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Single and Combined Effect of 17ß-Estradiol on *Leishmania tropica* Killing in Human Macrophages

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Abstract: 17β-estradiol (E_2), the predominant and most biologically active form of estrogen has been shown to modulate various macrophage activities, sometimes with opposite effects, related to experimental conditions. The aim of this study was to investigate the effect of E_2 alone or in combination with PKA inhibitor H-89 or recombinant human chorionic gonadotropin (rhCG) on leishmanicidal activity and cytokines production by human monocytes-derived macrophages infected with *Leishmania tropica in vitro*. Parasite killing by human monocyte-derived macrophages was microscopically evaluated by Giemsa dye. Nitric oxide release was measured by Griess reaction for nitrites. Whereas, pro-inflammatory cytokines release were evaluated by Enzyme-Linked Immunosorbent Assay (ELISA). Results showed that treatment with E_2 reduced the percentage of infected cells, as well as the parasite burden per cell. In addition, macrophages treated with E_2 prior to *L. tropica* infection produced more IL-12(p40), IL-6 and nitric oxide (NO) than *L. tropica* infected-macrophages. However, these leishmanicidal and immunomodulator activities of E_2 on *L. tropica*-infected macrophages were completely abolished when these cells were treated with E_2 in combination with rhCG plus IFNγ. On the other hand, E_2-induced phagocytic activity accompanied with cytokines production in *L. tropica*-infected macrophages greatly increased when the PKA inhibitor H-89 was added to inactivate intracellular PKA signaling pathway. From these data, we conclude that the implication of estrogen in combination with H-89 appears to be an effective strategy to enhance macrophage functions against *L. tropica in vitro*.
Key Words: *Leishmania tropica*, macrophage, estrogen (17β-estradiol), PKA pathway, Cytokines.

1. Introduction

Leishmaniasis is a complex disease with different clinical presentations and is caused by a parasite belonging to the *Leishmania genus* [1]. Although leishmaniasis is a worldwide vector-borne disease affecting 88 countries, studies show that 90% of cutaneous leishmaniasis (CL) cases occur in seven countries including Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. It is estimated that more than 15 million people are infected with this disease [2], with 1.5-2 million new cases per year [3]. Leishmaniasis is now considered as a severe public health problem particularly in developing countries [1, 4].The dimorphic life cycle of *Leishmania* parasites alternates between the flagellated promastigotes, transmitted by phlebotomine sandfly vectors into the vertebrates, and the intracellular amastigotes formed in the mammalian host tissue. *Leishmania* species are capable of parasitizing cells of the mononuclear phagocyte lineage [5], dendritic cells [6] and neutrophils [7], although their definitive host cell is the tissue macrophages. Macrophages play an essential role in innate immunity as well as adaptive immunity, by exerting specific functions, such as removal of debris, dead cells, and microorganisms and the production of both oxygen radicals and cytokines [8]. In addition, It is interesting that macrophages possess androgen [9], estrogen [10] and progesterone [11] receptors and the

treatment with these sex-associated hormones enhances macrophage phagocytic activity [12]. Macrophages, peripheral mononuclear cells, and T cells express surface receptors for 17β-estradiol, whereas B-lymphocytes express intracellular receptors for this molecule [13]. Two major signaling pathways, generally termed genomic and nongenomic, are known to mediate the effects of E_2 on cells [14]. The classical genomic pathways underlying the regulation of nuclear gene transcription by estrogens via the nuclear estrogen receptors alpha and beta (ERα and ERβ) [15], whereas the non-genomic pathways involving plasma membrane-associated ERs that activate a number of intracellular protein kinase-mediated phosphorylation signaling cascades [16]. 17β-estradiol also can bind to G-protein coupled estrogen receptor and mediates nongenomic action through kinase activation and intracellular signaling pathway [17].

Experimental models have shown that sex hormones can directly influence disease resistance or susceptibility and key immunological factors such as cytokines or NO can be differentially modulated as a result of gender or pregnancy. 17β-estradiol is the major circulating estrogen in pre-menopausal females and has a direct role in the modulation of innate immune function. It is widely accepted that 17β-estradiol are critically involved in the control of sexual dimorphism of the immune system [18]. Experimental studies indicated that increased concentrations of estrogen led to enhanced macrophage phagocytic activity [19] and that estrogen as well as progesterone and prolactin stimulated cytotoxic mechanisms through the release of reactive oxygen species [20], and NO production which is critical to *Leishmania* containment [21]. Previous study demonstrated that macrophages and neutrophils from hamster cultured with estrogen or progesterone increase NO production and *L. panamensis* killing [22]. Furthermore, bone marrow-derived macrophages (BMDMs) from female DBA/2 mice were found to be more resistant to *in vitro L. mexicana* infection than male BMDMs and E_2 treatment of both male and female BMDMs enhances parasite-killing and NO production [23]. 17β-estradiol was capable of enhancing the leishmanicidal activity of female BMDMs which was associated with higher levels of IFN-γRα expression, IL-12, IL-6 and NO production [24]. Although these observations, Immune–endocrine associations have not been sufficiently explored in human leishmaniasis. Studies investigating the immunologic mechanisms mediating sex-determined resistance have been mainly focused on animals and animals cells especially in murine and hamster models. Moreover, recent study was demonstrated that the outcome of infection depends on parasite pathogenicity and virulence and also on the genetic background of macrophages, the major target cells for parasite replication, and also involved in the early events of pathogen infection [25]. Therefore, This study was designed to determine if estrogen could enhance immune responses against *leishmania* by studying its effect on parasite killing and cytokines production by human monocytes-derived macrophages infected with the major cutaneous *leishmania* in Syria *L. tropica*. Furthermore, we have examined the efficacy of estrogen in combination with rhCG or PKA inhibitor H-89 to enhance the ability of human monocytes-derived macrophages to kill *L. tropica*.

2. Material and Methods

2. 1. Parasite culture

Leishmania was isolated from patients at the Dermatology Hospital of Damascus University in Syria. Parasites were cultured in semisolid culture medium (Agar, NaCl) containing antibiotics (penicillin/ streptomycin 100 U/ml, Cytogen, Sinn, Germany), followed by daily quantification. When promastigotes appeared in appropriate number (2×10^6), they were transferred to RPMI-1640 culture medium supplemented with L-glutamine (Sigma, Steinheim, Germany), penicillin/streptomycin 100 U/ml, and 10 % heat-inactivated fetal bovine serum (FBS) (Cytogen, Sinn, Germany). Infective metacyclic forms were selected from stationary phase (5th day) and used for macrophage infection. *Leishmania* was typed in our laboratory by PCR/Sequencing, and it showed that it was *L. tropica*.

2. 2. Isolation and differentiation of human monocytes-macrophages

Mononuclear cells were derived from Buffy coat of a healthy volunteer donors without previous leishmaniasis obtained from Blood Center in Damascus. Buffy coat was diluted with 4 volumes of 0.15 M NaCl, and fractionated on a Ficoll Hypaque gradient (1.077 g/ml, 800 xg, 30 min, room temperature (RT); GE Healthcare, Uppsala, Sweden). Mononuclear cells were collected from the interface between the plasma and the Ficoll, washed once with warm (37°C) saline buffer phosphate (PBS) without Ca^{+2} and Mg^{+2} (Sigma, Germany) and then collected by centrifugation at 400 xg for 15 min at RT. In this samples, the red blood cells were treated with lysis buffer (0.15 M NH_4Cl, 0.1 M $KHCO_3$, pH=7.2-7.4) for 2 min at RT and then washed thrice with PBS.

Monocytes were collected by centrifugation and suspended in warm Iscove's Modified Dulbecco's Media (IMDM) culture medium (Sigma, Steinheim, Germany) supplemented with 2 % heat-inactivated human AB serum (Sigma, Steinheim, Germany), and 100 U/ml penicillin/streptomycin (Cytogen, Sinn, Germany) and cell viability was determined by the Trypan dye. Monocytes were seeded onto 12-well plate (Cellstar, Greiner, Germany) at a concentration of 3×10^6 live cells/well in 2 ml of complete IMDM medium, and incubated in a 37 °C cell culture incubator (OSK, Tokyo, Japan) with 5 % CO_2/ 95 % air for 2 hours. Cells were rinsed three times with PBS to remove nonadherent cells, then IMDM culture medium containing 10 % heat-inactivated human AB serum was added. Medium was replaced with fresh media On the 2nd and 5th days of incubation. After 6 days, the monocytes had matured into macrophages.

2. 3. *In vitro* macrophages treatment and infection

Monocytes derived- macrophages were cultured for 5 min at 37 °C, 5% CO_2, 95 % air, in presence of increasing concentrations of 17β-estradiol (10, 1000, 2000 nM) (Sigma Chemical Co., St. Louis, MO, USA), or 5 ng/ml recombinant human interferon gamma (rHu-IFNγ) (sigma, Germany), followed after 6h by addition of 250 U rhCG (positive control) or in the absence of any estrogens (untreated control). In some experiments, 17 β-estradiol and rhCG+IFN-g were added together to the culture. The estrogen and rhCG were dissolved in absolute ethanol, and dilutions made in RPMI, 5 % FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. In other experiments, the PKA inhibitor H-89 (10 µM; Sigma, Germany) was added 2 h before the addition of 17β-estradiol to inhibit the intracellular PKA signaling pathway. After 2 days, macrophages were infected with stationary-phase promastigotes of *L. tropica* at a parasite/macrophage ratio of 5:1 for 2 h at 37°C and 5 % CO2. Extracellular parasites were then removed by gentle washing, and infected macrophages were maintained for a further 2 days. Supernatants were collected for NO detection and cytokine measurement.

2. 4. Evaluation of anti-leishmanial activity

Infected macrophages were fixed with methanol, washed, and then stained with Giemsa. The percentage of infected cells (i.e. number of infected cells per 100 macrophages) and the intensity of infection (i.e. number of parasites per 100 total host cells) were determined by light microscopy under oil (× 1000) by counting 300 cells per well.

2. 5. Measurement of nitric oxide production

For measurement of hormone-induced Nitric Oxide, NO concentrations in the supernatants were determined using Griess reagent kit (Oxford Biomedical, USA) according to the protocols supplied by the manufacturer. In short, 100 µl sample, 90 µl dH_2O, and 10 µl of 30 % $ZnSO_4$ were incubated at RT for 15 min and centrifuged at 800 xg for 5 min. The resulting supernatants were transferred to a tube containing 0.5 g dry granulated Cadmium and incubated at RT overnight with agitation. The samples were centrifuged again, and added to wells (20-100 µl) depending on the NO concentration in sample, then adjusted to 100 µl with dH_2O. Fifty microlitre of color reagent #1 were added followed by 50 µl of color reagent #2, and shaken for 5 min at RT. The absorbance was read at 540 nm by an ELISA reader (HumaReader HS, Human, Germany). Results were extrapolated from a standard curve prepared with Sodium Nitrite (Na NO_2) (0.5–100 µM).

2. 6. Assessment of cytokines production

Cytokines (IL-12 (p40), IL-6, and IL-10) from treated and untreated macrophages supernatants were quantified using sandwich ELISA kits (RayBiotech, Norcross, GA, USA) according to the protocol provided by the manufacturer. Briefly, 100 µl culture supernatants or standards were added to the capture antibodies coated wells of the 96 well ELISA plate. The plate was covered and incubated at room temperature for 2 h 30 min. The solution were then discarded and the wells were washed 4 times with wash buffer (300 µl). After that, 100 µl biotinylated anti-human cytokine antibody was added to each well and the plate was incubated at room temperature with continuous but gentle shaking. After 1 h, the wells were washed with wash buffer as previous. Then, 100 µl of horseradish peroxidase (HRP)–streptavidin conjugate solution was added and the plate was incubated for 45 min at room temperature with gentle shaking. Next, the wells were washed as previous and 100 µl 3,3,5,5′-tetramethylbenzidine (TMB) one-step substrate reagent was added followed by 30 min incubation at room temperature in dark. Then 50 µl of 0.2 mol/l sulphuric acid was added to each well to stop

the reaction. Absorbance was immediately measured at 450 nm and 490 nm using an ELISA reader (HumaReader HS, Human, Germany).

2. 7. Replication of Experiments and Statistical Analysis

All experiments were performed in duplicate and repeated at least three times. Differences between groups were compared by one-way ANOVA. The Kruskal-Wallis post hoc test was used to compare the means obtained from infection, NO and cytokines levels for the various treatment groups. P values ≤ 0.05 were considered statistically significant. Statistical software SPSS version 15 (SPSS Inc.,Chicago, IL, USA) was used in this data analysis.

3. Results

3. 1. Leishmanicidal activity of 17β-estradiol

In order to evaluate whether 17β-estradiol had an anti-leishmanial activity, rhCG was used as positive control against *L. tropica*. Our results showed that rHu-IFNγ-primed macrophages treated with rhCG at 250 U/ml (the more effective rhCG concentration) showed significant decrease in the median percentages of infected macrophages as well as the median number of amastigotes per macrophage. Also, as shown in Fig. 1a and 1b, E_2-treated macrophages displayed also significantly lower infection rates and contained fewer parasites compared to macrophage infected with *L. tropica*. The action of 17β-estradiol was dose-dependent, and the highest significant changes were observed in cells treated with E_2 at the concentration of 1000 nM. At this concentration, the median percentages of infected macrophages were decreased from 85 % to 54 % and the median number of amastigotes per macrophage were also reduced from 9 to 5.5. Thus, E_2 can also enhance macrophage phagocytic activity, but this effect was less potent than rhCG.

Intriguingly, this leishmanicidal activity of E_2 on *L. tropica*-infected macrophages was completely abolished in *L. tropica*-infected macrophages treated with 1000 nM E_2 in combination with 250 U/ml rhCG (see Fig. 1a and 1b).

In contrast, the median percentages of infected macrophages and the median number of amastigotes per macrophage were decreased by an average of 72 % and 64 %, respectively when *L. tropica*-infected macrophages were treated with 1000 nM E_2 in combination with 10 µM H-89 (the concentration used in most cell-based studies to inhibit PKA activity). This reduction in the infection rates and levels was much higher than in macrophages treated with either E_2 or hCG alone. (see Fig. 1a and 1b).

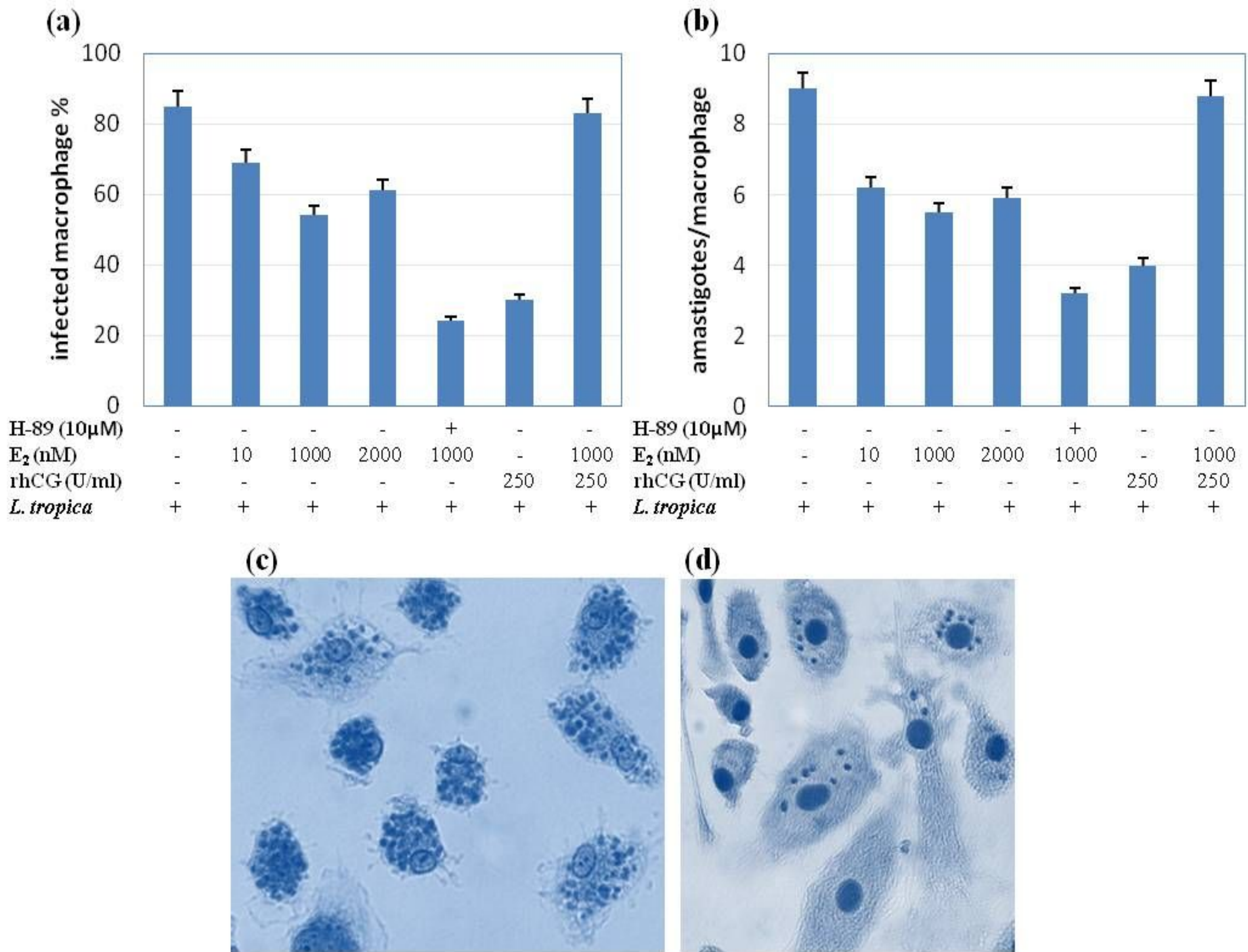

Figure 1. Macrophages were exposed *in vitro* for 5 min to estrogen (17-β estradiol; 0 – 2000 nM), primed with IFNγ (5 ng/ml) and after 6h rhCG (250 U/ml) was added, or treated with 1000 nM E_2 in combination with 250 U/ml rhCG. The PKA inhibitor H-89 (10 µM) was included 2h before estrogen treatment and macrophages were infected with *L. tropica* (ratio 1:5) 2 days after macrophages treatment with hormones. Within 2 days of culture, infection rates and levels were measured by microscopic examination of Giemsa-stained cells and counting 300 cells, at each E_2 concentration. Histograms showing both median percentages of *L. tropica* infected-macrophages (a) and median number of *L. tropica* amastigotes (b). Microscopic images of Giemsa-stained cells showing the number of E_2-untreated macrophages infected with *L. tropica* (c) compared to the number of macrophages treated with 1000 nM E_2 in presence of H-89 followed by *L. tropica* infection (d).

3. 2. Stimulation of NO Production by 17β-estradiol

Macrophage treated with rhCG produced NO after *L. tropica* infection comparing with untreated macrophages. Similar effect of rhCG on NO production was observed with increasing concentrations of 17β-estradiol (10, 1000, 2000 nM), but statistically significant differences of NO production were not found between different concentrations ($p > 0.05$). However, rhCG was more effective than E_2. Furthermore, when macrophages were cultured in the medium containing E_2 with rhCG plus IFN-γ, the E_2-stimulated NO production was completely inhibited ($p < 0.01$); the NO production was similar to that observed with the untreated *L. tropica* infected-macrophages.

On the other hand, H-89 at a dose of 10 µM had no significant effect on the estrogen-induced NO release (see Fig. 2).

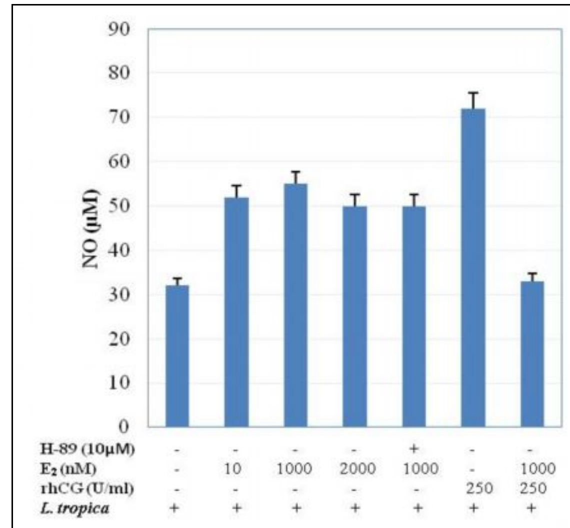

Figure 2. A diagram showing the effect of E_2 alone, in combination with rhCG, or in presence of H-89 on NO production by macrophages infected with *L. tropica*. Macrophages were treated with E_2 (10, 1000, 2000 nM) alone or in combination with rhCG (250 U/ml). 2 h before the addition of E_2 10 µM H-89 were added. Macrophages were infected with *L. tropica* then, after 2 days of culture, the culture supernatants were analyzed for NO production by Griess reagent kit. Histograms indicate mean values ± the standard deviation from three separate identical experiments.

3. 3. Modulation of cytokine secretion by 17β-estradiol

The concentrations of three cytokines were determined on the same supernatants used for nitric measurement. Our findings indicate that IL-12 (p40), IL-6 and IL-10 production was detected in the culture supernatant of *L. tropica*-infected macrophages, and the positive control stimulation, rhCG leads to a great increase of IL-12 (p40) and IL-6 production ($p < 0.05$). Similarly, increasing concentrations of estrogen caused a significant increase of both cytokines production from cells infected with *L. tropica*. This effect was dependent on hormone concentration (Fig. 3a and 3b). However, these effective doses of E_2 did not activate macrophage to produce IL-10 (see Fig. 3c). Moreover, This increase in the production of IL-12 (p40) and IL-6 was disappeared when cells treated with both steroids in combination (250 U/ml rhCG+ 1000 nM E_2) (Fig. 3a and 3b).

However, human macrophages that were treated with 1000 nM 17β-estradiol in presence of 10 µM H-89 then infected with *L. tropica* produced large amounts of IL-12 (p40) and IL-6, when compared to those observed in E_2-treated macrophages in absence of H-89 ($p < 0.01$) (see Fig 3a and 3b). In contrast, blockade of PKA with 10 µM H-89, caused no significant difference in the production of IL-10 in E_2-treated macrophages (see Fig 3c).

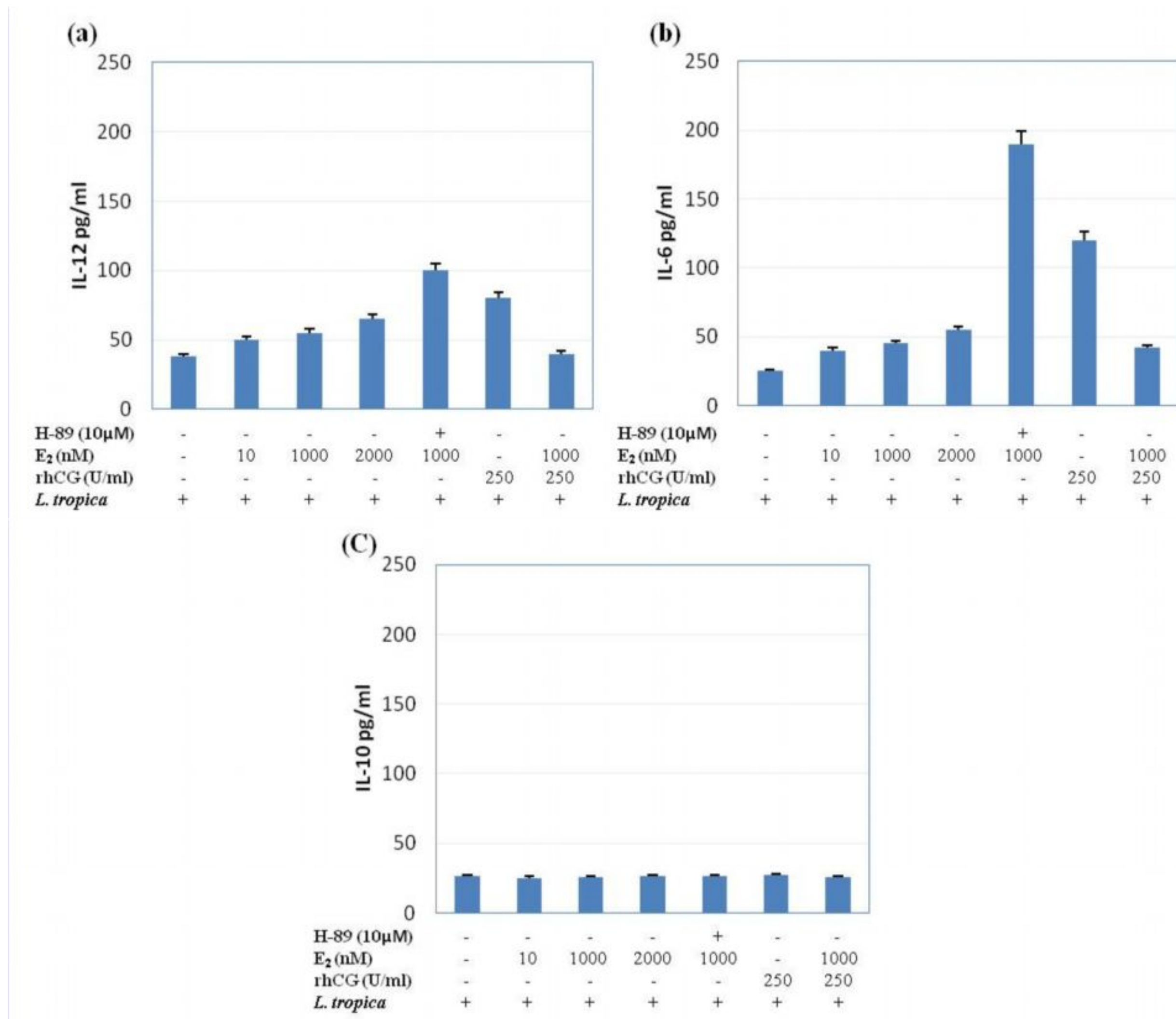

Figure 3. Macrophages were cultured for 5 min with increasing concentrations of 17-β estradiol, or 5 ng/ml IFNγ followed after 6 h by addition of 250 U⁄ml rhCG (positive control),or 1000 nM E_2 in combination with 250 U/ml rhCG. the PKA inhibitor H-89 (10 µM) was added 2 h before the addition 17β-estradiol to inhibit the intracellular PKA signaling pathway. After 2 days, macrophages were infected with *L. tropica*. Two days later, The supernatant, was harvested and assayed for IL-12 (p40) (a), IL-6 (b) and IL-10 (c) by ELISA assay. The histograms shown are representative of the mean ± the standard deviation of three separate identical experiments.

4. Discussion

Previous studies underscore the importance of hormones in the regulation of the immune response and support the concept that physiological changes that are associated with varying levels of circulating hormones can impact the balance between host and pathogens such as *Leishmania* [22]. Recently, we demonstrated that human chorionic gonadotropin (rhCG) has an leishmanicidal and immunomodulatory effect on human macrophages by increasing *L. tropica* killing and NO, IL-12(p40) and IL-6 production [26]. The best known function of hCG is to induce the production of progesterone and estrogen by the corpus luteum during early pregnancy [27]. Moreover, E_2 the major circulating estrogen in pre-menopausal females was found to regulates production of proinflammatory cytokines by macrophages [28]. For these reasons, we investigate in the present study the effect of estrogen on immune response against *Leishmania*. Moreover, estrogen and rhCG or estrogen and PKA inhibitor H-89 were combined, and all experiments were evaluated by studying intracellular *Leishmania* death and pro-inflammatory cytokines production by human macrophages infected with *L. tropica in vitro*.

Our results demonstrated that treatment of human macrophages for 5 min with increasing concentrations of E_2 (10–2000 nM) prior to *L. tropica* infection significantly increased the NO production, but there was no dose-dependent effect. 17β-estradiol has also been shown to decrease the number of infected macrophages as well as the number of amastigotes per macrophage in a dose-dependent manner. These data suggest that the E_2-mediated increase in NO production is not sufficient itself to affect parasite killing and that there must be other E_2-induced effector mechanisms that act additively or synergistically with the generation of NO. This is consistent with our results showed that the increased leismanicidal activity of human macrophages treated with E_2 prior to *L. tropica* infection was accompanied by significant increase in IL-12 (p40) and IL-6 secretion, typically associated with classical macrophage microbicidal activity and driving to the generation of a Th1 response characteristic of the resistance phenotype [29]. This immunomodulatory effect of E_2 was in a concentration-dependent manner. In contrast, treatment with all E_2 concentrations tested did not produce any significant response in IL-10 levels, which may be a result of the simultaneous induction of IL-12 production by E_2. IL-10 is the most anti-inflammatory cytokine known [30]. It inhibits the secretion of pro-inflammatory cytokines [31], suppresses phagocytosis [32] and intracellular killing leading to Th2 responses [33]. However, there is contradictory evidence about the role of estrogen in modulating pro-inflammatory cytokines and NO production. 17β-estradiol has been shown to exert pleiotropic effects on the production of pro-inflammatory cytokines and NO in murine macrophages *in vitro*; estrogen with low levels exerts a pro-inflammatory effect driving a Th1 response that results in resistance to *leishmania* infection. Whereas, with high levels, E_2 shows an anti-inflammatory effect with Th2 response characteristic of the susceptible phenotype [34]. However, In our study, we provide evidence that over a wide dose range of E_2 (10 – 2000 nM), a strong enhancement of IL-12 (p40) and IL-6 production was observed, which suggest that the timing of E_2 exposure could be more important than the dose of E_2 on macrophages responses. Previous data support this hypothesis and show that treatment with low to high doses of E_2 for short time increases IL-12 and IL-6 production and thus induced a strong enhancement of Th1 responses [35]. In contrast, other study found that high concentration of E_2 rendered macrophages from female C57BL/6 mice resistant to *L. mexicana in vitro*, but had no effect on male-derived C57BL/6 macrophages and this sex-associated resistance has been attributed to an enhancement of macrophage microbicidal activity and the modulation of cytokine production by 17β-estradiol [23, 24, 36]. Moreover, previous studies demonstrated that there are differences in macrophage NO production and regulation among different species [37, 38]; E_2-treated *L. mexicana* infected-macrophages from female C57BL/6 mice produced more NO, IL-12, and IL-6 than both the E_2-treated male-derived macrophages and untreated controls [24]. Whereas, the treatment by E_2 of *L. mexicana*-infected macrophages from DBA/2 mice, enhances leishmanicidal activity in both female and male DBA/2-derived macrophages by increasing NO production but not the pro-inflammatory cytokines IL-6, IL-12 (p70) or TNF-α [23]. Furthermore, macrophages from pregnant infected hamsters had enhanced capacity to restrict *Leishmania* replication, but this increased leismanicidal activity of hamster macrophages during pregnancy was accompanied by a general decrease in expression of IFN-γ, IL-12, and TNF-α [22]. It is possible that this difference in the ability of estrogen to transduce signals in macrophages isolated from different gender and species may be dependent on their differential expression of estrogen receptors (ERs) [39]. However, other studies showed that differences in macrophage responses was associated with differences in the *Leishmania* strain used. For example, Macrophages from nonpregnant hamsters infected with *L. panamensis* released high amounts of NO upon estrogen treatment [22]. Similarly, both female- and male-derived macrophages treated with E_2 were more resistant to *in vitro* infection with *L. mexicana* and produced more NO as compared with sham controls [23]. In contrast, pregnant C57BL/6 mice infected with *L. major* were more susceptible than nonpregnant controls [40]. Also, a few observations in humans indicated that women infected subclinically with *L. donovani* or *L. infantum* developed overt disease during gestation [41].

The other important observation of our study is that although E_2 alone or rhCG alone significantly increased leishmanicidal and immunomodulator activity in *L. tropica*-infected macrophages, these effects were inhibited when infected cells were treated with E_2 in combination with rhCG. These data are in agreement with Mor statements, which indicate that the pregnancy is associated with a unique immunological condition, characterized by decidual, as well as peripheral immune responses control in order to guarantee maternal tolerance to the foetus [42]. It is known that the hCG level peaks in the first trimester of pregnancy and then declines to a stable level in the remaining second and third trimesters, while progesterone and estrogen continue to increase until the end of the pregnancy [43]. Thus, the fact that the production of NO, IL-12 (p40) and IL-6 are down-regulated indicates that it may probably have harmful effects to fetal development and pregnancy progression. Furthermore, mechanisms limiting inflammation are crucial for maintenance of immune

homeostasis. Thus, application of such combination is a potential therapeutic approach for treating certain inflammatory diseases.

On the other hand, we investigated in this study the role of PKA inhibitor in the *leishmania* killing by human macrophages treated with estrogen. Our results showed that the PKA inhibitor H-89 was not able to change NO production in E_2 treated-macrophages, which implies that the PKA pathway is not implicated in the E_2-evoked NO production in *L. tropica*-infected macrophages. In fact, H-89 significantly raised the increased IL-12(p40) and IL-6 production in *L. tropica* infected-macrophages upon estrogen treatment, which was accompanied with further decrease in the level and the rate of infection as compared with E_2 treated *L. tropica* infected-macrophages in absence of H-89. This suggests that a different mechanism that act additively with the mechanism of E_2 action may be involved in H-89 induced pro-inflammatory cytokines production and *L. tropica* killing in human macrophages. This conclusion is supported by the following observations: First, PKA activation is a critical mechanism that *leishmania* exploit to survive within macrophages and the inhibition of PKA in *leishmania*-infected macrophages could restore and enhance human macrophage functions against this infection [44]. Second, recent study was demonstrated that H-89 alone exhibits leishmanicidal activity against both extracellular promastigotes and intracellular amastigotes within macrophages [45]. Third, early studies performed elsewhere showed that cAMP/PKA signaling pathway could inhibit the expression of pro-inflammatory cytokine genes of macrophages, such as IL-1, IL-6, TNFα, and IL-12 [46, 47]. In contrast, it could upregulate the expression of anti-inflammatory cytokines genes, such as IL-10 and IL-1 Rα [46]. fourth, PKA pathways have been reported to be involved in the nongenomic E_2 cascade in various types of cells [16]. But human monocytes and macrophages express soluble intracellular receptors (ERα and ERβ) that mediates genomic signaling pathway [48].

5. Conclusion

Given the reported importance of sex- and pregnancy-associated hormones in influencing the outcome of infectious diseases, the present study underscore the importance of the pregnancy-related hormones estrogen in the reinforcement of macrophage functions involved in host defense against *L. tropica* infection. The anti-leishmanial and immunomodulatory effects of estrogen were abolished in presence of rhCG, but were strongly enhanced in presence of the PKA inhibitor H-89. The results suggest that estrogen and the present inhibitor might be exploited as a target for new drugs against leishmaniasis.

Acknowledgments

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Conflict of Interests

The authors of this study declare that there is no conflict of interests related to the present work.

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