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PREPARATION AND EVALUATION OF CALCIUM- SILVER NANOCOMPOSITE FILMS ON CUTANEOUS WOUND HEALING IN RABBITS

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Abstract: The present study was conducted on eighteen rabbits divided into three groups of six animals comprising group-I control animals, group-II with calcium-silver (35:55) nanocomposite films applied animals and group-III with calcium-silver (45:45) nanocomposite films applied animals. Cutaneous wounds were created at the loin region of all the animals and the wounds were left unsutured in control group, same size nanocomposite films were cut and applied in group-II and group-III animals. Wounds were evaluated clinically by observing the appearance, extent of cicatrisation and determination of area of wound contraction in all the groups of animals at different time intervals. Compared to Group I and Group II, Early wound healing with maximum wound contraction was evident in calcium-silver (45:45) nanocomposite films treated animals. However nanocomposite films applied groups did not alter the physiological parameters. Based on these, it is concluded that calcium-silver nanocomposite films could be used safely for cutaneous wound healing without any adverse effects.

Keywords: Nanoparticles, wound healing, proliferation, inflammation, antimicrobial agents.

INTRODUCTION

Wound healing is an emerging area of research where Successful wound care involves optimizing patient's local and systemic conditions in conjunction with an ideal wound healing environment. All the preparations aimed at providing a pathogen-free, protected, and moist area for wound healing to occur. (Murphy and Evans 2012). Majority of the wound

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preparations contains combination of antibiotics, antifungal and corticosteroid agents, either in form of ointment or paste or gel or powders. However these preparations will have certain limitations like drug resistance, cost and local tissue reactions. Considering these points in mind research was diverted towards use of other materials in nano forms. Nanomaterials emerged as antimicrobial agents due to their high surface area per unit mass resulting in greater antimicrobial activity (Kemp *et al*., 2009). Silver has been used as an antimicrobial since 1800s but in the last two decades interest in silver for wound treatment resurged (Fong and Wood 2006).

Silver is one of the most powerful antiseptic materials available naturally and posses low toxicity towards the mammalian tissue (Sundaramoorthi *et al.,* 2009). The bactericidal property of silver is mainly due to its strong interaction with thiol groups present in the respiratory enzymes in the bacterial cell and also have interaction with structural proteins preferentially bind with DNA nucleic acid bases to inhibit replication (Church *et al.,* 2006).

Whereas Bishara *et al*., (2012) reported that topical silver compounds cause cytotoxicity to keratinocytes and fibroblasts in excessive doses however silver nanoparticles were preferred for external applications to other topical compounds as they offer slow, controlled release of silver ions and avoids excessive delivery, more over these are less susceptible to deactivation by the chlorides in the physiological media (Kemp *et al*., 2009).

Wound healing is a calcium mediated process which involves a cascade of events resulting in tissue repair where calcium plays a pivotal role as a secondary messenger (Kawai *et al*.,2011). Calcium has an established role in normal homeostasis of skin and is a modulator of keratinocyte proliferation and differentiation (Kawai *et al*.,2011). Influx of free calcium into the cell causes contraction of microfilaments in the cell and alters the cell shape which helps in wound healing (Stranistreet 1982). Calcium Nanoparticles show effective response over the other calcium preparations on wound healing. These calcium Nanoparticles are prepared from calcium chloride solution using beta glycerol phosphate and 0.2% acetic acid solutions (Kawai *et al*.,2011). In the present study nano calcium of phyto origin was coupled with nano silver for evaluation on the cutaneous wounds of rabbits.

MATERIAL AND METHODS

Preparation of Calcium-Silver Nanoparticles:

0.1moles of silver nitrate and calcium nitrate solution were prepared and made upto 100ml using double distilled water. Fresh leaves of pomegranate were collected from Horticultural Research Station, Achyuthapuram, Kadapa. Then the leaves were air dried and later kept in

the hot air oven at 60°C for 24 hours. Then the air dried leaves were ground and sieved using 2mm sieve. 10 gm of powder was mixed with 100ml of distilled water and treated to vigourous boiling. The extract was filtered and the filtrate was stored in refrigerator at - 4 °C for further usage. 20ml of plant extract was mixed with different proportions of 0.1 molar siver nitrate and calcium nitrate and heated at 95°C through vigorous stirring. After 1 hour change of colour was observed which was an indication for formation of calcium-silver nanoparticles. The hydrosol which was formed was transferred into a plastic container to carry out further characterization and application by TEM (Transition Electron Microscopy) analysis. The films were prepared by using Hydroxy propyl methyl cellulose polymer and ethylene glycol as plasticiser with different ratios of calcium and silver nanoparticles. Preliminary optimization of different calcium: silver combination were done and ideal combinations of films which gave satisfactory results (35:55 and 45:45) were selected for detailed study and evaluation.

Eighteen adult rabbits weighing 1.5-2 Kgs were selected and divided into three groups of six animals each (The protocols were approved by the IAEC of the institute). All the animals were prepared for anaesthesia and aseptic surgery Xylazine hydrochloride @ 5mg /kg was administered intramuscularly followed by Ketamine hydrochloride @35mg/kg to produce satisfactory anaesthesia . Cutaneous wounds of 1cm X 1cm at the loin region were created and the wounds were left unsutured (Fig-3), cleaned with normal saline and covered with steripad in group I which served as control group. A protective bandaging was applied and replaced once in two days till the wound showed healing in all groups. In group II the wounds were left unsutured, cleaned with normal saline. Calcium-silver (35:45) nanocomposite films (fig-1) cut into corresponding size of the wound and applied over the wounds (Fig-4) after making pores on the material with 22G needle and added a little normal saline to have a better adherence. In group III the wounds were left unsutured, cleaned with normal saline. Calcium-silver (45:45) nanocomposite films (fig-2) of corresponding size are applied over the wounds (Fig-5). The wound healing was assessed at seventh, fourteenth, twenty first day for the size of the wound, extent of cicatrisation, presence of discharges, formation of granulation tissue, scar formation and other complications if any.

Percentage Wound Contraction was measured to determine the reduction in wound area at different periods graphical method. Wound area was calculated on 7th, 14th, 21st post wounding day by counting number of squares of retraced wound area on graph paper. The

degree of wound healing was calculated as % closure of the wound area from the original wound using a formula according to Hima Bindu *et al.* (2011).

RESULTS

Gross changes during the healing process of cutaneous wounds in all the groups were recorded at 0, 7, 14 days post treatment. The nanocomposite films were intact, well adhered to the wound surface. Presence of wound fluid was observed up to 7 days in group I. None of the animals in group II and group III show inflammatory edema or exudates or erythema or local tissue reaction at any period of observation. External contamination and self mutilation of wounds and disturbing of external bandaging was not noticed in any group. One animal in group I showed purulent discharges in control group later on it was treated effectively. The healing process, wound contraction and other gross changes were recorded at regular intervals. None of the animals in group II and group III showed film rejection, edema formation at the sites and intense local tissue reaction. External bandaging was changed once in two days in all the groups. Group I, II and III animals showed complete healing in 20.42±0.24 (Fig-6), 16.00±0.18 (Fig-7) and 14.67±0.25 (Fig-8) days respectively (Table-1, Fig-9). Group I showed significant difference ($P<0.001$) with the other two groups.

The percentage of wound contraction at 7 and 14 days were 35.83±1.17 and 70.5±1.34 in group I, 48.33±1.23 and 85.5±1.18 in group II, 55.5±1.54 and 89.33±1.59 in group III respectively (Table-2, Fig-10). All the groups showed significant difference ($P<0.001$) between different time intervals. At day 7 all the groups showed significant difference ($P<0.001$) with highest value in group III and minimum value in group I. At day 14 group I showed significant difference ($P<0.05$) with the other two groups. At day 21 there was no significant difference. At third observation period all the groups showed complete healing.

DISCUSSION

The present work was planned in rabbits with similar managemental and hygienic conditions. Preliminary studies evaluated different combinations of calcium and silver nanocomposite films. Based on these results, optimization was done to prepare calcium-silver (35:55) nanocomposite films and calcium-silver (45:45) nanocomposite films. These combinations were compared with control group rabbits. In the present study equal size of cutaneous wound was created in rabbits of all the groups. The cutaneous wounds were washed with normal saline and protected with external bandaging to prevent contamination. In group II and group III, calcium-silver (35:55) nanocomposite films and calcium-silver (45:45) nanocomposite films were applied to the cutaneous wounds respectively. External bandaging

helped not only in protection of wound from contamination and self mutilation but also aided in immobilisation of wound edges during healing as reported by Jadon *et al.* (1985), Ansari *et al.* (1997). The nanocomposite films were closely adhered to wounds without complications. In the present study, blood clots and wound fluid was observed in control group post operatively which might be due to the haemorrhage at the wound area. None of the animals in treatment groups showed haemorrhage wound fluid. This might be attributed to haemostatic activity of the calcium (Barnett and Varley 1987). Wound fluid was absent in both the treatment groups which might be due to the rapid and uniform adherence of nanocomposite films conforming to wound bed topography preventing air or fluid pocket formation. The dressing was preferably permeable to water vapour so that a moist exudate under the dressing is maintained without pooling. Grossly none of the materials were rejected by the animals. In the present study, inflammatory reaction was absent in the animals treated with biomaterials i.e, calcium-silver (35:55) nanocomposite films and calcium-silver (45:45) nanocomposite films, which might be attributed to apoptosis of infiltrating inflammatory cells caused by silver nanoparticles (Nadworny et al. 2010). This gross finding not only explained the tolerance of the host tissue to the foreign material and also safety of the nanocomposite films. The nanocomposite films used in both the groups were found to adhere very firmly. In our study, two animals in the control group, showed purulent discharges which might be due to subsequent contamination of the wound, whereas infection was not evident in both the treatment groups. This finding was in agreement with the observations of Aldo and King (1966), Mackeen et al. (1987), Beam (1986), Bishara et al. (2012) who studied the antimicrobial effect of the silver and its composites on wound healing. However, in both the treatment groups silver being the common substance, which suggested that silver, as well as its derivatives and complexes were active against various microbes. The proposed antimicrobial mechanisms of silver include strong interaction with thiol groups present in the respiratory enzymes in the bacterial cell and also have interaction with structural proteins preferentially bind with DNA nucleic acid bases to inhibit replication (Church et al., 2006).

Early wound healing with maximum wound contraction was evident in calcium-silver (45:45) nanocomposite films treated animals. It might be due to acceleration of fibroblast differentiation, proliferation and collagen production by fibroblasts due to calcium in the films and aseptic environment created by the silver nanoparticles. In animals treated with calcium-silver (35:55) nanocomposite films the wound healing and wound contractions were delayed slightly compared to group III animals. This could be attributed to the lack of

synergism between the nanoparticles when compared to group II at this ratio. Analysis of physiological parameters like temperature , respiration and pulse rates in all the groups revealed no significant difference either between the groups or within the same group and were within the normal range of values as mentioned by Sirois (2005) and Kamalakar (2006) suggesting its safety in clinical usage.

CONCLUSION

Early wound healing was noticed in group III, followed by group II and group I. No host rejection, edema formation, local tissue reaction was observed in animals treated with nanocomposite films. Calcium-silver (45:45) nanocomposite films showed early cutaneous wound healing when compared to calcium-silver (35:55) nanocomposite films and control group.

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Table 1: Mean ± S.E values of Wound healing time (days) in control and calcium-silver nanocomposite films treated groups.

	ds Days taken for healing
Group-I	$20.42\pm0.24^{a***}$
Group-II	$16.00\pm0.18^{b***}$
Group-III	$14.67\pm0.25^{b***}$

Means bearing different superscripts differ significantly

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 2: Mean ± S.E values of Percent Wound Contraction at different time periods in 3 groups of rabbits under study

	Day 7***	Day 14*	Day 21
Group I***	35.83 ± 1.17^{aA}	70.5 ± 1.34^{aB}	100 ± 0^{C}
Group II***	48.33 ± 1.23^{bA}	85.5 ± 1.18^{bB}	100 ± 0^{C}
Group III***	55.5 ± 1.54^{cA}	89.33 ± 1.59^{bB}	100 ± 0^{C}

Means bearing different superscripts within a row (A,B..) and within a column (a,b..) differ significantly.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

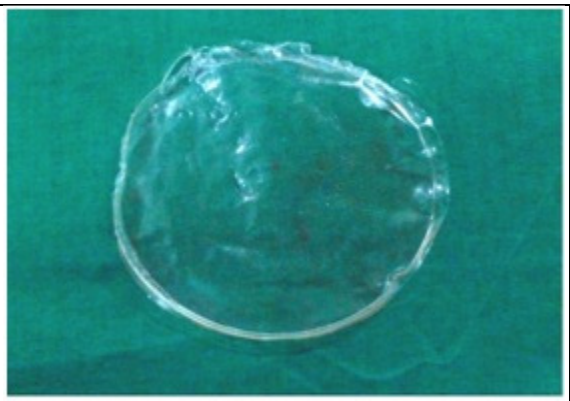

Fig-1: Photograph showing calcium-silver (35:55) nanocomposite films

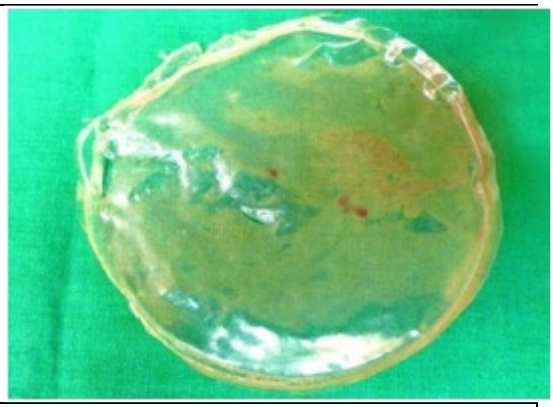

Fig-2: Photograph showing calcium-silver (45:45) nanocomposite film

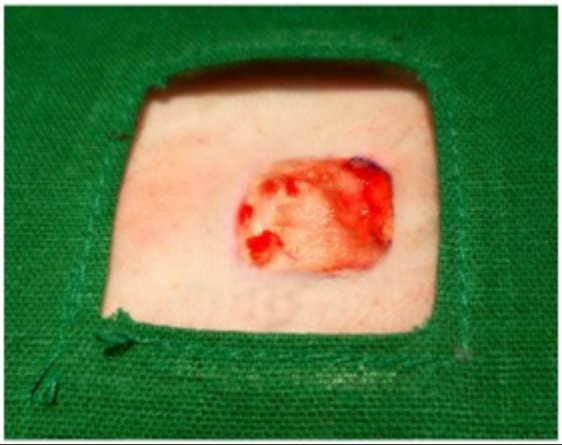

Fig-3: Photograph of gross appearance of control wound on day 0

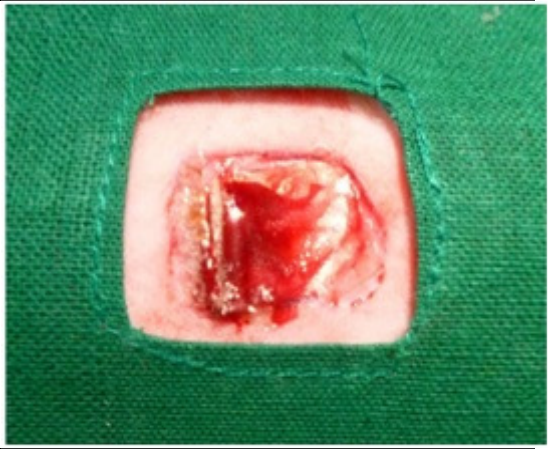

Fig-4: Photograph of gross appearance of calcium-silver (35:55) nanocomposite film applied wound on day 0

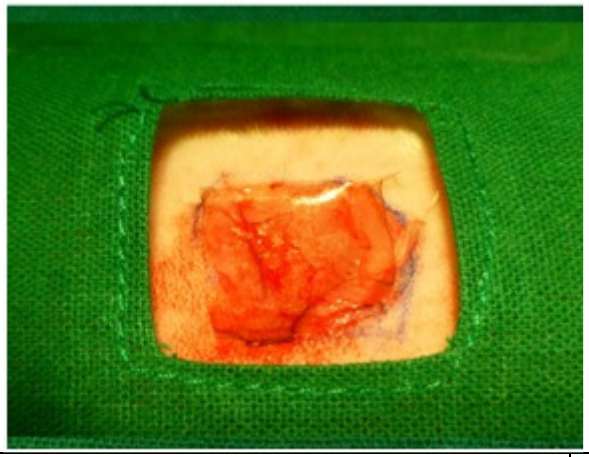

Fig-5: Photograph of gross appearance of calcium-silver (45:45) nanocomposite film applied wound on day 0

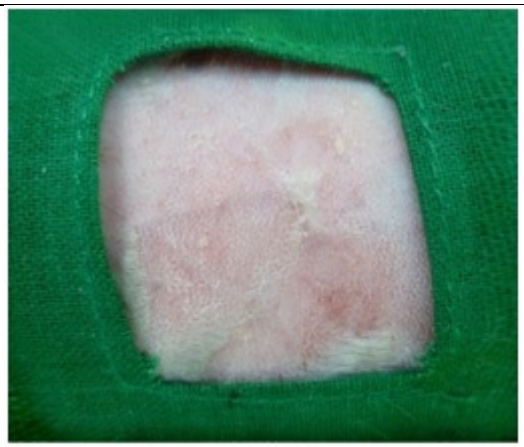

Fig-6: Photograph of gross appearance of control wound on day 20.
Note: Complete healing of wound

Fig-7: Photograph of gross appearance of calcium-silver (35:55) nanocomposite film applied wound on day 16
Note: Complete healing of wound

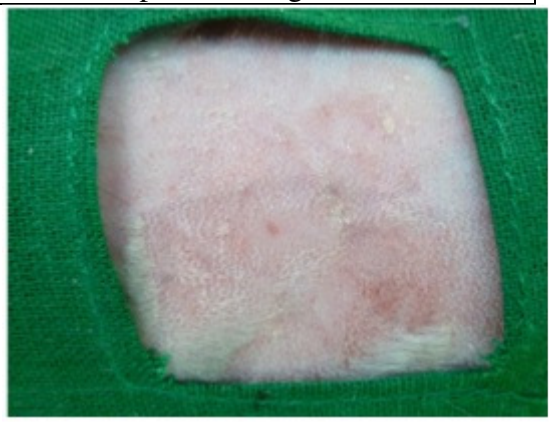

Fig-8: Photograph of gross appearance of calcium-silver (45:45) nanocomposite film applied wound on day 15
Note: Complete healing of wound

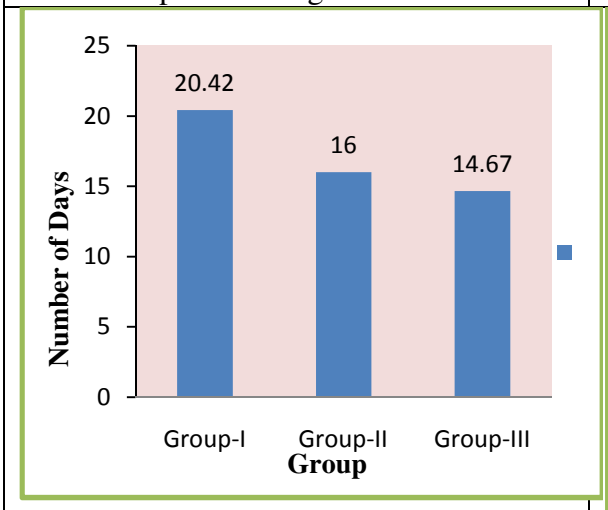

Fig-9: Graph showing the number of days taken for wound healing in 3 groups of rabbits under study

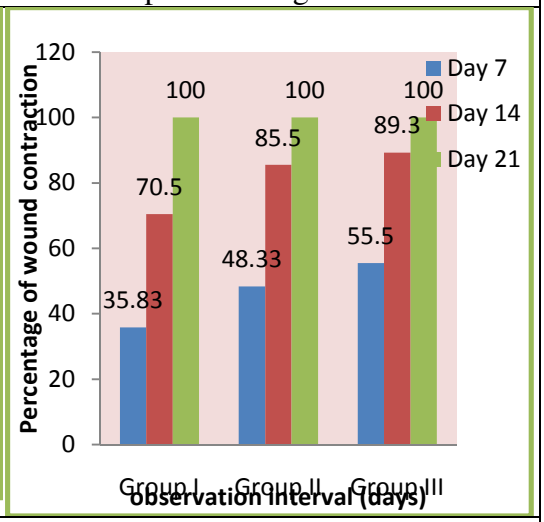

Fig-10: Graph showing the percentage of wound contraction in 3 groups of rabbits under study