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***In Vitro* Toxicity Evaluation of Silver Nanoparticles on *Entamoeba histolytica* trophozoite**

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Abstract

The protozoan parasite *Entamoeba histolytica* is a causative agent of amoebiasis, where it causes millions of cases of dysentery and liver abscess each year. Metronidazole is a drug of choice against amoebiasis. The drug is a choice because of its efficacy and low cost, but at the same time it causes several adverse side effects; therefore, it is important to find effective medications to treat amoebiasis without any complications or any side effects. The aim of this study is to evaluate the effectiveness of different concentrations (50, 75 and 100 µg/ml) of silver nanoparticle (AgNPs) against trophozoites stages of *E. histolytica in vitro*. The results showed a significant decrease ($p < 0.05$) in numbers of trophozoites stages after treated with AgNPs and metronidazole when it was compared with the control. Likewise, a significant difference ($p < 0.05$) was also observed between AgNPs groups and metronidazole drug, while it did not significantly differ between different concentrations of AgNPs. The mortality rate values of the *E. histolytica* trophozoites after 48h incubation with AgNPs at a concentration of 50, 75 and 100 µg/ml, and metronidazole were 37.2%, 42.4%, 46.7% and 100%, respectively. The microscopic studies confirmed that AgNPs were effective enough to induce apoptosis. Based on our results, the anti-parasitic activity of AgNPs at different concentrations will reduce the mean number of *E. histolytica* trophozoites.

Key word: Silver nanoparticles, toxicity, *Entamoeba histolytica*.

Introduction

Entamoeba histolytica is responsible for the human amoebiasis, cyst is the infective stage of *E. histolytica*. The parasite reaches human body by swallowing cyst infected water or food [1]. Annually, 40-50 million cases of amoebiasis and up to 100,000 death cases are recorded by WHO worldwide [2]. Metronidazole is a current therapy against amoebiasis, but resistance has been reported and the drug has unpleasant side effects. The protozoan flagellates *Giardia intestinalis* and *Trichomonas vaginalis* are resistant to this drug as well it has a low efficacy against asymptomatic cyst carriers in amoebiasis patient [3]. Metronidazole kills the trophozoites by alterations in the protoplasmic organelles of the amoeba, but it is not effective in the treatment of cyst passers [4, 5]. Therefore, new drugs with targets and modes of action different from those of metronidazole will be needed.

Nanoparticles have attracted significant attention in various fields of science in recent years. Owing to the unique chemical and physical properties of nanomaterials that appear when the materials reach

nanosize compared to bulky counterparts, bio-compatibility and tissue infiltration efficiency bio-ability [6]. Nano-medicine and nano-pharmacy are two growing fields of nanotechnology. In medicine, nanomaterials applied for disease diagnosis, drug delivery, and disease treatment [7, 8, 9]. Silver nanoparticles (AgNPs), as a type of metal nanoparticles, are a cluster of silver atoms having a size range from 1-100 nm. The antimicrobial activity of AgNPs has been investigated in many reports. The antifungal activity of AgNPs against *Candida albicans* and *Candida tropicalis* were evaluated [10], also AgNPs showed great antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [11]. Recently, the AgNPs were being used as anti-parasitic therapy [12]. However, little information focuses on the evaluating of cytotoxic effect of AgNPs on *Entamoeba*. The aim of the present study is to assess anti-parasitic activities of AgNPs on mortality and viability of *E. histolytica* trophozoites and to microscopically observe the parasite's morphology changes.

Materials and Methods

Sample isolation and maintenance of *E. histolytica*

E. histolytica trophozoite was kindly isolated from infected patient at the Al-Mahmudiyah Hospital Laboratory in Baghdad. Strain was xenically cultivated in diphasic Locke's egg (LE) medium modified by Boeck and Drobohlav (1925) at 37°C for 48h, medium was supplemented with rice starch and antibiotics 100 IU/ml of Procaine Benzylpenicillin, 2mg/ml of Streptomycin Sulphate, and 2mg/ml Nystatin [13]. For parasite maintenance, trophozoites were harvested at log-phase growth 48 and 72h post-inoculation and counted using haemocytometer.

Silver nanoparticle (AgNPs)

Silver Nano powder was commercially purchased from market, sky spring nanomaterial's Inc. Houston, TX. Purity of 99.95% and size distribution 20-30nm. AgNPs solution was prepared in Locke's solution. The solution was mixed by vortexing about two minutes then added to LE medium and autoclaved at 100°C for 5 min then again mixed by vortexing for one minute before each use.

***In vitro* assay and Mortality rate**

Three different concentrations of AgNPs were prepared 50, 75 and 100 µg/ml, then the 0.08x10⁶/ml *E. histolytica* trophozoites was added and incubated with the different concentrations of AgNPs in LE medium for 24 and 48h at 37°C. The standard metronidazole drug was also used in the study, 0.08x10⁶/ml trophozoites was treated with standard concentrations of metronidazole 17 µg/ml [14] for 24 and 48h at 37 °C. Control (untreated with AgNPs) was also prepared for each set of experiment.

The parasite number were counted using haemocytometer, in addition, morphology and viability were measured under the light microscope after staining by trypan blue dye exclusion method [15]. Moreover, the mortality rate was calculated using the following formula [16]:

Percentage of mortality rate = $100 \times \frac{\text{Test}}{\text{control}}$

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software, SPSS Inc.V.13 was utilized to statically analyzed results. Least Significant Difference (LSD) and Duncan Multiple Range Test were applied to calculate mean ± standard error (S.E.). *P* < 0.05 values were considered statistically significant.

Results

The results showed that the replication rate of trophozoites after 24 and 48h was significantly decreased ($P < 0.05$) when treated with AgNPs at concentrations (50, 75 and 100 $\mu\text{g/ml}$) compared with the control as observed in the Table. The mortality rate values for AgNPs after 24 and 48h incubation were 48%, 37.2% for 50 $\mu\text{g/ml}$, and 46.2%, 42.4% for 75 $\mu\text{g/ml}$, and 46.2%, 46.7% for 100 $\mu\text{g/ml}$, respectively.

Between groups, there was no significant difference between different concentrations of AgNPs after 24 and 48h of incubation. Significant inhibition replication rates ($p < 0.05$) of trophozoites were observed after the treated metronidazole (17 $\mu\text{g/ml}$) with high mortality rate that reaches to 100% after 48h incubation. Significant difference ($p < 0.05$) and decreased numbers of parasites were also observed when it was treated with mixed solution of AgNPs and metronidazole compared with the control group, and the mortality rate reach to 98%.

Table: The activity of AgNPs against *E. histolytica* trophozoites.

| Treatment concentration in $\mu\text{g/ml}$ | After 24 h | | After 48h | |
|---|---|--------------------|---|--------------------|
| | Means \pm standard error (Trophozoite $\times 10^6$) | Mortality rate (%) | Means \pm standard error (Trophozoite $\times 10^6$) | Mortality rate (%) |
| Control (containing only the parasites) | 0.173 \pm 0.014 A | - | 1.343 \pm 0.008 A | - |
| 50 AgNPs | 0.09 \pm 0.015 B | 48 | 0.843 \pm 0.069 B | 37.2 |
| 75 AgNPs | 0.093 \pm 0.021 B | 46.2 | 0.773 \pm 0.214 B | 42.4 |
| 100 AgNPs | 0.093 \pm 0.003 B | 46.2 | 0.716 \pm 0.250 B | 46.7 |
| 17 Metronidazole | 0.04 \pm 0.00 C | 76.9 | 0 C | 100 |
| 50 AgNPs + 17 Metronidazole | 0.016 \pm 0.003 C | 90.8 | 0.016 \pm 0.006 C | 98.8 |

*Different letters: significant difference ($P < 0.05$) between means

Light microscope images show many morphological changes in trophozoite after incubation with AgNPs. *E. histolytica* trophozoite seems to have spherical shape in control culture (Figure1.A), while AgNPs treated trophozoite revealed morphological changes in the membrane (Figure1.B), vacuole contain silver was seen inside trophozoite after 24h (Figure1.C). After 48h trophozoite was appeared with multiple large, round vacuoles filled with AgNPs in the cytoplasm (Figure1.D). Although the results showed that AgNPs were effective in inducing apoptosis by the presence of apoptotic body in trophozoite that confirmed the apoptotic rationale in the pharmacodynamics of AgNPs (Figure1.E & F).

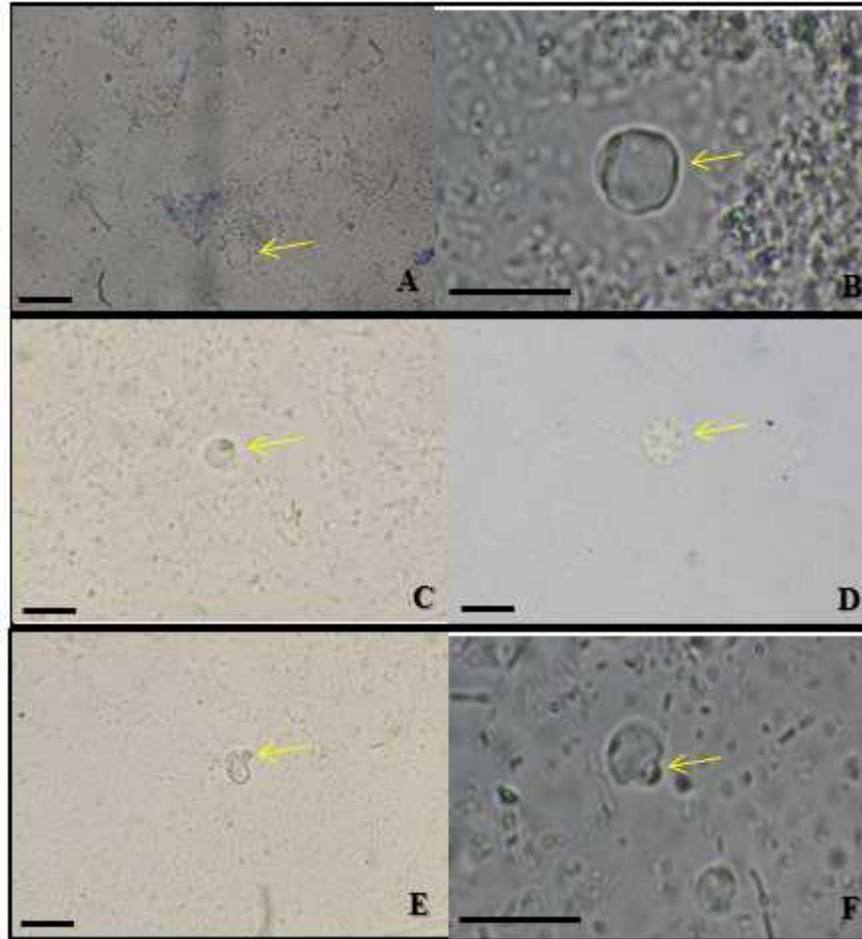


Figure (1): Light microscope views of the effects of AgNPs on *E.histolytica* trophozoite: (A). Spherical shape trophozoite in control culture (Yellow arrow), (B). Trophozoite (Yellow arrow) treated with silver nanoparticles, (C). Vacuole contain silver inside trophozoite after 24h incubation with AgNPs (Yellow arrow), (D). Multi vacuoles inside trophozoite (Yellow arrow) treated with AgNPs and stained by trypan blue after 48h. (E & F). *E.histolytica* undergoes apoptosis (Yellow arrows) after treated with AgNPs and observed apoptotic body.

Discussion

Entamoeba histolytica is one of the major problem diseases in the world. The inhibitory effect of AgNPs on *E. histolytica* trophozoite appeared after 24 h of incubation, where the parasite's viability was reduced almost to 50% with all concentrations (50, 75, 100 μ g/ml) used in the experiments. Furthermore, comparable results were noticed after 48 hours of incubation. The Saad *et al.*, reported a significant time and concentration-dependent toxicity effect of AgNPs on *E. histolytica* cysts after 3 hours of incubation about 51% were killed when the parasite exposed to 2.0 mg/l. Moreover, the mortality percentage increased to 100% when the particle concentration increased up to 4.0 mg/l. while no significant recorded when parasite exposed to very low concentration of AgNPs 0.5 and 1.0 mg/l [17]. However, investigators stated that particle's size is a relevant parameter to the toxicity of AgNPs. In fact, particle's size has an inverse relationship with antimicrobial activity [18]. So, as the particles size goes down the antimicrobial toxicity becomes higher.

In the literature, three main hypothesis were proposed for AgNPs antimicrobial activity. First, AgNPs have the ability to absorb and accumulate on microbe's cell wall, then the microbe's permeability and respiratory system destroyed [19]. Second, reactive oxygen species (ROS) can be produced on the surface of nanoparticles and interaction with respiratory enzyme. Excessive amount of ROS in the cell can attack plasma membrane, DNA and mitochondria and thus kill the cell [20]. Finally, silver ions that dissociate from AgNPs in aqueous phase [21] cause disruption of ATP and also might disturb and damage DNA replication through interaction of silver with phosphorous and sulfur groups on DNA and eventually lead to cell death [22]

The light microscopy images showed that the parasite actively uptakes the aggregate of AgNPs. Visible vacuole appeared inside the parasite after staining with trypan blue dye image (Figure 1.C & D) and as incubation time increased multiple vacuoles appeared in the cytoplasm. In addition to, a morphological changes appeared on the trophozoite's membrane and little membrane's bulbs appeared in the microscopic view indicating the parasite undergoes apoptosis process [6, 23] as an indicator for antiparasitic activity of AgNPs.

Our results indicate that the combination of AgNPs and metronidazole simultaneously showed positive results. It showed more antiparasitic activity than were treated with traditional drug alone after 24 hours and as effective as tradition drug after 48 hours. Authors suggest that the combination of AgNPs with drug could have potential antiparasitic activity against drug-resistance *Entamoeba*.

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تقييم سمية جزيئات الفضة النانوية على الاطوار المتغذية لاميبا الحالة للنسج في الزجاج

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الأميبا الطفيليات الابتدائية
سنوياً. و يعد عقار الميترونيدازول من العقاقير المختارة للقضاء على
لكن بنفس الوقت له عدة تأثيرات جانبية لذلك من المهم إيجاد علاج فعال ل
الهدف من هذه الدراسة هو تقييم فعالية و كفاءة تركيز مختلفة (50 75 100 مايكروغرام/) جزيئات النانوية
الاطوار المتغذية للاميبا الحالة للنسج في الزجاج. أظهرت النتائج انخفاض معنوي (p 0.05) في اعداد الاطوار المتغذية بعد معاملتها
بالجزيئات النانو و عقار ميترونيدازول مقارنة بمجموعة السيطرة. كما لوحظ ايضاً اختلافاً معنوياً بين المجاميع المعاملة بجزيئات النانو
و العقار في حين لا يوجد اختلاف معنوي بين التراكيز المختلفة للجزيئات النانوية. كما ان قيم فعالية القتل للاطوار المتغذية بعد 48
جزيئات النانوية بتركيز 50 75 100 مايكروغرام / ميترونيدازول 37.2 % 42.4 %
%46.7 % 100 على التوالي. كما أظهرت الدراسة المجهرية كفاءة جزيئات النانوية في حث عملية الموت المبرمج للاطوار
المتغذية، و اعتماداً على هذه النتائج تبين كفاءة جزيئات النانو الفضة كمضادات للطفليات ، اذ ان تراكيز مختلفة للجزيئات النانوية
اظهرت فعالية في الحد من مستوى نمو الاطوار المتغذية للطفيلي الاميبا الحالة للنسج في الزجاج.

الكلمات المفتاحية : جزيئات النانوية ، السمية، الاميبا الحالة للنسج.