ORIGINAL ARTICLE

Viability of human dental pulp stem cells: The potential of L-arginine-based culture media

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ABSTRACT

Dental pulp is built by proteins that have various roles in the biological process of pulp, such as structural protein, regulation protein, and catalytic protein. L-arginine, an amino acid and one of the building blocks of proteins, regulates pro-inflammatory and anti-inflammatory activity. Therefore, L-arginine-based culture has potential to promote dental pulp regeneration. This study aimed to investigate the potential of L-arginine-based culture in improving the viability of human dental pulp stem cells (hDPSCs). We evaluated the viability of hDPSCs in culture media supplemented with different concentrations of L-arginine amino acid (250, 300, 350, and 400 μmol/L) and Dulbecco's Modified Eagle Medium plus fetal bovine serum 10% (control) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 24-h incubation time. Statistical analysis was conducted using a one-way analysis of variance and *post hoc* least significant difference test. In qualitative analysis, the 4′, 6-diamidino-2-phenylindole staining method was used. The evaluation has shown a significant result when 250, 300, and 350 μmol/L concentration of L-arginine amino acid culture media compared with control, and 400 μmol/L has the best result and was not significantly different with control toward viability of hDPSCs.

Key words: Amino acid, human dental pulp stem cells, viability

INTRODUCTION

Studies on pulp biology have led to major progress in understanding of the structure and behavior of dental pulp in both healthy and unhealthy conditions.[1,2] Most of these studies have focused on the regenerative capacity of pulp tissue and the clinical application of regenerative treatment.[3] Studies on pulp tissue have identified 342 proteins in pulp tissue, with as many as 37 found specifically in pulp tissue.[4,5] These proteins were built by amino acids and have different roles in maintaining homeostasis.[4] L-arginine is an amino acid that serves as a building block for protein synthesis and as a precursor for multiple metabolites, including, polyamines, and nitric oxide (NO) that has strong immunomodulatory properties.[6]

In regenerative endodontic treatment, human dental pulp stem cells (hDPSCs) can proliferate and differentiate into different cell types required by pulpal tissue. Regenerative endodontic treatment applies a triad of tissue engineering methods, namely stem cells, scaffolds that act as cell growth media, and growth factors that induce pulp tissue regeneration.[7,8] Regenerative endodontic

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treatment needs strategies that can disinfect without harming the microenvironment while enabling stem cell differentiation.[9] Therefore, the search for irrigation solutions that have antibacterial abilities and do not reduce the viability of hDPSCs is the focus of much research.

L-arginine is a nonessential amino acid, but it is essential under conditions, such as in the growth process or inflammatory conditions.[10] It is found in food sources, such as meat and nuts, especially soybeans (*Glycine max*), which contain high levels, as shown by the results of a proteomic analysis.[7,11] L-arginine is metabolized by the arginine deiminase system, leading to the production of ammonia, which has antibacterial abilities.[12] L-arginine is a substrate for the enzyme NO synthase, which is responsible for the production of NO.[2] NO is an effective intercellular signaling molecule, regulates the activity of several transcription factors, and increases the proliferation of fibroblasts.[13] There is much research interest in L-arginine with several studies demonstrating its potential in dietary supplementation, wound healing process, and skin regeneration process.[14-16] Wound healing is impaired in patients who experience hemorrhagic trauma/shock due to decreased collagen synthesis. The risk of wound dehiscence after surgery is also higher in such patients. Arginine supplementation is often used in such cases to aid wound healing after surgery.[14] L-arginine has begun to be used in dentistry, especially in the field of caries prevention.[9] Toothpaste containing 1.5% arginine, insoluble calcium compounds, and 1450 ppm fluoride has been shown to reduce caries development compared to a regular toothpaste containing only 1450 ppm fluoride in children with low and moderate caries risk.[12] Inflamed dental pulp has the capacity to heal, but in actual clinical situation, the healing was impaired. L-arginine is an amino acid that serves as a building block for proteins and has antimicrobial and wound-healing potential that might help the healing process of dental pulp. The L-arginine-based amino acid solution used in this study is the product derived from herbal medicine (local soybeans). This is the first study of L-arginine-based amino acid solution toward hDPSC viability. Therefore, the aim of this study was to confirm the potential ability of L-arginine in increasing hDPSCs viability and determining the optimal dose of L-arginine for hDPSCs.

MATERIALS AND METHODS

This research is an *in vitro* study conducted at the Biotechnology Generating Laboratory and the Prodia Stem Cell Laboratory. L-arginine-based amino acid (ASMINO; Dermama Corp, Indonesia) solution was diluted with Dulbecco's Modified Eagle Medium (DMEM) to concentrations of 250, 300, 350, and 400 μmol/L. Evaluation of the effect of L-arginine (250, 300, 350, and 400 μmol/L) and DMEM (negative control) or fetal bovine serum (FBS) 10% (positive control) on the viability of hDPSCs was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 4′, 6-diamidino-2-phenylindole (DAPI) staining.[17] The hDPSCs in this study use biological stored raw material at passages 3–4 and incubated in a humidified atmosphere of 5% CO_2 at 37°C until reaching 80% confluence, and hDPSCs cells were divided into 96 well with 5×10^4 cells/well for MTT assay samples. hDPSC cultures were incubated for 24 h and then supplemented with 10 mM β-glycerophosphate, 50 μg/mL of ascorbic acid, and 10–8 M dexamethasone to create an osteogenic media. The cells were then starved by replacing the cell culture supplement with DMEM and 10% FBS for 24 h. The viability of the hDPSCs was analyzed using an MTT assay after 24-h incubation. After 24-h incubation, 10 μL of MTT reagent (ThermoFisher Scientific, Waltham, MA, USA) were incubated at 37° C in 5% CO_2 for 4 h. The medium was removed, and dimethyl sulfoxide solution (Sigma-Aldrich, Saint Louis, MO, USA) (100 μL/well) was added and incubated for 30 min at 37° C to dissolve the formazan crystals. The absorbance of each well was measured at a wavelength of 570 nm using an enzyme-linked immunosorbent assay reader. DAPI staining quality test was carried out after 24 h of culture. FBS was rinsed before fixation with 1 mL of methanol for 5 min; then, it was rinsed with phosphate-buffered saline 3 times before the application of 500 μL of DAPI dye. Next, it was incubated for 30 min before the picture was taken with fluorescence under microscope.

RESULTS

The quantitative data from the research were statistically analyzed, and the viability values of the control group and the test group were tested for normality and homogeneity. The distribution of normal data was then continued with the one-way analysis of variance (ANOVA) statistical parametric test, followed by the *post hoc* test using the least significant difference (LSD) test. This research was carried out with experimental laboratory tests to analyze the potency of L-arginine-based amino acid solution on the viability of hDPSC. The viability of hDPSC was assessed from the percentage of live cells and calculated using MTT assay after being given treatment for 24 h with different concentrations. The data obtained were then converted into the percentage of live cells or cell viability. Cell viability was calculated from the absorbance value (optical density) of each treatment group using the following formula:

$$\text{Cell Viability}\,(\%) = \frac{\text{Absorbance Value Treatment}}{\text{Absorbance Value Control}} \times 100\%$$

Statistical calculations were done using SPSS Statistics 22.0 software (IBM, Chicago, USA). The cell viability results at 24 h of observation obtained a normal data distributions (Shapiro–Wilk test; $P > 0.05$). The significance

test between the treatment and the control group was calculated using one-way ANOVA parametric test ($P < 0.05$); it was found that there is a significant difference ($P = 0.007$) in the viability of hDPSCs between the control group and the treatment group in 24-h observation [Table 1].

The highest mean value of cell viability of hDPSCs was found in the 400 µmol/L concentration group, which was 87.22% ±13.60%, then the 350 µmol/L concentration group 79.95% ±3.73%, the 300 µmol/L concentration group 75.71% ±7.33%, and the 250 µmol/L concentration group 68.26% ±8.52%. Statistical data analysis was continued with *post hoc* LSD test to determine differences between study groups at 24-h observation [Table 1]. It appeared that at a 24-h observation, time showed statistically significant differences in the viability values of concentrations of 350, 300, and 250 µmol/L compared to controls. The concentration of 400 µmol/L had no significant difference $P > 0.05$ with the control group. There was a significant difference between the concentration of 400 µmol/L compared to the concentration of 250 µmol/L $P < 0.05$ [Table 2].

Qualitative test of DAPI staining was carried out with the aim of confirming the viability test results of hDPSCs cells in the both groups; arrows indicate live hDPSCs [Figure 1]. The DAPI staining result in the control and the treatment groups of 400 µmol/L at 24-h incubation time will show the appearance of blue hDPSCs cells due to the binding of DAPI staining to cell DNA.

DISCUSSION

Cell viability is the ability of cells to survive and not to lose metabolic function or proliferative ability, and it can be measured by increasing the number of cells, the amount of protein, and DNA. Therefore, cell viability can be a sign of cytotoxicity of a material.[18] The viability test used in this study is the MTT assay; the MTT assay is one of the most used colorimetric assays to assess cytotoxicity or cell viability. According to ISO 10993-5:2009 guidelines, if a substance reduces the yield of cell viability below 70%, it is considered that the material has cytotoxicity to cells, and further tests are required. Soybeans (*Glycine max*) contain high levels of L-arginine, which is known from the results of proteomic analysis.[11]

In this study, 24-h observation was carried out because the time required for a cell to complete the cycle is approximately 24 h and to clarify the number of cell deaths that occur is not due to the apoptotic process. The results of the treatment group showed an average viability value above 70% except for a concentration of 250 µmol/L so that it was concluded that the amino acid L-arginine solution with a concentration of 300, 350, 400 µmol/L was not toxic to hDPSC according to ISO 10993–5:2009 guidelines. The concentration of 400 µmol/L showed the highest viability value of 88.2%, then the concentration group of 350 µmol/L was 79.95%, and the concentration group of 300 µmol/L was 75.71%. It showed an increase in the concentration of L-arginine-based amino acid solution also increased viability values of hDPSC cultures.[19] This may be because L-arginine is a substrate for the enzyme NO synthase, which is responsible for the production of NO.

NO supports collagen synthesis, which is important for wound regeneration, and NO is also a highly diffused intercellular signaling molecule that can activate several transcription factors. NO showed a proliferation-enhancing effect on BALB/c 3T3 fibroblasts

Table 1: Test the significance of the mean value of cell viability and standard deviation of various groups at 24-h of observation

Culture medium	Mean±SD	*P*
400 µmol/L	87.22±13.60	0.007*
350 µmol/L	79.95±3.73	
300 µmol/L	75.71±7.33	
250 µmol/L	68.26±8.52	
Control (DMEM +10% FBS)	100.00	

*$P<0.05$ means significantly difference, one-way ANOVA. SD: Standard deviation, DMEM: Dulbecco's Modified Eagle Medium, FBS: Fetal bovine serum, ANOVA: Analysis of variance

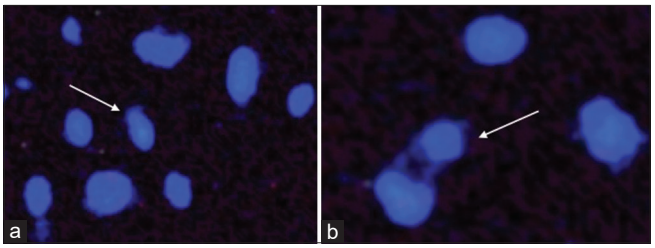

Figure 1: (a) hDPScs stained with DAPI in the Control Group, (b) hDPScs stained with DAPI in Treatment Group L-arginine 400 µmol/L

Table 2: The significance value of cell viability between groups at 24 h of observation

	Control (DMEM + FBS 10%)	400 µmol/L	350 µmol/L	300 µmol/L	250 µmol/L
Control (DMEM + FBS 10%)		0.081	0.012*	0.004*	0.001*
400 µmol/L			0.296	0.111	0.016*
350 µmol/L				0.535	0.106
300 µmol/L					0.284
250 µmol/L					

*$P<0.05$ means significantly difference, *post hoc* Mann–Whitney. DMEM: Dulbecco's Modified Eagle Medium, FBS: Fetal bovine serum

and endothelial cells. Studies have shown that L-arginine can increase the proliferation of fibroblast cells thought to be through the ERK1/2-CREB and PI3K/Akt pathways.[13] Other studies have shown that exogenous NO can maintain the viability of rat dental pulp stem cells and activate tumor necrosis factor-κB that will lead to odontogenic differentiation.[20] NO promotes angiogenesis and through perivascular and endothelial cell recruitment. Furthermore, NO has the potential to inhibit the growth of bacteria and prevent infection.[21] A study using a NO-releasing biomimetic nanomatrix gel found the formation of blood vessels as well as odontoblast-like cells in the beagle model.[22] NO has also been shown to influence the release of vascular endothelial growth factor during angiogenesis that occurs in bone remodeling.[23] The NO mechanism that might be responsible for amino acid L-arginine solution of 400 µmol/L concentration did not have a significant difference with the control group (DMEM + FBS 10%).

The best concentration in this study was a concentration of 400 µmol/L with a mean viability value of 88.2% and had no significant difference with the control group (DMEM + FBS10%) which is the gold standard of cell culture media.

Further research is needed to find the optimum concentration and effect of L-arginine-based solutions on the activity of hDPSCs. The amino acid L-arginine solution has potential clinical applications in the field of endodontics. The solution can be applied as an irrigation solution or a medicament for regenerative endodontic procedures that have antibacterial effects and can also maintain the viability of hDPSCs so that they are able to regenerate pulp tissue.

CONCLUSION

There are differences in potency of various amino acid concentrations of L-arginine on the viability of hDPSCs, with a concentration of 400 µmol/L being the highest concentration on the viability of hDPSCs. This is supported by a qualitative analysis of DAPI staining. Suggestions from the authors are that further research can be carried out with L-arginine amino acid concentrations higher than 400 µmol/L to find the optimum dose. Furthermore, viability studies can be carried out using the real-time polymerase chain reaction (PCR) method and propidium iodide staining to represent dead cells.

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Conflicts of interest

There are no conflicts of interest.

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