THE EFFECT OF ADVANCED GLYCATION END PRODUCT OF HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS ON VASCULAR ENDOTHELIAL GROWTH FACTOR

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ABSTRACT

Objective: To determine the effect of advanced glycation end product- bovine serum albumin medium of human umbilical cord mesenchymal stem cells on vascular endothelial growth factor secretion.

Methodology: This was an experimental study with post-test only control group design. Mesenchymal stem cells, isolated from a human umbilical cord were cultured and expanded up to passage 5. The subject groups were divided into a treatment group (aMEM+AGE-BSA medium) and a control group. Flowcytometry assessment was conducted in passage 4 by tripination and suspension of human umbilical cord mesenchymal stem cells in minimum essential medium. Samples collected on days 3, 6, 9, 12, 14, 17 and 21 were subsequently examined with enzyme-linked immunosorbent assay to observe the amount of vascular endothelial growth factor secretion.

Results: In the advanced glycation end product - bovine serum albumin group, the peak level of vascular endothelial growth factor secretion occurred on day 3 and gradually declined on day 21, whereas in the control group the peak level reached on day 6 and continued to decrease up to day 21.

Conclusion: This research indicated that advanced glycation end product-bovine serum albumin enhances vascular endothelial growth factor secretion by mesenchymal stem cells with maximum secretion occurring on day 3.

Key Words: Advanced glycation end product, Mesenchymal stem cells, Umbilical cord, Vascular endothelial growth factor

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INTRODUCTION

The prevalence of metabolic diseases especially diabetes mellitus has increased in Indonesia between 2007 and 2013[1]. Long-term increase in blood glucose levels in these patients leads to the formation of covalent additions of glucose with plasma proteins through a non-enzymatic process known as glycation. Protein glycation results in the formation of advanced glycation end products (AGE) which play an important role in the pathogenesis of complications of diabetes[2]. Pathologically high blood glucose increases glycosylation reactions resulting in large amounts of AGE products[3] which are also involved in the development of diabetes related osteoporosis. AGE products may also affect the activity of osteoblasts and osteoclasts, interfere with the bone remodeling process and reduce angiogenesis[4,5].

In prosthodontic treatments such as dental implants, angiogenesis plays a crucial role. New bone formation, bone regeneration and osseointegration after placement of dental implants are key to successful treatment. These processes require an adequate blood supply providing nutrients, oxygen and osteoprogenitor cells through newly-formed blood vessels. An interaction between angiogenesis and osteogenesis occurs in such a way that the regulation of angiogenesis can affect the bone remodeling process required for successful dental implant treatment[6].

Stem cell technology has developed rapidly in recent years to the extent that it is considered capable of offering new options for currently inadequate treatment of conditions such as diabetes and its complications[7,8]. The main source of mesenchymal stem cells (MSCs) for stem cell treatment is bone marrow, despite the fact that cell harvesting is a highly invasive procedure. In addition, the potential difference, the maximum number and the life span of MSCs declines with increased donor age[9]. Another potential source of MSCs is the umbilical cord due to its higher in vitro culture proliferation rate and supposed immunity to certain antigens. It is also a source

of MSCs that are usually discarded after childbirth, but whose collection and storge is developing in a global network bank. Human umbilical cord stem cells (hUCMSCs) possess multipotential properties and are able to differentiate into several types of cells such as adipocytes, osteoblasts, hepatocytes, chondrocytes, heart and nerve cells. They also cause beneficial effects such as angiogenesis[10,11].

Angiogenesis constitutes a dynamic process that is strongly influenced by signals from the extracellular matrix and serum contained in the micro environment. VEGF, angiopoietin, fibroblast growth factor and beta transformation growth factors are the most powerful angiogenic cytokines in the process of angiogenesis. VEGF is a specific mitogen vascular endothelial cell that stimulates endothelial cell proliferation and microvascular permeability, while also regulating several endothelial integrin receptors during the formation of new blood vessels[12,13].

At present, no in vitro studies exist regarding VEGF secretion by hUCMSCs in the AGE-BSA medium which constitutes the micro-environment in diabetic patients. Therefore, it is important to establish a theoretical basis for further research related to the application of hUCMSCs in the treatment of diabetes.The purpose of this study was, therefore, to determine the effect of AGE-BSA medium on hUCMSCs and, by extension, VEGF secretion.

METHODOLOGY

Ethical approval for this study was granted by the Komisi Etik Penelitian Fakultas Kedokteran Hewan, Universitas Airlangga, Indonesia (Number 2.KE.152.09.2018). The benefits of this study were explained to participants and an informed consent form was obtained.

Umbilical cord cells were extracted from the placenta of a healthy newborn by cesarean section with elective indications. The isolation and multiplication of hUCMSCs was conducted according to the standard procedures of the Stem Cell Research and Development Center, Universitas Airlangga, by modifying several stages previously implemented by Hendrijantini et al. (2015). The umbilical cord was cut into 10 cm long sections which were subsequently placed in sterile boxes lined with sterile gauze and washed three times with phosphate-buffered saline (PBS) in three different tubes to remove any residual blood. The umbilical cord was washed again with ringer lactate (RL) containing 2.5 µg/mL gentamicin and 1000U/mL amphotericin for 20 minutes before being transported to the laboratory in a cool box.

The umbilical cord was cut into 1 mm^3 sections, cleaned and separated from the arteries, veins and adventitia. It was subsequently immersed in a 0.75 mg/mL collagenase IV cone tube at 37°C and 0.075 mg/mL of DNAse I for 40 minutes before being placed on a medium hot plate stirrer for 15 minutes. In the subsequent step, the umbilical cord was filtered through a cell filter and the resulting pellets collected. The supernatant was removed before being centrifuged at 1800 rpm for six minutes, a procedure that was repeated twice. The pellets were transferred to a petri dish and stored in a 5% CO2 incubator at 37°C. Daily observation, conducted by means of an inverted microscope, monitored cell growth until it reached the confluent stage. Confluence constitutes a form of cell growth in a petri dish containing a large population so that the transition process can be completed. (Hendrijantini et al. 2015)

Flowcytometry assessment was conducted in passage 4 by tripination and suspension of hUCMSCs in αMEM medium, after which they were washed with PBS and fixed in 10% formaldehyde solution for ten minutes. They were subsequently covered with AGE-BSA solution for one hour, at which point the cells were incubated using the Human MSCs Analysis Kit (BD StemflowTM, BD Biosciences) with the addition of anti-human antibodies CD 73, CD 90, CD 105, and negative cocktails CD45, CD34 for 40 minutes. Unbonded antibodies were removed by means of PBS washing. The primary antibodies were labeled using Fluorescein isothiocyanate (FITC) conjugated anti-human antibodies by means of incubation for 30 minutes. The cells were then analyzed using a FACSCaliburflowcytometer (BD Biosciences, Franklin Lakes, NJ, USA). Modified AGE-BSA was produced by reacting BSA with glycolaldehyde under sterile conditions followed by extensive dialysis and purification. The doses were reduced gradually as follows: 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 mM. hUCMSCs were placed in 12-well plates, each containing a density of 1×105 cells per well. When the cells were ready, the culture media was replaced. hUCMSCs were then placed in an incubator at 37°C and 5% CO_2 for 21 days. It was necessary to replace the medium after 2-3 days depending on its condition indicated by changes in its color. If the medium turned yellow, it had to be replaced. During each replacement, the culture medium of the hUCMSCs was collected and centrifuged at 3000 rpm for 20 minutes. Supernatant was extracted and stored at -80°C. VEGF protein levels in the media were measured using an ELISA kit in accordance with the instructions of the manufacturer[16].

Statistical analysis was performed using statistical package for the social sciences software (SPSS) 24.0 edition (SPSS™, Chicago, United States). The data was analyzed using independent t-test.

RESULTS

During the first 24 hours, almost all cells were oval in shape. However, 24 hours later, they had attached themselves to the tube and assumed a spindle or fibroblast-like form. After three days, these cells grew to

reach 90 % confluence (see Figure 1).

It can be seen from figure 2 that the flowcytometry results for CD90 were 53% and 22.63% in the control group. This meant that the cells expressed CD90 positive. For Neg PE, the results were 0 and 22.63% in the control group, meaning that the cell state was Negative Neg PE.

Figure 3 shows the result for CD 105 in the control which were 90.28% and 21.16%, indicating that cells expressed CD 105 positivity.

From Figure 4, the results for CD 73 showed that cells expressing CD73 were positive with 6.08% and 3.83% in the control group.

Figure 1: hUCMSCs culture. (A) oval shaped. (B) spindle or fibroblast shaped cells

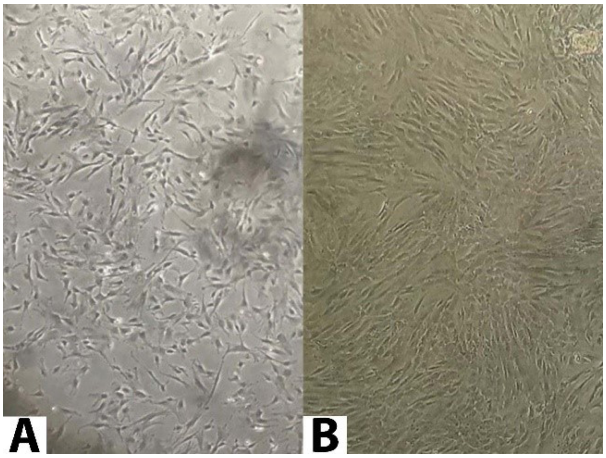
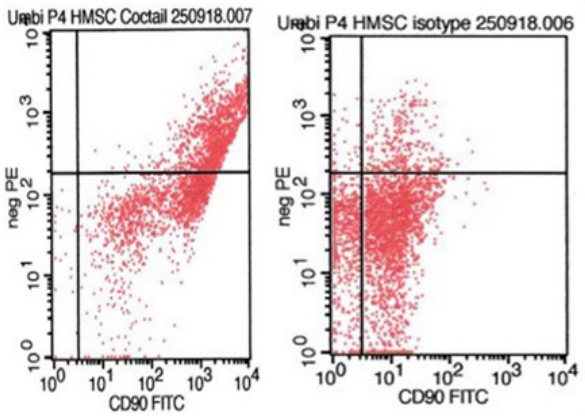


Figure 2: Flowcytometry results for CD90 with Neg PE



Collection of data relating to the amount of VEGF secreted by hUCMSCs in the medium was initiated during passage 5. Every change of medium color was observed and examined with an ELISA reader. Seven media were collected at a rate of one daily on days 3, 6, 9, 12, 14, 17 and 21. The data relating to these can be seen in figure 5.

In the AGE-BSA group, the peak level of VEGF secretion occurred on day 3, with a decrease from day 9. In the control group, VEGF secretion peaked on day 6 before reducing gradually between day 12 and day 21 though their levels were the same on days 6, 14, 17 and 21.

Figure 3: Flowcytometry result on CD105 with Neg PE

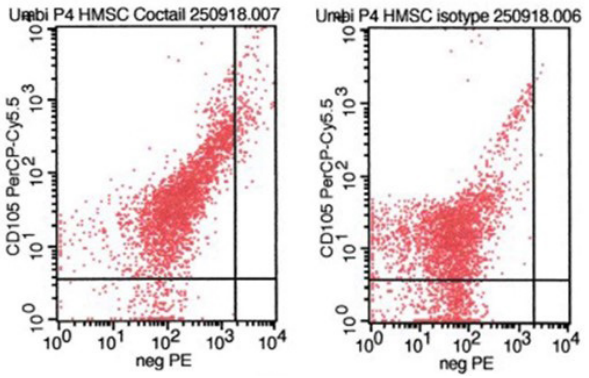
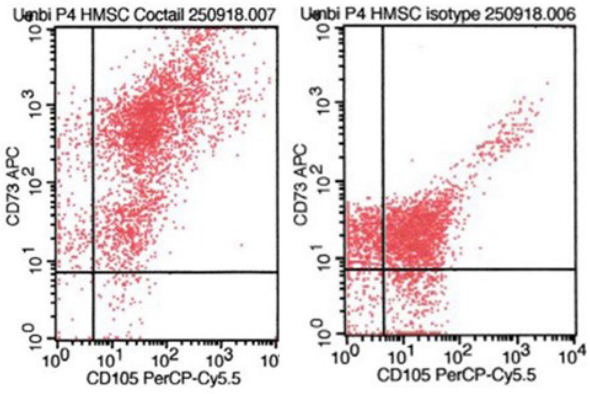


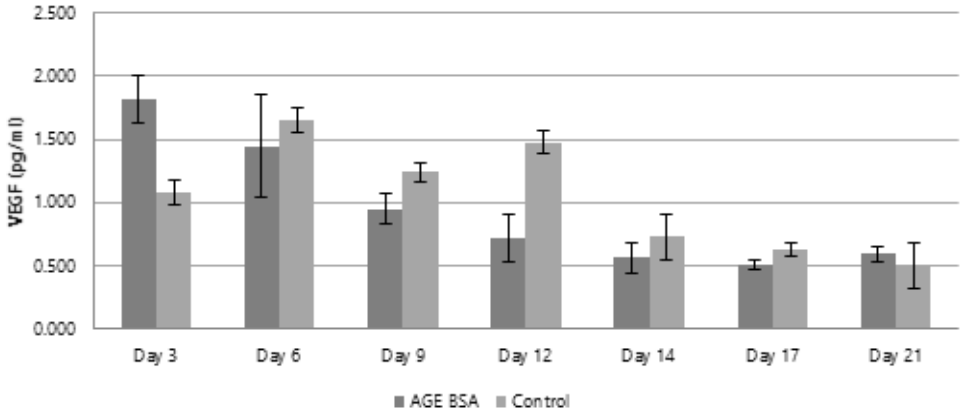
Figure 4: Flowcytometry result for CD105 with CD73



DISCUSSION

It has been reported that mesenchymal stem cells have the ability to differentiate into endothelial cells through a process of angiogenesis[28–31]. This angiogenic property was contributed by angiogenic cytokines such as VEGF that was released by the MSCs itself. VEGF is an important angiogenic factor that encourages migration and proliferation of endothelial cells to maintain blood vessel integrity. VEGF affects the formation of early

Figure 5: Means of VEGF secretion of control and treatment groups at various times



blood vessels and increases the formation of primitive blood vessel tissue[32]. VEGF production by MSCs may be an important factor responsible for the angiogenic potential of MSCs[33].

This study revealed that on day 3, VEGF secretion was higher in the AGE-BSA group than in the control group. This result supports the study conducted by Shoji et al. (2006) which found that AGE interactions can cause angiogenesis through autocrine vascular VEGF induction. However, the mechanism through which this occurs was not clearly understood at that time. In a more recent study, researchers found that AGE can increase VEGF secretion through its interaction with the RAGE receptor which regulates the expression of Cyr61, a cell matrix adjustment factor that plays an extremely important role in angiogenesis. Cyr61 can, in turn, activate the intergrin-PI3K / AKT signaling pathway, accelerate NF-κB nuclear translocation and promote VEGF secretion[35].

In another study, it was found that AGE interacts with RAGE to induce activation of the Ras-mitogen activated protein kinase (Ras-MAPK) activated by the generation of ROS-mediated NADPH oxidase which subsequently stimulates NF-kB translocation. The consequences lead to the transcription of the target gene, VEGF[36].

In the findings presented here, the peak rate of VEGF secretion by hUCMSCs in growth media occurred on day 6. This result is similar to that of a study conducted by Matsumoto et al. (2005) which found that the peak level of VEGF secretion in MSCs occurred on day 5.

The VEGF levels in AGE-BSA decreased earlier than un the control group. In the AGE-BSA group, the amount of VEGF decreased after day 9. On the other hand, the level of VEGF in the control group decreased by the 14th day. This finding might be due to AGE-BSA in the treatment group affecting the number of hUCMSCs through ROS production. The reaction between ROS and fat membrane will form MDA which demonstrates cell damage properties. Cell death caused by MDA is referred to as necrosis. ROS can also react with Fe / Cu ions to produce hydroxyl radicals (OH*). These radicals can translocate into the cell nucleus and cause damage to genetic components / DNA fragmentation. Fragmentation will cause cell death, known as apoptosis (Sudiana, 2017). The amount of hUCMSCs is reduced with the result that the VEGF level experiences a decrease.

CONCLUSION

AGE-BSA medium enhances the production of VEGF secreted by hUCMSCs. The peak secretion level was found to occur on day 3.

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CONTRIBUTORS

AB conceived the idea, wrote initial manuscript, collected data and supervised every step of the project. KM and AK helped in acquisition of data, interpretation and statistical analysis, corrections in the manuscript and finalization of bibliography. MK and NH went through the study protocol, carried out corrections in the process of peer review and prepared final manuscript after inserting and displaying data in results. All authors contributed significantly to the submitted manuscript.