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Experimental calculation of median effective concentration and assessment of healing potential of Hemin in diabetic wound model

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Abstract
Wound healing is an essential physiological process consisting of the collaboration of many cell strains and their products but it gets impaired and delayed in many pathological conditions, like diabetes mellitus. The aim of present study was to assess the healing potential and to calculate the EC50 of Hemin in diabetic wound model. Diabetes was induced in rats by injecting streptozotocin, and the 400 mm^2 wounds were created on the dorsal thoracic region. Ointments of different Hemin concentrations (0.05% Hemin, 0.1% Hemin, 0.5% Hemin and 2.5% Hemin) were applied topically on the wound, twice daily, for 7 days. In control group, only ointment base was applied. Wound area was photographed and measured by tracing its contour using a transparent sheet on days 0, 3, 5 and 7 post-wounding. Rats were sacrificed on day 7 post-wounding, and the granulation tissues were collected for assessing hydroxyproline and glucosamine levels. 7^{th} day wound contraction data was used to calculate EC_{50} by inverse linear regression analysis. Grossly there was considerable change in the wound size in the treatment groups compared to control group particularly from 5^{th} day onwards. Hemin-treated groups, specifically 0.5% and 2.5%, showed higher wound contraction on 5^{th} day onwards as compared to control group, and also there was increase in the levels of hydroxyproline and glucosamine in hemin-treated groups compared to control group. Further, in the present study, the experimental EC_{50} of Hemin is 0.96%. Based on our findings, it can be concluded that, in the present study, topical application of Hemin seemed to have played crucial role in improved wound contraction as well as better maturation of the healing tissue.

Keywords: Diabetic rats, Cutaneous wound healing, Hemin, EC_{50}

1. Introduction
The pathophysiologic relationship between diabetes and impaired healing is complex. The diabetic wounds get stuck in the inflammatory phase, which is characterized by continuing influx of neutrophils that release cytotoxic enzymes, free radicals and inflammatory mediators that cause extensive collateral damage to surrounding tissue. These destructive processes outbalance the healing process in such wounds and delay their repair (Falanga, 2000) [1]. Hemin, an inducer of HO-1, has a therapeutic value in conditions where higher level of HO-1 is desired like cutaneous wound healing. It has been reported that hemin through HO-1 activation enhances the wound healing process by protecting against inflammation, oxidative stress (Ndisang and Jadhav, 2009) [2], decreased angiogenesis (Grochot-Przeczek *et al.,* 2009) [3] and apoptosis of cells (Brouard *et al.,* 2000) [4]. It has been reported that hemin promotes early healing by enhancing the wound contraction and significantly increasing the hydroxyproline and glucosamine levels in granulation tissues, thus providing marked improvement in tissue tensile strength (Ahanger *et al*., 2010; Chandrashekara *et al*., 2017) [5] [6]. Bearing in mind, the wound healing potential of hemin and the impaired healing process in diabetes, the present study was planned to evaluate the cutaneous wound healing potential of different concentrations of hemin in diabetic rats, and to calculate the EC50 of hemin. The purpose of calculating the hemin EC_{50} is to conduct the combination studies with some other pharmacological agents in diabetic wound model in the future.

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.W.T. 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
The rats were randomly divided into five groups of 6 rats each. Hemin 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 experimental design

Group	Drug	Number of rats
I	Vehicle (control)	6
II	0.05% Hemin	6
III	0.1% Hemin	6
IV	0.5% Hemin	6
V	1.25% Hemin	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 ointments of different hemin concentrations, and it was applied on the wound topically, twice a day, for 7 days.

2.6 Wound contraction measurements
Wound surface area was measured on days 0, 3, 5 and 7 post-wounding by tracing its contour using a transparent sheet. 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 were expressed as percent wound contraction, which was calculated by Wilson's formula as follows:

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

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 hemin EC_{50}
The hemin EC50 was determined from the dose response curve, which was generated by inverse linear regression analysis of 7th day wound contraction data.

2.9 Collection of tissue
After euthanizing the rats on day 7 post-wounding, granulation tissues were harvested for estimating the hydroxyproline and glucosamine levels.

2.10 Assessment of hydroxyproline and glucosamine levels in granulation tissues
To indirectly assess the collagen and extracellular matrix (ECM) deposition in the granulation tissue, hydroxyproline and glucosamine levels were estimated in the granulation tissue by following Reddy and Enwemeka (1996) [7], and Rondle and Morgan (1955) [8] protocols respectively. The tissue samples were acid hydrolysed (50mg tissue/2ml 6N HCL) in a tube, and then it 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, on 3rd day post-wounding, there was no considerable change in the wound size in the treatment groups compared to control group, while, from 5th day onwards, the wound size was considerably reduced in the treatment groups particularly in 2.5% hemin-treated group. The same findings are illustrated in the Fig. 1.

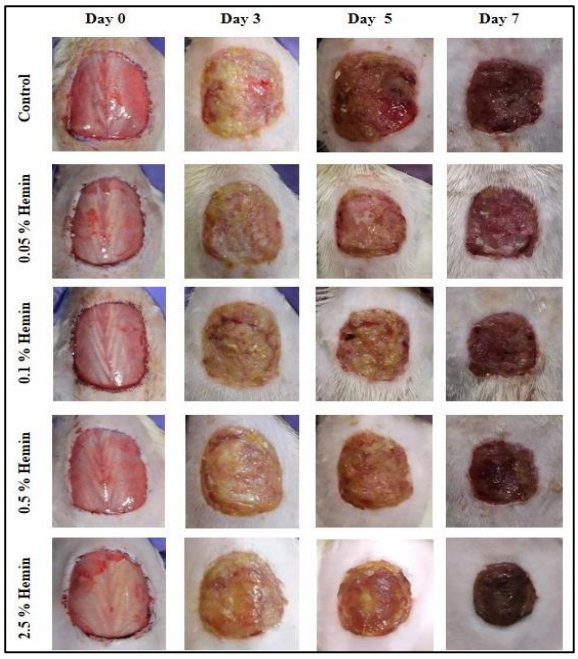

Fig 1: Representative gross images of wounds of control and Hemin-treated groups on days 0, 3, 5 and 7 post-wounding

4.2 Effect of topical application of Hemin on wound contraction

The results, presented in the Table 2 and Fig. 2, revealed that the Hemin-treated groups showed non-significantly higher per cent wound contraction on day 3 post-wounding compared to control group, while, on 5th day post-wounding, 0.5% and 2.5% Hemin-treated groups showed significantly higher wound contraction compared to control group. However, on day 7, the wound contraction was significantly increased in all the treatment groups except 0.05% Hemin-treated group. Moreover, there was significant difference in per cent wound contraction between 0.5% and 2.5% Hemin-treated groups on 7th day post-wounding.

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

Group/Day	Day 3	5 Day	Day 7
Control	1.88±1.01^{a}	12.35±0.88^{a}	29.02±2.50^{a}
0.05% Hemin	2.37±1.74^{a}	14.55±1.19^{a}	32.19±1.76^{a}
0.1% Hemin	3.92±1.36^{a}	17.38±1.99^{ab}	38.63±1.66^{b}
0.5% Hemin	5.25±1.60^{a}	21.67±2.32^{bc}	42.96±2.83^{b}
2.5% Hemin	6.58±0.96^{a}	27.85±1.46^{c}	57.51±2.74^{c}

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.

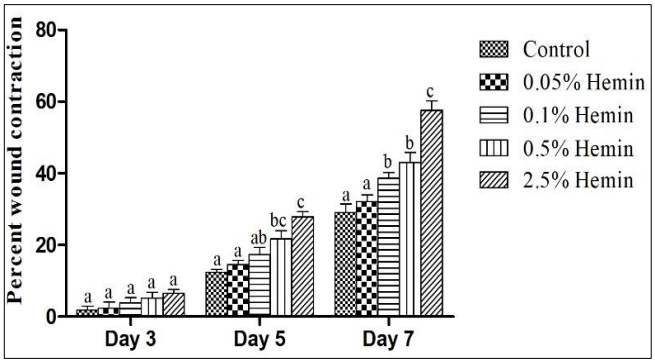

Fig 2: Per cent wound contraction in control and hemin-treated groups on different days. Data are expressed as mean±SEM (n=6). $P < 0.05$, statistically significant, when compared between groups. Bars bearing superscripts not in common differ significantly

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

There was significant increase in the hydroxyproline and glucosamine levels in the granulation tissue in 0.5% and 2.5% hemin-treated groups compared to control group (Fig. 3: Fig. 4). However, increased levels were non-significant in other hemin-treated groups as compared to control. Moreover, there was significant difference in hydroxyproline and glucosamine levels in 0.5% and 2.5% hemin-treated groups. The highest hydroxyproline (5.48±0.3313µg/mg tissue) and glucosamine (1.31±0.08 µg/mg tissue) levels were found in 2.5% hemin-treated group (Table 3).

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

Group	Hydroxyproline (µg/mg tissue)	Glucosamine (µg/mg tissue)
Control	2.73±0.05^{a}	0.33±0.02^{a}
0.05% Hemin	3.09±0.19^{ab}	0.47±0.05^{a}
0.1% Hemin	3.53±0.16^{ab}	0.53±0.02^{a}
0.5% Hemin	3.76±0.17^{b}	0.81±0.04^{b}
2.5% Hemin	5.48±0.33^{c}	1.31±0.08^{c}

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.

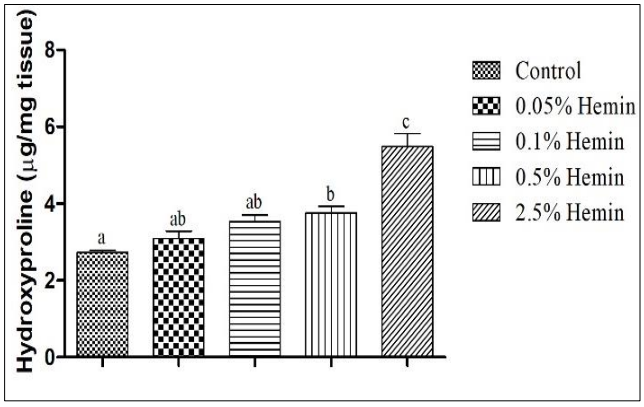

Fig 3: Effect of topical application of hemin on hydroxy proline level on day 7 post-wounding in granulation tissue. Data are expressed as mean±SEM (n=6). $p < 0.05$, statistically significant, when compared between groups. Bars bearing superscripts not in common differ significantly

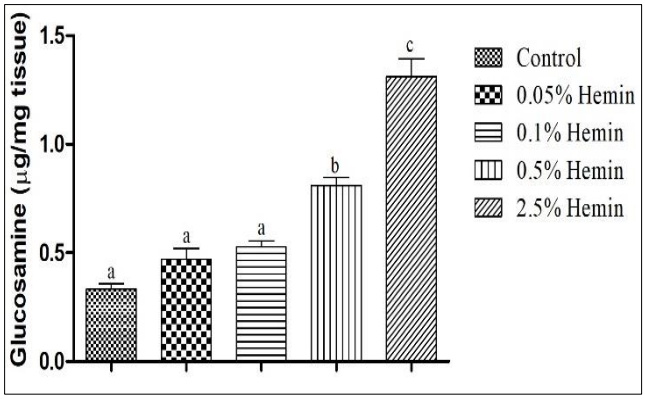

Fig. 4: Effect of topical application of hemin on glucosamine level on day 7 post-wounding in granulation tissue. Data are expressed as mean±SEM (n=6). $p < 0.05$, statistically significant, when compared between groups. Bars bearing superscripts not in common differ significantly

4.4 Calculation of hemin 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 hemin was determined from the dose response curve (Fig. 5), and it was 0.96%. The below equation was used to calculate the EC50.

$$Y = 5.9884\ln(x) + 50.421$$

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
0.05% Hemin	32.19±1.76^{a}
0.1% Hemin	38.63±1.66^{b}
0.5% Hemin	42.96±2.83^{b}
2.5% Hemin	57.51±2.74^{c}

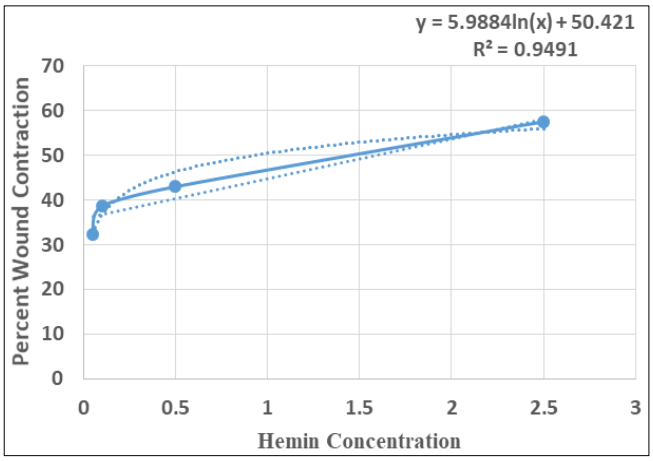

Fig 5: Dose response curve showing the effect of different concentration of hemin on wound contraction on day 7 post wounding

5. Discussion

The underlying mechanisms responsible for contraction are not fully understood, but there appears to be a complex interaction between contractile fibroblasts, sometimes referred to as "my fibroblasts" and the matrix components (Tomasek *et al*., 2002) [9]. However, in individuals with diabetes mellitus, the wound healing gets impaired due to alteration in macrophage function (Maruyama *et al*., 2007) [10], inhibition of fibroblast migration (Xuan *et al*., 2014) [11], less collagen deposition (Black *et al.*, 2003) [12], impaired epithelialization and diminished angiogenic response during proliferative phase of the healing process (Galiano *et al.*, 2004) [13] and many others, thus delay the wound contraction and healing process. In the present study, topical application of hemin on diabetic wound considerably reduced the wound size, and also there was higher wound contraction compared to control group. Further, there was increase in the levels of hydroxyproline and glucosamine in the granulation tissues of hemin-treated groups compared to control group. Previous studies (Ahanger *et al*., 2010; Chandrashekara *et al*., 2017) [5] [6], in support of our findings, also reported that hemin promotes early healing by enhancing the wound contraction, and by significantly increasing the hydroxyproline and glucosamine contents in granulation tissue. So, our findings suggests that hemin application on diabetic wound might have stimulated the cellular migration and proliferation, granulation tissue formation, transformation of fibroblasts to my fibroblasts and collagen synthesis as evidenced by higher wound contraction and increase in hydroxyproline & glucosamine levels.

6. Conclusion

The wound size was considerably reduced in the treatment groups particularly in 2.5% hemin-treated group compared to control group from 5th day onwards. Hemin-treated groups, specifically 0.5% and 2.5% groups, showed significantly higher wound contraction compared to control group from day 5 post-wounding. Further, there was increase in the levels of hydroxyproline and glucosamine in the granulation tissues of hemin-treated groups compared to control group. So, our findings suggests that hemin application on diabetic wound might have stimulated the cellular migration and proliferation, granulation tissue formation, transformation of fibroblasts to my fibroblasts, and collagen synthesis as evidenced by higher wound contraction and increase in hydroxyproline & glucosamine levels.

7. Acknowledgements

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