

## Iron-induced Oxidative DNA Damage in Rat Sperm Cells *In Vivo* and *In Vitro*

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We investigated whether acute iron intoxication causes oxidative DNA damage, measured in terms of 7-hydro-8-oxo-2'-deoxyguanosine, 8-oxodG, in nuclear DNA in testes and epididymal sperm cells *in vivo* and *in vitro* in rats. In addition, we investigated levels of the modified nucleoside in liver and kidney and measured its urinary excretion.

Sperm cells were isolated from the epididymides and the testes cells were isolated after homogenisation. *In vitro*, the sperm and testes cells were incubated with increasing concentrations of FeCl<sub>2</sub> ranging from 0 to 600 µM. The median (range) levels of 8-oxodG/10<sup>5</sup> dG in the epididymal sperm cells increased from 0.48 (0.42–0.90) to 15.1 (11.4–17.6) ( $p < 0.05$ ), whereas the level rose from 0.63 (0.22–0.81) to 8.8 (4.5–11.6) ( $p < 0.05$ ) at 0 and 600 µM, respectively, in the testicular cells.

*In vivo* groups of 7–8 rats received 0, 200 or 400 mg iron/kg as dextran i.p. After 24 h, epididymal sperm cells, testes, kidneys and liver were collected for analysis. Kidney and sperm DNA showed a significant increase in 8-oxodG in the iron-treated animals. The median (range) values of the 8-oxodG/10<sup>5</sup> dG in the epididymal sperm cells rose from 0.66 (0.38–1.09) to 1.12 (0.84–5.88) ( $p < 0.05$ ) at 0 and 400 mg iron/kg, respectively, whereas the values in the testes and liver showed no significant change. In the kidneys the 8-oxodG/10<sup>5</sup> dG median (range) values were 0.98

(0.73–1.24), 1.21 (1.13–1.69) and 1.34 (1.12–1.66) after 0, 200 and 400 mg iron/kg, respectively ( $p < 0.05$ ).

The 8-oxodG-excretion rate was measured in 24 h urine before and after iron treatment. The rate of urinary 8-oxodG excretion increased from 129 (104–179) pmol/24 h before treatment to 147 (110–239) pmol/24 h after treatment in the group receiving 400 mg iron/kg ( $p < 0.05$ ).

The results indicate that acute iron intoxication may increase oxidative damage to sperm and kidney DNA.

**Keywords:** Iron dextran, epididymal sperm cells, oxidative DNA damage, 7-hydro-8-oxo-2'-deoxyguanosine

**Abbreviations:** ROS, reactive oxygen species; 8-oxodG, 7-hydro-8-oxo-2'-deoxyguanosine; BW, body weight; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetraacetic acid disodium salt; 8-oxoGua, 8-oxoguanine

### INTRODUCTION

Numerous recent studies suggest that semen quality is deteriorating<sup>[1–3]</sup> but the matter is still

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widely debated.<sup>[4,5]</sup> Concurrently, increases in congenital abnormalities in the human male genitals along with an increase in the incidence of testicular cancer and in disorders in spermatogenesis have been observed (reviewed by Giwercman *et al.*, 1993<sup>[6]</sup>).

The formation of reactive oxygen species (ROS) has been proven in sperm and seems to affect the sperm function and male fertility.<sup>[7]</sup> ROS generation in spermatozoa may be a significant cause of male infertility<sup>[8]</sup> and strongly related to oligozoospermia.<sup>[9]</sup> Cigarette-smoking – a well-documented cause of increased ROS formation – appears to increase oxidative DNA damage as determined by 8-oxodG in spermatozoa.<sup>[10,11]</sup> Moreover, the level of 8-oxodG seems to be inversely correlated with sperm density and sperm number in the ejaculate.<sup>[12]</sup> The level of 8-oxodG in sperm DNA appears to be dependent on the concentration of vitamin C in seminal plasma.<sup>[13]</sup>

These observations are particularly interesting because of the special characteristics for semen DNA: tightly packed DNA,<sup>[14]</sup> inefficient DNA repair, and the suggestion that paternal pre-conception smoking may be associated with an increased risk of childhood cancer in the offspring.<sup>[15]</sup>

Overload of various metals has been associated with impaired reproductive functions.<sup>[16–18]</sup> Fe<sup>2+</sup> is involved in the generation of hydroxyl radicals via the Fenton reaction.<sup>[19]</sup> *In vitro*, iron is a potent inducer of oxidative DNA damage, including the mutagenic lesion 8-oxodG.<sup>[20]</sup> In testes DNA from iron-treated rats, the 8-oxodG level has been reported to be increased as has lipid peroxidation and antioxidant consumption.<sup>[21]</sup> This suggests that iron may inflict oxidative DNA damage on male reproductive cells.

The objective of this study was to investigate the formation of 8-oxodG in testicular and epididymal sperm cells *in vivo* and *in vitro* after acute iron overload. In the *in vivo* experiment the 8-oxodG levels in the liver and kidneys were also investigated along with its urinary excretion.

## MATERIALS AND METHODS

### Reagents

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

### Animals

Male Wistar rats, approximately 6 weeks old and 200–250 g BW, were used. The animals received standard laboratory diet and tap water *ad libitum*. The animals were housed in pairs in an environmentally controlled animal facility operating on a 12 h dark/light cycle at 22–24°C and 55% humidity.

### Isolation of Cells and Tissue

The rats were killed by cervical dislocation, the epididymis was excised and put in a petri dish containing a cold buffer solution of 150 mM NaCl, 10 mM Trizma base and 1 mM EDTA, 10% glycerol, pH 7.4. The epididymides were sliced open with a razor blade or a pair of sharp scissors and the sperm cells were flushed/squeezed out in 5 ml of buffer. The sperm cells were centrifuged at 1000× g for 10 min at 4°C. The buffer was removed and the cells were washed in saline.

Each testicle was homogenised in 16 ml Hepes buffer (250 mM mannitol, 70 mM sucrose, 5 mM Hepes (Boehringer, Mannheim, Germany), pH 7.4) on ice. The buffer was removed after centrifugation at 1000× g for 10 min at 4°C and the cells were washed in saline.

Liver or kidney samples weighing 600 mg were homogenised on ice in 30 ml Hepes buffer. The homogenate was centrifuged at 1000× g and 4°C for 10 min. The pellet was resuspended in 600 µl saline.

### *In Vitro* Design

Semen from 4 rats was pooled and divided into 8 samples. The sperm cells were incubated at 35°C

for 30 min in 3 ml of 600, 300, 200, 100, 50, 10, 1 or 0  $\mu\text{M}$   $\text{FeCl}_2$  (Merck, Darmstadt, Germany) in saline buffer as described above. Pooled testis cells from one animal were incubated under the same conditions. After incubation, the cells were washed with saline, with 10  $\mu\text{M}$  desferrioxamine (CIBA-GEIGY AG, Basel, Switzerland) in saline and with saline once more. The *in vitro* experiment was repeated 5 times.

Membrane integrity after incubation was tested in a single set of experiments. Sperm cells were embedded in an agarose gel and stained with YOYO-1 (Molecular Probes Europe BV, Leiden, The Netherlands) in PBS. No staining of cellular DNA was apparent in a fluorescence microscope whereas lysis of the membranes by the detergent Triton X-100 caused a maximum staining of the DNA.

### *In Vivo* Design

Male Wistar rats were housed alone in metabolism cages for 24 h before iron injection to allow acclimatisation. The animals were divided randomly into 3 groups of 7–8 rats, receiving 200 or 400 mg iron/kg BW as dextran (average molecular weight 5000) or saline (1 ml) i.p. After 24 h the animals were killed and testes, epididymides, kidneys and livers were excised. Kidneys and livers were stored at  $-20^\circ\text{C}$  until analysis was possible, whereas the epididymis and the testes were analysed immediately.

Urine was collected for 24 h before and for 24 h after injections. The urinary concentration of 8-oxodG was measured by HPLC-EC as previously described.<sup>[22]</sup>

### Analysis of 8-oxodG in Seminal and Testicular DNA

Sperm nuclei were prepared by adding 7 ml buffer containing 320 mM sucrose, 10 mM  $\text{MgCl}_2$ , 5 mM Trizma base and 1% Triton X-100, pH 7.6, to the sperm cells, followed by gentle rotation for approximately 10 min. The nuclei were obtained

by centrifugation at  $1000 \times g$  for 10 min at  $4^\circ\text{C}$ . The pellet containing the nuclei was carefully resuspended in 575  $\mu\text{l}$  ice-cold buffer, pH 6.5, with 5 mM sodium citrate and 20 mM sodium chloride. A total of 83  $\mu\text{l}$  Pronase E, 20 mg/ml, with 40 mM dithiothreitol was added and the mixtures vortexed. A buffer (pH 8.5) containing EDTA (20 mM), Trizma base (20 mM) and 1.5% N-lauroyl-sarcosine was added (650  $\mu\text{l}$ ) along with 75  $\mu\text{l}$  5% butylated hydroxyanisole (BHT) in methanol. The samples were incubated at  $40^\circ\text{C}$  overnight.

A buffer (650  $\mu\text{l}$ ) with Trizma base (10 mM) and EDTA (1 mM) (pH 8.5) along with 175  $\mu\text{l}$  ammonium acetate (7.5 M) was added to each sample, and the samples were mixed by inversion. A double amount of ice-cold ethanol (96%) was added and the samples were mixed carefully by inversion until signs of DNA precipitation. The DNA was left to precipitate for 1 h at  $-20^\circ\text{C}$ . After that the DNA was collected and washed in ice-cold ethanol (70%) and dried under a stream of nitrogen gas. The DNA was resuspended in 200  $\mu\text{l}$  sodium acetate 20 mM (pH 4.8) and digested to nucleosides by 20  $\mu\text{l}$  Nuclease P1 (5 Units), dissolved in 0.1 M  $\text{ZnCl}_2$ , 15% glycerol, 20 mM sodium acetate, pH 4.8, for 30 min at  $37^\circ\text{C}$ . Exactly 20  $\mu\text{l}$  alkaline phosphatase (1 Unit) was added and the DNA was incubated for another 60 min. The samples were centrifuged at  $5200 \times g$  for 35 min at  $4^\circ\text{C}$ .

An aliquot of the supernatant was injected into the chromatographic column (Beckman ODS 25-cm 5  $\mu\text{m}$  particle size). The column was eluted with acetonitrile 5% in sodium phosphate buffer, pH 4.5. The effluent was monitored by a UV-detector (Waters) (254 nm) for quantification of dG by an ESA Coulochem II electrochemical detector (Inc., Bedford, MA) with a 5011 analytical cell set for quantification of 8-oxodG. The chromatographic data were processed by Merck-Hitachi integration software. Quantification of 8-oxodG was performed by external standardisation from injection of known amounts of pure 8-oxodG and dG.

### Analysis of 8-oxodG in Liver and Kidney DNA

TE-buffer, pH 8.0, (10 mM Trizma base, 150 mM NaCl and 10 mM EDTA) 1.8 ml was added to 200  $\mu$ l liver or kidney cell suspension, and 200  $\mu$ l 10% sodium dodecyl sulphate (SDS) was added before mixing and rotation on an extraction bench for 15 min. The samples were incubated for 10 min at 37°C, 200  $\mu$ l 3 M sodium acetate, pH 5.2, and 550  $\mu$ l 5 M sodium perchlorate were added and the samples were mixed thoroughly immediately after. Chloroform:isoamyl alcohol (24:1) 2 ml was used to purify DNA. After rotation for 10 min, the top layer (the water phase) was isolated by centrifugation at 2100 $\times$  g for 10 min. Ice-cold ethanol (96%) 2 volumes were added slowly and the samples were left at -20°C overnight to precipitate.

The DNA was washed in ice-cold ethanol (70%) and dried under a nitrogen stream. The DNA was resuspended in 200  $\mu$ l 20 mM sodium acetate, pH 4.8, and the procedure was continued as described above, except that Whatman filters (30 kDa, Polysulphone, Maidstone, England) were used in the final centrifugation.

### Statistics

*In vitro* the 8-oxodG levels in the 8 FeCl<sub>2</sub> concentrations were compared by means of the Friedman test, if the test was significant the groups were compared by the paired Wilcoxon test.

In the *in vivo* experiment, groups were compared by means of the Kruskal-Wallis test. When *p*-values were significant according to the Kruskal-Wallis test, the groups were tested by means of the Mann-Whitney *U* test. BWs and urine samples were compared by the paired Wilcoxon test before and after injection of iron dextran, and the change in the 3 groups was compared by the Kruskal-Wallis test and the Mann-Whitney *U* test. A Spearman correlation analysis was performed on the urinary 8-oxodG excretion versus 8-oxodG/10<sup>5</sup> dG in kidneys or epididymal

sperm cells, and on the 8-oxodG/10<sup>5</sup> dG levels in kidneys versus epididymal sperm cells.

*p*-values less than 0.05 were considered statistically significant.

### RESULTS

*In vitro* the 8-oxodG levels in DNA from epididymal sperm cells and testes increased with the concentrations of FeCl<sub>2</sub> from 0 to 600  $\mu$ M. In both epididymal sperm cells and testes cells (Figure 1), 8-oxodG was elevated at FeCl<sub>2</sub> concentrations greater than 1  $\mu$ M (*p* < 0.05).

Acute iron overload in the *in vivo* experiment caused a significant decrease in BW in both experimental groups compared with the control group. The relative organ weights were calculated and the liver weights decreased in the iron-treated animals, whereas the kidneys, testes and epididymal weights remained unchanged.

The 8-oxodG/10<sup>5</sup> dG ratios in epididymal sperm cells, testes, kidneys and liver are illustrated in Figure 2. The median 8-oxodG level in the epididymal sperm cells rose by 70% in the experimental group receiving 400 mg iron/kg BW compared with the control group, *p* < 0.05. The median (range) values were 0.66 (0.38-1.09) and 1.12 (0.84-5.88) 8-oxodG/10<sup>5</sup> dG in the control and the experimental group, respectively. A similar increase in the group receiving 200 mg iron/kg BW failed to reach statistical significance, here the median (range) value was 1.14 (0.65-8.68) 8-oxodG/10<sup>5</sup> dG. The testes and the liver levels of 8-oxodG remained unchanged, whereas the ratio in the kidney increased significantly from 0.98 (0.73-1.24) in the control group to 1.21 (1.13-1.69) and 1.34 (1.12-1.66) 8-oxodG/10<sup>5</sup> dG in the groups receiving 200 and 400 mg iron/kg, respectively (*p* < 0.05).

The urinary excretion of 8-oxodG (Table I) increased by 14% in the group receiving 400 mg iron/kg from before to after the iron treatment (*p* < 0.05). However, the differences in urinary excretion of 8-oxodG between the control and the

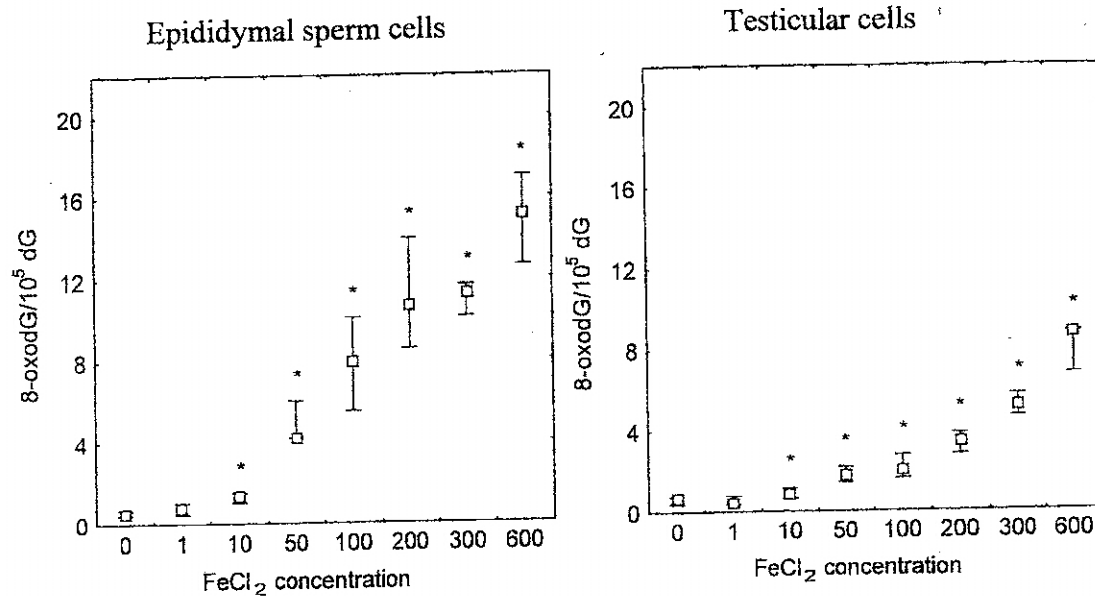


FIGURE 1 8-oxodG residues/10<sup>5</sup> dG after *in vitro* incubation with FeCl<sub>2</sub> in epididymal sperm cells or testicular cells from rat. \* Indicates that the group is significantly increased from the control ( $p < 0.05$ ). Values are median with interquartile range. The abscissa scale is not linear.

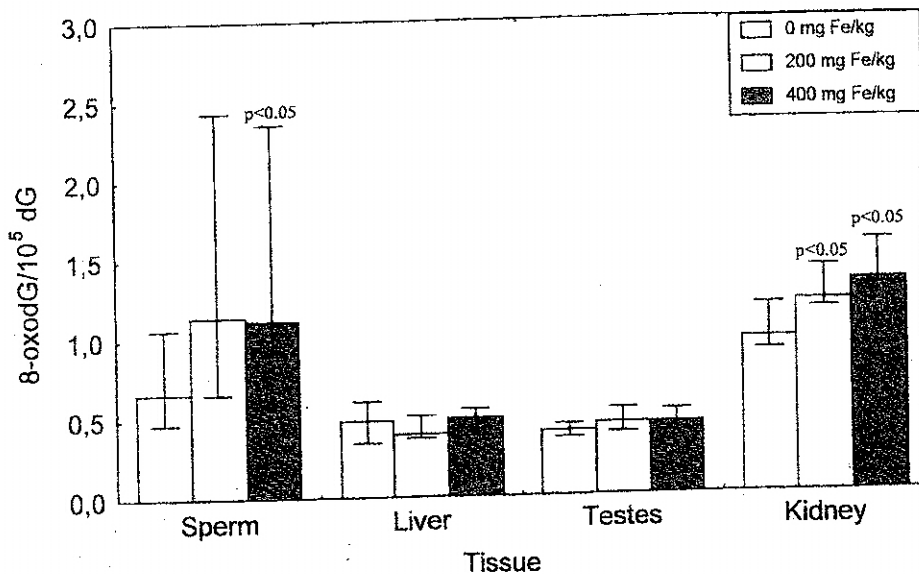


FIGURE 2 Effects of acute iron treatment *in vivo* on 8-oxodG levels in different tissue. Values are median with interquartile range.  $p < 0.05$  indicates that the group is significantly increased from the control.

200 mg iron-treated group were not statistically significant ( $p = 0.15$ ). The 24 h output of urine was not significantly changed by the iron treatment in any group. Thus, there is no reason to

suspect that changes in renal function could have affected the 24 h excretion of 8-oxodG.

There was no correlation between the urinary excretion of 8-oxodG and the 8-oxodG levels

TABLE I Effect of acute iron treatment on 8-oxodG excretion level in urine

	Control (7)	200 mg iron/kg BW (7)	400 mg iron/kg BW (8)
Before treatment	122.6 (94.4–156.0)	146.8 (60.7–198.4)	128.5 (104.4–179.2)
After treatment	110.5 (97.4–155.0)	142.6 (114.6–242.0)	146.9* (109.6–238.7)

Values are picomol 8-oxodG in 24 h urine per animal, median (range).

\*Significantly different from before treatment. The number of rats is shown in the parenthesis.

in kidneys ( $R_s = 0.22$ ,  $p = 0.33$ ) or in the epididymal sperm cells ( $R_s = 0.33$ ,  $p = 0.14$ ), or between the 8-oxodG/ $10^5$  dG ratios in kidneys and epididymal sperm ( $R_s = 0.34$ ,  $p = 0.12$ ).

## DISCUSSION

In this study we found that iron overload can induce oxidative DNA damage as determined by 8-oxodG in epididymal sperm cells *in vivo* and *in vitro*, whereas the 8-oxodG level increased significantly *in vitro* only in testicular cells. *In vivo* the kidney and urinary excretion level of 8-oxodG also increased whereas the liver level was unchanged after iron overload.

The present *in vitro* investigations indicate that an iron overdose can lead to oxidative DNA damage in testes and epididymal sperm. Recently another *in vitro* study on hydroxyl radical-induced DNA damage in human sperm by incubation with  $H_2O_2$  and  $FeSO_4$  showed a similar tendency.<sup>[23]</sup> The fact that oxidative DNA modifications are induced by iron *in vitro* is supported by studies on Leydig cells showing strand breaks<sup>[24]</sup> and increased levels of modified bases measured by GC-MS after incubation of murine hybridoma cells with iron sulphate.<sup>[25]</sup> In the latter study, the concentration-dependent increase of modified DNA bases decreased at high iron concentrations, in accordance with the results seen in the epididymal sperm cells and possibly in the testes cells in the present study. A possible explanation for these findings may involve several factors: (a) a further oxidation of the bases to base products not measured by HPLC

or GC-MS, (b) limited iron uptake in cells, or (c) accumulation of Fe(III).<sup>[25]</sup>

In agreement with the *in vitro* data, an increase in 8-oxodG was found in epididymal sperm cells after *in vivo* treatment. Lucesoli and Fraga<sup>[21]</sup> found a 25% rise in the 8-oxodG level in testes from iron-treated animals, and the non-significant increase in 8-oxodG levels of the testis tissue in our study is compatible with that. The slight rise in testes 8-oxodG in the iron-treated rats may be a consequence of increased damage in the immature sperm cells only. That would be consistent with the findings of this study and with the notion that late-phase immature mammalian sperm cells have no, or very little, DNA repair.<sup>[26]</sup>

Overdose of various metals is toxic to male reproductive organs. Recently  $CdCl_2$  was shown to induce DNA single strand breakage in isolated Leydig cells<sup>[24]</sup> and elevate the 8-oxodG level in rat testes, possibly by inhibition of 8-oxoGua repair activity.<sup>[27]</sup>

During spermatogenesis, somatic-type histones are replaced by protamines, which will constitute the majority of the nuclear sperm proteins.<sup>[28]</sup> In 1982, Balhorn<sup>[29]</sup> proposed an elegant model for the structure of chromatin in mammalian sperm, where binding of protamines would lead to a "side by side" package of DNA in a very compact manner. This package would probably largely inhibit the DNA damage, but at the same time prevent repair enzymes from repairing damaged DNA. Ultimately, the damage would be preserved until the sperm cell had fertilised the egg. Apart from the condensation of the chromatin in the last phase of spermatogenesis, the cytoplasm volume is also considerably

reduced, and the mRNA production is minimised, preventing the formation of repair enzymes. The level of antioxidants, particularly vitamin C in seminal plasma, therefore seems to be of crucial importance in the defence against ROS in sperm cells.<sup>[13,30]</sup> Even though vitamin C is generally considered to be beneficial in seminal plasma<sup>[31]</sup> it should be kept in mind that vitamin C possesses redox abilities that provides the means of redox cycling Fe(III) to Fe(II)<sup>[32]</sup> and therefore it may act as a pro-oxidant rather than an antioxidant.<sup>[33]</sup> The ratio between iron content and vitamin C may consequently play an important part when considering its antioxidant/damaging capacity.

Iron is believed to be one of the main mediators of the Fenton reaction and therefore also contributes to the formation of the hydroxyl radical – one of the most DNA damaging ROS. Iron is ubiquitous and transferrin (an iron transport protein) has been found in seminal plasma<sup>[34]</sup> implying the presence of iron and therefore the possibility of ROS formation. In spite of this ROS have not been detected in normally fertile men,<sup>[8]</sup> indicating an immediate neutralisation by the variety of defence systems found in sperm.

*In vivo* a single i.p. injection of iron dextran caused an increased formation of 8-oxodG in rat kidneys. Another iron derivative, ferric nitrilotriacetate (Fe-NTA) possibly possessing the same properties as iron dextran, accumulates in the kidneys and is a renal carcinogen that causes renal proximal tubular necrosis and may lead to renal adenocarcinoma in rats and mice.<sup>[35]</sup> Presumably, the mechanism involves formation of the hydroxyl radical by the Fenton reaction. Significant increases of 8-oxodG<sup>[36]</sup> and 8-oxoGua repair activity<sup>[37]</sup> were detected after a single Fe-NTA injection in rat kidneys.

Total body damage rate determined by 8-oxodG in urine significantly increased after iron treatment in the experimental group receiving 400 mg iron/kg. This strongly indicates an increased production of oxidative DNA damage in total body DNA and nucleotide pool. Animal

experiments have shown that injected 8-oxodG is readily excreted unchanged into the urine, whereas 8-oxodG from the diet and oxidation of dG during excretion do not contribute to the urinary 8-oxodG content.<sup>[38,39]</sup> The increase in urinary 8-oxodG in this experiment therefore presumably reflects a rise in the total body ROS exposure.

In humans, supplementation with iron and ascorbate increased the levels of oxidised DNA bases in white blood cells, supporting a pro-oxidant effect *in vivo*.<sup>[33]</sup> Nevertheless, analysis of liver DNA from haemochromatosis patients showed no elevated level of 8-oxodG<sup>[40]</sup> in spite of an iron accumulation in this organ. These negative findings are consistent with the findings of this study and may be related to the rather high content of iron that already exists in the liver.

ROS production in sperm may cause infertility.<sup>[7,41]</sup> These findings were supported by Iwasaki and Gagnon<sup>[8]</sup> that showed ROS levels in 40% of infertile men compared to no detectable ROS levels in fertile. Cigarette-smoking produces high levels (more than  $10^{15}$  organic radicals per puff) and a wide variety of ROS<sup>[42]</sup> and has been associated with low sperm density<sup>[43]</sup> and increased formation of oxidative DNA damage in sperm from humans.<sup>[10,11]</sup> Some studies even indicate an elevated risk of childhood cancer caused by paternal smoking,<sup>[15]</sup> whereas the effect of male smoking on fertility is less certain.<sup>[44,45]</sup>

In conclusion, this study suggests that the DNA-protecting systems in sperm can be overcome by acute iron intoxication, which could lead to base modifications, potentially resulting in mutations.

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