

## Cytotoxic effects of catechol to neuroblastoma N2a cells

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**Abstract.** The mechanisms of catechol-induced cytotoxicity were studied in cultures of neuroblastoma N2a cells. The minimal cytotoxic concentration after 72 h was 20  $\mu\text{mol}\cdot\text{l}^{-1}$ . The  $\text{EC}_{50}$  after 72 h was 38  $\mu\text{mol}\cdot\text{l}^{-1}$ . There was not a correlation between the cytotoxicity and the formation of quinones in the medium. Catechol-induced cytotoxicity was increased significantly when superoxide dismutase (SOD) was added. The addition of catalase did not protect cells, but this enzyme reverted the deleterious effect of SOD. The experimental studies showed a detrimental effect of deferoxamine on catechol-induced cytotoxicity suggesting that cells need iron to maintain its metabolism. NF- $\kappa$ B inhibitors increased the cytotoxicity, suggesting that this factor is also important for cell viability. L-cysteine and N-acetyl-L-cysteine protected cells significantly in a dose-dependent manner. The use of monochlorobimane showed that catechol induced reduced glutathione (GSH) depletion after 24 h, prior to cell death. The mode of cell death was studied by flow cytometry after double staining with annexin V and propidium iodide. Catechol induced apoptosis after 72 h. Furthermore, catechol also induced nuclear fragmentation. These data showed that catechol-induced cytotoxicity to N2a cell was not directly a consequence of reactive oxygen species production. Rather, it was due to GSH depletion followed by the induction of apoptosis.

**Key words:** Apoptosis — Catechol — Cytotoxicity — Glutathione — Neuroblastoma

### Introduction

Benzene is found in gasoline and this compound has been linked to cancer. Benzene was found and measured in the residential indoor air of subarctic homes, and it has been supposed that its primary source was gasoline in the home's attached garage (Isbell et al. 2005). An earlier article established that ethanol could interfere pharmacologically with benzene metabolism increasing its toxicity, and heightening the anemia and lymphocytopenia induced by this molecule, leading to the development of hematological diseases (Bermond II and Tose 2000). The aromatic hydrocarbon benzene is a well-recognized haematotoxin and carcinogen associated with malignancy in occupational environments. Atkinson has found that benzene metabolites are responsible

for its cytotoxicity, and are implicated in the progression to carcinogenicity (Atkinson 2008). 1,2-dihydroxybenzene, more commonly known as catechol, is one of its metabolites. In a previous study, although catechol did not affect  $^{59}\text{Fe}$  uptake into erythrocyte hemoglobin, it was decreased in a dose-dependent manner when this compound was given with phenol – another benzene metabolite (Snyder et al. 1989). However, despite of the catechol toxicity, this chemical group is present in endogenous biomolecules like 3,4-dihydroxy-L-phenylalanine (DOPA), which is also used as a drug in the treatment of Parkinson's disease. Furthermore, methods of treating neurodegenerative diseases have been patented, claiming the administration of new pharmaceutically effective compounds with additional drugs that are catechols like DOPA and some catechol-O-methyl transferase inhibitors (Chiang et al. 2007). Chromogenic catechols, including DOPA, may also be used as substrates to monitor the expression of screenable marker genes cloned in vectors and constructs, which encode enzymes like catechol dioxygenase (Bledig et al. 2008). The catechol group is

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also present in many natural products, including alkaloids obtained from marine organisms like lamellarin S obtained from the Australian tunicate *Didemnum* sp., or in synthetic compounds such as lamellarin Y (Fan et al. 2007). Hence, since the catechol group is largely distributed in nature, it is important to study its cytotoxicity.

Catechol has been used as a preservative at nontoxic dosages in pharmaceutical compositions patented to treat cancers, such as neuroblastoma (Lazar et al. 2007a,b, 2008). However, catechol is the most abundant phenol in tobacco smoke (80–400 µg/cigarette) that can be used as a stressor to study its effects on survival of neuronal cells such as neuroblastoma cell lines and primary retinal neuronal cells (Kubota 2008). Furthermore, catecholaminergic neuroblastoma cells can also be useful for analyzing Parkinson's disease pathogenesis *in vitro* using cell culture models (Lansbury and Liu 2007).

In our previous experiments we have demonstrated that catechol was cytotoxic to human glioblastoma cells *in vitro* via superoxide ( $O_2^-$ ) and quinone generation, killing 50% of these cells ( $EC_{50}$ ) when they were treated with 230 µmol·l<sup>-1</sup> of this substance for 72 h (Pereira et al. 2004). Other authors have shown that synthetic catechols diacetates were also cytotoxic to PC12-AC cells, and it has been supposed that these effects were due to the conversion of acetylated derivatives into catechols by intracellular esterases, with  $EC_{50}$  values ranging between 26–154 µmol·l<sup>-1</sup>, after 24 h (Chichirau et al. 2005). Catechol reacts with metal ions and can be used in coordination chemistry to form complexes with iron (III) or ruthenium (III).  $[Ru^{III}(NH_3)_4(catechol)]^+$  complex was less toxic than catechol to human glioblastoma GL-15 cells and also to rat astrocytes (Almeida et al. 2007). Thus, the present study was undertaken to assess the mechanisms of catechol-induced cytotoxicity to mouse neuroblastoma N2a cells. This study investigated whether reactive oxygen species (ROS) and quinones are involved in catechol-induced cytotoxicity to N2a cells. The induction of glutathione depletion and the kind of cell death were also studied.

## Materials and Methods

### Neuroblastoma cell cultures

Experiments were carried out using mouse neuroblastoma N2a cell line, which were kindly provided by Dr. L. D. G. Alvarez. The cells were cultured in Dulbecco's modified Eagle's medium/Ham F-12 (Cultilab, Campinas, Brazil), supplemented with 10% fetal calf serum (Cultilab), 100 IU·ml<sup>-1</sup> penicillin (Cultilab), and 100 µg·ml<sup>-1</sup> streptomycin (Cultilab). The cells were incubated in a humidified 5% CO<sub>2</sub> and 95% air atmosphere at 37°C. The culture medium was replaced three times a week. At the time of the experi-

ment, confluent cells were trypsinized and plated in 96-well plates (TPP, Trasadingen, Switzerland), or into tissue culture dishes (40 mm in diameter; TPP). N2a cells were plated at a density of 31,000 cells/cm<sup>2</sup>. Experiments were initiated 72 h after plating.

### *In vitro* evaluation of catechol-induced cytotoxicity

Catechol (Riedel-de Haen, Buchs, Switzerland) at concentrations between 1 and 200 µmol·l<sup>-1</sup> were used to examine its cytotoxic effects. Eight replicates were used for each dose per 96-well plate in seven independent experiments. Cells were exposed to catechol for 72 h. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, U.S.A.). MTT was dissolved at a concentration of 5 mg·ml<sup>-1</sup> in sterile phosphate buffered saline (PBS) at room temperature, and the solution was further sterilized by passing through a 0.2 µm filter and stored at 4°C in the dark. The final concentration of MTT added to each well was 1 mg·ml<sup>-1</sup>. After 2 h of incubation at 37°C, a same volume of lysis buffer was added. Lysis buffer was prepared as follows: 20% (w/v) sodium dodecyl sulfate was dissolved at 37°C in a solution of 50% (v/v) of *N,N*-dimethyl formamide and reagent grade water, pH 4.7. After an overnight incubation at room temperature, optical densities were measured at 580 nm. Cell viability was normalized to data measured under control conditions, without catechol. Catechol oxidation in the medium was monitored by method based on the formation of quinones, which absorb light at 405 nm. The time-course of catechol-induced cytotoxicity was studied by treating cells with 60 µmol·l<sup>-1</sup> of this compound. A total of 9 tissue culture dishes (40 mm in diameter) containing N2a cells were exposed to catechol. The dishes were divided into 3 separate groups ( $n = 3$  for each group), which were subjected for 24, 48, and 72 h of exposition, respectively. Three control dishes for each time were kept under the same conditions without being exposed to catechol. Cell viability was measured as described above.

### *Involvement of ROS generation in the cell environment on catechol-induced cytotoxicity*

In order to study the involvement of ROS generated by catechol oxidation in the environment in the induction of N2a cell death, superoxide dismutase (SOD; EC 1.15.1.1;  $3 \times 10^3$  U·ml<sup>-1</sup>;  $n = 8$ ; Sigma), and/or catalase (EC 1.11.1.6;  $10^5$  IU·ml<sup>-1</sup>;  $n = 8$ ; Sigma) were incubated with 60 µmol·l<sup>-1</sup> catechol for 72 h at 37°C. One unit of SOD is the amount of enzyme that inhibits the rate of reduction of cytochrome c by 50%. Each group was normalized to data measured under control conditions, without catechol and compared to the group treated with catechol alone.

### Exposure to drugs and protective assays

Cells were plated in 96-well plates and exposed to several drugs. All plates were exposed to various concentrations of several drugs ( $n = 8$  for each concentration) for 72 h in order to determine their  $EC_{50}$  by the use of the MTT assay. L-ascorbic acid (J. T. Baker, U.S.A.) was used because of its antioxidant properties (its concentration varied between 0.03 and 10  $mmol \cdot l^{-1}$ ); *N*-acetyl-L-cysteine (NAC; Proton, Jabaquara, Brazil) and L-cysteine chloride (Merck, Darmstadt, Germany) were used because of the antioxidant properties of their sulfhydryl groups (their concentrations varied between 0.1 and 30  $mmol \cdot l^{-1}$ ); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (CPTIO; Sigma) was used because it reacts with nitric oxide (NO) scavenging this radical, and the product of this reaction also inhibits NO synthase (its concentration varied between 0.06 and 1  $mmol \cdot l^{-1}$ ); curcumin (Sigma) was used due to its anti-inflammatory and anti-oxidant properties (its concentration varied between 0.6 and 200  $\mu mol \cdot l^{-1}$ ); deferoxamine mesylate salt (Sigma) – an iron chelator – was used at concentrations varying between 0.2 and 60  $\mu mol \cdot l^{-1}$ ; manganese(III)tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP; Calbiochem, San Diego, U.S.A.) – a cell-permeable SOD mimetic and peroxyxynitrite ( $ONOO^-$ ) scavenger were used at concentrations between 0.001 and 1  $mmol \cdot l^{-1}$ ; parthenolide (Sigma) – an anti-inflammatory agent that inhibits nuclear factor- $\kappa B$  (NF- $\kappa B$ ) activation was used at concentrations that varied between 0.3 and 100  $\mu mol \cdot l^{-1}$ ; (S)-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK; Sigma) was used because it also blocks the activation of NF- $\kappa B$  (its concentration varied between 1 and 300  $\mu mol \cdot l^{-1}$ ). In protective assays, cells were exposed to a cytotoxic concentration of catechol (60  $\mu mol \cdot l^{-1}$ ), and the concentration of these drugs was varied in a range below their effective cytotoxic concentrations.

### Morphological analysis and flow cytometry

#### Reduced glutathione (GSH) depletion evaluation by fluorescence microscopy

Monochlorobimane (MCB; Sigma) was used to evaluate GSH depletion. A stock solution of MCB was prepared at 100  $mmol \cdot l^{-1}$  in absolute ethanol and stored at  $-20^\circ C$  in the dark. After 72-h exposure to 60  $\mu mol \cdot l^{-1}$  catechol in tissue culture dishes (40 mm in diameter), N2a cells were washed three times with PBS following incubation with 1  $mmol \cdot l^{-1}$  MCB in 400  $\mu l$  of the medium for 40 min, under the same conditions described for cell cultures. After the incubation time, cells were washed again with PBS. DL-buthionine-(S,R)-sulfoximine (BSO; Sigma) was used at

the concentration of 1  $mmol \cdot l^{-1}$  to inhibit GSH synthesis in positive control group. The negative control group was not treated with any drug. Cells were observed by fluorescence microscopy (Olympus BX 51-URA2, San Jose, U.S.A.). The fluorescence mirror unit Olympus U-MWU2 was selected to observe cells. This mirror unity excites the fluorochrome in the band between 330–385 nm, it has a dichromatic mirror (400 nm), and an emission filter of 420 nm. Photographs were taken using an exposure time of 60 ms for all samples by an Olympus BX-2 camera attached to the microscope.

#### Phase contrast microscopy

Cell morphology was evaluated by phase contrast microscopy using an inverted microscope Eclipse TS100 (Nikon, Tokyo, Japan). Photographs were taken by a Coolpix 4300 digital camera (Nikon) attached to the microscope. Nikon View version 6.1.0 was used to transfer images from the camera to a computer to be edited. The only process used to edit images was to transform colour photographs into halftone pictures. A ruler with ticks every 10  $\mu m$  (Olympus, Tokyo, Japan) was photographed under the same conditions. A new layer containing the ruler was added to pictures using Photo Impression 4.0 (ArcSoft, Fremont, U.S.A.).

#### Annexin V FITC labeling

In this experiment, N2a cells treated with 60  $\mu mol \cdot l^{-1}$  catechol for 72 h, and untreated cells (control group) were trypsinized and centrifuged at  $10^3 \times g$  for 3 min. Cells were resuspended in 300  $\mu l$  of PBS and incubated with annexin V FITC and propidium iodide (PI) for 15 min according to manufacturer (Annexin V-FITC Kit, BioSource, Camarillo, U.S.A.). Non-apoptotic cells are not stained. Annexin V labels early apoptotic cells, but PI does not stain them. Necrotic cells or late apoptotic cells are double stained. Cells stained only by PI are considered as necrotic ones.

#### Flow cytometry

A fluorescence activated flow cytometer (Becton-Dickinson, San Jose, U.S.A.) was used to examine cells ( $3 \times 10^4$ ). Annexin V FITC, which has a high affinity for phosphatidylserine on the outer membrane of apoptotic cells, exhibits fluorescence that is measured in the green channel (FL-1) on flow cytometers. PI, which binds to DNA of necrotic cells or during the late stage of apoptosis, is measured in the red channel (FL-2) on flow cytometers.

#### Hoechst staining to detect nuclear fragmentation

Cultured N2a cells were exposed to 60  $\mu mol \cdot l^{-1}$  catechol for 72 h to examine if it could induce nuclear fragmentation

compared to untreated control cells. Then, the cells were incubated with  $9.4 \mu\text{mol}\cdot\text{l}^{-1}$  of the membrane-permeable dye Hoechst 33258 (Sigma) for 10 min at room temperature in the darkness, and images were obtained using an epifluorescence microscope as described previously (Movsesyan et al. 2002). Total and fragmented nuclei were counted in 10 different fields in both groups. The amount of cells with fragmented nuclei were expressed as a percentage of total cells.

### Statistical analysis

The data for concentration-response curves were fitted using nonlinear regression performed with GraphPad Prism software (San Diego, U.S.A.). This calculation was used to determine the  $\text{EC}_{50}$  of catechol and other drugs. The data for concentration-response curves were analyzed using one-way ANOVA and groups were compared by Student-Newman-Keuls test. Student's *t*-test was used to compare the viability of cells treated with catechol and their respective controls at different times. This test was also used to compare the effect of antioxidant enzymes with the viability of cells treated only with catechol.

## Results

### Catechol-induced cytotoxicity to neuroblastoma N2a cells

The effect of the concentration of catechol on the induction of cytotoxicity to neuroblastoma N2a cells was investigated (Fig. 1a). It was found that the minimal cytotoxic concentration was  $20 \mu\text{mol}\cdot\text{l}^{-1}$  as compared to control group, killing 24.8% of cells after 72 h. Catechol-induced cytotoxicity was fitted to Eq. (1):

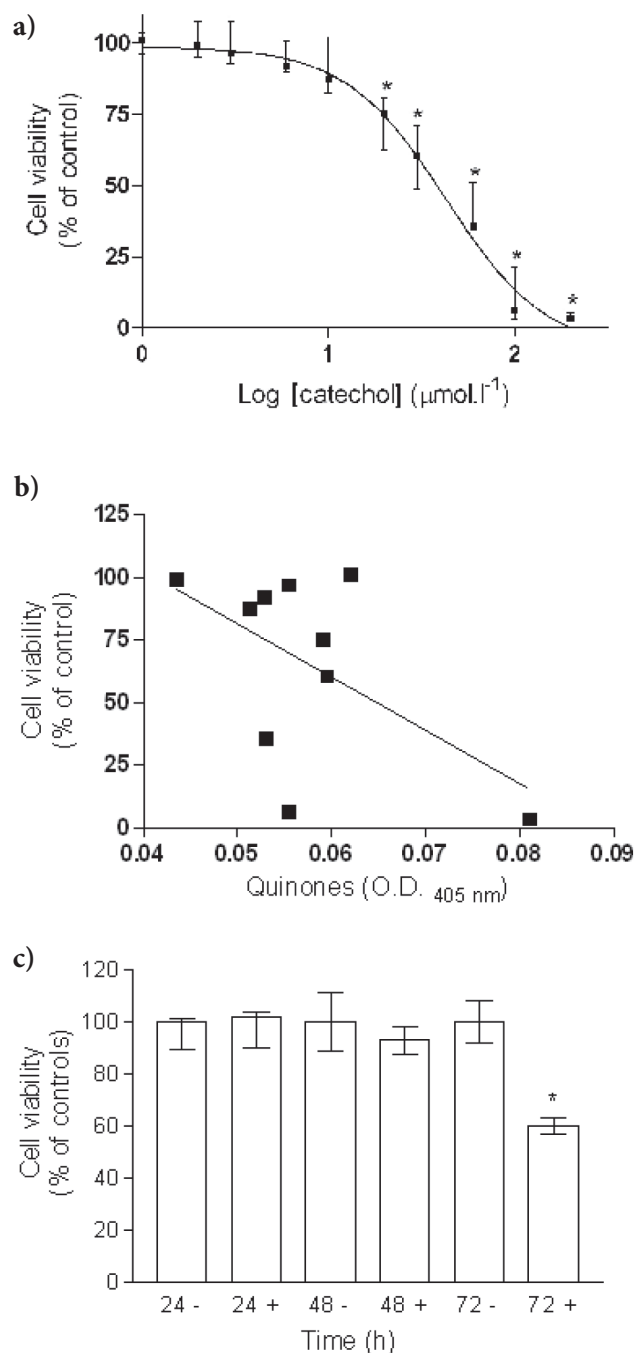
$$V = \{106.989 / [1 + 10^{(1.606 \log [c] - 2.624)}]\} - 8.279; \quad (1)$$

$(R^2 = 0.9920)$

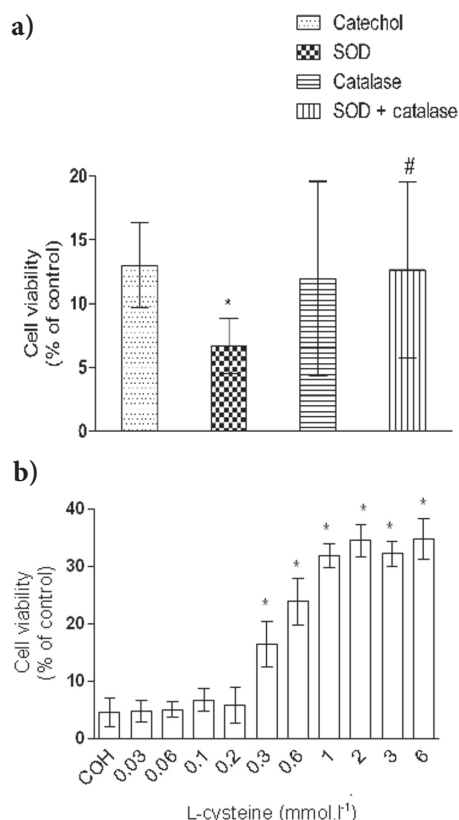
in which *V* corresponds to cell viability normalized to data measured under control conditions, and [*c*] is the catechol concentration. The calculated  $\text{EC}_{50}$  for catechol on N2a cells after 72 h was  $38 \mu\text{mol}\cdot\text{l}^{-1}$ . Fig. 1b shows the absence of correlation between cytotoxicity and quinone formation (Spearman  $r = -0.322$ ;  $p = 0.3679$ ). There was no difference between cells treated with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol and control ones until 48 h (Fig. 1c). However, cell viability was significantly lower after 72 h.

### The effect of antioxidants and other drugs on the protection against catechol-induced cytotoxicity

The catechol-induced cytotoxicity was increased significantly ( $p < 0.001$ ) in the presence of SOD (Fig. 2a). There was no



**Figure 1.** Catechol-induced cytotoxicity to neuroblastoma N2a cells. **a)** N2a cells were exposed to catechol ( $1\text{--}200 \mu\text{mol}\cdot\text{l}^{-1}$ ) for 72 h, at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , in 96-well plates. Seven independent experiments were carried out,  $n = 8$  for each concentration. Cell viability was normalized to data measured under control conditions, without catechol. \*  $p < 0.05$  versus control cells. **b)** Correlation between N2a cell viability and the formation of quinone (O.D.<sub>405 nm</sub>). **c)** Cytotoxicity of  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol to N2a cells after 24, 48, and 72 h. - control cells incubated in the absence of catechol; + cells exposed to catechol; \*  $p < 0.05$  versus control cells.



**Figure 2.** The effect of SOD, catalase and L-cysteine on catechol-induced cytotoxicity to N2a cells. **a)** N2a cells were exposed to  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol for 72 h in the absence of antioxidant enzymes, in the presence of  $3 \times 10^3 \text{ U}\cdot\text{ml}^{-1}$  SOD,  $10^5 \text{ IU}\cdot\text{ml}^{-1}$  catalase or both. \*  $p < 0.001$  compared to catechol by the Student's *t*-test. #  $p < 0.05$  compared to SOD. **b)** N2a cells were exposed to  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol (COH) for 72 h in the absence of L-cysteine or in the presence of this amino acid ( $0.03\text{--}6 \text{ mmol}\cdot\text{l}^{-1}$ ). \*  $p < 0.05$  compared to cells treated only with catechol by one way ANOVA followed by the post-hoc test of Student-Newman-Keuls.

difference between catechol-treated groups in the absence and in the presence of catalase, but this enzyme reverted the deleterious effect of SOD.

Table 1 shows the cytotoxic effects of treatments with several drugs to N2a cells. These drugs were further used in protective assays at concentrations beneath their toxic concentrations. Curcumin and NF- $\kappa$ B inhibitors showed the greatest cytotoxicities. Curcumin and TPCK were as toxic as catechol and parthenolide was more toxic than it. On the other hand, ascorbic acid, cysteine and its derivative, NAC, showed low toxicities. The  $\text{EC}_{50}$  of deferoxamine ( $0.2\text{--}3 \mu\text{mol}\cdot\text{l}^{-1}$ ) and Mn-TBAP ( $0.06\text{--}1 \text{ mmol}\cdot\text{l}^{-1}$ ) could not be determined in these ranges of concentration. However, since the minimal lethal concentration for deferoxamine was  $3 \mu\text{mol}\cdot\text{l}^{-1}$  (data not shown), this concentration was considered as a limit for protective assays. It seems that NO, ONOO<sup>-</sup>, and O<sub>2</sub><sup>-</sup> are not involved in catechol-induced cytotoxicity to N2a cells, since CPTIO and Mn-TBAP had no significant protective effect. Ascorbic acid and curcumin also failed to protect cells. On the other hand, inhibitors of NF- $\kappa$ B increased catechol-induced cytotoxicity. The same was observed with the iron chelator deferoxamine. Cysteine (Fig. 2b) and *N*-acetyl cysteine were the only agents that protected cells significantly in a dose-dependent manner. However, these amino acids were unable to fully protect cells.

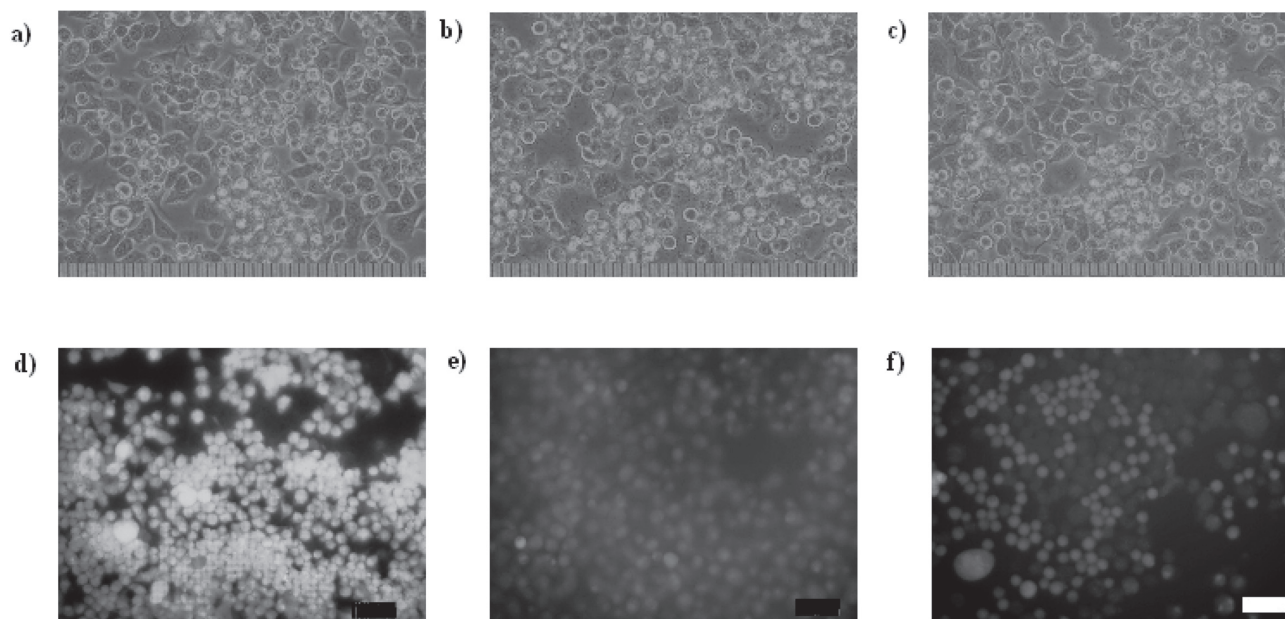
#### The effect of catechol treatment on GSH depletion

The morphology of cells treated with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol (Fig. 3b) or  $1 \text{ mmol}\cdot\text{l}^{-1}$  BSO (Fig. 3c) was not modified after 24 h when compared to the negative control group (Fig. 3a). However, the fluorescence was decreased in groups after 24-h exposure to catechol (Fig. 3e) or to BSO (Fig. 3f), with respect to negative controls (Fig. 3d).

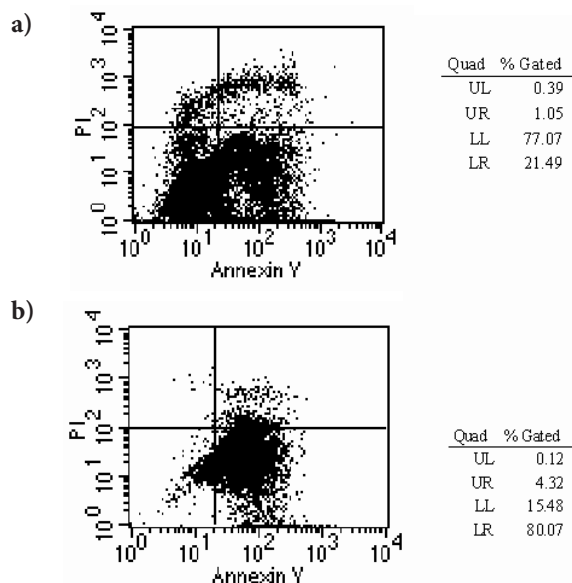
**Table 1.** Protective assays against catechol-induced cytotoxicity carried out using several agents below their effective cytotoxic concentrations

Drugs	$\text{EC}_{50}$ ( $\text{mmol}\cdot\text{l}^{-1}$ )	Concentration range for protective assays ( $\text{mmol}\cdot\text{l}^{-1}$ )	Protective assays	One way ANOVA
NAC	8.53	0.030 – 6.000	↑	$p < 0.001$
L-cysteine	12.07	0.030 – 6.000	↑	$p < 0.001$
Ascorbic acid	3.59	0.001 – 2.000	NS	$p > 0.05$
CPTIO	0.20	0.001 – 0.030	NS	$p > 0.05$
Curcumin	0.04	0.001 – 0.010	NS	$p > 0.05$
Mn-TBAP	NC	0.001 – 1.000	NS	$p > 0.05$
Deferoxamine	NC	$2 \times 10^{-5}$ – 0.003	↓	$p < 0.02$
Parthenolide	0.01	$6 \times 10^{-5}$ – 0.010	↓	$p < 0.05$
TPCK	0.04	$2 \times 10^{-4}$ – 0.030	↓	$p < 0.001$

NC, not cytotoxic in the range that was tested; ↑ significant protective effect against catechol-induced cytotoxicity; ↓ significant increase in catechol-induced cytotoxicity; NS, not significant effect.



**Figure 3.** The effect of the 24-h treatment with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol on N2a morphology (b) and GSH content (e). The morphology of negative control cells is seen in (a) and the fluorescence emitted by the product of the reaction of GSH with MCB is seen in (d). The morphology of positive control cells treated with  $1 \text{ mmol}\cdot\text{l}^{-1}$  BSO for 24 h is seen in (c), and the depletion of GSH is showed in (f). One unit in rulers showed in (a), (b), and (c) corresponds to  $10 \mu\text{m}$ . Bars in (d), (e), and (f) have  $100 \mu\text{m}$ .



**Figure 4.** The effect of 72-h treatment with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol on the mode of N2a cell death. **a)** Flow cytometry of control cells labeled with annexin V and PI. **b)** Flow cytometry of N2a cells treated with catechol and labeled with annexin V and PI. Quadrants (Quad): lower left (LL) – non-labeled alive cells; lower right (LR) – cells labeled only with annexin V (apoptosis); upper left (UL) – cells labeled only with PI (necrosis); upper right (UR) – double stained cells (necrosis or late apoptosis). The percentage of cells counted in each quadrant (% Gated) is shown beside the dot plot graphs.

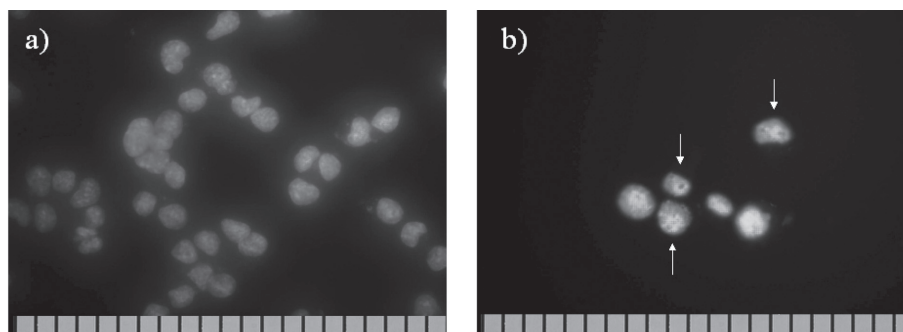
#### *The effect of catechol treatment on the mode of cell death*

To study the effect of catechol on the mode of cell death, cells were labeled with annexin V and PI (Fig. 4). Cells treated with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol for 72 h presented a decrease in the percentage of non-labeled alive cells (15.48%; Fig. 4b) when compared with control cells (77.07%; Fig. 4a). The percentage of cells labeled only with annexin V (apoptotic cells) was higher in the group treated with catechol (80.07%) than in control cells (21.49%). Treatment with catechol also increased the percentage of double stained cells (necrotic cells or late apoptotic cells).

Nuclear fragmentation is associated with apoptotic cell death. To study the effect of catechol on nuclear fragmentation, cells were labeled with Hoechst 33258 (Fig. 5). A total amount of 3337 nuclei were counted in 10 different fields of control cells, in which 818 of them were fragmented (24.5%). In cells that were treated with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol for 72 h, a total amount of 514 nuclei were counted in 10 fields, and 445 of them were fragmented (86.8%). These results suggest that catechol induces cell death by apoptosis.

#### **Discussion**

Despite significant research in myeloid leukaemias, there are limited biological and epidemiological studies on benzene and its metabolites in nonhaematopoietic cell types



**Figure 5.** The effect of 72-h treatment with  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol on the nuclear fragmentation. **a)** Control cells. **b)** Treated cells. Arrows show fragmented nuclei. One unity in rulers corresponds to  $10 \mu\text{m}$ .

(Atkinson 2008). In this study, N2a cells were used to determine the mechanisms of catechol-induced cytotoxicity. Neuroblastoma cells have been used to examine the effectiveness of therapeutic agents, such as interferon  $\beta$  and trichostatin A (Hamner et al. 2008). Mouse neuroblastoma N2a cells have been used in immunocytochemical studies to localize proteins in different cellular compartments (Mohrlüder et al. 2007), and also as a model to study scrapie (Chiang et al. 2007).

Catechol was cytotoxic to N2a cells after 72 h, at concentrations above  $20 \mu\text{mol}\cdot\text{l}^{-1}$ , with an  $\text{EC}_{50}$  of  $38 \mu\text{mol}\cdot\text{l}^{-1}$ . The comparison between data for catechol-induced cytotoxicity to N2a cells and those obtained in the literature for cells of glial origin (Almeida et al. 2007; Barreto et al. 2007; Pereira et al. 2004) shows that the former are more sensitive. Furthermore, the cytotoxic effects of catechols to glial cells were correlated to the formation of quinones. However, no correlation between the response of N2a cells to catechol concentrations and the formation of quinones was found in this study. It was supposed that glial cells are more resistant than neuronal cells because they express more antioxidant and drug metabolizing enzymes than neurons. Furthermore, since the cytotoxicity did not correlate to quinone generation, it was supposed that the cell death was not directly due to the formation of ROS, as it was previously described for glial cells.

The involvement of free radicals in catechol-induced cytotoxicity, the role of these toxic species and the antioxidant defense system have been studied. The results showed an increase in the cytotoxicity of catechol in the presence of SOD. SODs are specific antioxidant enzymes that dismutate  $\text{O}_2^-$ , forming hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The increase in catechol-induced cytotoxicity could be due to the generation of  $\text{H}_2\text{O}_2$  catalyzed by SOD. Furthermore, catalase reverted this deleterious effect of SOD. However, it seems that  $\text{H}_2\text{O}_2$  is not formed at toxic concentrations outside cells during the oxidation of catechol, because catalase alone did not protect them against catechol-induced cytotoxicity. 4-*t*-butylcate-

chol has also been shown to be cytotoxic to neuroblastoma cells at concentrations above  $30 \mu\text{mol}\cdot\text{l}^{-1}$  after 24 h, but at concentrations below  $10 \mu\text{mol}\cdot\text{l}^{-1}$  it was able to scavenge lipopolysaccharide-induced extracellular  $\text{O}_2^-$  generation (Lo et al. 2008). Catechol-mediated cytotoxicity to human glioblastoma GL-15 cells has been observed at concentrations above  $100 \mu\text{mol}\cdot\text{l}^{-1}$  after 72 h, and it was related to extracellular  $\text{O}_2^-$  generation (Pereira et al. 2004).  $\text{H}_2\text{O}_2$  has been used in retinal cell oxidative stress model system (Kubota 2008). However, since neither SOD nor catalase protected N2a cells, another mechanism must be thought to explain catechol toxicity to these cells.

It is known that ascorbic acid and curcumin may act as antioxidants. In the present study, treatment with these drugs did not show any significant protection against catechol-induced cytotoxicity. Additionally, the cell permeable SOD mimetic Mn-TBAP was also unable to protect N2a cells. This is probably indicative that intracellular  $\text{O}_2^-$  is not involved in the mechanism of toxicity. Furthermore, since both SOD and catalase are present inside cells, Mn-TBAP did not increase catechol-induced cytotoxicity as did SOD, when it was added alone to the extracellular medium. CPTIO, a NO scavenger, did not also protect N2a cells. Catechol is a suitable iron chelator group (Bergeron 2006). The experimental studies showed a detrimental effect of deferoxamine on catechol-induced cytotoxicity. It seems that N2a cells need iron from the medium to maintain its metabolism. Although iron chelators were able to protect PC12-AC cells against death induced by serum withdrawal, they were toxic at concentrations above  $10 \mu\text{mol}\cdot\text{l}^{-1}$  (Youdim et al. 2006). Iron chelators have been claimed to treat neuroblastoma (Bergeron 2006). NF- $\kappa$ B inhibitors increased the cytotoxicity of catechol, suggesting that this transcription factor is important for the viability of these cells. SK-N-AS neuroblastoma cells highly express the interleukin 1 signal transducer, also known as TRAF6, an activator of NF- $\kappa$ B (Padigaru 2006).

We have found that L-cysteine was toxic to N2a cells after 72 h with an  $\text{EC}_{50}$  of  $3.59 \text{ mmol}\cdot\text{l}^{-1}$ . However, L-cysteine

had a protective effect that was significantly dependent on the concentration, but the dose that effectively protects 50% of cells ( $ED_{50}$ ) was not attained. The dose ratio between toxic and therapeutic effects ( $EC_{50}/ED_{50}$ ) is known as the therapeutic index. Good therapeutic compounds exhibit large therapeutic indices (Welch and Zhuo 2007). Since the  $ED_{50}$  was not attained, we cannot say that L-cysteine was an effective protector, but these findings enabled us to hypothesize the possible mechanism of action of catechol. Catechol-induced cytotoxicity could be correlated with the depletion of intracellular biomolecules containing sulfhydryl groups, which are active as nucleophiles, since L-cysteine and *N*-acetyl-L-cysteine significantly protected cells in a dose-dependent manner. Oxidation of catechols generates *o*-benzoquinones that are able to react with the nucleophilic group in the side chain of cysteine. This reaction could lead to the loss of cellular GSH, a mechanism that induces neuron degeneration (Youdim et al. 2006). Furthermore, glutathione *S*-transferases (EC 2.5.1.18) are phase II drug metabolizing enzymes that conjugate reactive electrophiles, such as *o*-benzoquinones, with GSH, and have been implicated in its depletion, resulting in toxicological effects (Leone et al. 2007). The conjugation of quinones with GSH inside cells could be the reason why they were not observed in the medium, resulting in an absence of correlation between the cytotoxicity and quinone generation.

In this investigation, no difference was found between the morphology of cells exposed to  $60 \mu\text{mol}\cdot\text{l}^{-1}$  catechol or  $1 \text{ mmol}\cdot\text{l}^{-1}$  BSO for 24 h and those of the negative control group. However, we have observed a decrease in the fluorescence of cells treated with catechol or BSO, using MCB as a fluorochrome. BSO has been used to induce the depletion of GSH after 24 h treatment of astrocytes (Gabryel 2007) and rat brain endothelial cells (dos Santos et al. 2007). In this investigation, BSO also induced the depletion of GSH content in N2a cells. Furthermore, the depletion of GSH was observed in these cells treated with catechol for 24 h. This is important to note that the depletion of GSH occurred previously to catechol-induced cell death.

There were described two main different modes of cell death, apoptosis and necrosis. Necrosis is typically accompanied by increased permeability of the plasma membrane, swelling of the cell, and rupture of the plasma membrane. Apoptosis is typically characterized by membrane blebbing, cell shrinkage, the activation of endogenous nucleases, nuclear fragmentation, and the appearance of phosphatidylserine on the outer membrane. Previous studies have reported the use of annexin V binding for determining whether cells are apoptotic for screening neurological targets for drug discovery (Kubota 2008). On the other hand, the loss of plasma membrane integrity and cell lysis have been assessed by PI permeability (Law et al. 2007).

In this study, catechol increased the percentage of annexin V-labeled cells and also of nuclear fragmentation, but it did not increase the percentage of PI-labeled ones. An increase in the percentage of double stained cells was also observed. Furthermore, catechol also induced nuclear fragmentation. These results suggest that molecules bearing catechol groups could be cytotoxic to neuroblastoma cells by depleting its GSH content and inducing apoptosis. These findings can be useful in the design of new anti-tumor molecules. There is a significant need to enhance the arsenal of anti-tumor weapons, including molecules that destroy tumor cells *via* intracellular signaling leading to apoptosis (Elenbaas et al. 2007; Lazar et al. 2007c).

In conclusion, the present investigation shows that catechol possesses a cytotoxic effect to N2a neuroblastoma cells, which may be attributed to GSH depletion and to the induction of apoptosis.

**Acknowledgements.** The authors wish to thank the FAPESB, project ET 63/2004 for the research grant, and the CAPES for the M.Sc. Scholarship offered to R. M. F. L.

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Received: February 28, 2008

Final version accepted: September 9, 2008