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# Cytotoxicity of catechol towards human glioblastoma cells via superoxide and reactive quinones generation

*Citotoxicidade do catecol para células de glioblastoma humano via geração de superóxido e quinonas reativas*

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## key words

Catechol

Cytotoxicity

1,2-dihydroxybenzene

Glioblastoma

Superoxide

## abstract

It is known that the exposure to benzene in the petroleum industry causes lympho-haematopoietic cancer among workers. However, there is little data concerning the toxicity of benzene to the central nervous system. Benzene easily penetrates the brain where it is metabolized to catechol. Since catechol autoxidizes in physiological phosphate buffer, we hypothesized that it could be toxic towards glial cells due to the generation of reactive oxygen species and quinones. In this work we studied the cytotoxic properties of catechol towards human glioblastoma cells. We found that catechol was toxic towards these cells after 72 hours and this toxicity was related to the formation of quinones. Catechol at 230 $\mu$ M killed 50% of cells. The catechol-induced cytotoxicity was prevented by the addition of 100U superoxide dismutase, which also inhibited the formation of quinones. These data suggest that catechol induces cytotoxicity via the extracellular generation of superoxide and quinones.

## resumo

## unitermos

*Sabe-se que a exposição de trabalhadores ao benzeno na indústria petrolífera é uma causa de câncer do sistema linfo-hematopoiético. Pouco se sabe, contudo, a respeito da toxicidade do benzeno no sistema nervoso central. O benzeno penetra facilmente no cérebro, onde é metabolizado a catecol. Como o catecol se auto-oxida em tampão fosfato no pH fisiológico, supôs-se que esse composto poderia ser tóxico para células gliais por gerar espécies reativas do oxigênio e quinonas. Nesse trabalho estudou-se a citotoxicidade do catecol para células de glioblastoma humano. O catecol foi tóxico após 72 horas e essa toxicidade relacionou-se com a formação de quinonas. O catecol a 230 $\mu$ M matou metade das células em cultura. A toxicidade do catecol e a produção de quinonas foram inibidas por 100U de superóxido dismutase. Esses dados sugerem que a toxicidade induzida pelo catecol deve-se à produção extracelular de superóxido e quinonas reativas.*

Catecol

Citotoxicidade

1,2-diidroxi-benzeno

Glioblastoma

Superóxido

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## Introduction

Occupational hygienists have investigated the exposure to benzene<sup>(13)</sup> in the petroleum industry because it causes lympho-haematopoietic cancer among workers. The wastewater generated by the petroleum industry contains an appreciable amount of benzene, toluene, ethylbenzene and xylene (BTEX) that requires specific treatment to avoid environmental contamination<sup>(15)</sup> or to remove these compounds from the ground or surface water resources<sup>(6)</sup>. Certain tasks, such as the loading and unloading of petroleum products in the marine, railcars, trucks and so forth, produce a high risk of exposure to benzene<sup>(26)</sup>. Moreover, benzene is also present in tobacco smoke. The metabolism of benzene by human liver microsomes forms phenol, hydroquinone and catechol (1,2-dihydroxybenzene)<sup>(23)</sup>. Benzene, at concentrations up to 10mM, was neither genotoxic nor cytotoxic toward peripheral blood mononuclear cells *in vitro*. However, its metabolites benzenetriol and catechol are genotoxic at micromolar concentrations<sup>(9)</sup>.

Catechol easily autoxidizes to form potentially toxic quinones and reactive oxygen species (ROS) that induce stress proteins in *Acinetobacter calcoaceticus*<sup>(2)</sup>. Trace elements in tobacco smoke may act as catalysts for the formation of ROS and various free radicals by redox cycling of catechol, thus greatly enhancing the potential for tissue damage<sup>(24)</sup>. Catechol at micromolar concentrations induces a time-dependent release of iron from ferritin *in vitro* and causes lipid peroxidation in rat brain homogenates<sup>(1)</sup>. Furthermore, catechol and cigarette smoke condensates are reported to induce oxidative regulation of protein kinase C and influence the invasive capacity and metastatic spread of lung carcinoma cells<sup>(14)</sup>. Tobacco smoking is also a co-factor lowering the blood serum immunoglobulin concentration in subjects with occupational exposure to organic solvents containing BTEX<sup>(21)</sup>. Generally speaking, chronic smoking and exposure to BTEX are relevant problems to human health. In addition, the use of tobacco regularly associated with inhalation of solvents among street children has been reported as a social problem in Brazil<sup>(10)</sup>.

There is an increasing interest to study the toxicity of catechols toward cells from the central nervous system (CNS) since it has been speculated that the toxicity of these compounds is associated with neurodegenerative diseases. Among these cells astrocytes are the most numerous cellular elements in the CNS, where they play a critical role in physiological and pathological events. The dose-dependent toxicity of endogenous catechols like

L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine and also other drugs bearing a catechol group like apomorphine is well-documented in *in vitro* cell studies<sup>(7, 17, 18)</sup>. However, there are few data concerning the toxicity of catechol toward glial cells. Although the human glioma cell line GL15 is poorly differentiated, these cells preserve many characteristics of astrocytes<sup>(20)</sup> and proliferate rapidly in culture. Glioma cells have been largely used for the study of astrocyte properties<sup>(4)</sup>. In this study we evaluate the toxicity of catechol toward GL-15 cells and its relation with the production of superoxide by the measurement of mitochondrial dehydrogenases activity and quinones formation in cell cultures with and without superoxide dismutase (SOD) and catalase.

## Material and method

### Material

Catechol was from Riedel-de Haën, Buchs-Switzerland. SOD (EC 1.15.1.1) was purchased from ICN, OH, USA. Catalase (EC 1.11.1.6), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypsin (EC 3.4.21.4) were purchased from Sigma MO, USA. Dulbecco's modified Eagle's medium (DMEM) was purchased from Cultilab, SP, Brazil. All other chemicals were of guaranteed grade. Catechol solution (10mM) was prepared in 0.01M HCl. All solutions were prepared with deionized water.

### Measurement of catechol autoxidation in a cell-free system

Neutral aqueous solutions of catechol undergo spontaneous oxidative decomposition. Autoxidation is a multi-step reaction resulting in ROS and quinone derivatives formation. In the present study, the autoxidation rates were measured at 320nm in 1mM HCl, 50mM phosphate buffer (pH 7.4). The catechol oxidation (final concentration of 1mM) was carried out with or without 17U SOD or 100IU catalase in a volume of 3ml. Catechol oxidation was also measured by means of a Clark-type oxygen electrode fitted to an oxygen monitoring system (YSI model 53 oxygen monitor).

### Human glioblastoma GL-15 cell culture

The GL-15 cell line of clonal origin was established by Bocchini *et al.*, in 1991<sup>(3)</sup>. Cultures of these cells were prepared as previously described by Planchenault *et al.*, in

2001<sup>(22)</sup>. Experiments were initiated after cells had become confluent. Cell cultures were divided into seven groups. The control group was treated with  $2 \times 10^{-5}$ M HCl, which was used as the solvent of catechol. The other six groups were treated with concentrations of catechol ranging between 100 and 1000 $\mu$ M for 72 hours. In each group, cells were seeded into six Petri dishes (35mm in diameter) to obtain a final density of  $3 \times 10^5$  cells/dish. In order to study the role of the production of superoxide on the toxicity of catechol, cells were treated with this compound in the presence of 100U SOD for 72 hours. Catalase at 500IU was used to assess the role of hydrogen peroxide in the catechol-induced toxicity. The catechol oxidation in the medium was monitored spectrophotometrically at 405nm, based on quinones and melanin-like pigment formation<sup>(18)</sup>. The cell viability was quantified by the conversion of yellow MTT to purple MTT formazan by living cell mitochondrial dehydrogenases<sup>(16)</sup>.

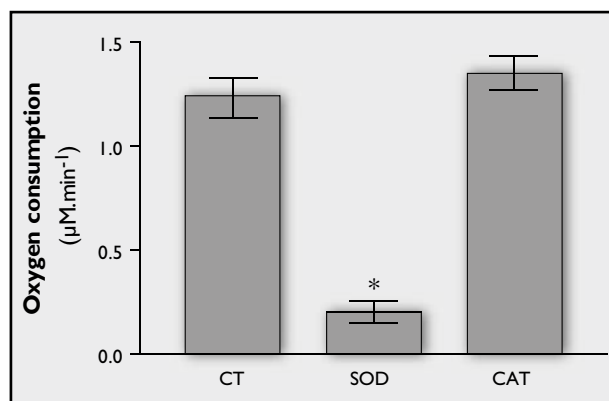
### Calculation

The rates of oxygen consumption and formation of quinones were obtained from a linear regression plot. Linear regression was carried out by using the least square method. This calculation was also used to determine the concentration of catechol that killed 50% of cells ( $IC_{50}$ ), since the plot was linear for catechol concentrations ranging between 100 and 500 $\mu$ M ( $y = -0.6085x + 63.99$ ;  $r^2 = 0.9927$ ). Data obtained for the catechol oxidation in a cell-free system were compared by the Student's *t* test. The significance of results obtained from toxicity studies was analyzed by ANOVA, with the Student-Newman-Keuls *post hoc* test.

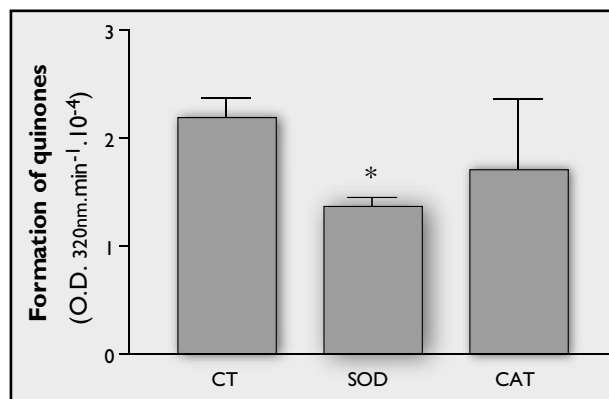
## Results

### Catechol autoxidation in a cell-free system

The oxygen consumption during autoxidation of 1mM catechol was estimated quantitatively. Catechol, at 1mM, oxidizes consuming oxygen in a rate of  $1.2 \pm 0.3\mu\text{M}\cdot\text{min}^{-1}$ . From **Figure 1** it can be seen that the addition of 17U SOD significantly inhibited oxygen consumption by 83.7%. The addition of 100IU catalase did not inhibit catechol autoxidation. The rate of quinones formation was also decreased by 37% in the presence of 17U SOD (**Figure 2**) but no significant difference was found when 100U catalase was added to the system. Altogether, these data suggest that superoxide is produced during the oxidation of catechol. On the other hand, the production of hydrogen peroxide was not evidenced by the use of catalase.



**Figure 1** – Oxygen consumption during autoxidation of 1mM catechol in 1mM HCl, 50mM phosphate buffer (pH 7.4) at 37°C. Catechol was tested alone (CT, n = 13), in the presence of 17U superoxide dismutase (SOD, n = 3), or in the presence of 100IU catalase (CAT, n = 3). Each value represents the mean  $\pm$  SEM; \* $p < 0.0005$



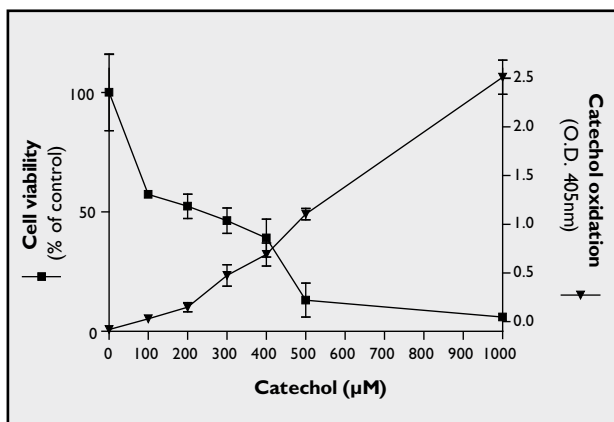
**Figure 2** – Formation of quinones during autoxidation of 1mM catechol in 1mM HCl, 50mM phosphate buffer (pH 7.4). Catechol was tested alone (CT, n = 6), in the presence of 17U superoxide dismutase (SOD, n = 4), or 100IU catalase (CAT, n = 3). Each value represents the mean  $\pm$  SEM; \* $p < 0.05$

### Catechol toxicity towards GL-15 cells

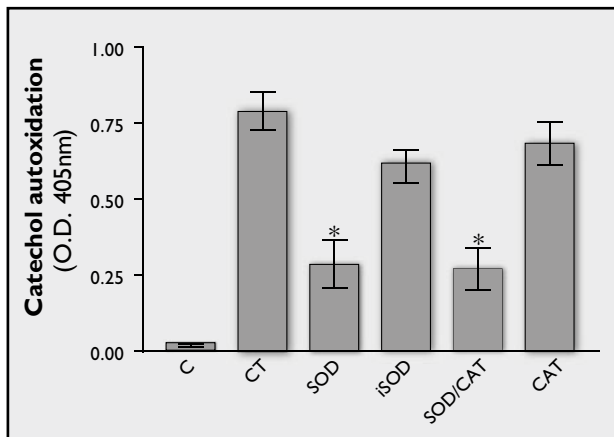
In order to determine the nature of cytotoxicity induced by catechol, GL-15 cells were treated for 72 hours with several concentrations of this compound. As shown in **Figure 3**, catechol treatment resulted in a marked loss of cell viability in a dose-dependent manner. The  $IC_{50}$  value for catechol-induced cytotoxicity towards GL-15 cells after 72 hours was 230 $\mu$ M. Autoxidation of catechol also formed quinones in the culture medium in a dose-dependent manner (**Figure 3**). The cytotoxic effects induced by catechol appeared to correlate with the formation of quinones in cell cultures.

The addition of 100U SOD to cells treated with 300 $\mu$ M catechol for 72 hours inhibited the formation of quinones by 63.5% (**Figure 4**). However, the addition of the same amount of heat-inactivated SOD (iSOD) or 500IU catalase did not inhibit catechol oxidation. Moreover, the addition of

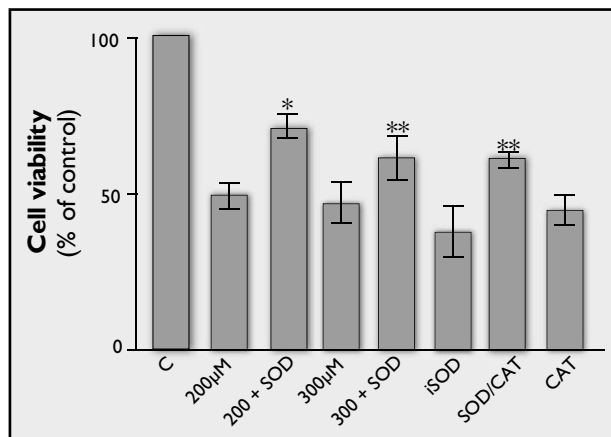
100U SOD combined with 500IU catalase did not increase the inhibition induced by SOD alone. The presence of SOD at 100U also significantly prevented catechol-induced cytotoxicity towards GL-15 cells (Figure 5). Cell survival increased from  $49.5\% \pm 4.2\%$  in cells treated with  $200\mu\text{M}$  catechol ( $n = 5$ ) to  $71.9\% \pm 3.9\%$  in the group incubated in the presence of 100U SOD ( $n = 5$ ). Cell survival also increased from  $47.4\% \pm 6.5\%$  in the group treated with  $300\mu\text{M}$  catechol ( $n = 5$ ) to  $61.6\% \pm 6.9\%$  in that incubated in the presence of SOD ( $n = 4$ ). The addition of iSOD or 500IU catalase did not protect the cells. Once again, SOD combined with catalase did not increase the protective effect of SOD.



**Figure 3** – Concentration-response curves for catechol-induced cytotoxicity and quinones formation in GL-15 cell cultures after 72h. Cell viability (■) was determined by the MTT test and expressed as a percentage of control treated with  $2 \times 10^{-5}\text{M}$  HCl. Each point represents the percentage of cell viability after treatment with catechol in several concentrations. The formation of quinones during the autoxidation of catechol was also measured by spectrophotometry at 405nm (▼). Data are represented by the mean  $\pm$  SD,  $n = 5$



**Figure 4** – SOD inhibits the formation of quinones in cultures of GL-15 cells. Cells were incubated for 72 hours with:  $2 \times 10^{-5}\text{M}$  HCl (C);  $300\mu\text{M}$  catechol alone (CT) or in the presence of 100U SOD (SOD), the same amount of heat-inactivated SOD (iSOD), 100U SOD combined with 500IU catalase (SOD/CAT) or 500IU catalase (CAT). Data are represented by the mean  $\pm$  SD,  $n = 6$ ; \* $p < 0.0001$ , when compared to cells treated with catechol alone



**Figure 5** – Effect of superoxide on catechol-induced cytotoxicity towards GL-15 cells. Cells were incubated for 72 hours with  $2 \times 10^{-5}\text{M}$  HCl (C) or with two different concentrations of catechol ( $200\mu\text{M}$  or  $300\mu\text{M}$ ). Prevention of cytotoxicity induced by these concentrations was observed in the presence of 100U SOD (SOD). Cells were also incubated with  $300\mu\text{M}$  catechol in the presence of heat-inactivated SOD (iSOD), 100U SOD combined with 500IU catalase (SOD/CAT) and 500IU catalase (CAT). Data are represented by the mean of the percentage of cell viability  $\pm$  SD,  $n = 6$ ; \* $p < 0.0001$ , when compared with cells incubated with  $200\mu\text{M}$  catechol; \*\* $p < 0.02$ , when compared with cells incubated with  $300\mu\text{M}$  catechol

## Discussion

The present results indicate that the reaction between catechol and oxygen is inhibited by SOD in a cell-free system. One of two phenolic hydroxyl groups of the catechol molecule is ionized in alkaline solutions and, therefore, electron transfer from this ionized group to a dissolved oxygen occurs easily<sup>(27)</sup>. Consequently, this results in superoxide formation during the catechol autoxidation. On the other hand, the formed superoxide reacts with catechol accelerating the formation of quinones, since this reaction was also inhibited by SOD. Since catalase did not inhibit the oxygen consumption or the quinone formation, this means that superoxide and not peroxide was the main ROS formed in the autoxidation. The oxidative property of catechol seems important because endogenous catechols like catecholamines and catechol estrogens<sup>(5)</sup> are produced in mammal brains and other tissues. Thus, catechol oxidation can be used for structure-activity relationship studies.

Reactive species produced during the autoxidation of catechols may also be toxic to cells. In this study catechol was toxic towards human GL-15 cells after 72 hours and this was related to the formation of quinones. The  $\text{IC}_{50}$  value for catechol-induced cytotoxicity was  $230\mu\text{M}$ . Some drugs that contain catechol moieties, like apomorphine, also have the same oxidative properties<sup>(8)</sup>. We have found that apomorphine-induced toxicity towards glial C6 cells depends on time and dose with an  $\text{IC}_{50}$  value of  $200\mu\text{M}$

after 48 hours<sup>(7)</sup>. Catecholamines are also toxic at high concentrations (> 50-100µM)<sup>(25)</sup>. The IC<sub>50</sub> values for L-DOPA- and dopamine-induced cytotoxicities towards SH-SY5Y neuroblastoma cells were 194 and 184µM respectively, after 24-hour drug exposure<sup>(17)</sup>. Thus, it seems that molecules bearing a catechol group are toxic to cells from the CNS *in vitro*. However, catechols at low concentrations (1µM), like norepinephrine and catechol, provide protection to dopaminergic neurons in mesencephalic cell cultures<sup>(25)</sup>.

In this study it has been shown that SOD protected cells against catechol-induced cytotoxicity and also inhibited quinones formation. Superoxide is the most common intracellular free radical leading to the formation of other reactive cell-damaging species, when present in excessive amounts. It is well accepted that SOD represents the first line of defense against oxygen toxicity. SOD also protected SH-SY5Y neuroblastoma cells against the neurotoxic properties of L-DOPA and dopamine-related compounds<sup>(17)</sup>. In the present study, catalase did not prevent catechol-induced cytotoxicity towards GL-15 cells. Similarly, catalase had no effect on semiquinone radicals formation in dopamine oxidation, although this enzyme showed a small preventive effect against dopamine-induced toxicity towards SH-SY5Y cells<sup>(17)</sup>. It has been determined both by animal and clinical studies that SOD protects the brain against injury by ischemic stroke and trauma<sup>(12)</sup>.

To increase the efficacy of L-DOPA in the treatment of Parkinson's disease, this agent is sometimes associated with inhibitors of catechol-O-methyltransferases (COMT, EC 2.1.1.6) and amino acid decarboxylase (AADC, EC 4.1.1.28). These inhibitors also present catechol groups in their structures. This association between L-DOPA with COMT or AADC inhibitors increased protein carbonyls, lipid peroxidation and oxidative DNA damage levels in the cerebral cortex of normal cynomolgus monkeys<sup>(19)</sup>. Consistent with these results, microdialysis experiments carried out in rats showed that the association between L-DOPA, carbidopa (an AADC inhibitor) and tolcapone (a central and peripheral COMT inhibitor) increased striatal hydroxyl radical production. Thus, it is thought that cell death induced by catechols involves oxidative damage.

In conclusion, these results revealed a possible mechanism of catechol-induced cytotoxicity in glioblastoma GL-15 cells. Catechol, which is a metabolite of benzene, probably exerts cytotoxicity mainly due to the generation of superoxide and quinones during its autoxidation.

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