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ORIGINAL PAPER

Differentiation-Dependent Effects of a New Recombinant Manganese Superoxide Dismutase on Human SK-N-BE Neuron-Like Cells

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Abstract

We have recently isolated a new isoform of recombinant manganese superoxide dismutase (*rMnSOD*) which provides a potent antitumor activity and strongly counteracts the occurrence of oxidative stress and tissue inflammation. This isoform, in addition to the enzymatic action common to all SODs, also shows special functional and structural properties, essentially due to the presence of a first leader peptide that allows the protein to enter easily into cells. Among endogenous antioxidants, SOD constitutes the first line of natural defence against pathological effects induced by an excess of free radicals. Here, we firstly describe the effects of our *rMnSOD* administration on the proliferant and malignant undifferentiated human neuroblastoma SK-N-BE cell line. Moreover, we also test the effects of *rMnSOD* in the all trans retinoic-differentiated SK-N-BE neuron-like cells, a quiescent "not malignant" model. While *rMnSOD* showed an antitumor activity on proliferating cells, a poor sensitivity to *rMnSOD* overload in retinoid-differentiated neuron-like cells was observed. However, in the latter case, in presence of experimental-induced oxidative stress, overcharge of *rMnSOD* enhanced the oxidant effects, through an increase of H_2O_2 due to low activity of both catalase and glutathione peroxidase. In conclusion, our data show that *rMnSOD* treatment exerts differential effects, which depend upon both cell differentiation and redox balance, addressing attention to the potential use of the recombinant enzyme on differentiated neurons. These facts ultimately pave the way for further preclinical studies aimed at evaluation of *rMnSOD* effects in models of neurodegenerative diseases.

Keywords Recombinant human manganese superoxide dismutase · Oxidative stress · Free radicals · SK-N-BE · Neurodegeneration

Abbreviations

ATRA	All-trans retinoic acid
Aβ	Beta-amyloid peptide
CAT	Catalase
Cy	Cyanine
DHE	Dihydroethidium
Glu	Glutamic acid
GSH-px	Glutathione peroxidase
MQ	Menadione quinone
MTT	3-[3,4-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
ROS	Reactive oxygen species
rMnSOD	Recombinant manganese superoxide dismutase

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Introduction

Oxidative stress is defined as an impaired balance between the production and the elimination of reactive oxygen species (ROS) [1]. These reactive molecules are produced in mitochondria and detoxified by enzymatic and non-enzymatic antioxidant defence systems including superoxide dismutase (SOD) [2], glutathione peroxidase [3] and members of the thioredoxin family [4].

To date, three different classes of SOD have been identified [5], differing in type of metals present at the active site: copper–zinc (Cu, Zn-SOD); iron (Fe-SOD); manganese (MnSOD). The latter, an enzyme located in the matrix of mitochondria, catalyzes the reaction of superoxide to the less reactive hydrogen peroxide [2] and is considered the key enzyme that forms the first line of defence against the ROS produced in the mitochondria itself. Lack of MnSOD causes accumulation of oxidative mitochondrial DNA damage as well as inactivation of respiratory chain and Krebs cycle enzymes [6, 7]. Furthermore, MnSOD can counteract mitochondrial dysfunction and exert an anti-apoptotic function following a highly toxic stimuli such as treatment with ionizing radiation and β-amyloid (Aβ) peptides [8], preventing the output from the mitochondrion of pro-apoptotic signalling such as cytochrome c [9] and Smac/DIABLO [10].

The efficacy of MnSOD in blocking apoptosis induced by inflammatory cytokines, ferrous sulphate and Aβ peptides was also demonstrated [10–12]. Conversely, knockdown of MnSOD in primary neuronal cultures increased excitotoxicity induced by Glu [13]. Finally, it has been reported that MnSOD enzymes control the inflammatory and immune responses by acting on the cell's transcriptional activity [14]. Some pieces of evidence indicate that SOD treatment could be an effective preventive strategy to alleviate inflammatory, infectious, respiratory, metabolic and cardiovascular diseases, giving rise to the question of its mechanism of action in these different pathological contexts. In particular, it is well known that the central nervous system is very sensitive to oxidative stress and recent findings show that selected brain regions (e.g. cortex, hippocampus) are particularly susceptible to a neurochemical challenge due to oxidative stress [15]. So, pharmacological use of antioxidants could be a tool to modulate neurodegenerative phenotypes with a wide spectrum of applications. Recently, the study of SOD led to the creation of a new isoform of this enzyme, named recombinant manganese superoxide dismutase (*rMnSOD*) [16]. This protein was synthesized from the cDNA of an MnSOD isoform secreted by a cell line of human liposarcoma (LSA), previously reported to secrete factor(s) exhibiting oncotoxic properties [17], which, in addition to the enzymatic action common to all the SODs, also shows special functional and structural properties. In fact, immunocytochemical studies revealed that *rMnSOD*, unlike the native form restricted to mitochondria is also localized in rough endoplasmic reticulum and in secretory vesicles of the LSA cells. Furthermore, according to mass spectrometry and nucleotide sequencing data, *rMnSOD* has a molecular weight (26 kDa) substantially higher than the wild-type form (24 kDa), due to the presence of a first leader peptide [18], which contains a 6-aa sequence that allows the protein to enter into cells, as a molecular carrier [19]. This latter feature considerably reduces the well-known poor bioavailability of exogenous SODs. Administration of *rMnSOD* showed a potent antitumor activity in the MCF7 adenocarcinoma cell line [18], while exhibited poor toxic activity in both MCF10A non-tumorigenic breast epithelial cells and MRC-5 fibroblasts [16]. In addition, *rMnSOD* was found to be radioprotective for normal as well as radiosensitizing tumor cells [20]; a recent study also showed that the *rMnSOD* has a protective effect in cirrhotic rats, decreasing liver fibrosis and portal hypertension [21].

In the present study, we investigated the effects of *rMnSOD* administration on proliferating and malignant human SK-N-BE neuroblastoma cell line and on all-trans retinoic differentiated cells from the same line, that present many neuron-like features.

Materials and Methods

Recombinant MnSOD

The LSA-type MnSOD was isolated from a human liposarcoma cell line and obtained in recombinant form *rMnSOD* as reported by Mancini et al. [16]. *rMnSOD* was diluted in the medium of cultured cells.

Cell Culture and Treatments

Human SK-N-BE cells, kindly supplied by Dr. G. Poiana (Sapienza University, Rome, Italy), were grown as a monolayer in the RPMI 1640 medium with L-glutamine (SIGMA) supplemented with 10% heat-inactivated (v/v) Foetal Bovine Serum (SIGMA), penicillin (100 U/ml, SIGMA), streptomycin (100 µg/ml, SIGMA), in a humidified atmosphere at 37 °C with 5% CO_2. Cell proliferation was estimated counting the total number of cells by hematocytometer. For differentiation, 2.0×10^4 neuroblastoma cells were seeded on coverslips (Menzel-Glaser) previously coated with poly-lysine (SIGMA). After 24 h, growth medium was replaced with RPMI 1640, 5% FBS and 10 µM all-*trans* retinoic acid (ATRA, SIGMA). The concentration of ATRA used is consistent with previous reports to induce differentiation in this cell type [22, 23]. Cells were cultivated up to 10 days, changing differentiation media every 3 days (0, 3, 6, 9).

To mimic in vitro neurodegeneration induced by glutamic acid (Glu), differentiated cells were treated with 0.5 or 1 mM Glu for 24 h in presence or absence of 0.75 µM *rMnSOD*. Differentiated cell cultures were also prepared and pre-treated with 0.75 µM *rMnSOD* for 1 h, then exposed to 50 and 25 µM menadione (MQ) for 1 h.

Measurement of Cell Growth

Undifferentiated cells were cultured in 60 mm dishes starting at an initial density of $1 \times 10^4/cm^2$. For counting, the cells were detached after a previous washing with phosphate-buffered saline (PBS), followed by a 10 min exposure at 37° to a solution of 0.05% trypsin in PBS (pH 7.2), and were then suspended. Cell proliferation was followed by counting the total number of cells in each dish using a haematocytometer chamber. A set of four independent experiments, each performed in triplicate, was carried out.

Measurement of Intracellular ROS

To measure superoxide ($O_2^{\cdot -}$) production in undifferentiated and differentiated cell cultures treated for 24 h with 0.75 µM *rMnSOD*, cells were incubated with 1 µM dihydroethidium (DHE, Molecular Probes) for 15 min at 37 °C [24]. Samples were then analyzed with a fluorescence-activated cell-sorting (FACS) flow cytometer (Becton Dickinson). The median values of fluorescence intensity histograms were used to provide semi-quantitative evaluation of $O_2^{\cdot -}$ production from eight independent experiments. The hydrogen peroxide (H_2O_2) content in cell extracts was determined by the Red Hydrogen Peroxide Assay Kit (Enzo Life Science, cat. N. ENZ-51004), according to manufacturer's instructions. Four independent experiments were performed.

Cell Viability Assay

Cell viability was determined by the mitochondria enzyme dependent reaction of 3-[3,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) both in undifferentiated and differentiated cell cultures [25]. To assess viability of undifferentiated cultures, proliferating SK-N-BE were plated in 24-wells tissue culture plates (density $1 \times 10^4/cm^2$ cells) and allowed to attach overnight. Cultures were treated with *rMnSOD* (at concentration 1.5, 0.75, 0.33 µM) and 20 h later MTT (0.5 mg/ml) was added. To evaluate MTT viability in differentiated neuron-like cultures, SK-N-BE (2×10^3) were plated in six-replicates in 96-well tissue culture plate, and then complete culture medium was replaced by differentiation medium for the following 10 days as described above. After MQ (50 or 25 µM), Glu (0.5 or 1 mM) and *rMnSOD* (0.75 µM) administration, MTT was added for 3 h to the neuron-like cells. We selected a concentration of 0.75 µM *rMnSOD* on the basis of preliminary experiments in which we established the optimal working concentration, as it was able to load cells with the enzyme, without affecting significantly cell viability. Metabolically active cells cleaved the yellow tetrazolium salt MTT to purple formazan crystals. The formazan formed was solubilized and the absorbance measured by a multiplate reader at 570 nm. Results of four independent experiments were expressed as percentage of the different neurodegenerative conditions.

Immunofluorescent Staining

Undifferentiated and ATRA-differentiated SK-N-BE cells were pre-treated with *rMnSOD* 0.75 µM, in complete medium for 3 h and fixed for 10 min or at room temperature in a buffered 4% formaldehyde solution freshly made from paraformaldehyde. Subsequently, cell preparations were incubated for 16 h at room temperature with primary polyclonal antibodies anti-*rMnSOD* leader peptide [21] diluted in PBS (1:100) and anti-GAP-43 monoclonal antibody (1:250, Sigma G9264); Cy2-conjugated anti-rabbit (1:100, Chemicon) and Cy3-conjugated anti-mouse (1:100, Chemicon) secondary antibodies, were incubated respectively for 1 h at room temperature. Nuclei were counterstain with DAPI (SIGMA).

SOD, CAT and GSH-px Activity Assays

The enzymatic activity of SOD (E.C. 1.15.1.1) was determined by quantification of pyrogallol auto oxidation inhibition, as previously described [26]. The results were expressed as units per mg of cell protein. One unit of enzyme activity was defined as the amount of enzyme necessary for inhibiting the reaction by 50% at 25 °C. Auto oxidation of 0.2 mM pyrogallol in air-equilibrated 50 mM Tris–cacodylic acid buffer, pH 8.2, containing 1 mM diethylenetriaminepentaacetic acid was measured by an increase in absorbance at 420 nm. The enzymatic activity of catalase (CAT, E.C. 1.11.1.6) was assayed by Aebi's method, whereby H_2O_2 decomposition to yield water and oxygen is measured at wavelength of 240 nm [27]. Results were expressed as units per mg cell protein. One unit of enzyme activity was defined as the amount of enzyme necessary to decompose 1 µmol of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 10 mM H_2O_2. A set of four independent experiments was performed. Glutathione peroxidase (GPx, EC 1.11.1.9) which catalyzes the reduction of a variety of ROOH and H_2O_2 using GSH as a reducing agent, has been detected according to Awasthi and colleagues [28]. The oxidation of NADPH to $NADP^+$ was followed by a decrease in absorbance at 340 nm, providing a spectrophotometric means for monitoring GSH-px enzyme activity. The rate of decrease in the A_{340} was directly proportional to the GSH-px activity in the sample. Results were expressed as milliunits per mg of protein. One unit is defined as 1 µmol of NADPH oxidized/min.

Image and Statistical Data Analysis on Cell Cultures

Images of fluorescent immunostained cells were carried out using Zeiss fluorescence microscope (Axioplan), and digitized by AxioCam (Zeiss). Statistical analysis was performed by InStat-3 statistical software (GraphPad Software Inc, San Diego, CA, USA) using one-way ANOVA test with Tukey–Kramer's correction for multiple comparisons. Significance was defined as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

rMnSOD Is Internalized by SK-N-BE Cells

In Fig. 1, the effects of 10 days of ATRA treatment on proliferating cells are displayed: after differentiation human neuroblastoma cell line SK-N-BE expressed the GAP-43 protein (E, F), a well-known marker of neural differentiation, which was undetectable in proliferating cultures (B, C). In addition, the neuronal differentiation induced a number of morphological changes, such as branching protrusions, resembling dendritic cell processes.

The following step was to assess if *rMnSOD* was able to enter within cells to perform its function. Both proliferating and ATRA-differentiated SK-N-BE cells were exposed to 0.75 µM *rMnSOD* for 3 h; presence of the recombinant protein was checked by immunocytochemistry using a specific antibody. As show in Fig. 2, a widespread positive staining throughout the cytoplasm and in cell dendrites is clearly evident in both undifferentiated (E) and differentiated cells (K) treated with the *rMnSOD*, indicating that the recombinant protein was able to cross plasmatic membrane and resettle within cells.

rMnSOD Affects Cell Viability in Undifferentiated SK-N-BE

To confirm previous evidences of *rMnSOD in vitro* antitumor activity, actively proliferating cells were treated with different *rMnSOD* concentrations, until 24 h of exposure, and cell viability, toxicity and morphology were analysed. As shown in Fig. 3, 1.5 and 0.75 µM *rMnSOD* treatments were able to induce an evident cell growth arrest of undifferentiated cultures, with a significant cell number reduction, already after 9 h of recombinant protein exposure. Conversely, 0.33 µM *rMnSOD* did not decrease the cell number. MTT assay (Fig. 4) showed a consistent and significant decrease of cell viability, with a clear dose response effect observed 24 h after *rMnSOD* treatment in undifferentiated and differentiated cells. In particular, cell viability of undifferentiated cell culture was significantly lowered by both 1.5 and 0.75 µM *rMnSOD* treatments respect to untreated cultures (-32% $p<0.001$ and -23.4% $p<0.01$, respectively) while no significant effect was observed respect to 0.33 µM *rMnSOD* treatment. Taking into consideration the effect of *rMnSOD* treatments respect to differentiated cultures, only 1.5 µM *rMnSOD*

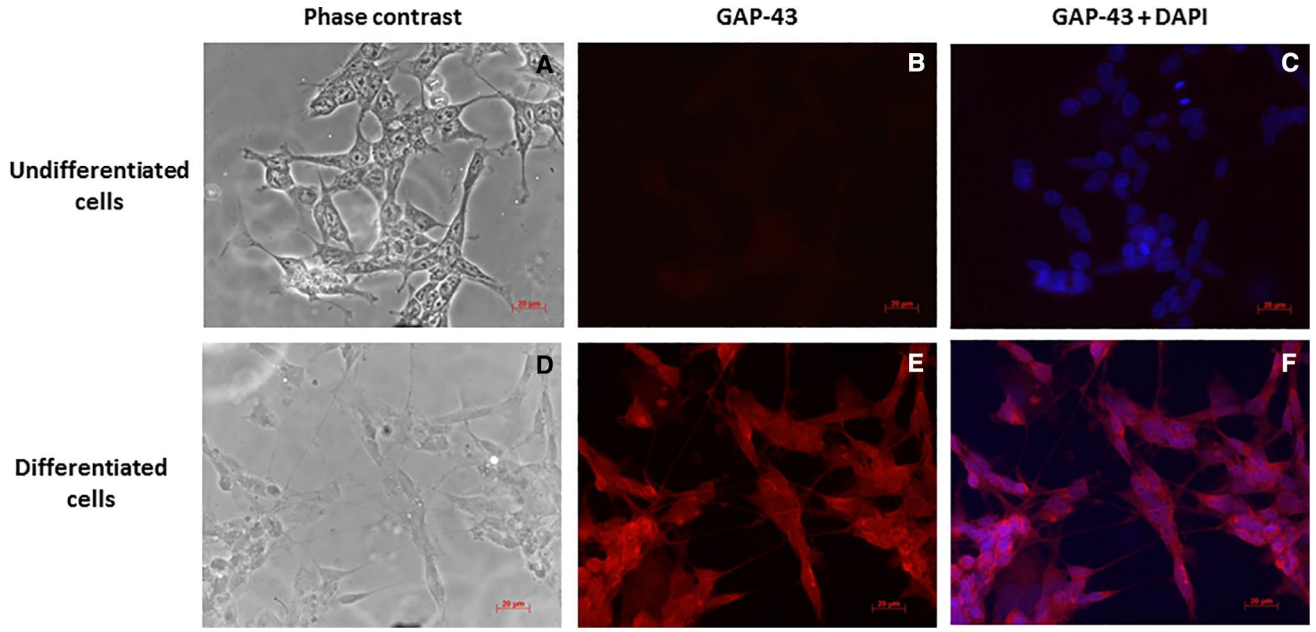

Fig. 1 SK-N-BE cells were able to express the GAP-43 protein, a biomarker for differentiation, marked in red (**e, f**) after a 10 days treatment with retinoic acid (**d–f**). No expression was appreciable by undifferentiated SK-N-BE (**a–c**), that were not treated with ATRA. DAPI-labelled nuclei in blue (**c, f**). Bar 20 µm. (Color figure online)

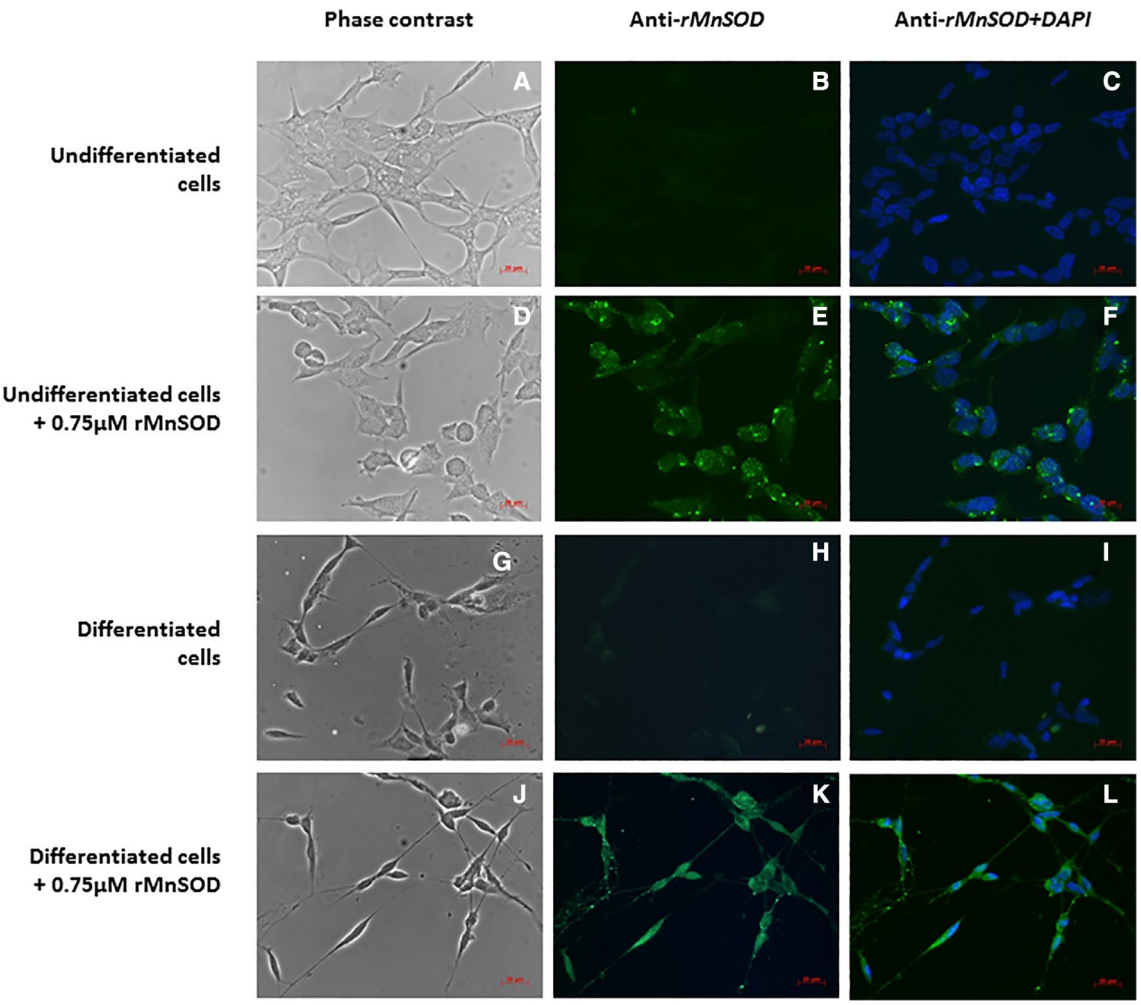

Fig. 2 Undifferentiated and differentiated SK-N-BE cells internalize *rMnSOD*. Untreated cell culture (**a–c, g–i**), cell culture treated for 3 h with the recombinant protein (0.75 µM; **d–f, j–l**). The recombinant protein, marked in green, is widely present in the cytoplasm and cell projection of treated cells (**e, k**), while untreated cultures showed no appreciable florescence (**b, h**). DAPI-labeled nuclei in blue (**c, f, i, l**). Images are representative of three independent experiments. Bar 20 µm. (Color figure online)

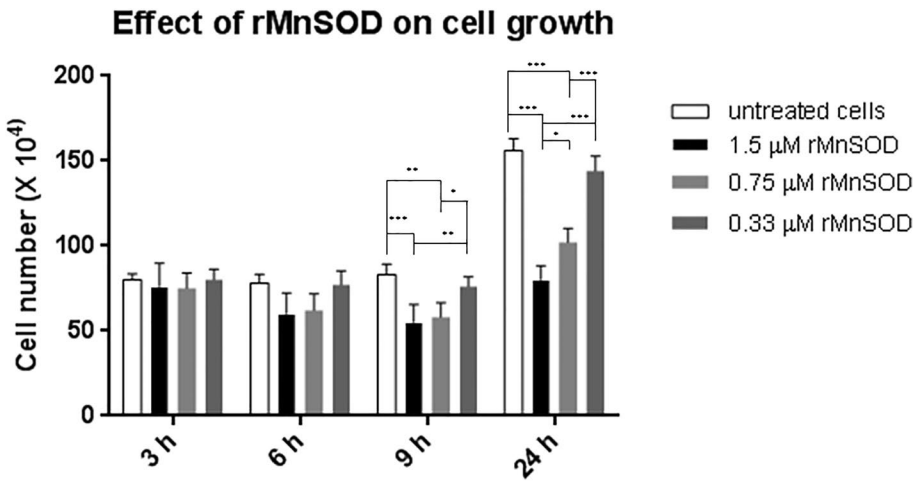

Fig. 3 Effect of *rMnSOD* on the growth of SK-N-BE cultures. Cells were initially seeded at the density of $10^4 \times cm^2$ on 60 mm plastic culture dishes, and were grown for 48 h in a CO_2 incubator. At each endpoint of *rMnSOD* treatment selected, cells were harvested and the cell number per dish was counted in a haematocytometer chamber. *rMnSOD* concentration-dependent reduction in cell number, with respect to the untreated cells, is appreciable

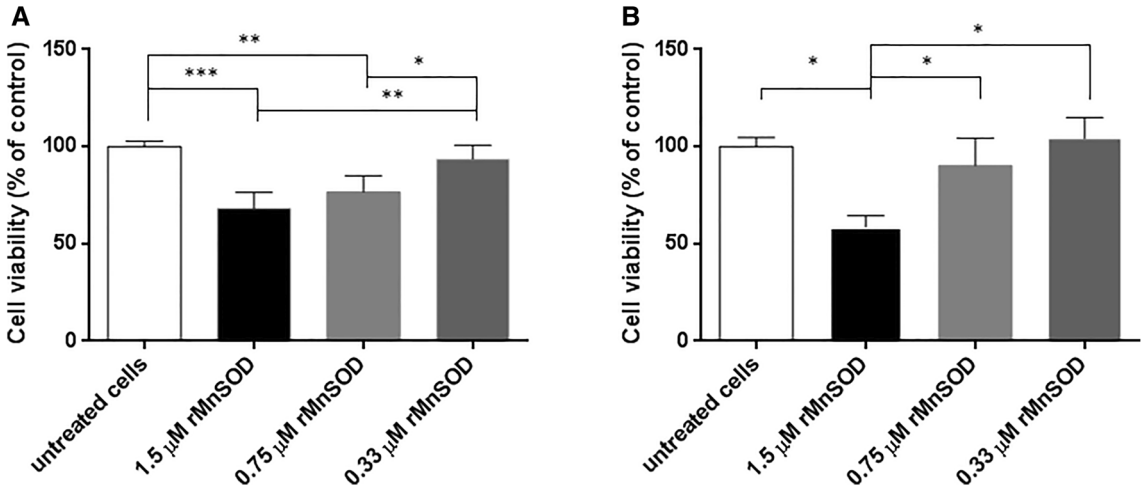

Fig. 4 To measure cell viability, MTT test was used in both undifferentiated (**a**) and differentiated (**b**) cell cultures. The treatment with 1.5 μM *rMnSOD* decreased significantly cell viability in both cell models. By contrast, 0.75 μM *rMnSOD* remains effective only in undifferentiated proliferating cells. The results are expressed as percent of untreated cells ± standard deviation of four independent experiments (treated vs. control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ANOVA test)

administration could significantly decrease cell viability respect to untreated cultures (− 42% $p < 0.05$). In the light of these results, treatment of differentiated cell cultures with 0.75 μM *rMnSOD* for 24 h was then chosen for further experiments to investigate mechanisms of *rMnSOD* that may modulate cell viability.

A morphological analysis (Fig. 5) of actively proliferating cells treated with 0.75 μM *rMnSOD* for 3 h showed that, besides the reported cell growth arrest and cytotoxicity, the recombinant protein also induced cell retraction and swelling (Fig. 5b). After 6 h of treatment, cell morphology appeared strongly altered (Fig. 5c) respect to control.

rMnSOD Treatment Does Not Increase ROS Levels Within SK-N-BE Cells

To verify if *rMnSOD* per se is involved in production of ROS, the levels of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$) in undifferentiated and differentiated cells were measured. As shown in Fig. 6a, b, basal levels of ROS within cells were not significantly affected by *rMnSOD* treatment.

rMnSOD Increase Intracellular Levels of H_2O_2 But Not $O_2^{\cdot -}$

A specific analysis of intracellular ROS after treatments with Glu or MQ, in presence or absence of pre-treatment with *rMnSOD*, was then carried out. As shown in Fig. 7a, no

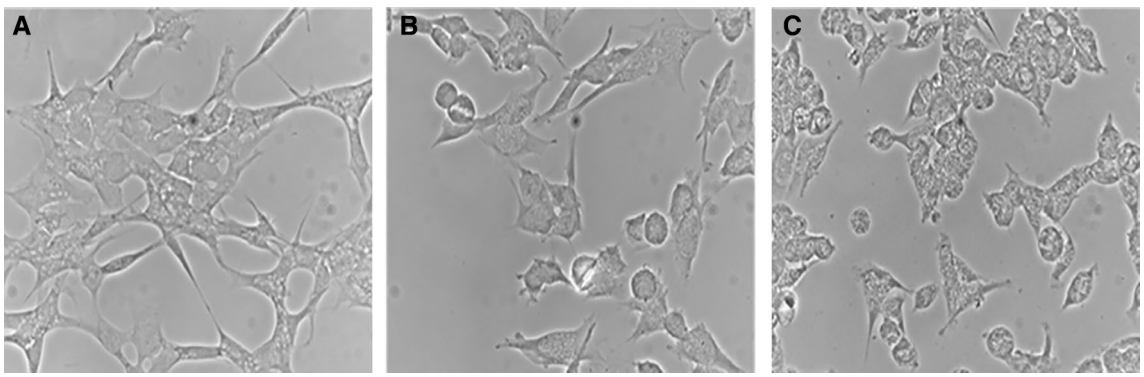

Fig. 5 Time course of the effect of *rMnSOD* (0.75 μM) on undifferentiated SK-N-BE cells. **a** Control cells. **b** Cells treated by the recombinant protein show the first signs of morphological changes, after 3 h. **c** Cell morphology was almost totally lost after 6 h. Phase contrast images; ×40 magnification

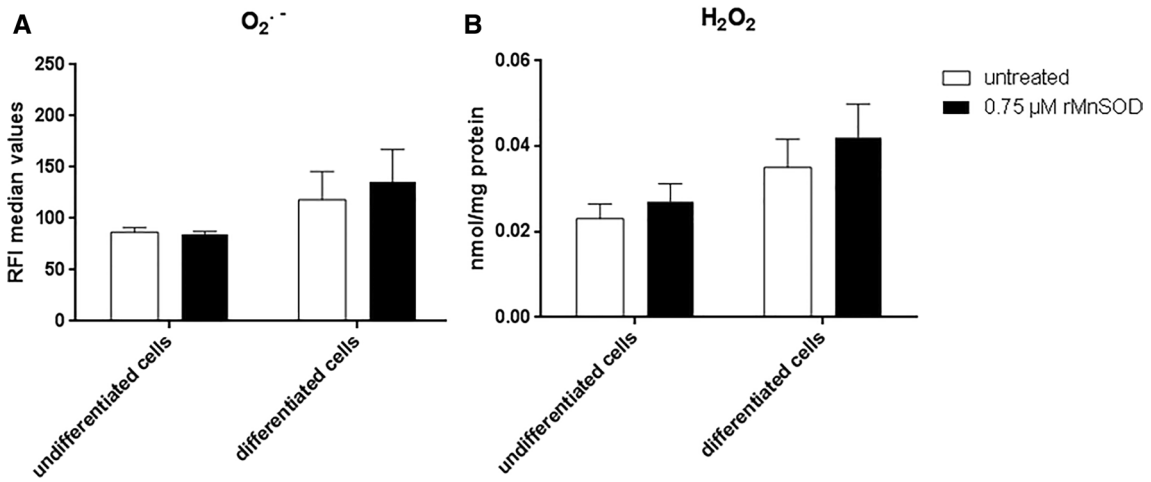

Fig. 6 Effect of *rMnSOD* on the ROS production in SK-N-BE cultures. Evaluation of (**a**) levels of superoxide anion and (**b**) levels of hydrogen peroxide after treatment with 0.75 µM *rMnSOD* in undifferentiated and differentiated cells. The values are the mean ± standard error deviation of eight independent experiments for superoxide (differentiated cells) and four independent experiments for all the other determinations

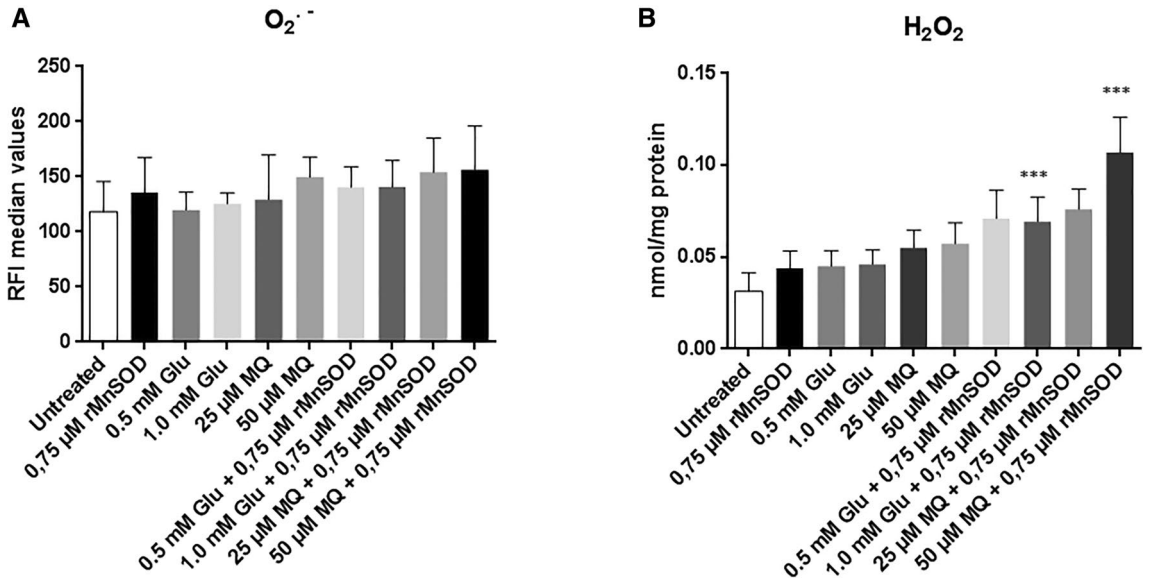

Fig. 7 Co-treatment of differentiated SK-N-BE cultures with MQ (50 µM) or GLU (1 mM) with 0.75 µM *rMnSOD*. The superoxide anion content in cells was determined by flow cytometry (**a**) and the hydrogen peroxide, by a specific colorimetric assay kit (**b**). The values are the mean ± standard error deviation of eight independent experiments for superoxide and four independent experiments for hydrogen peroxide (***$p < 0.001$ vs. untreated cells; ANOVA test)

significant changes in $O_2^{\cdot -}$ levels were appreciable in all experimental conditions (Fig. 7a). At the same time, a significant increase in H_2O_2 in cell extracts was evident following either combined coupling of treatments *rMnSOD* + Glu or *rMnSOD* + MQ (respectively 2.2 and 3.45-fold increase; $p < 0.001$ vs. untreated controls). Finally, only a slight increase of H_2O_2 was detectable in cells treated with Glu or MQ alone (Fig. 7b).

rMnSOD Treatment and Redox Enzyme Activities

Enzyme activities of SOD, CAT and GSH-px were measured, to evaluate the effect of *rMnSOD* on the redox profile of differentiated neuron-like cells. Enzymatic activity of SOD increased, with a peak of 2.78 and 2.97 fold ($p < 0.001$ for both) in the combined treatments respectively with Glu (1 mM) and MQ (50 µM)

and *rMnSOD* (0.75 µM) compared to untreated cells. A significant increase of SOD activity was also observed in cells exposed to the single treatments with *rMnSOD* (0.75 µM) and MQ alone ($p < 0.001$ vs. untreated cells; Fig. 8a). By contrast, no differences were evident in CAT activity (Fig. 8b). Changes in the equilibrium between formation of H_2O_2 from superoxide dismutation and its decomposition by CAT can be expressed by the ratio R = SOD/CAT; this ratio was significantly higher in cells treated with a combination of *rMnSOD* and Glu ($p < 0.05$) or MQ ($p < 0.01$), when compared with controls (Fig. 8c). Finally, a slight but significant ($p < 0.01$) increased activity of GSH-px was detected only in cells treated with *rMnSOD* + MQ (Fig. 8d).

rMnSOD Treatment Does Not Rescue the MQ Effect on Cellular Viability

Effects of MQ treatment, alone and/or in combination with *rMnSOD* on viability of differentiated SK-N-BE cells are shown in Fig. 9. A 30% reduction ($p < 0.01$) in cell viability was observed after treatment with 50 µM MQ (Fig. 9a), not

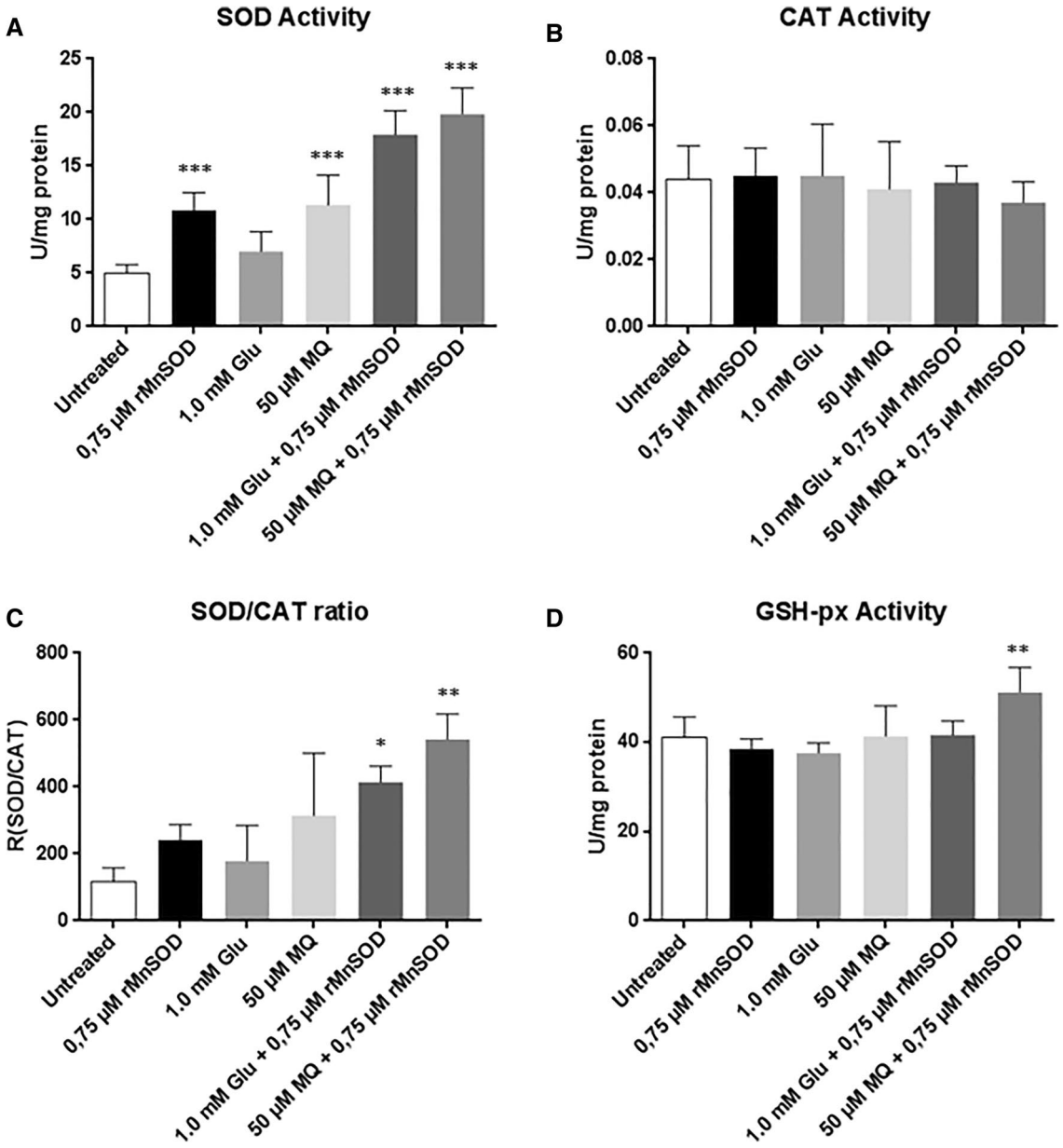

Fig. 8 Evaluation of oxidative stress in differentiated SK-N-BE treated with Glu, MQ or a combination of both. Evaluation of: **a** SOD activity; **b** CAT activity; **c** SOD/CAT ratio; **d** GSH-px activity. The results are expressed as mean ± standard error deviation of four independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. untreated cells; ANOVA test)

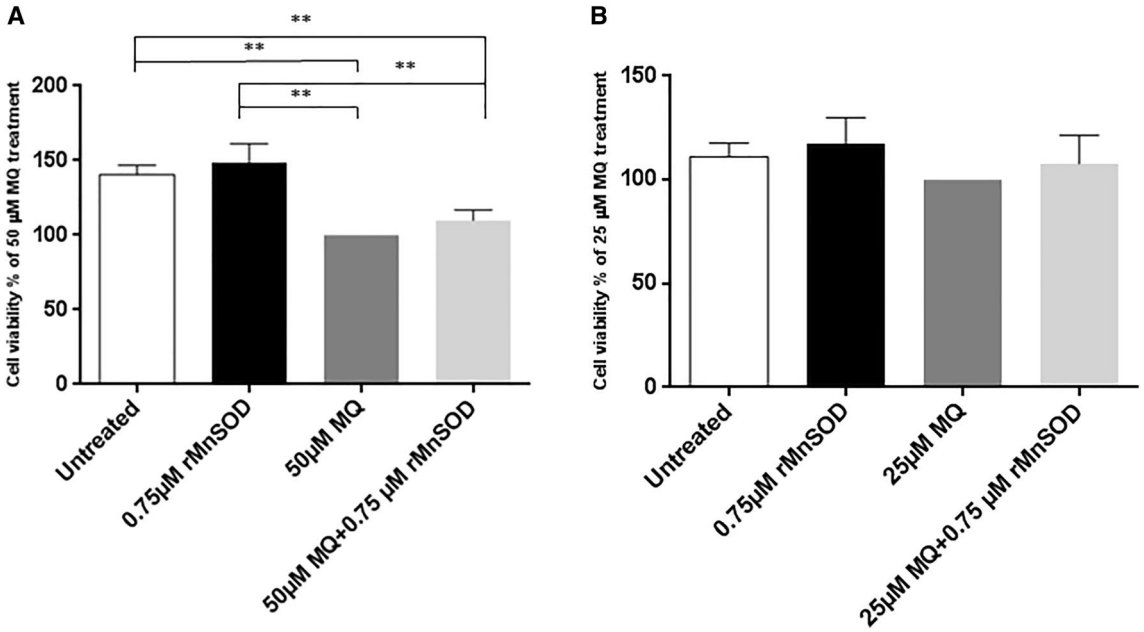

Fig. 9 Effect of 50 µM (**a**) and 25 µM (**b**) MQ and 0.75 µM *rMnSOD* co-treatment on cell viability (MTT assay). Cells were pre-treated with the *rMnSOD* for 1 h, exposed to 50 and 25 µM MQ for 1 h, then MTT was incubated for 3 h. Results are expressed as mean ± standard deviation of four independent experiments. **p < 0.01

with 25 µM MQ, suggesting a lower toxicity of the latter concentration (Fig. 9b). Pre-treatment with 0.75 µM *rMnSOD*, did not modify the effect induced by 50 µM MQ.

rMnSOD Treatments Does Not Rescue the Glu Effect on Cellular Viability

After 1 mM Glu treatment for 24 h, we observed a 16% increase in cell death compared to the control ($p < 0.05$; Fig. 10a), whereas 0.5 mM Glu did not affect cell viability

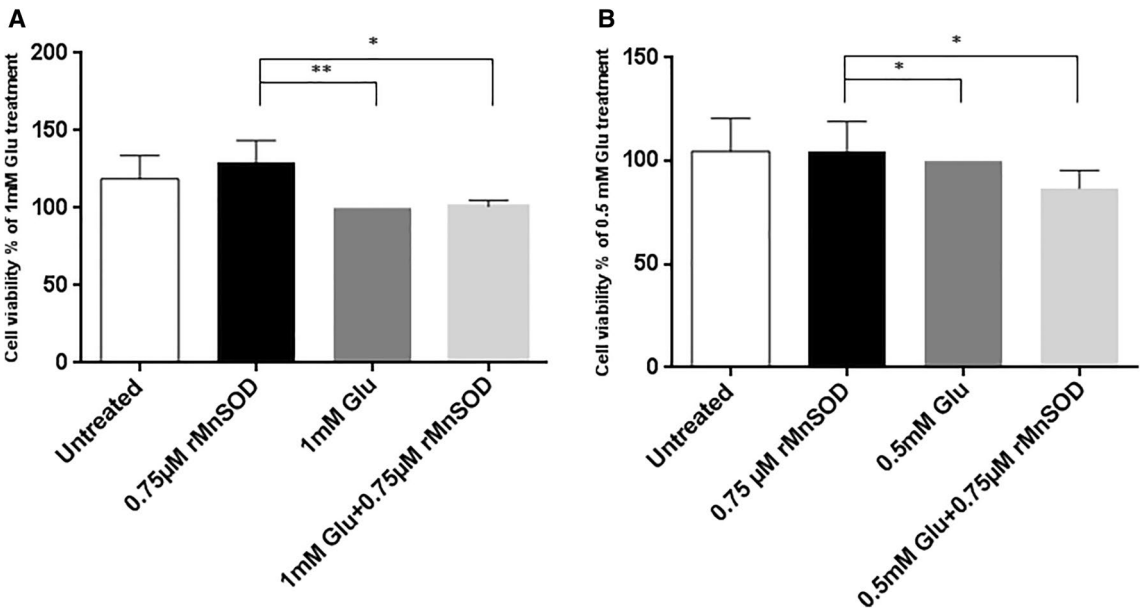

Fig. 10 Effect of 1 mM (**a**) and 0.5 mM (**b**) Glu and 0.75 µM *rMnSOD* co-treatment for 24 h (MTT was incubated for 3 h) on cell viability. The results are expressed as mean ± standard deviation of four independent experiments. *p < 0.05, **p < 0.01

(Fig. 10b). Following *rMnSOD* administration, cell viability did not increase in cell cultures treated with 1 mM Glu, whereas, paradoxically, lower Glu dose (0.5 mM) combined with *rMnSOD*, reduced cell viability (18%, $p < 0.01$).

Discussion

In the present study, we examined the effects of a new recombinant isoform of MnSOD in SK-N-BE neuron-like cells. Two different stages of differentiation of this cell line were considered: (a) the undifferentiated and actively proliferating neuroblastoma, and (b) the fully differentiated non-proliferating cells, more similar to neurons. Our aims were to verify the antitumor properties of the recombinant protein in the former model and to investigate the *rMnSOD* role in neuronal-like cells.

The starting step of our investigation was to verify the *rMnSOD* internalization by cells: the widespread positive immunoreactivity in the cytoplasm and cell processes demonstrated that *rMnSOD*, was rapidly internalized within neuron-like cells, thus crossing the plasma membrane. The intracellular localization of the recombinant protein is probably due to a so far unidentified membrane receptor binding the exogenous enzyme [18]. Internalization is a peculiarity of our *rMnSOD* molecule, intracellular delivery of other cloned SODs is actually prevented by both size and electric charge, thus reducing bioavailability within the cell and, consequently, potential therapeutic use.

We showed here for the first time that *rMnSOD* treatment alone on undifferentiated proliferating cells derived from neuroblastoma was toxic, inducing significant cell growth arrest, cell damage and finally cell death. These results fully agree with previous data concerning anti-tumoral effect of *rMnSOD* on systemic malignancies [16], suggesting that neuroblastoma could be a potential therapeutic target for this specific isoform of recombinant enzyme.

We also investigated the effect of the *rMnSOD* treatment on differentiated neuron-like cells using SK-N-BE cells induced to differentiate by ATRA. From a morphological point of view, differentiated cells showed poor sensitivity to *rMnSOD* treatment, without signs of cell shape alterations, cell loss and viability. Considering both the role of SODs in the redox machinery of cells and the one played by oxidative stress in neurodegeneration [29], we evaluated the interaction of *rMnSOD* with either a pro-oxidant drug (MQ) or an excitotoxic molecule (Glu). Previous data indicate that ATRA exposure, used to differentiate cells, also stimulates production of ROS, modifying antioxidant defense profile during neuroblastoma differentiation [30, 31]. In ATRA-differentiated SK-N-BE cells, basal levels of ROS produced by untreated cells were generally higher than the corresponding ones in undifferentiated cells, and *rMnSOD* administration did not modify this condition. Superoxide anion levels were not significantly modified by the administration of both Glu and MQ, and *rMnSOD* did not affect these outcomes. These results suggest that the differenced SK-N-BE cell cultures are quite refractory to the pro-oxidative effects of the drugs. However, the entity of this stress is strictly dependent on several factors such as, for example, the cell histotype and the number of cell passage, drug concentrations and time of exposure and so on. A possible explanation of our data could be linked to the low concentrations of Glu and MQ used in the study and to the relatively higher levels of superoxide produced by the ATRA treatments [32, 33]. By contrast, intracellular H_2O_2 concentration was significantly increased when the *rMnSOD* was administrated to the cell cultures along with high doses of Glu and MQ. According to the exogenous addition of *rMnSOD* enzyme, cell cultures showed increased activity of SOD that however was not accompanied by an increase of the CAT activity. In this context, GSH-px activity was also not influenced by treatments, with the exception of the cultures treated with *rMnSOD* + MQ. However, this slight increased activity was not able to counteract the increased levels of H_2O_2. In our experimental setting, *rMnSOD* was not able to revert the neurotoxic effect induced by the higher dose of MQ. Lortz and colleagues reported similar observations using a cell model with an impaired H_2O_2 detoxification system, after treatment with both MQ and MnSOD [34].

It is well known that in the nervous system the excess of Glu, an excitotoxic amino acid alters Ca^{2+} homeostasis, induces ROS mitochondrial dysfunction and apoptosis in the CNS [35]. In our setting, cell viability is only slightly reduced by Glu treatment and this effect is retained even in combination with *rMnSOD*. Interestingly, the lower dose of Glu did not alter cell viability, whereas co-treatment with *rMnSOD* elicited a neurotoxic response. Overexpression of SOD enzymes could be effective when combined with catalase activity, allowing removal of H_2O_2 produced during the dismutation of superoxide anion [29]. In our experimental model, when SK-N-BE cells were exposed to the pro-oxidant MQ or Glu concomitantly to MnSOD overload, although the superoxide anion levels remained constant, hydrogen peroxide levels significantly increased. This increase could be essentially due to the high activity of *rMnSOD*, which was not followed by an adequate activity of both catalase and GSH-px. The significant increase of the SOD/CAT ratio in both the combined treatments could confirm this hypothesis. The loss of cell viability after co-treatment with *rMnSOD* and low Glu dose could be explained by a synergic damaging effect exerted by the dose of Glu potentiated by the excess of H_2O_2 enzymatically produced within cells.

The mechanism of cell death in proliferating neuronal cells treated with the recombinant protein alone reproduces the oncotoxic pathway described for *rMnSOD* [36].

By contrast, when differentiated neuron-like cells were observed, *rMnSOD* treatment per se did not induce appreciable cell alterations; this could be due to the substantially unstimulated SOD activity, which did not generate the high levels of hydrogen peroxide needed to initiate cell damage. Conversely, in presence of a persistent condition of oxidative stress, such as those induced by MQ and Glu treatments, *rMnSOD* overcharge enhances the already present effects induced by the oxidants, through increased levels of H_2O_2, due to SOD/CAT unbalance. Since retinoids are a successful therapeutic option for oncologic patients, their interaction with *rMnSOD* appears to be of particular interest, potentially strengthening therapeutic efficacy. However, if ATRA-differentiated neuron-like cells are considered as neurons, the higher SOD/CAT ratio due to *rMnSOD* overload could be exacerbated, amplifying those situations in which oxidative imbalance plays a role in neurodegenerative processes.

In conclusion, our study showed that *rMnSOD* enzyme, easily internalized within SK-N-BE cells, exerts differential effects as a function of differentiation stage. These results (i) address attention to the potential use of the recombinant enzyme on differentiated neurons, (ii) pave the way for further preclinical studies aimed at evaluation of *rMnSOD* effects in models of neurodegenerative diseases.

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Compliance with Ethical Standards

Conflict of interest AM is the founder of Laedhexa Biotechnologies Inc. The others authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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