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Original papers

Gene expression of RNAP II, JBP1 and JBP2 in *Leishmania major* exposed to antimonials, amphotericin B and paromomycin

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ABSTRACT. Cutaneous leishmaniosis (CL) is treated with pentavalent antimony (SbV) as a first-line drug, while amphotericin B and paromomycin are potential alternatives in antimonial- resistant isolates. However, the mechanisms of drug resistance remain unclear. The present study analyses the gene expression of RNA polymerase II (RNAP II) and J-binding protein 1 (JBP1), and J-binding protein 2 (JBP2) in *Leishmania major* after exposure to drugs *in vitro*. *L. major* (MRHO/IR/75/ER) promastigotes were exposed to various concentrations of glucantime, paromomycin and amphotericin B for 72 hours. The RNA was then extracted and used for cDNA synthesis. The expressions of JBP1, JBP2 and RNAP II were analysed using SYBR Green real-time PCR. No change in JBP2 or RNAP II expression was associated with amphotericin B, but JBP1 expression decreased with increasing drug concentration. Paromomycin had no effect on JBP2 expression, but a 13.5-fold increase in JBP1 was observed at 100 µg/ml, and a decrease in RNAP II expression at 25 and 50 µg/ml. Exposure to glucantime resulted in 1.4-fold lower JBP1 expression at 5 µg/ml, and 333.33- to 500-fold lower RNAP II at concentrations of 5 to 15 µg/ml. As Base J synthesis requires both JBP1 and JBP2, RNAP II (encoding RNA polymerase II) could reduce expression. However, RNAP II was not expressed in all groups, indicating that the genes associated with drug resistance may be regulated in other ways.

Key words: *Leishmania major*, J-binding protein 1, J-binding protein 2, RNA polymerase II, glucantime, paromomycin, amphotericin B

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Introduction

Leishmania is a species of protozoan parasite which is known to cause leishmaniosis, with cutaneous leishmaniosis (CL) being the most common form. CL, including anthroponotic CL (ACL) and zoonotic CL (ZCL), is endemic in many parts of the world [1]. Due to the unavailability of a vaccine, chemotherapy is considered the main means of control. Pentavalent antimonials (SbV), including sodium stibogluconate, meglumine antimoniate or generic formulations, are the first-line treatment drugs. However, if antimony resistance occurs in endemic regions [2,3], second-line drugs such as amphotericin B deoxycholate are administered. The interaction between amphotericin B and the ergosterol present in the cell membrane alters cell permeability and causes cell death [4]. Another treatment for CL, particularly that caused by *L. major*, is paromomycin, which rapidly and

more completely cures the disease [5]. Overall, based on the shape and number of sores and the causative agent, treatment of CL may be local or systemic.

CL may develop drug resistance in response to several factors. The drug responsiveness of CL is known to be influenced by drug-host immune interaction, pharmacokinetic differentials and the causative species of *Leishmania*. In addition, response can be influenced by cell gene dosage, including intrachromosomal rearrangements [6], whole chromosome copy numbers [7] and aneuploidy [8].

In the Kinetoplastida, an important role in the silencing of an expression site is played by base J (β-D-glucosyl-hydroxymethyluracil). Base J is localized at terminator sites [9] and is critical for terminating RNA Polymerase II (RNAP II) transcription, which is responsible for the transcription of protein-coding genes. It is common throughout the genome, including stretches of repetitive DNA such as telomeric repeats [10]. Base J biosynthesis is therefore a potential target for the chemotherapy of pathogenic kinetoplastids [11].

Base J is synthesized by J-binding proteins 1 and 2 (JBP1 and JBP2) [12,13]. JBP1 is responsible for the first step of base J synthesis and JBP2 propagate its synthesis. JBP2 does not bind to J-DNA [13], but its activity is related to its SWI/SNF domain, which is essential for its activity [13]. Both JBP1 and JBP2 belong to the TET/JBP subfamily of dioxygenases, whose activity depends on Fe^{2+} and 2-oxoglutarate [14,15].

Although it seems that more than 90% of all base J in *Leishmania* is present in telomeric repeats [16], the remaining base J is present in chromosome-internal positions especially at RNAP II transcription termination sites [17]. The loss of base J is accompanied by excessive transcription of telomeres where the RNAP II present. As base J plays an important role in the promotion of correct gene expression by RNAP II, and base J synthesis is controlled by JBP1 and JBP2, the aim of the present study is to analyze the *in vitro* gene expression of JBP1, JBP2 and RNAP II in a standard strain of *L. major* (MRHO/IR/75/ER) exposed to glucantime, amphotricin B and paromomycin.

Materials and Methods

The following reagents were purchased: Glucantime (#M-2004; Sigma-Aldrich; MO; USA), amphotericin B (#A2411; Sigma-Aldrich; MO; USA), paromomycin (#P9297; Sigma-Aldrich; MO; USA) Arcturus® PicoPure® RNA Isolation Kit (#KIT0214; Applied Biosystems; CA; USA) for RNA extraction; High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (#4374966; Applied Biosystems; CA; USA) for cDNA synthesis; SYBR® Green PCR Master Mix (#4309155; Applied Biosystems; CA; USA) for Real-Time PCR SYBR Green master mix.

Leishmania major (MRHO/IR/75/ER) promastigotes was prepared from the Department of Parasitology and Mycology, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The standard strain was first cultured in NNN and then in RPMI 1640 supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), before being incubated at 25°C. Cultures were maintained in exponential growth by passages every three days to allow bulk cultivation.

L. major promastigotes (10^6/ml) were exposed to glucantime, paromomycin, and amphotericin B solutions for 72 hours *in vitro* during the logarithmic growth phase. Glucantime was administered at final concentrations of 5, 10, 15, and 20 µg/ml; paromomycin at concentrations of 25, 50, and 100 µg/ml; and amphotericin B at concentrations of 0.5, 10, and 15 mg/ml.

Total RNA was isolated using Arcturus® PicoPure® RNA Isolation Kit as described by the manufacturers. RNA integrity was analysed by electrophoresis in 1% agarose gels in 0.5× TBE (89 mM Tris borate, 2 mM EDTA, pH 8.3), stained with DNA Green Viewer (Parstous, Mashad, Iran) and visualized under UV light. The extracted RNA was quantified, and the absorbance measured at 260 and 280 nm by spectrophotometry. The purity of the extracted RNA was assessed according to the A260 nm/A280-nm ratio, with acceptable values being around 2.

First-strand cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, as described by the manufacturers. The products were stored at -20° C until the next step.

The gene expression of JBP1, JBP2 and RNAP II was assessed using SYBR Green Real-Time PCR. The endogenous control for the gene expression analysis was GAPDH. All primer pairs related to the JBP1, JBP2 and RN

AP II were designed using Primer3 software. The primer pair for GAPDH was used by Eslami et

Table 1. The primer name and their sequences for gene expression of JBP1, JBP2, RNAP II, and GAPDH [18] that were used in this study

Primer name	Sequences (5'-3')
JBP1-F	ATCTTTAACTTCCCGACCGCCA
JBP1-R	CTCGCAGCACAACACCAATGAT
GAPDHL-F	AGGACATTCTCGGCTTCACCAA
GAPDHL-R	GCCCCACTCGTTGTCATACCA
JBP2-F	CTCAACACGATGATCCAACTCTGC
JBP2-R	GCCGCCATCTTCCTCGTTCTTC
RNAP II-F	CGAAGCTGAGCATGAAAGAGGTG
RNAP II-R	TCGAAGACTTGGATGGAGAGCAG

al. [18]. The primer name and their sequences are listed in Table 1.

Amplifications were reacted in a total volume of 20 µl containing 2 µl cDNA, 10 µl SYBR Green I master mix and 200 nM each of primer pair (Table 1) using a StepOne thermocycler (Applied Biosystem, USA). The thermal conditions of reaction were 95°C for 10 s for first denaturation, followed by 40 cycles of 95°C for 10 s and 60°C for 10 s. The final extension was performed at 72°C for 10 minutes. The specificity of the amplicons was confirmed by melting curve analysis at temperatures between 60 and 95°C with a heating rate of 0.3°C/s. In addition, the size of the amplicons resulting in each specific primer pair was confirmed by analysis by staining with DNA Green viewer (3% agarose gels in 0.5×TBE), and visualization under UV light.

The determine the differential expressions, the mRNA level of each gene from the exposed groups was compared with its version in the unexposed strain. In this study, the GAPDH mRNA level was considered as a reference control. The 2^-delta delta CT method was used for analysis, calculated as delta Ct (exposed sample)-delta Ct (Reference sample).

The results were analysed by SAS software and the statistical analysis was performed using ANOVA. p-values less than 0.05 were considered as significant.

Results

Verification of cDNA synthesis

After RNA extraction, cDNA synthesis was performed. PCR was first performed with GAPDH primer pair to verify the process. Following this, the gene expressions in the different groups were assessed. A PCR product with a length of 200 bp was observed using 2% agarose gel electrophoresis (Fig. 1).

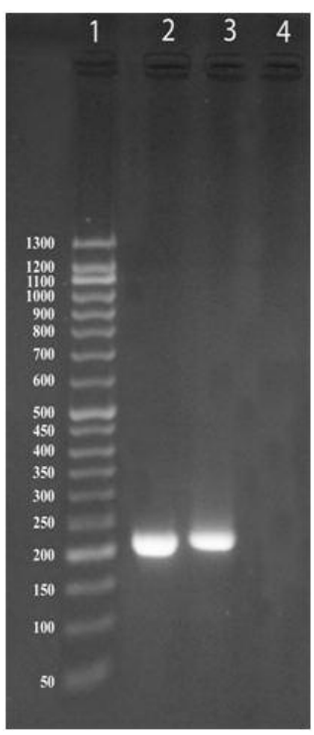

Fig. 1. The amplification of GAPDH fragment with the specific primer pair
Lane 1: 50 bp DNA ladder; Lanes 2 and 3: PCR product of GAPDH with the length of 200 bp; Lane 4: negative control.

Expression of JBP1 following exposure with amphotericin B, glucantime and paromomycin

The standard strain of *L. major* demonstrated different levels of mRNA expression following exposure to 0.5, 10 and 15 mg/ml amphotericin B (Fig. 2), with JBP1 expression decreasing with increasing concentrations of amphotericin B ($p<0.05$).

However, for glucantime, JBP1 mRNA levels

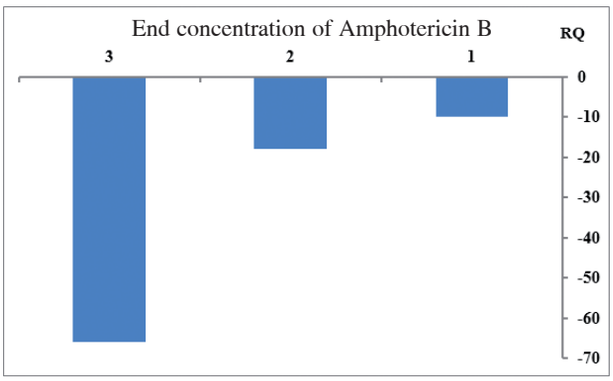

Fig. 2. The Relative Quantitative (RQ) of JBP1 from *Leishmania major* exposed to different concentration of amphotericin B
1: end concentration of 0.5 mg/ml; 2: end concentration of 10 mg/ml; 3: end concentration of 15 mg/ml

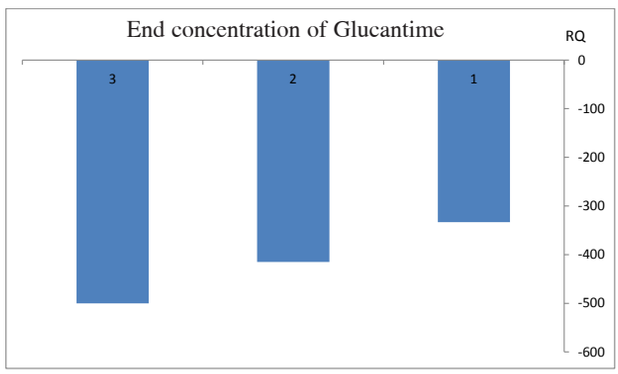

Fig. 3. The mRNA level of RNAP II from *Leishmania major* exposed to different concentration of glucantime B with end concentration of 5, 10, 15 µg/ml (right to left)

only varied after exposure to 5 µg/ml (1.4-fold lower than the unexposed strain; $p<0.05$); no difference was observed for 10, 15, and 20 µg/ml ($p>0.05$).

Finally, the promastigotes exposed to 100 µg/ml paromomycin showed a 13.5-fold increase in JBP1 mRNA compared to the unexposed strain ($p<0.05$). The other groups (25 and 50 µg/ml) showed no differences in mRNA level ($p>0.05$).

Gene expression of JBP2 following exposure to amphotericin B, glucantime or paromomycin

Regarding amphotericin B, JBP2 expression was found to be elevated 333.33-fold after exposure to 10 mg/ml compared with the unexposed group ($p<0.05$). No such significant increase was observed after exposure to 0.5 and 15 mg/ml ($p>0.05$).

No significant change in JBP2 expression was observed for any tested concentration of glucantime ($p>0.05$). Similarly, no significant change in expression was observed for any tested concentration of paromomycin (25, 50, and 100 µg/ml) ($p>0.05$).

Gene expression of RNAP II following exposure to amphotericin B, glucantime and paromomycin

Exposure to 0.5, 10 or 15 mg/ml amphotericin B caused no significant difference in RNAP II mRNA expression ($p>0.05$).

RNAP II mRNA level decreased significantly following exposure to 5, 10 and 15 µg/ml glucantime, with observed values ranging from 333.33- to 500-fold lower than those found for the unexposed group ($p<0.05$) (Fig. 3). No significant change in expression was observed for 20 µg/ml ($p>0.05$).

Finally, exposure to 25 and 50 µg/ml paromomycin significantly decreased RNAP II mRNA expression (20- and 50-fold), compared to the unexposed sample ($p<0.05$). No significant difference was observed for 100 µg/ml ($p>0.05$).

Discussion

Leishmaniosis is endemic in many tropical countries, many of which have no effective treatments and vaccines. The first-line treatment is pentavalent antimonial; however, other lines of drugs are indicated, such as amphotericin B and paromomycin, in the case of drug resistance. Therefore, to gain a clearer insight into the action of these drugs, the present study examines the expression of some important and novel genes, including JBP1, JBP2 and RNAP II in *L. major* following exposure.

Groups of *L. major* promastigotes were exposed to glucantime, amphotericin B or paromomycin at various concentrations, and the gene expression of JBP1, JBP2, and RNAP II was analysed. It was found that the expression of these genes varied depending on treatment.

The level of JBP1 expression was found to be inversely related to amphotericin B concentration, i.e. JPB1 expression decreased with increasing concentrations of amphotericin B. However, among the other tested groups, JBP1 was only expressed following treatment with 5 µg/ml glucantime and 100 µg/ml paromomycin. Being a member of the TET/JBP subfamily of dioxygenases, JBP1 is involved in the glycosylation of base J unique to

Kinetoplastida [13]; however, its action is dependent on Fe^{2+} and 2-oxoglutarate (2-OG) [14]. Other studies have shown that deletion of either JBP1 or JBP2 in *T. cruzi* and JBP2 in *L. tarentolae* results in the reduction of J levels which is the same in *T. brucei* mutants [19,20]. On the other hand, base J is present in chromosome-internal regions of RNAP II essential for transcription initiation [21]. Loss of base J at these chromosome-internal regions in *T. cruzi* leads to high RNAP II expression, resulting in greater transcription initiation, altered gene expression and increased parasite virulence [19,22].

Amphotericin B is an antifungal drug. It disrupts the permeability of *Leishmania* cell membranes in the same way as it does against fungal cell membranes [23]. The main portion of the membrane is ergosterol, which is essential for plasma membrane structure [24]. The synthesis of ergosterol in trypanosomatid protozoans is coded for by various genes. Amphotericin B is considered a first-line anti-leishmanial agent in patients harbouring antimony-resistant strains of *Leishmania* spp. and in ones with HIV-*Leishmania* spp. co-infections [25,26]. Goad et al. [27] characterized the sterols in *L. tropica*, *L. donovani*, *L. mexicana*, and *L. major* promastigotes by GC-MS [27,28]. The hydroxysteroid dehydrogenase/isomerase enzyme in *Leishmania* catalyses a reaction linking ergosta-7,22-dien-3β-ol to ergosterol [29].

Cholesterol and ergosterol are lipid rafts contained in detergent-resistant membrane (DRMs) [30]. Lipid-raft depletion in metacyclic promastigotes results in high degradation by complement *in vitro* and reduction of infectivity *in vivo* [29]. Our present findings indicate that increased concentrations of amphotericin B may lower JBP1 expression. It seems that increasing amphotericin B concentration could influence the ergosterol content of the *Leishmania* cell membrane. In response, the protozoon needs to increase ergosterol synthesis at the genetic level. Decreasing JBP1 may result in a decrease in base J synthesis by relaxing the chromosomal telomeres for more expression of RNAP II. However, our results indicate the amphotericin B exposure does not influence RNAP II gene expression. It seems that there are some other mechanisms that might stimulate the expression of enzymes for ergosterol synthesis, and that JBP1 may have other functions.

Paromomycin is an aminoglycosidic antibiotic that affects bacteria [31] and protozoa such as *Giardia* and *Entamoeba* [32], and *Cryptosporidium* [33]. It inhibits protein synthesis by binding to the main groove of 16S rRNA [31,34]. Paromomycin is useful against CL and visceral leishmaniosis [35,36]. It may act against *Leishmania* by changing the membrane fluidity, interacting with ribosomes, interfering with the mitochondrial membrane and inhibiting respiration [37].

The promastigotes exposed to 100 µg/ml showed a 13.5-fold increase in JBP1 expression compared to unexposed promastigotes. No differences were found in JBP2 expression, but RNAP II expression was found to be inhibited by exposure to paromomycin at 25 (20-fold) and 50 µg/ml (50-fold) compared with the unexposed control samples. Because paromomycin could alter protein synthesis, it is possible that low concentrations of paromomycin could result in low-level expression of RNAP II. At higher concentrations, JBP1 expression was increased, which may stimulate J base synthesis, thus preventing expression of RNAP II.

Glucantime is considered the first line of treatment for leishmaniosis, especially CL. Our results indicate that of the 5, 10, 15, and 20 µg/ml concentrations used, only exposure to 5 µg/ml glucantime significantly decreased JBP1 expression (1.4-fold). Although glucantime had no effect on JBP2 gene expression, exposure to glucantime concentrations between 5 and 15 µg/ml decreased RNAP II gene expression between 333.33- and 500-fold, suggesting that high concentrations of glucantime could stimulate RNAP II gene expression.

Overall, exposure to amphotericin B was not associated with any change in expression of JBP2 or RNAP II; however, significant differences were observed in JBP1 expression. JBP2 is not essential in *Leishmania* similarly as *Trypanosoma brucei*. Nevertheless, JBP1 has a key role in the biosynthesis of base J, and thus the termination of RNAP II transcription. Exposure to higher levels of glucantime and paromomycin increased the expression of RNAP II, reflected by higher RNAP II transcription, which may followed by up-regulation of the genes involved in either effluxing or deactivation of the drug. Our findings demonstrate that RNAP II, JBP1, and JBP2 can act against drugs such as paromomycin, amphotericin B and glucantime in different ways

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