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# AN *IN VIVO* STUDY ON THE WOUND HEALING ACTIVITY OF CELLULOSE-CHITOSAN COMPOSITE INCORPORATED WITH SILVER NANOPARTICLES IN ALBINO RATS

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**ABSTRACT**

Natural polymers are used as compounds for the designing of drugs in the treatment of different ailments. Chitosan and silver nanoparticle have proven wound healing properties individually. As both have wound healing property, the combination of these two may show improvement in wound healing activity. Thus, the composite were evaluated for various *in vitro* evaluation tests to ascertain the applicability of prepared combination for wound healing activity. These films were evaluated for water absorption capacity, antibacterial activity, tensile strength and *in vivo* wound healing studies by excision wound model using albino rats. The drug loaded films have shown significant difference in water absorption capacity and antibacterial activity when compared to optimized blank composite film. There was no significant difference in tensile strength of drug loaded films when compared to blank composite films. Percentage of wound contraction was better for wounds treated with gentamycin loaded composite film than blank composite film. With the above results, it was concluded that gentamycin loaded Chitosan-Cellulose-Silver nanoparticle composite(C-Ch-Ag) films had shown more wound healing properties than Chitosan-Cellulose-Silver nanoparticle blank composite film.

**KEYWORDS:** Gentamycin; Chitosan; Cellulose; Silver nanoparticle; antibacterial; wound healing; Tensile strength.

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## INTRODUCTION

Wound healing is the body's natural process of regenerating dermal and epidermal tissues. It is the process whereby the body restores the injured part to as near its normal condition as possible. Though wound healing takes place naturally on its own, some of complications like sepsis, disruption of tissue and skin layer, maggot's formation, extension of infection to adjacent and interior organs occur in major cases. To prevent extensive loss and damage to the tissue, skin grafting[1] and biological dressings[2] were developed. The ability of the skin to repair itself after a minor wound is remarkable, but when the damage is severe or occurs in large amounts of skin area, proper and immediate coverage of wound surface with an adequate dressing is needed to protect the wound and to accelerate wound healing. Ultimately the immediate wound coverage whether it is temporary or permanent, is one of the principal goals of wound management. For this films made with biomaterials are becoming popular due to many advantages.

Biomaterials are natural polymers and biodegradable. These are used in regenerative medicine, implantable materials, controlled release carriers or scaffolds for tissue engineering. Cellulose, chitin and chitosan are widely used natural polymers. Natural polymers when used as drug delivery carriers are degraded into biologically accepted compounds, often through the process of hydrolysis, which leave the incorporated medications behind[3]. The major advantages of natural polymers are good cytocompatibility, biodegradable and do not require any surgery for removal of polymers.[4]

Chitosan is nontoxic, biocompatible, [6-8] biodegradable polymer.[4,5,7-8] It is used in drug delivery, cell delivery systems, orthopedics, wound healing,[5] ophthalmology, and bone healing.[10]It enhances the function of polymorphonuclear cells, macrophages[11] and fibroblastic proliferation and migration.[12] It exhibits antimicrobial

activity against bacteria[13], fungi, and yeast. It is hypoallergenic, haemostatic, has rapid blood clotting property and acts as fat attractor by binding to dietary lipids.[14]

Silver has been used in the clinical setting as an antimicrobial for over a century, and silver nitrate is still a common antimicrobial used in the treatment of chronic wounds.[15] Silver is effective against a broad range of aerobic, anaerobic, Gram-negative and Gram-positive bacteria, yeast, filamentous fungi and viruses.[15,16]

Cellulose is an interesting material for wound dressing since it can control wound exudates and can provide moist environment to a wound resulting in better wound healing. However, Cellulose itself has no antimicrobial activity to prevent wound infection. To achieve an antimicrobial activity, in this work silver nanoparticles were impregnated into Cellulose through the reduction mechanism by immersing Cellulose-Chitosan composite in the reduced silver nitrate solution.

Biological dressings like, chitosan films[7] and application of silver nanoparticles[15] are popular for quicker wound healing. These polymers when used in combination such as chitosan-cellulose-silver nanoparticle had shown better results, than when used alone.

Impaired wound healing due to infections and other above mentioned complications spurred the search for drug loaded films.[17] The drug loaded films are prepared by incorporating drugs like antibacterial and antibiotics in the films. The drug loaded films act as barrier to micro-organisms when applied on to wound and thus prevent secondary infections to augment the process of wound healing by stimulating wound healing environment. Therefore, drug loaded film are more useful to avoid secondary infections on the wound for fast wound healing. The wound healing property of chitosan film was improved by addition of other polymers.

Thus, the present research work concentrated on the preparation and evaluation of composite films made with chitosan-cellulose-silver nanoparticle to ascertain the applicability of prepared combination for wound management. Moreover these findings are not yet reported in wound management so far.

Chitosan, Cellulose-Silver Nanoparticle combination shows many advantages when used in other preparations like sponges, scaffolds etc. This combination also has good compatibility. Thus the present work is aimed for the preparation of gentamycin loaded composite films to promote wound healing .

## MATERIALS AND METHODS

Gentamycin was obtained as a gift sample from Integrated Marketing Co., Hyderabad. Chitosan was extracted from crab shells. All other chemicals used were of good quality and analytical grade.

### Microorganisms

Four bacteria were tested for the evaluation of antimicrobial activity of films. These include two gram positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and two gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*). The bacterial strains were procured from Bethasda Hospital, Ambur, Tamilnadu. These were maintained in nutrient agar media.

### Extraction of Chitosan from crab shells

Crab shells were washed thoroughly to remove sand and other impurities and dried well. These were soaked in 5% sodium hydroxide solution, in 3N hydrochloric acid solution overnight for deproteinization and to remove calcium carbonate. The shells were treated with acetone for discoloration and dried. The resultant chitin was deacetylated to give chitosan and washed and dried after confirmation of complete deacetylation.[18]

### Water absorption capacity

It is of utmost importance, if they are used for biological applications and wound healing. It is used to measure the capacity of blank and drug loaded films to absorb wound exudates. Preweighed, one inch film was placed in 15ml. of distilled water and the weight of the film was noted periodically at first hour, second hour, third hour and 24th hour. Every time after noting the weight, the film was placed in fresh water. Water absorption capacity of the film was determined in triplicate and calculated using a formula

% Water absorption capacity= A1-A0/A0X100

A1 -Initial weight

A0 - Final weight

### Tensile strength

Tensile strength measures the ability of film to withstand rupture, mechanical pressures or the force required to break the film. Tensile strength of the blank films and optimized drug loaded film was determined by using the Instron tensile testing machine at SDDC section in CLRI Chennai, India. It was expressed in MPa units.

### Evaluation of antimicrobial activity

The measurement of the antimicrobial activity of chitosan-cellulose-silver nanoparticle composite films and drug loaded films was done by agar diffusion method using selected gram positive and gram negative organisms. The zone of inhibition was determined by placing a definite size of film into discs made in inoculated solidified nutrient agar medium in a Petri plate which were incubated for 24hrs at 37±1°C. This was done in triplicate with each film for each organism and an average diameter of zone of inhibition was noted.

**Evaluation of wound healing activity by *in vivo* studies**

The wound healing activity was evaluated by excision wound model in the adult albino rats. Pathogen free adult albino rats weighing 150-200 gms were selected for the study. The wound healing activity was conducted with the protocol as shown in table 2. The animal work was approved by institutional ethical committee.

The anaesthetized animal was placed on the operation table in normal position. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals on inter scapular region i.e., 5mm. away from ears. Full thickness skin from the demarked area was excised to get a wound area of 2 sq.cm. After achieving haemostasis, the wound was blotted with sterile gauze in control group (Group I) and the respective film on the wound of animals in treatment groups (Group-II and III). Then the following parameters were determined at specific time intervals.

**Percentage of wound contraction**

Wound healing is a process by which damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. It mainly depends on the repairing ability of tissue which may be reduced due to infections. It was measured to find the extent of reduction in wound area at different periods of treatment by graphical method. Wound area was calculated on 5th, 10th, 15th, 20th, and 25th 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:

% closure = 1-(Ad/A0) X 100

(A0 – Wound area on day zero, Ad – Wound area on corresponding days).

**Histopathological studies**

Understanding of microscopic changes is important in assessing efficacy of films in wound healing. The skin tissue samples collected by corneal trephiner at 5th, 10th and 20th day of post-treatment in different groups for evaluation of the extent of wound healing by studying the histopathological characteristics. Biopsy specimens were preserved in 10% buffered formalin. They were processed by routine paraffin embedding technique i.e., 5-6 microns thick sections were cut and stained with haematoxylin and eosin.[19]

**Photography**

The photographs of wound from different groups were taken at specific intervals for visual comparison.

**Tensile strength of skin sample**

The skin tissue samples of normal skin and treated skin were collected at the end of the study i.e., after the complete healing of the wound and tensile strength of these samples were measured to compare the skin after treatment with normal skin.

**Statistical Analysis**

The results are expressed as mean ± S.D. Statistical analysis was performed by analysis of variance (ANOVA) test and statistical significance was set accordingly at $P = 0.05$ level.

**RESULTS AND DISCUSSION**

Tensile strength of the blank film was found to be 1.54 MPa and optimized drug loaded film was 1.69 MPa which was determined by using the Instron tensile testing machine. Tensile strength measures the ability of film to withstand rupture, mechanical pressures or the force required to break the film. Significant variations were found between the blank and drug loaded composite film as shown in Table 1 due to the addition of gentamycin, which may stabilize the composite film.

The water absorption capacity of composite films was significantly increased with increase in Cellulose concentration, which may be due to hydrophilic and swelling properties of Cellulose. The water absorption capacity of drug loaded film was not significantly different with change in concentration of drug. It was found that, there was significant difference in water absorption capacity of blank composite film and drug loaded film as shown in Table 2. It indicated that the drug is decreasing the water absorption capacity of film which may be due to interference of drug in water absorption by polymers.

**Bioevaluation**

The zone of inhibition with various films against four different bacterial species was different in agar disc diffusion technique as shown in Table 3. There was no inhibition of growth with cellulose, which may indicate that the cellulose itself has no antimicrobial activity. As the concentration of drug in the film was increasing, the mean diameter of zone of inhibition was also increased with drug loaded film. Among biocomposite films, gentamycin loaded film has shown maximum inhibition against all tested bacteria. The antibacterial activity of drug loaded films was more than the blank composite films. It confirmed that the antibacterial activity of the drug loaded film is higher than blank films, so drug loaded films can show fast wound healing property than blank film.

***In vivo* studies**

The percentage of wound contraction in untreated and treated groups was measured and the results are shown in

Table 4. There was a significant difference in the percentage of wound contraction between wounds treated with optimized Chitosan-Cellulose-Silver nanoparticle composite(C-Ch-Ag) (Group-II) and drug loaded film (Group-III). It indicated that Chitosan-Cellulose-Silver nanoparticle composite(C-Ch-Ag) film and drug loaded composite film have improved wound healing activity than control group. Wound contraction was significantly increased in wounds treated with drug loaded composite film when compared to wounds treated with blank composite film and untreated wounds, which indicated that the composite film has shown more wound healing property than control group. Drug loaded film has shown increased wound contraction than blank film. The former indicated that loading of drug into composite films augmented the healing of wound than blank composite films. This may be due to broad antibacterial activity of gentamycin which reduces infections and thus fastens the healing of wound.

Damaged cells in epidermis and around the untreated wound was found whereas restoration and recovery of cells were observed in wounds treated with blank composite film (group II) and gentamycin loaded Chitosan-Cellulose-Silver nanoparticle composite (group III) compared to untreated wounds (group I) in 25th day photomicrographs. Further rapid epithelialization was found in photomicrographs of wounds treated with drug loaded composite films indicated the improvement in wound healing activity compared to wounds treated with blank composite film as shown in fig 1& fig 2. Almost normal cytoarchitecture of skin was observed in wounds treated with gentamycin loaded films on 25th day when compared to all other groups as given in Figure 3& Figure 4. This suggested that gentamycin drug loaded films may have more capacity for fast recovery and rapid epithelialization of skin than untreated and wounds treated with other film. It may be due to its antibacterial action which prevents further infections on wound supporting for fast epithelialization by stimulation of wound healing environment.

Based on the photographs shown in fig 5, it was found that the wound size was decreased by 10th day and wound treated with gentamycin loaded composite film has shown better result in wound healing than wounds treated with blank composite films. The size of wound in this group treated with gentamycin loaded film was more healing when compared to the other groups on respective days which indicated that loading of drug into composite films augmented the healing of wound than blank composite films. As per Table 5, increase in maximum extension of treated skin indicates that improved flexibility of skin after treatment. The effect of stress on elongation of skin has shown positive results of treated skin. The elongation percentage at break was higher in treated skin than normal skin, suggested that remodeling of skin was in progress. Maximum load and tensile strength of treated skin was less than normal skin, which indicated slow progress of the strength of treated skin near to normal skin.

## CONCLUSION

A composite film made with the combination of chitosan and Silver nanoparticles has shown improved water absorption capacity, tensile strength, wound contraction, histopathological characteristics and visual healing in invivo studies. The antibacterial activity against various bacterial species was improved by combination of Silver nanoparticle with chitosan as Silver nanoparticle can inhibit the growth of bacteria. Based on above findings it can be concluded that the composite film of chitosan and Silver nanoparticle is successful in wound dressing for wound management with improved wound healing properties. Further the gentamycin loaded films has shown improved antibacterial activity and wound healing properties in *in vivo* studies by releasing the drug completely without changing the water absorption capacity and the tensile strength of blank film.

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**Table 1 Tensile strength of composite films**

| S.No | Tensile strength parameters | C-Ch-Ag blank films | Gentamycin loaded C-Ch-Ag films |
|---|---|---|---|
| **1.** | Maximum load (N) | **41.27** | **30.57** |
| **2.** | Maximum extension(mm) | **4.67** | **2.17** |
| **3.** | Elongation at break (%) | **9.33** | **8.33** |
| **4.** | Tensile strength (MPa) | **1.54** | **1.69** |

**Table 2 water absorption capacity of the biocomposite films**

| S.No | Time (h) | C-Ch-Ag Blank films (%) | Gentamycin loaded C-Ch-Ag films (%) |
|---|---|---|---|
| 1. | 01 | 1236 | 993 |
| 2. | 02 | 1245 | 1104 |
| 3. | 03 | 1438 | 1112 |
| 4. | 24 | 1498 | 1241 |

**Table 3 Antibacterial activity**

| s.no | Name of the sample | Diameter of zone of inhibition (in cm.) (Mean±S.D.) | | | |
|---|---|---|---|---|---|
| | | ***Bacillus subtilis*** | ***Staphylococcus aureus*** | ***Escherichia coli*** | ***Pseudomonas aeruginosa*** |
| 1. | C-Ch-Ag blank films | 1.70±0.045 | 1.66±0.09 | 1.5±0.072 | 1.52±0.040 |
| 2. | Gentamycin loaded C-Ch-Ag films | 2.31±0.035 | 2.38±0.047 | 2.28±0.06 | 2.13±0.045 |

**Table 4. Protocol and percentage of wound contraction**

| S.N | Group | Name of the sample | Wound contraction (%) (mean ±S.D) | | | | |
|---|---|---|---|---|---|---|---|
| | | | **5$^{th}$ day** | **10$^{th}$ day** | **15$^{th}$ day** | **20$^{th}$ day** | **25$^{th}$ day** |
| 1. | I | Cotton gauze | 8.73±0.02 | 20.38±0.28 | 65.28±0.33 | 72.62±0.27 | 82.62±0.27 |
| 2. | II | C-Ch-Ag films | 21.31±4.2 | 59.80±2.95 | 75.91±2.10 | 82.56±2.01 | 92.46±1.11 |
| 3. | III | Gentamycin loaded C-Ch-Ag films | 59.49±3.61 | 83.15±2.05 | 93.01±0.32 | 95.6±0.08 | 99.68±0.06 |

**Table 5 Tensile strength of normal and tested skin sample**

| S.No | Tensile strength parameters | Normal skin sample. | Treated with C-Ch-Ag blank films | Treated with Gentamycin loaded C-Ch-Ag films |
|---|---|---|---|---|
| 1. | Maximum load (N) | 92.22 | 84.12 | 85.79 |
| 2. | Maximum extension (mm) | 13.83 | 15.17 | 14.67 |
| 3. | Elongation at break (%) | 95.24 | 101.23 | 103.33. |
| 4. | Tensile strength (MPa) | 6.4 | 6.00 | 6.33 |

Figure 1
5th Day (Group II)

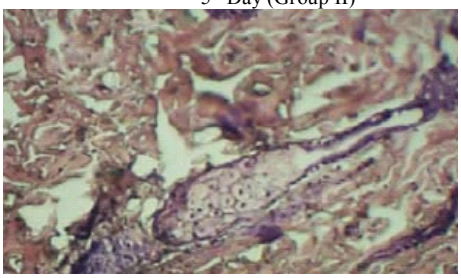


Figure 2
25th Day(Group II)

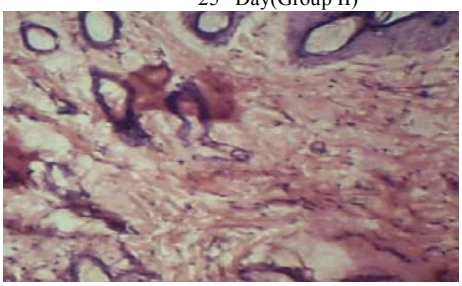


Figure 3
5th Day(Group III)

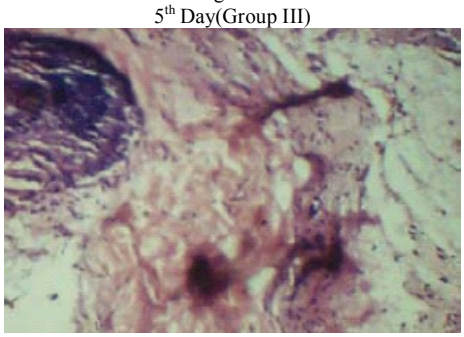


Figure 4
25th Day (Group III)

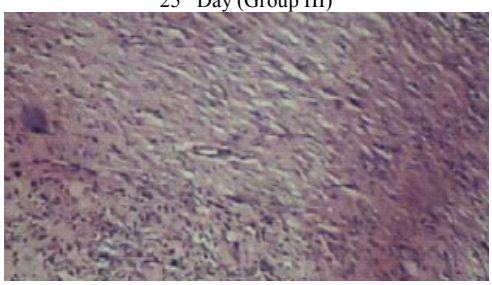
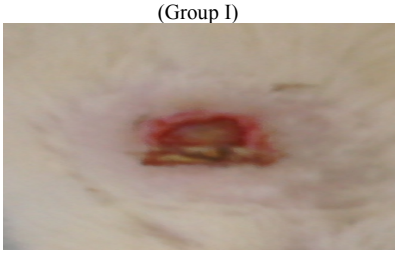


Figure 5

(Group I)



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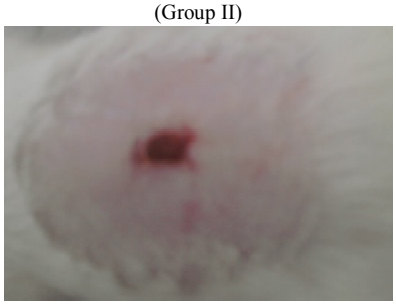
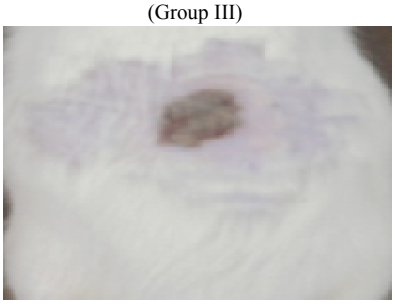


(Group III)



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