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Toxic effects of apomorphine on rat cultured neurons and glial C6 cells, and protection with antioxidants

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Abstract

Many catechol derivatives are currently used as drugs, even if they produce reactive oxygen species that may cause tissue damage. Among them, apomorphine, a potent dopamine agonist, displays efficient anti-parkinsonian properties, but the consequences of its oxidant and toxic properties have been poorly investigated on *in vitro* models. In the present work, we investigated apomorphine cytotoxicity by incubating cultures of rat glioma C6 cells and primary cultures of neurons with different concentrations of the drug. Apomorphine-promoted cell death was proportional to its concentration and was time-dependent. The ED_{50} of apomorphine on C6 cell death after 48 hr was about 200 μM. The cytotoxic effects induced by apomorphine were correlated to its autoxidation, which leads to the formation of reactive oxygen species, semiquinones, quinones, and a melanin-like pigment. C6 cells that underwent treatment with 400 μM apomorphine for 6 hr displayed features of necrosis, including loss of membrane integrity, degeneration of mitochondria, and DNA fragmentation. Thiols, such as cysteine, *N*-acetyl-L-cysteine, and glutathione, significantly protected cultured neurons and C6 cells against apomorphine-induced cytotoxicity. Thiols also inhibited apomorphine autoxidation. These data strongly suggest that apomorphine cytotoxicity towards neurons and C6 cells results from an intracellular oxidative stress.

Keywords: Apomorphine; Neurotoxicity; Free radicals; Necrosis; Thiols; Protein adducts

1. Introduction

The selective degeneration of dopaminergic neurons in the *zona compacta* of substantia nigra is the primary pathology of Parkinson's disease (PD) [1]. The precise mechanism responsible for degenerative damage remains unknown, but several observations suggest a cerebral oxidative stress in PD (for a review, see [2]). Accordingly, mitochondrial complex I dysfunction [3] resulting in an impairment of cerebral energy metabolism [4], decreased levels of GSH and increased free Fe^{2+} levels [5], as well as increased lipid peroxidation in substantia nigra [6], cerebral protein carbonyls [7], and DNA fragmentation [8], have been associated with PD. Moreover, several toxic conditions promoting side effects resembling PD symptoms, including poisoning with manganese [9] and exposure to the *N*-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) metabolite *N*-methyl-4-phenyl-pyridinium (MPP^+) [10] or to 6-hydroxydopamine [11], share with idiopathic PD oxidative events as a common neuropathological disorder.

Several catechol derivatives, especially levodopa, have been used as dopamine agonists for the therapy of PD, despite their well-known capacity for production of reactive oxygen species that may cause tissue damage. Both normal enzymatic catabolism through monoamine oxidases and non-enzymatic oxidative pathways result in the formation of

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Abbreviations: αMEM, alpha modified Eagle's medium; DHR123, dihydrorhodamine 123; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethyl formamide; DTT, DL-dithiothreitol; FBS, fetal bovine serum; levodopa, L-β−3,4−dihydroxyphenylalanine hydrochloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD, idiopathic Parkinson's disease; SFM, serum-free medium; and SOD, (Cu,Zn) superoxide dismutase.

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toxic products and reactive oxygen species (for a review, see [12]). Apomorphine is a potent dopamine agonist which displays anti-parkinsonian properties similar to those of levodopa [13]. Repeated subcutaneous injections of apomorphine reduce 'off' periods in fluctuating patients [14]. This compound, like other catechols, may undergo spontaneous autoxidation in neutral and alkaline solutions [15], and reactive metabolites may be produced during this non-enzymatic oxidative mechanism. Moreover, apomorphine stimulates DNA damage by Cu^{2+} in a cell-free system and also deoxyribose degradation by Fe^{3+}-EDTA + H_2O_2 *in vitro*, although it was also found that apomorphine scavenges peroxy radicals in an aqueous medium and inhibits microsomal lipid peroxidation [16,17]. Moreover, catechols may react with reactive oxygen species (ROS) in the presence of ferrous ions to form significant amounts of other neurotoxic compounds such as the neurotoxin 6-hydroxydopamine quinone [18].

We considered the possibility that apomorphine shares with levodopa and dopamine the mechanism of cytotoxicity related to the oxidative damage triggered by some degradation products of catechols, such as quinones and ROS [19, 20]. The purpose of this study was to examine and to characterize the mechanisms of apomorphine toxicity towards rat glioma C6 cells, which have been largely used for the study of astrocyte properties [21] and which are, like astrocytes, more resistant to oxidative stress than neurons. We compared the effects of the presence of different concentrations of apomorphine on the viability of both cultured primary neurons and C6 cells. Furthermore, we confirmed previous observations brought about in other *in vitro* systems on the mechanism of catechol-induced cell death. The effects of the presence of various antioxidant enzymes, reducing agents, metals, and metal chelators in the culture medium were also studied.

2. Materials and methods

2.1. Chemicals

DHR123, diethylenetriamine pentaacetic acid (DTPA), SOD, MTT, DTT, GSH, *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), SDS, and routine chemicals were purchased from Sigma. αMEM, amphotericin B, DMEM Mix F12, Dulbecco's PBS, penicillin/streptomycin, Trizol reagent, and trypsin were supplied by Gibco. FBS was purchased from Boehringer Mannhein. DNA ladder-plus (100 bp) was synthesized by MBI Fermentas. Iron sulfate was obtained from Merck. SeaKem Gold agarose was purchased from FMC Bioproducts. Treated disposable sterile multiple-well plates were from Corning Costar.

2.2. Culture of rat glioma C6 cells

Rat glioma C6 cells were obtained from the American Type Culture Collection (ATCC) (Interchim). After thawing, cells were suspended in DMEM mix F12/αMEM 1:1 (v/v) containing 10% (v/v) FBS, 100 μg/mL of streptomycin, 100 unit/mL of penicillin, 0.25 μg/mL of amphotericin B, 2 mM L-glutamine, and 1 ng/mL of BFGF and plated on 75-cm^2 flasks. The number of passages varied between 26 and 30, and the purity and viability of the cells were evaluated at each passage. Cultures were grown in an incubator with a humidified atmosphere of 5% CO_2 in air at 37°. At confluence, cells were incubated at 37° for 10 min in a culture medium containing 0.05 mg/mL of trypsin and 0.53 M EDTA, and further dissociated by mechanical agitation through a fire-polished Pasteur pipette. C6 cells were diluted and seeded into plates at a density of 3.1×10^4 cells/cm^2. After the cells had become confluent again, the medium was switched to SFM, and experiments were initiated 24 hr later. Both control and treated C6 cells were directly examined under phase-contrast microscopy after incubation.

2.3. Neuronal cell cultures

Neuronal cell cultures were obtained from 14-day-old rat embryo forebrain according to [22]. Pregnant female rats were anaesthetized with halothane, and living embryos were excised by cesarean surgery under sterile conditions. Whole embryos were placed in culture medium previously equilibrated at 37°, and forebrains were carefully collected. Brain tissues were dissected free of meningeal membranes and gently dispersed in a mixture of DMEM and Ham's F12 medium 1:1 (v/v) supplemented with 5% heat-inactivated FBS. After centrifugation at $700 \times g$ for 10 min, the pellet was dispersed in the same medium and passed through a 46-μm pore-size nylon mesh. The density of the cell suspension was measured, and aliquots were transferred into 35-mm Petri dishes precoated with poly-L-lysine in order to obtain a final density of 10^6 cells/dish. Cultures were kept at 37° in a humidified atmosphere of 95% air–5% CO_2 for 24 hr, and the medium was then replaced with a fresh hormonally defined SFM consisting of the DMEM/Ham's F12 mixture enriched with 1 mM human transferrin, 1 mM insulin, 0.1 mM putrescin, 10 nM progesterone, 1 pM estradiol, and 30 nM sodium selenite. Subsequent medium changes with this SFM were performed twice a week. Assays were carried out on 9- to 11-day-old cultures, and their purity was assessed by immunohistology using neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) antibodies. Cultures typically contained more than 92% NSE positive cells without GFAP positive cells.

2.4. Apomorphine autoxidation

Neutral aqueous solutions of apomorphine rapidly undergo spontaneous oxidative decomposition and turn green.

Autoxidation is a multi-step reaction resulting in quinone derivative formation [12], and a melanin-like polymer was postulated to be formed as an end product when apomorphine was incubated with a rat brain mitochondrial fraction [23]. In the present study, spectral modifications resulting from 330-μM apomorphine autoxidation were repeatedly recorded between 900 and 190 nm for 30 min. Autoxidation rates were measured at 343 nm in 50 mM phosphate buffer, pH 7.4. Assays were repeated in the presence of 320 U/mL of SOD, 1134 U/mL of catalase, and thiol solutions (0.5 mM DTT or 0.5 mM L-cysteine). Apomorphine oxidation was also monitored spectrophotometrically in rat glioma C6 cell cultures with a microplate reader at 405 nm, based on quinone and melanin-like pigment formation [19].

2.5. Assays of cell viability

Cells were incubated in SFM containing apomorphine and either antioxidant enzymes, reducing agents, metals, or metal chelators at different concentrations. For controls, monocellular layers were incubated under the same conditions, but without apomorphine. Concentrations of apomorphine higher than the human therapeutic dosage (micromolar range, but difficult to assess because of the very short half-life of the drug [24]) were used throughout to amplify the responses observed *in vitro*. Cell viability was quantified by the conversion of yellow MTT by mitochondrial dehydrogenases of living cells to a purple MTT formazan [25]. MTT was dissolved at a concentration of 5 mg/mL in sterile PBS at room temperature, and the solution was further sterilized by passing through 0.2 μm syringe filters and stored at 4° in the dark. The final concentration of MTT added to each well or dish was 1 mg/mL. After 2 hr of incubation at 37°, a same volume of lysing buffer was added. Lysing buffer was prepared as follows: 20% (w/v) SDS was dissolved at 37° in a solution of 50% (v/v) of both DMF and demineralized water, pH 4.7. After an overnight incubation at 37°, optical densities were measured at 580 nm. The viability of treated cultures was expressed as a percentage of the mean absorbance of control cultures, following subtraction of the background absorbance of wells without cells.

Proliferation of C6 cells was measured by counting viable cells, defined as the cells that remained adherent to culture wells and excluded trypan blue. At culture confluence, the medium was switched to SFM, and apomorphine was added 24 hr later. Cell viability was quantified again using trypan blue 48 hr later to confirm MTT assay. Nonadherent cells were removed by two washes with ice-cold PBS. Adherent cells were harvested by incubation with 0.05% trypsin–0.53 M EDTA for 10 min at 37°. Trypsin was neutralized by addition of DMEM mix F12/αMEM 1:1 (v/v), containing 10% (v/v) FBS. Cells were centrifuged for 5 min at 160 $\times$ *g* and resuspended in the same medium. Trypan blue 0.04% (w/v) was added for 10 min, and the number of viable cells excluding trypan blue was counted in a hemocytometer.

2.6. Electrophoresis of proteins and DNA

After growth to confluence, C6 cells were washed three times with PBS, and the culture medium was switched to SFM. Cells were incubated 24 hr later with different concentrations of apomorphine, and monocellular layers not subjected to apomorphine were used as control. Cells were lysed in Trizol reagent, then DNA and proteins were isolated according to the manufacturer's instructions (GIBCO-BRL). DNA samples were separated by electrophoresis on a 1% (w/v) agarose horizontal gel containing ethidium bromide. Protein was quantified by the method of Bradford [26] and separated on 12% SDS–PAGE slab gels. Conjugation of apomorphine with proteins was evidenced by UV transillumination at 312 nm before protein staining with Coomassie brilliant blue R-250.

2.7. Fluorescence microscopy of cells

DHR123 is a colorless, cell-permeant dye that is oxidized by reactive oxygen species to a fluorescent oxidation product, rhodamine 123. The presence of this oxidation product was used to visualize oxidative stress during apomorphine autoxidation in C6 cells cultured on cover slips in 24-well chambers. Cells were washed twice in PBS and then incubated in PBS containing DHR123 at 1 μg/mL. Then, cells were subjected to 400 μM apomorphine for 1 hr at 37°. Fluorescence of rhodamine 123 was measured at 530 nm under a 488-nm excitation wavelength. Cells without apomorphine or with apomorphine in the absence of the dye were used as controls.

2.8. Electron microscopy

Cells were cultured in 12-well chambers and fixed with 2.5% glutaraldehyde for 1 hr in 0.1 M cacodylate buffer, pH 7.2, and washed three times in this buffer. Samples were postfixed with 1% osmic acid (45 min), passed through a graded series of ethanol solutions (30, 50, 70, 80, 90, and 100% for 15 min each), and treated with a 1:1 (v/v) Epon–100% ethanol for 30 min, followed by 2:1 (v/v) Epon–100% ethanol for 30 min, and in pure Epon overnight. Epon polymerization was obtained at 56° for 48 hr. Ultrathin sections (~65 nm) were cut, stained with uranyl acetate and Reynold's dye, and examined with a Philips CM12 transmission electron microscope.

Cells that underwent treatment with 400 μM apomorphine were fixed 6 hr later. The cells which detached from wells were centrifuged at 300 $\times$ *g* for 5 min and were also examined. Cells treated with 100 μM were fixed 24 and 48 hr later. Control cells, which were not treated, were fixed after 6 and 48 hr.

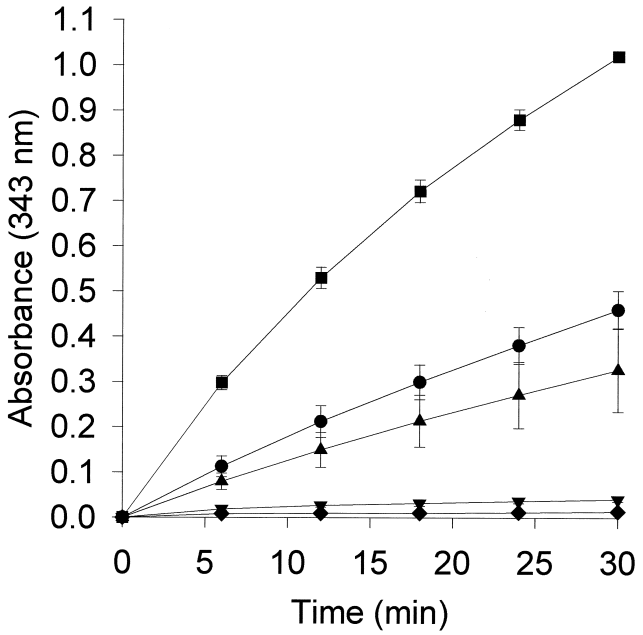

Fig. 1. Time–course of apomorphine autoxidation. The concentrations of reagents were: ●, 330 μM apomorphine; ■, 330 μM apomorphine + 320 U/mL of SOD; ▲, 330 μM apomorphine + 1134 U/mL of catalase; ▼, 330 μM apomorphine + 0.5 mM DTT; ▼, 330 μM apomorphine + 0.5 mM cysteine. Autoxidation rate was monitored by spectrophotometry at 343 nm. Data are expressed as the means ± SD of six separate assays for the autoxidation of apomorphine and of duplicate for the autoxidation in the presence of antioxidants.

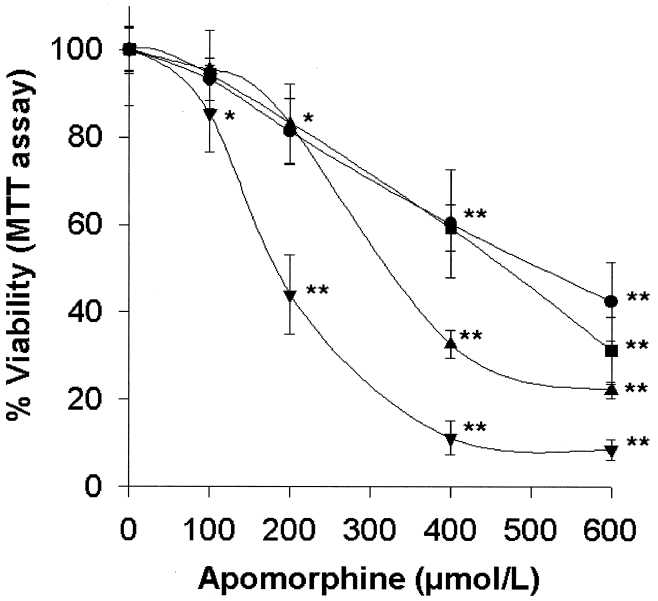

Fig. 2. Cytotoxicity of apomorphine towards rat glioma C6 cells as a function of the duration of the incubation. Incubations were run for either ●, 6 hr; ■, 12 hr; ▲, 24 hr; ▼, 48 hr. Cell viability was determined by the MTT test and expressed as a percentage of controls without apomorphine. Data are expressed as the means ± SD, N = 8. Data marked with two asterisks indicate a significant difference from the controls with a P value < 0.001, and a single asterisk indicates a P value < 0.01.

2.9. Data analysis

Results are expressed as means ± SD. Statistical analyses were performed using Student's t-test, and a P value <0.05 was considered significant.

3. Results

3.1. Non-enzymatic oxidation of apomorphine

The absorption spectrum of apomorphine in 0.01 M HCl exhibits two peaks at 229 and 264 nm. During autoxidation, two new peaks appeared in the absorption spectra that slowly increased as a function of time at 412 and 614 nm, with another of much greater absorbance appearing at 343 nm (not shown). Due to their rapid polymerization to melanin, we were unable to identify the oxidation products by mass spectrometry techniques. The autoxidation rate of apomorphine in 50 mM phosphate buffer, pH 7.4 at 343 nm was practically linear with time (Fig. 1). The reaction was slightly inhibited by 1134 U/mL of catalase and efficiently inhibited by 0.5 mM DTT and 0.5 mM cysteine, but the rate of autoxidation increased in the presence of 320 U/mL of SOD.

3.2. Effect of apomorphine on cellular viability

C6 cells displayed a typical growth curve, and cell confluence was usually obtained 72 hr after seeding. After treatment of cells with apomorphine concentrations up to 100 μM for 48 hr, there was no significant difference with controls in the number of viable cells when trypan blue was used to quantify cell viability. By contrast, almost all cells subjected to 400 μM apomorphine died after 48 hr (not shown). Fig. 2 shows the effect of various concentrations of apomorphine on cellular survival measured by the MTT assay. C6 cell survival was inversely correlated to apomorphine concentration. C6 cell viability became significantly lower 6 hr after treatment with 200 μM apomorphine (81.3 ± 7.5%, N = 8), or 48 hr after 100 μM apomorphine (85.3 ± 8.6%, N = 8). The cytotoxic effects of apomorphine were time dependent, and a maximal decrease in cell viability was obtained 48 hr after incubation with 400 μM apomorphine (11.2 ± 3.9%, N = 8). The ED_{50} of apomorphine on cell death was about 200 μM after 48 hr, and the cytotoxicity was correlated to the formation of a melanin-like pigment in the cultures (Fig. 3).

Primary cultures of rat neurons were more sensitive to apomorphine cytotoxicity than C6 cells (Fig. 4). Neuron viability decreased to 67.8 ± 20.4% and 28.1 ± 4.0% in the presence of 100 or 200 μM apomorphine for 6 hr, respectively (N = 5). These results were significantly different when compared to C6 cell viability, which was 93.2 ± 4.8% and 81.3 ± 7.5%, respectively, N = 8).

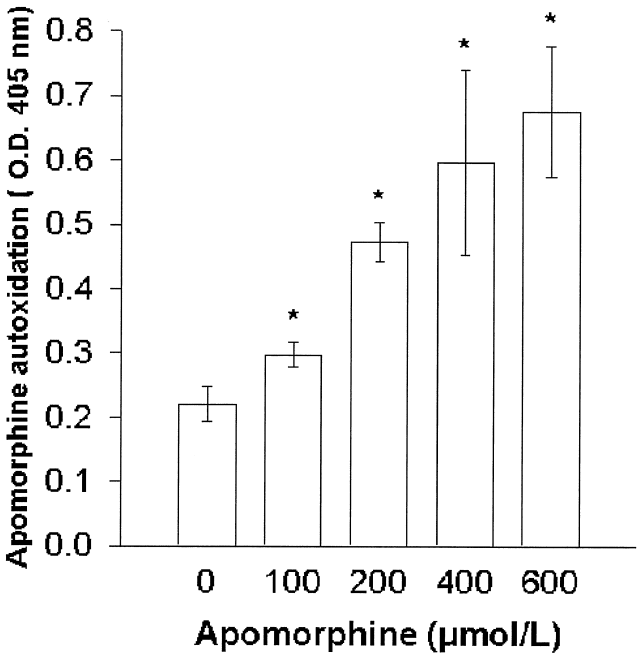

Fig. 3. Formation of a melanin-like pigment during apomorphine autoxidation. Rat glioma C6 cell cultures were incubated with 0–600 μM apomorphine for 48 hr. Measurement of pigment formation was monitored by spectrophotometry at 405 nm. Data are means ± SD, N = 8. Bars marked with an asterisk indicate a significant difference from the controls with a P value < 0.001, when compared with incubations without apomorphine.

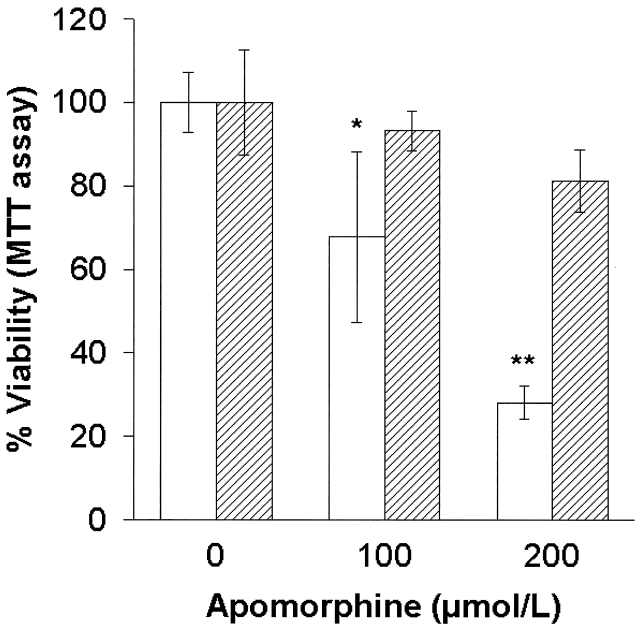

Fig. 4. Apomorphine cytotoxicity towards neurons (open bars, N = 5) and glioma C6 cells (hatched bars, N = 8) incubated with 100 and 200 μM apomorphine. Cell viability was determined by the MTT test and expressed as a percentage of controls without apomorphine. **: $P < 0.001$, *: $P < 0.02$.

3.3. Morphological observation of C6 cells

For phase-contrast microscopy, 3.1×10^4 C6 cells/cm^2 were plated in 96-well plates and incubated as described in the "Methods" section. For control culture, C6 cells were incubated for 48 hr in standard SFM without apomorphine. Fig. 5A shows that a confluent monocellular layer practically covered the whole surface of the dish. When 100 μM apomorphine was added to the medium, morphological degeneration of the cells progressed as follows: 6 hr later, cells began to be stained by the formation of a melanin-like pigment (Fig. 5B); 24 hr later, cells displayed a brown-greenish color and began to detach from the surface of the well (Fig. 5C); 48 hr later, confluence was lost and gaps representing areas where cells had detached from the monolayer were apparent (Fig. 5D).

When observed by fluorescence microscopy, control cells incubated with DHR123 in the absence of apomorphine exhibited a faint fluorescence when excited at 488 nm, presumably resulting from the basal production of reactive oxygen species during mitochondrial respiration and peroxidase activity (Fig. 6A). Cells incubated in the presence of apomorphine only were not fluorescent (not shown). Cells incubated with DHR123 in the presence of 400 μM apomorphine for 1 hr at 37° presented a bright fluorescence under excitation at 488 nm (Fig. 6, B and C).

When observed by transmission electron microscopy, control cells showed intact plasma membranes, numerous mitochondria, and ribosomes (Fig. 7, A and D). After treatment with 400 μM apomorphine for 6 hr, cells displayed features of necrosis, including loss of membrane integrity, alteration of mitochondria, and chromatin disintegration (Fig. 7, B and C). Although we also observed necrosis in some cells that were incubated with 100 μM apomorphine for 24 hr (not shown), the plasma membrane of most of these cells remained intact even after 48 hr (Fig. 7F) and the chromatin was preserved. A strong pigmentation outlined the formation of a melanin-like pigment (Fig. 7, E and F).

3.4. DNA electrophoresis

DNA damage caused by different concentrations of apomorphine (400, 300, or 200 μM) to C6 cells was examined by analysis of their genomic DNA on 1% agarose gel electrophoresis. C6 cells treated for 6 hr with apomorphine showed DNA fragmentation into small fragments of random size (Fig. 8, lanes 3–5). This smear pattern of DNA fragmentation is consistent with necrotic cell death. DNAs from control C6 cells showed a single band near the starting origin of electrophoresis. Densitometric analysis showed that areas beneath the curves between 100- to 500-bp bands increased proportionally to the concentration of apomorphine.

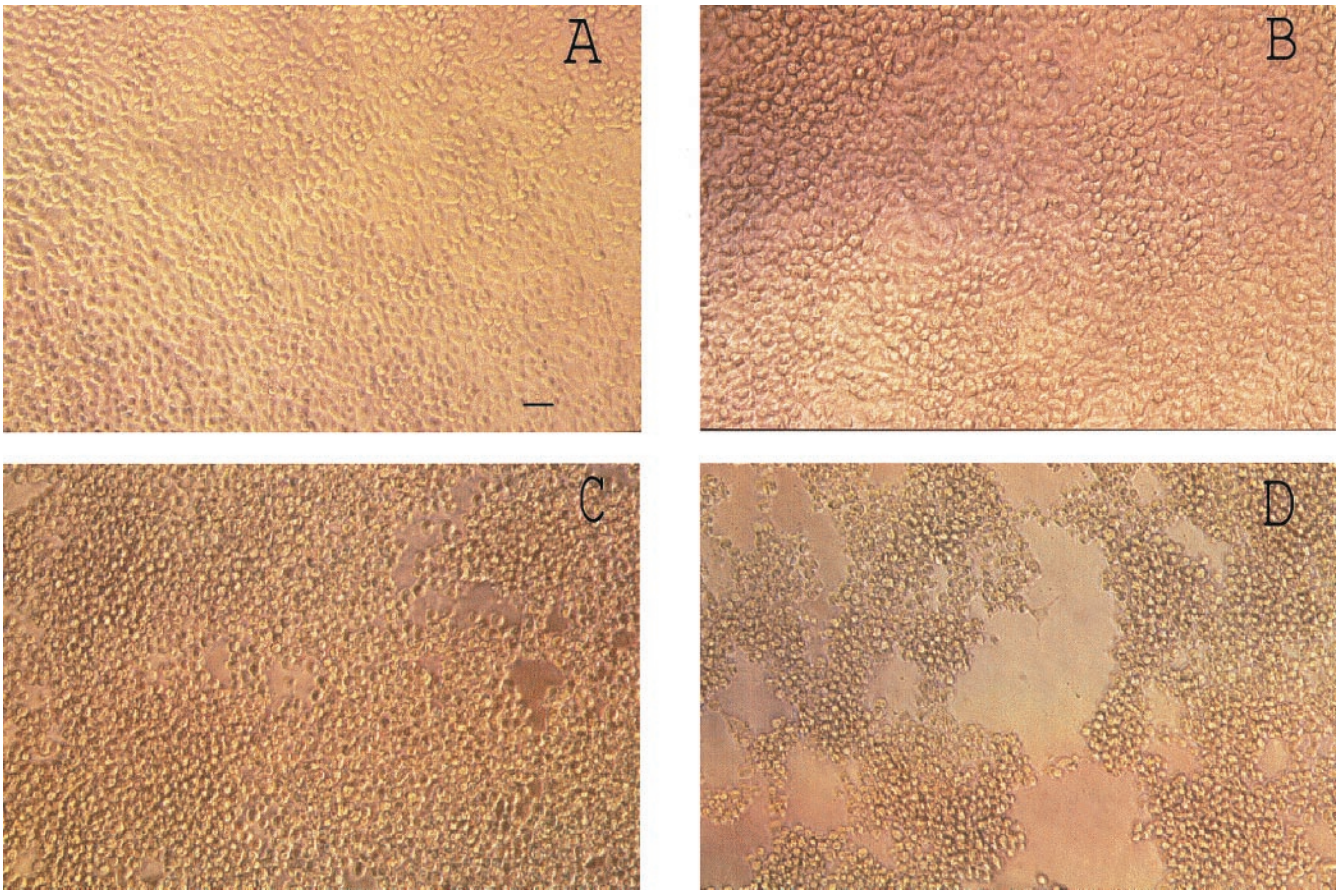

Fig. 5. Phase-contrast microscopy of control and apomorphine-treated rat glioma C6 cells. *Micrography A*: rat glioma C6 cells incubated with SFM for 48 hr in the absence of apomorphine display a normal monocellular layer. *Micrography B:* many of the cells subjected to 100 μM for 6 hr began to be stained by the formation of apomorphine autoxidation products. *Micrography C:* rat glioma C6 cells subjected to 100 μM apomorphine for 24 hr were all stained in a brown-greenish color and had began to detach from well. *Micrography D:* holes appeared in the monocellular layer when cells were subjected to 100 μM for 48 hr. Gaps in the monolayer represent areas in which cells have detached from the monolayer. Scale bar = 120 μm.

3.5. Protein electrophoresis

A previous report [27] described the formation of apomorphine–protein conjugates when apomorphine autoxidizes in the presence of serum albumin. To determine if apomorphine forms conjugates with intracellular proteins during autoxidation, we extracted proteins from cells incubated with apomorphine. After electrophoresis, the gels were observed under UV transillumination at 312 nm before staining with Coomassie blue. All proteins stained by Coomassie blue in control C6 cell extracts appeared green after incubation with apomorphine, suggesting a non-specific apomorphine–protein conjugate formation by binding of apomorphine to intracellular proteins (Fig. 9).

3.6. Effects of antioxidants and iron

Thiol compounds at concentrations of 1 and 10 mM significantly prevented apomorphine-induced cytotoxicity towards both neurons (Fig. 10) and C6 cells (Fig. 11A–C) after 6 hr of incubation. Thiols also decreased the formation of quinones and the melanin-like pigment (Fig. 11D). At 10 mM, L-cysteine or *N*-acetyl-L-cysteine efficiently protected C6 cells and neurons against 400 μM apomorphine cytotoxicity even after 48 hr of incubation. At this concentration, survival increased from 8% in cells incubated only in the presence of 400 μM apomorphine to 78% in cells incubated in the presence of apomorphine and thiols (not shown). These results are consistent with a thiol-mediated inhibition of apomorphine autoxidation by depletion of reactive oxygen species.

The presence of Fe^{2+} significantly aggravated apomorphine-induced cytotoxicity towards C6 cells (Fig. 12A), but did not increase the formation of quinones and of the melanin-like pigment (Fig. 12B). We also tested three iron chelators and showed that both DPTA (diethylenetriamine pentaacetic acid) and TPEN (*N,N,N′,N′*,-tetrakis (2-pyridylmethyl) ethylenediamine were cytotoxic to C6 cells at 100 and 10 μM, respectively. Deferoxamine at concentrations up to 1 mM neither protected cells against apomorphine-induced cytotoxic damage nor inhibited the formation of quinones and melanin-like pigment (not shown). These results strongly suggest that iron is not involved in the cytotoxicity or the synthesis of the melanin-like pigment, but its

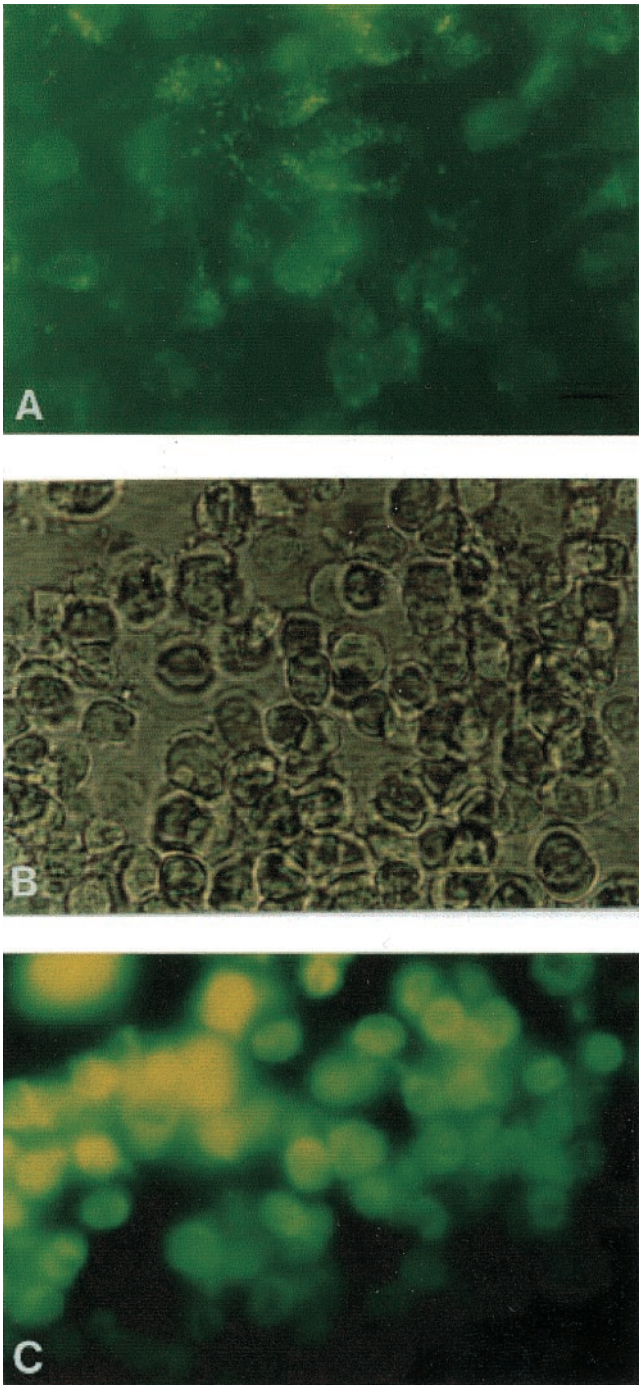

Fig. 6. Reactive oxygen species production by rat glioma C6 cells incubated in the presence of apomorphine. Rat glioma C6 cells were incubated in PBS containing 1 μg/mL of DHR123 and examined under phase-contrast fluorescence microscopy in the presence or not of apomorphine. *Micrography A:* control cells without apomorphine exhibited a faint fluorescence, probably due to mitochondrial respiration and peroxidase activity. *Micrography B:* cells subjected to 400 μM apomorphine for 1 hr at 37°. *Micrography C:* when the same field seen in *B* was photographed with a fluorochrome excitation at 488 nm, cells became fluorescent, evidencing the production of reactive oxygen species that oxidize DHR123 to the fluorescent rhodamine 123. Scale bar = 40 μm.

presence in the culture medium aggravates the cytotoxic effects of apomorphine.

Catalase up to 50 U/mL, SOD up to 500 U/mL, and the association of both enzymes at the same concentrations up to 50 U/mL did not protect C6 cells against the cytotoxic effects of apomorphine, suggesting that the extracellular release of hydrogen peroxide and superoxide during apomorphine oxidation does not play an important role in the toxic mechanism.

4. Discussion

In everyday clinical practice, the most effective treatment of PD remains levodopa therapy [28]. However, both levodopa and dopamine easily autoxidize to semiquinone and *o*-quinone derivatives [29] that may be activated by NADPH–cytochrome P450 reductase in the presence of oxygen, leading to the formation of superoxide [30,31]. On the other hand, post-mortem samples of brains from PD patients display features of oxidative stress, i.e. increased lipid peroxidation, protein carbonyls and 8-hydroxyguanine levels [6,7]. Therefore, levodopa therapy has been thought to enhance the progression of PD, as the beneficial response to levodopa decreases with time in most PD patients [28, 32]. Nevertheless, evidence for *in vivo* neurotoxicity of levodopa remains lacking [33], and the loss of efficacy of both levodopa and apomorphine may also be due to the normal loss of remaining neurons that can recycle dopamine and agonists. In fact, both levodopa and dopamine act *in vitro* either as oxidants or antioxidants, depending on their concentration. Levodopa at 100–250 μM is toxic to cultured neurons, and causes cell death by apoptosis or necrosis through the production of oxygen radicals, as suggested by the protective effect of thiol antioxidants [34]. By contrast, at lower concentrations (5–50 μM), levodopa displays scavenger properties [35].

Other catechols that are either dopamine agonists or catechol *O*-methyl transferase inhibitors, e.g. entacapone and tolcapone, are used for the therapy of PD [36]. Apomorphine is a potent D_1 and D_2 receptor agonist that quickly enters the brain and accumulates in the striatum [37]. As reported during levodopa therapy, a decline in the response to closely spaced administrations of apomorphine appears [38], also suggesting that it may either induce a desensitization of the receptors or aggravate the loss of dopaminergic neurons. By contrast, other studies did not report changes in apomorphine sensitivity after a long-term treatment [39]. Nevertheless, only a few data concerning its eventual cytotoxicity are available. Recently, low concentrations of apomorphine have been found to protect rat pheochromocytoma (PC12) cells from the toxic effects of H_2O_2 and 6-hydroxydopamine [16]. The same authors also found that at higher concentrations, apomorphine is cytotoxic (EC_{50} = 100 μM) and causes an almost complete cell loss in the presence of H_2O_2. In the same way, Ubeda *et al.* [17] described the ability of apomorphine to catalyze deoxyribose degradation through the generation of superoxide rad-

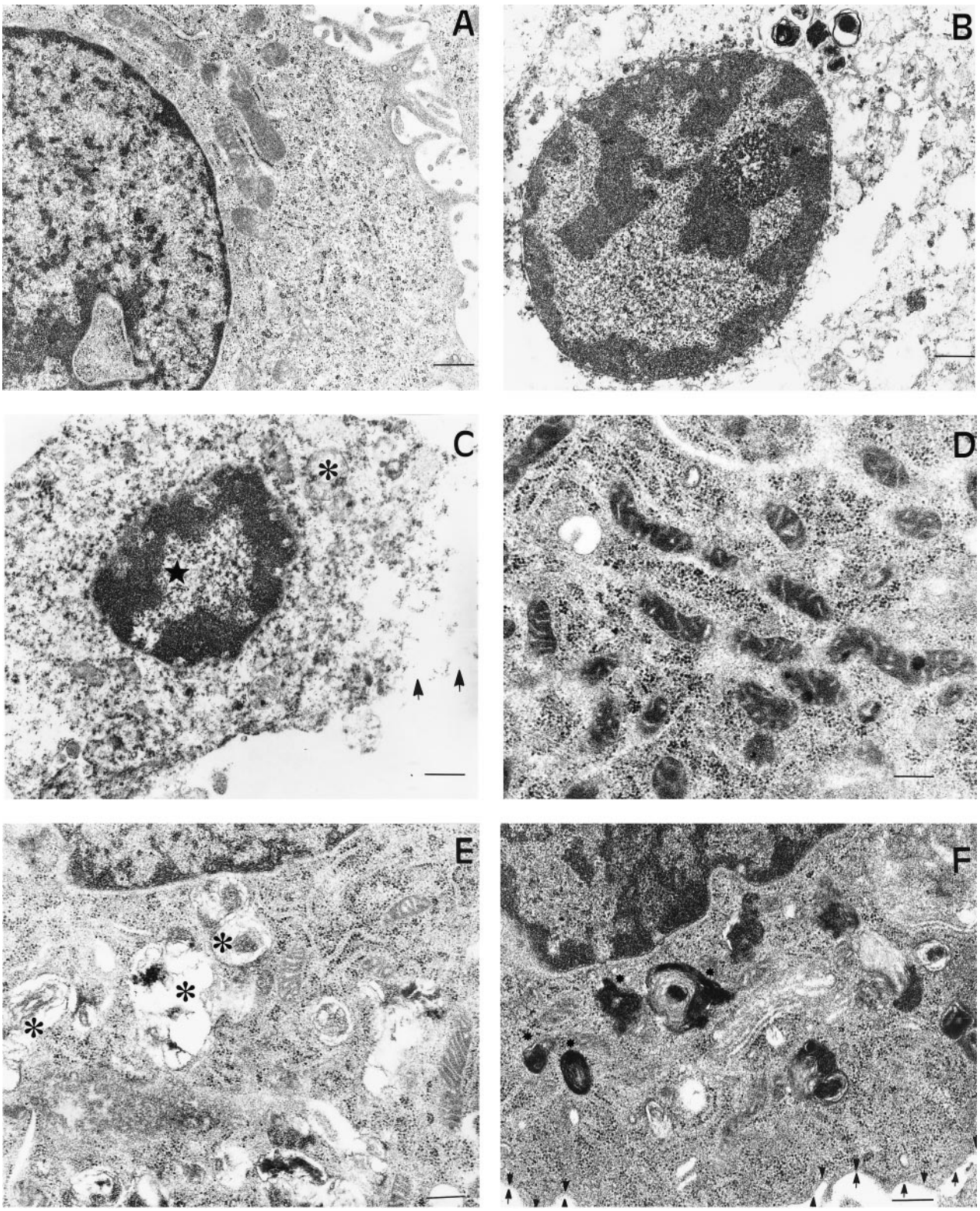

Fig. 7. Changes in cellular structure of rat glioma C6 cells after incubation with apomorphine. *Micrography A:* electron micrography of control C6 cells incubated without apomorphine for 6 hr (scale bar = 0.6 μm). *Micrography B:* dead C6 cell that remained adherent to the culture well after incubation with 400 μM apomorphine for 6 hr, showing features of necrosis (scale bar = 0.7 μm). *Micrography C*: non-adherent dead C6 cell incubated with 400 μM apomorphine for 6 hr. Typical features of necrosis include loss of plasma membrane integrity (↑), nuclear chromatin disintegration (★), and organelle degeneration (*) (scale bar = 0.6 μm). *Micrography D:* control C6 cell incubated without apomorphine for 48 hr (scale bar = 0.3 μm). *Micrography E*: rat glioma C6 cell incubated with 100 μM apomorphine for 24 hr. The cell contains degenerated organelles (*) and the number of normal mitochondria is reduced (scale bar = 0.3 μm). *Micrography F:* rat glioma C6 cell incubated with 100 μM apomorphine for 48 hr. Normal mitochondria have disappeared, and their degeneration leads to the formation of an important pigmentation (✱), although the cell still has an intact plasma membrane (↓) (scale bar = 0.3 μm).

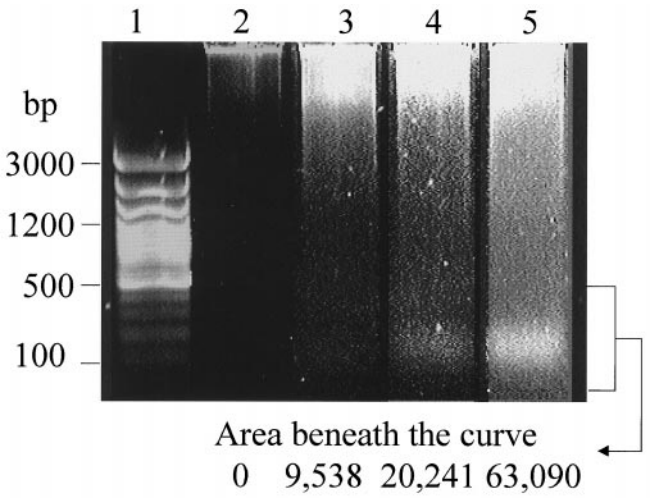

Fig. 8. Effect of different concentrations of apomorphine on rat glioma C6 cell DNA. One microgram of DNA extracted from C6 cells subjected to different apomorphine concentrations was separated by agarose gel electrophoresis. *Lane 1*: DNA marker; *Lane 2:* DNA obtained from control cells shows a single band near the starting origin of electrophoresis. *Lanes 3, 4, and 5:* DNAs isolated from cells incubated with either 200, 300, or 400 μM apomorphine for 6 hr, respectively. Densitometric analysis suggested that the degree of DNA disintegration, measured as a smear of 100- to 500-bp size, increased proportionally to apomorphine concentration. Densitometry values are arbitrary units.

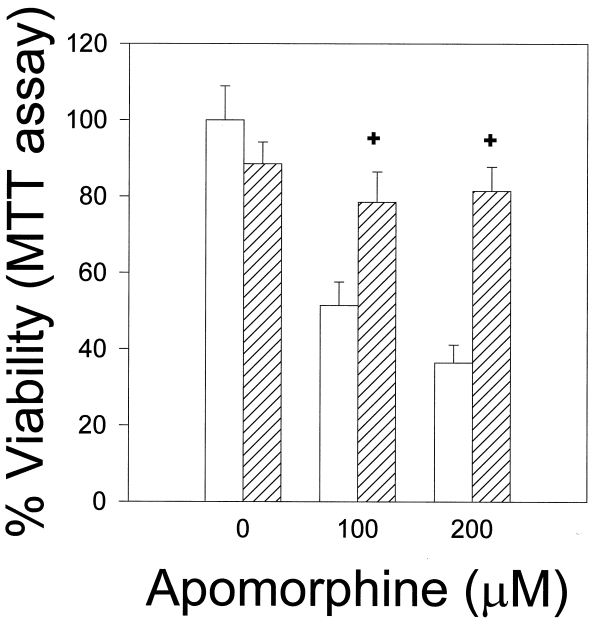

Fig. 10. Effect of *N*-acetyl-L-cysteine on the cytotoxicity of apomorphine towards cultured rat neurons. Neurons were incubated with apomorphine in the absence (open bars) or the presence of 10 mM *N*-acetyl-L-cysteine (hatched bars) as described in Fig. 4. Cell viability was determined by the MTT test and expressed as a percentage of controls without apomorphine. Data are expressed as the means ± SD, N = 10. (+), $P < 0.001$, compared with cells incubated only with apomorphine.

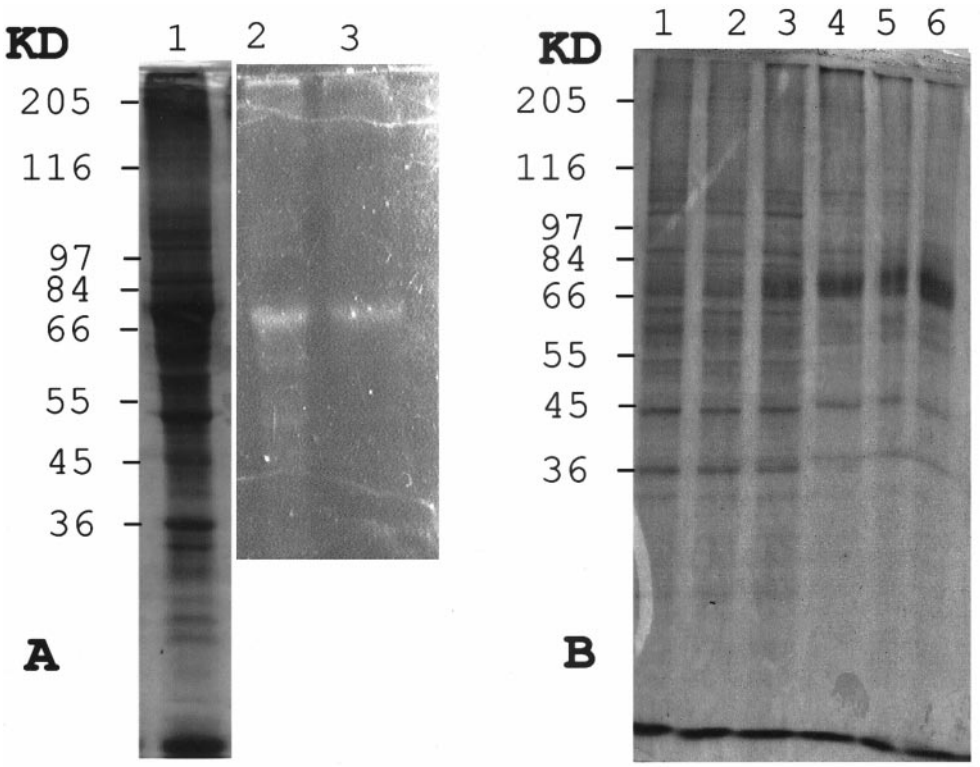

Fig. 9. Polyacrylamide gel electrophoresis of rat glioma C6 cell proteins from control and apomorphine-treated cells. (A) Fluorogram (right) and Coomassie blue staining (left) pattern showing proteins extracted from C6 cells treated with apomorphine 300 μM (lanes 1 and 2) and 400 μM (lane 3). Polyacrylamide (12%) gels were loaded with 45 μg protein. (B) Coomassie blue staining showing proteins extracted from C6 cells incubated without (lane 1) or with 50, 100, 200, 300, or 400 μM apomorphine for 48 hr (lanes 2–6, respectively). Polyacrylamide (12%) gels were loaded with 30 μg protein.

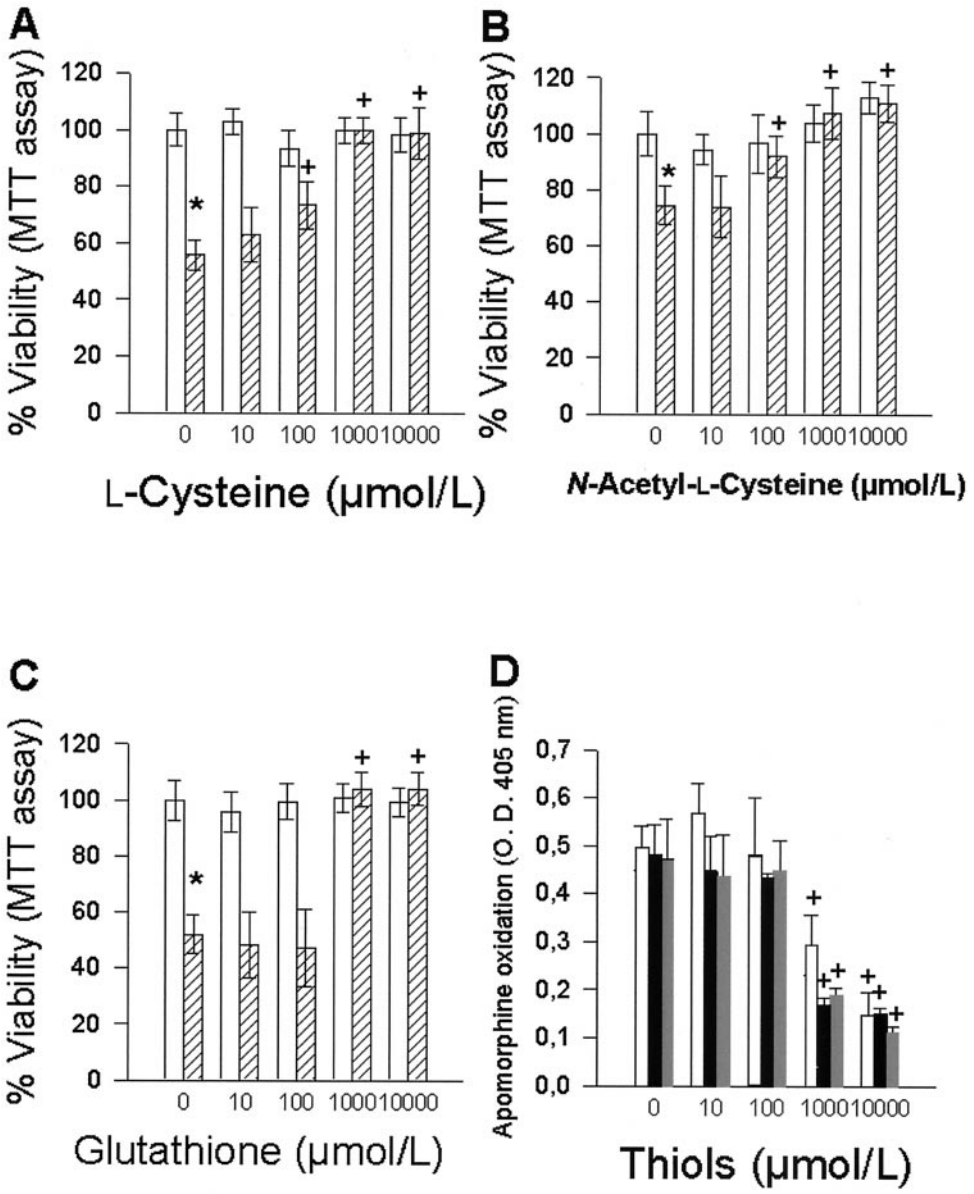

Fig. 11. Effect of thiols on the cytotoxicity of apomorphine towards rat glioma C6 cells. Cells were incubated with cysteine (*A*), *N*-acetyl-L-cysteine (*B*), and GSH (*C*) in the absence (open bars) or the presence of 400 μM apomorphine (hatched bars) for 6 hr. Cell viability was determined by the MTT test and expressed as a percentage of controls without apomorphine. (*D*) Effect of thiols on apomorphine autoxidation. Cells were incubated with cysteine (open bars), *N*-acetyl-L-cysteine (black bars), and with glutathione (gray bars) in the presence of 400 μM apomorphine. A melanin-like pigment that absorbs light at 405 nm was formed during apomorphine autoxidation. Data are expressed as the means ± SD, N = 8. (*), $P < 0.001$, compared with controls without added thiol reagents or apomorphine; (+), $P < 0.001$, compared with cells incubated with 400 μM apomorphine without thiol reagents.

icals and to increase DNA damage by Cu^{2+} ions, but noted that apomorphine inhibited Fe^{3+}-promoted lipid peroxidation. These observations suggest that like levodopa and dopamine, apomorphine displays scavenger properties at low concentrations, whereas it causes cell death at higher concentrations.

In aqueous solution, apomorphine undergoes spontaneous oxidation at neutral pH, forming both superoxide radicals and products that polymerize rapidly. This autoxidation process strongly resembles that already described for dopamine, levodopa, and 6-hydroxydopamine, with a subsequent formation of oxygen radicals, quinone derivatives, and neuromelanin associated with *in vitro* toxicity [19,20,40]. The apparent stimulatory effect of SOD on apomorphine autoxidation probably results from a favorable reduction potential of apomorphine oxidation products, resulting in a semiquinone:superoxide oxidoreductase activity of SOD [41]. On the other hand, apomorphine autoxidation was slightly inhibited by catalase, also indicating the involvement of H_2O_2 in the reaction, and it was largely inhibited by DTT and cysteine. Thiols probably prevented autoxidation of apomorphine by depletion of reactive oxygen species, even in cell cultures where their formation may occur intracellularly, as C6 cells became fluorescent when incubated with

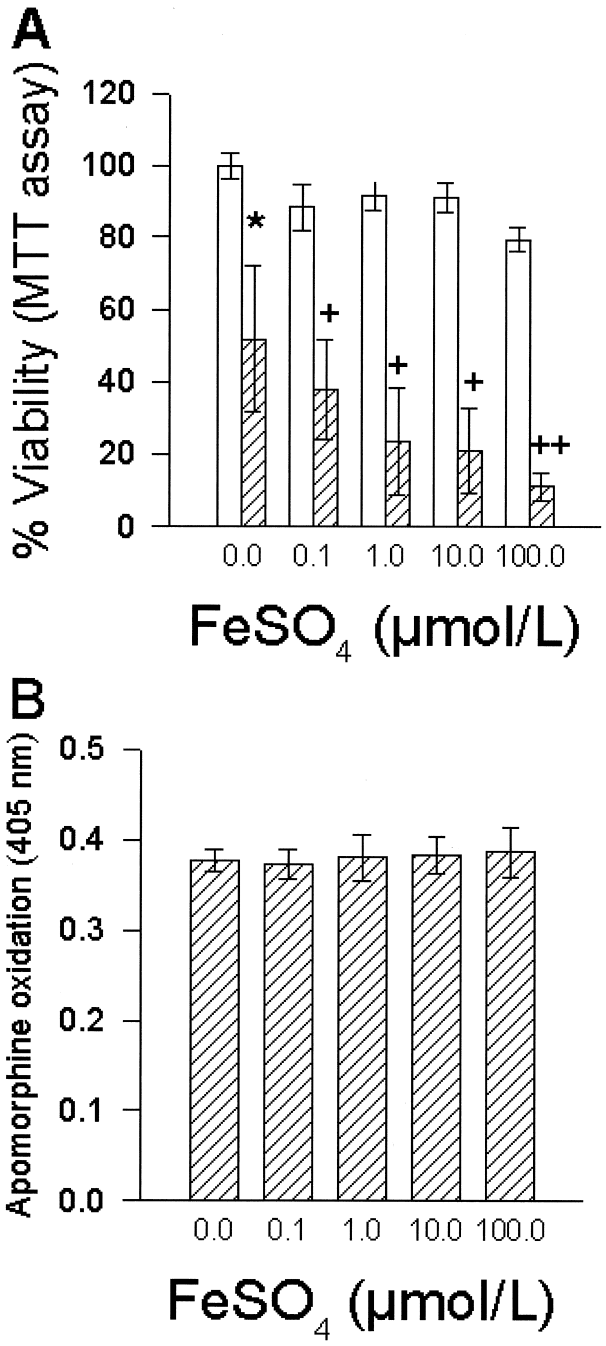

Fig. 12. Effect of Fe^{2+} on the cytotoxicity of apomorphine. The effects of the presence of Fe^{2+} on apomorphine cytotoxicity (*A*) and on apomorphine autoxidation (*B*) were studied by incubating rat glioma C6 cells with $FeSO_4$ in the absence (open bars) or the presence of 400 μM (hatched bars) for 6 hr. Data are the means ± SD, N = 8. (*): significantly different from the controls with $FeSO_4$, but in the absence of apomorphine, $P < 0.001$; (++): significantly different from controls without $FeSO_4$, but in the presence of 400 μM apomorphine, $P < 0.001$; (+): $P < 0.01$.

both apomorphine and DHR123. An intracellular protection against oxidative stress by thiols added to the culture medium is possible, as GSH can cross astrocyte membrane [42]. These results strongly suggest that apomorphine autoxidation initiates an intracellular oxidative stress, but does not exclude the toxicity of oxygen radicals formed during apomorphine autoxidation in the culture medium.

Apomorphine toxicity towards rat C6 glioma cells and neurons was clearly concentration- and time-dependent. Treated C6 cells showed fluorescent proteins that were not observed in controls, suggesting that apomorphine binds to proteins, as already shown in the central nervous system [27]. Apomorphine also irreversibly binds to albumin and liver microsomes in polyacrylamide gels, coloring proteins green [43]. Moreover, catecholamines can cross-link neurofilament proteins, resulting in the formation of Lewy bodies [44], and the formation of dopamine–protein adducts has been associated with dopamine toxicity *in vitro* [12]. The present work shows that C6 cell viability strongly depends on the mechanism of formation of a neuromelanin-like pigment during incubation with apomorphine. Thiol reagents significantly protected C6 cells and neurons, suggesting that quinone derivatives formed during apomorphine autoxidation react with sulfhydryls, resulting in adduct formation. In accordance with this hypothesis, very recent data showed the formation of levodopa and dopamine conjugates with cysteine and GSH during exposure to superoxide radicals [45].

A typical necrotic DNA degradation was found in C6 cells treated with 200 μM apomorphine for 6 hr. These results are consistent with DNA damage promoted by apomorphine in a cell-free system [17]. Several studies on dopamine and levodopa toxicity show that cultured neuron death occurs by necrosis or apoptosis [32,46]. The present results demonstrate that relatively high concentrations of apomorphine promote necrosis. This does not exclude the possibility that apoptosis may appear after exposition to lower concentrations of the drug, as previously suggested by studies on cultured neurons and on parkinsonian nigrostriatal samples [46].

Iron has been suggested as a catalyst of both catecholamine oxidation [18] and cytotoxic radical formation [47]. We observed that iron aggravates apomorphine cytotoxicity, but deferoxamine alone did not protect C6 cells (not shown). In the same way, neither SOD nor catalase protected C6 cells against apomorphine, although H_2O_2 is freely diffusible across the membrane, suggesting that the reactive oxygen species-promoted damage occurred before their efflux outside the cell.

Astrocytes contain more vitamin E, GSH, enzymes involved in GSH metabolism, and SOD than neurons [48], and are much less affected by an oxidative stress [49]. Accordingly, we showed that C6 cells are more resistant than neurons to apomorphine, in accordance with other results showing that C6 cells are more resistant than cultured pheochromocytoma (PC12) cells [16]. Dopamine and levodopa appear to be more cytotoxic towards neuroblastoma SH-SY5Y cells [19] than is apomorphine towards C6 cells, as cell viability was approximately 60 and 30% in the presence of 100 μM dopamine or levodopa, respectively. Apomorphine is also less toxic than menadione, as exposure

of astrocytes to 100 μM menadione killed all cells within 12 hr [50].

In conclusion, the present study shows that apomorphine induces cytotoxicity in both rat glioma C6 cells and rat cultured neurons *in vitro.* However, there is neither evidence of *in vivo* apomorphine neurotoxicity nor evidence that it alters the natural progression of the disease [39]. The present *in vitro* results also show that apomorphine autoxidation products irreversibly bind to intracellular proteins, forming fluorescent conjugates. As microdialysis assays showed that free apomorphine concentrates in brain cells [51], the true intraneuronal concentration (*i.e.* free *plus* protein-bound apomorphine) could be underestimated. As the parkinsonian brain contains high levels of unbound, redox-active iron [52] that should increase apomorphine toxicity, further studies are needed to characterize and establish the consequences of apomorphine accumulation and binding in the human brain.

Acknowledgments

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