Effect of trinitroglycerin therapy on serum zinc and copper levels and liver enzyme activities in BALB/c mice infected with *Leishmania major* MRHO/IR/75/ER

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**OBJECTIVES:** To evaluate the effect of trinitroglycerin (TNG) as nitric oxide donor agent on serum copper (Cu) and zinc (Zn) levels and liver enzymes in BALB/c mice infected with *Leishmania major* (L. major) MRHO/IR/75/ER.

**Materials and Methods:** Inbred female mice were divided into three groups: healthy group (uninfected naive mice), control group (infected with *L. major*), and test group (*L. major* infected mice treated with TNG). TNG (200 μg/μl) was inoculated subcutaneously into the mice of the test group. Serum Cu and Zn levels and liver enzymes activities were then evaluated by atomic absorption spectrophotometer and colorimetric methods, respectively.

**Results:** Serum Cu levels were significantly higher in the test group than in the control and naive groups (*P*-value <0.05), while Zn levels were higher in the test group than in the control group with no significant difference. Serum glutamic-oxaloacetic transaminase concentrations in the test group were significantly lower than those in other groups (*P*-value <0.05), while serum glutamate pyruvic transaminase concentrations were significantly higher in test compared with those in other groups (*P*-value <0.05). Moreover, alkaline phosphatase in the control and test groups were significantly lower than that in the naive group (*P*-value <0.05).

**Conclusion:** TNG treatment increased Zn and Cu levels and thus increased resistance to *Leishmania* because of the role of Zn and Cu; therefore, TNG therapy will be useful for treating cutaneous leishmaniasis. In addition, the decrease of serum glutamic-oxaloacetic transaminase activity can be an index of therapeutic process of TNG.

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**Introduction**

Leishmaniasis is a diverse group of diseases caused by a protozoan parasite of the genus *Leishmania*, that infect host mononuclear phagocytes and are transmitted by the bite of female sandflies (1). Cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis, mucocutaneous leishmaniasis (ML), and visceral leishmaniasis (VL) are the four major clinical forms of this disease which have different manifestations from localized infection to disseminated visceral disease (2).

CL caused by *Leishmania major* (L. major) and *Leishmania tropica* (L. tropica) is characterized by a skin ulcer which heals spontaneously and ultimately leaves a scar (3). This is the most common form of Leishmaniasis that is endemic in more than 80 countries worldwide endemic in more than 80 countries worldwide including Iran (2, 4).

This obligate intracellular parasite resides and multiplies in macrophage. After recognition of Leishmania species, macrophage activates "effector cell" and phagocytosis and destroys the invaders (5-6). Process of elimination of intracellular pathogens, such as leishmaniasis, requires a T-helper 1 (Th1) type immune response, whereas a dominant Th2 response leads to an exacerbated disease. Th2 responses limit Th1 functions which deactivate macrophages and nitric oxide (NO) (25, 30). The production of various cytokines, oxidative burst, and generation of NO are the cytotoxic mechanisms within macrophages for intracellular survival in human and experimental CL (30, 31). The production of reactive nitrogen intermediates is one of the predominant cytotoxic mechanisms against a variety of pathogens, including *L. major*, where Leishmania parasites effectively decrease to ensure its survival (5-8). Development
of protective immunity in experimental CL is dependent on the activation of infected macrophages to form nitric oxide (NO) and eliminate parasite. Both the endogenous and exogenous NO inhibit the development of intracellular and extracellular infections caused by L. major (8-10). NO plays a pivotal role as a leishmanicidal agent in mouse macrophages. In vivo and in vitro immunological studies showed that NO radicals within Leishmania lesions could reduce the parasite number (9, 10). Trinitroglycerine (TNG), a substance that causes vasodilatation by donation of NO and as an antianginal drug, is still used in the treatment of heart failure. TNG is an organic nitrate that is predominantly metabolized in the liver by nitrate reductase and generates NO to inhibit the catalytic activity of Leishmania; thus, as an exogenous source NO, it could be used for treatment of leishmaniasis. The treatment of CL with NO donor agents has been recently approached and the effect of NO on the parasites has been established (5, 9).

Trace elements including copper (Cu) and zinc (Zn) are involved in the activity of several enzymes that contribute to immune system response, including super oxide dismutase, catalase, and glutathione peroxidase (11, 12). Low serum Zn levels have been reported in parasitic diseases in which the immune system is affected. Low serum Zn levels could be associated with the acute phase response in VL (3, 11). One study showed that serum Zn concentrations in CL patients decrease during antimonial therapy (11, 13). Cu is a part of several metalloenzymes such as cytochrome oxidase, ferroxide, and amine oxidase that are required for oxidative metabolism (12, 14). Thus, changes in their levels are part of defense strategies of organisms essential for cell membrane stability, apoptosis, host metabolism, and enzyme activity (2, 6, 8).

Liver plays a major role in guarding against infection. Tissue macrophages (Kupffer cells) are key components in the prevention of hepatic infections (15, 16). Leishmania also multiply within macrophages in the liver, producing an active chronic hepatitis (17). Hepato-splenomaly is a result of infection with Leishmania. In VL patients, derangement of liver functions is common, and severe life-threatening hepatitis can occur occasionally (15, 18). Although hepatomegaly is common in patients with VL, the alteration of liver functions in these patients has been studied only in few studies (15, 19). Profound alteration of liver functions and severe jaundice are associated with poor prognosis of patients with VL. A significantly higher number of fatal cases also had altered liver functions as compared with nonfatal cases (18, 20).

TNG undergo enzymatic reduction to release NO within vascular smooth muscle; therefore it effectively reduces portal pressure and might potentially improve hepatic circulation. Although effect of TNG on cardiovascular disease has been widely studied, very few reports are available detailing alteration of liver functions in these patients. In one report the acute and chronic oral toxicities of TNG were studied in dogs, rats, and mice; it has been shown that normal rats fed 0.1% TNG had mild hepatic lesions similar to those seen in rats fed with larger doses (36).

In this study, we investigated whether TNG therapy, as a compound that generate NO, could affect essential trace elements (Zn and Cu) concentrations in vivo in susceptible inbred BALB/c mice infected with L. major (MRHO/IR/75/ER), and whether this drug could be a therapeutic agent against human CL. We also aimed to assess changes of liver enzymes including alkaline phosphatase (ALP), serum glutamate oxaloacetic transaminase (SGOT), and serum glutamate pyruvic transaminase (SGPT). Finally, we tried to determine whether TNG had therapeutic effects on these enzyme levels in BALB/c mice.

Materials and Methods

Mice

Inbred female BALB/c mice (6-8 weeks old with average body weight of (18.2 ± 1.3) g) were used in this study. All mice were housed at room temperature (20-23°C) on a 12 hr light/dark cycle, with unlimited access to food and water. Animal experiments were approved by the Ethical Committee of Pasteur Institute of Iran and were performed based on the ethical standards formulation in the declaration of Helsinki.

Parasite strains and maintenance

In this study, the L. major (MRHO/IR/75/ER) was used as the standard strain and the parasites were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) as determined by Nahrevanian et al (9). In this condition, the stationary phase of parasite growth was obtained in 6 days (2, 9). Promastigotes of L. major were harvested from culture media, collected, and used to infect BALB/c mice. The base of mouse tail was injected intradermally with inoculums of 2×10⁶ promastigotes. Infectivity of parasites was maintained by regular passage in susceptible BALB/c mice.

TNG drug

TNG for its ability to increase NO was inoculated subcutaneously to mice once a day for 15 days in the test group. Final concentration of TNG was 200 µg/ml prepared using normal saline as solvent. Preliminary experiments were applied in vitro on cultivated promastigotes and in vivo on uninfected BALB/c mice in order to optimize TNG dose and toxicity; it was selected according to the maximum efficacy and the minimum toxicity. Concentration of 500 µg/µl/60min of TNG was considered as lethal.
dose 50 (LD$_{50}$) based on killing of 50% of parasite in 100 promastigotes.

**Study design (grouping)**

Twenty-six BALB/c mice were used in this study, some of which died in stages before injection with TNG. The study was finally performed in 20 mice. The mice in the first group (n=6) were not infected with *L. major* and were kept un-infected as naive group. The mice in the second group (n=7) were infected by *L. major* promastigotes but untreated with TNG, and this group was considered as control group. The third group (n=7) was comprised of mice infected with *L. major* and treated with TNG as test group. Induction of CL infection in the latter two groups was confirmed by the laboratory demonstration of the parasite in the lesions at the end of the experimental period. Six weeks after progressing of infection in groups (control and test), normal saline and TNG were inoculated subcutaneously to the mice of the control and test groups for 15 days, respectively.

The effect of TNG and normal saline on serum Zn, Cu, and liver enzymes in the mice infected with *L. major* were assayed and the results were compared.

**Determination of Zn, Cu, and liver enzyme levels**

Whole blood was taken from anaesthetized mice by inhalation diethyl ether, and serum was prepared from by centrifuging the blood at 2,500 r/min for 10 min. The collected sera were stored at -70˚C until analysis. The serum samples were diluted with distilled and deionized water in a 1:10 proportion (2, 11). Cu and Zn concentrations were determined by flame atomic absorption spectrophotometry (Thermo Jarrel Ash, Germany); a UNICAM 929 model with a deuterium background correction. Zn and Cu values were expressed in mg/l. Absorbances were read at 324.7 and 213.9 nm wavelengths, for Cu and Zn respectively, and a 0.7 nm slit was used for both Cu and Zn. The samples analyzed in triplicate with integration time of 2 sec. Stock standard solutions (titrisol from Merck and for Cu and Zn) of 1000 mg/l for Cu and Zn were diluted with distilled and deionized water. Working standard solutions contained 0.1, 0.3, 0.5, 1.0, and 2.0 mg/l Cu or Zn. For accuracy, the standard solutions were run to test samples (12, 13).

All used chemicals were of analytical grade and acid washed glasswares were used throughout the study. Serum liver enzyme (SGOT, SGPT, and ALP) activities were determined photometrically using auto analyzer (Technicon, RA1000, USA) with commercial available kit.

**Statistical analysis**

SPSS software (version 16) was used for statistical analysis. All values are presented as mean ±SD. The differences among groups were determined by ANOVA and Student t-test. *P*-value less than 0.05 was considered significant.

**Results**

In this study, serum Cu and Zn levels were determined in three groups of inbred female BALB/c mice (naive, control, and test). The test group had serum Cu level of (1.39±0.05) mg/ml and Zn level of (1.45±0.15) mg/ml (Table 1). These results showed that serum Cu level in the test group (group that was infected with *L. major* and treated with TNG) was significantly higher than that in the naive and control groups (*P*-value <0.05), whereas no significant differences in serum Zn level were observed between these groups. However, the Zn level in the test group showed an increase as compared to that in the control group, but this change was not statistically significant. Zn deficiency in the infected BALB/c mice (control and test groups) was observed when compared to that in the naive mice, but these differences were not significant.

The lowest level of Cu/Zn ratio (0.717±0.09) was observed in the naive group (Table 1). As a result, therapy with TNG decreased this ratio and made it close to that in the naive group, but these changes were not statistically significant.

Before TNG therapy, catalytic concentration (activities) of SGOT, SGPT levels increased in infected mice in comparison with healthy naive, whereas ALP activity levels were lower in the infected group than in the naive group.

The evaluation of TNG therapy on the activities of liver enzymes showed that the serum SGOT activity was significantly lower in the test group than that in the control and naive groups (*P*-value<0.05). The SGOT activity in the control group was higher than that in the naive and test groups (Table 2).

Moreover, the SGPT activity in the test group was significantly higher as compared to that in the

![Table 1. Levels of Cu, Zn, and Cu/Zn ratio in the naive, control, and test groups](image1)

| Group   | Cu [µg/ml] | Zn [µg/ml] | Cu/Zn ratio |
|---------|------------|------------|-------------|
| A (naive)| 1.12±0.02  | 1.56±0.17  | 0.717±0.09  |
| B (control)| 1.3±0.08  | 1.18±0.12  | 1.10±0.12   |
| C (test) | 1.39±0.05  | 1.45±0.15  | 0.958±0.07  |

Data were presented as mean±SD. *P*-value<0.05 indicates statistically significant difference compared with the control and naive groups.

![Table 2. Activities of liver enzymes between the naive, control, and test groups](image2)

| Group   | SGOT [IU/l] | SGPT [IU/l] | ALP [IU/l] |
|---------|-------------|-------------|------------|
| A (naive)| 148.8±24.75 | 46.60±4.63  | 216.20±20.10|
| B (control)| 224±53.18 | 60.60±6.96  | 146±11.85   |
| C (test) | 347.5±22.25 | 365.33±96.17 | 144.33±14.07 |

SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamate pyruvic transaminase; ALP: alkaline phosphatase. Data are presented as mean±SD. *P*-value<0.05 indicates statistically significant difference the test group compared with the control group; *P*-value >0.05 shows statistically significant difference between control and naive groups; ◊ *P*-value<0.05 indicates statistically significant difference between the naive group.
control and naive groups (P-value<0.05) (Table 2). Based on the results, the serum ALP activity in the test and control groups were significantly lower than that in the naive group (P-value<0.05) (Table 2). Comparison of serum ALP activity between the three groups showed that TNG therapy in the infected mice led to the decrease of ALP activity.

Discussion

CL is the most common form of leishmaniasis that has been recognized as a major public health problem in several countries including Iran, where almost all cases are caused by either L. major or L. tropica (1, 21, 22).

Immunobiochemical changes are part of the host-defense strategies of organisms during the infection. NO dependent mechanisms by host macrophage and NO donor have been critical for control of Leishmania infection (5, 6, 23, 24). In one study, it has been shown that applying NO generating agents on lesions of mouse CL model leads to a modest efficiency, although these agents are able to cure human patients (9, 23).

Trace elements including Zn and Cu are directly involved in metabolic processes. Many immunological functions depend on these processes. Zn and Cu are essential in the function of immunocompetent cells, host metabolism, and enzyme activities that contribute to immune responses; thus, changes in their levels are part of the defense strategies of the organisms (14, 25).

In present study, the anti-leishmania effects of TNG as the NO donor Cu and Zn levels and liver enzyme activity in BALB/c mice infected with L. major was evaluated as a novel idea. However, TNG increased serum Cu concentration in the test group as compared to that in the control and naive groups, but Zn concentration in the control mice was lower than that in the naive and tested samples. Increased serum Cu in the test group could be associated with an increase in the system of Cu-binding proteins, ceruloplasmin, and production of cytokines. The result of this study is in accordance with the published reports by Kocyigit et al that demonstrated serum Cu increase in CL patients, and suggested that this increase is associated with ceruloplasmin and induced by interleukin-1 (11, 13). Exogenous NO donors can increase serum Zn level by releasing cytoplasmic Zn from metallothionein (26, 27).

Zn deficiency (decline) in infected but untreated BALB/c mice might be responsible for the inability of host to clear the parasite and the associated inflammation, due to impaired production of various cytokines and enzymes (2). Increased serum Zn level in mice treated with TNG could be related to the effect of TNG therapy on production of cytokines and activation of immune system.

Moreover, Zn is an activator of monocytes/macrophages; released interleukins of activated phagocytes cells lead to transfer of Zn from plasma to liver by metallothionein synthesis (25, 28). However, increasing IL-1 that resulted in increasing Cu is associated to ceruloplasmin synthesis (26). In a study carried out by Firooz et al in 2005, low serum Zn levels in CL patients could lead to disability of host to clear parasite despite of high level of INF-γ (32). The use of TNG also led to decrease in Cu/Zn ratio as compared to that in the control group, whereas this ratio in the control group was higher than that in the naive group, which in turn led to increased sensitivity to disease and its progressive course. This decrease could be related to the effect of TNG in clearing infection with activating defense system. Earlier reports suggested that Zn/Cu ratio imbalance could be a useful marker for immune dysfunction in leishmaniasis (12, 25). Thus, Cu/Zn ratios probably can be used as a marker of immune disorder during leishmaniasis, due to its role on cellular and humoral response against leishmania. This can be proposed as new strategy for therapy and prevention of human leishmaniasis.

In a report by Kocyigit et al, CL patients had lower Zn and Fe levels and higher serum Cu level as compared to the control subjects (13). Sorkhroodi et al evaluated the effectiveness of sodium selenite and zinc sulphate as known immunomodulator materials, in combination with Glucantime® in treatment of CL lesions resulting from L. major in a susceptible animal model. They showed that sodium selenite and zinc sulphate at mentioned doses and duration of treatment did not have any therapeutic effect on CL caused by L. major in BALB/c mice. Increasing the dose of supplements and considering the follow up period after treatment can lead to certain conclusion (29). Firooz et al have examined the therapeutic values of intralesional injections of 2% ZnSO₄ solution with meglumine antimoniate (Glucantime) and oral ZnSO₄ [10 mg/(kg.day)] on treatment of CL (32). Another study demonstrated that serum Cu concentrations were significantly higher in patients with acute and chronic CL as compared with those in control group (3), and the authors claimed that the changes could be a part of defense strategies of organisms induced by IL-1, TNF-α, and IL-6 (33). Kocyigit et al reported that Zn concentrations in CL patients increased during antimonial therapy (13). There were not similar studies about the relation between microelements, cytokines, and therapy with TNG.

In a study that was conducted on humans infected with VL in an Indian tertiary care center, mild increases in liver enzymes are often noted (15). Another study that was conducted by Ikeda-Garcia et al on dogs naturally infected by VL has showed changes of liver and renal functions after treatment with meglumine antimoniate However, unlike the kidneys, the liver is not a primary target organ. Infection can be associated with chronic
hepatitis. Hepatic enzymes present sensitivity in the identification of hepatobiliary disorders (18).

In the present study, the effect of TNG as a NO donor on SGPT and SGOT levels in BALB/c mice infected with L. major (MRHO/IR/75/ER) showed different results. TNG treatment led to the decrease of SGOT activity in the test group as compared with that in the control and naive groups, whereas TNG dramatically increased SGPT activity in the test group as compared with that in the other two groups. Thus, SGOT and SGPT could be considered as pathologic factors in hepatomegaly related with CL and also could be considered as hepatotoxic indexes (22, 24). Since TNG therapy led to the decrease of SGOT activity, it could be an index of therapeutic process of this drug and could be useful in following and monitoring CL treatment. These results are in accordance with the published report by Lawn et al that described both the cardiac and biochemical adverse effects of pentavalent antimonial treatment. Findings of this research, based on the enzyme activity results (about ALT activity) are in contrast to the results of Lawn et al that observed increased serum catalytic activity concentrations of ALT and AST above the upper limit of the normal range in 85% of CL and ML patients (34).

In contrast, TNG therapy does not have effect on ALP activity in mice with CL. However, the activity of this enzyme decreased as compared to that in the naïve group. Decreasing ALP level resulted in Zn deficiency and pernicious anemia (16, 35). Thus, ALP enzyme could be considered as a protective factor during infection. As a result, L. major inhibits the ALP activity and TNG does not have a role in increasing ALP and removing of inhibitory effect of L. major. In a study by Nassiri Kashani et al there was no significant alteration in laboratory values of liver, kidney, or pancreas indices before and after treatment with meglumine antimoniate at a dose of 20 mg/(kg.day) for 15 days in CL patients (20).

Based on FDA report about side effects of TNG on the liver in June 2014, 17 cases of 24022 people taking TNG and 3 cases of the same people have liver injury and liver tenderness, respectively (37). Thus, in regard to the effect of TNG treatment on liver function test, these factors could be used as a marker of liver function during treatment of CL with TNG or another drug.

Conclusion

Serum Zn, Cu, and liver enzyme concentrations are altered by some immunocytokines that are induced by applying TNG in mice with CL. It could be a defense strategy of host during infection and treatment, which could be considered as a parameter for treatment and vaccine strategy. TNG therapy leads to the increase of Zn and Cu levels in serum. As the increasing Cu level may in turn increase resistance to Leishmania and Zn plays a role in defense system, TNG therapy can be useful for treating CL. In addition, TNG therapy led to the decrease of SGOT activity that could be an index of therapeutic process of this drug for following and monitoring CL treatment. We can also suggest that supplements containing Zn and Cu in animal CL model may decrease the severity of the disease. Further investigations will be needed to study cytokines, enzymes, and metalloproteins, together with trace elements in CL and design a model function in determining a therapeutic method by applying oxidants that contribute in defense systems against this disease.

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Conflict of interest

Authors declare no conflicts of interest.

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