EFFECTS OF MANGANESE ON OXIDATIVE-STRESS IN CATH.a CELLS

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ABSTRACT

The present study addressed the effects of Mn on oxidative stress in a catecholaminergic CATH.a cell line. Oxidative stress was measured with the fluorescent dye, 2',7'-dichlorofluoroscein (DCFH). In the diacetate form the dye is taken up by the cells and cleaved by esterases, effectively trapping it within the intracellular space. DCFH is subsequently oxidized treatment in the presence of reactive oxygen species (ROS) to the fluorescent 2',7'-dichlorofluoroscein. The fluorescence was analyzed on an ACAS 470 Interactive Laser Cytometer. Treatment of CATH.a cells with $MnCl_2$ (up to 10 mM) from 10 min. up to 48 hrs. was not associated with increased intracellular ROS formation. While manganese (Mn) treatment alone did not increase the rate of ROS formation, when short-term (10 min.) Mn treatment was followed for the last 5 min. with treatment with H_2O_2, Mn (at concentrations > 5 mM) significantly increased ($p<0.05$) H_2O_2-induced ROS generation. Prolonged (24 hr.) Mn treatment prior to exposure to H_2O_2 was associated with a statistically significant ($p<0.05$) reduction in ROS generation compared with cells treated with H_2O_2 alone. This statistically significant decrease ($p<0.05$) in ROS generation was preserved in CATH.a cells that were treated for 48 hrs. with 10 and 100 μM Mn followed by H_2O_2 exposure. Although the trend for diminished ROS generation was also apparent with 500 μM and 750 μM Mn (48 hrs.), the decrease did not attain statistical significance. Combined these results suggest that Mn can act as both pro- and antioxidant, and that oxidative stress-related effects of Mn are dependent not only on the intracellular concentrations of the metal, but also the exposure duration, secondary oxidative challenges, and the overall oxidant "buffering" capacity of the cells.

INTRODUCTION

A free radical is defined as any atom or molecule containing an unpaired electron in the outermost orbit. This electrical configuration is unstable, necessitating that the free radical either take or donate an electron to an adjacent compound in order to restore its own orbital stability. The ensuing electron transfer not only disrupts the structure of the adjacent compound, but it may impair its own function (Zhang et al., 1994). Oxygen-free radicals are constantly produced in the central nervous system (CNS) where they may have beneficial or deleterious effects. The highly reactive superoxide (SO; $O_2^{\cdot-}$) free radical is formed during many normal biochemical events. Its overproduction, however, can lead to neuronal degeneration via its ability to mutate DNA and initiate a lipid peroxidation chain, in turn, leading to destruction of cell membranes. Arrayed against such damage is an extensive system of free radical defenses capable of scavenging and transforming oxygen-free radicals into non-toxic species.

Whether the affinity of Mn^{2+} for mitochondria and its inhibition of energy production are related to the distinctive pathology of excessive Mn exposure and the unique morphological damage to the globus pallidus and other CNS regions remains largely speculative. The brain consumes a full 20% of the total oxygen required by the human at rest (Sokoloff, 1974) and in active brain tissue approximately 40% of the total energy production is utilized to maintain ion gradients (Bradford, 1986). The globus pallidus and striatum, which preferentially accumulate Mn, are amongst the most active areas of brain, and, thus, may be highly vulnerable to its effects (Gavin et al., 1990). Should Mn-loaded neurons be put under strenuous metabolic conditions, necessitating an

increase in their firing rates, their compromised mitochondria might be unable to supply them with needed additional ATP. Thus, increased metabolic activity accompanied by elevated mitochondrial Mn^{2+}, and a decreased rate of ATP production just as the demand for energy is soaring, would compromise cell function (Gavin et al., 1999).

The CNS, like all other organs, shares an extensive system of free radical defenses, by scavenging and transforming oxygen-free radicals into non-toxic species (Bondy and LeBel, 1992). Included in these defensive systems is the enzymatic conversion of SO anions to hydrogen peroxide (H_2O_2) and subsequently to water (H_2O). Superoxide dismutase (SOD) plays a vital protective role by catalyzing the transformation of SO anion radicals into H_2O_2 (Reiter et al., 2001; Macmillan-Crow and Cruthirds, 2001). However, in the presence of unbound ions of iron, H_2O_2 is converted into potent and long-lasting hydroxyl ($^{\cdot}OH$) radicals that can cause damage to proteins, lipids and DNA.

SOD is a family of enzymes responsible for dismutation of $O_2^{\cdot -}$ to H_2O_2, thereby reducing the risk of $^{\cdot}OH$ formation (Reiter et al., 2001; Macmillan-Crow and Cruthirds, 2001). Three forms of SOD, encoded by 3 separate genes, are expressed in eukaryotic cells. Cu- and Zn-dependent SOD (CuZn-SOD) is localized to the cytoplasm. An extracellular CuZn-SOD is expressed in low concentrations in extracellular fluids, and a Mn-dependent SOD (Mn-SOD) is preferentially localized to the inner membranes of mitochondria. Given that production of oxygen free radicals through respiratory chain reactions is especially high in mitochondria, Mn-SOD would appear be of particular

importance in an antioxidant role (Zhang et al., 1994). Since the concentration of Mn-SOD is thought to correlate with free radical levels within the normal brain, its high content within the globus pallidus and substantia nigra may render these regions particularly vulnerable to free radical-induced damage should defensive mechanisms be compromised. Elevation in free radical levels is likely associated with activation of defense mechanisms against oxidative stress. In line with this concept, increased Mn-SOD activity has been noted in several conditions in which oxidative stress is suspected to occur (Zhang et al., 1994).

In addition to the ability of Mn-SOD to afford cellular protection, some Mn compounds can act as direct SODs. Examples include Mn^{2+}-pyrophosphate and Mn^{2+}-tartarate which can block lipid peroxidation mediated by $O_2^{\cdot -}$ and ionizing radiation (Cavallini et al., 1984; Donaldson et al., 1982; Archibald and Tyree, 1987). Free Mn^{2+} in solution, while it appears to have little or no ability to scavenge $^{\cdot}OH$ directly, can scavenge radicals produced by metabolic reactions, chemotherapeutic agents, or ionizing radiation (Wedler, 1993). Furthermore, a number of simple Mn^{3+} complexes are relatively stable and can selectively oxidize a variety of specific biological molecules.

Finally, some recent studies by Sziraki et al. (1995; 1999) and Van et al. (1999) suggest that Mn can completely suppress both acute lipid peroxidation in substantia nigra (a region of high dopamine content) and chronic degeneration of the neurons induced by infusion of ferrous citrate. As originally suggested by these authors, these *in vivo* data indicate that low-dose Mn is a potent antioxidant, which may activate antioxidative

defense mechanisms to protect brain neurons against oxidative stress induced by iron complexes. Only few studies have been carried out in cultured cells to address whether exposure to environmental Mn concentrations is associated with increased intracellular antioxidant levels, and whether pre-treatment of cells in culture with Mn affords protection and attenuates cytotoxicity. The most recent of these studies demonstrated that in HeLa cells high concentrations (1-2 mM) of Mn induced apoptosis and an increase in the production of ROS (Oubrahim et al., 2001).

The present study was undertaken to investigate Mn-induced oxidative stress in cultured CATH.a cell line. This cell line is catecholaminergic and synthesizes dopamine, a primary neurotransmitter in areas that accumulate Mn, such as the substantia nigra and globus pallidus. Hence, if dopamine, and its synthetic pathway which is regulated by TH is involved in Mn-mediated neurotoxicity, it was rationalized that this cell line would enable us to closely probe for the relationship between Mn, dopamine, and oxidative stress.

MATERIALS AND METHODS

Methodology

Cell Cultures and 2’,7’-dichlorofluoroscein (DCFH) fluorescence measurements:

Sterile equipment and technique to culture and treat the cells were employed. The cells were grown in a humidified environment at 37ºC, 95% air – 5% CO_2 in 75 ml and 325 ml T-flasks. A standard nutrient growth medium (RPMI-1640) was purchased from the Wake Forest University School of Medicine Core Cell Culture Laboratory. Cells were fed biweekly. Cells were divided weekly into new flasks or into 33mm plates for experiments. Plates were seeded at 70,000 cells and analyzed for fluorescence with an ACAS 470 Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI, USA). Cells were allowed at least 3 hrs for adherence prior to treatment with Mn. Oxidative stress was measured with a fluorescent dye. The method is based on the compound 2’,7’-dichlorofluoroscein (DCFH) which was added to the cells in a diacetate form. This allows the compound to enter the cells where intracellular esterases cleave diacetate, effectively trapping the dye within the intracellular space. DCFH is subsequently oxidized in the presence of reactive oxygen species to the fluorescent 2’,7’-dichlorofluoroscein. This fluorescence was then analyzed and the amount standardized to the protein content in each well. Protein content of 25 µl aliquots was determined using the bicinchoninic acid method (Pierce, Rockford, IL) after samples were neutralized with an equal volume of 1 M HCl. DCFH was added for the last 10 minutes of Mn exposure. Fluorescence was subsequently determined and expressed as the percentage of controls (i.e., Mn untreated cells).

In studies on oxidative stress with $MnCl_2$ alone, or in combination with stress agents, fluorescence was determined for the Mn effect at the specified times. Subsequently, the Hanks buffer was removed and a 200 μM solution of hydrogen peroxide (H_2O_2) in the absence of Mn was added to the cells. After the 5 minute H_2O_2 treatment, the fluorescence was determined again.

Statistical Analysis:

Statistical analysis of the data was determined with a one-way randomized ANOVA design. When the overall test of significance ($p<0.05$) led to a rejection of the null hypothesis, *post hoc* comparison (Neuman-Keuls; Weiner, 1971) were performed. Statistical analyses were performed with CSS: Statistica software (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Few reports are available on the absolute Mn concentration in cases of manganism. The most complete information is derived from a study in Indian red-haired monkeys (Suzuki et al., 1975). Three months of exposure to manganese dioxide (MnO_2) (0, 2.25, 4.5, and 9 gm), led to dose-related increases in CNS Mn concentrations (20.9, 91.0, 173.7, and 264 μM, in striatum; 35.3, 100.7, 241.7, 334.4 μM, in globus pallidus, respectively). The concentrations of Mn in human striatum and globus pallidus (primary target areas in manganism) are unknown. Mn cerebellar concentrations are available from Japanese accident victims, and at the time of autopsy they ranged between 9-10 μM (Sumino et al., 1975). Mn concentrations in various parts of control rat striatum have been reported to range from 4.4-18 μM (Ingersoll et al., 1999; Lai et al., 1999; Roels et al., 1997). In Mn-exposed rats, the levels of Mn in the striatum increase to 23-70 μM. As with MRI studies in non-human primates (Newland et al., 1989) and studies in rats (Sloot and Gramsbergen, 1994; Sloot et al.1996), actual Mn levels in the striatum and globus pallidus undoubtedly vary with the time after dosing. Despite inconsistencies in dosing regimens, both in monkeys and rats, a correlation exists between the severity of symptoms and Mn brain concentrations, substantiating that the rate and extent of Mn transport across the BBB affects the clinical outcome (Suzuki et al., 1975; Roels et al., 1997; Ingersoll et al., 1999).

Given the available reports on the absolute Mn concentration in cases of manganism (see above), a broad dose-response relationship curve was established for the effect of Mn on ROS generation. Figure 1 shows typical fluorescent images in control (A) and 10 mM

$MnCl_2$-treated CATH.a cells (B), respectively, captured with the ACAS 470 Interactive Laser Cytometer. The quantitative data derived from a broad range of Mn exposures are shown in Figure 2, depicting the effect of $MnCl_2$ on the generation of ROS in CATH.a cells. Although there appears to be a trend towards increased ROS with high Mn exposures, statistical analysis suggests that treatment of CATH.a cells with $MnCl_2$ (up to 10 mM) from 10 min. up to 48 hrs. is not associated with increased intracellular ROS formation.

Figure 3 depicts the short-term effect of Mn exposure ± H_2O_2 on ROS generation in CATH.a cells. As noted in figure 2, Mn alone at concentrations up to 10 mM did not significantly increase ($p>0.05$) the rate of oxidative stress in CATH-a cells. However, when the Mn exposure was followed with an additional 5 min. treatment with H_2O_2, Mn (at concentrations > 5 mM significantly increased ($p<0.05$) the effect of H_2O_2 on ROS generation.

Additional studies were carried out to determine whether prolonged treatment of CATH.a cells with Mn potentiates ROS generation by H_2O_2. CATH.a cells were treated with $MnCl_2$ for 24 and 48 hrs. followed by 5 min. exposure to H_2O_2. As shown in Figure 4, prolonged Mn treatment was associated with a statistically significant (*$p<0.05$) reduction in ROS generation after 24 hr exposure to Mn compared with cells treated with H_2O_2 alone. This statistically significant decrease (**$p<0.05$) in ROS generation was preserved in CATH-a cells that were treated with 10 and 100 μM Mn for 48 hrs. followed by H_2O_2 exposure. Although the trend for diminished ROS generation was also apparent with 500 μM and 750 μM Mn (48 hrs.), the decrease did not attain statistical significance.

It has previously been suggested that transition metals such as iron and manganese produce oxidative injury to the dopaminergic nigrostriatal system, which may play a critical role in the pathogenesis of Parkinson's disease (Sziraki et al., 1998). The present study is consistent with previous reports by Sziraki et al. (1998), demonstrating that elevated lipid peroxidation in the substantia nigra pursuant to intranigral injection of ferrous citrate can be dose-dependently diminished by Mn. The same authors have also shown that in CNS homogenates Mn (0 to 10 μM) concentration-dependently inhibited propagation of lipid peroxidation caused by iron (0 to 5 μM), suggesting that Fe and Mn mediate opposing effects in the nigrostriatal system, as pro-oxidant and antioxidant, respectively. Moreover, Mn (0-80 μM) concentration was also recently shown to retard diene conjugation of human low-density lipoproteins stimulated by 5 μM Cu^{2+} (Sziraki et al., 1999) in a concentration-dependent manner. The latter is also consistent with the anti-oxidative theory of Mn.

The present studies corroborate and extend the above conclusions. They suggest that Mn by itself does not induce oxidative stress in CATH.a cells, even at concentrations as high as 5-10 mM. Nevertheless, it should be pointed out that these studies are in disagreement with those by (Oubrahim et al., 2001), where Mn treatment was shown to lead to an increase in the production of ROS in HeLa cells, employing identical methodologies. The rationale for the differential response of HeLa cells (Oubrahim et al., 2001) vs. those employed by us (CATH.a) is unclear, but it might suggest a cell-specific sensitivity.

Treatment with Mn might increase the sensitivity of CATH-a cells to a second oxidative challenge, such that when CATH.a cells are exposed to H_2O_2 following short-term exposure to Mn (10 min.), Mn (at concentrations >5 mM) increases the intracellular generation of ROS. Conversely, when cells are exposed to Mn for 24 or 48 hrs., Mn reduces ROS formation in CATH.a cells in response to H_2O_2 challenge. Combined these results suggest that Mn can act as both pro- and antioxidant, and that oxidative stress-related effects of Mn are likely dependent not only on the intracellular concentrations of the metal, but also exposure duration, secondary oxidative challenges, and the overall oxidant "buffering" capacity of the cells.

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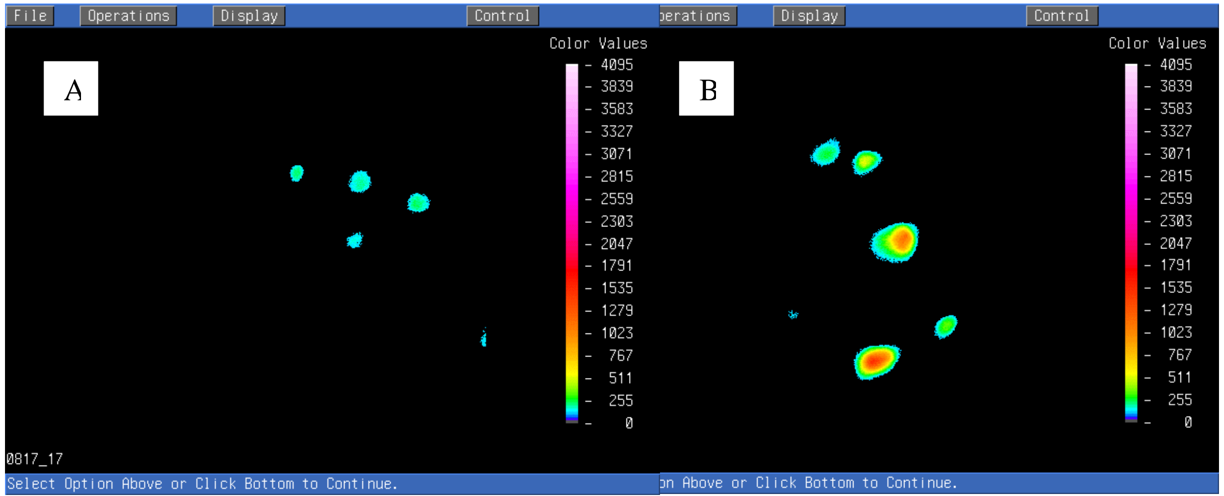
FIGURE LEGEND

Figure 1 Fluorescent images in control (A) and 10 mM $MnCl_2$-treated CATH.a cells (B). Treatments were carried out for 10 min. Plates were seeded at 70,000 cells and analyzed for fluorescence with an ACAS 470 Interactive Laser Cytometer. Cells were allowed at least 3 hrs for adherence prior to treatment with Mn. Oxidative stress was measured with a fluorescent dye. The method is based on the compound 2',7'-dichlorofluoroscein (DCFH). Fluorescence was then analyzed and the amount standardized to the protein content in each well. Protein content of 25 μl aliquots was determined using the bicinchoninic acid method after samples were neutralized with an equal volume of 1 M HCl. DCFH was added for the last 10 minutes of Mn exposure. Fluorescence was subsequently determined and expressed as the percentage of controls (i.e., Mn untreated cells).

Figure 2 Time- and dose-dependent ROS generation by $MnCl_2$ in CATH-a cells. For detailed methods refer to Figure 1 legend.

Figure 3 The effect of 10 min. treatment of CATH-a cells ± H_2O_2 (for the last 5 min.) on ROS (mean ± SEM; $*p<0.05$). For detailed methods refer to Figure 1 legend.

Figure 4 The effect of prolonged Mn treatments (24 and 48 hrs.) on H_2O_2 (for the last 5 min.) ROS generation in CATH-a cells (mean ± SEM; *$p<0.05$; **$p<0.01$). For detailed methods refer to Figure 1 legend.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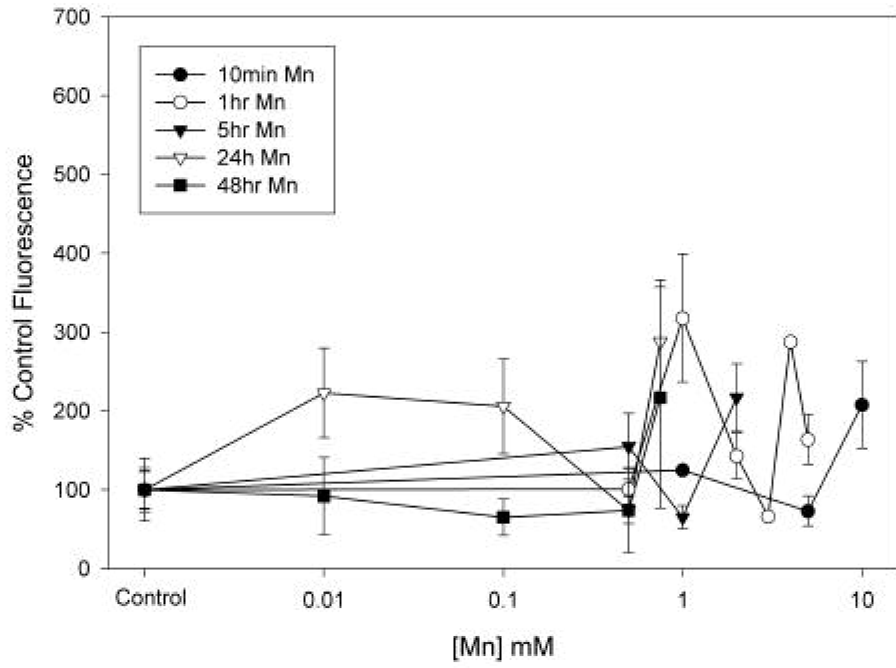

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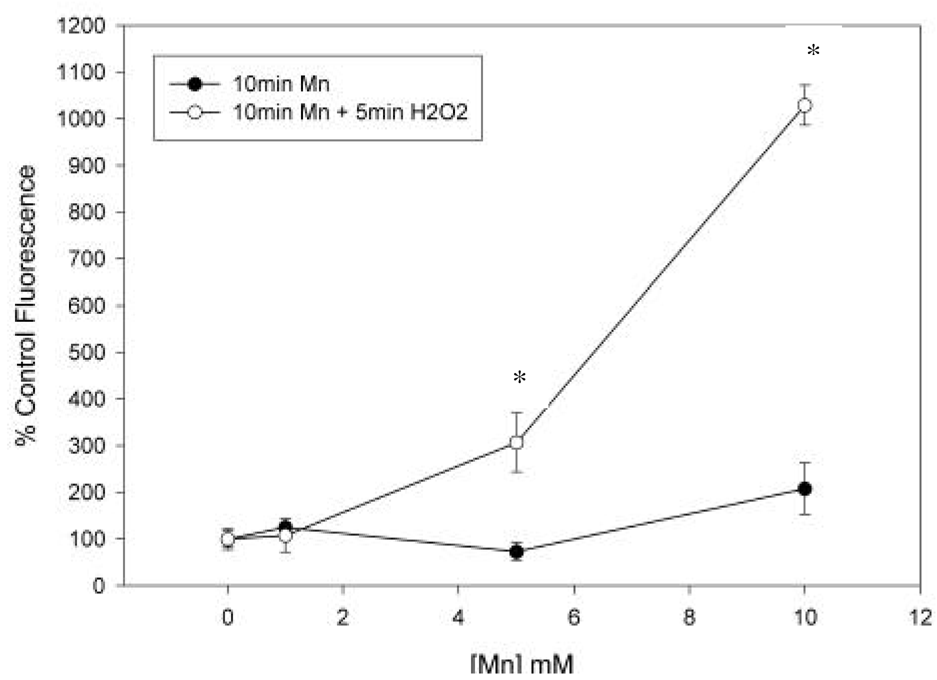
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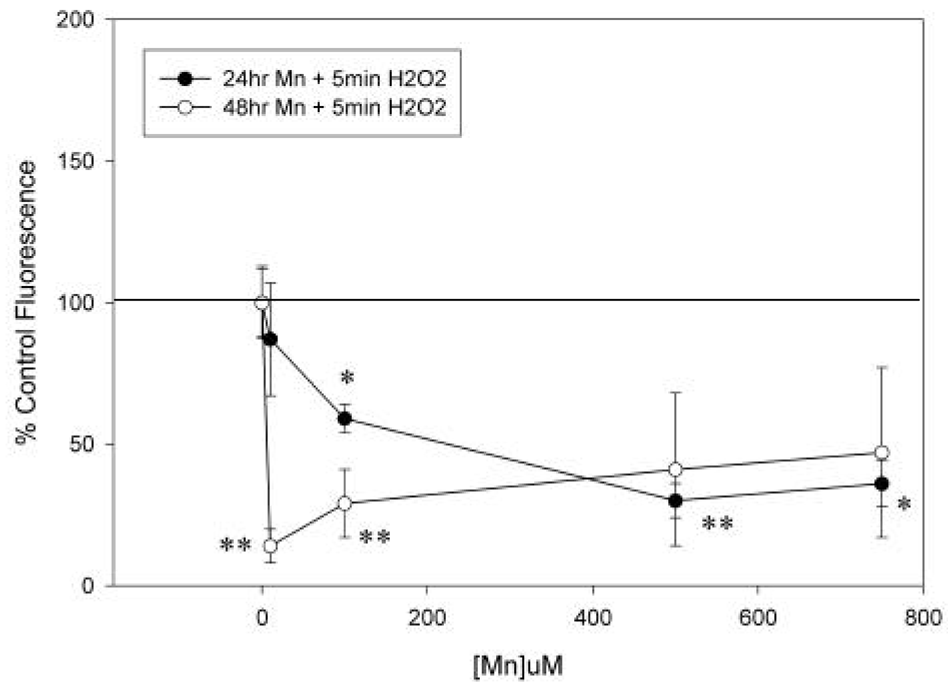

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