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# Magnesium oxide nanoparticles coated with glucose can silence important genes of *Leishmania major* at sub-toxic concentrations

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ABSTRACT

The aim of this study was to investigate the effect of magnesium oxide nanoparticles (MgO NPs) and MgO NPs coated with glucose (MONPCG) on *Leishmania* (*L*) *major*. First, the promastigotes of *L. major* were separately incubated with serial concentrations of MgO NPs and MONPCG for 24, 48, and 72 h at 37 °C. Then, the cell viability of promastigotes was evaluated by MTT assay. On the other hand, the relative expression of Cpb and GP63 genes was detected by quantitative-real time PCR. Based on results, the increase of concentration, both MgO NPs and MONPCG, and incubation time led to decrease of cell viability. Moreover, the expression of Cpb and GP63 genes was decreased with increase of concentration of MgO NPs and MONPCG. Also, the increase of incubation time led to decrease of their expression in MgO NPs treated promastogotes. But, in case of MONPCG treated promastogotes, the increase of incubation time did not change the expression of Cpb and GP63. Interestingly, MONPCG could silence Cpb and GP63 genes better than MgO NPs. Note, the capability was also seen at sub-toxic concentrations of MONPCG.


## 1. Introduction

*Leishmania* (*L*) *tropica* and *Leishmania major* are two important agents of cutaneous leishmaniasis (CL). Nowadays, glucantime is the first drug to treat CL. Importantly, this drug has no adequate efficacy, and its long-term usage can cause drug resistance [1]. It has been indicated that over-expression of ABC-efflux pumps is an important pathway in drug resistance [2,3]. Interestingly, *L. major* expresses two important surface molecules, gp63 and Cpb [4–6]. The products of these genes are good targets to design new drugs. Logically, the blocking of these products may lead to cell damage and even cell death. Moreover, parasite-macrophage interaction may be affected by such protocol. It must be noted that all blocking agents can be used in both naked and conjugated forms [7].

Natural or synthetic nanoparticles [8] can kill various pathogens, e.g., viruses, bacteria, fungus, parasites [9]. Based on our previous works, different metal and metal oxide nanoparticles can damage *L. major* [10–12]. Although both promastigotes and amastigotes are sensitive to metal and metal oxide nanoparticles, skin and macrophage cells can be also killed [10,13]. Several mechanisms have been suggested to describe the behavior of antimicrobial properties of nanoparticles. Ion release, reactive oxygen species (ROS) generation, enzyme degradation, protein denaturation, and DNA damage are some suggested mechanisms [14,15]. Since nanoparticles have high surface area and high chemical activity, they can easily penetrate into parasites and skin cells. Some molecules must be attached on the surface of nanoparticles, to target parasites [16] or toxins [17,18]. Based on our research, the killing of *L. major* by gold and silver nanoparticles was dose- and time-dependent [10]. The aim of this study was to investigate the effect of magnesium oxide nanoparticles (MgO NPs) and MgO NPs coated with glucose (MONPCG) on *L. major*. In this study, cell viability and expression of two genes, gp63 and Cpb, were evaluated after incubation with MgO NPs and MONPCG. Since these genes are two important surface molecules, they were selected in this study.

## 2. Materials and methods

### 2.1. Materials

RPMI1640, NNN medium, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), isopropanol, SYBR Green, and Hank's balanced salt solution (HBSS) were purchased from Sigma–Aldrich Chemical Co, (St Louis, MO). Also, RNA extraction

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kit, DNA-free kit, and cDNA Synthesis Kits were purchased from QIAGEN, Germany. MgO NPs, at range of 50 ± 5 nm, were sourced from ZFS Company, Yazd, Iran.

### 2.2. *Preparation* L. major *promastigotes and incubation with MgO NPs and MONPCG*

The standard isolates of *L. major* (MRHO/IR/75/ER) was provided from Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The isolate was first incubated for 48 h at 37 °C in NNN medium enriched by RPMI1640. After 48 h incubation, it was centrifuged at 3000 rpm, and washed with cold RPMI1640. Then, the quantity of promastigotes was adjusted to $2 \times 10^5$/mL by cell counter. In the next step, 0.1 g of MgO NPs was added to 1 mL of 100 mg/mL glucose, and then incubated for 1 h at 37 °C. After washing by distilled water (DW), 1 mL of *L. major* suspension was separately added to 1 mL of MgO NPs and MONPCG at concentration of 25–200 μg/mL. All experiments were done at standard conditions according to the National Institute of Health and ethics committee guidelines of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

### 2.3. *MTT assay*

First, 100 μL of serial concentrations (25–200 μg/mL) of MgO NPs and MONPCG was separately incubated with 100 μL of promastigotes ($2 \times 10^4$ cells) for 24, 48, and 72 h at 37 °C. After incubation, the promastigotes were washed with HBSS, and then 100 μL of RPMI1640 and 25 μL of 5 mg/mL MTT were added to washed cells, and incubated 3 h at 37 °C. In the next step, 100 μL of 70% isopropanol was added, and optical densities (OD) of each well were read by a micro-plate reader (Novin Gostar, Iran) at 490 nm. Finally, the cell viability was measured for each group. In negative control, the promastigotes were treated with normal saline [10].

### 2.4. *RNA isolation, cDNA synthesis, and real-time PCR*

Briefly, 100 μL of serial concentrations of MgO NPs and MONPCG was separately incubated with 100 μL of promastigotes ($2 \times 10^4$ cells) for 24, 48, and 72 h at 37 °C. Then, one mL of treated promastigotes ($2 \times 10^5$ cells) was centrifuged at 3000 rpm for 5 min, and washed by normal saline. Next, RNA extraction was done by RNA kit, and then it was treated with DNase master mix to remove possible DNA. Then, the mixture was incubated for 15 min at 95 °C to inactive the DNase. The OD of extracted RNA was read at 260 nm. Next, total RNA was reverse transcribed by cDNA Synthesis Kit with a random hexamer. Briefly, 1 μL of random hexamer was added to 10 μL of total RNA, and then incubated at 65 °C for 5 min. Then, 5 μL reaction buffer, 1 μL RiboLock RNase Inhibitor, 2 μL Revert Aid, and 1 μL transcriptase (200 U/μl) were mixed, and incubated at 42 °C for 60 min and then at 70 °C for 5 min by thermal cycler. Then, 2 μL of synthesized cDNA was added to 18 μL of SYBR Green (Sigma–Aldrich, USA), and entered into ABI 1 plus model of real-time PCR for 40 cycles. In this study, two genes (Cpb and GP63) were separately analyzed, and 18s rRNA was an internal control. All primers are shown in Table 1, obtained from the National Center for Biotechnology Information (NCBI). PCR amplification consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Finally, all data were normalized to control. In negative control, the promastigotes were treated with normal saline. At end, the comparative expression was measured by $2^{-\Delta\Delta Ct}$ method [19].

Comparative expression=$2^{-\Delta\Delta Ct}$ ΔΔCt = ((Ct of interested gene-Ct of internal control gene) in treated group-(Ct of interested gene-Ct of internal control gene) in control group).

**Table 1**
The primer sequences of Cpb, GP63,18s rRNA genes.

| Gene name | Sequences |
|---|---|
| Cpb | Forward: 5′-CCTATTTTACACCAACCCCCAGT-3′<br>Reverse: 5′-GGGTAGGGGCGTTCTGCGAAA-3′ |
| GP63 | Forward: 5′-CCGGTTTATATGAAGGAACAGT-3′<br>Reverse: 5′-GGGGAAGATAGAAGAACGAAA-3′ |
| 18s rRNA | Forward: 5′-GCCACAAAATAGGCGGGCTT-3′<br>Reverse: 5′-GGCCTTTTGTTTACTGCAAA-3′ |

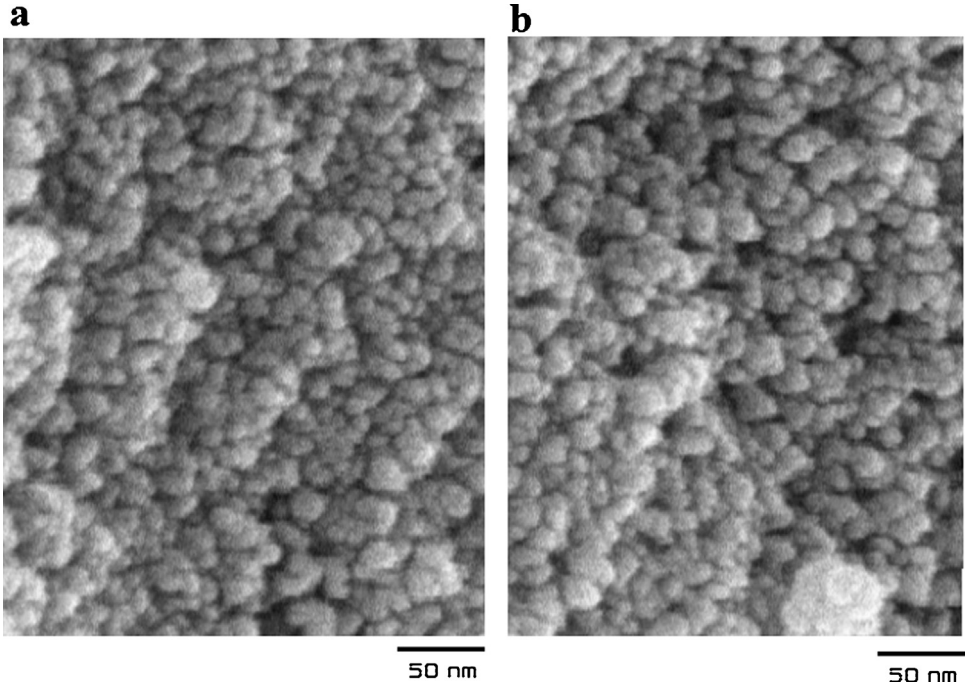


**Fig. 1.** The SEM image of MgO NPs (a) and MONPCG (b).

**Table 2**
The relative expression of Cpb gene after incubation with serial concentrations of MON and MONCG.

| Nanoparticles | Concentration (μg/mL) | Incubation time (hours) | | |
|---|---|---|---|---|
| | | 24 | 48 | 72 |
| MON | 25 | 1 ± 0.01[a,b] | 2 ± 0.1 | 5.3 ± 0.1 |
| | 50 | 1 ± 0.01 | 1.5 ± 0.02 | 2.2 ± 0.02 |
| | 100 | 0.1 ± 0.01 | 0.12 ± 0.01 | 0.12 ± 0.01 |
| | 200 | 0.1 ± 0.01 | 0.1 ± 0.01 | 0.12 ± 0.01 |
| MONCG | 25 | 0.1 ± 0.02* | 0.1 ± 0.5* | 0.1 ± 0.1* |
| | 50 | 0.15 ± 0.01* | 0.1 ± 0.02* | 0.1 ± 0.05* |
| | 100 | 0.01 ± 0.001* | 0.012 ± 0.001* | 0.012 ± 0.001* |
| | 200 | 0.001 ± 0.0001* | 0.001 ± 0.0001* | 0.002 ± 0.0001* |

[a] All data are demonstrated as Mean ± Standard deviation.
[b] The comparative expression was measured by $2^{-\Delta\Delta Ct}$ method.
* $P < 0.05$ compared with MON treated cells at same concentration, $n = 5$.

**Table 3**
The relative expression of GP63 gene after incubation with serial concentrations of MON and MONCG.

| Nanoparticles | Concentration (μg/mL) | Incubation time (hours) | | |
|---|---|---|---|---|
| | | 24 | 48 | 72 |
| MON | 25 | 1 ± 0.01[a,b] | 2 ± 0.1 | 5.3 ± 0.1 |
| | 50 | 1 ± 0.02 | 1.5 ± 0.02 | 2.2 ± 0.02 |
| | 100 | 0.1 ± 0.05 | 0.15 ± 0.05 | 0.12 ± 0.01 |
| | 200 | 0.1 ± 0.01 | 0.1 ± 0.01 | 0.14 ± 0.01 |
| MONCG | 25 | 0.1 ± 0.02* | 0.1 ± 0.5* | 0.1 ± 0.1* |
| | 50 | 0.12 ± 0.01* | 0.2 ± 0.02* | 0.3 ± 0.05* |
| | 100 | 0.01 ± 0.001* | 0.010 ± 0.001* | 0.015 ± 0.001* |
| | 200 | 0.002 ± 0.0001* | 0.001 ± 0.0001* | 0.002 ± 0.0001* |

[a] All data are demonstrated as Mean ± Standard deviation.
[b] The comparative expression was measured by $2^{-\Delta\Delta Ct}$ method.
* $P < 0.05$ compared with MON treated cells at same concentration, $n = 5$.

### 2.5. Statistical method

All tests were done five times and results were reported as mean ± standard deviation (SD). To detect significant differences between tested groups and control, One-way ANOVA was carried out by SPSS software (V.16.0 for Windows; SPSS Inc, Chicago, IL), and *P*-values less than 0.05 were considered as statistical significance.

## 3. Results

The SEM image of MgO NPs and MONPCG is shown in Fig. 1a and b, respectively. As seen, their shape was globular, and their average size was near 50 nm. Moreover, their distribution size was near 20–70 nm.

The viability of *L. major* promastigotes after incubation with MgO NPs and MONPCG is shown in Figs. 2 and 3, respectively. As seen, the increase of concentration and incubation time led to decrease of cell viability. In this test, significant differences were not observed between SNCCG and GNCCG ($P > 0.05$). The relative expression of Cpb and GP63 genes when exposed to serial concentrations of MgO NPs and MONPCG is shown in Tables 2 and 3, respectively. The expression of Cpb and GP63 genes was decreased with increase of concentration. Moreover, the increase of incubation time led to decrease of expression of Cpb and GP63 in MgO NPs treated promastogotes. But, in case of MONPCG treated promastogotes, the increase of incubation time did not change the expression of Cpb and GP63. As an important finding, MONPCG could silence Cpb and GP63 genes better than MgO NPs ($P < 0.05$). Interestingly, MONPCG could also decrease the expression of Cpb and GP63 genes at sub-toxic concentrations.

## 4. Discussion

The toxicity of naked and coated nanoparticles is considerably different, because their surface chemistry and physicochemical properties are different [20]. In the current study, we evaluated the effect of MgO NPs and MONPCG on *L. major*. Here, it was found that the exposure of *L. major* promastigotes to serial concentrations of MgO NPs and MONPCG led to decrease of cell viability.

It must be mentioned that some materials lead to enhanced cell viability after long exposure. The enhanced cell viability has been previously reported for some bacterial strains in presence of sub-toxic concentrations of antibiotics [21]. Also, the enhanced viability of peripheral blood mononuclear cells [22] and human skin carci-

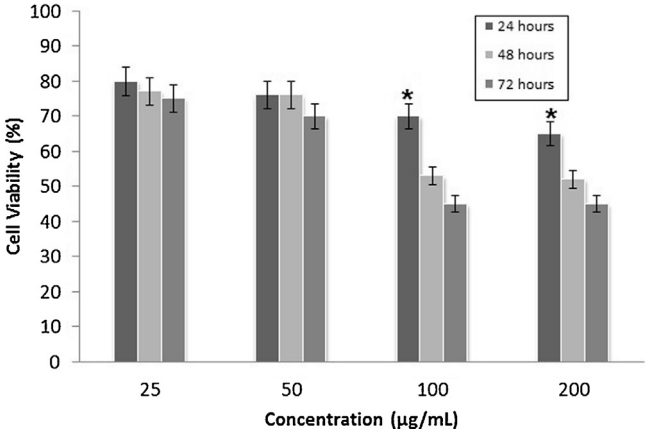


**Fig. 2.** The viability of *L. major* promastigotes after incubation with MgO NPs. * $P < 0.05$ compared with other treated groups at same concentration, $n = 5$.

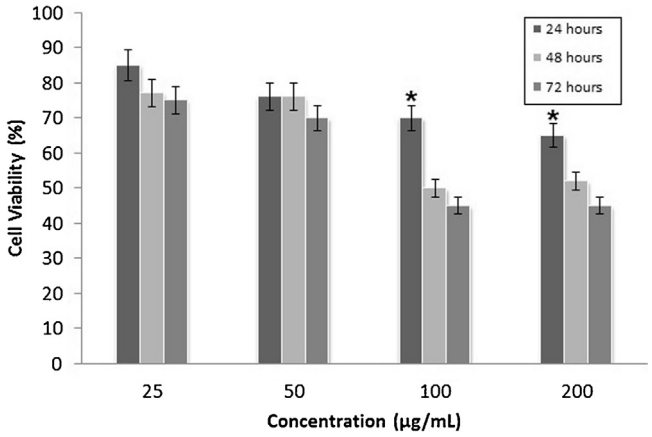


**Fig.3.** The viability of *L. major* promastigotes after incubation with MONPCG. $^{*}P < 0.05$ compared with other treated groups at same concentration, $n = 5$.

noma cells [23] at sub-toxic concentrations of silver nanoparticles had been previously reported. It is fact that both silver and gold nanoparticles are toxic, especially at high concentrations [24], but increased viability of promastigotes in presence of low concentrations of nanoparticles is a new finding. Mitochondrial dysfunction and induction of ROS lead to DNA damage, which are believed to be the main factors resulting in cell death [25]. DNA microarray analysis showed the effect of sub-toxic concentration of silver nanoparticles on cell division, proliferation, and DNA repair [26]. The authors suggest that sub-toxic concentrations of MONPCG may induce a repair pathway. In order to evaluate the biological effects of MgO NPs and MONPCG, the expression of two genes, Cpb and GP63, was investigated after exposure to promastigotes. Our studies showed that the expression of Cpb and GP63 genes was decreased with increase of concentration. But, the increase of incubation time led to decrease of expression in MgO NPs treated promastogotes. In case of MONPCG treated promastogotes, the increase of incubation time did not change the expression. It was found that MONPCG could silence Cpb and GP63 genes better than MgO NPs ($P < 0.05$). Interestingly, MONPCG could also decrease the expression of Cpb and GP63 genes at sub-toxic concentrations.

The enhanced viability of peripheral blood mononuclear cells [22] and human skin carcinoma cells [23] had been previously reported at sub-toxic concentrations of silver nanoparticles. MgO NPs are approximately toxic, especially at high concentrations [24], but increased viability of promastigotes in presence of low concentrations is an interesting phenomena. Mitochondrial dysfunction and induction of ROS lead to DNA damage, which are believed to be the main factors resulting in cell death [25]. Kawata et al. performed DNA microarray analysis to evaluate the effect of sub-toxic concentration. They found that it had negative effects on cell division, proliferation, and DNA repair [26].

## 5. Conclusion

It could be concluded that the increase of concentration and incubation time led to decrease of cell viability. Moreover, the expression of Cpb and GP63 genes was decreased with increase of concentration. Also, the increase of incubation time led to decrease of their expression in MgO NPs treated promastogotes. But, in case of MONPCG treated promastogotes, the increase of incubation time did not change the expression of Cpb and GP63. Interestingly, MONPCG could silence Cpb and GP63 genes better than MgO NPs.

## Conflict of interest

There is no conflict of interest to declare.

## Acknowledgment

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