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Effects of a Direct Injection of Liposoluble Iron into Rat Striatum. Importance of the Rate of Iron Delivery to Cells

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For a better understanding of the role of iron imbalance in neuropathology, a liposoluble iron complex (ferric hydroxyquinoline, FHQ) was injected into striatum of rats. The effects of two modalities of iron injections on brain damage, hydroxyl radical ($^{\bullet}$OH) production (assessed by the salicylate method coupled to microdialysis) and tissue reactive iron level (evaluated *ex vivo* by the propensity of the injected structure for lipid peroxidation) were examined. Rapid injection of FHQ (10 nmoles of 5 mM FHQ pH 3 solution over 1-min period) but not that of corresponding vehicle led to extensive damage associated with increased tissue free iron level in the injected region. Conversely, neither lesion nor free iron accumulation was observed after slow FHQ injection (10 nmoles of a 100 μM FHQ pH 7 solution over 1-h period) as compared to corresponding vehicle injection. Production of $^{\bullet}$OH was induced by slow FHQ injection but not by rapid FHQ injection, probably as a result of *in vivo* abolition of iron-induced $^{\bullet}$OH formation by acid pH. Indeed, rapid injection of FAC pH 7 (ferric ammonium citrate, 5 mM in saline) was associated with $^{\bullet}$OH formation whereas rapid injection of FAC pH 3 did not. Our results identify the rate of iron delivery to cells as an important determinant of iron toxicity and do not support a major role for extracellular $^{\bullet}$OH in damage associated with intracerebral iron injection.

Keywords: Iron; Glial activation; Hydroxyl radical; Salicylate hydroxylation; Neurodegeneration; Ischemia

INTRODUCTION

In brain as in other organs, the level of free iron, i.e. iron available for the synthesis of hemoproteins and other iron-containing molecules, is kept as low as possible because an excess of iron is toxic. Indeed, in the presence of free iron, superoxide anion and/or hydrogen peroxide, which are continuously produced by normal cell metabolism, give rise to hydroxyl radicals, an extremely cytotoxic oxygen radical species. At physiological pH and oxygen tension, ferrous ions are readily oxidized to ferric ions, which rapidly form essentially insoluble $Fe(OH)_3$ polymers. For these reasons, iron ions do not normally exist *in vivo* and free iron is chelated to low molecular weight species whose the nature has not clearly been established.[1] Two proteins which bind iron in a soluble and nontoxic form have a crucial role in the maintenance of free iron level below the toxic threshold and yet in concentrations allowing the satisfaction of metabolic needs: transferrin (Tf), the main iron transport protein from which cells acquire free iron and ferritin (Ft), the intracellular iron storage protein.[2]

Iron accumulation in the basal ganglia has been observed in brains of patients suffering from neurological diseases involving motor impairment such as Parkinson's disease,[3] Huntington's disease[4] and Friedreich ataxia.[5] In various kinds of experimental brain hypoxic/ischemic challenges, a progressive iron accumulation has been detected in regions affected by a process of delayed neuronal death.[6–8] Given the known ability of free iron to augment oxidative stress and evidence that oxidative stress leads to neurodegeneration, disruption of iron

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metabolism is suspected in these pathologies. However, since *postmortem* examination cannot distinguish whether iron accumulation caused the damage or resulted from damage, animal models in which iron is manipulated are informative.[9]

The content of brain iron does not change after manipulation of blood iron levels.[10] Thus, deposit of iron salts ($FeCl_3$, $FeSO_4$) and more recently of hydrosoluble forms of iron (ferrous citrate) into a given brain region of adult rats has been used to produce an excess of free iron in this region. In accordance with the toxic effect of free iron overload, irreversible brain damage occur in the region injected with iron.[11–16] However, the ability of iron salts and ferrous citrate to gain access to cells remains speculative. Given that in pathologic brains iron accumulates in the interior of the cells at site of injury, the potential relevance to a pathological state of the results is questionable. Therefore, to get further information on the role of brain iron imbalance in pathological processes, we considered it appropriate to examine the response of brain to direct intracerebral injections of a liposoluble form of iron, which conversely to iron salts or ferrous citrate enters brain cells by passive diffusion. Our study describes the effects of intrastriatal injections of liposoluble iron (10 nmoles) on neuronal damage, tissue reactive iron level and hydroxyl radical formation. The incidence of the rate of iron delivery to cells was evaluated by comparison of two modalities of iron injections: rapid injection (10 nmoles/min over 1-min period) and slow injection (0.16 nmoles/min over 1-h period).

MATERIALS AND METHODS

Male adult Wistar rats (300–350 g) were used throughout the experiments. All procedures were performed in accordance with the French Department of Agriculture guidelines (license 00776). Rats were housed in a temperature-controlled room (22–24°C) that was on a 12 h light/12 h dark cycle. Food and water were available *ad libitum*. Rats were anesthetized with chloral hydrate (400 mg/kg, 1 ml/100 g, i.p.) except when indicated.

Injection and Preparation of Iron Solutions

Iron was injected into the striatum of anesthetized rats by means of a stereotaxically placed cannula (26.5-gauge needle). The stereotaxic coordinates from the bregma were: +0.5 mm anterior–posterior, 3.5 mm medial–lateral, 6 mm dorsal–ventral, the bregma and the lambda being in the same horizontal plane. The entry of liposoluble iron within cells is directed by the concentration gradient across cell membrane. Thus, the higher the amount of iron delivered to striatum per unity of time, the more rapid is the diffusion of iron into cells. Therefore, modulation of iron rate acquisition by cells can be obtained by modulating iron delivery to cells. To do so, 10 nmoles of liposoluble iron (ferric hydroxyquinoline complex, FHQ) were administered either rapidly (rapid injection) or slowly (slow injection) by using a microinfusion pump (Harvard Apparatus 22). For rapid injections which delivered iron at a rate of 10 nmoles/min, 2 μl of 5 mM FHQ were administered over 1-min period. For slow injections which delivered iron at a rate of 0.16 nmoles/min, 100 μl of 100 μM FHQ were infused over 1-h period. Animals injected with the corresponding vehicle were used as controls (ethanol acidified to pH 3 with HCl for rapid injections and ethanol in pH 7 buffer for slow injections). In some experiments, hydrosoluble iron was rapidly injected according to the protocol used for liposoluble iron.

FHQ was extemporaneously prepared by mixing hydroxyquinoline (HQ) and $FeCl_3$ solutions. HQ (Sigma) was dissolved first in ethanol and further diluted in saline. $FeCl_3$ (Sigma) was dissolved in saline. Since highly concentrated FHQ solution was unstable in pH 7 buffer, FHQ solutions containing 1 or 5 mM iron were prepared by mixing (v/v) equimolar HQ and $FeCl_3$ solutions (the pH value of the 5 mM solution was 3). Conversely, 100 μM FHQ solution was prepared by adequate dilution of 1 mM FHQ with artificial cerebrospinal fluid (120 mM NaCl, 4 mM KCl, 1.3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 25 mM $NaHCO_3$, pH 7.4 with CO_2 bubbling).

Apomorphine-induced Rotating Behavior

After intrastriatal injections of iron or vehicle, rats were treated with the dopaminergic agonist apomorphine in order to evaluate the integrity of the dopaminergic neurotransmission. Apomorphine was dissolved in saline containing 0.1% ascorbate and was administered each morning (2 mg/kg, i.p.) during the 7 days following intrastriatal injections. Rats exhibiting a decrease in the number or in the function of dopaminergic receptors within striatum respond to apomorphine treatment by a homolateral rotating behavior.

Brain Examination

Brain examination, i.e. neuronal damage, microglial and astroglial activation was performed 7 or 30 days after striatal injections in rats previously treated with apomorphine. To do so, rats were transcardially perfused with isotonic saline solution and then with fixative FAM solution (formol 37%, acetic acid 100%, methanol 1/1/8 v). Brains were removed, postfixed in the same fixative for a week, dehydrated in ethanol and embedded in paraffin.

For visualization of neuronal damage, 10 μm-thick coronal sections including striatum were deparaffinized, rehydrated and stained with acid fuchsin and cresyl violet and coverslipped with Eukitt mounting medium. Microglial activation was evidenced on the adjacent paraffin-embedded sections used for neuronal damage. All sections were deparaffinized, rehydrated and treated simultaneously. They were first incubated for 15 min in PBS containing 0.1% Triton X100 and were subsequently incubated overnight at 4°C in PBS containing 10 μg/ml of peroxidase-conjugated isolectin B_4 derived from *Griffonia simplicifolia* seeds (L-5391, Sigma) which localizes microglia. The reaction product was visualized by using 0.06% 3,3′-diaminobenzidine tetrachloride as the chromogen in the presence of 0.02% H_2O_2. Then, the sections were lightly counterstained with Hematoxylin, dehydrated in graded ethanol and coverslipped with Eukitt mounting medium. Astroglial activation was also studied on the deparaffinized and further rehydrated sections adjacent to those used for microglia detection. All sections were treated simultaneously. Endogenous peroxidase activity was blocked for 20 min with 3% H_2O_2 solution. Non-specific binding was then blocked for 2 h with PBS containing 10% normal goat serum. The sections were incubated for 48 h at 4°C with a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (dilution 1:400, G-3893, Sigma), in order to specifically localize activated astrocytes. They were subsequently incubated with biotinylated anti-mouse IgG antibody for 2 h at room temperature, then with peroxidase conjugated streptavidine complex for 1 h at room temperature by using a Biostain Super ABC kit (Biomeda). The reaction product was visualized by using 0.06% 3,3′-diaminobenzidine tetrachloride as the chromogen in the presence of 0.02% H_2O_2. After the final wash, the immunostained sections were lightly counterstained with Hematoxylin, dehydrated in graded ethanol and coverslipped with Eukitt mounting medium.

Determination of Hydroxyl Radical Formation

Hydroxyl radical formation was measured by using the salicylate method coupled to microdialysis. This method which is commonly used for hydroxyl radical detection in response to various kinds of cerebral injury only detects hydroxyl radicals formed in the extracellular space. The dialysis probe with a 4 mm long dialysis membrane prepared according to a previously described technique[17] was implanted in additional anesthetized rats. For a better control of anesthesia which should be maintained for 5 h, pentobarbital (60 mg/kg, i.p in bolus followed by a 48 mg/h, i.v. infusion) was used instead of chloral as anesthetic agent. A catheter was inserted into the femoral artery for control of mean arterial blood pressure (MABP) throughout the experiment. Rectal temperature was maintained at 37°C by using a heating pad. Dialysis probes were continuously perfused at a constant flow rate (2 μl/min) with artificial cerebrospinal fluid (see above for composition) containing 5 mM salicylate. After introduction of the probe into the striatum, rats were dialyzed for 2 h, then the probe was removed and immediately replaced by the cannula for iron or vehicle injections. At the end of slow and rapid injections, the probe was again placed into the striatum instead of the cannula for a further 75-min dialysis period. During slow injections, salicylate 500 μM was co-administered with FHQ or vehicle in order to keep constant extracellular salicylate concentration throughout the whole experiment. The choice of this concentration (500 μM) is based on the recovery of the probe for salicylate (10% in our dialysis conditions). Importantly, addition of salicylate to FHQ cannot form DHBAs in cell free preparations because reductants are required for salicylate hydroxylation by ferric iron.[18]

Dialysates were collected every 15 min in Eppendorf tubes containing deferoxamine (100 μM final concentration) before iron or vehicle injections (4 samples) and after the arrest of injections (5 samples). Dialysate samples were immediately analyzed by HPLC for assessment of 2,3-DHBA and 2,5-DHBA levels, the main hydroxylated adducts of salicylate. The mobile phase (0.02 M trisodium citrate, 0.018 M sodium acetate, 5% methanol) flowed at 1 ml/min through an HPLC column (Lichrospher, 100-5RP 18, 250/4 mm, Macherey-Nagel). DHBAs were detected by a coulometric detector (200 mV, Coulochem II, ESA). Under these conditions, retention times were 5 min for 2,3-DHBA and 6.5 min for 2,5-DHBA. For each rat, means of the DHBAs concentration obtained from the 4 samples collected before iron injection corresponded to baseline values.

Evaluation of Free Iron in Brain Tissue

Since a linear relationship exists between endogenous iron content in brain regions and their ability to produced lipid peroxides *in vitro* and since iron chelating agents such as deferoxamine and dipyridyl are very effective in inhibiting lipid peroxidation,[19] the level of free iron in injected striatum was indirectly quantified by the measurement of the TBARS production induced by a 1 h incubation of striatal homogenates. Thus, additional groups of rats were treated with rapid or slow FHQ injections. Immediately after the arrest of the slow injection or 1 h after the arrest of the rapid injection, rats were sacrificed for removal of the two striata. Contralateral striatum was used as control. Striata were homogenized in artificial cerebrospinal fluid. TBARS

levels[18] were determined in the same homogenate before and after a 1 h incubation period at 37°C. Results were expressed as TBARS (nmoles) per gram of fresh tissue.

Effects of FHQ on Cerebromicrovascular Endothelial Cells (CECs) in Culture

Immortalized rat CECs were used to get arguments for a link between the level of intracellular free iron and the speed of FHQ delivery to cells. Such a cell line was developed by Muruganandam *et al.*[20] and were obtained as a kind gift of Dr D. Stanimirovic (IBS, NRC, Ottawa, Canada). CECs were cultured in M199 medium supplemented with 10% FBS, 0.5 mg/ml peptone, 2% glucose, 2% amino acids, 1% vitamins and 1% antibiotic–antimycotic solution. They were maintained at 37°C in an atmosphere of 5% CO_2 in air. All experiments were performed on confluent cells initially seeded in 24-well plates at a density of 40,000 cells/well.

Protocol 1 was conducted to assess the link between intracellular free iron level and cell death. The level of intracellular free iron was modulated by exposing the CECs for 2 h to different FHQ concentrations (2, 10, 20, 50 and 100 μM of iron). Vehicle treatment was done in parallel in similar conditions. The treatment was then withdrawn, the cells were quickly washed twice with prewarmed HBSS and further incubated in complete culture media up to 24 h at 37°C and 5% CO_2, for cell death measurement.

Protocol 2 was conducted to study the effect of the rate of FHQ delivery on cell death. FHQ was delivered according to two modalities in order to mimic those used *in vivo*. The first modality consisted of exposing the CECs for 1 h to 10 nmoles of iron by placing the cells in a buffered FHQ solution at 20 μM of iron, such a modality mimicking the *in vivo* rapid FHQ injection. The second modality consisted of exposing CECs to 10 nmoles of iron by placing the cells in a buffer (HBSS) in which was perfused a 100 μM FHQ solution at a delivery rate of 100 μl/h for 1 h, such a modality mimicking the *in vivo* slow FHQ injection. Corresponding vehicle conditions were carried out in parallel.

Cell death was measured by using the trypan blue exclusion method modified for spectrophotometric assessment of cell death.[21] Data were expressed as a percentage of cell death calculated from the maximal optical density obtained after H_2O_2 exposure (500 μM) which induced 100% of cell death.

Data Analysis

Morphological brain examination was performed by one person blinded to the experimental conditions. For comparison of the free iron level in brain tissue and the effect of increasing FHQ concentrations on CECs death, statistical significance was determined by using one-way analysis of variance (ANOVA) followed by *post-hoc* Newman–Keuls test. Student *t* test was used for comparison of the effects of rapid and slow FHQ injections on CECs death. One-way repeated analysis of variance followed by Dunnett *t* test was used for evaluation of hydroxyl radical production. The value $p < 0.05$ was considered to be significant.

RESULTS

Brain Damage and Effect of Apomorphine in Rats Treated with FHQ

At day 7 after rapid FHQ injection rats ($n = 5$) exhibited a severe destruction of the striatum involving one third to one half of the structure, as indirectly evidenced by extensive microglial activation (Fig. 1A). The core of the lesion was fully occupied by amoeboid microglia (Fig. 1B). No neurons were detected in the lesioned area after coloration with acid fuchsin and cresyl violet (data not shown), thus indicating that damaged neurons were already phagocyted by amoeboid microglial cells. The transitional area between necrotic core and intact tissue was invaded by a stellate-shaped microglial cells (Fig. 1C) and GFAP-immunostained astrocytes (Fig. 1D). The severe destructive effect of rapid FHQ injection has to be related to iron and not to acid injection because only a slight lesion restricted to the site of implantation was observed in rats treated with rapid vehicle (HCl) injection (Fig. 1E).

Slow injection of vehicle (100 μl of buffer over a 1 h period, $n = 5$) was associated at day 7 with a lesion not different from that obtained after rapid vehicle injection. As compared to vehicle injection, no more damage were observed after slow FHQ injection whatever the delay, 7 days ($n = 7$) or 30 days ($n = 5$) between injection and brain examination (data not shown).

Results concerning apomorphine-induced ipsilateral rotations confirmed histological data. Indeed, treatment with apomorphine did not induce rotating behavior in rats treated with vehicle (rapid or slow injection) or with slow FHQ injection. Conversely, injection of apomorphine to rats treated with rapid FHQ injection exhibited ipsilateral rotations (Fig. 2).

Effect of FHQ on Hydroxyl Radical Production

Hydroxyl radical production was evaluated by dialysate DHBA (2,3-DHBA + 2,5-DHBA) levels. A progressive decrease in MABP was generally

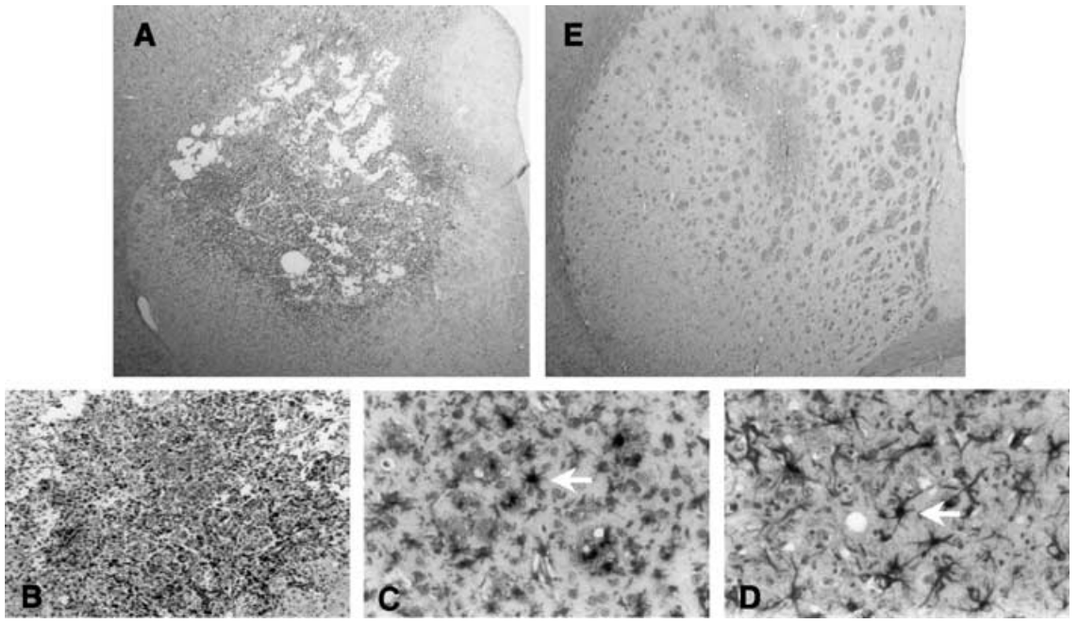

FIGURE 1 Representative microphotographs of microglial activation (Isolectin B_4 and Hematoxylin, A, B, C, E) and astroglial activation (GFAP and Hematoxylin, D) of striatum at day 7 post-injection. (A) Rapid FHQ injection induced severe tissue damage associated with extensive microglial activation, ×2.5, (B) amoeboid microglial cells within the core of the lesion, ×10, (C) the arrow shows a typical stellate-shaped microglial cell, ×40, (D) the arrow shows a typical activated astrocyte, ×40, (E) microglial activation was restricted to the site of implantation after rapid FHQ injection, ×2.5.

observed in dialyzed rats but MABP remained above 70 mm Hg throughout the dialysis period.

Hydroxyl radical formation was not induced by slow vehicle injection (Fig. 3A). Conversely, slow FHQ injection was associated with increased hydroxyl radical formation (Fig. 3B). Thus, DHBA level was found higher in dialysate collected after injection than in dialysate collected before injection, the difference reaching significance for sample 1 (+45%, $p < 0.01$) and for sample 5 (+40%, $p < 0.05$).

In rats treated with rapid FHQ injection (Fig. 3C), post-injection DHBA levels remained very closed to baseline values. This lack of effect of rapid FHQ injection on hydroxyl radical formation led us to suspect the existence of interplay between acid pH and iron-induced salicylate hydroxylation. To gain further information about this point, hydroxyl radical production was evaluated after rapid injection of ferric ammonium citrate (FAC) which can be prepared, conversely to FHQ, both in neutral and acid solutions. Thus, 5 mM FAC solutions were prepared either in saline or in saline acidified to pH 3 with HCl in order to obtain a FAC pH 7 solution and a FAC pH 3 solution, respectively. Rapid injection of FAC pH 7 was associated with an immediate and transient increase in hydroxyl radical formation. Thus, DHBA levels which were increased by 86% in the first post-injection sample ($p < 0.01$) progressively declined to baseline values in the following samples (Fig. 3D). Conversely, hydroxyl radical production was not induced by rapid FAC pH 3 injection (Fig. 3E), thus demonstrating that acid pH abolished iron-induced salicylate hydroxylation.

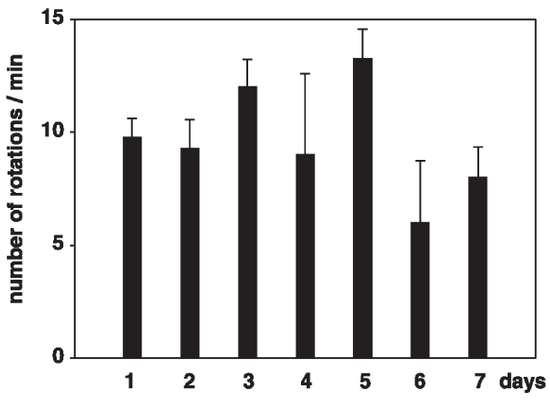

FIGURE 2 Apomorphine-induced rotating behavior in rats treated with rapid FHQ injections. Apomorphine (2 mg/kg, i.p.) was administered each morning during the 7 days following iron injection.

Effect of FHQ on Striatal Free Iron Level

Tissue reactive iron level was indirectly evaluated by the *ex vivo* propensity of the injected structure for lipid peroxidation. Rats were treated with a slow ($n = 5$) or a rapid FHQ injection ($n = 5$). Pre-incubation TBARS levels in striatal homogenates (nmol/g of fresh tissue) were not found significantly different between control striatum

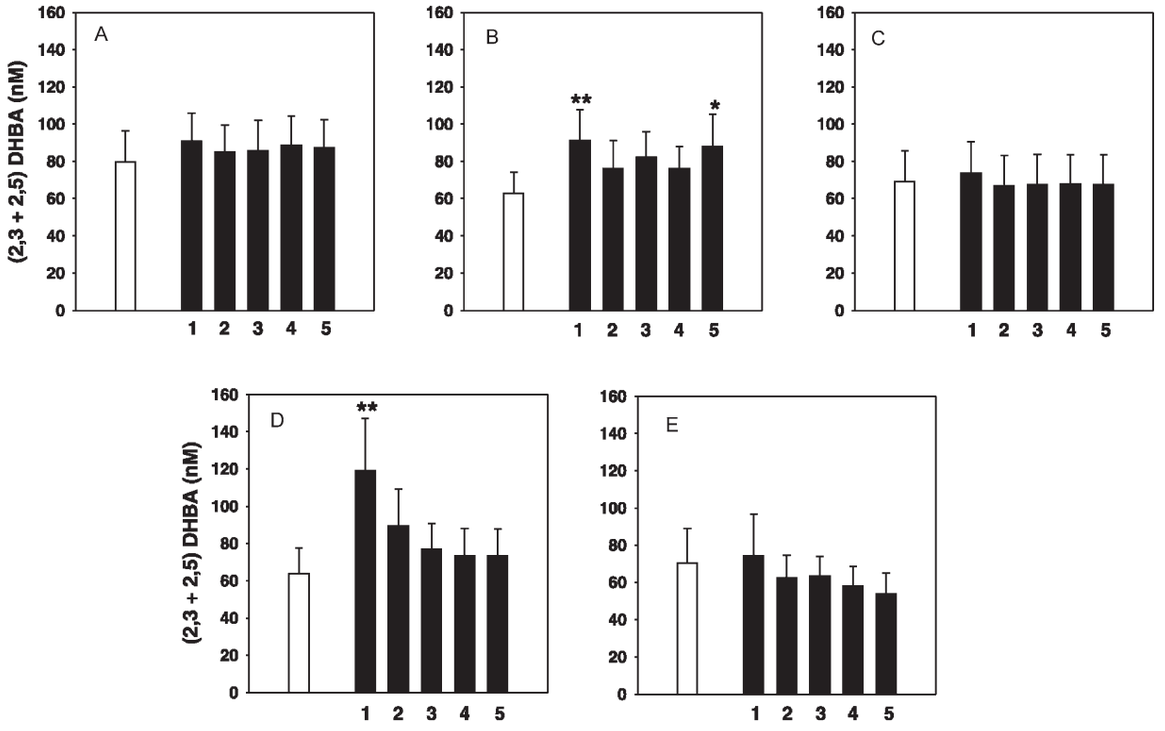

FIGURE 3 Effect of iron injection on hydroxyl radical formation. Rats were treated with (A) slow vehicle injection ($n = 5$), (B) slow FHQ injection ($n = 7$), (C) rapid FHQ injection ($n = 7$), (D) rapid FAC pH 7 injection ($n = 5$), (E) rapid FAC pH 3 injection ($n = 4$). Dialysates were collected before (baseline values, white bars) and after (5 dialysate samples, black bars). Hydroxyl radical production was evaluated by dialysate DHBA level. *$p < 0.05$, **$p < 0.01$, significantly different from baseline values after one-way repeated analysis of variance followed by Dunnett t test.

(36.6 ± 1.4, $n = 10$), striatum treated with slow FHQ injections (45.5 ± 3.8, $n = 5$) and striatum treated with rapid FHQ injections (38.9 ± 2.0, $n = 5$). However, as shown in Table I, a significant increase in incubation-induced TBARS formation was observed only after rapid FHQ injection, thus indicating that rapid FHQ injection led to enhanced tissue free iron level.

Effects of FHQ on CECs in Culture

The first experiment, consisting of an exposure of CECs to increasing concentrations of FHQ, showed that the higher the FHQ concentration, the more severe was the cell death. As compared with vehicle treatment ($5.7 \pm 1.9\%$), the cell death was enhanced in the presence of 2 μM ($8.3 \pm 1.4\%$, NS), 10 μM ($19.6 \pm 4.1\%$, $p < 0.05$), 20 μM ($73.5 \pm 8.0\%$, $p < 0.05$), 50 μM ($93.7 \pm 8.1\%$, $p < 0.05$) and 100 μM of FHQ ($97.2 \pm 9.4\%$, $p < 0.05$). These data indicated that the intensity of the cell death was dependent on the intracellular free iron concentration.

The results of the second experiment showed that the rate of iron delivery to cells influenced cell death. Thus, whereas a slow injection of FHQ did not affect the cell death (9.8 ± 0.9 vs. $8.6 \pm 2.4\%$ in the corresponding control, NS), a rapid injection of FHQ led to a severe mortality (85.5 ± 6.6 vs. $6.7 \pm 0.5\%$ in the corresponding control, $p < 0.05$).

From these *in vitro* experiments which strengthen the *in vivo* studies, we can conclude that the level of intracellular free iron is dependent on the rate of iron delivery to cells. After a slow FHQ injection, cells are able to deal with iron because they can maintain free iron below a toxic threshold. Conversely, cells exposed to a rapid FHQ injection die because intracellular free iron reaches toxic level.

DISCUSSION

In this study, FHQ as a liposoluble form of ferric iron (10 nmoles) was directly injected into striatum of rats. When iron was rapidly administered (10 nmoles/min for 1 min), FHQ induced severe histological damage. Conversely, no lesion was observed when the same amount of iron was slowly injected (0.16 nmole/min for 1 h). Importantly, only striatum treated with rapid FHQ injection exhibited increased free iron levels. These data identified the rate of iron delivery to cells as an important determinant of iron-induced neurotoxic effect.

FHQ is avidly taken up by cells and then distributed in a fairly homogeneous manner.[22]

The extensive lesion observed in the striatum of rats treated with rapid FHQ injection demonstrates the incapacity of brain cells of surviving from inappropriate iron delivery. However, iron acquisition by cells at a level above that required for satisfaction of metabolic needs appears to be not sufficient to induce cell death since slow FHQ injections failed to exert destructive effects. In fact, inappropriate iron delivery to cells appears to cause damage only if the rate of iron uptake by cells exceeds the ability for intracellular iron detoxification. An important component of the means for establishing iron homeostasis is through ferritin (Ft), the main intracellular iron storage protein. Thus, excess of intracellular free iron is not only stored by preexisting Ft but it is also capable of increasing the iron storage capacity of cell through the translation of Ft mRNA into the protein. In our study, free iron levels in striatal tissue are increased after rapid but not slow FHQ delivery. Since striatal iron content was analyzed in the acute period following iron injection, differences in free iron level between the two modalities of iron injection cannot be related to differences in Ft neosynthesis but rather to differences in iron storage capacity of preexisting Ft because translation of Ft mRNA into the protein requires at least 12 h. Thus, whereas free iron can be stored in preexisting Ft when it is delivered slowly to cells, acute iron delivery to cells appears to decrease the ability of Ft to inactivate iron, probably as a result of free iron-catalyzed oxidative damage. As hydroxyl radicals can attack all biological compounds including proteins, oxidative damage to heavy chains of Ft (H-Ft) may be involved in iron accumulation consecutive to rapid FHQ injection since H-Ft plays a crucial role in maintaining cell free iron level below toxic threshold. Indeed, H-Ft promotes the rapid incorporation of iron into Ft and H-Ft gene deletion has recently been reported to cause early embryonic lethality.[23] Furthermore, oxidative damage to Ft enhances its proteasomal degradation[24] and has the potential to cause further increases in free iron within cells, thereby enhancing iron toxicity.

Oxidative stress has largely been implicated in the toxicity of intracerebral iron injection because increased tissue lipid peroxidation was observed in structures injected with iron.[14,25–29] Furthermore, treatments with liposoluble antioxidants, including melatonin, 21-aminosteroids and alpha-tocopherol were reported to protect from iron injection.[30–32] However, although hydroxyl radicals and subsequent lipid peroxidation are thought to mediate the neurotoxic effects of iron, only one study has reported the effect of intracerebral iron injection on hydroxyl radical production.[25] Thus, it has been shown by these authors that a direct deposit of 8.2 nmoles of ferrous citrate into substantia nigra induced an immediate but transient increase in dialysate DHBA levels. At this dose, ferrous citrate injection was associated with both biochemical Parkinsonism and lipid peroxidation at the site of iron injection, leading the authors to suspect a link between extracellular hydroxyl radical formation and iron toxicity. Our data are not in favor of a damaging effect of extracellularly formed hydroxyl radicals. Indeed, rapid FHQ injection induced the partial destruction of the striatum but did not induce formation of hydroxyl radicals. Likewise, slow FHQ injection was associated with a mild but significant hydroxyl radical formation but the lesion was restricted to the site of implantation. The demonstration that iron-induced extracellular hydroxyl radical formation in brain is not predictive of iron toxicity but has parallels in isolated perfused rat heart studies. Thus, whereas a similar extracellular hydroxyl formation was observed after either liposoluble and hydrosoluble iron exposure, heart dysfunction was observed only after liposoluble iron treatment.[33] Similarly, in heart submitted to ischemia and reperfusion, application of deferoxamine (a hydrosoluble iron chelator) at the onset of reperfusion was reported to inhibit extracellular hydroxyl radical generation but not to attenuate myocardial injury.[34] This lack of relation between extracellular hydroxyl radical production and cell damage consecutive to iron injection or ischemia/reperfusion indicates that increased dialysate DHBA level has to be only considered as an index of the presence of reactive iron outside the cells.

Another important point highlighted in our study is the abolition by acidosis of iron-induced salicylate hydroxylation *in vivo*. Indeed, injection of FAC pH 7 but not injection of FAC pH 3 was associated with DHBA formation. This result has to be related to a recent *in vitro* study in which acid pH (below 3.5) was reported to abolish $FeSO_4$-induced

TABLE I Effect of FHQ injections on striatal free iron level

	Control ($n = 10$)	Slow FHQ injection ($n = 5$)	Rapid FHQ injection ($n = 5$)
% Increase in TBARS production induced by incubation	140.5 ± 12.4	120.3 ± 24.4	211.5 ± 27.7*

Rats received an unilateral slow or rapid FHQ injection into the striatum ($n = 5$ in each group). Immediately after the arrest of the slow injection or 1 h after the arrest of the rapid injection, the two striata were removed. Each striatum was homogenized. TBARS levels were then measured before and after a 1 h-period of incubation at 37°C. Increase in TBARS production induced by incubation (%) was taken as an index of the amount of free iron present within the striatum. Contralateral striatum was used as control. *$p < 0.05$, significantly different from control and slow FHQ injection.

hydroxylation of 2′-deoxyguanosine, another hydroxyl radical trapping agent,[35] in a cell free preparation. In our study, both the incapacity of iron to generate hydroxyl radicals and of salicylate to trap hydroxyl radicals in a pH 3 environment are possible mechanisms. Although we cannot favor either mechanism, our results probably explain the absence of published data on the effects of intracerebral iron salt injection on salicylate hydroxylation. Indeed, as a result of their insolubility at neutral pH, iron salts need to be injected in an acid vehicle. In addition, it is of interest to notice that the temporal profile of DHBA formation induced by FAC pH 7 was quite similar to that reported after ferrous citrate injection.[25] This indicates that the prooxidant effect of iron is independent on the redox status of added iron probably as a result of the presence of extracellular reductants to ensure adequate iron redox cycling. Furthermore, differences in the intensity of extracellular oxidative stress cannot explain why ferrous salts were found to have higher damaging effect relative to ferric salts.[12]

As indicated in the "Introduction" section a progressive iron accumulation has been detected in many neurodegenerative diseases as well as in brains subjected to various kinds of hypoxic/ischemic insults. Given the potency of iron in inducing oxidative stress and the evidence that oxidative stress contributes to cell death, a causal role of iron imbalance is suspected in these brain diseases.[36,37] In neurodegenerative diseases, inappropriate iron entry within cells as a result not only of the disruption in the production of Tf-R but also in the production of less well-known brain iron transport proteins, has recently been proposed as a cause of cell death.[38] Our results obtained in rats treated with slow FHQ injection indicate that cells can survive from iron acquisition at a level above that required for satisfaction of metabolic needs providing iron acquisition by cells be progressive. Thus, although we cannot exclude that extension of the duration of 100 μM FHQ injection would have led to the destruction of the injected region, our results suggest that inappropriate iron delivery to cells cannot alone cause cell death in neurodegenerative diseases. We rather propose that dysregulation of Ft metabolism plays a key role in pathogenesis of these diseases as suggested by studies conducted on knock-out mice for IRP2 (iron regulatory protein 2). IRP2 senses cellular iron status and regulates expression of ferritin. When iron level is above metabolic threshold, IRP2 is degraded, thereby increasing translation of Ft mRNA into the protein.[39] Thus, the inability of cells to adapt Ft content to iron status may explain why IRP2 deletion causes accumulation of ferric iron in the brain associated with Parkinson-like syndrome and neurodegeneration.[40] In brains subjected to hypoxic/ischemic insults, a late-onset iron accumulation has also been reported and was suspected to be of importance in some slowly progressive forms of neurodegeneration.[7] Again, abnormalities in iron storage capacity of neurons may be involved. In accordance with this hypothesis, microglial cells but not neurons overexpressed Ft in response to a brain ischemic insult.[41]

In summary, the main findings of our study are (i) the evidence that the rate of iron delivery to cells is an important determinant of the toxicity of intracerebral iron injection, (ii) the demonstration that iron-induced extracellular formation of hydroxyl radicals is not predictive of iron toxicity when iron is directly injected into a brain region.

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