The effect of iron overload on rat plasma and liver oxidant status *in vivo*

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There is ample evidence implicating reactive oxygen species in a number of human degenerative diseases such as atherosclerosis and haemochromatosis. Although lipid peroxidation underlies many of the toxic effects of oxidative stress, there is a lack of a sensitive and reliable method for its assessment *in vivo*. To understand the implications of oxidative stress *in vivo*, we have used dietary iron overload (IO) in the rat. Oxidant status in these animals was determined by assessing depletion of endogenous antioxidants and formation of various lipid peroxidation products, including acylated F_2-isoprostanes, a novel class of free-radical-derived prostaglandin-F_2-like compounds. IO led to a significant decrease in the concentrations of the antioxidants α-tocopherol and ascorbic acid in plasma, and α-tocopherol, β-carotene and ubiquinol-10 in liver. Whereas there was no significant lipid peroxidation in plasma, hepatic F_2-isoprostane levels were moderately but significantly increased in IO. In addition, IO caused a significant increase in plasma total and high-density lipoprotein cholesterol levels, an effect that was correlated with depletion of plasma ascorbic acid but not α-tocopherol. The data demonstrate that IO causes lipid metabolism disturbances and oxidative stress which is associated with substantial depletion of endogenous antioxidants and moderate lipid peroxidative damage.

INTRODUCTION

Iron-catalysed generation of reactive oxygen species has been implicated in the pathogenesis of many disorders including atherosclerosis [1,2], cancer [3], ischaemia reperfusion injury [4,5] and conditions of iron overload (IO) [6], such as haemochromatosis, which is one of the most prevalent genetic disorders in Western countries, affecting about 1 in 220 whites of northern European ancestry [7].

Under conditions of chronic IO, there is evidence that excess iron induces cellular injury and functional abnormalities in hepatocytes by the process of lipid peroxidation in lysosomal, mitochondrial and microsomal membranes (reviewed in [8]). Lipid peroxidation is a likely outcome of oxidative stress in biological systems, and its measurement is often used as a method of assessing the degree of oxidative damage. Towards this end, a number of analytical techniques which measure intermediates and end-products of lipid peroxidation have been used, but most of these assays lack sensitivity and specificity, which hampers accurate assessment of oxidative damage *in vivo*.

In this study, we have investigated the effects of dietary IO in the rat on endogenous antioxidant status and several parameters of lipid peroxidation in plasma and liver. Lipid peroxidation was assessed by measuring lipid hydroperoxides, thiobarbituric-acid-reactive substances (TBARS) and F_2-isoprostanes. The latter are a series of prostaglandin-F_2-like compounds derived from the free-radical-catalysed, non-enzymic peroxidation of arachidonic acid [9]. The *in vivo* levels of these F_2-isoprostanes have been shown to increase dramatically in acute hepatotoxicity [10].

The data presented here demonstrate that IO in the rat causes oxidative stress associated with depletion of endogenous antioxidants in plasma and liver. Although we found no conclusive evidence for lipid peroxidation in plasma, hepatic F_2-isoprostane levels were significantly increased in IO, showing that these compounds are good indicators of the oxidant status *in vivo*.

MATERIALS AND METHODS

Animals and diets

Male Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.) and housed in groups of six rats per cage. The rats were allowed diet and water *ad libitum*. Experimental chronic IO was produced in 12 rats by feeding a chow diet (Purina Lab Chow, no. 5001) enriched with 3% (w/w) reduced pentacarbonyl iron (a 99%, w/w, pure form of elemental iron, in the form of microscopic spheres of 4.5–5.2 μm diameter; Sigma Chemical Co.) for 10 weeks. Twelve weight-matched rats were fed the chow diet and were used as controls. At the conclusion of the study, the mass of the control rats was 296 ± 15 g (mean $\pm$ S.D., $n = 12$), and 274 ± 59 g ($n = 12$) for the IO rats. The relevant constituents of the chow diet were: cholesterol, 270 mg/kg; iron, 198 mg/kg; selenium, 0.2 mg/kg; carotene, 4.5 mg/kg; vitamin E, 40 i.u./kg; and no ascorbic acid.

At 10 weeks, after an overnight fast, experimental and control rats were killed by exsanguination via cardiac puncture while under light ether anaesthesia. Heparinized blood was used to obtain plasma. Two samples of liver from each rat were removed; one sample was thoroughly washed and frozen at -70 °C for later analysis, and the other was fixed in 10% (v/v) formal-saline, dehydrated with a graded series of ethyl alcohol and embedded in paraffin. Thin sections (5 μm) were cut and stained with hematoxylin and eosin and Perls' Prussian Blue stain for light microscopy analysis.

Serum iron and total iron-binding capacity

These parameters were measured in non-haemolysed serum samples by spectrophotometric analysis as described [11].

Abbreviations used: HDL, high-density lipoprotein; IO, iron overload; TBARS, thiobarbituric-acid-reactive substances; TIBC, total iron-binding capacity.

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Liver iron

Total liver iron was measured using atomic absorption spectroscopy as described in the 1984 Perkin-Elmer manual. Briefly, 0.1 g of liver was weighed and placed in iron-free glass tubes (washed with hydrochloric acid). The livers were dried in an oven at 115 °C for 5 h, 0.3 ml of concentrated sulphuric acid was added, and the samples were digested for 2 days. Subsequently, 0.1 ml of concentrated nitric acid and 50 μl of hydrogen peroxide were added, and the freed metal salts were determined using atomic absorption spectroscopy. The results are expressed as micrograms of Fe per gram of liver (wet weight).

F_2-isoprostanes

Fresh plasma and liver were analysed for acylated F_2-isoprostanes. Lipids were extracted by a modified Folch procedure and base-hydrolysed [9]. The resulting free F_2-isoprostanes were measured, after purification and derivatization, by gas chromatography/negative-ion chemical ionization m.s. as described [9].

TBARS

These substances, which exhibit an absorbance peak at 532 nm, were determined spectrophotometrically in n-butanol extracts of fresh plasma samples according to the methods of Halliwell and Gutteridge [12,13].

Lipid hydroperoxides

Neutral lipid hydroperoxides in hexane extracts of fresh plasma samples were measured by h.p.l.c. with isoluminol chemiluminescence detection as described [14,15].

Antioxidant status *in vivo*

Water-soluble antioxidants (ascorbic acid and uric acid) were measured in fresh plasma extracts using h.p.l.c. with electrochemical detection [16]. α-Tocopherol in fresh plasma was extracted into hexane and stored at -20°C until assayed by h.p.l.c. with electrochemical detection [17]. Because levels of circulating α-tocopherol are influenced by increased serum lipid levels [18], plasma α-tocopherol is expressed as the ratio of α-tocopherol (μM)/(total cholesterol + triacylglycerols) (mM) as described [19].

For analysis of lipid-soluble antioxidants in liver (α-tocopherol, β-carotene, lycopene and ubiquinols-9 and -10), 0.1 g of wet liver was weighed and homogenized in 1.0 ml of 10 mM PBS, pH 7.4. The homogenization solution also contained 5.0 mM of the metal chelator diethylenetriaminepenta-acetic acid in order to prevent *ex vivo* lipid peroxidation and loss of lipid-soluble antioxidants. The tissue homogenate was then extracted with 1.0 ml of methanol and 5.0 ml of hexane and the lipid-soluble antioxidants in the hexane extract were measured as described [17].

Plasma lipids

Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triacylglycerols were determined spectrophotometrically using Sigma kit numbers 352-20, 352-3 and 337-B respectively. The results are expressed in millimolar concentrations.

Statistical analysis

All results are expressed as means ± S.D. For comparisons between control and IO rats, the unpaired Student's t test was used. Pearson's correlations were used to determine relationships between covariates.

RESULTS

Effect of IO on plasma and liver iron levels and liver morphology

Dietary IO resulted in hepatic iron levels of 1391 ± 242 μg of Fe/g of tissue compared with 103.9 ± 14.6 μg of Fe/g in control rats. These data are comparable with published values in rats fed a 2–2.5 % carbonyl iron diet for 7 and 22 weeks [20]. Serum iron and total iron-binding capacity (TIBC) of control animals were not significantly different from those of IO rats (Table 1). Serum iron levels and TIBC in the control rats were comparable with the values reported in the literature [21]. However, to our knowledge, there are no reports of serum iron levels and TIBC in this model of IO in the rat.

Light microscopy analysis of the livers of the control group showed normal lobular architecture without stainable iron. At the time point studied, i.e. at the conclusion of the 10 week iron-feeding period, no histological evidence of inflammation or necrosis was observed in the livers of IO rats. In agreement with previous reports [22], Prussian-Blue-positive iron was present in the hepatocytes mainly with a periportal distribution, similar to that observed in idiopathic haemochromatosis.

Effect of IO on plasma antioxidant levels, lipid peroxidation and lipid profile

The plasma levels of α-tocopherol and ascorbic acid were significantly ($P < 0.001$) decreased (by 77 and 68 % respectively) in rats with IO compared with controls (Table 1). In addition to ascorbic acid, uric acid in plasma can act as a water-soluble antioxidant [23]. IO led to a slight but statistically non-significant decrease in plasma uric acid levels. β-Carotene, lycopene and ubiquinols, known lipid-soluble antioxidants present in human plasma lipoproteins [17], could not be detected in any of the rat plasma samples.

In rats with IO, there was a slight but non-significant increase in plasma F_2-isoprostane levels compared with control rats (Table 1). Lipid hydroperoxides, as measured by h.p.l.c. with chemiluminescence detection [14,15], were not detectable (< 2.0 nM) in plasma from 8 out of the 12 IO animals and 7 out of the 12 control animals. In the remaining plasma samples there

Table 1 Effect of IO on plasma antioxidant status and lipid peroxidation

Sprague–Dawley rats were fed either a carbonyl-iron-supplemented diet (12 rats) or a control diet (12 rats) for 10 weeks. Blood was removed and the plasma levels of the indicated compounds were measured. P values are calculated for comparisons of IO with control animals. ND, not detectable (detection limit < 0.03 μM); NS, non-significant difference. Results shown are means ± S.D.

	Control	IO	P
α-Tocopherol (μmol/mmol)*	1.17 ± 0.69	0.27 ± 0.23	< 0.001
Ascorbic acid (μM)	108.0 ± 25.0	34.7 ± 12.3	< 0.001
Uric acid (μM)	79.6 ± 37.0	64.7 ± 30.4	< 0.30
F_2-isoprostanes (pg/ml)	50.5 ± 18.9	61.1 ± 28.6	< 0.30
Lipid hydroperoxides (nM)†	50.8 ± 48.8	1380 ± 1590	< 0.20
TBARS	ND	ND	–
Serum iron (μg/dl)	133.6 ± 54.5	123.7 ± 46.0	NS
TIBC (μg/dl)	413.7 ± 20.6	411.7 ± 35.4	NS

* Values are expressed as the ratio α-tocopherol/(total cholesterol + triacylglycerols).

† Only those samples that contained detectable levels of lipid hydroperoxides are included; $n = 5$ for controls and $n = 4$ for IO animals.

Table 2 Effect of IO on plasma lipid profile

Sprague–Dawley rats were fed either a carbonyl-iron-supplemented diet (12 rats) or a control diet (12 rats) for 10 weeks. Blood was removed and the plasma levels of total cholesterol, HDL cholesterol and triacylglycerols were measured. *P* values are calculated for comparisons of IO with control animals. Results shown are means ± S.D.

	Concentration (mM)		
	Control	IO	*P*
Total cholesterol	1.05 ± 0.28	1.31 ± 0.24	< 0.05
HDL cholesterol	0.81 ± 0.12	1.28 ± 0.20	< 0.001
Triacylglycerols	0.17 ± 0.24	0.56 ± 0.67	< 0.10

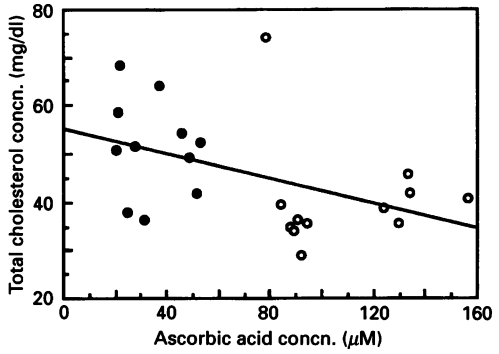

Figure 1 Correlation between total cholesterol and ascorbic acid levels in plasma

Sprague–Dawley rats were fed either a carbonyl iron-supplemented (●) or a control diet (○) for 10 weeks. Blood was removed and the plasma levels of total cholesterol and ascorbic acid were measured. $r = -0.46$; $P = 0.026$.

Table 3 Effect of IO on liver antioxidant status and lipid peroxidation

Sprague–Dawley rats were fed either a carbonyl iron-supplemented diet (12 rats) or a control diet (12 rats) for 10 weeks. The rats were then killed and 10 livers from each group were removed and assayed for the levels of lipid-soluble antioxidants, F_2-isoprostanes and total iron. *P* values are calculated for comparisons of IO with control animals. Results shown are means ± S.D.

	Control	IO	*P*
α-Tocopherol (nmol/g of liver)	253 ± 38.8	89.5 ± 45.0	< 0.001
β-Carotene (nmol/g)	342 ± 63.2	232 ± 64.0	< 0.002
Lycopene (nmol/g)	18.8 ± 3.0	25.2 ± 6.0	< 0.02
Ubiquinol-9 (nmol/g)	16.0 ± 3.9	25.5 ± 8.0	< 0.01
Ubiquinol-10 (nmol/g)	14.4 ± 2.7	4.0 ± 2.0	< 0.001
F_2-isoprostanes (ng/g)	4.59 ± 0.95	6.67 ± 2.19	< 0.02
Total iron (μg/g)	103.9 ± 14.6	1391 ± 242	< 0.001

were substantially higher levels of lipid hydroperoxides in the IO rats, but the difference was statistically not significant (Table 1). TBARS, however, were not detected in any of the plasma samples (Table 1). IO also led to a significant increase in total and HDL cholesterol (HDL is known to be the major cholesterol carrying lipoprotein in rat plasma) and a non-significant increase in triacylglycerol levels compared with control rats (Table 2). Because of previous reports linking ascorbic acid deficiency to

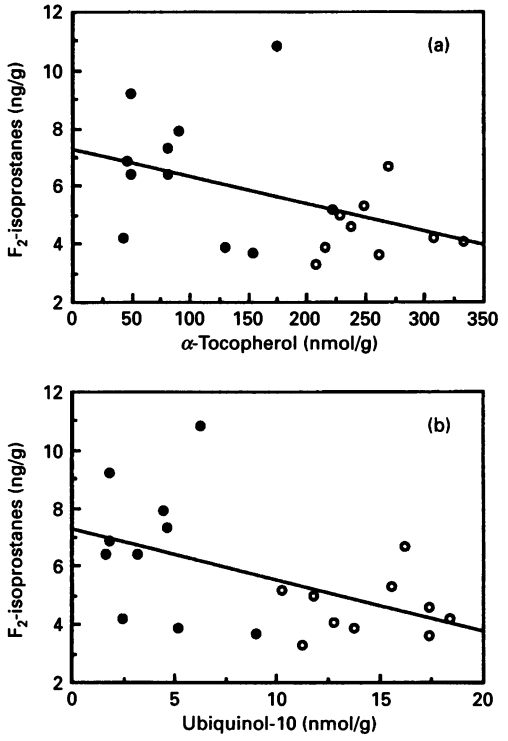

Figure 2 Correlation between F_2-isoprostane and α-tocopherol levels (a) and ubiquinol-10 levels (b) in liver

Sprague–Dawley rats were fed either a carbonyl iron-supplemented (●) or a control diet (○) for 10 weeks. The rats were then killed and the livers of 10 animals from each group were removed and assayed for the levels of acylated F_2-isoprostanes, α-tocopherol and ubiquinol-10. (**a**) $r = -0.45$; $P = 0.049$. (**b**) $r = -0.51$; $P = 0.023$.

hypercholesterolaemia [24], we examined the correlation between cholesterol and ascorbic acid levels in plasma. Interestingly, the increase in total cholesterol was inversely correlated with plasma ascorbic acid levels ($r = -0.46$; $P = 0.026$; Figure 1); no significant correlation was observed with plasma α-tocopherol levels (0.09–2.81 μM) ($r = -0.29$; $P = 0.167$).

Effect of IO on liver lipid-soluble antioxidants and lipid peroxidation

Dietary IO in the rats led to significant decreases in hepatic levels of α-tocopherol ($P < 0.001$), β-carotene ($P < 0.002$) and ubiquinol-10 ($P < 0.001$). Interestingly, there was an increase in lycopene ($P < 0.02$) and ubiquinol-9 levels ($P < 0.01$) (Table 3). These changes in antioxidant status were accompanied by a significant ($P < 0.02$) increase in hepatic lipid peroxidation as measured by the levels of F_2-isoprostanes (Table 3). The increase in F_2-isoprostane levels was significantly correlated with the decrease in α-tocopherol and ubiquinol-10 levels ($r = -0.45$; $P = 0.049$ and $r = -0.51$; $P = 0.023$ respectively; Figures 2a and 2b) but not with the decrease in β-carotene levels ($r = -0.23$; $P = 0.33$).

DISCUSSION

Dietary supplementation with carbonyl iron in the rat is a well established model of IO, which results in a predominantly hepatocellular iron deposition in a periportal distribution, a pattern analogous to that seen in hereditary haemochromatosis

and in African IO [22]. In the present study, dietary iron loading of rats led to a > 10-fold increase in hepatic iron levels; this is in agreement with published experimental data [20], and is comparable with the reported increase (10–40-fold) in hepatic iron levels in patients with hereditary haemochromatosis [25].

In humans, IO conditions are usually associated with increased serum iron levels and decreased TIBC [25]. Interestingly, in our study, serum iron levels and plasma TIBC in rats with IO did not differ significantly from control rats. Several studies on the condition of IO have indicated that the normal release of iron from tissues is impaired by ascorbic acid deficiency [26,27]. This leads to low serum iron and ferritin levels despite an increase in iron stores, a phenomenon which is reversed by ascorbic acid supplementation. As we observed severe ascorbic acid depletion in IO rats, this may explain why serum iron levels were not increased in these animals.

IO was associated with significant changes of the oxidant status in plasma and liver. Ascorbic acid concentrations in plasma of IO rats were significantly decreased compared with control animals. Ascorbic acid deficiency, as assessed by levels of leucocyte ascorbic acid, has been reported in patients with idiopathic haemochromatosis [28] and in conditions of secondary IO [29]. Wapnick et al. [29] demonstrated that administration of ascorbic acid to IO subjects was followed by only a small rise in the urinary ascorbic acid output, while the oxalic acid levels (measured in two subjects) showed a significant rise. The authors suggested, therefore, that ascorbic acid depletion results from the irreversible oxidation of ascorbic acid by iron. In ascorbic-acid-synthesizing species such as the rat, an alternative explanation of the decreased plasma levels of ascorbic acid may be that IO affected the rate of ascorbic acid synthesis in the liver.

Our observations that dietary IO led to a marked decrease in both plasma and hepatic α-tocopherol levels agree with previous data [30,31]. Although α-tocopherol was the only lipid-soluble antioxidant detectable in rat plasma, we also found carotenoids and ubiquinols in the liver. Carotenoids have been shown to exert antioxidant effects in a number of studies *in vitro* and in animal models *in vivo* [32].

In our study, hepatic β-carotene levels were significantly decreased ($P < 0.002$) in rats with IO compared with controls. This decrease was significantly correlated with decreased α-tocopherol levels ($P < 0.001$). These data suggest, therefore, that β-carotene acts as an antioxidant in this model of IO. In contrast, hepatic lycopene levels, which were $\sim$ 10-fold lower than β-carotene levels, were significantly increased in rats with IO. Given that it has been suggested that the bioavailability and storage of certain carotenoids may be affected by others [33], it is possible that the reduction in β-carotene levels has influenced hepatic lycopene levels.

Ubiquinols are recognized as antioxidants both *in vitro* and *in vivo* [34–36]. In our system of dietary IO, ubiquinol-10, but not ubiquinol-9, appeared to act as an antioxidant as evidenced by the reduced levels of ubiquinol-10 in the livers of IO rats compared with controls. The discrepancy between the effects of IO on ubiquinol-9 and -10 may be explained by the location of these ubiquinol homologues in different hepatocyte organelles in relation to the degree of lipid peroxidation. In dietary IO, mitochondrial lipid peroxidation was observed at mean hepatic iron concentrations of > 1000 μg of Fe/g of liver, whereas in microsomes lipid peroxidation did not occur until the hepatic iron concentration exceeded 2000 μg/g [20]. In the present study, the hepatic iron concentrations (1391 ± 242 μg/g) were in the range that is expected to cause lipid peroxidation in the mitochondrial but not the microsomal fraction. Ubiquinol-9 was shown to be present mainly in the cytosolic fraction of rabbit liver, whereas most of ubiquinol-10 was found in the mitochondrial fraction [37]. It is possible, therefore, that the preferential consumption of ubiquinol-10 in the present study is related to increased mitochondrial lipid peroxidation in the liver.

The decrease in plasma and hepatic antioxidant status in the IO rats was accompanied by increased lipid peroxidation in liver, but not plasma. There is a large body of indirect evidence implicating lipid peroxidation in patients and animals with IO. Most of this evidence concerns levels of TBARS in organs and organelles [8]. One study [38] has reported elevated plasma levels of TBARS in patients with IO who also had detectable 'catalytic' iron, i.e. iron capable of catalysing free-radical reactions, in their plasma. However, other indices of lipid peroxidation (diene conjugates and fluorescence in lipid extracts) were not changed in those plasma samples [38].

In contrast, in the present study, we were unable to detect TBARS in plasma from both IO and control rats. It is feasible that in the former study [38] the catalytically active iron in the plasma samples promoted lipid peroxidation during the assay itself. We have also used two methods of detecting lipid peroxidation that are considerably more sensitive and specific than the TBARS assay [9,14], but did not find conclusive evidence of increased lipid peroxidation in plasma of IO rats. This is not surprising in view of the normal serum iron levels and TIBC in these animals and the highly efficient antioxidant protective mechanisms of plasma [16,23]. However, we did observe increased lipid peroxidative damage in the livers of IO rats, as assessed by a novel and specific assay for lipid peroxidation, i.e. measurement of free-radical-derived F_2-isoprostanes [9,10]. This observation confirms and extends previous findings of increased hepatic lipid peroxidation in IO using indirect and less-specific assays for lipid peroxidation [8].

Finally, we observed that iron loading led to a significant increase in both total and HDL cholesterol levels in rat plasma. These findings are in agreement with those of Cunnane and McAdoo [39] who reported an increase in both cholesterol and triacylglycerol levels in plasma of rats given oral iron supplements for 12 weeks. However, the data are inconsistent with findings in β-thalassaemia major, a hereditary condition which, when treated with repeated blood transfusions, leads to secondary IO. In this disease, a lowering of total and HDL cholesterol was reported [40]. This effect is probably related to chronic liver disease, which is known to be associated with serum lipid disturbances [41] and can be caused by excessive liver iron loading exceeding a critical threshold value (> 22-fold increase) [22]. However, such chronic liver disease is not a likely event in the model of IO used in the present study, as the increase in hepatic iron levels was well below this critical threshold and histological analysis of the IO livers did not show inflammatory changes or necrosis. In addition, previous studies have demonstrated that dietary iron loading with comparable hepatic iron levels to our study does not lead to significant liver damage as assessed by serum alanine aminotransferase [42] and aspartate aminotransferase [43] activities. Therefore, it appears that during the early stages of iron loading, i.e. before the development of chronic liver disease, iron intake can influence the lipid composition in plasma by other mechanisms.

The increase in plasma cholesterol levels may be explained by the dramatic decrease in plasma ascorbic acid concentrations in IO rats, as the two parameters were significantly inversely correlated (Figure 1). Similarly, plasma as well as hepatic cholesterol levels were reported to be increased significantly in scorbutic guinea pigs [44]. In addition, strong clinical and experimental evidence suggests that chronic ascorbic acid deficiency leads to hypercholesterolemia [24]. The most critical effect

of chronic ascorbic acid deficiency on cholesterol metabolism appears to be the impairment of bile acid synthesis by cholesterol 7α-hydroxylase, an enzyme that requires ascorbic acid as a co-substrate for maximal activity [45].

In conclusion, our findings of decreased antioxidant levels in plasma and liver of IO rats are indicative of the presence of chronic oxidative stress in this condition. Although the endogenous antioxidants appeared to be capable of preventing significant lipid peroxidative damage in plasma, we found direct evidence for moderately increased lipid peroxidation products in liver. In addition to hepatic oxidative damage, IO also caused changes in the plasma lipid profile. It will be of interest to investigate whether these IO-induced changes in oxidant status and lipid metabolism can be ameliorated by antioxidant supplementation.

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