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EVALUATION OF A MUCIN-HONEY FORMULATION FOR HEALING IN FULL THICKNESS EXPERIMENTAL WOUNDS IN RATS

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ABSTRACT

In this work, the various topical formulations of mucin, honey and their admixtures (mucinated-honey) (MH) were investigated in wound treatment. Their wound healing effects, tissue re-epithelialization and efficacy in wounds were compared to those of silver sulphadiazine cream (SSD) which was employed as the standard. Gels were used and various quantities of mucin and honey were incorporated to give the required formulations. Histological examination coupled with visual scoring allowed the assessment of changes to the wounded area. MH showed enhanced wound healing when compared with mucin alone, honey alone or SSD. The combination resulted in up to 89% wound healing on day 15 compared to 74 % for honey and 80% for mucin alone. The wounds that were treated with gel preparations of MH had the fastest resolution of the inflammatory, proliferative and maturation phases of wound healing. The wounds showed early resolution of oedema, fever, minimal scar, early establishment of angiogenesis, fibroblast cells and high density keratinocytes than mucin, honey or SSD formulations. Organoleptically, the MH formulations had better stability characteristics when stored under use conditions for 12 months. MH accelerated wound healing in rats when compared with mucin, honey and SSD. This may be attributed to the synergy in mucin-honey admixture when applied to full thickness experimental wounds in rats. The relevance of MH in wound healing has been established in this study.

Keywords: *Gels, full thickness wound, mucinated-honey, wound healing.*

INTRODUCTION

Wound is an interruption in the continuity of the external surface of the body. Wound healing involves a well-orchestrated, complex process leading to repair of injured tissues. Wound healing can be delayed and this is more when an acute wound turns to chronic wound due to infection, non-ideal topical wound dressing preparation or underlying medical problems [1].

Mucins are mucoproteins secreted by cells. Mucins can raise the viscosity of the medium around them. Mucin is the major glycoprotein component of mucus [2]. They are conjugated proteins in which protein is combined with a polysaccharide containing hexosamines or glycoproteins as reported by Adikwu et al [3]. In a study by Adikwu *et al* [4] it was reported that snail mucin from the giant African snail, *Archachatina marginata* (Family Arionidae) has wound healing effect.

Honey is a carbohydrate-rich syrup produced by bees, primarily from floral nectars. The British Pharmacopoeia [5] defines purified honey as obtained by purification of the honey from the comb of the bee, *Apis mellifera L*, and other species of *Apis*. Honey has an extensive history of traditional human medicinal use, in a number of societies. Molan [6] stated that it may be used alone or in combination with other substances, and has been administered both orally and topically.

Gel as a semi-solid system contains components which include continuous phase, disperse phase, gelling agents, preservatives, antioxidants, colourants and fragrance. The continuous phase of a gel is the dispersion medium which may be aqueous or non-aqueous. The disperse phase consists either small inorganic particles or large organic molecules that are interpenetrated by a continuous phase (liquid). Generally most gels are produced in aqueous medium. Examples of gelling agents include acacia, gelatin, tragacanth, aluminum hydroxide and magnesium hydroxide.[7] Gels can be attacked by microbial organisms and preservatives like sorbic acid, benzoic acid etc are used to preserve them. Gels may have fragrance and colourant incorporated to enhance the aesthetic property. In this study, a combination of muicn and honey are dispersed in gels and evaluated as wound healing agents.

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MATERIALS AND METHODS

Materials

Snail mucin was obtained from a batch prepared in our laboratory (2.8 gm per kg body weight of the snails). Honey was procured locally from Nsukka central market. The following chemicals and materials were used as obtained from their manufacturers: succinyl alanyl-alanyl-alanyl-p-nitroanilide N3050, Folin Ciocalteu's phenol reagent (Sigma-Aldrich, Steinhein, Germany). Gelatin A powder (Fairlawn, New Jersey, USA), Mueller Hinton agar (Lancashire, U.K), flamazine cream (Hoechst, Germany), salicylic acid BP (Lewes, Sussex), sodium hydroxide, monobasic potassium phosphate (Bergoyne, India), disodium hydrogen phosphate anhydrous, sorbic acid, chlorocresol (BDH, Poole, England). Rabbits and albino rats were obtained from the Animal House of the Department of Pharmacology, University of Nigeria, Nsukka. Typed bacterial cultures were sourced from Pharmaceutical microbiology laboratory of University of Nigeria, Nsukka.

Methods

Standardization of honey

The honey used for the study was standardized to Pharmaceutical Codex (PC) by diluting the honey with sterile distilled water to a weight of 1.36 g/ml at room temperature (25 ± 1 ^{0}C).

Extraction of the mucin

Snails were purchased from the local market and washed with detergent. The shells were then broken open and fleshy portions collected and placed in one litre of distilled water containing 0.2 % w/v sodium metabisulphite to prevent excessive browning due to auto-oxidation. The washings were pooled together in another glass container and desolvated with twice its volume with acetone. The wooly precipitate was collected on a filter with a vacuum pump and dried under vacuum in vacuum desiccators. The dried materials were stored in vacuum desiccators until used. The yield was 2.75 gm per kg body weight of the snails.

Evaluation of the mucin

Various ratios of mucin were weighed and allowed to hydrate in distilled water. This was allowed to equilibrate for 6 h before the studies were carried out using a differential scanning calorimeter (DSC, ZetaSizer) to evaluate for purity using phase plot and correlelogram as the parameters.

Formulation of gels

To prepare the gels, the gel base was prepared by adding hot distilled water to 15 g of gelatin powder. This was then stirred to give a uniform mixture which was allowed to cool. The various quantities of mucin and honey were incorporated by trituration into the gel base respectively to give the required gel batch. A 0.5 g quantity of salicylic acid was incorporated into 25 g of the gels using fusion method for release studies. They were stored in wide mouthed glass jars. The standard. The 1 % silver sulphadiazine cream used in this study was Flamazine Cream^{R} (Hoechst, Germany). A 0.5 g of salicylic acid was added for release studies. Formula for all the formulations are shown in Table 1.

Table 1. *Formulation of the gel batches*

Bactches	1	2	3	4	5	6	7
Honey (Gm)	23.5	-	23.5	23.5	-	23.5	-
Mucin (Gm)	-	11.5	11.5	-	11.5	11.5	-
Gel Base (Gm)	-	-	-	76.5	88.5	65.0	-
Flamazine Cream	-	-	-	-	-	-	100

Absolute drug content

The absolute drug content of the gels was determined 30 min after formulation by assaying the quantity of salicylic acid in each batch of the ointment at 37 ± 1 ^{o}C with phosphate buffer solution pH 7.6 as the dissolution medium. In each batch 0.1 g of the gels was weighed and dissolved in the phosphate buffer solution. The volume was made up to 100 ml with the phosphate buffer solution. The solution was allowed to stand for about 30 min before the salicylic acid content was assayed at a wave length of 298 nm using a U.V. spectrophotometer (Coleman, U.V. Model 6/20A). This procedure was repeated five times for each batch and the average taken. The absolute salicylic acid content of each batch of the gels was determined from a Beer's plot. This was repeated for the standard.

Pharmacodynamic studies

Animal studies

All animal studies were carried out after approval by the ethical committee of the University.

Albino rats of both sexes, aged 3 - 4 months that had not been used for any studies and weighing between 168 g to 210 g were used for this study. They were housed in the animal house of the Department of Pharmacology, University of Nigeria, Nsukka. The animals were exposed to 12 h light and dark cycle with free access to water and food.

Conditioning and anaesthetizing of the rats

The rats were allowed free access to food and water before the commencement of the studies. They were anaesthetized with ketamine hydrochloride as the base anaesthetic at a dose of 6 mg/kg body weight intra-peritonealy and mantained with 0.04 mg/kg diazepam. Six rats were used for each batch of the formulations.

Surgical infliction of wounds

After the rat was anaesthetized, about 30 mm diameter circle was shaved with a surgical blade on the pelvic anterior region. The area was washed with sterile distilled water and allowed to dry. An area of 1257.14 mm^{2} was marked in the shaved region. Sterile surgical

forceps were used to lift the rat skin, which was then incised and excised, following the marked area. The rat was put into cage and allowed to recover from the anaesthesia. The dressing of the wound commenced 24 h post surgery. The temperature of the rat was monitored rectally daily for 2 weeks. This procedure was repeated six times for each batch of the preparations.

Wound healing studies

Each of the preparations was applied to cover the surface of the wound on alternate days after washing the wound surface with sterile distilled water. The initial diameter of the circular wound was measured and monitored to evaluate the rate of healing. The diameter of the wound was the average of the vertical and horizontal diameters of the wound area.

Wound enzymological studies

In this study, the modified Barisoni *et al* [8] model was used to determine the neutrophil elastase concentration in the wound area. This was determined by using succinyl alanyl-alanyl-alanyl-p-nitroanilide as substrate. The assay medium was 0.2 M Tris-HCl in a phosphate buffer solution of pH 7.6. Wound wash was collected by washing the surface of the wound with 5 ml sterile water. The substrate (1 ml) was mixed with various volumes (100-300 micromoles) of the wound wash and incubated at 37 ± 1 ^{0}C for different lengths of time (0-150 min). The reaction was stopped at intervals by adding 0.2 ml of acetic acid and the absorbance measured at 410 nm and that of protein at 750 nm using U.V. spectrophotometer. The concentration of neutrophile elatase was calculated by using the slope of pre-determined standard curve of p-Nitrophenol while that of protein was calculated using the slope of pre-determined standard curve of protein [9].

This procedure was repeated in all the wounds until healed, except for those wounds that could not heal after 21 days. The wound wash was collected before the wound was dressed with the particular batch.

Histological studies on the wounds

Rats numbering 160 were used for this study. The base line histopathological value of the wound was determined one day post-incision using modified methods of Subrahmanyam [10] and Ghaderi *et al* [11]. The wounded area and the edge tissue were excised and put in a phosphate buffer solution (pH 7.6) in a glass container. Ten animals were sacrificed each day after collecting wound biopsy. This was repeated in all the batches at 1, 4, 9 and 13 days post-incision. The 16 animals treated with batches (15% gelatin gel base) died on day 3 as a result of pyrexia and are not included in the study. Histological examination coupled with visual scoring allowed the assessment of changes to the wounded area. The histological score method was a modified procedure of Lee *et al* [12] under light microscopic examination. Numbers 0 to 5 were ascribed to the characteristics (oedema, angiogenesis, pus cells, granulation, fibroblast, collagen, epithelia cells) of the regenerated tissue repair in wounds. The percentage of the score is plotted against time in days. Each specimen was placed in pH 7.6, buffered phosphate solution for at least 48 h. The transverse section of the specimen against the skin surface was dehydrated with ethanol, embedded in paraffin wax, and stained with haematoxylin and eosin. Each wound sample was examined and evaluated under light microscope (Leica Diastar). Photomicrographs of the three randomly selected sites of each wounded sample were taken with a digital camera (Moticam 1000 1.3m Pixel). Histological changes in the wound tissue were evaluated.

Statistical analysis

The data on salicylic acid releases, and percentage skin permeation were compared using the analysis of variance (ANOVA). Reductions in wound diameter, neutrophil elastase and bioload were tested using Student's t-tests and SPSS ver. 10.

RESULTS AND DISCUSSION

Standardization of honey

The honey used was standardized to Pharmaceutical Codex standard of 1.36 mg/ml. It was observed for 14 months with no visible change in organoleptic properties as there was no foul odour or discolouration and no change in consistency of the standardized honey.

Evaluation of the mucin

The correlegram (Fig. 1) and phase plot (Fig. 2) studies indicate that the mucin was pure. The plots were, however, indicative of various fragments of the mucin and not just a homogenous uni-structural system. The size distribution is shown in Figure 3 below. The size distribution ranged from 5 nm to 10,000 nm after hydration for 4 h at various pH levels.

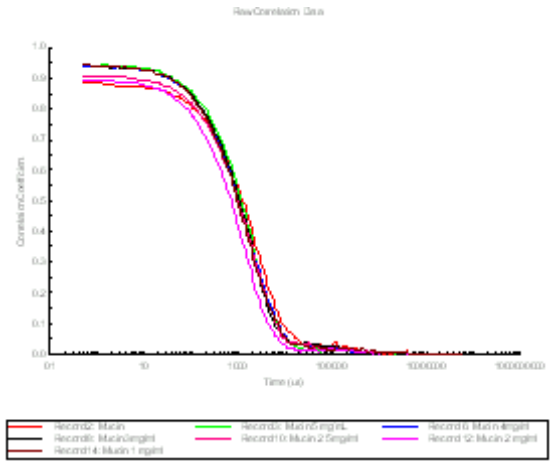

Fig. 1: *Correlegram of the mucin alone on a nanosizer*

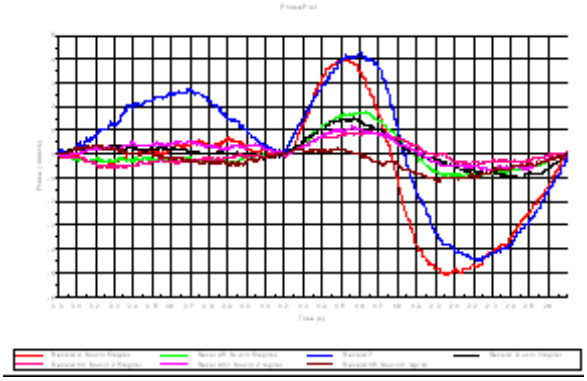

Fig. 2: Phase plot for mucin at various concentraions

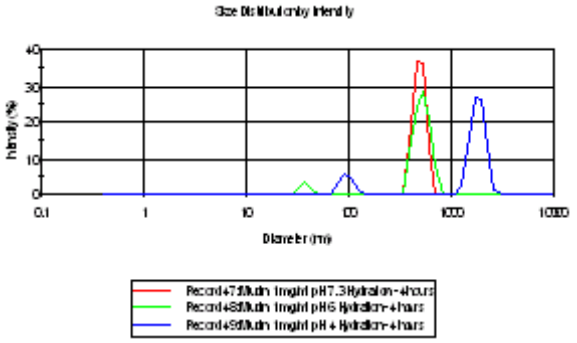

Fig. 3: Size distribution of the mucin

Absolute drug content

Absolute content of salicylic acid in the gels

The absolute drug content determination showed that the salicylic acid content of all the batches complied with British Pharmacopoeia[5] requirement with 0.5 g quantity of the formulations assessed containing approximately 10 mg of salicylic acid. Generally, the contents ranged from 94-97 % w/w of the gels.

Physical stability of the gels

There was no foul odour, discolouration or change in consistency of all batches of ointments and creams, after 14 weeks of storage at ambient temperature. Some of these parameters are shown in Table 2. There was change in the consistency of the gel batches as they became less viscous after three weeks of storage. Table 2 also shows that most of the gels were sticky to touch (adhesive). This is a good criterion for applications meant to be used on wound surfaces.

Pharmacodynamic studies

Wound healing rate

The wounds treated with the gel batches showed that the MH formulation had the highest % reduction in wound diameter, followed by formulations containing honey alone, mucin alone and SSD. The results showed that after 9 days of wound dressing, MH formulations exhibited superior wound healing ability to those of honey, mucin or standard (SSD). In comparative consideration of 9-15 days as to 3-9 days of wound dressing with the formulated products there was observed increase in the % wound reduction with time. MH had the highest % cumulative wound reduction followed by mucin, honey and SSD as shown in Table 3.

***Table 2:** Physical properties of selected gel formulations*

Batches	Minimum deviation (MID) mg	Maximum deviation (MAD) mg	Drug content (mg)	Colour amber	pH change	Texture
1. (23.5% honey)	(0.62)	(1.7)	9.65 ± 0.06	+	0.9	adhesive
2. (11.5% mucin)	(0.07)	(0.32)	9.68 ± 0.03	+	0.9	adhesive
3. (11.5% mucin: 23.5% honey)	(0.04)	(0.95)	9.84± 0.03	++	1.1	adhesive
6. (MH in gel)	(0.11)	(1.3)	9.55 ± 0.04	++	1.2	adhesive
7. (SSD)	(0.20	(1.5)	9.59 ± 0.04	white+++	0.1	nonadhesive

MID is minimum deviation in mg, MAD is maximum deviation in mg, ± is standard deviation, + is less amber colour, ++ is moderate amber colour and +++ is highly white. Nonadhesive means not sticky by touch while adhesive means sticky by touch.

***Table 3:** Percentage wound reduction at 3-15 days post dressing*

Batches	3-9 days post dressing	9-15 days post dressing
1. (23.5 % honey)	44.75 ±2.8 %	73.23 ± 1.1 %
2. (11.5 % mucin)	41.88 ± 3.2 %	74.75 ± 1 %
6. (11.5 % mucin: 23.5 % honey)	47.75 ± 2.6 %	80.88 ± 1.5 %
SSD (1 % silver sulph diazine cream)	23.25 ± 1.5 %	50.5 ± 1.8 %

In the wounds dressed with gel preparations, the same descending order of wound reduction was observed. The MH also showed the highest % reduction in wound followed by mucin, honey and SSD. At 50% reduction in wound diameter, the MH showed shorter days in wound healing than mucin, honey or SSD. In terms of 90% reduction in wound diameter MH was also better than the others.

The observed enhanced wound healing in the formulations containing MH formulations may be due to the ability of mucin to adhere to wound surface as reported by Fogelson[13] and increase the availability of the honey in the wound area. The acceleration in wound healing by MH is consistent with studies reported by Adikwu *et al* [14] that snail mucin dispersed in detarium gum gel accelerated wound healing in rats. Honey accelerates wound healing [6] and facilitates the regeneration of cells in wounds [11], and is reported [10] to heal wounds in superficial burns faster than SSD. The microenvironment in wound bed preparation is significant in overall wound healing rate and any topical agent that maintains favourable wound micro-environment will help in wound healing [15].

Characteristics of wounds treated with formulated products

The wounds showed early resolution of oedema, fever, minimal scar, early establishment of angiogenesis, fibroblast cells and high density keratinocytes than mucin, honey or SSD formulations. Some of the wound characteristics are shown in Table 4.

Table 4: *Some characteristics of wounds treated with gel batches 9-11 and SSD on day 13*

Batch	Oedema (days)	Fever (days)	Angio-genesis (days)	Fibroblast (days)	Granulation (days)	Kerat-inocytes	Scar
1. (23.5% honey)	< 5 ± 1.7	< 5 ± 1.1	< 5 ± 0.58	< 7 ± 1.5	< 9 ± 3.46	++	++
2. (11.75% mucin)	< 3 ± 2.31	< 4 ± 1.3	< 5 ± 2.89	< 7 ± 3.11	< 7 ± 0.74	++	+
6. (11.75% mucin: 23.5% honey)	< 3 ± 1.2	< 3 ± 0.58	< 5 ± 1.15	< 5 ± 0.63	< 5 ± 2.31	+++	-
SSD	< 8 ± 2.31	< 7 ± 1.1	< 9 ± 2.89	< 11 ± 3.46	< 110 ± .61	++	+++

SSD is 1 % silver sulphadiazine cream, ± is standard deviation, < is less than, - means minimal + is low, ++ means moderate and +++ means high.

The MH wound healing is able to show early resolution of oedema and fever. This resulted to early establishment of angiogenesis, fibroblast and granulation in wounds treated. Mucin [16] is reported to moderate neutrophil elastase resulting in early wound healing. The presence of the SH group which decreases enzyme activity on the mucin explain its ability to reduce the neutophil elastase activity in wounds.

Fibroblasts play multivariable role in wound tissue differentiation and granulation. Young *et al* [17] indicated that fibroblast is the most common support cell and is responsible for secreting the extracellular matrix in most tissues. One of the main functions of fibroblast is to maintain the integrity of supporting tissues by continous slow turnover of the extracellular matrix constituents. Fibroblasts in form of myofibroblasts also play an important role in contraction and shrinkage of the resultant scar tissue as in MH treated wounds. Therefore the early appearance of fibroblast cells in the wound treated with MH preparations would partly account for the accelerated wound healing observed in MH treated wounds.

The granulation substance (supporting tissue) fills the wound space for early wound healing as suggested by Herrick *et al* [18]. Elastin cells found in supporting tissues confer elasticity to enable recovery of tissue shape following normal physiological deformation. After granulation, re-epithelialization occurs and high density of keratinocyte cells indicates the maturation and healing of the wound as observed in the MH treated wounds.

The MH treated wounds also showed enhanced epithelialization and keratinocytes (+++) and a markedly diminished inflammatory response (<2days) unlike SSD that had low keratinocytes density (+) and reduced oedema in 8 days. Early resolution of inflammation during healing minimizes scar formation as observed in MH treated wounds. The studies on honey by Ghaderi *et al* [11], Efem [19] and Ali [20] also reported such wound characteristics but at less intensity compared to MH treated wounds.

Histological studies of the wound healing process using the various formulations

The histopathological evaluation results showed that MH healed wounds faster than honey, mucin or SSD when inflammatory reactions, pus cells, regenerated cells density and angiogenesis are considered.

Fig. 4 shows the histological scores of wounds treated with gel batches. The MH treated wound had drastic reduction in histological score within the 13 days followed by mucin, then honey and SSD (standard). Figure 5 shows the micrographs of the transverse sections of wounds 4 days post-treatment for ointment batches and SSD. It shows that for MH, within 4 days of dressing, the oedema (i) had resolved, granulation had reached an advanced stage (ii) and a high degree of angiogenesis had occurred (iii) as shown in Figures 5-7. The same was applicable to wounds treated with mucin but with less cell regeneration. Honey treated wounds still had the oedema present (i) while the granulation was less than the mucin treated wounds. The SSD had diffused oedema, less granulation and angiogenesis within the period under study. The micrographs in Figure 5 shows that the MH had reached the proliferative stage by day 9 as is characterized by high protein synthesis activity and blood vessels i, ii and iii, while mucin had high angiogenesis (i), squamous epithelial cells (ii) and blood cells (iii). The micrograph indicates honey treated wounds showed fewer angiogenesis and squamous epithelial cells than mucin. The SSD treated wounds (Fig. 6) were still trapped in the inflammatory stage of the wound healing as there were fewer squamous cells (ii) and less re-establishment of revascularization (i, iii). Fig. 7 shows that on the 13th day of dressing, the MH treated wounds had more defined squamous epithelial cells formed (ii), less revascularization (i) and less protein synthesis (iii). This is followed by mucin treated wounds with re-epithelialization (iii), high protein synthesis (ii) and blood vessels (i). The honey treated wounds had re-epithelialization (iii), high collagen formation (i) and high protein synthesis (ii). SSD treated wounds showed less re-epithelized cells (iii), less protein synthesis at the centre of the transverse section ii and high protein activity at the basal membrane (i). This suggests that the SSD wounds were still at the proliferative stage of the wound healing at day 13.

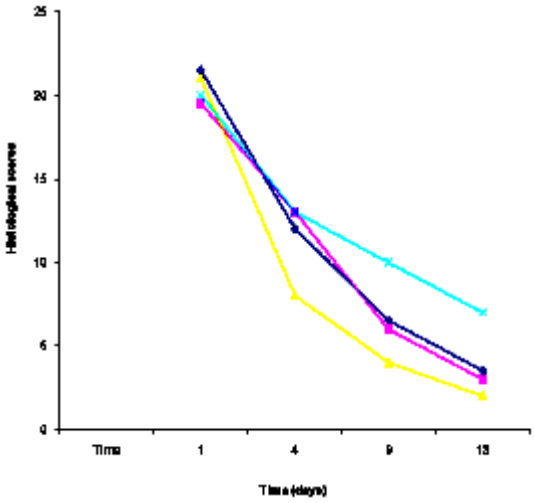

Fig.4. Plot of histological scores against time for gel batches.

Series1 Series2 Series3 Series4

Batch 1:23.5% honey Batch: 11.5% mucin Batch 3: (1:2) 11.5% mucin to 23.5% honey Batch 13: 100SSD%

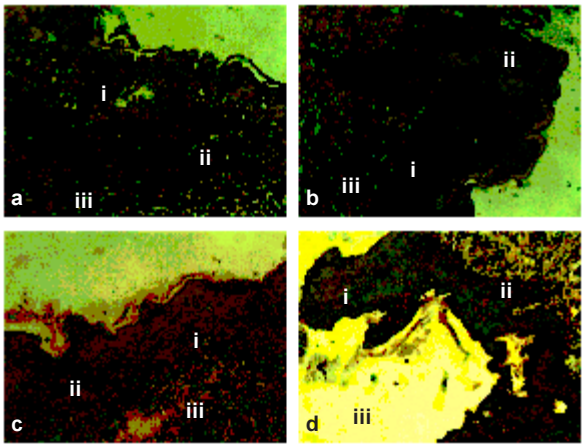

Fig. 5a: *Micrographs (a, b and c) of treated wounds for gel batches (1, 2 and 6) respectively and SSD (d) in 4 days. a, b, c and d are transverse sections of wounds treated with 23.5% honey, 11.5% mucin, mucinated-honey (11.5% mucin:23.5% honey) and 1% silver sulphadiazine cream respectively. Haematoxylin and Eosin stain x200*

The thickness of regenerating epidermis is uneven which a characteristic of regenerating skin[17]. This shows that new epidermis emanates from surviving islets of epithelial cells in the basal layer. Mucin in submucosa is known to enhance epidermal cell differentiation[21]. Gore *et al* [22] reported that topical delivery of wound medicament influences wound microenvironment. This favours rapid cell regeneration. Selby[23] observed that the histological examination of cell differentiation in wound healing shows the effect the medicament has

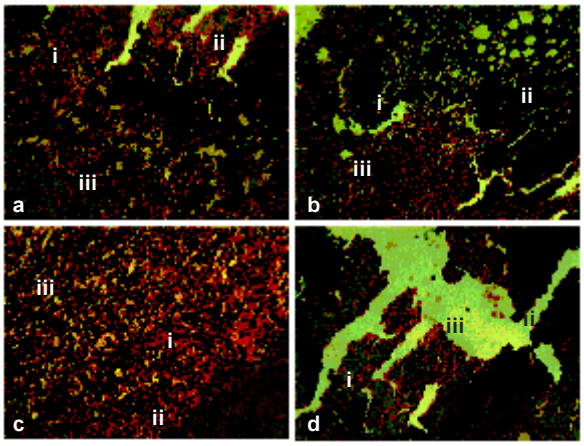

Fig. 6: *Micrographs (a, b and c) of treated wounds for gel batches (1, 2 and 6) respectively and SSD (d) in 9 days. a, b, c and d are transverse sections of wounds treated with 23.5% honey, 11.5% mucin, mucinated-honey (11.5% mucin:23.5% honey) and 1% silver sulphadiazine cream respectively. Haematoxylin and Eosin stain x200*

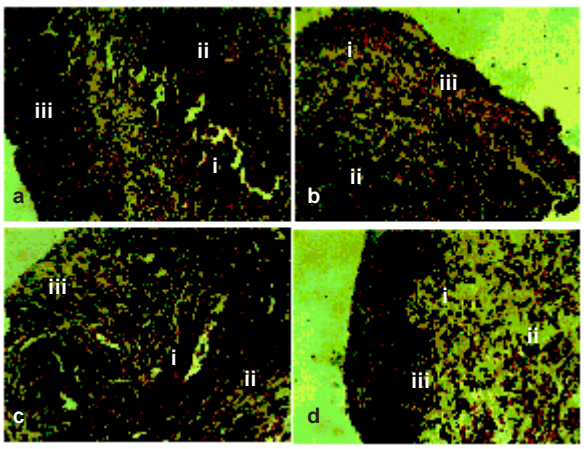

Fig. 7: *Micrograph (a, b and c) of treated wounds for gel batches (1, 2 and 6) respectively and SSD (d). a, b, c and d are transverse sections of wounds treated with 23.5% honey, 11.5% mucin, mucinated-honey (11.5% mucin:23.5% honey) and 1% silver sulphadiazine cream respectively. Haematoxylin and Eosin stain x200*

on the wound tissue. The newly regenerated cells move upward before moving sideways. The enhanced wound healing by MH implies that MH confers faster keratinocytes, endothelial cells, fibroblasts and inflammatory cells proliferation and migration to the site of injury and regeneration of cells.

As tissue cells differentiate into a stratified squamous epithelium above a newly generated basement membrane, the granulation tissue forms below the epithelium and are composed of inflammatory cells, fibroblasts and newly formed and forming vessels. Angiogenesis (i.e the generation of new capillary blood vessels from pre-existing vasculature to provide nutrients and oxygen to granulate tissue) is potentiated

in MH treated wounds, earlier than mucin, honey or SSD treated wounds (Fig. 5d). Young *et al* [17] pointed out that under physiological environment mucins (glycoprotein) appear to be involved in cell recognition phenomenon which may lead to the formation of intracellular adhesion and adsorption of molecules to the cell surface. In some situations it provides mechanical and chemical protection for the plasma membrane. The implication of this is that there will be early establishment of angiogenesis as seen in MH treated wounds. Li *et al* [24] reported that any exogenous agent that has the ability to stimulate angiogenesis accelerates wound healing. Santoro and Gaudino [25] observed that mucin (intergrin) molecules have the ability to facilitate the keratinocytes invasion of the wound bed for early wound healing while Falanga [26] explained that cells can only migrate over living tissue and that keratinocytes migration is best enhanced by moist wound environment compared to dry environment.

CONCLUSION

It can be concluded from this study that the combination of snail nucin with honey was more effective in wound healing than either muicn or honey alone. The combination offered additional advantage as the products were more stable than mucin products alone.

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