Journal of Animal and Veterinary Advances 7 (12): 1537-1545, 2008
ISSN: 1680-5593

Effects of Topical and Parenteral Application of Phenytoin on Cutaneous Wound Healing in Rabbits

[1]Aboutorab Tabatabaei Naeini, [2]Ahmad Oryan, [1]Seifollah Dehghani and [3]Behrooz Nikahval
[1]Department of Clinical Studies, [2]Department of Pathobiology,
School of Veterinary Medicine, Shiraz University, P.O. Box 1731, 71345 Shiraz, Iran
[3]Department of Veterinary Medicine, Islamic Azad University of Bojnourd,
Postal Code: 94176-94686 Bojnourd, Iran

Abstract: The effects of local and parenteral phenytoin therapy in the experimentally induced cutaneous wounds in rabbits are investigated by light and electron microscopy, biomechanical investigation and the extent of wound closure. Thirty skeletally mature rabbits were randomly divided into 2 experimental groups and a control group (each group n = 10). Under sterile conditions, surgical skin incisions were made perpendicular to the vertebral column in both sides of the paralumbar region. On left side only the skin was removed (superficial wound) but on the right side, the skin and the underlying tissues including subcutaneous fascia, panniculus carnosus were also excised (deep wound). One group received daily topical application of phenytoin and one group received daily intramuscular injection of phenytoin solution for 14 days. The 3rd group received no treatment following the operation. All the rabbits were euthanized 21 days post operation and samples were collected for histopathologic, electron microscopic and biomechanical investigations. There were no significant differences in wound closure time between the 3 groups and between the deep and superficial wounds. None of the biomechanical differences between the 3 groups and between the superficial and deep wounds in the same group were significant. The histopathological and ultrastructural results showed improvement in the architecture of the parenterally treated wounds compared to those treated topically. Concentration and the timing of the drug administration were considered to be the most important factors affecting the results. Lower doses seem to be more effective, although the reasons for this remain unclear.

Key words: Wound healing, phenytoin, topical, parenteral

INTRODUCTION

Wound healing, the result of a complex tissue repairing process, is a continuing challenge in rehabilitation medicine. The quest for better wound-healing agents is perhaps one of the oldest challenges for medical practice. One such agent that has been tried in wound healing is phenytoin. Phenytoin (diphenylhydantoin or Dilantin®) was introduced into therapy about 70 years ago for effective control of convulsive disorders (Kimball and Horan, 1939). Phenytoin has been investigated as a therapeutic agent for more than 100 disorders, such as epidermolysis bullosa (Fine and Johnson, 1988; Talas *et al*., 1997) pressure ulcers (Margolis and Lewis, 1995), venous stasis ulcers (Oluwatosin *et al*., 2000), diabetic ulcers (Muthukumarasamy *et al*., 1991), traumatic wounds (Modaghegh *et al*., 1989), linear scleroderma (Eubanks *et al*., 1996), discoid lupus erythematous (Rodriguez-Castellanos *et al*., 1995; Jessop *et al*., 2001) and neurologic pain (Wiffen *et al*., 2000; Jensen, 2002). Currently, much attention and faith are being placed on the development of expensive topical molecular factors for enhancement of wound healing. Phenytoin is inexpensive, easy to use and readily available. Clinical studies using phenytoin therapy suggest that it may be useful for the treatment of both acute and chronic wounds of various etiologies (Modaghegh *et al*., 1989; Muthukumarasamy *et al*., 1991; Margolis and Lewis, 1995). Although, these results are encouraging, the efficacy of topical and parenteral phenytoin therapy has yet to be confirmed by double-blind placebo-controlled studies. Phenytoin may promote wound healing through multiple mechanisms, including stimulation of fibroblast proliferation, facilitation of collagen deposition, glucocorticoid antagonism and antibacterial activity (Anstead *et al*., 1996). These apparent stimulatory effects of phenytoin on connective tissue may prove beneficial for treating wounds in a clinical situation.

Corresponding Author: Dr. B. Nikahval, Department of Veterinary Medicine, Islamic Azad University of Bojnourd, Postal Code: 94176-94686 Bojnourd, Iran

Therefore, this study was designed to assess the effects of local and parenteral phenytoin therapy on the morphological changes at light and electron microscopy, biomechanical properties and the extent of wound closure in the experimentally induced cutaneous wounds in rabbits.

MATERIALS AND METHODS

Animals: Thirty skeletally mature male White New Zealand rabbits, weighing between 3.3-4.6 kg were housed, individually in separate standard cages. When received, the rabbits were randomly assigned a number that was printed on the ear with indelible ink. The standard rabbit diet and water were available *ad libitum*. Temperature (25°C) and the ratio of daylight hours to non daylight hours (12 h light/12 h dark) were kept constant. The rabbits were randomly divided into 2 experimental groups and a control group (each group n = 10).

Care was taken to avoid unnecessary stress and discomfort to the animals throughout the experimental period. This study was approved by the ethical committee of Veterinary School of Shiraz University and the principles of laboratory animal care were followed.

Surgical procedures: All the rabbits were anesthetized by injection of Xylazine (3 mg kg^{-1} IM) (Rompun®, Alfasan International B.V. Woerden, Holland) and Ketamine (50 mg kg^{-1} IM) (Alfamine®, Alfasan International B.V.; Woerden, Holland). Before making incisions, the dorsal aspect of the lumbar area was shaved and washed with scrub solution of povidone iodine. Under sterile conditions, a 3×1 cm rectangular skin defect was created, the long side of the defect perpendicular to the vertebral column in both sides and the cranial border 1 cm caudal to the last rib. In left side, only the skin was removed (superficial wound) but in the right side the underlying tissues such as subcutaneous fascia, panniculus carnosus and go up to lumbar muscle (deep wound). The same procedures were performed for experimental and control groups. The scalpel blade used to create defects was changed after each animal. After operation and treatment (described below) had done, the wound areas were bandaged with sterile nonadherent pads and porous adhesive tapes. The duration of anesthesia was about 15 min for each rabbit. Carprofen (Rimadyl®, Pfizer Inc. Zaventem, Belguim) was injected to all animals (4 mg kg^{-1} SC) once just before the operation for pre-emptive analgesia and every 24 h for 3 days postoperatively.

Treatment regimes: In the control group after making, the incisions on both sides, no treatment was applied on the incisions and the incisions were remained intact. Bandages were changed daily for 21 days and wounds were rebandaged as described above. Daily observation performed and wound contraction was measured using a surgical ruler, for 21 days. The wound area of each lesion on each evaluation day was obtained by tracing the perimeter of the wound onto a sterile piece of clear acetate film with a special marking pen. The acetate was laid on the wound surface, smoothed and held flat and immobile by an assistant while the tracing was made by the examiner (ATN) wearing 2.5×loupes. The time that the skin edges of the length of each wound reached each other considered as wound-closure time, which was measured daily and described as mean (±S.D.). All wounds during the first 7 days were gently wiped with sterile sponges moistened in sterile saline. The amount and character of any wound fluid and any evidence of infection or other abnormalities were noted. The rabbits of topical phenytoin group received phenytoin powder, in a thin, uniform layer, directly on both wounds once daily before bandaging for a period of fourteen days. On each wound the powder content of a 100 mg phenytoin capsule (Dilantin®-Loghman Co. Iran) was administered entirely. The rabbits of parenteral phenytoin group received daily intramuscular injection of 25 mg kg^{-1} injectable phenytoin solution (1 mL of solution and rotate the site of administration in both legs) (Dilantin®, Desitin, Arzeneimittle, Germany) from 2 days pre-operation until 14 days post-operation. After 14 days of treatments the animals were then left untreated for another 7 days before their being sacrificed.

Sampling: At the end of the day 21 post operation all the rabbits were euthanized by IV injection of 40 mg kg^{-1} thiopental sodium (Nesdonal)® via marginal ear vein and sampling was done. Samples (1×1×1 cm^3) for histopatho-logical studies were taken from the edges of the wound, so that the sample contains both the lesion and its periphery including normal skin. Samples were collected for histopathologic and electron microscopic studies and biomechanical investigations. Samples from the skin of 5 animals of each group (including normal animals) were collected for histological, electron microscopic and the samples from the rest 5 animals were collected for tensile testing (Table 1). Similar skin samples from the intact skin of the lumbar region far from the site of initial excision of the same animal were excised as normal skin control.

Table 1: Division of animals per groups and number of samples for each group and subgroups

Groups	No. of perr animal group (n)	Wounds samples (n)	Samples* (n)	No. of samples per each subgroups
			Histopath.	5
			Biomech.	5
		Superficial (10)	EM	5
Topical			Histopath.	5
phenytoin			Biomech.	5
(n = 10)	10	Deep (10)	EM	5
			Histopath.	5
			Biomech.	5
		Superficial (10)	EM	5
Parenteral			Histopath.	5
Phenytoin			Biomech.	5
(n = 10)	10	Deep (10)	EM	5
			Histopath.	5
			Biomech.	5
		Superficial (10)	EM	5
			Histopath.	5
Control			Biomech.	5
(n = 10)	10	Deep (10)	EM	5

*EM: Electron Microscopic samples

Biomechanical studies: Skin from the 5 remaining animals in each group and their relevant contralateral skin were dissected for biomechanical tensile testing and stored at -20°C prior to testing. They were then thawed at room temperature before tensile testing (Oryan and Zaker, 1988; Moon *et al*., 2006; Oryan *et al*., 2007). Throughout, the testing period, the specimens were kept moist in saline soaked gauze. The specimens were mounted in an Instron-hydraulic tensile testing machine controlled by a personal computer. The proximal section was clamped to skin using a serrated interdigitating mechanical clamp. The distal section of the specimen was mounted in a cryo-clamp, using the method described by Riemersma and Schamhardt (1982); so that a constant specimen length of 1.5 cm was used in the test. Tensile testing to failure was performed by applying 10 kg force at a strain rate of 5 mm sec^{-1}. The ultimate and yield strength and strain, stiffness and maximum energy stored of the specimens were recorded by the computer.

Histopathological studies: Skin samples were taken from both treated and untreated wounds as well as comparable normal skin and fixed in 10% neutral buffered formalin. After fixation, the tissues were dehydrated using graded of ethanol, embedded in paraffin wax and 5 μm thickness sections were stained using Hematoxylin and Eosin (H and E), Alcian blue/PAS to visualize glycosamino-glycans, Masson's Green Trichrome and Van Gieson and Verhoeffs for collagen and elastic fibers. Ten zones were examined for each sample morphometrically through a calibrated ocular on a Nikon light microscope (Nikon, Tokyo, Japan) at a magnification of 400x. Histological examinations were performed in a double blind fashion. The criteria which were studied in histopathological sections consisted of hemorrhage, fibrin deposition, polymorphonuclear cell and mononuclear cell infiltration, reepithelialization, cornification of the epithelium, fibroblast content, glycosaminoglycan secretions, collagen content, revascularizations, necrosis, presence of fibrocytes and maturation and organization of collagen and elastic fibers.

Electron microscopic studies: Skin samples of 1×1×1 mm^3 were fixed using 2% cold glutaraldehyde and 2% paraformaldehyde in 0.125 M sodium cacodylate buffer at pH 7.4 (Karnovsky, 1965). The tissues were then post-fixed in 1% osmium tetroxide in 0.1 M buffer sodium phosphate, pH 7.4 for 1 h, dehydrated in graded ethanol and thereafter embedded in Spurr resin. Semithin sections of 1 μm in thickness were stained with 0.25% toluidine blue for 30 sec and observed with a Nikon Labophot AFX-II light microscope. Representative areas were selected and then ultra thin sections of 70-80 nm in thicknesses were stained with uranyl acetate and counter stained with lead citrate. After the staining processes, the samples were observed with an electron microscope (Phillips, M 300, Netherlands). Collagen fibrils in electron micrographs were seen as long and thin fibrils which were separate from each other. It was not possible to measure the length of the collagen fibrils in the sections but the diameters of the fibrils were measured. In each specimen the diameter of 500 collagen transverse sections were measured.

Statistical analysis: One way Analysis of Variance (ANOVA) and Duncan's multiple range test were used to evaluate the differences of biomechanical parameters between experimental groups (topical and parenteral phenytoin group) and between each experimental and control group (In each comparison, deep and superficial wound of experimental groups compared to the similar wounds of the other groups). Student's t-test was used to evaluate the significant differences of biomechanical parameters between normal skin samples and deep wounds and also between normal skin samples and superficial wounds. Differences were considered significant when $p<0.05$, using computer software SPSS version 11.5 for windows (SPSS Inc., Chicago, IL., USA).

RESULTS

Observations during daily wound care: On the first postoperative day, the wounds appeared clean. On the 2nd and 3rd days of treatment all the untreated wounds developed a moderately purulent-appearing exudate. The exudate over the treated wounds was less than that of the untreated wounds and disappeared over the first

Table 2: Mean (±S.D.) of wound-closure time

Group	n	Deep wound[a] (Right) (day)	Superficial wound[a] (Left) (day)
Control	10	17.50±3.02	15.50±3.33
Topical phenytoin	10	17.33±3.08	16.11±3.55
Parenteral phenytoin	10	15.40±2.41	14.20±2.82

n: Number of animals from which wound-closure values obtained; a: wound-closure time differences are not significant; d: day

8-10 days of follow-up. On the day 14 the wound size in both the topically and parenterally-treated and control groups was reduced. On the day 21 post-injury, the wound size in all treated and control lesions were diminished and the lesions showed proper re-epithelialization.

Wound-closure: There were no significant differences in wound closure time between 3 groups and between deep and superficial wounds (Table 2).

Histopathological findings: Both untreated deep and superficial lesions showed haemorrhage, fibrin deposition in dermis and also inflammatory cell infiltration mostly composed of neutrophils, macrophages, lymphocytes and plasma cells were also seen. The newly formed collagen fibers were still unorganized and showed a haphazardly distributed pattern. Many immature fibroblasts and fewer mature fibrocytes were also present in the untreated lesions. Alcian blue/PAS staining showed an increase in glycosaminoglycan and proteoglycan concentrations of the ground substance of both deep and superficial control wounds. Angiogenesis was evident in both deep and superficial untreated wounds and these lesions were well vascular. No elastic fiber was seen after staining the open superficial and deep control wounds with Van Geison and Verhoeffs. Both superficial and deep untreated lesions showed an optimal degree of re-epithelialization and cornification (Fig. 1a).

The lesions of the parenterally treated animals on the other hand showed significantly less inflammatory cells compared to those of the untreated lesions. Well formed epithelial layer with cornification was evident in the deep and superficial wounds of the parenterally treated group. However, the lesions of the parenteral group showed slight hypercellularity the cells were almost of mature fibrocytes and fewer immature fibroblasts (Fig. 1b and 2a). The newly collagen fibers of the treated animals of this group were relatively organized and the tissue alignment was greater as compared to other treated and untreated animals. No elastic fiber was seen after staining open superficial and deep control wounds of this group with Van Geison and Verhoeffs. The superficial wounds of this group were morphologically very close to those of the normal animals (Fig. 2a).

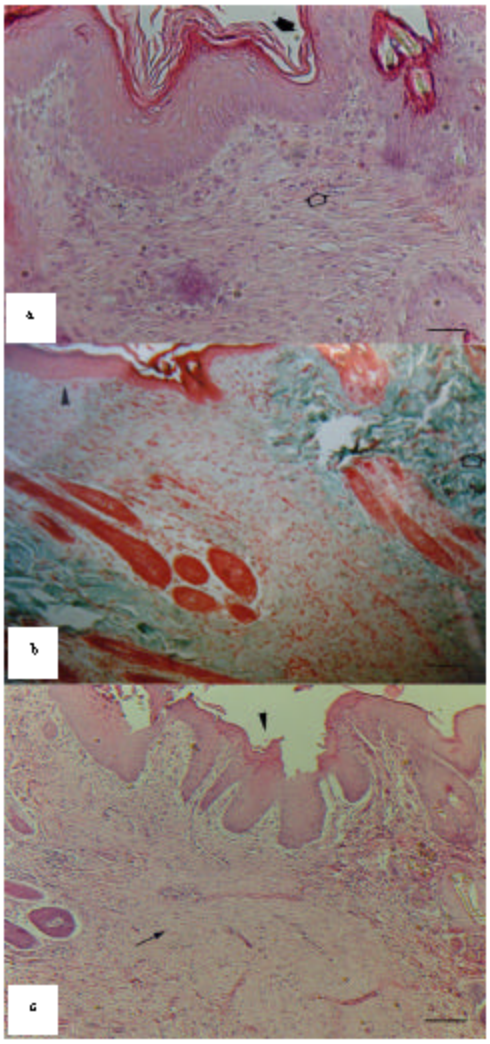

Fig. 1: (a) Deep wound in control group. Re-epithelialization and cornification (⧫). Newly formed connective tissue and blood vessels in dermis mildly infiltrated with leukocytes (◊). Haematoxylin and eosin (Bar = 60 μm) (b) Deep wound in parenteral phenytoin group. Re-epithelialization and tissue organization is seen (▲). Old connective tissue (◊). Masson's Green Trichrome (Bar = 100 μm) (c) Deep wound in topical phenytoin group. Re-epithelialization and newly formed keratin layer on the surface of epidermis (▼). The fibrous connective tissue of the dermis is still unorganized and is infiltrated with mononuclear cell infiltration (→). Haematoxylin and eosin (Bar = 100 μm)

The lesions of wounds treated topically showed hemorrhage and fibrin deposition and were hyper cellular with numerous immature and fewer mature fibroblasts

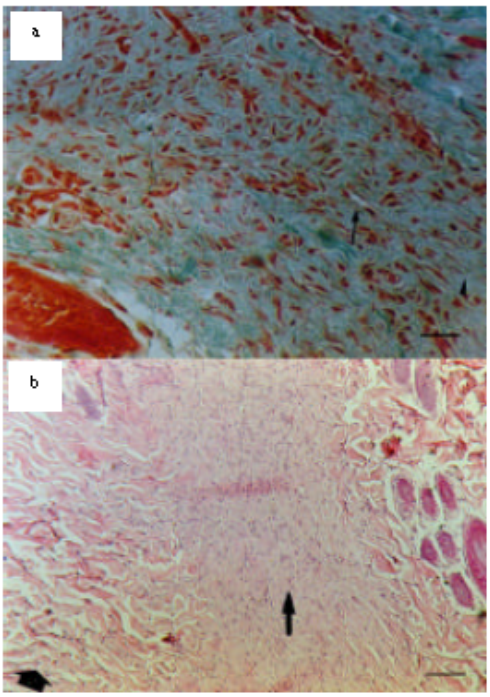

Fig. 2: (a) Superficial wound in parenteral phenytoin group. Newly formed collagen fibers (▲) and fibroblast cells (↑). Masson's Green Trichrome (Bar = 37.5 μm) (b) Superficial wound in topical phenytoin group. Newly formed connective tissue and blood vessels in dermis are unorganized (↑). Normal collagen exists at the margin of repair area (◗) Haematoxylin and eosin (Bar = 100 μm)

(Fig. 1c). Lymphocytes, plasma cells, macrophages and neutrophils were mildly infiltrated in the lesions. Inflammatory cell infiltration was greater in deep wounds of this group compared to those of the superficial ones. Re-epithelialization was complete and unique epithelial layer with cornification was formed. Angiogenic activity of this group was similar to those of the untreated control lesions. An increase in fibroblasts were seen but with rare fibrocytes. The results obtained from Van Geison and Verhoeffs staining showed no elastic fibers formation in the lesions. Superficial wounds showed the same characteristics with deep wounds except for less haemorrhage and fibrin deposition in superficial wounds (Fig. 2b).

Ultrastructural findings: At the ultrastructural level the collagen fibrils of the normal skin showed a bimodal distribution. Ninty eight percent of them were of large population with diameters of 127.70±29.15 nm and 2% of small diameter collagen fibrils of 28.10±2.40 nm (Fig. 3a).

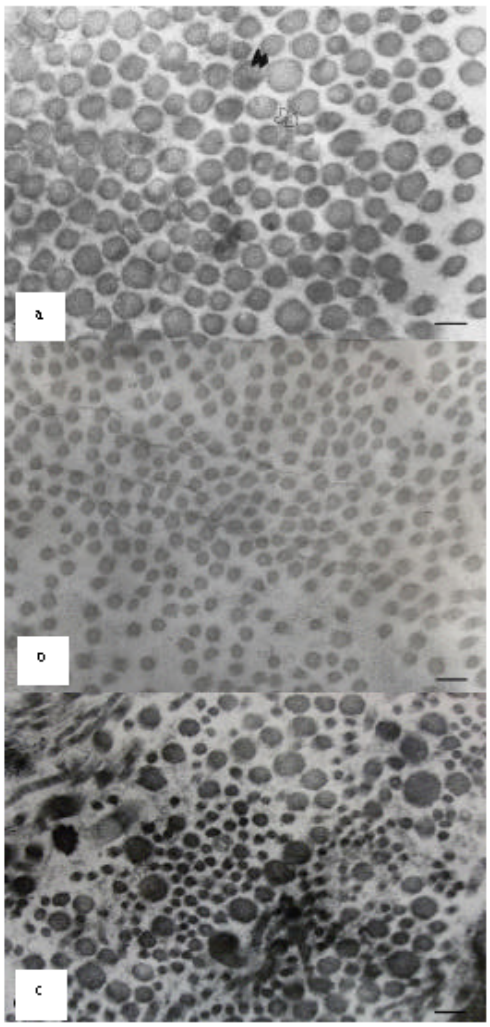

Fig. 3: (a) Ultra micrograph of normal rabbit skin of rabbits. The larger collagen fibrils (black arrow) and the smaller collagen fibrils (white arrow) (Bar=150 nm) (b) Superficial wound of topical phenytoin group. The collagen fibrils are unimodally distributed (Bar = 150 nm) (c) Deep wound of parenteral phenytoin group. Collagen fibril diameters are distributed bimodally (Bar = 150 nm)

Table 3: Diameter of collagen fibrils

Group	n	Deep wound (nm)*	Superficial wound (nm)*
control group	5	108.98±24.12	107.90±22.79
Topical group	5	100.52±22.52	104.48±26.71
Parenteral group	5	109.88±24.50	106.28±27.53

Mean (±S.D.) of diameter of collagen fibrils measured in nm in ultramicrographs, n: Number of animals per group, a: there are no significant difference between control and exp group, Mean diameter of collagen fibrils in normal skin: 127.70±29.15

Mean (±S.D.) of diameter of collagen fibrils are presented in Table 3. The lesions of the topically applied animals

Table 4: Mean±S.D. of biomechanical parameters

		Parenteral phenytoin			Topical phenytoin			Control		
Wounds criteria	Unit	Superficial (n = 5)	Deep (n = 5)	Intact skin* (n = 5)	Superficial (n = 5)	Deep (n = 5)	Intact skin* (n = 5)	Superficial (n = 5)	Deep (n = 5)	Intact skin* (n = 5)
Ultimate strength	kg	4.04±0.740	3.78±0.750	21.82±9.580	3.32±0.98	2.66±1.11	18.35±5.640	4.54±2.11	3.60±2.990	19.40±8.510
Yield strength	kg	3.69±0.680	3.51±0.850	13.88±4.170	2.99±1.15	2.30±1.15	10.99±3.390	4.41±2.17	3.30±2.970	15.25±6.590
Stiffness	kg cm^{-1}	3.77±1.590	4.32±0.660	7.67±4.610	3.42±1.05	3.12±1.34	5.63±2.590	3.81±1.83	3.32±2.410	6.16±2.400
Ultimate strain	(%)	40.16±13.33	22.81±11.72	72.64±15.20	29.64±3.45	23.03±5.88	59.48±21.44	32.98±5.12	28.48±12.63	64.24±10.63
Yield strain	(%)	36.77±11.85	23.35±5.650	53.50±21.16	25.30±1.51	17.61±4.59	48.60±13.49	30.68±4.70	24.10±9.060	52.17±7.270
Maximum energy stored	kg*cm	2.56±0.360	1.95±.0460	29.93±9.890	2.26±0.77	1.49±0.75	27.19±10.46	3.75±2.05	2.89±2.420	30.63±14.76

* Intact skin samples showed significantly higher biomechanical values as compared to the superficial and deep wounds of the both treated and untreated rabbits

showed a unimodal distribution comprising only of small sized collagen fibrils with diameters of 27.30±2.70 nm (Fig. 3b), while the systemically applied lesions showed a bimodal distribution of collagen fibrils but the small fibrils of 28.3±2.10 nm were the highest population and the large fibrils comprising about 20% of the sections had diameters of 98.10±21.10 nm (Fig. 3c). None of the collagen fibrils in either group reached its maximum diameter of normal skin.

Biomechanical findings: All the biomechanical findings are presented in Table 4. There were no significant differences between the 3 groups and between superficial and deep wounds in the same group. All the values in treated and untreated specimens were lower as compared to intact skin samples.

DISCUSSION

This study was designed to determine whether or not topical and systemic application of phenytoin is an effective means of promoting wound healing. Although, significant positive effects on wound healing using either of the aforementioned techniques for phenytoin administration were not detected, systemic therapy showed subjectively better results compared to the topical application. One possible reason for absence of any trend toward enhancement with topical application which previously has been stated is the concentration. It has been reported that lower concentrations is more effective. Moy *et al.* (1985) determined that the effect of phenytoin on human skin fibroblasts is both concentration and time dependent. At low phenytoin concentration (5 mg L^{-1}) and short incubation times (3 h), phenytoin markedly enhanced cell proliferation. However, at higher concentrations (>25 mg L^{-1}) and longer incubation times (>25 h), a decrease in fibroblast proliferation occurred. However, with the concentration we used (100 mg powder) not only didn't we observed a depression in fibroblasts, but we saw fibroblast multiplication and proliferation and differentiation, with minimal fibrocytes. Therefore, in the present experiment the concentration could have altered the fibroblasts maturation but not the proliferation. The exact reason for such contradictory findings remained unanswered to us. It needs further investigation working on the mechanisms by which phenytoin acts on fibroblasts. Lack of significant difference in morphological and biomechanical findings in the treated and untreated animals of the present study could be due to short duration of phenytoin therapy (3 weeks). This can be considered one explanation why systemic phenytoin (Lower doses) gave better results than topical phenytoin. Considering time of evaluations, our study was performed in a shorter term (3 weeks), compared to other studies such as the one carried out (Simpson *et al.*, 1965) in which the evaluations carried out in 13 weeks period. Our results showed that there was not significant deference between treated and untreated animals, whereas Simpson *et al.* (1965) reported the beneficial effects of phenytoin on morphological, biochemical and biomechanical properties of wound healing. This suggested that the effective changes of phenytoin in wound healing, reported by other researches, might have started lately in the wound healing process; proposing that prolonged or delayed use of phenytoin may be more useful. The ultrastructural findings, in which the new bimodally distributed collagen fibrils subsequently mature in a normal manner and give rise to a mixed population of small and larger diameter fibrils, could be considered as a sign of time passage by which the mechanical strength will increase with time. It seems it would be beneficial to evaluate the effects of phenytoin at different stages of wound healing including inflammatory, fibroplasia and remodeling phase of ordinary wound as well as more chronic wounds.

The other criterion to be considered is the method of application. The best method of delivery of topical application of phenytoin is not known. Some investigations have used pure phenytoin or phenytoin sodium powder, covered with gauze and others have used powder from phenytoin capsules, as we did. The powder from the capsules is reported to cause a white scar-like coating. Rhodes *et al.* (2001) reported that this could be

prevented by mixing phenytoin with NaCl (0.9%) and applying it with gauze. We did not observe this coating, this could be a result of the bandage we applied following administration, which resulted in mixing the powder substance with the wound secretions. One report suggests that the use of phenytoin suspension is more convenient, however, it contains several added ingredients. Injectable phenytoin has also a high pH (about 12) and should not be used topically because, it can damage the skin (Anstead *et al*., 1996). Modaghegh *et al.* (1989) compared four topical phenytoin formulations (gel, cream, phenytoin sodium powder and phenytoin powder) in a rat model of wound healing and concluded that the phenytoin powder showed the most favorable results. We used topical administration which supposed to be the most appropriate method of application and injectable phenytoin on which the least work has been carried out. The lack of any significant enhancement of wound healing as measured is this study could be related to a reduction in efficacy of phenytoin at higher doses as demonstrated in other studies and as used in this study (Moy *et al*., 1985; Modaghegh *et al*., 1989).

The exact mechanism by which phenytoin accelerates wound healing is unknown. *In vivo* and *in vitro* studies suggest that phenytoin may be involved in the healing process at different stages of fibroblastic proliferation, initiation, glycosaminoglycans and proteoglycans deposition, wound remodeling and vascularization, decreasing collagenase activity (by reducing its production or secretion or both), promoting deposition of collagen, elastin and reticulin components, decreasing bacterial contamination and shortening the inflammatory phase and resulting decreased wound exudate (McAnally and Thompson, 1992; Anstead *et al*., 1996; Talas *et al*., 1999). The inhibition of collagen breakdown may play a significant role in preservation of the architecture of the injured skin treated parenterally with phenytoin by preventing damage to some of the old fibrils. The continuing presence of the original aligned fibrils would then enhance the structural organization of the newly formed small diameter fibrils by providing a template for their growth (Oryan, 1995). It is also possible that phenytoin is in some way involved in the aggregation of collagen into larger diameter fibrils. Thus, if the lesions are studied few weeks later both preservation of existing and enhanced development of new, large diameter, fibrils may contribute to the greater tensile strength of the treated lesions in future. Shafer (1961) investigated the stimulatory effect of phenytoin sodium on cell lines from normal and neoplastic tissues of fibroblastic origin and showed that phenytoin increased proliferation 50-90% in 2 fibroblast cell lines, but had no effect on cells derived from heart, kidney, lung, intestine, squamous epithelium, or 4 of 5 tumor cell lines. Vijayasingham *et al.* (1991) found no stimulation of proliferation of human dermal fibroblasts or epidermal keratinocytes in culture. They proposed that phenytoin may act indirectly *in vivo* on keratinocytes by affecting membrane transport of cations, which in turn alters cytokine and growth-factor activities that affect inflammatory cells. Alternatively, it is possible that only certain subgroups of functionally-distinct fibroblasts respond to phenytoin. Phenytoin also reduced collagenase activity, not by direct enzyme inhibition, but by decreasing the synthesis centrally via the pituitary-adrenal axis or by competitive antagonism of the glucocorticoid receptor (Anstead *et al*., 1996).

Anti bacterial activity of phenytoin is another important factor that is reported to contribute in improving wound healing. A number of clinical studies indicate that phenytoin decreases the bacterial load of wounds (Modaghegh *et al*., 1989; Muthukumarasamy *et al*., 1991). Topical phenytoin is reported to eliminate *Staphylococcus aureus*, *E. coli*, *Klebsiella* sp. and *Pseudomonas* sp. from wounds within 7-9 days (Modaghegh *et al*., 1989). It is unknown if phenytoin has intrinsic antibacterial activity, or if the effect of phenytoin on the bacterial load of wounds is mediated indirectly by effects on inflammatory cells and neovascularization (Modaghegh *et al*., 1989). In this regard, this feature seems to be advantageous in clinically chronic wounds especially in dirty or infected ones. In our study the wounds were clean (incised under sterile condition and bandaged) and acute, so antibacterial effect of this agent probably had the least effects on the process. Nevertheless, there was not any bacterial load assessment or comparison between groups, so judgment on the antibacterial effect of phenytoin is not possible.

Local pain relief has also been observed with topical phenytoin therapy, which can be explained by its membrane-stabilizing action; the reduced inflammatory response may also contribute (Anstead *et al*., 1996; Rhodes *et al*., 2001). We used Carprofen as a sedative agent to prevent pain and discomfort during the first 3 days of experiment and local administration of phenytoin can enhance this effect. Nonetheless, we used carprofen in all groups to have the least differences between groups.

CONCLUSION

Both of the parenteral and topical application of phenytoin seems to have negligible positive effects on early stages of wound healing in experimentally induced

cutaneous wound healing in rabbits. However, the parenteral route resulted in a better tissue alignment, collagen fibrils differentiation and maturation, possibly if the animals were left for a longer period of time the mechanical properties of the treated tissues will be improved. This can be concluded subjectively, mostly on the EM and biomechanical results. Concentration and the time of application considered to be the most important factors affecting the results. Prolonged or delayed use may be more useful, regardless of the technique of administration, particularly in chronic or infected wounds. Lower doses, although with unknown mechanisms, seem to be more effective, but further experimental studies on longer term changes after treatment with parenteral route of phenytoin will be required to confirm this point.

REFERENCES

Anstead, G.M., L.M. Hart, J.F. Sunahara and M.E. Liter, 1996. Phenytoin in wound healing. Ann. Pharmacotherl., 30: 768-775. PMID: 8826558.

Eubanks, L.E., E.I. McBurney, W. Galen and R. Reed, 1996. Linear scleroderma in children. Int. J. Dermatol. 35: 330-336. DOI: 10.1111/j.1365-4362.1996.tb.03633.x. PMID: 8734654.

Fine, J.D. and L. Johnson, 1988. Efficacy of systemic phenytoin in the treatment of junctional epidermolysis bullosa. Arch. Dermatol., 124 (9): 1402-1406. PMID: 3415285.

Jensen, T.S., 2002. Anticonvulsants in neuropathic pain: Rationale and clinical evidence. Eur. J. Pain, 6: 61-68. DOI: 10.1053/eujp.2001.0324. PMID: 11888243.

Jessop, S., D. Whitelaw and F. Jordaan, 2001. Drugs for discoid lupus erythematosus (Review). Cochrane Database Sys. Rev., 1: CD002954. DOI: 10.1002/14651858.CD002954. PMID: 11289785.

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol., 27: 137.

Kimball, O.P. and T.N. Horan, 1939. The use of Dilantin in the treatment of epilepsy. Ann. Int. Med., 13: 787-793.

Margolis, D.J. and V.L. Lewis, 1995. A literature assessment of the use of miscellaneous topical agents, growth factors and skin equivalents for the treatment of pressure ulcers. Dermatol. Surg., 21 (2): 145-8. DOI: Wiley.com/10.1002/term.101. PMID: 7894932.

McAnally, L.E.A. and D. Thompson, 1992. Use of phenytoin for wound healing. Hosp. Pharm., 27: 649-650.

Modaghegh, S., B. Salehian, M. Tavassoli, A. Djamshidi and A. Shiekh Rezai, 1989. Use of phenytoin in healing of war-and non-war wounds. Int. J. Dermatol., 28: 347-350. DOI: 10.1111/j.1365-4362.1989.tb01363.x. PMID: 2666326.

Moon, D.K., S.L.Y. Woo, Y. Takakura, M.T. Gabriel and S.D. Abramowitch, 2006. The effects of refreezing on the viscoelastic and tensile properties of ligaments. J. Biomech., 39: 1153-1157. DOI: 10.1016/j.jbiomech.2005.02.012. PMID: 16549103.

Moy, L.S., E.M.L. Tan, R. Holness and J. Uitto, 1985. Phenytoin modulates connective tissue metabolism and cell proliferation in human skin fibroblast cultures. Arch. Dermatol., 121: 79-83. PMID: 2981518.

Muthukumarasamy, M.G., G. Sivakumar and G. Manoharan, 1991. Topical phenytoin in diabetic foot ulcers. Diabetes Care, 14: 909-911. PMID: 1773690.

Oluwatosin, O.M., J.K. Olabanji, O.A. Oluwatosin, L.A. Tijani and H.U. Onyechi, 2000. A comparison of topical honey and phenytoin in the treatment of chronic leg ulcers. Afr. J. Med. Med. Sci., 29: 31-34. PMID: 11379464.

Oryan, A., 1995. Induction of soft connective tissue formation by various prosthetic implants. Transplant Proc. A., 27: 2754-2755. PMID: 7482902.

Oryan, A., A. Khalafi-Nezhad, N. Toloo and M.N. Soltani Rad, 2007. Effects of 4-Chloro-2, 6-bis (2-hydroxyl-benzyl) phenol on healing of skin wounds and growth of Bacteria. J. Vet. Med. A., 54: 585-591. DOI: 10.1111/j.1439-0442.2007.00984.x. PMID: 18045344.

Oryan, A. and S.R. Zaker, 1988. Effects of Topical Application of Honey on Cutaneous Wound Healing in Rabbits. J. Vet. Med. A., 45: 181-188.

Rhodes, R.S., C.A. Heyneman, V.L. Culbertson, S.E. Wilson and H.M. Phatak, 2001. Topical phenytoin treatment of stage II decubitus ulcers in the elderly. Ann. Pharmacother., 35: 675-681. DOI: 10.1345/aph.10229. PMID: 1408983.

Riemersma, D.R. and H.C. Schamhardt, 1982. The cryo-jaw, a clamp designed for *in vitro* rheology studies of horse digital flexor tendons. J. Biomech., 15: 619-620. PMID: 7142228.

Rodriguez-Castellanos, L.A., J.B. Rubio, J.F.B. Gomez and A.G. Mendoza, 1995. Phenytoin in the treatment of discoid lupus erythematosus. Arch. Dermatol., 131: 620-621. PMID: 7741559.

Shafer, W.G., 1961. Effect of Dilantin sodium on various cell lines in tissue culture. Proc. Soc. Exp. Biol. Med., 108: 694-696. PMID: 13911106.

Simpson, G.M., E. Kunz and J. Sulafat, 1965. Use of diphenylhydantoin in treatment of leg ulcers. N.Y. State J. Med., 65: 886-888. PMID: 14265330.

Talas, G., T.S. Adams, M. Eastwood, G. Rubio and R.A. Brown, 1997. Phenytoin reduces the contraction of recessive dystrophic epidermolysis bullosa fibroblast populated collagen gels. Int. J. Biochem. Cell Biol., 29: 261-270. DOI: 10.1016/S1357-2725(96) 00132-X. PMID: 9076961.

Talas, G., R.A. Brown and D.A. McGrouther, 1999. Role of phenytoin in wound healing-a wound pharmacology perspective. Biochem. Pharmacol., 57 (10): 1085-1094. DOI:10.1016/S0006-2952(98)00363-3.PMID:11265677.

Vijayasingham, S.M., P.J. Dykes and R. Marks, 1991. Phenytoin has little effect on *in vitro* models of wound healing. Br. J. Dermatol., 125: 136-139. DOI: 10.1111/j.1365-2133.1990.tb04410. PMID: 1911296.

Wiffen, P., H. McQuay, D. Carroll, A. Jadad and A. Moore, 2000. Anticonvulsant drugs for acute and chronic pain. Cochrane Database Sys. Rev. CD001133. DOI: 10.1002/14651858.CD001133.pub2. PMID: 10796752.