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Glucose-6-phosphate dehydrogenase (G6PD) activity can modulate macrophage response to *Leishmania major* infection

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PDH) ultimately plays a critical role in macrophage functions used against infectious agents. The present study investigated whether changes in G6PDH activity could influence the resistance of infected macrophages against *Leishmania major* infection. Mouse peritoneal and J774 macrophages were infected, respectively, ex vivo and in vitro, with *L. major* and then exposed to an inhibitor (6-aminonicotinamide) or activator (LPS + melatonin) of G6PDH activity for 24 h. Cell viability [using MTT assay] was measured to assess any direct toxicity from the doses of inhibitor/activator used for the macrophage treatments. Nitric oxide (NO) produced by the cells and released into culture supernatants was measured (Griess method) and cell G6PDH activity was also determined. Moreover, the number of amastigotes form Leishmania in macrophages that developed over a 7-d period was evaluated. The results showed that an increase in G6PDH activity after treatment of both types of macrophages with a combination of LPS + melatonin caused significant increases in NO production and cell resistance against *L. major* amastigote formation/survival. However, exposure to 6-aminonicotinamide led to remarkable suppression of G6PDH activity and NO production, events that were associated with a deterioration in cell resistance against (and an increase in cell levels of) the parasites. The results suggested that activation or suppression of G6PDH activity could affect leishmanicidal function of both mouse peritoneal and J774 macrophages. Thus, regulation of macrophages via modulation of G6PDH activity appears to provide a novel window for those seeking to develop alternative therapies for the treatment of leishmaniasis.

1. Introduction

Leishmaniasis, caused by protozoan parasites of the *Leishmania* genus, is a group of diseases with a wide range of clinical manifestations that has a high worldwide morbidity and mortality. The World Health Organization (WHO) estimates that leishmaniasis has affected ≈12 million people [1,2]. The parasites have an obligatory intracellular form (amastigotes) in mononuclear phagocytes [3]. A persistence of amastigotes in dermal tissues is vital for the development of cutaneous leishmaniasis (CL), a non-fatal skin lesion mainly caused by *Leishmania major* [4,5]. During leishmaniasis, the main target for therapy is the intracellular amastigotes that survive and divide in tissue macrophages [6]. However, conventional therapies have the major drawbacks of various adverse reactions, high resistance rates, general ineffectiveness, as well as toxicity [7,8]. These important disadvantages have led many researchers to seek novel more efficient and safe therapeutic strategies against *Leishmania* infection [9].

In the body, immunologic resistance against *Leishmania* parasites is mediated primarily via macrophages. After their phagocytic uptake, promastigotes transform into amastigotes within the acidic environment of the macrophage phagolysosomes [10,11]. In turn, various cellular processes are initiated leading to macrophage activation; these include enhanced NADPH oxidase activity (leading to increases in reactive oxygen species formation) and increases in formation of nitric oxide (NO) [4,12]. The survival of the amastigotes in macrophages is mainly determined by the balance between the cell ability to be activated and the parasite to resist cytotoxic mechanisms used in/by the cells [13,14].

Glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49), a

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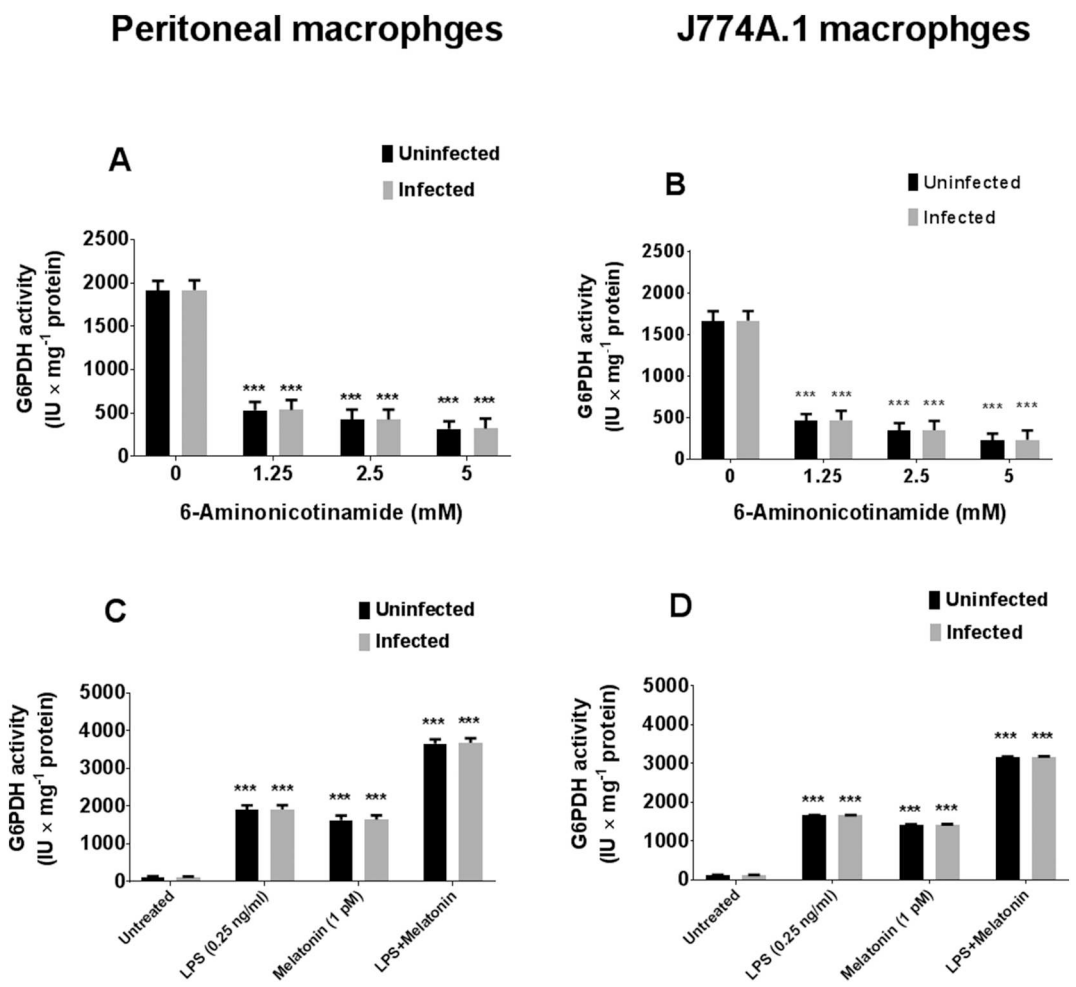

Fig. 1. G6PDH (IU × mg^{-1}) activity in supernatants of peritoneal and J774 macrophages after treatment with (A) stimulant (a combination of LPS 0.25 ng/ml and melatonin 1 pM) or (B) inhibitor (6-aminonicotinamide 1.25–5 mM) for 24 h. In each group, the amount of G6PDH activity was measured in lysates of cells. Results shown are mean G6PDH (U/mg protein) activity [± SD] (n = 3/treatment). ***$p < 0.001$ vs. all other treatment regimens within corresponding untreated cells.

rate limiting enzyme of the pentose phosphate pathway of carbohydrate metabolism, aids in re-generation of NADPH (Nicotinamide Adenine Dinucleotide Phosphate-oxidase) cofactor from $NADP^+$ [15,16]. NADPH is essential for the formation of reactive oxygen species (ROS) that evolve after activation of NADPH oxidase [17,18]. In mouse peritoneal and J774 macrophages, stimulation with pathogen-associated molecular patterns (PAMP) elicited G6PDH activity [19]. As would be expected with any impaired NADPH formation due to reduced/deficient G6PDH activity (and so deficits in macrophage function), there is a link to increases in the incidence of infections [20–25]. As NADPH is critical to the conversion of arginine to citrulline (and hence, NO formation), pharmacologic inhibition of G6PDH using 6-aminonicotinamide (6-AN), a potent G6PDH competitive inhibitor, would significantly impaired their NO production [26–28]. Moreover, a combined treatment of monocytes with melatonin and LPS could synergistically enhance G6PDH activation [29–32].

Because of the roles of ROS and NO in immune responses to *L. major* in infected macrophages, the present study sought to assess whether any changes in macrophage G6PDH activity might impact the development/survival of *L. major* amastigotes. Using activators and inhibitors of G6PDH, the studies here employed both mouse primary and cell line macrophages to discern if modulation of G6PDH activity could potentially considered as a new target for alternative therapies in the treatment of leishmaniasis.

2. Materials and methods

2.1. Mice

Balb/c mice (male, 6–8-wk-old) were obtained from the Pasteur Institute (Tehran, Iran). All mice were kept in facilities under specific pathogen-free conditions in rooms maintained at 25 ± 5 °C and with a 12-h day/night cycle. All mice had ad libitum access to standard commercial rodent chow pellets and filtered water. All studies received the approval of the Ethics Council of the Tarbiat Modares University (Tehran, Iran).

2.2. Preparation of peritoneal macrophages

Macrophages were collected from the peritoneal cavities of naïve mice by the aspiration of peritoneal liquid under sterile conditions. Harvested macrophages were suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (all materials from Sigma, St. Louis, MO). Cells were counted and viability determined using Trypan blue exclusion.

2.3. Culture of J774 macrophages

The murine J774 macrophage cell line (Pasteur Institute, Iran) cultured in complete RPMI 1640 medium (as above) in a humidified

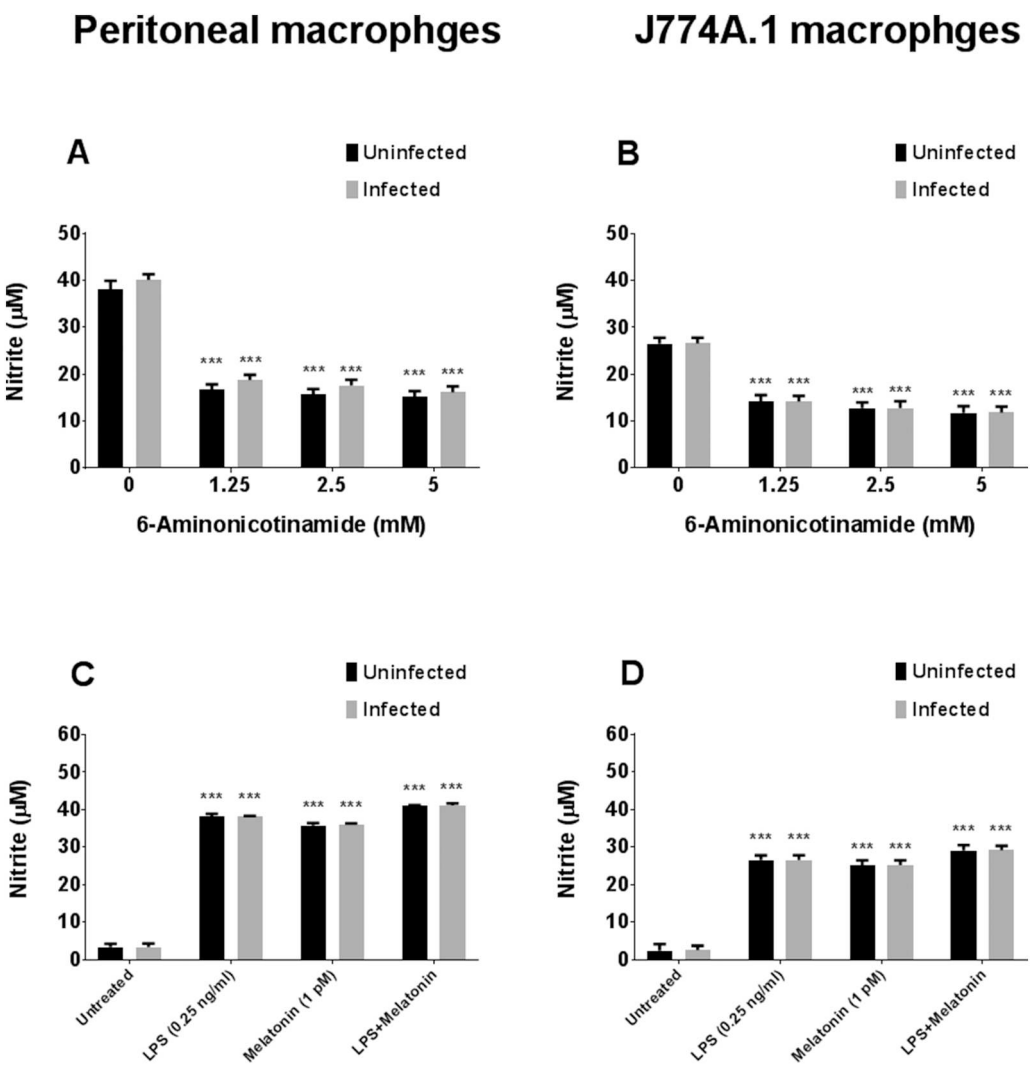

Fig. 2. Evaluation of NO production by peritoneal and J774 macrophages after 24 h of treatment with (A) stimulant (a combination of LPS 0.25 ng/ml and melatonin 1 pM) or (B) inhibitor (6-aminonicotinamide 1.25–5 mM) for 24 h. In each group, the amount of NO was measured in supernatants collected after 24 h of treatment. Results shown are mean NO (μM) formation [± SD] (n = 3/treatment). ***p < 0.001 vs. all other treatment regimens within corresponding untreated cells.

95% air: 5% CO2 atmosphere at 37 °C. After harvest from the culture flasks, cell viability determined using a Trypan blue exclusion method.

2.4. Treatment

For experiments where the macrophages (and G6PDH) were to be activated by lipopolysaccharide (0.25 ng LPS/ml; Type O26:B6 from *Escherichia coli*, Sigma, St. Louis, MO), the cells were first treated with 1 pM melatonin (Sigma, St. Louis, MO) for 8 h prior to receiving the LPS [29–32]. In studies requiring inhibition of the G6PDH, 6-aminonicotinamide (1.25–5 mM; Sigma, St. Louis, MO) was used.

2.5. Isolation and culture of L. major promastigotes

Stationary-phase promastigotes (2×10^6) of *L. major* (MRHO/IR/75/ER; WHO designation) were injected subcutaneously into the base of the tail of naïve Balb/c mice and were then allowed to develop for 8 wk. Mice were then euthanized and *L. major* parasites in the lesions were isolated and maintained as promastigote were grown at 24 °C in complete RPMI medium (containing 20% heat-inactivated FBS) for up to five passages. Cultures were then collected at the middle of the logarithmic (or beginning of the stationary) phase [33].

2.6. Macrophage infection with L. major

For all experiments where the cultured cells were to be infected, macrophages were seeded into dedicated wells and incubated at 37 °C for 4 h. Promastigotes (1:10 cell:parasite ratio) were added to the adherent cells for 24 h. After a further 24 h, the medium was replaced and the cells were used in the assays described below.

2.7. Assessment of cell viability using MTT assay

The effect of induced changes on growth/proliferation of uninfected macrophages was assessed via mitochondrial respiration-dependent reduction of MTT [3-(4,5 di-methylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] to formazan [34]. In brief, cells (40,000/well, in 200 μl) were incubated with the activator or inhibitor. Control cells were incubated only in medium. After a 24 h incubation, MTT solution (5 mg/ml) was added to each well; 3 h later, the medium was removed and cells lysed by addition of 100 μl dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) to dissolve formazan crystals that had formed in viable cells. The optical density was measured at 545 nm using a microplate reader (Convergent Technology, Germany). Cell viability (%) was calculated as 100 X [OD treated/OD control].

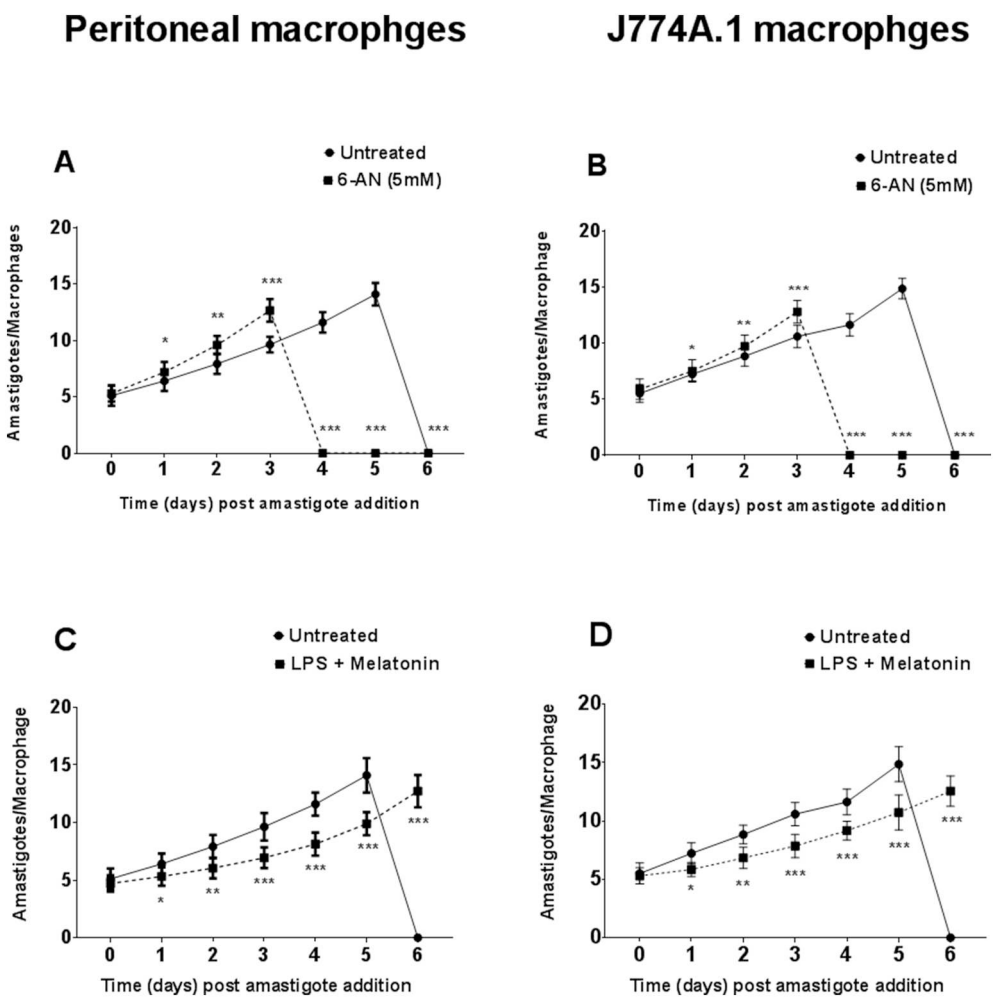

Fig. 3. Effects of modifiers of G6PDH activity upon *L. major* amastigote survival in peritoneal and J774 macrophages. Stimulant (a combination of LPS 0.25 ng/ml and melatonin 1 pM) or (B) inhibitor (6-aminonicotinamide 5 mM). Each column represents the mean number of amastigotes/cell [± SD] (n = 3 replicates/treatment shown). *p < 0.05, **p < 0.01, or ***p < 0.001 vs. corresponding Day 0 value within a given regimen. 6-AN; 6-Aminonicotinamide.

2.8. Measurement of cytosolic G6PDH activity

Assessments of G6PDH activity were performed in cell extracts as previously described [35]. Macrophages were detached from the wells (by gentle scraping) and washed (200 × *g*, 10 min, 4 °C) twice with phosphate-buffered saline (PBS, pH 7.4). The cells were then sonicated (6 times, 10-sec bursts in 1-min intervals); clear extracts were obtained by centrifugation at 12000 × *g* for 20 min at 4 °C. Lysate protein levels were estimated using a Bradford assay. Enzyme activity was determined by combining 100 µl supernatant with a mixture of Tris buffer (pH 7.8) containing 4.7 µM G6P and 8.7 µM NADP + (both Merck, Germany) and measuring the rate of increase in absorbance at 340 nm reflecting the conversion of $NADP^+$ to NADPH - using the plate reader. One international unit (IU) of G6PDH activity was defined as the amount of enzyme that catalyzed formation of 1 µmol NADPH/min/mg protein present.

2.9. Determination of nitric oxide production

Uninfected/infected J774A.1 and peritoneal macrophages (5×10^5 cells/well) were incubated with various concentrations of compounds for 24 h. Nitrite accumulation (indicator of NO synthesis) was measured in the culture medium using Griess reagent [36]. Briefly, equal amount of culture supernatants were mixed with Griess reagent and incubated at RT for 20 min. Thereafter, the absorbance at 545 nm in each well was measured in the microplate reader. Nitrite concentration (µM) was extrapolated from a sodium nitrite standard curve generated in parallel using nitrite standards.

In a separate set of assays, uninfected/infected macrophages were assessed for effects of known modifiers of iNOS activity (all from Sigma, St. Louis, MO), i.e., SNAP (100 µM) as a NO donor, recombinant interferon (rIFN)-γ (20 ng/ml) as a potent activator of macrophages, and L-NG-monomethyl arginine citrate (L-NMMA) as a non-selective inhibitor of iNOS.

2.10. Assessing levels of intracellular amastigotes in infected macrophages

Macrophages were seeded onto 16-well chamber slides (Nunc™ Lab-Tek™, NY) and infected with late log-phase *L. major* promastigotes (at 1:10 cell:parasite ratio). After 4 h of culture at 37 °C, the medium containing non-phagocytosed promastigotes was replaced with fresh medium containing inhibitor (6-AN, 5 mM) or activator (LPS + Melatonin). The medium was then removed and the slides were fixed with absolute methanol and stained with Giemsa solution. Each slide was then examined in a light microscope and 100 macrophages/well were counted (in triplicate) for numbers of infected cells and number of amastigotes/macrophage.

2.11. Statistical analysis

Data are reported as mean ± SD values of three independent determinations. Statistical analysis was performed using a one-way

analysis of variance (ANOVA) test; multiple comparisons were made using a Bonferroni's test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects on macrophage viability

Both peritoneal and J774 macrophages were exposed to activator/inhibitor for 24 h and cell viability then assessed using MTT assay. Neither the activator nor inhibitor (except for 10 mM 6-AN) caused significant toxicity (Supplementary Fig. 1).

3.2. Effects on G6PDH activity

Basal activity of G6PDH in lysates of unstimulated peritoneal and J774 macrophages was 120.54 [± 2.84] and 111.40 [± 2.22] IU × mg^{-1} protein, respectively. When the effect of 6-AN (1.25–5 mM) was examined, it was shown that G6PDH activity after 24 h was significantly decreased at all 6-AN tested concentrations - of peritoneal and J774 macrophages (Fig. 1A and B). Cell G6PDH activity was significantly increased (compared to the untreated group) after treatment with stimulator (Fig. 1C and D).

3.3. Effects on nitric oxide production

Basal NO levels with unstimulated peritoneal and J774 cells were, respectively, 3.3 [± 0.9] and 2.4 [± 0.7] μM. Use of 6-aminonicotinamide (1.25–5 mM) significantly inhibited LPS-induced NO production by peritoneal and J774 cells (Fig. 2A and B). Also, bacterial LPS (0.25 ng/ml) + melatonin (1 pM) caused considerable increases in NO formation by both peritoneal and J774 macrophages (Fig. 2C and D).

Comparative study showed that incubation of peritoneal and J774 macrophages with the combination of SNAP and IFN-γ and also LPS and IFN-γ significantly increased, and L-NMMA remarkably decreased, the amounts of NO produced (Supplementary Table 1).

3.4. Effects on intracellular L. major amastigotes in infected macrophages

Leishmanial infectivity (as number of intracellular amastigotes) in untreated peritoneal and J774 macrophages were, respectively, 5.11 [± 0.10] and 5.50 [± 0.10] amastigotes/cell. In comparison with untreated macrophages, treatment with 6-AN (at 5 mM) increased the number of peritoneal and J774 macrophages harboring amastigotes (Fig. 3A and B). In fact, levels of the parasite increased from Day 1, reaching significantly greater levels by Day 2 and thereafter. By Day 4, overall cell survival was compromised regardless of cell type being analyzed (i.e., figures indicate no cells to analyze due to overt cell death). Use of activator (a combination of LPS and melatonin) caused strong inhibitory effects on amastigote growth/survival in the peritoneal macrophages (Fig. 3C). The effect in the J774 macrophages was not statistically apparent until Day 5 after post-infection (Fig. 3D).

4. Discussion

Cutaneous leishmaniasis is an important cause of morbidity and mortality all over the world. Some drawbacks of current anti-leishmania drugs including increasing resistance and toxicity has restricted their usage. Therefore, alternative therapeutic strategy with few side and superior curative effect must be devised [9].

Macrophages are the principal host cells for *Leishmania major* parasites and utilize effector mechanisms to fight against intracellular amastigotes. In interactions between the parasite and infected macrophages, production of nitric oxide (NO) profoundly impact the elimination or persistence of intracellular amastigotes [10,14].

Nitric oxide generated by activated macrophages is primarily mediated by inducible nitric oxide synthase (iNOS) activity, a metabolic process that requires NADPH as cofactor [36,37]. In macrophages, G6PDH is the key enzyme that control cytosolic NADPH formation and also ultimately plays a pivotal role in NO generation [38,39]. This has been demonstrated not only here but in multiple lines of earlier studies.

Immunological stimulation of macrophages with pathogen-associated molecular patterns (PAMPs), including LPS is associated with enhanced G6PDH and iNOS activity, and ultimately, elevated production of NO [40]. Based on the above, it is not surprising that many studies have reported an association between reduced/deficient G6PDH activity in macrophages and an increased incidence of host infections [20–25].

In the field of parasitology, there are many who believe that the development of an effective treatment against leishmaniasis is feasible; however, to date, there is no available treatment against any form of leishmaniasis in humans [8,9]. Considering the importance of G6PDH in the generation of agents in/by macrophages for leishmanicidal functions, the present study aimed to investigate the potential utility of G6PDH modulation in macrophages as a novel target for therapeutic intervention during *L. major* infection.

To achieve this goal, in the present study, mouse peritoneal and J774 macrophages were infected in vitro with *L. major* promastigotes and changes in parasite load as a function of changes induced in cell G6PDH activity (i.e., inhibition by 6-AN or activation by LPS + melatonin) was assessed. Changes in NO release by macrophages were also evaluated since intracellular killing of *L. major* depends primarily on NO production as a result of NADPH-dependent iNOS activity [4,12]. Moreover, treatment with the combination of LPS and melatonin increased considerably the NO production by macrophages as it was previously reported [29–31].

Obtained results demonstrated that when G6PDH activity was increased by treatment with LPS + melatonin, infected macrophages were considerably able to increase their NO production and also suppress intracellular amastigotes formation/survival. Therefore, we can deduce that G6PDH activation could potentially augment host resistance against *L. major* infection. This approach gained support by the finding here that inhibition of G6PDH with 6-AN led to significant reductions in NO release and the ability of the amastigote to be killed by macrophages.

Taken together, the present study confirms the crucial role of G6PDH activity in resistance against leishmania infection. These findings are encouraging especially because they not only suggest an involvement of G6PDH in the leishmanicidal function of macrophages but provide a novel target for potential therapeutics that could be developed to treat leishmaniasis [41].

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.028.

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Declaration of interest

The authors declare no conflicts of interest.

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