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# 6-HYDROXYDOPAMINE RELEASES IRON FROM FERRITIN AND PROMOTES FERRITIN-DEPENDENT LIPID PEROXIDATION

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**Abstract**—Iron was released from ferritin by the catecholamine analog, 6-hydroxydopamine. Iron release was more efficient under nitrogen than in air, suggesting that the hydroquinone has the major role in the process. Superoxide dismutase, alone or in combination with catalase, strongly inhibited 6-hydroxydopamine oxidation and greatly enhanced the amount of ferritin iron release. Catalase alone had a similar, but lesser effect. Iron released from ferritin accelerated the autoxidation of 6-hydroxydopamine. This occurred by a mechanism that was inhibited by a combination of catalase and a chelator, and to a lesser extent by superoxide dismutase. 6-Hydroxydopamine was a good promoter of metal-catalysed lipid peroxidation, and ferritin-iron participated in the process. Superoxide dismutase, and to a lesser extent catalase, stimulated peroxidation catalysed by adventitious levels of iron, but in the presence of ferritin, each enzyme was inhibitory. It appears that the greatly enhanced iron release seen under these conditions accelerated the autoxidation of 6-hydroxydopamine so that less was available to participate in peroxidative reactions. However, when 6-hydroxydopamine autoxidation was prevented by a combination of superoxide dismutase and catalase, lipid peroxidation was also inhibited, suggesting that some intermediate of autoxidation is a further requirement for the process.

The catecholamine analog 6-hydroxydopamine (6-OHDA)† is a neurotoxic agent which can accumulate in catecholamine-containing neurons, promoting degeneration of nerve terminals [1–3] and other adverse effects [4, 5]. Recent investigations [6, 7] have implicated 6-OHDA in the pathophysiology of Parkinsonism and schizophrenia. 6-OHDA autoxidizes at neutral pH, with production of $H_2O_2$ and $O_2^-$ [8]. $H_2O_2$ and the *p*-quinone of 6-OHDA are produced in stoichiometric amounts [9] as represented by Eqn 1:

$$QH_2 + O_2 \rightarrow Q + H_2O_2 \qquad (1)$$

where 6-OHDA is represented by $QH_2$ and its *p*-quinone by Q.

$O_2^-$ as well as $H_2O_2$ can drive 6-OHDA autoxidation [10, 11], Heikkila and Cohen [10] have shown that the reaction with $O_2^-$ is catalytic (reactions 2 and 3):

$$QH_2 + O_2^- + H^+ \rightarrow QH^\cdot + H_2O_2 \qquad (2)$$

$$QH^\cdot + O_2 \rightleftharpoons Q + O_2^- + H^+ . \qquad (3)$$

Superoxide dismutase (SOD) inhibits 6-OHDA autoxidation, but only transiently. This inhibition is characterized by an initial lag phase followed by rapid oxidation involving reactions 3 and 4, as the *p*-quinone accumulates [12]:

$$Q + QH_2 \rightleftharpoons 2QH^\cdot . \qquad (4)$$

Catalysis of 6-OHDA autoxidation by transition metal ions has been extensively studied [12–15]. In the presence of even adventitious levels of iron or copper, autoxidation via a reaction involving a ternary complex (either 6-OHDA–metal–oxygen or 6-OHDA–metal–peroxide) can predominate and inhibition by SOD not to be observed [15]. Catalase and chelators inhibit this mechanism and autoxidation is the dependent on propagation by $O_2^-$.

Metal ions may not only promote 6-OHDA autoxidation but also react with the $H_2O_2$ so formed to produce the hydroxyl radical ($^\cdot OH$). Formation of $^\cdot OH$ has been observed and many studies implicate $^\cdot OH$ or a species with similar reactivity in 6-OHDA toxicity [7, 8, 16–18].

"Free" iron is not generally available physiologically, but a possible source of such iron is the storage protein, ferritin. Ferritin iron can be released by $O_2^-$ [19] and other reducing radicals, e.g. bipyridyl, nitroaromatic and semiquinone radicals [20, 21]. Furthermore, the cytotoxic pyrimidines, dialuric acid, divicine and isouramil reduce and release ferritin iron directly [22]. This study was undertaken to determine whether 6-OHDA is able to release iron from ferritin, either directly or through radical generation. We also investigated the ability of 6-OHDA to use the iron released to promote lipid peroxidation. The effects of SOD and catalase on iron release and lipid peroxidation were also examined and compared with their effects on autoxidation of 6-OHDA.

## MATERIALS AND METHODS

Enzymes and other biochemicals were purchased from Sigma Chemical Co. (St Louis, MO). $FeCl_3$ was from BDH (Poole, U.K.). Catalase was shown to have no detectable SOD activity in the cytochrome

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† Abbreviations: 6-OHDA, 6-hydroxydopamine; DTPA, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

c reduction assay of McCord and Fridovich [23], at the concentrations used in the present study. Ferritin (from horse spleen) contained 0.74 nmol iron/μg.

Solutions were prepared in deionized water (Milli Q system), and buffers were stirred with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA) to decrease the levels of contaminating transition metals. 6-OHDA was prepared daily as a 10 mM solution in 0.1 M HCl that had been bubbled with oxygen-free nitrogen for 5 min. Solutions were stored under nitrogen at 0°.

*Iron release from ferritin.* Ferritin (200 μg/ml) was incubated at 37° in phosphate buffered saline (PBS), pH 7.3, with ferrozine (200 μM), the desired concentration of 6-OHDA (25–125 μM) and other additives, when required. The appearance of the $Fe^{2+}$–ferrozine complex was continuously monitored at 562 nm in a Pye-Unicam PU 8000 spectrophotometer. Rates measured during the first minutes of the reaction were calculated using $\varepsilon_{562}$ 27,900/M/cm [24]. Reactions under anaerobic conditions were performed in a tonometer (total volume 100 ml) in which solutions were pre-equilibrated with oxygen-free nitrogen for 5 min. 6-OHDA was then added through a rubber septum with a microsyringe and $A_{562}$ was monitored.

Total changes in $A_{562}$ occurring during 30 min incubation were also measured.

*Lipid peroxidation.* Phospholipids were extracted from fresh lamb brains [25]. Multilamellar liposomes were prepared daily by adding phospholipid (10 mg/ml) to 0.15 M NaCl, pH 7.0, and shaking with glass beads. Liposomes (1 mg phospholipid in a total vol. of 1 ml PBS) were incubated at 37° in air for 30 min with 6-OHDA (25–100 μM) with or without ferritin and other additives when required. Peroxidation was determined by measuring the formation of 2-thiobarbituric acid reactive species (TBARS) [26]. Butylated hydroxytoluene (0.03 vol. in 2% ethanol) was added to the thiobarbituric acid reagent to prevent further lipid peroxidation occurring during the assay procedure.

*Autoxidation of 6-OHDA.* Oxygen uptake by 6-OHDA solutions (50 μM, total vol. 4.0 ml) was measured at 37° using a Clark-type $O_2$ electrode (Model 25, Yellow Springs Instruments, Yellow Springs, OH) in the absence or presence of SOD (10 μg/ml); catalase (60 μg/ml); ferritin (200 μg/ml); ferrozine (200 μM) and diethylenetriaminepentaacetic acid (DTPA) (50 μM).

*Formation of the p-quinone.* Formation of the *p*-quinone was followed by monitoring the increase in $A_{490}$. The rates of *p*-quinone formation were measured during the first minute of the reaction and were calculated using $\varepsilon_{490}$ 2100/M/cm [1].

## RESULTS

Iron was released from ferritin on incubation with 6-OHDA in PBS, pH 7.3, at 37° (Fig. 1). In air, iron release terminated after 3–4 min, whereas under nitrogen, rates remained constant for a longer period. Initial rates were slightly higher under nitrogen than in air, and under both conditions increased with increasing 6-OHDA concentration (Fig. 2).

The *p*-quinone of 6-OHDA, prepared by allowing

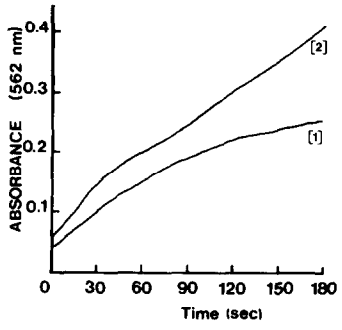


Fig. 1. Time-course of formation of $Fe^{2+}$–ferrozine complex (followed at 562 nm) due to iron mobilization from ferritin by 6-hydroxydopamine (1) in air and (2) in nitrogen. Experimental conditions: PBS, pH 7.3, ferrozine (200 μM), 6-hydroxydopamine (100 μM) and ferritin (200 μg/ml), incubated at 37°.

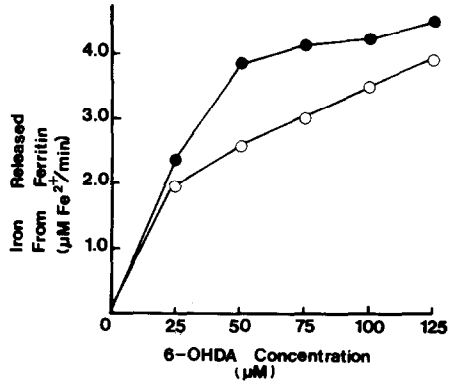


Fig. 2. Initial rates of iron release from ferritin as a function of 6-hydroxydopamine concentration in air (○) or nitrogen (●). Experimental conditions were as in Fig. 1 with 6-hydroxydopamine (0–125 μM).

6-OHDA (100 μM) to autoxidize completely by incubating for 15 min at pH 7.3 [21], also released iron from ferritin, but only at approximately 1/20 of the rate measured with 6-OHDA under nitrogen (an initial rate of 0.24 μM/min compared with 4.20 μM/min).

The effects of SOD and catalase on the initial rates of aerobic iron release and on total release over 30 min were determined. SOD and catalase stimulated the initial rate of iron release by 73% and 54%, respectively, with the combination having no greater effect than SOD alone (Table 1). The same trends were observed after 30 min but the enhancement, especially with SOD, was much greater (Table 1). Thus, SOD and catalase not only increased the initial rates of iron release but also extended the time during which it occurred. Adding GSH, which should scavenge the 6-OHDA radical and regenerate 6-OHDA, enhanced the initial rate of iron release in air, from 2.10 μM to 2.72 ± 0.06 μM (N = 4) with 0.6 or 1 mM GSH. GSH alone gave no significant release over this time period.

Table 1. Effects of SOD and catalase on the release of iron from ferritin under aerobic conditions*

| Additions | Iron release | |
|---|---|---|
| | Initial rate ($\mu$M/min) | Amount released in 30 min ($\mu$M $Fe^{2+}$) |
| None | 2.3 | 5.3 |
| SOD | 3.9 | 29.9 |
| Catalase | 3.5 | 17.3 |
| Catalase + SOD | 4.0 | 32.6 |

* Reaction mixtures contained in a final volume of 1.0 ml, 6-OHDA (50 $\mu$M for initial rate measurements, 100 $\mu$M for 30 min release), ferritin (200 $\mu$g/ml) and ferrozine (200 $\mu$M) in PBS with SOD (10 $\mu$g/ml) and catalase (60 $\mu$g/ml) added where indicated. Measurements of $Fe^{2+}$–ferrozine complex appearance were made at 37°. Results are means of duplicates which agreed within 10%.

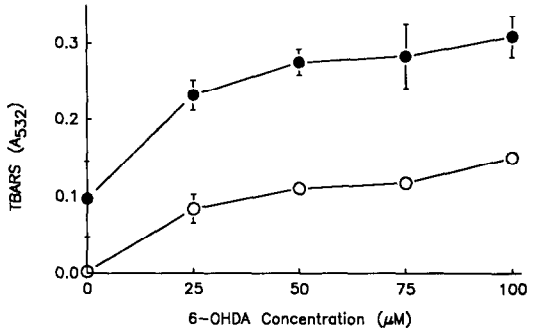


Fig. 3. Effects of increasing concentrations of 6-hydroxydopamine on lipid peroxidation of liposomes (TBARS production) in the presence of adventitious iron (○) or ferritin (50 $\mu$g/ml) (●). Experimental conditions: PBS, pH 7.3; liposomes (1 mg/ml), 6-hydroxydopamine (0–100 $\mu$M), incubated in air for 30 min at 37°. Assays were performed in triplicate. Error bars represent SD, except where they fall within the symbol.

*Lipid peroxidation mediated by 6-OHDA and ferritin*

Liposomes incubated with 6-OHDA underwent lipid peroxidation (Fig. 3, open circles) that was enhanced in the presence of 50 $\mu$g/ml ferritin (Fig. 3, solid circles). Peroxidation increased with increasing concentration of 6-OHDA. Desferrioxamine added either in the absence or presence of ferritin gave almost total inhibition. This indicates that peroxidation in the absence of ferritin was due to adventitious iron and in the presence of ferritin depended on released iron. Peroxidation catalysed by adventitious iron was enhanced by catalase as well as by SOD, but was strongly inhibited by a combination of the two enzymes (Table 2). In the presence of ferritin, however, SOD and catalase alone or in combination were inhibitory (Fig. 4).

To try to explain why SOD was in some situations stimulatory and in others inhibitory, its effect on lipid peroxidation mediated by 6-OHDA and added iron was examined (Fig. 5). Increasing concentrations of iron gave increasing levels of lipid peroxidation. In the presence of SOD, however, peroxidation reached a maximum with 0.5 $\mu$M added iron then gradually declined. Thus, the enhancing effect of SOD on lipid peroxidation was much greater at the lower iron concentrations, and the trends in Fig. 5 suggest that at even higher iron concentrations, SOD could be inhibitory.

*Effects of ferritin, SOD, catalase and chelators on autoxidation of 6-OHDA*

The effects of SOD and catalase on iron release from ferritin were compared with their effects on 6-OHDA autoxidation, measured either as oxygen uptake or formation of the *p*-quinone. Both methods gave comparable results (Table 2). Autoxidation of 6-OHDA in buffer was inhibited approximately 50% by either catalase or DTPA. Ferrozine had a similar effect to DTPA, indicating that it also inhibits autoxidation catalysed by adventitious metal ions. SOD inhibited initial rates of autoxidation by approximately 80% and the combination of SOD and catalase or a chelator almost completely inhibited the reaction for at least 10 min. Thus, in agreement with previous observations [12, 15], autoxidation in the presence of chelator occurred only by an $O_2^-$-dependent mechanism, whereas catalase-inhibitable oxidation required the additional presence of metal ions. In another study [15], "pure" catalase did not affect the autoxidation of 6-OHDA in the presence of a chelator, and the authors suggested that inhibition by catalase preparations under these conditions is indicative of contamination by SOD. Even though we could not detect contamination by the cytochrome *c* assay (see Materials and Methods), this is a possible explanation for the modest increase in inhibition of 6-OHDA autoxidation (41 to 54%) by our catalase in the presence of ferrozine.

Ferritin gave some enhancement of 6-OHDA autoxidation (Table 2), implying that released ferritin-iron provides additional catalysis of the reaction. Ferrozine or DTPA gave some inhibition but additional catalase was required to decrease the rate to that seen with chelator and no ferritin. SOD alone was less inhibitory in the presence of ferritin, but in combination with a chelator suppressed most of the autoxidation. The combination of SOD and catalase in the presence of ferritin gave more inhibition of 6-OHDA autoxidation than did each enzyme alone.

Table 2. Effects of ferritin, ferrozine, DTPA, SOD and catalase on 6-OHDA autoxidation

| Additions | Oxygen consumption* (Initial rates μM/min) | p-Quinone formation† (Initial rates μM/min) |
|---|---|---|
| None | 43 ± 4 | 87.3 ± 2.5 |
| Catalase | 21 ± 1.4‡ | 44.2 ± 2.0 |
| DTPA | 22 ± 2.8 | 59.9 ± 1.3 |
| Ferrozine | 23 ± 1.9 | 51.9 ± 5.4 |
| Ferrozine + catalase | ND | 40.4 ± 3.3 |
| SOD | 8 ± 1.4 | 16.6 ± 1.4 |
| SOD + catalase | 2 ± 0.5 | 5.2 ± 0.0 |
| Ferrozine + SOD | ND | 0.5 ± 0.0 |
| Ferritin | 60 ± 0.0 | 94.4 ± 4.4 |
| Ferritin + ferrozine | 48 + 2.8 | ND |
| Ferritin + DTPA | ND | 74.1 ± 3.5 |
| Ferritin + catalase | ND | 65.8 ± 5.6 |
| Ferritin + DTPA + catalase | ND | 48.9 ± 2.9 |
| Ferritin + ferrozine + catalase | 19.5 ± 0.7‡ | ND |
| Ferritin + SOD | ND | 58.2 ± 2.9 |
| Ferritin + DTPA + SOD | ND | 16.2 ± 3.3 |
| Ferritin + ferrozine + SOD | 4.6 ± 0.2 | ND |
| Ferritin + SOD + catalase | ND | 29.2 ± 2.3 |

Reaction mixtures contained 50 μM 6-OHDA* or 100 μM 6-OHDA†, ferritin (200 μg/ml); ferrozine (200 μM); SOD (10 μg/ml); catalase (60 μg/ml) and DTPA (50 μM) in PBS. Measurements were made in air at 37°. Results are means of triplicates ± SD.

‡ The values of $O_2$ uptake obtained with catalase were multiplied by two because catalase gives back half of the oxygen consumed.

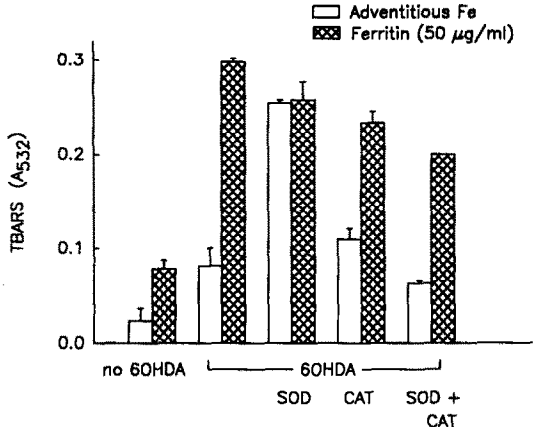


Fig. 4. Effects of superoxide dismutase and catalase on lipid peroxidation mediated by 6-hydroxydopamine. Reaction conditions are the same as in Fig. 3 except the 6-OHDA concentration was 50 μM and SOD (10 μg/ml) or catalase (60 μg/ml) was present where indicated. Results are means of triplicates, error bars indicate SD.

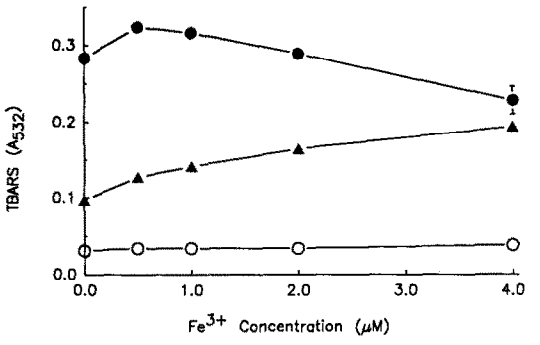


Fig. 5. Effects of increasing concentrations of iron on lipid peroxidation of liposomes (TBARS production) (○); in the presence of 6-hydroxydopamine (50 μM) with (●) or without (▲) superoxide dismutase (10 μg/ml). Experimental conditions: PBS, pH 7.3, liposomes (1 mg/ml), $Fe^{3+}$ (0–4.0 μM), incubated for 30 min in air at 37°. Assays were performed in triplicate. Error bars represent SD, except where they fall within the symbol.

These results imply that some of the 6-OHDA oxidation in the presence of chelator is caused by chelated iron and can be inhibited by catalase. Since the effect of catalase compared with that of SOD was much greater than seen in the absence of ferritin, it probably represents $H_2O_2$ scavenging by the enzyme rather than contaminant SOD activity.

From the results presented in Table 2, it can be deduced that under the conditions used for measuring iron release from ferritin to ferrozine, catalase should inhibit slightly, and SOD with or without catalase inhibit markedly 6-OHDA oxidation. Lipid peroxidation was studied with no chelator added. Under these conditions, SOD or catalase alone should only partially inhibit 6-OHDA autoxidation but in combination should be more effective.

## DISCUSSION

The present study has shown that 6-OHDA is able to release iron from ferritin. The iron is released in the ferrous form and can be chelated by ferrozine. Initial rates were at least 100 times faster than

observed previously with physiological reductants such as ascorbate or glutathione [27, 28], and many fold faster than with the cytotoxic dihydroxypyrimidines, dialuric acid, divicine and isouramil [22]. Thus, a role for ferritin iron in the pathophysiology of 6-OHDA can be proposed.

Iron release could occur through direct reduction by the hydroquinone form of 6-OHDA, or via its autoxidation intermediates, $O_2^-$ and the semiquinone. The relatively greater efficiency measured in $N_2$ compared with air suggests that direct reduction by 6-OHDA is the predominant pathway. Iron release from ferritin mediated by $O_2^-$ and other reducing radicals has been observed previously [19–21]. The strong stimulatory effects of SOD, both on initial rates and total amounts of iron release rule out a significant role for $O_2^-$ in 6-OHDA-mediated release. However, a minor role for the semiquinone radical cannot be discounted. The semiquinone could be formed anaerobically from 6-OHDA reacting with ferritin (or traces of $O_2$), and the greater iron release under $N_2$ could be explained by the lack of $O_2$ to compete with ferritin for the semiquinone. However, adding GSH to scavenge the semiquinone enhanced rather than inhibited iron release, which supports 6-OHDA itself as the better reductant of ferritin iron. The *p*-quinone product of 6-OHDA can undergo further oxidation to indole derivatives [1]. Our results suggest that this reaction may also reduce ferritin iron, but at a much slower rate than with 6-OHDA itself.

SOD, and to a lesser extent catalase, increased the efficiency of ferritin iron release, mainly by extending the period over which it occurred. These effects are explicable on the basis that iron release requires 6-OHDA in its reduced form. This would be maintained by SOD and to a lesser extent catalase, as a result of their inhibitory effects on 6-OHDA autoxidation.

The iron released from ferritin increased the autoxidation rate of the 6-OHDA. This occurred by a mechanism that was inhibited by a combination of catalase and a chelator, and to a lesser extent by SOD. This is consistent with the mechanism proposed for iron-catalysed 6-OHDA autoxidation [12].

Iron released from ferritin should be available to initiate other free radical reactions with either 6-OHDA or its autoxidation products. We have shown that 6-OHDA is a very good promotor of metal catalysed lipid peroxidation, and that ferritin-iron can participate in this process. 6-OHDA-dependent lipid peroxidation has been described previously [7]. Our studies suggest a mechanism in the absence of ferritin in which there is no absolute requirement for $O_2^-$ or $H_2O_2$, since SOD and catalase each enhanced the process. It seems unlikely that the effect of catalase on lipid peroxidation was due to contaminating SOD, in view of the strong inhibition it caused in the presence of excess SOD. The enhancement by SOD observed with adventitious iron became progressively less as the iron concentration increased. The rate of 6-OHDA autoxidation in the presence of SOD also increases with increasing iron concentration [12], as the reaction became less $O_2^-$-dependent. Thus, the highest rates of lipid peroxidation occurred when autoxidation was slow. The results suggest, therefore, that a requirement for lipid peroxidation is the presence of reduced 6-OHDA, probably because it can maintain iron in a reduced form [29]. However, some intermediate of 6-OHDA autoxidation appears also to be involved, since very little lipid peroxidation occurred when autoxidation was inhibited by the combination of catalase and SOD.

With ferritin-dependent lipid peroxidation SOD and catalase, alone or in combination, caused some inhibition. Thus the enhanced iron release from ferritin that was seen with catalase and SOD was not accompanied by increased peroxidation. Inhibition by SOD, however, is what would be predicted from the results in Fig. 5 extrapolated to higher iron concentrations. The mechanism of lipid peroxidation is obviously complex, but it may be that when there is high iron release (up to 30 $\mu$M in the presence of catalase and SOD is indicated in Table 1) the autoxidation of 6-OHDA is accelerated to an extent that its availability as an iron reductant becomes limiting. Thus, although ferritin can interact with 6-OHDA to cause lipid peroxidation, excessive iron release apparently inhibits the process.

A number of *in vitro* studies have shown that iron can interact with autoxidizing 6-OHDA to give the $^{\cdot}OH$ radical or a similar highly reactive oxidant [7, 8, 16–18]. Others have implicated iron in cytotoxic or neurotoxic reactions of 6-OHDA [5, 8, 13]. Our results suggest that ferritin could be a source of this iron and contribute to the toxicological action of 6-OHDA.

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