Iranian Biomedical Journal 7 (4): 161-166 (October 2003)

A Study on the Effects of Modulation of Intracellular Calcium on Excisional Wound Healing in Rabbit

Mohammad Hassan Pipelzadeh[*1], Mohammad Reza Pipelzadeh[2] and Popak Husseinzadeh[1]

[1]*Dept. of Pharmacology and* [2]*Anaesthesia, Medical School, Ahwaz University of Medical Sciences, Ahwaz, Iran*

Received 23 September 2002; revised 12 October 2002; accepted 21 April 2003

ABSTRACT

An *in vitro* study on the role of intracellular calcium ions in healing of excisional wound in rabbit was undertaken. We employed two drugs namely, glibenclamide and nitroglycerin that are topically applied *in vivo* to modulate the activity of intracellular calcium. Our model consisted of a 15 × 15 mm excisional wound. Seven groups of New Zealand rabbits were used. The first three groups served as untreated, Vaseline- and lubricating jell vehicle-treated as controls. The remaining groups received topically 0.5 g of nitroglycerine (2 % in Vaseline base) or glibenclamide (1, 2 and 4% in lubricating jell) on the day of excision and continued for 11 days. Using wound surface area measurement, complemented with measurement of the breaking strength and histological assessment, the results showed that inhibition of intracellular calcium ion had a favorable effect on wound healing. The mean wound half-lives and breaking strength were significant and concentration dependently reduced in glibenclamide-treated group. In contrast, in nitroglycerin-treated group the rate of wound healing and breaking strength were increased relative to untreated control and Vaseline treated groups. Histological findings revealed more organized collagen fibers and angiogenesis in nitroglycerin-treated wounds. The present study demonstrated that intracellular calcium ion has an important role on the overall process of wound healing. This information may be utilized in further studies to assess its role in healing of human wounds. *Iran. Biomed. J. 7 (4): 161-166, 2003*

Keywords: Excisional wound, Nitroglycerin, Glibenclamide, Wound surface area, Tensile strength

INTRODUCTION

Accumulating evidences from biomedical research implicate calcium ion as a key intracellular signal that modulates the expression of cellular function and gene expression in a variety of systems including the developing ones [1]. As early as 1983, Chapman [2] demonstrated changes in calcium gradient following chemical or physical stimulation that correlated with activation of calcium channels across the cell membrane. Cell culture studies on keratinocytes and fibroblasts demonstrated the capacity of local calcium to modulate cellular proliferation, modification, maturation and the creation of epidermal lipid function through signal transduction and gene expression [3]. Such functional capabilities implicate calcium ion as a central role in epidermal regeneration and reconstruction in wound healing [4].

In the wound site, calcium concentration consistently changes with the biological events of the healing process [5]. Experimental studies have shown that calcium channel blockers, verapamil and nifedipine to abolish terminal differentiation of cultured keratinocytes [6]. On the other hand, calcium concentration in the extracellular media was found to be inversely proportional to cellular motility [7]. Furthermore, excessive calcium concentration in the wound environment induced reduction in proliferation and chemotactic responses and is believed to lead to delay in healing [8].

Topical application of verapamil to chronic wounds in experimental animals resulted in improved wound contraction and healing [9]. In contrast, diltiazem was not found useful in treatment of fibrous tissue formation in conjunctival wounds in rabbit [10]. All these observations are likely to have important clinical implications, but the available evidence is equivocal.

*Corresponding Author;

[DOR: 20.1001.1.1028852.2003.7.4.3.7]

Grzesiak and Pierschbacher in 1995 [11], suggested that the level of extracellular Mg^{++} and Ca^{++} could have a distinct impact on the adhesion and migratory activities of many cell types. The experimental models support the notion that management of calcium is essential for successful epidermal reconstruction [12], the benefits and possible implications under *in vivo* conditions of wound healing has received little attention. Therefore, we used an *in vivo* setting to assess the role of intracellular calcium on the healing of an experimentally induced excisional wound. For this purpose, we topically applied two drugs known to modulate the intracellular calcium level namely: glibenclamide, an antidiabetic drug known to increase the intracellular calcium ion via blocking ATP-dependent potassium channel (K_{ATP}) [13], and nitroglycerin, a potent coronary vasodilator that increases cGMP level and inhibits the intracellular calcium activity [14].

MATERIALS AND METHODS

Animals and treatment. Thirty-five white New Zealand rabbits of an average weight of 2.5 Kg (range 2.3 to 2.8 Kg) were utilized in this study, and divided randomly into seven groups of five animals each. The groups were allocated into the following categories: untreated control, vehicle 1 control (lubricating jell) (Abazar Darman Company, Tehran), vehicle 2 control (Vaseline) (Tehran Chemi Company), nitroglycerin (Berenguer-Infale, Germany) (2 %, in Vaseline base), and 1, 2 and 4 % glibenclamide (Gifted by Chemidaru Pharmaceutical Company, Tehran) in lubricating jell. The vehicles and treatment groups received 0.5 g of topical treatment daily commenced on the day of wounding and continued for 11 days post injury.

Wounding procedure. A 15 × 15-mm full thickness excisional wound was made on the lower flank of each animal according to the model proposed by Cross *et al.* [15]. The animals were held in standard crouching position, and the lower right flank were clipped with scissors and wet shaved with a scalpel blade. The area to be excisioned was outlined using a 15 × 15 mm template and then was locally anaesthetized. After established local anesthesia, by a subcutaneous injection of 1-2 ml of 2% lidocaine solution, a full-thickness wound was made by excising the skin within the confines of the template square down to the level of lose subcutaneous fascia.

Wound measurement. While the animals were held in standard crouching position, five wound boundaries for each wound were traced by a fine-tipped permanent marker onto an acetate transparency. This procedure was performed daily starting from day 0, the day of wounding and continued for 11 days post wounding. The areas were quantified by placing the transparency film over graph paper and counting the squares. The areas for each day after wounding were converted into percentage of wound surface area relative to day 0, which was considered 100%. The degree of effectiveness of drug treatments on the overall process of wound healing was quantified and compared by extrapolation of wound half-lives [16]. Furthermore, the area-under-the-curve (AUC) for all treatments relative to vehicle control was calculated [17].

Histological observations. At the end of the wound measurement period on day 11, the wound area together with 0.5 cm of intact skin from the outer boundary was removed. Half of the samples were selected for histological assessment. For this purpose, the skin samples were placed in 10 % formalin in normal saline. The tissues were processed by normal standard procedures of embedding in paraffin, sectioning, dehydration, and staining with haematoxylin and eosin, and finally mounted on a microscopic slide. The following parameters were assessed microscopically: the apparent number of polymorphonuclear leucocytes and fibroblastic-shaped cells, the relative extent of development new vascular beds (or angiogenesis) and the organization of collagen fibers were compared in different treatment groups.

Measurement of wound tensile strength. The remaining half of the removed wound samples was used for measurement of the tensile strength. For this purpose, a simple manual method was employed, by which the tissue was hanged by cotton threads vertical to the wound surface and fixed at one end to a metal bar and the other end to a 1 g aluminum envelope. With an aid of a large fixed magnifying glass and drop-wise addition of tap water, the point at which detachment of the wound surface from the skin or a break within the body of the wound tissue was considered the end point for breaking. The envelope was removed and weighed which reflected the force necessary for breaking the wound tissue.

Statistical analysis. The data are expressed as mean ± standard error of means (s.e.m.). Wound

[DOR: 20.1001.1.1028852.2003.7.4.3.7]

half-lives and breaking strengths between different groups of animals were compared using analysis of variance (ANOVA) for multi-group comparison. Where ANOVA between two groups was significant (P<0.05), then further comparisons using Dunnett t-test was performed.

RESULTS

The general observation from measurement of wound surface area showed a progressive reduction in the wound surface area with time in all groups. The data collected are illustrated in Figure 1, and revealed the mean half-lives were significantly different in untreated-control as compared to both glibenclamide and nitroglycerin-treated groups. Glibenclamide produced a dose-dependent delay in the wound surface area compared to untreated and vehicle 2 (lubricating jell) (P<0.01). Wound half-lives were 6.5, 8.9, and 10.3 days for 1, 2 and 4 % glibenclamide-treated groups respectively (Fig. 1). The half-life for lubricating jell (vehicle) treated group was 6 days and similar to untreated control group (P>0.05). In comparison, the nitroglycerin-treated group showed an acceleration in the rate of healing producing a reduction of wound half-life of 3.52 days, statistically significance compared to both the untreated control (P<0.01), and vehicle (Vaseline) treated groups (P<0.05).

For vaseline-treated group, the wound half-life was 5.1 days, in contrast to 6 days for untreated control group (P<0.05). Calculation of the AUC for all treatment groups relative untreated control group are summarized in Table 1. Where the values obtained reflected the relative extent of the rate of healing on the overall process of wound healing for each treatment relative to untreated control, and a negative or a positive sign represents the direction of this effects, i.e. decrease or increase respectively (17). These data were correlated with the times for 50% reduction in wound surface area measurements (Fig. 1).

Histological observations. Haematoxylin and eosin staining of 4 µm thick paraffin embedded sections, taken from the glibenclamide-treated wounds, revealed delayed epithelialization and a dose-dependent increase in the inflammatory polymorphonuclear leukocytes and dis-organized collagen fibers (Figs. 2e and f) compared to untreated control (Fig. 2a).

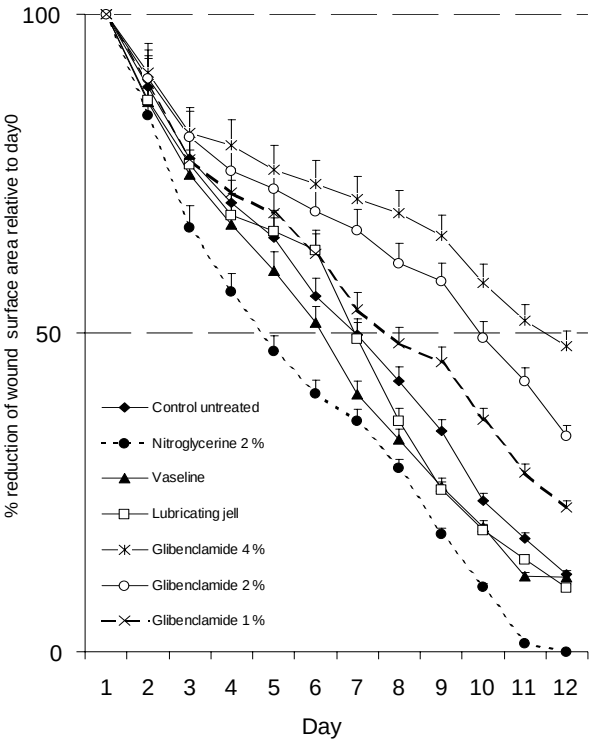

Fig. 1. Graphic representation of the changes in excisional wound surface area in the rat over the study period for untreated control and various treatment groups.

In contrast, in nitroglycerin-treated wounds complete epithelialization and the collagen fibers were organized parallel to the surface of the skin together with prominent new vascular tissues (Fig. 2d) compared to other treatment groups (Figs. 2b and c). In Vaseline-treated wounds, there were fewer inflammatory cells relative to the control untreated wounds (results not shown), possibly suggesting the mere physical occlusion of the wound surface had favorable outcome on the process of wound healing.

Table 1. Summary of the area-under-the-curves (A.U.C.) for assessment of the effectiveness of different treatment protocols on the overall process of wound healing relative to control untreated group [17].

	Vaseline	Jell	Ntg	Glb 4%	Glb 2%	Glb 1%
A.U.C.	+325.8	-1.03	+785.9	-1772	-992.9	-237.2

Jell (lubricating jell vehicle); Ntg (Nitroglycerin 2 % in vaseline base); Glb (Glibenclamide in lubricating jell). The plus and minus signs indicate an increase or decrease rate of wound surface area reduction relative to the control, respectively.

[DOR: 20.1001.1.1028852.2003.7.4.3.7]

Table 2. Summary of the means ± s.e.m. of the tensile breaking strengths of the excisional wound granulation tissues taken from different treatment groups of rats.

	Control	Vaseline	Jell	Ntg	Glb 4%	Glb 2%	Glb 1%
Mean ± sem	16.7±1.2	18.5±1.4	16.6 ±1.2	35.1*± 2	6.98*±0.9	11 *±0.7	14.5 ±1.3

*P<0.001 relative to no treatment control group, ANOVA followed by Dunnet's test. Jell (lubricating jell vehicle); Ntg (Nitroglycerin 2 % in vaseline base); Glb (Glibenclamide in lubricating jell).

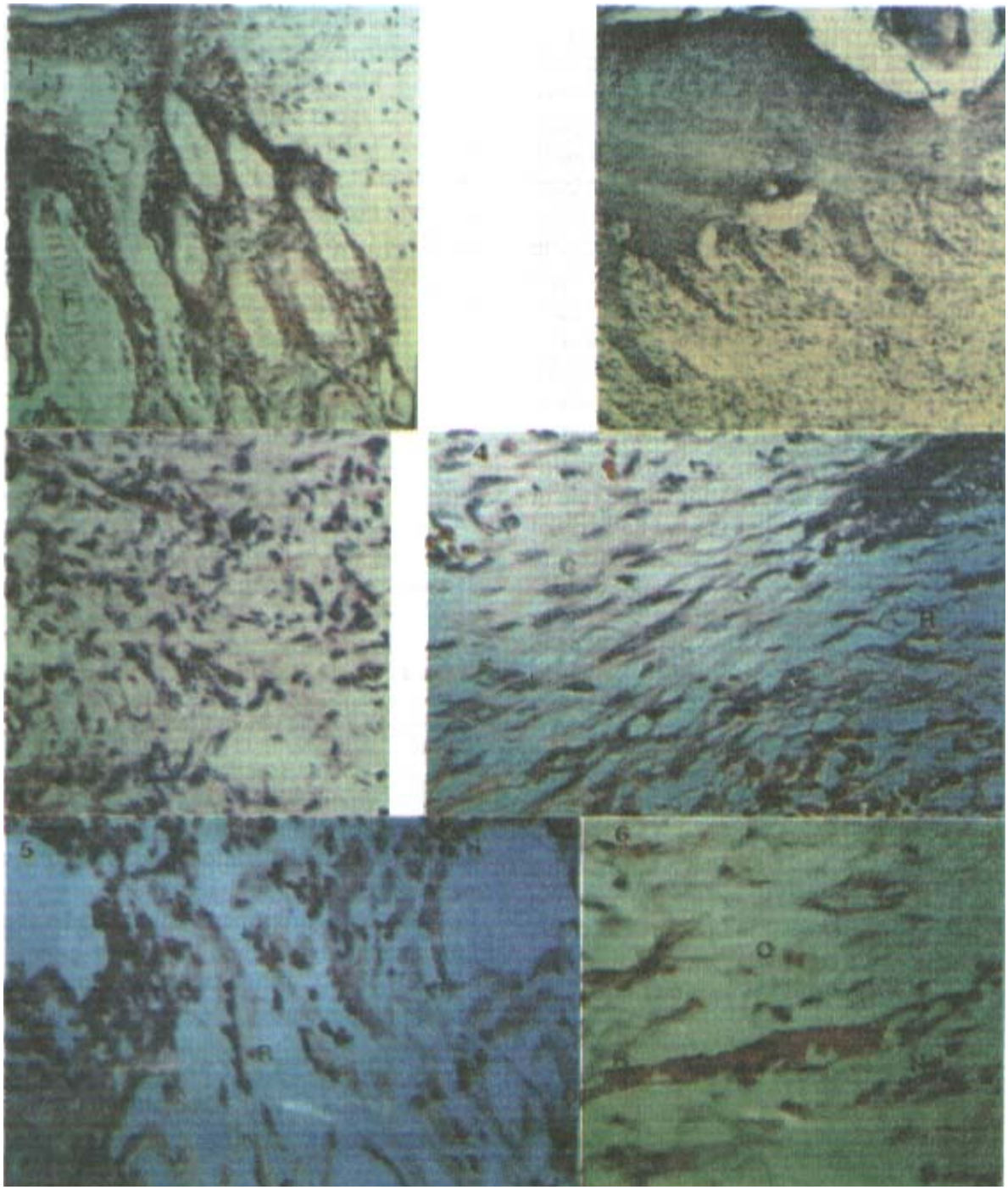

Fig. 2. Photomicrographic illustrations of haemotoxylline and oesin stained rabbit skinsamples taken from (**a),** (100 ×) normal skin; **(b)**, (100 ×) and **(c)**, (400 ×) untreated control; **(d)**, (400 ×): nitroglycerin-treated and **(e)** and **(f)**, (400 ×): 4 % glibenclamide-treated wound granulation tissues 11 days posr wounding. A, angiogenesis; C, collagen fibres; E, epidermis; F, fibroblasts; K, keratin layer; N, neutrophil; O, oedema; R, red-blood cell and S, scab.

[DOR: 20.1001.1.1028852.2003.7.4.3.7]

Wound tensile strength measurements. The tensile strengths measurements were found to be closely correlated with both the histological and wound surface area measurements. The greatest tensile strength measured was for the nitroglycerin-treated group, while there was a dose dependent and significant reduction in the tensile strength in the glibenclamide-treated group (Table 2).

DISCUSSION

This study demonstrated that modulation of the intracellular calcium ions causes marked changes in the overall process of wound healing. Glibenclamide, a drug known to increase the level of intracellular calcium concentration via inhibition of ATP-dependent potassium channels (K_{ATP}) [13], produced adverse effects on the wound healing. In contrast, nitroglycerin a drug known to act via activation of cGMP, an intracellular intermediate that inhibits the calcium activity [14], was found to favorably promote wound healing. All these observations suggest a paradoxical role for calcium ions compared to those observed for smooth muscle cells.

Smooth muscle cells rapidly lose the ability to contract when exposed to calcium-free medium. On the other hand, cell culture studies demonstrated loss in motility with reduction in calcium concentration in culture media [10]. In this study, we found that topical application of nitroglycerin induced an increase in the rate of healing. This finding suggests that contraction of wound granulation tissue may be independent of calcium ions or the inhibition of intracellular calcium promotes other important factors that govern wound-healing process. Since nitroglycerin is known to induce vasorelaxation and our histological findings showed a correlated increase in development of new vascular tissues compared to other treatments, together cause more nourishment of wound granulation tissues. Therefore, these findings suggest that the process of angiogenesis plays a more vital role than cellular migration. It seems that, in these complicated phenomena, wound contraction mediated by the myofibroblast, is a secondary effect to angiogenesis [15].

In one study, only nitroglycerine was employed in rat as an agent that caused acceleration in wound healing and increased the wound breaking strength relative to untreated control [16]. However, no histological evaluation was made, and four 15 × 15 mm excisional wounds seemed to be a large area for a relatively small animal.

If excessive calcium, under pathological conditions such as tissue injury, promotes inflammation [17], then various methods can be used to limit its manifestations. One way for limiting this stage of wound healing would be the application of calcium channel blockers, such as diltiazem [9] or verapamil [8], or, as in our study, the use of agents that nullify its effect, by employing nitroglycerin. Recently, it has been reported recently that inflammatory phase is not as vital as previously assumed for normal healing to proceed in adult wound healing [18]. In this study, topical application of nitroglycerin resulted in the production of organized collagen fibers and dampening of inflammatory responses, by reducing the number of polymorphic nucleoside. Thus, it may be capable of reducing scar formation [18]. Also, nitroglycerin could help faster healing and producing stronger wounds. Whatever the exact underlying mechanism that governs these effects the data suggest that modulation of calcium can have important role in wound healing.

Since there is no universally accepted method in interpretation of the data obtained from wound surface area, we utilized two methods for evaluating the changes in wound surface area, the time for 50% reduction in wound surface area [19] and AUC [17]. We found that both methods were positively correlated.

Measurement of glucose level on day 11 did not show a significant change in the blood glucose level (results not shown). This suggests that the effects produced by glibenclamide were solely local. Glibenclamide was found to produce reduction in both wound breaking strength and slowed the rate of closure of the wound surface area, suggestive to adverse effects on wound healing process. In addition, the histological observations showed correspondingly disorganized collagen and increased polymorphonuclear leucocytes, indicating an abnormality in collagen synthesis and persistent inflammation at the site of the wound. Whether healing of wounds in diabetic patients, who are under glibenclamide treatment, is also adversely affected warrants further clinical or experimental assessment.

Additional pharmacological effects, other than K_{ATP} channel blockage and via still unknown mechanism attributed to glibenclamide, were reported [20, 21]. Glibenclamide was found to cause depolarization effect on the cardiac muscles during the ventricular fibrillation [20]. It also produced relaxant effect on isolated rat aorta.

[DOR: 20.1001.1.1028852.2003.7.4.3.7]

Although, we did not measure changes in the intracellular calcium, it seems the calcium level increases since all the parameters considered in this study were opposite to those observed for nitroglycerin, a drug known to produce inhibition of intracellular calcium activity. However, the exact cells affected by glibenclamide cannot be allocated from these findings, and deserve further studies.

ACKNOWLEDGEMENTS

The authors would like to sincerely thank Mrs. Massoumeh Bassirzadeh for her useful comments and valuable help during preparation of the manuscript, and Miss Marjan Pipelzadeh in preparing the photomicrographic illustrations.

REFERENCES

1. Boitano, E.R., Dirksen, E.R. and Sanderson, M.J. (1992) Intracellular propagation of calcium waves mediated by inositol triphosphate. *Sceince 258: 292-295*.
2. Chapman, D. (1983) Trigger processes, receptors and membrane dynamics. *Trends Pharm. Sci. 46: 233-238*.
3. Dlugsosz, A.A. and Yuspa, S.H. (1994) Protein kinase C regulates kertinocyte transglutiminase (TG_k) gene expression in cultured primary mouse epidermal kertinocytes induced to terminally differentiate by calcium. *J. Invest. Dermatol. 102: 409-414*.
4. Trump, B., Berenesky, I.K., Sato, T., Laiho, K.U., Phelps, P.C. and Declaris, N. (1984) Cell calcium, cell injury and cell death. *Eniviron. Health Perspect. 89: 530-538*.
5. Hunt, T.K., Hopf, H. and Hussein, Z. (2000) Physiology of wound healing. *Adv. Skin Wound* Care 13: 6-11.
6. Resing, K.A., Al Alawi, N., Blomquist, C., Fleckman, P. and Dale, B.A. (1993) Independent regulation of two cytoplasmic processing stages of intermediate filament-associated protein filaggrin and role of Ca^{++} in the second stage. *J. Biol. Chem. 268: 25139-25145*.
7. Menson, G.K. and Elias, P.M. (1991) Ionic calcium reservoirs in mammalian epidermis: ultrastructural location by ion-capture cytometochemistry. *J. Invest. Dermatol. 84: 508-512*.
8. Sank, A., Chi, M., Shima, T., Reich, R. and Marin, G.R. (1989) Increased calcium levels after cellular and molecular events in wound healing. *Surgery 106: 1147-1148*.
9. Oruk, S., Orhan, D., Orhan, M., Irkec, M., Basxi, N., Barun, S. and Bozkurt, A. (2000). The pharmacokinetics and effects of diltiazem in rabbit. *Eur. J. Ophthalmol. 10: 46-50*.
10. Grzesiak, J.J. and Pierschbacher, M.D. (1995) Shifts in the concentrations of magnesium and calcium in early porcine and rat wound fluids activate the cell migratory response. *J. Clin. Invest. 95: 227-233.*
11. Landsdown, B.G. (2002) Calcium: a potential central regulator in wound healing in the skin. *Wound Repair Regen. 10: 271-285*.
12. Zunkler, B.J., Lenzen, S., Manner, K., Panten, U., and Trube, G. (1988) Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic B-cells. *Naunyn Schmiedebergs Arch. Pharmacol. 337: 225-230*.
13. Molina, C., Andresen, J.W., Rapaport, R.M., Waldman, S.A. and Murad, F. (1987) Effects of *in vivo* nitroglycerin therapy on endothelium-dependent and -independent relaxation and cyclic GMP accumulation in rat aorta. *J. Cardiovasc. Pharmacol. 10: 371-378*.
14. Cross, S.E., Naylor, I.L., Coleman, R.A. and Teo, T.C. (1995) An experimental model to investigate the dynamics of wound contraction. *Br. J. Plastic Surgery 48: 189-197*.
15. Gabbiani, G., Hirschel, F., Rayan, G.B., Statkov, P.R. and Majno, G. (1972) Granulation tissue as contractile organ: A study of the structure and function. *J. Exp. Med. 135: 719-733*.
16. Heggers, J.P., Elzaim, H., Garfield, R., Goodheart, R., Listengarten, D., Zhao, J. and Philips, L.G. (1997) Effect of combination of *Aloe vera*, nitroglycerine, and L-NAME on wound healing in rat excisional model. *J. Altern. Compelement Med. 3: 140-153*.
17. Pipelzadeh, M.H., Jouyban, A., Khodadadi, A. and Naylor, I.L. (2001) A new mathematical model to investigate drug treatment on the overall process of wound healing. *Iran. Biomed. J. 5: 61-68*.
18. Wilgus, T.A., Vodovotz, Y., Viitadini, E., Clubbs, E.A. and Oberyszyn, T.M., (2003) Reduction of scar formation in full-thickness wounds with celecoxib treatment. *Wound Repair Regen. 11: 25-34*.
19. Snowden, J.M. (1981) Wound contraction: A quantitative interpretation. *Aust. J. Exp. Biol. Med. Sci. 59: 203-217*.
20. Lomuscio, A. (1994) Effect of glibenclamide on ventricular fibrillation in non-insulin-dependent diabetes with acute myocardial infarction. *Coron. Artery Dis. 25: 767-771*.
21. Chan,W., Yao, X., Ko, W. and Huang, Y. (2000) Nitric oxide mediated endothelium-dependent relaxation induced by glibenclamide in rat isolated aorta. *Cardiovasc. Res. 46 (1): 180-187*.

[DOR: 20.1001.1.1028852.2003.7.4.3.7]