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Original Research Article

The Role of β2 Antagonist (Timolol) and β2 Agonist (Salbutamol) on Cell Migration *in vitro.*

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Abstract
Cell migration is a complex and dynamic biological process, it's the movement of the cell from one area to another, generally in response to a chemical signal. It is an essential feature of living cells for functions such as wound repair, tissue regeneration, cell differentiation, and immune response. Keratinocytes have the enzymatic machinery to generate catecholamines, they can synthesize endogenous epinephrine, which could be locally secreted into the wound and function in an autocrine manner. To determine whether beta2-adrenergic receptor (β2AR) antagonist and agonist altered endothelial cells (EC) migration, single-cell migration (SCM) assays were performed with human umbilical vein endothelial cells (HUVEC) in the presence and absence of β2AR agonist and antagonist. Screening the tested drugs revealed that the salbutamol (β2AR agonist) significantly decreases migration rate on (HUVEC) compared with control group, while timolol (β2AR antagonist) significantly increases the migration rate. In conclusion the administration of timolol increase single cell migration resulting and enhancement of re-epitheliazation, while administration of salbutamol decrease single cell migration resulting in inhibition of wound re-epitheliazation.

Key Words: Migration, salbutamol, timolol, HUVEC , *In vitro*

الخلاصة
إن هجرة الخلايا عبارة عن عمليه ديناميكية بايلوجية معقدة، وهي حركة الخلية من منطقة الى أخرى وبصورة عامة استجابة الى المحفزات الكيميائية. وهي ظاهرة مهمة لوظائف الخلايا الحية في شفاء الجروح، توليد الانسجة، تمايز الخلايا، والاستجابة المناعية. تمتلك الخلايا المولدة للكيراتين قابلية لانتاج الامينات الكاتيكولية وكذلك لها القابلية على تكوين الابينفرين وافرازه موضعيا ليعمل ذاتيا في الجروح. ولغرض تحري في ما لو ان لمحفزات وكابحات مستقبلات ب-٢ الأدرينالينية دور في تغيير هجرة الخلايا الطلائية صممت تجربة هجرة الخلية المفردة للخلايا الطلائية للحبل السري البشري بوجود وغياب محفزات وكابحات مستقبلات ب٢ الادرينالية.

اظهر تحري تاثير العقاقير المختبرة الى ان السالبيوتامول قد قلل هجرة الخلايا الطلائية للحبل السري البشري، بينما زاد عقار التيمولول وبصورة معنوية هجرتها.

وإستنتاجا فان أعطاء كابحات مستقبلات ب-٢ الدرينالينية كالتيمولول ، يزيد من هجرة الخلايا المفردة ويحفز عملية اعادة تكوين الطبقة الطلائية بينما يقلل اعطاء محفزات مستقبلات ب-٢ الادرينالينيه كالسالبيوتامول من هجرة الخلايا وينتج في وقف عملية التكون الطلائي للجروح.

الكلمات المفتاحية: الهجرة، سالبيوتامول، تيمولول، هيوفك، في الزجاج.

Introduction
Migration is a complex and dynamic biological process, it's the movement of cell from one area to another generally in response to a chemical signal. It's an essential feature of live cells and for functions such as wound repair, tissue regeneration, cell differentiation, embryonic development, immune response, and disease processes such as cancer metastasis and inflammation [1]. Endothelial cell (EC) migration is a critical process in angiogenesis and re-epithelialization [2].

The cells often move as a tightly or loosely associated cohesive group, in collective cell migration in which cells are influenced by the interaction with their neighbors [3]. Virtually all living tissue is constructed by collective cell migration, which plays an important role in the initial symmetry breaking and leader-

follower organization of cell groups during embryonic development [4].

Collective migration has also been recognized as an important mechanism for wound healing and cancer invasion when cells in a group interact with the same chemotactic and mechanical signals as those of isolated cells, they may respond differently in a collective fashion [5].

In the commonly used Madin-Darbey Canine Kidney cell (MDCK) cells in a monolayer migrated directionally to the anode in an electric field (EF), whereas isolated cells displayed random migration in an EF of the same strength [6].

Traction forces, which play an important role in cell migration, also occurred in different patterns in single cells compared with the cell monolayer. For a single cell, traction forces are generated by the attachment of the actin network to the substrate at the leading edge of the cell, whereas at the trailing edge, the forces are a result of the actin network slipping over the substrate [7, 8]. Collective cell migration is one of the hallmarks of wound healing. Through wound healing, epithelial cells migrate collectively as a coherent sheet to heal wounds. Wounding an epithelial monolayer prompts directional migration of a cell sheet, that keeps tight intercellular adhesion [9, 10]. Cells found in the stratified epithelium similarly migrate in mass following injury [10]. During skin wound healing, epithelial cells proliferate and migrate collectively into the center of the wound [11]. Quantitative analysis demonstrated that over 95% of the cells moved at similar migration speeds and trajectories with very little change in their relative position [12].

These endothelial sprouts are guided by highly polarized tip cells that protrude long actin-rich filo podia and sense the local high concentration of vascular endothelial growth factor-A (VEGF-A) secreted by astrocytes [13, 14]. The extracellular VEGF gradients control the directional collective migration of endothelial cells in angiogenesis [14]. In wound healing, the primary goal for epithelial cells is to restore the epithelial barrier [15]. The application of most growth factors or other migration-stimulating chemicals usually has the potential detrimental effect of "scattering" cells in epithelial sheets, thus compromising epithelial barrier function. Epidermal growth factors (EGFs) reduce barrier function and decrease the dispersal of cells [16, 17]. Drugs that can enhance wound-induced endogenous Electronic Funds Source (EFs) may be incorporated into a bandage that can be applied to skin wounds. Those drugs can then constantly exert effects on epithelial cells of local skin wound edges and may promote the collective migration of epithelial sheets to heal wounds [18, 19].

Materials and Methods

Human umbilical ventricular endothelial cell line (HUVEC) was purchased from National Cell Bank Iran (NCBI). The entire cell lines were maintained in its specific medium; Endothelial cell growth medium (211-500), this cell lines were adapted to Ham's F12 + Roswell Park Memorial Institute medium (RPMI-1640) (Gibco/UK) (I/IV) and fetal bovine serum (FBS 10%) at NCBI and 1% pen/strep (Penicillin/streptomycin) (Sigma-Aldrich, Germany) was added to those media to make complete growth medium as mentioned in the growth medium sheet provided with cell line. Polylysine purchased from Sigma-Aldrich (Germany) was used to coat the flask that used to culture the HUVEC cell for 48hrs in the CO_2 incubator. Salbutamol and timolol were prepared by dissolving the powdered samples in the medium used for culture of the cell line (in concentration of 0.5 mg /ml for each drug). The control group wells received 200µl of medium, while the wells of tested agents received 200µl medium with 0.5mg/ml conc. of each agent, then incubated at 37˚C, with 5% CO_2 for 48hrs [20].

Cell Culture Wound Closure Assay (scratch assay)

Among the numerous ways to study cell migration, the cell wound closure assay is useful to determine the migration ability of whole cells mass. This assay which was used in this study according to Liang *et al* [21] and Zhang methods with slight modification [22].

The method was a wound closure technique applied on cell culture of human umbilical vein endothelial cells (HUVEC). Wound width in a micrometer (um) was measured during (12) hours every three hours after

dividing the samples into three groups; control, salbutamol group , and timolol group. The first group included only the growth media, the second group was treated with 0.5 mg/ml salbutamol and the third group was treated with 0.5 mg/ml timolol

1. Cells were detached from the tissue culture flask using 0.25% trypsin-EDTA solution. Pellet the cells in a 15 ml conical tube by centrifugation, the supernatant decanted out, and the cells are re-suspend in culture medium. Appropriate number of cells was distributed in a 6-well plate and incubated until it reach 100% confluence in about 24 hours. Coating of the plates with collagen I prior to cell seeding offers the possibility to study migration on different substrates.
2. In a sterile environment typically a biosafety cabinet, a scratch was made by using 200 µl pipette tip to press firmly against the cell monolayer of the tissue culture plate and swiftly a vertical wound down through the cell monolayer was done.
3. Carefully aspirated the media and cell debris. Slowly add, enough culture medium alone, 0.5 mg/ml of salbutamol and 0.5mg/ml of timolol, against the well wall of the control and test wells to cover the bottom of the wells and detaching additional cells were avoided. Following the generation and inspection of the wound an initial picture was taken. Then place the tissue culture plate in CO2 incubator at 37 °C.
4. At three hours' time interval, the plate was taken off from the incubator and examined by the inverted microscope, snapshot picture was taken and for checking of wound closure.
5. The approximation rate of the wound edge are snapshotted and the distance of one side of the wound to the other side was measured by using a scale bar. Wound closure rate can be analyzed over a time using a scatter plot or bar graph.

Statistical analysis

Data were collected, summarized, analyzed and presented using three statistical software programs; the statistical package for social science (SPSS version 22), Microsoft Office Excel 2013 and MedCalc 2014. Comparison of mean values between two groups was carried out using Mann Whitney U test whereas comparison of mean values within the same group on different occasions was carried out using Wilcoxon test. P-value was considered significant when it was equal to or less than 0.05 and highly significant when it was equal to or less than 0.01 [23].

Results:

The results are shown in figures (1) and (2). The wound width distance in timolol group shown highly significant wound edge approximation after 12 hours than the control group ($P<0.01$) whereas the wound width distance in salbutamol group was less significantly approximated, after 12 hours than the control group ($P<0.05$). The rate of wound closure was highly significant in timolol group than in control group, 58.33 versus 33.33 µm/hr ($P<0.01$). The rate of wound closure was none-significantly in salbutamol group in comparison to control group, 25.00 versus 33.33 ($P<0.05$), as shown in table (1).

Table (1): Wound closure assay in control and study groups.

Time (hours)	Control	Salbutamol	Timolol
	M±SD Mm	M±SD Mm	M±SD Mm
0	1000	1000	1000
3	900	1000	800
6	800	900	600
9	700	800	500
12	600	700	300
Rate of wound closure (µm/hr)	33.33	25.00*	58.33†

* Significantly (<0.05) lower than control; † highly significant (<0.01) greater than control.

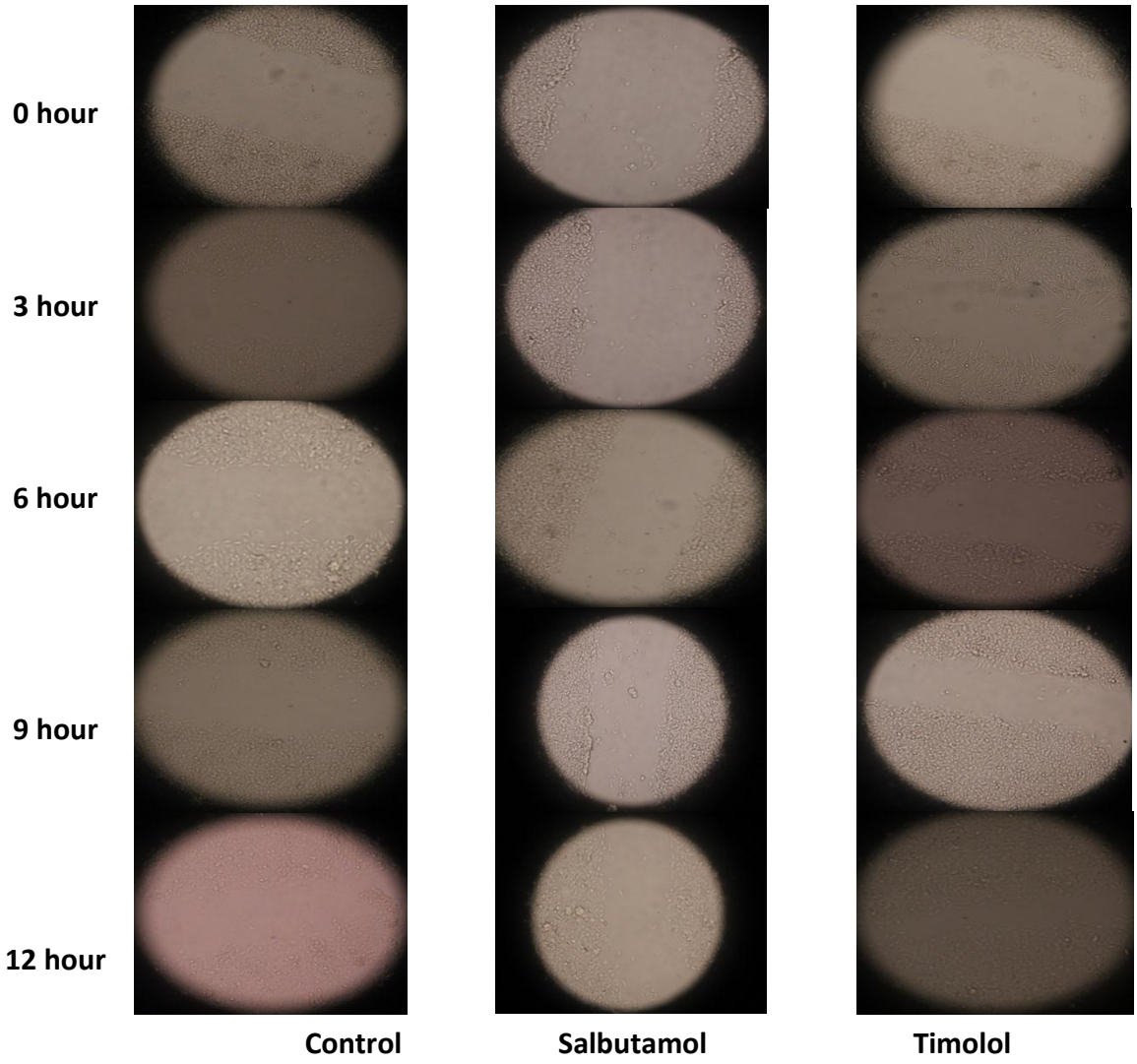

Figure (1): Wound closure assay during 12 hours of control and study groups by inverted microscope (power 20x).

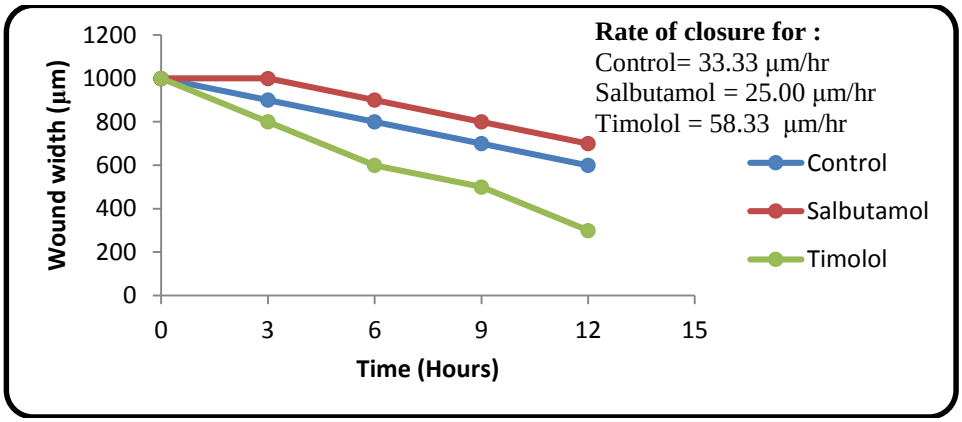

Figure (2): Wound closure assay in control and study groups.

Discussion

In the present study, the rate of wound closure was highly significant in timolol group than in control group. The rate of wound closure was

none significant in salbutamol group than in control group.

In agreement with results of present study, Sivamani *et al*. stated that epinephrine (B2 agonist) levels in the culture medium was elevated to the level that was found in burn stressed animals [24], the rate of migration of both cultured murine and human keratinocytes is impaired (reduced by 76%, in humans, $p < 0.001$, and by 36%, in mice, $p = 0.001$). However the current study the β2 agonist (salbutamol) resulted in 32 % reduction in migration rate and this is less than that observed by Sivamani *et al.* The cause may be attributed to different tissue used in culture, since the cell line used in Sivamani *et al*. was neonatal human foreskin whereas the cell line used in the present study was HUVEC; the second cause may be due to the use of a different β2 agonist since the drug used in the present study was salbutamol while the drug used in the experiment of Sivamani *et al*. was isoproterenol.

Sivamani *et al*. also examined the effect of highly selective β2AR antagonist (ICI-118,551), found that the β2AR-selective antagonist increased keratinocyte migration rate [24], as a similar finding of the present study.

The explanation for the role of β2AR antagonist in increasing the migratory rate of cultured cells may be explained as following: the keratinocytes express (β2AR), Which is activated by the catecholamine, isoproterenol as (β2AR) agonist) decreasing the cell migration speed, interfere with healing of an in vitro scratch wound in keratinocyte monolayers and delays healing of acute surgical wounds *in vivo* [25,26]. These effects are inverted by β-adrenergic receptor (βAR) antagonists (timolol) involving a βAR-mediated mechanism, as shown in the present study and in the study of Sivamani *et al*. Activation of the β2AR in keratinocytes enhancing the Protein phosphatase 2 A (PP2A) and extracellular-signal-regulated kinase (ERK) to the receptor and results in minimizing the ERK phosphorylation that is required for migration [27]. On the other hand, several studies proved that the phosphatidyl inositol 3 kinase (PI3K)/ Protein Kinase B (AKT) pathway is a downstream mediator of β2AR activation; this pathway regulates migration through actin mediated pathway [27].

Ghoghawala *et al*., conducted a study on the effect of *β*-AR agonist and antagonist on the rate of migration of cultured of murine cornea epithelial (AMCE) and concluded that: The *β*-AR agonist isoproterenol decreased (AMCE) cell migration velocity to about 70% than in untreated control groups [28], the result was is similar to that of the present study; however the reduction rate in the current study was 32% which is less than that of Ghoghawala et al. study. On the other hand, Ghoghawala *et al* found that treatment with the *β*-AR antagonist (timolol) enhance the healing speed by about 35% which is also similar to the finding of the current study, although the increment in the rate was 75%. Probably the difference in reduction of the rate by *β*-AR agonist and increment in rate by the β-AR antagonist is due to the use of a different cell line, in the present study it was HUVEC while in Ghoghawala *et al*. Study it was AMCE. Ghoghawala *et al*. also found that *β*-AR agonist resulted in a 0.60-fold lowering in concentration of activated phospho-ERK (P-ERK) [28] and that the *β*-AR antagonist (timolol) resulted in a P-ERK 2.4-fold increment ($P < 0.05$).

References

1. van Roosmalen W, Le Devedec SE, Golani O, Smid M, Pulyakhina I, Timmermans AM, Look MP, Zi D, Pont C, de Graauw M, Naffar-Abu-Amara S, Kirsanova C, Rustici G, Hoen PA, Martens JW, Foekens JA, Geiger B, van de Water. Tumor cell migration screen identifies SRPK1 as breast cancer metastasis determinant. *J Clin Invest*. 2015;125(4): 1648-1664.
2. Eming SA, Brachvogel B, Odorisio T. Regulation of angiogenesis: wound healing as a model. Progr Histochem Cytochem. 2007;42:115–70.
3. Friedl P, Weigelin B. Interstitial leukocyte migration, and immune function, Nat. Immunol. 2008; 9:960–969.

4. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration, and cancer. Nat Rev Mol Cell Biol. 2009;10:445-57.
5. Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. Dev Cell. 2008; 14:570-81.
6. Poujade M, Grasland-Mongrain E, Hertzog A, Jouanneau J, Chavrier P, Ladoux B, Buguin A, Silberzan P. Collective migration of an epithelial monolayer in response to a model wound, Proc. Natl. Acad. Sci. U.S.A. 2007; 104: 15988–15993.
7. Renkawitz J, Sixt M. Mechanisms of force generation and force transmission during interstitial leukocyte migration. EMBO Rep 2010; 11:744-50.
8. Fournier MF, Sauser R, Ambrosi D, Meister JJ, Verkhovsky AB. Force Transmission in migrating cells. J Cell Biol. 2010;188:287-97.
9. Fong E, Tzlil S, Tirrell DA. Boundary crossing in epithelial wound healing. Proc Natl Acad Sci US A. 2010;107:19302-7.
10. Vitorino P, Hammer M, Kim J, Meyer T. A steering model of endothelial sheet migration recapitulates monolayer integrity and directed collective migration. Mol Cell Biol. 2011;31:342-50.
11. Gipson IK, Danjo Y. Actin 'purse string' filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound, providing coordinated cell movement. J Cell Sci. 1998;111:3323-32.
12. Zhao M, Song B, Pu J, Forrester JV, McCaig CD. Direct visualization of a stratified epithelium reveals that wounds heal by unified sliding of cell sheets. Faseb J. 2003; 17:397-406.
13. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 2003;161:1163-77.
14. Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P. Dll4 signaling through Notch1 regulates the formation of tip cells during angiogenesis. Nature, 2007; 445: 776-80.
15. Rorth P. Collective guidance of collective cell migration. Trends Cell Biol. 2007;17:575-9.
16. Unoki N, Murakami T, Nishijima K, Ogino K, van Rooijen N, Yoshimura N. SDF-1/CXCR4 contributes to the activation of tip cells and microglia in retinal angiogenesis. Invest Ophthalmol Vis Sci. 2010;51:3362-71.
17. Li L, Hartley R, Reiss B, Sun Y, Pu J, Wu D. E-cadherin plays an essential role in the collective directional migration of large epithelial sheets. Cell Mol Life Sci 2012; 69: 2779-89.
18. Fong E, Tzlil S, Tirrell DA. Boundary crossing in epithelial wound healing. Proc Natl Acad Sci US A. 2010;107:19302-7.
19. Omelchenko T, Hall A. Myosin-IX A regulates collective epithelial cell migration by targeting RhoGAP activity to cell–cell junctions. Curr. Biol. 2012; 22:278–288.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65:55-63.
21. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat. Protoc. 2007;2:329–333.
22. Zhang Y. Comparative study of 3D morphology and functions on genetically engineered mouse melanoma cells. Integr. Biol. (Camb).
23. Daniel WW. Biostatistics a foundation for analysis in the health sciences. 9th ed.; 2009. Chapter seven: 7.10, determining sample size to control type II errors. P. 278.
24. Sivamani RK, Pullar CE, Manabat-Hidalgo CG. Stress-Mediated Increases in Systemic and Local Epinephrine Impair Skin Wound Healing: Potential New Indication for Beta Blockers. Davidson J, ed. PLoS Medicine. 2009; 6(1):e1000012. doi:10.1371/journal.
25. Pullar CE, Chen J, Isseroff RR. PP2A activation by beta2-adrenergic receptor agonists: novel regulatory mechanism of keratinocyte migration. J Biol Chem. 2003; 278:22555–22562.
26. Pullar CE, Isseroff RR. The beta 2-adrenergic receptor activates promigratory and pro-proliferative pathways in dermal fibroblasts via divergent mechanisms. J Cell Sci. 2006; 119:592–602.
27. Selvaraj N, Budka JA, Ferris MW, Plotnik JP, Hollenhorst PC. Extracellular Signal-Regulated Kinase Signaling Regulates the Opposing Roles of JUN Family Transcription Factors at ETS/AP-1 Sites and in Cell Migration. Molecular and Cellular Biology. 2015; 35(1):88-100. doi:10.1128/MCB.00982-14.

28. Ghoghawala SY, Mannis MJ, Pullar CE, Rosenblatt MI, Isseroff RR. β2-Adrenergic Receptor Signaling Mediates Corneal Epithelial Wound Repair. Investigative ophthalmology & visual science. 2008; 49(5):1857-1863. doi:10.1167/iovs.07-0925.