Membrane permeability of redox active metal chelators; an important element in reducing hydroxyl radical induced NAD depletion in neuronal cells

T. Jayasena[a], R.S. Grant[b], N. Keerthisinghe[a], I. Solaja[a] and G.A Smythe[a]*

[a]Bioanalytical Mass Spectrometry Facility, [b] Department of Physiology & Pharmacology, Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia.

* Corresponding Author: Tel: +61-2-93852952 E-mail: G.Smythe@unsw.edu.au

Abstract

There is substantial evidence implicating increased production of the hydroxyl radical and oxidative stress in the pathogenesis of Alzheimer's disease (AD). Amyloid beta deposits and neurofibrillary tangles contain redox-active iron facilitating the production of hydroxyl radicals via Fenton chemistry. Increased iron levels within the cytoplasm of vulnerable neurons suggest that this may also be an important site of oxidative activity. We investigated the likelihood that intracellular, rather than extracellular, metal chelators (ferrous Vs ferric) may be more effective in reducing oxidative stress and preserving cell viability in a neuronal cell line (SK-N-SH). Using intracellular NAD measurements as an indicator of cell viability we found that membrane permeable ferrous chelators were most efficient in preserving cellular NAD levels. Hydrophilic, ferrous or ferric chelators and lipophilic ferric chelators were essentially ineffective in preventing cellular NAD depletion when added at physiological concentrations. We propose that lipophilic ferrous chelators, due to their actions inside the cell, are effective in moderating neuronal damage in conditions such as AD where oxidative stress plays a significant role in disease pathology.

Keywords: Hydroxyl radical, iron chelation, NAD depletion

.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that results in the progressive and irreversible loss of neurons in specific areas of the brain. The disease is characterised pathologically by senile plaques which are composed mainly of β-amyloid (Aβ), neurofibrillary tangles and an increased level of cellular markers for oxidative stress such as lipid peroxidation, protein oxidation and DNA damage (Atwood et al., 2003). Oxidative stress results from an imbalance in pro-oxidant/antioxidant homeostasis leading to an excess of reactive oxygen species (ROS) (Zambrano et al., 2004).

The majority of ROS are generated from the reaction of molecular oxygen with the redox-active metals copper and iron (Bush, 2002) via the Fenton reaction, where iron(II) is oxidized by hydrogen peroxide (H_2O_2) to iron(III), producing the highly reactive hydroxyl radical (Smith et al., 1997). The hydroxyl radical can efficiently attack DNA causing strand breaks resulting in the activation of the DNA repair enzyme poly ADP-ribose polymerase (PARP) which uses nicotinamide adenine dinucleotide (NAD) as a substrate. Over-activation of PARP will result in a massive depletion of cellular NAD (Ha and Snyder, 2000). NAD is necessary

for cell survival and participates in a variety of essential metabolic processes including DNA repair, cell signalling and as a cofactor for a number of dehydrogenase enzymes. NAD also plays an essential role in the synthesis of ATP through its role as an electron transporter in glycolysis and oxidative phosphorylation. A critical reduction in NAD levels therefore results in disrupted cellular metabolism and energy depletion leading ultimately to cell death (Ha and Snyder, 2000).

A significant feature of AD is the restriction of damage to the cell bodies of vulnerable neurons. While Aβ deposits and neurofibrillary tangles do contain redox-active iron (Perry et al., 2003), there are also significant increases in iron concentration within the cytoplasm of neurons themselves (Smith et al., 1997; Sayre et al., 2000). Whereas redox-active iron is scarcely detectable in the cytoplasm of normal controls, ultra structural localization of iron in AD tissues shows diffuse deposits associated with the cytoplasm (Perry and Smith, 1998).

These observations suggest that in AD brain, excess iron accumulation in neuronal cytoplasm, in addition to that observed in amyloid plaques, may play a significant role in the generation of ROS and subsequent AD pathology. Thus, one strategy for reducing excess cellular iron in AD and thereby minimising oxidative damage is through the use of selective iron chelation.

Clinical studies using the hydrophilic chelate drug desferrioxamine, showed a significant reduction in the rate of decline of daily living skills in AD subjects compared with normal controls (Finefrock et al., 2003). However the clinical utility of this hydrophilic drug is reduced due to the development of significant side effects such as anaemia caused by non specific chelation of extracellular iron.

The hydrophobic metal chelator, clioquinol (CQ), currently in phase II clinical trials is able to bind a range of metal ions and can cross cell membranes including the blood brain barrier effectively. In a transgenic mouse model of AD CQ as been shown to reduce Aβ burden by almost 50% (Cherny et al., 2001), and does not seem to result in some of the side effects shown by other less membrane permeable chelating drugs.

These observations suggest that, while metal chelators have the potential to moderate or even prevent free radical formation generated by free metal ions, effective targeting of the specific site of free radical generation by these agents may be important in reducing the non specific side effects and thus provide a more effective therapy.
As the cytoplasm of vulnerable neurons in AD appears to be an active site of oxidative stress due to the accumulation of intracellular iron, in this study we tested the hypothesis that lipophilic chelators were more effective at reducing cell NAD losses during Fenton induced oxidative stress than corresponding hydrophilic chelators.

Materials and Methods

RPMI1640, fetal bovine serum, glutamax-I, trypsin, and dulbecco's phosphate buffered saline (DPBS) were obtained from Invitrogen Life Technologies, Melbourne, Australia. Ethylenediaminetetraacetic acid (EDTA), iron (II) sulphate, ascorbic acid, hydrogen peroxide (H_2O_2), ethanol, methanol, sodium hydroxide and formic acid were obtained from AJAX chemicals, Auburn, Australia. Sodium dodecyl sulphate (SDS) was obtained from BDH chemicals, Victoria, Australia. Picogreen dsDNA quantitation reagent was obtained from Molecular Probes Inc., Eugene, USA. Picolinic acid, picolinamide, desferrioxamine mesylate salt (DFO), dipyridyl (DP), 1,10-phenanthroline (OP), bathophenanthroline sulfonic salt (BPS), melatonin, α-tocopherol acetate (vitamin E), nicotinamide, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), bicine, β-nicotinamide adenine dinucleotide (β-NAD), alcohol dehydrogenase (ADH), γ-globulins, trifluoroethanol (TFE), trifluoroacetic acid (TFA) and 3,4-dihydroxyphenyl acetic acid (DOPAC) were obtained from Sigma-Aldrich, Castle Hill, Australia. Phenazine methosulfate and p-hydroxyphenyl acetic acid (HPAA) was

obtained from ICN Biochemicals Inc., Ohio, USA. Bradford reagent and urea were obtained from Biorad, Hercules, CA, USA. 3,4-dihydroxyphenyl-d3 acetic-2,2-d2 acid (d5-DOPAC) was obtained from Merck Sharp and Dohme, Montreal, Canada.

2.1 Cell Culture

Human neuroblastoma cells (SK-N-SH) were maintained in RPMI1640 cell culture medium supplemented with 10% foetal bovine serum, 2 mM l-glutamine, 1% penicillin/streptomycin, at 37 ^{o}C in a humidified atmosphere containing 95% air/5% CO_2. Before experimentation cells were seeded into 24 well culture plates to a density of approximately $5X10^5$ cells and were incubated for 24 hrs before experimentation.

On the day of the experiment, the culture medium was aspirated and discarded. Cells were then washed twice with 500 µL DPBS before addition of 1 ml DPBS/well containing 10 µM iron, 10 µM ascorbic acid (to maintain iron in its Fe^{2+} oxidation state), and 100µM H_2O_2. The iron was incubated with the chelator for 5 mins before the addition of H_2O_2. All treatments were incubated for 30 mins.

2.2 NAD(H) Assay

Damaged cells show mitochondrial dysfunction, which results in decreased cellular NAD levels. Intracellular NAD(H) concentration was quantified using the thiazolyl blue microcycling assay established by Bernofsky and Swan (1973) adapted to 24 well plate format.

Total protein Assay

Total protein was determined using the Bradford assay method (Biorad, Sydney), to correlate NAD levels with cell number.

2.3 Hydroxyl Radical Production in Cell homogenates

To test whether sufficient intracellular iron was available for initiation of the Fenton reaction subsequent production of hydroxyl radicals in cell homogenates were evaluated. HPAA was used as the substrate to which generated hydroxyl radicals would react forming the product, 3,4-dihydroxyphenyl acetic acid (DOPAC) as previously described (Wang and Smythe 2003). DOPAC formation, measure by gas chromatography/mass spectrometry (GC/MS) after derivatisation, is directly proportional to the amount of hydroxyl radicals produced.The cell culture media was replaced with DPBS and cells were sonicated for approximately 10 sec, 1 mM EDTA, 1 mM ascorbic acid and 20 mM HPAA were added to the samples and incubated at 37^{o}C for 30 mins. Tris buffer (pH 8.6) was then added to the solution, followed by the addition of approximately 200 mg aluminium oxide. Samples were vortexed, then filtered through a glass column (pasteur pipette) packed with lint free tissue. The alumina was then washed with 6 ml milliQ water. DOPAC was eluted from the alumina with 1 ml methanol:water:formic acid (20:4:1) solution. The samples were then dried down under vacuum (Savant speedvac), the residues mixed with TFE and TFA, and heated at 70^{o}C for 30 mins to produce the DOPAC ester. This product was again dried and re-dissolved in 1 ml of 1%TFAA in toluene. 1 µL samples were then injected into the GC/MS. Standard curves were prepared using varying concentrations of standard DOPAC and a constant amount of internal standard (d5-DOPAC). The ions monitored by the GC/MS were m/z 345 for DOPAC and m/z 350 for d5-DOPAC.

2.4 Extracellular Hydroxyl Radical Production

Extracellular hydroxyl radical production (i.e in cell culture media) during treatment was assessed using the following method. 200 µM HPAA was added to all wells for 30 min and incubated as described previously (see 2.1 above). After the incubation period the media was removed and assayed for DOPAC production as previously described (see 2.3 above).

2.5 Hydroxyl Radical Production in Cell-free Buffer

The potential for the iron chelate complexes themselves to produce hydroxyl radicals was tested using a cell free system. Hydroxyl radical production was initiated using a reaction mixture consisting of an iron-EDTA chelate in

the presence of ascorbic acid and HPAA. 50 µM Fe and 1mM EDTA were used with 2 mM ascorbic acid as the control. 150 µM of selected chelators and/or the antioxidant melatonin was used, plus 20 mM HPAA was added to all samples.

GC/MS
GC/MS was performed on an Agilent Technologies 6890 gas chromatograph interfaced with an Agilent 5973 mass selective detector (Agilent Technologies, Ryde, Australia). The instrument was operating in electron capture negative ionisation (ECNI) mode, using methane as the reagent gas, set at a flow control setting of 40% as previously described (Wang and Smythe, 2003).

2.6 DNA Damage Assay
Cellular DNA damage following H_2O_2 exposure was determined using the method described by Batel et al., 1999. Briefly, cells were grown in 96 well microplates (3000 cells/well). After a 30 min exposure to 1-100 µM H_2O_2 in PBS, the buffer was aspirated and replaced with 25 µL TE buffer and 25 µL lysing solution (4.5M urea, 0.2M EDTA, 0.1% SDS supplemented with PicoGreen). Cells were then incubated for 40 mins on ice in the dark. DNA denaturation was initiated by the addition of 250 µL NaOH (pH 12.4). The rate of denaturation was quantified by measuring fluorescence of the dsDNA-picogreen complex (485 nm excitation, 530 nm emission) after10 mins at room temperature.
Double stranded DNA percentages were calculated relative to control values at 0-time denaturation after blank correction .

2.7 Statistics
All values are presented as means ± SEM. Statistical comparison were performed using the two-tailed student t-test with equal variance. Differences between treatment groups were considered statistically significant if $P < 0.05$.

Results
3.1 DNA Damage
Treatment of neuroblastoma cells with H_2O_2 (1-20 µM) for 30 min significantly increased DNA strand breaks compared to controls in a dose dependent manner (fig 1).

3.2 Hydroxyl Radical Production
3.2.1 Cell Homogenates
A marked increase in hydroxyl radical (OH•) production was observed in the DOPAC reaction mixture to which neuroblastoma cell homogenates were added whereas no OH• production was observed in the presence of reaction mixture alone (fig 2), this indicated that sufficient quantities of transition metals (e.g. iron) were available within the neuroblastoma cell to initiate hydroxyl radical production.

3.2.2 Cell Free System
As iron chelate complexes themselves may generate OH• radicals we tested the capacity of all chelators used to generate the OH• radical in the presence of iron by using a known initiator of Fenton chemistry, the Fe^{3+}-EDTA complex in the presence of ascorbic acid (fig 3).
In this cell free system, using the free radical scavenger melatonin as a positive control, we found that all chelators, with the exception of DFO, significantly reduced the formation of the OH• (fig 3).

3.2.3 Cell Media
We investigated DOPAC production in the cell culture media (i.e extracellular hydroxyl radical production) during treatment with the chelators (fig. 9). The addition of iron caused a significant increase in OH• production. This free radical production was reduced by all the chelators tested with the exception of EDTA which as expected dramatically increased extracellular OH• production by 20 times the control.

3.3 Iron chelators
Intracellular OH• generation is a key contributor to Alzheimer's pathogenesis with DNA damage resulting in PARP activation and increased NAD turnover playing a significant role. We therefore tested chelators with demonstrated OH• attenuating activity (see section 3.2), but with different cell permeability characteristics, for their ability to attenuate OH• induced NAD depletion.

3.3.1 Hydrophilic (cell impermeable) Ferric (Fe^{3+}) chelators

Treatment with the hydrophilic ferric chelators DFO and EDTA up to 100 μM did not prevent NAD depletion in neuroblastoma cells following exposure to 30 min H_2O_2 (fig 4). However at 1 mM DFO did significantly moderate NAD depletion (fig 4), whereas EDTA, further depleted NAD levels compared to treatment with H_2O_2 + Fe alone (fig 4).

Cells incubated in the presence of up to 1mM of either EDTA or DFO alone, without H_2O_2, did not show evidence of toxicity throughout the experimental period and did not result in changes to NAD levels, compared to control.

3.3.2 Hydrophilic (Cell impermeable) Ferrous (Fe^{2+}) chelator

Similarly, treatment with the hydrophilic ferrous chelator bathophenanthroline (BPS), at concentrations up to 100 μM, did not attenuate the drop in intracellular NAD, requiring at least 1 mM to produce a modulating effect (fig 5).

Neuroblastoma cells incubated in the presence of up to 1 mM of BPS alone, without H_2O_2, did not affect NAD levels and showed no evidence of toxicity throughout the duration of the experiment.

3.3.3 Lipophilic (Cell permeable) Ferrous (Fe^{2+}) chelators

The lipophilic ferrous chelators orthophenanthroline (OP) and dipyridyl (DP) were effective at modulating NAD depletion at minimum effective concentrations (MECs) as low as 10 μM in a dose dependent fashion (fig 6). Complete abrogation of the NAD depleting effect of H_2O_2 and Fe^{2+} was achieved at the maximum dose of 100 μM of drug (OP or DP).

Cells incubated in the presence of these drugs alone, without H_2O_2 did not show evidence of toxicity or reduced NAD levels compared to controls.

3.3.4 Lipophilic (Cell permeable) Ferric (Fe^{3+}) chelator

The lipophilic ferric chelator, PIH was also tested and found to be unable to prevent NAD depletion at concentrations below 1 mM (fig 7).

3.3.5 Lipophilic (Cell permeable) Copper, Zinc and Iron chelator

The lipophilic chelator Clioquinol (CQ) will chelate a variety of metal ions with different affinities. CQ was effective at preventing NAD depletion caused by peroxide treatment at concentrations similar to MECs observed for OP and DP (i.e 10 μM, fig 8).

Discussion

In AD, Aβ is implicated as a source of the oxidant H_2O_2 which is capable of reacting with available iron to produce the highly reactive hydroxyl radicals. Extracellular OH• production is unlikely to produce the intracellular oxidative stress seen in this disorder. Due to its extreme reactivity the OH• radical can only diffuse nanometer distances, indicating that the source of ROS activity must be in close proximity to the damage site, i.e. within the cell.

Intracellular iron has been shown to be increased in AD. The H_2O_2 produced by Aβ easily diffuses across the cell membrane and is available to react with this excess free metal. The available H_2O_2 and Fe^{2+} will react intracellularly leading to increased production of the hydroxyl radical.

Consistent with the model of OH• damage in vivo in AD using our cell culture model we observed that when H_2O_2 was applied extracellularly at physiological concentrations in the presence of Fe^{2+} significant levels of OH• were generated both intracellularly (fig 2) and extracellularly (fig 9) with DNA damage and NAD depletion. We observed high levels of OH• production in homogenates of neuroblastoma cultures (fig 2). Indicating sufficient available intracellular iron (or copper) for Fenton chemistry and OH• production.

However, only intracellular OH• generation resulted in significant DNA damage leading to NAD depletion. This loss of cellular NAD was prevented most efficiently by lipophilic (membrane permeable), ferrous (Fe^{2+}) chelating drugs (figs6 & 8).

The cause of the NAD depletion may have been due to a direct intracellular oxidative event or

potentially indirectly via a cell membrane initiated cascade leading to intracellular oxidative stress responses and DNA damage. We investigated the potential source of DNA damage and NAD depletion by correlating OH• production in the culture medium to NAD depletion. All chelators, regardless of their cell permeability were found to significantly reduce hydroxyl radical production in the cell culture medium (Fig 9), with the exception of EDTA. The cell permeability of the chelators had no effect on their ability to prevent extracellular hydroxyl radical production as lipophilic chelators were the most effective at reducing NAD depletion. This suggests NAD depletion was caused by intracellular OH• production (fig 2) leading to DNA damage (fig 1) and activation of the NAD catabolic enzyme poly(ADP-ribose) polymerase (PARP).

The lipophilic (i.e. membrane permeable) ferrous iron chelators OP and DP were found to effectively prevent NAD depletion in a dose dependent manner at concentrations as low as 10 μM, (fig 6) whereas the hydrophilic (reduced membrane permeability) chelators, DFO (ferric), and BPS (ferrous) only attenuated NAD loss at the highest concentration tested (1 mM). As mentioned previously these results support the hypothesis that OH• production and damage occurs intracellularly. Removal of intracellular free Fe^{2+} using membrane permeable chelators is then an efficient way of reducing oxidative stress.

However at high concentrations the hydrophilic chelators also attenuated NAD depletion (figs 4 & 5). This may be explained as once effective intracellular concentrations are reached, depending on the affinity of the chelator for the transition metal, available ions such as Fe^{2+} (and Cu^{+}) can be rendered unavailable for participation in intracellular Fenton reactions. The hydrophilic chelators DFO and BPS, have lipid/water partition coefficients which limit their access through cell membranes. Therefore extracellular concentrations of greater than 100 times that needed for the lipophilic chelators OP and DP were required before effective intra cellular concentrations of these drugs were obtained.

Importantly no evidence of cell toxicity was observed over the short duration (30 min) of the experiment for any of the chelators tested. NAD levels were also not affected by adding chelators alone in the absence of added H_2O_2. This is consistent with transition metal chelation and reduced OH• production being directly involved in the mechanism of NAD depletion rather than non-specific toxic effects on cell biochemistry.

In contrast to the other drugs tested, the hydrophilic chelator EDTA significantly reduced NAD levels at 1 mM. Although hydrophilic, at high concentrations a proportion of EDTA will cross the cell membrane. The intracellular chelate complex formed could potentially initiate OH• production consistent with observation in the cell free system (fig. 3) leading to increased DNA damage and a further decrease in NAD.

Interestingly, the lipophilic chelator PIH which complexes with predominantly the redox inactive (i.e. ferric) form of the iron was also able to prevent NAD depletion at 1 mM, the highest concentration tested (fig 7). PIH does enter cells rapidly (Barbouti et al., 2001) resulting in very high intracellular levels of this chelator within the experimental timeframe. As with most chelators, PIH will bind both redox forms of iron but has a greater affinity for the redox inactive ferric form. At very high concentrations PIH will likely bind enough of the ferrous iron to prevent Fenton generated OH• production, thereby inhibiting DNA damage and attenuating NAD depletion.

The lipophilic chelator Clioquinol (CQ) was also tested in our cell culture system currently under investigation in phase II clinical trials (Ritchie et al., 2003) resulting from its ability to reduce Aβ load in the Tg2576 transgenic mice model of AD (Cherny et al., 2001).

CQ has the capacity to bind both extracellular and intracellular copper, zinc and iron. When tested in our cell culture system CQ

significantly reduced NAD depletion caused by H_2O_2 (fig 8), at a similar effective concentration to that observed for the lipophilic chelators DP and OP. This supports the idea that the mechanism behind the effect of CQ in AD pathology may not to be due entirely to its capacity to solubilise plaques but may also be due to its ability to chelate intracellular iron and/or copper resulting in a decrease in intracellular OH• mediated damage.

It is important to note that none of the chelators used in this study are specific chelators of iron. Chelators have a range of affinities for various metal ions and while they may preferentially bind one metal over another that does not exclude the possibility that other metals may also be coordinated. This is particularly true at high concentrations where the amount of chelator is vastly in excess of the concentration of the various metal ions found in either the cell culture medium or cytoplasm. Therefore although Fe^{2+} was placed in excess in the culture system, both available Fe^{2+} and Cu^{+} will likely be chelated to some degree resulting in the observed attenuation of OH• mediated effects.

In conclusion, this study demonstrates for the first time that selected metal chelators are effective in reducing critical losses of the essential nucleotide NAD in conditions consistent with conditions where H_2O_2 is produced in excess (e.g. AD, inflammatory disease). These observations are consistent with a previous report showing that lipophilic chelators are effective in preventing H_2O_2 induced DNA damage (Barbouti et al., 2001).

This study showed that the lipophilic chelators, OP, DP and CQ attenuated the effect of OH• induced NAD depletion at concentrations 100 times lower than the hydrophilic chelators DFO and BPS. Two factors therefore emerge as important in determining the efficacy of a chelating agent's ability to moderate NAD depletion, 1) the ability of the chelator to complex transition metals intracellularly and 2) the ability of the chelator to complex the redox active form of iron (i.e. ferrous).

As the intracellular environment is a major site of oxidative damage, the effectiveness of membrane permeable chelators, (as seen in this study), suggests this class of drug may be useful in the treatment of AD. Effective targeting of the specific site of free radical generation by selected chelation drugs may be helpful in reducing non specific side effects and thus provide a more effective therapy.

Acknowledgements

The authors thank Dr Gilles Guillemin (Centre for Immunology, St Vincent's Hospital, Sydney, Australia) for his kind donation of the SK-N-SH cells. We also thank Prof Des Richardson (Children's Cancer Research Institute, Sydney, Australia) for his donation of PIH. This work was supported in part by grants from the Australian Government Systemic Infrastructure Initiative and Major National Research Facilities Program (UNSW node of the Australian Proteome Analysis Facility) and by the UNSW Capital Grants Scheme. One of the authors (T. Jayasena) wishes to thank Mr Ray Williams for scholarship support.

References

Atwood, C. S., Obrenovich, M. E., Liu, T., Chan, H., Perry, G., Smith, M. A. and Martins, R. N., 2003. Amyloid-beta: a chameleon walking in two worlds: a review of the trophic and toxic properties of amyloid-beta. Brain Research Brain Research Reviews 43, 1-16.

Barbouti, A., Doulias, P. T., Zhu, B. Z., Frei, B. and Galaris, D., 2001. Intracellular iron, but not copper, plays a critical role in hydrogen peroxide-induced DNA damage. Free Radic Biol Med 31, 490-498.

Batel, R., Jaksic, Z., Bihari, N., Hamer, B., Fafandel, M., Chauvin, C., Schroder, H. C., Muller, W. E. and Zahn, R. K., 1999. A microplate assay for DNA damage determination (fast micromethod). Analytical Biochemistry 270, 195-200.

Behl, C., Davis, J. B., Lesley, R. and Schubert, D., 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77, 817-827.

Bush, A. I., 2002. Metal complexing agents as therapies for Alzheimer's disease. Neurobiology of Aging 23, 1031-1038.

Cherny, R. A., Atwood, C. S., Xilinas, M. E., Gray, D. N., Jones, W. D., McLean, C. A., Barnham, K. J., Volitakis, I., Fraser, F. W., Kim, Y., Huang, X., Goldstein, L. E., Moir, R. D., Lim, J. T., Beyreuther, K., Zheng, H., Tanzi, R. E., Masters, C. L. and Bush, A. I., 2001. Treatment with a copper-zinc chelator markedly

and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. Neuron 30, 665-676.

Finefrock, A. E., Bush, A. I. and Doraiswamy, P. M., 2003. Current status of metals as therapeutic targets in Alzheimer's disease. Journal of the American Geriatrics Society 51, 1143-1148.

Ha, H. C. and Snyder, S. H., 2000. Poly(ADP-ribose) polymerase-1 in the nervous system. Neurobiology of Disease 7, 225-239.

Kicic, A., Chua, A. C. and Baker, E., 2001. Effect of iron chelators on proliferation and iron uptake in hepatoma cells. Cancer 92, 3093-3110.

Millar, A. D., Rampton, D. S. and Blake, D. R., 2000. Effects of iron and iron chelation in vitro on mucosal oxidant activity in ulcerative colitis. Aliment Pharmacol Ther 14, 1163-1168.

Perry, G. and Smith, M. A., 1998. Is oxidative damage central to the pathogenesis of Alzheimer disease? Acta Neurologica Belgica 98, 175-179.

Perry, G., Taddeo, M. A., Petersen, R. B., Castellani, R. J., Harris, P. L., Siedlak, S. L., Cash, A. D., Liu, Q., Nunomura, A., Atwood, C. S. and Smith, M. A., 2003. Adventiously-bound redox active iron and copper are at the center of oxidative damage in Alzheimer disease. Biometals 16, 77-81.

Richardson, D. R. and Ponka, P., 1998. Development of iron chelators to treat iron overload disease and their use as experimental tools to probe intracellular iron metabolism. Am J Hematol 58, 299-305.

Ritchie, C. W., Bush, A. I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., Kiers, L., Cherny, R., Li, Q. X., Tammer, A., Carrington, D., Mavros, C., Volitakis, I., Xilinas, M., Ames, D., Davis, S., Beyreuther, K., Tanzi, R. E. and Masters, C. L., 2003. Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. Arch Neurol 60, 1685-1691.

Sayre, L. M., Perry, G., Harris, P. L., Liu, Y., Schubert, K. A. and Smith, M. A., 2000. In situ oxidative catalysis by neurofibrillary tangles and senile plaques in Alzheimer's disease: a central role for bound transition metals. Journal of Neurochemistry 74, 270-279.

Smith, M. A., Harris, P. L., Sayre, L. M. and Perry, G., 1997. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proceedings of the National Academy of Sciences of the USA 94, 9866-9868.

Wang, X. and Smythe, G. A., 2003. Assessment of hydroxyl radical generation and radical scavenging activity of Chinese medicinal herbs using GC-MS. Redox Report 8, 223-228.

Zambrano, C. A., Egana, J. T., Nunez, M. T., Maccioni, R. B. and Gonzalez-Billault, C., 2004. Oxidative stress promotes tau dephosphorylation in neuronal cells: the roles of cdk5 and PP1. Free Radical Biology and Medicine 36, 1393-1402.

Tables

Table 1: Binding properties of iron chelators tested

Chelator	**Hydrophilic (extracellular)**		**Hydrophobic (intracellular)**		**References**
	Ferrous	***Ferric***	***Ferrous***	***Ferric***	
Desferrioxamine (DFO)		■			(Millar et al., 2000) (Kicic et al., 2001)
Ethylenediaminetetraacetic acid (EDTA)		■			(Kicic et al., 2001)
Bathophenantroline (BPS)	■				(Kicic et al., 2001)
1,10-Phenanthroline (OP)			■		(Barbouti et al., 2001) (Millar et al., 2000)
Dipyridyl (DP)			■		(Kicic et al., 2001)
Pyridoxyl isonicotinoyl hydrazone (PIH)				■	(Richardson and Ponka, 1998)

Figures

Fig 1: DNA damage in neuronal cells caused by increasing concentrations of H_2O_2

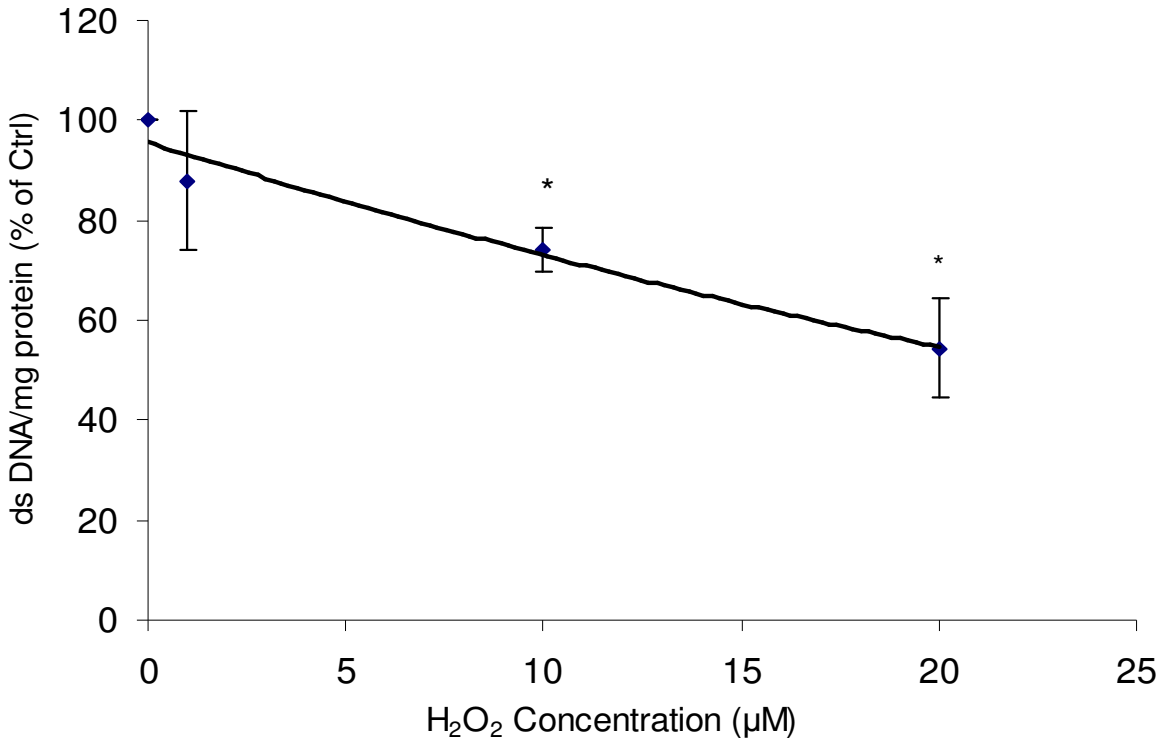

Fig 2: DOPAC production in cell free buffer and in cell homogenates of human neuroblastoma cells. Cell free solutions contained either, phosphate buffered saline (PBS) alone or, PBS plus 100 μM ethylenediaminetetraacetic acid (EDTA), and 200 μM ascorbic acid (AA). Results are expressed as mean ± SEM from n=5-6. *$p < 0.05$ vs PBS Only

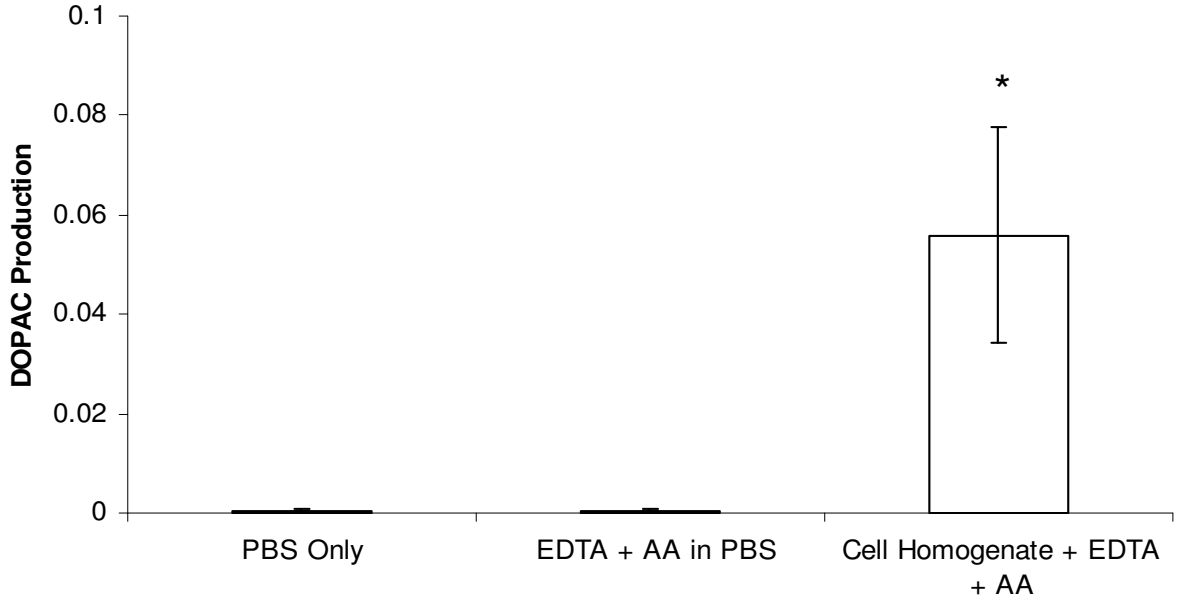

Fig 3: DOPAC production by iron chelate complexes in cell free buffer. Results are expressed as mean ± SEM. *$p < 0.05$ vs Ctrl.

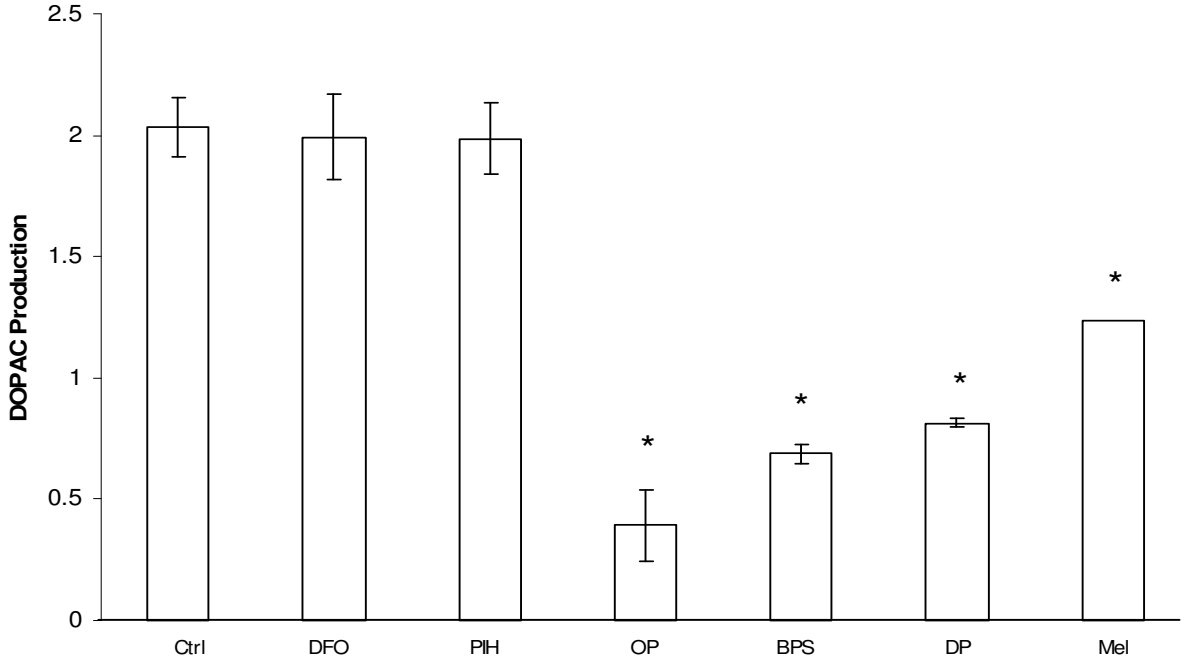

Fig 4: Dose response relationship between the hydrophilic chelator desferrioxmine (DFO) and EDTA and intracellular NAD in human neuroblastoma cells following treatment with H_2O_2 in the presence of ascorbic acid and ferrous iron. Results are expressed as mean ± SEM from n=14-16. *$p < 0.05$ vs Ctrl, **$p < 0.05$ vs H_20_2 + Fe ***$p < 0.05$ vs H_20_2 + Fe

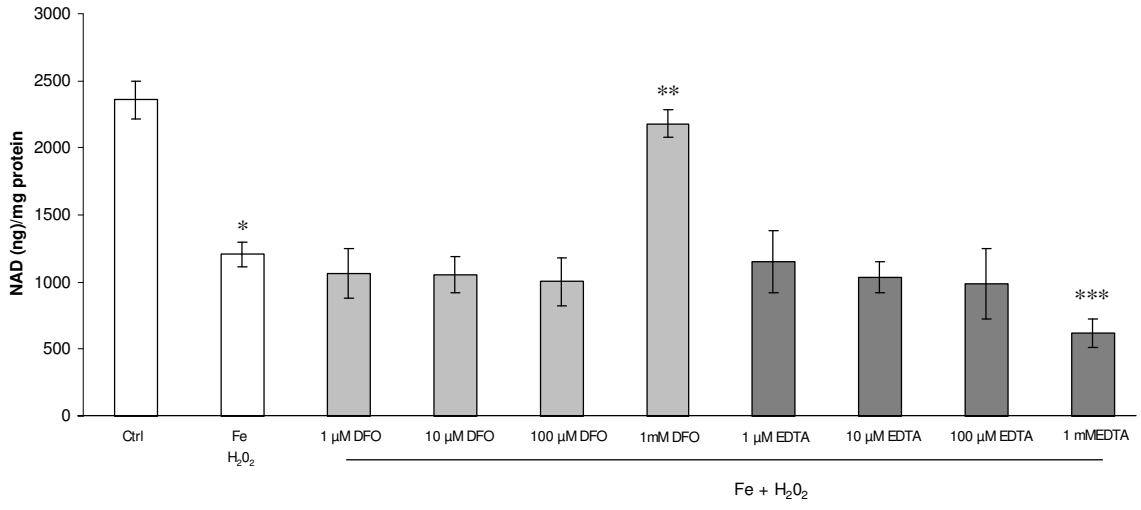

Fig 5: Dose response relationship between the hydrophilic chelator BPS and intracellular NAD in human neuroblastoma cells following treatment with H_2O_2 in the presence of ascorbic acid and ferrous iron. Results are expressed as mean ± SEM from n=8-12. *$p < 0.05$ vs Ctrl, **$p < 0.05$ vs H_20_2 + Fe.

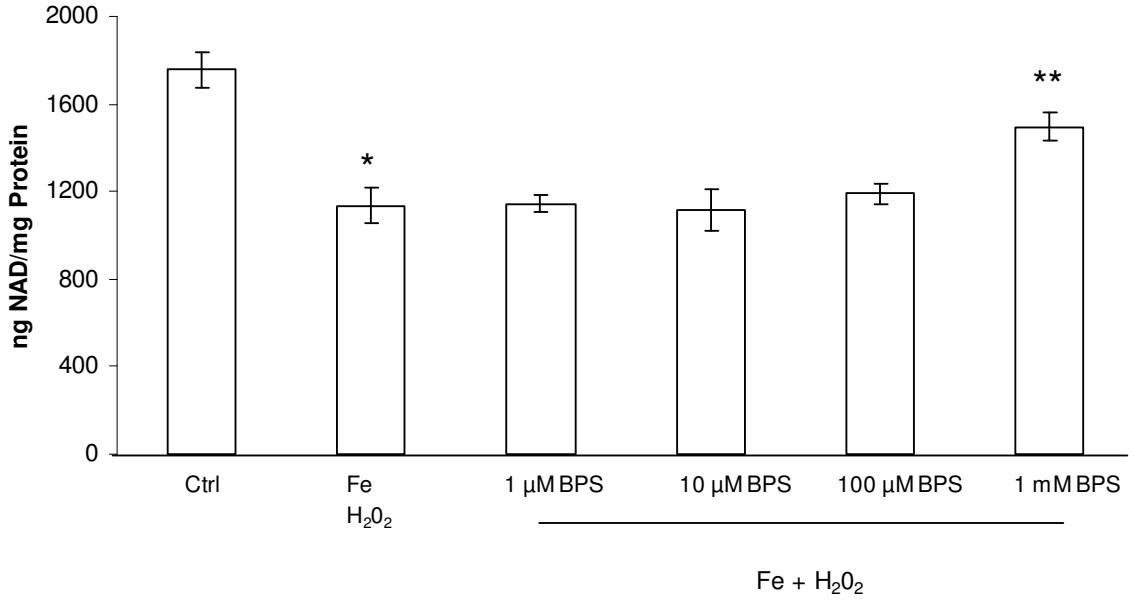

Fig 6: Dose response relationship between the lipophilic chelators orthophenanthroline (OP) and dipyridyl (DP) and intracellular NAD in human neuroblastoma cells following treatment with H_2O_2 in the presence of ascorbic acid and ferrous iron. Results are expressed as mean ± SEM, n=8-10. *$p < 0.05$ vs Ctrl, **$p < 0.05$ vs H_20_2 + Fe, ***$p < 0.05$ vs 10 µM OP + H_20_2/Fe, #$p < 0.05$ vs 100 µM H_20_2 + Fe , ##$p < 0.05$ vs 10 µM DP + H_20_2 + Fe.

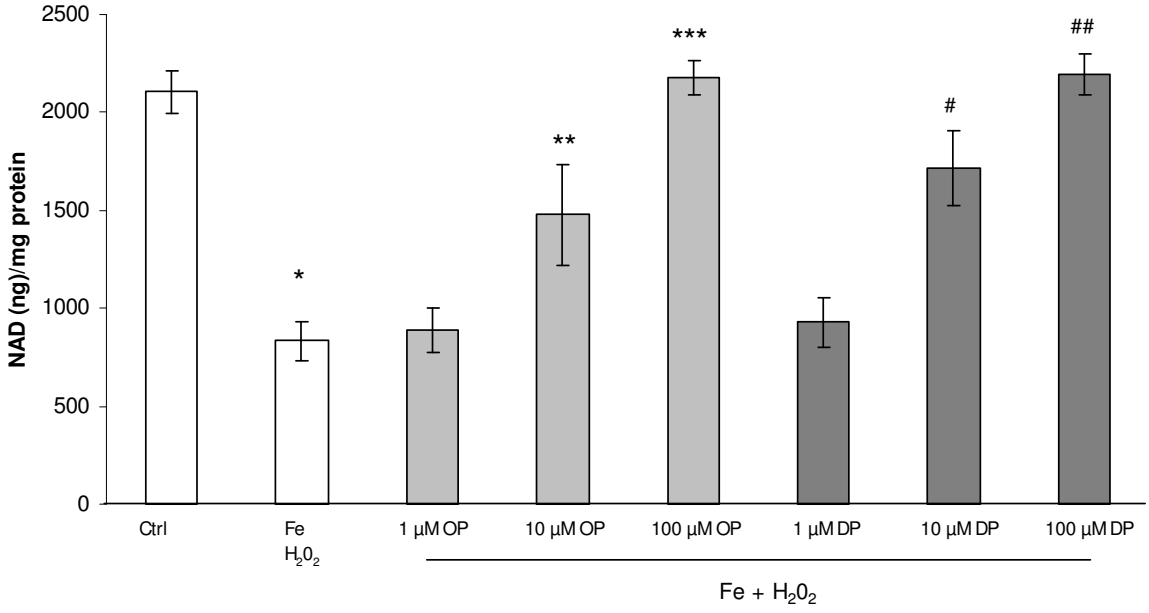

Fig 7: Dose response relationship between the lipophilic chelator PIH and intracellular NAD concentrations in human neuroblastoma cells following treatment with H_2O_2 in the presence of ascorbic acid and ferrous iron. Results are expressed as mean ± SEM from n=8. *$p < 0.05$ vs Ctrl, **$p < 0.05$ vs H_20_2 + Fe

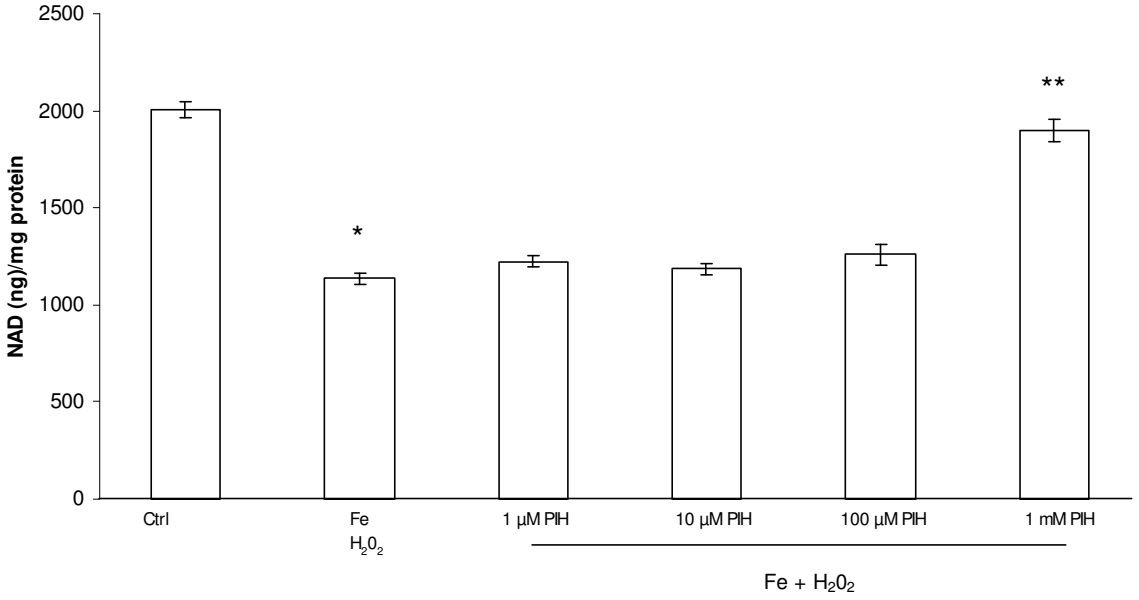

Fig 8: Dose response relationship between the lipophilic chelator CQ and intracellular NAD concentrations in human neuroblastoma cells following treatment with H_2O_2 in the presence of ascorbic acid and ferrous iron. Results are expressed as mean ± SEM from n=3. *$p < 0.05$ vs Ctrl, **$p < 0.05$ vs H_20_2 + Fe, ***$p < 0.05$ vs 10 µM CQ + H_20_2 + Fe

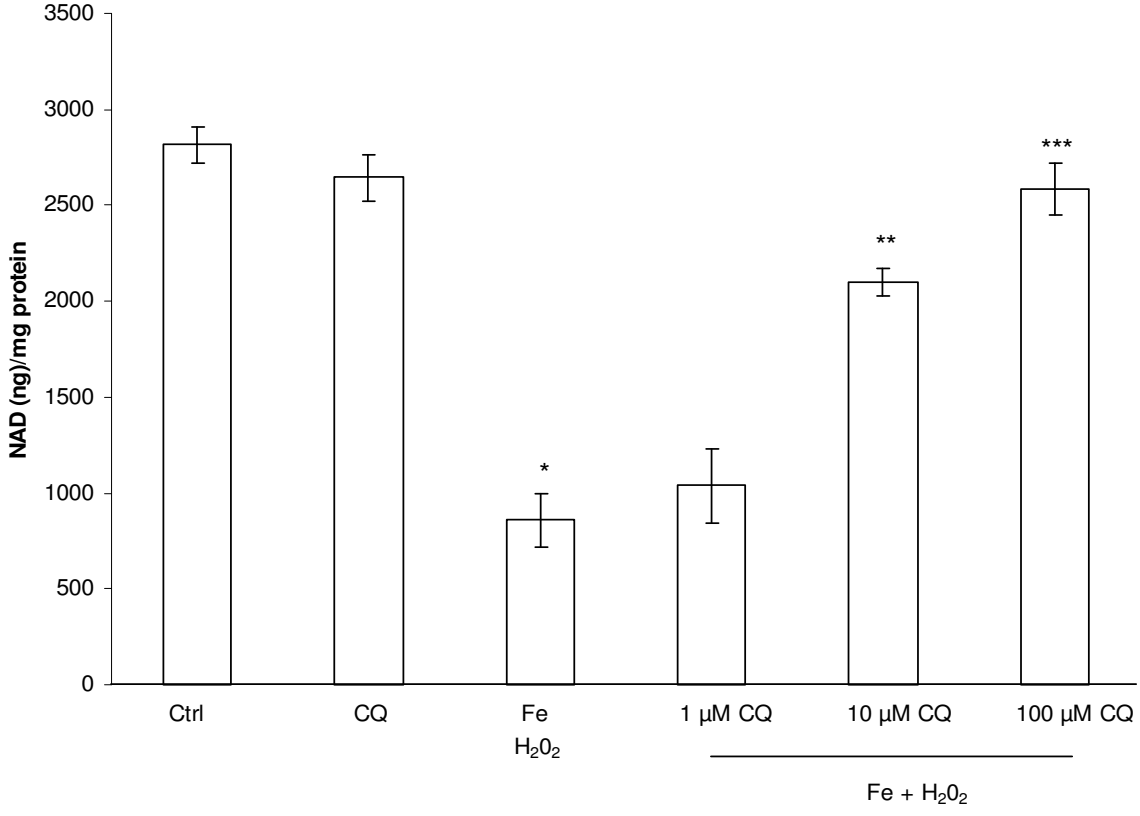

Fig 9: DOPAC production in cell culture medium during treatment with chelators in the presence of H_2O_2, ascorbic acid and ferrous iron (ie extracellular hydroxyl radical production). Results are expressed as mean ± SEM from n=3-6. *$p < 0.05$ vs H_20_2 + Fe.