Transient knockdown of *Nucleoside transporter 4* gene expression as a therapeutic target in *Leishmania major* by antisense RNA: *In vitro* and *in vivo* studies

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ABSTRACT

Background & objectives: Leishmania parasites cause various clinical symptoms in humans such as cutaneous ulcers and fatal visceral diseases. These parasites cannot synthesize purine rings *de novo* and must uptake purines from their hosts *via* salvage. Salvage is regulated by permeases in the cell membrane. There are hundreds of membrane transporter proteins to receive nutrients in *Leishmania.* Nucleoside transporter 4 (NT4) is one of the purine transporters that is involved in enhancing the uptake of adenine in *Leishmania major*. They are important new drug targets for the treatment of leishmaniasis because they can be used to transport toxic purine analogs to kill parasitic cells, thus preventing the progression of the infection. The present study was conducted to silence the NT4 nucleobase involved in the salvage pathway to interrupt purine nucleotide membrane transport in the cells of *L. major.*

Methods: In this study, a 502 bp segment of *NT4* gene sequence was selected and designed as antisense transcripts after insertion in the parasite. The NT4 construct was transfected into *L. major* promastigotes for *in vitro* study of gene expression. Then, BALB/c mice infected with transgenic *Leishmania* and wild-type strain along with the number and size of lesions were studied *in vivo*.

Results: The study showed that relative expression of *NT4* gene in mutant *Leishmania* was lower than in the control on Day 3 to 20. The percentages and the number of amastigotes in infected macrophages with wild-type strain *L. major* were more than infected macrophages with mutant parasites. Infected BALB/c mice with transgenic *Leishmania* showed a lower number and size of lesions than the BALB/c mice infected with wild-type strain.

Interpretation & conclusion: The results of the study indicated that the use of antisense RNA reduced *NT4* gene expression in *L. major*. Further, studies are needed to ascertain that the use of antisense can be considered as a new treatment for leishmaniasis.

Key words Antisense RNA; gene expression; *Leishmania major; Nucleoside transporter 4* gene; transient knockdown

INTRODUCTION

Leishmaniasis, caused by different species of the protozoa *Leishmania* manifests various clinical symptoms, such as dermal ulcers and fatal visceral diseases in humans[1]. Prevalent in around 98 countries of the world[2], it is estimated to account for >12 million infection, with >350 million people at risk[3]. The life-cycle of these parasites appears in two forms: Promastigote[2] in the gut of sandflies at neutral pH, and amastigote[4] in mammalian macrophages under an acidic pH of 5.5. These parasites cannot synthesize purine rings *de novo* and must uptake purines from their hosts[5–7]. This process of importing purines is carried out *via* salvage pathways that are regulated by permeases in the cellular membrane[7]. There are hundreds of membrane transporter proteins to transport nutrients in *Leishmania*, such as the purine transporters. Some of these transporters are polytopic proteins or symporters. The purines are essential for life, and they are precursors to activated forms of lipids and carbohydrates and nucleotide derivatives of vitamins[5]. The nucleoside transporter 4 (NT4) is one of the purine transporters in *Leishmania major* that increases adenine uptake. The purine transporters can also uptake purine analogs, such as allopurinol, which are toxic for *Leishmania*[5]. There are four genes in the *Leishmania* genome that are related to purine transport: NT1, NT2, NT3 and NT4. *Leishmania major* expresses two nucleoside transporters, NT3 and NT4 (LmaNT3,

LmaNT4), which has an amino acid homology of 33% between each other. The efficiency of LmaNT4 transporter is very low at neutral pH but is activated at acidic pH[8]. The permeases acts as a pipeline to import purines into the promastigotes and amastigotes of invertebrate hosts. For the NT4 transporter, K_m values related to select nucleobases, such as hypoxanthine, adenine, guanine, and xanthine is less than mM[7–9]. In invertebrate hosts, NT4 permease activity is higher in promastigotes than in amastigotes. Studies have shown that purine transporters are important targets for major drugs in the treatment of leishmaniasis[9]. For example, purine analogs, such as allopurinol, are used to treat the disease because they can enter the cells through purine transporters and cause cell death. Meglumine antimoniate (glucantime) is currently used to treat the patients but drug resistance to this drug is increasing[10–11]. The present research was conducted to inhibit the NT4 nucleobase permease involved in the salvage pathway to disrupt the transport of purine in *L. major,* thereby resulting in the death of the parasite[6].

MATERIAL & METHODS

NT4 antisense preparation

The length of *NT4* gene sequence is 1653 bp based on GeneBank (Accession No.: XM_001681471). The 502 bp sequence (5'-end) of the *NT4* gene was selected as antisense site and cloned into the pcDNA 3.1 Zeocin vector[12] by Hind III and the EcoRI restriction enzymes. The recombinant plasmid was transformed into the *E. coli* strain TOP10. It was extracted and confirmed by Hind III and the EcoRI restriction analysis. PCR was used to confirm the 500 bp cloned fragment. The NT4 primers used were: F: 5'-TACTGCCCGCGCAAGGTTGT-3'; R: 5'-GAGTCG-GCCAAGTAGCGGCA-3'.

Parasite culture

Leishmania major promastigotes (MRHO/IR/75/ER) were grown in complete RPMI-1640 culture medium containing10% inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. These cultures were incubated at 25 °C, and after 24–48 h, the obtained cells were used for electroporation[13].

Transfection (Transient transfection)

Leishmania major promastigotes, which were collected from cultures during the stationary phase at a concentration of 1×10^6/ml, were washed twice with PBS buffer and then suspended in an electroporation buffer (20 mM HEPES, pH 7.2, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose). Promastigotes (350 μl,

1×10^6/ml) were transferred to electroporation 0.2 cm cuvettes (Eppendorf, Germany) and 10 μl of plasmid DNA containing the antisense *NT4* gene at a concentration of 2 ng/μl was added. Electroporation was performed (Eppendorf, Germany) with a 250 V/cm and 15 μs capacitor. The transfected cells were incubated on ice for 10 min, and the cells were transferred to 2 ml of complete RPMI-1640 broth medium without antibiotics and incubated at 25 °C for a period of 20 to 24 h. On Day 2, the culture was centrifuged, and the pellet was immediately dissolved in 1 cm³ of complete RPMI-1640 medium containing 30 μg/ml Zeocin antibiotics (Gibco, UK). The culture was then incubated at 25 °C[13–14].

RNA extraction, cDNA synthesis and real-time qRT-PCR

Total RNA was extracted from the transgenic and wild-type *Leishmania* promastigotes using a total RNA purification kit (Jena Bioscience, Germany) according to the manufacturer's instructions on Days 3, 7, 10, 15 and 20 after the electroporation, transfection was confirmed by PCR after 3 days (Fig. 1). Then, the RNA concentration was determined using a Nanodrop spectrophotometer. Following the manufacturer's instructions, the cDNA was synthesized using AccuPower RT PreMix (Bioneer, Korea) at a volume of 20 μl by using a PCR cycler for 71 min with the following program: Denaturation at 55° C for 30 sec and amplification for 12 cycles at 20 °C (30 sec), 42 °C (4 min), and 95 °C (5 min). cDNA was amplified with specific primers of the *NT4* gene (forward: 5'-TACTGCCCGCGCGTTGT-3', reverse: 5'-GAGTC-
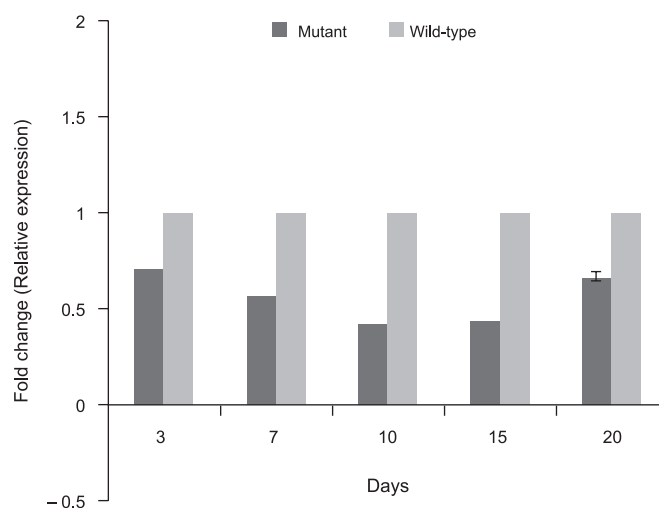

Fig. 1: NT4 gene expressions (Real-time PCR results) in transgenic *Leishmania major* on Days 3, 7, 10, 15, and 20 after transfection were 0.714 ± 0.921742155 (p <0.703910869), 0.574 ± 0 (p <0.05), 0.42 ± 0.020611515 (p <0.00063237, 0.442 ± 0.010854053 and 0.671 ± 0.016451669 (p < 0.0012508), respectively.

GGCCAAGTAGCGGCA-3'). Real-time PCR was performed in a volume of 10 μl, with 1 μl of cDNA, 2 μl of 5 pmol primers, 5 μl of 2 × Greenstar qPCR master mix and 2 μl of deionized water. The real-time PCR reactions were performed in duplicate for each sample using the Rotor gene 6000 (Qiagen, Germany) by the following program: 94 °C for 2 min and 94 °C for 20 sec, 40 cycles at 54 °C for 20 sec, and 72 °C for 30 sec. The *rRNA 45* gene of *Leishmania* was used as a reference gene (forward: 5'-CCTACCATGCCGTGTCCTTCTA-3', reverse: 5'-AACGACCCCTGCAGCAATAC-3')[15]. A comparative method of Ct was performed for *NT4* gene expression, and relative quantity was obtained by the formula $2^{-\Delta\Delta Ct}$.

Western blot analysis

The pellets of the transgenic and the wild-type promastigotes were collected on Days 3, 7, 10, 15 and 20 after transfection by centrifugation (3000 rpm for 15 min) and then washed twice with PBS buffer. The parasites were lysed with a protein lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 25% v/v glycerol, 0.01% w/v bromophenol blue, 5% v/v 2-mercaptoethanol), which was added to the pellets on ice and sonicated for three times (each time for 20 sec).

The sonicated cells were then boiled for 10 min. Equal amount of total protein extracted from 1×10^6 cells of wild and mutant strain promastigotes were subjected to western blot analysis with anti-NT4 Ab. Electrophoresis was performed on a 10% polyacrylamide gel (Fig. 2)[16]. The protein bands were transferred to a nitrocellulose membrane. NT4 specific sheep antibody was used as a primary antibody with titer of 1:1000 (GenScript, USA). Rabbit anti-sheep IgG antibody conjugated with Horseradish peroxides (at a 1:10,000 titer) was used to detect the protein bands.
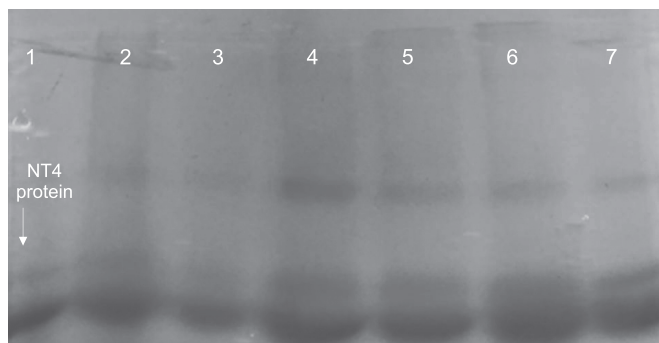

Fig. 2: SDS-Page of *NT4* gene expression in transgenic *Leishmania major* on Days 3, 7, 10, 15 and 20 after transfection. Lane 1: Negative control; Lane 2: Wild-type as positive control; Lanes 3–7: Mutant strains on Days 3, 7, 10, 15 and 20, respectively after electroporation.

Macrophage infection assay

The murine macrophages cell lines J774 at a concentration of 2×10^5 were cultured in a DMEM medium containing 10% inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 24-well plates. The plates were placed in incubators at 37 °C with 5% CO_2[17]; after 5 h, the suspension of macrophages was centrifuged and the supernatant was separated. Fresh complete DMEM medium was then added to the cell pellet to produce a homogenous suspension. After 24 h of culturing, the mutant and wild-type *Leishmania* promastigotes (1×10^6) were added to the 24-well plates containing cell line J774. The plates were transferred into incubators at 37 °C with 5% CO_2. Two slides were prepared from the plates at 24, 48, and 72 h of culturing, and these slides were stained with Giemsa. The macrophages (n = 100) were counted on each slide, and then the percentage of the infected macrophages and the number of amastigotes per infected macrophage was calculated.

Infectivity assay in BALB/c mice

For this eight female BALB/c mice were used, aging 4–6 wk and weighing between 20 and 25 g. The mice were divided into two groups (n = 4 in each group). The groups were infected with promastigotes of the wild-type and the mutant of *L. major*. Concentrations of 1.5×10^8 promastigotes[18] in a 0.1 ml volume were subcutaneously injected into the base of the tail of the mice[19–20]. Mice were evaluated for ulcer number, diameter, and incubation period macroscopic examination for groups. It should be noted that in the *in vivo* study, the examination was performed on >4 mice in each group, but unfortunately, some mice were excluded from the study, because they lost the test conditions. Eventually the work was conducted with four mice in each group.

Statistical analysis

The statistical analysis was done using SPSS software ver. 22.0, and an ANOVA was performed. Statistical differences were considered significant at $p < 0.05$.

Ethical statement

This study was conducted based on the International Guiding Principles for Biomedical Research involving Animals as issued by the Council for the International Organizations of Medical Sciences. The Ethics Committee of laboratory animals at Kerman University of Medical Sciences approved the details of this project (Code No. 92/78, 2013). Nevertheless, all attempts were made to minimize animal pain and suffering.

RESULTS

The gene expression of NT4

Real-time RT-PCR was carried out on Days 3, 7, 10, 15 and 20 after transfection. The results showed that fold change of the relative *NT4* gene expression in transgenic *Leishmania* gradually reduced on Days 3 to 20. The effect of antisense RNA on *NT4* gene expression in transgenic *Leishmania* samples caused down regulated gene expression. The differences were considered significant on Days 7, 10, 15 and 20 post-transfection ($p < 0.5$) (Fig. 1).

Protein production by western blot

Western blot analysis showed that the protein was not expressed in the transgenic promastigotes 15 days after electroporation, evidenced by no band observed on the nitrocellulose paper (Fig. 3).

Macrophages infection

The results showed that the percentage of the infected macrophages with the mutant parasites at the three time points were lower than that of the wild-type strain. The statistical analysis showed a significant difference ($p < 0.05$) between percentages of the infected macrophages in the mutant *L. major* and the wild-type strains at three time points (Fig. 4).

The number of amastigotes infected macrophages with mutant *L. major* was lower than that of the wild-type strains. The statistical analysis showed a significant difference ($p < 0.05$) between the number of amastigotes infected macrophages in the mutant *L. major* and the wild-type strains (Fig. 5).
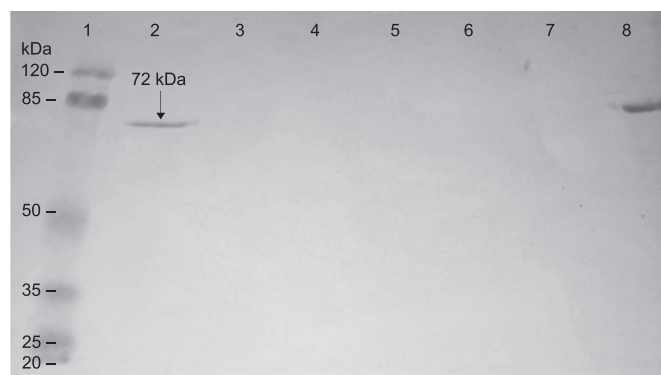

Fig. 3: Western blot analysis of *NT4* gene expression in transgenic *Leishmania* on Days 3, 7, 10, 15 and 20 after transfection. Lane 1: Protein marker; Lane 2: Mutant strain on Day 20; Lanes 3–6: Mutant strains on Days 15, 10, 7 and 3, respectively post-transfection; Lane 7: Negative control; and Lane 8: Wild strain used as positive control. The result of this experiment shows that protein was produced in transgenic promastigotes just on Day 20 after electroporation; and in other cases, probably there was no gene expression or the expression was too low, as no bands were observed.
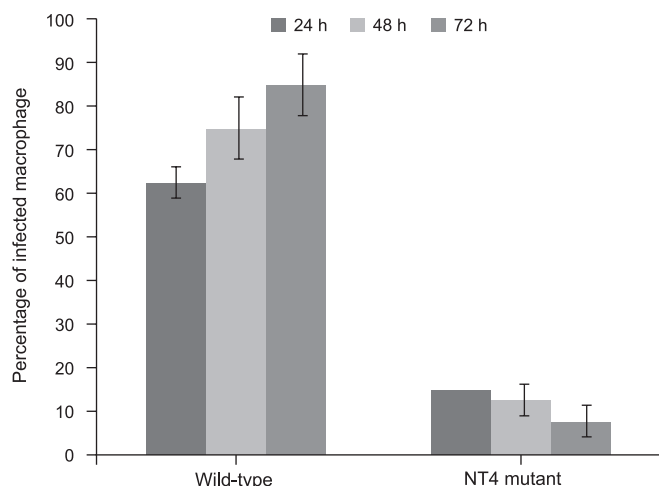

Fig. 4: Percent of infective macrophage with the transgenic *Leishmania major* and the wild strains. The percentage of infected macrophages in the mutant strains was 15 ± 0 (n = 2), 12.5 ± 3.5 (n = 2), and 7.5 ± 3.5 (n = 2) at 24, 48 and 72 h, respectively. In contrast, the percentage of infected macrophages in the wild-type was 62.5 ± 3.5 (n = 2), 75 ± 7.07 (n = 2), and 85 ± 7.07 (n = 2) at 24, 48 and 72 h, respectively.

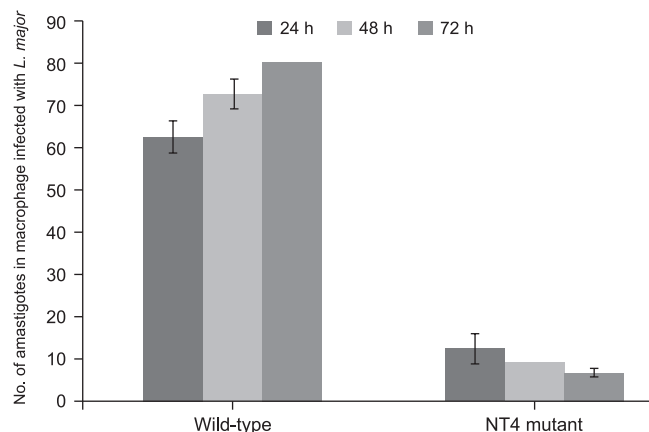*Fig. 5:* The number of amastigotes in macrophages infected with the mutant (transgenic) strain *Leishmania major* was 12.5 ± 3.5 (n = 2), 10 ± 0 (n = 2), and 7.5 ± 3.5, (n = 2) at 24, 48 and 72 h, respectively; on the other hand, the number of amastigotes in the wild-type was 62.5 ± 3.5 (n = 2) at 24 h, 72.5 ± 3.5 (n = 2) at 48 h, and 80 ± 0 (n = 2) at 72 h.

The lesion(s) in BALB/c mice, were observed within 30 days in the mice infected with the wild-type (Fig. 6a) but in the BALB/c mice infected with mutant parasites, no lesions were perceived within this period (Fig. 6b). It was developed within >30 days in both the mice infected with mutant (Fig. 6c) and wild-type parasite (Fig. 6d). Many lesions with a diameter >5 mm were found in the mice receiving the wild-type, while one ulcer with a diameter of <5 mm was observed in the mice infected with the mutant strain.
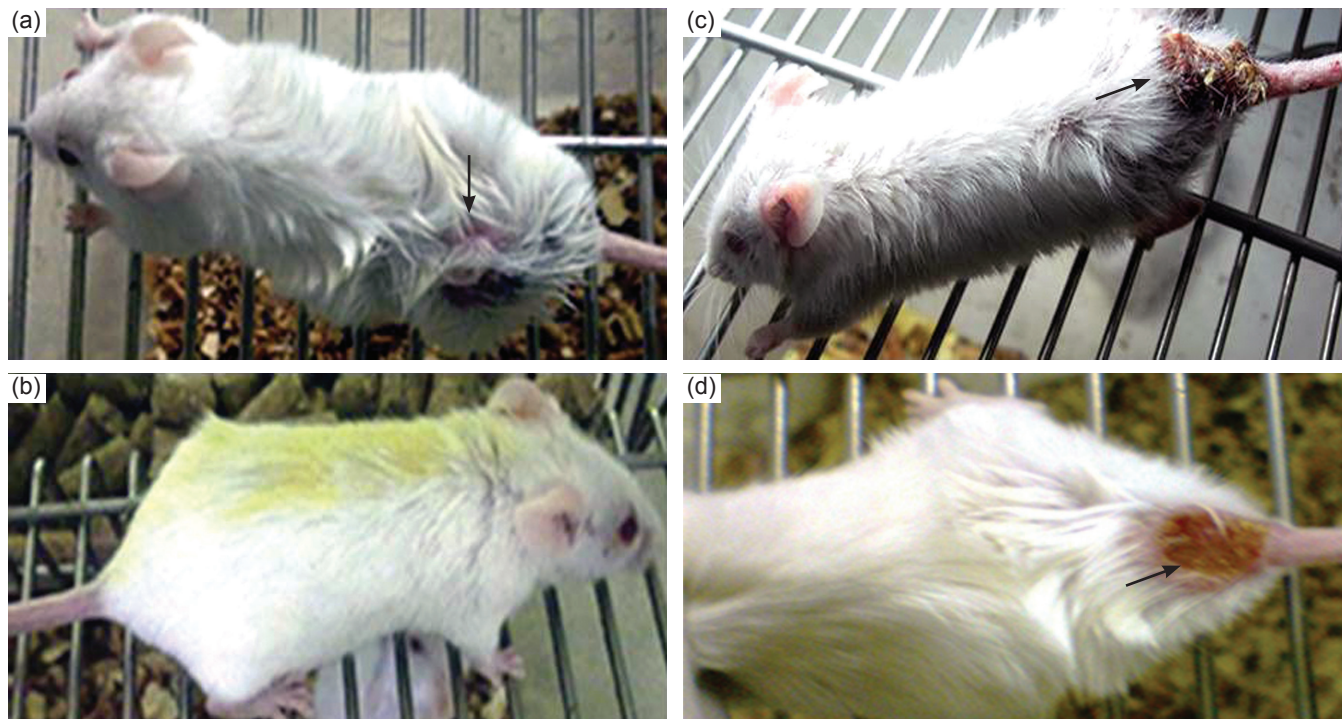
Fig. 6: Comparison of wounds/lesions in BALB/c mice infected with wild strain and transgenic *Leishmania major* parasites—(a) The lesion(s) were observed within 30 days in the mice infected with the wild-type; (b) No any lesions were seen within 30 days in the BALB/c mice infected with the mutant parasites; (c) One ulcer with a diameter of < 5 mm was developed within 70 days in the mice infected with the mutant parasites; and (d) Many lesions with a diameter > 5 mm were seen within 70 days in the mice infected with the wild-type.

DISCUSSION

Purines are essential for different cellular and metabolic processes, including energy production and cell signaling. *Leishmania* parasite can not synthesize purines and must salvage them from the surroundings. The lack of purine causes a 45–85-fold increase of mRNA translation and salvage enzymes expression to facilitate the production of purine transporters in *Leishmania*. At present, purine transporters are considered the primary target in the treatment of leishmaniasis, because parasite mortality is improved by transmission of toxic purine analogs. In *Leishmania,* purine transporters are the targets of new molecular therapeutics research. Nucleoside transporter 4 (LmaNT4) is one of the most important nucleoside transporters that is expressed in *L. major*[8]. Studies have shown that LmaNT4 has a major role in the biology of *L. major* but has a less important role in the absorption of purines in promastigotes close to neutral pH[21]. LmaNT4 is adenine transporter, but has a lower affinity for nucleobases[7–8, 21]. In *Leishmania,* the compulsory nature of purine salvage makes its therapeutic effect more prominent[22]. In this study, antisense RNAs were used to investigate their effect on the reduction of *NT4* gene expression in *L. major*, using both *in vitro* and *in vivo* models. Antisense RNA bind to complementary RNA sequences, which causes hybridization and binding to RNA, preventing target protein translation. Antisense RNA may block the ribosomal 40S unit and other signals from the start of translation[23]. The results of this study showed a decrease in the expression of *NT4* gene in mutant *Leishmania*. Western blot results analysis also confirmed this as the protein of this gene was not produced, or the expression was too low, since there was no banding until Day 20. On Day 20, NT4 protein was produced with a molecular weight of 72 kDa in the mutant and the wild-type parasites. Western blot was repeated twice. The antibody was designed to detect a specific epitope of *NT4* gene; the *NT4* gene may have other variants, one of which was expressed on Day 20. In this study, percentage of infected macrophages and the number of amastigotes inside the macrophages infected with the wild strain was more than the mutant parasites (Fig. 3). In other words, the ability of mutant parasites to proliferate in macrophages was less than the wild-type strain, which is in concordance with earlier studies on *L. donovani*[18] and *L. braziliensis*[19]. The LmaNT4 is a necessary permease for the survival of the parasites, and presumably, reduced viability within the macrophage might be related to reduction of its gene expression. It was a noteworthy that deletion of *LmaNT4* gene cause deaths to amastigote inside macrophages of mice[8]. This is in agreement to other studies also[24–26].

The *in vivo* study indicated a reduction in diameter, number, and duration of ulcers in BALB/c mice infected with the transgenic parasites as compared to the mice infected with the wild-type strains similar to the results of experiment conducted by Mottram *et al*[27]. Also, the *in vivo* results showed that ability to develop ulcers in the mice infected with mutant *L. major* was lower than those infected with wild-type parasites. In wild-type infected mice, a large number of wet wounds with a diameter of >5 mm were produced in 30 days, while there were no wounds in the mutant mice for 30 days; however, a small ulcer (<5 mm) was observed within 70 days. No difference was seen in the shape and size of amastigotes in the mutant parasite and the wild-type strain by eyepiece micrometer light microscopy. The data generated show that the reduction of ulcers in the mice may be related to the down-regulating expression of the *NT4* gene, decreasing the parasites virulence and viability. A reduction in ulcers was seen in BALB/c mice.

CONCLUSION

In the present study, antisense RNA was applied for observing the expression of the *NT4* gene in *L. major.* It was found that the expression NT4 was down-regulated by antisense RNA. The NT4 down-regulation causes reduction in the percentage and the number of the infected macrophages; and parasites virulence and the viability of parasites in BALB/c mice.

ACKNOWLEDGEMENTS

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