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Oxidative Stress and Antioxidant Enzyme Activities in Patients with Cutaneous Leishmaniasis in Endemic Region of Hatay (ANTIOCH)

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Leishmania is the collective name for a number of disease caused by protozoon flagellates of genus *Leishmania* which have diverse clinical manifestations. In this study, we investigated the effect of free radicals and antioxidants on cutaneous leishmaniasis (CL). Venous blood samples of patients and controls were collected and analyzed for serum nitric oxide (NO) and malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase (CAT) activities. 53 treated CL patients, 42 untreated patients and 30 healty control subjects were enrolled in this study. The levels of MDA and NO showed a significant increase ($p < 0.05$) in the untreated patients when compared to the treated patients and control groups. In the untreated group, the activities of both SOD and CAT were found to be decreased ($p < 0.05$) compared with values in the control and treated groups. After the treatment, NO and MDA levels were decreased whereas the activities of CAT and SOD were increased. These results provide some evidence for a potential role of increased lipid peroxidation and decreased enzymatic antioxidants in cutaneous leishmaniasis. These results suggested that oxidant stress plays an important role in the pathogenesis of cutaneous leishmaniasis.

Key Words: Cutaneous *Leishmania*, Lipid peroxidation, Antioxidant, Hatay (Antioch).

INTRODUCTION

Leishmaniasis is a widespread disease affecting about 12 million people in 88 countries, with 1.5-2 million new cases each year. Leishmania widely distrubuted in tropical and subtropical regions throught the world, including Turkey[1,2]. Two forms are observed in Turkey. One is *Leishmania infantum* responsible for visceral leishmaniasis (VL) and the other is *Leish-*

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mania tropica, which is widespread throughout Southeastern Anatolia, for several years, *causes* cutaneous leishmaniasis (CL)[3]. In Hatay province, the Mediterranean coast in south-eastern Turkey, human cutaneous leishmaniasis has posed an important public health problem for many years. Between 1998 and 2002, 742 cases were recorded in this province by the Ministry of Health[4].

The multiplication of the parasite within macrophages leads to ulcerative lesions in the skin and mucosa, which frequently results in permanent disfiguration of patients. All parasites of genus Leishmania are intracellular parasites that infect cells of the mononuclear phagocyte lineage of their vertabrate hosts. They exist as non-motile intracellular amastigotes and are responsible for such causes mentioned above[3]. Several infection agents are carcinogenic to humans. Most of these are releated with viruses and bacterial and parasitic infection. To survive sucessfully and multiply within these two disprate biological environments, the parasites must undergo profound biochemical and morphological adaptations[5]. Also induction protective immunity against leishmaniasis is generally thought to depend on the production of interleukin (IL)-12. IFN gama in turn mediates protection by inducing nitric oxide sytnhase (NOS) expression and nitric oxide (NO) production[5].

Stimulation of phagocytes by particulate agents or soluble mediators such as chemoattractians trigers the generation of large amounts of reactive oxygen species (ROS), such as hydroxyl radicals (·OH), superoxide anion radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2)[6-8]. Activated macrophages kill intracellular parasites by generating ROS and NO. Cellular damage induced by these cytotoxic oxygen free radical species is represented by lipid peroxidation of unsaturated fatty acids[9]. Lipid peroxidation occurs at low level in all tissues and leads to the formation of numerous degradation products, including malondialdehyde (MDA)[10]. To minimize the damaging effects of ROS, aerobic organisms evolve both enzymatic and non-enzymatic antioxidant defences. Purely enzymatic defences such as superoxide dismutase (SOD) and catalase (CAT) and peroxidases provide protection by directly scavenging superoxide radicals and hydrogen peroxide, converting them to less reactive species. The presence of intracellular *Leishmania* amastigotes in mononuclear phagocytes may impair the oxidant-antioxidant balance[11,12].

NO is an important bioregulatory mediator and process many physiological functions[13-15]. NO and reactive nitrogen intermediates (RNI) are implicated in macrophage derived cytostatis/cytotoxicity against tumour cells and various intracellular and extracellular pathogens. Mononuclear phagocytes are the exclusive host cell of the intracellular protozooan parasite leishmaniasis[11,14,15].

In the present study, we investigated the role of free oxygen radicals and antioxidants on cutaneous leishmaniasis. For this purpose, serum levels of MDA and NO, SOD and CAT activities were measured in treated and untreated CL patients in Hatay region.

EXPERIMENTAL

Patients were selected from endemic area for leishmaniasis in Hatay. Diagnosis was confirmed by parasitology laboratory upon demonstration of the parasite in the lesions by direct smears. Lesions were cleaned with ethanol and punctured at the margins of the lesion with sterile lancet. Material was smeared, dried in air and fixed by methanol. The smears were stained with Giemsa's stain for examination by light microscopy. Patients with a negative smear were diagnosed by culture of the lesion (culture of fine needle aspirate on NNN(Novey-MacNeal-Nicolle) medium (with rabbit blood) and incubated at 24°C. Culture were observed for 4 weeks before being considered as negative[3,16,17]. 95 Patients in the age between 5-72 years were included to the study. They were divided into two groups. Untreated 42 active CL patients (18 females, 23 males) and healed 53 CL patients (27 females, 26 males) were treated using the drug with pentavalent antimonal compounds. Antimonate solution was adminestered intramusculary once a day for 15-20 d (20 mg/Sb/kg/d) by a single injection. 50 Healthy subjects (24 females and 24 males) from the same area who were not exposed by *Leishmania* parasites were used as controls. After dignosis, 10 mL of venous blood samples from all patients and healthy controls were withdrawn and transferred into tubes without anticoagulants to measure the level of oxidants and antioxidant activity. These blood samples were centrifugated for 15 min at 3500 rpm. Serum was removed and stored at -20°C for biochemical assays. All subjects were informed about the content of the study prior to tests and their written consents were obtained. The study protocol was approved by the ethical committee of the Mustafa Kemal University Hospital.

Biochemical assay

MDA determination: The levels of homogenized tissue MDA were determined as an index of lipid peroxidation by thiobarbituric acid reaction according to the Yagi[18].

NO determination: The oxidized end products of NO (nitrite and nitrate) were analyzed by a photometric endpoint determination (nitrite/nitrate, colorimetric method; catalogue no. 1-746-081, Roche Diagnostics GmbH, Mannheim, Germany). Nitrate was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase. The resultant nitrite reacted with sulphanilamide and N-(1-naphthyl)-ethylene-diamine dihydrochloride producing

red-violet diazo dye. The absorbance of the diazo dye was measured spectrophotometrically at 540 nm.

Enzyme assay: SOD activity was measured on the basis of the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system[19]. One unit of SOD activity was defined as the amount of protein causing 50 % inhibition of the NBT reduction rate.

Catalase activity of tissues was determined according to the method suggested by Aebi[20]. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm, resulting from enzymatic decomposition of H_2O_2. The difference in absorbance per unit time is a measure of catalase activity. The enzyme activities were given in U/mL.

Statistical analysis: Statistical evaluation of data was performed using the SPSS 10.0 statistical program. Mann-Whitney U test was used for the comparison of groups. All data were reported as mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

In this study, we investigated oxidant and antioxidant levels in patients with Cutaneous Leishmaniasis in endemic area in Hatay. The levels of MDA and NO and activities of SOD and CAT in the serum of control group and in the patients before and after treatment are reported in Table-1. MDA and NO levels were found to be significantly higher ($p < 0.05$) in CL patients than those of control and treatment groups. There was no significant difference between the control and treatment groups. In the untreated group, the activity of both SOD and CAT were found to be decreased ($p < 0.05$) compared to the values in the control and treatment groups. No significant difference in SOD activity was obtained between the control and treatment groups whereas in the untreatment group, CAT activity was significantly different from control group. After treatment, NO and MDA levels were decreased although the activities of CAT and SOD were increased.

TABLE-1
COMPARISION OF PARAMETERS OF CONTROLS AND PATIENTS WITH CL (MEAN ± SD)

Parameters	Control (n = 50)	Untreated (n = 42)	Treated (n = 53)
MDA (μmol/L)	1.39 ± 0.35	2.91 ± 1.17[a,b]	1.45 ± 0.62
NO (μmol/L)	17.90 ± 1.23	30.02 ± 2.05 [a,b]	20.18 ± 1.38
SOD (U/mL)	9.08 ± 1.05	4.13 ± 0.72 [a,b]	8.09 ± 0.65
CAT (U/mL)	68.15 ± 5.73	22.05 ± 6.08 [a,b]	44.12 ± 7.12 [a]

[a]$p < 0.05$ compared with control group.
[b]$p < 0.05$ compared with treated group.

There has been some evidence that the oxidant-antioxidant system is altered in human Cutaneous Leishmaniasis. ROS or oxidants are formed in oxidative processes that normally occur at relatively low levels in all cells and tissues. Under normal conditions, a variety of antioxidant mechanisms serve to control this ROS production. In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules. Furthermore, decomposition of peroxidized lipid yields a wide variety of end-products, including malondialdehyde (MDA)[21]. Elevated levels of MDA observed in the serum of the patients with CL compared to treated patients and controls in this report. Vural *et al.*[22] and Serarslan *et al.*[8] have observed a significant increase in MDA levels in the erythrocytes and serum of the CL patients, respectively. Increased levels of MDA suggesting increased lipid peroxidation in CL patients.

Over the past decade or so, it becomes evident that the free radical nitric oxide ($NO^{\bullet}$) acts as a novel transcellular messenger molecule in many key physiological and pathological processes[23]. $NO^{\bullet}$ is double-edged role in specialized tissues and cells, which is an essential physiological signaling molecule mediating various cell functions but also induces cytotoxic and mutagenic effects when present in excess. $NO^{\bullet}$ reacts rapidly with superoxide anion to form peroxynitrite, which may be cytotoxic by itself or easily decompose to the highly reactive and toxic hydroxyl radical and nitrogen dioxide ($NO^{\bullet}_2$)[23]. The present study showed that serum levels of $NO^{\bullet}$ in patients with CL were significantly higher than the treated patients and controls. Similarly, Serarslan *et al.*[8] reported that patients with CL had higher NO levels than those of control and treatment groups. In a previous study, plasma nitrite and nitrate concentrations were also found to be higher in CL patients than those in healthy patients[24]. Increased NO levels found in present CL patients might participate in this process by reacting with superoxide anion, producing highly cytotoxic peroxynitrite with a consequent lipid peroxidation. It appears, therefore, that oxidative stress and the increased NO synthesis may be involved in the pathogenesis of CL.

The destructive chain reaction initiated by ROS can be broken by antioxidants, which are able to convert ROS into harmLess derivates. SOD and CAT are antioxidant enzymes which are used in this study. SOD activities in CL patients are controversial, as some reports show decreased[8] while others show increased activity levels[24]. The present results of serum SOD activity are similar to the report of Serarslan *et al.*[8], which showed significantly lower levels in CL patients than in the other two groups. CAT activities of CL patients were found to be decreased in the present study. The results for CAT activity are in aggrement with that obtained by Erel *et al.*[24]. The lower SOD and CAT activities may have been due to the response to

increased ROS production, which with elapsing time may be inadequate to detoxify high levels of ROS. The impaired antioxidant system may favour accumulation of free radicals. Alternatively, it is possible that the antioxidant system is impaired as a consequence of an abnormality in the antioxidative metabolism due to the CL.

In conclusion, on the basis of increased lipid peroxidation and decreased levels of antioxidant and enzymes in serum, the patients with cutaneous leishmaniasis are subject to oxidative stress. These results are consistent with the underlying hypothesis that there is an imbalance between ROS production and the antioxidant defence system in cutaneous leishmaniasis disease.

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