# The Role of Overproduction of Nitric Oxide in Apoptosis of BALB/C Mice Macrophages Infected with *Leishmania Major* in Vitro

**Shabnam Kharazi[1], Ahmad Zavaran Hosseini[1], and Taghi Tiraihi[2]**

*[1]Department of Immunology, Tarbiat Modarres University, Tehran, Iran*
*[2]Department of Anatomical Sciences, Tarbiat Modarres University, Tehran, Iran*

**ABSTRACT**

Nitric oxide (NO) derived from activated macrophages has been shown to be crucial for the host's leishmanicidal activities. Excess NO, however, can induce apoptosis in some cell types, including macrophages. In the present investigation, we studied the role of NO in inducing apoptosis of BALB/c mice macrophages infected with *Leishmania major* in vitro. The peritoneal macrophages were harvested and cultured with or without *L.major* in the presence of a donating reagent (s-Nitroso-N-Acetylpenicillamine (SNAP)) or an inhibitor of NO synthase (NG –Methyl-L-Arginine (NMMA)). The concentration of NO in culture supernatants was measured after 18 hours incubation. Simultaneously, macrophages undergoing apoptosis were identified by fluorescence and electron microscopy. The results showed an increase in apoptosis rate in parallel to nitrite production in macrophages cultured in the presence of SNAP. Although macrophages infected with *L.major* had no significant increase in NO production, they showed a significant increase in apoptosis rate. Besides, macrophages cultured with NMMA, had a decreased NO production but the apoptosis rate increased. Therefore, mechanisms involved in apoptosis induction in the last two groups may be different from NO overproduction.

**Key words:** Apoptosis, *Leishmania major*, Nitric Oxide.



## INTRODUCTION

Macrophages play a dual role in the diseases caused by Leishmania parasites: they both serve as potentially safe habitats for the parasites, and when activated by cytokines, they produce NO, which is a potent effector molecule against extra- and intracellular Leishmnia (Assreuy et al., 1994; Mauel & Ransijn, 1997; Bogdan & Rollinghoff, 1998; Bogdan et al., 2000). However, it is also true that the cytotoxic effect of NO is so strong that it kills not only the target cells but also the NO producing macrophages. In different studies, the involvement of NO in apoptosis induction has been demonstrated (Albina et al., 1993; Shimaoka et al., 1995; Gotoh & Mori, 1999), but it has not yet been shown whether the NO produced during the infection of macrophages with Leishmania parasites, has any effect on the producing cells. So, we investigated the role of the overproduction of NO on apoptosis of BALB/c mice macrophages infected with *L.major* in vitro.

**Corresponding author:** Dr. Ahmad Zavaran Hosseini, Dept. of Immunology, Tarbiat Modarres University, Tehran, Iran. Fax: (+98 21) 8013030, E-mail: zavarana@modares.ac.ir.

## MATERIALS AND METHODS

### Reagents

All chemicals used were from Sigma Chemical Co. (St. Louis, USA), unless otherwise indicated. Sterile tissue culture plasticware were from NUNC (Roskilde,

Denmark).

**In vitro culture of parasites and macrophages**

A cloned line of *Leishmania major* (WHO designation: MRHO, IR, 75, ER) was used in all experiments. *L.major* parasites, were freshly isolated from infected BALB/c mice and were maintained as promastigote cultures for less than five passages. Macrophage infection was done as described previously (Handel-Fernandez & Lopez, 2000). Briefly, murine peritoneal cells were harvested from inbred female BALB/c mice without elicitation. The cells were washed, resuspended in complete culture medium (RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 µg/ml streptomycin and 100 IU/ ml penicillin) and plated in 24- well ($1\times10^6$ cell/1 ml) culture plates (for electron microscopy investigation). Peritoneal cells ($2\times10^6$ cell/2 ml) were also grown on glass coverslips (15-mm diameter) in 6-well culture plates (for fluorescence microscopy investigation). After a 2 hour incubation, nonadherent cells were removed by extensive washing. Adherent cells were then infected with stationary-phase promastigotes of *L.major* at a 1:10 or 1:20 cell: parasite ratio and were incubated with or without SNAP (1000 µM) (100 µM SNAP gives 1.4 µM NO/min at 37°C (Assreuy et al., 1994)), or NMMA (1000 µM), an inhibitor of NO synthase (Mebmer et al., 1995). After culture for 18 hours, supernatants were harvested for nitrite analysis and adherent cells were prepared for fluorescence and electron microscopy.

**Measurement of nitrite**

Cell-free culture fluids were obtained by centrifugation and assayed for the stable end product of NO, nitrite, using the Griess reaction, as described elsewhere (Moshage et al., 1995). Briefly, 100 µl of culture supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid/ 0.1% naphthylene-diamine dihydrochloride) at room temperature for 10 minutes. Absorbance was measured at 540 nm with an ELISA reader and compared with a standard curve generated with sodium nitrite (1-300 µM).

**Morphological investigation of apoptosis**

Morphological evidence of apoptotic cell death was obtained by fluorescence microscopy analysis (Kim et al., 1998): 10 µl of a mixture of 100 µg/ml each of acridine orange and ethidium bromide were added to the adherent cells grown on glass coverslips, then they were examined by fluorescence microscope (Axiophot, Zeiss), under which apoptotic and viable cells had a green appearance (they can be distinguished by the morphological features of apoptotic cells) while late apoptotic or necrotic cells showed an orange colour.

The number of apoptotic cells was determined by fluorescence microscopy.

We used electron microscopy (EM) analysis for confirming apoptosis induction (Albina et al, 1993). For EM analysis preparation, adherent macrophages were harvested by incubating the plates on ice and gentle scraping (Handel-Fernandez & Lopez, 2000). Cells were then centrifuged and fixed in phosphate buffer saline containing 0.25% glutaraldehyde at pH 7.2. After mixing with 2% agar, samples were postfixed with 1% osmium tetroxide, dehydrated and embedded in Epon 812 resin (TAAB, Berkshire, England). Ultrathin (50-70 nm) sections were stained with uranyl acetate and lead citrate and examined by a Zeiss-900 electron microscopy.

**Statistical analysis**

The Mann-Whitney test was used to determine the statistical significance of the inter-group comparisons; $p< 0.05$ was considered to indicate significance.

## RESULTS

**Kinetics of nitrite production**

After 18h incubation, macrophages cultured together with SNAP showed an increased nitrite production ($p$=0.0002). In contrast, the addition of NMMA, reduced the amount of nitrite produced by macrophages ($p$=0.03). Cells cultured with *L. major* alone, showed no significant increase in nitrite production (Table 1 and Figure 1).

**Apoptosis induction**

Macrophages undergoing apoptosis were identified by fluorescence microscopy. The pictures presented in figures 2-4 exemplify the nuclear and cytoplasmic changes that were detected in cells undergoing apoptosis. These changes, which are characteristic of apoptosis, include nuclear and cytoplasmic condensation, a reduction in cell volume, nuclear fragmentation, formation of cell surface blebs and shedding of membrane-bound apoptotic bodies.

We used EM analysis for confirming the induction of apoptosis in macrophages. The results are exemplified in Figures 5 and 6.

In agreement with the results of others (Albina et al., 1993), the apoptotic changes appeared to be asynchronous. Macrophages presenting the previously described nuclear and cytoplasmic alterations were found in the same microscopic field as other cells exhibiting grossly normal appearance and yet others that were clearly necrotic (Figure 7). Table 1 and figure 8 show the percentage of apoptotic cells in differ-

**Table 1.** Concentration of nitrite in culture supernatants and percentage of apoptotic macrophages after 18 hours incubation in the presence of *L. Major*/SNAP/NMMA.

| Cells and Materials | Macrophage | Macrophage+ L. major | Macrophage+ L. major+SNAP | Macrophage+ L. major+NMMA | Macrophage +SNAP |
|---|---|---|---|---|---|
| Nitrite Concentration (micromolar) | 33.09+1.76 | 33.33+2.09 | 259+49+5.44 | 26.96+1.67 | 264.1+7.82 |
| %Apoptosis | 2.5 | 21.5 | 21 | 5.4 | 25.1 |

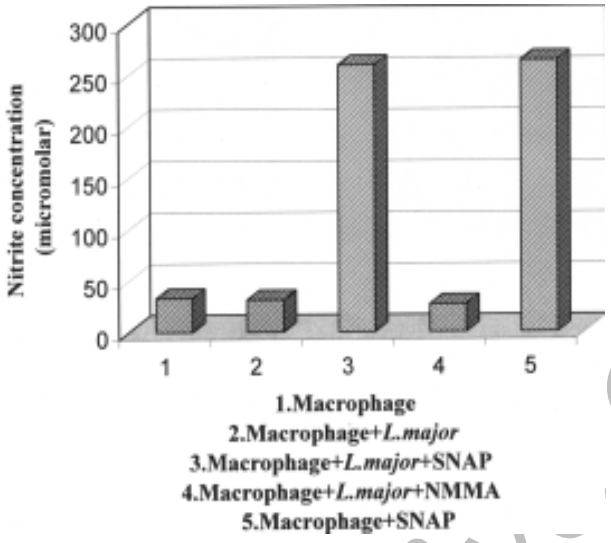


**Figure 1.** Nitrite produced from BALB/c mice macrophages after 18 hours incubation with *L. major*/SNAP/NMMA.

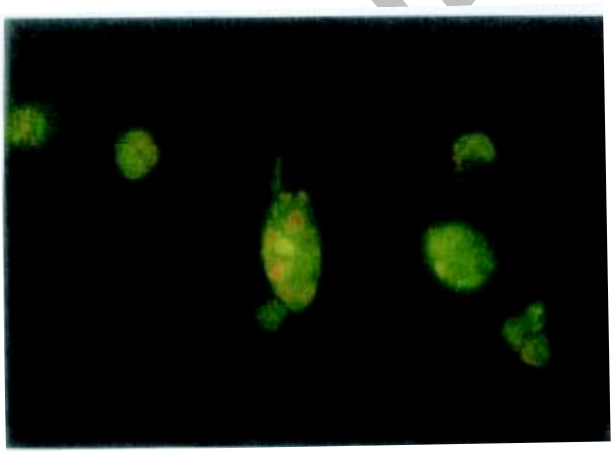

**Figure 2.** Infected viable and apoptotic macrophages in the presence of SNAP. A typical apoptotic macrophage is seen in the middle of the picture from which an apoptotic body is being separated. The orange granules are parasites. Other cells are viable (*100)

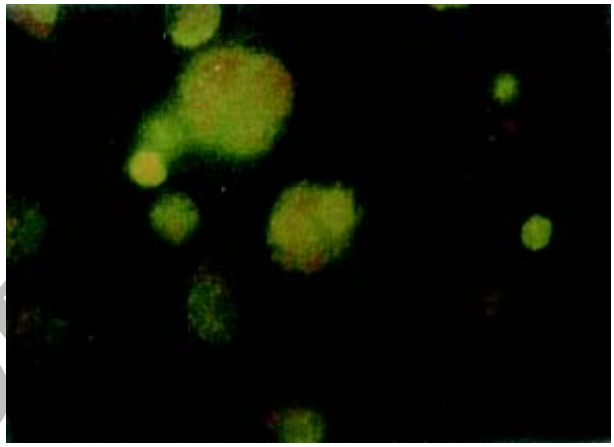

**Figure 3.** Infected viable and apoptotic macrophages. One apoptotic macrophage can be seen in the middle of the picture characterized by fragmented nucleus (*100).

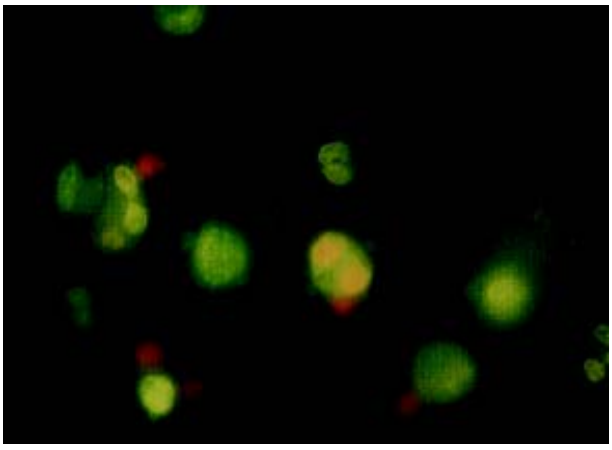

**Figure 4.** Not infected apoptotic and viable macrophages in the presence of SNAP. Apoptotic cells show nuclear fragmentation, cell blebbing and apoptotic body formation (*100).

**Figure 5.** A macrophage in the early stages of apoptosis: chromatin condensation led to formation of nuclear caps (Λ) and a cell surface bleb (< ) appeared on the plasma membrane (*7000).
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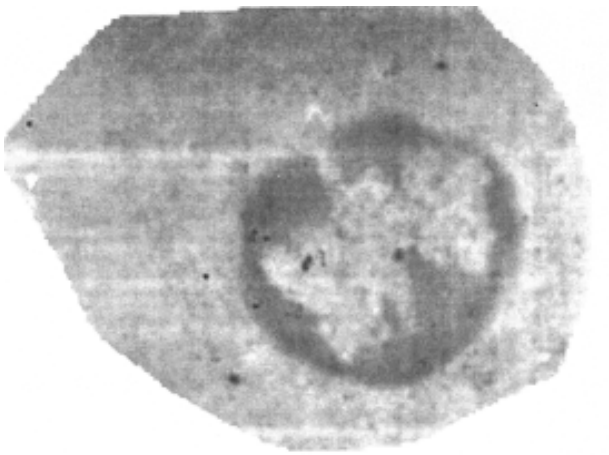

**Figure 6.** A macrophage in the late stages of apoptosis, showing prominent cytoplasmic and nuclear condensation, formation of nuclear caps, reduction in cell volume and organelle free cytoplasm (*4400).

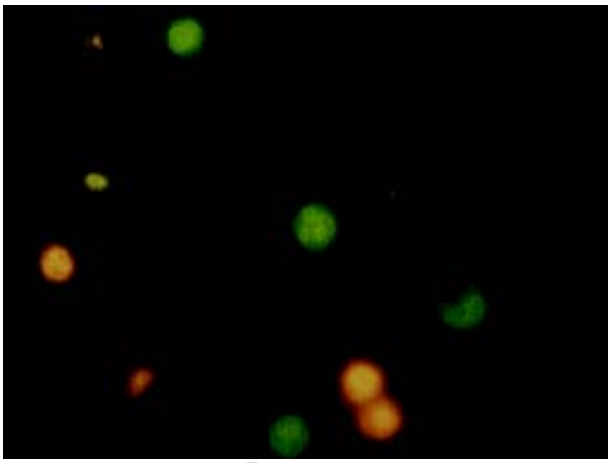

**Figure 7.** Viable (green), apoptotic (green) and necrotic (orange) macrophages. Two apoptotic cells are seen along a straight line in the middle of the picture, which are smaller than viable cells and have fragmented and condensed nuclei (*400).

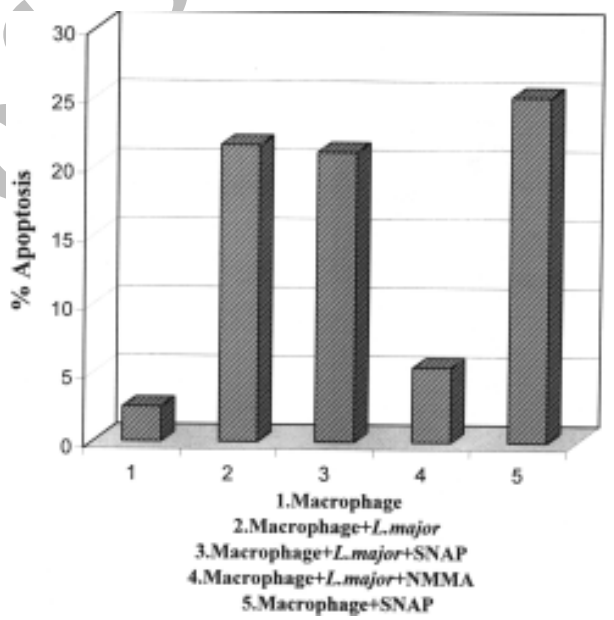


**Figure 8.** Percentage of Apoptotic Macrophages after 18 Hours Incubation with *L. major*/SNAP/NMMA.

ent groups. All groups had a significant increase in apoptosis rate compared to the control group ($p$<0.001).

## DISCUSSION

The present results demonstrate that the amount of NO produced in macrophages infected with *L. major*, had no significant difference from that of the control group, but the apoptosis rate increased (p=0.0007). The low production of NO in these macrophages was probably due to the active suppression of the synthesis of NO by Leishmania parasites: It has been shown that infection of macrophages with *L. major* promastigotes prior to stimulation with interferon-gamma and lipopolysaccharide partially inhibit the release of NO. This effect is mimicked by addition of glycoinositolphospholipids from *L. major*, which are abundantly expressed on the surface of both the promastigote and amastigote parasite forms. Another

parasite molecule, which might downregulate inducible nitric oxide synthase (iNOS) actively in infected macrophages, is the lipophosphoglycan-associated kinetoplastid membrane protein-11. At amino acid position 45, this protein contains NG-monomethyl-L-arginine,a structural analogue of L-arginine and well known inhibitor of iNOS (Bogdan et al, 1996; Bogdan & Rollinghoff, 1998). These mechanisms can lead to the low production of NO in infected macrophages.

Unrestricted replication of Leishmania amastigotes in macrophages is assumed to cause the eventual burst of the host cell (Rittig & Bogdan, 2000), accompanied by the release of macrophage intracellular compounds, some of which are proapoptotic for viable cells. This phenomenon is possibly responsible for the increased rate of apoptosis seen in these cells.

In this study, NO production in macrophages cultured in the presence of SNAP was much higher than the control group ($p$=0.0002), which correlated with an increase in apoptosis rate in these cells ($p$<0.0007). These results are in agreement with previous studies which showed that induction of apoptosis correlated with NO production (Sarih et al., 1993; Albina et al., 1993; Albina & Reichner, 1998).

Indeed, only about 23% of these macrophages demonstrated morphological changes compatible with apoptosis when examined by fluorescence microscopy at 18h. This relatively low number may have resulted from the efficient removal of cells undergoing apoptosis by the remaining viable macrophages or by the progression of the process into a phase of secondary necrosis. Providing support for both these outcomes, apoptotic macrophages were seen that contained apoptotic nuclei, and few cells with typical apoptotic morphology were present at 24h (Figures 9A and 9B).

The amount of NO produced by macrophages treated with NMMA decreased ($p$=0.03) but the number of apoptotic cells increased ($p$= 0.001). Both NMMA itself and the release of the macrophage intracellular compounds mentioned before, may contribute to this increased rate of apoptosis.

In summary, we report here that although apoptosis rate increased in the BALB/c peritoneal macrophages infected with *L.major* in vitro, this is not correlated with endogenous NO production by macrophages.

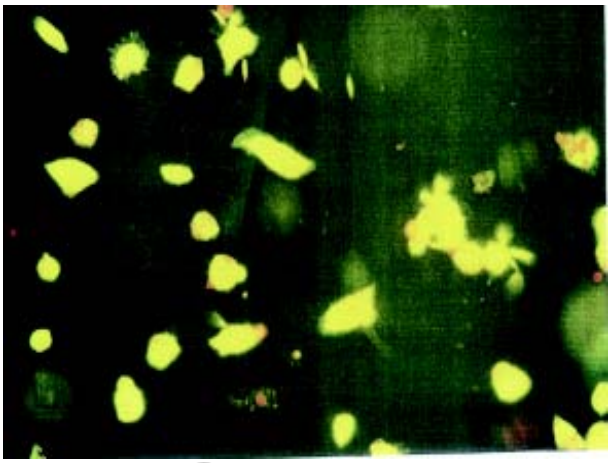

**Figure 9A.**

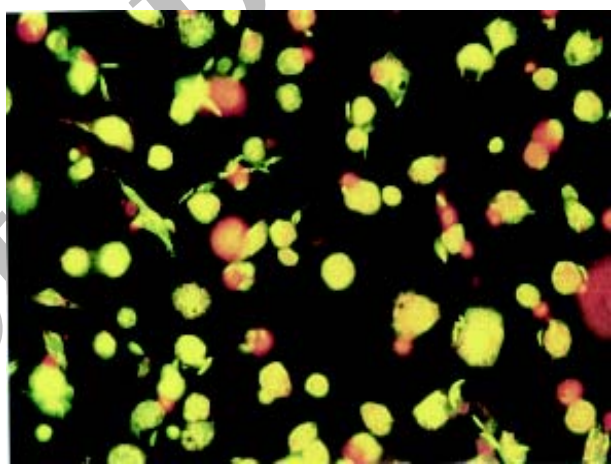

**Figure 9B.**

**Figure 9.** Infected macrophages cultured in the presence of SNAP (A) and NMMA (B); as it is shown, the number of macrophages (and Leishmania parasites) decreased in the presence of SNAP, probably due to the increased production of NO (×400).



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