1996 (2000-50000 cPs))[23]. Based on the calculations, the flow properties of the formula are thixotropic in the manufacture of creams according to the reference flow properties expected in the manufacture of creams.

Stability test

There are two stability determinations; the freeze and thaw test and the mechanical test with also including all the parameters such as organoleptic observation, homogeneity test, pH test. There were no physical changes in the base and cream in the form of texture, color,

odor, and homogeneity, which were affected by changes in extreme temperatures from 5 °C to 25 °C for three cycles. This result follows previous studies where the microemulsion preparation is very stable to temperature changes [24].

The selection of Formula 2 is based on the acquisition of the level of preference in the previous aroma test, where Base 2 has the highest preference point. Therefore, testing was continued for Formula 2 only.

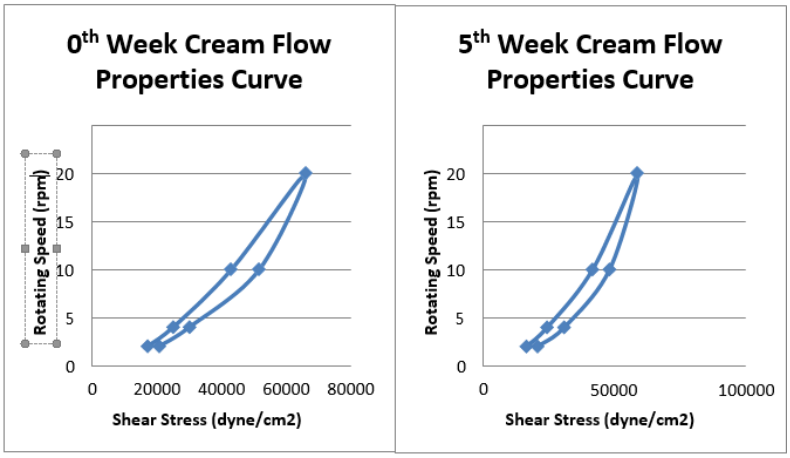

Fig. 2: The results of the viscosity test, showing the thixotropic flow properties curve of CM-MSC cream at weeks 0 and 5

Table 4: The result of the freeze and thaw test and mechanical test

Formulas	Stability test		1st cycles	2nd cycles	3rd cycles
2	*Freeze and thaw* test	Texture	Soft	Soft	Soft
		Color	Ivory	Ivory	Ivory
		Smells	Aromatic	Aromatic	Aromatic
		Homogeneity	Homogenous	Homogenous	Homogenous
	Mechanical test		(-)	(-)	(-)

1st cycle: 25 °C in 24 h; -5 °C in 24 h

2nd cycle: 25 °C in 24 h; -5 °C in 24 h

3rd cycle: 25 °C in 24 h; -5 °C in 24 h: There is no phase separation.

The mechanical test used centrifugation at 3750 rpm for 10 min. This test uses vibration to see the break in the preparation. The results found that formulas did not experience phase separation despite a strong vibration.

Clinical trial on human skin (No. KEPK/005/04/2021)

An irritation test was carried out on eight volunteers to determine whether the cream was safe. Volunteers willing to be the research object fill out the irritation test readiness sheet first. The test was carried out on the inner forearm with an area of 1 cm for 24 h. After that, observe the changes, including redness, swelling, and itching. A good cream does not irritate the volunteer's skin. Based on observations, it was found that the cream was safe and did not cause irritation to the skin. After being declared safe in the irritation test, the effect test was continued by examining skin conditions using a skin analyzer at Derma Q Skin Clinic. Although the moisture has not seen a significant increase in elasticity and collagen, there is an improvement in skin condition.

Characterization and measurement of FGF concentration by ELISA test and microplate reader

Based on the results, the presence of FGF in the preparation of mesenchymal stem cell secretome cream was indicated by a change in the color of the solution. In addition, the concentration of FGF in 5% mesenchymal stem cell secretome preparations with a microplate reader was 61.143 pg/ml. FGF has increased cellular proliferation, migration, differentiation, and regeneration in various tissues. Based on the study's results, the presence of FGF in the preparation of mesenchymal stem cell secretome cream was qualitatively characterized by a change in the color of the solution. Quantitatively, the concentration of FGF in 5% mesenchymal stem cell secretome preparations with a microplate reader was 61,143 pg/ml±0.404; FGF has increased proliferation, migration, differentiation, and cellular regeneration in various tissues.

Table 5: Parameters of before and after (mean±SD) using a cream formulation with secretome

Parameter	Before	After (8 w)
Moisture	24.375±11.97	25.125±7.1
Elasticity	40.375±8.39	48.5±9.09
Collagen	48.25±13.54	56.5±8.63

DISCUSSION

The conditioned medium obtained from adipose tissue contains many growth factors, cytokines, extracellular macromolecules, and vesicles. It also includes microvesicles and exosomes that can stimulate multiple biological reactions, especially in modulating new tissue formations. These factors play an essential role in communication between cells and are involved in various physiological processes, including signal transduction to provide biological responses [25].

The use of the conditioned medium as an efficacious ingredient in cosmetic preparations has previously been investigated. For example, Kim (2020) studied the clinical effectiveness of cosmetics

containing human stem cell conditioned medium [9].

This study formulated secretome into a cream dosage form and checked for effectiveness. The cream is formulated using different scents, such as orange and patchouli oil. In addition to providing a calming aroma, this essential oil is added to help penetrate protein in the secretome [26]. The three cream preparations had similar consistency, and there were no visible physical changes. Based on the evaluation, including the aroma test, the second formula was chosen as the final formula for the follow-up test. The stability test showed that the cream could maintain its integrity during the accelerated stability test for three cycles without any instability.

The condition of the volunteers' facial skin is shown in table 5, where three parameters were checked with a skin analyzer, with an increase in facial elasticity and collagen. A study by Moon (2018) reported that the moisture increased by 11.1% after an experiment in a control group in which spicules moisture serum was applied [27]. In research by Kim (2020), the use of ADSC secretome with a concentration of 5% for cosmetic preparations has proven its effectiveness for 28 d by looking at the increase in water content, decreasing the value of Transepidermal Water Loss (TEWL), and repairing wrinkles and whitening effects on the faces of women aged 30-50 Y [9].

Growth factors are plentiful in adipose tissue, such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), and collagen. These four factors play a role in wound healing and fibroblast activity, leading to increased collagen biosynthesis and reduced wrinkling [9]. Activation of FGF promotes effects in delaying aging by increasing skin elasticity and inducing collagen and elastin synthesis [28].

CONCLUSION

This study focuses on clinical trials of secretomes formulated in cream dosage forms and checked for effectiveness and protein content. The cream dosage formula containing 5% CM-MSC with additional ingredients Olivem® 1000 and orange oil meets the requirements as a cosmetic through several evaluations of the preparation. The cream preparation is an oil-in-water type with good physical stability. The cream preparation has a potential antiaging activity based on the difference between the parameters of the antiaging effect test before and after using the cream on volunteers, including moisture, elasticity, and collagen values. In this study, the ROS test was not performed; it can be added to further research to support this claim. There was an increase in moisture from 24.375±11.97 to 25.125±7.1; besides that, the skin elasticity also increased from 40.375±8.39 to 48.5±9.09, and the collagen value increased from 48.25±13.54 to 56.5±8.63. An ELISA test shows that the cream contains a 61.143 pg/ml concentration of FGF.

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AUTHORS CONTRIBUTIONS

Marlina Marlina, Conceptualization, Data curation, Reysa Pradifta, conceptualization, resource, writing-original draft, Henny Lucida, conceptualization, resources, Ikhwan Resmala Sudji, methodology, writing–review, Hana Nurul Salsabila, resources, writing-original draft, Nur Elida, resources, writing-review and editing, Popy Ayu Namira, writing-original draft, editing.

CONFLICT OF INTERESTS

Declared none

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