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# Protection of U937 Cells from Free Radical Damage by the Macrophage Synthesized Antioxidant 7,8-dihydroneopterin

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Interferon-$\gamma$ stimulation of human macrophages causes the synthesis and release of neopterin and its reduced form 7,8-dihydroneopterin (7,8-NP). The purpose of this cellular response is undetermined but *in vitro* experiments suggests 7,8-NP is an antioxidant. We have found 7,8-NP can protect monocyte-like U937 cells from oxidative damage. 7,8-NP inhibited ferrous ion and hypochlorite mediated loss of cell viability. $Fe^{++}$ mediated lipid peroxidation was effectively inhibited by 7,8-NP, however no correlation was found between peroxide concentration and cell viability. Hypochlorite was scavenged by 7,8-NP, preventing the loss of cell viability. 7,8-NP was less effective in inhibiting $H_2O_2$-mediated loss of cell viability with significant inhibition only occurring at high 7,8-NP concentrations. Analysis of cellular protein hydrolysates showed none of the oxidants caused the formation of any protein bound DOPA or dityrosine but did show 7,8-NP prevented the loss of cellular tyrosine by HOCl. Our data suggests macrophages may synthesize 7,8-NP for antioxidant protection during inflammatory events *in vivo*.

*Keywords*: Neopterin, macrophage, antioxidant, free-radical, peroxidation, hypochlorite

*Abbreviations*: 7,8-NP, 7,8-dihydroneopterin; AAPH, 2,2'-azobis(amidinopropane) dihydrochloride; HPLC, high performance liquid chromatography; IFN-$\gamma$, interferon-$\gamma$; TBARS, thiobarbitaric acid reactive substance

## INTRODUCTION

Interferon-$\gamma$ (IFN-$\gamma$) acts to prime macrophage cells as part of the immune response. IFN-$\gamma$ binding promotes the expression of a number of genes within macrophages including cyclohydrolase 1, which causes the breakdown of guanosine triphosphate to 7,8-dihydroneopterin-triphosphate.[1] In non-primate macrophages the 7,8-dihydroneopterin-triphosphate is converted to 5,6,7,8-tetrahydrobiopterin, the cofactor for inducible nitric oxide synthase. Primate, and therefore human monocytes and macrophages, lack the enzymes required for the production of

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5,6,7,8-tetrahydrobiopterin causing the intracellular concentration of 7,8-dihydroneopterin-triphosphate to increase. The action of non-specific phosphorylase converts the triphosphate to 7,8-NP which can leak from the cells into circulation.

The purpose of IFN-$\gamma$ stimulated 7,8-NP production by macrophages is unknown. The fact that it is produced as part of macrophage priming by IFN-$\gamma$ suggests a role in the cytotoxic or phagocytic mechanisms during the immune response. There has been some suggestion that neopterin, the oxidation product of 7,8-dihydroneopterin acts to enhance the cytotoxicity of reactive oxygen species.[2,3]

We have previously shown 7,8-NP is a potent antioxidant, inhibiting the oxidation of linoleic acid, low density lipoproteins,[4] lysis of red blood cells,[5] and the oxidation of tyrosine residues to dityrosine.[6] Others have shown 7,8-NP to inhibit $H_2O_2$-induced luminol chemiluminescence,[7] neutralise chloride species such as chloramine-T,[2,8] inhibit linoleic acid oxidation[4] scavenge peroxyl radicals,[9] and superoxide anions.[10,11] Together, these findings suggest 7,8-NP may function as an extracellular antioxidant, protecting macrophages from their own and neutrophil derived reactive oxygen species.

To explore the possibility that 7,8-NP is a cellular antioxidant we have exposed the human derived monocyte-like cell line, U937, to three different reactive oxygen species and quantified the degree of protection extracellular 7,8-NP gives to the cells. U937 cells do not produce 7,8-NP, even when stimulated with IFN-$\gamma$ and therefore make an ideal model system with which to examine the antioxidant activity of 7,8-NP on macrophage like cells. We have limited the time of cellular exposure to the ROS before analysis to restrict our investigation to the initial cellular damage. Incubating the cells for longer periods of time would allow activation of cellular repair mechanisms or apoptosis.

## MATERIALS AND METHODS

All reagents used were of AR grade or better and obtained from the Sigma Chemical Company (USA) or BDH Chemicals New Zealand Limited. Tissue culture media and plasticware were supplied from Gibco (USA) through Life Technologies (NZ). MTT reagent (Thiazolyl blue) was supplied by Sigma. Dityrosine was prepared by oxidation of tyrosine with horseradish peroxidase and purification on DEAE-sephacel (Sigma).[12] All solutions were prepared using ion-exchanged ultrafiltered water prepared in a NANOpure ultrapure water system from Barnstead/Thermolyne (Iowa, USA). HOCl was purchased from Cloro-o-ogene (Petone, New Zealand). The concentration was determined using the extinction coefficient of $350\,M^{-1}cm^{-1}$ at 292 nm at pH 12.[13]

## TISSUE CULTURE AND CELL VIABILITY

U937 cells were cultured in RPMI 1640 media with 5% heat inactivated foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were prepared for experiments by washing twice in phosphate buffered saline (PBS) to remove the culture media and suspended in RPMI 1640 media without phenol red indicator or Earle's Balanced Salt Solution (EBSS). The cells were incubated in a humidified incubator containing 5% $CO_2$ at 37°.

Following exposure to an oxidant, cell viability was assessed by either trypan blue dye exclusion,[14] lactate dehydrogenase (LDH) leakage into the media[14] or MTT reduction. Cell viability measurements by MTT reduction were carried using the method of Mosmann[15] with modifications. Briefly, 1 ml of cells at $5 \times 10^6$ cells/ml was removed from each well under aseptic conditions. The cells were washed twice in warm PBS and suspended in 1 ml of RPMI 1640 with no phenol red in 12 well plates. One hundred μl of MTT reagent (5 mg/ml thiazolyl

blue (MTT) in RPMI 1640) was added to each well and incubated for three hours. One ml of 10% SDS was added to each well and mixed with a pipette tip to dissolve the formazan crystals formed by the cell mediated MTT reduction. The resulting cell extract was measured spectrophotometrically at 570 nm.

Hydrogen peroxide concentration was determined by the ferric-xylenol orange hydroperoxide assay (FOX 2 assay).[16]

The protein concentration of the U937 cells was determined using the BCA method (Kit supplied by Pierce, USA) with BSA as a standard.

## HPLC ANALYSIS OF LIPID AND PROTEIN OXIDATION

Cellular lipid peroxidation was determined as TBARS. Cells were removed from the plates with a cell scraper. Butylated hydroxytoluene (BHT) in methanol was added to the cell suspension to prevent further lipid oxidation[17] and TCA to precipitate cellular protein. The samples were immediately sonicated before centrifugation at 23,100 × g. The cellular supernatants were reacted with TBA and analysed for TBARS by reverse phase HPLC using fluorescence detection of the TBA conjugate.[18] The HPLC was a fully automated Shimadzu Sil-10A sytem equiped with a cooled autosampler and RF-10Axl spectrofluorometer connected to a PC for peak integration using Shimadzu Class LC10 software.

During our initial studies we found the addition of foetal calf serum or bovine serum albumin to the culture media generated additional TBARS when the U937 cells were incubated with $FeSO_4$. Cell viability was not affected by the exclusion of a protein source in the media. We subsequently conducted all cellular experiments in media without protein supplementation.

Oxidation of tyrosine residues was measured by acid hydrolysis of the cellular proteins followed by HPLC analysis of the hydrolysate. U937 cells were washed with PBS and lysed by suspension in water with sonication. Two hundred μL of cell lysate was tranferred to an autosamplier vial and dried under vacuum in a speed vac (Savant, USA). The vials were placed into a Pico-Tag hydrolysis reaction vessel (Millipore, USA) and hydrolysed for 24 hours at 110 °C with 6 M HCl containing 1% w/v phenol as previously described.[19,20] The hydrolysates were dried under vacuum and dissolved in 200 μl of 0.1% trifluoroacetic acid (TFA), before transferring to autosampler vials. Twenty μL of the sample was injected onto an Econosphere RP C-18 250 × 4.6 mm, 5 μm column (Alltech Associates Inc., USA) using the same HPLC system described for the TBAR analysis. The column was developed with a gradient starting at 5% methanol in 0.1% TFA pH 2.5 pumped at 1 ml/min, increasing to 15% methanol over 16 minutes. The tyrosine and oxidation products were detected by fluorescence emission using a Shimadzu RF-10Axl fluorescence detector set at an excitation wavelength of 280 nm and emission detection wavelength of 320 nm. After the elution of the tyrosine peak the emission wavelength changed to 410 nm to detect dityrosine.

## RESULTS AND DISCUSSION

### Iron Mediated Oxidation

Iron salts in solution catalyse the peroxidation of lipids via a fenton mechanism. Exposure of U937 cells to increasing concentrations of iron salt caused an increase in cellular lipid peroxidation, measured as TBARS by HPLC as previously described by Fuhrman *et al.*[21] and a decrease in cell viability. After examination of this data we selected an iron concentration of 50 μM to examine the protective effect of 7,8-NP. Addition of between 20 to 200 μM 7,8-NP to iron containing media caused a highly significant increase in cell viability (Figure 1). The MTT assay was the most sensitive of the assays showing a 20% drop

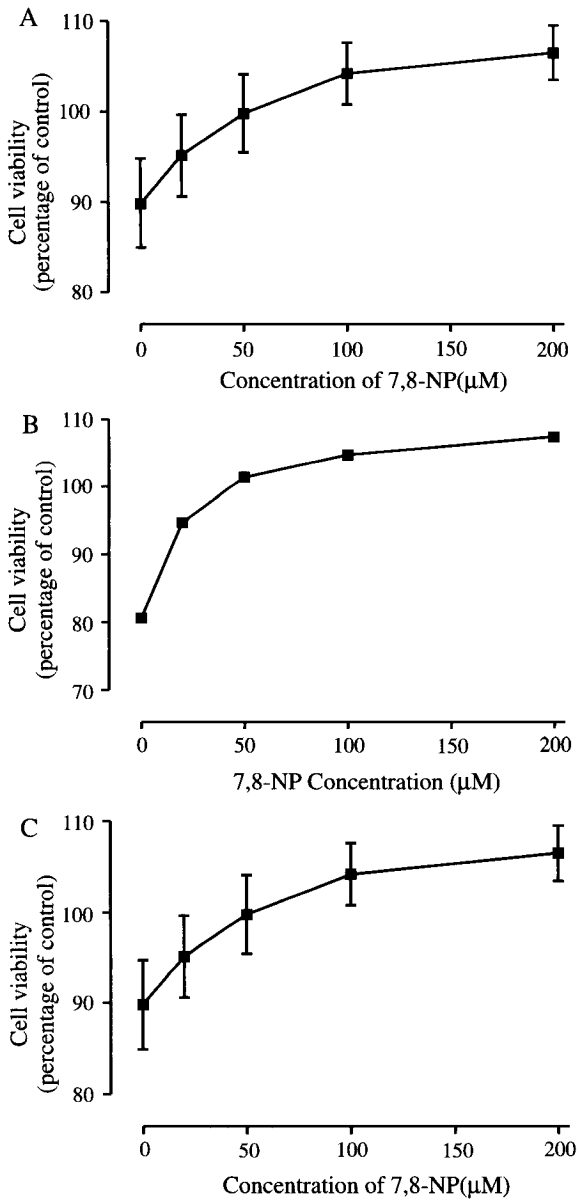


FIGURE 1 Inhibition of $Fe^{++}$ mediated loss of U937 cell viability by 7,8-dihydroneopterin. U937 cells at $5 \times 10^6$ cells per ml in RPMI 1640 media were preincubated with a range of 7,8-NP concentrations before the addition of 50 μM $FeSO_4$. After four hours of incubation the cell viability was measured by (A) trypan blue dye exclusion; (B) MTT reduction; (C) LDH leakage from cells into the media. All concentrations of 7,8-NP showed a significant effect on cell viability, $p < .01$.

in cell viability which was restored to 100% by the addition of 50 μM 7,8-NP (Figure 1B). The MTT assay measures the cells ability to reduce MTT to the formazan by NADPH oxidase enzymes. This makes the assay very sensitive to changes in the cells metabolic state. The trypan blue exclusion and LDH leakage assays measure the failure of the cell membrane and tends to be less sensitive (Figures 1b and 1c).

In all three cell viability assays, the addition of 100 and 200 μM 7,8-NP increased the cell viability to above 100%. 7,8-NP was found not to react with the MTT or trypan blue reagent nor interfere with the LDH enzyme assay. The addition of 7,8-NP to cells without iron salt also showed a small increase in viability suggesting the increase above the control cells was due to a real increase in cell viability and activity. Examination of the control U937 cells under phase contrast showed a small but significant number of the U937 cells with some bleb like structures on their cell surfaces. It would appear a small percentage of the U937 cell population is undergoing cell death in the restricted media. 7,8-NP appears to reduce the number of cells dying and therefore increase the cell viability by up to 10% above the control.

As expected, the presence of iron salts in the media did cause a large increase in cellular lipid peroxidation, measured as TBARS by HPLC analysis. The addition of 50 μM 7,8NP caused a significant reduction in cellular TBARS with complete inhibition of the iron mediated lipid oxidation with 200 μM 7,8-NP (Figure 2). Previously we have shown 7,8-NP is equally effective in inhibiting copper mediated LDL oxidation.[4] Our study indicated 7,8-NP was able to scavenge the peroxyl radical. This was later confirmed by an independent electron spin resonance study of 7,8-NP.[9] With the U937 cells, 7,8-NP is most likely scavenging the peroxyl radicals formed by radicals generated by the iron mediated fenton reaction.

Intriguingly, the 50 μM 7,8-NP restored cell viability to 100% (Figure 1) yet the cells still contained high levels of lipid peroxides. Fifty μM 7,8-NP reduced the TBAR level to only half that of the 50 μM iron control (Figure 2). There

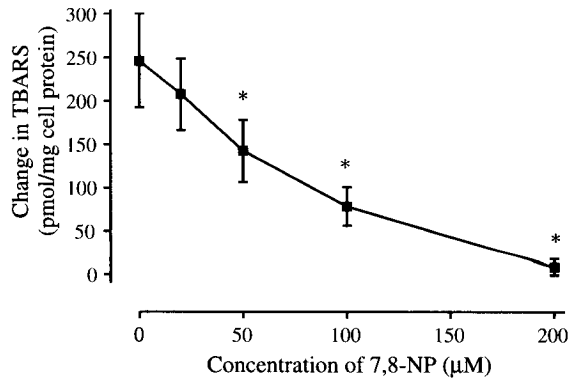


FIGURE 2 Inhibition of $Fe^{++}$ mediated lipid hydroperoxide formation by 7,8-dihydroneopterin. U937 cells at $5 \times 10^6$ cells per ml in RPMI 1640 media were preincubated with a range of 7,8-NP concentrations before the addition of 50 μM $FeSO_4$. The cells were incubated for four hours before HPLC TBARS analysis. * Values significantly different from control, $p < .01$.

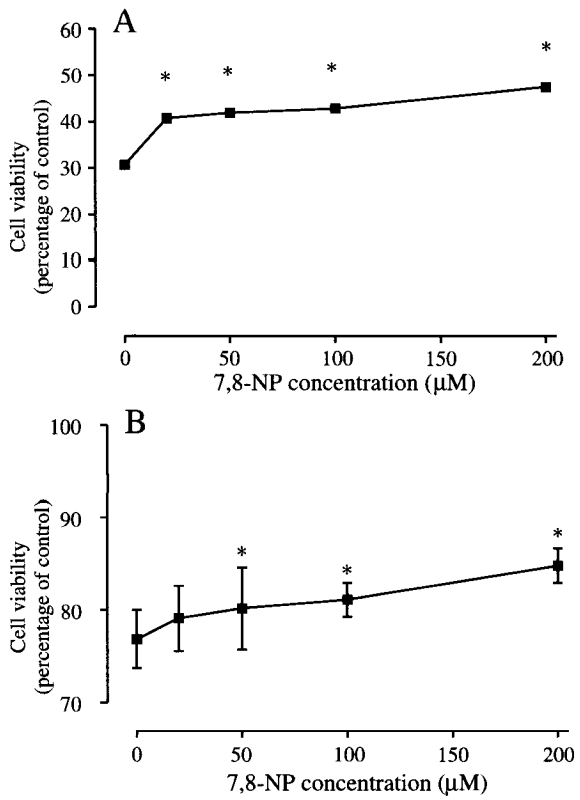


FIGURE 3 Inhibition of hydrogen peroxide induced reduction in cell viability by 7,8-dihydroneopterin. U937 cells at $5 \times 10^6$ cells per ml in RPMI 1640 media were preincubated with varying concentrations of 7,8-NP before the addition of 10 mM hydrogen peroxide. After four hours of incubation the cell viability was measured by (A) MTT reduction; (B) LDH leakage from cells into the media. * Values significantly different from control, $p < .01$.

appears to be little correlation between cell viability and the level of lipid peroxidation measured. Using azo radical initiators and hydrogen peroxide on red blood cells, we found no correlation between lipid peroxidation and cell lysis.[6] Though 7,8-NP does inhibit the formation of lipid peroxides by iron salts, it appears unlikely to be the mechanism of the increase in cell viability. This raises the possibility that protein rather than lipid damage was the cause of the loss in cell viability but HPLC analysis of cellular protein hydrolysates failed to show the formation of the tyrosine oxidation products DOPA or dityrosine, (data not shown). Iron and copper ions reacting with peroxides can cause the formation of these amino acid residue derivatives on samples of pure protein.[19,22] However, the intracellular enviroment of the U937 cell does not appear to favour these reactions.

### Hydrogen Peroxide Treatment

U937 cells were relatively unaffected by exposure to hydrogen peroxide (Figure 3). This may have been due in part to the rapid loss of hydrogen peroxide in the presence of the U937 cells. Using the FOX-2 assay we found 80% of the hydrogen peroxide was lost in the first hour of incubation with no hydrogen peroxide detected after 2 hours (data not shown). This may partially explain why 2 mM hydrogen peroxide had no significant effect on the cell viability. To obtain a reasonable level of cell death with which to measure 7,8-NP protection, we chose to use 10 mM hydrogen peroxide which caused a 70% reduction in cell viability as measured by the MTT assay (Figure 3a). The LDH leakage assay only showed a 24% reduction, highlighting its lower sensitivity (Figure 3b).

7,8-NP had a modest yet highly significant protective effect on hydrogen peroxide mediated loss of cell viability. Two hundred μM 7,8-NP

only raised the MTT measured cell viability to 45%, an increase of only 15% of total cell viability. We did not examine the effect of high concentrations of 7,8-NP as we felt the data would have little physiological significance.

The reaction between 7,8-NP and hydrogen peroxide is relatively slow[5] and likely to be slower than the damaging reactions occurring within the cell. This suggests that 7,8-NP is a poor scavenger of hydrogen peroxide.

Surprisingly cellular TBARS concentrations were not significantly elevated by the hydrogen peroxide treatment. The maximum TBARS obtained by hydrogen peroxide was only 35 pmol/mg of cell protein, 14% of that obtained with 50 μM iron. Similarly our previous study on the lysis of red blood cells with hydrogen peroxide also showed a lack of significant lipid peroxidation.[5] It would appear lipid oxidation is a not a significant mechanism of hydrogen peroxide mediated cell damage. Analysis of the U937 cells' protein after hydrogen peroxide treatment also failed to show the formation of DOPA or dityrosine. We have previously identified dityrosine in hydrogen peroxide treated red blood cell ghosts[5] but there may be insufficient iron available in the U937 cells to cause tyrosine residue oxidation.

Hydrogen peroxide is known to inhibit cellular ATP production by specifically inactivating the glycolytic enzyme glyceradehyde-3-phosphate dehydrogenase and the mitochondrial ATPase-synthase complex.[23,24]

For 7,8-NP to be effective against hydrogen peroxide it must rapidly react with and neutralise the hydrogen peroxide extracellularly or directly compete with the hydrogen peroxide intracellularly. The data presented here shows 7,8-NP provides only limited protection against hydrogen peroxide, (Figure 3) suggesting 7,8-NP has only a limited capacity to enter cells. 7,8-NP synthesising cells such as the human monocyte like THP-1 cell line,[1] may be resistant to hydrogen peroxide toxicity when stimulated with interferon-γ to produce *de novo* 7,8-NP intracellularly.

## Hypochlorite Treatment

HOCl is a potent reactive oxygen species which rapidly oxidises many biomolecules within the cell. *In vivo* it is formed by the action of the neutrophil enzyme myeloperoxidase. We found U937 cells were remarkably resistant to the toxic effects of HOCl over the one hour of analysis. After treatment with 1 mM HOCl on $5 \times 10^6$ cells/ml cell viability dropped to 76% as measured by trypan blue dye exclusion and 82% as measured by MTT reduction (Figure 4). In comparison, Schraufstatter *et al.* using the murine macrophage cell line P388D1, observed a 70%

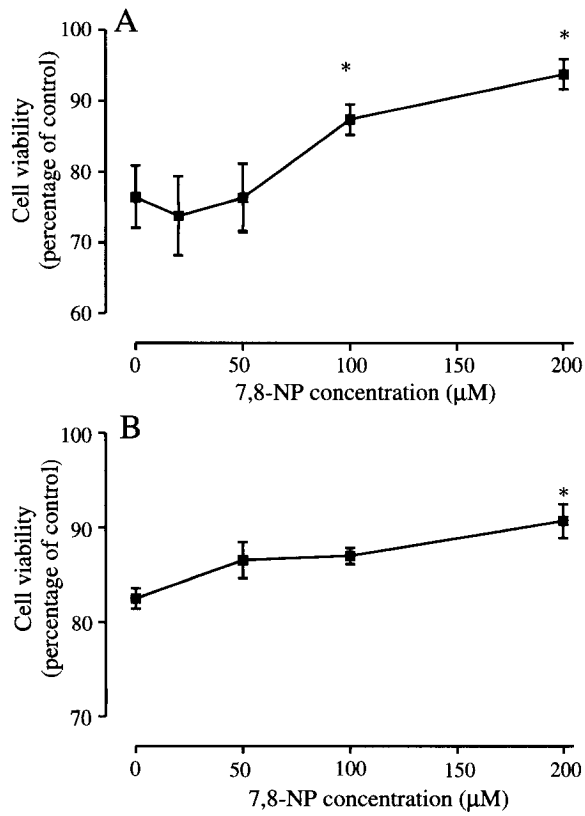


FIGURE 4 Inhibition of hypochlorite mediated loss of U937 cell viability by 7,8-dihydroneopterin. U937 cells at $5 \times 10^6$ cells per ml in HBSS media were preincubated with varying concentrations of 7,8-NP before the addition of 1 mM HOCl. The cells were then incubated for one hour before analysis. (A) Cell viability as measured by trypan blue dye exclusion assay; (B) Cell viability as measured by MTT reduction. *Values significantly different from the control, $p < .05$.

loss of cell viability using 75 μM HOCl on $2 \times 10^6$ cells/ml, also after one hour of treatment.[23] LDH leakage was not measured as the HOCl can denature the released LDH, masking the degree of plasma membrane damage. Longer incubation after the HOCl exposure would have shown lower cell viability due to the induction of apoptosis. However the aim of our study was to measure the initial damage and immediate effects of 7,8-NP as an antioxidant, rather than any possible interaction with apoptotic mechanisms. 7,8-NP at 100 μM significantly inhibited the loss of cell viability measured by trypan blue (Figure 4a). The effect on MTT reduction was only significant at 200 μM 7,8-NP (Figure 4b). Due to a misunderstanding of the literature, we examined HOCl treated cells proteins for the formation of dityrosine. Though no dityrosine formation was found, we did observe a massive loss in cellular tyrosine. HOCl reacts with tyrosine to produce the aldehyde *p*-hydoxyphenylacetaldehyde.[25] Chlorotyrosine is only a minor product of the reaction. The observed loss of cellular tyrosine was inhibited by 50 μM 7,8-NP (Figure 5). This was an interesting result as few antioxidants have been shown to protect proteins from ROS in whole cells. Tyrosine oxidation by 7,8-NP was completely inhibited when the 7,8-NP concentration was half the HOCl concentration suggesting a direct scavenging reaction of the 7,8-NP on the HOCl. In previous studies we have shown there is a direct and very rapid reaction between 7,8-NP and HOCl resulting in the formation of neopterin.[5]

$$\text{HOCl} + \text{7,8-NP} \rightarrow \text{neopterin} + \text{HCl} + \text{H}_2\text{O}$$

This reaction may be fast enough to scavenge the HOCl so preventing the HOCl mediated oxidation of the tyrosine.

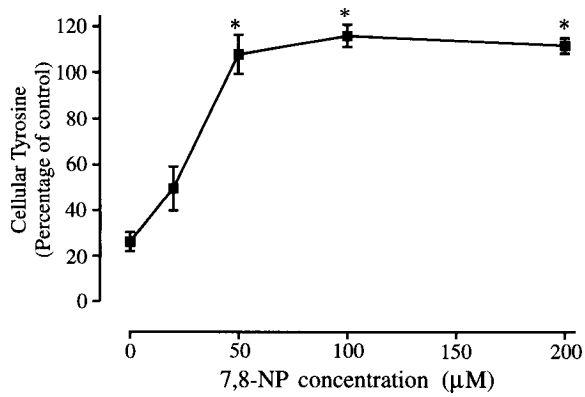


FIGURE 5 Protection of cellular tyrosine residues by 7,8-dihydroneopterin from HOCl oxidation U937 cells at $5 \times 10^6$ cells/ml in HBSS media were preincubated with varying concentrations of 7,8-NP before the addition of 100 μM HOCl. The cells were then incubated for one hour before washing, acid hydrolysis and HPLC analysis. * Values significantly different from that obtained with 100 μM HOCl with no 7,8-NP, $p < .01$.

## Cellular Protection by 7,8-NP

This study shows for the first time the protective effect of the antioxidant 7,8-NP on monocyte-like cells exposed to reactive oxygen species. 7,8-NP acted as an effective antioxidant against fenton generated reactive oxygen species (ferrous ion oxidation) and HOCl, inhibiting or suppressing the loss of cell viability and damage to cellular biomolecules. 7,8-NP was less effective against hydrogen peroxide, though the U937 cells showed remarkable resistance to hydrogen peroxide toxicity. Our current studies using tert-butyl-hydroperoxide on the monocyte-like THP-1 cells have also shown 7,8-NP to have a low protective effect against peroxides, (data not shown). Using a cell line more more susceptible to hydrogen peroxide may have shown the addition of 7,8-NP to have a greater effect as a lower hydrogen peroxide concentration could have been used. We previously found lysis of red blood cells exposed to 2 mM hydrogen peroxide was effectively inhibited by 7,8-NP addition.[5]

The protective effects seen in this study with ferrous ions and HOCl is consistant with our hypothesis that macrophage cells synthesize and release 7,8-NP to protect them from free radical mediated cellular damage during inflammatory events *in vivo*. The effectiveness of 7,8-NP *in vivo* as a cellular antioxidant will depend on its concentration. As yet there is no data

available on the concentration of 7,8-NP or neopterin within inflammatory sites.

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