Alterations in the Clinical Manifestations of Cutaneous Leishmaniasis in Various Total Antioxidant Capacities: An Animal Study Using BALB/c Mice

Mojtaba Yousefi, Seyed Masoud Zolhavarieh, Alireza Nourian, Hossein Rezvan, Ali Sadeghi-nasab

Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran. Department of Clinical Sciences, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran.

ABSTRACT

The severity of the clinical manifestations of cutaneous leishmaniasis can vary depending on various factors, such as the Leishmania species involved as well as hosts and their immune response. This study aimed to investigate the relationship between the severity of different clinical signs, histopathological changes, and genetic indicators with TAC in mice experimentally infected with Leishmania major. A total of 105 eight-week-old BALB/c mice of both sexes were assigned to seven experimental groups (15 in each) as follows: 1) healthy mice, 2) Leishmania-infected mice treated with 100 mg/kg/day of SC glucantime until complete healing, 3) mice which received 20 IU/kg/day of vitamin E (SC for 10 days) to increase TAC prior to infection and further treatment with glucantime, 4) Leishmania-infected mice which received both vitamin E and glucantime daily until complete healing, 5) mice which received 20 IU/kg/day of vitamin E (SC for 10 days) before infection, and 6) Leishmania-infected mice which received 20 IU/kg/day of SC vitamin E up to the end of the trial, and 7) mice which received daily vitamin E until the end of the experiment. Wound size, expression of pro-inflammatory cytokines (IFN-γ and TNF-α) and healing genes (KGF and EGF), histopathological findings, and mortality rate were assessed three times on days 31, 38, and 72 post-infection. Approximately, 31 days after the parasite inoculation, dermal lesions were developed in all infected mice. In group 3, the clinical manifestations, healing time, and histopathological changes were significantly more favorable, while group 4 showed the worst situation in terms of the evaluated indicators. A high level of TAC before the onset of the disease has an effective role in the recovery indicators. However, its simultaneous elevation at the beginning of infection will decrease the body’s ability to effectively clear the parasite, heal the tissue, and improve the clinical manifestations of the disease.

Keywords
Antioxidant capacity, Clinical manifestations, Cutaneous leishmaniasis, Leishmania, IFN-γ, TNF-α

Abbreviations
EGF: Epidermal growth factor
OH: Hydroxyl radical

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Introduction

Various species of Leishmania are obligate intracellular protozoa that replicate within macrophages after being phagocytized by these cells. This parasite has an extracellular promastigote form in its arthropod vector, sandfly, and an intracellular form inside the mammalian macrophages known as amastigote. Upon the sandfly bite, the parasites trans- fer into the dermis and are subsequently phagocytized by neutrophils, which are immediately called to the site. Within 2 days after the arrival of monocytes/macrophages, as the second wave of inflammatory cells infiltration, the parasites are engulfed mainly by these mononuclear phagocytes, lose their flagella, and differentiate into amastigotes [1, 2].

Macrophages are equipped with microbicidal mechanisms, from which, the intracellular microorganisms must escape to survive [3]. During leishmaniasis, the germicidal processes may occur in two stages. First, during the initial phagocytosis of promastigotes, the macrophage can show a fast oxidative response stimulated by the phagocytic event. Second, once infection with amastigotes is established, the quiescent macrophages can slowly be activated to potentially destroy the intracellular Leishmania [4].

Efficient escape from microbicidal molecules produced at each stage of infection is important for Leishmania to initiate and maintain the host cell infection. Two important macrophage-derived oxidants are critical in controlling Leishmania infection. During the early stage of infection, the free radical superoxide anion (O2−) is produced as a part of the macrophage respiratory burst in response to the phagocytized cell [5, 6]. Superoxide production is catalyzed by NADPH oxidase, a heme-containing cytochrome that comprises cytosolic and membrane-bound components. After assembly, the oxidase transfers an electron from NADPH to molecular oxygen and produces O2−. Promastigotes are susceptible to being killed by exposure to O2− and hydroxyl radical (OH•) produced from H2O2 [7, 8].

The second anti-leishmanial oxidant produced by macrophages is NO• [4]. Unlike O2−, which is generated during parasite phagocytosis, NO• is produced after macrophage activation by IFN-γ and TNF-α and is closely associated with the intracellular killing of amastigotes [9].

Neutrophils normally have a short lifespan (less than a day) and undergo spontaneous apoptosis. This period may be elongated when these cells are infected with microorganisms [10]. L. major can suspend neutrophil apoptosis for up to two days by inducing the secretion of anti-apoptotic cytokines, such as granulocyte-macrophage colony-stimulating factor and IL-8 [11]. It has also been reported that infected neutrophils undergoing apoptosis release more macrophage inflammatory protein 1 beta to attract macrophages to the site of infection and prepare a safe and silent entry to these cells [12]. In other words, the prevention of neutrophil apoptosis is an important mechanism used by Leishmania to subvert its death [12, 13]. This silent entry into macrophages is reminiscent of the Trojan Horse scenario [2, 14] as promastigotes suspend the neutrophils’ apoptosis process until macrophages arrive at the site of infection, and also suppress O2− and NO• mediated microbicidal responses [11, 12]. Infected neutrophils are engulfed by macrophages and allow promastigotes to multiply and transform into amastigotes in macrophage phagosomes.

Many studies emphasize the key role of parasite proliferation and host inflammatory responses in leishmaniasis and the impact on the clinical course of the disease [9, 15, 16]. It has been shown that skin wounds and tissue destruction are necessary for effective parasite clearance [3]. Therefore, the clinical manifestations of leishmaniasis, which range from skin lesions to potentially fatal visceral disease [17], are caused by parasite replication and the host’s inflammatory responses [9, 18]. In a clinical study, it was shown that the TAC level in leishmaniasis patients suffering from unhealed chronic wounds is significantly higher than in healed patients [19]. Leishmania causes inflammation by stimulating the connective tissue mast cells and the resultant production of reactive oxygen species (ROS) pro-inflammatory mediators. The production of ROS and NO• during an inflammatory response leads to oxidative damage to cells. On the other hand, similar to the lipophosphoglycan of the promastigote membrane, the intracellular amastigotes disrupt IFN-γ signaling and therefore, significantly inhibit the activity of superoxide dismutase and catalase (CAT) [20]. In other words, during leishmania infection, on one hand, free radicals are created during an inflammatory response, and on the other hand, the TAC level decreases simultaneously with the healing of skin wounds and improvement of other clinical manifestations [21]. The present study was conducted to investigate the relationship between the severity of leishmanial lesions with different levels of TAC to better understand the course of the disease and improve the treatment process.

Abbreviations-Cont’d

IL-1α: Interleukin-1 alpha
IFN-γ: Interferon-gamma
KGF: Keratinocyte growth factor
NADPH: Nicotinamide adenine dinucleotide phosphate
ROS: Reactive oxygen species
O2−: Superoxide
TAC: Total antioxidant capacity

Cutaneous leishmaniasis and total antioxidant capacity in BALB/c mice
Results

Leishmaniasis lesions in different groups

On average, after 31 ± 2 days, a *Leishmania* wound was observed in the parasite inoculation area (Figure 1). In this study, it was observed that the wounds in all groups except for group 2 (LT) became wider until the second sampling time (day 38) and then, their size gradually decreased. The smallest size of the wounds was in group 3 (20.2 mm² ± 23.5), while the largest was in group 2 (*p* < 0) (Figure 2). Moreover, in the last sampling time, the wounds of group 3 had the highest percentage of healed area (83.9%), and the lowest percentage was observed in group 4 (47.5%) (*p* < 0) (Figure 3). In the groups administered with glucantime with or without vitamin E, after approximately 72 days, the cutaneous wounds were completely healed and no nodules or remnants were observed.

TAC measurement

While group 3 (ALT) had the lowest amount of TAC compared to other groups (Figure 4), wound healing in this group showed a significant inverse relationship with the amount of TAC (64.5 mm² ± 45.1) ([Pearson correlation (P-value) respectively] (Table 1). Furthermore, in group 5 (AL), it was observed that the total area of the wound had an inverse relationship with the level of [-0.5 (0.001)] [Pearson correlation (P-value) respectively] (Table 1).

RT-PCR results

It was found that IL-1α and IFN-γ genes were not expressed simultaneously in groups infected with *Leishmania*, and as soon as glucantime was used in them, the concurrent expression of inflammatory genes was observed. In group 3, which showed the smallest size of cutaneous wound and the highest proportion of healed area, the inflammatory genes were expressed without any significant differences between sampling times, and the expression of healing genes

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**Figure 1.** Cutaneous wounds caused by the *Leishmania* major parasite.

**Figure 2.** Changes in total wound area in different groups (mm²). Groups 1 and 7 are not included in this diagram, because they had no wounds.

**Figure 3.** Changes in the ratio of healed areas to the initial size of the wound in each group (percentage).

**Figure 4.** Changes in the TAC levels (μM).

**Figure 5.** The occurrence of death in healthy and test groups.
Table 1.
Changes in the expression pattern of the healing and pro-inflammatory genes in relation to changes in the TAC amount and the healed area of the wound

<table>
<thead>
<tr>
<th>Healed</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL or INF</td>
<td>EGF or KGF</td>
</tr>
<tr>
<td>70.2 ± 19.5*</td>
<td>0</td>
</tr>
<tr>
<td>31.3 ± 26.0**</td>
<td>44.2 ± 30.2</td>
</tr>
<tr>
<td>(0.000)</td>
<td>(0.000)</td>
</tr>
<tr>
<td>72.6 ± 29.7</td>
<td>45.4 ± 38.4</td>
</tr>
<tr>
<td>27.1 ± 31.9</td>
<td>81.5 ± 18.7</td>
</tr>
<tr>
<td>(0.000)</td>
<td>(0.001)</td>
</tr>
<tr>
<td>49.4 ± 26.9</td>
<td>31.0 ± 36.4</td>
</tr>
<tr>
<td>24.4 ± 22.1</td>
<td>51.2 ± 7.2</td>
</tr>
<tr>
<td>(0.009)</td>
<td>(0.033)</td>
</tr>
<tr>
<td>56.5 ± 26.3</td>
<td>100 ± 0.00</td>
</tr>
<tr>
<td>18.0 ± 16.3</td>
<td>38.4 ± 25.9</td>
</tr>
<tr>
<td>(0.000)</td>
<td>(0.000)</td>
</tr>
<tr>
<td>51.4 ± 28.3</td>
<td>24.5 ± 35.5</td>
</tr>
<tr>
<td>29.9 ± 0.00</td>
<td>60.0 ± 15.7</td>
</tr>
<tr>
<td>(0.000)</td>
<td>(0.006)</td>
</tr>
</tbody>
</table>

The numbers in the first row: the mean ± standard deviation of the group in which none of the genes are expressed. The numbers in the second row: the mean ± standard deviation of the group in which at least one of the two genes is expressed. Numbers in parentheses are probability values (P-values). Groups 1 and 7 were not shown in this diagram because they had no wounds.

Figure 6.
Histopathological changes in the skin, G1: The normal microscopic structure of the skin, G3: Regeneration of the epidermis (arrowhead), G4: Partial regeneration of the epidermis (arrowhead) and the presence of edema and inflammation in the dermis (D) and hypodermis (H), G6: The presence of edema and severe inflammation in the dermis (D) and hypodermis (H) and the presence of a large number of amastigote forms of the parasite (arrow) in these parts (inset).
increased from the second sampling time (results not shown). In addition, in group 6 (LA), which showed the highest levels of TAC, although the expression of the KGF gene increased at the last sampling, the expression of pro-inflammatory genes was very low. Meanwhile, our results showed that the rise in the ratio of healed area to the total wound area was greatly related to the expression of pro-inflammatory genes rather than to the expression of healing genes (Table 2).

**Mortality rate**

Although the death that occurred in some groups was not statistically significant, there was no death in groups 3 and 5, and the deterioration of the lesions in these groups was less than in others (clinical observation) (Figure 5).

**Histopathologic findings**

Histopathological examination of the skin and spleen tissue samples showed the most promising results in group 3 (ALT) and the least in group 6 (LA). Although treated with glucantime, group 4 indicated impaired microscopic architecture in the spleen tissue (Figures 6 and 7). The absence of granulomatous lesions in all groups was a remarkable finding in this study.

**Discussion**

Considering the destructive effects of the oxidant systems and the interaction of these microbial mechanisms with the proliferation of the Leishmania parasite, this study aimed to evaluate the outcomes of *L. major* infection in association with different levels of TAC. Therefore, the most important criterion of this investigation was the clinical presentation of the disease. In all experimental groups, the wound size increased first, and then, gradually decreased. This phenomenon did not occur in group 2, and its reason has not yet been determined by the authors.

*Leishmania* down-regulates the pro-inflammatory genes [16, 19, 20], but once treatment with glucantime is started, the expression of main pro-inflammatory genes (IL-1α and IFN-γ), as important and influential factors in the immune response against *Leishmania*, is resumed [19]. Contrary to what was expected in group 4 (LTA), the expression of pro-inflammatory genes was not resumed, which might have been due to the use of glucantime. It was observed that in this group, the expression of pro-inflammatory (p = 0.019) and healing (p = 0.001) genes declined. In other words, raising the TAC level together with treatment with glucantime may delay the immune system to reach the threshold for the production of essential pro-inflammatory cytokines, such as IL-1α and IFN-γ [11, 21]. Therefore, it seems that parasite survival is facilitated by increasing exogenous TAC levels in the host.

Interleukin-1α and IFN-γ, as "warning cytokines", are the main pro-inflammatory cytokines secreted by macrophages. It has been reported that IL-1α induces the expression of adhesion molecules on the surface of endothelial cells and leukocytes, and initiates and propagates the host’s inflammatory response [17, 23]. Several studies have emphasized the crucial role of IL-1α in the control of inflammatory and immune responses in leishmaniasis for changing the clinical course of this disease. This function is conferred by T-helper cell type-1 lymphocytes, which limit the

Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>TAC</th>
<th>Total Wound Area</th>
<th>Healed Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>1.7 ± 0.3§</td>
<td>1.7 ± 0.3*</td>
<td>50.7 ± 0.3§§</td>
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<tr>
<td>G3</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.3</td>
<td>49.2 ± 35.5</td>
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<tr>
<td>G4</td>
<td>41.1 ± 27.8</td>
<td>46.1 ± 34.9</td>
<td>46.1 ± 34.9</td>
</tr>
<tr>
<td>G5</td>
<td>42.8 ± 29.6</td>
<td>59.1 ± 60.8</td>
<td>49.9 ± 27.9</td>
</tr>
<tr>
<td>G6</td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>49.9 ± 27.9</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation of TAC
** Mean ± standard deviation of the total wound area
§Mean ± standard deviation of the healed part of the wound
§§Mean ± standard deviation of the percentage of the healed part of the wound
***The Pearson correlation coefficient and the numbers in the parentheses are the P-value.

Groups 1 and 7 were not shown in this diagram because they had no wounds.
spread of the parasite and lead to wound healing [17, 22, 23]. It seems that wound healing cannot be coordinated as long as the number of neutrophils in the wound exceeds the number of lymphocytes and macrophages. Therefore, the prolongation of the acute inflammatory phase and the delay in the replacement of acute inflammatory cells (granulocytes) by chronic cells (mononuclear) in this group can be due to the lack of (or very low) expression of IL-1α and IFN-γ genes [24]. In the third group, the presence of an inverse statistical relationship between TAC levels and wound healing ($p = 0.011$) strongly supports the fact that rising TAC levels in leishmaniasis worsen the clinical manifestations of the disease [25].

Granuloma formation is another feature of tissue pathology in wound healing as well as fibrous transformation due to excessive collagen deposition and resultant scar tissue. Not only granulomas can keep microorganisms alive, but also they prevent the spread of infection. The granulomatous reaction occurs in response to infection by some *Leishmania spp.* as fibroblasts migrate into the area and change the normal tissue structure. However, in the histopathological examination of the spleen and skin samples, no organized amastigotes containing granulomatous lesions were detected in any of the groups. According to other studies, the absence of granuloma formation is associated with the dissemination of cutaneous leishmaniasis. In other words, the severity of the disease depends on the ability of the host to develop a granulomatous reaction [24]. In the present study, there were no signs of disease spread and visceralization, and it is difficult to assess whether the non-spreading behaviour of the disease is the cause or the result of IL-1α and IFN-γ genes expression. Because the expression of other inflammatory genes was not investigated in this study, further evaluations and tracking of more pro-inflammatory cytokines might be helpful.

In groups 3 and 5, the mortality rate was zero (Figure 5). This result can probably be related to the protective role of vitamin E against the occurrence of some co-existing infections that otherwise may lead to the deterioration of the patient’s condition and death. When administered systemically, vitamin E has been shown to increase the resistance of wounds to infections. However, no effects other than inhibiting collagen synthesis have been found for this vitamin when administered topically [26, 27].

**Figure 7.**
Histopathological changes in the spleen, G1: Normal microscopic structure of the spleen with normal white (WP) and red (RP) pulps, G3: The structure of the organ is observed normally, G4: Disruption of the normal structure of the organ, reduction in the size of white pulp and severe necrosis in white and red pulps, G6: Severe necrosis (arrowhead) in white and red pulps.
Conclusion

In cutaneous leishmaniasis, a delicate balance between tissue pathology and infection control determines the clinical presentation of the disease. T-cells are the main infiltrating lymphocytes in the skin lesions of leishmaniasis to control the parasite proliferation as well as tissue destruction. Upon the inoculation of mice with *L. major*, the epidermis is damaged as a result of tissue destruction by neutrophils, macrophage necrosis, and keratinocyte apoptosis mediated by FasL/TRAIL [3]. Consequently, in the treatment of cutaneous leishmaniasis, it is necessary to control both parasite proliferation and tissue damage [3, 9, 16-18]. It was concluded from the results of this study that in the BALB/c mouse model, by increasing TAC levels before infection with *L. major*, the severity of the clinical manifestations of the disease will be reduced.

Materials & Methods

This study was approved by the Medical Ethics Committee of Bu-Ali Sina University, Iran (protocol number: 8-13/02/1399) based on international protocols for working with laboratory animals). A total of 105 BALB/c mice of both sexes at the age of 8 weeks old were randomly allocated into seven groups (n=15 in each group) as follows: 1) healthy mice (N), 2) *Leishmania*-infected mice treated with 100 mg/kg/day of SC glucantime (Sanofi Aventis, France) until complete healing (LT), 3) mice which received 20 IU/kg/day of SC vitamin E (Aburaihan, Iran) for 10 days to increase TAC prior to infection and further treatment with glucantime (ALT), 4) *Leishmania*-infected mice which received both vitamin E and glucantime daily until complete healing (LAT), 5) mice which received 20 IU/kg/day of vitamin E (SC) for 10 days before infection, 6) *Leishmania*-infected mice which received 20 IU/kg/day of vitamin E (SC) until the end of the experiment, and 7) mice which received daily vitamin E until the end of the experiment to increase TAC levels (A). An inoculate of 10⁶ *L. major* promastigotes MHOM/76/ER was injected intradermally (tail base) in each mouse [17]. The animals were housed in polycarbonate cages under standard conditions of cycles of 12 hours of light-dark and at a temperature of 25°C±1°C. Animals were fed ad libitum with a balanced diet and tap water. Mortality was checked daily.

Wounds were photographed by placing a one-centimeter marker next to the lesion. The lesion size was calculated using ImageJ with Java 1.8.0_172 after the calibration of photographs. At the first appearance of the ulcerative lesion (31 ± 2 days), 7 days later (day 38), and at the time of recovery, five mice from each group were euthanized and sampled (blood, skin, and spleen). Blood samples were collected immediately after euthanizing with chloroform, and skin and spleen tissue samples were surgically harvested. The tissue samples were cut in half for molecular and histopathological analyses. All the collected blood and half of tissue samples (skin and spleen) were stored at -80°C for further molecular analysis of healing (KGF and EGF) and pro-inflammatory (IL-1α and IFN-γ) genes (Table 3). The remaining half of the skin and spleen tissue samples were placed immediately in 10% neutral buffered formalin and processed to obtain hematoxylin and eosin-stained tissue sections. The sections were then examined independently by a veterinary pathologist using a light microscope equipped with a digital camera (Olympus DP25, Germany).

**Measurement of TAC**

In this study, the TAC level in the sera was measured using a commercial enzyme-linked immunosorbent assay kit (Kiazist, Iran) according to the ferric-reducing antioxidant power method.

**Statistical analysis**

After testing the normality and homogeneity of variances at the level of groups and different stages of the experiment, repeated measures analysis of variance and Tukey’s test as a follow-up test were performed by the SPSS statistical software (version 19). The significance level was considered less than 0.05.

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Conflict of interest

The authors declare that there is no conflict of the interest.

References


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