L-Ascorbic acid affect the DNA methytransferase expression in Mouse Embryonic Fibroblasts

Leila Mohammadi Amirabad [a*], Mohammad Massumi [b, c], Mohammad Reza Alivand [d]

[a] School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [b] Department of Physiology, University of Toronto, Toronto, Ontario, Canada; [c] Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada; [d] Department of Medical Genetics, Tabriz University of Medical Sciences, Tabriz, Iran

Corresponding author:** Leila Mohammadi Amirabad, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ***Tel: +982122439847; ***E-mail:*** Leila.mo@nigeb.ac.ir

Submitted: 2017-08-01; **Accepted:** 2017-08-20

Introduction: Induced pluripotent stem cells (iPSCs) can be generated from different source of cells with different efficiencies. Two DNA methyltransferases DNMT1 and DNMT3A have been shown to regulate epigenetically the gene expression involved in cell viability and reprogramming. L-ascorbic acid (L-AA) is a chemical factor that can accelerate reprogramming. Here, we sought to investigate the effect of L-AA on DNMT1 and DNMT3A expressions. **Materials and Methods**: First, mouse embryonic fibroblasts at passage 3 were cultured in the presence of 10 µg/ml L-AA days for 5 days. Then, DNMT1 and DNMT3A expressions were determined using real-time PCR at days 3 and 5. **Results:** It was showed that L-AA could enhance DNMT-1 expression which involve in cell viability and decrease the DNMT3A which involve in cell differentiation. **Conclusion:** The results therefore suggest a new insight into L-AA mechanism impact on reprogramming process.

Keywords: Induced pluripotent stem cells; DNA methyltransferases; DNMT1; DNMT3A; reprogramming; L-ascorbic acid

Introduction

Induced pluripotent stem cells (iPSCs) can be generated from different sources of cells using a defined combination of transcription factors (1). However, the efficiency of iPSC generation from mouse fibroblasts even using four Yamanaka's factors, i.e. OCT4, SOX2, c-MYC and KLF4 (OSKM) is about 1% of the starting population (2). There are many unknown chemicals and epigenetic factors can enhance the efficiency of somatic cell reprogramming.

L-AA is a chemical factor which can accelerate reprograming of the human and mouse cells (3). The studies showed that this role of L-ascorbic acid (L-AA) in enhancing reprogramming efficiency is unrelated to its antioxidant activity (3, 4). L-AA enhances stemness marker gene expression and thus regulate the cell senescence in an unclear way. This anti-aging effect of L-AA may be promoted by epigenetic mechanism that proceed the further changes.

DNA methylation is an epigenetic modification which can regulate the cell cycle and stem cell differentiation (5). To obtain successful nuclear reprogramming DNA methylation patterns should be modified at the sites of the promoters of genes such as pluripotency genes and genes specified for differentiation in reverse manner. The partially reprogramming will be achieved if loss of DNA methylation is not obtained (6). Studies on DNA methylation during the reprogramming process has been shown that DNA methyltransferases activities alter during reprogramming (6).

DNMT1 and DNMT3A are two kinds of DNA methyltransferases essential for stem cell differentiation during embryonic development(7). The studies showed that in knockout mice without Dnmt3A, blastocyst stage embryos were survived but could not differentiate into mesoderm and died several weeks after birth (8, 9). While, in contrast deletion of DNMT1 resulted in rapid cell death in human embryonic stem cells (7). This finding showed the DNMT1 can play an anti-aging role. Moreover, it has been shown that silencing the Dnmt3 expression can enhance reprogramming into induced pluripotent stem (iPS) cells by an ectopic expression of the OSKM factors (10). In another study, nuclear reprogramming of mouse embryonic fibroblasts (MEF) was achieved in the absence of Dnmt3A (11). However, it has not been resolved whether de novo DNA methylation is important or might even be essential for nuclear reprogramming of somatic cells to a pluripotent state.

In this study, we sought to investigate the impact of L-AA in of Dnmt1 and Dnmt3a expression and therefore its effect on enhancing reprogramming.

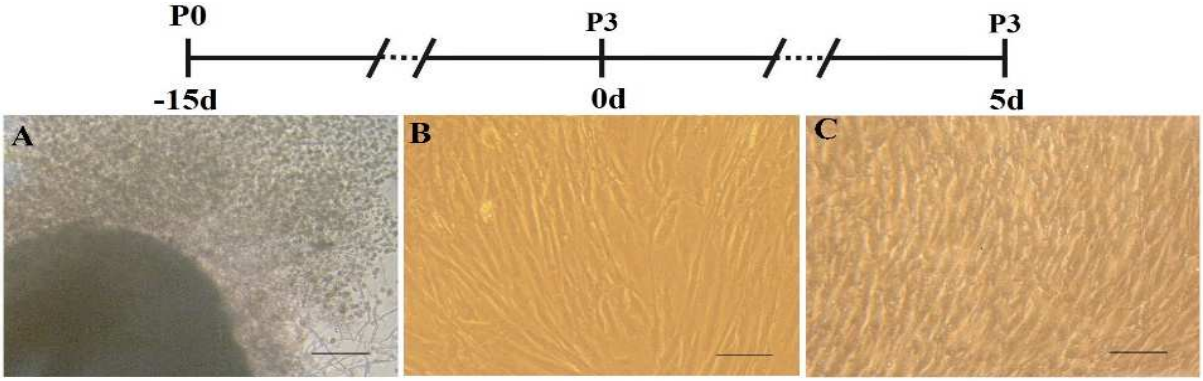

Figure1. (A) the explant culture of mouse embryonic fibroblast cells 3 days after placing the mouse embryonic pieces into T75 flasks at passage 0 (P0). (B) The MEF cells at passage 3 (P3). (C) The MEF cells 5 days after exposing the cells with L-AA. Scale bars A= 250µm, B and C=100µm

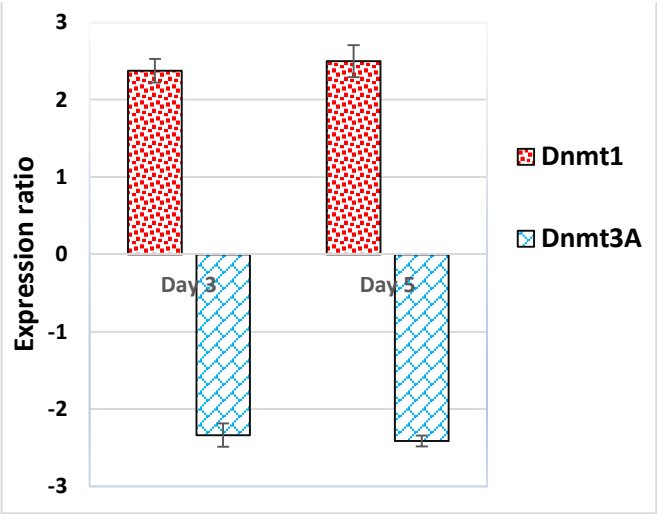

Figure 2. The Gene expression levels of Dnmt1 and Dnmt3A in MEF cells cultured in MEF media in the presence of L-AA at days 1, 3

Materials and Methods

Cell Culture

Mouse embryonic fibroblasts (MEFs) were isolated form13.5-day embryos. After washing the embryos with PBS, placental and all innards were removed. Then, the embryos were minced with a razor blade and incubated in trypsin/EDTA. After 30 minutes, the trypsin was inactivated in MEF media (DMEM 10% FBS) and the cellular suspension was transferred to T75 flask. The cells incubated for 5 days and then the medium was exchanged every two days. The cells at passage 3 (10^4 cells) were cultured in each well of 6-well plates in MEF media +50µg/ml of L-AA (Sigma Aldrich) for 5 days. The cells with similar concentration cultured in MEF media were considered as a control group.

RNA extraction and cDNA synthesis

To extract total RNA, the cells were washed twice with PBS and then were subjected to vigorous pipetting in 1 ml RNX-plus (Cinnagen). Then, the total RNA concentration was measured by means of NanoDrop 2000 instrument (Wilmington, USA) at 260/280nm. The RNA quality was assessed by loading them on agarose gel 2% and staining them with ethidium bromide. The sharp bands of 18S and 28S without any smear showed the high quality of RNA. To synthesize cDNA, by using of random primer, 11 µl of total mRNA (200ng/µl) was subjected to reverse transcription into first-strand cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's protocol described previously.

mRNA quantitation by real-time PCR

Real-time PCR for Dnmt1 and Dnmt3A genes was set up on Termocicler Rotor-Gene™6000 Corbett Research (Australia) by using of the SYBR Premix Ex Taq II kit (Takara). For synchronization the differences in the total RNA volume of each reaction, we used β-actin as an endogenous control.

Primer pairs for Dnmt1, Dnmt3A and β-actin were designed by using of primer express software. The primer sequences were listed in table 1.

Real-time PCR was performed on 1 µL cDNA templates using 0.4 µL pairs of primer (200 nM) and 2 µL of the SYBR® Green II Master Mix in 10 µL of final reaction volume. The condition of real-time PCR for each cycle for β-actin, Dnmt1, Dnmt3A genes was 5 seconds at 95°C, 15 seconds at 60°C, and 20 seconds at 72°C. The amplification cycle number was adjusted at 45 cycles. The emission of fluorescent from each sample tube in threshold line was considered as the threshold cycle (C_t).

Table S1. List of used primers used in real-time PCR. T_m values for all primers were about 60°C

Genes	Forward sequence 5'--> 3'	Reverse sequence 5'--> 3'	Product size (bp)
Dnmt1	ACA CCG TTC CCG TTC AG	TCA TCC ACA GCA TCC TCA G	154
Dnmt3A	GAG CAC GGC AGA ATA GC	AAC ACC CTT TCC ATT TCA G	146
B-actin	CTT CTT GGG TAT GGA ATC CTG	GTG TTG GCA TAG AGG TCT TTA C	95

Statistical Analysis

The efficiency and Ct of each reaction were achieved from LinReg software (12) and the results were analyzed using REST 2009 software based on Pfaffl method. The equation used for analysis is as follows:

$$R = \frac{(E_{\ target})^{\Delta Ct_{target}\ (Mean\ normal - Mean\ sample)}}{(E_{ref}\ )^{\Delta Ct_{\ ref}(Mean\ normal - Mean\ sample)}}$$

Where, R shows the relative expression ratio of *Dnmt1* and *Dnmt3A* in MEFs cultured in the presence of L-AA in comparison to control group, E implies to real-time PCR efficiencies, and *ΔCt* refers the Ct difference between MEFs cultured in the presence of L-AA against MEFs cultured in MEF media. The statistical significance of expression ratios was determined using randomization tests and Taylor algorithm was used to calculate the standard errors. (13-15).

Results

Cell culture

The cells were isolated using explant culture (Figure 1A, B) and then exposed to L-AA for 5 days (Figure 1C). After 5 days, the cells were seemed to become shorter and less elongated. This morphology change is maybe because of the initiation of dedifferentiation in MEF cells.

Real-time PCR for Dnmt1 and Dnmt3A

In this study, we performed real-time PCR on cDNA synthesized from mRNA extraction of the MEF cells cultured in MEF media in the present and absent of L-AA at days 3 and 5. Figure 2 shows the average gene expression of Dnmt1 and Dnmt3A in test samples compared with the control group.

We found both Dnmt1 and Dnmt3A were expressed in MEFs. At day 3, the expression of Dnmt1 was upregulated in the presence of L-AA by mean a factor of 2.37, while the expression of Dnmt3a downregulated in the presence of L-AA by a mean factor of 2.337. The results showed that the expression of Dnmt1 increased by a mean factor of 2.5 and Dnmt3A decreased by a mean factor of 2.414 at day 5.

L-AA is a component showed many anti-aging, anti-cancer effects and abundant in our daily diet. During iPSC Generation the studies showed that L-AA can improve iPSCs generation efficiency by alleviation of the senescence roadblock (3). L-AA anti-aging activity promoted by some unclear ways seems to be irrelevant to its antioxidant function. L-AA promotes gene expression during reprogramming and thus induce iPSC generation. It is possible that L-AA promotes epigenetic modifications such as DNA methylation and demethylation. For example a study showed that, L-AA is a cofactor in reaction with histone demethylases and HIF (hypoxia-inducible factor) prolyl hydroxylases (16), and can influence reprogramming by modulating the expression and activity of these proteins. Expression of Nanog, an important pluripotent transcription factor, is modulated by histone demethylases (17). Hence, L-AA might accelerate the reprogramming by facilitating histone demethylation or even DNA methylation in the site of some gene promoter. During nuclear reprogramming, DNA methylation patterns should be altered. DNA methylation de novo in mammals is mediated by Dnmt1 and Dnmt3A and their action is necessary for development (18). The studies have shown that DNMT3 induced differentiation of human embryonic stem cells and knockdown of this DNA methyltransferase promotes reprogramming of the cells into induced pluripotent stem (iPS) cells by introducing the OSKM factors. In a study, nuclear reprogramming of MEF was accelerated with deletion of Dnmt3A gene (11). While, in contrast knockout of DNMT1 resulted in rapid cell death in human embryonic stem cells [6]. This finding showed the DNMT1 can play an anti-aging role while DNMT3A induces differentiation of stem cells has a crucial role for imprinting in development (19). These findings are consistent with the results of enhanced expression of Dnmt1 and reduced expression of Dnmt3A in the presence of L-AA.

Conclusion

The findings confirm that the L-AA could modify the expression of DNA methyltransferses which might accelerate the cell reprogramming. These results are important for clarification of the pathways through which L-AA can enhance reprogramming the cells into iPSCs. Collectively, these results indicate that DNMT3A and DNMT1 might act as an inhibitor and accelerator of iPS reprogramming, but verifying this conclusion need further research.

Acknowledgement

The authors gratefully acknowledge the support of the Stem Cell Technology Research Center, and all of the participants who took part in this study.

Conflict of Interest: 'None declared'.

References

1. Mohammadi Amirabad L, Massumi M, Shamsara M, Shabani I, Amari A, Mossahebi Mohammadi M, et al. Enhanced Cardiac Differentiation of Human Cardiovascular Disease Patient-Specific Induced Pluripotent Stem Cells by Applying Unidirectional Electrical Pulses Using Aligned Electroactive Nanofibrous Scaffolds. ACS applied materials & interfaces. 2017;9(8):6849-64.
2. Silva J, Barrandon O, Nichols J, Kawaguchi J, Theunissen TW, Smith A. Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS biology. 2008;6(10):e253.
3. Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, et al. Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell stem cell. 2010;6(1):71-9.
4. van der Reest J, Gottlieb E. Anti-cancer effects of vitamin C revisited. Cell research. 2016;26(3):269.
5. Wagner JR, Busche S, Ge B, Kwan T, Pastinen T, Blanchette M. The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. Genome biology. 2014;15(2):R37.
6. Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, et al. Dissecting direct reprogramming through integrative genomic analysis. Nature. 2008;454(7200):49.
7. Liao J, Karnik R, Gu H, Ziller MJ, Clement K, Tsankov AM, et al. Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. Nature genetics. 2015;47(5):469-78.
8. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99(3):247-57.
9. Jackson M, Krassowska A, Gilbert N, Chevassut T, Forrester L, Ansell J, et al. Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. Molecular and cellular biology. 2004;24(20):8862-71.
10. Wongtrakoongate P, Li J, Andrews PW. DNMT3B inhibits the re-expression of genes associated with induced pluripotency. Experimental cell research. 2014;321(2):231-9.
11. Pawlak M, Jaenisch R. De novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells to a pluripotent state. Genes & development. 2011;25(10):1035-40.
12. Ruijter J, Ramakers C, Hoogaars W, Karlen Y, Bakker O, Van Den Hoff M, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009;37(6):e45-e.
13. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 2001;29(9):e45-e.
14. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;30(9):e36-e.
15. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology. 2002;3(7):research0034.
16. Shi Y. Histone lysine demethylases: emerging roles in development, physiology and disease. Nature reviews Genetics. 2007;8(11):829.
17. Cloos PA, Christensen J, Agger K, Helin K. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes & development. 2008;22(9):1115-40.
18. Fatemi M, Hermann A, Gowher H, Jeltsch A. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. The FEBS Journal. 2002;269(20):4981-4.
19. Kaneda M, Okano M, Hata K, Sado T. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature. 2004;429(6994):900.
20. Personna YR, Slater L, Ntarlagiannis D, Werkema D, Szabo Z. Electrical signatures of ethanol–liquid mixtures: Implications for monitoring biofuels migration in the subsurface. Journal of contaminant hydrology. 2013;144(1):99-107.
21. Yang F, Murugan R, Ramakrishna S, Wang X, Ma Y-X, Wang S. Fabrication of nano-structured porous PLLA scaffold intended for nerve tissue engineering. Biomaterials. 2004;25(10):1891-900.
22. Wan Y, Cao X, Zhang S, Wang S, Wu Q. Fibrous poly (chitosan-g-DL-lactic acid) scaffolds prepared via electro-wet-spinning. Acta biomaterialia. 2008;4(4):876-86.

Please cite this paper as: Mohammadi Amirabad L, Massumi M, Alivand MR. L-Ascorbic acid affect the DNA methytransferase expression in Mouse Embryonic Fibroblasts. Regen Reconstr Restor. 2017;2(1):30-33.