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Antiparasitic Effect of Carbonnanotubes on *Leishmania donovani in vitro*

Khawla H. Zghair*, Entsar J. Saheb , Ban Noori AL-Qadhi
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Although several drugs are used against *Leishmania* infection but they are associated with several adverse complications. Therefore, a new effective treatment needed to be found. In this study, the effect of carbonnanotubes nanoparticles (CNTs NPs) on *Leishmania donovani* promastigotes was assessed. Viability of promastigotes after adding different concentrations of carbonnanotubes (CNTs) nanoparticles (0.05, 0.1, 5, 10, 20, 40, 60 and 80 µg/ml) to the parasite culture was evaluated by growth rate, viability rate assay and morphological changes. The results indicated that the effect of CNTs NPs on growth rate of promastigotes form. After exposed to 80 µg/ml of CNTs, the growth rate of promastigotes clearly decreased compared with promastigotes treated with the same concentrations of pentostam drug (the standard antileishmanial drug) and the control group. The inhibitory concentration (IC50) of CNTs NPs on promastigotes growth rate was 59.30µg/mL after 72 hours. In addition, CNTs NPs exert cytotoxic effects on *L. donovani* promastigotes through the induction of their death when exposed to 80µg/ml of CNTs NPs and pentostam. The inhibitory concentration (IC50) of CNTs NPs on *L. donovani* promastigotes was 53.79µg/mL after 72 hours. The antiparasitic effect increased with the increasing of the CNTs NPs concentration, while the viability curve of the parasite dropped. In addition, a visual inspection by light microscopy has shown that CNTs induces morphological changes in *L. donovani* promastigotes in comparison to the morphology of the untreated promastigotes. Our data determine the superiority of CNTs as a novel leishmanicidal effect against *L. donovani* infection over pentostam *in vitro*.

Keywords: *Leishmania donovani*, carbonnanotubes, antiparasitic effect, nanoparticles.

تأثير انابيب الكربون النانومترية على الليشمانيا الحشوية في المختبر

خولة حوري زغير* ، انتصار جبار صاحب ، بان نوري القاضي
جامعة بغداد، كلية العلوم، قسم علوم الحياة، بغداد، العراق

الخلاصة

على الرغم من استخدام العديد من الأدوية ضد عدوى طفيلي الليشمانيا لكنها تتسبب بالعديد من المشاكل والتعقيدات ولذلك، فإن من الضروري البحث عن علاج جديد فعال. في هذه الدراسة تم تقييم تأثير الأنابيب المتناهية الصغر للكاربون على الطور المسوط لليشمانيا الاحشائية. تم تقييم فعالية هذه الأنابيب النانومترية الكربونية بتراكيز مختلفة منها (0.05، 0.1، 5، 10، 20، 40، 60 و 80 ميكروغرام / مل) عن طريق اختبارمعدل النمو وفحص الحيوية والتغيرات المظهريه. أشارت النتائج إلى تأثير الأنابيب النانومترية الكربونية على معدل نمو الطفيلي بعد التعرض لتركيز 80 ميكروغرام/مل من الأنابيب النانومترية الكربونية ،انخفظت

*Email: entsarpar73@yahoo.com

اعداد الطورالسوطي بوضوح مقارنة مع معدل نمو السوطيات المعاملة مع نفس التراكيز من عقار البنتوستام ومجموعة السيطره. تم احتساب IC50 للأنابيب النانوية الكربونية لمعدلات نمو الطور السوطي لليشمانيا الاحشائيه وقد سجلت 59.30ميكروغرام/مل بعد 72 ساعة. وبالإضافة إلى ذلك، كان للانابيب النانومترية الكربونية تاثير سمي على الطورالسوطي للشمانيا الحشوية من خلال تاثيرها على حيوية الطفيلي عندما معاملتها بتركيز 80 ميكروغرام / مل. تم احتساب IC50 للأنابيب النانوية الكربونية لحيوية الطور السوطي لليشمانيا الاحشائيه وقد سجلت 53.79 ميكروغرام/مل بعد 72 ساعة. وبالإضافة إلى ذلك، فقد أظهر الفحص البصري بواسطة المجهر الضوئي أن الأنابيب النانوية الكربونية يؤدي الى تغييرات شكلية في سوطي اللشمانيا الحشوية مقارنة باشكال السوطيات غير المعالجة. تبين نتائج هذه الدراسة افضلية الأنابيب النانوية الكربونية على عقار البنتوستام في التأثير السمي على الليشمانيا الاحشائية في الزجاج.

Introduction

Leishmaniasis is a vector- borne infection caused by obligate intra-cellular parasite, *Leishmania donovani* [1, 2]. It is transmitted to humans by different species of phlebotomines flies [3]. There are mainly two clinical diseases caused by various species of *Leishmania*: Cutaneous leishmaniasis and Visceral leishmaniasis (VL) [4]. The life cycle of *Leishmania* include two forms, amastigote which is round to oval bodies and found only in the macrophages of infected vertebrate hosts and the other form is the promastigote which is seen in the gut of the sand fly, it is motile with a single anterior flagellum [5, 6].

Treatment of VL relies on specific antileishmanial drugs. Over than 60 years, the first chemotherapy against cutaneous or visceral leishmaniasis has been the pentavalent antimonials [7]. The treatment against leishmaniasis has been caused several side effects, such as: nausea, abdominal pain, myalgia, pancreatic inflammation and hepatitis, thus leading to the reduction or cessation of treatment [8]. Also, the long course treatment allows antileishmanial levels of the drug to accumulate in tissues, particularly in liver and spleen. Sodium stibogluconate (Pentostam) was first used following by the glucantime, meglumine antimoniate [9]. Pentostam have been produces to decrease the high cost of the treatment. However, it causes fatal cardio toxicity [10]. Pentoxifylline is a drug used to treat peripheral vascular disease [11]. Therapy with pentoxifylline led the lesions to heal faster than therapy with the antimonial alone, however the side effects reported were nausea, arthralgia, dizziness, abdominal pain and diarrhea [12]. It was recommended to replace the antimonials by amphotericin B [13]. Miltefosine was approved *in vitro* activity on *Leishmania* amastigotes in 1987 [14]. It also causes several side effects including gastrointestinal disturbances and renal toxicity [15].
Nanoparticles can be defined as substances ranging in size from 1- 100 nm. They have exclusive physicochemical properties such as tiny size, great surface area; electrical charge and character [16] which is vary significantly from the bulk material [17]. The nanoparticles are used in drug delivery and cancer treatment [18]. The ability of CNTs to cross cell membranes and to transport peptides, proteins and nucleic acids into cells makes them useful as vehicles for drug delivery against intracellular targets. Studies have demonstrated that f-CNTs are taken up by lymphocytes and macrophages without affecting cell viability [19]. In addition to size and surface characteristics, the shape of nanoparticles is the key factor influencing circulation time, bio-distribution, cellular uptake, as well as targeting in cancer drug deliver [20]. These nano-scale particles provide more effectiveness, lower toxicity, extend the product life cycle and eventually reduce health care costs. Nanotechnology can be a useful tool for synthesize new drugs against infectious diseases [21]. Nanoparticles antileishmanial activity of drugs are preferable as compared to any other parasitic disease mainly due to the fact that *Leishmania* parasite resides within the macrophages which are responsible for clearance of liposomes *in vivo* [22].

Materials and Methods

Parasite culture

L. donovani parasites (DUAA/IQ/2005/MRU15) kindly provided from 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.

Preparation the CNTs NPs

Nanoparticle CNTs powder was purchased from Selekchem Company, USA. The average size of the particle was 20 nanometers (nm). The stock of CNTs NPs was dispersed by sonication at 100W and 40 kHz for 40 min in ultrapure water to obtain homogeneous suspensions. The NPs were then diluted in sterile ultrapure water and furthermore sonicated for 40 min. To avoid aggregation of particles, small magnetic bars were used in the suspensions for stirring during dilution [23].

Anti-parasitic assay of *L. donovani* promastigote *in vitro*

CNTs powder dissolved in dimethyl sulphoxide (DMSO) at 2.5 mg/mL and was used as stock solutions. Promastigotes of *L. donovani* was cultured sterile screw tube vials containing 5 ml of M199 media. The promastigotes stage was added ($1x10^5$cell /ml) as triplicate in different concentrations of the drug (0.05, 0.1, 5, 10, 20, 40, 60 and 80 µg/ml) of CNTs and were incubated at 26 °C.

Pentostam drug, an injectable ampoules (100 mg/ml) manufactured by (Glaxo Operations UK Limited Castle, Member of the Glaxo Smith Kline Group companies) were used in this study. They were kindly provided from Al-Karama Teaching Hospital in Baghdad. A stock solution of pentostam was used to prepare, the following concentrations (20, 40, 60 and 80 µg/ml) immediately before used. To evaluate the parasite survival, the multiplication of the promastigotes was determined by counting the cells by hemocytometer chamber after adding the nanoparticles after 24, 48 and 72 hr of incubation. In control group, promastigotes were cultured as triplicate without CNTs NPs. GraphPad prism was used to determine the IC_{50}.

MTT assay

Leishmania donovani promatigotes (2 x 10^5 parasites/well) and test compounds (CNTs NPs and pentostam drugs) were prepared and dispensed in a flat-bottom 96-well microtiter plate in the presence of 30, 60, 90 and 120 µg/ml of CNTs NPs and were incubated at 25˚C. These cultures were repeated in triplicate. The microtiter plate was incubated at 25˚C for three days. After 24, 48 and 72h incubation periods of the wells, 10 µl of MTT solution was added per well to achieve final concentration of 0.45 mg/ml. The microtiter plate was incubated for 4h at 25˚C. The media was removed and 100µl of DMSO solution was added in order to solubilize the formazan crystals. The microtiter plate was stirred gently then left for 15 minutes. The optical absorbance of these plates was measured by the ELISA reader in at 570 nm. Viability percentage was calculated by: [(AT-AB) / (AC-AB)] ×100. Where, AB is OD of blank well, AC is OD of negative control and AT is OD of treated cells [23].

The microscope observations

The *Leishmania* promastigotes were treated with different concentrations of CNTs (0.05, 0.1, 5, 10, 20, 40, 60 and 80 µg/ml) and were incubated at 26 °C. After 72hr incubation periods the microscope observations were made as following: the liquid of the parasite culture was transferred to a clean glass slide and allowed to dry on a slide and then stained with Giemsa stain and examined microscopically.

Statistical Analysis

The Statistical Analysis System program was used to effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means and Chi-square test was used to significant compare between percentages in this study.

Results

The effect of CNTs on promastigotes stage growth

The results showed the effect of low concentrations (0.05, 0.1, 5, 10µg/ml) (figure 1) and high concentrations (20, 40, 60, and 80 µg/ml) of CTNs NPs on promastigotes growth rate after 24, 48 and 72 h Figure- 2. The parasites treated with high concentrations of CNTs NPs (20, 40, 60, and 80 µg/ml) after 72h incubation showed significant ($P<0.05$) was cytotoxic in the growth rate of the promastigotes compared with the promastigotes treated with penostam and the untreated promastigotes Table- 1. The number of the parasites were (22, 21, 25 and 12 $\times10^5$ cell/ml) respectively after 24 hr in comparison with the number of promastigotes treated with the same concentrations of pentostam which was (49, 40, 41, 46×10^5 cell/ml) respectively and untreated parasites which was 34×10^5 cell/ml, as shown in Figures -2A and 3A . After 48 and 72h, the number of promastigotes treated with high concentrations of CTNs decreased clearly reaching to (32, 29, 30, 31×10^5 cell/ml) and (33, 30,16,and 9×10^5 cell /ml) respectively, compared with promatigotes treated

with the same concentrations of pentostam which count (78,78,70, and 31×10^5 cell/ml) and (69,70, 43, and 21×10^5 cell/ml), respectively, and with untreated parasites which recorded 49×10^5 cell/ml and 82×10^5 cell/ml respectively. The IC50 of CTNs NPs and pentostame was measured 59.3µg/ml and 60.14µg/ml after 72h respectively Figure -2B and 3B.

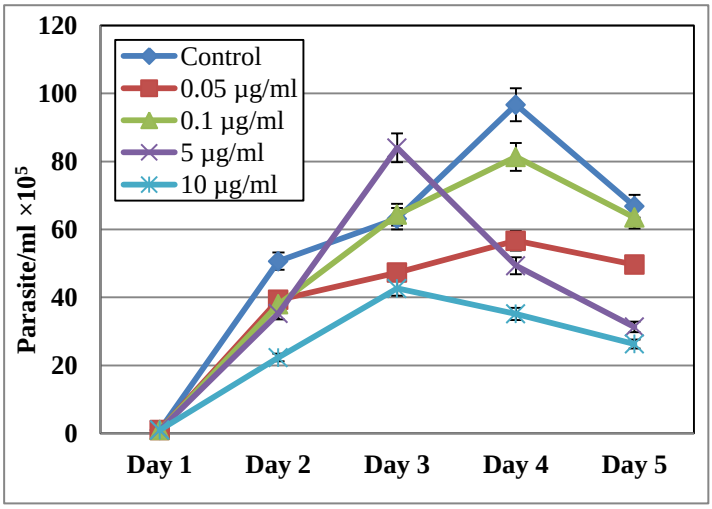

Figure 1- Mean number (Cell/ml) of viable parasites after treating with low concentrations of CNTs NPs (0.01-10µg/ml)

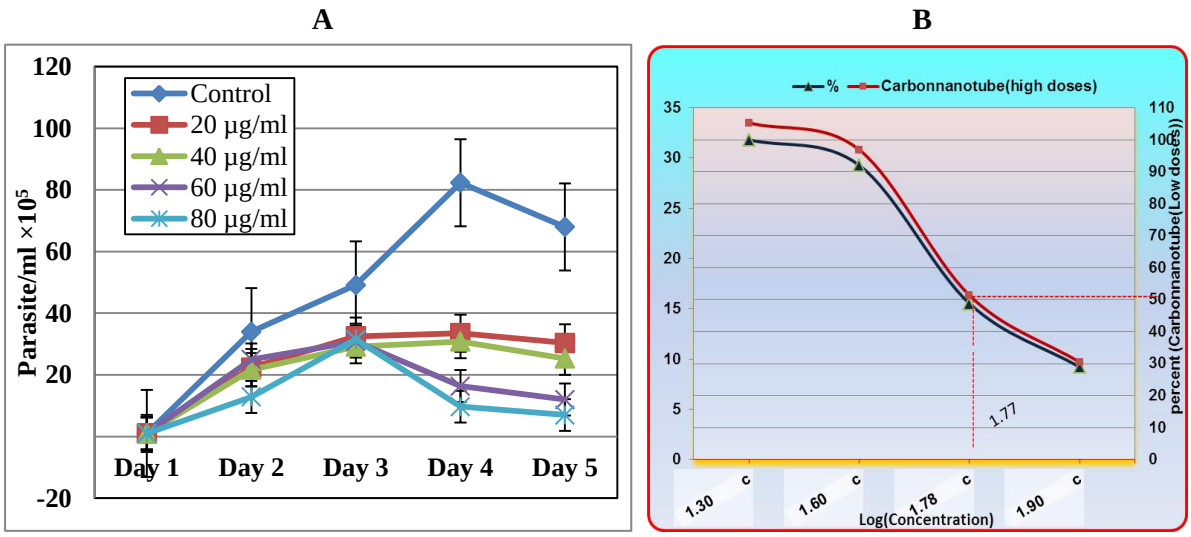

Figure 2- A) Mean number (Cell/ml) of viable parasites treated with high concentrations of CNTs NPs (20-80µg/ml). **B)** The IC50) of CNTs NPs on *L. donovani* promastigotes was 59.30µg/mL after 5 days.

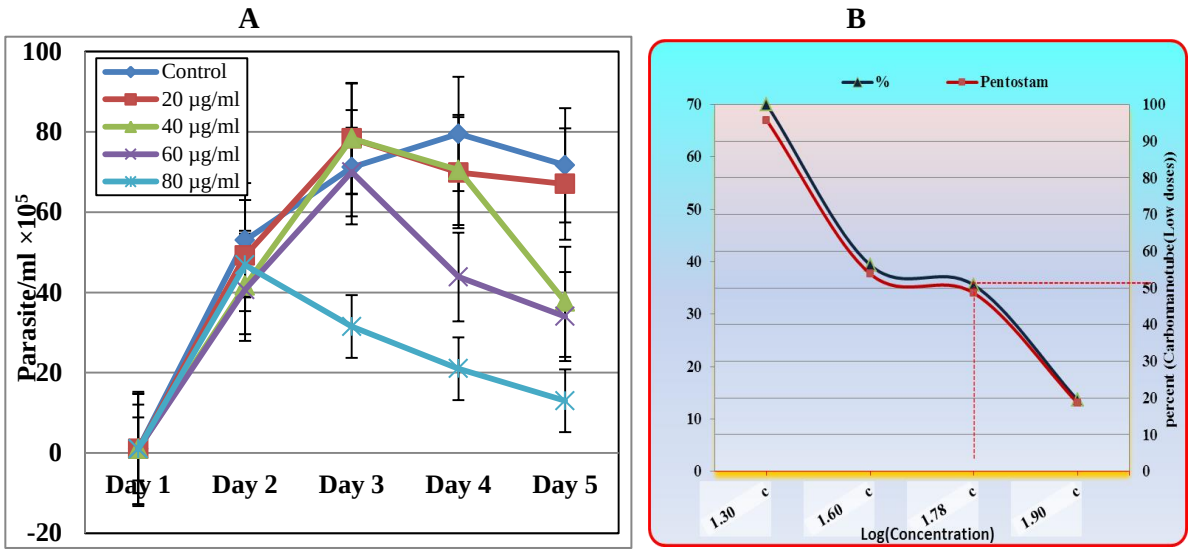

Figure 3- A) Mean number (Cell/ml) of viable parasites after adding different concentrations of petostame drug (20-80µg/ml). **B)** The IC50 of petostame on *L. donovani* promastigotes was 60.14µg/mL after 5 days.

Table 1- Compare between CNTs NPs and pentostam in growth rate ($x10^5$)

Conc. (µg/ml)	Carbonnanotube	Pentostam	LSD value
Control	68.00	71.67	5.49 NS
20	30.33	67.00	7.91 *
40	25.33	37.67	6.44 *
60	12.00	34.00	6.92 *
80	7.00	13.00	5.024 *
* (P<0.05).			

Cell cytotoxicity by colorimetric assay (MTT) *In vitro*

In the present study, *L. donovani* promastigotes treated with different concentrations (20, 40, 60 and 80 µg/ml) of CNTs NPs and pentostam, their viability were determined by colorimetric assay (MTT) after 24, 48 and 72h. The MTT assay is based on the capacity of the mitochondrial enzyme, succinate-dehydrogenase of viable cells to convert the MTT tetrazolium salt into a blue colored product. Table-2 revealed that promastigotes viability after 24 h of drugs exposure showed significant ($p< 0.05$) differences between them, except the concentration (20 µg/ml), which have the values of (80.87%) and (82.67%) of viable cells for CNTs NPs and pentostam respectively, while other higher concentrations like 40, 60 and 80 µg/ml recorded lower percentage of viability which were [(64.17%) & (83.85%)], [(49.84%) & (83.84%)] and [(42.37%) & (61.74%)] for both drugs respectively. The IC50 of CTNs NPs and pentostam was measured 53.79 µg/ml and 78.33µg/ml after 72h respectively Figure -4 A and B.

Significant ($p< 0.05$) differences have been shown in promastigotes viability after 48h of exposure to CNTs and pentostam drug (Table 2). Parasites exposed to 20 µg/ml of CNTs and pentostam recorded (71.52%) and (96.12%) of viable cells respectively, while when exposed to 40 µg/ml of the two drugs the percentage of viability recorded (54.43%) and (85.37%) respectively. The concentration 60 µg/ml of both drugs showed (35.33%) and (80.06%) of viable cells respectively, and the percentage of promastigotes viability decreased to (17.14%) and (71.96%) when exposed to 80 µg/ml for both drugs respectively.

Table-2 also revealed that promastigotes viability after 72h was higher when exposed to low concentrations of both drugs. Parasites exposed to 20µg/ml of CNTs NPs and pentostam recorded (49.92%) and (89.69%) of viable cells respectively, and 40 µg/ml of the two drugs recorded (30.14%)

and (82.63%) of viable cells respectively. When using the drug concentration 60µg/ml, the percentage of viable cells was (23.05%) and (79.82%) for CNs and pentostam respectively, and the percentage of promastigotes viability reach to (5.92%) and (42.08%) when exposed to 80µg/ml for both drugs respectively.

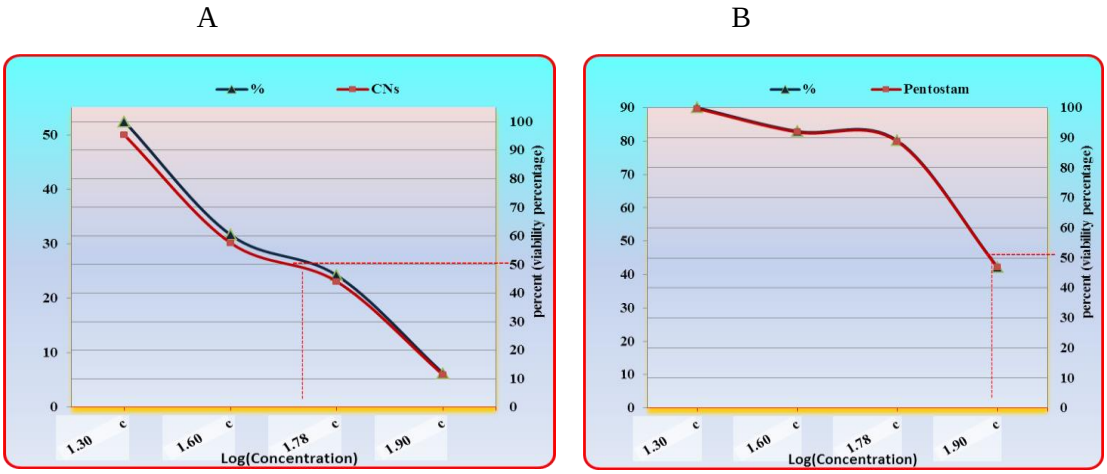

Figure 4- A) The IC50 of CNTs NPs on *L. donovani* promastigotes viability was 53.79µg/mL after 72 hours. **B)** The IC50 of petostame on *L. donovani* promastigotes viability was 78.33µg/mL after 72 hours.

Table 2-Compare between CNTs NPs and pentostam treatments effect in viability percentage.

Treatment (Conc.)	Time (hours)			LSD value
	24	48	72	
CNs 20 (µg/ml)	80.87	71.52	49.92	9.663 *
CNs 40 (µg/ml)	64.17	54.43	30.14	8.758 *
CNs 60 (µg/ml)	49.84	35.33	23.05	8.125 *
CNs 80 (µg/ml)	42.37	17.14	5.92	9.516 *
Pentosam 20 (µg/ml)	82.67	96.12	89.69	8.407 *
Pentosam 40 (µg/ml)	83.85	85.37	82.63	6.449 NS
Pentosam 60 (µg/ml)	83.84	80.06	79.82	6.392 NS
Pentosam 80 (µg/ml)	61.74	71.96	42.08	8.509 *
LSD value	7.629 *	9.033 *	9.815 *	----
* (P<0.05), NS: Non-significant.				

CNTs NPs effect on the parasites morphology

A visual inspection by light microscopy revealed that CNTs induces morphological changes in *L. donovani* promastigotes in comparison to the morphology of the untreated promastigotes. In the high concentration (80µg/ml) of pentostam drug after 72h the parasite morphology had less changing compared with CNTs NPs in the same concentration and it was more likely to the untreated parasites. *L. donovani* promastigotes cultured in fresh complete medium had elongated forms, while cells exposed to CNTs NPs in the following concentrations 5, 10, and 20 after 72 h, showed rounded forms and cell shrinkage, increased particularly at the higher doses**.** In the concentrations 40µg/ml and 60µg/ml of CNTs NPs after 72 h showed more rounded forms and the cell lose their movement gradually. In the concentration 80µg/ml of CNTs NPs the promastigotes forms more rounded and the flagellum disappeared Figure- 5.

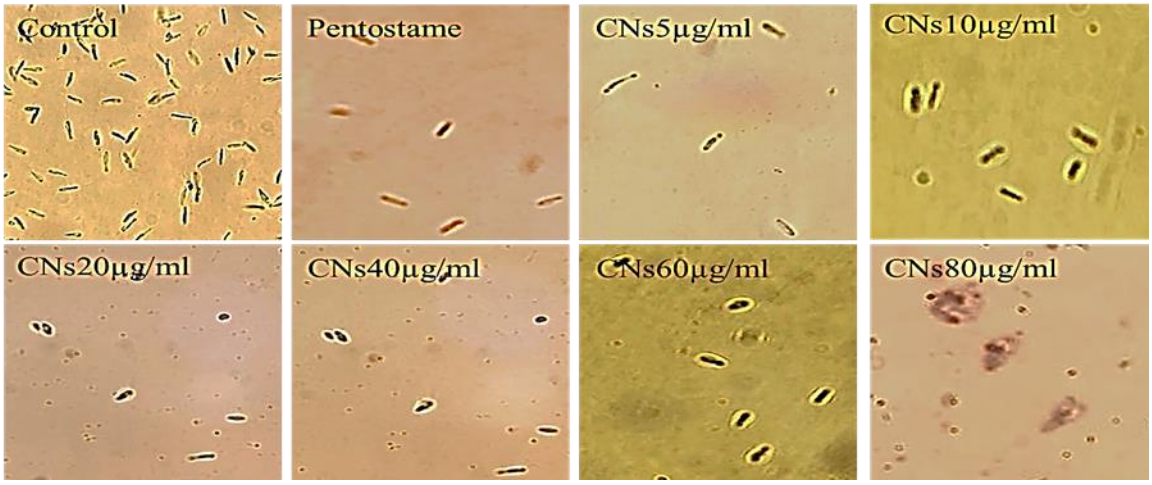

Figure 5- Morphological changes of promastigotes under light microscopy (magnification =400), (control) without treatment, Pentostam and different concentration of CNs NPs (5, 10, 20, 40, 60, 80 µg/ml) after 72hours.

Discussion

leishmaniasis is one of the significant causes of morbidity and mortality in numerous countries. This disease affects people worldwide [2]. Current treatments for *Leishmania* parasite often have many side effects. A novel technique to kill this parasite is by using nanoparticles. This novel technique is opening up new possibilities for the treatment of pathogenic parasites and other infectious pathogens as well.

The results of this study have shown lower efficacy of pentostam on *L. donovani* promastigotes in all using concentrations while CNTs NPs have shown higher efficacy through all days of treatment with concentrations (20, 40, 60 and 80 µg/ml), this may be due to the fact that the pentavalent antimonials are required biological reduction to the trivalent form (SbIII) for antileishmanial viability. The site of infection and mechanism of reduction remain controversial. However, several studies have stated that axenic amastigotes are susceptible to pentostamSb (V), whereas promastigotes are not thus, some parasite stage specific reduction occurs in this life cycle stage, the mechanism by which amastigotes reduce Sb(V) is not clear [24-27]. While, both glutathione and trypanothione cannon-enzymatically reduce Sb(V) to Sb(III), particularly under acidic conditions [28-32].

Nanoparticles have unique physicochemical properties such as shape, small size, great surface area, and electrical charge [16]. The nanoparticles are frequently used in medicine in drug delivery and cancer therapy [18]. Carbon nanotubes (CNTs), as a nanomaterial, have developed as a new candidate drug tool for transporting therapeutic molecules against different microorganisms [33] due to their distinctive physical and chemical properties. In the present study we examined CNTs antiparasitic activity on the Iraqi strain of *L. donovani*. CTNs nanoparticle in all using concentrations showed effectiveness on *L. donovani* promastigotes viability. In addition, the present study showed the effect of CNTs concentrations on the morphology in the higher concentrations in comparison to the morphology of the untreated promastigotes. A study demonstrated that functionalized carbon nanotubes – amphotericin B has significantly greater antileishmanial efficacy than amphotericin B and had no significant cytotoxic effects [34]. These results were agreed also with previous study confirmed the affectivity of CNTs *in vitro* against *Leishmania* species [35]. CNTs are useful as vehicles for drug delivery against intracellular targets due to their ability to cross cell membranes and to deliver nucleic acids, peptides, and proteins. Studies demonstrated that f-CNTs are taken up by lymphocytes and macrophages without affecting their viability *in vitro* [19]. But antileishmanial mechanism of CNTs NPs is yet unknown. Other studies have been shown that the nanoparticles drug has lethal effects against cancer cells and fibroblasts. Nanosilver induces apoptosis in human alveolar adeno carcinoma cells [36]. Other studies have been reported that ZnO NPs can induce apoptosis in human dermal fibroblasts and cell death in human mesothelioma [37].

Our data determine the superiority of CNTs NPs over the standard drug pentostam *in vitro*. This provides a possibility of eventually using these CNTs NPs as a candidate drug target with better

antileishmanial efficacy. Furthermore, its production is favorable in comparison with pentostam with low-cost production. Further studies are needed to develop CNTs NPs as an antileishmanial drug and to explore its potential for oral administration.

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