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Iron Mobilisation and Cellular Protection by a New Synthetic Chelator O-Trensox

Nafissa Rakba, Fouad Aouad,† Christophe Henry,† Catherine Caris,‡ Isabelle Morel,§ Paul Baret,‡ Jean-Louis Pierre,‡ Pierre Brissot,*‖ Roberta J. Ward,† Gérard Lescoat* and Robert R. Crichton†¶*

*INSERM U 49, Unite de Recherches Hepatologiques, Rennes, France; †Unite de Biochimie, Universite Catholique de Louvain, Louvain-la-Neuve, Belgium; ‡CNRS UMR 5616, Laboratoire d'Etudes Dynamiques et Structurales de la Selectivite, Universite Joseph Fourier, Grenoble, France; §INSERM U 456, Detoxication et Reparation Tissulaire, Faculte de Pharmacie, Rennes, France; and ‖Clinique des Maladies du Foie, CHRU Pontchaillou, Rennes, France

ABSTRACT. We tested a new synthetic, 8-hydroxyquinoline-based, hexadentate iron chelator, O-Trensox and compared it with desferrioxamine B (DFO). Iron mobilisation was evaluated: (i) *in vitro* by using ferritin and haemosiderin; DFO mobilised iron much more rapidly from ferritin at pH 7.4 than did O-Trensox, whereas at pH 4, ferritin and haemosiderin iron mobilisation was very similar with both chelators; (ii) *in vitro* by using cultured rat hepatocytes which had been loaded with ^{55}Fe-ferritin; here DFO was slightly more effective after 100 hr than O-Trensox; (iii) *in vivo* administration i.p. to rats which had been iron-loaded with iron dextran; O-Trensox mobilised 51.5% of hepatic iron over two weeks compared to 48.8% for DFO. We also demonstrated the effect of O-Trensox in decreasing the entry of ^{55}Fe citrate into hepatocyte cultures. The protective effect of O-Trensox against iron toxicity induced in hepatocyte cultures by ferric citrate was shown by decreased release of the enzymes lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotranferase (ALT) from the cultures and, using electron paramegnetic resonance (EPR) measurements, decreased production of lipid radicals. O-Trensox was more effective than DFO in quenching hydroxyl radicals in an acellular system. BIOCHEM PHARMACOL **55**;11:1797–1806, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. hepatocyte cultures; iron chelators; Fe-citrate; Fe-ferritin; O-Trensox

As McCance and Widdowson [1] suggested from early studies, iron balance in man is essentially determined by iron absorption, because excretory pathways are extremely limited. It follows that in several disorders of human iron metabolism, such as thalassaemia, which are treated by regular blood transfusions, iron loading of various tissues, notably the liver, occurs progressively. This secondary haemochromatosis can lead to the development of fibrosis and cirrhosis after many years of iron overload: some patients will develop a hepatocellular carcinoma.

In this pathology, the only way to remove iron is chelation therapy, and at present the only drug used in treatment is DFO**, or Desferal®. However, this siderophore isolated from *Streptomyces pilosus* is ineffective by oral administration because its half-life in serum is very short (12 min) Desferal is administered by overnight s.c. infusion to permit sufficient iron excretion. In addition to its high cost and problems of patient compliance, the drug also presents some long-term toxic effects.

There is thus an important need to develop the search for a new, cheaper and orally active alternative iron chelator to treat patients with secondary iron overload. In this area, our groups have recently reported investigations on compounds such as the bacterial siderophores pyoverdin [2–4] and desferrithiocin [5, 6] as well as synthetic hydroxypyrid-4-ones [7, 8], of which one derivative, CP20, has been proposed as an oral iron chelator for the treatment of secondary iron overload [9, 10].

In the present study, we evaluated the iron chelation and hepatic protection of a new synthetic chelator O-Trensox. O-Trensox is a synthetic water-soluble tripodal iron-sequestering agent (Fig. 1) constituted by 3 units of 8-hydroxyquinoline connected together by appropriate linker groups [11]. It is a strong complexing agent for both ferric and ferrous ions and does not induce radical damage. The ferric complex exhibits highly promising properties for plant nutrition [12], and the free ligand seems to be well-suited for iron chelation therapy. Preliminary studies indicate that it is effective in protecting rat hepatocytes against the toxic effects of iron both by decreasing iron

¶ Corresponding author: Prof. R. R. Crichton, Unité de Biochimie, Université Catholique de Louvain, Place Louis Pasteur 1, B-1348 Louvain-la-Neuve, Belgium. Tel. (32) 10-472794; FAX (32) 10-472796. E-mail: crichton@bioc.ucl.ac.be.

** *Abbreviations:* ALT, alanine aminotranferase; AST, aspartate aminotransferase; DFO, desferrioxamine B; DMPO, dimethylpyrroline *N*-oxide; EPR, electron paramagnetic resonance; LDH, lactate dehydrogenase.

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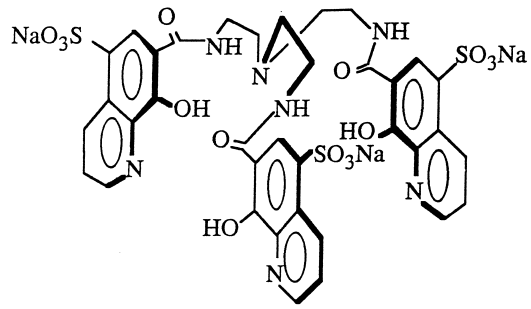

FIG. 1. Structure of O-Trensox.

uptake and increasing iron release by the cells [13]. Because the liver is a major target of iron overload toxicity, hepatocyte culture systems represent a good *in vitro* experimental model to assess the effects of O-Trensox on hepatic iron overload. However, when hepatocyte cultures are loaded with iron from ferric-citrate, the iron loading turns out not to be stable with time [8, 14, 15] and also to be toxic to the cells. In contrast, temporal stability of iron loading can be achieved by using ^{55}Fe-labelled ferritin, without any toxic effects on the hepatocyte cultures [14, 15]. This is why, in the present study, we assess the iron mobilising effects of Trensox using rat hepatocyte cultures loaded with ^{55}Fe-labelled rat liver ferritin and its protective effects against iron-induced toxicity using ferric citrate.

MATERIALS AND METHODS

Chemicals

Horse spleen ferritin was prepared by the method of [16] modified as in [17]. Haemosiderin was prepared as described in [18]. DFO was from Novartis (Basle, Switzerland) and O-Trensox was supplied by P. Baret (CNRS UMR 5616; Laboratoire d'Etudes Dynamiques et Structurales de la Sélectivité, Université Joseph Fourier, Grenoble, France). Ferric citrate solutions were prepared according to the method of [19]. Tri-sodium citrate dihydrate (Merck) was used to maintain ferric iron in a soluble state. To 2.94 g of tri-sodium-citrate-dihydrate, dissolved in 100 mL of sterile water, was added 270 mg of iron (III) chloride hexahydrate (Merck). The final concentration of ferric iron in the stock solution was 10 mM and the molar ratio iron to citrate was 1/10. This solution was filtered before use, then diluted in the culture medium to obtain a final medium iron concentration of 20, 50 or 100 μM. For the preparation of rat liver ferritin labelled with ^{55}Fe, Wistar male rats weighing 250 g were injected i.p. with 300 μCi of $^{55}FeCl_3$ and sacrificed 24 hr later. Ferritin was isolated according to the method of [5].

In Vivo *Model*

Male rats (Wistar strain) 270 g in weight were iron-loaded by intraperitoneal injection of iron dextran (Vifor) at a dose of 100 mg/kg, 3 times/week for 2 weeks. Subsequently, groups of 6 animals were maintained on an iron-free diet (Fe content 0.003 g of Fe/kg) for 2 weeks either without chelation (iron-loaded control group) or with the administration of the iron chelators desferrioxamine or O-Trensox i.p. at 10 mg/kg every second day for 14 days (i.e. 7 administrations). At the end of the treatment period, the animals were sacrificed as described in [7], the livers removed, and total hepatic iron determined by electrothermal atomic absorption [20].

Iron Mobilisation from Ferritin and Haemosiderin

Ferritin was incubated in 0.2 M of MOPS (Sigma M-1254) buffer pH 7.4 or in 0.2 M of formate with an iron concentration of 300 μM (corresponding to a protein concentration of 10^{-7} M) and a chelator concentration of 1 mM. Samples were incubated at 37° for 6 hr and iron release was measured at regular intervals using λ_{max} of 428 nm and 595 nm and ϵ (L/mol.cm) of 2,800 and 5,200 for DFO and O-Trensox, respectively. Results are presented as means ± SEM for 5 samples at each time point. Haemosiderin was solubilised in a solution of 20 mM of tetramethylammonium hydroxide (Sigma T-7505) and the pH adjusted to pH 4.0 buffered with 0.2 M of formate. The iron concentration was 300 μM. Samples were incubated at 37° for 6 hr with vigourous stirring. Before each measurement, samples were centrifuged for 5 min at 10,000 *g*, the absorbance of the supernatent was measured, and the supernatent was mixed with the pellet and reincubated at 37°. Results are means ± SEM for 3 samples at each time point.

Hepatocyte Isolation and Culture for Fe-Citrate Overload Study

Adult hepatocytes were isolated from 2-month-old Sprague–Dawley male rats by cannulating the portal vein and perfusing the liver with a collagenase solution (0.025% collagenase from Boehringer Mannheim, 0.075% $CaCl_2$ buffered with 0.1 M of HEPES, pH 7.6 from Calbiochem) according to Seglen's method with some modifications [21–23]. Rats were maintained on a 12 hr light/dark cycle and were given standard food and tap water *ad lib.* The hepatocytes were collected in Leibovitz medium containing per mL: 1 mg of bovine serum albumin and 5 μg of bovine insulin. The cell suspension was filtered through gauze and allowed to sediment for 20 min to eliminate cell debris, blood, and sinusoidal cells. The cells were then washed three times by centrifugation at 50 *g* and tested by trypan blue dye exclusion for viability that was always in the range of 85–95%. The hepatocytes were then suspended in a mixture of 75% Eagle's minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum containing per mL: streptomycin (50 μg), penicillin (7.5 IU), bovine insulin (5 μg), bovine serum albumin (1 mg) and $NaHCO_3$ (2.2 mg). Usually, 3.0×10^5 hepatocytes were suspended in 1 mL of medium in multiwell tissue

culture plates (well area = 3.8 cm^2). The medium was changed 3–4 hr later. The effects of iron and iron chelators were studied in cultures maintained in medium deprived of fetal calf serum but supplemented with dexamethasone (10^{-7} M).

Hepatocyte Isolation and Culture for Fe-Ferritin

Rat hepatocytes were isolated and cultured by a combination of several techniques [21–24]. The suspension of hepatocytes was filtered at 0° through 3 Perlon filters, of 100 μm, 63 μm and 28 μm, respectively and then centrifuged for 2 min at 50 *g* in order to remove nonparenchymal cells. The cells were then washed three times at 0° in Dulbecco's modified Eagle's medium (Life Technologies). The centrifugation times after each washing were 105, 90 and 75 sec, respectively. The hepatocytes were then suspended in 100 mL of culture medium and counted in a Bürker cell. Cellular viability, as judged by trypan blue exclusion was greater than 90%. Two milliliters of the cellular suspension at a density of 10^6 cells/mL in Dulbecco's Modified Eagle's Medium containing 10% (v/v) of fetal calf serum (Life Technologies), 4 mM of glutamine, 20 mM of glucose, 100 units/mL of penicillin and 100 μg/mL of streptomycin, buffered in 10 mM of HEPES, pH 7.4 was transferred to polystyrene culture dishes (Nunc) which had been precoated with collagen. The coating involved incubation of the culture dishes for at least 5 hr at 37° with 2 mL of Vitrogel 100 (Collagen Corporation) diluted in PBS at a concentration of 30 μg/mL.

The hepatocytes were cultivated at 37° in a water-saturated atmosphere under CO_2/air (7:93 v/v). After 4 hr incubation, almost all of the cells had adhered to the collagen membrane and the culture medium was replaced by Williams E medium (Life Technologies) to which was added insulin (10 μg/mL, Life Technologies), glucagon (10 μg/mL, Sigma), epidermal growth factor (50 μg/mL, Life Technologies), oligoelements (M_n, M_o, N_i, S_i, S_n, V), ascorbate (0.1 mM) and tocopherol (10 μg/mL)—all from Life Technologies, and prolactin (20 munits/mL), somatotropin (10 μunits/mL), linoleic acid complexed to albumin and dexamethasone (1 μM)—all from Sigma. In some cases, penicillin (100 units/mL) and streptomycin (100 μg/mL) were added to the culture medium. The cultures were maintained in the control condition (with neither iron nor chelators present), in the presence of iron alone, in the presence of chelators alone, or in the presence of iron plus chelators (DFO or O-Trensox). Iron or chelators were added on day 1 (i.e. 24 hr after culture establishment).

^{55}Fe Uptake by Hepatocyte Cultures

Hepatocyte cultures were maintained for 1 day in the presence of 0.02 μM of ^{55}Fe-citrate (Amersham). In order to demonstrate that the chelators were able to decrease iron uptake by the hepatocytes, 20 or 40 μM of O-Trensox was added to the culture medium on day 1 at the same time as ^{55}Fe. The iron chelator effect was compared with that of a control which consisted of culture medium without addition of chelators. To count intracellular radioactivity, the cultures were rinsed with phosphate saline buffer in order to minimize the nonspecific adsorption of radioactive iron at the membrane level; then, the cells were sonicated in 1 mL of the same buffer and the radioactivity measured.

^{55}Fe Release from Hepatocyte Cultures

For the iron mobilisation experiments following ferritin loading, hepatocyte cultures were incubated in hormono-defined Williams E medium on a coating of 60 μg of rat collagen. Hepatocytes were iron-loaded by using ^{55}Fe-labelled rat ferritin at a concentration of 20 μg for 24 hr. After iron loading, the hepatocytes were incubated with the chelators at a concentration of 50 μM, and ^{55}Fe iron release was measured over a period of 100 hr.

Protein Assay

To determine the total protein per well, the media were decanted and the cell cultures extensively rinsed with phosphate-buffered saline. Cells were sonicated for 15 sec. The protein content was determined according to the method of Bradford [25] or Lowry *et al.* [26] by using BSA as standard.

Albumin and Transferrin Assay

Standard rat albumin or transferrin and antisera used were obtained from Cappel Laboratories. At the end of the treatment, the media from the culture dishes were withdrawn and stored at −80°. Albumin and transferrin were quantified by laser immunonephelometry [27]. Standard albumin or transferrin solutions or incubation media were mixed with an appropriate dilution of antiserum against albumin or transferrin in 0.9% NaCl containing 4% polyethylene glycol. Light scattering was measured after 2-hr incubation at room temperature. The limit of sensitivity of the assay was 2 μg/mL.

Enzyme Assays

ALT, AST, and LDH activities were measured in the culture medium and intracellulary as indexes of cytotoxicity, employing LDH, ALT and AST kits (Bayer Diagnostics) adapted to the ALCYON 300 analyzer (Alcyon). Extracellular enzyme activity was measured on an aliquot cell-free medium obtained by centrifugation of the medium at 50 *g* for 2 min. Intracellular enzyme activity was evaluated on hepatocytes previously lysed in phosphate saline buffer by sonication for 15 sec. Experimental results were expressed in terms of ALT, AST and LDH releases into the medium expressed as a percentage of the total activity of the culture.

EPR Measurements

The EPR of the lipid-derived radical adducts was measured in hepatocyte cultures, using the spin-trap α-(4-pyridyl 1-oxide)-*N*-*tert*-butyl-nitrone (POBN). For this EPR analysis, the culture media were removed and the hepatocytes washed twice with 0.01 M of phosphate buffer, pH 7.45. The cells were resuspended in 3 mL of the same buffer and centrifuged at 50 *g* for 2 min at 5°. The buffer was discarded and the hepatocytes were kept on ice and lysed by ultrasonic homogenizer. Each sample was then analysed by EPR. Before placing the homogenates into the EPR spectrometer cavity, 160 mM (final concentration) of α-(4-pyridyl 1-oxide)-*N*-*tert*-butyl-nitrone was added to the cell homogenate. The mixture was gently mixed and then transferred to a Pasteur pipette adapted for EPR analysis.

A method involving a spin-trapping technique in conjunction with EPR spectroscopy was used to measure the ability of O-Trensox to scavenge free radicals such as hydroxyl radicals, generated in an acellular system by hydrogen peroxide and UV photolysis, using the spin-trap DMPO. Briefly, 30 μL of H_2O_2 aqueous solution (20 mM) and 60 μL of DMPO aqueous solution (53 mM) were added in that order into a small glass tube. After mixing, the solution was irradiated under a UV light at 254 nm (Bioblock) for 6 min. Using this method, the antioxidant activity of the iron chelator O-Trensox was investigated and compared with that of DFO.

EPR spectra were recorded on a Bruker ESP 106 Spectrometer at ambient temperature operating at *ca.* 9.74 GHz (× band) frequency under the following instrument conditions: sweep width 55 G, microwave power 20 mw, modulation amplitude 1.8 G, modulation frequency 100 KHz, receiver gain 1×10^6, time constant 163 msec, sweep time 335 sec, three scans accumulated. The results were obtained as arbitrary units given by computer double integration of the low field doublet of the spectra; the results of lipid radical adducts were related to per mg protein content for each sample. At the end of analysis, the remaining cell homogenate was used for protein content estimation according to Bradford [25].

RESULTS

Iron Mobilisation from Ferritin and Haemosiderin by O-Trensox

Mobilisation of iron from ferritin at pH 7.4 was significantly slower and less efficacious with O-Trensox than with DFO (Fig. 2a). However, at pH 4.0 the two chelators were observed to be more or less equivalent (Fig. 2b). When iron release from haemosiderin was analysed (Fig. 3), O-Trensox was found to be somewhat slower than DFO initially, but the amount of iron mobilised after 6 hr was similar to DFO.

Iron Release from Iron-loaded Hepatocytes In Vivo *and* In Vitro *by Chelators and Their Inhibition of Iron Uptake*

The mobilisation of iron by DFO and O-Trensox, at a chelator concentration of 50 μM, from rat hepatocyte cultures which had been loaded *in vitro* with ^{55}Fe-ferritin is presented in Fig. 4. DFO is slightly more efficient than O-Trensox. In order to study the effect of O-Trensox on iron uptake by the hepatocytes, the cultures were maintained for one day in a medium containing 0.02 μM of ^{55}Fe without chelator or with 20 or 40 μM of O-Trensox. In the absence of O-Trensox, a high uptake of iron by the hepatocytes was observed; in the presence of O-Trensox, this uptake was decreased, and the decrease was dose-dependent ($P < 0.001$; Fig. 5). We also carried out studies in our *in vivo* model of iron overload, in which both DFO and O-Trensox were administered i.p. at a dose of 10 mg/kg every second day for 2 weeks to rats which had been loaded with iron dextran. When the results were compared with untreated iron-loaded animals, we found that DFO resulted in a decrease of total hepatic iron of 48.8%, whereas O-Trensox reduced hepatic iron by 51.5%.

Protective Effect of Trensox

In the cultures treated with 20 μM of iron, a highly significant release of LDH, AST, ALT was observed ($P < 0.001$; Fig. 6a, b, c). The addition of O-Trensox protected against this iron-induced toxicity, and the decrease in the enzyme release was dose-dependent ($P < 0.001$; Fig. 6a, b, c). This protective effect of O-Trensox was also seen on the decrease in the albumin and transferrin secretion induced by iron overload ($P < 0.05$ for Tren 20 μM; $P < 0.01$ for Tren 40 μM; Fig. 7). O-Trensox alone (20 or 40 μM) was not toxic after one day of culture (Figs. 6 and 7).

In the hepatocyte cultures treated with 20 μM of iron, a high level of production of lipid radical adducts measured by EPR was observed ($P < 0.001$; Fig. 8). The addition of 20 or 40 μM of O-Trensox at the same time as iron significantly decreased lipid radical adduct production, and the protective effect of O-Trensox on this toxicity was also dose-dependent ($P < 0.001$; Fig. 8). O-Trensox alone (20 or 40 μM) was without effect on lipid radical adduct production when compared to the control (Fig. 8).

The scavenging effect of 100 or 200 μM of O-Trensox on hydroxyl radical production was evaluated by EPR in an acellular system in which hydroxyl radicals were generated by UV-induced H_2O_2 decomposition. In the absence of chelators (control), an important EPR signal corresponding to the generation of hydroxyl radicals was observed (Fig. 9). Addition of O-Trensox led to a significant decrease in the EPR signal when compared to the control or to DFO (Fig. 9). We also observed that O-Trensox at 200 μM scavenged hydroxyl radicals better than at 100-μM concentration ($P < 0.05$ for 100 μM; $P < 0.001$ for 200 μM; Fig. 9) and

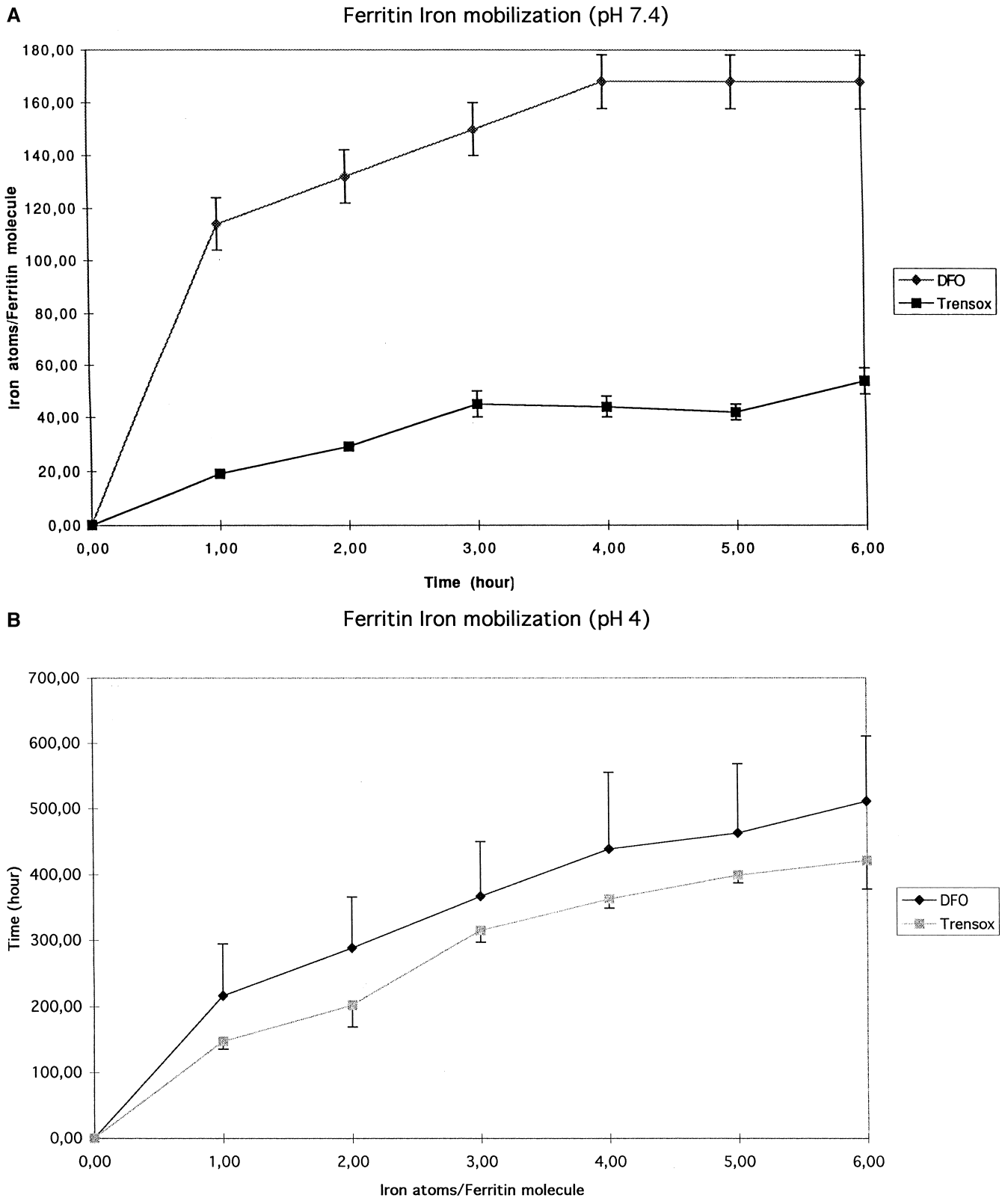

FIG. 2. Mobilisation of iron by 1 mM of DFO or O-Trensox from ferritin at (A) pH 7.4 or (B) pH 4.0. Results are means ± SEM for 5 samples at each time point.

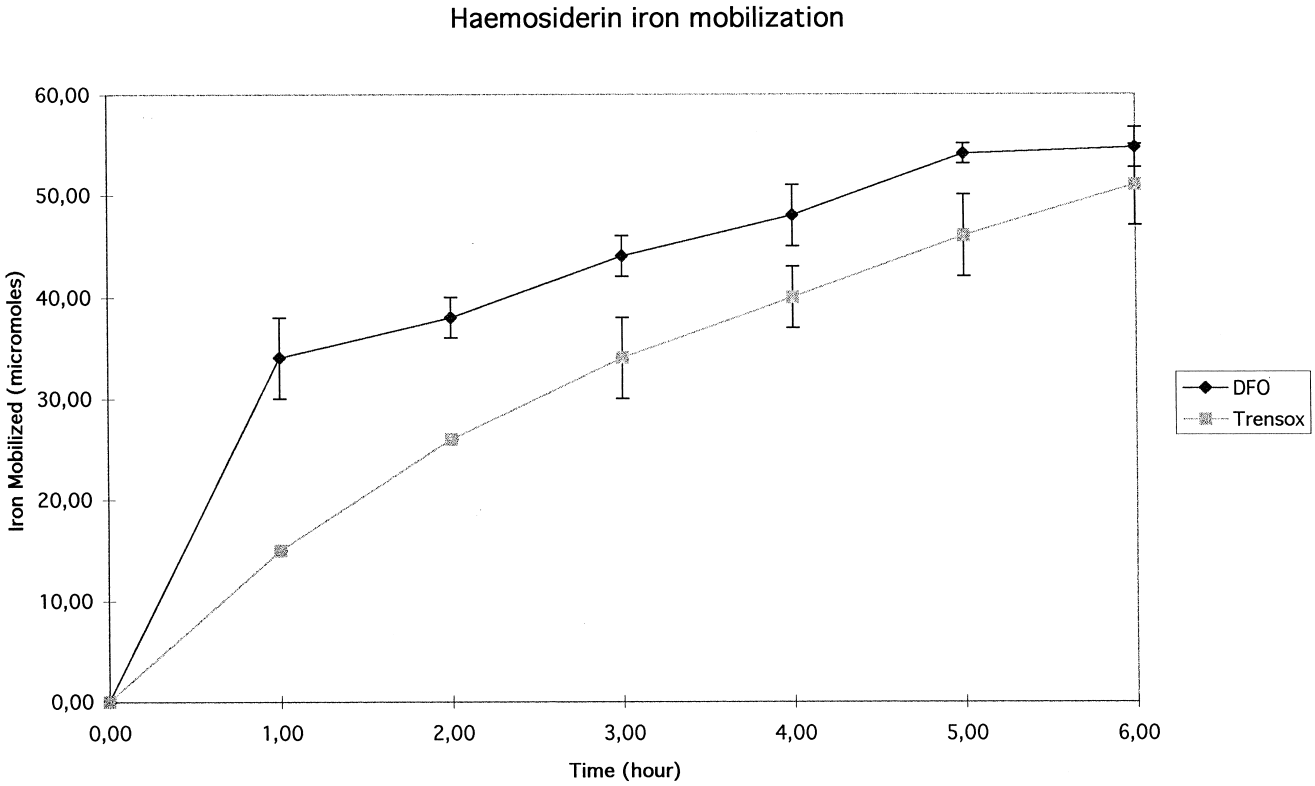

FIG. 3. Mobilisation of iron by 1 mM of DFO or O-Trensox from haemosiderin at pH 4. Results are means ± SEM for 3 samples at each time point.

with more efficiency than 200 μM of DFO, which was without significant effect (Fig. 9).

DISCUSSION

It is clear that iron uptake by mammalian cells is not only dependent on transferrin-bound iron uptake by its receptor, but also on other mechanisms which involve nontransferrin pathways [28]. Some of these have been more clearly charted than others. For example, cells such as hepatocytes

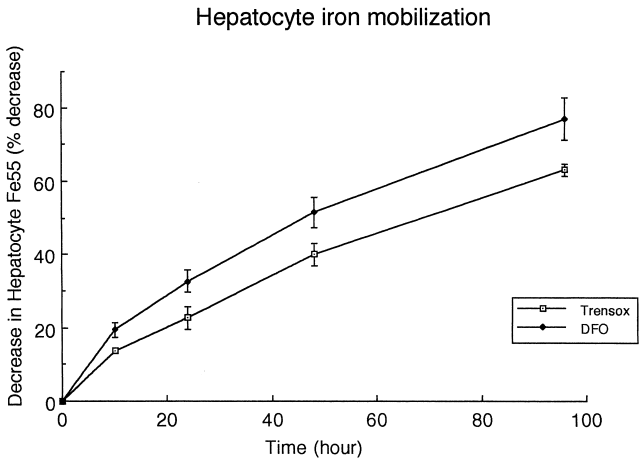

FIG. 4. Mobilisation of iron from ^{55}Fe-ferritin preloaded rat hepatocyte cultures by 50 μM of DFO or O-Trensox. Results are means ± SEM for 3 samples at each time point.

can capture iron from circulating ferritin via specific receptors [29, 30]; this might be the way in which iron accumulated by macrophages, under conditions of secondary iron overload such as transfusion treatment for thalassaemias, could be recycled from the reticuloendothelial system to the parenchymal cells [31]. Other examples of iron uptake from nontransferrin sources are illustrated by congenital disorders such as haemochromatosis and atransferrinemia [28]. The former, an autosomal recessive disorder, is caused by a defect in the regulation of intestinal iron absorption [32], and this is reflected by a 100% transferrin saturation; despite this intestinal iron absorption continues. Nontransferrin bound iron is rapidly cleared resulting in excessive parenchymal iron accumulation [33, 34]. This excess iron deposition may be associated with cirrhosis, liver cancer, cardiomyopathy, diabetes mellitus, arthropathy and endocrine deficiencies [35, 36]. In atransferrinemia, a rare condition found in humans and rodents, the undetectable levels of transferrin result in anemia due to iron-limited erythropoiesis, but also in progressive iron overload [37–41].

Hepatocytes are crucially important not only in general intermediary metabolism, but also in iron metabolism. In many ways, iron uptake from nontransferrin sources may prove to be extremely important in determining to what extent parenchymal iron loading may be either relatively easily handled, or in other cases lead to toxicity manifested by the consequences of oxidative stress provoked by the potential for Fenton chemistry of iron.

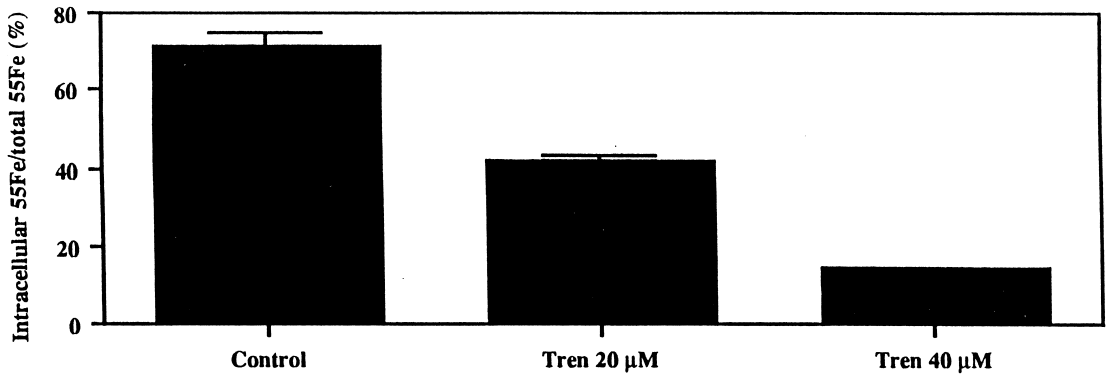

FIG. 5. O-Trensox effect on ^{55}Fe uptake by rat hepatocyte cultures maintained in the presence of 0.02 µM of ^{55}Fe-citrate alone (control) or ^{55}Fe plus 20 or 40 µM of O-Trensox (Tren 20 µM; Tren 40 µM). Results are expressed as means ± SEM of three independent experiments in which each experimental condition was carried out in quadruplicate.

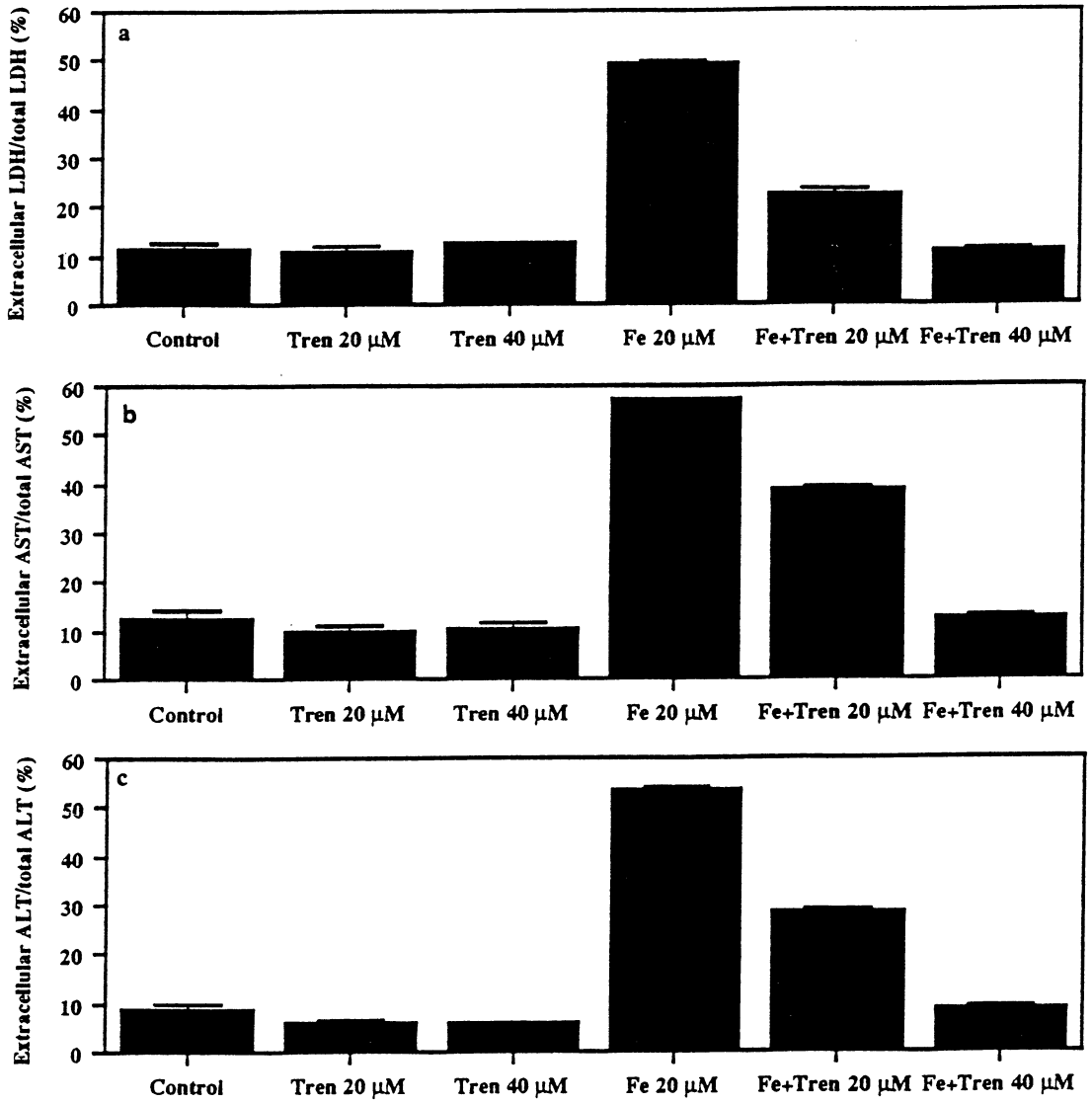

FIG. 6. Releases of LDH (a), AST (b) and ALT (c) in rat hepatocyte cultures maintained for 24 hr under the control condition (control), in the presence of O-Trensox alone (Tren 20 µM; Tren 40 µM), iron citrate alone (Fe 20 µM) or iron citrate plus O-Trensox (Fe + Tren 20 µM; Fe + Tren 40 µM). Results are expressed as means ± SEM of three independent experiments in which each experimental condition was carried out in quadruplicate.

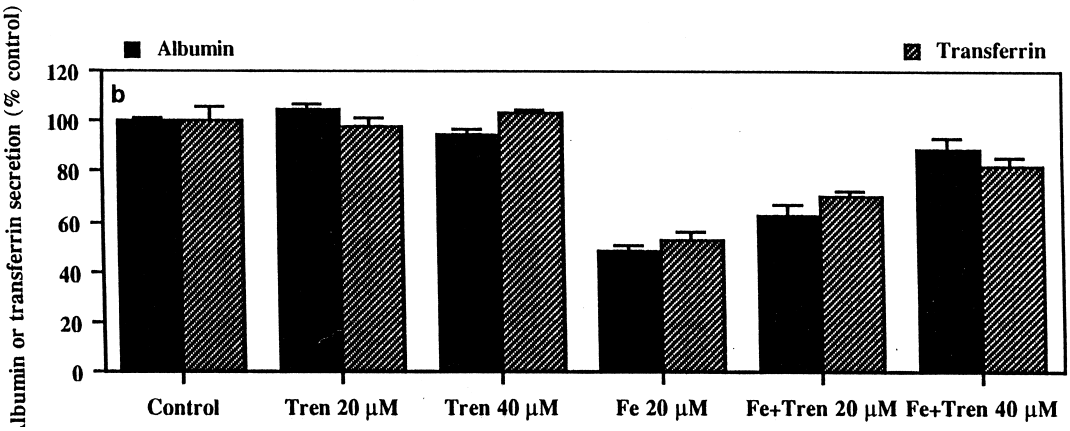

FIG. 7. Albumin and transferrin secretions in rat hepatocyte cultures maintained for 24 hr under the control condition (control), in the presence of O-Trensox alone (Tren 20 μM; Tren 40 μM), iron citrate alone (Fe 20 μM) or iron citrate plus O-Trensox (Fe + Tren 20 μM; Fe + Tren 40 μM). Results are expressed as means ± SEM of three independent experiments in which each experimental condition was carried out in quadruplicate.

Therefore, there is clearly a need for *in vitro* systems which allow the efficacy of new classes of iron chelators to be evaluated before their utilisation in animal models. In the present study, we have compared the effects of the chelator which is presently used clinically in man, DFO, with O-Trensox, the water-soluble version of a new class of synthetic iron chelators based on the 8-hydroxyquinoline molecule. Like DFO, O-Trensox is a hexadentate chelator. When tested on iron mobilisation from ferritin, O-Trensox was less effective than DFO in mobilising ferritin iron at pH 7.4, but was as good as DFO at pH 4.0. When it was examined with the lysosomal iron storage protein haemosiderin at pH 4.0, it was as good as DFO. The potential of O-Trensox is further substantiated by its performance in removing iron *in vitro* from rat hepatocyte cultures which had been loaded with rat liver ferritin, where it was found to be almost equal to DFO. It is also similar to DFO in its capacity to remove iron from the *in vivo* iron dextran model of iron overload [18]. Previous studies which addressed the use of hepatocytes for the evaluation of iron chelators used iron labelling *in vitro* with transferrin [42–44], or alternatively *in vivo* with iron dextran [45] or carbonyl iron [46]. The advantages of the ferritin-loaded hepatocyte model are that it has proved to be both stable with time and to be nontoxic to the hepatocytes [14, 15]. It is based on the uptake of ferritin by hepatocytes via a receptor-mediated pathway [29, 30], which allows much more iron to be assimilated than via the transferrin receptor-mediated pathway. The ferritin iron accumulates in the mitochondria and the lysosomes, from where it is progressively transferred to cytosolic ferritin [47].

In contrast, the loading of rat hepatocytes with ferric

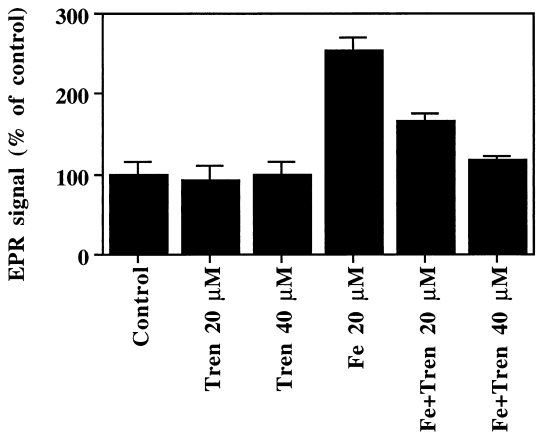

FIG. 8. Lipid radical adduct production in rat hepatocyte cultures maintained for 24 hr under the control condition (control), in the presence of O-Trensox alone (Tren 20 μM; Tren 40 μM), iron citrate alone (Fe 20 μM) or iron citrate plus O-Trensox (Fe + Tren 20 μM; Fe + Tren 40 μM). Lipid-POBN-radical adducts were detected by EPR. Results are expressed as means ± SEM of three independent experiments in which each experimental condition was carried out in duplicate.

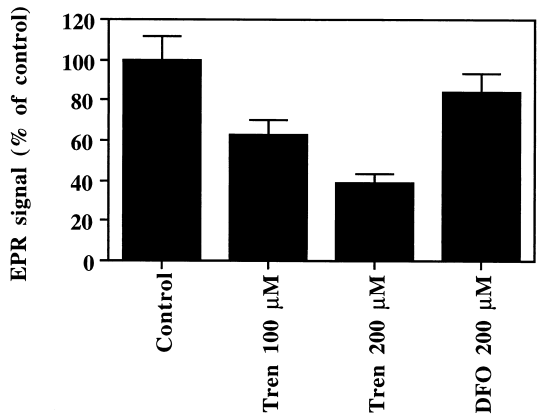

FIG. 9. O-Trensox (Tren 100 μM; Tren 200 μM) and DFO (DFO 200 μM) scavenging effect on hydroxyl radicals: hydroxyl radicals were generated by H_2O_2 UV decomposition and then stabilised by DMPO spin trapping. °OH-DMPO adducts were detected by EPR. The EPR signal decrease corresponds to a scavenging effect of °OH radicals. Results are expressed as means ± SEM of three independent experiments in which each experimental condition was carried out in duplicate.

citrate leads to an iron loading which is not stable with time [14], but which clearly provokes toxic effects, as measured by release of a number of hepatic enzymes (LDH, AST, ALT) into the culture medium, together with a decreased secretion of albumin and transferrin and an increased production of oxygen-based free radicals with concomitant lipid peroxidation [8]. We observed that O-Trensox was effective in protecting hepatocytes from prooxidant-induced cell injury [46, 48–50]. Furthermore, O-Trensox was able to scavenge hydroxyl radicals with a higher efficacy than DFO.

We have previously demonstrated that loading of hepatocytes with ferritin represents a nontoxic way of loading the cell, and is an attractive *in vitro* model for the evaluation of the capacity of new iron chelators to mobilise hepatic iron, particularly since it gives results which are similar to those obtained in our previously validated *in vivo* model [7, 20]. In contrast, ferric citrate represents a potentially toxic route for introduction of iron into hepatocytes, perhaps because it is in many ways analogous to the nontransferrin bound iron, reported previously to be responsible for hepatic iron toxicity in genetic haemochromatosis [51].

The present data demonstrate that Trensox was able to mobilise iron from ferritin, haemosiderin or hepatocytes, and to protect the hepatocytes against the toxic effect of iron overload. In conclusion, O-Trensox functions as a hepato-protector against the toxicity of iron overload, and new investigations concerning its oral efficacy will be required to estimate its therapeutic potential.

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