In vitro cultivation of axenic amastigotes and the comparison of antioxidant enzymes at different stages of *Leishmania tropica*

Bahrami, S.[1*], Hatam, G.R.[2], Razavi, M.[3] and Nazifi, S.[4]

[1] Department of Parasitology, Faculty of Veterinary Medicine, Shahid Chamran University, Ahvaz, Iran
[2] Center of Basic Researches in Infectious Disease, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[3] Department of Parasitology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[4] Department of Clinical Studies, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[*] Corresponding author email: bahrami.somayeh@gmail.com

Abstract. The present study aimed to establish a simple method to yield large amounts of *Leishmania tropica* amastigote-like forms in axenic cultures and to compare the superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymes at different stages of *L. tropica*. Different culture conditions were tested to find the optimum condition of axenic amastigotes generation. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities were determined at logarithmic and stationary phases and axenic amastigote stage of the parasite. A high proportion (88%) of amastigote-like forms of *L. tropica* was observed in BHI medium supplemented with 20% FCS, pH 4.5, and incubated at 37ºC with 5% CO_2. The results showed that SOD activity was at the lowest level in the logarithmic phase of promastigotes and increased towards the stationary phase of promastigotes and amastigote stage. The results showed that the optimum condition for differentiation of *L. tropica* promastigotes to axenic amastigotes was BHI medium containing 20% FCS at pH 4.5, incubated at 37ºC in the presence of 5% CO_2. It seems that SOD, but not GPX is a major determinant of intracellular survival of the parasite.

INTRODUCTION

Leishmania species are responsible for a wide spectrum of diseases, including cutaneous, mucocutaneous and fatal visceral leishmaniasis. Leishmaniasis is endemic in 88 countries and about 350 million people in the world are at risk (Mirjalili *et al.*, 2003). Two million new cases and 57,000 deaths are estimated to occur each year. Due to the significant impact of the disease on global public health, it is listed among the World Health Organization's (WHO) tropical disease priorities (de Oliveira Silva *et al.*, 2008). Leishmaniasis has received renewed interest because of an upsurge of cases in traditional endemic areas and the emergence of new foci of the disease (Ashford, 2000; Desjeux, 2001).

Cutaneous leishmaniasis (CL) is a disfiguring and debilitating disease that is prevalent in Iran, its neighboring countries and many other countries throughout the world (Katakura, 2009). *Leishmania tropica* and *Leishmania major* are the main etiologies of cutaneous leishmaniasis in Iran.

Leishmania are trypanosomatid flagellates with two distinct morphological stages. They exist as flagellated promastigotes in the insect vectors and as intracellular non-flagellated amastigote forms that infect mammals and replicate within their mononuclear phagocytes. In the mammalian hosts the flagellated promastigotes are delivered into a blood pool at the site of the sand fly bite. The promastigotes are then ingested by

mammalian macrophages (Sacks, 1992). In transforming from the promastigotes to amastigotes, the *Leishmania* parasites undergo numerous biochemical changes in order to adapt to their new environment. Numerous genes have been shown to be differentially expressed in the promastigote and amastigote stages and many morphological and metabolic changes have been documented (Plewes *et al.*, 2003). Upon infection, macrophages undergo a respiratory burst, producing reactive oxygen intermediates such as H_2O_2, ·OH radical, superoxide anions (O_2^-) and peroxynitrate as part of an oxygen dependent mechanism to kill invading microorganisms (Beaman & Beaman, 1984). Efficient evasion of the toxic microbicidal molecules produced at each stage of infection is important for *Leishmania* to be able to initiate and maintain host cell infection (Gantt *et al.*, 2001). The axenic logarithmic promastigote as the procyclic promastigote is a motile, flagellated form that multiplies in the sand fly gut. The axenic stationary phase as the metacyclic promastigote is a nondividing infective form that resides in the sand fly mouthparts and finally the axenic amastigote as the intracellular amastigote is a non motile form that lives and replicates in the phagolysosomal compartment of the mammalian macrophages.

The present study aimed to establish a simple method to yield large amounts of *L. tropica* amastigote-like forms in axenic cultures and to compare the superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymes at different stages of the parasite.

MATERIALS AND METHODS

Parasite and culture
The *Leishmania* strain used in this study was *L. tropica* (MHOM/ AF/ 88/ KK27). The promastigotes were grown at 26ºC in BHI medium plus 10% heat- inactivated fetal calf serum (FCS), pH 7.0 and 1 % of Penicillin (50 u/ml) Streptomycin (50 µg/ml) solution (Sigma, St. Louis, Mo., USA). The methods used for *in vitro* transformation of *L. tropica*

promastigotes to amastigotes mimic the parasites environmental condition in macrophage phagolysosomes. Different culture conditions for *L. tropica* promastigote transformation including medium (BHI, RPMI and NNN- Schneiders Drosophila), pH (4.5 and 5.5), temperature (30, 34 and 37ºC), FCS concentration (10 and 20%) and incubation with or without CO_2 were tested.

The cultures were initiated by 5 ml of the medium with a concentration of 5×10^6 promastigotes/ml in 25 cm^2 tissue flasks. To disrupt the aggregated forms in the cultures; the parasites were passed through a 25 gauge needle before counting and parasite number was calculated using a haemocytometer. To evaluate the transformation of promastigotes to amastigotes, three criteria including roundness, loss of flagellum and viability were checked every 12 hours. The viability of axenic amastigotes was tested by both Trypan blue (vital dye) and their differentiation back to promastigotes by shifting them to grow in pH 7.0 at 25ºC. To evaluate the morphology of the transformed parasites, the axenic amastigotes were harvested from the cultures, centrifuged at 400 g with 50 mM phosphate buffered saline (PBS) and applied to microscope slides. The slides were stained by Giemsa and examined by a light microscope (Olympus Optical, NY, model BX60, Tokyo, Japan) at x1000 magnification.

Determination of SOD and GPX activities
Twenty samples of different stages comprising of 1×10^7 parasites/ml were prepared. The logarithmic and stationary phases of organisms were obtained 3 and 10 days after cultivation respectively and the axenic like forms were harvested after 3 days. The culture tubes were centrifuged at 2000 g for 20 min at 4ºC, the supernatants discarded and the pelleted organisms were washed three times in cold-PBS. An equal volume of hypotonic aqueous solution of enzyme stabilizers containing 1 mM amino-n-caproic acid, 1 mM dithiotheritol and 1 mM EDTA (Sigma, St. Louis, Mo., USA) was added into the pellets and mixed thoroughly. Freeze/ thaw cycles were performed for five times.

The extracts were centrifuged at 18000 g for 30 min at 4ºC and the supernatants stored at -70ºC until use.

The determination of SOD activity was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5- phenyltetrazolium chloride to form a red formazon dye. The absorbance was measured at 505 nm and the SOD activity was then calculated according to the manufacturer's instruction (Ransod®-Randox Lab, Antrim, UK). GPX activity was determined based on the fact that GPX catalyzed the oxidation of glutathione by cumen hydroperoxide. In the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione was immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. The absorbance was measured at 340 nm and the GPX activity was then calculated according to the manufacturer's instruction (Ransel®- Randox Lab, Antrim, UK). The enzymes activities were expressed as U/ml. Data were analyzed statistically by One-Way ANOVA using SPSS 17 (SPSS Inc., Chicago, IL, USA). Differences of *P<0.05* were considered significant.

RESULTS

The results of promastigote transformation to axenic amastigotes at different conditions are presented in Table 1. As shown, the optimum condition for differentiation was BHI medium containing 20% FCS at pH 4.5, incubated at 37ºC in the presence of 5% CO_2. Differentiation was started after 12 h of incubation and a high proportion (88%) of living amastigote like forms was observed after 48 h. The axenic amastigotes could differentiate back to promastigotes by shifting them to grow at pH 7.0 at 25ºC within 48 h. At temperatures and media tested, the amastigotes grew slower at pH 5.5 than pH 4.5. The light microscopy of Giemsa-stained amastigote-like forms revealed oval or pyriform cells with flagellum either absent or truncated.

The SOD activity was observed at different stages with the highest level in axenic amastigotes. The activity was 12.34±1.37, 32.71±0.87 and 107.90±0.57 U/ml for logarithmic promastigotes, stationary promastigotes and axenic amastigotes, respectively. GPX activity was not detected at different stages.

Table 1. *L. tropica* promastigotes transformation (%) to axenic amastigotes at different conditions after 48 h of incubation. Data shown are the mean values of triplicate experiments

Composition of culture medium			Incubation condition					
Basic medium	FCS (%)	pH	30ºC		34ºC		37ºC	
			5 % CO_2	No CO_2	5 % CO_2	No CO_2	5 % CO_2	No CO_2
BHI	10	4.5	60	60	81	76	79	76
		5.5	45	41	50	48	52	50
	20	4.5	65	63	84	76	88	82
		5.5	45	43	53	51	54	51
RPMI	10	4.5	51	49	62	58	68	65
		5.5	43	43	59	54	67	63
	20	4.5	54	53	69	63	72	72
		5.5	49	48	67	65	69	67
NNN-Schneiders	10	4.5	56	54	66	65	69	67
		5.5	38	34	53	52	55	55
	20	4.5	58	57	70	67	73	70
		5.5	46	45	64	63	70	69

DISCUSSION

The protozoa of the genus *Leishmania* display three distinct physiological stages including, the flagellated procyclic promastigotes which infect the sand fly vector, the mammalian infective metacyclic promastigote forms and the non-flagellated amastigotes which reside in mammalian macrophage phagolysosomes (Saar *et al.*, 1998; Habibi *et al.*, 2008). Amastigotes are responsible for all clinical manifestations in the vertebrate hosts; therefore vaccines and chemotherapeutic targets need to be developed against this stage of the parasite. Most information on *Leishmania* have been derived from studies on the promastigote stage because culturing and maintaining of promastigotes are easy in different media. In contrast, the difficulty in obtaining large numbers of amastigotes free from host cell contaminant has hampered the investigation on them (Gupta *et al.*, 2001). The establishment of axenic cultures of the amastigote stage of *Leishmania* is important for the understanding of the mechanisms regulating the differentiation, development and pathogenecity of the parasite with a view to develop and identify molecular, immunological and chemotherapeutic targets. The availability of *L. tropica* axenic amastigotes which grow slowly and produce small lesions in animal models would facilitate drug screening, biological and chemical studies (Nasereddin *et al.*, 2010).

The first aim of the present study was to establish the optimum conditions for obtaining large amounts of *L. tropica* axenic amastigotes. The study was carried out with a 100% promastigote population which was cultured in different media at various temperatures, FCS concentrations, pH and presence or absence of CO_2. For *L. tropica*, a high proportion (88%) of the amastigote-like forms was observed in BHI medium supplemented with 20% FCS at pH 4.5 after 48 h of incubation at 37ºC in the presence of 5% CO_2. Several attempts have been directed to cultivate the amastigotes in the cell free media (Bates, 1993; Pan *et al.*, 1993), focusing on the *in vitro* transformation of promastigotes to amastigote like forms in

response to elevated temperature and acidic pH. Our findings are similar to those reported by Gupta *et al* (2001) for *L. donovani*, *Leishmania mexicana* and *Leishmania amazonensis*. In another study, optimal conditions for generation of *L. tropica* axenic amastigotes have been reported using RPMI medium, pH 5.5 at 36ºC (Nasereddin *et al.*, 2010). Balanco *et al* (1998) have reported transformation of the *L. braziliensis* promastigotes to amastigote forms in axenic culture using a UM-54 medium at 34ºC and pH 6.3. Saar *et al* (1998) primarily subjected the promastigotes of *L. donovani* to 37ºC in the presence of 5% CO_2 for 24 h and the differentiation to axenic amastigotes was then completed in 120 h by shifting the pH.

The *Leishmania* parasite occurs in the vertebrate hosts as the amastigote in the macrophage phagolysosomes. Upon infection macrophages apply various defense mechanisms to eliminate the parasite. These include production of reactive oxygen species (ROS), reactive nitrogen species (RNS), acidification of phagolysosomes and digestion by hydrolytic enzymes. The primary ROS include H_2O_2, OH radical and superoxide anion (O_2^-). The RNS defense mechanisms include nitric oxide and peroxynitrite ($ONOO^-$) as part of the oxygen-dependent mechanism to destroy the invading microorganisms. Overproduction of toxic oxygen intermediates may lead to modification of the macromolecules inside the cells such as proteins, lipids and DNA, which eventually leads to cell shrinkage and DNA fragmentation and causes programmed cell death or necrosis (Samali *et al.*, 1999; Tiwari *et al.*, 2002; Wochna *et al.*, 2005). On the other hand, the microorganism has evolved various mechanisms to inhibit the oxidative burst and nitric oxide production, and to scavenge and detoxify ROS and RNS. One of the antioxidant mechanisms that protect the micro-organisms against reactive oxygen intermediates is the enzymatic defense which includes SOD, catalase, peroxidoxins, flavo hemoglobins and glutathione S-transferase/GPX coupled to glutathione reductase (Chance *et al.*, 1979; Ursini *et al.*, 1995; Landis & Tower, 2005; Jirata *et al.*, 2006).

414

In the present study, two antioxidant enzymes were measured at different stages of *L. tropica*. SOD is a key enzyme that appears to act as a first line defense against ROS. The role of SOD is to accelerate the dismutation of the toxic superoxide radical ($O_2^.$) during oxidative energy processes to hydrogen peroxide and molecular oxygen. Our findings demonstrated a higher level of SOD activity in axenic amastigotes of *L. tropica*. It seems that this high level of SOD activity is crucial for survival of the parasite against the macrophages defense mechanisms. A higher level of SOD activity was also evident in the stationary phase promastigotes compared to the logarithmic phase. The higher level of SOD in the stationary phase compared to the logarithmic phase suggests that SOD is more important in the stationary phase. In terms of virulence, one difference between stationary and logarithmic phase is that the former is more infective than the latter. Therefore promastigotes undergo a developmental change as they progress from logarithmic to stationary phase, which is accompanied by an increase in resistance to ROS and increase in virulence. Two types of SOD gene (SOD-A and SOD-B) have been cloned from *Leishmania chagasi*. Overexpression of SOD in *L. chagasi* parasites conferred enhanced protection against the free radical generating agents paraquat and nitroprusside (Paramchuk *et al.*, 1997). Ghosh *et al* (2003) demonstrated that SOD-deficient promastigotes of *L. tropica* have enhanced sensitivity to menadione and hydrogen peroxide in axenic culture. They concluded that SOD was a major determinant of intracellular survival of *Leishmania* (Ghosh *et al.*, 2003).

In most eukaryotes, glutathione dependant peroxidases play a key role in the metabolism of peroxides. Our results showed no activity of GPX in all stages of *L. tropica* which was in accordance with the findings of others who have reported the deficiency of this enzyme in different trypanosomatids (Docampo, 1990; Mehlotra, 1996). In eukaryotes, peroxide detoxification mainly depends on glutathione and involves glutathione peroxidase and glutathione

reductase. The medically important parasitic Kinetoplastida, genera *Leishmania* and *Trypanosoma*, are different and rely on a polyamine peptide conjugate N1, N8- bis (glutathionyl) spermidine called trypanothione. This key metabolite regulates intracellular thiol balance, contributes to defense against oxidant stress and provides reducing equivalents for peroxide metabolizing systems and ribonucleotide reductase for nucleic acid synthesis (Fairlamb & Cerami, 1992; Flohe *et al.*, 1999). However, Wilkinson *et al* (2000) showed GPX1 in *T. cruzi* which was actually tryparedoxin-dependent peroxidase with low and non physiological activity with glutathione. Konig & Fairlamb (2007) showed that the genome of *L. major* contains three almost identical genes encoding putative glutathione peroxidase, but the relevant recombinant protein showed negligible activity with glutathione.

In conclusion, our results showed that BHI medium supplemented with 20% FCS at pH 4.5 and incubated at 37ºC in the presence of 5% CO_2 was the optimum condition for differentiation of *L. tropica* promastigotes to axenic amastigotes, and SOD, but not GPX, plays a key role in the metabolism of peroxides in *L. tropica*.

REFERENCES

Ashford, R.W. (2000). The leishmaniases as emerging and reemerging zoonoses. *International Journal of Parasitology* **30**: 1269-1281.

Balanco, J.M.F., Pral, E.M.F., da Silva, S., Bijovsky, A.T., Martara, R.A. & Alfieri, S.C. (1998). Axenic cultivation and partial characterization of *Leishmania braziliensis* amastigote like forms. *Parasitology* **116**: 103-113.

Bates, P.A. (1993). Axenic culture of Leishmania amastigotes. *Parasitology Today* **9**: 143-146.

Beaman, L. & Beaman, B.L. (1984). The role of oxygen and its derivatives in microbial pathogenesis and host defense. *Annual Reviews of Microbiology* **38**: 27-48.

Chance, B., Sies, H. & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* **59**: 527-605.

de Oliveira Silva, F., Morais Teixeira, E. & Rabello, A. (2008). Antileishmanial activity of Azithromycin against *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania chagasi*. *American Journal of Tropical Hygiene* **78**: 745-749.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **95**: 239-243.

Docampo, R. (1990). Sensitivity of parasites to free radical damage by antiparasitic drugs. *Chemico-Biological Interactions* **73**: 1-27.

Fairlamb, A.H. & Cerami, A. (1992). Metabolism and functions of trypanothione in the Kinetoplastida. *Annual Reviews of Microbiology* **46**: 695-729.

Flohe, L., Hecht, H.J. & Steinert, P. (1999). Glutathione and trypanothione in parasitic hydroperoxide metabolism. *Free Radical Biology and Medicine* **27**: 966-984.

Gantt, K.R., Goldman, T.L., McCormick, M.L., Miller, M.A., Jeronimo, S.M.B., Nascimento, E.T., Britigan, B.E. & Wilson, M.E. (2001). Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. *Journal of Immunology* **167**: 893-901.

Ghosh, S., Goswami, S. & Adhya, S. (2003). Role of superoxide dismutase in survival of Leishmania within the macrophage. *Biochemical Journal* **369**: 447-452.

Gupta, N., Goyal, N. & Rastogi, A.K. (2001). In vitro cultivation and characterization of axenic amastigotes of Leishmania. *Trends in Parasitology* **17**: 150-153.

Habibi, P., Sadjjadi, S.M., Owji, M., Moattari, A., Sarkari, B., Naghibalhosseini, F., Hatam, G.R. & Kazemian, S. (2008). Characterization of in vitro cultivated amastigote like of *Leishmania major*: A substitution for in vivo studies. *Iranian Journal of Parasitology* **3**: 6-15.

Jirata, D., Kuru, T., Genetu, A., Barr, S., Hailu, A., Aseffa, A. & Gedamud, L. (2006). Identification, sequencing and expression of peroxidoxin genes from *Leishmania aethiopica*. *Acta Tropica* **99**: 88-96.

Katakura, K. (2009). Molecular epidemiology of leishmaniasis in Asia (focus on cutaneous infections). *Current Opinion in Infectious Diseases* **22**: 126-130.

Konig, J. & Fairlamb, A.H. (2007). A comparative study of type I and type II tryparedoxin peroxidases in *Leishmania major*. *The FEBS Journal* **274**: 5643-58.

Landis, G.N. & Tower, J. (2005). Superoxide dismutase evolution and life span regulation. *Mechanisms of Ageing and Development* **126**: 365-79.

Mehlotra, R.K. (1996). Antioxidant defense mechanisms in parasitic protozoa. *Critical Reviews in Microbiology* **22**: 295-314.

Mirjalili, A., Alimohammadian, M. & Madani, R. (2003). Presence of immunoglobulins on the surface of *Leishmania major* as a tool for isolation of amastigote stage from *BALB/ c* mice. *Iranian Biomedical Journal* **7**: 155-160.

Nasereddin, A., Schweynoch, C., Schonian, G. & Jaffe, C.L. (2010). Characterization of *Leishmania (Leishmania) tropica* axenic amastigotes. *Acta Tropica* **113**: 72-79.

Pan, A.A., Duboise, S.M., Eperon, S., Rivas, L., Hodgkinson, V., Traub- Cseko, Y. & McMahon-Pratt, D. (1993). Developmental life cycle of Leishmania cultivation and characterization of cultured extracellular amastigotes. *Journal of Eukaryote Microbiology* **40**: 213-223.

Paramchuk, W.J., Ismail, S.O., Bhatia, A. & Gedamu, L. (1997). Cloning, characterization and overexpression of two iron superoxide dismutase cDNAs from *Leishmania chagasi*: role in pathogenesis. *Molecular and Biochemical Parasitology* **90**: 203-221.

Plewes, K.A., Barr, S.D. & Gedamu, L. (2003). Iron superoxide dismutases targeted to the glycosomes of *Leishmania chagasi* are important for survival. *Infection and Immunity* **71**: 5910-5920.

Saar, Y., Ransford, A., Waldman, E., Mazareb, S., Spector, S., Plumblee, J., Turco, S.J. & Zilberstein, D. (1998). Characterization of developmentally regulated activities in axenic amastigotes of *Leishmania donovani*. *Molecular and Biochemical Parasitology* **5**: 9-20.

Sacks, D.L. (1992). The structure and function of the surface lipophosphoglycan on different developmental stages of Leishmania promastigotes. *Infectious Agents and Disease* **1**: 200-206.

Samali, A., Nordgren, H., Zhivotovsky, B., Peterson, E. & Orrenius, S. (1999). A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. *Biochemical and Biophysical Research Communications* **255**: 6-11.

Tiwari, B.S., Belenghi, B. & Levine, A. (2002). Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant Physiology* **128**: 1271-1281.

Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K.D., Roveri, A., Schomburg, D. & Flohe, L. (1995). Diversity of glutathione peroxidases. *Methods in Enzymology* **252**: 38-53.

Wilkinson, S.R., Meyer, D.J. & Kelly, J.M. (2000). Biochemical characterization of a trypanosome enzyme with glutathione-dependent peroxidase activity. *Biochemical Journal* **352**: 755-761.

Wochna, A., Niemczyk, E., Kurono, C., Masaoka, M., Majczak, A., Kędzior, J., Słomińska, E., Lipiński, M. & Wakabayashi, T. (2005). Role of mitochondria in the switch mechanism of the cell death mode from apoptosis to necrosis-Studies on{rho}0 cells. *Journal of Electron Microscopy* **54**: 127-138.