Synthesis of Copper Nanoparticles Using Aspergillus Niger and Their Efficacy Against Pathogenic Staphylococcus Aureus

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Abstract

The attention has been paid in recent years to the biosynthesis of copper nanoparticles (CuNPs) and their important in medical applications. This study was conducted to synthesis of CuNPs using Aspergillus niger supernatant, study its characteristic and their antibacterial activity alone or with Ciprofloxacin against Staphylococcus aureus isolated from wounds infection. Then, its activity on the healing wounds was experimentally induced with Staphylococcus aureus using laboratory Hamster., followed by assaying of some blood parameters. The results indicated that Aspergillus niger supernatant was optimal for converting the CuNO3 to produce of CuNPs. The CuNPs were confirmed by UV spectrum, revealed an absorption at 225 nm, and the average size was by 20 nm after assay with the scanning electron microscopy (SEM). The mixing between CuNPs and ciprofloxacin had increased by 60% in the antibacterial activity against Staphylococcus aureus, more than for single inhibition activity of each treatment. The CuNPs or ciprofloxacin singly or in combination treated of induced experimentally contaminated wounds with Staphylococcus aureus appeared that synergism healing effects of the mixed treated with a short time compared with single treatments. The treatment of induced wounds with Staphylococcus aureus were significantly (p<0.05) increased in number of the WBCs and decreased of platelets number. While, the treatments with CuNPs or Ciprofloxacin singly or in combination were not significantly different with the same parameters in control group. Furthermore, the values of urea and creatinine further of the IgG and IgA for all animals’ groups were reduced compared with the same values in infections animal group. GOT, GPT of liver enzymes values were also showed a significant decreasing.

Key words: Aspergillus niger, Copper nanoparticles, Staphylococcus aureus, wounds.

1. Introduction

Nanoscience is a relatively new research science, including Technology, Engineering and Mathematics, disciplines, are involved in nanomaterial synthesis of around 1–100 nm. At the nanoscale level, the materials have unique chemical, physical, optical, magnetic and electrical properties due to their greater area to volume ratio [1]. Multidrug-resistant (MDR) microbes are growing problems in the treatment of infectious diseases as a result of the widespread use of broad-spectrum antibiotics, the majority of which resulted in the production of antibiotics resistance to many human bacterial pathogens [2]. Advances in nanotechnology have opened new line horizons in nanomedicine, enabling the synthesis of nanoparticles (NPs) that are already considered a better alternative to antibiotics, and there seems to be a high potential to solve the problem of microbial multidrug resistance development [3]. The synthesis of high-performance copper nanostructures was depending on the method used, where good control over particle size, shape and spatial distribution is of key importance [4]. Thus, the fungus synthesized procedure has been applied which is economical, applicable, and nontoxic for biomedical applications [5]. The treatment of wounds infections in laboratory animals, a large number of antibiotics are used [6]. Recently, due to the huge widespread use of antibiotics in the treatment of infected wounds, pathogenic bacteria have become more resistant to chemotherapy, which it will need to use a new antibiotics at all times. As a result, the use of novel therapies such as NPs is critical in overcoming this issue [1,6].

The aim of this study was conducted to evaluate the effect of copper nanoparticles (CuNPs) after synthesized by Aspergillus niger supernatant singly or in combination with antibiotics against Staphylococcus aureus that contaminated the wounds in...
laboratory hamsters on some blood parameters such as CBC, some liver enzyme, urea and creatinine and some type of immunoglobulins.

2. Materials and Methods

2.1. Identification of filamentous fungus Aspergillus niger (A. niger)

Isolates of *A. niger* was obtained from laboratory of food science department in Agriculture College, University of Tikrit. Further diagnostic was confirmed using their diagnostic keys and charactereristics according to [7]. Non-cultured soil was the source of *A. niger*.

2.2. Collection of pathogenic isolates

The wound samples of patients were obtained from Salah-Adin General Hospital. The samples were collected from different ages and genders during the period from 1st of Nov. 2019 to Feb. 2020. After cultivation on the enriched, selective and differential media the isolates were identified throughout the morphological, microscopic and biochemical test as well as VITEK2 compact system. Subsequently, all bacterial cells were stained with grams’ stain for further confirmation [8].

2.3. Synthesis of nanoparticles by *A. niger*

Colonies of the *A. niger* were sub-cultured on Potato Dextrose Agar plates and incubated at 28 °C for 6 days. It was used to inoculate 300 ml of Potato Dextrose broth contain 3 grams Glucose and 3 ml of Ampiclox antibiotic in Erlenmeyer flasks. The flasks were incubated at ~28 °C for 7 days with shaking at 150 rpm. Mycelial pads were harvested aseptically and washed 3 to 4 times with distilled water. Then, 60 g of biomass was weighed and crushed with a sterile ceramic mortar, then divided into three conical flasks, each one containing 100 ml of sterile deionized water and left in the shaking incubator for 96 hours. The biomass was filtered using sterile filter paper No.1, and 100 ml of supernatant was taken from each flask. Fungal supernatant was used for the synthesis of nanoparticles [9]. Some modifications in incubation time and concentration optimizing of CuNO3 (1 and 5 mM) were selected. The fungal supernatant was added to 100 ml of the CuNO3 at 1 and 5 mM (v/v), then incubated for 96 hours at 30 °C with agitation at 150 rpm in completely dark conditions until the color changed which indicated the synthesis of CuNPs [9].

2.4. Characterization of CuNPs

From each synthesized solution 5 ml were used to detect the wave length that absorbent by nanoparticles synthesized used the UV–Visible absorption spectrum from 200-800 nm [1]. Whereas, the shape and diameter of CuNPs manufactured were shown using the SE-SEM microscope at the University of Kashan /Iran.

2.5. Antibacterial activity of CuNPs against pathogenic isolates

The agar well diffusion ethod was using Mueller Hinton Agar Plate the sensitivity of the bacterial isolates against CuNPs Different dilutuions were made usi ng sterilised water and various concentrations (10, 20, 40, 50, 60, 70 and 100 %). Approximately (0.2) ml were placed in each well. After streaking (0.1) ml of fresh bacterial suspension on plates by sterilized cotton swab, and then the plate was left in the incubator at 37 °C for 24 hours. Next day, the diameter (mm) of the inhibition zone was measured [10]. *A. niger* supernatant was included as control.

2.6. Activity of CuNPs with Ciprofloxacin antibiotics

Well diffusion method was employed to screen the synergistic effect of CuNPs with Ciprofloxacin antibiotic on Mueller Hinton Agar Plate. The following concentrations were used: 1/1, 1/2, 1/3, 2/1, and 3/1. Each well contains approximately (0.2) ml per hole after streaking (0.1) ml of fresh bacterial suspension (1.5 x 10^8 cell per ml) on the gar surface by sterilized cotton swab, and then left the plate in the incubator for 24 hours at 37 °C After that the diameter (mm) of the inhibition zone was measured[10].

2.7. Laboratory animal initialization

The laboratory hamster were used as model animals under standardized laboratory conditions in suitable ventilation and temperature within (27 ±3 °C) and photoperiod at cycle 12 light and 12 hours of dark [11]. The diet was prepared according to [12]. In this experiment, 15 male laboratory hamsters were included with some standarized features such as the age was
approximately 6 months, healthy, and their weight between 210 to 230 g. The animals were divided into five groups with polypropylene cages: G1: control animals with wounds only. G2: Animals with infection wounds with *S. aureus*. G3: Animals wounds infected with *S. aureus* and treated with CuNPs. G4: Animals wounds infection with *S. aureus* and treated with Ciprofloxacin. G5: Animals wounds infection with *S. aureus* and treated with CuNPs + Ciprofloxacin.

The back at end dorsal of the animal were shaved. Two symmetrically placed full-thick skin wounds were made by 6 mm using sterile surgical blades (24 size). Linear cutaneous wounds of 6 mm in diameter were formed at the level of the subcutaneous fat in the dorsal end of the hamster [11].

2.8. Treatment of wounds used the ciprofloxacin antibiotic at 10 µg/ml and CuNPs at 60%

After wounding made, the 50 µl of the solution were taken from each Ciprofloxacin or CuNPs according the groups treatment was applied on wounds, the treatments were daily performed after the infections were appeared.

2.9. Healing Time of Wounds

Complete healing of each wound was considered when no scab was visible and complete epithelialization occurred. [11].

2.10. Statistical analysis

Data were analyzed for statistical significance using two-way ANOVA analysis with Bonferroni's multiple-comparison posttest (GraphPad Prism 7). A probability value that considered with significant was 0.05.

3. Results

3.1. Identification of *A. niger*

Colonies of *A. niger* on PDA grow relatively rapidly, Macroscopic observation of *A. niger* reveals that their growth were initially white but they changed to black after a few days when producing conidial, the edges of the colonies appeared pale yellow and producing radial fissures, the reverse plate showed as white to yellow. A microscopic view of *A. niger* reveals that has smooth colored conidiophores and conidia. The conidiophores are protrusions from a septate and hyaline hypha. The conidial heads appeared radial and they split into columns (biseriate). The conidiophore vesicle produces sterile cells known as matulae which supported the phialides on the conidiophores, the matulae and phialides cover the vesicle as clear in Figure 1.

![Figure 1](image-url) Microscopic examination of *A. niger*. (A) Magnification was 10 ×, (B) Magnification was 40×. Small pieces of fungal colony (incubated at 28 °C for 9 days) were placed on a slide then one drop of sterile distilled water was added, mixed, covered with a cover slip visualised under the microscope.

3.2. Synthesis and characterization of CuNPs by *A. niger* supernatant

The results were indicated that the *A. niger* isolate was able to transformed successfully the CuNO$_3$ to CuNPs at 5 mM after 96 hours of incubation by changing the color from blue to dark green and the peak spectra of UV-vis experiment (Figure 2).
Figure 2. (1) Change color of test sample after 96 hours of incubation. (A) before incubation (B) after incubation. (2) UV-Visible absorption spectrum of CuNPs. (a) Supernatant of *A. niger* control. (b) *A. niger* supernatant plus 5mM Cu(NO₃)₃ test sample before incubation. (c) Test sample after incubation. Inset figure: Color change of solution from pale white (b) to light green (treated) after synthesis of CuNPs (c). Incubation temperature was 30 °C and incubation time was 96 hrs. at 150 rpm.

The wavelength scan of UV- vis spectra revealed an absorption peak at 225 nm (Figure 2.2) indicating a surface plasmon resonance (SPR), having nanoparticles with sizes less than 100 nm.

Furthermore, CuNPs have confirmed using scanning electron microscopy. The shape and size the CuNPs have studied. Field Emission Scanning Electron microscopy (FE-SEM) was used to determine the morphology and size of the CuNPs synthesized. The FE-SEM micrograph shows presence of CuNPs magnified 150,000 times and they have mainly spherical and oval shapes. showed the nanoparticles are agglomerated in some amount due to sticky nature of the fungus supernatant (Figure 3.A and B).

therefore, the data obtained from FE-SEM micrographs confirm that the synthesized CuNPs have the size ranging from 3 to 70 nm and the size distribution was 20 nm as average.

Figure 3. (A) FE-SEM for CuNPs synthesized by *A. niger*. (B) Size distribution of nanoparticles in FE-SEM micrograph. Magnification was 150,000X. 100 ml of fungal filtrate added to 100 ml of Cu(NO₃)₃ (5 mM) and incubated for 96 hours at 30 °C at 150 rpm.

3.3. Isolation and identification of pathogenic bacteria

Samples of surgical and non-surgical wounds (accidents) from a total of 72 clinical different wound samples from human were collected from General Hospital of Salah-Adin in Salah-Adin governorate /Iraq. The samples were collected from different gender during the period from Nov. 2019 to Feb. 2020.
The results revealed that surgical wound samples were 60 samples, while accident wound samples had a lower number at 12 samples (Figure 4).

![Figure 4](image-url)

**Figure 4.** Number and percentage of wound samples according to sources.

Thirty-nine (54.2%) of the samples showed with bacteria growth for wounds, while the others 33 (45.8%) did not show bacteria growth. Patients’ samples from a total of 72 different wound samples were collected. Age grouped between (1-70 years). The findings revealed that 46 (64%) of wound samples belonged to male, while 26 (36%) belonged to female. Wound infection in relation with demographic characteristics of the patients, from total samples of male patients among them 21 (46%) samples showed aerobic bacterial growth, while others 25 (54%) shown negative growth, and female 17 (71%) samples were positive for aerobic bacterial growth but others 7 (29%) appeared to be invisible aerobic bacterial growth.

The morphological, microscopically and biochemical characteristics were conducted to identify the bacterial isolates that contaminated of wound inflation of patients [13]. Fifty-six isolates were found out of seventy-two samples screened. The majority of isolates were appeared as gram positive bacteria at 50 (89%), while the gram negative was at 6 (11%) of the samples. Among 56 bacterial isolates, *Staphylococcus epidermidis* was the most predominant bacteria which appeared at 22 (39.3%) isolates followed by *Staphylococcus aureus* 17 (30.4%) isolates, *Staphylococcus saprophyticus* 6 (10.7%) isolates, *Staphylococcus lugdunensis* 5 (8.9%) isolates, *E. coli* 5 (8.9%) isolates, and *Pseudomonas aeruginosa* 1 (1.8%) isolates. This results were similar to the Upreti et al., [14].

### 3.4. Susceptible of antibiotic against some species of *Staphylococcus*

Antibiotics are classified into many categories based on their mechanism of action, one antibiotic was taken from each group. The sensitivity of the bacterial isolates was investigated for 4 antibiotics. The results showed that the isolates were differed in their sensitivity to the antibiotics under study between resistance, Intermediate and Sensitivity, Figure (5), according to (CLSI) [15].

![Figure 5](image-url)

**Figure 5.** The sensitivity ability of *staphylococcus* spp against some antibiotics.

*S. aureus* and *S. epidermidis* isolates were had a 41 and 64% resistance respectively to the Trimethoprim antibiotic. While the *S. saprophyticus* isolates showed at 83% resistance, and *S. lugdunensis* at 60% resistance to Trimethoprim antibiotic.
Chloramphenicol as a type of non β-lactams (inhibit protein synthesis) showed a high inhibitory activity against S. aureus isolates when only (6%) of them were resistance. This came in accordance with the results of Daini and Akano, [16]. S. epidermidis was found to be 50% susceptible to chloramphenicol, while S. lugdunensis was found to be 80% susceptible to the same antibiotic. S. saprophyticus, on the other hand, was found to be chloramphenicol-resistant in 67% of cases. It's possible to come up with a new way to avoid antibiotics. The Ciprofloxacin antibiotic was only fluroquinolone used and the action activity was to inhibit the DNA replication and transcription, shows relative efficacy that only S. saprophyticus shown (50%) of the tested isolates develop resistance against this type of antibiotic and 33% intermediate. This finding is consistent with that of Al-Ruaily and Khalil [17], who found that ciprofloxacin to be the least active antibiotic during their research. S. epidermidis reported no significant in sensitivity or resistance to antibiotics of ciprofloxacin. Vancomycin is an antibiotic that active on inhibits the cell wall synthesis, on the other hand, was found to be the most effective antibiotic in this samples, with 82% of S. aureus, 64% of S. epidermidis, and 83% of S. saprophyticus being susceptible to it. S. lugdunensis, on the other hand, 60% were vancomycin resistant.

3.5. Inhibition effect of CuNPs singly or in combination with ciprofloxacin against S. aureus

Antibacterial effect of CuNPs was evaluated against bacterium S. aureus. In comparison to other antibiotics, the bacterial isolate was chosen because it was less susceptible to ciprofloxacin for S. aureus (intermediate 20 mm). The different concentrations of CuNPs that produced by fungal supernatant was revealed that S. aureus was susceptible to lower concentration of 6 mm of zone inhibition which was mean that lower values as at 60% (Table 1).

| CuNPs Concentration | Zone Inhibition diameters (mm) |
|----------------------|-------------------------------|
| %                    |                               |
| 10                   | 0                             |
| 20                   | 0                             |
| 40                   | 0                             |
| 50                   | 0                             |
| 60                   | 6                             |
| 70                   | 6.5                           |
| 100                  | 7                             |

The ability of inhibition of CuNPs synthesized with A. niger supernatant when combined with ciprofloxacin at different concentration were also showed a high inhibition activity against the S. aureus (Figure 6) when compared with individual activity (6 and 20 mm) and the mix of CuNPs with ciprofloxacin at 1/1, 1/2, 1/3, 2/1 and 3/1 were exhibited the zone inhibition diameter against S. aureus at 35, 36, 35, 36 and 35 mm respectively (Table 2).

| CuNPs/ Ciprofloxacin combination | Zone Inhibition diameters (mm) |
|----------------------------------|-------------------------------|
| 1/1                              | 35                            |
| 2/1                              | 36                            |
| 1/3                              | 35                            |
| 2/1                              | 36                            |
| 3/1                              | 35                            |

Figure 6. The inhibition zone diameters of a CuNPs/Ciprofloxacin combination.
3.6. Effects on wounds healing

Each critical changes in wound size during the wound healing progression were investigated (Figure 7). The healing potential was evaluated using the optical images of the wound on different days after surgical intervention. The wounds in the group one G1, in the day of surgery and after 3 days, where non visible difference in appearance was observed, complete closure was observed after 14 days of wound healing, but it has the scars. The contaminated wounds with *S. aureus* in G2 were appeared in Figure (7), that has no difference in wound size at day 3, but the wounds show that healed more slowly with left scars compared to the other groups. Group 3 was illustrated the effects of treated with CuNPs synthesized by fungal supernatant on the periods of healing the wounds G3. The contaminated wounds in group three were treated with CuNPs synthesized by fungal supernatant; the results were appeared that show a few changes in the size or the healing of wounds, while the treatment after 14 days, made the healing faster compared with the control groups.

The effect of antibiotic as ciprofloxacin when use to treat the contaminated wounds with *S. aureus*, were caused a few change in the wound size at 3 days. while the injured wounds were healed faster compared with the control sample at day 14. Also, there was no scars observed of G4. Also, the used of CuNPs synthesized by fungal supernatant mixed with Ciprofloxacin antibiotic in G5, were investigated, the treatments triggered the healing to be recovered rapidly at 3 days without scarring and completed healing were observed in the 14 days.

![Figure 7. The healing wounds and size occurred during treated with CuNPs or Ciprofloxacin synergistically together, through 1, 3 and 14 days contaminated with *S. aureus*. G1: The wounds healing with 14 days. G2: wounds with *S. aureus* contamination. G3: CuNPs derived from fungus supernatant treated the wounds infected with *S. aureus*. G4: *S. aureus* contaminated wound on treatment with Ciprofloxacin. G5: CuNPs with fungi synthesized with ciprofloxacin against *S. aureus*.](image)

3.7. Effects on hematological parameters

Complete blood count standards one of the fundamental tests that provide an indication of the animal’s physiological and health status by estimating the total a count of major molecules in the blood, including White blood cells (WBCs), Red blood cells (RBCs), hemoglobin level (Hb), Hematocrit (PCV) and platelets parameters. The results in table (3) showed that the WBCs counts in G2-S6 group of laboratory hamsters that experimental wounds were contaminated with *S. aureus* shown a significant increased (p<0.05) and appeared at 13.2 (cells/mm3) compared with its accounts in the control group (G1) which at 12.13 (cells/mm3). The WBCs of the hamsters that’s wounds contaminated by *S. aureus* and treated with the CuNPs synthesized with *A. niger* supernatant singly or with Ciprofloxacin did not reveal significant differences in the WBCs count in
groups G3 S6, G4 S6 and G5F S6 that appeared at 12.2, 12.03 and 12.11 (cells/mm3) compared with normal level 12.13(cells/mm3). The experimental hamsters wounds groups that treated with CuNPs and infected with *S. aureus*, G3-S6 to G5-S6 did not display significant differences at all groups of RBCs, Hb and PCV values. Compared with the values in the control group that appeared at 5.2, 11.2 and 34.3 respectively. The platelet count of the infected laboratory hamsers’ wounds that contaminated with *S. aureus* was shown significantly increased of the platelet count 314.3 cells/mm3 compared with the count in the control group G1 (222.3 cells/mm3). The treatment of laboratory hamsers with the NPs types singly or in combination with the ciprofloxacin G3, G4 and G5, the platelet accounts were non-significatly differ from the control group. The treatment of infected animals with Ciprofloxacin in G4 resulted in non-differences in its values with the control group.

**Table 3.** Effect of CuNPs on some blood parameters of Hamsters with experimental induced wounds contaminated with *S. aureus*.

| Groups   | WBCs (cells/mm³) | RBCs (cells/mm³) | Hb (g/dl) | PCV (%) | Platelet (cells/mm³) |
|----------|------------------|------------------|-----------|---------|----------------------|
| G1       | 12.13 a          | 5.2 a            | 11.2 a    | 34.3 a  | 222.3 b              |
| G2 S6    | 13.2 b           | 5.08 a           | 11.8 a    | 35.0 a  | 314.3 a              |
| G3F S6   | 12.2 a           | 5.82 a           | 10.9 a    | 34.6 a  | 276.2 b              |
| G4 S6    | 12.03 a          | 5.95 a           | 11.5 a    | 35.4 a  | 217.3 b              |
| G5F S6   | 12.11 a          | 5.27 a           | 11.8 a    | 35.8 a  | 238.2 b              |

G1: represent control group only wounds. G2 S6: represent control group, infected wounds with *S. aureus*. G3F S6: represent treated infected wound with *S. aureus* by CuNPs produced from fungal supernatant. G4 S6: represent treated infected wound with *S. aureus* by ciprofloxacin. G5F S6: represent treated infected wound with *S. aureus* by combination between CuNPs produced from fungal supernatant and ciprofloxacin. Different letters in the two adjacent columns indicate significant differences at the level of P < 0.05.

3.8. Effects on some biological parameters

The effect of CuNPs singly or combined with ciprofloxacin on some serum biological parameters were illustrated in table (4). The contamination of hamster’s wounds with *S. aureus* (G2) was shown significantly increased (p<0.05) the level of C-reactive protein (CRP) and became at 12.13 mg/dl compared with the value of G1 at 8.13 mg/dl. The treatment groups G3, G4 and G5 were affected on recovery of the parameters value to became non-significantly increased of the platelet count 314.3 cells/mm3 compared with the control group G1 which appeared at 44.6 and 32.1 U/L respectively. The effects of the CuNPs were synthesized by *A. niger* supernatant singly or combined with ciprofloxacin as G3, G4 and G5 were affected on recovery of the parameters value to became non-significantly compared with the control group, except the G4 were significantly increased 50.1 and 42.2 U/L respectively.

**Table 4.** Effect of CuNPs on some serum biological parameters of Hamsters with experimental induced wounds contaminated with *S. aureus*.

| Groups | CRP (mg/dl) | GPT (U/L) | GOT (U/L) | B. urea (mg/dl) | Creatinine (mg/dl) |
|--------|-------------|-----------|-----------|-----------------|-------------------|
| G1     | 8.13 a      | 44.6 c    | 32.1 c    | 42.3 b          | 0.98 b            |
| G2     | 12.13 b     | 56.6 a    | 48.6 a    | 48.2 a          | 1.03 a            |
| G3     | 8.55 a      | 42.6 c    | 32.0 c    | 44.3 b          | 0.96 b            |
| G4     | 8.04 a      | 50.1 b    | 42.2 b    | 44.3 b          | 0.97 b            |
| G5     | 8.75 a      | 43.3 c    | 34.7 c    | 43.3 b          | 0.95 b            |

G1: represent control group only wounds. G2: represent control group, infected wounds with *S. aureus*. G3: represent treated wound infection with *S. aureus* by CuNPs produced from fungal supernatant. G4: represent treated infected wound with *S. aureus* by ciprofloxacin. G5: represent treated infection wound with *S. aureus* by combination between CuNPs produced from fungal supernatant and ciprofloxacin. Different letters in the two adjacent columns indicate significant differences at the level of 0.05.

The urea and creatinine parameters which indicated for the kidney state appeared significantly increased with the laboratory hamsers that experimental induced wounds were contaminated with *S. aureus* and appeared at 48.2 and 1.03 mg/dl respectively compared with the same parameter’s values in control group at 42.3 and 0.98 mg/dl respectively. The effects of CuNPs singly or in combination with ciprofloxacin as G3, G4 and G5 were became non significantly different with the values of the same parameters in control group (G1).
3.9. Effects on IgG and IgA parameters

The table (5) was investigated the effect of CuNPs singly or combination with ciprofloxacin on IgG and IgA parameters.

**Table 5.** Effect of CuNPs on IgG and IgA of Hamsters with experimental induced wounds contaminated with *S. aureus.*

| Groups | IgG mg/dl | IgA mg/dl |
|--------|-----------|-----------|
| G1     | 980 c     | 202 b     |
| G2     | 1643 a    | 280 a     |
| G3     | 1110 b    | 215 b     |
| G4     | 1116 b    | 210 b     |
| G5     | 1179 b    | 208 b     |

G1: represent control group only wounds. G2: represent control group, infected wounds with *S. aureus.* G3: represent treated infection wound with *S. aureus* by CuNPs produced from fungal supernatant. G4: represent treated infection wound with *S. aureus* by ciprofloxacin. G5: represent treated infected wound with *S. aureus* by combination between CuNPs produced from fungal supernatant and ciprofloxacin. Different letters in the two adjacent columns indicate significant differences at the level of 0.05.

The contamination of laboratory hamster experimentally induced wounds with *S. aureus* (G2) was increased significantly (p<0.05) the level of IgG and IgA parameters and became at 1643 and 280 mg/dl compared with the value of G1 at 980 and 202 mg/dl. The treatment groups that as G3, G4 and G5 were enhanced the IgG and IgA values to became non significantly different in comparsion with the control group.

4. Discussion

Filamentous fungus *A. niger* has been extensively investigated worldwide. However, no more sufficient studies of CuNPs synthesis using *A. niger* are available in Iraq. *A. niger* is the most common fungi species in relation to its morphology, physiology, benefits, and effects. Due to this, it is well known to be less pathogenic to humans and animals [18].

The results were shown that the most common bacterial isolates from wound infections was *Staphylococcus* spp, then *E. coli* isolates, while one isolate was identified of *Pseudomonas aeruginosa.* These results were differed from study of Khalil [19], who found *E. coli* isolates were isolated from wounds infection with the highest percentage (77.27), while *Pseudomonas aeruginosawas* less count in wound infection (42.21%). The reason of dominate of *Staphylococcus* spp isolates over the other gram negative bacteria in wounds infection may be due to these isolates have the ability to invade and spread to skin and soft-tissue, such as surgical site, abscesses, carbuncles, and boils. Furthermore, its ability to produce many enzymes and toxins require to cause infections [20, 37], also represent some of these isolates (*S. aureus*) a large part of the normal flora of the human body, as they are found in many areas such as throat, nose, gut, genital tract, and the skin, if they move from the body to the affected areas they will cause wound infections, also the possibility of transmission through carriers from healthy people [21]. The possible reason for the availability of negative gram bacteria in samples of wound infection might be these isolates are commonly found in the hospital environment, so whenever damage in skin and soft tissue, they can easily disseminate [22].

The bacterial isolates were capable to develop the resistance for Trimethoprim may be by developing the new enzymes such as Dihydrofolate reductase that have a low affinity for the trimethoprim antibiotic. The bacterial resistance is based on a change in the target molecule of substrate for Dihydrofolate reductase enzyme. Also, bacteria may able to develop various strategies to overcome antibiotics as a result of the widespread use of trimethoprim in infection treatment. The reason behind resistant is the isolates to chloramphenicol this may be because of its have yet to evolve a particular mechanism to overcome chloramphenicol bacterial modified mechanisms. According to Evers and Courvalin [23], *The van genes* that are responsible for resistance (van genes) are inducible and transferable and confer high-level resistance to vancomycin. The size of the nanoparticle plays a major role in the antibacterial activity, the fact that the cell membranes in bacteria have nano size holes, while the nanoparticles have a smaller size than the size of the holes, so they can pass through the cell membrane holes without any a hindrance. For this reason the CuNPs produced by fungal supernatant, were 3-70 nm in size [24], which illustrates the relationship between the size of nanoparticles and their Inhibition activity against bacterial species [38].

When CuNPs synthesized from fungal supernatant were combined with ciprofloxacin and used against *S. aureus,* the zone inhibition diameters were increased when compared to individually action, confirming that the activity was synergistic. At day 14, of the animals treated, the wounds in group one had slowly healed, and leaving scars. While in group two, the wounds that infected with *S. aureus* bacteria was showed slow healing, because the healing of wounds were depended on period of infection and the efficiency of animal immune to destroy the microbes [25]. The treatment of wounds with the CuNPs were shown obvious impact in healing wounds in G3., it was active as encouraging angiogenesis, blood clotting,
enhancing proliferation of fibroblasts, and facilitating collagen deposition [26]. Also, the CuNPs have acting as a strong antibacterial effect; however, excessive accumulation of these nanoparticles may result in microbial cell death [27]. The use of the antibiotic ciprofloxacin which has a broad antibacterial spectrum [28], and they were probably causing inhibition the *S. aureus*, results in healing the wounds rapidly without leave any scars in G4. In comparison to the control groups, wounds treated with CuNPs and antibiotics individually or synergistically showed fresh skin layer from day 3 and healing was completed at day 14, according to Choi *et al.* [29] examined wound healing by observing re-epithelialization, inflammatory cell infiltration, collagen regeneration and pattern, and granulation tissue, granulation tissue and skin appendage.

Due of the inflammation in the hamster body that is usually occurs after a bacterial infection and caused the increased of WBCs. The host’s immune response serves as a line of defense, which was capable to combating bacterial invasion. The occurrence of an infection with pathogenic bacteria that stimulated the immune system to form defensive cells has been attributed to the increase in WBCs. These cells are important in the body’s immunity due to their ability to secrete immune chemicals that elimination microorganisms [30]. Non-significance numbers of WBCs in the groups of animals that were infected with the *S. aureus* and treatment by CuNPs in G3 due to the positive effect of these CuNPs in eliminate the negative impact of bacteria and restore the values of normal parameters. Inhibiting the pathogenic bacteria by using CuNPs due to their ability to penetrate the bacterial cell membrane without any hindrance because of its small size and breaking down the cell membrane, it leads directly to the breakdown of minerals, proteins, and genetic material causing cell death, as noted by Adams *et al.* [31].

Red blood cells (RBCs) and Hemoglobin (Hb) and package red blood cells (PCV) are all linked together, and appeared that the treatments with the factors used in this study affected in recover the parameters to became at the same values of each parameter in control group. Because PCV is a measure of the volume of red blood cells, its level is stable relation with RBCs. The reason for this stability may be to these findings indicate that simply finding bacteria commonly associated with surgical infection is less important than the quantitative amounts of bacteria involved. Platelets are one of the major components of blood, and their function is to form clots to prevent bleeding in blood vessels. Infection group shown increase in level of platelets while all other groups were decreased due to effect of solution that used in treatment, a decrease or increase in the level of these platelets causes thrombocytopenia.

These recovery effects of CuNPs individually or in combination with ciprofloxacin may be referred to the effects of its factors on the inhibition against the *S. aureus*, and the values were in normal range. The CRP assay is an acute phase protein which reflects a measure of the acute phase response include fever, leukocytosis, and a change in the hepatic synthesis of acute phase proteins [32]. The results showed that’s significantly increased of the GPT and GOT enzymes values in the laboratory hamsters that infected with *S. aureus* singly or combined with ciprofloxacin was used as treatment, except the G4 that were significantly increased and became at 50.1 and 42.2 U/L respectively. The treatment of infected animal groups with CuNPs had no obvious effect on GPT and GOT enzyme levels, which were comparable to those in the control group. The effectiveness of these treatments is dependent on the nanoparticles' ability to reduce the harmful effects of bacteria by acting as antibacterial agents [33], the recovery of the enzymes GPT and GOT indicates the normal re-activity of the liver. The reason is due to the high level of immunoglobulins (IgG, IgA) upon infection, as IgG as this type of antibodies are passable from blood serum due to their small size, and they become in great contact and adhesion to the cell. [34]. While IgA is present in the blood by 30% and is produced locally in the urinary tract and the digestive system in case of infection, as it acts to prevent the attachment of bacteria to the epithelial cells of the urinary tract and prevents them from penetrating the tissues. Furtermore, its presence and raise in the blood serum coincides with the presence and increase of IgG antibody. The results of the current study were agreement with the results of Khalf, [35], who found an increased in the concentration of the two immunoglobulins IgG and IgA in patients that were infected with bacterial infection, and also these results comparable with the results of Al-Sakr, [36], who found a significant increase in the concentration of IgA and IgG antibodies in patients with bacterial infection.

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