PHARMACOLOGICAL EVALUATION OF WOUND HEALING POTENTIAL OF *JASAD BHASMA* USING WISTAR RATS: A MECHANISTIC APPROACH

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Summary

Jasad bhasma ointments (10 and 20%) were made in simple ointment base B.P.). The ointments were made extemporaneously by levigating the *jasad bhasma* (JB) in Simple ointment base B.P. The ointments formed (10 and 20%) were evaluated for the acute dermal toxicity and dermal irritation study in male Wistar rats as per OECD guidelines. Wound healing potential of *Jasad bhasma* was evaluated by *in vivo* rat excision and resutured incision wound models (n=6) against povidone-iodine ointment. The excision and incision (Resutured incision wound model) were inflicted under aseptic conditions using thiopentone anesthesia. The parameters studied were percent wound closure, period of re-epithelization and scar area formation in excision wound model while tensile strength was measured in resutured incision wound model by continuous water flow technique. Results obtained were statistically compared using one-way ANOVA followed by Dunnett's test. Further the *Jasad bhasma* was evaluated for fibroblast proliferation in NIH3T3 cell line. Statistical analysis indicated dose dependent significant ($p<0.01$) healing responses in *in vivo* models. Also a dose dependent effect on fibroblast proliferation was exhibited by *Jasad bhasma*. Increased wound contraction and tensile strength, decreased period of re-epithelization and scar area along with proliferative activity on fibroblasts support the use of *Jasad bhasma in* the cutaneous wound healing.

Keywords: *Jasad bhasma*, wound healing, excision, incision, fibroblasts proliferation
Running title: Wound healing activity of *Jasad bhasma*

Introduction

Wound can be defined as disruption of cellular and anatomical or functional continuity of living tissues. The healing of wounds caused by accident, assault, welfare and surgical operations has always been a central consideration in surgical practice because any breach in continuity of skin or mucous membrane exposes the deeper tissues to the danger of infections. Cutaneous healing may be defined broadly as the interaction of a complex series of phenomena that eventuates in the resurfacing, reconstitution and proportionate restoration of tensile strength of wounded skin (1).

The wound repair phenomena involves various steps such as vascular and inflammatory phase, re-epithelization, granulation tissue formation which includes fibroplasia and matrix formation along with neovascularization, wound contraction and matrix and collagen remodeling (2). Scar is the ultimate outcome of all these complex mechanisms.

Ayurveda explains *bhasma* as anything organic or inorganic incinerated into its ash (3). These are generally end products of incineration process. The starting material undergoes an elaborate process of purification *(shodhana)*. This process is followed by the reaction phase, which involves incorporation of some other mineral and herbal extracts. Then the material in pellet form is incinerated in a furnace. The end product is expected to be non toxic material.

Traditionally *Bhasma*s have been indicated for various disorders such as *Loha Bhasma* (Iron oxide) in anemia, *Muktashukti Bhasma* (consisting of pearl, Aloe vera and vinegar) as anti inflammatory (4), *Abhrak Bhasma* (Mica) as Hepatoprotective (5), *Sankha Bhasma* (Oxide of conch shell) as antiulcerogenic. *Jasad bhasma* or *Yasad Bhasma* is one of the members of this class of Ayurvedic pharmaceuticals, which has been mentioned in Ayurveda. The major metal present in this *bhasma* is zinc. It is administered systemically in diseases like sprue, diabetes, leucorrhoea and hyperhydrosis. The role of the *Jasad bhasma* in arresting myopia has been examined in one such study (6). Zinc as a micronutrient has been reported as a co-factor of metalloproteinase enzymes like collagenase, which plays a critical role in wound bed remodeling. More recent studies have shown unequivocally that topical zinc therapy reduces wound debris and advances epithelialization in surgical wounds in the rat (7, 8). Considering the role of zinc in the wound healing process, the above study was planned to evaluate the wound healing activity of ayurvedic pharmaceutical product JB in an ointment base for local application.

Methods

Jasad bhasma ointment

Jasad bhasma was obtained as a gift sample from Shree Dhootpapeshwar Limited, New Mumbai, India. Assay by Atomic absorption spectroscopy revealed the percent content of zinc as 65.08% with the Quality control limit of 65-75%. Simple ointment base B.P. was chosen as a vehicle to prepare JB ointment for topical application. Two strengths of 10% and 20%were prepared by levigating the required quantity of bhasma in the ointment base.

Animals

The study was approved by Institutional animal ethical committee, IAEC (UICT/PH/IAEC/0405/10). Healthy Wistar rats of either sex weighing 180-200 gm sex were procured from Nicholas Piramal Ltd, Mumbai, India and used in the study. They were acclimatized to standard conditions of temperature (23 $\pm$ 1^{o}C) and humidity, for a period of 8 days. The animals were allowed free access to food consisting of standard pellet diet and water *ad libitum*. The animals were exposed to 12hrs day and night cycle. Rats were housed in a group of six animals in clean cages at room temperature. The bedding material of the cages was changed everyday. The wounds were inflicted under thiopentone (50mg/kg) anesthesia. During the study the animals were housed one per cage. Acute dermal toxicity and acute dermal irritation studies were performed as per the OECD guidelines (guideline number 402 and 404 respectively).

Wound healing activity

In vivo Excision and Resutured incision wound models were used for evaluating the wound healing activity in Wistar rats. The *in vitro* mechanistic fibroblast proliferation activity was measured in NIH3T3 cell line.

Excision wound model (9)

Wound was inflicted in rats as described by Morton and Malone. The animals were divided into four groups of 6 each. Group 1 served as control group and received no treatment. Group 2 was topically treated with the marketed povidone-iodine ointment and served as positive control. Group 3 and 4 were treated with 20% and 10% ointment respectively. The wound was dressed with the drugs applied on the 2cm X 2cm cotton patch and the dressings were secured by the adhesive paper tapes. The wound was dressed every 24 hrs and was swabbed daily with the isopropyl alcohol. Control group was also swabbed with isopropyl alcohol. Wound contraction was studied by tracing the raw wound area on 0^{th}, 4^{th}, 8^{th}, 12^{th}, 16^{th} day. Area was assessed using transparent paper and a permanent marker. The wound areas were measured using a graph paper. Number of days required for falling of eschar without any residual raw wound gave the period of epithelization. Area of scar formed after complete healing was also recorded using same technique.

Calculation of wound contraction

The wound contraction percentage was determined from the measurements using the following formula:

$$\text{Percent wound contraction} = 1 - \frac{\text{Wound area on Corresponding Day}}{\text{Wound area on Day } 0^{th} \text{ day}} \times 100$$

Incision wound model

Continuous water flow technique (10) was adopted to evaluate the tensile strength of the wound. The animals were divided into five groups of 6 each. Group 1 served as control group and received no treatment. Group 2 was topically treated with the marketed povidone-iodine ointment and served as positive control. Group 3 was given the placebo treatment of Simple Ointment Base B.P. Group 4 and 5 were treated with 20% and 10% ointment respectively. The wound was dressed with the drugs applied on the 2cm X 2cm cotton patch and the dressings were secured by the adhesive paper tapes. The wound was dressed every 24 hrs and was swabbed daily with the isopropyl alcohol. Control group was also swabbed with isopropyl alcohol. Under anesthesia, two para-vertebral incisions of about 5cm long were made through the entire thickness of the skin on the either side of the vertebral column with the help of sharp blade and scissor. The incision was closed with interrupted suture using 4-0 silk thread. Sutures were removed on 8^{th} post-wounding day and the tensile strength was determined on 10^{th} post wounding day using continuous water flow technique.

Fibroblast Proliferation Assay (11)

NIH3T3 cell line was procured from National Centre for Cell Science, Pune, India. The cells were grown as a mono-layer culture at 37°C in 5% CO_2 in minimal essential medium supplemented with 10% fetal calf serum. Cells were seeded onto 96-well plates at a density of 2×10^5 cells per well. Plates were incubated for a further 24 h, after which the medium was replaced with 200µl of Dulbecco's modified Eagle's medium containing 10% fetal calf serum in the presence or absence of different concentrations of JB. The plates were incubated in a carbon dioxide incubator at 37°C and cell growth determined after treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) test.

Statistical analysis

All the results were analyzed by one-way ANOVA test followed by Dunnett's test (Compares all groups v/s control) (n=6). Results of the activity were discussed as Mean ± SEM. The level of significance was set at ($p<0.05$).

Results

Wound contraction

A significant increase in the percent wound closure (Table 1) was seen at the end of the 4th day with both 10% and 20% ointment as compared to control. Also a significant wound contraction was seen with 20% ointment when compared to the marketed preparation i.e. povidone iodine ointment. Similar results obtained at the end of 8th and 12th day indicated higher efficiency of both 10% and 20% ointment than the marketed preparation of povidone iodine as compared to the control. An excellent wound healing was shown in terms of percent wound contraction at the end of 16th and 20th day by both 10% and 20% JB ointment when compared to marketed preparation ($p< 0.05$ and $p< 0.01$ respectively)

Period of re-epithelization

20% ointment produced quickest re-epithelization than both marketed preparation (17.83 $\pm$ 0.38 days) and 10% ointment group (17.83 $\pm$ 0.74 days). (Table 1). The control group took a longer time to produce re-epithelization than the other groups.

Scar area measurement

It was observed that 20% JB ointment produced least scar area (better epithelization) whereas control exhibited higher scar area among the groups. Both 10% and 20% ointment exhibited significantly reduced scar area than the marketed preparation. ($p<0.05$). (Table 1).

Table 1: Effects of topical application of *Jasad bhasma* ointment on wound contraction in excision wound model

Percent Wound Closure				
Post wounding days	Control Group	Marketed Formulation	20% Ointment	10% Ointment
0	0	0	0	0
4	13.66 ± 2.79	26.39± 1.27**	44.28± 3.21**	22.53 ± 1.91*
8	50.88 ± 6.05	61.90 ± 2.58*	78.64± 1.82**	65.87 ± 1.46**
12	69.17 ± 1.93	75.87 ± 1.46**	90.83± 0.91**	80.46 ± .28**
16	77.09±1.99	83.21±1.58**	97.37 ± 0.60**	89.31 ± 1.56**
Period of re-epithelization (days)	23.83 ± 0.87	17.83 ± 0.38**	13.83 ± 0.67**	17.83 ± 0.74**
Scar area (mm^2)	137.66 ± 8.63	110.83 ± 6.86*	19.33 ± 2.63**	89.00 ± 9.66**

Results indicate as percent wound contraction ± S.E.M. Results statistically compared using one way ANOVA followed by Dunnett's test. (n=6). P<0.05 considered as significant (*), p<0.01 as very significant (**) and p<0.001 as highly significant (***).

Resutured incision wound model

A significantly higher tensile strength was produced by both 10% and 20% ointment than the marketed preparation. (Table 2)

Table 2: Effects of topical application of Jasad bhasma ointment in incision wound model

Groups	Volume of Water (ml)
Control	155.00 ± 10.61
Vehicle	148.33 ± 9.00
Marketed Formulation	210.00 ± 7.67**
20% Ointment	352.50 ± 8.77**
10% Ointment	265.83 ± 11.13**

Results indicate as ml of water ± S.E.M. Results statistically compared using one way ANOVA followed by Dunnett's test. (n=6). P<0.05 considered as significant (*), p<0.01 as very significant (**) and p<0.001 as highly significant (***).

Fibroblast Proliferation Assay

The Fibroblast proliferation assay showed a dose dependent proliferation till 1000mcg/ml of *jasad bhasma*. (Table 3)

Table 3. Fibroblast Proliferation Assay (MTT Assay)

No	Group	Absorbance	% Proliferation
1	Vehicle	1.63 ± 0.08	0
2	*Jasad Bhasma* (250mcg/ml)	1.79 ± 0.10	9.90 ± 3.06
3	*Jasad Bhasma* (500mcg/ml)	1.85 ± 0.13	14.35 ± 20.37
4	*Jasad Bhasma* (1000mcg/ml)	1.95 ± 0.01	20.06 ± 9.15

Results indicate as Average Absorbance ± S.E.M. Results statistically compared using one way ANOVA followed by Dunnett's test. (n=6). $P<0.05$ considered as significant (*), $p<0.01$ as very significant (**) and $p<0.001$ as highly significant (***).

Discussion

From the earlier research work it has been found that rats fed with a zinc-deficient diet have poor wound healing (12, 13), probably due to involvement of zinc in metalloproteinases that cleave propeptides of procollagen molecules (14), in a step that determines the rate of collagen synthesis (15). Evidence for the functional role of zinc in repair systems is provided by demonstration of zinc metalloenzymes like alkaline phosphatase, RNA and DNA polymerases, and metalloproteinases (16). All these marker enzymes are involved in angiogenesis, connective tissue proliferation and cellular proliferation. Such literature endorses the role of zinc in wound healing. Conventionally antibacterial agents are used to treat and heal the wounds. Taking into consideration the important role of zinc in wound healing we decided to evaluate an ayurvedic preparation; the zinc containing bhasma for its wound healing activity on local application. Such a zinc containing bhasma is available as an ayurvedic proprietory preparation in India and hence we decided to formulate the same in an ointment base for its evaluation for wound healing activity locally. The excision wound majorly heals by wound contraction to reduce the wounded area for its rapid healing. Similarly re-epithelization rather than scar formation indicates better healing of wound. Thus parameters under consideration were wound contraction, period of re-epithelization and scar area to evaluate the healing of wound.

The results of the excision wound model clearly indicate an excellent wound healing activity of both 10% and 20% ointment under evaluation, as compared to the marketed povidone-iodine ointment, for all three parameters under consideration.

The tensile strength of a tissue is dependent on collagen synthesis and is a measure of healing of wound. The results of resutured wound model indicated excellent healing by both 10% and 20% ointment under test even better than the marketed povidone-iodine ointment. The simple ointment base did not show any such healing in this model and thus confirming the wound healing activity of JB.

A dose related increase in the proliferation of fibroblasts indicates effect of JB on the proliferation of fibrocytes. Since fibrocytes are major site of collagen synthesis, the wound healing of JB may be attributed to its proliferative action on fibrocytes.

Conclusions

Traditionally prepared JB was evaluated for its wound healing activity. The JB exhibited excellent wound healing activity by both excision wound model and resutured incision wound model. It also produced proliferative activity on fibroblasts in NIH3T3 cell line in dose dependent manner; which may be the reason for increased tensile strength in resutured incision model. Thus traditional ayurvedic proprietory *Jasad bhasma* is a promising novel candidate as a wound healer.

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