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An experimental study for assessing the wound healing potential and for calculating the median effective concentration (EC_{50}) of deferoxamine in diabetic wistar albino rats

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Abstract
The scientific literature shows that deferoxamine (DFO) has the potential to accelerate the healing process both in diabetic and non-diabetic condition. So, in the present study, we have evaluated the effect of different concentrations (0.01%, 0.05%, 0.25% and 1.25%) of DFO on percent wound contraction for assessing its healing potential, and for calculating its median effective concentration (EC_{50}). Diabetes was induced in rats by injecting streptozotocin. An open excision type wound, of area 2 x 2 cm^2, was created on the dorsal thoracic region under ketamine and xylazine combination anaesthesia. Ointments of different DFO concentrations, were applied topically on the wound, twice daily, for 7 days. In control group, only ointment base was applied. Wounds were photographed and its area was measured on days 0, 3, 5 and 7 post-wounding for assessing the healing potential. After sacrificing rats on day 7, the granulation / healing tissue was collected for assessment of hydroxyproline and glucosamine levels. For calculating the EC_{50} of DFO, 7^{th} day wound contraction data was utilised to generate a dose response curve by inverse linear regression analysis. Grossly the wound size was progressively reduced in DFO-treated groups compared to control group. DFO-treated groups showed higher per cent wound contraction compared to control group. Further, DFO-treated groups showed higher content of hydroxyproline and glucosamine in granulation tissue compared to control group. The dose response curve generated by inverse linear regression analysis revealed that the EC_{50} of DFO is 0.04%. In conclusion, in the present study, a greater wound contraction, considerable reduction in wound size and significantly higher content of hydroxyproline and glucosamine levels in DFO-treated groups compared to control group suggests that DFO have accelerated the diabetic wound healing process by promoting collagen synthesis and deposition in ECM.

Keywords: Diabetic rats, cutaneous wound healing, deferoxamine, EC_{50}

1. Introduction
Many cytokines, growth factors and enzymes are involved in initiating and directing the phases of wound-healing to complete normal tissue repair after damage (Singer and Clark, 1999) [1]. However, there are many factors that can affect healing process by interfering the one or more phases, thus causing improper or delayed tissue repair. One such factor is the diabetes, which affects the healing process by defective immune cell responses or impaired cellular recruitment within the injured site. In individuals with diabetes mellitus, the rate of wound repair is slow due to reduced migration and proliferation of keratinocytes and fibroblasts (Gibran *et al*., 2002) [2], decrease production of growth factors, prolonged inflammation, diminished angiogenic response (Falanga, 2005) [3], alteration in macrophage function, less collagen deposition and delayed granulation tissue formation (Snyder, 2005) [4], increased levels of proteinases and reduction in ECM deposition and its remodelling (Lobmann *et al*., 2002) [5].
Preclinical studies have demonstrated that DFO topically applied improved wound healing in diabetic mice with increased granulation tissue and neovascularization (Glotzbach *et al*., 2010; Ram *et al*., 2016) [6, 7]. Moreover, DFO (transdermal patch) treated wounds demonstrated increased collagen density and improved neovascularization (Duscher *et al*., 2015) [8]. The available scientific literature shows that DFO have the potential to accelerate the wound healing process, both in diabetic and non-diabetic condition, by regulating the expression of certain endogenous factors involved in healing process. So, in the present study, we have

evaluated the effect of different concentrations of DFO on percent wound contraction for assessing its healing potential, and for calculating its median effective concentration (EC_{50}). The purpose of calculating the DFO EC_{50} is to evaluate the wound healing potential of DFO in combination with some other pharmacological agents in the future studies.

2. Materials and Methods

2.1 Experimental animals

The study was conducted in apparently healthy adult male Wistar rats (150-170g). Rats were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute (IVRI), Izatnagar (U.P.). They were housed in clean polypropylene cages with chopped wheat straw as the bedding material, and were provided free access to standard feed and water. They were maintained under standard management condition, and handled as per the Institute Animal Ethics Guidelines. Before commencement of the experiment, rats were kept in the laboratory condition for a minimum of 7 days for acclimatization.

2.2 Induction of diabetes

Diabetes was induced by injecting streptozotocin (STZ). Before inducing diabetes, rats were starved for overnight, and their fasting blood glucose level was measured using glucometer (On Call plus Blood glucose meter, ACON Lab., Sane Diago, USA). STZ, dissolved in citrate buffer solution (0.1 M, pH 4.5), was administered intraperitoneally @ 53 mg/kg b.wt. After 72 hours of administration of STZ, rats were monitored again for blood glucose level, and the rats, having more than 300 mg/dl fasting blood glucose level, were selected for further study. The diabetic rats were kept under observation for 10 days, and then wound was created.

2.3 Experimental design

In the present study, the effect of different concentrations of DFO, on percent wound contraction in diabetic rats, was evaluated for assessing its wound healing potential, and for calculating its EC_{50}. The rats were randomly divided into five groups of 6 rats each. Dose range is selected based on the previous studies including that conducted in our laboratory. The same is presented in the tabular form below (Table 1).

Table 1: Details of trial conducted for assessing healing potential, and for calculating the EC_{50} of DFO

Group	Drug	Number of rats
I	Vehicle (control)	6
II	0.01% DFO	6
III	0.05% DFO	6
IV	0.25% DFO	6
V	1.25% DFO	6

2.4 Creation of wound

The rats were anesthetized by injecting ketamine (50mg/kg) and xylazine (5mg/kg) combination intraperitoneally. An open excision type wound, of area 2 x 2 cm^2 (400 mm^2), was created on the back (dorsal thoracic region) of the rats to the depth of loose subcutaneous tissue. After recovery from anaesthesia, rats were housed individually in properly disinfected cages.

2.5 Drug preparation and application

Ointment base consisting of soft paraffin (90%), hard paraffin (5%) and lanolin (5%) was used to prepare the ointment of different DFO concentrations, and it was applied topically, twice a day, for 7 days on the wound.

2.6 Wound contraction measurements

Wound surface area was measured by tracing its contour using a transparent sheet on days 0, 3, 5 and 7 post-wounding. The area (mm^2) within the boundaries of each tracing was determined planimetrically, and compared with that of day 0 area. The results of wound measurements on various days were expressed as percent wound contraction, which is calculated by Wilson's formula as follows:

$$\% \text{ wound contraction} = \frac{\text{0 day wound area - wound area on particular day}}{\text{0 day wound area}} \text{ X } 100$$

2.7 Photographic evaluation

Wounds were photographed on days 0, 3, 5 and 7 post-wounding by using digital camera, and images were observed to assess the quality of wound healing.

2.8 Calculation of DFO EC_{50}

7th day wound contraction data was utilised to generate a dose response curve by inverse linear regression analysis. The EC50 of DFO was determined from the dose response curve.

2.9 Collection of tissue

On day 7 post-wounding, rats were euthanized for harvesting the granulation tissues for estimating hydroxyproline and glucosamine levels.

2.10 Assessment of hydroxyproline and glucosamine levels in granulation tissues

To indirectly assess the collagen and ECM deposition in the granulation tissue, pro-healing parameters like hydroxyproline and glucosamine levels were estimated in the granulation tissue by following Reddy and Enwemeka (1996) [9], and Rondle and Morgan (1955) [10] protocols respectively. The tissue samples were acid hydrolysed (50mg tissue/2ml 6N HCL) in a tube, which was tightly sealed and autoclaved at 50 pound pressure for 3 h. The hydrolysate obtained was used for estimating hydroxyproline and glucosamine levels.

3. Statistical analysis

Appropriate statistical tests were applied to analyse the data. A value of $p<0.05$ was considered statistically significant.

4. Results

4.1 Gross photographic evaluation of wound healing

Grossly the wound size was progressively reduced in time dependent manner in DFO-treated groups compared to control group. On 3rd day post-wounding, there was no considerable difference in the wound size in the treatment groups compared to control group, while, on 5th day, the wound size was considerably reduced in the highest two DFO concentration groups compared to control group. However, on 7th day, the wound size was markedly reduced, in a concentration dependent manner, in all the treatment groups compared to control group. The same findings are depicted in the Fig. 1.

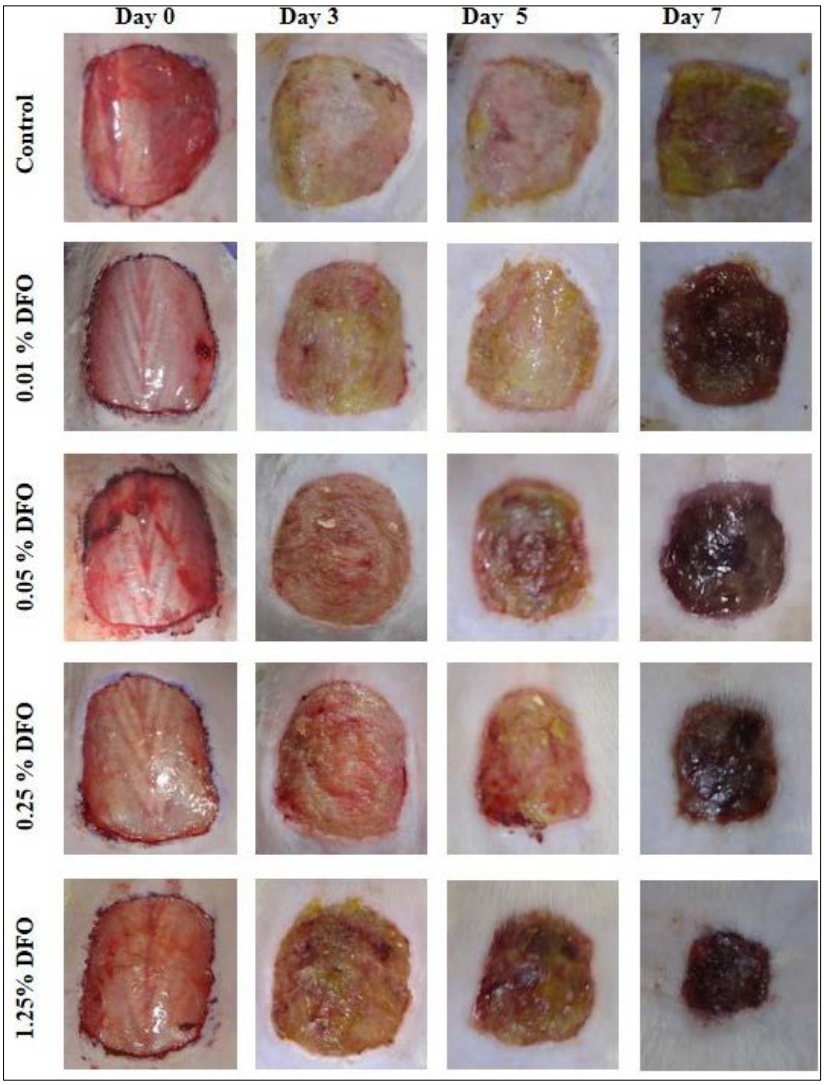

Fig 1: Representative gross images of wounds of control and DFO-treated groups on days 0, 3, 5 and 7 post-wounding.

4.2 Effect of topical application of DFO on wound contraction

As evident from the Table 2 and Fig. 2, the DFO-treated groups revealed non-significantly higher per cent wound contraction on day 3 post-wounding compared to control group**,** while, on 5th day post-wounding, 0.25% and 1.25% DFO-treated groups showed significantly higher wound contraction compared to control group. On day 7, the wound contraction was significantly increased in all the treatment groups except 0.01% DFO-treated group compared to control group. Additionally, there was significant difference in per cent wound contraction between 0.25% and 1.25% DFO-treated groups on 5th and 7th day post-wounding.

Table 2: Effect of topical application of DFO on wound contraction

Group/Day	Day 3	Day 5	Day 7
Control	1.94±0.98^{a}	19.56±1.58^{a}	38.4±3.50^{a}
0.01% DFO	2.69±1.07^{a}	21.71±2.31^{a}	44.54±2.09^{ab}
0.05% DFO	2.93±1.22^{a}	25.76±1.74^{ab}	52.50±2.75^{bc}
0.25% DFO	3.37±3.12^{a}	30.25±1.70^{b}	55.34±3.55^{c}
1.25% DFO	5.56±1.81^{a}	38.63±2.90^{c}	64.21±2.67^{d}

Data are expressed as mean± SEM (n=6). $p<0.05$, statistically significant, when compared between groups. Values bearing superscripts not in common differ significantly.

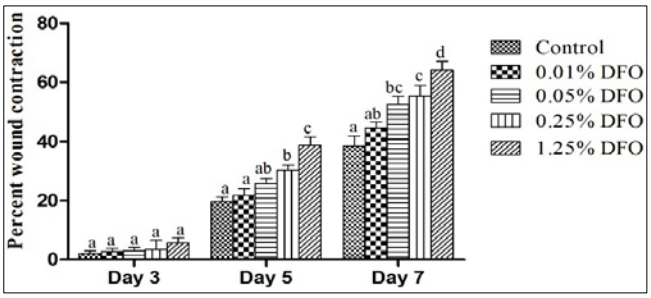

Fig 2: Per cent wound contraction in control and DFO-treated groups on different days. Data are expressed as mean± SEM (n=6). $p<0.05$, statistically significant, when compared between groups. Bars bearing superscripts not in common differ significantly.

4.3 Effect of topical application of DFO on hydroxyproline and glucosamine levels on day 7 post-wounding in granulation tissue

The results obtained revealed that DFO-treated groups showed significantly higher content of hydroxyproline in granulation tissue compared to control group in a concentration dependent manner. The highest hydroxyproline content (6.52±0.34µg/mg tissue) was found in 1.25% DFO-treated group, and lowest (2.26±0.13µg/mg tissue) was in control group (Table 3; Fig. 3). Glucosamine content in granulation tissue was significantly increased in two highest

DFO concentration-treated groups, while, it was non-significantly higher in other DFO-treated group as compared to control group. The highest glucosamine content (1.62±0.12 µg/mg tissue) was found in 1.25% DFO-treated group, and lowest (0.36±0.08 µg/mg tissue) was in control group (Table 3; Fig. 4).

Table 3: Effect of topical application of DFO on hydroxyproline and glucosamine levels on day 7 post-wounding in granulation tissue

Group	Hydroxyproline (µg/mg tissue)	Glucosamine (µg/mg tissue)
Control	2.26±0.13^{a}	0.36±0.08^{a}
0.01% DFO	3.20±0.17^{b}	0.42±0.05^{a}
0.05% DFO	3.70±0.20^{bc}	0.66±0.11^{a}
0.25% DFO	4.30±0.17^{c}	1.20±0.13^{b}
1.25% DFO	6.52±0.34^{d}	1.62±0.12^{b}

Data are expressed as mean± SEM (n=6). $p<0.05$, statistically significant, when compared between groups. Values bearing superscripts not in common differ significantly.

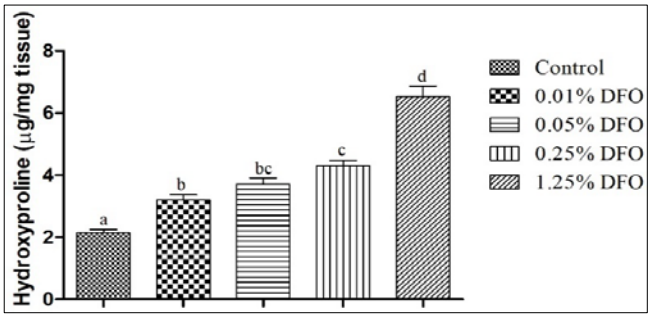

Fig 3: Effect of topical application of DFO on hydroxyproline level on day 7 post-wounding in granulation tissue. Data are expressed as mean± SEM (n=6). $p<0.05$, statistically significant, when compared between groups. Bars bearing superscripts not in common differ significantly

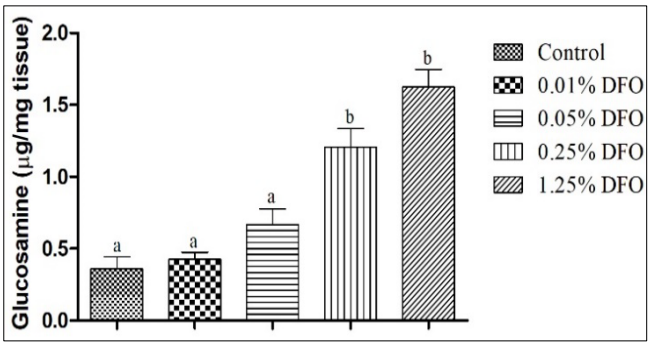

Fig 4: Effect of topical application of DFO on glucosamine level on day 7 post-wounding in granulation tissue. Data are expressed as mean± SEM (n=6). $p<0.05$, statistically significant, when compared between groups. Bars bearing superscripts not in common differ significantly

4.4. Calculation of DFO EC_{50}

7th day wound contraction data (Table 4) was utilised to generate a dose response curve by inverse linear regression analysis. The EC50 of DFO was determined from the dose response curve (Fig. 5), and it is 0.04%. The below equation was used to calculate the EC_{50}.

$Y = 4.2878\ln(x) + 63.817$

Where

Y indicates 50% response. So, accordingly we have calculated X value i.e EC50.

Table 3: 7th day wound contraction data

Group	Day 7 wound contraction
0.01% DFO	44.54±2.09
0.05% DFO	52.50±2.75
0.25% DFO	55.34±3.55
1.25% DFO	64.21±2.67

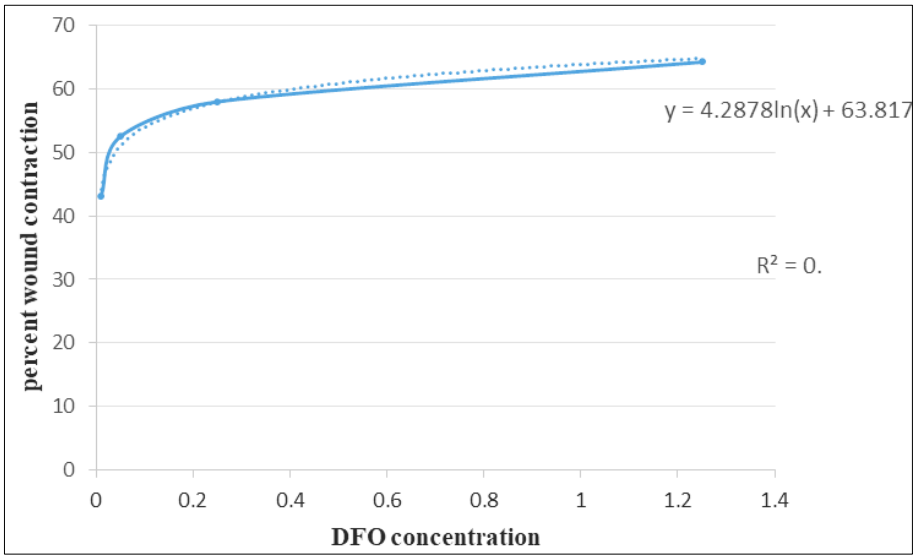

Fig 5: Dose response curve showing the effect of different concentration of DFO on wound contraction on day 7 post wounding

5. Discussion

Wound contraction is the movement of wound edges towards each other in a centripetal fashion (Tejero-Trujeque, 2001) [11]. Contraction of the wound begins soon after wounding and peaks at 2 weeks. During granulation tissue formation, fibroblasts are gradually modulated into myofibroblasts (Majno, 1979) [12]. The wound undergoes physical contraction throughout the entire wound healing process, which is believed to be mediated by contractile fibroblasts (myofibroblasts) that appear in the wound (Gosain and DiPietro, 2004; Campos *et al*., 2008) [13, 14]. Myofibroblasts are the predominant mediator of this contractile process

because of their ability to extend and retract. Fibroblasts themselves generate tractional forces *in-vitro*, possibly sufficient to initiate wound closure (Ehrlich and Rajaratnam, 1990) [15]. However, in individuals with diabetes mellitus, the rate of wound repair is slow, and high blood glucose level inhibits fibroblast migration (Xuan *et al*., 2014) [16]. In the present study in diabetic rats, we found a greater wound contraction, and also, there was considerable reduction in wound size in DFO-treated groups compared to control group. Our findings are in agreement with a recent study where it has been found that DFO accelerated diabetic wound closure on gross examination, and significantly increased per cent wound contraction on day 7 compared to control group (Ram *et al.*, 2016) [7]. Therefore, greater wound contraction and considerable reduction in wound size in DFO-treated groups suggests that DFO has the stimulatory effect on granulation tissue formation, fibroblast infiltration, transformation of fibroblasts to Myofibroblasts and increased expression of smooth muscle differentiation markers of α–smooth muscle actin, smooth muscle myosin and desmin.

During proliferative phase of healing process, fibroblasts infiltrate, proliferate and secrete the collagen into the extracellular wound environment, and its levels continue to rise for approximately 3 weeks (Stadelmann *et al*., 1998) [17]. Once in the extracellular space, collagen polymerizes into collagen fibers and covalently cross–links to increase tissue tensile strength. Hydroxyproline, a major component of the protein collagen (Szpak, 2011) [18] comprising roughly 13.5% of mammalian collagen, is an important biochemical marker for assessing the collagen synthesis and deposition in the tissue, and its higher level in the injured tissue is a positive indication of the progression of healing (Kokane *et al*., 2009; Nayak *et al*., 2011) [19, 20]. Hydroxyproline and proline play key roles for collagen stability (Nelson, 2005) [21]. They permit the sharp twisting of the collagen helix (Brinckmann *et al*., 2005) [22], thus promotes the wound contraction. The rapid production of hyaluronic acid by fibroblasts in the early stages of wound healing may be of crucial importance as hyaluronic acid stimulates the migration and mitosis of mesenchymal and epithelial cells (McCarty, 1996) [23]. Glucosamine availability appears to be rate-limiting for hyaluronic acid synthesis, thus promoting ECM deposition (McCarty, 1996) [23]. However, in diabetes, all phases of the healing process are disrupted, which leads to delayed collagen synthesis, impaired epithelialization and reduced angiogenesis during the proliferative phase of the healing process (Falanga, 2005) [3]. In the present study, there was significantly higher content of hydroxyproline and glucosamine levels in granulation tissues of DFO-treated groups compared to control group, which suggests that DFO, by stimulating the hydroxyproline and glucosamine synthesis, might have accelerated the wound healing process by promoting collagen synthesis and deposition in ECM.

6. Conclusion

Grossly the wound size was progressively reduced in a time dependent manner in DFO-treated groups compared to control group. DFO-treated groups showed significantly higher wound contraction compared to control group. Moreover, DFO treated groups showed higher content of hydroxyproline and glucosamine in granulation tissue compared to control group. Hence based on our findings, it can be concluded that greater wound contraction, considerable reduction in wound size and significant increase in hydroxyproline and glucosamine synthesis in DFO-treated groups suggests that DFO has accelerated the wound healing process by stimulating granulation tissue formation, fibroblast infiltration, transformation of fibroblasts to Myofibroblasts and promoting collagen synthesis and deposition in ECM, which is necessary for wound contraction.

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