Research Square

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Evaluation of immune cellular enzymes, oxidative and nitrosative Stress in Saudi Patients with Cutaneous Leishmaniasis in Al-AHssa, before and after treatment

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Research article

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Abstract

Background Macrophages, within which Leishmania sp. replicate, generate large amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to kill these parasites.

Methods The aim of the present study was to assess oxidative, nitrosative stresses, and some immune enzymes in blood of cutaneous leishmaniasis (CL) patients before and after treatment as well as in control individuals. Serum activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidas (GSH-Px) and the levels of reduced glutathione, malondialdehyde (MAD) and nitric oxide (NO) as well as L-arginase, myeloperoxidase (MPO), adenosine deaminase (ADA) have been studied.

Results The activities of the L-arginase, MPO and ADA, the levels of MDA and NO are significantly elevated ($P < 0.001$), while that of SOD, CAT, and GSH-Px, and GSH level were significantly ($P < 0.001$) reduced in untreated patients compared with the corresponding activities of the treated and control individuals. The treatment ameliorated these agents in comparison to the untreated group but there was still variations between the values of treated and control groups.

Conclusion These results suggested that oxidative and nitrusative stress may play an important role in the pathogenesis of untreated cutaneous leishmaniasis

Background

Leishmaniasis is a disease caused by several species of the flagellated protozoa belonging to the genus Leishmania, and is transmitted by the bite of the female sand fly [1]. Leishmania are widely distributed in tropical and subtropical regions through the world, including Al-Ahsa, Eastern province of Saudi Arabia [2]. Infectious leishmaniasis, which infect and replicate within macrophages and other phagocytic cells, is accompanied by generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which contribute to the regulation of the inflammatory response controlled by the cellular antioxidant defense system [3].

Myeloperoxidase (MPO) is a heme-containing peroxidase. It is the most abundantly expressed in immune cells, such as neutrophilic polymorphonuclear leukocytes (neutrophils). The antibacterial activities of MPO involves the production of reactive oxygen and reactive nitrogen species, as well as hypohalous acids and so is involved in the killing of several micro-organisms and foreign cells [4]. It has been reported that MPO is one of the best inflammatory and oxidative stress markers among these commonly occurring diseases [5].

L-arginine, essential amino acid, acts as a double-edged sword so that while it is needed for nitric oxide synthase (iNOS) to produce nitric oxide (NO)-mediated parasite killing and/or inhibiting parasite growth, it can also be hydrolyzed by L-arginase generating polyamine or collagen-mediated parasite replication [6]. Several studies have shown that the activities of both (L-ARG) and iNOS are significantly increased in Leishmania parasites infection in mice and in lesions of patients with cutaneous leishmaniasis 7, 8].

Adenosine deaminase (also known as adenosine aminohydrolase, or ADA, E.C.3.5.4.4.) is widely distributed in human tissues and is higher in macrophages and lymphoid tissues. It is mediates deamination of the anti-inflammatory nucleoside adenosine to inosine and thus it has a role in maintenance of cellular immunity [9]. It was reported that high serum ADA activities were observed in patients with acute hepatitis, chronic active hepatitis, and in sera and lymphocytes of CL patients [10].

Various therapeutic modalities have been used for treating cutaneous leishmaniasis. Intralesional pentavalent antimonial injection is one of the effective therapeutic modalities and remain the main therapeutic agents for various forms of leishmaniasis. The 2 available preparations, sodium stibogluconate (Pentostam), and meglumine antimonate (Glucantime), have similar efficacy [11].

Therefore, the present study would evaluate the oxidative, nitrosative and the immune cellular enzymes in Saudi patients infected with cutaneous leishmaniasis before and after treatment. For this purpose, blood activities of SOD, CAT, GSH-Px, L-arginase, ADA and MPO as well as the levels of MDA and NO were estimated in healthy control groups, untreated and treated patients group in Al-Hssa, Eastern region in Saudi Arabia.

Methods

Chemicals: Most of parameters under study were measured by using kits of high quality.

Ethics approval and consent to participate: Written informed consent was obtained from all participants enrolled in the study. The study protocol was approved by the ethical committee of College of Medicine, King Faisal University, Al-Ahsa in collaboration with the King Fahd Hospital, Saudi Arabia.

Subjects

The present study included 50 male subjects infected only with cutaneous leishmania selected from Center for the Control of Vector Control Leishmania and Malaria, Al-Ahsa City, Saudi Arabia while their age and gender matched controls (N=30) were randomly selected from those attending other clinics or accompanying patients and exhibiting no cutaneous lesions or prior CL history. The duration of illness for CL subjects 4-6 months. Fasting 10 ml blood in the morning were withdrawn from both groups separately at the leishmanial center, Al-Ahssa, Saudi Arabia and immediately transferred from the center of leishmania to our laboratory at the College of Medicine, King Faisal University in an icebox. Each sample was centrifuged at 4000 rpm and the serum was separated and stored at -20^{o}C until analysis. After withdrawing the blood samples from the untreated patients, they were treated by intramuscular injection of sodium stibogluconate (20 mg/kg/day intramuscularly) for 28 days. After 60 days after stop treatment (i.e. after 28 days treating), 10 ml blood freshly samples were withdrawn from the treated patients. The number of the subjects who followed up after treatment is 41. All subjects did not have any medicine for at least a month before taking the blood sample.

Inclusion Criteria: The study was performed in the steady state, i.e., free of acute clinical illness. Furthermore, the patients included in the present study suffered from only CL, while patients who suffered from other diseases CL were excluded.

Biochemical Analysis

I- Assays of immune enzymes

i) Estimation of L-Arginase activity (mU/g protein)

The blood arginase activity (ARG, EC. 4.2.1.11) was determined by the method of Iyamu et al. [12]. The L-ornithine produced from L-arginine by ARG was measured to determine the activity of the ARG. ARG activity was expressed in µmol L-ornithine/ min/ per g protein [U/g].

ii) Estimation of Myeloperoxidase activity

Myeloperoxidase activity was measured spectrophotometrically according to the method described by Krawisz et at., [13]. 0.1 ml of serum was combined with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml 0-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured with a Boeco S-20 spectrophotometer (Boeco S-20 Spectrophotometer, Hamburg, Germany). One unit of MPO activity was expressed as U/g protein

iii) Estimation of Adenosine deaminase activity:

Serum ADA activity was measured spectrophotometrically according to the method of Giusti and Galanti [14], in which NH_3 generated via the effect of ADA on Adenosine (its substrate). The blue color formed from the reaction of NH_3 with indophenol is measured spectrophotometrically at 628 nm. ADA levels were calculated and expressed in unit per g protein (U/g protein).

II- Assays of Antioxidants/Oxidant products

i) Estimation of Superoxide dismutase (SOD) activity. The method described by Halliwell and Gutteridge [15], was used to estimate the total SOD activity spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) by using test kit obtained from SpinReact Biodiagnostic, Cairo, Egypt. The activity was expressed as percentage of inhibition of formazan/g protein.

ii) Estimation of Catalase (CAT) activity. CAT activity was measured measured spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) according to the Johansson and Borg [16], method using a standard CAT assay kit Biodiagnostic, Cairo, Egypt, through following the decomposition rate of H_2O_2 at 240 nm. The results were expressed as U/g protein.

iii) Estimation of Glutathione peroxidase (GSH-Px) activity. GSH-Px activity was assayed based on the decrease of NADPH absorbance at 340 nm according to the method of Flohé and Günzler [17], by using a diagnostic kit provided by Biodiagnostic, Cairo, Egypt. The results were expressed as mU/g protein.

iv) Determination the reduced glutathione (GSH) concentration

Reduced glutathione (GSH) concentration was determined using the method described by Beutler et al. employing 5,5'-dithiobis(nitrobezoic) acid forming with glutathione thiol groups colored adduct, with spectrophotometric measurement at 412 nm. The results are expressed as µmol/g protein 18].

v) Determination of malondialdehyde (MDA) level. MDA, an end product of Lipid peroxidation, was measured by the method as outlined by Esterbauer et al., [19] in which MDA reacts with thiobarbutiric acid (TBA), forming a colored thiobarbituric acid reactive substance (TBARS) complex which was quantified spectrophotometrically at 535 nm by using a diagnostic kit supplied by Biodiagnostic, Cairo, Egypt. The amount of TBARS was calculated using an extinction coefficient of 1.56 × 10−5/M/cm and expressed in nanomoles of MDA per gram protein.

vi) Determination of NO level: The produced nitric oxide (NO) was determined indirectly by measuring the nitrite levels based on Griess reaction [20].

vi) Serum Protein Estimation: Serum protein content was determined according to the method of Bradford [21] using bovine serum albumin as standard.

Results

Table-1: Cellular immune enzymes' activities: L-arginase, Myeloperoxidase (MPO), and Adenosine deaminase (ADA) in blood of untreated, treated cutaneous Leishmaniosis patients and control group

Parameters	Control (n = 30)	Untreated (n = 50)	Treated (n = 41)
L-Arginase	14.64 ± 2.84	58.78 ± 7.97[a,b]	16.57 ± 4.10[c]
MPO	12.19 ± 2.35	165.59 ± 15.07[a,b]	32.83 ± 9.93 [c]
ADA	18.67 ± 2.62	102.90 ± 17.28[a,b]	25.65 ± 7.89 [c]

Values expressed as Mean ± SD

[a]P < 0.001 highly significant compared with values of control group

[b]P < 0.001 highly significant compared with values of treated group

[c]P < 0.01 Significant for L-arginase, and [c]P<0.001 for MPO & ADA compared with the values of control group

The activities of L-arginase, MPO and ADA in the serum of the subjects before and after treatment and of control healthy group are represented in Table-1. The activities of these enzymes were high significantly

elevated in blood of untreated patients group compared with the corresponding values of control[a] and treated subjects' groups[b]. The activities of these enzymes were found to be significantly decreased (Table-1) in follow-up cases (n = 41) after 30 days with sodium stibogluconate treatment at a dose of 20 mg/kg/day intramuscularly compared with that of untreated patients[b]. The activities of these enzymes in treated group were significantly different from that of the control group[c].

Table-2: Levels of GSH, NO and MDA in blood of untreated, treated Human Cutaneous Leishmaniosis patients and control group

Parameters	Control (n = 30)	Untreated (n = 50)	Treated (n = 41)
GSH	67.54 ± 21.45	16.33 ± 2.39[a,b]	55.80 ± 16.37 [c]
NO	28.19 ± 8.53	100.28 ± 29.95[a,b]	40.01 ± 16.45 [c]
MDA	32.53 ± 6.98	188.18 ± 29.4[a,b]	43.58 ± 15.66 [c]

Values expressed as Mean ± SD

[a]P < 0.0001 highly significant compared with values of control group

[b]P < 0.001 highly significant compared with values of treated group

[c]P < 0.001 Significant for GSH, and NO, and [c]P < 0.0001 for MDA compared with the values of control group

Table-2 showed a significant decrease in the levels of GSH (P < 0.001), while the levels of NO, and MDA were significantly higher (p < 0.001) in the untreated patients compared with the values of control[a] and treatment groups[b]. The GSH level was significantly increased, while the levels of both MDA and NO were significantly decreased after 30 days in intramuscularly treated patients group with sodium stibogluconate compared with the values of untreated patients group[b]. There was a significant difference of these parameters between the control and treatment groups (Table-2).

The blood activities of SOD (Figure-1-A), CAT (Figure-1-B), and GSH-Px (Figure-1-C) were high significantly (P < 0.001) reduced in untreated patients group infected with CL compared with the corresponding activities of control[a] and treated patients[b] groups. The activities of these enzymes in intramuscularly treated patients group with sodium stibogluconate compared with the values of untreated patients group[b]. There as a significant difference in the activities of these enzymes was obtained between the control and treatment[c] groups.

Discussion

The present study was aimed to verify the occurrence of oxidative stress in untreated patients infected by CL and how can the drug used could ameliorate this oxidative stress. A significant reduction in the antioxidants, e.g. the activities of SOD, CAT and GSH-Px as well as the GSH levels (Figs. 1-A, 1-B, & 1-C respectively) with the elevation of oxidative products, e.g. MDA (Table 2), and nitric oxide (NO) have been reported in the blood of untreated CL patients of the present study. The depletion of these protective antioxidants may be attributed to the overproduction of reactive oxygen species (ROS), e.g. superoxide (O_2^-.), hydrogen peroxide (H_2O_2) and hydroxyl radicals (.OH) and reactive nitrogen species (RNS), e.g. NO, in response to phagocytosis by monocytes/macrophages as a host defense mechanism for killing of engulfed Leishmania [22, 23]. Furthermore, the reduction in the blood GSH-Px activity in untreated CL patients compared to the results of control and treated patients groups (Fig. 1-C) may be attributed to the reduction of Selenium which is required for the activity of GSH-Px[24]. This argument has been shown in the study of Koçyiĝita et al., [25] which has found a significant decrease in serum Selenium and its related enzyme glutathione peroxidase in patients with Cutaneous Leishmaniasis. Furthermore, the decreased GSH-Px activity in the present study may be also due to the reduction in GSH concentration because GSH is in conjunction with glutathione peroxidase [26].

4 strategies of organisms to kill the parasite) with the depletion of the protective antioxidants (Table 2) and thus are capable of damaging lipids and other biomolecules [22, 29]. Furthermore, in the present study, NO level is significantly elevated in blood of untreated CL patients than in control or treated patients (Table 2). It has reported that NO reacts rapidly with superoxide anion ($O_2^{\cdot -}$) producing highly cytotoxic peroxynitrite (is unique as a lipid oxidant), which may potentiate lipid peroxidation increasing MDA 30]. In addition, the elevation of MDA may be due to the increased activity of MPO in untreated CL patients (Table 1). This argument has been reported by the study of Zhang et al., which found that MPO can oxidize tyrosine and nitrite into tyrosyl and nitrogen dioxide ($^{\cdot}NO_2$) radicals (i.e. reactive intermediates) which in turn, can oxidize lipids in plasma and the cell membrane leading to elevation of MDA [31].

Myeloperoxidase (MPO) is believed to be a front-line defender against phagocytosed microorganisms via generation of reactive oxygen and reactive nitrogen species 32]. Furthermore, MPO is released into the extracellular fluid after oxidative stress and different inflammatory responses. Thus, the elevation of MPO in untreated CL patients group (Table 1) is related to the remarkable increase in the production of oxygen and nitrogen metabolites in response to phagocytosis by macrophages as a host defense mechanism for killing of engulfed cutaneous leishmaniasis and may amplify the leishmanicidal activity in infected patients [33].

The results of this study showed that serum L-arginase activity and the NO level are elevated in CL patients (Table 1). The infected macrophages with CL metabolize arginine by two enzymes; inducible nitric oxide synthase (iNOS) or L-arginase. It is reported that the balance between these two enzymes are competitively regulated by type 1 (Th1) and type 2 (Th2) T helper cells respectively via their secreted cytokines [34]. Furthermore, both Barbosa et al., and Papadogiannaki et al., [35, 36], showed the balance

between Th1 and Th2 cytokine profile in the blood and tissues of dogs infected by leishmania infantum respectively. The type 1 cytokine (notably IFN-γ and TNF-α) induces expression of iNOS, while the type 2 cytokine (including IL-4, IL-10 and IL-13) induces L-arginase activity [37]. Thus, the elevation of serum L-arginase activity and NO levels may be attributed to the balance in Th1 and Th2 cytokines production in inflammatory cells infected with CL [34]. Furthermore, the results of the study of Miralles et al., [38] indicated that mRNAs for IFN- γ, IL-2, IL-4, and IL-10 were all induced by L. donovani infection.

It has been reported that adenosine deaminase (ADA) is a cytoplasmic enzyme which activity is elevated in in disorders that stimulate cells involved in the immune system [39]. The elevation of serum ADA activity in untreated CL patients may be attributed to the elevation of adenosine, the substrate of ADA which has been confirmed by other studies [10, 40]. On the other hand, the increased serum ADA activity in patients with cutaneous leishmaniasis may be a reflection of induction of phagocytosis of macrophages and the increased cellular immunity by IFN-γ and IL-2, which are synthesized by Th1 cells [41].

Our findings revealed that the injection of CL patients with Sodium stibogluconate and meglumine antimonite ameliorated the activities of SOD, CAT, GSH-Px, MPO, ADA and L-arginase, and the levels of MDA, NO and GSH nearly to corresponding values of both control and treaded patients groups (Tables 1, 2, and Figs. 1A, 1B, 1C). This amelioration effect of the present drug may be due to that Sodium stibogluconate and meglumine antimonite possess antileishmanial potency by enhancing non-specific immunity by macrophages activation and inducing NO, interferon-γ and tumor necrosis factor (TNF)-γ during treatment, thereby those chemicals and cytokines produce fundamental host defense system and kill the invading parasite [42].

Conclusions

These findings indicate that the enhanced capability of activated macrophages to resist infection is related to the remarkable increase in the production of reactive oxygen and nitrogen species (ROS and RNS) in response to phagocytosis to kill the parasite.

Evaluation of the activity of ADA and L-arginase in serum of patients with cutaneous leishmaniasis can be considered a useful tool for monitoring their clinical status. Therefore, both ADA and L-arginase may be predictive and sensitive parameters of leishmaniasis treatment

Furthermore, it is found that ADA activity increases in diseases where cellular immunity is involved as in leishmaniasis. So, the assay of ADA activity in the serum and other biologic fluids is very useful for an accurate diagnosis of many pathological conditions.

MPO concentrations in blood may be considered as a marker of neutrophil activation and degranulation [43].

Abbreviations

CL: cutaneous leishmaniasis

SOD: superoxide dismutase

MDA: Malondialdehyde

CAT: catalase

GSH-Px: glutathione peroxidase

GSH: reduced glutathione

NO: nitric oxide

MPO: myeloperoxidase

ADA: adenosine deaminase deaminase

ROS: reactive oxygen species

RNA: reactive nitrogen species;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' Contributions

M.A.S., and H.I.M. conceived the scientific idea of the present manuscript, H.I.M. collected the blood samples, M.A.S. performed the experiment, with the assistance of the technician, Mr. Hussain Alnwaisser,

carried out the data analysis, discussed the experimental results, wrote the manuscript, and reviewed the manuscript.

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Figures

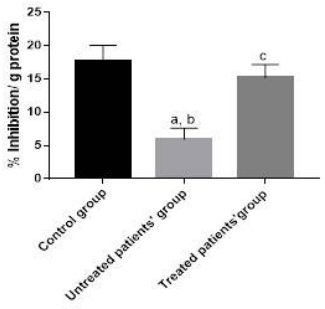

Figure -1-A: Activity of superoxide dismutase (SOD) in blood of control health group, untreated and treated Human Cutaneous Leishmaniosis patients

[a]P < 0.001 Highly significant compared with values of control group

[b]P < 0.001 Highly significant compared with values of treated group

[c]P < 0.01 Highly significant compared with the values of control group

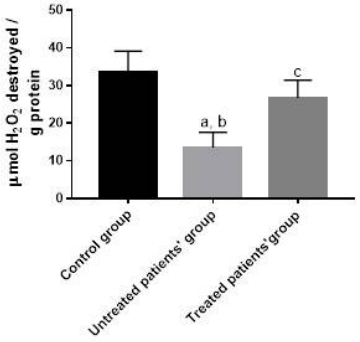

Figure -1-B: Activity of Catalase (CAT) in blood of control health group, untreated and treated Human Cutaneous Leishmaniosis patients

[a]P < 0.001 Highly significant compared with values of control group

[b]P < 0.001 Highly significant compared with values of treated group

[c]P < 0.01 Highly significant compared with the values of control group

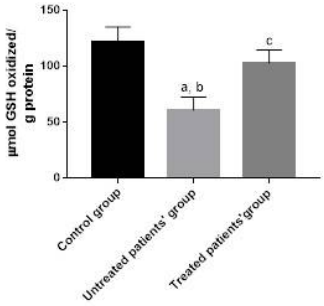

Figure -1-C: Activity of Glutathione peroxidase (GSH-Px) in blood of control health group, untreated and treated Human Cutaneous Leishmaniosis patients

[a]P < 0.001 Highly significant compared with values of control group

[b]P < 0.001 Highly significant compared with values of treated group

[c]P < 0.01 Highly significant compared with the values of control group

Figure 1

(caption included in figure)