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HISTOPATHOLOGICAL AND BIOCHEMICAL CHANGES IN CUTANEOUS WOUNDS OF RABBITS GRAFTED WITH CALCIUM-SILVER NANOCOMPOSITE FILMS

P Ravi Kumar[1]*, RV Suresh Kumar[2], N Dhana Lakshmi[3], Ch Srilatha[4], M Vidyavathi[5], P Veena[6] and TNKVV Prasad[7]

[1]Assistant Professor, Department of Veterinary Surgery and Radiology,
NTR College of Veterinary Science, Gannavaram- 521 102(AP)
[2]Professor, Department of Veterinary Surgery and Radiology,
[3]Professor, Department of TVCC,
[4]Professor, Department of Veterinary Pathology,
[6]Professor, Department of Veterinary Surgery and Radiology,
College of Veterinary Science, Tirupati, (AP)
[5]Professor, School of Pharmaceutical Sciences
Sri Padmavathi Mahila University, Tirupati
[7]Senior Scientist, Soil Science,
Institute of frontier Technology, Tirupati (AP)
E-mail: ravikumarpallitvm1018@gmail.com (**Corresponding Author*)

Abstract: The study was conducted on eighteen rabbits divided into three groups of six animals comprising group-I as control animals, group-II with calcium-silver (35:55) nanocomposite films applied, 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, nanocomposite films were applied in group-II and group-III animals. Wounds were evaluate by biochemical and histological studies. Hydroxyproline in granulation tissue showed significant changes among the groups and also between the groups with lower value on day 7 and higher value on day 14. Hexosamine values showed significant changes between the groups and also among the groups but with higher value on day 7 which were decreased on day 14. None of the animals showed a positive reaction for C- reactive protein at different periods of observations in all the groups. Early fibroplasia, vascularization and epithelialisation were noticed in group III compared to other two groups. It was 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 for many scientists due to varied reasons. Newer drugs were for early healing properties and safety. Majority of the wound preparations contains combination of antibiotics, antifungal and corticosteroid agents, either in form of ointment or paste or gel or powders. However these preparations have certain limitations like drug resistance, cost and local tissue reactions. Nanomaterials emerged as antimicrobial

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agents due to their high surface area per unit mass resulting in greater antimicrobial activity (Kemp *et al*., 2009). 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). Silver decreases the activity of matrix metalloproteinase which are more in burn wounds and chronic wounds there by promoting wound healing (Fong and Wood 2006). Several scientists prepared silver nanoparticles by acticoat method (physical vapour deposition) using argon gas (Fong and Wood 2006), biosynthesized from *Aspergillus niger* (Sundaramoorthi *et al*., 2009), biosynthesized from *Streptomyces aureofaciens* (Sundaramoorthi et al., 2010), and studied the effects. Calcium plays a pivotal role as a secondary messenger involved in several signalling cascades in wond healing (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. 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

Experimental design:

Eighteen adult rabbits weighing 1.5-2 Kgs were randomly selected and divided into three groups of six animals each. (The protocols were approved by IAEC of the institution). Xylazine hydrochloride @ 5mg /kg was administered intramuscularly followed by Ketamine hydrochloride @35mg/kg to produce satisfactory anaesthesia in all the rabbits. Cutaneous wounds of 1cm X 1cm at the loin region were created and the wounds were left unsutured, cleaned with normal saline and covered with steripad in Group I rabbits. In group II rabbits, Calcium-silver (35:45) nanocomposite films (fig-1) cut into corresponding size of the wound and applied over the wounds after making pores on the material with 22G needle and added a little normal saline to have a better adherence. In group III rabbits, Calcium-silver (45:45) nanocomposite films (fig-2) cut into corresponding size of the wound and applied over the wounds after making pores on the material with 22G needle and added a little normal saline to have a better adherence. Steripad was applied over the nanocomposite film and covered

with protective bandage. The bandages were replaced with new steripad and bandage once in two days until the wound showed healing.

Procedure for Hydroxyproline estimation

Estimation of hydroxyproline and the aminoacid present in the collagen fibres of granulation tissue was done in the connective tissue of the wound as per the procedure of Woessner (1961).

Granulation tissue weighing around 300 mg was homogenized in 10 ml of 6N HCl using glass homogenizer in 25 ml glass ampoules. After homogenization, the contents were transferred to the test tubes. The test tubes were sealed and hydrolysed for 3 hr at 130ºC. After hydrolysis, the test tubes were opened and the contents were collected in graduated glass cylinders. The tubes were washed thoroughly with distilled water and the washings were combined with the hydrolysate. To the above glass cylinder 2 drops of methyl red were added and shaken thoroughly. This was followed by the addition of 2.5N NaOH, drop by drop, till the pink colour changes yellow. The p^H was adjusted to 6–7 by the addition of 0.01N HCl. The sample was diluted to 50 ml with distilled water. To 2 ml of the above sample, 1 ml of chloramine –T was added. The contents were mixed and allowed to stand for 20 minutes at room temperature. To this 1 ml of perchloric acid was added and the contents were mixed and allowed to stand for 5 minutes at room temperature. To all the test tubes 1 ml of PDAB solution was added and shaken until no colour was observed. All the tubes were placed in hot water bath at 60ºC and heated for 20 minutes, then cooled under running tap water. The absorbance of the purple coloured solution was measured at 570 nm. Standard curve was prepared and the amount of hydroxyproline was expressed as mg / gm wt. of tissue.

Procedure for estimation of Hexosamine:

Granulation tissue weighing around 300 mg was homogenized in 10 ml of 6N HCl using homogenizer in 25 ml glass ampoules. After homogenization, the contents were transferred to the test tubes. The test tubes were sealed and hydrolysed at 98ºC for 8 hr. After hydrolysis, the test tubes were opened and the contents were collected in graduated glass cylinders. The tubes were washed thoroughly with water and the washings were combined with the hydrolysate. The p^H of the hydrolysate was adjusted to 7 using 4 N NaOH. The sample was diluted to 50 ml with distilled water. To 2 ml of above sample, 1 ml of 2% acteylacetone was added and heated to 96ºC for 40 minutes. The mixture was cooled, and 5 ml of 96% ethanol was added followed by the addition of 1 ml of ehrlich´s reagent. The solution was thoroughly

mixed and kept at room temperature for 1 hour. The absorbance of the pink colour solution was measured at 530 nm. The amount of hexosamine was determined by comparing with a standard curve. Hexosamine content has been expressed as μg / gm wt. of the tissue.

HISTOPATHOLOGICAL STUDIES

Representative tissue samples for histopathological studies were fixed in 10 per cent buffered formalin and were processed routinely for histopathological examination. 4 to 6μ thickness sections were made and stained with Haematoxylin and Eosin (Carleton and Drury 1965).

RESULTS

The changes in premean±S.E values of hydroxyproline of group I, group II and group III were 25.70±4.01, 27.44±1.58and 14.61±0.62 respectively (Table-1, Fig-1). Group I animals had value of 15.0±1.38 which increased significantly ($p<0.01$) to 32.91±5.13 on day-14. Group II animals had value of 18.48±1.38 on day-7 which increased significantly ($p<0.05$) to 28.43±2.65 on day-14. Group III animals showed the value mean of 26.45±2.10 on day-7 which decreased significantly ($p<0.05$) to 14.22±0.59 on day-14. The values differed significantly among three groups at day-7 ($p<0.01$) and day14 ($p<0.05$).

The premean±S.E values of hexosamine in different groups were 9.12±0.88, 13.31±1.40and 11.36±1.32 respectively (table-2, Fig-2). The values decreased non significantly from day 7 to day 14 in all the three groups and did not differ significantly day at different periods of observation.

Histopathological sections prepared out of the samples collected at different periods of observations were studied and the findings were compared for evaluating the wound healing process in all the groups.

Seventh day:

Histopathological sections of group I animals showed mild hemorrhage with more number of fibroblasts and few number of inflammatory cells. No initiation of epithelium was observed (Fig-3). Group II animals showed moderate amount of collagen and fibroblasts. Minimal number of inflammatory cells and thin epithelium were seen (Fig-4). Group III (calcium-silver animals showed no hemorrhage, Well matured collagen deposition with few number of fibroblasts and inflammatory cells besides well formed epithelium and marked proliferation of capillaries (fig-5). Compared to group II, group III showed more vasculature with less number of fibroblasts.

Fourteenth day:

Histopathological sections of group I animals showed few number of inflammatory cells, poor collagen formation and fibroblast proliferation. Improper epithelialisation with few numbers of capillaries was seen. No initiation of hair follicle and gland formation was noticed (Fig-6). Group II animals showed thin epithelial layer along with initiation of hair follicles and glands and few numbers of fibroblasts with thin collagen deposition in stroma (Fig-7). Group III animals showed thick and well formed epithelium, hair follicles and glandular structures with marked proliferation of capillaries and Stroma containing few numbers of fibroblasts with thick collagen deposition (fig-8). None of the animals showed giant cells or any other abnormal cells. No metaplastic changes were observed in any of the sections studied at different periods of observation.

Discussion

Based on preliminary studies, 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. 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) and Sreenu *et al.* (2002). 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 neither haemorrhage nor 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), 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). The mean hydroxyproline values increased significantly in group I and group II animals and decreased significantly in group III animals with a significant difference between the groups and within the group. There was a gradual increase in hydroxyproline content in group I and group II. Increased levels of hydroxyproline were indicative of an increased amount of collagen deposition. The collagen content in healing tissue was directly correlated with the laying down of fibroblasts and was suggestive of an early healing response. But in group III there was a decrease in value indicating the early completion of healing process. These results were in accordance with the findings of Jadon *et al*. (1985), Canseven *et al*. (1996), Ansari *et al*. (1997) Gomathi *et al.* (2003), Gangwar *et al.* (2006) and Nithya *et al.* (2011).

In the present study, a higher concentration of hexosamine was seen on day 7 and which gradually decreased upto day 14. The higher concentration of hexosamine content in the initial stage of repair could be attributed to its non- utilisation since granulation tissue was being laid down. This might be due to the young fibroblasts that are responsible for the secretion of mucopolysaccharides, which accumulate in the granulation tissue in large quantities at the beginning of the healing process. As the healing progressed, the concentration of hexosamine gradually decreased. Similar observations in healing tissue have been repored by Jadon *et al.* (1985), Ansari *et al.* (1997), Gangwar (2006) and Nithya *et al.* (2011).

C-reactive protein is a marker for evaluating pain and inflaammation of the animals. A change in C-reactive protein levels confirms the presence of stress and inflammation. In the

present study, none of the animals in all the groups showed a positive reaction to the C-reactive protein. These findings suggest that there was no inflammatory response in the treatment groups as reported by Khadeer Basha (2007). But this finding is in contrary with the observations of Kamalakar (2006) who reported increased C-reactive protein levels with fibrin-chitosan biocasings.

The wound healing was evaluated microscopically in biopsy specimens collected from wound at 7^{th}, 14^{th} day after treatment. In the present study, control animals showed mild haemorrhage, few inflammatory cells, fibroblast proliferation and poor collagen formation, whereas in animals treated with calcium-silver (45:45) nanocomposite films showed well matured collagen deposition with few number of fibroblasts and inflammatory cells by day-7. Early epithelialisation and hair follicles and glandular structures with marked proliferation of capillaries was evident in calcium-silver (45:45) nanocomposite films treated group which was correlated with the values of hydroxyproline, hexosamine and also with the levels of trace minerals estimated in the present study. In group II, animals treated with calcium-silver (35:55) nanocomposite films, showed moderate amount of collagen, fibroblasts and minimal number of inflammatory cells with initiation of hair follicle and gland formation by day-14. Similar observations were reported by Stone *et al.* (2000), Sezer *et al.* (2007), Burkatovskaya *et a.l* (2008), Dai *et al.* (2009) and Dai *et al.* (2011). Histopathologically, the tissue changes were early in animals treated with calcium-silver (45:45) nanocomposite films, suggesting early remodelling compared to other groups.

Conclusion

On the basis of macroscopic, biochemical and histopathological changes of wounds it was concluded that the treatment of wounds with calcium-silver (45:45) nanocomposite films enables the wounds to heal early in comparison with calcium-silver (35:55) nanocomposite films and control group of animals.

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Table 1: Mean ± S.E values of Hydroxyproline at different time periods in 3 groups of rabbits under study

	Day-7**	Day-14*
Group-I**	15.00 ± 1.38^{bA}	32.91 ± 5.13^{aB}
Group-II*	18.48 ± 1.38^{abA}	28.43 ± 2.65^{abB}
Group-III*	26.45 ± 2.10^{aA}	14.22 ± 0.59^{bB}

Means bearing different superscripts within a row (A,B..) and within a column (a,b..) differ significantly. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

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

	Day-7	Day-14
Group-I	10.48±1.11	7.75±0.89
Group-II	15.77±1.77	10.85±0.74
Group-III	13.06±1.51	9.66±1.89

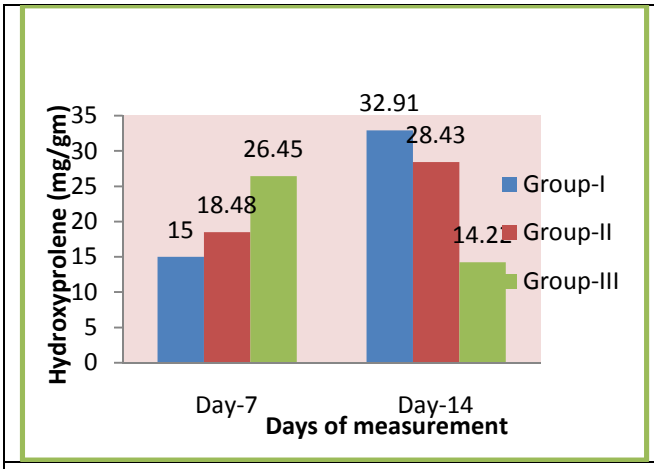

Fig-1: Graph showing the hydroxyproline values at different time intervals in 3 groups of rabbits under study

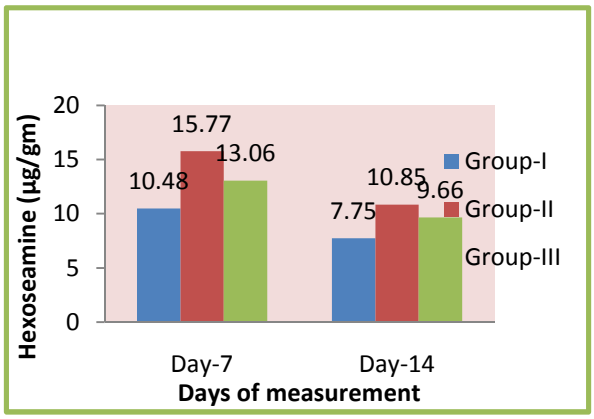

Fig-2: Graph showing the hexosamine values at different time intervals in 3 groups of rabbits under study

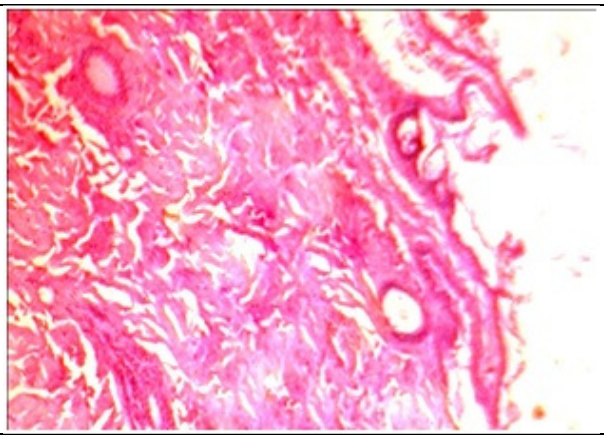

Fig-3 Control wound on day-7 Section showing mild hemmorage, thin collagen and absence of epithelium. H&EX280

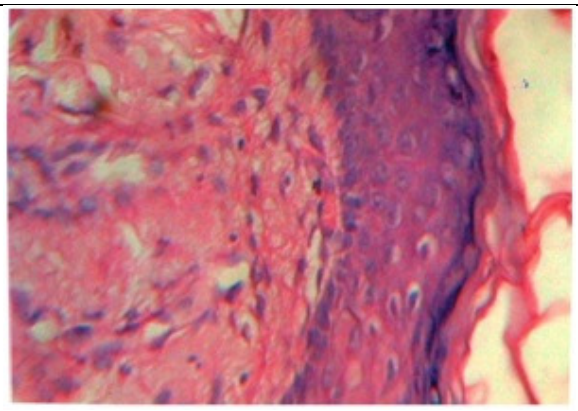

Fig-4: Calcium-Silver (35:55) Nanocomposite film applied group on day-7.
Section showing thin epithelium, moderate amount of fibroblasts and collagen with minimum number of inflammatory cells. H&EX70

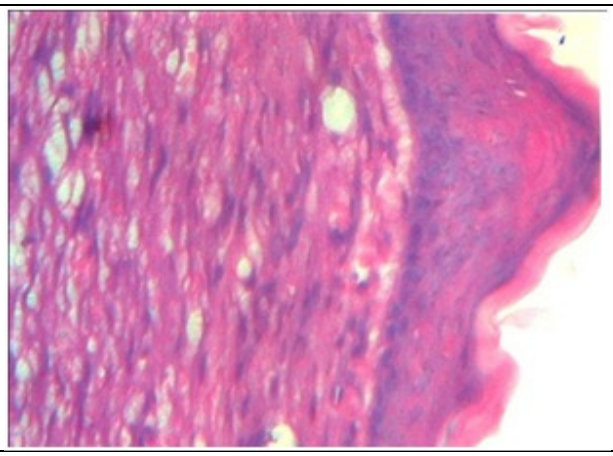

Fig -5: Calcium-Silver (45:45) Nanocomposite film applied group on day-7.

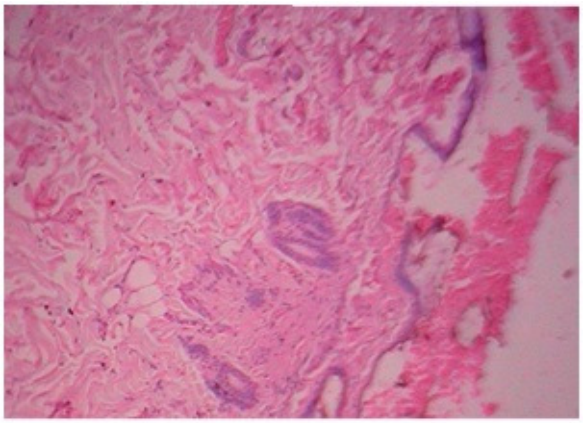

Fig-6 Control wound on day-14
Section showing haemmorage, poor collagen

Section showing well matured collagen deposition, thick epithelium, fibroblasts and few inflammatory cells. H&EX280	formation and fibroblast proliferation with improper epithelisation. H&EX280
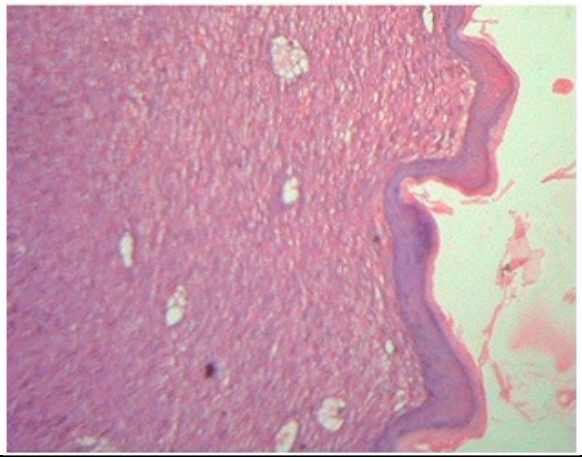	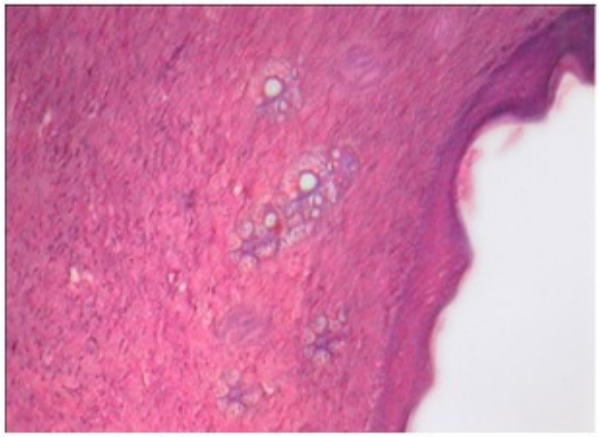
Fig-7: Calcium-Silver (35:55) Nanocomposite film applied group on day-14. Section showing moderate amount of mature collagen and fibroblasts, thin epithelium absence of inflammatory cells with initiation of hair follicles and glandular structures formation. H&EX70	Fig–8: Calcium-Silver (45:45) Nanocomposite film applied group on day-14.Section showing more amount of mature collagen and less amount of fibroblasts, well formed epithelial layer with very few inflammatory cells. formation of hair follicles and glandular structures. H&EX70