EFFICACY OF PENTAVALENT ANTIMONIAL INJECTION IN IMPROVING THE OXIDATIVE, NITROSATIVE STATUS AND IMMUNE CELLULAR ENZYME ACTIVITY IN TREATING SAUDI PATIENTS WITH CUTANEOUS LEISHMANIASIS

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Abstract

Macrophages, within which Leishmania sp. replicate, generate large amounts of reactive oxygen species and reactive nitrogen species to kill these parasites. The present study aimed to assess the oxidative and nitrosative stresses and activities of key immune response enzymes in the serum of patients with cutaneous leishmaniasis (CL) before and after treatment with sodium stibogluconate as well as in the control individuals. Serum activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and levels of reduced glutathione, malondialdehyde (MDA), and nitric oxide (NO) as well as those of L-arginase, myeloperoxidase (MPO), and adenosine deaminase (ADA) were studied. The activities of L-arginase, MPO, ADA, and MDA and levels of NO were significantly elevated (P < 0.001), whereas those of SOD, CAT, and GSH-Px and levels of GSH were significantly reduced (P < 0.001) before treatment compared with those after treatment and in control individuals. Treatment ameliorated these agents in comparison with the untreated group, but there were still variations between the values of the treated and control groups. Thus, oxidative and nitrosative stresses may play an essential role in the pathogenesis of untreated CL.

Keywords: Cutaneous leishmaniasis; Antioxidants; Nitric oxide; Adenosine deaminase; Myeloperoxidase; Lipid peroxidation

Introduction

Leishmaniasis is a disease caused by several species of flagellated protozoa belonging to genus Leishmania and transmitted by the bite of female sand fly (Burza et al, 2018).

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and causes skin lesions, mainly ulcers, on exposed parts of the body, such as the forearms, legs, and face, where sand fly bites occur most often, leaving life-long scars and serious disability or stigma. CL is widely distributed in the tropical and subtropical regions, including AlAhsa, the Eastern Province of Saudi Arabia (Abuzaid et al, 2017). Leishmaniasis, which infects and replicates within macrophages and other phagocytic cells is accompanied by the enzymatic generation of the reactive oxygen species (ROS) and reactive nitrogen species (RNS), which contribute to the regulation of inflammatory response controlled by the cellular antioxidant defense system (Paiva and Bozza, 2014).

Myeloperoxidase (MPO), a heme-containing peroxidase is most abundantly expressed in immune cells, such as neutrophilic polymorphonuclear leukocytes (neutrophils). Antibacterial activities of MPO involved the production of ROS and RNS, as well as hypohalous acids, and thus is involved in the killing of microorganisms and foreign cells (Faith et al, 2014). MPO is one of the effective inflammatory and oxidative stress markers for such commonly occurring diseases (Gogoi et al, 2016).

L-arginine, an essential amino acid, acts as...
a double-edged sword in infection, needed by nitric oxide synthase (iNOS) to enable nitric oxide (NO)-mediated parasite killing and/or inhibiting parasite growth, it can also be hydrolyzed by L-arginase generated by polyamine or collagen-mediated parasite replication (Olekhnovitch et al, 2014). The activities of L-arginase and iNOS significantly increased in cutaneous leishmaniasis infected mice and patients’ lesions (Mortazavi et al, 2016).

Adenosine deaminase (known as adenosine aminohydrolase, or ADA, E.C.3.5.4.4.) was widely distributed in human tissues especially in macrophages and lymphoid tissues. ADA mediates the deamination of the anti-inflammatory nucleoside adenosine to inosine and thus maintaining cellular immunity (Kaljas et al, 2017). High serum ADA activities were in patients with acute hepatitis, chronic active hepatitis, as well as in cutaneous leishmaniasis (Rai et al, 2016).

Various therapeutic modalities were used to treat CL. Pentavalent Antimonial compound (Pentostam) intra-lesion or intramuscular injection proved an effective therapeutic modality and the main therapeutic drug for different forms of leishmaniasis in Saudi Arabia (Sebai et al, 1975), Egypt (Saleh et al, 2017) and Brazil (Ventin et al, 2018).

Two available preparations, namely, sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) more or less have same efficacy (Frézard et al, 2009).

The present study aimed to evaluate the activity of oxidative, nitrosative, and immune cellular enzymes in CL infected Saudi patients before and after treatment.

Materials and Methods
The serum activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), L-arginase, ADA, and MPO as well as levels of malondialdehyde (MDA) and NO were estimated in treated patients in AlAhsa as compared with healthy controls.

Ethics Statement: The study protocol was approved by the Ethical Committee of the Department of Biological Sciences, Faculty of Sciences and Humanities, Shaqra University (Approval letter SH19-2020 on 12/ 04/ 2020) and in collaboration with the College of Medicine, King Faisal University, and King Fahd Hospital, Saudi Arabia.

Besides, a written informed consent was obtained from all the participants.

The present study included 50 male CL infected patients selected from the Center for the Control of Vector Control Leishmania and Malaria, AlAhsa City, who did not suffer from any other diseases Also, age and sex-matched 30 controls were selected from those attending other clinics or accompanied patients, with neither active CL nor history of previous infection or any other diseases.

Sheets were filled out on each one, including name, and CL duration in the last 6 months. A 10ml fasting blood sample was taken in labeled tubes (BD Vacutainer®, Gribbles Pathology, VIC, Australia) from both patients and controls at AlAhsa Leishmania and Malaria Center, and immediately transferred in an icebox to the laboratory, College of Medicine, King Faisal University. Each tube was centrifuged at 4000 rpm to separate sera was separated, which were stored at -20°C until analyzed. After blood sample collection from the controls, patients were given sodium stibogluconate (20mg/kg/day) as intramuscular injection for 60 days. After 60 days. After 28 days of treatment and 10 ml fresh blood samples were taken from the41 treated patients followed up, none of whom had received any medicine at least a month before for biochemical analysis.

Diagnosis: CL diagnosis was based on clinical pictures and the parasite detection in Giema stained smears. Also, scrapping was carefully taken by midpoint sterile blood lancet from suspected lesion for histopathological studies. Material was smeared on a sliding glass, fixed with methanol acetone free, stained with Giemsa, and microscopically examined (SMZ-2B, Nikon, Rhodes, Australia) for lamastigotes (Saab et al, 2015).

Assays of immune enzymes: Serum L-arg-
inase (EC. 4.2.1.11) activity of both healthy controls and patients with CL was determined (Iyamu et al, 2008). L-ornithine from L-arginine was measured to determine activity, which was expressed in µmol L-ornithine/min/g protein (U/g). MPO activity was spectrophotometrically measured (Krawisz et al, 1984). Serum (0.1mL) from controls and patients was combined with 2.9ml of 50 mM phosphate buffer, pH6.0, containd 0.167mg/ml 0-dianisidine hydrochloride and 0.005% hydrogen peroxide. Absorbance change at 460nm was otometrically measured (Boeco S-20, Hamburg, Germany). A unit of MPO activity was express-ed as U/g protein. Sera ADA activity was spectrophotometrically measured (Giusti and Galanti, 1984), in which NH₃ was generated via ADA effect of on adenosine (substrate). Blue color formed by NH₃ reaction with ind-ophenol was measured at 628nm. ADA lev-els were unit per gram protein (U/g protein).

Assays of antioxidants/oxidant products: Halliwell and Gutteridge (2007) method was adapted to estimate total SOD activity by using kits (Spin-Reac Biodiagnostic, Cairo, Egypt). Activity was expressed as an inhibition percent of formazan/g protein. CAT activity was spectrophotometrically measured (Johansson and Borg, 1988), followed decomposition rate of H₂O₂ at 240 nm, and expressed as U/g protein. GSH-Px activity was assayed based on NADPH decreased absorbance at340 nm (Lasota et al, 2004) and expressed as mU/g protein. Reduced glutathione (GSH) concentration was deter-mined using 5,5’-dithiobis (nitrobezoic) acid formed with glutathione thiol groups colored adduct and spectrophotometric measurement at 412nm expressed as µmol/g protein (Beutler et al, 1963).

Determination of MDA Level: MDA, an end product of lipid peroxidation (LPO), was measured where MDA reacted with thiobarbituric acid (TBA), forming a colored TBA reactive substance (TBARS) complex, quantified spectrophotometrically at 535nm and calculated by extinction coefficient of 1.56×10⁶/M/cm and expressed in MDA/gm protein nanomoles (Esterbauer et al, 1992).

Determination of NO Level: The produced NO was determined indirectly by measuring the nitrite levels based on the Griess reaction (Cortas and Wakid, 1990).

Protein Estimation: Serum protein content was determined (Bradford, 1976) using bovine serum albumin as a standard.

Statistical analysis: Data were analyzed by Statistical Package for Social Sciences program as mean ± standard deviation. Linear regression, paired t-test, and two-way analysis of variance compared between groups. Significance level was taken as the P < 0.05.

Results

The results were given in tables (1, 2, & 3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 30)</th>
<th>Patients with CL before treatment(n = 50)</th>
<th>Patients with CL after treatment (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginase</td>
<td>14.64 ± 2.84</td>
<td>58.78 ± 7.97 abc</td>
<td>16.57 ± 4.10 c</td>
</tr>
<tr>
<td>MPO</td>
<td>12.19 ± 2.35</td>
<td>165.59 ± 15.07 ab</td>
<td>32.83 ± 9.93 c</td>
</tr>
<tr>
<td>ADA</td>
<td>18.67 ± 2.62</td>
<td>102.90 ± 17.28a,b</td>
<td>25.65 ± 7.89 c</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of patients with cutaneous leishmaniasis and healthy controls

Over one year, 50 male CL patients were monitored, mean age of 21 years, and weight was ~60 kg. 41/50 patients were previously treated with antimonials.

Table 2: Enzyme activities of L-arginase, myeloperoxidase (MPO), and denosine deaminase (ADA) in sera of untreated and CL treated patients with and controls.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Health (n = 30)</th>
<th>Patients with CL before treatment (n = 50)</th>
<th>Patients with CL after treatment (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases (80)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>All cases are males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.95 ± 4.99</td>
<td>21.87 ± 5.11</td>
<td>21.57 ± 4.93</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63 ± 14.8</td>
<td>57 ± 13.7</td>
<td>59.5 ± 14.8</td>
</tr>
</tbody>
</table>

*P < 0.001 highly significant compared with controls, †P < 0.001 highly significant compared with controls, ‡P < 0.01, significant for L-arginase, and §P < 0.001 for MPO and ADA compared with controls.
Activities of L-arginase and ADA in patients’ sera before and after treatment as well as healthy controls, showed enzymes were significantly elevated in CL-patients before treatment compared to CL-patient post-treatment and control. Enzymes activities significantly decreased in the 41 follow-up cases after 60 days sodium stibogluconate treatment at 20mg/kg/day as compared to CL patients before treatment. Enzymes activities in CL-patients after treatment were significantly different from controls.

Table 3: Levels of GSH, NO, and MDA in the serum of untreated and treated patients with CL and that of the control group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 30)</th>
<th>Patients with CL before treatment (n = 50)</th>
<th>Patients with CL after treatment (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>67.54 ± 21.45</td>
<td>16.33 ± 2.39a,b</td>
<td>55.80 ± 16.37 c</td>
</tr>
<tr>
<td>NO</td>
<td>28.19 ± 8.53</td>
<td>100.28 ± 29.95a,b</td>
<td>40.01 ± 16.45 c</td>
</tr>
<tr>
<td>MDA</td>
<td>32.53 ± 6.98</td>
<td>188.18 ± 29.4a,b</td>
<td>43.58 ± 15.66 c</td>
</tr>
</tbody>
</table>

*a* P < 0.0001 highly significant compared with control group values, *b* P < 0.001 highly significant compared with treated group values, *c* P < 0.001 significant for GSH and NO and *d* P < 0.0001 for MDA compared with control group values.

The GSH levels significant decrease (P < 0.001), but NO & MDA levels significantly increased (P < 0.001) in CL patients before treatment as compared controls and post-treated patients. 60 days post-treatment, GSH level increased significantly, but MDA & NO decreased significantly as compared to untreated patients, with significant parameters difference between controls and CL post-treated patients.

Fig. 1: Activity of (A) superoxide dismutase (SOD), (B) catalase (CAT), and (C) glutathione peroxidase (GSH-Px) in serum of control healthy group and untreated and treated patients with cutaneous leishmaniosis aP < 0.001, highly significant values compared with controls. bP < 0.001, highly significant values compared with treated group. cP < 0.01, highly significant compared values with controls.

Activities of SOD, CAT, & GSH-Px were reduced significantly (P < 0.001) in CL-patients before treatment as compared to controls and post-treated CL-patients. Activities in treated patients were significantly elevated as compared to them before treatment, with significant difference between controls and post-treated CL-patients.

**Discussion**

In the present study, significant reduced levels in antioxidants assessed the activities of SOD, CAT, and GSH-Px as well as the level of GSH together with the elevated levels of oxidative products, e.g., MDA and NO in the serum of untreated patients with CL. The depletion of these protective antioxidants was attributed to the overproduction of ROS, e.g., superoxide (O2\*), hydrogen peroxide (H2O2), and hydroxyl radicals (.OH), and of RNS, e.g., NO, in response to phagocytosis by monocytes/macrophages as a host defense mechanism for the killing of the engulfed leishmania (Serarslan et al, 2005; Aşkar et al, 2008). The reduction in GSH-Px activity in the serum of untreated patients with CL compared with that in the control and treated patients was attributed to the reduction of selenium, which is required for the activity of GSH-Px (Baker et al, 1993). This agreed with Koçyiğit et al. (1999) who found a significant decrease in selenium levels and GSH-PX in CL patients.

In the present study, decreased in GSH-Px activity was due to the GSH concentration reduction in Px activity, which agreed with Gokce and Dag (2017).
GSH acts as a free radical trapping factor and as a substrate for several enzymes, including GSH-Px & glutathione-S-transferase (Romão et al., 2006). A significant depletion of GSH level was in untreated patients' sera. Reduction in GSH levels was due to high production of ROS by activated phagocytic cells (Neupane et al., 2008).

LPO is caused by ROS oxidative damage of unsaturated fatty acids in the lipid membrane. A common marker of LPO is MDA, which has been frequently used as a marker of oxidative stress in response to different agents such as infection. Thus, the elevation of MDA in the untreated patients with CL in this study might be attributed to the over-production of ROS (ROS are an essential part of host defense strategies of organisms to kill the parasite) with the depletion of the protective antioxidants and subsequent oxidative damaging of lipids and other biomolecules (Serarslan et al., 2005; Mustafa et al., 2018).

In the present study, the NO level was significantly elevated in the sera of untreated patients with CL in comparison with that in the control or the treated patients. The NO reacted rapidly with superoxide anion (O$_2^-$) producing highly cytotoxic peroxynitrite (which is unique as a lipid oxidant) and may potentiate LPO by increasing levels of MDA (Mustafa et al., 2018). Besides, the elevation of MDA levels was due to the increased activity of MPO in untreated patients with CL. This agreed with Zhang et al. (2002) who found that MPO can oxidize tyrosine and nitrite into tyrosyl and nitrogen dioxide (NO$_2^-$) radicals (i.e., reactive intermediates), which in turn oxidized lipids in the plasma and the cell membranes leading to the MDA elevation.

MPO is a front-line defender against phagocytosed microorganisms by generation of ROS and RNS (Klebanoff et al., 2013). Also, it released into the extracellular fluid after oxidative stress and different inflammatory responses. Thus, MPO elevation levels in untreated CL-patient group was related to remarkable increase in production oxygen and nitrogen metabolites in response to phagocytosis by macrophages as a host defense mechanism for killing the engulfed Leishmania and amplified leishmanicidal activity in patients (Ndrepepa, 2019).

The results of this study showed that serum L-arginase activities and NO levels were elevated in patients with CL. The CL-infected macrophages metabolize arginine via two enzymes; iNOS and L-arginase. The balance between these two enzymes is reported to be competitively regulated by type 1 (Th1) and type 2 (Th2) T helper cells, respectively, via their secreted cytokines (Munder et al., 1999). Barbosa et al. (2011) and Papadogiannaki et al. (2015) showed the balance between Th1 & Th2 cytokine profile in the serum and tissues of dogs infected with Leishmania infantum, respectively. Type 1 cytokines (notably IFN-$\gamma$ & TNF-$\alpha$) induce expression of iNOS, whereas type 2 cytokines (including IL-4, IL-10, & IL-13) induce L-arginase activity (Kropf et al., 2005). Thus, elevation of serum L-arginase activity and NO levels was attributed to the balance in Th1 & Th2 cytokines production in inflammatory cells infected with CL (Munder et al., 1999). Miralles et al. (1994) indicated that expression of IFN-$\gamma$, IL-2, IL-4, and IL-10 mRNA was induced by L. donovani infection.

ADA is a cytoplasmic enzyme whose activity is elevated in disorders that stimulate the cells involved in the immune system (Turel et al., 2018). The elevation of serum ADA activity in the untreated patients with CL was be attributed to the elevation of adenosine, the substrate of ADA, which was confirmed by other studies (Rai et al., 2011; 2016). But, increased serum ADA activity in CL patients was a reflection of induction of the macrophages phagocytosis and increased cellular immunity stimulated by IFN-$\gamma$ & IL-2 synthesized by Th1 cells (Khan et al., 2013).

In the present study, the sodium stibogluconate and meglumine antimoniate to CL pa-
tients activated SOD, CAT, GSH-Px, MPO, ADA, & L-arginase that changed values of MDA, NO, & GSH to be normal. Mechanism of meglumine antimoniate role in amelioration the present parameters as an anti-leishmanial agent depended on several targets. One of which was that the pentavalent antimony, Sb(V) reduced the bioenergetics of the Leishmania parasites by inhibiting glycolysis and β-oxidation of fatty acids resulting in decreased reducing equivalents for antioxidant defense (Roberts et al, 1995).

This also inhibited phosphofructokinase, resulting in decreased synthesis of ATP, needed for parasite survival. The diminished energy production eventually led to parasite death (Monzote, 2009). Besides, antimony altered the thiol-redox potential in parasites by actively promoting efflux of thiols, glutathione, and trypanothione, rendering the parasite more susceptible to oxidative stress and DNA topoisomerase I inhibition (Wyllie et al, 2004; Ameen, 2007). The Sb (V) acts as a prodrug that reduced within organism into a more toxic and active trivalent antimony compound (Shaked-Mishan et al, 2001), that induced DNA fragmentation, with appearance of apoptosis (Sereno et al, 2001). Apoptosis in Leishmania indicated that the intracellular Ca²⁺ played a role in parasite clearance (Sudhandiran and Shaha, 2003), a phenomenon proved in the oxidative-stress-induced apoptosis-like death in L. donovani promastigotes (Mukherjee et al, 2002). Also, Sb (V) killed intracellular parasites by activating host innate & adaptive immune system, which elected the host anti-leishmanial immune response (Murray et al, 1991).

Fortunately, Abuzaid et al. (2020) reported that the kingdom of Saudi Arabia with the Pentostam® brought the visceral leishmaniasis cases from 100s during 1980s &1990s to zero case in 2019.

Conclusions

Enhanced capability of activated macrophages against infection was related to marked increase in ROS and RNS in response to phagocytosis to kill the parasite. ADA & L-arginase evaluated activity of in CL patients’ sera monitored the clinical status. So, ADA and L-arginase are predictive and sensitive agents for CL treatment. Also, ADA increased leishmaniasis’ cellular immunity, and valuable in diagnosing several pathogenesis.

Author contributions: All authors equally contributed in all aspects of this study.

Conflicts of interest: The authors did not have conflicts of interest.

Data Availability Statement: All generated and analyzed data were here in included.

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References


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