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Original Article

Overexpression of Iron Super Oxide Dismutases A/B Genes Are Associated with Antimony Resistance of *Leishmania tropica* Clinical Isolates

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Received 23 Feb 2022
Accepted 19 May 2022

Keywords:
Superoxide dismutase; Gene expression; Antimony resistance; *L. tropica*; Polymerase chain reaction

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Abstract

Background: Pentavalent antimonial has been a drug of choice against leishmaniasis, despite the emergence of treatment failure. Identification of resistance markers is urgently needed to design new therapeutic strategies. Iron-Superoxide dismutases (Fe-SODs) are antioxidant enzymes contributing to detoxify reactive oxygen species to prevent a cell from oxidative stress. Since antimonial compounds induce oxidative stress, in this survey, the expression of *SOD* genes was investigated to identify their expression pattern in clinical resistant isolates.

Methods: This cross-sectional survey was done in Mashhad City, northeast of Iran during 2014 to 2019. The RNA expression level of mitochondrial (*SODA*) and glycosomal (*SODB*) superoxide dismutase was investigated in 25 antimony responsive (n=15) and unresponsive (n=10) anthroponotic cutaneous leishmaniasis (ACL) patients. Total RNA extraction and cDNA synthesis, the qRT-PCR approach was utilized to investigate the relative RNA expression level.

Results: The transcript level of *SOD*s was over-expressed in the most resistant isolates. Gene expression analysis demonstrated the over-expression of *SODA* and B by a factor of 3.8 and 4.81, respectively, in resistance isolates vs. sensitive ones.

Conclusion: Aberrant expression of *SODA/B* in unresponsive parasites could potentially implicate in detoxifying antimony-induced oxidative stress. Moreover, *SOD*s might be considered as potential predictive markers of the response to antimonials in ACL patients in endemic areas.

Introduction

Leishmaniasis is a group of infectious vector-borne tropical diseases caused by protozoan parasites belonging to the *Leishmania* genus transmitted to humans by the bites of infected sand flies (1). Clinical manifestations could be ranged from cutaneous, mucocutaneous to visceral forms, fatal if left neglected (2). Cutaneous leishmaniasis (CL) is one of the most frequent types of disease (3). Both forms of cutaneous leishmaniasis, including anthroponotic and zoonotic CL and Mediterranean visceral leishmaniasis are distributed in more than half of the provinces of Iran (4). The causative agents of anthroponotic cutaneous leishmaniasis (ACL) patients and zoonotic cutaneous leishmaniasis (ZCL) are *Leishmania tropica* and *L. major*, respectively (5). The first-line therapy for leishmaniasis is pentavalent antimonials (SbV), such as meglumine antimoniate (glucantime®) (6). In 2002 and 2004, the outbreaks of ACL occurred in Mashhad and Bam counties in the northeast and center of Iran, respectively (7). In recent decades, implicit evidence proposes growing number of patients with CL are unresponsiveness to antimonials (8). In this line, the result of a cross-sectional study in Mashhad revealed that almost 12% of the patients did not respond to antimonial drugs (9).

Based on the results obtained from laboratory-generated parasites, various mechanisms such as lower activation of sbV to sbIII, mitigation of drug uptake, and promoted drug efflux could be involved in the resistance (10-13). However, several additional contributing factors have also been suggested to develop natural antimony resistance. In this context, proteomics results from two responsive and unresponsive *L. tropica* field isolates suggested that molecular markers involved in oxidative stress such as superoxide dismutase might be implicated in this issue (14).

Superoxide dismutases (SODs) contain a set of antioxidant metalloenzymes comprising iron (FeSOD), manganese (MnSOD), nickel (NiSOD), as well as copper and zinc (Cu/Zn SOD) that protect the cell against reactive oxygen spices (ROS). These enzymes catalyze the superoxide ($O_2^{-\circ}$) into hydrogen peroxide (H_2O_2) and ordinary molecular oxygen (O_2) (15, 16). FeSOD has been identified in kinetoplastids such as *Leishmania* spp. It is contributed to the long-term survival of parasites during respiratory bursts of macrophages and thereby establishing the infection (16, 17). Because Fe-SOD is not found in humans, it could be introduced as a potential drug target of leishmaniasis (17). Two types of *SOD* genes have been reported in *Leishmania* parasites; *SODA* exists in mitochondria, and *SODB* is localized in the glycosome (15, 18, 19). FeSODA accelerates *Leishmania* parasites' virulence through protection against mitochondrion-produced oxidative stress and via supporting the reactive oxygen species (ROS)-mediated pathway mechanisms essential for the survival and differentiation of infective types (20).

Furthermore, *SODB* protein is identified as a cofactor that facilitates the persistence of *L. major* parasites in macrophages and promotes virulence in mice (21). Since SODs are involved in the scavenging of reactive oxygen species (ROS), they can reduce the effectiveness of drugs. Given that one of the predominant mechanisms of the antimonial compound is the generation of ROS (20), the potential role of antioxidant enzymes like SOD in antimony resistance should be considered.

We aimed to assess the RNA expression of the *SODA/B* gene in antimony-sensitive and -resistant *L. tropica* field isolates from northeast Iran, the endemic area for ACL.

Materials and Methods

Study area and clinical isolates

This cross-sectional survey was done in Mashhad City, one of the most important regions for ACL due to *L. tropica* located in the northeast of Iran during 2014 to 2019. Samples were collected from patients with positive smear skin lesions admitted to the parasitology laboratory of Imam Reza Hospital, Mashhad, Iran. Skin biopsies samples were obtained from lesions of 25 CL patients. Samples were divided into two groups, including 15 responsiveness and 10 unresponsiveness to treatment with glucantime®. Characterization and distribution of responses to treatment based on clinical signs, duration of glucantime, and clinical outcome are given in Table 1. Unresponsiveness cases were patients who were administrated to receive at least 3 courses of glucantime; however, they still presented active lesion(s), and responsiveness cases were patients who were cured after treating with one course of glucantime (9).

Moreover, *L. tropica* strain MHOM/IR/10/175 with IC_{50} of 6.7 ± 1.2 µg/ml was used as an antimony sensitive reference strain to normalize the data. In addition, an isolate MHOM/IR/10/827 with IC_{50} values of 56. 7 ± 5.8 µg/ml was considered as a resistant reference strain (12).

This study was approved by the Ethics Committee of Tehran University of Medical Sciences, and written informed consent was signed from all patients.

Table 1: Characterization and distribution of responsive and unresponsive cases by clinical signs, drug administration duration, and clinical outcome

Patients (Nos)	*Clinical signs Number of lesions/Duration of lesions (Nos)*	*Duration of using glucantime®*	*Clinical outcome*
Responsive to glucantime (n=15)	1-2 lesions /3-4 months(n=10) ≥3 lesions/4-6 months (n=5)	One course ≤ 20 days	Healing
Unresponsive to glucantime (n=10)	1 lesion /> 2 years (n=4) 2-3 lesions/ > 3 years (n=3) Lupoid form/ > 5 years (n=3)	Two courses ≤ 30 days More than 3 courses ≤ 100 days More than 3 courses ≤ 180 days	Non-healing after 2 years Non-healing after 3 years Non-healing>5 years

Skin lesion sampling and Parasite culture

The serosity samples were first cultured to Novy, Mac -Neal & Nicolle (NNN) culture medium. After 5-8 days, promastigotes were propagated and transferred to RPMI 1640 (Gibco-Invitrogen) supplemented with 15% fetal bovine serum (FBS) and 1% pen-strep (100 U/ml penicillin G, 100 µg/ml streptomycin sulfate) at the temperature of 24–25 ◦c. The growth of promastigotes was monitored daily by the inverted microscope (22).

DNA extraction and PCR-RFLP

For characterization of the *Leishmania* species, DNA of 1 × 10^6 stationary phase promastigotes was extracted by Genomic DNA extraction was processed with the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The DNA samples were preserved at -20 °C until used.

For PCR-RFLP, first Internal transcript spacer 1 of ribosomal RNA (ITSI-rRNA) fragment was amplified using the following forward and reverse primers: 5'- CTG-

GATCATTTTCCGAT-3' and 5'-TGATACCACTTATCGCACTT-3'. Afterward, 10µl of amplicons were digested with fast digestion HaeIII (BsuRI) enzyme (Fermentas, Germany), according to the manufacturer's instructions, and digested fragments were visualized on 3% agarose gels. *Leishmania* species were identified based on obtained patterns alongside reference species, including *L. tropica* (GenBank accession number EF653267) and *L. major*. PCR-RFLP analysis confirmed all clinical isolates were *L. tropica* as described elsewhere (13, 23).

RNA extraction and cDNA synthesis

Total RNA was prepared from 10^8 metacyclic promastigotes of sensitive and resistant samples from the mid-log phase using Tripure reagent (Roche, Mannheim, Germany) (13). The quality and concentration of the isolated RNA were assessed by electrophoresed on agarose gel and NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific Fisher, US). RNA was subjected to DNase I (Fermentas, Burlington, Canada) to remove any genomic contamination as described by the manufacturer.

Three independent RNA specimens were utilized for each real-time PCR run. Synthesis of complementary DNA (cDNA) was performed using 1 µg RNA template, 2.5 µmol/µl oligo-dT using a cDNA Synthesis Kit (Roche, Germany) concerning the manufacturer's recommendations. The cDNA integrity was evaluated with alpha-tubulin primers as a housekeeping gene. The PCR reaction protocol was performed by 35 cycles of 94 °C (30 s), 49 °C (30 s), and 72 °C (45 s). The eventual extension was done by 72 °C (5 min) (24).

Real-time RT-PCR analysis

For real-time reverse transcriptase-PCR (RT-PCR) assays, 1 µl cDNA template was utilized in a total amplification reactions volume of 20 µl, with 1x SYBR® Premix Ex Taq™ II (Takara, Tokyo, Japan), forward and reverse primers (100 nM), in Real-Time System, Applied Biosystems (ABI), Foster City, CA, USA). PCR primers of genes of interest for the real-time method were designed using Primer 3 software version 0.4.0 (Table 2). cDNA was quantified in duplicate and a 2-step PCR protocol. The program of PCR cycling reaction was the first step at 95 °C (3 min), followed by 40 cycles at 95 °C (10 s) and 60 °C (32 s).

The specificity of the reaction was confirmed by a generation of the melting curve. For normalization purposes, the housekeeping alpha-tubulin gene was used (Table 2).

Table 2: Designed primers for real-time RT-PCR

Target genes	*Name*	*Primer sequences*	*PCR product (bp)*
α-tubulin (internal control)	*ALT* F	CAGGTGGTGTCGTCTCTGAC	119
	ALT R	TAGCTCGTCAGCACGAAGTG	
Superoxide dismutase A (*SODA*)	*SODA* F	TGTGCTACCATACCCTTCCTC	146
	SODA R	CCGAGCCTGTTCAACTTGT	
Super oxide dismutase B (*SODB*)	*SODB* F	ATG TCGAAG GAGCAGGTCAC	127
	SODB R	CAG ACTTGATGATGTCCAC-CAG	

Gene expression analysis

The resulting data are expressed as the fold change in each target gene expression according to the observed threshold cycle (Ct) value in the *L. tropica* field isolates normalized to that of reference genes (α-tubulin) and in relative to the sensitive reference isolate S175 im-

plemented in the REST 2009 software, as described formerly (25).

Statistical Methods

All experiments were taken at least two times and expressed as the mean ± standard deviations (SDs). The expression ratio results of the investigated transcripts were tested for significance by a statistical model (Pair Wise Fixed Reallocation Randomization Test). They were plotted using standard error estimation through a complex Taylor algorithm produced by REST. Statistical significance between two groups was evaluated using a two-tailed unpaired *t*-test. The acceptable *P* values for statistically significant were ≤ 0.05 (26).

Results

Species Identification

DNA extracted from culture-positive samples yielded an amplicon of 300–350 bp. All the clinical isolates were characterized as *L. tropica* using PCR-RFLP. After digestion *L. tropica* showed three bands of 200 bp, 60 bp, and 50 bp, while the pattern of L. major exhibited 220 and 180bp fragments (Fig. 1).

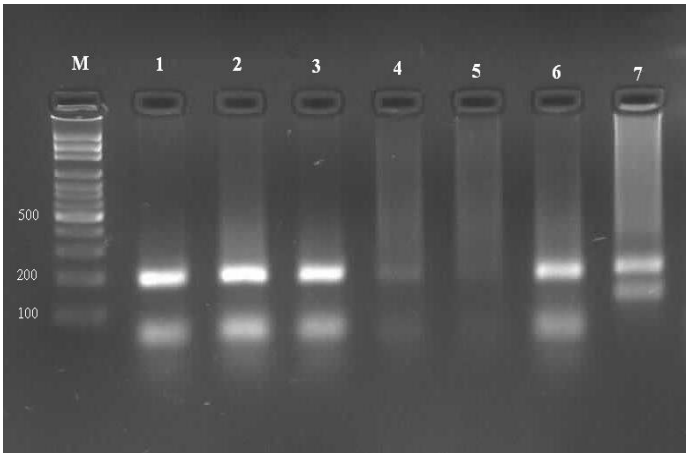

Fig. 1: ITS-PCR RFLP banding patterns were obtained from patient samples (lane1-5), lane 6, *L. tropica* reference strain, lane 7, *L. major* reference strain, lane M, and molecular size standard marker (100bp)

Gene expression of L. tropica isolates

The relative gene expression values of target genes in 15 responsive and 10 unresponsive isolates, as well as a resistant standard isolate, were expressed concerning to the reference sensitive isolate (MHOM/IR/10/Mash-175). Moreover, the specificity of the reaction was confirmed by the generation of the melting curve. Melting peaks analysis on the PCR products showed that there were no primer-dimers and non-specific products. Only a single peak was visible for each gene in the melting peak chart (Fig. 2).

SODA gene expression

The transcript level of *SODA* was significantly increased in 6 resistant isolates by 2.46 to 3.84-fold ($P<0.05$), whereas in remaining unresponsive samples, it did not alter substantially versus reference isolate S128. In addition, in standard resistant isolates MHOM/IR/10/827 (number 26), it was over-expressed significantly by a magnitude of 2.98-fold. On the other hand, in 2 responsive isolates, it was upregulated slightly by 2.23-fold and 2.57-fold, and in one sensitive isolate, it was downregulated (-2.13-fold); in other sensitive isolates, no significant differences in mRNA expression level was detected (Fig. 3). In addition, the mean expression value of

SODA in resistant and sensitive isolates were 2.16 and 0.579, respectively, and a significant up-regulation (3.73-fold) was detected in resistant isolates versus sensitive ones.

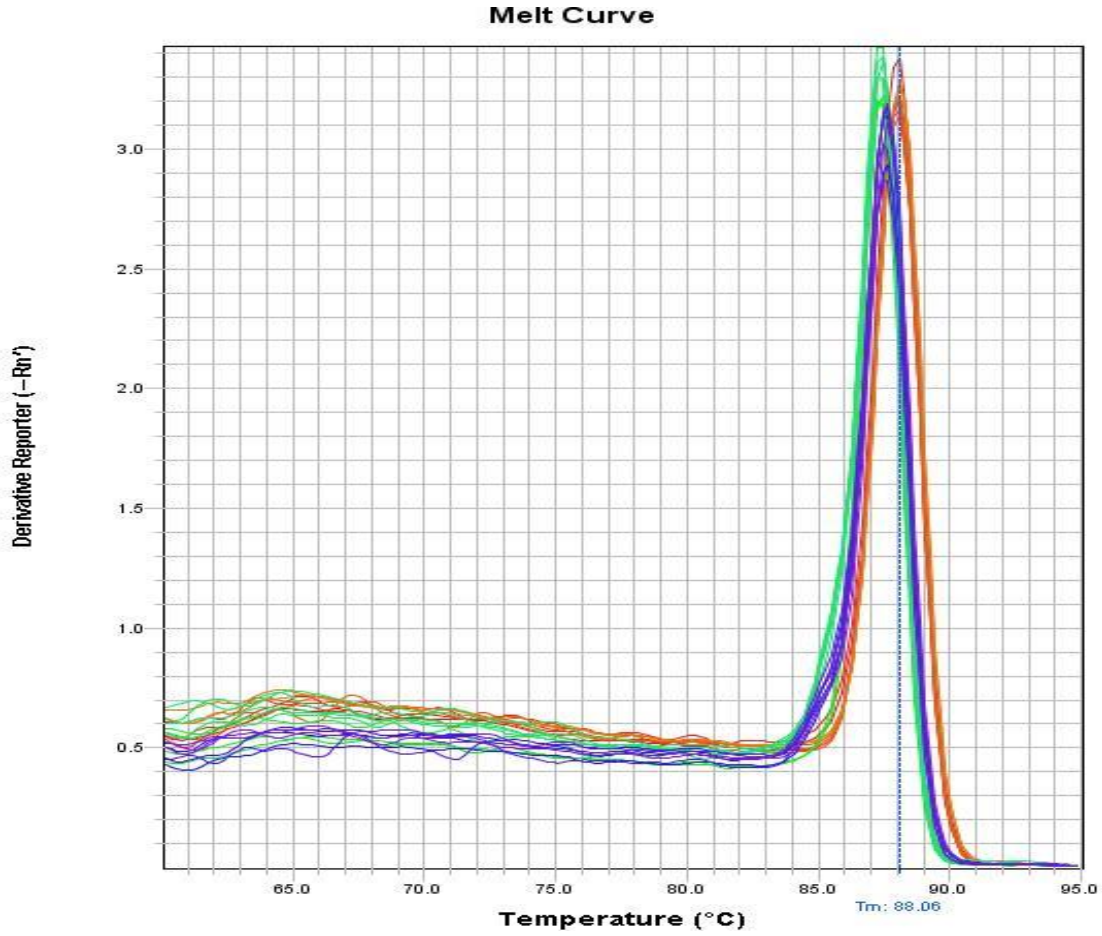

Fig. 2: Melting Curve analysis on the PCR product of *SODA*, *SODB*, and *ALT* genes. Only a single peak was visible for each specific PCR product. Orange, green, and purple peaks are related to *ALT*, *SODA*, and *SODB*, respectively

SODB gene expression

The expression of *SODB* was significantly upregulated in 8 unresponsive isolates by 2.389 to 4.924-fold, and in the remaining resistant isolates, it yielded normal expression compared to reference strain S175; also, in the standard resistant isolates MHOM/IR/10/827 it was increased (3.997-fold). The RNA amplification of *SODB* was elevated in three sensitive isolates by 2.41 to 2.92-fold and decreased significantly in one sensitive sample (-2.23-fold); in the remaining specimens, it exhibited normal expression similar to reference strain S175 ($P > 0.05$; Fig. 3). Furthermore, the mean expression value of *SODB* in unresponsive and responsive isolates were 3.8 and 0.79, respectively, which was significantly 4.81-fold higher in resistant isolates than sensitive ones.

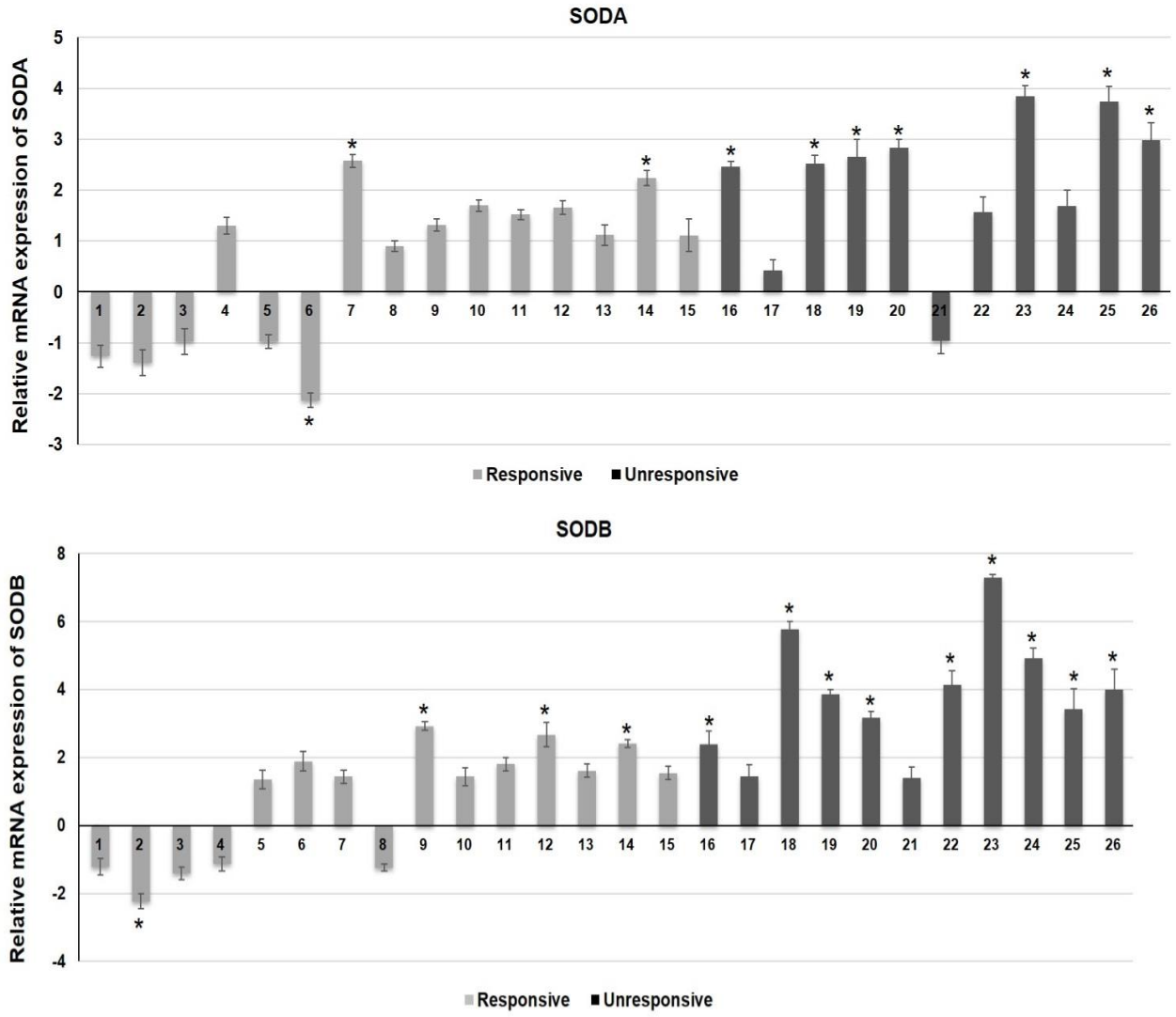

Fig. 3: Relative mRNA expression patterns of SODA and SODB in L. tropica isolates from responsive and unresponsive by Real-Time RT-PCR. The expression of alpha-tubulin was used to normalize the data. The values are the mean ± SD of two independent experiments, *; Significantly different (P<0.05)

Discussion

In the last decade, the number of treatment failures to pentavalent antimonials is increasing due to the development of resistant parasites (9). Several investigations on transcriptome and proteome of the resistant parasite have been clarified that drug resistance is complex, and different pathways could be involved (12, 28). In this concept, it was suggested that the redox pathway could play an important role in natural antimony resistance. One of the important antioxidant proteins in this pathway is *SOD*s, which protect the cell from free-radical toxicity (15, 16). In the current study, the transcription levels of *SODA* and *SODB* genes were determined at mRNA level in parasites isolated from responsive and unresponsive ACL patients.

Accumulating evidence indicated that FeSODs are critical for the replication and persistence of *Leishmania* in macrophages through protecting against oxidative stress. SODA

plays a vital role in reducing superoxide generation in mitochondria and preventing their toxic effect (15). In this line, the overexpression of *SODA* in *L. chagasi* leads to increased resistance to causative agents of free radicals such as nitroprusside and paraquat, suggesting that *SOD*s can neutralize free radicals induced by drugs (18). The high-regulation of *FeSODA* protects *L. donovani* from cytotoxic effects of miltefosine by decreasing superoxide generation in mitochondria (19). Inconsistent, in laboratory-generated miltefosine-resistant strains of *L. donovani*, the transcript level of *SODA* was increased significantly, suggesting its role against mitochondrial-derived ROS damage and apoptosis (28).

Moreover, in antimony-resistant *L. braziliensis* and *L. infantum* lines, which were selected in vitro, *FeSODA* transcript and protein expression were similar compared to wild type; though, the activity of superoxide dismutase enzyme was higher in SbIII-resistant laboratory-induced resistant cell lines (29). This result proposed the involvement of *SODA* in antimony resistance induced by drug pressure. Recently, Veronica et al (30) revealed upregulation of *FeSODA* in the miltefosine-resistant *L. donovani* strain using proteomics approach as well as higher enzymatic activity. In addition, silencing of *SODA* sensitized cells to miltefosine suggesting the critical importance of *SODA* in monitoring drug resistance. In consistent with previous studies (28, 30), in current study the mean expression value of *SODA* was 3.73-fold higher in resistant isolates; the transcription level was raised in six out of ten unresponsive isolates and decreased in one sensitive isolate; however, in contrast, in two sensitive isolates, it was up-regulated slightly. These discrepancies in expression level in individual clinical isolates were also reported in former studies conducted on different target genes of various *Leishmania* species suggesting that different genes might be contributed to natural antimonial resistance of even each isolate of the identical species (12, 31, 32).

The other gene that was investigated is *SODB*, which is essential for the detoxification of oxidative radicals in glycosome (15, 33). Plewes and co-workers demonstrated that knockout of the *SODB* gene in *L. chagasi* reduced parasite survival in macrophages and increased sensitivity to oxidative stress; these data suggested that *SODB* is essential for parasite virulence and persistence in macrophages (16). Besides, overexpression of *SODB* in *L. chagasi* resulted in an increase of resistance to agents that produce free radicals such as nitroprusside and paraquat (18). In addition, *SOD* has been reported to eliminate oxidative stress induced by arsenic (34). Moreover, in the present study, the mean expression level of *SODB* was 4.81-times more in unresponsive *L. tropica* isolates versus the responsive ones.

Additionally, 80% of unresponsive isolates presented a significant up-regulation in the mRNA level of *SODB* and one sensitive isolate displayed a down-regulation. On the other hand, 3 responsive isolates showed slight up-regulation of *SODB*; these discrepancies in a few isolates suggesting a multiplicity of underlying mechanisms participate in drug resistance. In addition, the elevation of *SODA/B* in a few sensitive isolates proposed that different vital roles of *SOD*s besides antimony resistance should be considered in this alteration.

Conclusion

Overall, our results revealed the upregulation of *SODA* and *SODB* genes in resistant clinical isolates; suggesting that these alterations could potentially contribute to detoxifying reactive oxygen species and oxidative stress induced by antimonials. Furthermore, they could be developed as potential predictive markers of the response to antimonials in ACL patients, especially in endemic areas. Further study on a large scale is suggested to validate our findings for developing novel

tools to predict treatment efficacy and assist in controlling disease propagation and resistance dynamics.

Acknowledgements

The authors are grateful to Dr. Maryam Nakhaee, Mr. Mahmood Paryan, and Miss. Bahare Abdollahi for their kind cooperation. This project was financially supported by the Vice-Chancellor for Research, Tehran University of Medical Science, Iran (Project NO: 27047).

Conflict of interest

All authors of this manuscript declare that they have no conflict of interest related to the content of this manuscript.

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