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ORIGINAL ARTICLE

Comparative effect of selenium and glycine on hydrogen peroxide-induced cell death and activation of macrophage U937 cells

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Abstract The effects of selenium and glycine (either separately or in combination) on hydrogen peroxide-induced cell death on U937 cells and activation of U937-derived macrophages were investigated. In the first instance, U937 cells were incubated with or without selenium (Se) or glycine (GLY) or both (Se + GLY) for 24 h before exposure to hydrogen peroxide. Control cells were not incubated with Se, GLY or exposed to hydrogen peroxide. Cell viability was later assessed via trypan blue and MTT assays. For the other experiment, U937 cells were transformed to the macrophage form using phorbol 12-myristate 13-acetate before incubating with or without Se, GLY, Se + GLY. Contents were subsequently exposed to hydrogen peroxide and 24 h later assessed for the production of TNF-α, IL-1, IL-6 and the expression of iNOS and NF-κB. The results revealed that hydrogen peroxide caused significant cell death which was ameliorated by both Se and GLY. Pre-incubation of the cells with both Se and GLY did not significantly enhance cell numbers compared to GLY ($p > 0.05$). On the other hand, Se and GLY reduced hydrogen peroxide-mediated production of TNF-α, IL-1, IL-6 and expression of iNOS and NF-κB. Incubating the U937-derived macrophages with Se + GLY significantly ameliorated hydrogen peroxide-mediated activation of macrophages when compared to pre-treatments with Se or GLY ($p < 0.05$). The findings demonstrate that both Se and GLY reduced hydrogen peroxide-induced alterations in U937 cells and U937-derived macrophages. Implications of the findings are discussed.

1. Introduction

Reactive oxygen species (ROS) are constantly produced in the body as a consequence of cellular metabolic activities though their deleterious activities are kept in check by the endogenous antioxidant defenses which could be enzymatic and nonenzymatic. However, there could be serious pathological conse-

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quences should an imbalance occur between the production of ROS and antioxidation. Hydrogen peroxide is a non-radical ROS produced spontaneously during respiratory burst, oxidative phosphorylation in the mitochondria and peroxisomes under normal physiological conditions [9]. Though considered less reactive compared to other ROS, hydrogen peroxide diffuses throughout the mitochondrion and across the cell membrane oxidizing various targets such as thiol groups and DNA disrupting cellular pathways and subsequently producing many types of cellular/tissue injury which are implicated in cancer, diabetes, neurodegradative, cardiovascular and thyroid disorders [25,29,31,35].

Selenium is an essential trace element found in cereals, dietary supplements and multivitamins [28]. Other sources of the trace element are crabs and shell fishes [20]. Selenium in a nonenzymatic antioxidant whose activity has been attributed to the antioxidation of selenoproteins which are thought to protect the mitochondrion, plasma membrane and DNA from oxidative damage [4,34]. In the current study, the effect of selenium and glycine (either separately or in combination) on hydrogen peroxide-induced alterations of the human monocyte cell line U937 and macrophages was studied. Though glycine is considered a nonessential amino acid, available evidence suggests that the amount synthesized in the body is insufficient to meet metabolic needs [36]. This simplest amino acid has been shown to be cytoprotective in various experimental models [15,32,40].

2. Materials and method

2.1. Chemicals and reagents

Glycine, selenium (as sodium selenite), *L*-glutamine, RPMI-1640, hydrogen peroxide, 3-(4,5-dimetylthiazol-2-yl),-2,5-diph enyl-tetrazolium bromide (MTT) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (USA). Penicillin, streptomycin and all antibodies were purchased from Invitrogen. The cell line U937 was a product of European Collection of Cell Cultures (Ssalisbury). All other chemicals were of analytical grade and commercially available.

2.2. Cell culture

The cell line U937 was grown in RPMI-1640 media supplemented with *L*-glutamine (2 mM), 10% fetal calf serum (heat inactivated), penicillin (100 U/mL) and streptomycin (100 mg/mL). Cell numbers were maintained at 5×10^4 cells/mL and grown at 37 °C in an atmosphere at 5% CO_2.

2.3. Cytotoxicity studies

Cells were incubated with glycine at different concentrations (100 μM, 300 μM, 600 μM) or selenium (0.5 μM, 1 μM, 3 μM) in order to assess for any potential toxicity. To evaluate the effect of glycine and selenium on hydrogen peroxide-induced alterations, cell cultures were supplemented with either glycine or selenium or both (Se + GLY) for 24 h before exposure to 100 μM hydrogen peroxide (HPO). For control experiments, neither glycine (GLY) nor selenium (Se) was added rather an equivalent amount of RPMI-1640 was delivered to the cells. Thus the groups were normal controls, HPO only, Se + HPO, GLY + HPO, Se + GLY + HPO. Cell viability was later determined via the trypan blue dye exclusion and MTT assays 1 h after adding hydrogen peroxide. The method of Zhou et al. [41] was used for the determination of cell death via MTT reduction. Briefly, 10 μL of MTT solution was added to each culture to a final concentration of 0.5 mg/mL and incubated for 1 h at 37 °C. The MTT was removed and each culture supplemented with DMSO (Dimethyl sulfoxide). Absorbance was measured at 570 nm using a microplate reader.

2.4. Assessment of macrophage activation

Cultured U937 cells were transformed to the macrophage form by treatment with PMA for 24 h [26]. Supernatants were removed and solutions of glycine and selenium were mixed with the cells and incubated for 24 h before exposure to hydrogen peroxide (as in Section 2.3). The supernatants of each culture were analyzed for the production of TNF-α, IL-1 ad IL-6 via cytokine capture ELISA as described by Okoko and Oruambo [26].

2.5. Expression of iNOS and NF-κB

Total RNA was extracted from cells (after removing supernatants as in Section 2.4) using TRIzol reagent (Invitrogen) and quantified by measuring absorbance at 260 nm. The cDNA was synthesized using a Revert Aid cDNA synthesis kit according to the manufacturer's protocol. For RT-PCR, 1 μg of the resulting cDNA was used to amplify regions specific to iNOS and NF-κB in an ABI Prism 7500 system (Applied Biosciences) with primer pairs listed in Table 1. PCR products were run on 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. Real-Time PCR data were analyzed and presented as fold change in expression to the GAPDH housekeeping gene of same sample.

2.6. Statistical analysis

Values are expressed as mean ± SEM (n = 6), data were analyzed using either a students' *t*-test or analysis of variance followed by Duncan's multiple range test. Significance was set at $p < 0.05$.

3. Results

3.1. Effect of selenium and glycine on cell proliferation

The cells were treated first with different concentrations of selenium (0.5 μM, 1 μM, 3 μM) and glycine (100 μM, 300 μM, 600 μM) for 24 h to investigate their effect on cell

Table 1 Primer sequences for RT-PCR.

mRNA	Primer sequence (5′-3′)
iNOS	FP: GTGCCACCTCCAGTCCAG
	RP:GCTGCCCCAGTTTTTGATCC
NF-κB	FP:GCCTTGCATCTAGCCACAGAG
	RP:GATGTCAGCACCAGCCTTCAG
GAPDH	FP:GTCGGAGTCAACGGATTTGGTC
	RP:CTTCCCGTTCTCAGCCTTGAC

viability. We decided to use concentrations that reflect actual levels of Se and Gly in blood however, reference values depend on sex, age and instruments. According to Laposata [16], the reference value of selenium in whole blood is 0.74–2.97 μM while for glycine, it is 120–560 μM in plasma. There was no effect on cell viability following exposure to either selenium or glycine thus they were classified as nontoxic at those concentrations (data not shown). Fig. 1 shows the effect of selenium and glycine on hydrogen peroxide-induced alterations in cell viability. Treating the cells with hydrogen peroxide (100 μM) resulted in significant cell death as assessed by the trypan blue dye-exclusion and MTT reduction assays (i.e. 19.54 ± 3.34% and 25.41 ± 2.26% viability via trypan blue and MTT reduction assays respectively). Both selenium and glycine significantly ($p < 0.05$) reduced hydrogen peroxide-induced cell death which was concentration-dependent for selenium. However, there was no significant difference in viability when the cells were pretreated with 300 μM and 600 μM glycine (Fig. 1B). Thus for the other experiments, selenium was used at 3 μM (final concentration) while glycine 300 μM (final concentration). Cell viability in the Se + GLY group was not significantly higher ($p > 0.05$) than the GLY group (Fig. 2).

3.2. Effect on cytokine secretion

The ability of selenium (3 μM) and glycine (300 μM) to inhibit hydrogen peroxide-induced production of pro-inflammatory cytokines in U937-derived macrophages was assessed by cytokine capture ELISA (Fig. 3). Both selenium (i.e. Se group) and glycine (i.e. GLY group) inhibited hydrogen peroxide-induced production of TNF-α, IL-1 and IL-6 but the pre-treatment of selenium and glycine (i.e. Se + GLY) was better at reducing cytokine production than their separate responses ($p < 0.05$).

3.3. Effect on gene expression levels

The effect of selenium and glycine on hydrogen peroxide-induced expression of iNOS and NF-κB genes was determined by the amplification of total RNA via RT-PCR. The level of mRNA when the cells were treated with hydrogen peroxide (i.e. HPO group) was significantly higher ($p < 0.05$) than control cells (Fig. 4). However, selenium and glycine reduced mRNA levels closer to control values. The combined pre-treatment of selenium and glycine (Se + GLY) resulted in further reduction in expression than their separate effects.

4. Discussion

The production of ROS is indispensable for life since they are used by phagocytic cells to fight infectious agents. However, excessive upregulation of phagocytic cells such as neutrophils, macrophages and monocytes could result in tissue damage because of excessive production of ROS [14]. Though the body has efficient mechanisms to scavenge these ROS, serious pathological imbalances could be unavoidable should there be an imbalance in favor of the oxidants. Diseases associated with such imbalance include malaria, shock, diabetes, and cancer [14,39]. In addition to phagocytosis, molecular oxygen is used by aerobes for energy production, and ROS (as by-products of oxygen metabolism) such as hydrogen peroxide, and hydroxyl radical are produced [7]. Hydrogen peroxide is

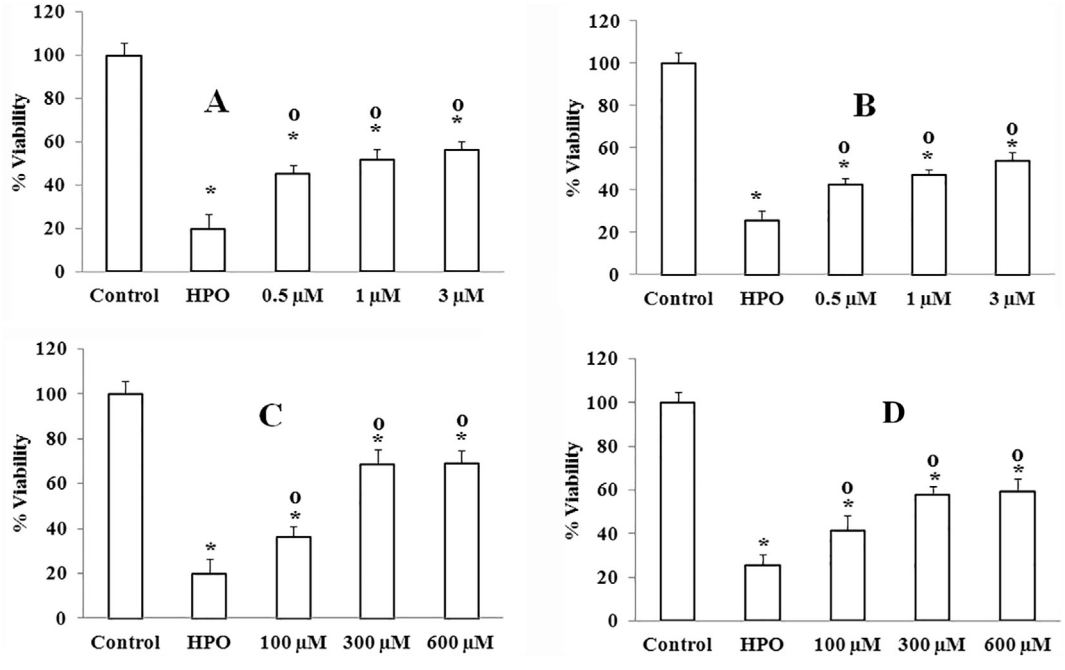

Figure 1 Effect of selenium and glycine on hydrogen peroxide-induced cell death. Effect of (A) selenium on cell viability assessed by trypan blue assay (B) selenium on cell viability assessed by MTT reduction assay (C) glycine on cell viability assessed by trypan blue assay (D) glycine on cell viability assessed by MTT reduction assay. HPO, cells treated with hydrogen peroxide only. Each bar represents mean ± SEM of six replicates.* Significantly different from control cells and ° Significantly different from cells treated with H_2O_2 only ($p < 0.05$).

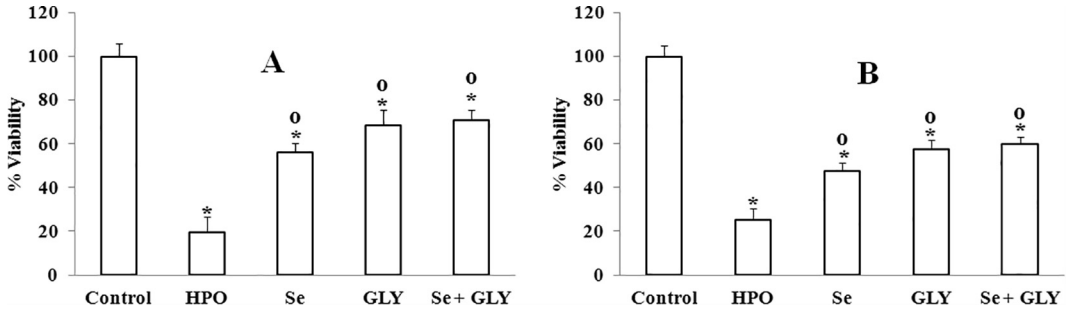

Figure 2 Effect of selenium and glycine (separately or in combination) on cell death. Viability was assessed via (A) trypan blue and (B) MTT assays. HPO, cells treated with 100 μM hydrogen peroxide only; Se, cells treated with hydrogen peroxide before incubation with selenium (3 μM); GLY, cells treated with hydrogen peroxide before incubation with glycine (300 μM); Se + GLY, cells were treated with hydrogen peroxide before incubation with both selenium and glycine (3 μM and 300 μM respectively). Each bar represents mean ± SEM of six replicates.* Significantly different from control cells and ° Significantly different from cells treated with H_2O_2 only ($p < 0.05$).

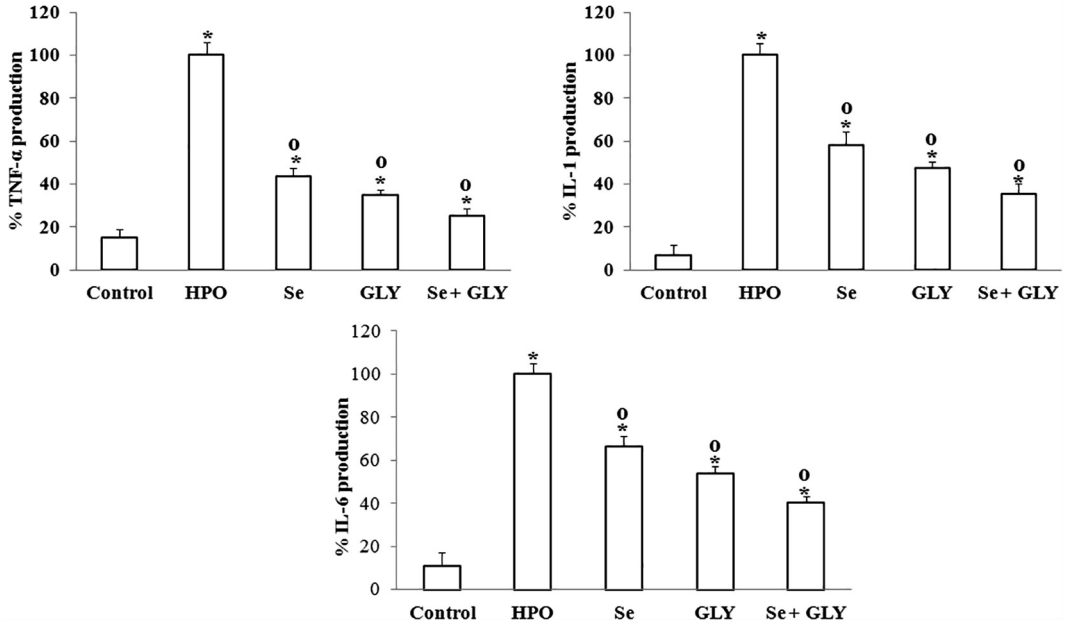

Figure 3 Secretion of TNF-α, IL-1 and IL-6 in U937-derived macrophages. HPO, transformed cells treated with 100 μM hydrogen peroxide only; Se, transformed cells treated with hydrogen peroxide before incubation with selenium (3 μM); GLY, transformed cells treated with hydrogen peroxide before incubation with glycine (300 μM); Se + GLY, transformed cells were treated with hydrogen peroxide before incubation with both selenium and glycine (3 μM and 300 μM respectively). Each bar represents mean ± SEM of six replicates.* Significantly different from control cells and ° Significantly different from cells treated with H_2O_2 only ($p < 0.05$).

produced in peroxisomes in reactions catalyzed by xanthine oxidase, NAD(P)H oxidase and diffuses across the plasma membrane and attack proteins, lipids, carbohydrates, DNA which can result in cell death [5,39]. The mechanism of hydrogen peroxide-induced cell death might not be unconnected with the imbalance of intracellular calcium and the decrease in the mitochondrial membrane potential thus could impair the selective permeability of the membrane [18,19,39].

From the experiment, treatment of the cells with hydrogen peroxide alone caused significant cell death as evidenced from the trypan blue and MTT reduction assays (Figs. 1 and 2). But both glycine and selenium reduced cell death however glycine gave a better response at the concentrations used. Glycine has been shown to be cytoprotective in various experiments. Ruiz-Maena et al. [30] reported that glycine protects post-ischemic reperfusion cell injury by inhibiting mitochondrial permeability transition, however Nishimura and Lemasters [24] reported that the amino acid protects chemical hypoxia-induced cell death in endothelial cells by blocking a death channel. This may suggest that glycine could interfere with the diffusion of hydrogen peroxide across membranes which might probably be mediated by its unique transporter [15]. It has also been suggested that amino acids such as glycine could serve as cofactors with Mn^{2+} to metabolize hydrogen peroxide [37]. Though selenium has been reported to be cytoprotective [3], the mechanism is still unclear though it may not be unconnected with antioxidation of selenoproteins [4].

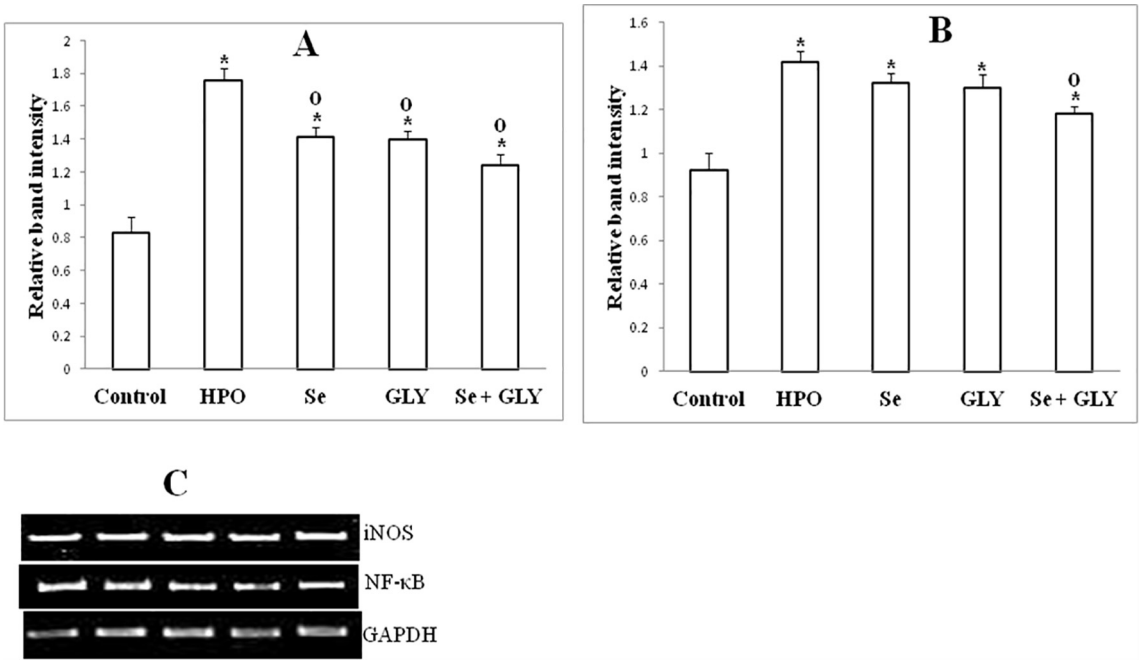

Figure 4 Expression of iNOS and NF-κB in U937-derived macrophages. (a) Ratio of intensities of (A) iNOS and (B) NF-κB over housekeeping gene GAPDH for RT-PCR of mRNA isolated from U937-derived macrophages. (C) Agarose gel analysis of RT-PCR of mRNAs. Se, transformed cells treated with 100 μM hydrogen peroxide before incubation with selenium (3 μM); GLY, transformed cells treated with hydrogen peroxide before incubation with glycine (300 μM); Se + GLY, transformed cells were treated with hydrogen peroxide before incubation with both selenium and glycine (3 μM and 300 μM respectively). Each bar represents mean ± SEM of six replicates.*Significantly different from control cells and º Significantly different from cells treated with H_2O_2 only ($p < 0.05$).

Excessive release of pro-inflammatory cytokines by macrophages has been implicated in a number of pathological conditions such as organ failure, septic shock, rheumatoid arthritis, asthma, cancer, chronic obstructive pulmonary diseases, viral infections, autoimmune diseases, hypotension and other systemic responses which could be traceable to the production of ROS [10,17,21,22].

It has been demonstrated that hydrogen peroxide stimulates macrophages to release cytokines and other vasoactive amines (11,18). This supports the production of TNF-α, IL-1, IL-6 and the expression of inducible nitric oxide synthase (iNOS) and nuclear factor kappa B (NF-κB) mRNA by stimulated U937 treated with hydrogen peroxide (Figs. 3 and 4).

Nuclear factor kappa B (NF-κB) plays a central role in inflammation which is stimulated by pro-inflammatory cytokines, bacterial toxins and some compounds though it may also depend on the cell type [6,31,33]. The ability of hydrogen peroxide to upregulate the transcription of iNOS and NF-κB is in correlation with other studies [8,11,27]. The expression of iNOS is dependent on the translocation of NF-κB into the nucleus. In other words, the activation of NF-κB is central to the expression of iNOS. A number of radicals are produced by hydrogen peroxide-stimulated cells such as nitric oxide. Nitric oxide is a signal molecule synthesized from *L*-arginine in a reaction catalyzed by iNOS thus improper upregulation of the transcription factor results in excessive production of nitric oxide which could alter cellular redox balance. This has been implicated in a number of inflammatory conditions such as neurodegradation, and cancer [13,23].

Glycine has been reported to reduce oxidative stress in various studies [1,2,12]. In this current work, glycine reduced hydrogen peroxide-mediated induction of the pro-inflammatory cytokines and the expression of NF-κB and iNOS in U937-derived macrophages. This further reveals the anti-inflammatory ability of glycine. Previous findings in our laboratory reveal selenium significantly reduced hydrogen peroxide-induced monocyte cell death and activation of U937-derived macrophages (in press) so it was thought that the combined action of selenium and glycine could produce a very significant response than their individual responses. Even though their combined action of the antioxidants did not produce a significantly higher response on cell viability, the combined pre-treatment of the transformed cells (i.e. U937-derived macrophages) with the antioxidants produced a response higher than their individual responses though it was neither additive nor synergistic. It has been revealed that the glycine transporter is essential for cellular protection against oxidative damage [15]. Glycine activates a ligand-gated chloride channel causing an influx of chloride ions which hyperpolarizes the plasma membrane. This subsequently blocks cytokine production [38]. This study further corroborates the immense antioxidant and immunomodulatory potential of glycine and perhaps selenium which can be exploited pharmacologically.

5. Conclusion

The findings reveal both selenium and glycine reduced hydrogen peroxide-induced cell death and the activation of U937-derived macrophages. In all experiments glycine produced a better response than selenium at the concentrations used. Elevation of glycine in blood has a remarkable improvement in cancer, and shock [38] and this could justify the

immense anti-inflammatory potential of glycine at reducing the hydrogen peroxide-mediated activation of the cells which was further enhanced by selenium. Liu et al. [18] reported that the inhibition of NF-κB is a major mechanism antioxidants protect against hydrogen peroxide-induced cell death. In addition to this, we suggest that the inhibition of the production of the pro-inflammatory cytokines protects against cell death.

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