TOPICAL APPLICATION OF DUNG-CAKE ASH AS INNOVATIVE THERAPY IN SKIN WOUND HEALING IN RABBIT MODEL

Hamid Zia Shaikh, Din Muhammad Shaikh
Department of Physiology, Isra University Hyderabad, Pakistan.

Background: The pharmacologicl effects of different types of ashes containing different elements and trace elements are explainable. These effects poteintiate and help in repair and regenerative processes in rabbit skin wound. **Method:** For this proposes ash from buffalo dung-cake, was prepared and analyzed. The ash was applied besides Polymyxin B-Bacitracin Zinc ointment, one of the topical applications as control and the process of healing was observed for two weeks physically and histologically. **Results:** A significant difference between the effects in healing was observed in control and experimental. Moreover, the skin tissue repairing rate by ash is faster. **Conclusion:** This study confirms that the ash played a very positive role and repaired the wound within a period of 7–11 days. This consistency between ointment and ash expresses the ability of ash in healing the wounds. Hence ash could be considered as natural medicine and possesses unique properties.

Keywords: Ashes, Polymyxin B, Bacitracin, Rabbit skin tissue

INTRODUCTION

Wound healing disorders are therapeutic problem of extensive clinical importance as wound healing involves multiple complicated events. Even in modern times wound healing agents continue to puzzle medicals scientists. Thus it is reasonable to search for simple and easily available or otherwise treatment to establish novel therapeutic approaches on other hand. Since the time of Holy Prophet (PBUH) application of ash is reported categorically in *Sahih Bukhari*.[1–3]

Re-epithelization is central to wound closure and driven by movements of epithelial keratinocytes. After a lag period of several hours after the skin injury, these movements are initiated starting with epidermal migration.[4] Subsequently the injury mediated loss of epidermal keratinocytes is replaced by these generations of keratinocytes from the newly formed highly proliferative epithelia at the wound margins which now feed the moving epithelial tongue. Growth factors[5] and cytokines participate in the close regulation of keratinocytes behaviour during these processes. Thus EGF and KG factors essentially during skin repair.[4–7]

The inflammatory phase of wound healing is believed to be instrumental in supplying the growth factors, cytokines and chemokines that orchestrate the cells movement necessary for wound repair.

One of the advantages of animal models is that wounding can be performed in a standardized manner in addition the normal healing process is accelerated (in rats[8]) which enable the course of study within days and not over weeks as required in humans. The rabbit for present work is selected for its easy availability, low cost and reasonable size to observe the skin wound healing visually and histologically.

Egyptians had also made concoction of naturally occurring products *viz* honey, animal fat, vegetable fibre and oils. As various ingredients in mixture were found successful.[9] They have also used the faeces of cow and donkey besides the charred wood for topical application.

Physiological affect of different topical ointments available are well documented through out the literature. Even the mechanism of some of those is also known. However, at present no single wound care product provides the optimal environment for healing all types of wounds.

Skin wound healing in skin depends upon the availability of appropriate metals and trace elements as enzyme cofactors and structural component in tissue repair also needed.[10]

Ashes provide an appropriate requirement of trace metals on one hand and also antioxidant, anti inflammatory and anti infective coat on other hand. Thus provides favourable internal environment for regeneration and proliferation.

The present study was aimed to examine the influence of buffalo dung-cake ash, containing appropriate mixture of metals and trace metals, in surgical induced skin wounds in rabbit models as it enables the study the course within days and not over weeks are required in humans.

MATERIAL AND METHODS

Ash collection and analysis

Dried buffalo dung-cakes were procured from the village adjacent to the Isra University. The type of fodder was also noted. The Ash was collected after complete burning and was also dried at 105 °C in the hot air oven. Replicate 1.9 gm to 2.0 gm samples of dried ash were weighed in 100 ml conical flasks and treated with 5.0 ml of nitric acid. Five ml of nitric

acid was also added to an empty flask. The flasks were covered with watch glasses and their contents were heated to reflux gently on an electrical plate. After refluxing for one hour the contents of the flasks were treated with 5.0 ml more of nitric acid, 2.0 ml of 35% hydrogen peroxide was added and the heating at gentle reflux was continued for another hour. The watch glasses were removed from the flasks and the heating was continued until the volume of their contents was reduced to 2–3 ml. The contents of the flasks were cooled, diluted with de-ionized water and filtered through Whatmann #42 paper into 25.0 ml volumetric flasks and brought to volume with de-ionized water and examined by atomic absorption spectrophotometry (Hitachi Model 180-50) for the sodium, potassium, calcium, magnesium, iron, zinc, manganese, and copper levels (Table-1).

Animals:
Adult wild type rabbits (1000–1250 gm body weight) were used in this study. They were bred in the animal house of Isra University. They were housed under controlled conditions of 30±5 °C, 55–60% relative humidity and 12 hour day/night cycles as specified in AAALAC international institutional animal care and use program guide, 1996. The animals were housed individually in stainless steel cages. Alfalfa fresh and tap water were provided ad libatum.

Experimental details
Incisional full thickness skin wounds (10 mm long) were made surgically with #15 scalpels in the closely shaved dorsal skin of rabbits under conditions of local anaesthesia. The rabbits were shaved on the dorsal aspect of their skin after clipping the dense hair. A centimetre scale and skin pen were used to mark four sites in the cranio-caudal direction, two on each side of the spine, each was two centimetre distant on the contra-lateral side and also on the ipsilateral aspect. The area was disinfected using spirit as standard disinfectant. The rabbits were injected with 1.0 mg/ml diazepam i.m., for relaxation and sedation and the previously marked sites were anesthetized using 2% lignocaine+0.00001% adrenaline (w/v) taken in 1.0 cc syringes having needle gauge of 29. Each site of proposed incision was injected with this local anaesthetic subcutaneously taking precaution that none of the deeper structures or blood vessels was penetrated. The animals were allowed 10 minutes for the drugs to take effect. The incision on each site was made as cleanly as possible and deep enough to cut through the panaculus carosus muscles. However the sub-cutaneous tissue covering the lumbo-dorsal fascia was spared. Four different coloured permanent markers were used to colour code the different wounds by smudging the hair on cranial aspect of each wound. Ashes were smeared on the two wounds on the cranial side and one wound on the caudal side, the remaining wound was covered liberally with Polymyxin-Bacitracin skin ointment (Polyfax^{TM}, GlaxoSmithKline Pakistan Ltd). The ash was deliberately filled into the wounds, so that the cut edges could not be approximated and the wounds were left to heal by secondary intention. The wound in every case was left open to air and without any dressing material to access the barrier function. Taking the operation day as Day 0, three rabbits each were sacrificed on days 1, 3, 5, 7, 9, 11 and 13 post-wounding. The entire wound area along with 45 mm of the surrounding skin were lifted by excision and places within previously marked containers, in 10% formaldehyde as preservative for haematoxyline and eosin staining and histology.

Histological procedure and microscopy:
The manual procedure was adopted to process the formaldehyde-preserved tissue, which included dehydration, clearing, impregnation, embedding, and cutting followed by staining of section carried out on alternate days. Microscopic photography was also done.

RESULTS

Visual and microscopic examination of post wound sites:

By day 1:
Post wounding: There was scab formation on the control wound while the test site showed a crust of ashes on the wounds. There was no reduction in the size of wounds. Mitotic activity was observed in epidermal cells and wound margins and dermal fibroblasts. The control wound did not exhibit any such activity. RBC, WBC, Monocytes appearance indicated inflammatory phase.

By day 3:
The control wound remained the same where as there was a decrease in wound length by about 2mm in test site. Neutruphils and monosites infilteration was seen with considerably large population of eosinophils. Increase in fibroblast was also initiated.

By day 5:
The control wound appeared to dry out while the test site still had the thin crust of ash but the wound area had decreased further by another, 2 mm in experimental animals. Crusting and hardening of the wound was evident. After this time, progressive loss of the superficial ash covering was observed and prominent hair growth was also observed.

By Day 7:
The control wound had decreased by approx. 2 mm but the test sites had decreased by a total of 5 mm or half the original size, also the thin crest of ash was extruded from wound applied with dung-cake ash. Epidermis showed up to three times than normal thickness with numerous mitosis in basal layers.

The wound also exhibited healthy granulation tissue on test site by day 7 while there was still a crust on the control wound. Healing in the test wound from day 7 on words was associated with pronounced decline in the inflammatory cell infiltrate in a near the wound margins and a prominent reduction in the crater formation and wound debris and realignment of epidermal cells, by pit-like configuration and hyperkeratosis.

There was no evidence of repair of panculus carosus muscle but collagen deposition (pale staining) was evident at this site.

By day 9:
The control wound had decreased by another 1 mm but there was protrusion of the scabs above the wound margin. The test site continued to exhibit healthy granulation tissue, which appeared to loose its reddish colour and become progressively paler.

By day 11:
The control wound had shed its scab and showed red to pale granulation tissue. The test wounds also showed granulation tissue. The test wound showed prominent growth of hair and a very transparent pinkish skin covering the entire wound area except the very centre, which still showed red to pale granulation tissue.

By Day 13:
The control wound showed appearance of a thin pinkish skin on top of the granulation tissue. However, the test sites were completely covered by what appeared to be furry (hairy) skin. However, the control wound did show this appearance

By Day 20:
The test wounds showed complete normalization of the lining epidermal however, there was still presence of a few monocytes and eosinophils in the deep dermis and around the panculus carosus muscle.

The lining epithelium still showed hyperkeratosis in the control wound along with a considerable leukocyte infiltration in the dermis and hypodermis.

Table-1: Relative concentrations (ppm) of trace elements in dung-cake ash

Na^{+}	K^{+}	Ca^{++}	Mg^{++}	Fe^{++}	Zn^{++}	Mn^{++}	Cu^{++}
0.27	1.3	5.2	1.02	1.2	5.0	1.3	0.91

Table – 2: Approximate recovery (%) by Polymyxin-Bacitracin ointment and dung cake ash on re-epithelization

S. No	Days	Count	Recovery %	Dung Ash	Recovery %
1	1	1620		1657	
2	3	1730	22	1862	25.0
3	5	1830	30	2080	37.0
4	7	2056	55	2586	65.0
5	9	2226	68	2843	79.0
6	11	2330	75	2923	87.50
7	13	2363	78	2943	90.50

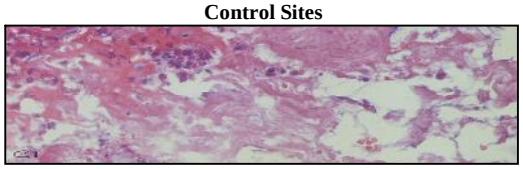
Table-3: Comparative effect of Polymyxin-Bacitracin ointment and Buffalo dung-cake ash on skin healing after 13 days

Application of treatment.	1 day	13^{th} day	Difference	Recovery %
Control group (+Ointment), No. of Fibroblasts	1620	2363	743	45.86
Experimental group (+Ash), No. of Fibroblasts	1620	2943	1323	90.5

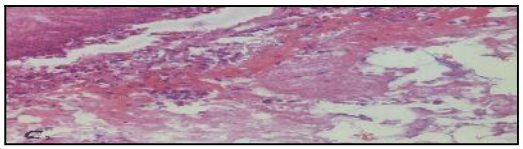
Note: *Overall 30.6% recovery above the control.*

Figure-1: Histological Observations

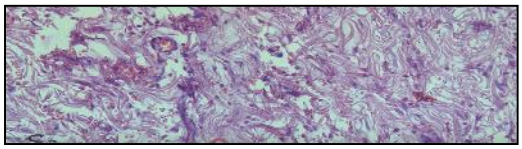
Control Sites



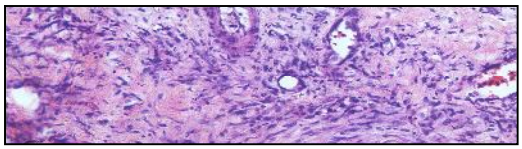
Day 01: Numerous RBCs and PMN, infiltrate at the wound site with edematous fluid.



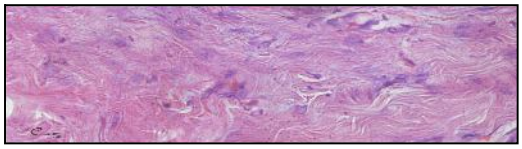
Day 03: Formation of pus (PMNs) and acute inflammatory. Infiltrate and numerous RBCs also seen. Beginning of fibroblast proliferation.



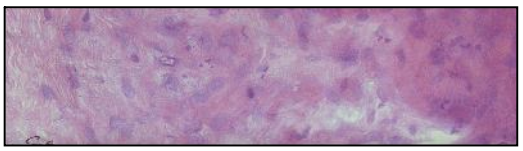
Day 05: Numerous RBCs and leukocytes infiltrate along with fibroblast proliferation (enhanced)



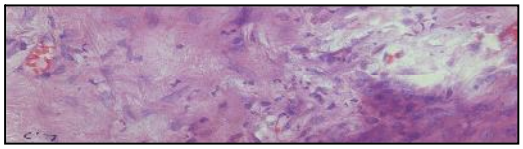
Day 07: Large vessels with few RBCs and fibroblasts proliferating. Fairly high deposit of inflammatory infiltrate.



Day 09: Fewer inflammatory cells in presences of myofibroblasts. The tissue still appears edemaotus.

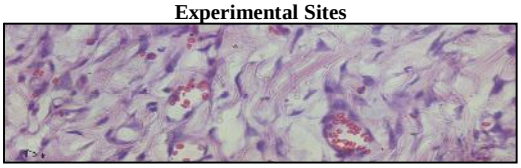


Day 11: Thick vesicular nuclei of myofibroblasts seen. However presence of pus is also visible with faded cellular out line just below the neoepithelial surface

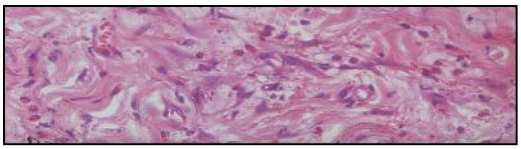


Day 13: Edematous neoepithelialization with vesicular cells and normal appearing epithelium. However the presence of inflammatory; cells pus cells beneath below with fairly heavy infiltration at the wound edge

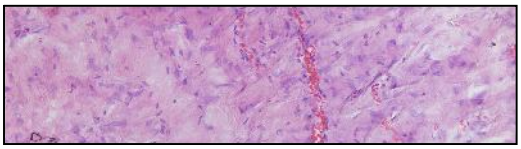
Experimental Sites



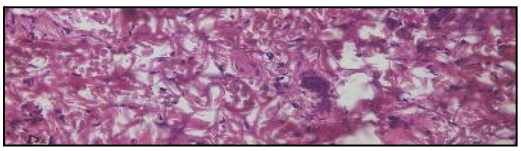
Day 01: Large no of RBCs in dilated blood vessels fibroblasts start to proliferate. Edematous tissue with leukocyte infiltrate.



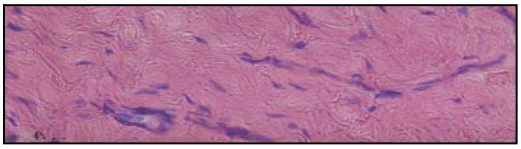
Day 03: Edemaotus tissue with PMN leukocyte, eosinophils prederminate. Fibroblast proliferation increased



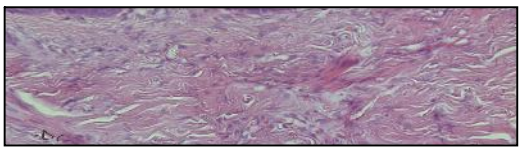
Day 05: Fibroblast increasing and forming sheets. Large area showing neo – vascularization.



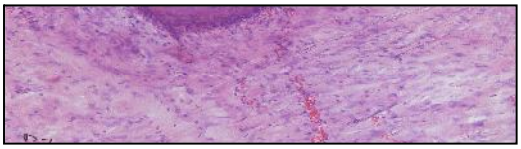
Day 07: Appearance of myofibroblasts with edematous spaces the leukocyte infiltrate deceases



Day 09: Fibroblast laying down ground substance with fair increase in myofibroblast having dark staining elongated nucleus.



Day 11: Same as previous day with appearance of epitheliazation along with neovascularizaiton



Day 13: The epithelization appears advanced showing definite arrangement while the underlying stroma appears stable and well vascularized.

DISCUSSION

Traditionally since centuries the natural products are being used till today specially to cure the skin injuries.[9,11,12] Ash, one of these products is also in used in the country on the whole and in Sindh in particular. However, no attempt for scientific observations are made and reported to work out its mechanism of action. Undoubtedly different ashes *viz* wood ash, charcoal ash and dung ash, clays and oils are being used beside the Bentonite (*Multani mitti*).[6,9,12,] The application of Bentonite on skin wound healing in rat model is comparatively safe and feasible. The skin is the largest organ and contributes 10% of whole body and is exposed to physical and mechanical assaults daily. Injury of skin causes immediately local inflammation and clot formation a hallmark of the wound healing that orchestrate the cell movements necessary for wound healing. The present study is an attempt to observe the visual and microscopic details of healing process stepwise by the application of buffalo dung cake ash. Surprisingly ash contains many metals and trace elements required in healing cascade (Table-1). This highlights its clinical importance. The deficiency in availability of metals leads to metabolic defects. The imbalance in the relative concentrations in Cu^{+2}, Zn^{+2} and Ca^{+2} etc. are potential causes of impaired wound healing.[13–16] Our histological observations are consistent to the study of Vizram[17] and Singer[18] who have reported no microscopically visible changes in epidermis during the first 18 hours. In present study the epidermis regeneration showed up to 3 times thickness compared to control. Mitotic changes were also observed in adjacent areas in test sites in comparison to control where low activity was seen.

Metaloenzymes have an important role in repair and regeneration process in skin wounds.[10] The modulation of trace elements is likely to involve carrier proteins, cytokines and growth factors[4–6,19] but concentration requirement varies from phase to phase. In our study, high concentrations of calcium and zinc in dung cake ash probably play a major role in early healing. It is reported that skin wound healing requires progressive increase in zinc, calcium and manganese till 5th day of post wounding. Zinc requirement is further increased till 10th day. Topical administration of zinc appears to be superior to oral therapy due to its action in reducing any super infection.[19] These observations coincide with present study and is evident from normalization with the increased in number of fibroblasts from day 3 till 11th day. Calcium has a potential central role in skin wound repair and regeneration. Calcium present in ash might play a key role in this homeostasis process. Zinc is now known to regulate calcium and zinc ratio. The presence of low copper level in ash also support and help the normal wound healing generation and defence. Undoubtedly, the trace elements also play an important function to stabilize proteins besides cellular tissue defence against oxidants and is consistent to the studies of Lars[20] and Clarke[21].

Through the visual and histological observation throughout the wound healing period we experienced the sequential changes and normalization of the repair of skin wound in our rabbit model. This is consistent with metaloenzymes requirements in maturation of keratinization. Zinc and calcium ratio is involved in motivated epidermis cell proliferation and maturation.[20] The ultimate differentiation of the regeneration of epithelial cells proceeds near the wound margins before epitheliazation is completed. This in our study was from 7 to 11 days where significantly maximum number of fibroblasts (2943) were found and recovery rate is 90% (Table-3).

CONCLUSION

We assume that the appropriate combination and utilization of metals/trace elements present in the dung ash will make a constituent in metaloenzymes.

We conclude that ash contents provide almost all metals required in healing cascade. As ash is easy, cheap and cost free alternate remedy for skin wound healing. However, data from animal study, if tried on human subjects, may provide a sliver line so that it could be recommended for skin injury treatment in human subjects.

The present findings, the ash as a potential therapeutic drug, can accelerate the clinical testing and commercialization. This will also provide patent-protected market position and promising therapeutic targets at all levels.

ACKNOWLEDGMENT

Author to thank Mr. Asif Ali Mashori, Computer Operator for making tables and graphs, and to Mr. Ghulam Farooq for scanning the photographs.

REFERENCE

1. Bukhari AMI. Kitab ul Jihad. In: Bukhari AMI Sahehe Bukhari. Ala ed. Lahore: Muktaba-e-Rehmania 1999;2:p 280.
2. Muslim AAM. Kitab ul Jihad. In: Muslim AAM. Sahehe Musim. Ala ed. Lahori: Muktaba-e-Rehmania 1999;1:p 602.
3. Ibn-kyeum AJ. Care of wound in prophetic medicine. In: Ibn-Keeum Aj. Prophetic medicine. 1st ed. Lahore: Muktaba-e-Quddusia, 2000;1: p 81.
4. Martin P. Wound healing aiming for perfect skin regeneration. Science. 1997;276 (5309): 75–81.
5. Grazul-Bilska, Anna T, Johnson ML, Bilski JJ, Redmer DA. Reynolds P, *et al*. Wound healing the role of growth factors. Drugs of today 2003;39(10)787–800.
6. Fowler JF Jr. A skin moisturizing cream containing quaternium-18-Bentonite effectively improves chronic hand dermatitis. J Cutan Med Surg. 2001;5(3):201–5.

7. Grotendorst G. Chemoattractants and growth factors. In wound healing; Biochemical and clinical aspects (ed. Cohen Ik, Diegelmann RF, Lindblad WJ), pp. 237–46. Philadelphia: W B Saunders. 1992.
8. Wanda A, Dorsett-Martin. Rat models of skin wound healing.. Atif D. Agaiby and Mary Dyson. Immuno inflammatory cell dynamics during cutaneous wound healing. (2004).
9. Peter Sipos, Hedvig Gyory, Krisztina Hagymasi, Pal Ondrejka, Amna Blazovics. Special wound healing methods used in ancient Egypt and the mythological background. Published online: January 8, 2004.
10. Lansdown AB, Sampson B, Rowe A. Sequential changes in trace metal, metallothionein and calmodulin concentrationsin healing skin wounds. J Anat. 1999;195(Pt 3):375–86.
11. Bayoumi AH, Gaskell S and marks R. Development of a model for Transepidermal elimination. Br. J Dermatol 1978;99(6):611–20.
12. Emami-Razavi1 SH, Esmaeili1 N, Forouzannia SK, Amanpour1 S, Rabbani1 S, Alizadeh1 AM, Mohagheghi1 MA. Effect of bentonite on skin wound healing experimental study in the rat model. Acta Medica Iranica 2006;44(4):235–40.
13. Lansdown AB. Physiological and toxicological changes in the skin resulting from the action and interaction of metal ions. Crit Rev Toxicol 1995;25(5):397–462.
14. Lansdown AG. Influence of zinc oxide on the closure of open wounds: an experimental study. Int J Cosmet Sci. 1993;46:83–5.
15. Klevay LH. The relationship of zinc to copper of diets in the united states. Nutr Rep Int. 1975;11:223.
16. Vicanova J, Boelsma E, Mommaas AM, Kempenaar JA, Forslind B, Pallon J, *et al*. Normazation of epidermal calcium distribution profile in reconstructed human epidermis is related to improvement of terminal differentiation and stratum corneum barrier formation. J Invest Dermatol 1998;111(1):97–106.
17. Viziam CB, Matoltsy AG, Mescon H. Epithelialization of small wounds. J Invest Dematol 1964;43:499.
18. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999;341(10):738–46.
19. Lansdown AB, Mirastschijski U, Stubbs N, Scanlon E, Agren MS. Zinc in wound healing theoretical, experimental, and clinical aspects. Wound Repair Regen 2007;15(1):2–16.
20. Klotz LO, Kröncke KD, Buchczyk DP, Sies H. Role of copper, zinc, selenium and tellurium in the cellular defence against oxidative and nitrosative stress. Journal of Nutrition, 2003 (CAB Abstract at http://www.cababstractsplus.org/abstracts/Abstract.aspx?AcNo=20043015347)
21. Clark Raf. Wound repair: overview and general consideration. In the molecular and cellular biology of wound repair, 2nd ed (ed. Clark RAF). New York, London: Plenum press. 1996, pp 3–50.

Address for Correspondence:

Dr. Din Muhammad Sheikh, Department of Physiology, Isra University, Hyderabad.
Email: dr_dinmuhammadshaikh@hotmail.com