The protective effect of myo-inositol on human thyrocytes.

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Short title: Myo-inositol and thyrocytes

Abstract

Patients affected by autoimmune thyroiditis reached positive effects on indices of thyroid autoimmunity and/or thyroidal function, after following a treatment with selenomethionine (Se) alone, or Se in combination with Myo-inositol (Myo-Ins).

Our purpose was to investigate if Myo-Ins alone, or a combination of Se+Myo-Ins, is effective in protecting thyroid cells from the effects given by cytokines, or hydrogen peroxide (H_2O_2).

We assessed the interferon (IFN)-γ-inducible protein 10 (IP-10/CXCL10) secretion by stimulating primary thyrocytes (obtained from Hashimoto's thyroiditis or from control patients) with cytokines in presence/absence of H_2O_2.

Our results confirm: 1) the toxic effect of H_2O_2 in primary thyrocytes that leads to an increase of the apoptosis, to a decrease of the proliferation, and to a slight reduction of cytokines-induced CXCL10 secretion; 2) the secretion of CXCL10 chemokine induced by IFN-γ+tumor necrosis factor alpha (TNF)-α has been decreased by Myo+Ins, both in presence or absence of H_2O_2; 3) no effect has been shown by the treatment with Se.

Therefore, a protective effect of Myo-Ins on thyroid cells has been suggested by our data, which exact mechanisms are at the basis of this effect need to be furtherly investigated.

Keywords: myo-inositol, selenomethionine, hydrogen peroxide, thyrocytes, cytokines, CXCL10.

1 Introduction

Primary hypothyroidism is a frequent disease, accounting per year an incidence of about 250/100,000 and a prevalence of about 5% in the adult population, that are both increasing [1, 2].

Hashimoto's thyroiditis (HT) is the leading cause of primary hypothyroidism, whose annual frequency is increasing during the years starting from the beginning of 90's [3-5].

Several studies showed an increase of the oxidative stress in autoimmune thyroid diseases (AITD) [6-9]. Either the overproduction of the hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), as well as its decreased degradation, contribute to the pathogenesis of the inflammation in AITD, and to the apoptosis linked to AITD of thyroid cells [10, 11].

H_2O_2 is actually involved in the regulation of multiple inflammation signalling pathways [12]. Indeed, in order to induce oxidative stress, several experiments have been performed by culturing human or animal cells with H_2O_2, including thyrocytes [11], gingival fibroblasts [13], peripheral blood mononuclear cells (PBMC) [9, 14, 15], neurons [16], glia cells [17, 18], cardiomyocytes [19], pancreatic beta-cells [20, 21], myoblasts [22], retinal pigment epithelium [23], stem cells [24], and embryos too [25]. Environmental factors are able to induce intrathyroidal oxidative stress [26].

The main features of AITD are a lymphocytic infiltration in the thyroid, and high production of cytokines by lymphocytes and thyrocytes, including chemokines, whose secretion is induced by pro-inflammatory cytokines themselves [27, 28].

The interferon gamma (IFN-γ)-inducible chemokines, such as IFN-γ-inducible protein 10 (IP-10/CXCL10), and monokine induced by IFN-γ (MIG/CXCL9), and IFN-inducible T-cell alpha chemoattractant (ITAC/CXCL11), act by binding the same receptor [(C-X-C motif) receptor 3 (CXCR3)], and contribute to the pathogenesis of several diseases [organ specific autoimmune disorders (as Graves' disease (GD) and ophthalmopathy, type 1 diabetes mellitus), or systemic autoimmune disorders, (as Sjogren syndrome, systemic sclerosis, mixed cryoglobulinemia, or systemic lupus erythematosus)] [29-33].

IFN-γ stimulates CXCL9, CXCL10, and CXCL11 secretion by CD4+, CD8+, and natural killer (NK). CXCL10 is also released by thyroid cells or other cell types under the IFN-γ stimulation [34, 35]. Elevated CXCL10 or CXCL9 levels in peripheral fluids are therefore a marker of a T helper (Th)1 orientated immune response [36-38]. In fact, CXCR3 chemokines levels are significantly higher in HT patients than in those affected by non-autoimmune nodular goiter or healthy subjects [39].

Furthermore, these chemokines were significantly higher in HT patients affected by a more severe thyroiditis, particularly in presence of hypothyroidism and a hypoechoic pattern [39].

Several studies investigated about the use of Selenomethionine (Se) [40-43], plus Myo-inositol (Myo-Ins) [44], or L-carnitine [45, 46] (by a nutraceutical approach) in the management of AITD. The antioxidant activity is the common feature of these substances [40, 47-49].

HT patients treated with Se (usually at 200 μg/d) for three to twelve months, showed a decline in thyroperoxidase autoantibodies (AbTPO) [40], even if thyroid function was not changed. A better outcome was reached by the supplementation with Se in comparison to that of sodium selenite.

Positive effects on indices of thyroidal function and autoimmunity have been reported in AbTPO positive women in treatment with Se plus Myo-Ins [42, 44].

Therefore, the aim of this study was to stress thyroid primary cells (ThyC) from healthy patients (c-ThyC), or Hashimoto's thyroiditis patients (HT-ThyC), with cytokines, or H_2O_2, and then to check whether, in the presence of cytokines, or H_2O_2, the addition of equimolar concentrations of Se alone, Myo-Ins alone, or their combination could protect ThyC from the effects given by cytokines, or H_2O_2.

2 METHODS

2.1 General outline of the experiments

Experiments were carried out in order to stress ThyC from c-ThyC, or HT-ThyC, with cytokines, or H_2O_2, and then assess whether, in the presence of cytokines, or H_2O_2, equimolar concentrations of Se alone, Myo-Ins alone or combined could protect ThyC from the effects given by cytokines, or H_2O_2. Se was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and Myo-Ins was obtained by LO.LI Pharma S.r.l (Italy). Both Se and Myo-Ins were prepared in sterile phosphate buffered saline (PBS) before the utilization.

We evaluated ThyC viability, proliferation, and apoptosis, and also CXCL10 secretion.

2.2 Thyroid follicular cells

Surgical thyroid tissue was obtained from **3 patients with HT and 3 benign nodular thyroid, euthyroid at the time of surgery**. Thyroidectomy was adviced to these patients mainly because of the presence of a large goiter and/or thyroid nodules. The study has been conducted along the lines of the Declaration of Helsinki (2000) on the ethic in clinical study. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and national laws; the patients gave their informed consent to it [50].

Thyrocytes were prepared as reported previously [51, 52].

Surgical tissues were minced with scissors and digested with collagenase (1 mg/mL; Roche, Mannheim, Germany) in RPMI 1640 (Gibco BRL, Paisley, UK) for 1 h at 37 °C. Semi-digested follicles were removed, sedimented for 2 min, washed, and cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM glutamine, and 50 μg/mL penicillin/streptomycin at 37 °C and 5% CO_2 in plastic 75 cm^2 flasks (Sarstedt, Verona, Italy).

2.3 CXCL10 secretion assay

For CXCL10 secretion assays, 3000 cells were plated in 96-well plates in growth medium. After 24 h, the growth medium was removed, cells were accurately washed in PBS, and incubated in phenol red and serum-free medium. Cells were incubated (24 h) with IFN-γ (R&D Systems, Minneapolis, MN; 500, 1000, 5000, 10000 IU/ml) and 10 ng/mL tumor necrosis factor (TNF)-α (R&D Systems), alone or in combination [53].

The concentration of TNF-α was chosen to obtain the highest responses, according to previously conducted experiments. After 24 h, the supernatant was collected and frozen at -20 °C until chemokines assay.

To investigate the effect of Myo-Ins and Se on cytokines, we used three concentrations for each of them (0.1, 0.25 and 1.0 μM) (alone or in combination), in presence or absence of IFN-γ and/or TNF-α (see above).

To investigate the effect of Myo-Ins and Se on H_2O_2, we used three concentrations for each of them (0.1, 0.25 and 1.0 μM) (alone or in combination), in presence or absence of cytokines, or 200 μM H_2O_2 (see above), added at the same time as cytokines, for 24 h.

We used a quantitative sandwich immunoassay (R&D Systems), with a sensitivity range of 0.41–4.46 pg/mL, to assess the CXCL10 levels in cell culture supernatant.
The absorbance was evaluated at 450 nm (with 540 nm as correction wavelength), by a plate reader (VICTOR™ X4, Perkin Elmer, Waltham, Massachusetts, USA). Experiments were performed in triplicate. The intra- and inter-assay coefficients of variation were 4.5 and 7.3% for CXCL10.

2.4 Cell viability and proliferation assay

To determine cell proliferation, we used the WST-1 assay (Roche Diagnostics, Almere, The Netherlands), a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide used in the MTT assay [54-56]. To investigate the effect of Myo-Ins and Se we used three concentrations for each of them (0.1, 0.25 and 1.0 μM) (alone or in combination), in presence or absence of cytokines, or 200 μM H_2O_2 (see above), **all added at the same time to cell cultures**. **The treatments were conducted for 24 h.**

The absorbance was measured after 2 h from the start of the tetrazolium reaction. All experiments were performed in triplicate for each cell preparation.

2.5 Proliferation assay: cell counting

The proliferation was evaluated also using the cell number counting [54-56].

2.6 Apoptosis determination- Hoechst uptake

ThyC were seeded (35000 cells/mL in a final volume of 100 μL) in each well of a 96-well plates. Then, cultures were incubated for 48 h with Myo-Ins and Se [for each of them we used three concentrations (0.1, 0.25 and 1.0 μM) (alone or in combination)], in presence or absence of cytokines, or 200 μM H_2O_2 (see above)] in a humidified atmosphere (37 °C, 5% CO_2), and stained with Hoechst 33342 [56]. The apoptosis index (ratio between apoptotic and total cells) x100 was calculated.

2.7 Statistics

Data was reported as mean ± SD for normally distributed variables or as the median and interquartile range. Mean group values were compared using one-way ANOVA for normally distributed variables or by the Mann-Whitney U or Kruskal-Wallis test. Proportions were compared by the $\chi 2$ test. Post hoc

comparisons of normally distributed variables were carried out using the Bonferroni-Dunn test. P values lower than 0.05 were considered statistically significant, whereas between 0.10 and 0.05 as borderline significant.

3. RESULTS

3.1 IFN-γ and TNF-α modulation of CXCL10

In the supernatants obtained from cultures of HT-ThyC or c-ThyC, the levels of CXCL10 were undetectable.

CXCL10 was released in a dose-dependent manner by IFN-γ in HT-ThyC (CXCL10: 0, 141±54, 376±67, 421± 84, and 495±96 pg/mL at the following IFN-γ concentrations of 0, 500, 1000, 5000, and 10,000 IU/mL, respectively; ANOVA, $p < 0.001$). Similar results were observed in c-ThyC, without any significant difference with respect to HT-ThyC (data not shown).

TNF-α had no effect on CXCL10 secretion, indeed it remained undetectable after addition of TNF-α in the cultures. The combination of IFN-γ (1000 IU/mL) plus TNF-α (10 ng/mL) had a significant synergistic effect on the CXCL10 secretion by HT-ThyC [CXCL10, 1541±77 vs 289±69 pg/mL with IFN-γ (1000 IU/mL) alone; ANOVA, $p < 0.0001$], in agreement with previous results [57]. Similar results were observed in c-ThyC, without any significant difference with respect to HT-ThyC (data not shown).

3.2 CXCL10 Modulation by H_2O_2, Se, Myo-Ins

CXCL10 secretion induced by IFN-γ+TNF-α was significantly reduced by H_2O_2 (200 μM) in HT-ThyC (**Figure 1**).

Se (0.1, 0.25 and 1.0 μM) had no effect on CXCL10 secretion induced by IFN-γ+TNF-α, in presence (**Figure 2A**) or absence (**Figure 2B**) of H_2O_2 (200 μM) in HT-ThyC.

Myo-Ins (0.1, 0.25 and 1.0 μM) reduced dose dependently and significantly CXCL10 secretion induced by IFN-γ+TNF-α, in presence (**Figure 3A**) or absence (**Figure 3B**) of H_2O_2 (200 μM) in HT-ThyC.

The combination of Myo-Ins (1.0 μM) plus Se (1.0 μM) reduced significantly CXCL10 secretion induced by IFN-γ+TNF-α, in presence (**Figure 4A**) or absence (**Figure 4B**) of H_2O_2 (200 μM). However the CXCL10 reduction induced by Myo-Ins (1.0 μM) plus Se (1.0 μM) was not significantly different from that obtained with Myo-Ins (1.0 μM) alone in HT-ThyC (**Figure 4**).

The c-ThyC cells subjected to similar experiments behaved in the same way as the HT-ThyC.

3.3 Proliferation and apoptosis

IFN-γ+TNF-α had no effect on proliferation (cell growth 99% with respect to control, expressed as 100%) or apopotosis (2.4% of control cells were apoptotic, and the percentage was 2.6% after the treatment with IFN-γ+TNF-α; $P > 0.05$, ANOVA) in HT-ThyC.

Proliferation was slightly reduced by H_2O_2 (**Figure 5A**; 50 μM, 100 μM, or 200 μM), while apoptosis increased (**Figure 5B**), in HT-ThyC.

Se (1.0 μM) had no effect on proliferation or apoptosis changes, induced by H_2O_2 (200 μM), in presence or absence, of IFN-γ+TNF-α, in HT-ThyC [Se (1.0 μM) + H_2O_2 (200 μM), 98% vs H_2O_2 (200 μM) alone].

Myo-Ins (1.0 μM) had no effect on proliferation or apoptosis changes, induced by H_2O_2 (200 μM), in presence or absence, of IFN-γ+TNF-α, in HT-ThyC [Myo-Ins (1.0 μM) + H_2O_2 (200 μM), 97% vs H_2O_2 (200 μM) alone].

The combination of Myo-Ins (1.0 μM) plus Se (1.0 μM) had no effect on proliferation or apoptosis changes, induced by H_2O_2 (200 μM), in presence or absence, of IFN-γ+TNF-α, in HT-ThyC [Myo-Ins (1.0 μM) + Se (1.0 μM) + H_2O_2 (200 μM), 96% vs H_2O_2 (200 μM) alone].

The c-ThyC cells subjected to similar experiments behaved in the same way as the HT-ThyC.

The proliferation was evaluated also using the cell number counting, that confirmed the above mentioned results (data not shown).

4. Discussion

Our findings confirm the toxic effect of H_2O_2 in primary thyrocytes, leading to a decreased proliferation, increased apoptosis, and a slight reduction of cytokines-induced CXCL10 secretion. Moreover, we first show that Myo-Ins reduces the secretion of CXCL10 chemokine induced by IFN-γ+TNF-α, in presence or absence of H_2O_2, while Se has no effect, in HT-ThyC, or in c-ThyC. These data suggest a protective effect of Myo-Ins on thyroid cells.

The fact that H_2O_2 reduces the secretion of CXCL10 chemokine under the influence of cytokines, can be explained by the reduction of proliferation, and increase of apoptosis in ThyC. In this specific case, the results on chemokine production can be accounted on the toxic effect of H_2O_2 on ThyC vitality. On the contrary, since Myo-Ins is not inducing any change in proliferation or apoptosis of ThyC, the reduction of the CXCL10 secretion under the influence of the pro-inflammatory cytokines IFN-γ, and TNF-α, can be accounted as a protective effect of Myo-Ins on the thyroid cells themselves.

The involvement of Myo-Ins and phosphatidylinositol(s) (PtdIns) in physiological and pathological conditions of the thyroid gland has been shown by various experimental researches and clinical trials.

PtdIns have a significant role in the intracellular signaling linked with thyroid-stimulating hormone (TSH) in thyrocytes [58]. Two different signals are related to the TSH intracellular signaling pathway, one involving cyclic AMP (cAMP) as second messenger, implicated in thyroxine (T4), triiodothyronine (T3) release, and in cell growth and differentiation, the second one depending on inositol [59, 60], and regulates the iodination mediated by H_2O_2 [59]. Furthermore PtdIns is involved in thyroid autoimmunity [61, 62].

The important role performed by iodine and Se in thyroid autoimmunity has been shown [27, 63]. Indeed, an elevated prevalence of autoimmune thyroiditis (AT) has been observed in regions **with** severe Se deficiency. This is caused by a reduced activity of Se-dependent glutathione peroxidase activity in thyroid cells. In addition, Se-dependent enzymes are important in the regulation of the immune system. A number of papers have shown that even mild Se deficiency could play a role in the development and maintenance of AITD [40, 64, 65]. Some investigations have been carried out in AITD patients treated with sodium selenite, or Se, showing the reduction of AbTPO [40].

Other studies showed that patients with subclinical hypothyroidism, due to AT, after treatment with Myo-Ins+Se obtained a significantly decline of the TSH levels as well as of the antithyroid autoantibodies levels [44, 66-68]. The Myo-Ins treatment showed also the reduction of the CXCL10 serum levels, confirming the immune-modulatory effect of this substance [67]. This finding agrees with the present *in vitro* results, showing that Myo-Ins reduces the secretion of CXCL10 chemokine induced by IFN-γ + TNF-α, in presence or absence of H_2O_2, in thyroid cells.
Conversely our data show Se has no effect on chemokine levels, and agree with those of a recent study that demonstrates that the short-term Se supplementation has a limited impact on the natural course in euthyroid HT, and has no effect on CXCL10 circulating levels [69].

Our findings are in line with that of an *in vitro* study aiming to assess if PBMC obtained from HT and control women, were protected by the oxidative stress caused by H_2O_2 after an antioxidant treatment. The study involved eight HT women and three healthy women, whose PBMC were treated with the addition of H_2O_2 (200 μM) alone, then with H_2O_2 plus Myo-Ins (0.25, 0.5, or 1.0 μM), or Se (0.25, 0.5, or 1.0 μM), or their combination (0.25+0.25, 0.5+0.5, 1.0+1.0 μM) [70]. Treatment with H_2O_2 alone leads to a decrease of PBMC proliferation that is furtherly decreased in a dose-dependent manner in each group (especially in that with Myo-Ins+Se in HT). It has been observed a decrease of the PBMC vitality of 5% in the controls group and of 10% in the HT group by H_2O_2; while a rescue of the vitality has been obtained in both groups after the addition of Myo-Ins, Se, or Myo-Ins+Se. The Comet score rised to +505% above baseline in controls, and +707% in HT women after the addition of H_2O_2 alone. In both group, each addition contrasted genotoxicity in a dose-dependent manner. H_2O_2 alone increased chemokines concentration especially in the group of HT woman in comparison to that of the controls. Chemokines levels decreased dose-dependently in both groups after each addition, especially after Myo-Ins+Se treatment, reaching -80% of baseline. Therefore, it was concluded that the Myo-Ins and Se have positive effects on PBMC in presence of oxidative stress caused by H_2O_2 *in vitro*, in controls as in HT women; and that the Myo-Ins+Se combination is the most effective [70]. In ThyC Myo-Ins was not able to rescue the damage induced by H_2O_2, that was observed in lymphocytes in the previous study

[70]. However, it is of note that since the Myo-Ins, and or Se, or the combination of both, are not able to change the proliferation, or the apoptosis induced by H_2O_2, the change in the secretion of chemokines after cytokines stimulation, cannot be due to an interference on cell vitality, but to a modulation induced by Myo-Ins in ThyC **responsiveness** to cytokines.

As the data obtained with Myo-Ins, and or Se, in HT-ThyC are similar to those observed in c-ThyC, this is in agreement with the results of other studies showing a similar behavior of chemokine production under cytokines stimulation in ThyC obtained from normal, or from GD, thyroid [51].

In conclusion, we have first shown that Myo-Ins reduces the secretion of CXCL10 chemokine induced by IFN-γ+TNF-α, in presence or absence of H_2O_2, in primary thyrocytes. These data suggest a protective effect of Myo-Ins on thyroid cells; other studies will be needed to evaluate the exact mechanisms.

Compliance with Ethical Standards

Funding: The authors have nothing to declare.

Research involving Human Participants: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest: The authors declare that they have no conflict of interest.

Figure legends

Figure 1. CXCL10 secretion induced by IFN-γ+TNF-α was significantly reduced by H_2O_2 (200 μM) in HT-ThyC. * = P < 0.05 by ANOVA. Bars are mean ± SEM.

Figure 2. Se (0.1, 0.25 and 1.0 μM) had no effect (P > 0.05 by ANOVA) on CXCL10 secretion induced by IFN-γ+TNF-α, in presence (**Figure 2A**) or absence (**Figure 2B**) of H_2O_2 (200 μM) in HT-ThyC. Bars are mean ± SEM.

Figure 3. Myo-Ins (0.1, 0.25 and 1.0 μM) reduced dose dependently and significantly CXCL10 secretion induced by IFN-γ+TNF-α, in presence (**Figure 3A**) or absence (**Figure 3B**) of H_2O_2 (200 μM) in HT-ThyC. * = P < 0.05 by ANOVA. Bars are mean ± SEM.

Figure 4. The combination of Myo-Ins (1.0 μM) plus Se (1.0 μM) reduced significantly CXCL10 secretion induced by IFN-γ+TNF-α, in presence (**Figure 4A**) or absence (**Figure 4B**) of H_2O_2 (200 μM). * = P < 0.05 by ANOVA. Bars are mean ± SEM.

Figure 5. Proliferation (**Figure 5A**) was slightly reduced by H_2O_2 (50 μM, 100 μM, or 200 μM), while apoptosis (**Figure 5B**) increased, in HT-ThyC. * = P < 0.05 by ANOVA. Bars are mean ± SEM.

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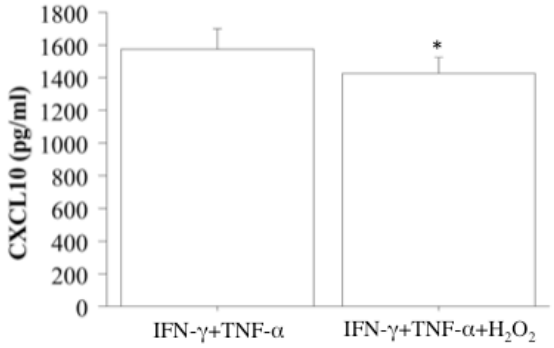
1800
1600
1400
1200
1000
800
600
400
200
0
CXCL10 (pg/ml)
*
IFN-γ+TNF-α
IFN-γ+TNF-α+H2O2

Fig. 1

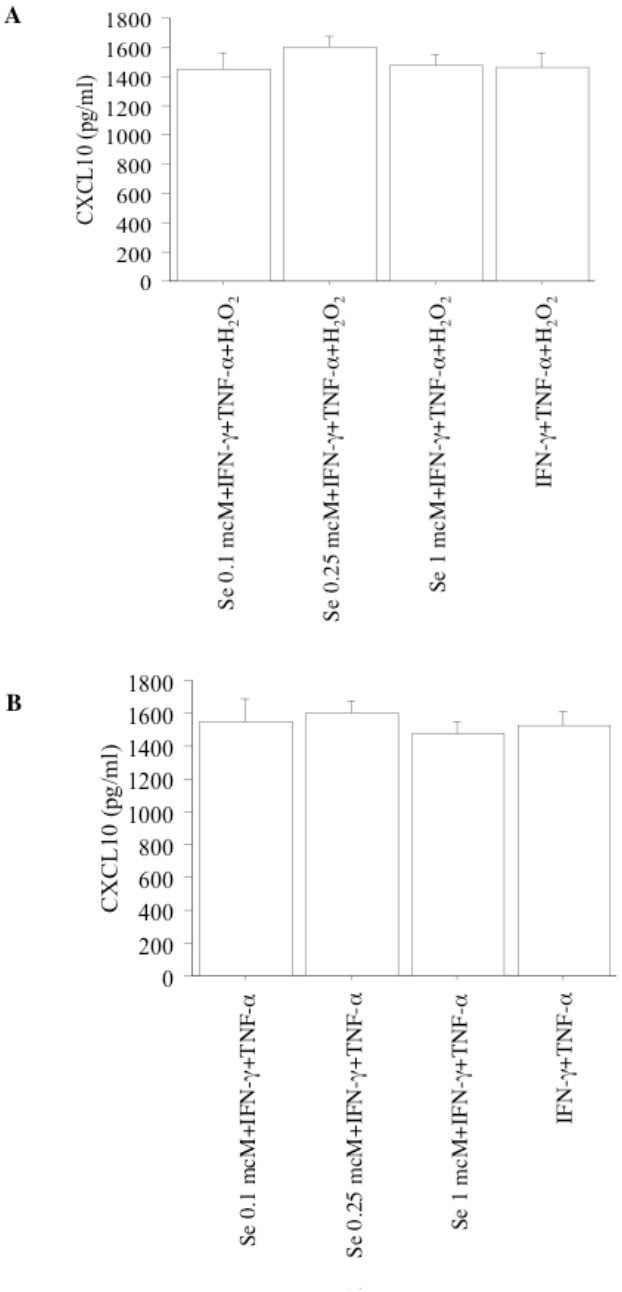
A
CXCL10 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
Se 0.1 mcM+IFN-γ+TNF-α+H2O2
Se 0.25 mcM+IFN-γ+TNF-α+H2O2
Se 1 mcM+IFN-γ+TNF-α+H2O2
IFN-γ+TNF-α+H2O2
B
CXCL10 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
Se 0.1 mcM+IFN-γ+TNF-α
Se 0.25 mcM+IFN-γ+TNF-α
Se 1 mcM+IFN-γ+TNF-α
IFN-γ+TNF-α

Fig. 2

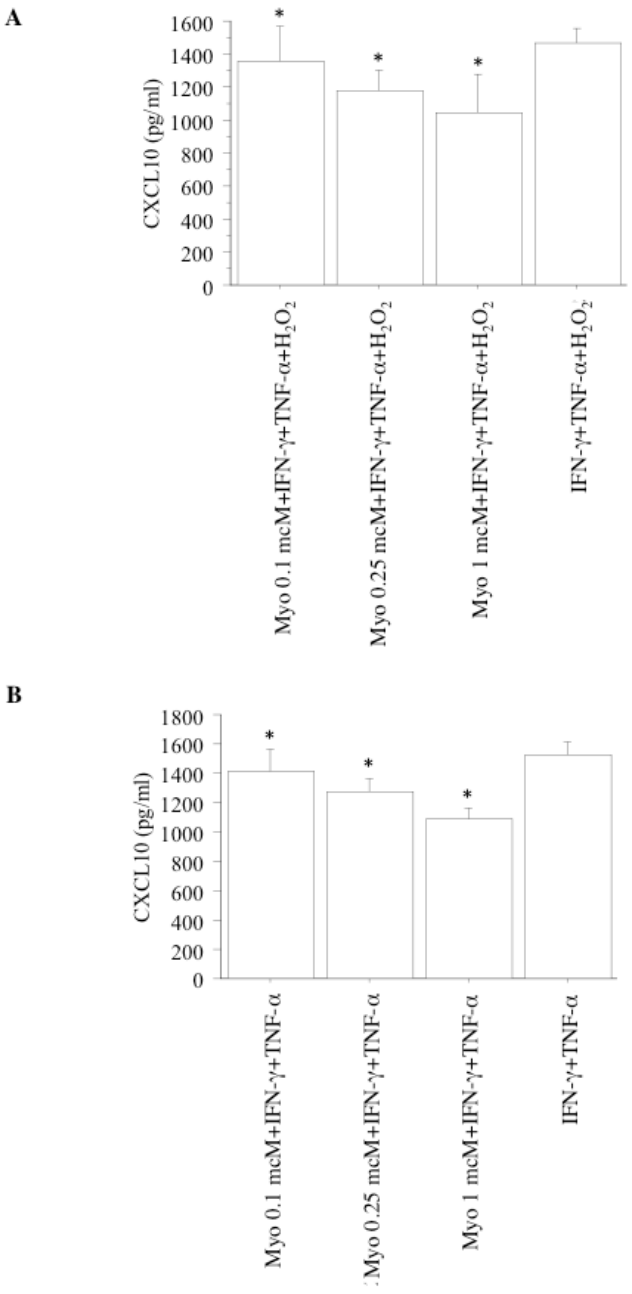
A
CXCL10 (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
*
*
*
Myo 0.1 mcM+IFN-γ+TNF-α+H2O2
Myo 0.25 mcM+IFN-γ+TNF-α+H2O2
Myo 1 mcM+IFN-γ+TNF-α+H2O2
IFN-γ+TNF-α+H2O2
B
CXCL10 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
Myo 0.1 mcM+IFN-γ+TNF-α
Myo 0.25 mcM+IFN-γ+TNF-α
Myo 1 mcM+IFN-γ+TNF-α
IFN-γ+TNF-α

Fig. 3

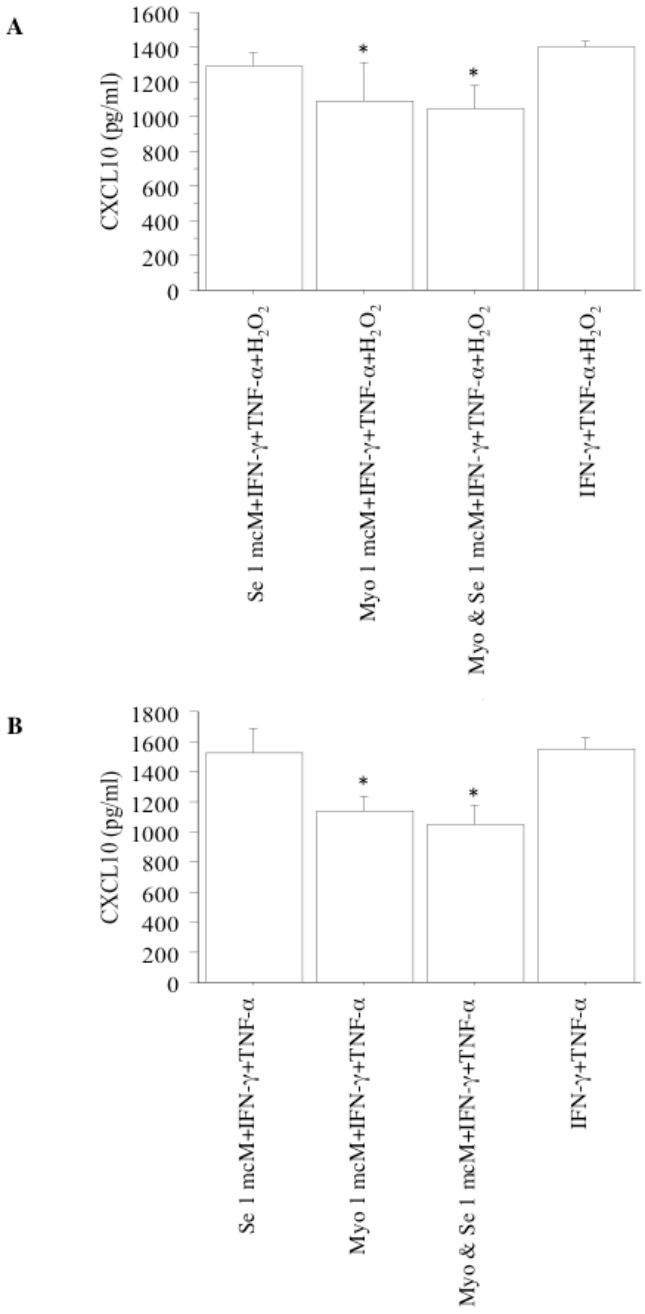
A
CXCL10 (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
*
*
Se 1 mcM+IFN-γ+TNF-α+H2O2
Myo 1 mcM+IFN-γ+TNF-α+H2O2
Myo & Se 1 mcM+IFN-γ+TNF-α+H2O2
IFN-γ+TNF-α+H2O2
B
CXCL10 (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
Se 1 mcM+IFN-γ+TNF-α
Myo 1 mcM+IFN-γ+TNF-α
Myo & Se 1 mcM+IFN-γ+TNF-α
IFN-γ+TNF-α

Fig. 4

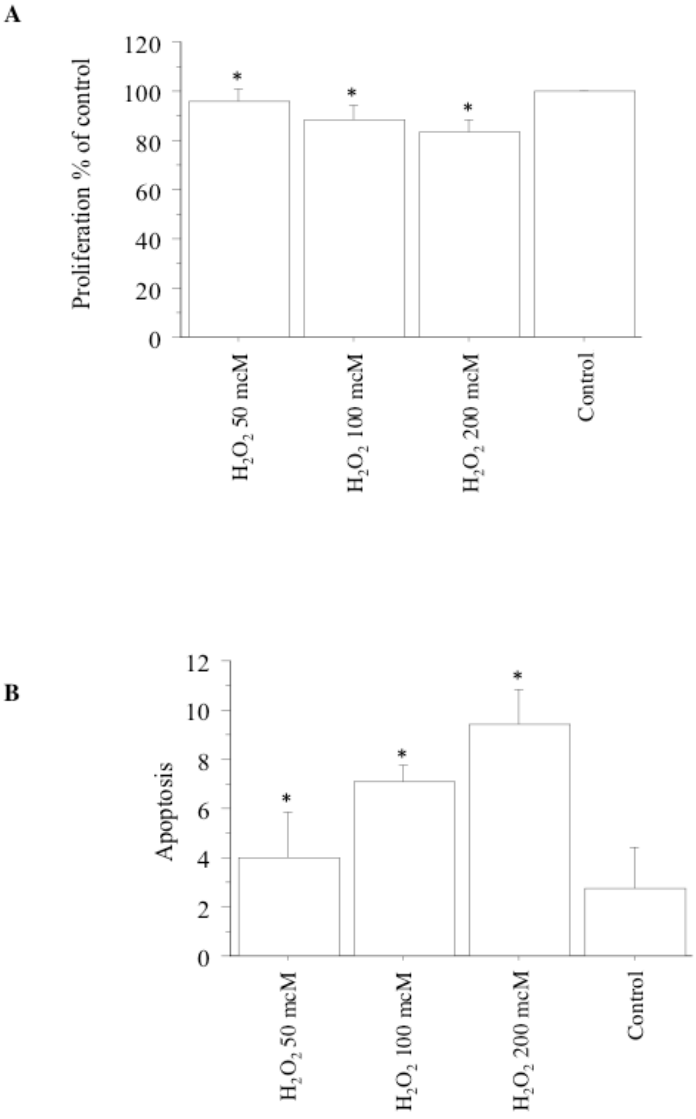
A
Proliferation % of control
120
100
80
60
40
20
0
*
*
*
H_2O_2 50 mcM
H_2O_2 100 mcM
H_2O_2 200 mcM
Control
B
Apoptosis
12
10
8
6
4
2
0
*
*
*
H_2O_2 50 mcM
H_2O_2 100 mcM
H_2O_2 200 mcM
Control

Fig. 5