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APPLICATION OF LYSOZYME AND DEXTRAN CONJUGATED LYSOZYME AS NATURAL ANTIMICROBIAL AGENTS IN THE TREATMENT OF EXPERIMENTAL SKIN WOUND IN MICE

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ABSTRACT:

Background: Skin wound infected by *Staphylococcus aureus (S. aureus)* is a common and painful lesion. Tetracycline is commonly used as the antibiotic of choice for its treatment.

Aim: to determine the effectiveness of hen egg white lysozyme and dextran conjugated lysozyme in decreasing the bacterial count of experimentally induced skin wound infection in mice.

Methods; Lysozyme was conjugated with dextran under mild Maillard reaction conditions. Lysozyme and conjugated lysozyme were tested *in vitro* for their effectiveness against *S. aureus* and *E. coli*. Mouse model of skin wound infection was prepared in 6 group of mices (n=5) by 1cm × 1cm paravertebral laceration, extending to fascia created by scalpel. Five minutes after wounding, 10 µl culture media suspension containing 10^6 CFU⁄cm^2 *S. aureus* was inoculated. Lysozyme and dextran conjugated lysozyme were applied as a 400µg/ml eucerin ointment base in treatment groups. Tetracycline was applied topically in the third group of mice.

Results: Both Lysozyme and conjugated lysozyme exhibited *in vitro* antibacterial effect against *S. aureus* at 400µg/ml, but only the conjugated lysozyme was effective against *E. coli*. Results showed a 2, 2.1 and 3.5 log 10^6 CFU ⁄cm^2 decrease in *S. aureus* count after 12 days in lysozyme, conjugated lysozyme and tetracycline treated mice, respectively.

Conclusion: Although tetracycline appears to be more effective than lysozyme, due to increased prevalence of antibiotic resistance, application of natural antibacterials such as lysozyme and its derivatives is suggested in the treatment of infected skin lesions.

INTRODUCTION: *Staphylococcus aureus* is the most common causative agent of primary and post – operative skin infections [1, 2]. Certain skin and soft tissue infections may lead to severe complications such as sepsis.

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S. aureus has become increasingly resistant to the available antibiotics. Frequently seen are methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus* which are dilemma for health and science [3]. The outbreak of antibiotic resistance in infectious agents has urged pharmacologists and pharmaceutical industries to look for novel antimicrobial agents. An important stage in testing the potential of natural products as antimicrobial drug candidates is to establish their effectiveness in an animal model system [4]. A useful study should be clinically relevant, experimentally easy, ethically

acceptable and convenient to perform and should provide reliable and reproducible results. Additionally, extensive side effects of drugs have led many countries to move towards searching for natural antimicrobials. Lysozyme is a well-known enzyme that has the ability to lyses bacterial cells [5, 6]. Lysozyme is abundantly found in nature and is produced by bacteria, fungi, plants, birds and mammals. Some viruses even contain genetic code for lysozyme [7].

Chicken egg white lysozyme is a polypeptide with 129 amino acids and possesses the enzymatic activity against ß–1, 4 glycoside linkages between N–acetyl muramic acid and N–acetyl glucosamine found in peptidoglycan, the major component of the outer membrane of Gram positive bacteria. Its effect against Gram negative bacteria is limited due to the presence of a lipid layer in the membrane of these bacteria [8].

The purpose of this investigation was to prepare and assess the antimicrobial effects of lysozyme and its dextran conjugate as topical ointments for the treatment of *S. aureus* infected skin in mice.

MATERIALS AND METHODS: Lysozyme was provided by Canadian Inovatech (Abbotsford, British Columbia). Dextran (M_r 10,000) and Sephadex G–100 were obtained from Sigma (St Louis, Mo). Protein molecular – weight markers were from Fermentas (Molndal, Sweden). TSB broth, McConkey agar, Blood agar, Baird Parker agar, Mannitol salt agar media were obtained from Merck (Darmstadt, Germany). *E. coli* IFO 1399 and *S. aureus* IFO 1112 were obtained from the Persian Type Culture Collection (Tehran, Iran). All other chemicals were purchased from local suppliers. Four week old male mice were bought from Razi Institute (Shiraz, Iran).

Preparations of Lysozyme Dextran Conjugate: Four hundred mg lysozyme and 2 g dextran were mixed in 5 ml 0.1 M sodium phosphate buffer (pH 7.0) and lyophilized as described by Scaman et al 2006[9]. The mixture was incubated at 60°C and 78.9 % relative humidity provided by saturated KBr for 1 week. A control (without dextran) was treated under the same conditions. The prepared lysozyme-dextran conjugate was separated from the untreated lysozyme by gel permeation chromatography, using a Sephadex G-100 column.

The column was equilibrated and eluted with 0.05 M phosphate buffer (pH 7.4), and then 100 mg of conjugated lysozyme was dissolved in 1ml of 0.05 M phosphate buffer (pH 7.4). The solution was gently mixed and centrifuged at 2500×g for ten minutes. The supernatant was applied to the Sephadex G-100 column (90 × 1.5 cm), and the column was connected to the buffer reservoir.

The protein content in each fraction was determined by measuring the absorbance at 280 nm. All fractions containing the lysozyme dextran conjugate were pooled and lyophilized. Fifty mg of un-conjugated lysozyme was treated similarly. The soluble protein content of all samples was determined by the method of Lowery et al 1953 [10].

Electrophoresis: Sodium dodecyl sulfate (SDS) slab gel electrophoresis was performed according to the method of Laemmli, 1970 [11]. Protein samples were added to the loading buffer to give final concentration of 1mg⁄ml protein, 10% glycerol, 0.1 M Tris-HCl, 0.004% bromophenol blue and 0.4% SDS.

The running gel consisted of 10% polyacrilamide gel in 1.2 M Tris-HCl and 0.3% SDS. The electrode buffer comprised 0.025 M Tris-HCl, 0.192 M glycine and 0.15% SDS. Electrophoresis was performed at a constant current of 15 mA. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and destained with 7% methanol and 10% acetic acid.

***In vitro* antimicrobial activity:** The antimicrobial activities of lysozyme and its conjugated derivatives were determined against *E. coli* as a representative of Gram negative and Gram positive bacteria representative *S. aureus*. Microorganisms were incubated in TSB broth at 37°C for 24 hours and then ten-fold diluted to give a final approximate number of 10^6 CFU⁄ml. TSB broth medium (1.5 ml) containing 800 µg⁄ml lysozyme or modified lysozyme were diluted to give final concentrations of 50, 100, 200 and 400µg⁄ml.

A volume of 0.75 ml TSB containing suspension of bacteria was added to 0.75 ml solutions contains various concentrations of lysozyme or conjugated lysozyme. Mixtures were incubated at 37°C for 24 hours and their optical densities were recorded every hour for 20 hours at 600 nm.

Mouse model of skin wound infected by *S. aureus*: Thirty specific–pathogen free male Balb/c mice weighting 20 to 30 g were allocated into 6 groups. In each group 5 mice were used. Treatment groups were designated as follows: Group1- control, wound without inoculation and treatment, group 2- infected wound without any treatment, group 3- infected wound with eucerin as ointment base treatment, group 4- infected wound with 400 µg/ml lysozyme ointment treatment, group 5- infected wound with 400 µg/ ml lysozyme-dextran ointment treatment and group 6- infected wound with tetracycline ointment treatment.

Before experimental induction of wound, mice were anesthetized with intraperitoneal injection of a cocktail composed of 100mg/ml ketamine and 10mg/ml xylazine and the body was then shaved. Animal infection experiments were performed in accordance with institutional and national guidelines [12]. Paravertebral lacerations (1cm × 1cm) extending to fascia were created with scalpel. Five minutes later, an aliquot of 10 µl suspension containing 10^8 CFU/ml *S. aureus* was inoculated over the whole surface by the pipette tip [13].

Wound managements: Lysozyme and modified lysozyme were formulated in eucerin ointment base at a concentration of 400 µg/ml. Tetracycline was used as a commercial 3% ointment. Drugs were administered topically for 9 days. Additional groups of mice received eucerin ointment or remained untreated as infected and uninfected control groups. Swab samples were collected from infected wounds and were suspended in a known volume of sterile saline. Bacterial populations in experimentally infected wounds were counted on days 3, 7 and 12 after infection. Aliquots of each sample was cultured on Baird Parker and mannitol salt agar and incubated overnight at 37°C.

Statistical analysis: Data were analyzed by the ANOVA procedure of COSTAT. Comparison of means was performed using Duncan multiple range test and $P < 0.05$ was considered as significant.

RESULTS:

Conversion of lysozyme to lysozyme dextran conjugate: Incubation of chicken egg white lysozyme at 60° C and 79% relative humidity for 7 days resulted in pale browning of protein powder, indicative of Maillard reaction. **Figure 1A** shows the elution profile of lysozyme-dextran conjugate from gel permeation chromatography on Sephadex G–100 column. Compared with unmodified lysozyme, the glycosylated lysozyme was eluted in void volume of G-100 gel permeation chromatography column. SDS-PAGE results are shown in **Figure 1B** which indicates the appearance of diffused bands.

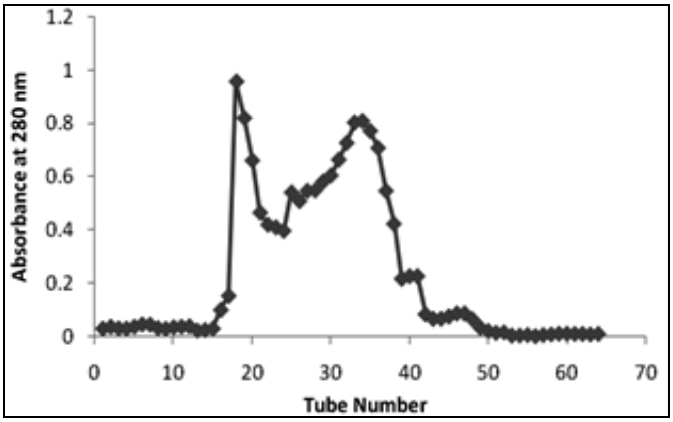


FIGURE 1(A)

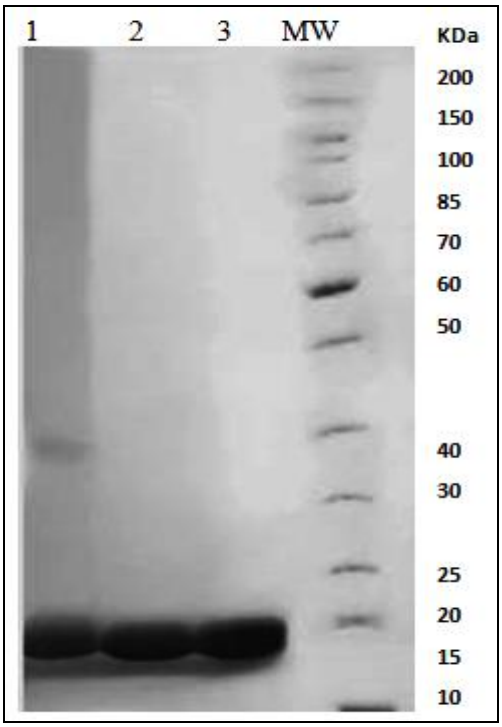


FIGURE 1(B)

FIGURE1: (A) Elution of lysozyme-dextran conjugate and lysozyme from a Sephadex G-100 column (90 by 1.5). Glycosylated lysozyme was prepared at pH 7.0 and 60°C, for 1 week. The column was equilibrated and eluted with 0.05M sodium phosphate buffer (pH 7.4.). **(B) SDS –PAGE results of dextran-conjugated lysozyme (10%gel, 20µg of protein per well).** 1: Fractin I from Figure 1: Dextran-conjugated lysozyme, 2: Fraction II from Figure 1 (unreacted lysozyme). 3: Native lysozyme. MW: Molecular mass markers**.**

Antimicrobial activity of the conjugated lysozyme: Figures 2A and 2B show that the activity of lysozyme and lysozyme dextran conjugate on *S. aureus* is a function of enzyme concentration. The antimicrobial effects of lysozyme dextran conjugate against *S. aureus* were not remarkably different from that of unmodified lysozyme. **Figure 2C** shows that lysozyme did not have significant antimicrobial effect against *E. coli*, but a concentration dependent activity of modified lysozyme against *E. coli* was observed (**Figure 2D**). For the conjugate derivative the highest antimicrobial activity was observed at 400µg /ml.

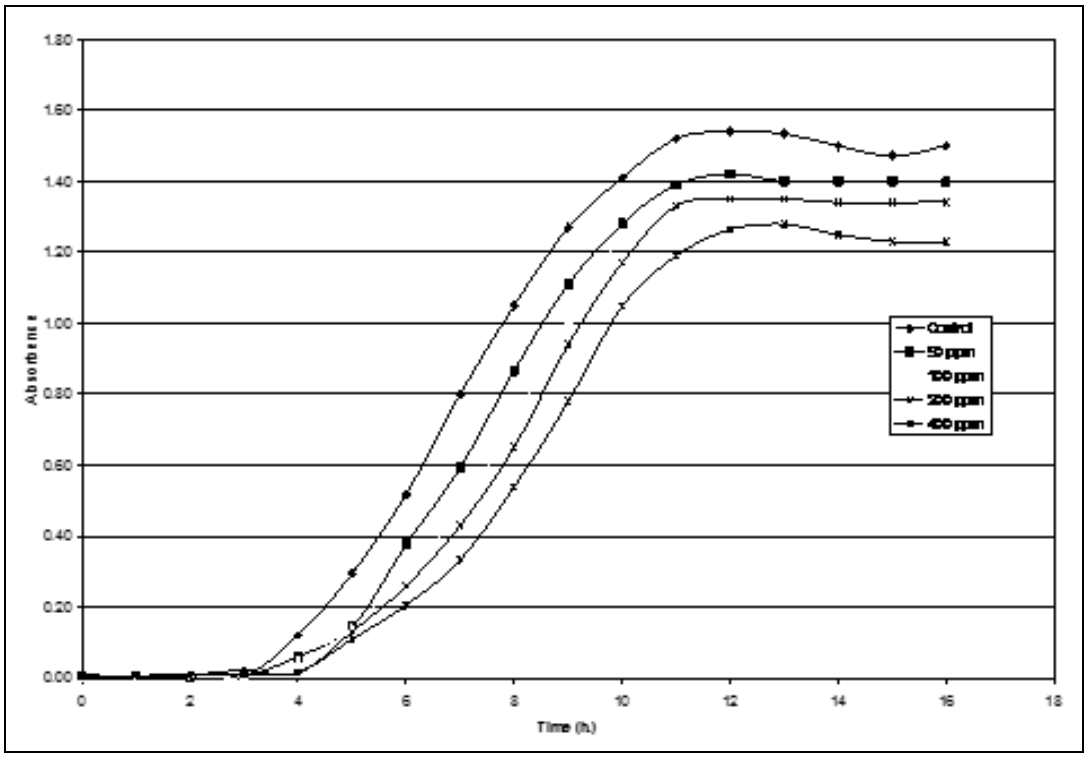


FIGURE 2A

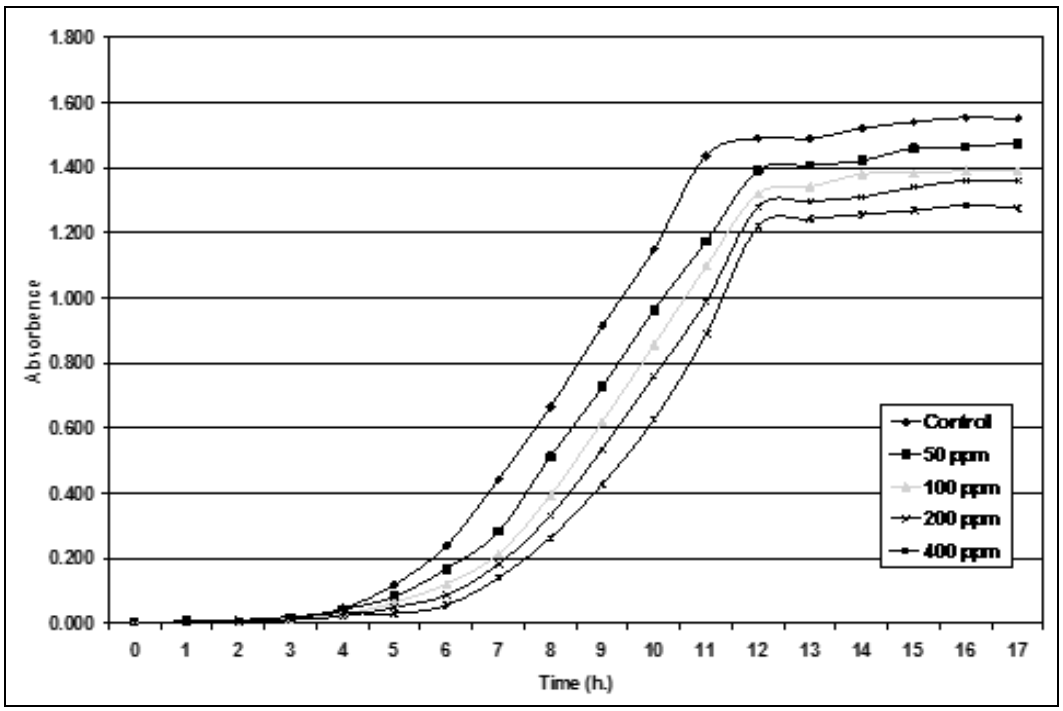


FIGURE 2B

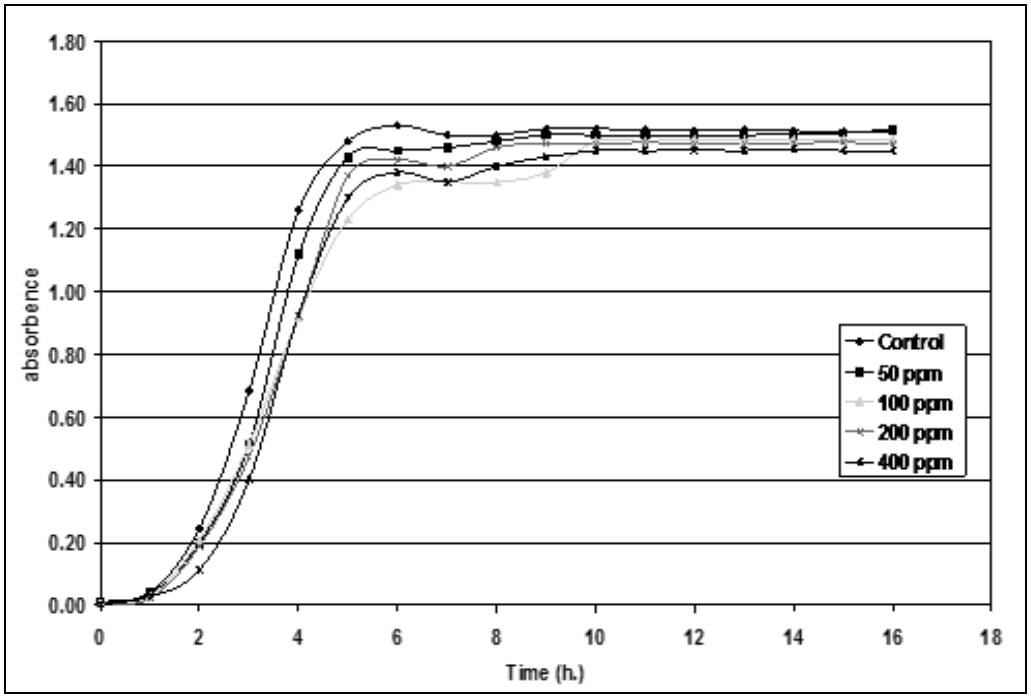


FIGURE 2C

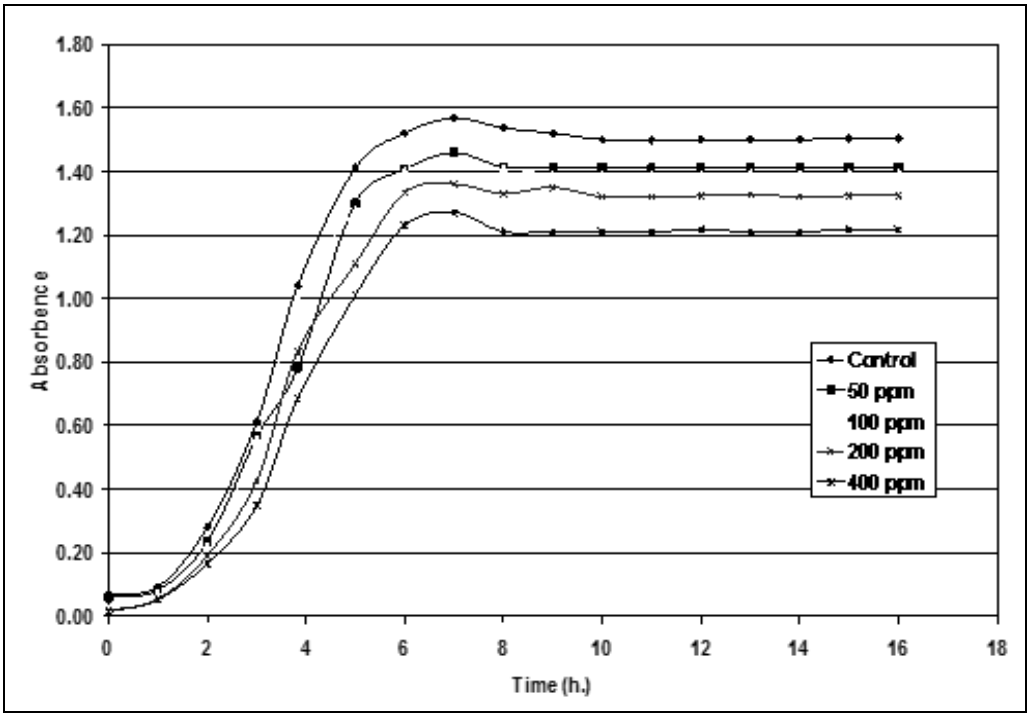


FIGURE 2D

FIGURE 2: Antibacterial effect of lysozyme and dextran-conjugated lysozyme on *S. aureus* and *E. coli*. (A) Lysozyme - *S. aureus*. (B) dextran-conjugated lysozyme- - *S. aureus*. (C) Lysozyme - *E. coli*. (D) dextran-conjugated lysozyme- *E. coli*. The bacterial culture was incubated in the presence of different concentrations (50-400ppm) of lysozyme or dextran-conjugated lysozyme at 37°C for 24 hours and the absorbance at 600 nm was recorded every hour. Control no Lysozyme.

Antimicrobial activity of lysoyme and lysozyme-dextran topical ointment on the infected wounds: Applying 10^6 CFU/ cm^2 *S. aureus* on skin wound of adult mice caused visible inflammation, redness and swelling of the skin initiated 3 days post infection. Therapeutic agents were administered topically for 9 days. At day 3 following contamination with *S. aureus* (before any treatment) number of bacteria in the samples obtained from group 1 (2.37 CFU/cm^2) were significantly ($P<0,005$) less compared to other groups (4.77 CFU/cm^2) (**Figure 3**).

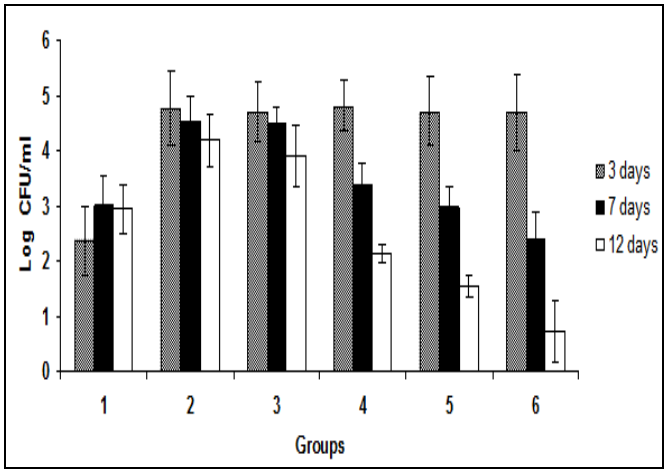
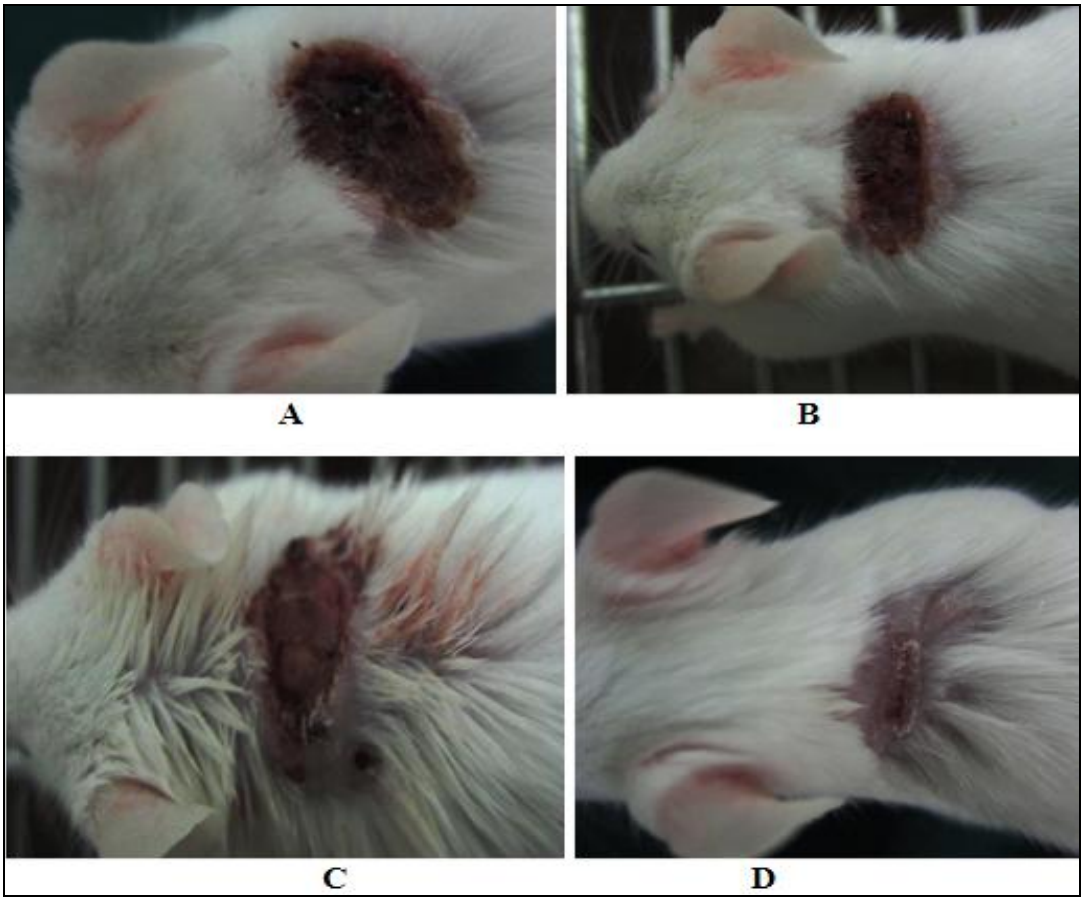


FIGURE 3: Bacterial count (log CFU/ml) of *S. aureus* in different groups of mice. 1- control, wound without inoculation and treatment, 2- infected wound without any treatment, 3- infected wound with eucerin as ointment base treatment, 4- infected wound with 400 µg/ml lysozyme ointment treatment, 5- infected wound with 400 µg/ ml lysozyme-dextran ointment treatment and 6- infected wound with tetracycline ointment treatment.

Other groups contained 4 $\log_{10}$ CFU/cm^2 in wounds. At day 7, number of bacteria from control animals was not different from that seen at day 3. However, after 12 days administration of lysozyme and lysozyme dextran conjugate in ointment, significant reduction ($P< 0.05$) of bacterial count is seen compared to those in the untreated and eucerin groups (Figure 3). No significant difference in the numbers of CFU/cm ^{2} between untreated and eucerin treated animals were seen.

Conjugated lysozyme was more effective in the reduction of bacterial count than unmodified lysozyme. Tetracycline is shown to be superior to lysozyme and lysozyme dextran.

At day 12 no significant difference in the numbers of CFU/cm^2 between untreated and eucerin treated animals was observed. Lysozyme and lysozyme dextran ointment reduced the mean bacterial count to 2.13 ± 0.17 and 1.55 ± 0.20 $\log_{10}$m respectively. Treatment with tetracycline 0.72±0.56 $\log_{10}$ ointment was more effective than two other treatments and reduced the bacterial count less than that in the control group. **Figure 4** shows the results of treatment with lysozyme, dextran conjugated lysozyme and tetracycline on wound infected with *S. aureus* after 7 and12 days treatment. These results indicate all therapeutic agents were effective in wound healing and dextran conjugated lysozyme was almost as effective as tetracycline.



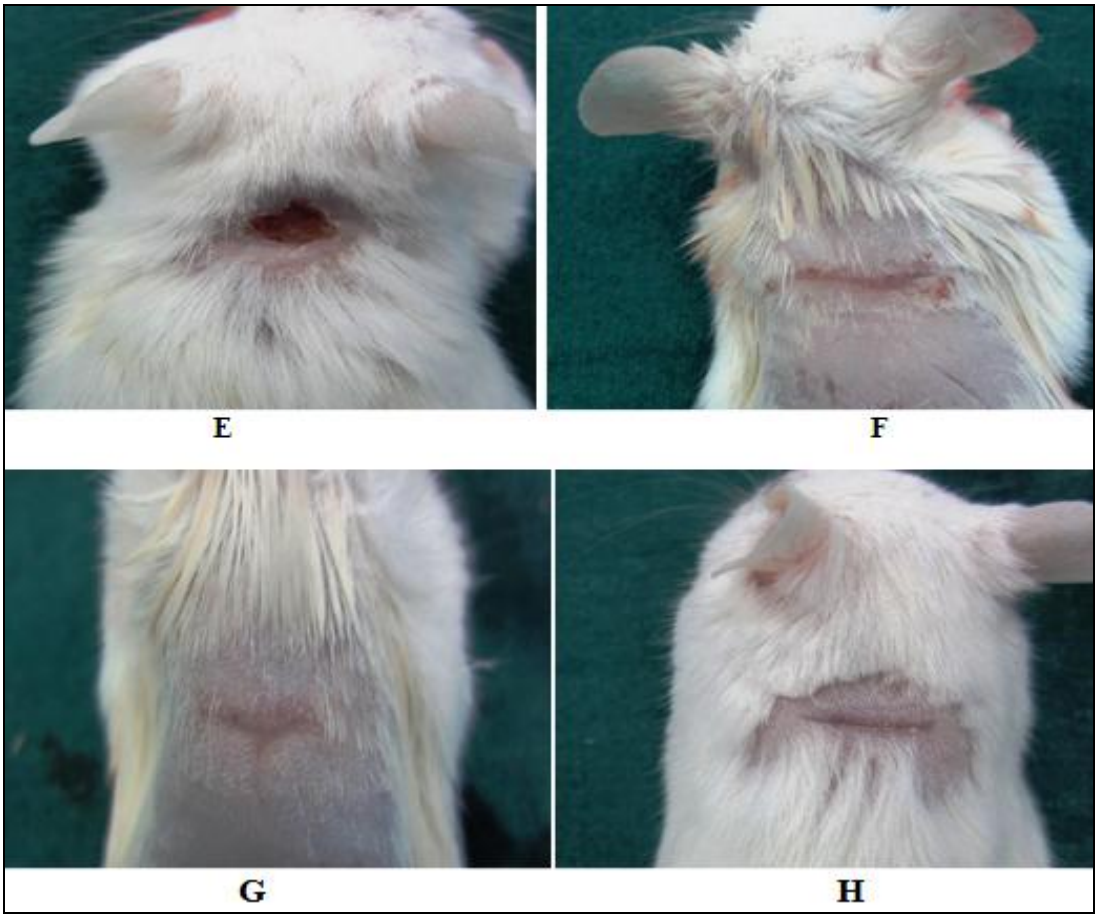


FIGURE 4: EFFECT OF TREATMENT ON THE SKIN WOUND HEALING INDUCED BY *S. AUREUS* IN MICE AFTER 7 (A-D) AND 12 DAYS (E-H). (A) infected wound without any treatment, **(B)** infected wound treated with 400 µg/ml lysozyme , **(C)** infected wound treated with 400 µg/ ml dextran-conjugated lysozyme-, **(D**) infected wound with tetracycline, **(E)** infected wound without any treatment, **(F)** infected wound treated with 400 µg/ml lysozyme , **(G)** infected wound treated with 400 µg/ ml dextran-conjugated lysozyme-, **(H)** infected wound with tetracycline.

DISCUSSION: This study was undertaken to prepare and apply a modified form of lysozyme as a novel antimicrobial agent. Modification was performed by conjugation of lysozyme with dextran under mild conditions. Dextran is a complex, branched polysaccharide made of many glucose molecules, composed of chains of varying lengths (from 3 to 2000 kilodaltons). It is used medicinally as an antithrombotic to reduce blood viscosity and as a volume expander in anemia. It is also used in some eye drops as a lubricant, and in certain intravenous fluids to solubilize other factors, e.g. iron (=iron dextran such as Cosmofer) [14].

Dextran is synthesized from sucrose by certain lactic-acid bacteria, the best-known being Leuconostoc mesenteroides. G-100 gel permeation chromatography was used to separate the dextran-conjugated lysozyme from un-conjugated constituents.

The appearance of large molecular weight proteins in the void volume suggests an increase in the size and molecular mass of lysozyme due to the covalent attachment of dextran. The diffused bands in SDS-PAGE further confirm the formation of conjugated products with wide distribution of molecular weights. Similar results have been reported by other investigators [9, 15-20].

It is well known that lysozyme has antimicrobial effects against Gram positive bacteria[6]. Figures 3 and 4 show the effect of the lysozyme and lysozyme dextran conjugate on *S. aureus* and *E. coli*.

While lysozyme did not have significant antimicrobial effect against *E. coli*, a concentration dependent activity of modified lysozyme was observed. The antimicrobial effects of lysozyme dextran conjugate against *S. aureus* were not remarkably different from that of unmodified lysozyme.

For the conjugate derivative the highest antimicrobial activity was observed at 400µg /ml. Destruction of the outer membrane of Gram-negative bacteria is a result of strong surface activity of the conjugated lysozyme, which might enhance the lytic activity of lysozyme toward peptidoglycan layer in the inner membrane. Due to the improvement of lysozyme dextran solubility at different pH values and better emulsion and foam stability, lysozyme dextran is a suitable candidate for antimicrobial activity [15, 20, 21].

The studies on mice proved the improvement of antibacterial activity of lysozyme in wound healing due to conjugation. When applied topically to *S. aureus*-infected skin wound of adult mice, a significant decrease in the bacterial counts was observed. Therapeutic agents were administered for 9 days. Several days after administration of lysozyme and lysozyme dextran conjugate, significant reduction of bacterial count is seen compared to those in the untreated and eucerin groups (Figure 3). Conjugated lysozyme was more effective in the reduction of bacterial count than unmodified lysozyme.

Tetracycline is shown to be superior to lysozyme and lysozyme dextran. After 12 days lysozyme and lysozyme dextran reduced the mean bacterial count by more than 2 and 3 log cycle, respectively. Treatment with tetracycline was more effective than two other treatments and reduced the bacterial count less than that in the control group. Figure 4 show that infected wounds were healed after12 days treatment with lysozyme, dextran conjugated lysozyme and tetracycline. These results indicate effectiveness of dextran conjugated lysozyme which is comparable with tetracycline. The improved antimicrobial effects of conjugated lysozyme have been reported by other investigators [15-19, 22, 23].

CONCLUSION: Taken together, the results of this study show that lysozyme, as a natural antimicrobial agent, can be considered as a suitable replacement for synthetic antibiotics. Conjugations with polysaccharides, such as dextran, can improve the lysozyme activity particularly against Gram negative bacteria. Lysozyme or lysozyme conjugated dextran reduce bacterial count in the infected skin wound which make these potentially useful for wound healing.

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