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

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**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*

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**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-spore 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 100°C for 10 minutes. Eppendorf tube transmitted to ice then centrifuged at 12000 rpm 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 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.

Nanoparticles solution
Prepared by dissolving of nanoparticles by vortex and final concentrations were: 2, 4, 8, 16, 32, 64, 128 and 256 µg /ml.

Antibiotic/ nanoparticles solution mixture
A 1 µg antibiotic: 1µg Nano were prepared with the final concentrations: 2, 4, 8, 16, 32, 64 and128 µg /ml.

Determination of inhibitory zone
100 µl of 1.5x10^8 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 1.5x10^8 CFU/ml) and nanoparticles were added in concentration 2, 4, 8, 16, 32, 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 scalp and 1ml of 1.5x10^8 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>ACCAGGTGGC</td>
</tr>
<tr>
<td>3</td>
<td>OP M-01</td>
<td>GTTGGTGGCT</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>TCTGTCGCTC</td>
</tr>
<tr>
<td>7</td>
<td>OP R-10</td>
<td>CCATTCCTCA</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. *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) Inhibitory diameter zone</th>
<th>Antibiotic + CF NPs (µg /ml) Inhibitory diameter zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>128</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>128</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Ceftraixon</td>
<td>64</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Amikacin</td>
<td>32</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>8</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Setreptomycin</td>
<td>32</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>32</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>32</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>16</td>
<td>8</td>
<td>22</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).

Figure 4: Agarose gel electrophoresis of RAPD- PCR products. M: 100 bp DNA ladder, lines (1-10) positive result of P. aeruginosa with 10 different primers, A: before treatment with Nanoparticles, B: after treatment with Nickel Ferrite NiFe$_2$O$_4$ Nanoparticles.

Figure 5: A cross section in skin of rat infected with P. aeruginosa, showed, Hemorrhage H, edema in dermis A, infiltration of inflammatory cells B. H&E. X400.
Nanoparticles of nickel ferrite and cobalt ferrite showed thick keratin layer A, edema in dermis B, rupture in Stratum granulosum, hemorrhage H, infiltration of inflammatory cells D, degeneration of hair follicles E. H&E. X400.

Figure 6: A cross section in skin of rat infected with P. aeruginosa and exposed to nickel ferrite NiFe₂O₄. Nanoparticles showed thick keratin layer A, edema in dermis B, rupture in Stratum granulosum, hemorrhage H, infiltration of inflammatory cells D, degeneration of hair follicles E. H&E. X400.

Figure 7: A cross section in skin of rat infected with P. aeruginosa and exposed to gentamycin /NiFe₂O₄. Nanoparticles showed normal multi layers of epidermis and dermis, normal adipose tissue and hair follicles. H&E. X100.

The results of the current study revealed that P. aeruginosa was the predominant bacteria isolated from wound infections in different types of animals. This result was in agreement with the previously study (4,5).

The predominance of Pseudomonas may be due to the ability of Pseudomonas to live in wide range of the temperatures, highly distributed in the environment, high antibiotic resistance and it possess many types of the virulence factors which facilitates the pathogenesis of this bacterium (15,16).

Nanoparticles of nickel ferrite and cobalt ferrite showed antibacterial activity against P. aeruginosa, and it is clear in the results of MICs and inhibition zones by nanoparticles. The result of this study showed similar results in a previous study (5). The antibacterial activity of Cobalt ferrite is due to their particular small size which makes a perfect attachment to the membrane of the microorganisms (1). This antimicrobial activity is known to be the function of surface area which is adhesion to the microorganisms. The small size and the 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 H₂O₂ and super-oxide O²⁻ 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.
Acknowledgments

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Conflict of interest

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

Reference


تقييم الأمان والفاعل التأزري للأجسام النانوية NiFe$_2$O$_4$ مع المضادات الحياتية ضد الزوائف الزنجارية

حلا محمد مجيد 1، هبة يونس خلف 2، هالة عبد الخالق عوض 3، بشار صادق نومي 2، نهاد عبدالحسين جعفر 2 و خالد احمد هادي 2

1 كلية الطب، جامعة ابن سينا، 2 كلية الطب البيطري، 3 كلية العلوم، جامعة تكريت، تكريت، العراق

الخلاصة

تهدد وتعوق المقاومة للمضادات الحياتية مستقبل السيطرة والعلاج على الجراثيم ذات الانتشار الواسع، ولهذا السبب فقد استهدفت الدراسة تقييم الأمان والفاعل التأزري للأجسام النانوية NiFe$_2$O$_4$ مع المضادات الحياتية ضد الزوائف الزنجارية. تم دراسة 112 إصابة بالجروح، ثم طبقت المحاولة الجديدة كعلاج لتنوع الجراثيم المؤثرة على الجروح. أظهرت النتائج أن الزوائف 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 المثبطة. اظهر اختبار مؤشر التضاعف العشوائي تأثيراً واضحاً للجراثيم النانوية على المادة الوراثية للزوائف الزنجارية وذلك من خلال ظهور وانخفاف عدد من الحزم قبل وبعد المعاملة إضافة إلى الزيادة في وضوحها وتحكمها.

تهدف وتعوق المقاومة للمضادات الحياتية مستقبل السيطرة والعلاج على الجراثيم ذات الانتشار الواسع، ولهذا السبب فقد استهدفت الدراسة تقييم الأمان والفاعل التأزري للأجسام النانوية NiFe$_2$O$_4$ مع المضادات الحياتية ضد الزوائف الزنجارية.