Evaluation the safety and synergistic effect of NiFe$_2$O$_4$ nanoparticles with antibiotic against *Pseudomonas aeruginosa*

H.M. Majed$^{1,3}$, H.Y. Khalef$^{2,3}$, H.A. Awadh$^3$, B.S. Noomi$^2$, N.A. Jafar$^2$ and K.A. Hadi$^2$

$^1$College of Medicine, Ibn Sina University, $^2$College of Veterinary Medicine, $^3$Collage of Science, Tikrit University, Tikrit, Iraq

**Abstract**

The antimicrobial resistance currently impedes and threatens the future of effective prevention and treatment of the continually expanding range of infections caused by bacteria. This study aimed to identify the bacterial causes the wound infection among animals and using the antibiotic/nanoparticles mixture as a new attempt for the treatment the wound infection induced in rats. For this purpose, 112 swabs wound infection cases in the different animal types (36 sheep, 21 goats, 12 cows, 4 horses, 8 dogs, 9 rabbits, 7 genies pigs and 15 rats) were studied in the for bacterial isolation. The *Pseudomonas aeruginosa* was tested for its sensitivity to the antibiotics and the nanoparticles (CoFe$_2$O$_4$ and NiFe$_2$O$_4$) in vitro by using the MIC method. Also the wound infection was induced in the rats and the effect of nanoparticles/antibiotics mixture were tested in vivo. The results showed that *P. aeruginosa* was the predominant bacterial type that the caused wound infection. The minimum inhibitor concentration of NiFe$_2$O$_4$ and CoFe$_2$O$_4$ nanoparticles were 32 µg /ml and 16 µg /ml respectively. A clear synergistic effect of antibiotic/nanoparticles as antibacterial were noticed which appear as a decrease in MIC and increase of the inhibitory diameter zone. According to the result of Random Amplification of Polymorphic DNA test, the nanoparticles effects on genetic material of *P. aeruginosa* observed as an appearance/disappearance of bands, increase in thickness and clarity of the bands.

**Keywords:** Evaluation, Synergistic, Nanoparticles, Antibiotic, *Pseudomonas aeruginosa*

**Correspondence:**
H.M. Majed
m.hala17@yahoo.com

**Article information**

**Article history:**
Received November 24, 2019
Accepted February 21, 2020
Available online September 11, 2020

**Keywords:** Evaluation, Synergistic, Nanoparticles, Antibiotic, *Pseudomonas aeruginosa*

**Introduction**

Skin is the body's first line of defense against to different types of bacteria, it is protected by a thin, acid film and normal flora that help in pathogens prevention by entering in to the body (1).

The wound infections are frequent complications following lacerations, injuries, penetrating trauma, animal fights and bites (2). Wounds can be classified as an acute and chronic. The acute wounds either traumatic or surgical, chronic wounds that need longer period for treatment no matter the cause (3).

There are many types of bacteria causing wound infection, but the most prevalent of them is *P. aeruginosa* (4,5).

*P. aeruginosa* is a gram negative bacteria, non-spor forming, obligate aerobes, motile by one or more polar flagella, most isolates are the oxidase and catalase positive (6). It is a saprophytic bacterium in soil, water and plants, in addition apart of the normal flora in animals and human (7).

*P. aeruginosa* has many the virulence factors which help it in skin infection like protease, lipases, phospholipases, haemolysin, motility, pigments, outer membrane and exoenzyme (8).

The wound infection is mainly treated with antibiotics and antimicrobials. Excessive or inappropriate use of these antibiotics can lead to the emergence of resistant bacteria which do not respond to antibiotic treatment, as seen in recent decades (9). Nanoparticles (NPs) have been established as a promising approach to solve this problem.
NPs are the materials that have at least one dimension 1-100 nm and have demonstrated the broad-spectrum antibacterial properties against both gram-positive and gram-negative bacteria (10). This study aimed to identify the bacterial causes of wound infection among animals and using of antibiotic/nanoparticles mixture as a new attempt for the treatment of wound infection induced in rats.

**Materials and methods**

**Samples**
112 swabs were collected from a wound infected from 36 sheep, 21 goats, 12 cows, 4 horses, 8 dogs, 9 rabbits, 7 genie pigs and 15 rats.

**Bacterial diagnosis**
Bacterial isolation and identification done on all swabs cultured in three culture media included blood agar, mannitol salt agar and MacConkey agar, and incubated aerobically at 37°C for 48 hours. The gram stain and group of biochemical tests were applied to diagnose of isolates (6).

**Genetic study**
DNA extraction done by using one *Pseudomonas* colony was dissolve in 200 μl of DNase free water then heated in water bath at 100c for 10 minutes. Eppendorf tube transmitted to ice then centrifuged at 12000c/m for 20 seconds. The supernatant was taken and kept in -20°C. (11). Compounds used in preparation of reaction mixture are Taq PCR Master Mix KIT, which contain Taq DNA Polymerase, PCR Buffer with 3mM MgCL2, 200μM dNTP, 23.0µl PCR Master Mix KIT, which contain Taq DNA Polymerase, Compounds used in preparation of DNA template 3.0 µl and DNA free water 21.4µl.

**Thermocycler programs**
The program began by initial denaturation step of 94°C for 4 min, then 30 cycles, each cycle consists of 3 steps; a denaturation step of 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 90 s; and a final extension step of 72°C for 10 min.

**Preparation of nanoparticles**
Nickel Ferrite NiFe₂O₄, Cobalt Ferrite CoFe₂O₄ prepared in the Department of Chemistry, College of Science, Tikrit University by Sol-Gel Auto-combustion methods. (1) Inhibition solution: three types of solution were prepared and (2) Antibiotic solution: antibiotics powders were diluted in the concentration 2, 4, 8, 16, 32, 64 and 256 μg /ml.

**Antibiotic/ nanoparticles solution mixture**
A 1 μg antibiotic: 1 μg Nano were prepared with the final concentrations: 2, 4, 8, 16, 32, 64 and 128 μg /ml.

**Determination of inhibitory zone**
100 µl of 1.5x10⁶ CFU/ml of bacterial suspension was disseminate in agar media. Holes in the plate were done aseptically with the sterile cork borer. Then 100µl of antibiotic or/ and NPs were put in hole and incubated at 37°C for 24h. The inhibition zones were measured using caliper (11).

**Determination of MIC**
One isolate of *P. aeruginosa* inoculated into brain heart infusion broth (final concentration 1.5X10⁸ CFU/ml) and nanoparticles were added in concentration 2, 4, 8, 16, 32, 64, 128 and 256 μg/ml, then incubated at 37°C for 24h. The lowest concentration tube in which growth not seen consider as MIC (12).

**The pathological effect of *P. aeruginosa***
24 adult Rats were used, 20 rats of them were hair removed and skin scratched with sterile scalpel and 1ml of 1.5x10⁶ CFU/ML of bacterial suspension were disseminated on the induced wound.

After appearance of infection, they divided to 5 groups. First group: 4 infected rats were killed for the histopathological study. Second group: 4 infected rats treated with 16 μg /ml (Nickel Ferrite NiFe₂O₄ carried on vaseline as a vehicle. Third group: 4 infected rats treated with 4 μg /ml gentamycin carried on vaseline as a vehicle. Fourth group: 4 infected rats treated with antibiotic/nanoparticles (Nickel Ferrite NiFe₂O₄ gentamycin (1:1) in concentration 4 μg /ml carried on Vaseline as a vehicle. The other 8 rats divided in two groups.

Fifth group: 4 rats' hair removed and treated with 64 μg / ml of Nickel Ferrite NiFe₂O₄ Nanoparticles. After 7 day killed and skin taken for histopathological study. Sixth group: 4 rats leaved as a control.

**Effect of nanoparticles study**
Effect of nanoparticles on skin: studied as refer in Fifth group. Effect of nanoparticles on *P. aeruginosa* RAPD technique (Random Amplification of Polymorphic DNA) used with 10 primers (Table 1).

**Results**

**Bacterial isolation**
Table 2 shows that seven types of bacteria were isolated from wound infection, and *P. aeruginosa* is the dominant bacterial Spp. With isolation ratio of 36.8%.
Table 1: The primers sequences used in RAPD technique

<table>
<thead>
<tr>
<th>Number</th>
<th>Primer code</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OP G-5</td>
<td>CTGAGACGGA</td>
</tr>
<tr>
<td>2</td>
<td>OP H-14</td>
<td>ACCAGTTGCT</td>
</tr>
<tr>
<td>3</td>
<td>OP M-01</td>
<td>GTTGTTGGCT</td>
</tr>
<tr>
<td>4</td>
<td>OP J-01</td>
<td>CCCGCCATAA</td>
</tr>
<tr>
<td>5</td>
<td>OP P-04</td>
<td>GTGTCTCAGG</td>
</tr>
<tr>
<td>6</td>
<td>OP Q-02</td>
<td>TCTGTGCCTG</td>
</tr>
<tr>
<td>7</td>
<td>OP R-10</td>
<td>CCATTCCCCAC</td>
</tr>
<tr>
<td>8</td>
<td>OP V-20</td>
<td>CAGCATGGTC</td>
</tr>
<tr>
<td>9</td>
<td>OP U-12</td>
<td>TCACCAGCCA</td>
</tr>
<tr>
<td>10</td>
<td>OP W-17</td>
<td>GTCCTGGGTT</td>
</tr>
</tbody>
</table>

Table 2: Number and ratio of bacterial isolates from wound infection

<table>
<thead>
<tr>
<th>No</th>
<th>Type of bacteria</th>
<th>Isolate No.</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pseudomonas aeruginosa</td>
<td>38</td>
<td>36.8%</td>
</tr>
<tr>
<td>2</td>
<td>Staphylococcus spp.</td>
<td>31</td>
<td>30.0%</td>
</tr>
<tr>
<td>4</td>
<td>Escherichia coli</td>
<td>19</td>
<td>18.4%</td>
</tr>
<tr>
<td>5</td>
<td>Proteus spp</td>
<td>11</td>
<td>10.6%</td>
</tr>
<tr>
<td>6</td>
<td>Klebsiella spp</td>
<td>2</td>
<td>1.9%</td>
</tr>
<tr>
<td>7</td>
<td>Salmonella spp</td>
<td>2</td>
<td>1.9%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>103</td>
<td>100%</td>
</tr>
</tbody>
</table>

All the *P. aeruginosa* isolates are appearing as a blue green color on the surface of MacConkey agar (Figure 1), and showed a positive result in the catalase, oxidase, VP and citrate utilization tests, while the *P. aeruginosa* isolates appeared the negative result in the indole and methyl red tests.

The colonies of *Proteus spp* appeared as a pale in color with swarming on MacConkey agar, and gave positive results for catalase, methyl red, citrate utilization tests and negative results for indole, oxidase, and voges proskauer tests. *E. coli* colonies appear as pink color on the surface of MacConkey's agar and metallic green on eosin methylene blue agar, and given positive results for catalase, indole, and methyl red tests and negative results for oxidase, voges proskauer, and citrate utilization tests. *Klebsiella spp.* colonies appear as pink color and are viscous/mucoid on MacConkey's agar, and gave positive results for catalase, voges proskauer, and citrate utilization tests and negative results for indole, oxidase, and methyl red tests. *Salmonella spp.* colonies were colorless, usually with black center on surface of Salmonella Shigella agar. The *Salmonella* isolates gave positive results for catalase, methyl red, citrate utilization, and H₂S tests and negative for indole, oxidase, and voges proskauer tests.

**Polymerase chain reaction result**

PCR test were used to confirm the results of culture and biochemical test of *P. aeruginosa* (Figure 2).

**Minimal inhibitory concentrations of nanoparticles**

The MIC of NiFe₂O₄ and CoFe₂O₄ were 32 µg /ml and 16 µg /ml respectively (Table 3).

**Synergistic effect of nanoparticles with antibiotic**

Table 4 shows the MIC of antibiotic for *P. aeruginosa* which ranged from 8 µg /ml to 128µg/ml, and these values decreased when antibiotic mixed with nanoparticles to became about 2-64 µg /ml. Figure 3A and 3B shows the inhibitory diameter zones of antibiotics with and without Nanoparticles.

**Result of RAPD**

The present study shows the effect of Nickel Ferrite NiFe₂O₄ on the genetic material of *P. aeruginosa* which occurred as appearance and disappearance of bands, increase in thickness and clarity of bands (Figure 4).
Table 3: Minimal inhibitory concentration of CoFe$_2$O$_4$ and NiFe$_2$O$_4$ for *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Effect of nanoparticles (µg /ml)</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt Ferrite</td>
<td></td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Nickel Ferrite</td>
<td></td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

Figure 3: Inhibitory diameter zone of antibiotic, A: without Nanoparticles, B: with Nickel Ferrite NiFe$_2$O$_4$ Nanoparticles.

Table 4: Minimal inhibitory concentrations of antibiotic with and without nanoparticles on *P. aeruginosa*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC of antibiotic (µg /ml)</th>
<th>Antibiotic +NF NPs (µg /ml)</th>
<th>Antibiotic + CF NPs (µg /ml)</th>
<th>Inhibitory diameter zone (µg /ml)</th>
<th>Inhibitory diameter zone (µg /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>128</td>
<td>32</td>
<td>8</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>128</td>
<td>32</td>
<td>8</td>
<td>64</td>
<td>6</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64</td>
<td>8</td>
<td>18</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Ceftraixon</td>
<td>64</td>
<td>8</td>
<td>16</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Amikacin</td>
<td>32</td>
<td>4</td>
<td>26</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>8</td>
<td>2</td>
<td>28</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8</td>
<td>2</td>
<td>30</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Setreptomycin</td>
<td>32</td>
<td>8</td>
<td>20</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>32</td>
<td>8</td>
<td>18</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>32</td>
<td>16</td>
<td>14</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>16</td>
<td>8</td>
<td>22</td>
<td>8</td>
<td>18</td>
</tr>
</tbody>
</table>

MIC: minimal inhibitory concentration; NF NPs: Nickle ferrite nanoparticles; CF NPs: Cobalt ferrite nanoparticles.

**Pathological study**

This section of the study shows the pathological change that occurred after experimental infection, and after exposure to high dose of Nickel Ferrite NiFe$_2$O$_4$ as in figure 5 and figure 6, and after exposure to gentamycin /nanoparticle mixture as in figure 7.

**Discussion**

In this study, *P. aeruginosa* isolated from all animal samples with different levels. This result was in agreement with the study of Al-Hadithi (13) who isolated *P. aeruginosa* from different animal samples in Baghdad. The highest isolation rate was recorded to *P. aeruginosa* from wound of infection and this was in agreement with Doge *et al.* (14).
antimicrobial activity is known to be the function of surface area which is adhesion to the microorganisms. The small size and high surface to volume ratio. Large surface area enhances the interaction between the nanoparticles and the microbes to carry out a broad range of probable antimicrobial activities (17). The lag phase of the growth cycle may be prolonged and the generation time of the organisms increased because of the nanoparticle binding to membranes of microorganisms (18).

Nanoparticles can interact with membrane lipids and disorganize the membrane structure, which leads to loss of membrane integrity, malfunction, and finally to bacterial death (19,20). Moreover, nanoparticles can react with the thiol group (-SH) of the proteins in the bacterial cell wall, causing inactivation of transport proteins nutrients, reducing cell permeability and causing death. The antibacterial activity of the nanoparticles used may be related to the binding of the nanoparticles to the outer membrane causing inhibition of active transport and eventually inhibiting RNA, DNA and protein synthesis, leading to cell death. Nanoparticles may lead to release of reactive oxygen species (ROS) such as hydrogen peroxide H$_2$O$_2$ and super-oxide O$_2^-$ generated from the surface of nanoparticles (21).

Research has been conducted indicating the possible mechanisms involved in the interaction of nanoparticles with biological macromolecules, which indicates that bacteria have a negative charge, while the metal oxide nanoparticles have a positive charge. This causes an attraction between bacteria-nanoparticles and leads to oxidation of the bacteria (22,23).

The RAPD PCR results refer to the occurrence of genetic change, that’s may be due to ability of nanoparticles to penetrate bacterial cells at a nanoscale level and result in the production of toxic oxygen radicals, which cause damage to DNA (24).

A synergistic effect of nanoparticles with antibiotics were observed in the current study. Similar founding reported by other authors (25,26). The mechanism of the synergism is still under investigation; however, several mechanisms have been proposed between Ag nanoparticles and gentamycin. It has been reported that as compared to AgNPs alone the combination of antibiotics and AgNPs complexes will release Ag$^+$ at a higher rate, moreover it has also been proposed that the combination of antibiotic with AgNPs through the active groups of antibiotics such as hydroxyl group and amine group will result in conjugation of both the molecules. This will result in the increase in the effective concentration of antibiotics at a specific site (27-29).

Pathological study showed clear pathological effect of pseudomonas on infected Rats skin that’s agreement with (30).

Conclusions

Many variations were observed on the studied bacterial isolated including the appearance and disappearance of DNA
and its different numbers when treated with nanoparticles. As for the results of the histopathological, it was found that the injury of mice with *P. aeruginosa* antibiotic resistance emerged after about five days and the symptoms were heat, redness and swelling of the skin and the release of yellow and green purulent secretions from the place of injury. When treated mice infected with nanoparticles and antibiotics together the time of the healing was faster than the time of the healing of nanoparticles treated only.

**Acknowledgments**

Author wish to thank Knowledge University for providing and supporting facilities.

**Conflict of interest**

Hala M. Majed declares that he has no conflict of interest regarding publishing or funding this article.

**Reference**


تقييم الأمان والفاعل التأزري للأجسام النانوية NiFe₂O₄ مع المضادات الحياتية ضد الزواحف الزنجارية

حلا محمد مجيد 1، هبة يونس خلف 1، هالة عبد الخالق عوض 1، بشار صادق نومي 2، نهاد عبدالحسين جعفر 2، خالد احمد هادي 2
1 كلية الطب، جامعة ابن سينا، 2 كلية الطب البيطري، كلية العلوم، جامعة تكريت، العراق

الخلاصة
تهدد وتعوق المقاومة للمضادات الحياتية مستقبل السيطرة والعلاج على الجراثيم ذات الانتشار الواسع، ولهذا السبب فقد استهدفت الدراسة حلا لمجيد، هبة خلف، هالة عوض، بشار صادق، نهاد جعفر، و خالد هادي. تم دراسة 112 إصابة بالجروح، ثم طبقت المحاولة الجديدة كعلاج لنوع الجراثيم الرئيسي المسبب لعدوى الجروح. أظهرت النتائج أن جراثيم الزواحف الزنجارية هي المسبب الرئيسي لعدوى الجروح. إن التركيز المثبط الأدنى لكوبالت الحديد وكوبالت النيكل 32 ميكروغرام / مل و 16 ميكروغرام / مل على التوالي. كما أظهرت الدراسة تأثير تأزري واضح للأجسام النانوية مع المضادات الحياتية وذلك من خلال انخفاض التركيز المثبط الأدنى وزيادة منطقة القطر المثبط. اظهر اختبار مؤشر التضاعف العشوائي تأثيراً واضحاً للجراثيم النانوية على المادة الوراثية للزواحف الزنجارية وذلك من خلال ظهور وختفاء عدد من الحزم قبل وبعد المعاملة إضافة إلى الزيادة في والنقصان سمكها ووضوحها.