Hygeia.J.D.Med. July 2017 – November 2017 ISSN 2229 3590
Hygeia :: journal for drugs and medicines
July 2017
Open Access www.hygeiajournal.com
Research article section: Pharmacology
A Half Yearly Scientific, International, Open Access Journal for Drugs and Medicines
DOI:10.15254/H.J.D.Med.9.2017.163 Research Article

CURATIVE POTENTIAL OF HEMIN: AN INDUCER OF HEME OXYGENASE-1 ON EXPERIMENTALLY INDUCED EXCISION WOUNDS IN RATS

CHANDRASHEKARA H H*, KUMAR D, WASIF AHMED, KATARIA M, TANDAN SK, RANJITH D, AND SANDHYA S

Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Keywords: Heme oxygenase, Hemin ointment, Wound healing, Angiogenesis

Correspondence
Chandrasekhara H.H MVSc
Veterinary Officer, Pig Breeding Station, Izatnagar, Bareilly, Uttar Pradesh India.

Received: 5 December 2016,
Revised: 20 January 2017
Accepted: 13 April 2017,
Available online: 15 July 2017

ABSTRACT

Plan: *Hemin is a critical component of hemoglobin and induces heme oxygenase (HO), an enzyme which degrades heme in a rate limiting manner and has an important role in cellular protection against oxidative stress. This HO inducer like Hemin may be of potential therapeutic value in the wound healing and inflammation. The present study was proposed to investigate the effect of hemin on experimentally induced excision wounds in rats.*

Preface: *A multitude of cellular and biochemical processes are set in motion upon occurrence of wound. The sequential influx of inflammatory cells production of an extra-cellular matrix is essential for efficient healing. Wound healing is a burning problem and constitutes an important aspect in rehabilitation medicine. The acute induction of HO has been shown to have beneficial effects including wound healing process.*

Methodology: *To identify the beneficial activity of HO vis a vis wound healing, hemin was used as inducer of HO in rats using a full-thickness cutaneous excision wound model. A 400 mm^2 excision wound was created under normal conditions. The route of hemin ointment administration was topical in normal wound model. The topical Povidone Iodine ointment was used as a standard drug of treatment for wound healing. The assessment of healing process was ascertained by physical (contraction measurements) and chemical (hydroxyproline and glucosamine content of granulation tissue) studies.*

Outcome: *Heme oxygenase-1 pathway when activated can modulate wound healing process for early and fast healing. Hemin showed a strong pro-healing potential as it induces HO-1 and therefore, triggers the pro-healing convergence pathway. Histopathological studies indicated the process of angiogenesis has occurred in granulation tissue of the treated groups.*

Corresponding author email: vetcharangowda@gmail.com
Mob No: +91-9449913314

1. INTRODUCTION

Wound is an injury to the body caused by physical, chemical, thermal or microbial means resulting in disruption of continuity in the body structures. Normal wound healing involves four temporarily overlapping phases i.e. haemostasis, inflammation, proliferation and remodeling [1]. Cutaneous wounds in addition to causing pain and discomfort they predispose the patients to superficial and chronic infections, involving significant cost associated with long term therapy [2]. Despite some recent advances in understanding basic principles of wound healing, problems due continue to cause significant morbidity and mortality, particularly in animals [3]. A large variety of modalities are available for the wounds (application of antibiotics, occlusive layers, bandages, poultices) but they all have one drawback in common; that all will enhance wound healing by supporting the body mechanisms. To tide over such situations, a treatment modality is desired that speeds up the healing by actively regenerating the skin (dermis and epidermis). Hemin, an inducer of heme oxygenase (HO), a rate limiting enzyme in the catabolism of heme which leads to formation of equimolar concentration of bile pigment biliverdin, carbon monoxide (CO) and free iron (Fe^{2+}) [4] and also upregulates HO expression [5]. Hemin, an iron containing porphyrin (protoporphytin IX) having ferric iron ion and chloride ligand. Heme oxygenase -1 and catabolism of heme plays crucial decisive role in preventing injury from many diseases [6-10]. HO-1 has anti-inflammatory, angiogenic and cytoprotective activity in cardio vascular diseases [11-13]. The formed biliverdin will gets transformed to bilirubin by NADPH reductase enzyme. Further, bilirubin and biliverdin are powerful antioxidants [14], carbon monoxide executes multifarious functions including reduction of oxidative stress and regulation of inflammation [15]. To validate this hypothesis, the present study had been designed to investigate the effect of hemin on experimentally induced excision wounds in rats.

2. EXPERIMENTAL

2.1. Preparation of ointment

Hemin was procured from Sigma, USA and a simple ointment in concentrations of 0.25%, 0.5% and 1% were prepared using ointment base on a pill tile.

2.2. Experimental animals

The animals used in the present study were Wistar albino rats (150-180 g) obtained from Laboratory Animal Resource Section of Indian Veterinary Research Institute (IVRI). The animals were housed in polypropylene cages at a room temperature of 22 ± 2ºC. A balanced feed procured from Feed Technology Unit of IVRI, Izatnagar were used throughout the study period. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee (IAEC), Indian Veterinary Research Institute, Izatnagar and conforms to the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.3. Evaluation of wound healing activity by excision wound model

Male Wistar albino rats weighing 150-180gwere starved overnight with free access to water. Rats were anaesthetized by sodium pentobarbitol (50 mg/kg i.p). A square shaped piece of skin (400 mm^2) in its full thickness was excised from the back region of rats. Wounds were not dressed or covered. The animals were divided into five groups of five animals each. Group I(Control group)animals were applied topically with simple ointment base, Group II(Positive Control)animals were applied with povidone iodine (1% w/w) ointment, Group III(Treatment) animals were applied with Hemin ointment 0.25%topically, Group IV(Treatment) animals were applied with Hemin ointment 0.5% was applied topically, Group V(Treatment) animals were applied Heminointment1%. All the animals were treated once daily for 14 days. No antibiotics were used during entire experimental period.

2.4. Measurement of wound area

The wound area of each animal was measured at predetermined interval of time starting at 3 hour interval after the creation of wound. This interval was considered as 0 day measurement and the delay of 3 hours after the creation of wound for measurement was allowed to accommodate the wound stretching that occurs due to the struggle of animal during recovery. The subsequent measurement was taken on days 2, 4, 7, 10 and 14 after the creation of wound. The measurement of wound studied by use of a firm but flexible transparent polythene rectangular (3x3 cm^2) sheet was held just over the wound and its margins were marked with a permanent marker on sheet and the animal was released back to the cage. The area demarcated on the transparent sheet was estimated planimetrically in which a standard quality card paper was used to convert the area of the wound on the transparent sheet into the weight of the card paper with same area.

2.5. Tissue harvesting

After the last measurement was taken, the animals were sacrificed with an overdose of Diethyl ether and the healed skin was carefully lifted, freed of adhesions and excised out along with about 2 mm adjacent normal skin so as to differentiate between the healed and normal skin. The healed tissue was divided in to two fractions; first one was kept for Biochemical parameters and were preserved at -80ºC in an ultra-low freezer (Nuaire, USA) till further use. The second fraction was immediately preserved in 10% formalin for histological sectioning and staining.

2.6. Tissue processing for the estimation of hydroxyproline / glucosamine

About 50 mg of the preserved healed skin from each sample was subjected to acid hydrolysis by adding 1 ml 6N HCl in a tube which was tightly sealed and autoclaved at 50 pound pressure for 3 hours. The hydrolysate so produced was used for estimating hydroxyproline [16] and glucosamine [17].

2.7. Histological Studies

The granulation tissue preserved in 10% formalin was subjected to sectioning and 6 µm thickness sections were stained with hematoxylin and eosin and visualized for histological changes under light microscope.

2.8. Statistical Analysis

Results are expressed as Mean±S.E. with n equal to number of animals. Data were analyzed by one-way ANOVA followed by Dunnet's test and two-way ANOVA followed with Bonferroni's multiple comparision test. A value of $P<0.05$ was considered to be statistically significant [18].

3. RESULTS AND DISCUSSION

3.1. Effect of topical application of hemin on wound contraction in rats (wound area)

The results obtained depict absolute measurement of wounds which clearly depicts significant differences in wound contraction by various concentration of hemin than the control and Povidone (Table 1). The increased rate of wound contraction in hemin-treated group might be attributed to increased proliferation and transformation of fibroblasts into myofibroblasts affected through carbon monoxide (CO) and bilirubin. The pro-healing role of Heme Oxygenase-1 (HO-1) has also been substantiated by inhibition of HO-1 through use of tin protoporphyrin-IX which led to retardation in the closure of wound in rat and mice [19].

Table 1: Effect of topical application of hemin on wound contraction in rats (wound area)

Day	Average wound area Mean ±SEM (mm^2)					
	0 Days	2 Days	4 Days	7 Days	10 Days	14 Days
Control	402.45±3.76	376.95±6.37	354.73±7.40	226.47±17.03	96.169±8.11	44.84±3.47
Povidone	402.37±3.18	377.92±4.34	354.24±10.79	230.36±12.84	93.43±10.15	46.83±7.58
Hemin0.25%	402.45±3.76	346.40±6.56**	289.56±4.29***	138.77±6.76***	60.24±2.46**	26.18±4.67**
Hemin0.50%	402.45±1.83	340.03±2.86**	277.67±1.37***	127.80±3.86***	50.37±5.89**	18.87±6.54**
Hemin 1.0%	402.45±2.78	335.28±7.28**	267.91±9.47***a	111.57±10.78***a	40.10±5.43**a	15.54±4.38**

n = 5, $^{a}P<0.05$, as compared to hemin 0.25%. **P<0.01, ***P<0.001, as compared to control.

3.2. Effect of topical application of hemin on wound contraction in rats (percent values)

The hemin-treated groups were all the way ahead in the wound contractions far as level of significance is concerned in comparison to the control group. Table 2 depicts absolute measurement of wounds in percent values. The increased rate of wound contraction in hemin-treated group can be attributed to anti-apoptotic, anti-inflammatory and cytoprotective action of CO which results in conditions conducive for proliferation, transformation of fibroblasts into myofibroblasts, and re-epithelialization [20].

CO potentially attenuates ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) which are invariably present in the microenvironment of wounds; therefore, the presence of CO at such places may synergize the process of healing process [21].

Table 2: Effect of topical application of hemin on wound contraction in rats (percent values):

Day	Per cent wound contraction				
	2 Days	4 Days	7 Days	10 Days	14Days
Control	6.33±1.25	11.85±2.58	43.72±4.34	76.10±2.88	88.85±3.85
Povidone	6.07±0.67	11.96±3.66	42.74±2.58	76.77±0.94	88.36±3.54
Hemin 0.25%	13.92±1.66**	28.05±3.79***	65.51±6.22***	85.03±1.52**	93.49±1.87**
Hemin 0.50%	15.41±2.82**	31.11±3.35***	68.74±5.78***	88.41±1.49**	95.66±1.34**
Hemin 1.00%	16.68±2.04**	33.43±2.63***a	72.27±6.70***a	90.036±1.46**a	96.13±1.23**

n = 5, [a]P<0.05, as compared to hemin 0.25%. **P<0.01, ***P<0.001, as compared to control.

3.3. Effect of topical application of hemin on the hydroxyproline content of the granulation tissue of excision wounds in rats

Table 3 reveals all the concentration of hemin, significantly higher content of hydroxyproline as compared to the control group, though highest content was in hemin 1% group as compared to control, Hemin 0.5%, hemin 0.25% and Povidone-treated groups of rats, respectively. The early re-epithelialization and faster wound closure in hemin-treated group might be attributed to increased keratinocyte proliferation and their migration to the wound surface [22].The observed increased levels of hydroxyproline and glucosamine in treatments with hemin provides the strength to the regenerated tissue. Hemin over all seemed to promote both epithelialization as well as granulation tissue proliferation better than other treatments probably because of its action is through HO-1 which triggers the pathway and invokes effects as envisaged in normal physiological process.

Table 3: Effect of topical application of hemin on the hydroxyproline content of the granulation tissue of excision wounds in rats

Treatment	Hydroxyproline(mg/g tissue)
Control	9.006±0.49
Povidone	13.89±0.42**
Hemin 0.25%	17.47±0.41**
Hemin 0.50%	18.10±0.31**
Hemin 1.00%	19.30±0.41**

n = 5, **P<0.01, as compared to control

3.4. Effect of topical application of hemin on the glucosamine content of the granulation tissue of excision wounds in rats

Glucosamine content of the healing wound in response to various concentration of hemin depicted in Table 4 which showed similar trend to that of hydroxyproline except that povidone treatment did not reveal any difference from control group. The highest content of glucosamine was shown byhemin1%-treated groups.

Table 4: Effect of topical application of hemin on the glucosamine content of the granulation tissue of excision wounds in rats:

Treatment	Glucosamine(mg/g tissue)
Control	4.73±0.19
Povidone	5.36±0.19
Hemin 0.25%	6.41±0.13**
Hemin 0.50%	7.02±0.12**
Hemin 1.00%	7.44±0.18**

n = 5, **P<0.01, as compared to control

3.5. Histological findings

The H & E stained sections of healing wound are represented in Fig. 2. Evidently, control group showed loose granulation tissue with little neo-vascularization and few necrotic patches (Fig. 2A&B). Povidone iodine treated wound showed relatively better granulation tissue but there was inflammatory infiltration. Epithelialization was also better than control group (Fig.2 C&D). Various concentrations of hemin revealed compact and mature granulation tissue, particularly hemin 0.5% group (Fig.2 G&H) and hemin 1% group (Fig.2 I&J). Hemin 0.25% group (Fig.2 E&F) showed relatively loose but mature granulation tissue.

Angiogenesis during wound repair serves the dual function of providing the nutrients required by the healing tissue and contributing to structural repair through the formation of granulation tissue [23]. VEGF (Vascular Endothelial Growth Factor) improves angiogenesis during wound healing by stimulating the migration of endothelial cells through the extracellular matrix [24]. Histological evaluation indicated enhanced blood vessel formation in the granulation tissue of the treatment groups.

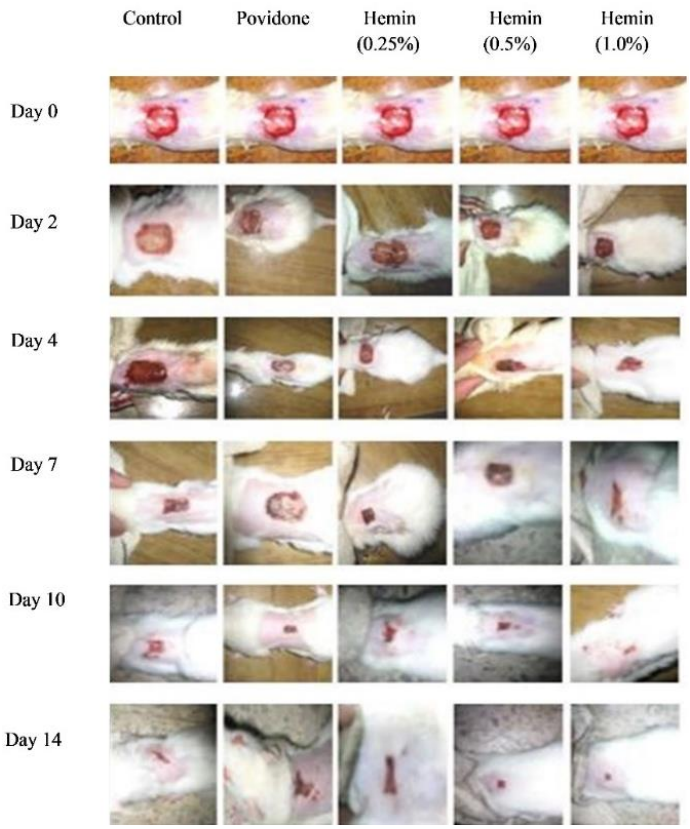

Figure 1: Photographs of Wound Healing (Hemin)

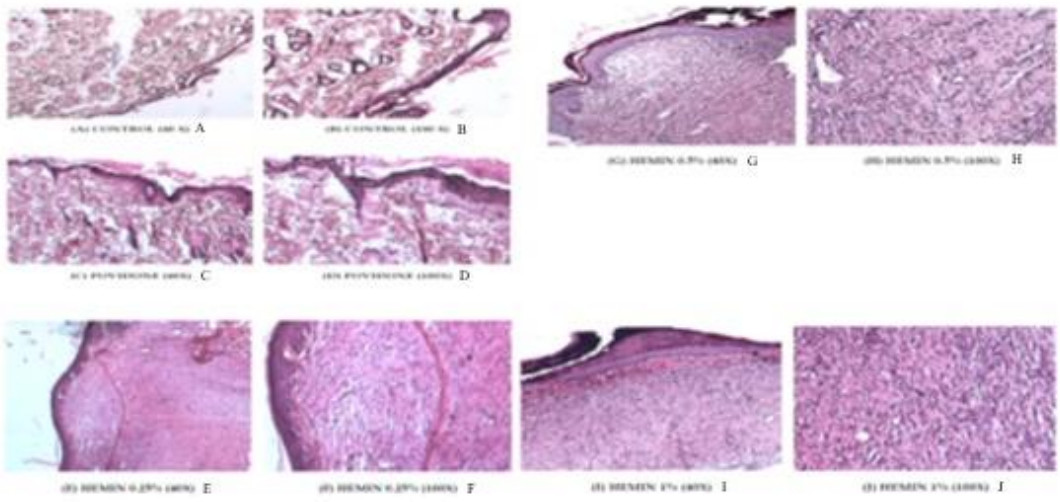

Figure2: Histological findings of healed skin tissue (hemin treated group)

4. CONCLUSION

In conclusion hemin, an activator of HO-1 (Heme Oxygenase-1) pathway can modulate wound healing, Topical application of various concentrations of hemin and in normal wounds in rats revealed early and fast healing effect. Hemin approach in wound healing showed better effects in the healing process because it induces the entire effectors of HO-1 pathway in a more natural and balanced way.

ACKNOWLEDGEMENT

The authors express their deep sense of gratitude to Indian Veterinary Research Institute for the help rendered in completion of this work.

REFERENCES

1. Donald M., Zachary JF. Pathological Basis of Veterinary Disease, Elsevier Publication, USA, 4th edn, **2004**.
2. Campos AC., Groth AK., Branco AB. Assessment and nutritional aspects of wound healing.*Curr. Opin. Clin. Nutr. Met. Care.***2008**, 281–288. CrossRef PMid:18403925
3. Fine N., Mustoe T. Wound Healing. Philadelphia: Lippincott Williams & Wilkins.**2006**; 76-97.
4. Abraham NG., Kappas A. Pharmacological and clinical aspects of heme oxygenase. *Pharmacol. Rev* **2008**; 60:79-127. CrossRef PMid:18323402
5. Immenschuh S, Ramadori G. Gene regulation of hemeoxygenaseas a therapeutic target. *Biochem. Pharmacol.***2000**; 60:1121-1128. CrossRef
6. Bach FH. Heme oxygenase-1 and transplantation tolerance. *Human Immunology* **2006;** 67(6): 430–432.
7. Öllinger R., Wang H., Yamashita K. Therapeutic applications of bilirubin and biliverdin in transplantation. *Antioxidants & Redox Signaling* **2007**; 9(12): 2175–2185.
8. Otterbein LE, Soares MP, Yamashita K., Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends in Immunology* **2003**; 24(8): 449–455.
9. Soares MP, Bach FH. Heme oxygenase-1 in organ transplantation. *Frontiers in Bioscience* **2007**; 12(1): 4932–4945.
10. Wu ML, Ho YC, Yet SF. A central role of heme oxygenase-1 in cardiovascular protection. *Antioxidants & Redox Signaling* **2011**; 15(7): 1835–1846.
11. Loboda A, Jazwa A, Grochot-Przeczek A. Heme oxygenase-1 and the vascular bed: from molecular mechanisms to therapeutic opportunities. *Antioxidants & Redox Signaling* **2008**; 10(10): 1767–1812.
12. Dulak J, Deshane J, Jozkowicz A, Agarwal A. Heme oxygenase-1 and carbon monoxide in vascular pathobiology: focus on angiogenesis. *Circulation* **2008**; 117(2): 231–241.
13. Józkowicz A, Dulak J. Effects of protoporphyrins on production of nitric oxide and expression of vascular endothelial growth factor in vascular smooth muscle cells and macrophages. *Acta Biochimica Polonica* **2003**; 50(1): 69–79.
14. Jansen T., Hortmann M., Oelze M. Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. *Journal of Molecular and Cellular Cardiology* **2010**; 49(2): 186–195.
15. Ryter SW., Choi AM. Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy, *American Journal of Respiratory Cell and Molecular Biology* **2009**; 41(3): 251–260, 2009.
16. Woessener JF. The determination of hydroxyproline in the tissue and protein sample containing small proportion of this iminoacod. *Arch BiochemBiophys*1**961**; 93:440-447.CrossRef
17. Rondle CJM, MorganWIJ. The determination of glucosamine and galactosamine. *Biochem. J.***1955**; 61:586-589. CrossRef PMid:13276341 PMCid:PMC1215838
18. Snedecor GW, Cochran WG. Statistical Methods, 8th ed. Oxford and IBH Publishing Company, New Delhi, **1989**.

19. Grochot-Przeczek A., Lach R., Mis, J., Skrzypek, K., Gozdecka, M., Sroczynska,P., Dubiel, M., Rutkowski, A., Kozakowska, M., Zagorska, A., Walczynski, J., Was, H., Kotlinowski J , Drukala, J., Kurowski K, Kieda, C., Herault, Y, Dulak, J. and Jozkowicz, A. Heme oxygenase-1 accelerates cutaneous wound healing in mice. *PLoS ONE,* **2009**; 4:e5803. CrossRef PMid:19495412 PMCid:PMC2686151
20. Zhang X., Shan P., Alam J., Davis RJ, Flavell RA., Lee PJ. Carbon monoxide modulates Fas/Fas ligand, caspases and Bcl-2 family proteins via the p38a mitogen-activated protein kinase pathway during ischemia-reperfusion lung injury. *J.Biol.Chem*.**2003**; 24:22061-22070. CrossRef PMid:12690100
21. Cepinkas G, Katada K., Bihari A., Potter FR. Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *Am. J. Physiol. Gastrointest. Liver Physiol*.**2007**; 1: G184-G191. CrossRef PMid:17991708
22. Moulin V., Auger FA., Garel D., German L. Role of wound healing myofibroblasts on re-epithelialization of human skin. *Burns* **2000**; 26: 3–12. CrossRef
23. Martin A., Komada MR., Sane DC. Abnormal angiogenesis in diabetes mellitus. *Med. Res. Rev*.**2003**; 23:117–45.CrossRef PMid:12500286
24. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. *J.Mol.Med*.**1999**; 77: 527-543. CrossRef

Chandrashekara H H*, kumar D, Wasif Ahmed, Kataria M, Tandan SK, Ranjith D, and Sandhya S. Curative potential of hemin: an inducer of heme oxygenase-1 on experimentally induced excision wounds in rats. *Hygeia.J.D.Med* **2017**; 9(1):43-51. Available from http://www.hygeiajournal.com , DOI: 10.15254/H.J.D.Med.9.2017.163