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Rat brain endothelial cells are a target of manganese toxicity

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Abstract

Manganese (Mn) is an essential trace metal, however exposure to high Mn levels can result in neurodegenerative changes resembling Parkinson's disease (PD). Information on Mn's effects on endothelial cells of the blood-brain barrier (BBB) is lacking. Accordingly, we tested the hypothesis that BBB endothelial cells are a primary target for Mn-induced neurotoxicity. The studies were conducted in an in vitro BBB model of immortalized rat brain endothelial (RBE4) cells. ROS production was determined by F₂-Isoprostane (F₂-IsoPs) measurement. The relationship between Mn toxicity and redox status was investigated upon intracellular glutathione (GSH) depletion with diethylmaleate (DEM) or L-buthionine sulfoximine (BSO). Mn exposure (200 or 800 μM MnCl₂ or MnSO₄) for 4 or 24h led to significant decrease in cell viability vs. controls. DEM or BSO pre-treatment led to further enhancement in cytotoxicity vs. exposure to Mn alone, with more pronounced cell death after 24h DEM pre-treatment. F₂-IsoPs levels in cells exposed to MnCl₂ (200 or 800 μM), were significantly increased after 4h and remained elevated 24h after exposure compared with controls. Consistent with the effects on cell viability and F₂-IsoPs, treatment with MnCl₂ (200 or 800 μM) was also associated with a significant decrease in membrane potential. This effect was more pronounced in cells exposed to DEM plus MnCl₂ vs. cells exposed to Mn alone. We conclude that Mn induces direct injury to mitochondria in RBE4 cells. The ensuing impairment in energy metabolism and redox status may modify the restrictive properties of the BBB compromising its function.

Keywords

RBE4 cells; manganese neurotoxicity; oxidative stress; glutathione; F₂-Isoprostanes; mitochondria cytotoxicity

1 - Introduction

Manganese (Mn) is an essential trace metal required for normal lipid, protein and carbohydrate metabolism (Hurley and Keen, 1987). Mn plays an important role in numerous enzyme families including oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Fitsanakis

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et al., 2005). Despite its essentiality, exposure to excessive Mn levels is associated with an irreversible brain disease characterized by psychiatric and motor disturbances (Chia et al., 1993; Pal et al., 1999; Stredrick et al., 2004). The disorder, referred to as manganism, is associated with elevated brain levels of Mn, especially the caudate-putamen, globus pallidus, substantia nigra, and subthalamic nuclei (Aschner et al., 1999; Dobson et al., 2004). Typical symptoms of chronic Mn exposure include tiredness, sleep disturbances, aggressiveness and behavioural disturbances (“manganese madness”). Delayed neurological disturbances encompass the extrapyramidal system, characterized by walking difficulties, dystonia and kinesia resembling many clinical features of PD (Mena et al., 1967). Mining, steel manufacturing and welding represent occupational exposures that are associated with Mn exposures to high Mn levels (Tanaka and Lieben, 1969; Cook et al., 1974; Myers et al., 2003). An organic Mn compound, methylcyclopentadienyl manganese tricarbonyl (MMT), used as an octane booster or anti-knock agent in gasoline, has also been shown to cause adverse health effects in humans in Canada and elsewhere (Abbott et al., 1987; Sierra et al., 1995; Dobson et al., 2004).

The BBB plays a major role in maintaining homeostasis in central nervous system (CNS), restricting passage of substances from the bloodstream. This is achieved by specialized endothelial cells and their interactions with glia (Davson and Segal, 1996; Rubin and Staddon, 1999; Fitsanakis et al., 2006; Moser et al., 2007). In order for blood-borne macro and micro-molecules to enter the brain parenchyma they must first cross the endothelial cells of the BBB. The high electrical resistance, reflecting upon the physical coupling of the tight junctions that link brain endothelial cells prevents any paracellular passage across the barrier in physiological conditions.

The BBB regulates the transport of essential trace metals including Mn (Bradbury, 1992) via facilitated diffusion (Rabin et al., 1993), active transport (Aschner and Gannon, 1994), divalent metal transport 1 (Erikson et al., 2002), ZIP8 and transferrin-dependent transport mechanisms (Aschner and Gannon, 1994; Au et al., 2008). Among different endothelial cell lines, the RBE4 line has shown great promise as an *in vitro* BBB model (Reichel et al., 2000; Aschner et al., 2002; Yang and Aschner, 2003; Fitsanakis et al., 2006). It is derived from immortalized (with the plasmid pEIA-neo) rat brain microvascular endothelium (Roux et al., 1994; Abbott et al., 1995). Immortalized RBE4 cells express enzymes and transporters localized in the BBB endothelium, with similar characteristics to those noted *in vivo* (Roux and Couraud, 2005). These cells were used in a wide range of studies including transferrin receptor-mediated (Huwlyer et al., 1999), 3,4-dihydroxy-L-phenylalanine (Sampaio-Maia et al., 2001), choline (Friedrich et al., 2001) and serotonin transport (Brust et al., 2000), and P-glycoprotein activity (Bendayan et al., 2002). Using this model, Fitsanakis et al. (2006) characterized various aspects of Mn transport.

Brain tissue/cells are dependent on glucose-derived oxidative metabolism not only for energy needs, but also for the synthesis of tricarboxylic acid (TCA) cycle-related neurotransmitters, such as glutamate, aspartate, GABA, and acetylcholine (Clark and Lai, 1989). One proposed mechanism of Mn neurotoxicity is energy failure due to Mn inhibition of ATP synthesis. This Mn-induced impairment in energy metabolism could be attributed to decrease in the activities of enzymes of glucose oxidative metabolism. Interestingly, Mn is taken up by mitochondria via its high affinity for the calcium uniporter (Gavin et al., 1990; Gavin et al., 1999) and preferentially accumulates in these organelles. Enzymes located in the mitochondrial matrix are known to be vulnerable to Mn toxicity and inhibition of the TCA cycle enzyme, aconitase, has been suggested as one of the mechanisms underlying Mn neurotoxicity (Zheng et al., 1998). It was also demonstrated that Mn can directly inhibit ketoglutarate dehydrogenase complex activities in both liver and brain mitochondria and induce a dose-related lowering of the activities of two other TCA cycle enzymes, namely citrate synthase and malate

dehydrogenase, further corroborating that mitochondria constitute a major subcellular target for Mn toxicity (Lai et al., 1999). Thus, Mn exerts inhibitory effects on TCA cycle (Lai et al., 1999; Malthankai et al., 2004) and on oxidative phosphorylation (Gavin et al., 1992).

Mitochondria are an important cellular source of ROS and are highly susceptible to oxidative damage. Under resting conditions between 2 and 5% of molecular oxygen consumed by mitochondria is partially reduced by the electron transport chain to form superoxide and subsequently hydrogen peroxide. Elevated mitochondrial ROS generation can inhibit one or more of the components of the respiratory chain, further accelerating the rate of superoxide formation (Turrens and Boveris, 1980), ultimately leading to cell death (Diemer et al., 2003). Interestingly, Mn preferentially localizes within these organelles (Liccione and Maines, 1988; Brown and Taylor, 1999), reflecting its high affinity for the calcium uniporter (Gavin et al., 1990; Gavin et al., 1999). Moreover, superoxide produced in the mitochondrial electron transport chain (ETC) catalyzes the transition shift of Mn^{2+} to Mn^{3+} through a set of reactions analogous to those mediated by SOD, resulting in increased oxidant capacity of Mn (HaMai and Bondy, 2004; Gunter et al., 2006).

Oxidative damage is a consequence of excessive oxidative stress, insufficient antioxidant potential or a combination of both (Chapple, 1997). High ROS levels produce tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation, protein oxidation and depletion of cellular thiols (Halliwell and Gutteridge, 2005). Several cellular antioxidant systems afford protection against free radical damage including α -tocopherol, ascorbic acid, glutathione (GSH) as well as antioxidant enzymes, such as glutathione peroxidase (GPx) and superoxide dismutase (SOD). When ROS levels exceed the cellular redox capacity, cells are subjected to oxidative stress ensuing in cell damage. A delicate balance exists between cell death and survival, in that oxidative stress pushes the system toward damage while antioxidant defence mechanisms promote survival (Schulz et al., 2000; Sayre et al., 2008). GSH is the most abundant cellular antioxidant (Mytilineou et al., 2002) and the principal thiol for maintaining optimal redox balance.

Prostaglandin- F_2 -like compounds, termed F_2 -isoprostanes (F_2 -IsoPs), are a complex family of compounds produced from arachidonic acid via a free radical catalyzed mechanism (Morrow et al., 1990). The measurement of F_2 -IsoPs is the most reliable approach to assess oxidative stress status *in vivo* and can be used as authentic biomarkers of lipid peroxidation (Basu, 2004; Montuschi et al., 2004; Davies et al., 2007). Another consequence of increased oxidative stress is the induction of the mitochondrial permeability transition (MPT), a Ca^{2+} -dependent process characterized by the opening of the permeability transition pore in the inner mitochondrial membrane (Jacobson and Duchen, 2002; Keil et al., 2006), which consequently leads to a collapse of the mitochondrial inner membrane potential and reduced ATP generation (Rama Rao and Norenberg, 2004).

While the precise mechanism of Mn-induced neurotoxicity remains to be fully defined, altered bioenergetics and oxidative stress are known critical factors in mediating its pathogenesis. Consistent with these effects Mn accelerates the oxidation of dopamine and other catecholamines and concurrently amplifies ROS generation (Ali et al., 1995; Sloot et al., 1996). Furthermore, divalent Mn catalyzes Fenton-like reactions generating hydroxyl radical, proteolytic degradation and protein turnover (Wedler, 1993; HaMai and Bondy, 2004).

Virtually nothing is known about Mn's effects on capillary endothelial cells and the potential for these cells to contribute to Mn-induced neurotoxicity. Accordingly, the principal aims of our study were to extend our previous observations and determine if endothelial cells of the BBB are directly targeted by Mn and to investigate the underlying mechanism of cytotoxicity using the following endpoints of toxicity: i) reduction of mitochondrial membrane potential as

a predictive biomarker of oxidative stress; ii) increased F₂-IsoPs as indicators of lipid peroxidation, resulting from increased ROS generation and iii) redox (GSH) status as a mediator as cellular viability with the decrease of GSH as a potential indicator of the increase of ROS. A series of studies was carried out to address Mn's ability to induce ROS, alter mitochondrial membrane potential and determine the role of GSH in modulating Mn-induced endothelial cells toxicity.

2 - Results

BSO and DEM potentiate Mn-induced RBE4 cell death

Fig. 1 and Fig. 2 depict the effects of MnCl₂ or MnSO₄ (200 or 800 μM) on RBE4 cell viability (measured by the MTT). A significant concentration-dependent decrease ($p < 0.05$ for the 200 and $p < 0.001$ for the 800 μM) in cell viability was noted for cells exposed to MnCl₂ or MnSO₄ for 4 or 24h compared with controls.

The effect of MnCl₂ or MnSO₄ (200 and 800 μM) was also evaluated after 24h pre-treatment with DEM (600 μM) or BSO (1mM). As shown in Fig. 1A, in cells exposed to MnCl₂ for 4 h immediately after DEM or BSO, cell viability was indistinguishable from cells exposed to MnCl₂ (200 or 800 μM). Cells exposed under similar conditions to MnSO₄ (Fig. 1B) responded in a manner analogous to cells exposed to MnCl₂.

As shown in Fig. 2A, RBE4 cell viability was significantly reduced ($p < 0.001$) when exposure to Mn (200 or 800 μM) was extended to 24h pre-treatment with DEM (compared with controls and MnCl₂ alone). Similarly, 24h pre-treatment with BSO prior to MnCl₂ treatment (24h) was associated with a significant reduction in cell viability ($p < 0.05$) compared with controls, but the effect was limited to the higher MnCl₂ concentration (800 μM compared with MnCl₂ alone). Similar trends and effects were noted for treatments of RBE4 cells with MnSO₄ alone and after DEM or BSO pre-treatment (Fig. 2B).

As discussed above, in initial experiments we used 2 divalent Mn salts, the chloride and the sulphate in order to ascertain whether different physical-chemical properties might influence the extent of cytotoxicity in RBE4 cells (Fig. 1 and Fig. 2). As no significant differences between the two salts were noted in the viability assays, in further experiments we solely focused on the chloride salt. Notably, the chloride salt solution has higher water solubility (*vs.* the sulphate salt) and it has been more extensively used in experimental Mn models, both *in vivo* (Weber et al., 2002; Zhang et al., 2003) and *in vitro* (Rama Rao and Norenberg, 2004; Milatovic et al., 2007; Yin, et al., 2008).

Determination of F₂-IsoPs in RBE4 cells exposed to MnCl₂

As shown in Fig. 3, in cells exposed for 4h to MnCl₂ (200 or 800 μM), there was a significant ($p < 0.05$) and concentration-dependent increase in F₂-IsoPs levels compared with controls. The effect of MnCl₂ on F₂-IsoPs was more pronounced after 4h exposure, yet remained elevated and statistically significant *vs.* controls ($p < 0.05$) also after 24h exposure to MnCl₂.

Measurement of mitochondrial membrane potential

Exposure of RBE4s to MnCl₂ (200 or 800 μM) for 2h led to a significant decrease ($p < 0.001$) in mitochondrial membrane potential compared with controls (Fig. 4A and B). These results are consistent with those obtained on cell viability and F₂-IsoPs (Fig. 1, Fig. 3, and 5). Further decrease in fluorescence was noted in cells exposed to MnCl₂ ($p < 0.001$) plus DEM. As shown in Fig. 4A and B, DEM treatment alone had no effect on TMRE fluorescence in RBE4s when compared with controls. MnCl₂, both at 200 or 800 μM led to a significant decrease in TMRE

fluorescence when compared with controls. This effect was more pronounced and significant ($p < 0.001$) in RBE4s treated for 2h with $MnCl_2$ plus DEM (at both Mn concentrations).

3 - Discussion

Given its essentiality in multiple metabolic functions, Mn homeostasis is vital for the optimal CNS functioning. Accordingly, its transport across the BBB is tightly regulated. However, the potential for endothelially localized Mn to elicit neurotoxicity and modulate barrier function has yet to be reported. The present study extends upon our earlier report (Marreilha dos Santos et al., 2008) on the sensitivity of the BBB to Mn, providing evidence Mn affects endothelial cell redox status and mitochondria function.

The mechanism of Mn toxicity is not fully understood, yet altered bioenergetics and oxidative stress play a critical role in its neurotoxicity (Zheng et al., 1998; Malecki, 2001; Milatovic et al., 2007). Moreover, it was hypothesised that Mn toxicity is associated with mitochondrial dysfunction given its propensity to preferentially accumulate in the mitochondrial matrix (Liccione and Maines, 1988; Brown and Taylor, 1999), reflecting upon its high affinity for the calcium uniporter (Gavin et al., 1990; Gavin et al., 1999). Since cultured RBE4 cells preserve the characteristics of brain endothelial cells offering a unique *in vitro* BBB model for studying both solute transport and permeability (Rist et al., 1997; Aschner et al., 2002; Roux and Couraud, 2005), we specifically addressed whether Mn-induced endothelial toxicity is associated with compromised redox status.

As shown in Fig. 1 and Fig. 2, our studies establish that Mn chloride and Mn sulphate-induced ROS generation leads to loss of mitochondrial integrity and redox status in RBE4 cells. These effects are concentration- and time-dependent, and are further augmented when intracellular GSH levels are depleted (Fig. 1 and Fig. 2). After 24h of Mn exposure, a trend towards increased toxicity at the higher concentrations (800 μM) was observed for both the Mn chloride and sulphate salts. These results are consistent with earlier observations in other cell types, corroborating that Mn toxicity is dependent upon intracellular GSH levels and that impairment of mitochondrial function is a key mechanism for Mn toxicity (Desole et al., 1997; Stredrick et al., 2004; Dukhande et al., 2006). However, several other mechanisms can be invoked to explain Mn-induced mitochondrial dysfunction. For example, Mn^{2+} can substitute for Fe^{2+} in cytochromes of the cellular respiratory chain (with structure similar to haemoglobin; Missy et al., 2000), leading to incomplete reduction of O_2 and the formation of free radicals and oxygenated compounds such as O_2^- and H_2O_2 . Mn was also shown to directly inhibit enzymes of the mitochondrial ETC (Husain et al., 1976; Singh et al., 1979). It might be expected that this inhibition would also enhance the rate of radical production, thereby aggravating the effects on ETC inhibition. The increase in radical production would be exacerbated by the Mn-induced reduction in GPx and SOD activities (Liccione and Maines, 1988).

Our study also established that BSO and DEM pre-treatment potentiate the effects of both Mn salts on RBE4 cells, with more pronounced effects in DEM pre-treated cells. While BSO inhibits the rate-limiting enzyme of GSH synthesis γ -glutamylcysteinyl synthetase, it does not eliminate the antioxidant activity of this thiol (Meister, 1995). In contrast, DEM is a GSH depletor both *in vivo* and *in vitro*, given its reaction with the thiol group of GSH (Plummer et al., 1981; Deneke and Fanburg, 1989; Meister 1991). Furthermore, BSO is more poorly transported across the BBB compared with DEM (Jain et al, 1991). Accordingly, the ability of DEM to exert a greater effect on Mn-induced cellular death is consistent with its increased potency in reducing intracellular GSH levels. Analogous to observations in other cell types, Mn toxicity in RBE4 cells is also dependent on the cellular redox status (Stredrick et al., 2004), corroborating the role of ROS generation as a primary mechanism for Mn-induced

toxicity. Concerning the effects of BSO and DEM on cell viability of RBE4s, no significant difference was observed after comparing with control cells (data not shown).

We also directly evaluated the ability of $MnCl_2$ to induce ROS formation in mitochondria of RBE4 cells by measuring lipid peroxidation rates and the collapse of the mitochondrial inner membrane potential. Major mitochondrial targets of ROS are the polyunsaturated fatty acids and protein components of the radical chain reaction (Cadenas and Davies, 2000). To our knowledge, this report is the first to establish increased oxidative stress/injury in RBE4 cells by measuring F_2 -IsoPs, the most sensitive biomarker of oxidative stress (Kadiiska et al., 2005). Measurements of F_2 -IsoPs have several advantages over other quantitative markers of oxidative stress. They are chemically stable, and substantially increase in various animal models of oxidant injury (Montuschi et al., 2004). Upon exposure of RBE4s to $MnCl_2$ (200 or 800 μM) F_2 -IsoPs levels increased peaking at 4h post exposure (107% and 193% increase for 200 and 800 μM , respectively), and declining after 24h (yet remaining significantly elevated, with $p < 0.05$, compared with controls, Fig 3). These results are consistent with observations in astrocytes (Milatovic et al., 2007), where following exposure to low $MnCl_2$ concentration (100 μM), levels of F_2 -IsoPs were elevated at 2h, but indistinguishable from controls at 6h post-treatment. Corroborative evidence reflective of this pattern is also noted in a rat carbon tetrachloride in vivo model, with F_2 -IsoPs levels peaking 4h after treatment and remaining significantly elevated albeit lower at 24h (Morrow et al., 1992; Basu, 1999; Morrow and Roberts, 1999; Sodergren et al., 2001; Kadiiska et al., 2005).

The centrality of mitochondria in Mn-induced damage was further evaluated by direct measurements of the mitochondrial membrane potential, representing a common final pathway in many conditions associated with oxidative stress (Keil et al., 2006). Increased ROS formation and a subsequent change in mitochondrial permeability transition reflect a Ca^{2+} -dependent process characterized by the opening of the permeability transition pore in the inner mitochondrial membrane. This process results in increased permeability to protons, ions and solutes (Zoratti and Szabo, 1995; Rama Rao and Norenberg, 2004), leading to collapse of the mitochondrial inner membrane potential. In turn, colloid osmotic swelling ensues with movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis and further generation of ROS, thus unabatedly feeding forward a vicious cycle. Fig. 4 A and B show a significant decrease in the mitochondrial membrane potential after exposure of RBE4s to $MnCl_2$ (200 and 800 μM) for 2h vs. control cells. This effect is more pronounced in RBE4 cells treated with $MnCl_2$ after GSH depletion (co-exposure with DEM at both concentrations).

Mn exposure also leads to a concentration-dependent decrease in TMRE fluorescence indicative of mitochondrial membrane potential loss. The mechanisms mediating Mn-induced opening of the mitochondrial permeability transition pore have yet to be defined. However, increased production of ROS and associated oxidative stress are generally considered major contributing factors. Other studies in astrocytes, cerebellar granule neurons and PC12 cells (Seyfried et al., 1999; Wullner et al., 1999; Rama Rao and Norenberg, 2004; Milatovic et al., 2007; Yin et al., 2008;) have shown that depletion of mitochondrial and cytoplasmic GSH result in increased generation of ROS, disruption of the mitochondrial membrane potential and rapid loss of mitochondrial function. In addition, Zhang et al. (2004), showed that high levels of $MnCl_2$ (1mM) caused a significant dissipation of the mitochondrial membrane potential in isolated rat brain mitochondria consistent with alteration of the mitochondria permeability transition. Notably, an association between defects in mitochondrial energy production and neurodegenerative diseases is well established (Wallace et al., 1992; Blass and Gibson, 1999; Castellani et al., 2002; Ogawa et al., 2002) and these deficits are associated with ROS damage to the mitochondrial genome. Given that many metabolically active cell types, particularly tonically active motor neurons such as those in the substantia nigra and globus

pallidus, two areas with propensity to accumulate Mn as well as endothelial cells require high levels of ATP to optimally function and survive (Klockgether et al., 1989) the effects of Mn would render these areas and cells, respectively, especially susceptible to ROS.

In summary, we extended our previous observations, showing that endothelial cells are a target of Mn-induced cytotoxicity and mitochondrial dysfunction mediates its toxic effect. In this study, we investigated oxidative stress as a possible mechanism for Mn toxicity, addressing the relationship between Mn cytotoxicity and biomarkers of oxidative stress namely GSH levels, F2-Isopts and mitochondrial membrane potential. Decreased cell viability in cells with compromised GSH status (induced by BSO and DEM) along with the increase in F2-Isopts (a marker of lipid peroxidation), corroborate the role of ROS generation as a key mediator of Mn toxicity in RBE4 cells. In addition, the centrality of mitochondria in Mn-induced damage was further evaluated by direct measurements of the mitochondrial membrane potential, representing a common final pathway in many conditions associated with oxidative stress. We suggest that direct Mn-induced injury to mitochondria and the ensuing impairment in their energy metabolism can modify the properties of the BBB and lead to changes in its permeability, which may facilitate the entrance into the brain parenchyma of endogenous and exogenous macro and micro-molecules resulting in brain toxicity. Future focus on direct measurements of BBB permeability upon Mn exposure should be pursued to determine whether injury to the BBB is associated with changes in nutrient and macromolecular transport from blood to brain.

4 - Experimental Procedure

Chemicals

Manganese chloride ($\text{MnCl}_2 \cdot \text{H}_2\text{O}$), manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), buthionine sulfoximine (BSO), dimethyl maleate (DEM) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO); heat inactivated fetal bovine serum (FBS), penicillin/streptomycin, alpha medium, F-10 Nutrient Mixture, penicillin/streptomycin and basic fibroblast growth factor from Gibco/Invitrogen, geneticin (G418) were purchased from Boehringer Mannheim (Indianapolis, IN) and tetramethylrhodamineethyl ester (TMRE) was purchased from Invitrogen (Carlsbad, CA).

RBE4 cell culture and treatment

RBE4 cells were grown in T-75 collagen I-coated flasks until confluent. Cells were bathed in alpha-type medium containing 50% (v/v) alpha minimum essential medium (MEM), with 50% F-10 Nutrient Mixture supplemented with penicillin/streptomycin (100 U/ml), 10% heat-inactivated fetal bovine serum, 300 $\mu\text{g}/\text{ml}$ geneticin (G418) and basic fibroblast growth factor (1 ng/ml). The cultures were maintained in a humidified 37°C, 5% CO_2 air incubator. The medium was changed every 2 days. Upon confluence, the cells were transferred to 96-well collagen-I coated plates where they were assayed. The experiments were conducted at confluence with cell densities between 10^5 and 10^6 cells per well.

Exposure protocol of RBE4 cells to MnCl_2 or MnSO_4 and MTT assay

Confluent 96-well plates of RBE4s were treated with 1000 μM BSO or 600 μM DEM for 24h, and then exposed to MnCl_2 or MnSO_4 solutions (200 and 800 μM) for 4 or 24h (in fresh medium absent BSO or DEM). Other cells were treated with Mn salts for 4 or 24h in the absence of BSO or DEM treatments. After the exposures, viability was evaluated by the MTT assay according to the manufacturer's protocol (Sigma). After the various experimental treatments, and at the end of the respective incubation periods, 100 μl of MTT solution was added to each well, followed by incubation of the plates for 4h at 37°C in a CO_2 incubator. The reaction was terminated by removal of the media, and 20 minutes shaking at 70 rpm/min addition after 100

μ l of HCl/isopropanol to each well. Levels of reduced MTT were determined by measuring the absorbance at 570 nm using a plate reader (Zenith 3100). Each experiment was independently repeated 3 times with a minimum of 3 plates/experiment.

Determination of F₂-IsoPs

Cells were plated in 6-well collagen-I coated plates. At confluence, the cells were treated with MnCl₂ and MnSO₄ (200 and 800 μ M), for 4 or 24h. Next, cells were resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated and then subjected to chemical saponification using 15% KOH to hydrolyze bound F₂-IsoPs. The cell lysates were adjusted to a pH of 3, followed by the addition of 0.1 ng of 15-F_{2 α} -IsoPs-d4 internal standard. F₂-IsoPs were subsequently purified by C18 and silica Sep-Pak extraction by thin layer chromatography. Next, pentafluorobenzyl ester, a trimethylsilyl derivative was added and the cells were analyzed by gas chromatography, negative ion chemical ionization-mass spectrometry. Gas chromatography (GC) was performed using a 15 m, 0.25 mm diameter, 0.25 μ m film thickness, DB1701 fused silica capillary column. The column temperature was programmed from 200 to 300°C at 20°C/min after the first minute. Methane was used as the carrier gas at a flow rate of 1 ml/min. The ion source temperature was maintained at 250°C, electron energy at 70 eV, and filament current at 0.25 mA. Negative ion chemical ionization (NICI) mass spectrometry (MS) was performed with a Hewlett Packard HP5989A GC/MS instrument interfaced with an IBM Pentium III computer system (Morrow and Roberts, 1999). Protein content of RBE4 cells was measured by the method of Bradford (1976) with a Bio-Rad Protein Assay kit (Bio-Rad Lab, Richmond, CA). Bovine serum albumin was used as the standard. Each experiment was performed in duplicates and independently repeated three times.

Evaluation of mitochondrial membrane potential

The mitochondrial membrane potential was measured with the fluorescent dye TMRE (Patenaude et al., 2004). At confluence, RBE4 cells grown in 6-well collagen coated plates were treated with MnCl₂ (200 and 800 μ M) alone and MnCl₂ (same concentrations) plus DEM, for 2 h. Next, the culture medium was removed and the cells were loaded for 30 min with TMRE at a final concentration of 50 nM (in HEPES buffer at 37°C in a 5% CO₂ incubator). At the end of treatment, cells were rinsed with PBS and examined with a Zeiss inverted fluorescent microscope (Zeiss Axiovert S100, Carl Zeiss MicroImaging Inc.) equipped with a cooled digital camera (Photometrics CoolSNAP, Roper Scientific Photometrics, Tucson, AZ). Images were captured at 10 \times magnification with a digital camera. Fluorescent intensities were calculated in 8 randomly selected fields per experiment and were analysed with the NIH software (Scion Incorporation, Frederick, MD). In each image field, the total number of pixels was quantified on a gray scale (0255 counts) and the mean pixel value in each image field was obtained and expressed as means \pm SEM. The fluorescent intensities were expressed as percent fluorescence change over control. Each experiment was performed in duplicate plates and independently repeated three times.

Statistical Analysis

All data from the experiments were presented as mean \pm SEM of three to four independent experiments. The data were analysed by one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison test with the statistical significance set at $p < 0.05$. The Bonferroni test was used to correct for multiple comparisons and assure the strictest analyses. All analyses were carried out using Graph Pad Prism 4.02 for Windows (Graph Pad Software, San Diego, CA, USA).

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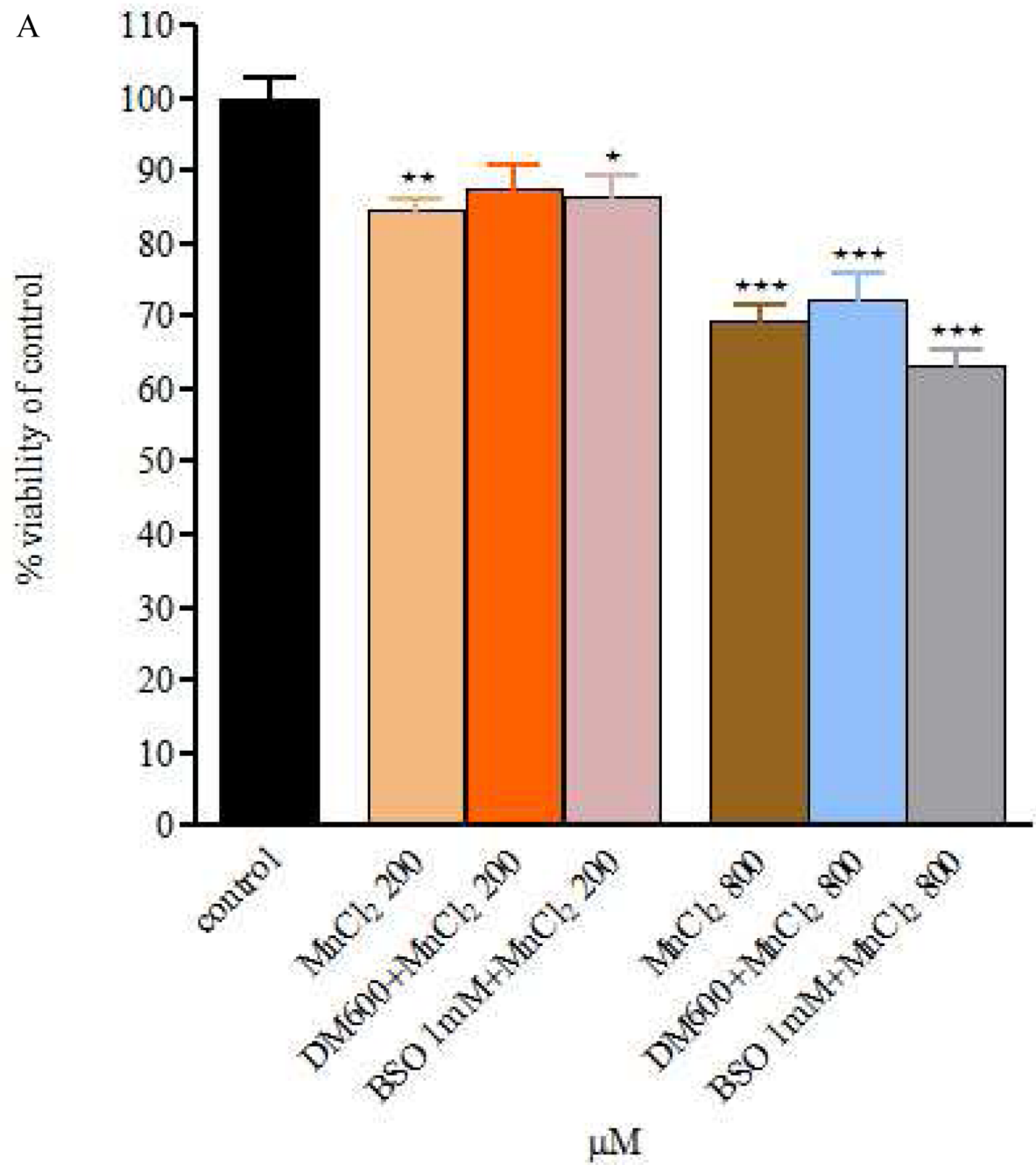
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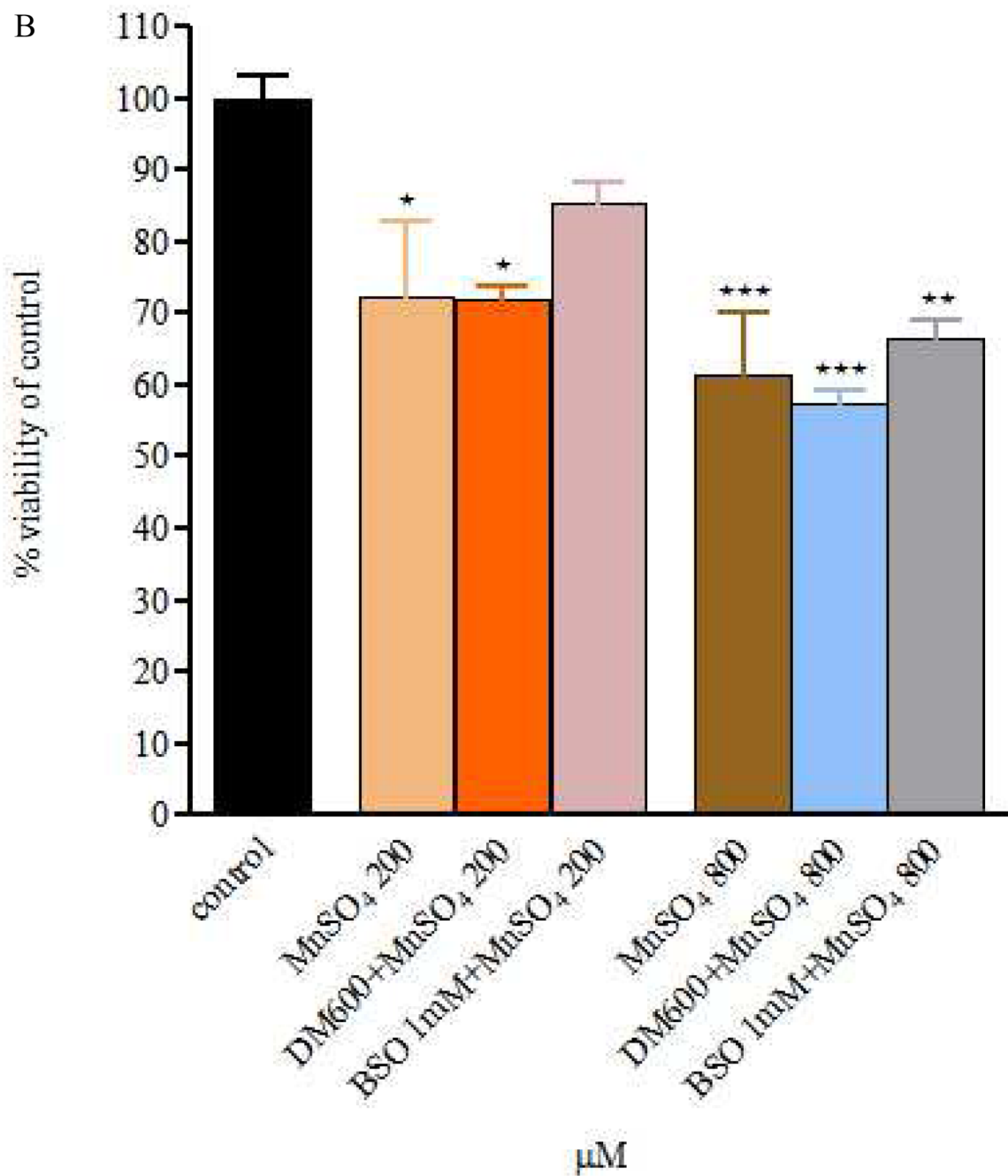
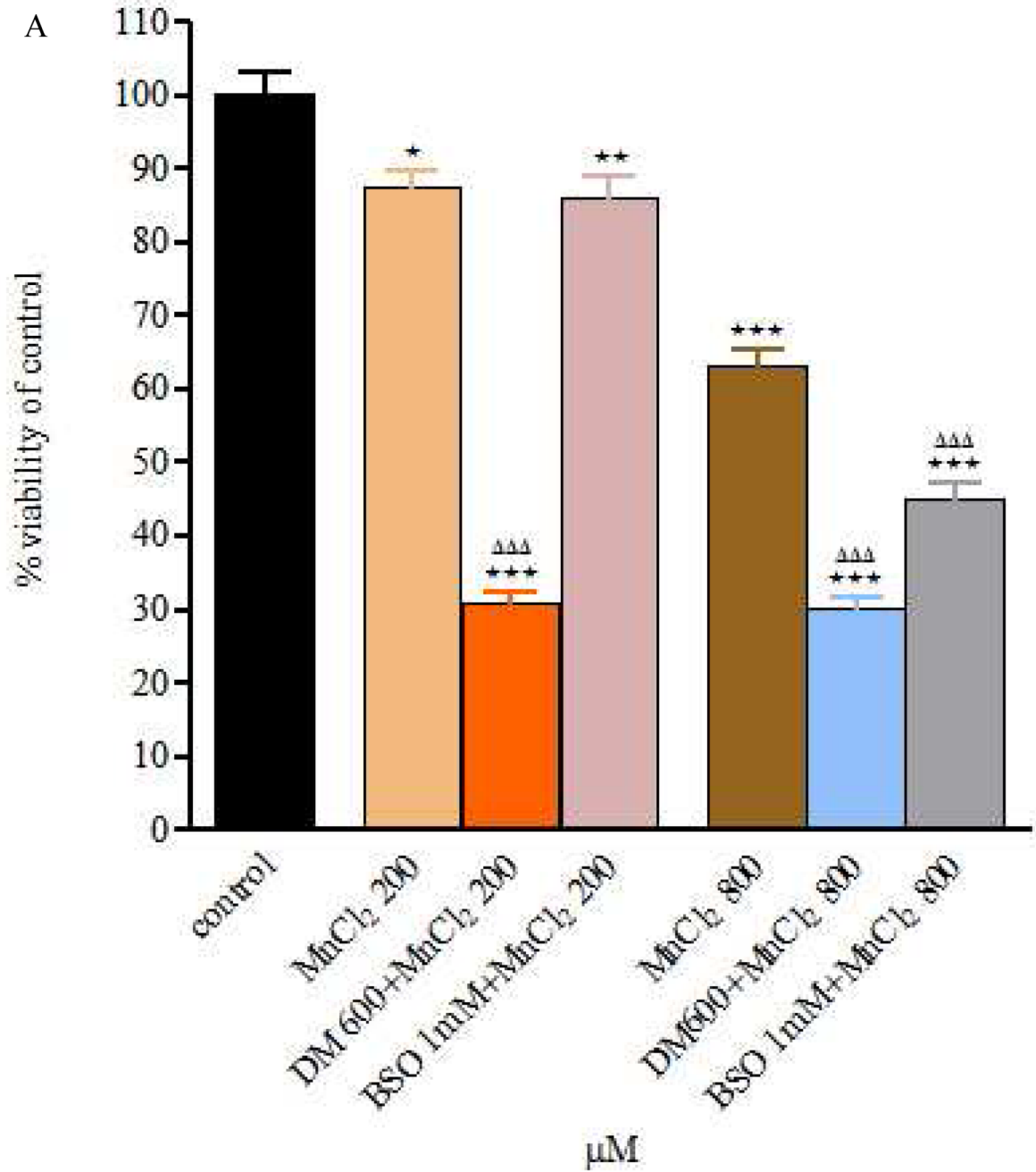


Figure 1.

Figure 1 A and B – Viability of RBE4 cells exposed to MnCl₂ or MnSO₄ during 4h (200 and 800 μM), after a 24h DEM 600 μM and BSO 1mM treatment. Values are expressed as means ± SEM from 3 independent experiments. * p< 0.05, ** p< 0.01, *** p< 0.001 compared to control; There are no statistical significant differences among the treatment groups.



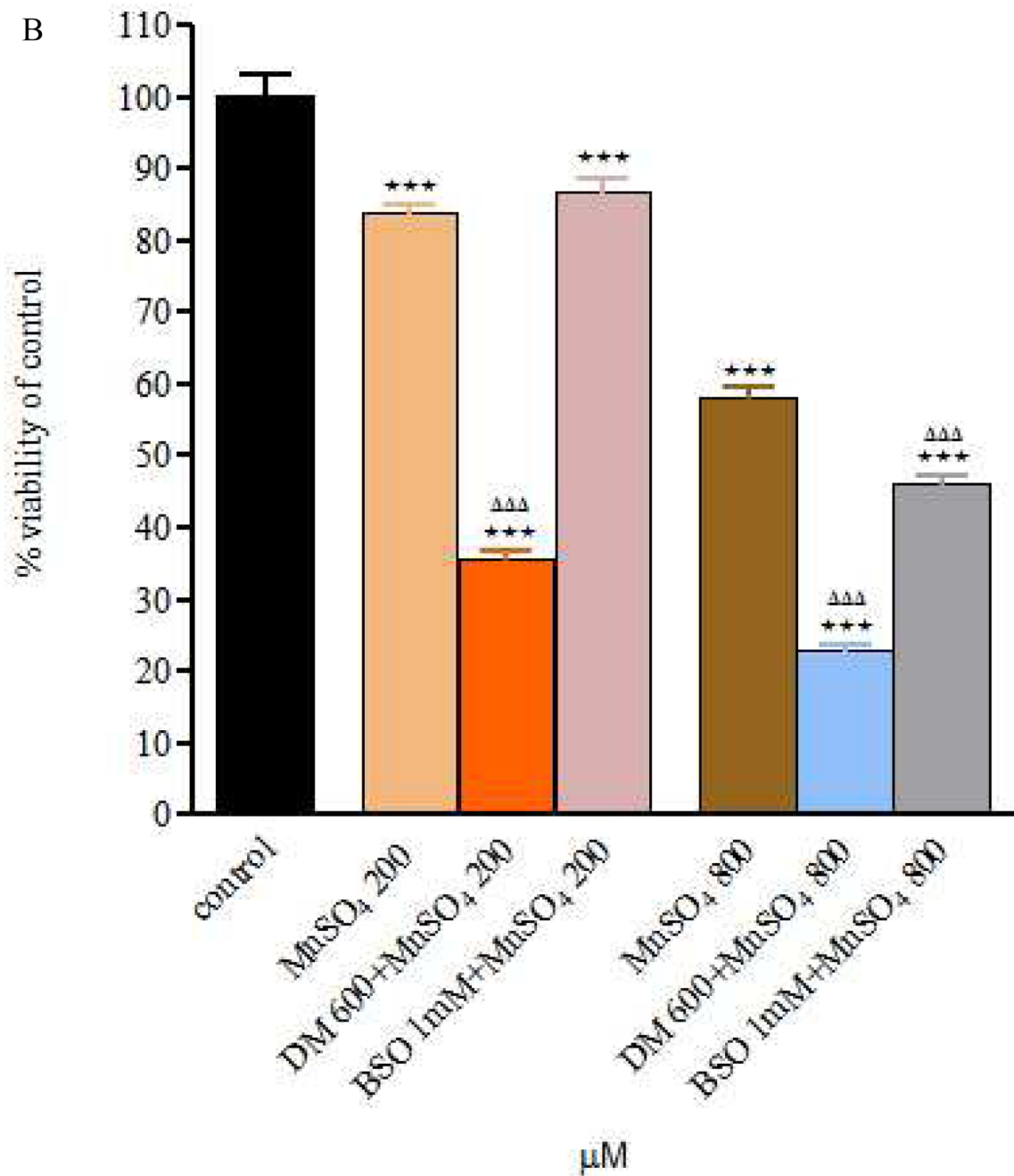


Figure 2.

Figure 2 A and B – Viability of RBE4 cells exposed to MnCl₂ or MnSO₄ during 24h (200 and 800 μM), after a 24h DEM 600 μM and BSO 1mM treatment. Values are expressed as means ± SEM from 3 independent experiments. * p<0.05, ** p<0.01, *** p<0.001 compared to control; ΔΔΔ p<0.001 compared to Mn 200 in Mn 200 μM group or Mn 800 in Mn 800 μM group, respectively.

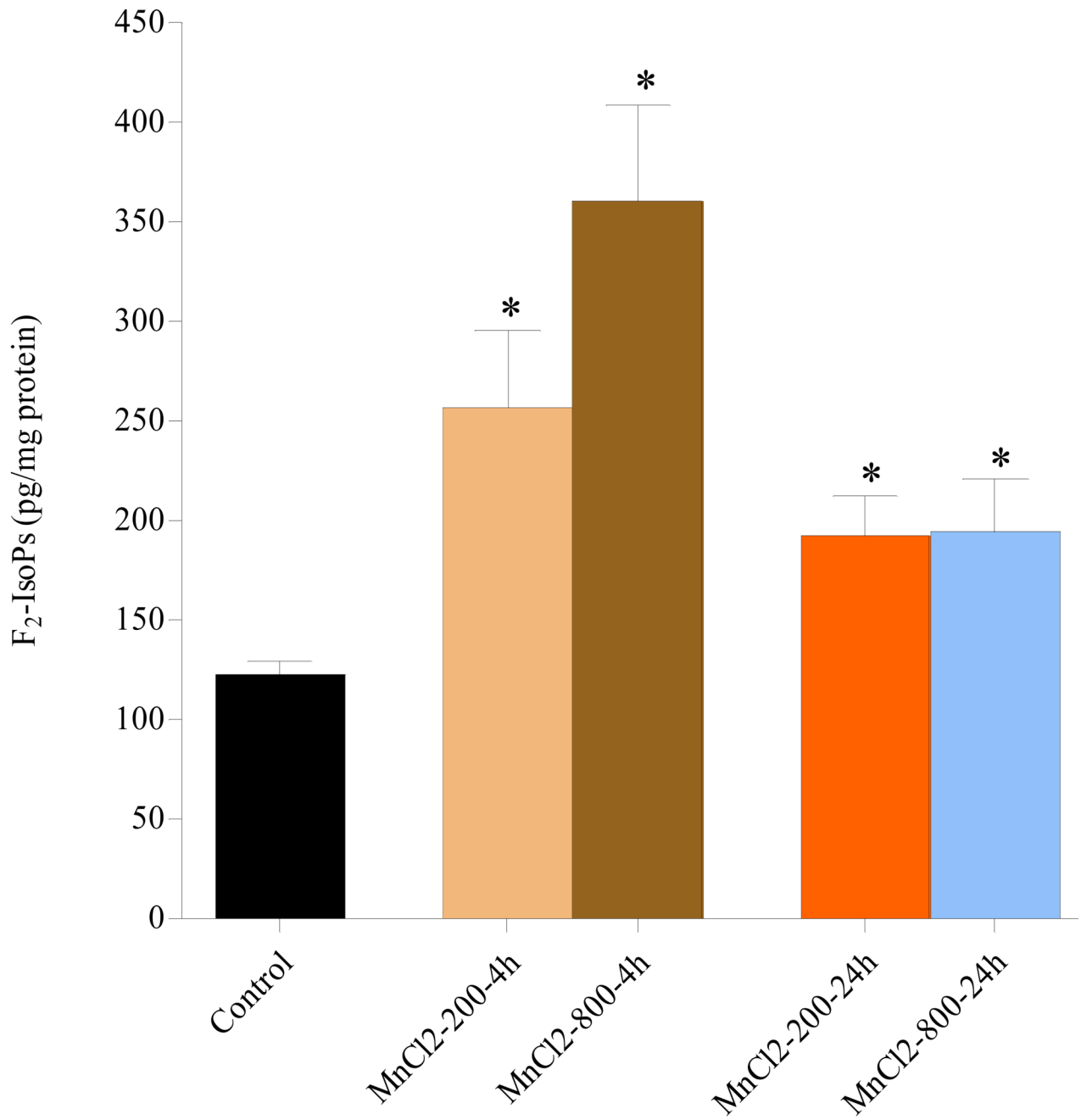
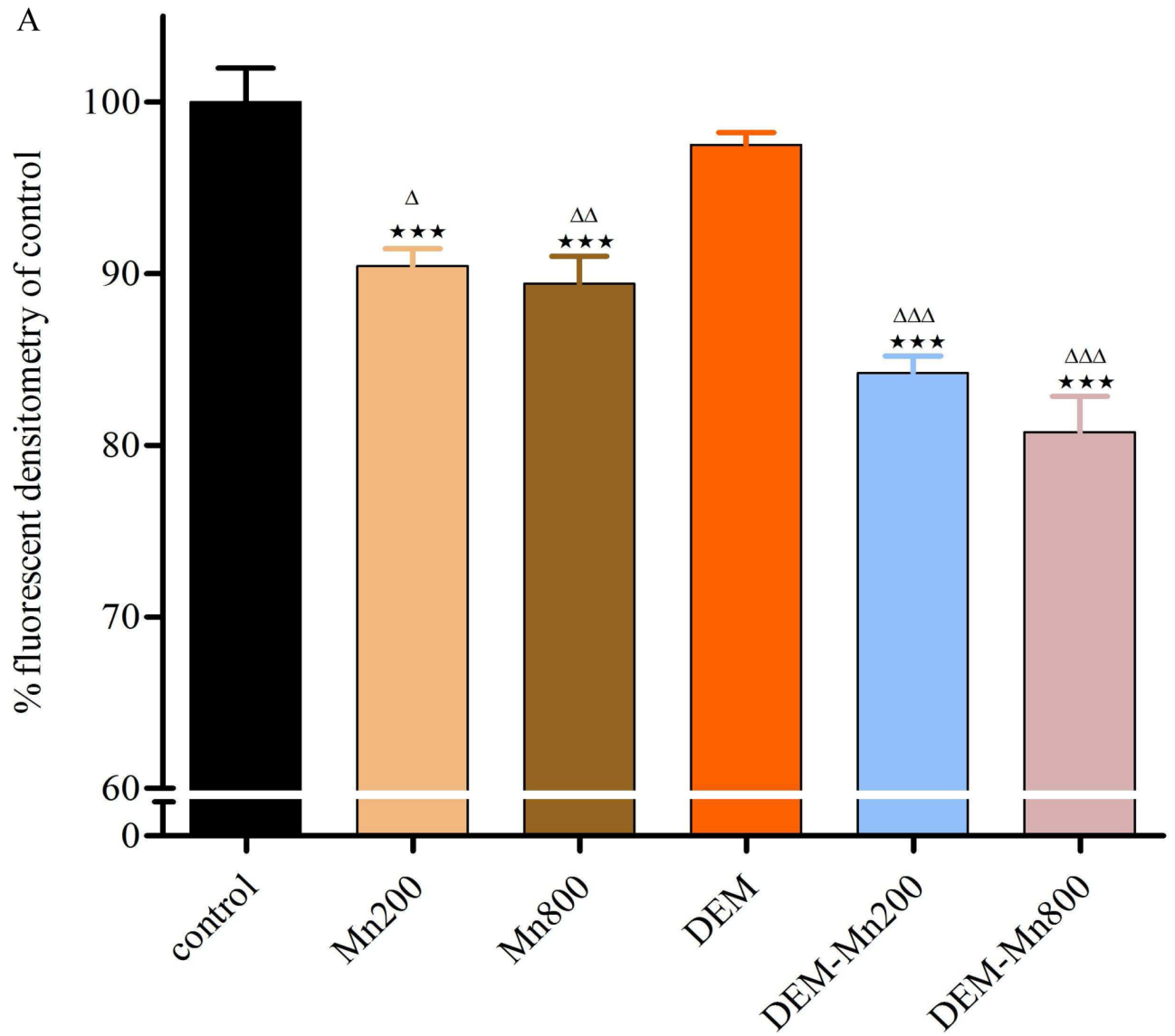


Figure 3. Quantitation of F₂-IsoPs levels in RBE4 cells exposed to MnCl₂ 200 and 800 μ M for either 4 or 24h. * $p < 0.05$ compared to control.



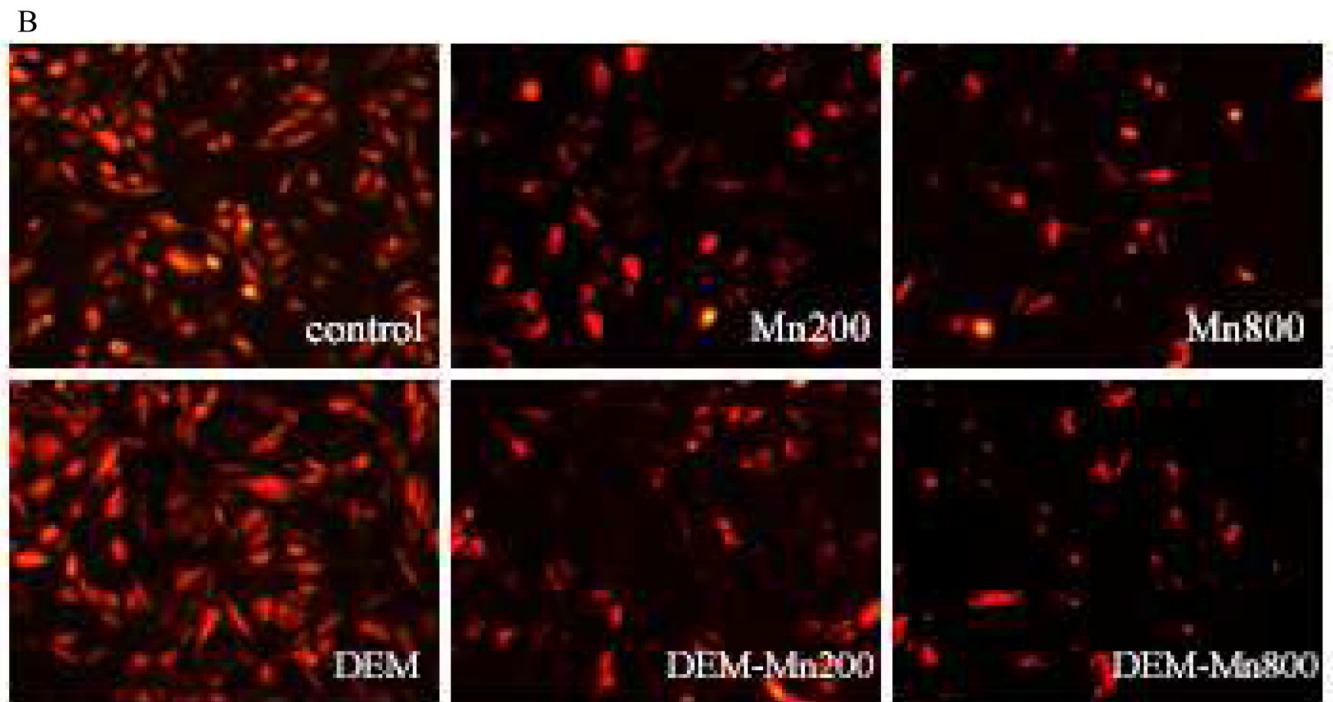


Figure 4.
Figure 4 A and B – Quantitation of TMRE fluorescent intensities. Cultured RBE4 cells exposed to Mn at various concentrations (200 and 800 μ M) with/without DEM for 2h. Values are expressed as mean \pm SEM of 12 random fields in each group from 3 independent experiments. *** $p < 0.001$ vs. control; Δ $p < 0.05$, $\Delta\Delta$ $p < 0.01$, $\Delta\Delta\Delta$ $p < 0.001$ vs. DEM; $\blacktriangle\blacktriangle$ $p < 0.01$ vs. Mn 200 or Mn 800 μ M.