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Stimulation of Macrophage Cells Against Cutaneous Leishmaniasis Using Silver Nanoparticles

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Abstract:

Cutaneous leishmaniasis is a disease caused by *Leishmania tropica* parasite. Current treatments for this parasite are undesirable because of their toxicity, resistance, and high cost. Macrophages are key players against pathogens. Nitric oxide (NO), a molecule produce by immune cells, controls intracellular killing of pathogens during infection. Silver nanoparticles (Ag NPs) demonstrated broad-spectrum activity against various types of infectious diseases. It has the ability to stimulate oxygen species production. This study aims to analyze the macrophages activation through NO production and estimate the cytotoxicity based on the lactate dehydrogenase (LDH) release upon exposure to *L. tropica* and Ag NPs. Serially concentrations of Ag NPs were used under two conditions during and following macrophages exposure to *L. tropica.* MTT assay was used to determine the cytotoxicity of Ag NPs on *L. tropica* amastigotes during infection of macrophages *in vitro*. The results showed that by increasing the Ag NPs concentrations, the viability percentage of *L. tropica* amastigotes decreased and reached to 21.7 ± 0.64 % during infection compared with the control. The 50% inhibitory concentration of Ag NPs on amastigotes was 2.048µg/ml during infection. Moreover, post-phagocytosis study involved the assessment of NO and LDH release by macrophages upon exposure to *L. tropica.* It have shown that untreated macrophages released low levels of NO while in the presence of Ag NPs, macrophages were activated to produce higher levels of NO under all experimental conditions. On the other hand, macrophages were capable of controlling cytotoxicity and decreasing LDH levels during phagocytosis of *L. tropica* amastiogotes. Taking together, these findings suggest that Ag NPs can enhance macrophages NO production which provides a method for the identification of Ag NPs ligands with microbicidal and anti-cytotoxic properties against *L. tropica* pathogens.

Key words: Lactate dehydrogenase, *Leishmania tropica*, Macrophages, Nitric oxide, Silver nanoparticles.

Introduction:

Leishmaniasis is an infection caused by the parasitic protozoan *Leishmania* and it spreads by sand fly insect vector (1).The lifecycle of *Leishmania* spp. involves a series of differentiation processes; the procyclic promastigote procedures, infective metacyclic promastigotes in the invertebrate sand fly host, which then differentiate into amastigotes in the vertebrate host (2). The most spread type of leishmaniasis is cutaneous leishmaniasis (CL), and its infections frequently give rise to serious skin lesions and scars (3). CL is a global healthcare trouble with medical influence(4).CL still represents harmful consequences in Iraq (5).

Leishmania has recognized strategies for efficient uptake into macrophages and it is capable to regulate phagosome progress for parasite development and to escape destruction.

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Subsequently, macrophage resistances is enhanced either by oxidative damage through nitric oxide (NO) and reactive oxygen species (ROS), antigen presentation, immune activation and apoptosis(6). Nevertheless, *Leishmania* decreases the NO production(7).

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme. It has a role in the anaerobic glycolytic pathway through transferring H^+. The oxidation of Lactate to pyruvate is catalyzed by LDH in the existence of nicotinamide adenine dinucleotide as hydrogen acceptor (8). In parasitic diseases, raised serum LDH levels have been described in patients with Sarcocystis (9), toxoplasmosis (10) and leishmaniasis (11).

Recent antileishmanial drug showed resistance by the parasites and the patients had problems with drug poisonousness (12).While pentavalent antimonial drugs are the most frequently prescribed treatments for leishmaniasis, they yield severe side effects, including

hepatotoxicity and cardiotoxicity. Extra compounds, such as amphotericin B, miltefosine and pentamidine are the second choice drugs but they furthermore produce side effects that can threaten the patient's life. Nanotechnology has performed as an attractive alternative due to its enhanced bioavailability and lower toxicity, and extraphysical appearance which is important to reduce the burden of the diseases (13, 14). Silver nanoparticles (Ag NPs)have attained a special focus (15), as an example, the production of ROS by silver nanoparticles is an antibacterial effect (16).Therefore, this study aimed to study the stimulus effect of Ag NPs on macrophages ability to inhibit *L. tropica* infection via evaluating the level of NO production and LDH release *in vitro*.

Material and Methods

Parasite Culture

Leishmania tropica was obtained from patients attended to Al Karama Hospital, Baghdad, Iraq. The parasite was maintained at Biology Department, College of Science, University of Baghdad. The promastigotes were cultivated in 199 medium containing 50 µg/ml penicillin and 10% HI-FBS and incubate at 26 °C (17).

Culture of Macrophage Cells

To evaluate the antileishmanial activity of Ag NPs against the intracellular amastigotes, macrophage model, was used. The macrophage cells were isolated from the peritoneum of BALB/c mice by injection the Thioglycollate. The isolated macrophages were counted and the appropriate size completed using DMEM which supplemented with10% fetal bovine serum. Macrophage cells were then seeded into flat- bottom 96 well microtiter plates for 24 hrs. at 37^{o}C in a humidified 5% CO_2, 95% air incubator. Flouting cells were removed by washing with the medium (18).

Raising Infection of Metacyclic Stage Parasites *in vitro*

Leishmania culture were centrifuged, washed two times with PBS (pH7.2) and re-suspended in complete RPMI medium. FBS (5%) was added to the parasite solution and incubated for 30 min at 37˚C in order to gain the infective metacyclic-stage parasites. After 6 days, the metacyclic stage parasites were isolated from the cultures (19).

Preparation of Ag NPs Concentrations

Ag NPs were imported from NANO pars SPADANA Technology. The concentration of Ag NPs was 4000mg/L. The stock solution of Ag NPs was serially diluted in serum free DMEM media yielding concentrations (0.25, 0.5, 1, 2, 4, and 8µg/ml). For establishing a homogeneous suspension, the solution was sonicatedat 100W and 40 kHz for 40 min and small magnetic bars was used for stirring during dilution to avoid aggregation of the particles (20).

Determination Viability Percentage of *L. tropica* and Macrophages after Treatment with Ag NPs

The antileishmanial effectiveness of Ag NPs was tested during macrophages exposure to *L. tropica* promastigotes, 100 µl of promastigotes (2×10^4 cells/well) were treated with 50 µl serially diluted Ag NPs (0.25, 0.5, 1, 2, 4, and 8 µg/ml) and incubated for 4 hrs. at room temperature. Macrophage cells and the parasites were counted using the hemacytometer. Macrophages culture were infected with Ag NPs-treated promastigotes at a ratio of 1:10 (macrophage/parasites) and incubated at 37°C for 48 hrs. Macrophages exposed to *L. tropica* promastigotes only was used as control. Viability percentage of the parasites and macrophages during infection were measured using the calorimetric MTT assay (21). A solution of MTT (Thiozolyl blue tetrazolium bromide) (Bio-World, USA) was added and incubated for 4 hrs. at 37°C, the MTT solution was removed. The residual crystals in the wells were solubilized by the addition of 130 µl of DMSO medium then incubated at 37°C for 15 min with shaking. The absorbency was measured on a microplate reader at 584 nm.

Macrophages Activation and CytotoxicityAssay

Macrophages activation and cytotoxicity were measured by NO production and LDH release. The antileishmanial effectiveness of Ag NPs was tested under two conditions, during and following macrophages exposure to *L. tropica* promastigotes. For the (during) treatment, 100 µl of promastigotes (2×10^4 cells/well) were treated with 50 µl serially diluted Ag NPs (0.5, 1, 2, 4, and 8 µg/ml) and incubated for 4 hrs. at room temperature. Macrophages culture was infected with Ag NPs-treated promastigotes at a ratio of 1:10 (macrophage/parasites) and incubated at 37°C for 24 and 48 hrs. For the (following) treatment, culture was infected with 100 µl of promastigotes (2×10^4 cells/well) at a ratio of 1:10 (macrophage/parasites) and incubated at 37°C for 4 hrs. After their incubation, 50 µl serially diluted Ag NPs (0.5, 1, 2, 4, and 8 µg/ml) were introduced to each culture and incubated for 24 and 48 hrs. Cell culture supernatants (50µl) were assayed for NO using Griess assay (Promega, USA) and compared with $NaNO_2$ as a standard (22). Briefly, 50µl of cell culture supernatant was mixed with 50µl of Griess reagent (0.1% (w/v) N-(1 naphthyl) ethylenediaminedihydrochloride and 1% (w/v)

sulfanilamide in 5% (v/v) phosphoric acid). The samples were left at room temperature for 15 min and the absorbance was measured at 490 nm using plate reader. Cell cytotoxicity was analyzed using a CytoTox96® kit (Promega, Inc., Madison, WI, USA). This kit allows measuring LDH in the culture supernatant (23). LDH is released when cells are lysed upon toxic effects of studied agents. In the kit, tetrazolium salt is converting to a red formazan product. The amount of color in the supernatant is related to the LDH release. LDH activity was measured at 490 nm.

The Statistical Study

To find IC_{50} using excel application, X-axis represent logarithm of concentration and Y- axis represent MTT density relative (Y_0), by interpolation method between two concentrations (X_1 more than Y_0, X_2 less than Y_0) get IC_{50}. The Statistical Analysis System- SAS (2012) program was used to study the difference factors in study parameters. Least significant difference–LSD test was utilized for the significant compare between means in this study.

Results:

The Impact of Ag NPs on Infected Macrophages

The viability of infected macrophages treated with Ag NPs increased with significant ($P<0.05$) differences. The lowest used concentration of Ag NPs (0.25 µg/ml) recorded (92.05 ± 3.79) of viable cells, while the highest concentration (8 µg/ml) recorded (103.02 ± 5.07) of viable cellscompare with the control group (macrophages infected with *L. tropica* and not treated with Ag NPs) which was (67.461 ± 2.83)(Table 1).

Table 1. Macrophages viability percentage after treatment and infection with *L. tropica*.MØ: Macrophages.

Ag NPs (µg/ml)	MØ viability %
0.25	92.05 ± 3.79
0.5	94.17 ± 3.62
1	96.63 ± 3.55
2	98.98 ± 4.92
4	99.85 ± 4.31
8	103.02 ± 5.07
Control	67.461 ± 2.83
LSD value	8.563 *
* (P<0.05)	

The Impact of Ag NPs Against *L. tropica* Amastigotes During Infection of Macrophages

The viability percentage of *L. tropica* amastigotes treated with Ag NPs during infection of macrophages decreased with significant ($P<0.01$) differences. After 48 hrs., the low concentration of Ag NPs had the highest percentages of viable cells (57.83 ± 2.37), while the highest concentration recorded lower percentage of viability (21.7 ± 0.64) of *L. tropica* amastigotes compare with the control group (macrophages infected with *L. tropica* and not treated with Ag NPs)(Table 2).The IC_{50} value of Ag NPs on *L. tropica* amastigotes during infection of macrophages was 2.048 µg/ml.

Table 2. *Leishmania tropica* viability percentages after 48 hrs. of treatment with An NPs.

Ag NPs (µg/ml)	*L. tropica*viability %
0.25	57.83 ± 2.37
0.5	32.7 ± 1.72
1	29.03 ± 1.44
2	29.06 ± 1.36
4	24.79 1.03
8	21.7 ± 0.64
Control	89.371 ± 3.75
LSD value	7.577 **
** (P<0.01)	

Nitric Oxide (NO) Production

The differences of NO production from macrophage cells treated with Ag NPs (during) infection with *L. tropica* for 24 and 48 hrs. were tested. The first control included only macrophages and the second control was included macrophages infected with *L .tropica* and not exposed to Ag NPs. During infection, the results showed no-significant differences of NO production after 24 and 48 hrs. of treatment with the low concentrations of Ag NPs, while the high concentrations (8µg/ml) of Ag NPs displayed significant differences of NO production after 24 and 48 hrs. of treatment, the readings were (0.170 ± 0.01) and (0.250 ± 0.03) respectively (Table 3).

Table 3. Nitric Oxide production after 24 and 48 hrs. of treatment (during) infection [(*L. tropica*+AgNPs) 4h+ MØ]. MØ: Macrophages.

Ag NPs (µg/ml)	24 hrs.	48 hrs.	LSD value
0.5	0.106 ± 0.01	0.159 ± 0.02	0.059 NS
1	0.110 ± 0.01	0.177 ± 0.01	0.076 NS
2	0.129 ± 0.02	0.181 ± 0.02	0.072 NS
4	0.129 ± 0.01	0.210 ± 0.02	0.077 *
8	0.170 ± 0.01	0.250 ± 0.03	0.069 *
MØ	0.147 ± 0.01	0.233 ± 0.02	0.083 *
MØ+ *L. tropica*	0.124 ± 0.02	0.203 ± 0.02	0.071 *
LSD value	0.077 NS	0.083 *	---
*(P<0.05), NS: Non-significant			

In addition, the results revealed differences of NO production from macrophages treated by Ag NPs (following) infection with *L. tropica* for 24 and 48 hrs. After treatment with the concentrations of 0.5 and 1µg/ml of Ag NPs, the results showed no-

significant differences of NO production, while the concentrations of 2, 4 and 8µg/ml of Ag NPs showed significant differences of NO production after 24 and 48 hrs. (Table 4).

Table 4. Nitric Oxide productions after 24 and 48 hrs. of treatment (following) infection [(*L. tropica*+ MØ) 4h+ AgNPs]. MØ: Macrophages.

Ag NPs (µg/ml)	24 hrs.	48 hrs.	LSD value
0.5	0.136 ± 0.02	0.203 ± 0.01	0.072 NS
1	0.137 ± 0.03	0.203 ± 0.03	0.069 NS
2	0.140 ± 0.02	0.220 ± 0.01	0.071 *
4	0.152 ± 0.02	0.239 ± 0.04	0.068 *
8	0.204 ± 0.03	0.304 ± 0.03	0.082 *
MØ	0.147 ± 0.01	0.233 ± 0.02	0.062 *
MØ+ *L. tropica*	0.124 ± 0.01	0.203 ± 0.03	0.069 *
LSD value	0.082 NS	0.105 NS	---

* (P<0.05), NS: Non-significant.

Lactate Dehydrogenase (LDH) Release

The differences of LDH release from macrophages treated with Ag NPs during infectionwith *L. tropica* for 24 and 48 hr were tested.After treatment with the Ag NPs during infection, the results showed significant (P<0.05) differences. The lowest concentration of Ag NPs recorded (0.265 ± 0.02) and (0.514 ± 0.03) after 24 and 48 hrs.However, the highest concentration of Ag NPs recorded (0.042 ± 0.00) and (0.343 ± 0.02) after 24 and 48hr (Table 5).

Table 5. LDH release after 24 and 48 hrs. of treatment during infection [(*L. tropica*+ Ag NPs) 4h+ MØ]. MØ: Macrophages.

AgNPs (µg/ml)	24 hrs.	48 hrs.	LSD value
0.5	0.265 ± 0.02	0.514 ± 0.03	0.114 *
1	0.137 ± 0.01	0.490 ± 0.03	0.194 *
2	0.063 ± 0.01	0.406 ± 0.02	0.225 *
4	0.062 ± 0.01	0.389 ± 0.02	0.192 *
8	0.042 ± 0.00	0.343 ± 0.02	0.216 *
MØ	0.129 ± 0.01	0.372 ± 0.02	0.109 *
MØ+ *L. tropica*	0.292 ± 0.02	0.547 ± 0.04	0.126 *
LSD value	0.114 *	0.109 *	---

* (P<0.05), NS: Non-significant

Also, the differences of LDH release from macrophages treated by Ag NPs following infectionfor 24 and 48 hrs. were significant (P<0.05). Only the concentration (1µg/ml) of Ag NPs showed no-significant differences between 24 and 48 hrs. The lowest concentration of Ag NPs recorded (0.27 ± 0.02) and (0.41 ± 0.02) of LDH release after 24 and 48 hrs. of treatment. However, the highest concentration of Ag NPs recorded (0.05 ± 0.00) and (0.23 ± 0.01) after 24 and 48 hrs. of treatment (Table 6).

Table 6. LDH release after 24 and 48 hrs. following infection [(*L. tropica*+ MØ) 4h+ Ag NPs]. MØ: Macrophages.

Ag NPs (µg/ml)	24 hrs.	48 hrs.	LSD value
0.5	0.27 ± 0.02	0.41 ± 0.02	0.136 *
1	0.19 ± 0.02	0.32 ± 0.02	0.144 NS
2	0.11 ± 0.01	0.29 ± 0.02	0.136 *
4	0.08 ± 0.01	0.27 ± 0.02	0.132 *
8	0.05 ± 0.00	0.23 ± 0.01	0.141 *
MØ	0.13 ± 0.01	0.37 ± 0.02	0.133 *
MØ+ *L. tropica*	0.29 ± 0.02	0.55 ± 0.04	0.145 *
LSD value	0.163 *	0.142 *	---

*(P<0.05), NS: Non-significant

Discussion

Recent antileishmanial chemotherapy applications have clear side effect (24).Ag NPs have been used as an attractive alternative treatment due to its enhanced bioavailability and lower toxicity (15). This study aimed to test investigatethe stimulation effect of Ag NPs on macrophages to inhibit *L. tropica* infection *in vitro*. When the macrophages treated with Ag NPs during infection, the MTT results have shown the antileishmanial effects of Ag NPsdue to the decreasing of the *L. tropica* amastigotes viability. On the other hand, the viability of macrophages increased when treated by Ag NPs during infection compare with control. The Ag NPs have been revealed to be cytotoxic at a concentration more than 6 µg/ml (25). Although in this study, the concentration 8 µg/ml of Ag NPs was non-toxic. It was found that low concentrations such as 1, 5, and 10 µg/ml of Ag NPs had no toxic effects on macrophages in the dark or under UV light (16). Ag NPshave presented certain capability in generating high amounts of reactive oxygen species (ROS) in macrophages, the host cells for *Leishmania* parasites. ROS can cause oxidative stress and DNA damage and ultimately apoptosis of the parasite(26).

The results of (27) showed the effectiveness of Ag NPs through decreased viability of *L. tropica* axenic amastigotes to 10.58% in the concentration 2.1 µg/ml. These findings suggest that Ag NPs can be used to support macrophages to eliminate parasite. Macrophages can produce sufficient amounts of ROS to kill microbial agents (28). However, *Leishmania* prevent this production through the inhibition of the enzymatic pathway that responsible for producing ROS, and *Leishmania* can live inside macrophages without exposed to any damage (29). Therefore, it may be

suggested that to prevent *Leishmania* parasites with an ROS-based treatment, these oxygen products must be produced in a physical way such as using Ag NPs, as an alternative of the enzymatic way that can be obstructed by the parasites.

This study reveals that Ag NPs stimulates macrophages to increase the production of NO (during) and (following) infection with *Leishmania***.** Stimulation of macrophages and the following NO production reflect the main antileishmanial element for macrophages. It has an important role in infection control, chiefly during the first stages and before the parasite protection mechanisms are started (30).Moreover, the results displayed the effectiveness of Ag NPs due to the decrease LDH release in macrophages infected with *L. tropica.* This indicates the ability of Ag NPs to inhibit *L. tropica* inside the macrophage and increase macrophages viability. Taher *et al.*(2016)(31) showed that there was an increase in LDH enzyme activity in visceral Leishmaniasis patients in comparison with healthy controls. Determining LDH enzyme is advantageous for cytotoxicity assays, especially if cells are exposed to the test compound and studied agents for a long time (32). Taking together, this study demonstrate that Ag NPs stimulate NO production and decrease LDH level by macrophages upon exposure of *L. tropica*. The more effective concentration ofAg NPs was (8µg/ml) to enhance NO production under (during) and (following) to *L. tropica* exposure.

In brief, cutaneous leishmaniasis still represents a healthcare trouble with medical and social impact. Recent drugs that are used to treat leishmaniasis infection are accompanying with several side effects (24). Ag NPs demonstrated broad-spectrum activity against diverse types of causative agents of infectious diseases which creates the possibility to apply Ag NPs not only for treatment purposes but also for control the infections (33).

Conclusion:

The results of this study proved that Ag NPs is a candidate targets against *L. tropica* infection through its effect to stimulate NO production and decrease the cytotoxicity that caused by *L. tropica* amastiogotes on macrophage cells.

Conflicts of Interest: None.

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تحفيز الخلايا البلعمية ضد الاصابة باللشمانيا الجلدية باستخدام جزيئات الفضة المتناهية الصغر

رؤى فارس حسين السعيدي **انتصار جبار صاحب**

قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة:

داء الليشمانيات الجلدي هو مرض يتسبب فيه طفيلي الليشمانيا المداري*L. tropica*. الخلايا البلعمية لها دور اساسي ضد مسببات الأمراض. أكسيد النيتريك (NO)،هو جزيء ينتج بواسطة الخلايا المناعية ، يتحكم في قتل مسببات الأمراض أثناء العدوى. تمتلك جسيمات الفضة المتناهية الصغر (Ag NPs)القدرة على تحفيز إنتاج أنواع الأكسجين. تهدف هذه الدراسة إلى تحليل تنشيط البلاعم من خلال إنتاجأكسيد النيتريك وتقدير السمية الخلوية المستندة إلى إطلاق نازعة الهيدروجين (LDH) عند التعرض لطفيلي الليشمانيا المداريو جسيمات الفضة المتناهية الصغر. في هذه الدراسة تم استخدام تراكيز مخففة منجسيمات الفضة المتناهية الصغرخلال وبعد تعرض البلاعم لطفيلي الليشمانيا المداري. تم استخدام اختبار MTT لتحديد السمية الخلوية لـجسيمات الفضة المتناهية الصغرعلىالطور عديم السوط لطفيلي الليشمانيا المداريأثناء إصابة البلاعم في المختبر. أظهرت النتائج أنه من خلال زيادة تركيزاتجسيمات الفضة المتناهية الصغر انخفضت نسبة حيوية الطور عديم السوط لطفيلي الليشمانيا المداري إلى 21.7 ± 0.64٪ خلال العدوى مقارنة مع السيطرة. كان التركيز التثبيطي 50 ٪ لـجسيمات الفضة المتناهية الصغرعلى الطور عديم السوط لطفيلي الليشمانيا المداري 2.048ميكروغرام / مل خلال العدوى. علاوة على ذلك، اشتملت دراسة ما بعد البلعمة على تقييم إفرازأكسيد النيتريكونازعة الهيدروجينبواسطة البلاعم عند التعرض لطفيلي الليشمانيا المداري وقد أظهرت النتائج أن البلاعم التي لم يتم علاجها قد انتجت مستويات منخفضة من أكسيد النيتروجين أثناء وجودجسيمات الفضة المتناهية الصغر، وقد تم تنشيط البلعمات لإنتاج مستويات أعلى منأكسيد النيتروجين تحت جميع الظروف التجريبية. من ناحية أخرى، كانت البلاعم قادرة على التحكم في السمية الخلوية وتقليل مستوياتنازعة الهيدروجينأثناء عملية البلعمة للطور عديم السوطلطفيلي الليشمانيا المداري. تشير هذه النتائج إلى أنجسيمات الفضة المتناهية الصغريمكن أن تنشط الخلايا البلعمية لانتاج أكسيد النيتروجين. ان هذه النتائج يوفر المعلومات حول الالية التي من خلالها تستطيع جسيمات الفضة المتناهية الصغرالسيطرة على الاصابة بالميكروبات وتحفيز الخلايا المضادة للسمية ضد مسببات الليشمانيا المداري.

الكلمات المفتاحية: نازعة الهيدروجين، الليشمانيا المدارية، البلاعم، أكسيد النيتريك، جسيمات الفضة المتناهية الصغر.