Original Article

Role of *Iron Overload* in Apoptosis of Balb/c Mice Macrophages Infected with *Leishmania Major In Vitro*

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Abstract

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Objective: Although Iron is a crucial element for many metabolic pathways of the body, the excess iron may induce apoptosis in some cell types such as macrophages**.** In the present investigation, the role of iron overload in inducing apoptosis of Balb/c mice macrophages infected with L. major in vitro, as a selective model, was studied.

Materials and Methods: The peritoneal macrophages were harvested and cultured with different concentrations of iron to refuse its cytotoxic effect. Then, the macrophages were harvested and cultured with or without Leishmania in the presence of iron or donated reagent [S-Nitro-N-Acetylpenicillamine(SNAP)] or an inhibitor of NO, synthase [NG-Methyl-L-Arginine(NMMA)]. The concentration of NO as an immunological mediator in culture supernatants was measured after 18 hour incubation. Simultaneously, macrophages undergoing apoptosis were identified by fluorescence and electron microscopy.

Results: The findings showed that there is a statistically significant relationship between iron overload and apoptosis ($p<0.05$). Apoptosis rate had also increased in the macrophages cultured with iron, SNAP, NMMA, as compared with control group.

Conclusion: Iron influences the apoptosis rate and NO production in the macrophages infected with L. major.

Keywords: Apoptosis, Iron Overload, Nitric Oxide, Leishmania Major, *In Vitro*

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Introduction

Iron is ubiquitous in living organisms and works as an essential trace element for the synthesis of enzymes that are central to respiration, DNA synthesis and detoxification (1). It has been addressed by several writers that iron modulates immune-affecting mechanisms such as cytokine activities, nitric oxide (NO) formation or cell proliferation and thus host immune surveillance (2). The amount of iron within the cell is carefully regulated, because, disruption of iron metabolism can lead to iron deficiency anemia or iron overload. In iron overload diseases, such as hereditary haemachromatosis, iron accumulates in parenchymal organs, namely, in the liver. Untreated iron overload leads to liver fibrosis, cirrhosis and cancer (3). In addition, it has been suggested that iron overload causes immune abnormalities, including defective chemotaxis and phagocytosis by neutrophils and macrophages, decreased natural killer cell activity, decreased TNF-α production by macrophages, decreased numbers of $CD4^+$ T cells, NK cells, and an increased IL-4/IL-10 production in $CD8^+$ T cells (4). The mechanism of its toxicity has not been fully clarified and it is probably

the easy oxidation that releases a single electron with the production of damaging reactive oxygen species molecules, as it occurs in the Fenton reaction. Free radicals initiate lipid peroxidation of cell membrane and oxidative damage of proteins, which in turn cause changes in membrane fluidity, disruption of microsomes, lysosomes, accumulation of peptide fragments and cross-linked protein aggregates. This ultimately leads to deregulation of cellular processes, cell dysfunction and eventually to apoptosis or necrosis (3).

Recent studies have indicated that iron-induced free radicals are involved in various pathological disorders such as inflammation (5), organ transplantation, carcinogenesis (6), and ageing (7). Iron is a potent generator of oxidative damage whose levels increase with age, potentially exacerbating age-related diseases (8), because, iron-induced free radicals lead to the dysfunction of mitochondria and decreased ATP production by mitochondria and the enhancement of apoptosis and oxidative stress (9). Apoptosis can be induced by a variety of stimuli such as ionizing radiation, glucocorticoids, chemotherapeutic agents and lymphokine deprivation. Oxidative stress has been suggested to play a role as a common mediator of apoptosis and recent independent observations in divers systems support a role for oxidative mechanisms in the induction of apoptosis (7).

Studies of severe iron overload modeling haemachromatosis have reported increased hepatic lipid peroxidation, nuclear DNA damage and mitochondrial dysfunction (10). Also recent findings have demonstrated iron-induced apoptosis of renal proximal tubules after iron- nitrilotriacetate (Fe-NTA) injuries in vivo, where free radical injuries occurred (11). Even in viral hepatitis, iron may play a role in liver cell injuries (12).

A number of studies have described the complex relationship between iron and NO (13). Also, the involvement of NO in apoptosis induction has been demonstrated (14). It has been reported that iron modulate NO-mediated cell death and apoptosis in at least some tumour cells (15). Therefore, in the present study, the role of iron overload on apoptosis of Balb/c mice macrophages infected with leishmania major was investigated.

Materials and Methods

Reagents

All chemicals were obtained from sigma Chemical Co. (St, Louis, USA), unless otherwise indicated. Sterile tissue culture plasticwares were purchased from NUNC (Roskilde, Denmark).

Measurement of iron cytotoxicity (LD50 determination)

Briefly, the murine peritoneal cells were harvested from five of 6-8 weeks 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 mg/ml streptomycin and 100 IU/ml penicillin) and plated in 96-well (1×10^5cell/100μl) culture plates in triplicates. After 2 hours incubation, nonadherent cells were removed by extensive washing. The adherent cells were then cultured with 100, 150, 200, 250, 300, 350 and 400μM concentrations of iron. After 1, 2 and 3 hours of incubation, cell death percentage in the detached cells was determined by trypan blue. The current study was approved by the Institutional Review Board (IRB) of Faculty of Tarbiat Modares University. The animal use protocol was approved by the Institutional animal care of Tarbiat Modares University.

In vitro culture of parasite and macrophages

A cloned line of Leishmania major (WHO designation: MRHO, IR, 75, ER) was used in all the experiments. L. major parasites were freshly isolated from the infected Balb/c mice and maintained as promastigote cultures for less than five passages. The harvested macrophages, as described previously, were plated in 24-well (1×10^6cell/1ml) culture plates (for electron microscopy investigation) and also grown on glass coverslips (2×10^6cell/2 ml) in 6-well culture plates (for fluorescence microscopy investigation). After 2 hour incubation, nonadherent cells

were removed by extensive washing. The adherent cells were then infected with the stationary-phase promastigotes of L. major at a 1: 10 or 1: 20 cell: parasite ratio and incubated with or without SNAP (1000 μM) as a donating (16), or NMMA (1000 μM), an inhibitor of NO synthase (17). After being cultured for 18 hours, the 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 method, as described elsewhere (18). Briefly, 100 μl of the culture supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid/0.1 % naphtylene – diamine dihydrochloride) at room temperature for 10 minutes. OD values was measured at 540 nm using an ELISA reader and compared with a standard curve generated by sodium nitrite (1-300 μM).

Morphological investigation of apoptosis

Morphological evidence of apoptosis cell death was obtained by fluorescence microscopy analysis: 10 μl of a mixture of 100 μg/ml either of acridine orange and ethidium bromide was 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 color.

The number of apoptotic cells was determined by fluorescence microscopy. Electron microscopy (EM) analysis was used for confirming apoptosis induction (19). For EM analysis preparation, the adherent macrophages were harvested by incubating the plates on ice and gentle scraping. The cells were then centrifuged and fixed in phosphate buffer saline containing 0.25% glutaraldehyde at pH 7.2. After mixing with 2% agar, the samples, postfixed with 1% osmium tetroxide, 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 significant differences.

Results

Kinetic of iron cytotoxicity and nitrite production

After 1, 2 and 3 hours, calculated cell death percent showed 300 μm concentration of iron as a LD50 dose ($p=0.0001$). The amount of nitrite produced by the macrophages cultured together with iron, SNAP or NMMA showed an increased nitrite production ($p=0.02$) (Table 1, Fig 1).

Apoptosis induction

The macrophages undergoing apoptosis were identified by fluorescence microscopy. The pictures presented in Figures 2-5 exemplify the nuclear and cytoplasmic changes that were detected in the cells undergoing apoptosis.

Table 1. Concentration of nitrite in the culture supernatants and percentage of apoptotic macrophages after 18 hours incubation in the presence of iron/L. major/SNAP/NMMA. The data are presented as Mean ± SD, p<0.05.

Cells and Materials	Nitric Concentration (micromolar)	Apoptosis %
Macrophage	31.65±0.81	3.9±1.34
Macrophage+L.major	32.58±0.79	5±1.76
Macrophage+L.major+Iron	47.70±0.26	9.2±3.50
Macrophage+L.major+SNAP+Iron	119.09±1.40	13.71±3.81
Macrophage+L.major+NMMA+Iron	76.56±1.8	15.9±3.68

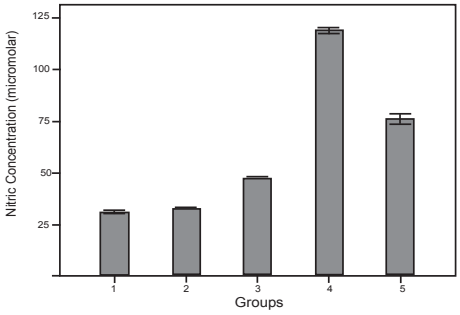

Fig 1: Nitrite produced fro the Balb/c mice macrophages after 18 hours incubation with L. major/Iron/SNAP/ NMMA.1. Macrophage
2. M-acrophage+L. major
3. Macrophage+L. major+ Iron
4. Macrophage+L. major+SNAP+Iron
5. Macrophage+L. major+NMMA+Iron.
The results are expressed as Mean±SD, n=3, p<0.05.

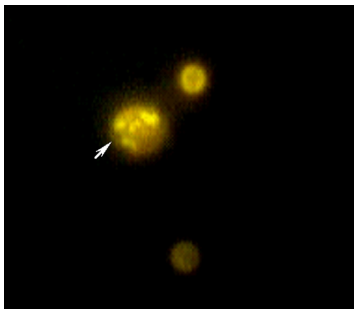

Fig 2: No infected apoptotic macrophage can be seen in the middle of the picture ch-aracterized by nuclear fragmentation to ind-uced by nitrosamide deamination or inducti-on of lipid peroxidation (arrow)(x100).

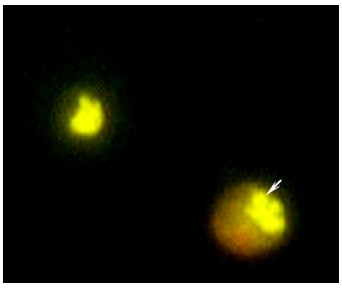

Fig 3: Infected apoptotic macrophage with clover leaf shaped nucleus and orange gran-ules in the presence of NMMA(arrow) (x100).

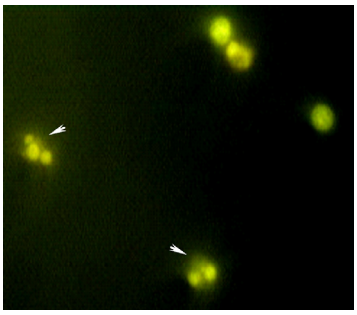

Fig 4: Viable and apoptotic macrophages in the presence of SNAP. Nuclear fragmentat-ions by NO-mediated nitrosamide deaminati-on are clearly seen in the apoptotic macropha- ges (arrow)(x100).

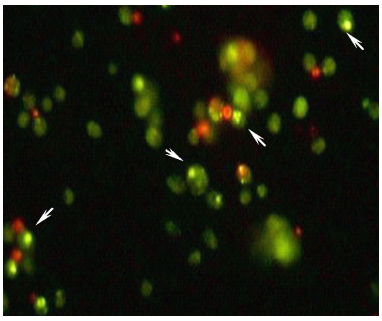

Fig 5: Viable (green), apoptotic (green) mac-rophages with shined nuclear fragments. Apo-ptotic bodies can be seen by a small fragment- of nuclear with low volume of cytoplasm (arrow)(x40).

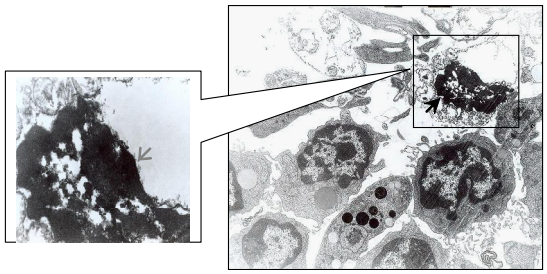

Fig 6: Electron microscopic observation shows chromatin aggregation on the nuclear membrane (Gray arrow) and disin-tegration of the mitochondria crista(Black arrow)(x4400).

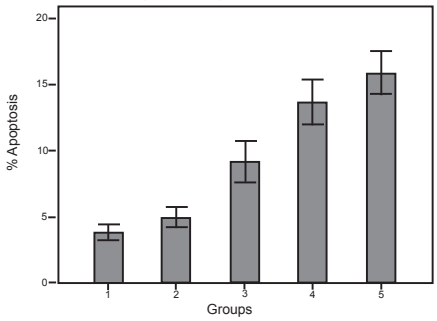

Fig 7: Percentage of the apoptotic macrophages after18 hours incubation with L. major/Iron/SNAP/ NMMA. 1. Macrophage, 2. Macrophage+L. major, 3. Macrophage+L. major+Iron, 4. Macrophage+L. major+ SNAP+Iron, 5. Macrophage+L. major+ NMMA+Iron. The results are expressed as Mean±SD, n=5, p<0.05.

These changes, which are characteristics of apoptosis, induced nuclear and cytoplasmic condensations, 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 the macrophages. The results are exemplified in Figure 6. Table 1 and Figure 7 show the percentage of the apoptotic cells in different groups. All the groups had a significant increase in

apoptosis rate compared with the control group (p=0.009).

Discussion

The result of this study demonstrated that the apoptosis rate and NO production in the infected macrophages cultured in the presence of iron was significant as compared with the control group (p=0.025). This might be attributed to oxidative stress, nitric oxide production, cytokine signaling, and many other unknown pathways which in itself can increase the apoptosis process(3). It has been demonstrated that intracellular free iron catalyzes the formation of highly reactive compounds such as superoxide ($O_2^{\cdot}$) and nitric oxide (NO) that those are precursors of reactive oxygen and nitrogen species such as hydroxyl radical, peroxide hydrogen, and peroxynitrite. Therefore, the increase in the free iron has caused cytotoxicity due to oxidative stress and eventually led to apoptosis (20-22), but we did not measure the level of free radicals in the culture supernatants. Free radicals also promote cytochrome c release from mitochondria, activation of caspases, production of chemokines (IL-8) and adhesion molecules, inflammation, fibrosis and cancer (23). It has been suggested that poly (ADP- ribose) polymerase and some heat shock proteins may be associated with iron-induced apoptosis (11). In addition, it has been known that iron affects NO production due to the increased nuclear factors (NF-KB, NF-IL-6) and iNOS induction. However, controversy remains as to the influence and significance of iron on NO production, likely, related to the different types of iron used in the studies. It has been shown that in addition to the effect of NO in formation of free radicals, there are signal pathways by which NO directly induce apoptosis, proton leak in mitochondria and an increase in oxygen consumption leading to cell death (21).

Recently, Hinda *et al* reported in liver kupffer cells preloaded with colloidal iron a strong iNOS induction, increased NO level in the liver and blood, and increased IL-6, IL-1β and TNF-α (24, 25). Apoptosis in astrocytes mediated by IL-1β is also associated with NO production and also nitric oxide synthase (NOS) play an important role in TCR-mediated apoptotic death in the thymus. Thus, NO has been recognized to be involved in the oxidative stress induced in the liver during the reperfusion or in hepatocytes by cytokines and lipopolysacharide (LPS) (15). The rate of apoptosis in the macrophages cultured with SNAP (NO donor) was greater than in the control group (p=0.009). Feger *et al* demonstrated that the addition of NO donor to the culture medium of the tumor cells resulted in depletion of iron in the cells, suggesting that it could be the principal mechanisms for the NO induced apoptotic cell death. In addition, p38 MAP kinase acted as a major mediator for the NO-induced apoptotic cell death of neural progenitor cells (6). In other study, it has been demonstrated that the addition of NO donor to burkitt's lymphoma cell lines generates effects which partially mimic those were induced by iron addition (20).

The proapoptotic effect of NO in different cell types is referred to up regulation of FasL, generation of peroxynitrite, inhibition of ATP synthase in mitochondria and inactivated antioxidant enzymes. In addition, it has been shown that NO mobilized iron from ferritin (anti apoptotic factor) in a GSH-dependent manner. NO could also facilitate iron release, when iron is delivered by a phagocytic route independently of the iron acquired from transferin (15). Thus, deprivation of iron has also been reported to induce apoptosis (11). In the present research, in the group that NMMA was added into the cell culture, NO production was significantly higher than that of the control group (p=0.021). This finding might be attributed to the fact that it couldn't completely inhibit NO production in this group. Studies carried out by Kuo demonstrated that NMMA supplementation decreased GSH content in hepatocytes leading to an increase in the NO-induced oxidative stress. Thus, NO and GSH depletion are now well-known to induce heme oxygenase expression that catalyzes heme degradation, which leads to increased free iron (15).

Conclusion

According to our findings, iron can induce apoptosis which is clinically important. In the present research, apoptosis rate was increased in the Balb/c mice peritoneal macrophages infected with L. major in vitro, which was not contributed to the direct effect of iron but it seems that another molecule Such as NO can affect the apoptotic pathways. The results are also in consistent with the results of other studies (20, 21, 26).

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