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Original paper

Detection of artemisinin effect on macrophage inducible nitric oxide gene expression in macrophage infected with *Leishmania donovani*

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ABSTRACT. Leishmaniosis is a parasitic infection spreads to humans by sand flies. Over 20 different species of *Leishmania* are responsible for the disease, which infect over 14 million people around the world. Chemotherapy is one of the most effective treatments for leishmaniosis, however it is restricted by the high cost and/or toxicity. In this study, the possible effect of artemisinin (ART) was detected on intracellular amastigotes of Iraqi strain of *Leishmania donovani* in *ex vivo* condition in U937 macrophage cell line. Gene expression of inducible nitric oxide synthase (iNOS) in U937 macrophage was investigated, before and after treatment with artemisinin. Kinetic result by real-time PCR demonstrated that the iNOS expression folding reached the maximum at concentration of 500 μM after 24 hours, at 750 μM after 48 hours and at 1000 μM after 72 hours, which was 56, 11, and 6, respectively. The copy number of iNOS gene expression was also significantly higher in treated infected U937 cells compared to both non-treated-infected cells and intact macrophages, under different concentration of ART along the three times of follow-up. Moreover, stained macrophages with fluorescent DAPI proved that the percentage of intracellular infective amastigotes was decreased to the minimum in treated U937 cells, in comparison to non-treated cells. The minimal amastigote-invasion percentage was recorded at 1000 μM, which was 26%, 27%, 21% compared to 61%, 87%, 75% in untreated cells after 24, 48, 72 hours respectively. These findings demonstrated ART positive efficacy against iNOS expression and this compound can be further studied as novel therapeutic rather than toxic available chemotherapies.

Keywords: *L. donovani*, U937, artemisinin, DAPI stain, inducible nitric oxide synthase

Introduction

Leishmaniosis is a serious worldwide health problem and it is considered a neglected tropical disease (NTD) which is endemic in 98 tropical regions [1]. According to the World Health Organization (WHO), two million cases of leishmaniosis are recording annually, exposing 350 million individuals at risk of infection [2]. The disease is found in 90 countries, Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, Syria, and Tunisia having the highest prevalence [3,4]. Chemotherapy is still the most common treatment for leishmaniosis; antimonials are currently the most used treatment, followed by miltefosine, amphotericin B and its liposomal forms, paramomycin, and several WHO-recommended combination treatments [5,6]. However, various complications were reported including high costs, considerable toxicity and the development of drug-resistant parasites, which limit their use [7]. *Artemisia* is a type of medicinal herbs of the Asteraceae family with antibacterial properties [1]. Many researchers have recently identified that *Artemisia* extracts, such as artemisinin (ART) and its derivatives have antiparasitic, antioxidant, antibacterial, anti-inflammatory, and anticancer activities [8,9]. According to studies [10,11], ART has a multifunctional role in the control of leishmaniosis,

it enhances NO and iNOS synthesis in uninfected macrophages and regulates the immune system indirectly by stimulating Thl cytokine expression. It is been used to treating numerous diseases, for example, angiogenesis inhibitors are used to treat cancer [12,13] as well as psoriasis and diabetic retinopathy treatment [14]. The generation of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS) enzyme has been shown to have a vital role in the *in vitro* and *in vivo* killing of *Leishmania* in activated macrophages [15]. The iNOS gene is increased after the T-cell derived cytokine IFN-γ activates mammalian macrophages, resulting in the production of NO, which is fatal for *Leishmania* and a number of intracellular infections such as *Plasmodium falciparum*, *Toxoplasma gondii*, and others [16,17]. Stimulation of iNOS expression in human macrophages appears to be more regulated than in mouse macrophages, making its role in human leishmaniosis more remarkable [17,18]. However, human macrophages have been shown to kill *Leishmania* in an NO-dependent mechanism in different studies and the detection of iNOS enzyme in a human tissue granuloma containing *L. donovani* had been reported [17]. Producing activated oxygen radicals and nitric oxide are two main pathways for eradicating intracellular parasites [19]. NO generation by iNOS can be regulated at the transcriptional level, most cells have extremely low levels of iNOS protein, but activating these cells with cytokines or growth factors causes increased iNOS gene transcription and consequent synthesis of high quantities of NO [20,21]. This finding improves the theory that iNOS has a role in human leishmaniosis, identifying the mechanism of controlling the disease and other infections via macrophages depending-iNOS expression [22]. The mechanisms and pathways that stimulate *Leishmania*-infected macrophages are having more attention because they could be used to develop novel anti-leishmanial drugs [21]. In this study, the kinetic effect of ART on iNOS expression was investigated in *L. donovani*-infected macrophage.

Materials and Methods

Parasite and macrophages cell line U937 used in this study

Iraqi strain of *Leishmania donovani* was kindly provided from the laboratory of Parasitology, graduate studies, Department of Biology, College of Science, University of Baghdad and was previously diagnosed by PCR [23]. Macrophage U937 cell line was purchased from Rawafid-Aleloom laboratories, Hillah, Iraq.

Promastigote culture

Axenic procyclic promastigotes (logarithm phase) were cultivated in Novy-MacNeal-Nicolle (NNN) medium before being transferred to RPMI cell culture media. This medium was supplemented with 10% HIFCS, 100 IU/ml pen/strep [6]. The parasites were concentrated to 1×10^6 parasites/ml and put into 5 ml universal tubes and stored at 26°C after the culture promastigote forms was counted using a Neubauer chamber enhanced bright-line hemocytometer.

Amastigotes differentiation from promastigotes, *in vitro*

Transferring 1×10^6 promastigotes/ml to a fresh RPMI1640 medium supplemented with 20% HIFBS, pH 5.5, and incubating at 35°C for two days to create infectious metacyclic promastigotes was used to promote the formation of axenic amastigotes [24].

Macrophage host U937 cell line culture

The cell line U937 was grown in RPMI1640 medium with 10% HIFBS and 1% pen/strep at 37°C in a humidified incubator with 5% CO_2.

Artemisinin preparation

Artemisinin was made by dissolving 5 mg of ART powder in 3.5 ml of dimethyl sulfoxide, as directed by the manufacturer (TOCRIS bioscience /UK) (DMSO).

Ex vivo infection of U937 macrophages with L. donovani amastigotes

Cells were scraped from the bottom of a healthy culture flask and counted using a haemocytometer before cultured on a 6 well-plate (Falcon/USA). The cells were washed twice with PBS, then re-suspended at a final concentration of 1×10^5 cell/ml in RPMI media supplemented with 10% HIFBS, 1% pen/strep in a humidified incubator at 37°C. *L. donovani* amastigotes was added counted and added in a ratio of 1:10. The following concentrations of artemisinin were added to the wells (1000, 750, 500, 250, and 125) μM. Triplicate plates were prepared and incubated for three time of follow-up (24, 48, 72) hours [25]. Plates were fixed and stained with DAPI stain after the specified incubation time;

Table 1. Primers used for RT-PCR of iNOS [42]

Primer	Seq.	Annealing temp. (ºC)	Product size (bp)
iNOS-F	5`-ATGCCAGATGGCAGCATCAGA-3`	60	371
iNOS-R	5`-ACTTCCTCCAGGATGTTGTA-3`		

another plate was prepared as control where no ART was added.

DAPI staining and fluorescent microscopy

DAPI stain powder was prepared and used according to Sigma-Aldrich®, dissolved in D.W to a final concentration of 1 to 5 mg/ml as stock solution. Dye stock solution was diluted with methanol to a final concentration of 1 µg/ml and was added to each well for 5 minutes, then washed three times in PBS, triplicates were prepared for each well. 100 macrophages were counted in random fields under the fluorescent microscope to calculate the number of infected and non-infected cells, the total number of parasites in 100 macrophages, and the number of parasites in infected macrophages, the following formula was used to obtain the mean number of parasites per cell [26].

Parasite/cell = Total number of amastigote load in 100 macrophages/number of infected cells

Real-time polymerase chain reaction (*Quantitative-PCR One step*) *of iNOs gene expression in U937 macrophages*

Macrophage RNA was extracted using TRIzol Reagent (Thermo Scientific, USA) according to the manufacturer's instructions [43]. Pellet was rehydrated in 50 µl of nuclease free water then incubated in a water bath set at 55–60°C for 10–15 minutes.

This experiment was done using real time thermal cycler Mic qPCR cycler one step version 2.10.0 and the results were analyzed using special software for this device. Amplification for gene at the different conditions. The cycles were run correctly according to the plot of delta fluorescence/delta temperature (df/dt) with the number of cycles.

Real-time PCR primer pair for iNOS was purchased from Macrogen Company in a lyophilized form (Tab. 1). Lyophilized primers were dissolved in a nuclease free water to give a final

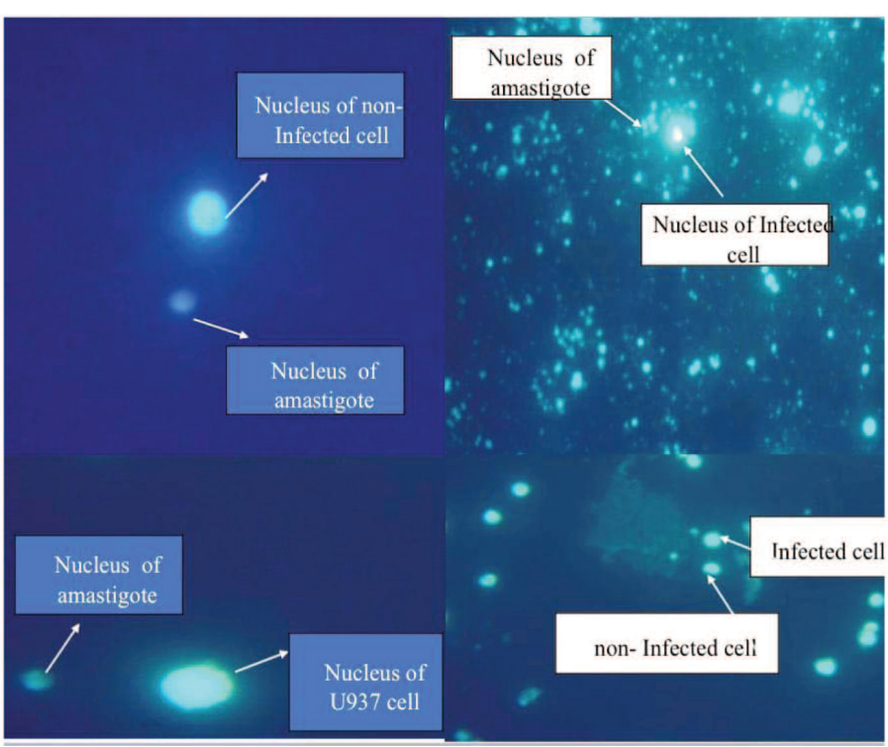

Figure 1. *Ex vivo* infection of U937 with *L. donovani* amastigotes, in different concentration of ART and stained with DAPI stain, picture was taken by fluorescent microscope (40×)

Table 2. *Ex vivo* infection of U937 with *L. donovani* amastigotes after 24, 48 and 72h (cell counting with DAPI stain)

Parameter	Percentage of infected MØ			Percentage of amastigote/100 MØ cell			Percentage of amastigote/MØ cell		
Concentration of ART µM	24h	48h	72h	24h	48h	72h	24h	48h	72h
1000	26	27	21	43	36	32	1.6	1.3	1.5
750	17	23	47	21	46	63	1.2	1.4	1.3
500	23	14	51	33	23	59	1.4	1.6	1.1
250	38	47	60	76	117	70	2	2.4	1.1
125	41	75	56	89	123	89	2.1	1.6	1.5
Control	61	87	75	135	234	120	2.2	2.6	1.6

concentration of 100 pmol/µl as a stock solution. The Master mix components was prepared as the following: 5 µl qPCR Master Mix, 0.25 µl RT mix, 0.25 µl $MgCL_2$, 0.5 µM/µl of each forward or reverse primer, 2.5 µl nuclease free water, 1 ng/µl RNA, 10 µl total volume. 9 µl of Master mix per tube and add 1 µl of template was added. Real-time PCR (Bio Molecular System, Australia) for cDNA amplification was set up for 40 cycles as the following: 95°C/20 sec, 60°C/20 sec and 72°C/20 sec.

Statistical analysis

Significant difference was calculated by GraphPad Prism v7.0 in which t-test was used at $P \leq 0.05$ significant, mean ± SEM.

Results and Discussion

Nucleic acid staining and fluorescent microscopy

Different artemisinin concentrations were examined against U937 macrophages infected with *L. donovani* amastigotes. DAPI is a fluorescent dye that binds to double-stranded DNA with high selectivity and creates highly fluorescent DNA-DAPI complexes. It is blue fluorescence stands out in stark contrast to other structures' green, yellow, or red fluorescent probes [27]. Figure 1 shows the inhibitory efficacy of artemisinin against the ability of amastigotes to invade macrophages in treated and untreated cells after 24, 48, and 72 hours of follow up. Infection of U937 cells started in 24 hours, and efficacy was improved after 48 and 72 hours, this could indicate that ART is a dose- and time-dependent inhibitor [6].

According to the table 2, *ex vivo* infection of U937 macrophages at concentrations of (1000, 750, 500, 250, 125) µM revealed a significant difference between test and control after 24, 48 and 72 hours, with the minimal invasion percentage in 1000 µM, which was 26%, 27%, 21% compared to 61%, 87%, 75% in the control plate in 24, 48, 72 hours, respectively. The percentages of infected macrophage were reduced in 24, 48, and 72 hours of incubation, indicating the effect of artemisinin on *Leishmania* infectivity.

Similar study by Hassan and Ali [28], found that *L. tropica* promastigotes count was reduced in, *in vitro* culture, after 48 hours and gradual decrease was continued after 72 hours. They also found that the infectivity of intracellular *L. tropica* amastigotes was highly decreased after 48 hours *ex vivo* infection. Another study by Bok et al. [29] investigated the infectivity of different pathogens, such as Norovirus, on RAW264.7 and detected nuclear morphology using DAPI staining. Another study by Amoa-Bosompem et al. [30] reported of investigation the efficacy of three anti-trypanosome agents against *Leishmania* were employed DAPI stain to detect any phenotypic changes in *Leishmania hertigi* parasites. Colineau et al. [31] studied *L. donovani* effectors that support macrophage infection in another investigation, THP-1 cells infected with *Leishmania* nuclei were identified with DAPI, which detected both macrophages and *Leishmania* nuclei, this enabled for the counting of the number of infected cells per total cells (infection rate) and the number of *Leishmania* per infected macrophage (parasite burden) on each image, as well as the calculation of

averages. Al-HAlbosiy et al. [32] reported when use DAPI stain after 24 and 48 hours of ART treatment, the infectivity and amount of intracellular Leishman bodies in treated murine peritoneal macrophages were evaluated.

Measuring expression of iNOS gene in U937 by real-time PCR (*qPCR*)

iNOS gene expression was investigated by qPCR to determine the expression in both infected-treated and infected non-treated cell under different concentration of artemisinin.

In this study, the use of artemisinin increases endogenous iNOS gene expression in *L. donovani*-infected U937 macrophages: RT-PCR results demonstrated in two ways relative and absolute. The highest folding expression of iNOS was recorded at concentration of 500 µM after 24 hours, at 750 µM after 48 hours and at 1000 µM after 72 hours, which was 56, 11, and 6, respectively. The copy number of iNOS gene expression was also significantly higher in treated infected U937 cells compared to both non-treated-infected cells and intact macrophages, under different concentration of ART along the three times of follow-up. that iNOS gene expression was much higher in *L. donovani*-infected U937 cells compared to both non-treated-infected cells and cell control. Figure 2, 3 and 4 show the absolute expression of iNOS mRNA, figures 5, 6 and 7 show relative expression of iNOS gene expression in which significant increase in *L. donovani* infected macrophages treated with artemisinin in different concentration (1000, 750, 500, 250, 125) µM was observed. iNOS gene expression was found to be enhanced in infected cells treated with artemisinin at concentrations of 1000, 750, 500, 250, and 125 after 24, 48, and 72 hours, respectively. In contrast levels of mRNA was significantly lower in non-infected U937 cells and the expression index showed in the infected group without artemisinin exposure and also non- infected U937 cell line were lower than test groups.

Artemisinin has proven therapeutic potential against malaria and have also demonstrated effectiveness in experimental models of leishmaniosis, schistosomosis and *Clonorchis* infections [33]. The formation of free radicals and cell death in the presence of iron sources is proposed as a mechanism for ART in *Leishmania*, demonstrating that the endoperoxides (EP) bond is required as a potential pharmacophore [34]. ART also affect in several ways, including as inhibiting

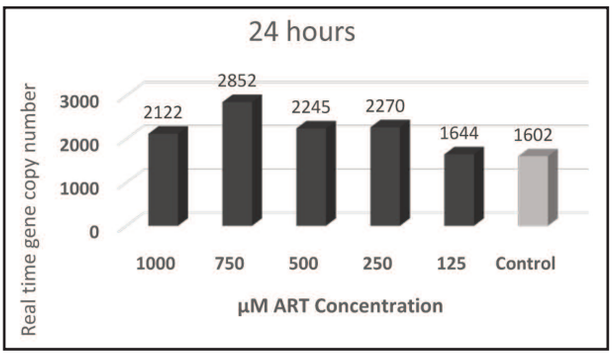

Figure 2. Absolute (copy number) of iNOS gene in U937 treated with ART after 24 hours of incubation

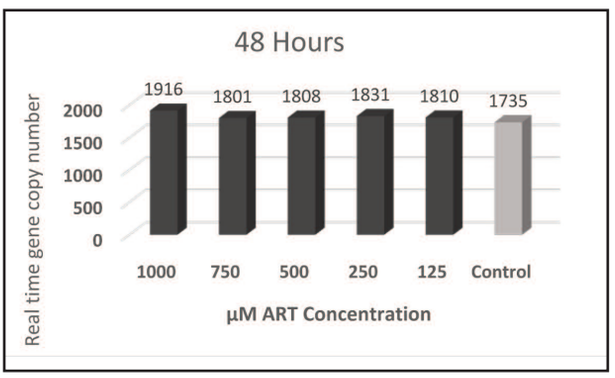

Figure 3. Copy number of iNOS gene in U937 treated with ART after 48 hours of incubation

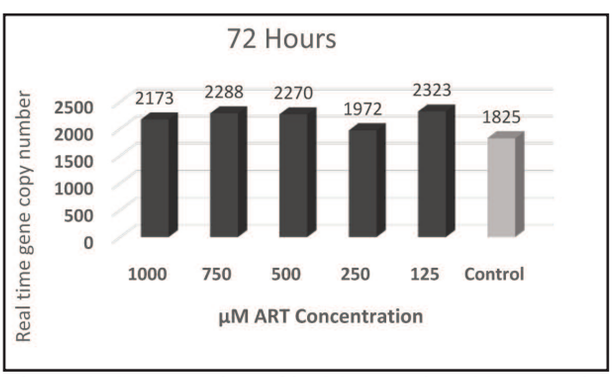

Figure 4. Copy number of iNOS gene in U937 treated with ART after 72 hours of incubation

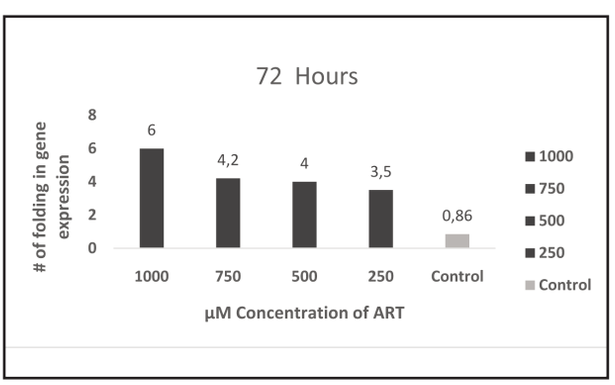

Figure 5. Relative (folding) of iNOS gene in U937 treated with ART after 24 hours of incubation

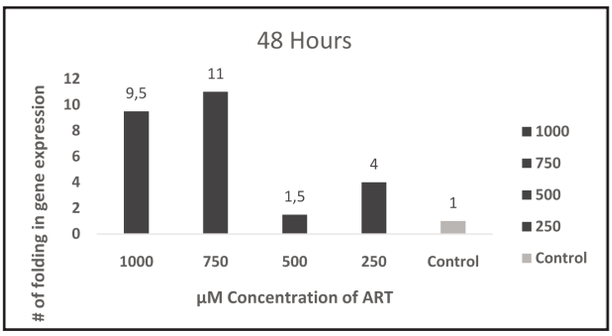

Figure 6. Relative (folding) of iNOS gene in U937 treated with ART after 48 hours of incubation

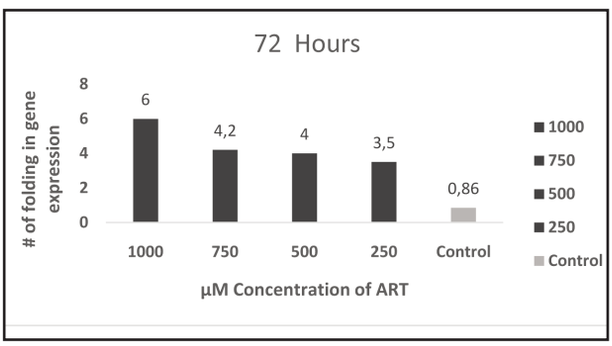

Figure 7. Relative (folding) of iNOS gene in U937 treated with ART after 72 hours of incubation

certain parasite enzymes in a peroxide-independent manner or causing mitochondrial damage [35]. The macrophage is programmed to have an M1 pattern [36]. It produces large quantities of reactive oxygen species (ROS), nitric oxide (NO), and pro-inflammatory cytokines including IL-12 and TNF as a consequence of this activation, leading to enhanced microbicidal capacity [37]. This activation is required for the parasite *Leishmania* to be eradicated [38]. Santos-Pereira et al. [36] found NO generation was higher in C3H/He cells in the absence of a stimulus, and these cells also had a reduced infection rate and parasite load, NO generation elevated and infection rate reduced when C3H/He and BALB/c macrophages were activated with LPS and IFN-γ. *In vitro* experiments with *L. amazonensis*-infected macrophages have revealed that inhibiting inducible nitric oxide synthase (iNOS) increases parasite load inside the cells [39]. Pessenda and da Silva [40] detected the spleen gene expression of iNOS and arginase, enzymes involved in both nitric oxide (NO)-mediated parasite killing and polyamine-mediated parasite replication, respectively. Sen et al. [41] reported that ART was cytotoxic to uninfected macrophages and that it increased the creation of macrophage nitric oxide, which is necessary for the parasite's elimination. Furthermore, a related study showed that ART had a very minor effect on uninfected macrophage [13]. In this study, *Leishmania* infection caused a decrease in the production of NO that, following addition of artemisinin, translated into a significant increase, but importantly only to levels comparable with uninfected macrophages. Furthermore, infection translated into lowered mRNA expression of iNOS that was restored by artemisinin [41]. This research proved the activity of ART in the *ex vivo* treatment of visceral leishmaniosis, and detection the role of iNOS in infection may help to improve new anti-*Leishmania* drugs.

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