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Research Article

Evaluation of Therapeutic Efficacy of Copper Nanoparticles in *Staphylococcus aureus*-Induced Rat Mastitis Model

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The current study was devised to develop alternative, nonantibiotic, and economically viable treatments for bovine mastitis. The concentration of 6.25 μg/mL (25 nm) CuNPs was selected as intramammary (IM) treatment in S. aureus-induced mastitis in rats as this concentration showed a significant zone of inhibition through the in vitro sensitivity test and minimal cell toxicity on fibroblast cell lines. After, this in vivo study was conducted, and rats were divided into four groups of 6 rats each: group I (healthy control/deionized water), group II (disease control), group III (CuNPs), and group IV (gentamicin). Injection of gentamicin IM for 5 days was selected on the basis of an antibiotic sensitivity test. The therapeutic efficacy of CuNPs was assessed on the basis of clinical signs, mammary gland architecture, bacterial load, oxidative stress parameters, and histopathology of mammary glands. The clinical signs of mastitis in group III ameliorated within 3 days of treatment while in group IV clinical signs ameliorated within 4 days of initiation of treatment. On the 5th day after randomization, bacterial load, mammary gland weights, TOS (Total Oxidant Status), and OSI (oxidative stress index) were significantly lower in the CuNPs group compared to the disease control group and commercial antibiotic group. Similarly, TAS of group III was significantly higher compared to that of groups II and IV indicating that CuNPs have better ameliorative efficacy in mastitis. Treatment with IM, CuNPs @ 6.25 μg/mL showed early recovery, reduced bacterial loads, and amelioration of oxidative stress indices henceforth resulting in marked amelioration in histopathological changes compared to rats in group IV. From this study, it may be concluded that CuNPs may provide a potential alternative therapeutic regimen for the treatment of bovine mastitis.

1. Introduction

Bovine mastitis has created an enormous loss to the dairy industry across different parts of the globe and is considered a disease of prime importance in the dairy sector [1]. The disease has caused a quantum of economic losses which range from a decrease in milk production to the cost involved in the prevention and treatment of the disease which accounts for approximately $35 billion globally and 167 billion in India [2, 3]. The disease has an average incidence of about 20%, and in most instances, more than one-quarter of udder gets affected by the disease [4]. With the introduction of high-yielding breeds of cattle, control and treatment of mastitis have remained a chronic challenge for dairy producers and veterinarians, and many researchers have attributed these challenges to the multifactorial nature of the disease [5]. Various studies have identified the most commonly found infectious agent for causing mastitis as *Staphylococcus*, *Streptococcus*, and Coliforms [6]; among them, the leading etiological agent for mastitis includes *S. aureus* reported across different geographical regions of the world [2]. In J&K also, the major mastitis-causing bacteria (66.67%) have been found to be S. aureus [7]. Recently, different studies have found that S. aureus shows resistance against most commonly used antibiotics, and the possible explanation put forward by researchers includes confinement to extracellular space and the long replication period within polymorph nuclear leukocytes referred to as dormant bacteria [8, 9]. Furthermore, for effective resolution of mastitis, we need to use high-end techniques and technologies and protocols which include bacterial isolation and antibiotic sensitivity testing so that effective antibiotic can be selected against mastitis [10–12]. The main drawback associated with antimicrobial therapy involves the emergence of multiple drug-resistant microbes, excretion of antibiotic residues in food items, and the emergence of superbugs which are very difficult to treat [7]. These complexities and resistance profile of the bacteria have propelled investigators to search for an effective non-antibiotic-based therapeutic regimen. The search has begun in this decade, and metal-based nanoparticles (NPs) have aroused some interest in the research fraternity, and these novel molecules are an appropriate agent for investigation [13]. In addition to this, NPs of diverse materials are gaining great importance in therapeutic use [14].

Among the various metal NPs available like silver, gold, copper, zinc, and iron, CuNPs have been found to have high antibacterial and antifungal effects [15, 16]. The therapeutic efficacies with special reference to the bactericidal effect of CuNPs are intrinsic attributes of these molecules, which include small size and henceforth high surface-to-volume ratio, which ensures the close interaction of NPs with the microbial framework [17–19]. In addition to the use of NPs in health care, these novel molecules are finding their applicability as titanium dioxide NP desalination of marine waters [20], zinc oxide NPs for improving the quality of textile and textile products [21], zinc oxide NPs for enhancing the efficacy of solar cells [22], titanium dioxide NPs for improving the efficacy of photosynthesis [23], as efficient catalytic reagents and in oil industries [24, 25], zinc oxide NPs for determining the purity of compounds [26], titanium dioxide as antimicrobial [27], and zinc oxide for enhancing fermentation by microorganisms. It has been reported that experimental mastitis induced either by intramammary infusion of *E. coli* or *Staphylococcal* endotoxin results in oxidative stress in dairy cows [28–31]. Animal modeling of mastitis in rat/mouse has been found to be an appropriate simulation of disease and henceforth allows an in-depth understanding of the pathogenic hotspots [32–35]. The added advantage of using a rat model includes easy handling, effective mimicking of human/animal diseases, conventionally ease handling of organs, and economic viability [34, 35]. Since the bovine model is expensive, the use of an appropriate, sensitive, and reliable animal model is essential to study mastitis and to evaluate possible ameliorative drugs against mastitis. Based on the above findings, the present study was conducted to evaluate the ameliorative effects of CuNPs against mastitis in a rat model. The advantages offered by this model include simulation and resemblance of mastitis in a rat model to a bovine model due to the similar anatomical structure of the mammary gland as that of cows. The study was conducted to answer the following problems: (i) to evaluate CuNPs in terms of cytotoxicity and antimicrobial activity in an in vitro model, (ii) to design an alternative therapy against mastitis with minimal/no use of antibiotics, and (iii) to evaluate CuNPs as ameliorative agents against mastitis in terms of modulation of clinical symptoms, oxidative stress, and tissue architecture/histopathological changes.

2. Material and Methods

The current interventional laboratory animal study was undertaken with the following objectives which include quantification of and safe antimicrobial concentration of CuNPs against pure culture of Staphylococcus aureus, evaluation of the therapeutic potential of IM CuNPs in the S. aureus-induced rat mastitis model, and investigation of oxidative stress and histopathological changes in the S. aureus-induced rat mastitis model.

2.1. Place of Work. The research work was conducted in the Division of Clinical Veterinary Medicine Ethics and Jurisprudence, F.V.Sc and A.H., SKUAST-K, Shuhama, Srinagar.

2.2. Antibiotic Susceptibility Testing of S. aureus Isolates. Pure culture of S. aureus (ATCC, 25293) was procured and stored at -20°C. The preserved culture was revived and subjected to conventional biochemical tests to confirm purity of culture. Confirmed ATCC, 25293 S. aureus isolates were evaluated for antibiotic susceptibility testing by using antibiotic discs (HiMedia Lab, Mumbai) as per standards procedure (CLSI, 2013) by the disc diffusion method of [19]. The test was performed by applying bacterial inoculums (1.5×10^8 CFU/mL) having the same turbidity as that of 0.5 McFarland standard (HiMedia Lab, Mumbai) as per the standard procedure [19]. The sensitivity or resistance of isolates for a particular antibiotic was examined by

Table 1: Experimental trial design.

Group	Group designate	Interventions
Group I ($n = 6$)	Healthy control	Sterile deionized water (10 μL) given via intramammary route and sacrificed on the same day as group II.
Group II ($n = 6$)	Diseased control (infection induced with 10 μL of 1.5×10^4 CFU/mL of *S. aureus*)	No medication given. Sacrificed after the appearance of clinical signs.
Group III ($n = 6$)	Treatment group I (infection induced with 10 μL of 1.5×10^4 CFU/mL of *S. aureus*)	Treated with copper nanoparticles (10 μL at a conc of 6.25 μg/mL) via intramammary route s.i.d. for 5 days and sacrificed on the 6th day after the start of treatment.
Group IV ($n = 6$)	Treatment group II (infection induced with 10 μL of 1.5×10^4 CFU/mL of *S. aureus*)	Treated with gentamicin (0.25 mg/10 μL/gland) via intramammary route s.i.d. for 5 days and sacrificed on the 6th day after the start of treatment.

measuring the degree of inhibition (diameter), and the degree of resistance/sensitivity was determined from the standard scale [36].

2.3. Preparation of CuNP Suspension. Stock suspension of CuNPs (size 25 nm) (procured from Sigma Aldrich) was prepared by suspending 250 mg of metallic CuNPs in 50 mL of sterile deionized water. These CuNPs were characterized by the manufacturer; to further validate, we characterized CuNPs by the spectrophotometric method. The suspension was then subjected to ultrasonication by using an electric sonicator with a thick probe for 1 hour. The stock suspension was diluted with deionized water to make suspensions of different concentrations (100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, and 6.25 μg/mL).

2.4. In Vitro Antimicrobial Activity of Copper Nanoparticles (CuNPs). The antimicrobial activity of CuNPs was assessed on pure culture of S. aureus (ATCC, 25293) by the standard method of agar well diffusion. Freshly prepared Muller Hinton Agar (MHA) Petri plates were inoculated with pure culture of S. aureus. Wells were punched on MHA with the base of 1 mL microtip (5 mm in diameter), and 100 μL of different concentrations of CuNP suspensions was poured into the different wells, which were subsequently incubated at 37°C for one day. The zone of inhibition (diameter) was interpreted as the degree of antibacterial activity as per the standard method [37]. More than ten plates were used to obtain the average zone of inhibition for each concentration.

2.5. In Vitro Cytotoxicity Assay for CuNPs. The cytotoxicity assay of CuNPs was evaluated using the MTT assay in chicken embryo fibroblasts (CEFs) of a 9-day-old chicken embryo and was cultured as per standard procedure [15]. When approximately 60-70% confluence of CEFs was established, plates were removed. Following this, these plates were treated with 100 μL DMEM (5% FBS + 0.1% penicillin and streptomycin) containing copper nanoparticles at concentrations (W/V) of 0.0, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL in six replicates. This was followed by incubation of plates at 37°C and 5% CO_2 for 24 and 48 hours, respectively, to evaluate cytotoxicity after 1 and 2 days posttreatment. Following the standard procedures and protocols like aspiration and washing of plates, these plates were then examined for optical density (OD) at 570 nm in a multimode microplate reader (Biotek, Cytation™ 3, and Winooski, VT, USA), and a similar procedure was repeated on the 2nd instance after 48 hrs posttreatment with NPs. Control wells (0.0%) and blank wells (without cells) were utilized for calculating the cell viability by using the following formula % cell viability. % cell viability is measured by subtracting sample absorbance from blank absorbance divided by absorbance of control minus absorbance of blank multiplied by 100.

2.6. Preparation of Inoculum for Inducing Infection. The bacterial concentration of pure culture of S. aureus (ATCC, 25293) in brain heart infusion (BHI) broth was standardized to an optical density of 0.5 at 620 nm (approximately 1.5×10^8 CFU/mL) using the McFarland scale. The inoculums were subjected to serial fold dilutions till they reached the concentration of 1.5×10^4 CFU/mL. The total numbers of CFUs were estimated by inoculation of culture on agar media for overnight incubation followed by counting of colonies.

2.7. Experimental Therapeutic Study

2.7.1. Experimental Animals. Twenty-four pregnant Wistar rats were procured from the Central Animal House Indian Institute of Integrative Medicine (IIIM), Jammu (Registration no: 67/CPCSEA) and were housed individually in ventilated cages. The animals were housed in a laboratory animal house under standard laboratory conditions. The clinical trial was carried out on lactating rats after proper approval from the Institutional Animal Ethics Committee (IAEC).

2.7.2. Experimental Design. Following parturition, these lactating animals were divided into four groups (groups I-IV) of 6 rats each as illustrated in Table 1, with identical husbandry conditions. Pups were removed from lactating females at the age of approximately 10 days, 1 hr prior to infection. Group I served as the healthy control in which sterile deionized water was given via the IM route. In groups II, III, and IV, infection was induced with S. aureus (1.5×10^4 CFU/mL). Group II served as the mastitis control group in which no medication was given. In group III, pregnant females were treated with CuNPs IM. In animals of

Table 2: Sensitivity pattern of *S. aureus* isolates to different antibiotics.

Antibiotic discs	Average zone of inhibition (mm)	Interpretive standards (mm)		
		Sensitive	Intermediate	Resistant
Enrofloxacin	30.3 ± 0.14	18	15-17	14
Ceftriaxone	26.7 ± 0.32	21	14-20	13
Gentamicin	27.4 ± 0.49	15	13-14	12
Amoxyclav	19.6 ± 0.42	20	14-17	19
Penicillin G	11.3 ± 0.42	29	—	28
Oxytetracycline	29.2 ± 0.12	22	19-21	19

group IV, gentamicin was given via the IM route; this treatment was given single time per day for 5 days in all groups (Table 1).

2.7.3. Induction of Mastitis. The rats were anesthetized using ketamine (Aneket, Neon Laboratories limited, Mumbai) @ 22-25 mg/kg body weight through the intraperitoneal route and placed on the dorsal surface. The ventral surface was exposed and observed under a magnifying lens. Prior to bacterial inoculation, the mammary gland area was cleaned with 70% ethyl alcohol, and 10 μL (1.5×10^4 CFU/mL) of S. aureus culture was inoculated in left fourth, left fifth (L4, L5) and right fourth, right fifth (R4, R5) teats. Six rats (group I) were inoculated with 10 μL of sterile deionized water in left fourth, left fifth (L4, L5) and right fourth, right fifth (R4, R5) teats and served as the normal control group.

2.8. Evaluation of Treatment Efficacy. The treatment efficacy and recovery of rats were assessed on the basis of the weight of mammary glands, enumeration of bacterial load, estimation of oxidative stress, and histopathological examination of mammary glands.

2.8.1. Weight of Mammary Glands. After euthanizing rats in each group, mammary glands were aseptically dissected and collected as per the standard procedure.

2.8.2. Enumeration of Bacterial Load. L5 mammary glands from all the rats were aseptically removed and homogenized in phosphate buffer saline (0.2 M, pH 7.4). After overnight incubation at 37°C, colonies were counted using a colony counter, and values were expressed as CFU/g.

2.8.3. Evaluation of Oxidative Stress. R4 mammary gland homogenate was evaluated for Total Oxidant Status (TOS), Total Antioxidant Status (TAS), and oxidative stress index (OSI) as per the standard procedure [31]. Total Oxidant Status (TOS) was measured using the automated colorimetric method [38]. Total Antioxidant Capacity (TAS) was measured using the automated direct method of ABTS radical cation [38].

2.9. Histopathological Studies. R5 mammary glands were collected for histopathological studies. The tissue for histopathological studies was collected as per the method of [39]. These samples were subjected to formalin treatment after the removal of blood from the tissue framework. After 24 hrs of 10% formalin treatment, the tissue samples were transferred to new 10% formalin solution and were kept there for 21 days; after 21 days, these samples were stained by routine H and E technique as per the method of [39].

2.10. Statistical Analysis. the data was collected and analysed by SPSS version 20. Categorical variables were analysed by the chi-squared test, and numerical data was analysed by using the appropriate statistical test. Values were considered significant if they varied at 5% of significance.

3. Results

3.1. Antibiogram of S. aureus. The in vitro antibacterial activity was evaluated by measuring zones of inhibitions on at least 10 inoculated MHA plates. S. aureus isolates were found to be susceptible to Oxytetracycline, Enrofloxacin, gentamicin, and Ceftriaxone. The isolates showed a significant degree of resistance to penicillin G and intermediate resistance to Amoxyclav (Table 2, Figures 1(a)–1(g)). The average zone of inhibition shown by Enrofloxacin was 30.3 ± 0.14 mm, Ceftriaxone 26.7 ± 0.32 mm, gentamicin 27.4 ± 0.49 mm, Amoxyclav 19.6 ± 0.42 mm, penicillin G 11.3 ± 0.42 mm, and Oxytetracycline 29.2 ± 0.12 mm (Table 2).

3.2. In Vitro Antimicrobial Activity of Copper Nanoparticles against S. aureus. The present study indicates that all concentrations of CuNPs used in this study (6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL) had antimicrobial activity against S. aureus with a dose-dependent increase in inhibition zones on agar plates. The average zones of inhibition shown by CuNPs against S. aureus at 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL were 15.5 ± 0.5 mm, 20.5 ± 0.5 mm, 23.2 ± 0.51 mm, 29.10 ± 0.38 mm, and 33.10 ± 0.48 mm, respectively. The mean values were statistically significant ($P < 0.05$) from each other (Table 3, Figures 1(a)–1(g)) with higher zones of inhibition found at higher concentrations of CuNPs.

3.3. In Vitro Cytotoxicity Assay of CuNPs. The mean values of cell viability after 24 hours of exposure to CuNPs (W/V) at 0 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL were 105.47 ± 2.73, 104.23 ± 2.89, 102.6 ± 2.88, 91.93 ± 1.64, 79.86 ± 2.54, and 69 ± 1.65, respectively, as shown in Table 4. The mean values differed nonsignificantly ($P > 0.05$) from each other, but the viability of cells

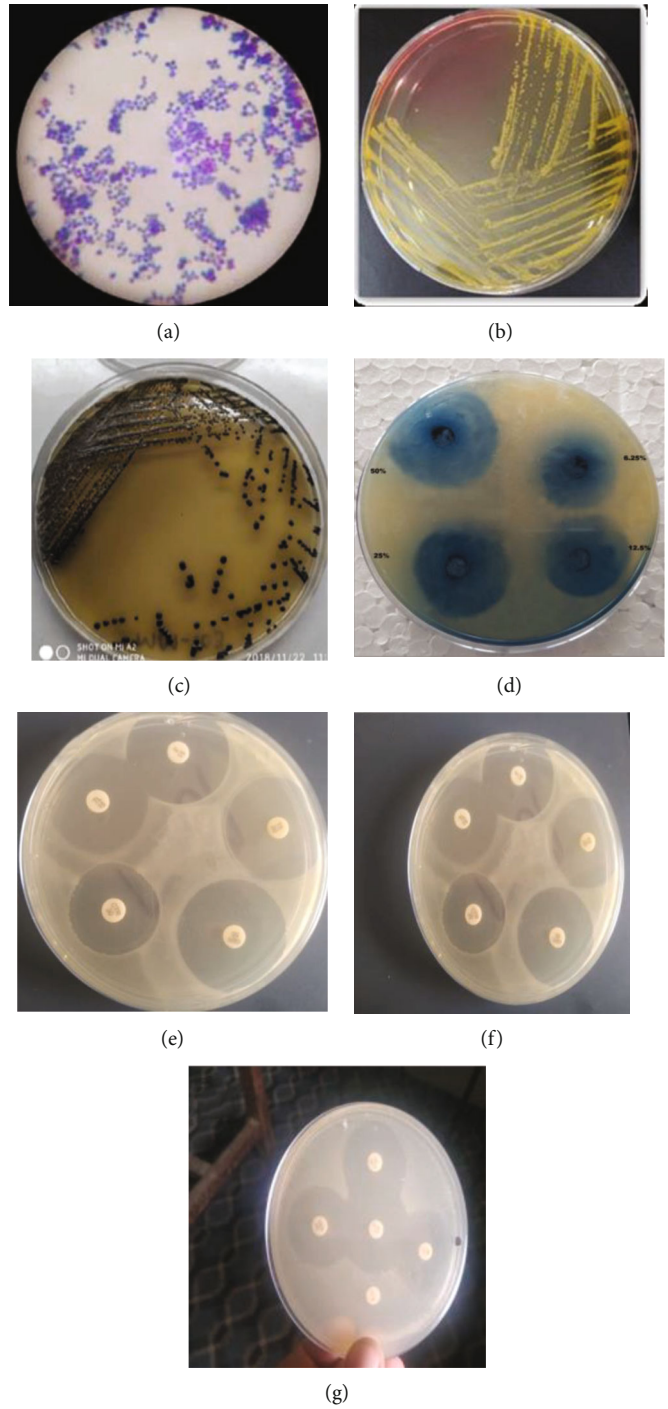


Figure 1: (a) Gram-positive cocci arranged in irregular clusters (Staphylococcus) seen on Gram staining. (b) Growth of S. aureus on MSA. (c) Growth of S. aureus on Baird Parker. (d) In vitro antimicrobial activity of copper nanoparticles. (e–g) Antibiogram of S. aureus.

decreased with the increase in the concentration of CuNPs. A similar pattern of decreased viability with increasing concentration was seen after 48 hrs of exposure, and the mean values did not differ significantly. The mean values of cell viability at different concentrations of CuNPs (W/V) at 0 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL were 117.03 ± 2.02, 115.81 ± 2.78, 111.51 ± 3.76, 79.98 ± 1.58, 41 ± 2.87, and 27.89 ± 1.20, respectively. The mean values did not differ significantly when the 24 hrs of incubation was compared to the 48 hrs of incubation at respective concentrations as shown in Table 4 and Figure 2(a).

3.4. Therapeutic Trial. The ideal concentration of CuNP suspension on the basis of in vitro antimicrobial property (15.5 ± 0.5 mm) and minimal cytotoxicity (104.23 ± 2.89) concentration were established. After confirmation of infection in groups II, III, and IV, the concentration of 6.25 μg/mL of CuNPs was selected for the treatment.

Table 3: Average zones of inhibition shown by CuNPs against *S. aureus*.

Conc. of CuNPs	Average zone of inhibition (mean ± S.E.)
6.25 μg/mL	15.500 ± 0.5^{a}
12.5 μg/mL	20.500 ± 0.5^{b}
25 μg/mL	23.200 ± 0.51^{c}
50 μg/mL	29.100 ± 0.38^{d}
100 μg/mL	33.100 ± 0.48^{e}

Mean data with different superscripts vary significantly ($P < 0.05$).

Table 4: Cell viability at different concentrations of copper nanoparticles at 24 hrs and 48 hrs (mean ± SE).

Concentration of CuNP solution (W/V)	% viability	
	24 hrs	48 hrs
No exposure	105.47 ± 2.73^{Da}	117.03 ± 2.02^{Db}
6.25 μg/mL	104.23 ± 2.89^{Da}	115.81 ± 2.78^{Db}
12.5 μg/mL	102.6 ± 2.88^{Da}	111.51 ± 3.76^{Db}
25 μg/mL	91.93 ± 1.64^{Ca}	79.98 ± 1.58^{Cb}
50 μg/mL	79.92 ± 2.54^{Ba}	41.12 ± 2.87^{Bb}
100 μg/mL	69.86 ± 1.65^{Aa}	27.89 ± 1.20^{Ab}

Mean values with different superscripts vary significantly ($P < 0.05$): lowercase alphabets down the table and uppercase alphabets across the table.

3.4.1. Clinical Signs. The clinical signs of mastitis were observed after 6, 12, 24, and 48 hrs of IM inoculation of S. aureus culture in rats. Clinical observations observed in the present study included reddening of the mammary gland skin to the obvious swelling and oozing out of pus. After inoculation of infection, obvious signs of infection/mastitis were observed in groups II, III, and IV after 24-48 hours of infection. The clinical signs started reducing in group III animals within 2 days after initiation of treatment with CuNPs and completely disappeared after 3 days while in group IV, the clinical signs disappeared after 3-4 days of initiation of treatment with IM gentamicin.

3.4.2. Mammary Gland Weight. The mammary gland weights (g) were significantly higher in group II as compared to group I (Table 4). Also, mammary gland weights in group III and group IV were statistically different from each other and also from group II (Table 5). The mean values of mammary gland weights of group III did not differ significantly from group I indicating that the weight of mammary glands after treatment with CuNPs was almost in concurrence with group I.

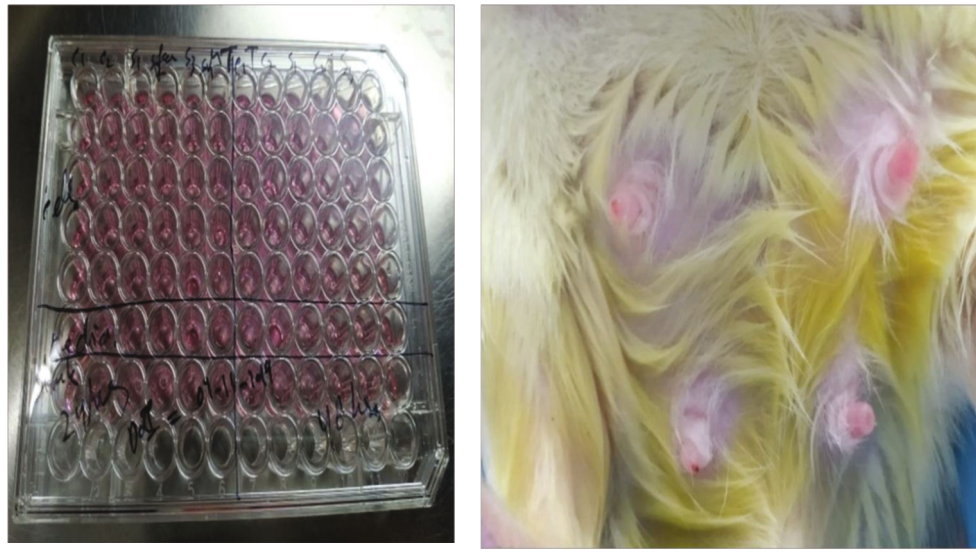
3.4.3. Bacterial Load of Mammary Glands. L5 mammary glands from all the rats were taken for estimating the bacterial loads after homogenizing the samples, and bacterial loads (log10 CFU/g) are presented in Table 5. The bacterial load (log10 CFU/g) in groups I to IV was 0.000 ± 0.000, 8.208 ± 0.029, 7.674 ± 0.029, and 7.968 ± 0.029, respectively (Table 6). The bacterial load in group II was significantly higher than that in groups III and IV. The mean values for bacterial load in groups III and IV were statistically significant from each other with lower values in group III than group IV indicating higher efficacy of CuNPs in reducing the bacterial load in group III compared to gentamicin.

3.4.4. Effect of Treatment on Oxidative Stress Indices. The total oxidative stress (TOS) (μmol H_2O_2 equivalent/L) of different treatment groups is presented in Table 7. The TOS differed significantly between group I and group II, with values being significantly elevated in group II when results were compared to values in group I. The total oxidative status of groups III and IV differed significantly from that of groups I and II. In groups III and IV, the TOS decreased with treatment although the difference was not statistically significant, but the TOS decreased more in group III after treatment with CuNPs than group IV indicating better efficacy of CuNPs in reducing the TOS.

(1) Total Antioxidant Stress. The TAS (μmol Trolox equivalent/L) was 0.48 ± 0.06 in healthy lactating rats (group I), 0.16 ± 0.02 in the mastitis group (group II), 0.37 ± 0.03 in group III (treated with CuNPs), and 0.29 ± 0.01 in group IV (treated with gentamicin). The TAS differed significantly between group I and group II with values being significantly elevated in group I when values were compared to values in group II. The TAS values of groups III and IV differed significantly from groups I and II. In groups III and IV, the TAS increased with treatment although the difference in increase between the two groups was not statistically significant, but a higher gradient of TAS was observed in group III compared to group IV indicating better efficacy of CuNPs in increasing the TAS.

(2) The Total Oxidative Stress Index (TOS/TAS). The values of TOS/TAS are presented in Table 6. The TOS/TAS differed significantly between group I and group II with values being significantly elevated in group II compared to values of these indices in group I. The TOS/TAS of group III differed significantly from group II, but no significant difference was observed when values were compared with group I. The TOS/TAS of group IV differed significantly from groups I and II, but no significant difference was observed when these values were compared with the values of group III. In groups III and IV, TOS/TAS decreased with treatment, and the difference in the decrease between the two groups was statistically significant, but the TOS/TAS decreased more in group III after treatment with CuNPs, and values were closer to group I than group IV indicating better efficacy of CuNPs in reducing the TOS/TAS.

3.5. Histopathology of Mammary Glands. Grossly, no appreciable changes were observed in the rats of the control group (group I). In the mammary glands of group II, lesions like swelling and severe congestion were observed, and glands were firm to palpate (Figure 2(b)). No appreciable gross lesions were seen in the rats of group III and group IV except mild congestion. Histopathological alterations of the mammary gland are shown in Figure 3. Histopathological changes of mammary glands of group I did not reveal any lesions as the alveolar tissues and ducts were normal.



(a) (b)

Figure 2: (a) ELISA plate (cell line toxicity). (b) Reddening and swelling of mammary glands in mastitis group.

Table 5: Effect of treatment on mammary gland weights.

Groups	Mammary gland weight in grams (mean ± S.E.)
GI	1.233 ± 0.0084^{a}
GII	1.390 ± 0.0012^{c}
GIIII	1.255 ± 0.0082^{a}
GIV	1.292 ± 0.0088^{b}

Values with different superscripts differ significantly ($P < 0.05$) from each other.

Table 6: Effect of bacterial load of mammary glands (mean ± SE).

Groups	No. of colonies (mean ± SE)	Bacterial load (log10 CFU/g) (mean ± Std.error)
GI	0.00 ± 0.00^{a}	0.000 ± 0.000^{a}
GII	163.625 ± 11.31^{d}	8.208 ± 0.034^{d}
GIIII	48.75 ± 4.27^{b}	7.674 ± 0.038^{b}
GIV	94.375 ± 6.36^{c}	7.968 ± 0.029^{c}

*Values with superscripts (a–c) differ significantly ($P < 0.05$).

Mammary glands of rats in group II revealed massive infiltration of inflammatory cells predominantly neutrophils (abscess), hemorrhagic congestion of blood vessels, and alveolar atrophy showing discontinuous epithelial and luminal cell lining. The mammary glands of animals in group III showed a marked reduction in polymorphonuclear cells and mild alveolar damage but regeneration at their ends along with vascular degeneration indicating recovery. Mild infiltrations of polymorph nuclear cells and mild congestion of blood vessels and superficial inflammation at the point of inoculation of infection were also observed in group IV rats, and the alveolar cavities appeared clear without any bacterial rods in both the groups (groups III and IV).

4. Discussion

4.1. In Vitro Antimicrobial Activity of CuNPs against S. aureus. In the present study, antimicrobial activity of CuNPs was evaluated by in vitro antimicrobial activity on pure culture of S. aureus (ATCC, 25293) which is one of the major mastitis-causing pathogens identified and isolated from clinical cases of bovine mastitis in Kashmir valley as reported from earlier studies [40, 41]. On antibiogram analysis, pure culture of S. aureus showed susceptibility to Oxytetracycline, Enrofloxacin, gentamicin, and Ceftriaxone but showed a high degree of resistance to penicillin G and intermediate degree of resistance to Amoxyclav (Table 2). So, based on the resistance profile of S. aureus, the present study was devised in order to find an alternative nonantibiotic therapeutic regimen against S. aureus. When we compared the effectiveness of CuNPs against S. aureus, we found that these nanoformulations are effective against mastitis in laboratory animal models. Although earlier studies of [15, 42, 43] also reported that CuNPs were effective against Salmonella choleraesuis, Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus, and Bacillus subtilis and also yeast species such as Candida albicans, majority of these studies are based on in vitro studies.

4.2. In Vitro Cytotoxicity Assay for CuNPs. The MTT assay in the present study was conducted based on findings of earlier studies that compared four commonly used in vitro cytotoxicity assays to determine their ability to detect early cytotoxic events and concluded that MTT assays are the most sensitive assays in detecting cytotoxic events compared to LDH leakage, neutral orange, and protein assay [40, 44–46]. Furthermore, the dose-dependent cytotoxicity of CuNPs reported in the present study is in concurrence with [47, 48], who reported the genotoxic potential of copper oxide nanoparticles on human pulmonary epithelial cell line

Table 7: Effect of treatment on TOS, TAS, and OSI in rat mastitis model.

	Group I	Group II	Group III	Group IV
TOS	6.73 ± 0.22^{a}	16.00 ± 0.53^{c}	10.95 ± 0.49^{b}	12.20 ± 0.46^{b}
TAS	0.48 ± 0.06^{c}	0.16 ± 0.02^{a}	0.37 ± 0.03^{b}	0.29 ± 0.01^{b}
OSI	15.27 ± 1.99^{a}	106.51 ± 13.68^{c}	31.19 ± 3.53^{ab}	42.55 ± 2.54^{b}

*Values with the same superscripts (a–c) do not differ significantly ($P < 0.05$).

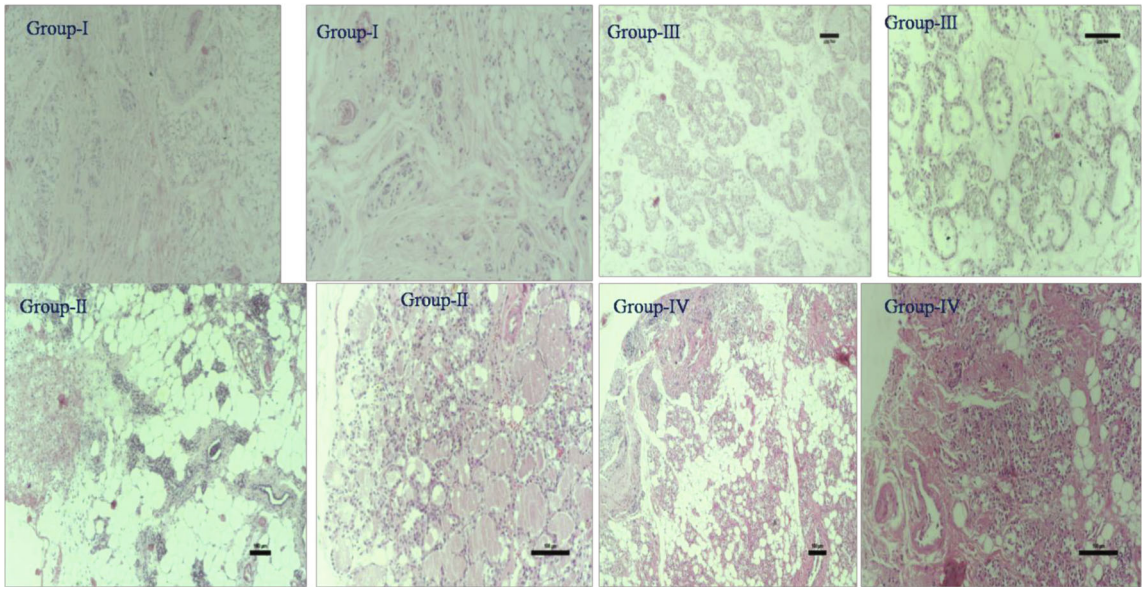


Figure 3: Group I: normal alveolar tissues and ducts. Group II: massive infiltration of neutrophils (abscess), hemorrhagic congestion, and alveolar atrophy. Group III: marked reduction in polymorph nuclear cells, mild alveolar damage, but regeneration at the ends. Group III: mild infiltrations of PMNs, mild congestion of blood vessels, and superficial inflammation at the point of inoculation of infection.

(A549) and concluded that CuONPs induced cytotoxicity in a dose-dependent manner. In addition, the same study reported that CuO NPs induce DNA damage in human lung epithelial cells (A549) through lipid peroxidation.

4.3. Therapeutic Efficacy

4.3.1. Clinical Signs. The clinical signs of mastitis were observed after 6, 12, 24, and 48 hrs of IM inoculation of S. aureus culture in four groups of rats. On the basis of amelioration of clinical signs of mastitis in group III earlier than group IV, treatment with IM copper nanoparticles proved to be efficient than IM gentamicin. Hence, it can be postulated from the present study that CuNPs are efficient in arresting the inflammatory processes as can be seen from the significant reduction in mammary gland weights. Our findings of assessing clinical recovery on the basis of a significant decrease in mammary gland weights after treatment with CuNPs are in accordance with the findings of [49].

4.3.2. Bacterial Load of Mammary Glands. The bacterial load in group II was significantly higher than groups III and IV. The mean values for bacterial load in groups III and IV were statistically significant from each other with lower values in group III than group IV indicating higher efficacy of CuNPs in reducing the bacterial load in group III than gentamicin in group IV. Similarly, the number of colonies counted on nutrient agar plates was significantly higher in group II (Table 6). The number of colonies reduced significantly in group III than group IV indicating the better antimicrobial potential of CuNPs in combating infection than gentamicin. These findings are in agreement with the findings of [49]; they reported bactericidal activity of nanosilver particles in the S. aureus-induced murine mastitis model. The bactericidal activity of the CuNPs may be attributed to their intrinsic activity of causing minute pores in bacterial cell walls and disruption of protein synthetic machinery [50].

4.3.3. Effect of Treatment on Oxidative Stress Indices. In groups III and IV, the TOS decreased with treatment, although the difference in reduction was statistically nonsignificant, but TOS was found to be decreased more in group III after treatment with CuNPs than in group IV treated with commercial antibiotics, indicating better efficacy of CuNPs in reducing the TOS. These findings are of special importance as [49] reported that during the pathogenesis of S. aureus, mastitis oxidative stress plays a key role in the progression of diseases. Similar findings of enhanced oxidative stress parameters in milk of mastitis cows have been

reported by [51]. Furthermore, to support these propositions, mastitis has been found to result in alterations in redox potential characterized by an increase in oxidative free radicals and a concurrent decrease in protective antioxidant enzymes [52]. Owing to consistent occurrence of these findings in dairy mastitis, these biochemical markers can serve as effective and potential markers for monitoring the health status of the udder. Similarly, various antioxidants have demonstrated a beneficial role in the amelioration of mastitis symptoms [53]. The mechanistic pathways involved in mastitis and oxidative damage initiate by the recruitment of activated neutrophils in udder parenchyma which causes the release of a large quantum of free radicals in order to destroy ingested foreign organisms [39]. To further support the role of the antioxidant defense mechanism, [54] proposed that there occurs a pronounced imbalance in antioxidant defense in dairy animals suffering from mastitis due to the imbalance in free radical production and consumption of antioxidant enzymes from inflammatory processes of the mastitis mammary gland. The antioxidant activity of CuNPs may be attributed to their effective blocking of the free radical cascade involved in the pathogenesis of mastitis [53] and scavenging of free radicals produced by polymorphonuclear lymphocytes, which support their therapeutic action against mastitis.

4.4. Histopathology. The microscopic findings of our study are in concurrence with [49]; these findings have reported pronounced proinflammatory cell infiltration and subsequent damage to epithelial cells due to induction of inflammation with Staph aureus in the experimental murine mastitis model. Furthermore, [25] reported increased levels of proinflammatory markers which include IL-1, IL-2, and TNF-α in udder tissue of animals infected with S. aureus. In the present study, the mammary glands of animals in group III showed a marked reduction in polymorph nuclear cells and mild alveolar damage but regeneration with vascular degeneration indicating progression towards recovery. Mild infiltrations of polymorph nuclear cells and mild congestion of blood vessels and superficial inflammation at the point of inoculation of infection were also observed in group IV rats. The alveolar cavities were found to be clear without any infection/infectious organisms in both groups (groups III and IV). The microscopic findings indicate resolution of inflammation in group III as similar findings were reported by [49]. They reported reduction of neutrophil infiltration, underrepair secretory cells, and normal alveolar epitheliums as well as normal interalveolar septa in histopathological sections of mammary tissues of mice infected with S. aureus treated with silver nanoparticles. Our findings are in agreement with [55–57] who also reported antimicrobial property of nanocopper gel in the treatment of clinical mastitis against Gram-positive and Gram-negative bacteria.

5. Conclusion

CuNPs inhibit the growth of Staph aureus (which is one of the major mastitis-causing pathogens) and exhibit minimal toxicity on fibroblast cell lines at concentrations as low as 6.25 μg/mL. From the results of this study, it can be postulated that IM treatment with CuNPs at a concentration of 6.25 μg/mL showed better efficacy than IM gentamicin treatment of Staph aureus-induced mastitis in rats on the basis of clinical signs, mammary gland weights, bacterial load, and histopathology. Thus, from safety, efficacy, and economics of the compound, CuNPs could provide a promising alternative for the fabrication of a novel therapeutic regimen against bovine mastitis. With further research in this direction, a new window for research on the use of NPs as antimicrobial can be conducted against the wide spectrum of microorganisms which can drastically reduce the use of antibiotics in veterinary and humans. Although encouraging results have been observed from the present preclinical study, in order to obtain a clear picture, there is a need for case-controlled clinically randomized placebo study in actual clinical settings.

6. Future Prospectus/Significance of Research

The study found amelioration of mastitis in a rat model by using CuNPs; the study can be of use in following future prospectuses.

(i) With further research in the actual clinical setting, the use of conventional antibiotics can be drastically reduced which will result in the reduction of antibiotic residues in food items derived from animals

(ii) The fabrication and standardization of the therapeutic regimen based on CuNPs will help in controlling the emergence of antibiotic-resistant bacteria and will avert the calamity-like emergence of superbugs

(iii) Based on the results of the present study, CuNPs should be evaluated against other animal and human diseases caused by a wide spectrum of bacterial species

(iv) A cocktail of different NPs with diverse physiological properties beneficial for health can be developed which will be effective for the welfare of human and animal health

(v) This indicates that we need to understand the therapeutic activity of CuNPs at cellular, subcellular, and genomic levels in order to develop effective medical preparation for diverse diseases

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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