Considering Heal Promoters in Estimating Wound Age in Rats

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Abstract. Wound healing is a great issue that is very important in forensic medicine with its great medicolegal importance in crimes, through understanding a wound characters and estimating its age, the wound can be determined if it is ante-mortem or post-mortem. In this study, fifty-four male albino rats (eight weeks old, 250-300gm) were grouped into three groups and used for studying the forenc aspect of wound and wound healing using products from natural origin (Panthenol and Contractubex) in loose and ischemic wounds. Group I kept as positive control (wound surface moistured topically by saline only), group II (Panthenol group, wound was topically covered with a thin film of Panthenol) and group III (Contractubex group, wound was topically covered with thin film of Contractubex) daily for three weeks. The site of wound was monitored macroscopically during this period. Histopathological examination of the skin was done to examine the healing process and determines the effects of Panthenol and Contractubex. Also, vascular endothelial growth factor (VEGF) was used as a parameter for the healing process. Results revealed that Panthenol and Contractubex accelerated wound healing process compared with control groups; the effect was more marked in Panthenol group. Using of these products may mislead the forensic decision about estimating the wound age.

Key words: forensic, wound healing, loosing wound, ischemic wound, VEGF, Panthenol, Contractubex.

Introduction

Impaired the skin integrity leads to the progress of a wound that could include tissues from the epidermis to deeper layers (Gorecki et al., 2010: 1525-1534). The integrity of healthy skin plays an important role in retaining body physiological homeostasis. Although wound healing cascade and specific cell functions in wound repair mechanisms have been defined, many underlying pathophysiological actions are still unobvious and it could be possible to invent new and effective therapies for wound healing if we understand better this multifaceted relationship (Morton and Phillips, 2016: 589-605).

Wound is of great medico legal importance in forensic medicine and crimes, the wound can be determined if it is ante-mortem or post-mortem through determining its characters and estimating its age (Ohshima, 2000: 153-164). The pathophysiology of the wounds and healing events became the focus of the forensic investigation (Grellner and Madea, 2007: 150-154). Determining the age of a wound can lead to arrest of suspects. Forensic scholars have tended to focus on evaluating wound vitality and determining the time elapsed since the wound was occurred (Na et al., 2018: 1-10).

The variety of wound types has resulted in a wide range of wound dressings with new products frequently introduced to target different aspects of the wound healing process. The ideal dressing should achieve rapid healing at reasonable cost with minimal

inconvenience to the injured (Vairamon and Mary, 2009: 273-279). Ischemic wound has been defined as cellular injury resulting from the reperfusion of blood to a previously ischemic tissue (Pretto, 1991: 1912-1914).

When a tissue has been depleted of its blood supply for a significant time, the tissue may reduce its metabolism to preserve function (Kubes, 1995: 235-244). Vascular endothelial growth factor (VGEF) is an angiogenesis factor of major importance for skin vascularization (Detmar, 1994: 1141-1146).

VEGF plays an important role in mediating angiogenesis during development, as well as in a number of inflammatory and neoplastic diseases that are associated with revascularization (Dvorak, 1995: 1029-1039). In healing wounds and in other skin diseases characterized by enhanced angiogenesis and targeted overexpression of VEGF in the epidermis of transgenic mice resulted in enhanced skin vascularization with increased numbers of tortuous and leaky blood vessels (Brown et al., 1992: 1375-1379).

As wound healing research involving phyto-chemical and naturally derived substances progresses (Vermeulen et al., 2004). Naturally derived unpurified extracts are a complex mixture of many different chemicals that can individually have antioxidant, anti-inflammatory, antigenic and cell synthesis-modulating properties. This can lead to a myriad of beneficial effects over a single extract (Eshghi et al., 2010: 647). Panthenol is an alcohol derivative of pantothenic acid, a constituent of the vitamins B complex and an important element of a normally functioning epithelium and it is established in every living cell and has antioxidant and anti-inflammatory activities (Wan et al., 2016: 1757-1763).

Panthenol has high local concentrations and good penetration, it is used in many topical medications, such as ointments, gel and lotions for treatment of dermatological disorders to relieve itching or stimulate healing. Dermatological effects of the local use of Panthenol contained improved fibroblast proliferation and enhanced re-epithelialization in wound healing. Furthermore, it acts as a topical moisturizer, protectant and has revealed anti-inflammatory characters (Biro et al., 2003: 80-84).

Onion extract preparation in the form of onion juice showed anti-inflammatory properties due to thiosulfates (Dorsch et al., 1990: 39-42). The onion extract in the form of oil had an antimicrobial activity against Gram-positive bacteria (Zohri et al., 1995: 167-172). Contractubex (MerzPharma, Frankfurt, Germany) (CTBX) is a gel comprising principally Allium cepae (onion) extract as well as 50 IU sodium heparin and 1% allantoin (Hosnuter et al., 2007: 251-254). The water soluble gel base mediates the deep penetration of the active ingredient into the skin, there by affording an intensive local try to use it in wound healing process.

Describing the critical process underlying wound healing using animal model with natural products to accelerate the healing process could misguide the forensic doctor on estimating the wound age. Therefore, the aim of the present study is to estimate medico legal aspects of wound healing process with and without presence of medications from natural origin (Panthenol and Contractubex) in two types of wounds (loose and ischemic).

Materials and methods

Experimental animals

Fifty-four male albino rats (eight weeks old, with average body weight 250-300g). They were obtained from the animal house lab, Faculty of veterinary medicine, Suez Canal University, Ismailia. The animals kept three weeks for adaption prior to study. Animals were clinically examined specially for their skin and hair and all were apparently healthy and had normal skin and hair. Animals were housed and maintained on standard ration (72%corn, 72% soya bean and 1% fish meal) they kept with feed and water *ad*

libitum. The surgical interventions were carried out under aseptic conditions using ketamine / Xylazine anesthesia (50 mg/kg body weight ketamine and 6 mg/kg body weight xylazine) intra-peritoneal (Hall et al., 2001: 341-366). The skin area had been shaved prior to the experiment.

Loosing wound model

The rats were anesthetized with intra-peritoneal ketamine / Xylazine then full thickness skin loosing wound was created on the back of rats using 5mm biopsy punch under aseptic condition (Fig. 1).

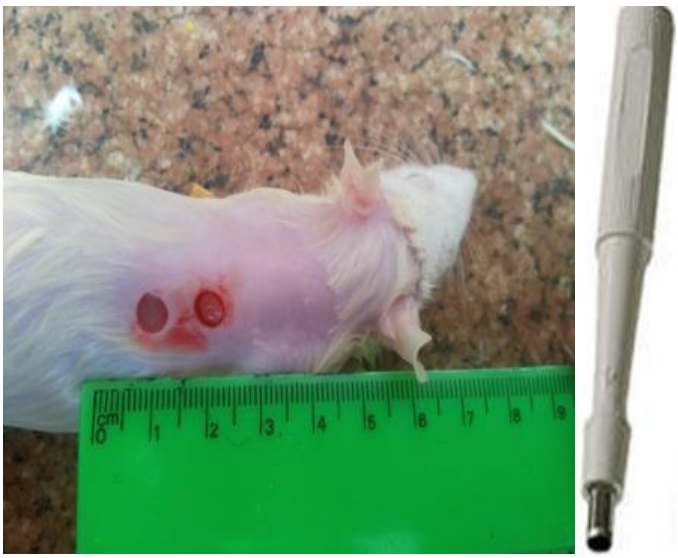

Fig. 1. Loosing wound model

Ischemic wound model

The rats were anesthetized with intra-peritoneal ketamin / Xylazine then the ischemic wound was created using a sharp scalpel. A window shape wound (U shape) was made by making three vertical lines of incised wound including full thickness of the skin. The wound dimensions were (1cm*1cm*1cm) for each line on the back, 5 cm from the ear of the anaesthetized rat (Fig. 2).

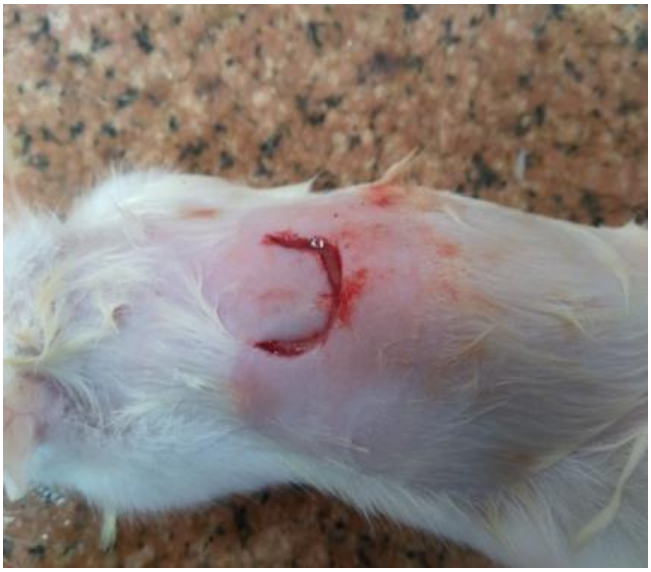

Fig. 2. Ischemic wound model

Study groups

In this study three groups of albino male rats were used for studying the forensic aspect of wound and wound healing using products from natural origin (Panthenol and Contractubex), each group has eighteen rats. They were sub-divided into two groups (nine rats for ischemic wound and the other nine rats for loose wound).

Group one was a positive control (wound surface was moistured by saline only), group two wound was covered topically with a thin film of Panthenol daily for three weeks and group three wound was covered topically with a thin film of Contractubex daily for three weeks.

Estimating wound dimensions

The histo-morphometric evaluation technique was used to calculate the wound width. This was based on the previously described techniques (Langemo et al., 2008: 42-45; Angela et al., 2011: 18). Each type of wound dimensions were estimated using a ruler weekly, for three successive weeks, the differences in the wound dimensions and degree of healing were photographed and scaled.

Histopatholgical examination

Each week, 3 rats from each group were sacrificed for histopathological examination and a piece of skin containing the wound area were obtained then fixed in 10% formaline, dehydrated in ascending grades of alcohol, cleared in xylol and embedded in paraffin. The paraffin blocks were sectioned at 5 µm. The obtained sections were stained by hemat-oxylin and eosin stain (H&E) (Bancroft and Gamble, 2008: 345-378).

Immunohistochemical staining and evaluation

Formalin fixed paraffin embedded specimens were cut into 5 µm sections and mounted on slides. After de-paraffinization, sections were heated with an autoclave in 0.1 mm-EDTA Tris/HCL buffer (pH 9.0) for 1 hour at 121^{o}C for antigen retrieval. The sections were then incubated with primary antibody at 4^{o}C overnight. This was followed by sequential 60-minute incubations with secondary antibodies (Envision+System-HRP Labelled Polymer, Dako) and visualization with the Liquid DAB + Substrate Chromogen System (Dako). All slides were evaluated blindly with an independent pathologist. Assessment of wound healing was done based on a qualitative assessment method; as previously described (Deyhimiet al., 2016: 43-48). For the following changes: epidermal cell proliferation, amount of collagen, vascularity, and amount of inflammatory cells.

Statistical analysis

Appropriate analysis according to (Steel and Torrie, 1981: 234-245) using the one-way analysis of variance (ANOVA) was done to analyze the wound area during three weeks of treatment followed by post hook Fishers. Computer program software (SPSS) was used for data analysis the data. Data were considered significant at ($P \leq 0.05$).

Results

Macroscopic findings of wound healing

Loosing wound model

Results of macroscopic appearance and wound areas (mm^2) during three weeks of treatment in the three groups were showed in Table 1 and Table 2, in Fig. 3 and Fig. 4.

At zero day: the wound area was almost the same at the three groups, after one week: of treatment there was significant decrease in wound area (mm^2) in Panthenol group, while the decrease in case of Contractubex was not significant compared with positive control group, after two weeks: the Panthenol caused significant decrease in

wound area and after three weeks: there was no significant difference between the wound areas in all groups.

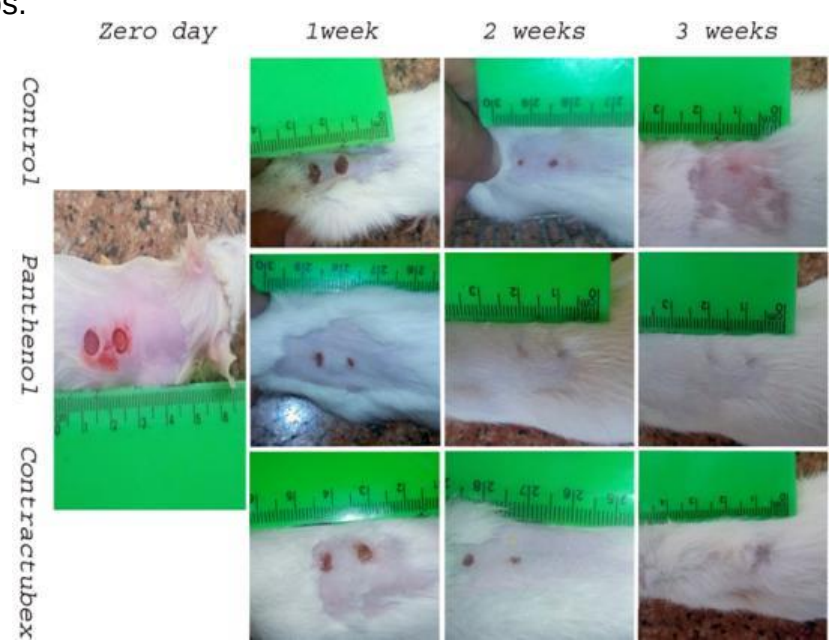

Fig. 3. Macroscopic appearance of loosing wound model in different healing stages in rats

Table 1. Loosing wound area (mm^2) for control, Panthenoland Contractubex groups during three weeks

Groups	Wound area (mm^2)			
	Weeks			
	0	1	2	3
Control	19.9±0.15[a]	17.5±0.61[a]	4.77±0.32[a]	0.80±0.46[a]
Panthenol	20.0±0.24[a]	13.4±0.61[b]	2.53±0.50[b]	0.13±0.13[a]
Contractubex	19.8±0.32[a]	15.9±0.31[a]	5.00±0.31[a]	0.30±0.15[a]

Table 2. Loosing wound contraction % for control, Panthenol and Contractubex groups during three weeks

Groups	Wound Contraction (%)			
	Weeks			
	0	1	2	3
Control	0	11.7	76.0	96.0
Panthenol	0	32.9	87.3	99.3
Contractubex	0	19.8	75.0	98.5

***Wound contraction % = (area at first day-area at biopsy day) / (area on first day) × 100

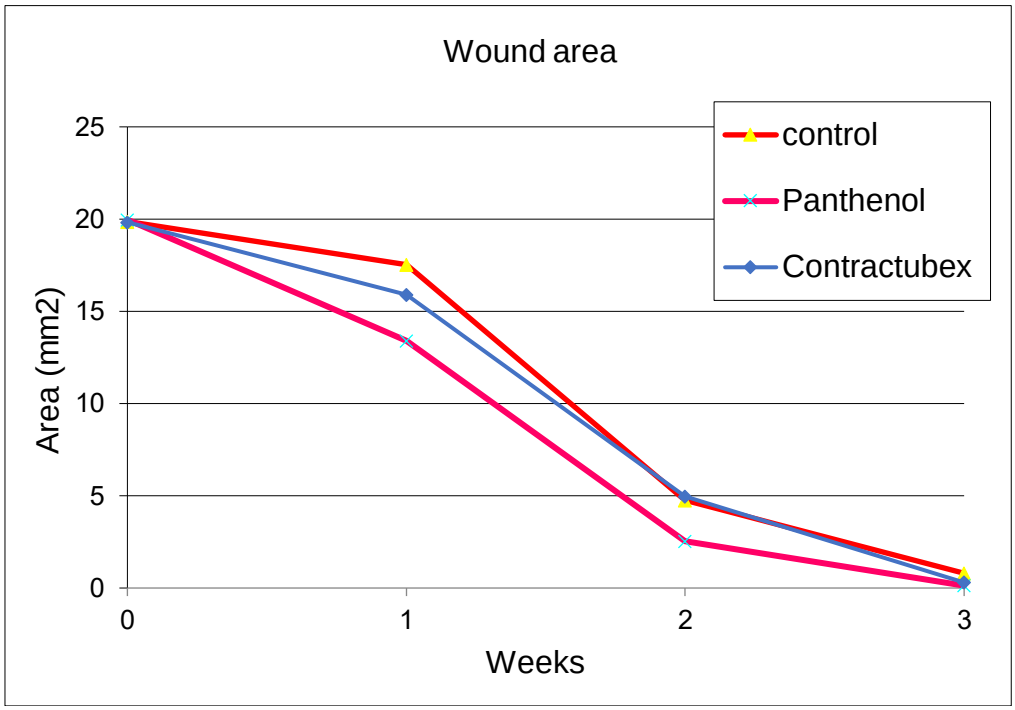

Fig. 4. Loosing wound area (mm2) for control, Panthenol and Contractubex groups during three weeks

Ischemic wound model

Regarding macroscopic appearance and wound areas (mm^2) in the three groups during three weeks of treatment were showed in Table 3 and Table 4, in Fig. 5 and Fig. 6. At zero day: the wound area was the same at the three groups, after one week of treatment: there was significant decrease in wound areas (mm^2) in both Panthenol and Contractubex groups compared with positive control group while, there was no significant difference between effects of Panthenol and Contractubex on wound area, after two weeks: there were significant differences in wound areas between the three groups. The decrease in wound area was marked in Panthenol group than in Contractubex group compared with positive control group and after three weeks: there was significant decrease in wound areas (mm^2) in both Panthenol and Contractubex groups compared with positive control group but there was no significant difference between Panthenol and Contractubex groups.

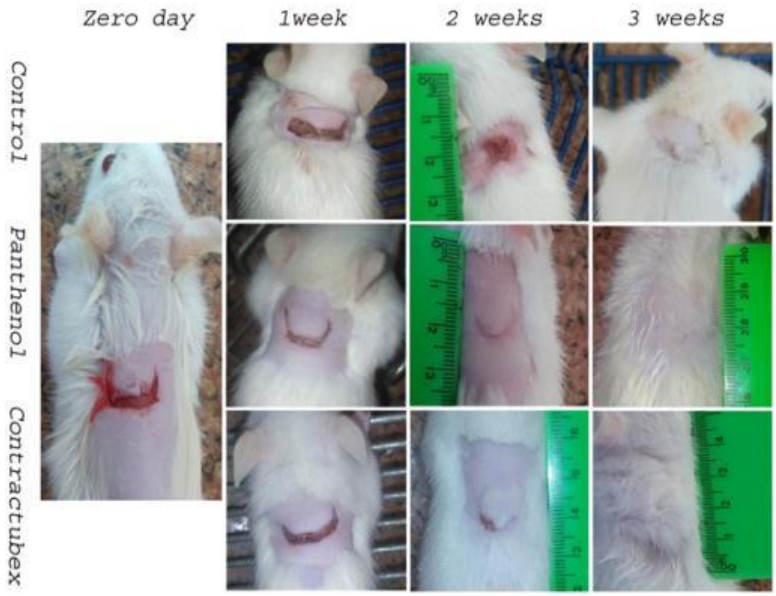

Fig. 5. Macroscopic appearance of ischemic wound model in different healing stages in rats

Table 3. Ischemic wound area (mm2) for control, Panthenol and Contractubex groups during three weeks.

Groups	Wound area (mm^2)			
	Weeks			
	0	1	2	3
Control	38.7±1.99[a]	41.7±2.47[a]	26.1±2.38[a]	7.90±2.32[a]
Panthenol	39.1±1.77[a]	24.6±1.83[b]	7.83±1.74[c]	1.57±1.07 [b]
Contractubex	39.1±1.75[a]	29.1±1.42[b]	16.1±0.99[b]	2.63±0.48[b]

Table 4. Ischemic wound contraction % for control, Panthenol and Contractubex groups during three weeks

Groups	Wound Contraction (%)			
	Weeks			
	0	1	2	3
Control	0	7.65	32.6	79.6
Panthenol	0	37.0	79.9	95.9
Contractubex	0	25.5	58.9	93.3

***Wound contraction % = (area at first day-area at biopsy day) / (area on first day) × 100

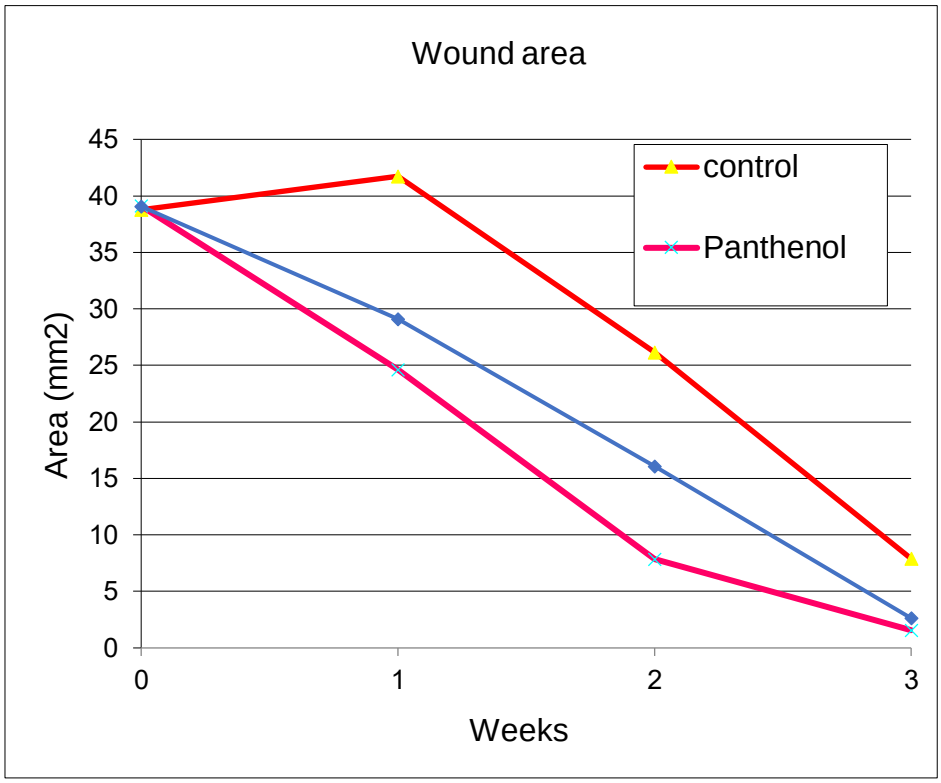

Fig. 6. Ischemic wound area (mm2) for control, Panthenol and Contractubex groups during three weeks

It was noticed that Panthenol was more effective on healing process while, hair growth was more obvious with Contractubex Fig. 7.

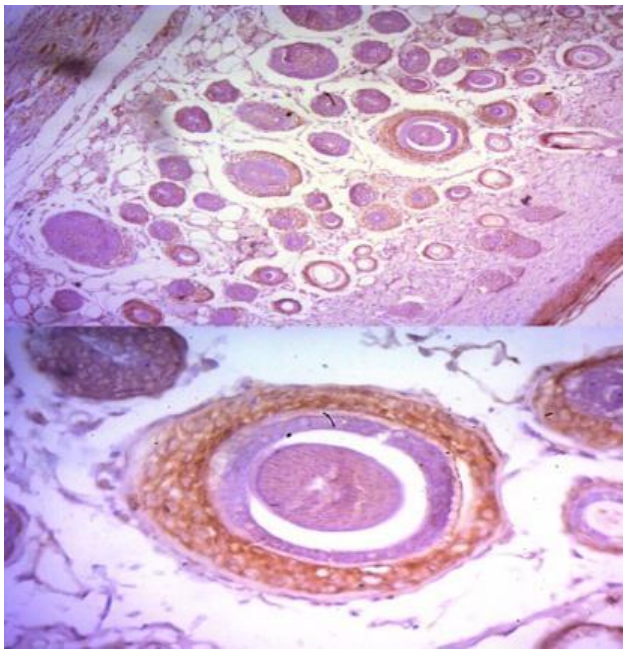

Fig. 7. Effect of Contractubex on hair growth (H&E, 10&40 xs)

Histopathological findings

Epidermal cell proliferation, amount of collagen, vascularity, and amount of inflammatory cells were examined microscopically for the three groups to determine the stages of healing process.

Positive control group

The histopathological findings of positive control group were showed in Fig. 8.

Loosing wound model

First week sections examined revealed partially denuded epidermis, covered by scab (Black arrow), with an underlying cleft between epidermis and dermis, containing cell debris and inflammatory cells (Blue arrow) and overlying granulation tissue reaction (Red arrow), second week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows) and third week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrow) with the underlying dermis showing dermal appendages (Red arrow). Healing was in the grade 1, 4 and 4 in the first, second and third weeks, respectively.

Ischemic wound model

First week Sections examined revealed partially denuded epidermis, covered by scab (Black arrow), and overlying an inflammatory tissue reaction (Red arrow), Second week sections examined revealed partially epithelized epidermis (Red arrow), covered by scab (Black arrow). The underlying dermis is partially filled with granulation tissue, with collagen (White arrow). A space cleft is still separating epidermis from underlying dermis indicating incomplete healing to dermis (Blue arrow) and third week sections examined revealed partially denuded epidermis, covered by scab (Black arrow), with abscess formation (Blue arrow) and overlying granulation tissue reaction (Red arrow). The healing was in the grade 1, 4 and 1-2 in the first, second and third weeks, respectively.

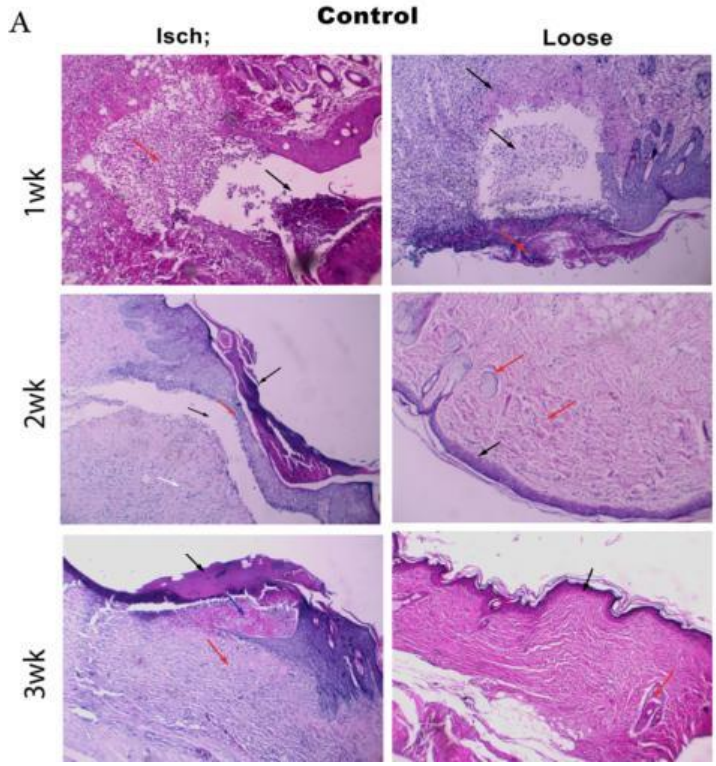

Fig. 8. Healing stages of control group in loose and ischemic wound models during three weeks (H&E, 10 xs)

Contractubex group

The histopathological findings of control group were showed in Fig. 9.

Loosing wound model

First week sections examined revealed partially denuded epidermis, (Black arrow), and overlying granulation tissue reaction (Red arrow), Second week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows)and Sections examined revealed mild keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows).The healing was in grade 1-2, 4 and 4 in the first, second and third weeks, respectively.

Ischemic wound model

First week sections examined revealed partially denuded epidermis, covered by scab (Black arrow), and overlying granulation tissue reaction (Red arrow), Second week Sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrow). And third week Sections examined revealed mild keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows). The healing was in grade 1-2, 4 and 4 in the first, second and third weeks, respectively.

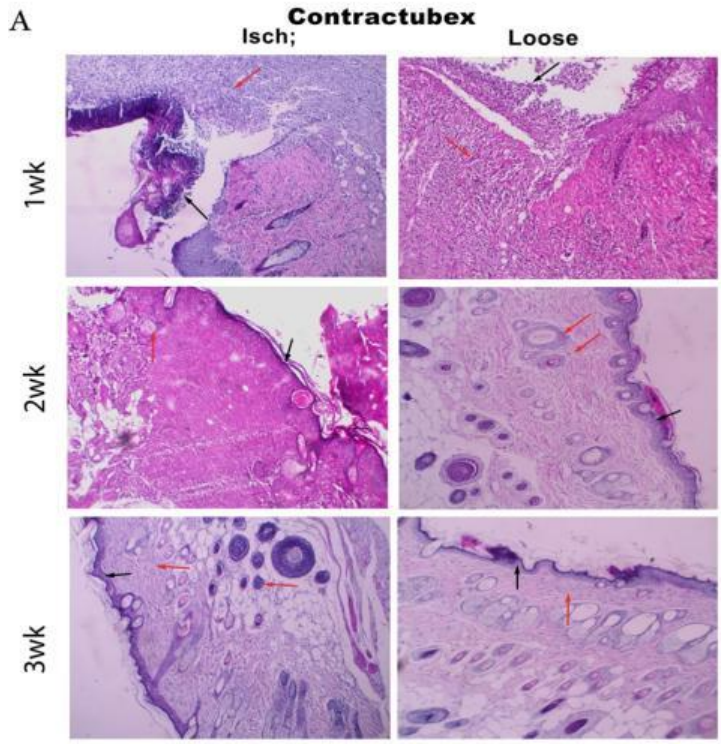

Fig. 9. Healing stages of Contractubex group in loose and ischemic wound models during three weeks (H&E, 10 xs)

Panthenol group

The histopathological findings of Panthenol group were showed in Fig. 10.

Loosing wound model

First week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows), with mild acanthosis and proliferation into the underlying dermis, forming small uniform nests lined with squamous and basal cells within dermis (Red arrows). Second week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis

showing dermal appendages (Red arrows). and third week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows) (H&E, 10x). The healing was in grade 4 for the first, second and third weeks.

Ischemic wound model

First week sections examined revealed partially epithelized epidermis (Red arrow). The underlying dermis is partially filled with granulation tissue, with collagen (White arrow). A space cleft is still separating epidermis from underlying dermis indicating incomplete healing to dermis (Blue arrow), Second week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows), and third week sections examined revealed keratinizing uniform stratified squamous epidermal covering (Black arrows) with the underlying dermis showing dermal appendages (Red arrows). The healing was in 2, 4 and 4 in the first, second and third week, respectively.

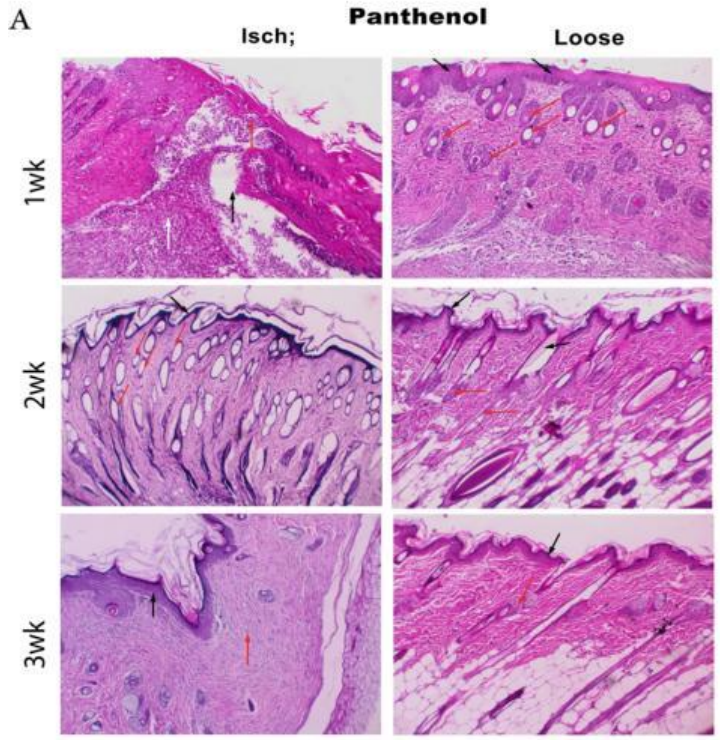

Fig.10. Healing stages of Panthenol group in loose and ischemic wound models during three weeks (H&E, 10 xs)

Immuonohistochemistryfindnigs (VEGF)

The results indicated that the VEGF reaction was moderate at the first week in both wound models in control group while at third week it was weak in both wound models, The reaction was strong at the first week in both types of wound in Panthenol group while at the third week it was negative in both types of wound, the reaction was moderate in two types of wound at the first week in Contractubex group while at the third week it was weak in loose wound and negative in ischemic wound (Fig. 11, Fig. 12, Fig. 13).

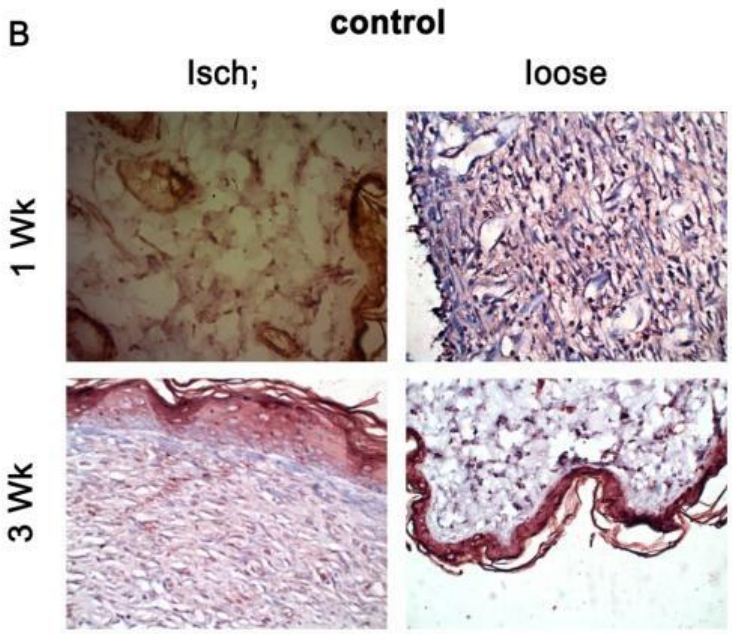

Fig. 11. The VEGF reactions in control group at first and third weeks

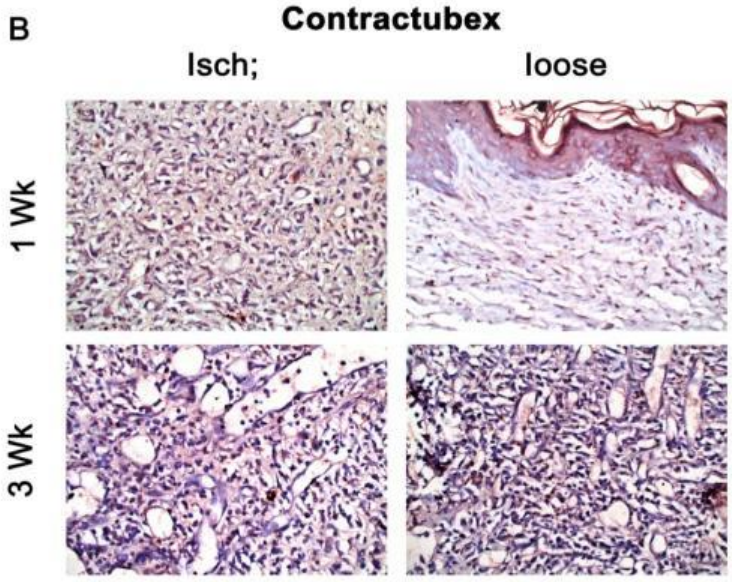

Fig. 12. The VEGF reactions in Contractubex group at first and third weeks

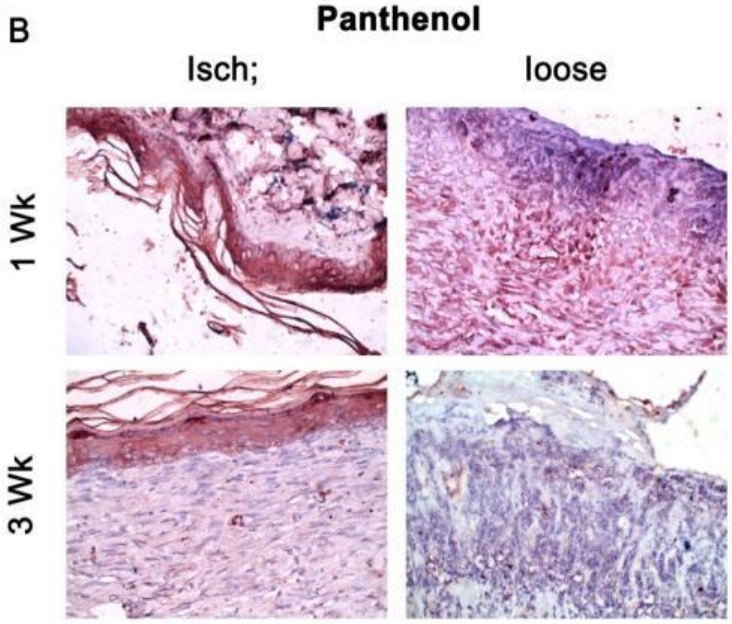

Fig. 13. The VEGF reactions in Panthenol group at first and third weeks

Discussion

In this study the wound healing model provides an in vivo method for studying the healing process of wounds in domestic animals. The dorsal surface of the rat appears to be a proper model for the wound healing study and we have used this model because of its simplicity. The approaches in this study were related to those in preceding experimental and clinical wound studies (Hosnuter et al., 2004: 121-125; Han et al., 2005: 624-627; Durmus et al., 2009: 135-139; Yaman et al., 2010: 619-624).

In the present study, the effects of Contractubex (CTBX) and Panthenol on wound healing of two types of wounds (loosing and ischemic) in rats were evaluated. Although a meaningful healing was observed with the individual application of CTBX and Panthenol, the effect of the Panthenol was more pronounced than CTBX. These results may be mainly related to their anti-inflammatory and anti-proliferative effects.

The findings in this study using CTBX were parallel to those in previous experimental and clinical wound studies that carried out (Maragakis et al., 1995: 199-206; Dollery, 1999: 765-769). It was reported that the efficacy of heparin in minimizing the duration of the wound healing process, promoting the erythema and resulted in smooth healed skin without scars or contractures could be shown in clinical investigations.

Heparin encourages capillary endothelial cells to migrate into ischemic tissues where they proliferate and form new capillary blood vessel systems which, on perfusion with blood, renovate blood stream into the ischemic tissues (Saliba, 2001: 349-258; Venakatachalapathy et al., 2007: 189-198). Heparin was mentioned to primarily speed up collagen production and deposition, and in the second phase slowed and reabsorbed collagen, which would tend to inhibit fibrin accumulation and formation of a scar (Venakatachalapathy et al., 2007: 189-198). These findings came with agreement with ours where CTBX treated rat group showed rapid healing in ischemic wounded area than loose one with smooth new skin and rapid hair growth after 3 weeks (Zohri et al., 1995: 167-172). Allantoin is a supporting agent with its supportive properties on primary and secondary wound healing progression. The mechanism of action involves blocking excessive connective tissue synthesis and therefore preventing the progression (Koc et

al., 2008: 1507-1514; Karagoz et al., 2009: 1097-1103; Temiz et al., 2009: 387-392; Muangman, 2011: 442-446).

On the other hand, DexPanthenol (DEX) is an alcoholic analog of pantothenic acid, a member of the B complex vitamins (vitamin B5). It is enzymatically oxidized to pantothenic acid, which is widely distributed in tissues as coenzyme A (Ebner et al., 2002: 427-433).

Histopathological observations were found to be parallel to each other. Within the skin, dexPanthenol (pro-vitamin B5) is broke down into pantothenic acid (vitamin B5), which is important for the normal active epithelial cells, particularly during the first phase of epithelial regeneration (during the first - four days). These results came in agreement with our histopathological findings which were in accordance with the immune-histochemical results eventually as healing process was enhanced in rats treated with Panthenol than Contractubex and control groups (Wong, 2013: 2933-2948).

In the present the results revealed that the Panthenol group showed partially epithelized epidermis in first week that turned to keratinizing uniform of stratified squamous epithelium at the third week and this came in accordance to the positive role of DexPanthenol on wound healing and epithelization (Raczynska, 2003: 175-178).

The Contractubex group was found histologically to show mild immune-reactivities of laminin, fibronectin, and VGEF as well as produce less histological evidence of Scarring and this was in agreement with our results that showed moderate to weak immunoreactions (Sahin et al., 2012: 74-81).

VEGF provides arteriogenesis, lumen expansion, and collateral vessel formation. Angiogenesis and functional vessels play a critical role in wound healing and granulation formation. VEGF is essential for early vessel formation and angiogenesis. VEGF level (which is a marker of angiogenesis) is significantly higher and its involvement in tissue increases. VEGF expression has been observed to increase in healing of acute cutaneous wound healing, this was in agreement with the present results in the immune-histochemical examination conducted after 7-day treatment, and VEGF immunostaining in the skin of the dermis region was more significant in the Panthenol group compared to the Contractubex group. This suggests that Panthenol increases angiogenesis and thus presents more effectiveness in wound healing. VEGF expression has been reported to decrease in wounds that were almost closed (Biçer et al., 2016: 757-765).

Medico-legal injury report and scientific inferences drawn on its basis form a strong foundation for legal interpretation. With rapid growth in transportation, victims receive immediate treatment and leads to healing of such wounds. But many times routine examination fails to differentiate the manner, especially if the wound is in the stage of healing (Garg et al., 2009: 2). So, interfering time of normal wound healing process by using such treatment will cause misleading in forensic medical inspection and will result in a fake medico legal report.

Conclusion

Panthenol and Contractubex have been shown to improve wound healing. This study showed that the effects of Contractubex on wound healing are comparable to those of Panthenol. From the obtained results we could conclude that topical application of Panthenol and Contractubex accelerate wound healing process in both Ischemic and loose skin wounds, which may misguide the forensic doctor in estimating wound age. It was noticed that Panthenol was more effective on the healing process of the wound than Contractubex and positive control groups while, hair growth was more obvious with Contractubex. VEGF has very important role in healing process.

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