Potential Use of L-Arginine Amino Acids towards Proliferation and Migratory Speed Rate of Human Dental Pulp Stem Cells

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ABSTRACT

Objective: L-arginine is a semi-essential amino acid produced by the body which has an important role in the process of stem cell regeneration. However, under inflammatory conditions, denaturation of pulp amino acids and proteins occurr resulting in a decrease in the ability of stem cells to self-renew. Therefore, in this study, L-arginine was added *in vitro* to the culture media Dulbecco's Modified Eagle Medium – (DMEM) of human dental pulp stem cells (hDPSCs) to analyse the potential of L-arginine on migration and proliferation by comparing between 3 concentrations, namely 300, 400, 500 µmol/L and control group (DMEM), to obtain the most optimal concentration for proliferation and migration.

Methods: Serum-starved hDPSCs were divided into four groups: control: hDPSCs in DMEM; hDPSCs in 300 µmol/L of the L-Arginine based culture media group; hDPSCs in 400 µmol/L of the L-Arginine based culture media group; and hDPSCs in 500 µmol/L of the L-Arginine based culture media group, which were added in two separate 24-well-plates (5×10^4 cell/well) for proliferation and migration evaluation. The proliferation of all groups was measured by using a cell count test (haemacytometer and manual checker) after 24 h. The migratory speed rate of all groups was measured by using cell migration assay (scratch wound assay) after 24 h. Cell characteristics were evaluated under microscope that was then evaluated using image-J® interpretation. This image J represented the measurement of migratory speed rate (nm/h) data. Statistical analysis was conducted using one-way ANOVA and *post hoc* Bonferroni ($p<0.05$) for proliferation and *post hoc* LSD ($p<0.05$) for migration.

Results: There was a statistically significant difference in hDPSCs proliferation among various concentration groups of the L-Arginine based solution (300, 400 and 500 µmol/L) compared to the control group ($p<0.05$). There was a statistically significant difference in the migratory speed rate of hDPSCs at 500 µmol/L of the L-Arginine based solution group compared to lower concentrations and control group ($p<0.05$).

Conclusion: All three concentrations of L-arginine can induce proliferation of hDPSCs. L-arginine at 500 µmol/L can induce higher hDPSCs proliferation and faster migration at 24 hours compared to lower concentrations and control.

Keywords: Amino acid, cell migration assay, cell proliferation, dental pulp, L-Arginine, stem cell

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HIGHLIGHTS

- L-Arginine is a semi-essential amino acid in which the production is insufficient under oxidative stress and inflammation.
- L-Arginine is the sole substrate for nitric oxide synthase (NOS) to produce nitric oxide (NO), which plays an important role in cell proliferation and migration.
- L-Arginine has the potential to increase hDPSCs proliferation and migration.

INTRODUCTION

The pulp responds to stimuli by generating reactive oxygen species (ROS) and inducing inflammatory response. Oxidative stress induced by various stimuli can increase expression of pro-inflammatory factors such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) (1). The presence of oxidative stress can cause tissue regeneration or necrosis, depending on the duration and intensity. ROS regulate cellular homeostasis and act as major modulators of cellular dysfunction that contributes to the pathophysiology of the disease (1). ROS play a role as a center for regulation of inflammatory signaling, especially nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) (2). At lower concentrations, ROS has positive effects, namely tissue repair and cell differentiation. However, in higher concentrations, ROS induces uncontrolled cell activity, leading to mitochondrial dysfunction, mutagenesis, and apoptosis (3).

A total of 123 proteins have been identified in the dental pulp: 66 in healthy pulp, 66 in inflamed pulp, and 91 in necrotic pulp (4). Amino acids are the building blocks of protein, and also play an important role in pulp biological processes, such as cell communication, growth and maintenance, immune responses, metabolism, and transportation (5). The number of proteins and amino acids decrease in inflamed pulp, associated with amino acid sequence alteration due to the activation of the mitogen-activated protein kinase (MAPK) pathway by ROS (6).

L-Arginine is a semi-essential amino acid, the production of which is insufficient under oxidative stress and inflammation. It is mainly found in human cells, organs, and body fluids, including saliva (7). L-Arginine based medications had been developed as wound dressing and food supplement (8). L-Arginine can be obtained endogenously from protein synthesis of citrulline and exogenously from food, such as meat and beans (7). L-Arginine is cationic in nature; hence, it has antibacterial properties and supports wound healing bioactivity by modulating inflammation reactions, collagen formation, angiogenesis, and tissue remodeling (9). It is the sole substrate for nitric oxide synthase (NOS) to produce nitric oxide (NO) (10). NO is known to have an important role in cell proliferation and migration of fibroblasts (11).

Wound healing is initiated by hemostasis and followed by cell migration to wounded area to begin proliferation (12). Cell migration processes can be quantitatively measured by the migration rate through comparing the difference of initial and final wound length to time (13). Rhoads et al. (14) showed that the addition of 400 μmol/L of extracellular L-Arginine in fetal bovine serum (FBS) increased the migration rate by 1.7 fold. A significant increase in intestinal stem cell migration was observed in a minimum concentration of 200 μmol/L; the maximum effect was observed at 400 μmol/L (15).

Cell proliferation is a fundamental, complex, and organized biological process, as two daughter cells result from one cell (15). It is an increase in the number of cells after cell division and can be measured by different methods, such as cell counts, BrdU, and MTT-assay (15, 16). Tan et al. (17) reported that the addition of 100 μmol/L and 350 μmol/L of L-Arginine increased intestinal stem cell proliferation after 48 and 96 hours. Kim et al. (18) showed that the most effective trophectoderm cell proliferation occurred using 200 μmol/L of L-Arginine after 4 days. Fujiwara et al. (19) studied L-Arginine supplementation toward skin fibroblast proliferation after 6, 12, and 24 hours. Maximum proliferation was observed at 600 μmol/L of the L-Arginine group after 12 and 24 hours (19).

The present study aimed to analyze the potency of L-Arginine in different concentrations that had been added to culture media to evaluate the proliferation and migration of hDPSCs.

MATERIALS AND METHODS

This study was approved by the Ethics Committee on March 7th, 2022, with number as followed No. 3/Ethical Exempted/FKGUI/III/2022; No. Protocol: 050190222. hDPSCs were cultured from previous research, with number as followed No.49/Ethical Approval/FKGUI/IX/2020 (amendment); No. Protocol: 070260820 and conducted in accordance with The Declaration of Helsinki. This study was conducted at Prodia Stem Cell (ProStem) Laboratory, Jakarta, Indonesia. This *in vitro* study consisted of several stages; hDPSCs culture and serum starvation induction, L-arginine based solution dilution, then the hDPSCs were divided for migration test and proliferation test.

Human Dental Pulp Stem Cell Culture

The hDPSCs at passage 5 (cultured from biological stored raw material from previous study) and were incubated in a humidified atmosphere of 5% CO_2, at 37°C until reaching 80% confluence. Cells were then starved by replacing the cell culture supplement with DMEM (Gibco, Thermo Fisher Scientific, Massachusetts, USA) with 1% FBS (Gibco, Thermo Fisher Scientific, Massachusetts, USA) for 24 hours.

Preparation of L-arginine Amino Acids Solution

L-Arginine based amino acid solution (ASMINO, Dermama Biotech Lab, Surakarta, Indonesia) at concentration 800 μmol/L before dilution) were incubated for 24 hours at 37°C. Later, the solution was centrifuged at 1000 rpm for 10 minutes, and filtered (Minisart filters, Sigma-Aldrich, Massachusetts, USA), then diluted with DMEM (Gibco, Thermo Fisher Scientific, Massachusetts, USA) to obtain L-Arginine concentrations of 300, 400 and 500 μmol/L.

Migration of hDPSCs

Migration test procedure was based on previous study on human dental pulp stromal cells migration. hDPSCs cells were divided into 24 well plate (NUNC, Thermo Fisher Scientific, Massachusetts, USA) each containing 2×10^5 cells. A single continuous scratch was made on confluent cell surfaces using a P200 pipette tip (Dragon Lab, DLAB, Beijing, China) to create a 'wound'. Initial wound lengths were observed under microscope magnification and measured using ImageJ Software (ImageJ, U.S. National Institutes of Health, Maryland, USA). After that, 300, 400 and 500 μmol/L of L-Arginine solution was added and then incubated in a humidified atmosphere of 5% CO_2, 5% CO_2 at 37°C. After 24 hours, final wound lengths were measured using the same software. After that, migration rates were calculated by dividing the differences of final and initial lengths to time (24 hours).

TABLE 1. *Post-hoc* analysis of migration speed rate of hDPSCs cultured with 300, 400, 500 μmol/L L-Arginine

Treatment	L-Arginine 300 μmol/L	L-Arginine 400 μmol/L	L-Arginine 500 μmol/L	Control (DMEM)
L-Arginine 300 μmol/L		0.951	0.001*	0.695
L-Arginine 400 μmol/L			0.001*	0.740
L-Arginine 500 μmol/L				0.001*
Control (DMEM)				

*: p<0.05; *post hoc* LSD test. hDPSCs: Human dental pulp stem cells, DMEM: Dulbecco's Modified Eagle Medium, μmol/L: Micromol per liter

Human Dental Pulp Stem Cell Proliferation

hDPSCs cells were divided into 24 well plate each containing 2×10^5 cells. 300 μmol/L, 400 μmol/L, and 500 μmol/L of L-Arginine solution was added and incubated in a humidified atmosphere of 5% CO_2, at 37°C. After 24 hours, quantitative analysis was done using cell count test with hemocytometer (Blaubrand™ Neubauer Counting Chamber, Thermo Fisher Scientific, Massachusetts, USA) and manual checker under microscope magnification (Carl Zeiss, Zeiss, Oberkochen, Germany).

Statistical Analysis

Data analysis was done using IBM SPSS Statistics Software (version 26.0, IBM Corp., New York, USA). This study used two different statistical analysis. hDPSCs migration data was analysed using one-way ANOVA, followed by LSD *post hoc* to compare between study groups. hDPSCs proliferation data was analysed using one-way ANOVA, followed by Bonferroni *post hoc* to identify comparisons between groups. The level of significance was set at 0.05 (p=0.05).

RESULTS

Human Dental Pulp Stem Cells Migration

For migration analysis, the measurement was done by a single observer. Inter-rater reliability test was done with intraclass correlation coefficient (ICC) of 0.946. (IBM SPSS Statistics Software, version 26.0, IBM Corp., New York, USA). In this study, wound lengths were measured shortly after scratching (initial wound length) and after 24 hours (final wound length). Microscopic images (Fig. 1) were obtained during observation, and then processed and measured using ImageJ Software. (ImageJ, US National Institutes of Health, Maryland, USA) The narrowest final wound length was observed in 500 μmol/L group, which was 1.09×10^{-3} μm. According to one-way ANOVA analysis there were significant differences between test groups (p<0.05). LSD *post-hoc* analysis showed that there was a significant difference between final and initial wound length in 500 μmol/L group (p<0.05). Migration speed rate was calculated by dividing the difference between final and initial wound length to time (24 hours). According to migration speed rate formula, the rate of hDPSCs migration was 7.7 times faster in 500 μmol/L group compared to control. However, 300 and 400 μmol/L group were not significantly different with control group (Table 1 and Fig. 2).

Human Dental Pulp Stem Cells Proliferation

For proliferation analysis, the measurement was done by a single observer. Inter-rater reliability test was done with intraclass correlation coefficient (ICC) of 0.996. There were significant differences (p<0.05) in the potency of various concentrations of the amino acid L-Arginine on the proliferation of hDPSCs (Table 2). The highest average yield was obtained at the concentration of L-Arginine amino acid culture medium 500 μmol/L.

TABLE 2. Analysis of hDPSC Proliferation in 24 Hours (Cell Count)

Treatment	Mean (SD)	p
Control	250.000 (13.228)	<0.001*
300 μmol/L L-Arginine	376.667 (24.664)	
400 μmol/L L-Arginine	366.667 (28.431)	
500 μmol/L L-Arginine	436.667 (32.145)	

*: One-way ANOVA test, p<0.05. hDPSCs: Human dental pulp stem cells, SD: Standard deviation, μmol/L: Micromol per liter

DISCUSSION

This study examined the potency of the amino acid L-Arginine against hDPSCs. L-Arginine acts as a central metabolic module where L-Arginine metabolism directly and indirectly participates in many biological phenomena such as vasodilation, calcium release, adenosine triphosphate (ATP) regeneration, neurotransmission, cell proliferation and the immune system (9) The purpose of this study was to determine the potential differences of various concentrations of L-Arginine amino acid solution on the proliferation and migration of hDPSCs. hDPSCs were used in this study because of the important role in tissue regeneration and immunomodulatory functions that control inflammation to achieve tissue homeostasis (20). hDPSCs have a rapid proliferation rate, non-specialized cells and can differentiate into other types such as osteoblasts, odontoblasts, chondrocytes, neurons and adipocytes (21). hDPSCs showed greater proliferation than other stem cells such as bone marrow stem cells (BMSCs) and adipose-derived stem cells (ASCs) (22). In this study, serum starvation was also performed on hDPSCs to conform to the biological niche of pulpal inflammation (reversible pulpitis) so that the proliferation potential of hDPSCs could be determined after treatment with L-Arginine.

Migration of hDPSCs towards wound area involved a complex process which was not fully known. Two of many signaling pathways that expected to have a role in hDPSCs migration are stromal cell-derived factor-1/ CXC chemokine receptor-4 (SDF-1/CXCR4) axis and focal adhesion kinase (FAK) (23). L-Arginine was the sole substrate of nitric oxide synthase (NOS) for secreting nitric oxide (NO) (9). NO is a reactive free radical.

Microscopic Images (Original)

a b c d

e f g h

Microscopic Images (Processed)

i j k l

m n o p

Figure 1. Qualitative data of hDPSCs migratory speed rate; original microscopic images (a-h) and processed with ImageJ Software (Image j, US National Insitutes of Health, Maryland, USA) (i-p). hDPSCs after scratch (0 hour) in 300, 400, 500 μmol/L of L-Arginine solution and control (a-d, i-l). hDPSCs after 24 hours in 300, 400, 500 μmol/L of L-Arginine solution and control (e-h, m-p)

hDPSCs: Human dental pulp stem cells

According to Jobgen et al. (24), the addition of 50–500 μmol/L extracellular L-Arginine could increase NO synthesis in various cells. NO could activate focal adhesion kinase (FAK) signalling pathway, which internalises integrin and regulates focal adhesion assembly and disassembly (23). For a cell to migrate, there should be an interaction between focal adhesion (FA) molecule and acin molecule on cytoskeleton. Meanwhile, cell migration would be disrupted, if FAK pathway was inhibited (8). Polyamine, which is one of L-Arginine metabolites, is needed during migration process, due to its ability to create lamellipodia, stress fibre and integrin subunit (11).

In this study, hDPSCs migration rate treated with 500 μmol/L of L-Arginine was significantly faster than control. Mean-

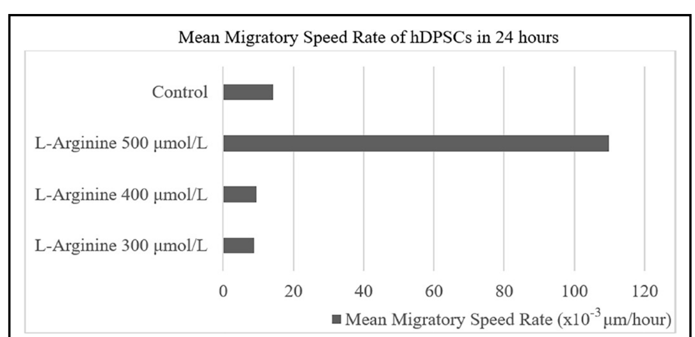

Figure 2. Mean values of hDPSCs migration rate of each group in 24 hours. hDPSCs in 500 μmol/L of L-Arginine solution at 0 and 24 hours (c). hDPSCs in DMEM (control) at 0 and 24 hours (d)

while, 300 and 400 µmol/L group showed no statistical differences to control. According to Rhoads (2004), at minimum concentration of 200 µmol/L, migration of intestinal stem cells were significantly risen, than lowered at concentration of 2000 µmol/L (14). This might be influenced by L-Arginine absorption from various concentrations into cells. Intracellular L-Arginine concentration was known to be higher than micro-extracellular environment, ranged from 100–1000 µmol/L (9). Whereas the concentration of L-Arginine in plasma varied from 72.4 to 113.7 µmol/L (25). Cellular absorption of L-Arginine could not occur passively since the lipid membrane not permeable to cationic L-Arginine molecule. Hence, active cationic amino acids transport system is needed, such as cationic amino acid transporters-1 and -2 (CAT-1 and -2) (9). There was no previous studies that reported the minimum concentration of L-Arginine needed in stimulating hDPSCs migration. Therefore, further study should be done to find the optimum dose.

The choice of concentrations of 300, 400 and 500 mol/L was based on previous studies, although studies were conducted on cells other than hDPSCs. Tan et al. (17) tested the addition of L-Arginine concentrations of 100 and 350 mol/L (physiological concentrations in plasma) resulted in an increase in intestinal cell proliferation at 48 hours and 96 hours. Fujiwara et al. (19) conducted a study using L-Arginine supplementation with a concentration of 0–7 milliMol (700 mol/L) on the proliferation of skin fibroblasts at 6, 12 and 24 hours, with the results of L-Arginine at a concentration of 6 milliMol (600 mol/L) resulted in maximum stimulation of proliferation and an increase in the number of fibroblast cells at 12 and 24 hours by MTT-assay and cell counting with trypan blue. Greene et al. (26) studied L-Arginine 200 mol/L (physiological) and 800 mol/L (supraphysiological) on human endometrial carcinoma cell line (RL95–2) for 2 and 4 days and showed higher cell proliferation at both concentrations especially at L-Arginine 800 mol/L. In this study, the use of a concentration of 200 mol/L due to the plasma concentration of L-Arginine in humans physiologically so the researchers determined the use of L-Arginine concentrations between 200 mol/L and 800 mol/L (26).

From this study, the highest proliferation of hDPSCs average yield was obtained at the concentration of L-Arginine amino acid culture medium 500 mol/L. This shows that the amino acid L-Arginine supports the potential for cell proliferation. In this case, hDPSCs were observed at 24 hours which is the time for cells to cycle through the G1-S-G2-M phase (27). The cell cycle is the process by which all cells reproduce. The speed of the cell cycle depends on the stage of development and the type of cell. The cell cycle is divided into 2 basic phases, namely Interphase and M phase (mitosis), with the full cell cycle in about 24 hours. Duration of G1 lasts for 8–10 hours, S phase is 6–8 hours, G2 usually lasts for 4–6 hours and M phase less than an hour (28).

In proliferation and migration evaluation, the control group (DMEM) and the L-Arginine group of 300, 400 and 500 mol/L showed significant differences. This can occur because L-Arginine functions as a precursor to various biologically active compounds such as NO, ornithine, proline and polyamine, creatine to phosphocreatine, and agmatine. Ornithine is a stimulator of cell growth and differentiation. L-Arginine is the only substrate used for NO synthesis and NO is synthesized during inflammatory conditions (10). L-Arginine is converted to NO through the action of NOS, while polyamines are produced through the conversion of L-Arginine to ornithine via arginase (12). These polyamines and NO have important roles in cellular processes and cell signaling. NO and polyamines stimulated cell proliferation and had a positive effect on progression through the cell cycle (26). Thus, the proliferation potential of hDPSCs in the L-Arginine group was significantly different from that of the control group.

L-arginine supplementation is important in wound healing, facilitated by NO synthesized from L-arginine through the action of NOS during the wound healing process (29). L-arginine stimulates fibroblast proliferation through G protein-coupled receptor family C group 6 member A - extracellular signal-regulated kinase 1/2 (GPRC6A-ERK1/2) pathway and phosphoinositide 3 kinase - protein kinase B (PI3K/AKT) pathway, regulates the cytokine environment in the wound area, produces an anti-apoptotic effect, and reduces interleukin 6 (IL-6) present in the wound area so that L-arginine reduces the harmful effects of increased inflammation in the wound. wound area (19). In addition, L-arginine supplementation increases protein synthesis, decreases protein degradation and has a protective effect against lipopolysaccharides (LPS) induced damage through the mechanic target of rapamycin (mTOR) and toll-like receptor 4 (TLR4) signaling pathways (17).

CONCLUSION

This study shows that 500 µmol/L of L-Arginine can induce higher hDPSCs proliferation and migration in 24 hours compared to lower concentrations and control.

Disclosures

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Ethics Committee Approval: This study was approved by the Ethics Committee on March 7th, 2022, with number as followed No. 3/Ethical Exempted/FKGUI/III/2022; No. Protocol: 050190222. We were using hDPSCs from previous research, with number as followed No.49/Ethical Approval/FKGUI/IX/2020 (amendment); No. Protocol: 070260820 and conducted in accordance with The Declaration of Helsinki.

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