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PRE-CLINICAL TRIAL STEM CELL METABOLITES DERIVED FROM PLACENTA FOR WOUND HEALING

Purwati[1,2,3], M. Yulianto Listiawan[4], P. Ardhiah Iswanda[4], Fedik A. Rantam[1], Helen Susilowati[1], Eryk Hendrianto[1], H. Afif Nurul[4], Ernawati[5], Imam Susilo[2] and R. Diah Puspita[2]

[1]Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia.
[2]Faculty of Vocational Education, Universitas Airlangga, Surabaya, Indonesia.
[3]Department of Biotechnology, Asia University, Taichung, Taiwan.
[4]Department of Dermatology and Venereology, Universitas Airlangga – Dr. Soetomo General Hospital, Surabaya, Indonesia.
[5]Department of Obstetrics and Gynecology, Universitas Airlangga – Dr. Soetomo General Hospital, Surabaya, Indonesia.

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ABSTRACT : Previous research focuses on in vitro study of stem cell metabolites derived from placenta for wound healing. This study, however, is an advanced stage which focuses on testing the efficiency and efficacy of stem cell metabolites in rats (*Rattus novergicus*). The tests carried out examined the blood levels with ELISA instruments and integument histology by observing the activity of polymorph nuclear and monocyte cells in the control and treatment groups. In the control group, the rats were injured in the anterior and posterior back skin with a 1×1 cm incision wound, (only antibiotics), while the treatment group uses antibiotics and 4 mL injections of stem cell metabolites. Each group was repeated three times with the samples observed for blood levels using ELISA Interleukin-4, Interleukin-10 and Tumor Necrosis Factor-α, with integument histology at pre-injection, in days 1, 3 and 6. These were used to compare the development of inflammatory cells, polymorphonuclear and monocytes between the control and treatment groups. Stem cell metabolites are significantly effective and efficient with the ability to inhibit the inflammatory process in tissues in terms of examining the blood levels of rats using ELISA Interleukin-4, Interleukin-10 and Tumor Necrosis Factor-α. Interleukin-4 and Interleukin-10 (anti-inflammatory) tend to significantly increase the treatment group, while Tumor Necrosis Factor-α (pro-inflammation) increases the control group. Histology results showed a decrease in the activity of polymorphonuclear and monocytes inflammatory cells in the treatment group compared to the control, which indicated that the stem cell metabolites were able to significantly inhibit the inflammatory process. It is concluded that stem cell metabolites derived from placenta are effective and efficient for wound healing in rats. Clinical study is needed for further research for it to be used on humans.

Key words : Stem cell metabolites, placenta, wound healing, *in vivo* study, *Rattus novergicus*.

INTRODUCTION

Wound healing is a dynamic process consisting of four continuous, overlapping and precisely programmed phases, which includes complex interactions among cells, growth factors and extracellular matrix molecules to sequentially achieve hemostasis, cell proliferation, angiogenesis, re-epithelialization and tissue remodeling (Hu *et al*, 2014; Ya Hui *et al*, 2016). Each phase need to occur in a regulated manner as interruptions, aberrancies or prolongation has the tendency of delaying the process (Guo *et al*, 2010). A successful wound healing process is followed by skin grafting from autograft, allograft and synthetic tissue, resulting in infectious, scar, immune rejection and rigid skin (Ya Hui *et al*, 2016).

Stem cells with their unique characteristics to self-renew and differentiation are emerging as a promising therapy for wounds where conventional treatments failed. Preclinical trials have demonstrated that accelerated wound healing is conducted using stem cell treatment via topical or systemic delivery (McFarlin *et al*, 2006; Badiavas *et al*, 2003). All subjects showed clinical improvement in their wounds within days with decrease in size without side effects associated with the delivery of mesenchymal stem cells (Dash *et al*, 2009; Aggarwal *et al*, 2005).

Stem cell metabolites secreted a variety of cytokines such as IL-10, IL-4, EGF, GM-CSF and TGF-β to promote dermal fibroblast proliferation, angiogenesis and collagen deposition to achieve regeneration (Gnecchi *et al*, 2008). These cytokines penetrates the skin layer to stimulate the growth of new cells, increase nutrition, accelerate skin metabolism, stimulate new proteins, collagen and elastic fibers, also it induces basal cells to proliferate, which results in the growth of epidermal keratinocytes (Kraich *et al*, 2006; Francisco-Cruz *et al*, 2014; Massagué, 2012, Voehringer, 2012).

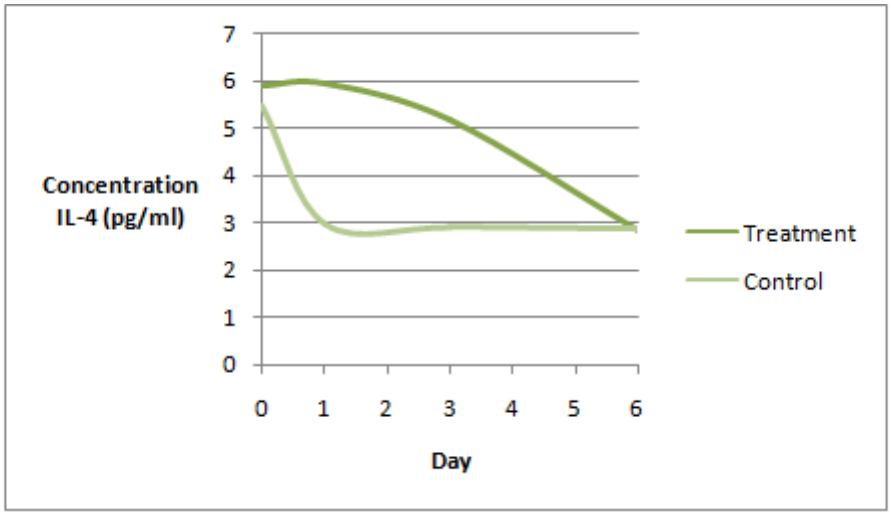


Fig. 1 : Blood level examination chart for 6 days with ELISA IL-4.

In this study, stem cell metabolites were obtained from human placenta which contains more hematopoietic stem cell populations and mesenchymal precursor compared to adult blood or bone marrow. Validation of stem cell metabolites were conducted before use which includes plasticity, purity and contamination. Furthermore, cells are free of infectious diseases such as HIV, herpes, hepatitis, BSE, gonorroe, and cancer. In addition, its viability and phenotype levels need to be in accordance with the target (Rantam *et al*, 2009).

Previous research focuses on the in vitro study of stem cell metabolites derived from placenta for wound healing. The characterization of stem cell metabolites in vitro were conducted, which includes cytotoxicity, cytokine detection and apoptosis assays. In conclusion, stem cell metabolites are not toxic in terms of test results with a percentage of cell viability which exceeds 50%. Furthermore, stem cell metabolites do not cause any systemic immune response to surrounding tissue in terms of decreased levels of cytokine which is released, excluding the induction apoptosis in terms of an increased percentage of expression of Hsp70 (anti-apoptotic gene) which decreases with p53 and caspase-3 (pro-apoptotic gene) in the treatment samples (with metabolites) compared to the controls (Purwati *et al*, 2019).

This is an advanced which focuses on the efficiency and efficacy of stem cell metabolites in rats (*Rattus novergicus*) for wound healing. The tests carried out examined the blood levels in rats using ELISA instruments, with antibiotics and stem cell metabolites used to observe the activity of PMN and monocyte cells in control samples.

METHODS

This study received ethical clearance from the Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia, following the regulatory guidelines of the country.

Sample preparation : Sample preparation consisted of placenta isolation, cell culture of mesenchymal stem cells (MSCs) and validation. MSCs were derived from human placenta tissue using aspiration and separation on Histopaque-1.077. The harvested cells were cultured in Dulbecco's Modified Eagles Medium containing 1.0 g/L glucose, with Penicillin-Streptomycin and Fungizone as antibiotic and anti-fungi. Furthermore, MSCs validation were performed and expressed asCD105, CD73, CD90, CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR surface molecules. Stem cell metabolites are a medium used in cell culture (Purwati *et al*, 2019).

Animal Trial : Efficiency test of stem cell metabolites in vivo was carried out in adult rats (*Rattus novergicus*). The rats were injured in the anterior and posterior back skin with a 1×1 cm incision wound. Previously, the wounds were cleaned using providine iodine and applied with gentamycin as an antibiotic in the control group. While in the treatment group gentamycin and 4 mL injection intra-muscular of stem cell metabolites were applied, with each group repeated three times.The samples were observed for blood levels using ELISA IL-4, IL-10 and TNF-α, along with a integument histology at pre-injection in day 1, 3 and 6 to compare the development of inflammatory cells, which are polymorphonuclear (PMN) and monocytes (MN) between the control and treatment groups.

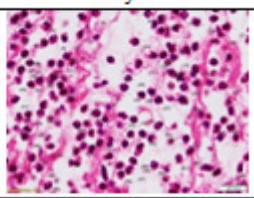
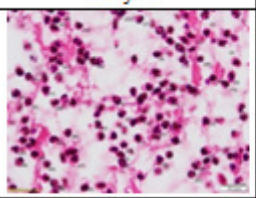
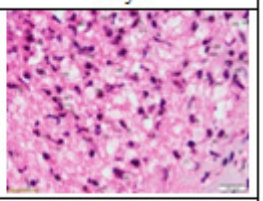
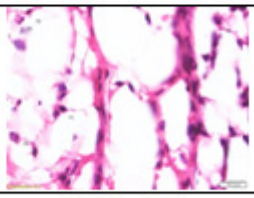
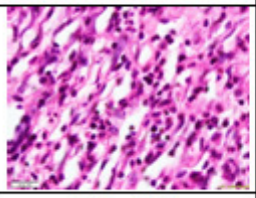
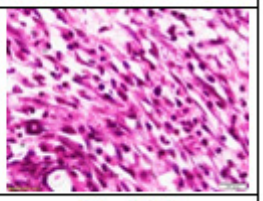
RESULTS

Blood level examination : The results of blood levels examination in rats in the control and treatment groups for 6 days are presented in Table 1, Figs. 1, 2 and 3.

Table 1 : Results of blood level examination in rats with ELISA instruments.

Standard/Sample	ELISA IL-4		ELISA IL-10		ELISA TNF-á	
	OD	Concentration (pg/ml)	OD	Concentration (pg/ml)	OD	Concentration (pg/ml)
Std 1	3.448	200	1.121	250	1.084	1000
Std 3	0.839	50	0.360	62.5	0.268	250
Std 5	0.258	12.5	0.157	15.6	0.104	62.5
Std 7	0.105	3.2	0.106	3.9	0.071	15.6
Pre-1	0.122	5.91	0.181	31.42	0.100	60
Pre-2	0.113	5.47	0.171	29.68	0.085	51.8
Day 1-P	0.123	5.95	0.177	30.72	0.101	60.69
Day 1-K	0.098	2.98	0.152	15.1	0.101	60.69
Day 3-P	0.107	5.18	0.170	29.51	0.091	54.68
Day 3-K	0.096	2.92	0.142	1.1	0.102	61.29
Day 6-P	0.093	2.83	0.148	14.7	0.089	53.48
Day 6-K	0.095	2.89	0.139	3.81	0.098	58.89

Table 2: Polymorphonuclear cells (PMN) in histology of the rat skin tissues.

	Day 1	Day 3	Day 6
Histology (Control)			
Σ Cells	81.8 ± 3.27	78.4 ± 4.39	32.6 ± 3.28
Histology (Treatment)			
Σ Cells	24.4 ± 2.61	12.0 ± 2.54	5.6 ± 2.07

Integument histology : Observation and calculation of the number of PMN and monocyte cells in the control and treatment groups are presented in Tables 2 and 3.

DISCUSSION

Wound healing constitutes a complex process, where different cells and molecules act in an orchestrating way. The stem cell is a promising therapy in skin wound where conventional treatments failed (Ya Hui Tang, 2016). The mechanism enhances wound healing in the metabolites secret anti-inflammatory cytokines and attenuate secretion of the pro-inflammatory. These anti-inflammatory properties make stem cells beneficial to wounds by advancing it past a chronic inflammatory state into the healing stage.

In this study, stem cell metabolites were extracted from human placenta containing 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-10, Interleukin-4, EGF (Epidermal Growth Factor), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor) and TGF-β (Transforming Growth Factor Beta). These growth factors tends to penetrate the skin layer to stimulate the growth of new cells increase nutrition, accelerate its metabolism, stimulate the production of proteins, collagen and elastic fibers. It also induces basal cells resulting in the growth of epidermal keratinocytes.

Previous research focuses on in vitro study of stem cell metabolites derived from placenta for wound healing, with its characterization conducted using cytotoxicity, cytokine detection and apoptosis assays. The study concludes that stem cell metabolites are not toxic with the cell viability percentage which exceeds 50%. Furthermore, it does not lead to any systemic immune response to surrounding tissues, fails to induce apoptosis in terms of the percentage of Hsp70 expression (as anti-apoptotic gene), p53 and caspase-3 expression (as pro-apoptotic gene). This study is an advanced stage which

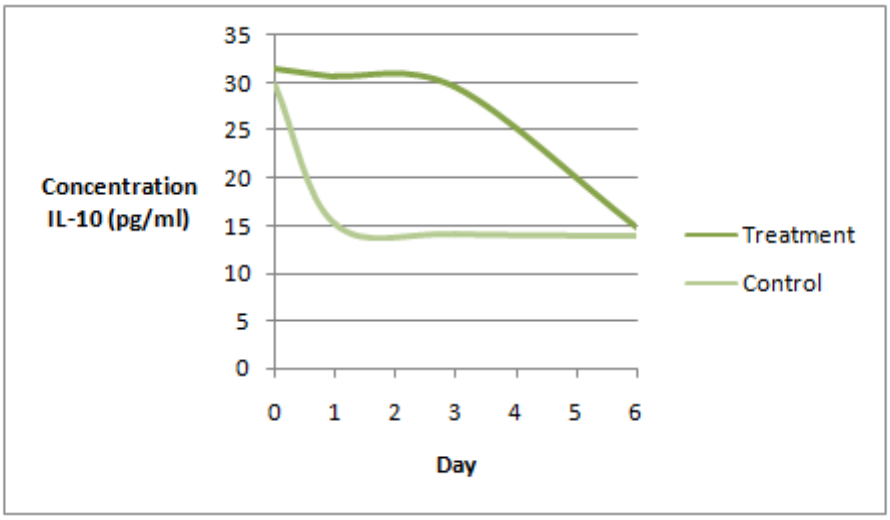


Fig. 2 : Blood level examination chart for 6 days with ELISA IL-10.

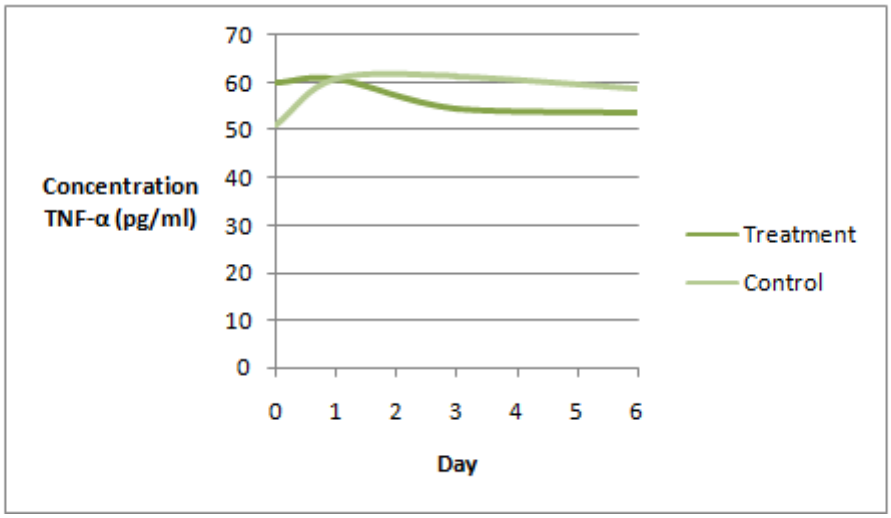


Fig. 3 : Blood level examination chart for 6 days with ELISA TNF-á.

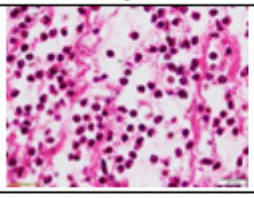
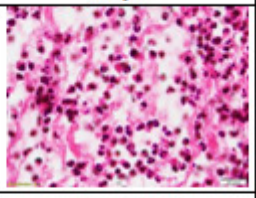
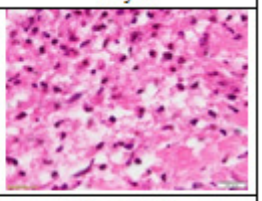
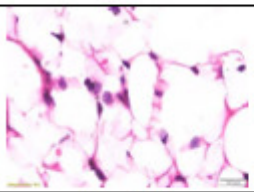
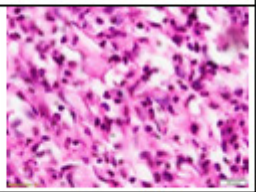
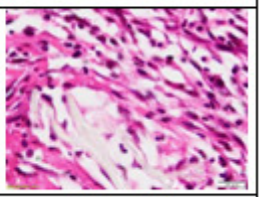
focuses on the efficiency and efficacy of stem cell metabolites in rats (*Rattus novergicus*) with tests conducted by examining their blood levels in using ELISA instruments and integument histology.

Growth factors are signaling proteins that function as ligands to specific reseptor tyrosine kinase on target cells. These reseptors in turn trigger intracellular pathways to promote key processes such as cell growth, division, differentiation and angiogenesis. In this study, ELISA kits which consist of IL-4, IL-10 and TNF-α were used to identify the inflammation process in cells. IL-4 and IL-10 are anti-inflammatory cytokine agents, while TNF-á is pro-inflammatory. The results obtained, shows that the concentration of IL-4 and IL-10 (pg/ml) tends to increase significantly in the treatment group compared to the control group from day 1 to 6. While different results are shown on examination using ELISA TNF-α. The TNF-α (pro-inflammatory) concentration tends to be high in the control group and the graph rises to the 6th day. This shows that metabolite stem cell products are effective and efficiently inhibit the inflammatory process in tissues.

After blood levels examinations, histological observations were carried out on animal model using microscope and the technique of providing tissue samples, which begins with surgery, biopsy or autopsy. The tissue is then processed with fixative to avoid damaging the dosage with formalin (10% formaldehyde dissolved in water) known as the most common animal tissue. The fixed tissue sample is immersed in multilevel alcohol for removing water (dehydration) and transferred to toluene to extract alcohol (alcoholization). The final step is to insert tissue samples into hot paraffins which infiltrate it for 12 to 16 hours thereby, making it hard and easier to cut using a microtome to produce a layer with a thickness of 5 µm. This layer is then placed on the object's glass and colored (Maura *et al*, 2017).

The histology test was conducted to observe

Table 3 : Monocytes cells (MN) in histology of the rat skin tissues.

	Day 1	Day 3	Day 6
Histology (Control)			
Σ Cells	10.6 ± 2.07	9.2 ± 3.63	5.8 ± 1.09
Histology (Treatment)			
Σ Cells	5.2 ± 1.48	3.0 ± 1.41	2.6 ± 0.89

inflammatory cell activity, which neutrophils consisting of polymorphonuclear, leukocyte, and monocytes which are a part of white blood cells from the granulocyte group. Neutrophil granules are bluish red with three lobes, which are clearly visible in each cell. PMN is related to the body's defense against bacterial infections and inflammatory processes and is the first cell to be present during an infection. Furthermore, the phagocytic properties, which are similar to the macrophages, enables PMN to attack the pathogens in the respiratory using variety of toxic substances containing strong oxidizing agents, including hydrogen peroxide, oxygen free radicals, and hypochlorite (Viera *et al*, 2010). While monocytes (mononuclear) are white blood groups that are part of the immune system and identified by the cell nucleus color. When inflammation occurs, monocyte migrates to the site of infection and replaces the damaged cells, by dividing or changing into one of these cells. PMN and MN are important indicators to determine the level of inflammation in tissues (Sallusto *et al*, 2010; Ginhoux *et al*, 2014).

In this test, samples were divided into the control group with only antibiotics and the treatment groups with antibiotics and stem cell metabolites with a total of 14 rats. The observation time was according to the growth of skin tissue and inflammation at pre-injection on day 1, 3 and 6. The skin tissue of the anterior and posterior muscular parts with a 1x1 cm incision wound was taken and made in the form of preparations and then stained with Hematoxilin Eosin (HE). The making of histological preparations was repeated 5 times for each group followed by the proper analysis of the data.

This study used a manual approach to determine the average number of inflammatory cells. Tables 2 and 3 show the average number of PMN and MN, which tends to decrease significantly from day 1 to 6 in the treatment group, compared to control. Cytokines in stem cell metabolites such as IL-10, IL-4, EGF, GM-CSF and TGF-β have respective roles to play in inhibiting the inflammatory process. IL-10 has an essentially inhibitory effect on the immune response, while IL-4 stimulates the growth and development of the immune cells. EGF is able to penetrate the skin layer to stimulate new cell growth, increase nutrition, accelerate metabolism, to produce new protein and elastic fibers. GM-CSF is able to induce basal cells to proliferate resulting in the growth of epidermal keratinocytes, while TGF-β works on damaged tissue by removing debris from old collagen.This reaction is automatic and results in activities which result in fibroblasts being instructed to produce more collagen and elastin.

CONCLUSION

In conclusion, stem cell metabolites are effective and significantly inhibit the inflammatory process in tissues. Experiments were carried out to the blood levels examination in rats using ELISA IL-4, IL-10 and TNF-α. IL-4 and IL-10 (anti-inflammatory) tend to increase significantly in the treatment group, while TNF-α (pro-inflammation) is higher in the control group. Histology results showed a decrease in the activity of PMN and MN inflammatory cells in the treatment group compared to control, which indicated that the stem cell metabolites were able to significantly inhibit the inflammatory process. Clinical study is needed for further research so this product can be used in human.

Ethics approval

This study received ethical clearance with No. 2.KE.023.02.2019 from Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia following the regulatory guidelines of the country.

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