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### Original Article

## Expression of Pro-inflammatory Genes in Lesions and Neutrophils during *Leishmania major* Infection in BALB/c Mice

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### **Abstract**

**Background:** Leishmaniasis is a worldwide disease prevalent in tropical and sub-tropical countries in the world. Characterization of inflammatory responses produced in cutaneous leishmaniasis has not yet been completed.

**Methods:** The specific primers were designed for ten pro-inflammatory genes including CCL4, CCL3, TNF- $\alpha$ , IL-1 $\alpha$ , IL-12P35, IL-12P40, CCL5, CCR5, IL-1 $\beta$  and IFN- $\gamma$  and their expression were assessed and compared using RT-PCR in the lesion and peripheral blood neutrophils in *Leishmania* infected BALB/c mice.

**Results:** None of the pro-inflammatory genes was expressed in the healthy tissue and except IFN- $\gamma$  others were down-regulated by the parasite in the lesion in untreated mice. In mice treated with anti-Leishmanial drugs, the expression of the pro-inflammatory genes restarted. The figure of pro-inflammatory gene expression in neutrophils was different from the lesions in treated and untreated mice.

**Conclusion:** *Leishmania* is capable to suppress the expression of pro-inflammatory genes in the lesions but not in neutrophils. The expression of TNF- $\alpha$  in the lesions and down-regulation of IL-1 $\beta$  in neutrophils could be accounted as an indication for healing of cutaneous leishmaniasis. The results open a new window on characterization of *Leishmania* lesions and clarifying the role of neutrophils in *Leishmania* infections.

### Introduction

**L** *eishmania* parasites are obligatory intracellular protozoa living in macrophages of humans and many other vertebrates. *L. major* is one of the main causes of the cutaneous form of the leishmaniasis in the world

(1). Most of *Leishmania* studies have yet been focused on *Leishmania* itself or the immunogenicity and pathogenicity of the parasite when characterization of the lesion produced in cutaneous form of the disease is rarely addressed

in these studies. Identifying the pro-inflammatory genes preferentially expressed during the parasite infection would help to elucidate the mechanisms controlling gene regulation and intracellular survival of the parasite where identifying and characterizing the expressed genes and the molecular mechanisms underlying their regulation can be considered as therapeutic targets (2).

Studies using biopsy samples from human cases have suggested different chemokine patterns linked to the phenotype of leishmaniasis (3). In mice, various inflammatory mediators such as TNF- $\alpha$ , IL-1 $\alpha$  and CC and CXC chemokines have been detected in in vitro and in vivo studies during *L. major* infections (4) however, it is still little known about the expression profiles of these mediators in *Leishmania* infection.

The profile of gene expression in macrophages varies in *Leishmania* infection, entirely related to mechanisms applied by the macrophages to control the parasite (5). In *Leishmania* lesions, pro-inflammatory cytokines such as IL-1beta, CCL3 and CCL4 activates macrophages enhancing parasite killing (6) where the expression of pro-inflammatory genes is shown to be associated with time evolution of the lesions (7). On the other hand, neutrophils also play a crucial role in inflammatory responses and *Leishmania*-specific immune responses to control the infections (8) and there is no report on the expression of pro-inflammatory genes in neutrophils in *Leishmania* cases.

In the present study, the expression of pro-inflammatory genes in *Leishmania* infected mice in the lesion and neutrophils compared to see whether the situation of the lesion in *Leishmania* cases can be predicted by determining the expression of these genes for therapeutic purposes.

## Materials and Methods

### *Leishmania*

The standard Iranian *L. major* promastigotes (MRHO/IR/75/ER) were obtained from Pas-

teur Institute of Iran and cultured in biphasic Nicol Mac NalNeavy (NNN) culture at 25 °C and pH 5.5 for 4-7 d. The culture supernatant containing parasite promastigotes was transferred into RPMI- 1640 containing 100 u/ml of penicillin, 100  $\mu$ g/ml of Streptomycin and 200  $\mu$ l/ml of FBS.

### Primers

Specific primers for ten pro-inflammatory genes including CCL4, CCL3, TNF- $\alpha$ , IL-1 $\alpha$ , IL-12P35, IL-12P40, CCL5, CCR5, IL-1 $\beta$  and IFN-  $\gamma$  were designed using the gene bank primer software. The sequences of the primers are shown in Table 1.

### Animals

8-12 week female BALB/c mice were obtained from Pasteur Institute of Iran and bred at Bu-Ali Sina University animal house. All animals were housed in accordance with Bu-Ali Sina University Codes of Practice for the housing and care of animals (Code Number 92-385).

### *Leishmania parasites and infection*

Eight-weeks BALB/c mice were purchased from Pasteur institute of Iran and housed in BU-Ali Sina university animal house according to the standard of ethical agreement for keeping inbred animals. *L. major* promastigotes strain MHOM/76/IR obtained from Pasteur institute of Iran and cultured in RPMI-1640 medium supplemented with 10% FCS.  $1 \times 10^7$  *Leishmania* promastigotes were intradermally injected onto the base of the tail and the animals were divided into two groups of five mice. The first group was treated with 20 mg/kg glucantime half in the lesions and half intramuscularly. The second group was injected with PBS. The mice were then monitored in two days interval for 3 weeks. The size of the lesions was calculated using the following equation:

$$P = (D + d) / 2$$

(D = the large diameter, d = the small diameter, P = the average size of the lesion)

**Table 1:** Sequence of primers designed for pro-inflammatory genes

| Accession          | Sequence                       | Gene                |
|--------------------|--------------------------------|---------------------|
| NM_013652.2        | F: CAG CCC TGA TGC TTC TCA CT  | CCL4/MIP-1 $\beta$  |
|                    | R: GGG AGA CAC GCG TCC TAT AAC |                     |
| NM_011337.2        | F: TCT GCG CTG ACT CCA AAG AG  | CCL3/MIP-1 $\alpha$ |
|                    | R: GTG GCT ATC TGG CAG CAA AC  |                     |
| M38296.1           | F: TAT AAA GCG GCC GTC TGCAC   | TNF- $\alpha$       |
|                    | R: TCT TCT GCC AGT TCC ACG TC  |                     |
| CCDS16725          | F: CAG TTC TGC CAT TGA CCA TC  | Il-1 $\alpha$       |
|                    | R: TCT CAC TGA AAC TCA GCC GT  |                     |
| M86672.1           | F: ATG ATG ACC CTG TGC CTT GG  | IL-12P35            |
|                    | R: CAC CCT GTT GAT GGT CAC GA  |                     |
| M86671.1           | F: CTG CTG CTC CAC AAG AAG GA  | IL-12P40            |
|                    | R: ACG CCA TTC CAC ATG TCA CT  |                     |
| NM_013653.3        | F: GTG CTC CAA TCT TGC AGT CG  | CCL-5               |
|                    | R: AGA GCA AGC AAT GAC AGG GA  |                     |
| NM_009917.5        | F: ATT CTC CAC ACC CTG TTT CG  | CCR5                |
|                    | R: GAA TTC CTG GAA GGT GGT CA  |                     |
| <b>CCDS16726.1</b> | F: TTG ACG GAC CCC AAA AGA TG  | IL-1 $\beta$        |
|                    | R: AGA AGG TGC TCA TGT CCT CA  |                     |
| MN-008337          | F: GCT CTG AGA CAA TGA ACG CT  | IFN- $\gamma$       |
|                    | R: AAA GAG ATA ATC TGG CTC TGC |                     |

### *Isolation of neutrophils*

Heparinized blood samples taken from the heart of the mice were diluted with the equal volumes of NaCl 0.85%. Meglumine compound (megluminediatrizoate 66%, sodium diatrizoate 10%) was diluted in 3 times of volume of NaCl 0.85% and used for isolation of neutrophils. Five ml of the diluted blood were slowly added to 4ml of the diluted meglumine compound being careful not mixing the two phases of the blood and the meglumine compound. The samples were centrifuged for 15 min at 250 g and the supernatant was replaced with 0.5ml of PBS. The pellet was homogenized by slow pipetting. Two 25-seconds hypotonic lyses were applied with distilled water and NaCl 2.55% and the cells were then centrifuged for 5min at 200 g replacing the supernatant with NaCl 0.85%.

### *RNA Isolation*

Total cell RNA was isolated using DENAzist total RNA isolation kit according

to the manufacturer's instruction with modification. Briefly, 100 mg of tissue or cell pellet was sonicated for 20 min. One ml of G1 lysing buffer was added and the sample was vortexed for 15 sec followed by incubation at room temperature for 5 minutes and centrifugation at 12,000 g for 10 minutes at 4 °C. The supernatant was then transferred into a new tube and 200  $\mu$ l chloroform was added and vortexed for 15 sec. The tubes were incubated at room temperature for 3 min and then spined at 12,000 g for 15 min at 4 °C. The top phase was transferred into a new tube and an equal volume of isopropanol was added. An equal volume of G2 buffer was added, mixed and incubated at room temperature for 10 min and then centrifuged at 10000 g for 10 min at 4 °C. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellet. After a quick vortex, the tubes were spined at 10000 g for 5 min at 4 °C. The supernatant was then discarded and 30-100  $\mu$ l of nuclease-free water was added.

### RT-PCR

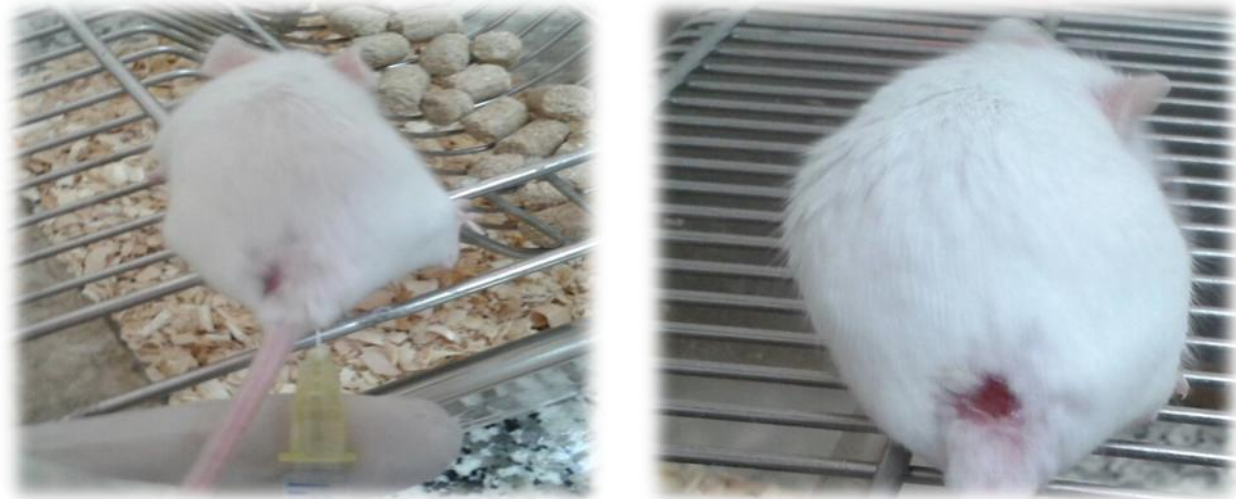
RT-PCR was performed using DENAzist kit according to the manufacturer's instruction with slight modification. RNA-primer mixtures (total RNA 10µg, oligodT primer 1µl, dNTP 1µl, nuclease-free water top up to 10µl) was prepared and mixed. The mixture was incubated at 65 °C for 5 min and chilled on ice for 2 min. The cDNA Synthesis mixture (10X Buffer M-MuLV 2µl, M-MuLV reverse transcriptase 100 unit, nuclease-free water top up to 10µl) was prepared and 10µl of the cDNA synthesis mixture was added to each RNA-primer mixture and incubated at 42 °C for 60 min. The tubes were then incubated at 85 °C for 5 min and chilled on ice. The PCR was programmed as 40 cycle of denaturation at 95 °C for 30s, annealing temperature in accordance with melting temperature, lasted for 30sec, extension at 72 °C for 20sec. An initial denaturation step at 95 °C for 10 min before the cycles and a final extension step at 72 °C for 20sec after the cycles were also applied.

### Results

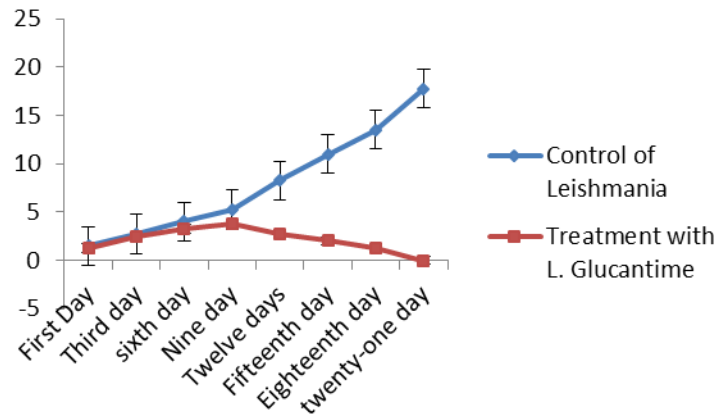
#### *Leishmania major* lesions

*Leishmania* lesions were produced in two groups of BALB/c mice by injecting standard Iranian *L. major*. To evaluate the characterization of the lesions during the progressive and healing periods in terms of the lesion size and expression of pro-inflammatory periods, one group treated with Glucantime (group test) and the other group with PBS (group control) (Fig.1).

A week after injection of the parasites, lesions were appeared on the site of injection. The size of the lesions were measured and compared every two days in both groups. The results clearly showed an expansion in the size of lesions in group control but in group test, the lesions became smaller over the time until disappeared (Fig. 2).



**Fig. 1:** *Leishmania* lesions in BALB/c mice  $1 \times 10^7$  *L. major* promastigotes were intradermally injected onto the base of the tail of BALB/c mice. The mice were divided into two groups of five animals. The first group was treated with 20mg/kg glucantime and the second group was injected with PBS for three weeks. A) BALB/c mice with *Leishmania* lesions in group test, treated with glucantime B) BALB/c mice with *Leishmania* lesions in group control, injected with PBS



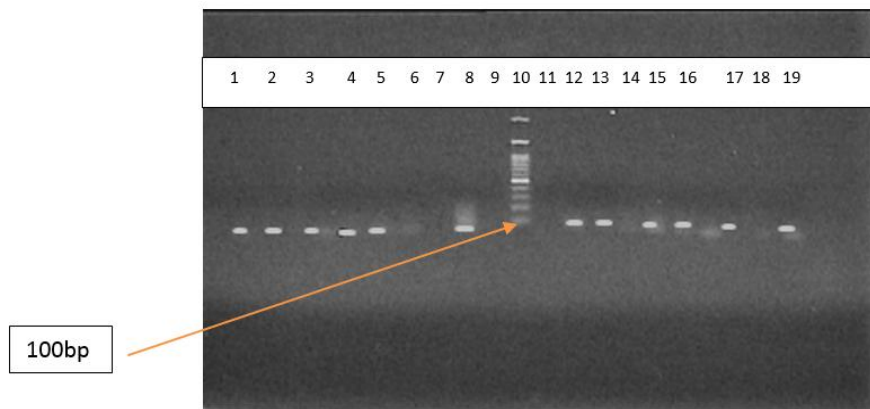
**Fig. 2:** Comparison of the size of lesions caused by *L. major* BALB/c mice treated with glucantime or PBS

The size of *Leishmania* lesions in two groups of five mice was measured every two days for three weeks. The first group was treated with glucantime and the second group with only PBS. After three week, all mice in group test cured and all mice in group control died.

### Expression of pro-inflammatory genes in *Leishmania* lesions

The expression of ten pro-inflammatory genes (CCL4, CCL3, TNF- $\alpha$ , IL-1 $\alpha$ , IL-12 P35, IL-12 P40, CCL5, CCR5, IL-1 $\beta$ , IFN- $\gamma$ ) in *Leishmania* lesions and neutrophils were evaluated in BALB/c mice infected with *L. major* and treated with either glucantime or PBS (Fig. 3). In uninfected mice, no sign of expression in pro-inflammatory genes was observed in the lesions and neutrophils. However, in early

stages of infection (before treatment) or in the group received PBS in the lesions, IFN- $\gamma$  and in neutrophils, CCL3, IL-12 P35, IL-12 P40, CCL5, CCR5, IL-1 $\beta$ , IFN- $\gamma$  were expressing. In the first week after treatment in the group test (the group received glucantime), the pro-inflammatory genes started expressing in both lesions and neutrophils where in the lesions, except IL-1 $\beta$ , CCR5 and IL-1 $\alpha$  and in neutrophils except IL-1 $\beta$  و CCR5 و CCL5 و CCL4 other genes were expressing. In the last week in this group, in the lesions except IL-1 $\beta$  و CCR5 other genes were highly expressed but in neutrophils IL-1 $\beta$ , IL-1 $\alpha$  and CCL4 were switched off and the other genes were expressing (Table 2).



**Fig. 3:** Expression of pro-inflammatory genes in mice infected with *L. major* (lesion & neutrophils)

**Table 2:** Expression of pro-inflammatory genes in mice infected with *L. major* (lesion & neutrophils)

|                                      | Sampling Time    | Sampling site | CCL4 | CCL3 | TNF- $\alpha$ | IL-1 $\alpha$ | IL-12P35 | IL-12P40 | CCL5 | CCR5 | IL-1 $\beta$ | IFN- $\gamma$ |
|--------------------------------------|------------------|---------------|------|------|---------------|---------------|----------|----------|------|------|--------------|---------------|
| Healthy Tissue                       |                  | wound         | -    | -    | -             | -             | -        | -        | -    | -    | -            | -             |
| Group control (treated with PBS)     | Before treatment | wound         | -    | -    | -             | -             | -        | -        | -    | -    | -            | +++           |
|                                      | week 1           | Neutrophils   | -    | +    | -             | -             | ++       | ++       | ++   | ++   | +            | +++           |
|                                      |                  | wound         | -    | -    | -             | -             | -        | -        | -    | -    | -            | +++           |
|                                      | Week 2           | Neutrophils   | -    | +    | ++            | +             | +        | +        | +    | -    | +            | +             |
|                                      |                  | wound         | -    | -    | -             | -             | -        | -        | -    | -    | -            | -             |
| Group test (treated with glucantime) | Before treatment | wound         | -    | -    | -             | -             | -        | -        | -    | -    | -            | +++           |
|                                      |                  | Neutrophils   | -    | +    | -             | -             | ++       | ++       | ++   | ++   | +            | +++           |
|                                      | week 1           | wound         | +    | +    | +             | -             | ++       | ++       | +++  | -    | -            | ++            |
|                                      |                  | Neutrophils   | -    | +    | +             | +             | ++       | ++       | -    | -    | -            | ++            |
|                                      | Week 2           | wound         | +    | +    | +             | +             | ++       | ++       | ++   | -    | -            | ++            |
|                                      |                  | Neutrophils   | -    | +++  | +             | -             | ++       | ++       | +    | +    | -            | -             |

The thickness of bands shown in the gel electrophoresis was marked from 1 to 3 plus

BALB/c mice were injected with  $1 \times 10^7$  *L. major* promastigotes and the expression of pro-inflammatory genes in the lesions and neutrophils was evaluated by RT-PCR. The figure shows the expression of pro-inflammatory genes in lesions and neutrophils in *Leishmania* infected mice treated with glucantime (group test) after two weeks of treatment. Bands 1-8 shows the expression of TNF- $\alpha$ , IL-1 $\alpha$ , IL-12P35, IL-12P40, CCL5, CCR5, IL-1 $\beta$ , IFN- $\gamma$  in the lesions, band 9 negative control, band 10 standard DNA, 11-19 the expression of CCL4, CCL3, TNF- $\alpha$ , IL-1 $\alpha$ , IL-12P35, IL-12P40, CCL5, IL-1 $\beta$ , IFN- $\gamma$  in neutrophils

## Discussion

The main goal of this study was to examine the expression of ten pro-inflammatory genes including IL-12P35, IL-1 $\alpha$ , CCL4, CCL3, CCL5, IL-12P40, CCR5, IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$  in lesions and neutrophils of BALB/c mice infected with *L. major* to see whether or not there is a relation between the expression of the genes in the lesion and neutrophils in *Leishmania* infection.

The results clearly showed that in uninfected mice, none of the pro-inflammatory genes was expressed in healthy tissues and blood derived neutrophils. Similar figure was in the lesions of infected mice with no treatment at early stages of the infection where except IFN- $\gamma$  other pro-inflammatory genes were not expressed in the lesions however, in the neutrophils, CCL3, IL-12P35, IL-12P40, CCL5, CCR5, IL-1 $\beta$  and IFN- $\gamma$  were expressed. At late stages of the infection in this group, TNF- $\alpha$  and IL-1 $\alpha$  were also expressed where the expression of IFN- $\gamma$  in the lesions and CCR5 in neutrophils stopped. Recent studies have shown a down-regulation in IFN- $\gamma$  and TNF- $\alpha$  produced by NK cells in diffuse cutaneous leishmaniasis caused by *L. mexicana* (9). Down-regulation of IFN- $\gamma$  is also reported in *L. braziliensis* cases co-infected with *Mycobacterium leprae* (10). Another study indicated an inhibition of IL-12 through CR3 engagement by *L. major* (11). We have also already showed the down regulation of MHC I in DCs infected with *L. mexicana* (in press). Lack of expression of the pro-inflammatory genes was in-line with other studies indicating a similar potency for *L. major* in managing the immune

response and controlling the expression of not only IL-12 but also the other pro-inflammatory genes including IFN- $\gamma$ . We already showed that early expression of IFN- $\gamma$  has direct relation and key role in with *Leishmania* immunity in BALB/c mice (12) therefore, down-regulation of IFN- $\gamma$  in *Leishmania* infection results in exacerbation of the disease.

The role of neutrophils in inducing resistance against *Leishmania* parasites have been reviewed already (13). To the best of our knowledge, there is no report on the relation between the expression of pro-inflammatory genes in the lesion and blood neutrophils in *Leishmania* infection. The expression of pro-inflammatory genes in blood neutrophils indicates that the neutrophils are primed by the parasite before migrating to the infected tissue. Various cytokines and chemokines including IL-17, IL-8, IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF and CCL5 play prominent roles in the recruitment, activation and survival of neutrophils at the site of inflammation (14-15). CCL5 has also negative effect on the extravasation of neutrophils and monocytes into the peritoneal cavity (16). It is known that most of neutrophils in the *Leishmania* lesion migrate from the blood. The difference between the expression profile of pro-inflammatory gene in lesions and blood neutrophils of *Leishmania* infected mice indicated that interaction of neutrophils with the parasite affects the expression profile of the genes in neutrophils migrated to the infected tissue. After treatment with anti-leishmanial drugs, a high level of expression in most of the pro-inflammatory genes including CCL4, CCL3, TNF- $\alpha$ , IL-12P35, IL-12P40, CCL5 and IFN- $\gamma$  in *Leishmania* lesions was observed confirming the inhibitory effect of *Leishmania* infection on the expression of the pro-inflammatory genes in the *Leishmania* lesions.

On the other hand, IL-1 $\beta$  expressed in untreated or control mice only in neutrophils but not the lesion and after beginning of treatment with antileishmanial drug, the expression was down regulated. Amastigotes

are capable to induce IL-1 $\beta$  in PBMCs in *L. (V.) braziliensis* (17) where production of IL-1 $\beta$  promotes host resistance to *Leishmania* infection (18). Our results for the first time demonstrate that *L. major* similar to *L. (V.) braziliensis* directly stimulates production of IL-1 $\beta$  in neutrophils.

None of the control mice was expressed CCL4 and TNF- $\alpha$  either in the lesion or neutrophils. However the gene was only expressed in groups received treatment and when the lesions began healing, CCL4 in the lesions and TNF- $\alpha$  in both lesions and neutrophils started expression. Therefore, TNF- $\alpha$  can be candidate as an indicator for the healing of *Leishmania* lesions.

## Conclusion

*L. major* can strongly suppress the expression of pro-inflammatory genes in the lesions. This may help the parasite control the immune responses induced by the host. The expression of pro-inflammatory genes in the lesions restarted when the lesions were treated with anti-leishmanial drugs. Expression of pro-inflammatory genes in peripheral neutrophils was not suppressed by the parasite indicating the important role of these cells in *Leishmania* infections. The expression of TNF- $\alpha$  in the lesions and down-regulation of IL-1 $\beta$  in neutrophils were only observed after treatment with anti-Leishmanial drugs and could be accounted as an indication for healing of cutaneous leishmaniasis.

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The authors declare that there is no conflict of interest.

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