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The role of stem cell metabolites derived from placenta for skin regeneration: An in vitro study

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ABSTRACT

Background: The role of stem cells in skin aging is to repair injured tissue or replace other cells in programmed cell death. Stem cell metabolites are rich in growth factors including IL-10, IL-4, EGF, GM-CSF, and TGF-β that can induce the skin production of protein and elastic fibers, leading to the improvement of skin appearance.

Aim: This study aimed to assess the characteristics of stem cell metabolites in vitro.

Methods: Cytotoxicity assay was performed using MTT reagents and optical cell densities were determined using ELISA reader to find the percentage of living cells. Cytokine detection assay was performed by analyzing the cytokine levels in the peripheral blood mononuclear cell (PBMC) and mesenchymal stem cell (MSC) using ELISA. Apoptosis assay was performed using the double staining method with the markers identified were Hsp70, p53, and caspase-3.

Results: All samples showed the percentage of living cells that exceed 70%. Cytokine detection assay showed a decrease of IL-12 and IFN-γ in both PBMC and MSC groups. The apoptosis assay of human adipose mesenchymal stem cells using a fluorescence microscope showed most of the green light was lost in control cells without metabolites. We found that the expressions of Hsp70 were increased while the expression of p53 and caspase-3 were decreased in the stem cell metabolites samples.

Conclusion: These results showed that stem cell metabolites are non-toxic, do not cause a systemic immune response to surrounding tissue, and able to inhibit the occurrence of apoptosis.

Keywords: stem cell metabolites, placenta, skin regeneration, in vitro study

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INTRODUCTION

Aging can be defined as a progressive functional decline or a gradual deterioration of physiological function with age. Human aging is associated with a wide range of physiological changes that not only make a person more susceptible to death but also limit the normal functions of body and increase the susceptibility to many diseases.[1] The functional decline is related to various systems in the body, such as decrease of memory, muscle weakness, hearing impairment, vision loss, psychological and physical appearance changes, and other decrease in biological functions.[2]

Skin aging is a natural process where renewing skin cells process and collagen production slows down, as well as the weakening of the internal support structure and the natural protective layer of the skin. The deterioration of the skin renewal process combined with environmental and lifestyle factors, such as pollution, can cause signs of aging to appear earlier.[3] Premature aging often occurs in someone who has a dry skin type because sebum, which is an oil content to protect the skin from pollution and moisturizes the skin, is very lacking.

Several anti-aging therapies are commonly used, including cosmetological treatments such as cosmetic products that protect from UVA and UVB, and topical agents such as tretinoin to stimulate the synthesis of type I collagen and vitamin C for antioxidants. Anti-aging therapies may also include the use of systemic agents (such as vitamins C and E, glutathione, polyphenols, melatonin and selenium) and procedural therapy which offers services such as laser therapy.[4]

The role of stem cells in skin aging is to repair injured tissue or replace damaged cells that undergo apoptosis (programmed cell death). In this way, stem cells keep a person healthy and prevent premature aging. These stem cells act like "microscopic doctor forces" in the human body.[5] The derived products of stem cells for skin regeneration are then called stem cell metabolites.

Human placenta is an alternative source of stem cell metabolites. The placenta contains more hematopoietic stem cell populations and mesenchymal precursor cells when compared to adult blood or bone marrow. Stem cell metabolites are rich in growth factors including interleukin (IL)-10, IL-4, epidermal growth factor (EGF),

granulocyte-macrophage-colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)-β. These growth factors induce the skin production of protein and elastic fibers and new proteins, that will return the elasticity of the skin, reduce black pigments, and induces basal cells to proliferate resulting in the growth of epidermal keratinocytes.

Stem cell metabolites must be validated before use. The validation process includes assessment of potential (plasticity) which shows the ability of stem cells to differentiate, purity to prove that these cells are true stem cells, and contamination by both fungi and other microorganisms. Cells must be free from infectious diseases such as HIV, herpes, hepatitis, BSE, gonorrhea, and free from cancer cells. The level of cell viability and cell phenotype must also suit the desired target.[6] Stem cell metabolites are also characterized, both in vitro and in vivo, before finally being applied to humans. This study aimed to assess the characteristics of stem cell metabolites in vitro, using cytotoxicity assay, cytokine detection assay, and apoptosis assay.

METHODS

Cytotoxicity assay

The cytotoxicity of stem cell metabolites can be identified with the MTT assay using human adipose cells. The cytotoxicity assay was performed using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] reagents according to Vybrant MTT Cell Proliferation Assay Protocol by Thermo Fisher Scientific. This study used three variations of samples: dialysis medium only without stem cell metabolites (as control), stem cell metabolites in 1x dilution dialysis medium, and stem cell metabolites in 10x dilution dialysis medium. Each sample was repeated ten times to improve the accuracy in the experiment. Optical cell densities were determined using ELISA Reader to find the percentage of living cells. A material is said to be non-toxic and compatible with human body if the percentage of living cells is more than 70%.[7]

Table 1. The result of cytotoxicity assay in stem cell metabolites

Repetition	Media Control	Cell Control	Dialysis Medium	Medium 1:1	Medium 1:10
1	0.060	0.521	0.318	0.509	0.478
2	0.059	0.500	0.317	0.528	0.521
3	0.058	0.513	0.213	0.493	0.471
4	0.060	0.503	0.316	0.450	0.500
5	0.059	0.521	0.320	0.542	0.500
6	0.059	0.543	0.291	0.528	0.526
7	0.058	0.533	0.316	0.519	0.523
8	0.060	0.561	0.280	0.515	0.595
9	0.059	0.533	0.291	0.527	0.571
10	0.057	0.533	0.316	0.527	0.497
Total	0.589	5.261	2.978	5.138	5.182
Mean	0.059	0.526	0.298	0.514	0.518
Std.			0.033105	0.026038	0.039063
% Living Cells			51.13 ± 3.31	97.37 ± 2.60	98.31 ± 3.91

Cytokine detection assay

The inflammatory response in skin regeneration is an important process that affects the performance of stem cell metabolites. This assay aimed to reveal the role of cytokines IL-12 and INF-γ in rejuvenation, by analyzing the differences in cytokine levels in the peripheral blood mononuclear cell (PBMC) and mesenchymal stem cell (MSC) groups. All cytokine ELISA kits were purchased from Thermo Fisher Scientific, and the experiment was conducted based on their protocols. Samples were then matched with Fisher's exact test. Cytokine levels were measured using the ELISA technique from human peripheral venous blood samples. A multivariate analysis was performed to see the role of cytokines together between groups.

Apoptosis assay

Apoptosis observation was performed using the double staining method with the markers identified were Hsp70 (70 kilodalton heat shock protein), p53 (protein 53), and caspase-3 (caspase protein-3). All apoptotic kits were purchased from Thermo Fisher Scientific, and the methods were carried out using their protocols. Hsp70 plays a role in maintaining homeostasis (anti-apoptosis), while p53 and caspase-3 play a role in inducing apoptosis (pro-apoptosis). Human adipose cells were used in this assay. A statistical analysis of immunopositive cells was performed to obtain the percentage of expression of Hsp70, p53, and caspase-3.

RESULTS

Cytotoxicity assay

The viability of stem cell metabolites was calculated using ELISA reader. Based on the results, all samples were not toxic which was shown in the percentage of living cells that exceed 70% (Table 1). Sample with stem cell metabolites in 1x dilution dialysis medium had cell viability of 97.37% ± 2.60%, while sample with stem cell metabolites in 10x dilution dialysis medium had cell viability of 98.31% ± 3.91%. The best results were found in the dilution

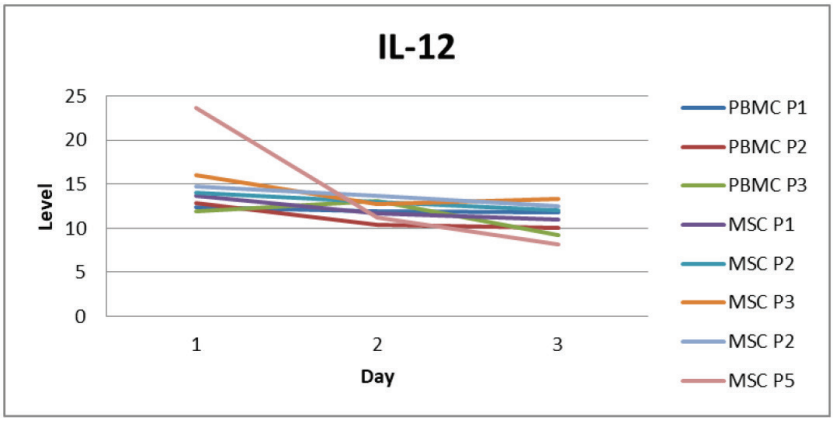

Figure 1. The decrease of IL-12 levels in the peripheral blood mononuclear cell (PBMC) and mesenchymal stem cell (MSC) groups

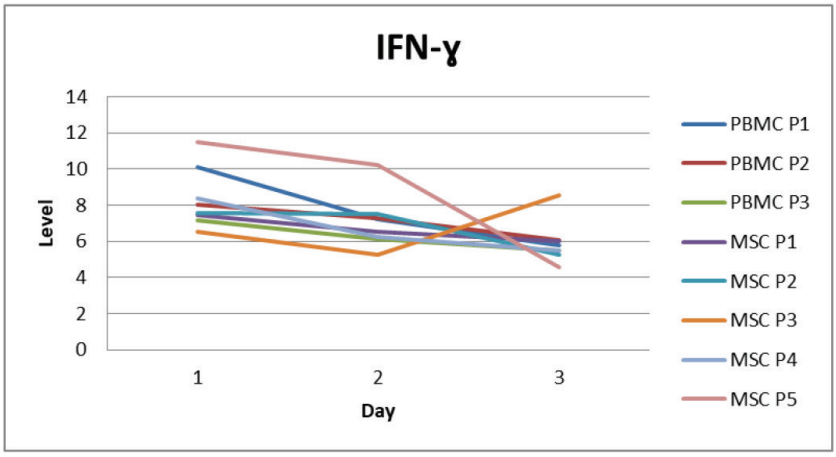

Figure 2. The decrease of IFN-ɣ levels in the peripheral blood mononuclear cell (PBMC) and mesenchymal stem cell (MSC) groups

dialysis medium 1:10, but the results were not much different from medium 1:1.

Cytokine detection assay

Figure 1 and 2 shows the secretion of IL-12 and interferon (IFN)-γ. All the mesenchymal stem cells in stem cell metabolites (MSC passage-1, MSC passage-2, MSC passage-3, MSC passage-4, and MSC passage-5) did not elicit the systemic immune response to surrounding cells or tissues, as shown in the decreased levels of cytokine release in three days of observation. This result shows that stem cell metabolites can enhance immune responses.

Apoptosis assay

The apoptosis assay of human adipose mesenchymal stem cells using a fluorescence microscope showed most of the green light was lost in control cells without metabolites (Figure 3.A). This result indicates the loss of membrane permeability in most cells as an indicator of cell death and visible fragmentation of the cell nucleus which later became apoptotic bodies. The cells given the treatment of stem cell metabolites were seen to be green fluorinated (Figure 3.B). This could be because most of the cells absorbed acridine orange, and only a small percentage of cells absorbed ethidium bromide, indicating the integrity of the cells were still good.

The percentage of immunopositive cells was calculated to obtain the average percentage data of Hsp70, p53, and caspase-3 expressions as shown in Table 2. The percentage of Hsp70 expression as anti-apoptosis in stem cell metabolites was 61.23% ± 0.78%, which was higher compared to control (20.14%± 0.71%). The percentages of p53 and caspase-3 expression as pro-apoptosis were 21.96% ± 1.15% and 9.08% ± 0.77%, respectively. It was lower compared to control (43.68% ± 1.85% and 21.3% ± 1.95%, respectively). In the apoptotic induction pathway assay, cells that express immunopositive cells (Hsp70, p53, and caspase-3) appear as brown cytoplasm cells, whereas immunonegative cells are purplish blue in their nucleus with clear cytoplasm (Figure 4).

DISCUSSION

Stem cells are undifferentiated cells with high potency to develop into many different types of cells in the body. Stem cells also function as a repair system to replace damaged cells. When stem cell undergoes cell division, each new cell has the potential either to remain as stem cells or to become other types of cell with more specialized functions. Stem cells have the most significant potential in regenerative therapy. These stem cells may be obtained from all layers of the skin, starting from the hair follicle, the interpholic epidermal layer, the papilla dermis, and the perivascular cavity. Numerous studies have stated that these layers have the potential for skin regeneration.[5,7]

Stem cells develop into new cells and lead to the rejuvenation process. Gradually, stem cells may reduce dull and wrinkled skin due to the decrease in collagen production caused by the degenerative process. Some practitioners claimed that stem cell therapy might produce more elastic skin and reduce pigmentation and increase the overall skin appearance of the patient. The derived products of stem cells for skin regeneration are known as stem cell metabolites.

Metabolites are any substances involved in metabolism, either as metabolic products or needed for metabolism. Metabolites are essential substances for chemical changes in cells or organisms that produce energy and necessary materials needed for important life processes, such as the function

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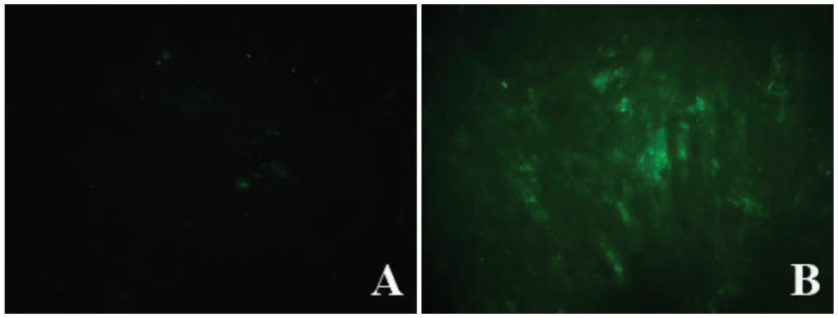

Figure 3. (A) Cell apoptosis in control samples; (B) Cell apoptosis in samples treated with stem cell metabolites

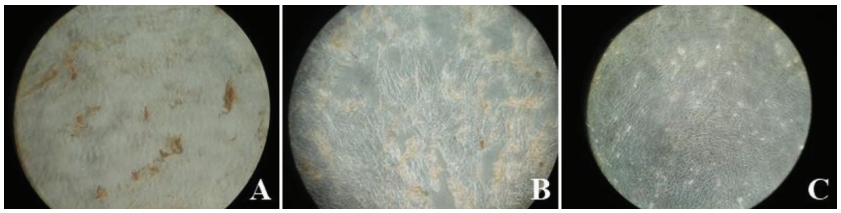

Figure 4. Cells after immunohistochemical staining using: (A) Hsp70; (B) p53; (C) caspase-3

Table 2. The percentage of Hsp70, p53, and caspase-3 expression in human adipose mesenchymal stem cell (MSC)

Sample	Hsp70 expression (%)	p53 Expression (%)	Caspase-3 expression (%)
Stem cell metabolites	61.23 ± 0.78	21.96 ± 1.15	9.08 ± 0.77
Control	20.14 ± 0.71	43.68 ± 1.85	21.3 ± 1.95

of structures, signals, stimulation and inhibitory effects on enzymes, catalytic activity (usually as a cofactor of enzymes), defense, and interaction with other organisms (e.g. pigments, odorants, and pheromones).[8]

Metabolites can be divided into two types, primary metabolites, and secondary metabolites. Primary metabolites are directly involved in normal growth, development, and reproduction. While secondary metabolites are not directly involved in the process, they usually have important ecological functions.[9] Some antibiotics use primary metabolites as precursors, such as actinomycin which is created from the primary metabolites of tryptophan and stem cell metabolites derived from stem cells in various sources such as bone marrow, fat tissue, cord blood cells (placenta), and others.

Stem cell metabolites are obtained from human placenta. The placenta contains more hematopoietic stem cell populations and mesenchymal precursor cells when compared to adult blood or bone marrow. Stem cell metabolites are rich in growth factors including IL-10, IL-4, EGF, GM-CSF, and TGF-β. These growth factors can induce the skin production of protein and elastic fibers and new proteins. They allow the return of skin's elasticity, reduce black pigments, and induce basal cells proliferation resulting in the growth of epidermal keratinocytes.

Stem cell metabolites must be validated prior use. The validation process includes assessment of plasticity which shows the differentiation ability of stem cells, assessment of purity to ensure the cells are true stem cells, and assessment of contamination by both fungi and other microorganisms to ensure the cells are free of infectious diseases. The level of cell viability and cell phenotype must also be suited to the desired target.[6] Stem cell metabolites are also characterized, both in vitro and in vivo before finally being applied to humans. This study focuses on the characterization of stem cell metabolites in vitro, including cytotoxicity assay, cytokine detection assay, and apoptosis assay.

MTT assay is a colorimetric assay for measuring cell metabolic activity. The principal of MTT assay is the ability of living cells to convert MTT (tetrazolium dye) into formazan crystals. NADPH-dependent cellular oxidoreductase enzymes can reduce MTT to its insoluble formazan, which has a purple color. Since this process occurs in mitochondria of living cells, this assay, therefore, can measure cell viability in terms of reductive activity, although reducing agents and enzymes located in other organelles, such as the endoplasmic reticulum are also involved.[10,11] In the MTT assay, a solubilization solution is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer.

MTT method is broadly used to analyze cell proliferation and viability. It is taken up by cells via endocytosis and is reduced by mitochondrial enzymes as well as endosomal/lysosomal compartments. The endocytosis of MTT did not cause obvious lesion or induce cell death, but the metabolism and exocytosis of MTT could damage the cells. After the reduction of MTT, it is then transported to cell surfaces to form needle-like MTT formazans. The appearance of MTT formazan crystals could activate apoptosis-related factors, such as caspase-8, caspase-3, or accelerate the leakage of cell contents. The main advantage of the MTT assay is the gold standard for cytotoxicity testing.[10,12,13] In this study, cell viability of stem cell metabolites was calculated using ELISA reader. The percentage of living cells in all samples were >70%, indicating that all samples are not toxic.

Cytokines are protein molecules released by cells when activated by antigens. Cytokines are involved in cell communication, acting as mediators to enhance immune responses through interactions with specific cell surface receptors on leukocytes.[14] IL-12 is a regulator of cytokines which has an important function in the initiation and regulation of cellular immune responses. IL-12 can regulate the differentiation of naïve T cells that are important in determining resistance and the type of response that will arise for specific pathogens. IL-12 is produced by macrophages, monocytes, genetics, and β-cells as immune responses to intracellular bacteria and parasites. IL-12 then produces IFN-γ and tumor necrosis factor (TNF)-α from NK cells and T helper cells. IL-12 induces IFN-γ to increase phagocytic secretion, the production of nitrogen oxide (NO), and oxidative explosions which cause damage to pathogens. IL-12 is also a marker in suppressing Th-2, such as IL-4 and IL-10. IL-12 plays an integral role in the immune response in disease pathogenesis.[15]

In this study, the decreased levels of cytokine in the three days of observation showed that MSC in stem cell metabolite products did not cause a systemic immune response to surrounding cells or tissues. This result proves that stem cell metabolites are not pathogenic and do not cause excessive inflammatory responses to tissues.

Apoptosis is a programmed cell death that does not cause inflammation and injury to the tissue. Apoptosis observation was performed using the double staining method using ethidium bromide-acridine orange with the markers identified are Hsp70, p53, and caspase-3. Hsp70 is a protein molecule that plays a role in maintaining cell homeostasis and function, both in normal circumstances and in stressful situations. Hsp70 has anti-apoptotic effects and improves damaged cells. As a cell guard, the function of p53 is to monitor stress from cells and induce apoptosis. In damaged tissue, p53 will initiate apoptosis which will destroy the damaged cell. Similar to p53, caspase-3 also plays a role in inducing apoptosis. Caspase-3 contains a prodomain that is short and serves as an effector, splitting various dead substrate causing morphological and biochemical changes seen in apoptotic cells.[16,17,18]

The apoptosis assay of human adipose mesenchymal stem cells using a fluorescence microscope showed that in control cells without metabolites most of the green light was lost. This result indicates the loss of membrane permeability in most cells as an indicator of cell death and visible fragmentation of the cell nucleus which later became apoptotic bodies. While the cells given the treatment of stem cell metabolites were seen to be green fluorinated because most of them absorb acridine orange, only a small percentage of cells absorb Ethidium bromide because cell integrity is still good. Stem cell metabolites with certain levels can inhibit the occurrence of apoptosis.

The percentage of immunopositive cells was calculated to obtain the average percentage data of Hsp70, p53, and caspase-3 expressions. Control of apoptosis is associated with genes that regulate the cell cycle. The results showed that stem cell metabolites may inhibit the occurrence of apoptosis in terms of the increase in Hsp70 (anti-apoptotic gene) expression and the decrease in p53 and caspase-3 (pro-apoptotic gene) expression in metabolite samples compared to control samples.

CONCLUSION

In this study, it can be concluded that stem cell metabolites are not toxic in terms of the toxicity test results with a percentage of cell viability that exceeds 70%. Stem cell metabolites do not cause a systemic immune response to surrounding tissue in terms of decreased levels of cytokine release. Stem cell metabolites also do not induce apoptosis in terms of the increased percentage of expression of Hsp70 (anti-apoptotic gene) and the decrease in the percentage of expression of p53 and caspase-3 (pro-apoptotic gene) in the stem cell metabolites groups compared to controls.

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DISCLOSURE

The author reports no conflicts of interest in this work.

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