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RESEARCH ARTICLE

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Investigation of the Effectiveness of Cl-Amidine on Wound Healing: an in Vitro Study

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Abstract

Objective: Peptidylarginine deiminases (PADs) are enzymes converting the arginine to citrulline. They play a role in embryogenesis and cell signaling activities. But excessive or dysregulated PAD levels were determined to be associated with disorders and to increase in many diseases. It has been shown that Chloramidine (Cl-amidine) used as a PAD inhibitor suppresses increased PAD activity and shows anti-cancer, anti-inflammatory and antioxidant activities. Anti-inflammatory and antioxidant properties play an important role in wound healing. In this study, the possible efficacy of Cl-amidine on wound healing in the keratinocyte cell line was investigated by considering these parameters.

Methods: Cell proliferation evaluations of Cl-amidine concentrations (500, 125, 31.25 and 7.81 µM) determined according to the results of MTT method on HaCaT keratinocyte cells were performed using Real-Time Cell Analysis System (RTCA DP). COL1A1 mRNA expression levels were analyzed by RT (Real Time)-PCR (Polymerase Chain Reaction) method at the concentrations where proliferation was achieved (125, 31.25 µM). Migration effects of Cl-amidine on cells were evaluated by performing scratch analysis. MTT results were statistically analyzed with one-way ANOVA and Tukey test, and $p<0.05$ was accepted as significant. RTCA DP and RT-PCR results were evaluated using device software programs.

Results: In the study, it was found that certain concentrations of Cl-amidine had a proliferative effect on HaCaT keratinocyte cells. It was determined that Cl-amidine increased the amount of type 1 collagen, which is an important parameter for wound healing, by RT-PCR method. In addition, according to scratch analysis, it was detected that it positively affected cell migration in relation to wound closure.

Conclusion: This research shows that Cl-amidine may have a significant potential for wound healing.

Key words: Peptidyl arginine deiminase (PAD), Cl-amidine, wound healing, HaCat keratinocyte cell line

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INTRODUCTION

Peptidyl (protein) arginine deaminases (PADs) provide the conversion of peptidyl arginine to peptidyl citrulline in the presence of calcium with posttranslational modification (1). Increased PAD activity has been observed in many diseases, including rheumatoid arthritis, multiple sclerosis, ulcerative colitis, lupus, Alzheimer's, Parkinson's, and many cancers (2-5). There are five identified types of PAD in other mammals, including humans: PAD1, PAD2, PAD3, PAD4, and PAD6 (6-8). In animal experimental models of diseases with high PAD values, it has been shown that PAD inhibitor agents reduce the severity of disease symptoms. Cl-amidine (Chlor-amidine), which is a PAD inhibitory substance, suppresses all PAD activities (9).

It has been shown that PAD1, PAD 2 and PAD3 are expressed in epidermis cells. Although PAD1 is found in all keratinocyte layers of the epidermis, it increases from the basal to the granular layer. PAD2 is expressed in all keratinocytes, least in basal cells and most in granular layer cells. PAD3 is specifically expressed by granular keratinocytes. Cl-amidine treatment in human primary keratinocyte cell culture causes a dose-dependent reduction in the total amount of deiminated proteins (10).

Wound is a term that refers to the deterioration of skin epithelial integrity due to surgical procedure or trauma (11). Wound healing requires the formation of four sequential stages to restore the histological and functional properties of the skin: hemostasis, inflammation, proliferation and remodeling. The healthy functioning of these stages is essential for optimal wound healing. Among these, re-epithelialization and fibroblast activity play an important role in the proliferation stage, where cellular activity is dominant (12,13).

In our study, it was aimed to investigate the wound healing activity of Cl-amidine, a PAD inhibitor, in human HaCaT keratinocyte cells.

METHODS

Cell Culture and Treatment

HaCaT cells, human skin keratinocytes, were supplied by Professor Dr. Arzu Onay Besikci, Ankara University. HaCaT cells were grown in DMEM medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37ºC in a humidified incubator with a 5 % CO2 atmosphere. Cl-amidine was dissolved in dimethyl sulfoxide (DMSO) as a stock solution.

Determination of Cytotoxicity by MTT Method

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that specifically binds to the succinate-dehydrogenase enzyme in the mitochondria of living cells and converts to water-insoluble formazan salts. The amount of formazan formed directly indicates metabolically active (live) cells in culture (14).

HaCat cells were seeded in 96-well plates at 10,000 cells per well. Different concentrations of Cl-amidine (3.90, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µM) were applied to HaCat cells. At the end of 48-hour incubation period, it was incubated with 100 µl MTT for 3 hours. At the end of the incubation, absorbances were read at a wavelength of 540 nm in an ELISA reader device, with 8 wells in each group. Experiments were performed as 3 independent repetitions. The results were calculated according to the viability formula and determined as % viability.

Determination of HaCaT Cell Proliferation in Real-Time Cell Analysis System (RTCA DP)

RTCA DP detects cell viability by measuring electrical impedance and creates real-time data by continuing this measurement at desired intervals. The values received by the system from the E-plate are calculated as the unitless 'cell index (CI)' value accepted in the literature. This value increases in parallel with the electrical response as the cells cover the bottom of the E-plate and multiply (15). Cl-amidine concentrations (7.81, 31.25, 125 and 500 µM) applied to HaCat cells in Real-Time Cell Analysis System (RTCA DP) were determined according to MTT method. HaCat cells were seeded into each well of E-plate as 10,000 cells in 100 µl of medium. After 24 hours, concentrations of Cl-amidine were applied to E-plate wells in 100 µl of medium. The results were analyzed using RTCA DP Software 1.2.1 program and IC_{50} values were determined in the same program.

Wound healing with scratch assay

In order to determine the effects of Cl-amidine on wound healing, HaCaT keratinocyte cells were seeded in 6-well plates as $1x10^6$ cells and waited for 24 hours for adhesion. Then, for creating an *in vitro* wound model with HaCaT keratinocyte cells reaching approximately 90% density, a linear opening of approximately 1 mm was created using a 200 ml sterile pipette tip and the wells were washed with PBS. This opening was accepted at 0 hour and photographed. Cl-amidine concentrations (31.25 and 125 µM), which provided proliferation more than the control group according to RTCA DP proliferation results, were applied to determine cell migration by scratch wound healing method. After 48 hours of incubation, the effects of Cl-amidine concentrations on the amount of closure of the wounds were visualized under an inverted light microscope (Leica DM 300 invert microscope) (16).

Determination of COL1A1 mRNA Expression Levels by Real-time Polymerase Chain Reaction Method

RNA was isolated from HaCaT cells treated with Cl-amidine concentrations (31.25 ve 125 µM). Total RNA isolation was performed on MagNA Pure LC 2.0 system (Roche, Germany). From each RNA population, 500 ng total RNA was used for cDNA synthesis (Transcriptor High Fidelity cDNA Synthesis Kit).

Quantitative real time polymerase chain reaction (qRT-PCR) was used to assess mRNA levels of collagen type I alpha 1 chain (*COL1A1*, Assay ID;100861, Roche) gene in relation to wound healing. As an internal positive control, glucose-6-phosphate dehydrogenase (G6PD) mRNA levels were used. Results were analysed by advanced relative quantification with LightCycler® 480 System's software (version 1.5.0.39).

Statistical Analysis

Using GraphPad Prism 6.0 analysis program, % cell viability graphs of the groups compared to the control were drawn and statistical analyzes were realized. The obtained data were analyzed by applying one-way ANOVA and post-hoc Tukey tests. Statistical significance values were evaluated as; $p>0.05$ no difference, $p<0.5$*, $p<0.01$**, $p<0.001$*** and $p<0.0001$****.

RESULTS

Evaluation of Cytotoxic Effects of Cl-amidine in HaCaT Keratinocyte Cells by MTT Method

The cytotoxic effects of Cl-amidine in HaCaT cells determined by MTT method using 3.90, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µM concentrations are shown in **Figure 1**. When MTT results were evaluated, it was determined that Cl-amidine decreased cell viability in 250 and 500 µM concentrations in HaCaT cells compared to the control group.

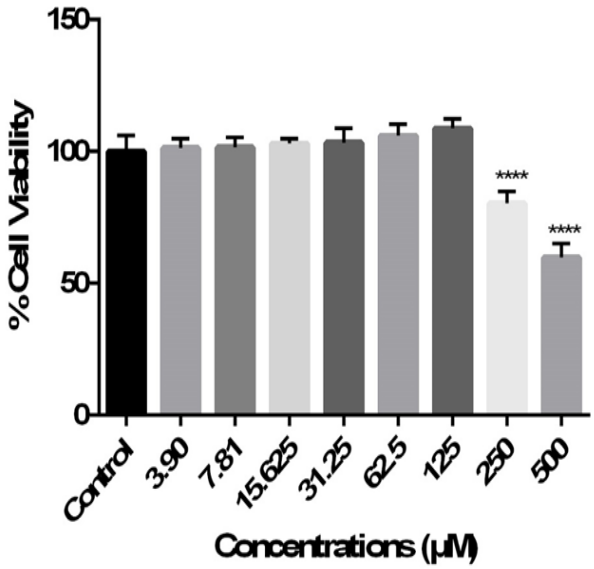

Figure 1. Viability (%) values of Cl-amidine concentrations calculated according to MTT Method in HaCat cells at 48 hours and statistical evaluation (Mean±SD, solvent control: % 0.1 DMSO, n=8)

Evaluation of Cell Proliferation Using Real Time Cell Analysis System (RTCA DP)

Proliferation and IC50 concentration determination studies in HaCaT cells at 7.81, 31.25, 125 and 500 µM concentrations of Cl-amidine (determined according to MTT method) were performed in Real-Time Cell Analysis System (RTCA-DP). According to the results, it was determined that 31.25 and 125 µM concentrations of Cl-amidine had an increasing effect on HaCat cell proliferation compared to

the control group (Figure 2). In addition, IC50 concentration of Cl-amidine in HaCaT cells at 48 hours was determined and calculated as 432 µM using the RTCA DP system (Figure 3).

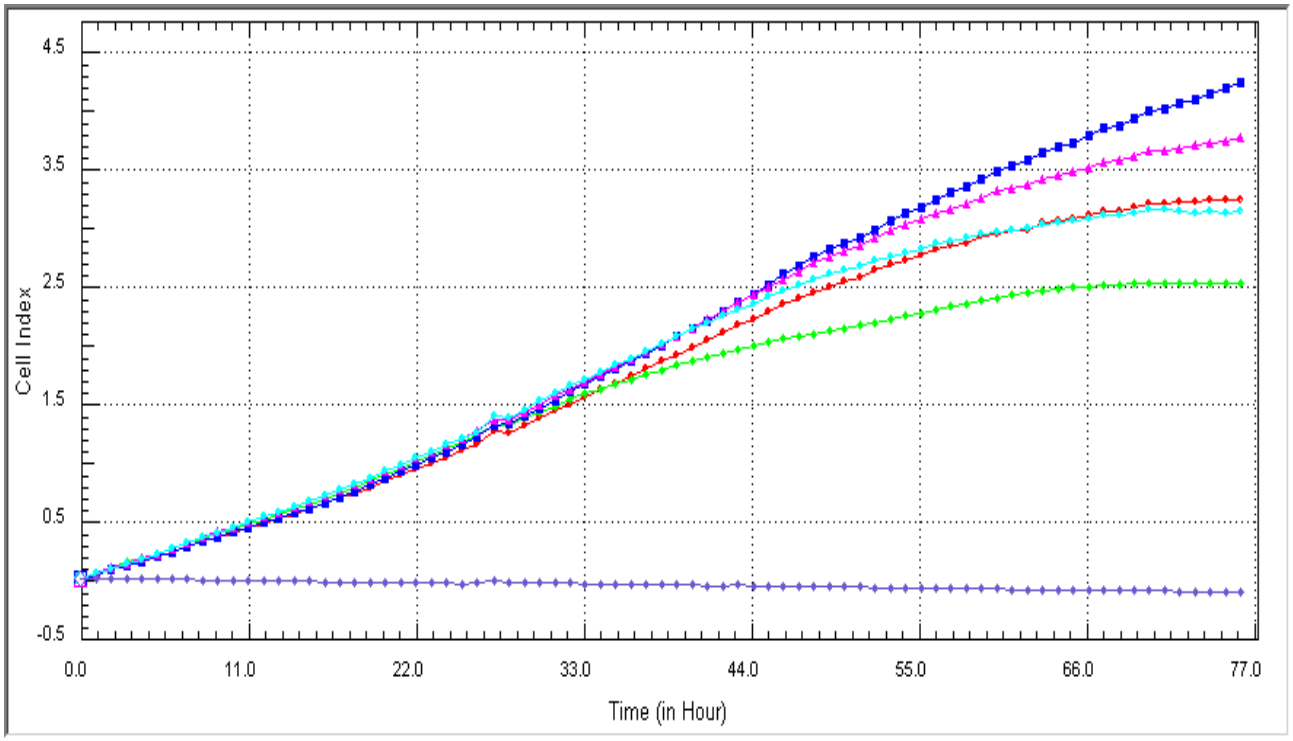

Figure 2. 48-hour proliferative effects of Cl-amidine in HaCaT cells and slope graph in RTCA-DP system (n=6, mean± standard deviation).

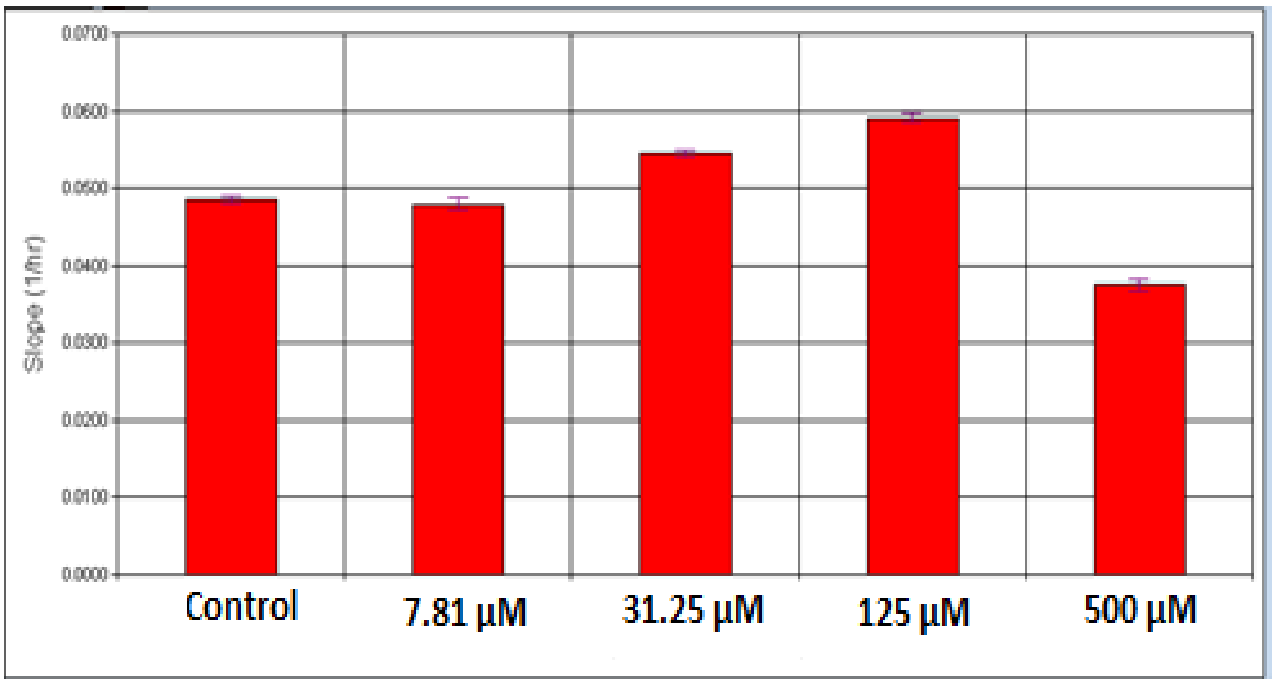

Figure 3. IC50 value of Cl-amidine on HaCaT keratinocyte cell line at 48 hours in Real-Time Cell Analysis System (IC50: 432 µM).

Assessment of Wound Healing

The growth and migration effects of Cl-amidine on HaCaT cells were investigated using the strach wound healing method.

The ability of cells to migrate in the wound area is another important parameter for wound healing. According to the results of the scratch wound healing method performed in vitro for this purpose, it was determined that Cl-amidine increased HaCaT cell migration at both 31.25 and 125 µM concentrations. It was determined that this effect was higher especially at the concentration of 31.25 µM (Figure 4).

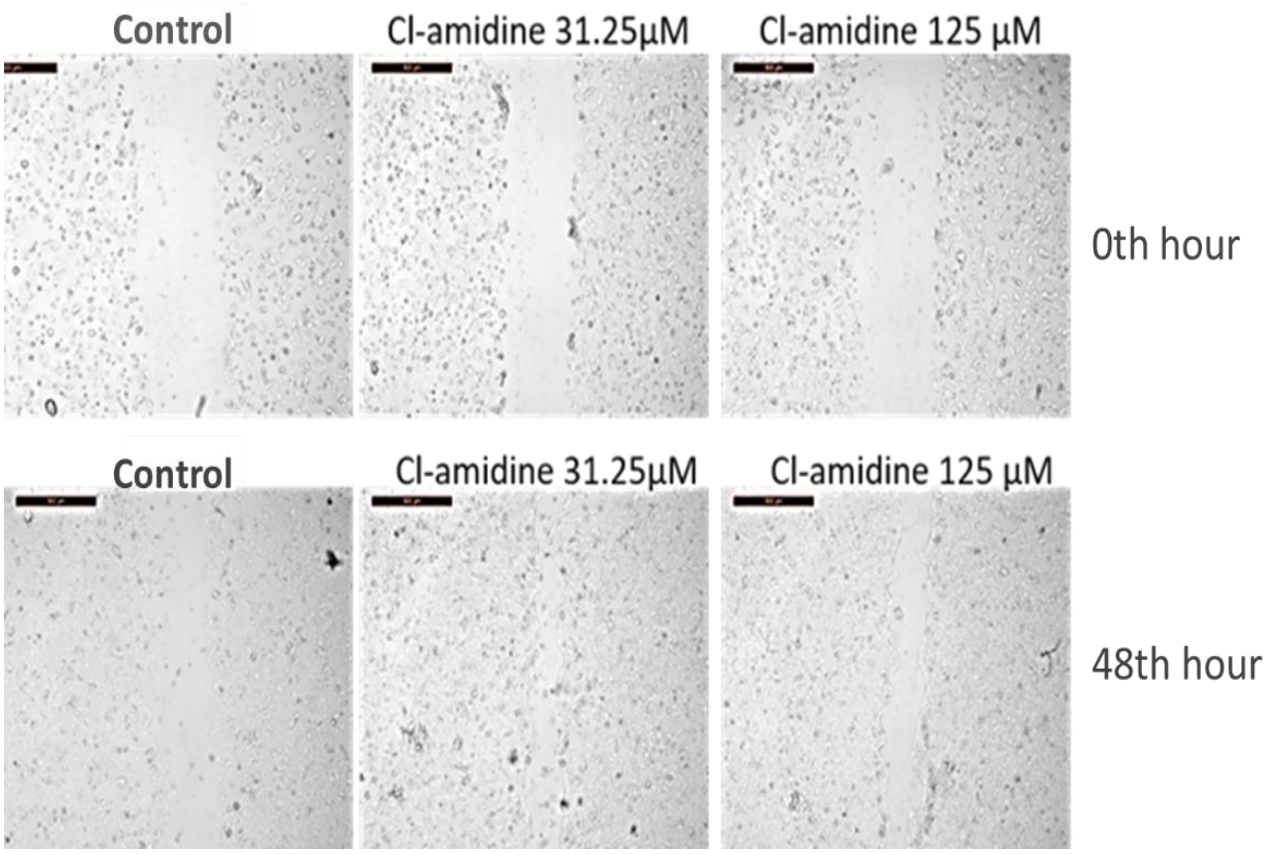

Figure 4. Light microscope images of HaCAT cell migration determined by scratch wound healing method (10X)

Evaluation of *COL1A1* mRNA Expression Levels by RT-PCR Analysis

The effects of different concentrations of Cl-amidine (31.25 and 125 µM) on *COL1A1* mRNA expression levels in HaCaT cells were determined by RT-PCR method. According to these results, it was determined that *COL1A1* mRNA level, which is an important marker in wound healing, was approximately twice (1.74) higher than the control, especially at 31.25 µM concentration (**Table 1** and **Figure 5**).

Table 1. Target/reference ratios and normalized values of *COL1A1* gene in HaCaT cells incubated for 48 hours with Cl-amidine concentrations

Sample Name	Target Name		Tgt Cp	Ref Cp	Ratios	
	Target	Reference	Mean	Mean	Tgt/Ref	Norm
Control	*COL1A1*	G6PD	32.05	30.61	0.3688	**1.000**
Cl-amidine 125 µM	*COL1A1*	G6PD	31.74	29.71	0.2449	0.6642
Cl-amidine 31 µM	*COL1A1*	G6PD	31.26	30.62	0.6432	**1.744**

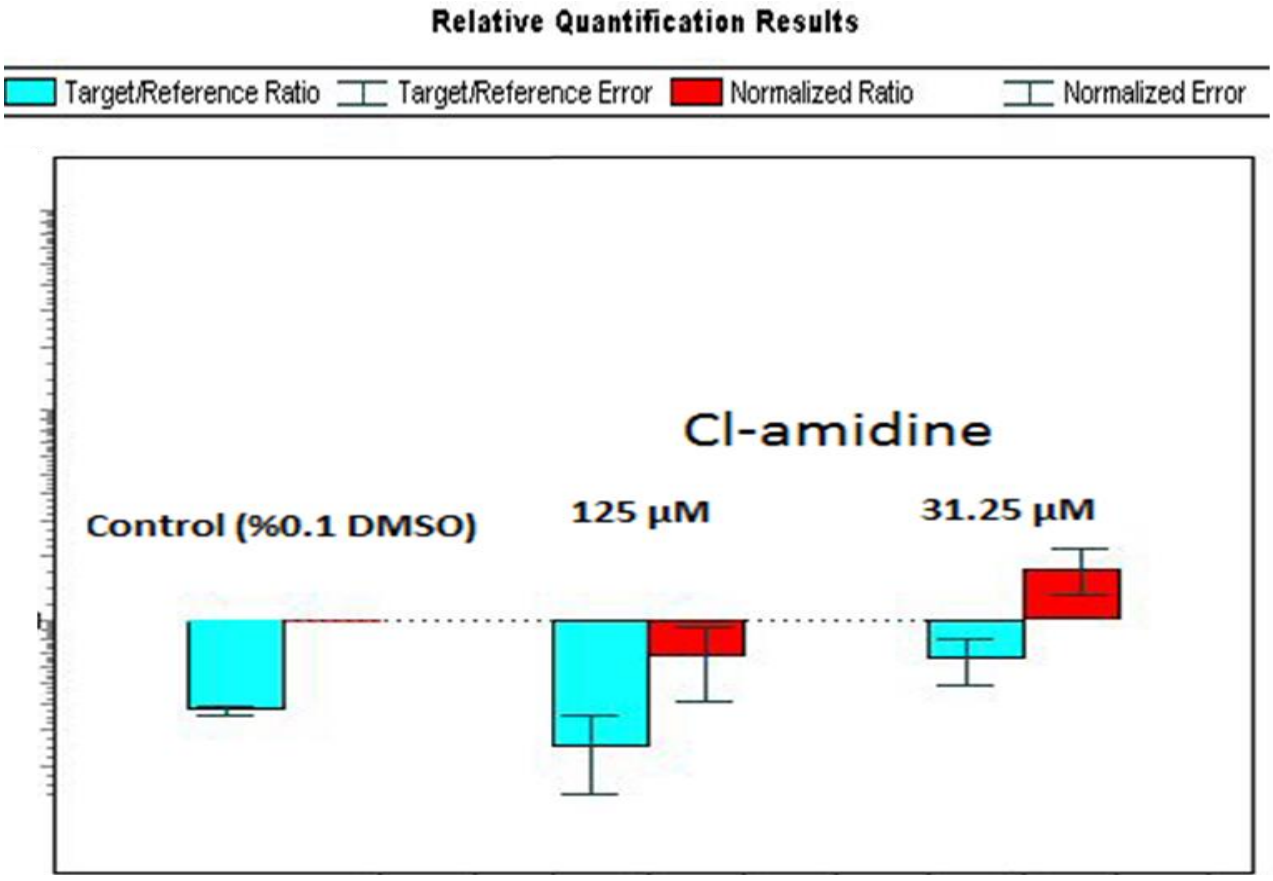

Figure 5. Normalized value graph of *COL1A1* gene mRNA levels in HaCaT Cells.

DISCUSSION

Peptidyl arginine deiminases (PADs) are enzymes that convert arginine to citrulline (1). They play a role in embryogenesis and cell signal transduction activities. However, it has been determined that excessive or dysregulated PAD levels increase in many diseases and may be associated with diseases (2-5). It has been reported that Cl-amidine, which is used as a PAD inhibitor substance, suppresses increased PAD activity and exhibits anti-cancer, anti-inflammatory and antioxidant activities. Anti-inflammatory and antioxidant properties play an important role in wound healing (2, 4-8).

Re-epithelialization is an important step in the wound healing process in order to restore the barrier function of the skin (17). Following

injury, keratinocytes migrate from each other and break their connections in the basal lamina. Then, the basement membrane is reconstructed by the proliferation of keratinocytes that come to the environment to carry out the formation of the epidermis. Basal layer keratinocytes at the wound margin show excessive proliferation in the days following wound formation (18). Some phenotypic changes occur in proliferating keratinocytes to migrate; cell shapes change, intracellular tonofilaments shorten, intercellular desmosomes dissolve, and the connection between epidermis and dermis is broken. Keratinocytes migrate to the wound area by ameboid movements. In addition to the division activity, the cells synthesize the basement membrane components, type 4 collagen and heparin sulfate, gradually repair the basement membrane, return to their normal shapes and connect to each other and to the basement membrane. Keratinocytes divide and differentiate to form layers of the epidermis and connect the newly formed epidermis with the basement membrane and dermis (12, 19).

HaCaT cell line has a similar migration index to primary human keratinocytes and mimics many features of normal keratinocytes (20, 21). For this reason, HaCaT cell line was used as an in vitro wound healing experiment model in our study.

In our study, the concentrations of Cl-amidine increasing HaCaT cell proliferation at 48 hours were determined as 31.25 and 125 µM by Real-Time Cell Analysis System. Then, these determined concentrations were evaluated with the strach wound healing model, which is a model used to investigate cell migration in wound healing, and it was determined that Cl-amidine was effective at both 31.25 and 125 µM concentrations. Especially, the most significant increase in HaCaT cell migration was observed at 31.25 µM concentration at 48th hour. When we look at the results of COL1A1 mRNA expression levels, which support these results, it was determined that the highest gene expression increase was at 31.25 µM Cl-amidine concentration (approximately 2 times) compared to the control, and our experimental results show parallelism.

CONCLUSION

In the study, it was found that Cl-amidine, which is a PAD inhibitor, has a proliferative effect on HaCat keratinocyte cells. The effects of Cl-amidine on type 1 collagen, which is an important marker for wound healing, were determined by the increase in COL1A1 mRNA gene expression levels by RT-PCR method. In addition, according to scratch analysis, it was determined that it positively affected cell migration in relation to wound closure. This study shows that Cl-amidine may have significant potential in wound healing.

Ethical Approval: Ethics committee approval is not required in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept: PNO Design: PNO Literature search: PNO Data Collection and Processing: SEO, MD Analysis or Interpretation: SEO, MD, PNO Writing: PNO

Conflict of Interest: No conflict of interest was declared by the authors.

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REFERENCES

1. Jones JE, Causey CP, Knuckley B, Slack-Noyes JL, Thompson PR. Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. Curr Opin Drug Discov Devel. 2009; 12(5): 616-27.
2. Chen CC, Isomoto H, Narumi Y, Sato K, Oishi Y, Kobayashi T, Yanagihara K, Mizuta Y, Kohno S, Tsukamoto K. Haplotypes of PADI4 susceptible to rheumatoid arthritis are also associated with ulcerative colitis in the Japanese population. Clin Immunol. 2008; 126: 165–171.
3. Moscarello MA, Mastronardi FG, Wood DD. The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis. Neurochem Res. 2007; 32: 251–256.
4. Ishigami A, Ohsawa T, Hiratsuka M, Taguchi H, Kobayashi S, Saito Y, Murayama S, Asaga H, Toda T, Kimura N, Maruyama N. Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. J Neurosci Res. 2005; 80: 120–128.
5. Chang X, Han J, Pang L, Zhao Y, Yang Y, Shen Z. Increased PADI4 expression in blood and tissues of patients with malignant tumors. BMC Cancer. 2009; 9(40): 1-11.
6. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays. 2003; 25(11): 1106-1118.
7. Chang X, Han J. Expression of peptidylarginine deiminase type 4 (PAD4) in various tumors. Mol Carcinog. 2006; 45(3):183-96.
8. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest. 1998; 101: 273–281.
9. Luo Y, Arita K, Bhatia M, Knuckley B, Lee YH, Stallcup MR, Sato M, Thompson PR. Inhibitors and Inactivators of Protein Arginine Deiminase 4: Functional and Structural Characterization. Biochemistry, 2006; 45 (39): 11727–11736.

10. Cau L, Takahara H, Thompson PR, Serre G, Méchin M, Simon M. Peptidylarginine Deiminase Inhibitor Cl-Amidine Attenuates Cornification and Interferes with the Regulation of Autophagy in Reconstructed Human Epidermis. J Invest Dermatol. 2019; 139(9): 1889-1897.
11. Fredrikkson C. Keratinocytes in tissue engineering of human skin: in vitro and in vivo studies. Ġsveç: Linköping University. 2008.
12. Shaw TJ, Martin P: Wound repair at a glance. J Cell Sci. 2009; 122: 3209-3213.
13. Sgonc R, Gruber J. Age-related aspects of cutaneous wound healing: A mini-review. Gerontology. 2013; 59: 159-164.
14. Carmicheal J, DeGrafit WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of tetrazolium-based semiautomated colorimetric assay: Assasment of chemosensivity testing. Cancer Research. 1987; 47: 936-942.
15. Engür-Öztürk S, Dikmen M. Proteasome inhibitor immunotherapy for the epithelial to mesenchymal transition: assessing the A549 lung cancer cell microenvironment and the role of M1, M2a and M2c ‘hydrocortisone-polarised’macrophages. Molecular Biology Reports. 2022; 49(6): 4777-4793.
16. Yuksel SN, Dikmen M, Canturk Z. Evaluation of Real Time Cell Proliferation, Anti-Inflammatory and Wound Healing Potential of Helenalin on HaCaT Keratinocytes Treated with Lipopolysaccharide Stimulated Monocytes. Indian Journal of Pharmaceutical Sciences. 2021; 83(2); 219-229.
17. Safferling K, Sütterlin T, Westphal K, Ernst C, Breuhahn K, James M, Jäger D, Halama N, Grabe N. Wound healing revised: A novel reepithelialization mechanism revealed by in vitro and in silico models. Journal of Cell Biology. 2013; 203(4): 691-709.
18. Evans ND, Oreffo ROC, Healy E, Thurner PJ, Man YH. Epithelial mechanobiology, skin wound healing, and the stem cell niche. Journal of the Mechanical Behaviour of Biomedical Materials. 2013; 28: 397-409.
19. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Frontiers in Bioscience. 2004; 9: 283-289.
20. Sandt J, Roguet R, Cohen C, Esdaile D, Ponec M, Corsini E, Barker C, Fusenig N, Liebsch M, Benford D, Fraissinette AB, Fartasch M. The use of human keratinocytes and human skin models for predicting skin irritation. Alternatives to Laboratory Animals. 1999; 27: 723-743.
21. Ranzato E, Patrone M, Burlando B. Platelet lysate stimulates wound repair of HaCaT keratinocytes. British Journal of Dermatology. 2008; 159: 537-545.