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Induction of Oxidative Stress by Organic Hydroperoxides in Testis and Epididymal Sperm of Rats In Vivo

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Abstract

The present study describes the extent and pattern of oxidative stress induction in testis and epididymal sperm of rats following in vivo exposure to repeated sublethal doses of 2 model pro-oxidants, namely, t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP). Single sublethal (1/40, 1/20, and 1/10 LD_{50}) doses of hydroperoxides (HP) administered intraperitoneally to male rats (CFT-Wistar strain) failed to induce any significant increase in malondialdehyde or reactive oxygen species (ROS) levels in testis or epididymal sperm. However, repeated doses for 1 or 2 weeks induced a marked dose-related enhancement of lipid peroxidation (LPO) and ROS levels in both testis and epididymal sperm. Further evidence, such as significant perturbations in both enzymic and nonenzymic antioxidants and enhanced levels of protein carbonyls in testis, suggested induction of oxidative stress. In testis, moderate depletion in reduced glutathione levels and marked diminution in ascorbic acid and α-tocopherol content were accompanied by increased activities of various antioxidant enzymes, namely glutathione peroxidase, glutathione-S-transferase, and catalase, in both the HP treatments. Furthermore, significant alterations in the specific activities of testicular enzymes such as LDH-X, G-6-PDH, and SDH indicated altered testicular physiology. Both HP at higher doses induced significant DNA damage (determined by fluorimetric analysis of DNA unwinding assay) in testis and epididymal sperm. Increased total iron levels in testis of HP-treated rats are indicative of the possible

involvement of iron-mediated free radical reactions in this model. These findings provide an account of early oxidative damage in testis and epididymal sperm following short-term exposure to HP in vivo, and this model is being further exploited for understanding the consequences of chronic oxidative stress-mediated alterations for the physiology of male reproductive system and its implications for fertility.

Key words: Rat testis, lipid peroxidation, ROS levels, antioxidant defenses, DNA damage

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Understanding the consequences of repeated oxidative stress in the male reproductive milieu is gaining wide attention (Cummins et al, 1994; Sikka, 2001; Saleh and Agarwal, 2002; Agarwal and Said, 2005). Earlier epidemiological evidence suggests that both the quality and the quantity of semen in humans has declined progressively over the past half a century (Carlsen et al, 1992; Augur et al, 1995) in concert with a general increase in the incidence of male reproductive pathologies, including cancer. Free radical production and lipid peroxidation (LPO) are known to be important mediators in testis physiology (de Lamirinde et al, 1997). However, elevated levels of reactive oxygen species (ROS) in testis in vivo can result in altered tissue physiology, or induce oxidative damage to DNA, which is of potential risk to reproduction. Recent findings have led to the proposal that oxidative stress can play a vital role in the etiology of male infertility (Aitken, 1995; Aitken et al, 1998; Ong et al, 2002). Further, certain situations of oxidative stress in humans, such as ascorbic deficiency and smoking, have been shown to cause an increase in oxidation of sperm DNA and poor antioxidant levels in semen, clearly emphasizing a potential relationship between oxidative damage to testis and sperms and male reproductive dysfunctions (Fraga et al, 1996; Saleh et al, 2002).

Various animal models (Oteiza et al, 1995; Lucesoli and Fraga, 1999; Lucesoli et al, 1999; Doreswamy et al, 2004) have been deployed to investigate oxidative stress-mediated testicular dysfunctions. However, data on biochemical dysfunctions in testis following short-term multiple exposures to pro-oxidants is limited. Such studies are vital, as elevated ROS levels may influence some transcription factors, enzyme activities, modulate cell proliferation, and various important signal transduction pathways, leading to male reproductive dysfunctions (Agarwal and Saleh, 2002; Kaur et al, 2006).

Organic hydroperoxides (HP) have not been widely used to induce oxidative stress in vivo, although t-butyl hydroperoxide (tbHP) has often been employed as a prototypic inducer of oxidative stress in various in vitro systems (Sakida et al, 1991; Latour et al, 1995; Rajeshkumar and Muralidhara, 2002). Earlier, we employed HP as model pro-oxidants to induce oxidative stress in testis of mice and investigated the associated genotoxic implications (Rajeshkumar and Muralidhara, 1999; Rajeshkumar et al, 2002). Recent studies have amply demonstrated cHP- and tbHP-induced peroxidative damage in the male reproductive system in rodents (Li et al, 2006; Kaur et al, 2006). However, the underlying biochemical mechanisms by which HP induce oxidative damage in testis in vivo are not well understood.

In the present study, we describe an in vivo model of oxidative stress in rat testis employing organic HP with an emphasis on determination of various biochemical dysfunctions, such as induction of lipid peroxidation in testis and epididymal sperm, perturbations in antioxidant defenses (enzymic/nonenzymic), DNA damage, and specific physiological alterations. The results obtained indicate that HP at sublethal doses induces a significant degree of oxidative stress in testis of rats, which is probably mediated by iron-catalyzed Fenton reaction. The enhanced lipid peroxidation was accompanied by protein oxidation, DNA damage in testis and epididymal sperm, and specific biochemical perturbations in testis, which are likely to have a significant effect on testicular

physiology and function.

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Chemicals

Organic HP (tbHP, 70% aqueous, CAS number 75-91-2, and cumene hydroperoxide, 80% aqueous, CAS number 80-15-9), 1,1,3,3-tetraethoxypropane, ethidium dibromide, enzyme substrates, and other chemicals, including HPLC solvents, were procured from Sigma Chemical Co (St Louis, Mo).

Animals and Care

Adult male rats (CFT-Wistar strain, 10–12 weeks old, 200–210 g) were randomly drawn from the stock colony of the CFTRI animal house facility and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12-hour light/dark cycles, 50% ± 5% humidity, and an ambient temperature of 25° ± 3°C). The rats were acclimatized for 1 week prior to the start of the experiment. Rats were maintained on commercial pelleted diet (Gold Mohur, supplied by Lipton India Pvt Ltd, Bangalore, India) ad libitum and had free access to water. All procedures with animals were conducted strictly in accordance with approved guidelines by the Institute Animal Ethical Committee regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. During the experiments, maximum care was taken to minimize animal suffering, and in addition, the number of rats used was kept at a minimum.

Animal Treatment and Experimental Protocol

A preliminary study was conducted to determine the median lethal dose (LD_{50}, intraperitoneal) of HP in adult rats (n = 6). Acute graded doses of HP were administered at dosages ranging from 50–400 µmol/100 g of body weight (tbHP) and 50–200 µmol/100 g of body weight (cHP). The mortality data obtained was subjected to probit regression analysis to compute the LD_{50} values. The statistically computed LD_{50} values were for tbHP, 300 µmol/100 g and for cHP, 150 µmol/100 g of body weight respectively.

Initially, to assess the extent of oxidative damage, in the first study (acute exposure) adult rats (n = 4) were administered (intraperitoneally) with HP at single doses equivalent to 1/40, 1/20, and 1/10 LD_{50} (tbHP, 7.5, 15, and 30 µmol/100 g of body weight and cHP, 3.75, 7.5, and 15 µmol/100 g of body weight). In the second study (repeated exposures), HP were administered once daily (0900 to 1100 hours) at similar doses for either 1 or 2 weeks. Both the control and HP treated rats were killed either at 24 hours or 1 or 2 weeks after dosing for various biochemical analyses. Body weights were recorded on the day of treatment and at autopsy. Immediately after euthanizing, testes and epididymis were excised and weights were recoded. While LPO and ROS levels in testis and epididymal sperm were measured at 1 and 2 weeks, all other determinations were made only at the end of treatment (2 weeks).

Tissue Sampling and Pathology of Testis

To prepare homogenates, testes were decapsulated, weighed, and homogenized in 10 volumes of 1.15% KCl, tris-HCl (10 mM, pH 7.4) at 4°C. For histopathologic studies, 1 testis was fixed in Bouin solution for 24 hours and embedded in paraffin, processed by standard histological techniques, stained with hematoxylin-eosin, and examined by light microscopy. To obtain sperm from epididymis,

the caudae were excised, rinsed, and teased, followed by gentle mincing in phosphate buffered saline. The fragments were allowed to sediment, and the sperm were filtered and collected for biochemical assays. The epididymal sperm counts were enumerated according to the procedure described in the World Health Organization manual (1999).

Assessment of Lipid Peroxidation and ROS Generation in Testis and Epididymal Sperm

Induction of oxidative stress was assessed by monitoring the degree of LPO in testis and epididymal sperm. LPO was quantified by measuring the formation of thiobarbituric acid reactive substances as described earlier (Ohkawa et al, 1979), using 1,1 3,3-tetramethoxypropane as the standard, and the data was expressed as malondialdehyde equivalents (nm MDA/g tissue). Determination of ROS was based on a modified fluorometric assay (Driver et al, 2000) using 2', 7'-dichlorofluorescin diacetate (DCFH-DA) as the probe. Briefly, testis homogenate (5 mg/mL) or aliquots of epididymal sperm were preincubated for 15 minutes with DCFH-DA (10 µmol) at room temperature to allow the probe to be incorporated into any membrane-bound vesicles, and the diacetate group cleaved by esterases. After 30 minutes of incubation, the conversion of DCFH to the fluorescent product dichlorofluorescein (DCF) was measured using a fluorescence spectrophotometer with an excitation at 485 nm and emission at 530 nm.

Determination of Protein Carbonyl and Iron Content

Protein carbonyls were determined in supernatants (obtained by centrifugation of testicular homogenate at 10 000 rpm for 15 minutes) by the most common and reliable method based on the reaction of carbonyl groups with 2,4-dintrophenylhydrazine (DNPH) to form a 2,4-dintrophyenylhydrazone (Levine et al, 1990). Tissue iron concentrations were measured by atomic absorption spectrophotometry (AAS) using a Perkin-Elmer Model 5000 spectrophotometer following digestion of testis with concentrated nitric acid.

Measurement of Nonenzymic Antioxidants

Testis was homogenized in trichloroacetic acid (5%, w/v), and the deproteinized supernatant was used for GSH assay (Hissin and Hilf, 1976). Ascorbic acid levels were determined by the HPLC method as described earlier (Behrens and Madere, 1987). Briefly, testis was homogenized (10%) in 3% metaphosphoric acid and centrifuged at 10 000 rpm for 10 minutes at 4 °C. To an aliquot (100 µL) of the supernatant, TCA was added (final concentration 2%), then centrifuged at 10 000 rpm for 10 minutes at 4 °C. Supernatants were separated, and suitable aliquots were injected to the chromatographic column (C18) with UV detector at 245 nm. Vitamin E was estimated by the method of Zaspel and Csallany (1983) with minor modifications. Then 100 mg of testis was homogenized in 20 volumes of acetone and centrifuged. The pellet was re-extracted with acetone, and supernatant fractions were pooled, reduced to 1 mL volume under N_2 at 30 ° – 40 °C, and filtered into a 1-mL volumetric flask. Suitable volume of this filtrate was injected into a reverse-phase HPLC system using C18 column, and the tissue tocopherol was detected by UV detector at 280 nm.

Measurement of Enzymic Antioxidants

The activities of antioxidant enzymes, namely, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST), were assayed in 1000 x *g* supernatants of testis homogenates. Total SOD activity was determined by monitoring the inhibition of the reduction of ferricytochrome c at 550 nm, using the xanthine-xanthine oxidase system as the source of $O_2^{\bullet -}$. One unit of SOD is defined as the amount of the enzyme required to inhibit 50% of the rate of cytochrome c reduction (Flohe and Otting, 1984). CAT activity was measured by following the rate of H_2O_2 consumption spectrophotometrically at 240 nm (Aebi, 1984) and expressed as µmol

H_2O_2 oxidized/min/mg protein. GPX activity was determined by following the enzymatic NADPH oxidation at 340 nm (Flohe and Gunzler, 1984). GST activity was measured by determining the rate of conjugate formation between GSH and 1-chloro-2, 4-dinitrobenzene (Guthenberg et al, 1985), and the enzyme activity was expressed as µmol of product (S-2, 4-dinitrophenylglutathione) formed/min/mg protein (ϵ_{340} = 9.6 $mM^{-1}cm^{-1}$)

Quantification of DNA Damage

The fluorimetric analysis of DNA unwinding (FADU) was performed according to the procedure described by Birnboim (1990). Testicular cell suspensions were prepared as described earlier, and the final cell pellet was suspended in Krebs-Ringer bicarbonate solution (Romrell et al, 1976). An aliquot of testicular cell suspension (~1 x 10^6 cells) or epididymal sperm (~2 x 10^6) was transferred into test tubes, and cell lysis was performed for 10 minutes. The pH was increased by adding, successively and carefully, the alkaline solutions in order to allow DNA unwinding. Following neutralization, the percentage of double-stranded DNA (ds DNA) formed was detected by measuring the fluorescence of samples after addition of ethidium bromide. Measurements were performed in a Shimadzu F-2000 fluorescence spectrophotometer with 520 nm and 575 nm as excitation and emission wavelengths, respectively. The percentage of ds DNA remaining after the unwinding process was calculated by the ratio (unwound DNA fluorescence – denatured DNA fluorescence)/(native DNA fluorescence – denatured DNA fluorescence).

Activity of Functional Enzymes in Testis

Testis was homogenized in ice-cold Tris buffer (10 mmol, pH 7.0, containing 0.1% cetyltrimethyl-ammonium bromide). The crude homogenates were centrifuged (10 000 rpm for 30 minutes at 4 °C), and the supernatants were used for the enzyme assays. Lactate dehydrogenase (isoenzyme X) was assayed according to the method of Goldberg and Hawtrey (1987) using α-ketovalerate as the substrate. The activities of glucose-6-phosphate dehydrogenase (G6PDH), isocitric dehydrogeanse (ICDH), and sorbitol dehydrogeanse (SDH) were determined following the standard methods (Bergmeyer, 1974) and the activities expressed as µmol of product formed/min/mg protein. Protein content in testis and epididymal sperm was determined according to the method of Lowry et al (1951), using bovine serum albumin as the standard.

Statistical Analyses

Data are expressed as the mean ± SD, and differences between the control and treatment groups were analyzed by ANOVA using the SPSS software package for Windows (SPSS Inc, Chicago, Ill).

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Body Weights, Testicular Weights, Pathology, and Sperm Counts

Single or repeated doses of HP failed to induce any distinctive clinical signs of toxicity or mortality. Data on the body and testicular weights presented in Table 1 revealed no treatment-related effects. Microscopic examination of both testis and epididymis showed no histopathologic alterations among the HP treated rats (data not shown). Further, caudal sperm counts of HP treated rats were in the normal range except for a significant decrease at the highest dose of tbHP.

View this Table 1. *Body weights, testicular weights, epididymal weights and caudal sperm*

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*counts of rats treated organic hydroperoxides for 2 weeks**

Oxidative Damage in Testis; LPO and ROS Generation

Single doses of HP failed to induce any significant increase in the endogenous MDA levels (an index of in vivo LPO) in the testis measured at 24 hours post-injection (data not shown). However, with repeated doses, significant increases in MDA levels in testis were evident at 1 and 2 weeks (Table 2). Although the degree of induction of LPO was moderate during week 1, the increase in LPO was subsequently higher and dose-related with both HP (Figure 1A). At week 2, tbHP treatment enhanced the testicular MDA levels by 12%, 54%, and 98% over the endogenous levels, while cHP treatment increased the MDA levels by 17%, 45%, and 66% at the administered doses (Table 2). While the low doses of both HP caused only a marginal increase in LPO at both weeks (Figure 1B), higher doses resulted in a significant increase in ROS levels. At week 2, tbHP treatment enhanced ROS levels by 36% and 63%, while cHP treatment increased by 76% and 100% at higher doses.

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Table 2. *Malondialdehyde (MDA) levels, protein carbonyl and total iron content in testis of rats treated with organic hydroperoxides for 2 weeks**

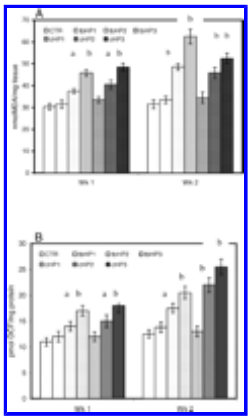

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Figure 1. Status of lipid peroxidation measured as malondialdehyde (MDA) levels **(A)** and ROS levels **(B)** (measured using DCF–DA) in testis of rats treated with repeated doses of hydroperoxides (tbHP, 7.5, 15, and 30 µmol/100g b.w; cHP, 3.75, 7.5, and 15 µmol/100g bw/d for 1 and 2 weeks) (values are expressed as mean ± SD; n = 4; data analyzed by ANOVA, [a] $P < .01$; [b] $P < .001$).

Oxidative Damage in Epididymal Sperm: Lipid Peroxidation and ROS Levels

Single doses of HP failed to induce any significant increase in the MDA levels in epididymal sperm (data not shown). However, with repeated doses, significant dose-related elevation in MDA levels was observed at both weeks (Figure 2A). At week 2, tbHP treatment enhanced the sperm MDA levels by 15%, 32%, and 52% over the basal levels, while cHP treatment increased the levels by 22%, 59%, and 82% at

the administered doses. Further, ROS levels in epididymal sperm were significantly elevated at both weeks. At week 1, higher doses of HP induced a marked increase in ROS levels (tbHP, 53%, 82%; cHP, 60%, 75%), while at week 2, the levels were further elevated (tbHP, 58%, 84%; cHP, 70%, 98%) indicating the susceptibility of sperm in the epididymal milieu (Figure 2B).

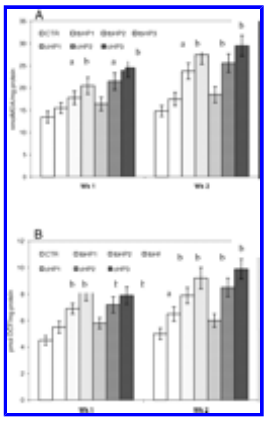

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Figure 2. Status of lipid peroxidation measured as malondialdehyde (MDA) levels **(A)** and ROS levels **(B)** in epididymal sperm of rats treated with repeated doses of hydroperoxides (tbHP, 7.5, 15, and 30 µmol/100g bw; cHP, 3.75, 7.5, and 15 µmol/100 g bw/d for 1 and 2 weeks) (values are expressed as mean ± SD; n = 4; data analyzed by ANOVA, [a] $P < .01$; [b] $P < .001$).

Protein Carbonyl and Iron Content in Testis

Protein carbonyls in testis were markedly increased in rats treated with higher doses of HP at week 2 (Table 2). While tbHP treatment enhanced carbonyl content by 40% and 78% over the basal levels, cHP treatment increased the carbonyl content by 35% and 50% at the higher doses. Interestingly, the total iron content measured in testis at the end of treatment was significantly increased in both the HP treated groups (Table 1).

Status of Nonenzymic Antioxidants in Testis

Data on the effect of HP treatment on nonenzymic antioxidants, namely GSH, ascorbic acid, and α-tocopherol, in testis measured at week 2 is presented in Table 3. While no depletion of reduced GSH occurred at the lowest dose, significant decreases were evident at higher doses of HP. The percent decrease in reduced GSH levels over the basal levels was for tbHP, 13% and 17%, and for cHP, 22% and 25%. The ascorbic acid levels were markedly reduced, and the percent decrease over the basal levels were for tbHP, 28% and 60%, and for cHP, 30% and 60%. Further, the α-tocopherol levels were also significantly diminished with HP treatment (tbHP, 27% and 47%; cHP, 18% and 22%).

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Table 3. *Reduced GSH and non–enzymic antioxidant levels in testis of rats treated with organic hydroperoxides for 2 weeks**

Activity of Enzymic Antioxidants in Testis

Data on the activities of testicular antioxidant enzymes in rats treated with HP is presented in

Table 4. While there was no change in the SOD activity, specific activities of GPX, GST, and CAT were found to be significantly elevated at higher doses. Elevation in the activity of GST was most evident with both HP (tbHP, 24% and 68%; cHP, 37% and 48%). Likewise, CAT activity was also enhanced at higher doses (tbHP, 14% and 32%; cHP, 24% and 60%). Further, the activity of GPX was also marginally increased at higher doses (tbHP, 15% and 22%; cHP, 18% and 27%). In contrast, the GR activity was significantly decreased over the control at the highest dose (tbHP, 29%; cHP, 20%).

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Table 4. *Specific activity of antioxidant enzymes in testis of rats treated with organic hydroperoxides for 2 weeks**

Activity of Dehydrogenases in Testis

Data on the activities of several dehydrogenases in testis determined at the end of treatment is presented in Table 5. While no appreciable effect was evident at the lowest dose, both the HP caused marked alterations in the activities of dehydrogenases at higher doses. The activity of G6PDH was significantly elevated at higher doses (tbHP, 24% and 61%; cHP, 34% and 50%). Further, the activities of other dehydrogenases also showed varying degree of increase, suggesting altered testicular physiology.

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Table 5. *Specific activity of dehydrogenases in testis of rats treated with organic hydroperoxides for 2 weeks**

DNA Damage in Testis and Epididymal Sperm

The effect of HP treatment on DNA damage (measured by FADU assay) in testis and epididymal sperm at the end of treatment is presented in Figure 3. While both HP at the lowest dose caused no alteration, the formation of increased DNA strand breaks were more pronounced at higher doses, as is evident from the decreased percentage of ds DNA in testis and ES.

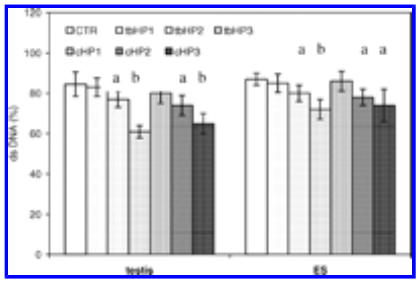

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Figure 3. Effect of repeated doses of hydroperoxides (tbHP, 7.5, 15, and 30 µmol/100g bw; cHP, 3.75, 7.5, and 15 µmol/100g bw/d for 2 weeks) on the percentage of double-stranded DNA in testis and epididymal sperm of rats (values are expressed as mean ± SD; n = 4; data analyzed by ANOVA, [a] $P < .01$; [b] $P < .001$).

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Discussion

Organic HP such as tbHP have been employed as a prototypic inducer of oxidative stress in a variety of in vitro (Sakida et al, 1991) and in vivo systems (Younes and Weiss, 1990; Kaur et al, 2006; Li et al, 2006). Previously, we investigated the propensity of HP to induce oxidative damage in the male reproductive system of mice and studied its genotoxic implications (Rajeshkumar and Muralidhara, 1999; Rajeshkumar et al, 2002). However, the underlying mechanisms and associated biochemical consequences in testis were not examined. Hence, in the present investigation we have focused on induction of oxidative damage in testis and epididymal sperm perturbations in enzymic/nonenzymic antioxidants, oxidative damage to protein, DNA, and functional status of testis.

In the present study, HP treatment induced a significant increase in the lipid peroxidation and enhanced ROS generation in testis after 1- and 2-week exposure, clearly suggesting their potential to induce significant oxidative stress in the reproductive milieu of rats. These results are consistent with the earlier data on HP-induced oxidative damage in rat liver (Younes and Weiss, 1990) and in testis of mice (Rajeshkumar et al, 2002; Kaur et al, 2006) and rats (Li et al, 2006). In this study, although only a moderate increase in LPO was evident after 1-week exposure, the elevation was marked at the end of treatment, clearly suggesting the cumulative effect of HP. Further evidence of HP-induced oxidative stress in testis was discernible in terms of depletion of reduced GSH/nonenzymic antioxidant molecules, perturbations in the activities of antioxidant enzymes, and higher protein carbonyls.

In general, an imbalance in pro-oxidant and antioxidant status could produce oxidative stress, and a change in the antioxidant enzyme activities is frequently used as an important indicator in a given tissue in vivo (Ong et al, 2003). In the present model, HP treatment significantly enhanced the activities of various antioxidant enzymes in testis. The concomitant increase in the activities of CAT and GPX suggests that HP may increase the level of hydrogen peroxide, the substrates for these enzymes. GSTs are a group of primary phase II detoxification enzymes that provide protection against products of oxidative stress whose abundance and protective role in germ cells has been adequately demonstrated (Aravinda et al, 1995; Hayes and Guilford, 1995; Rao and Shaha, 2000). The marked increase in the activity of GST observed in the current study is consistent with the earlier findings in vitro (Rajeshkumar and Muralidhara, 2002; Hemchand and Shaha, 2003), suggesting its vital role under HP intoxication. Since GST activity was markedly enhanced, we speculate they play a major role in the regulation of oxidative stress products in this model. Additionally, the reduced GSH pools in the cytosol were also significantly depleted in testis, suggesting the protective role of GSH and related enzymes. GSH is the major cellular sulfhydryl compound that serves as an effective reductant and a nucleophile that interacts with numerous electrophilic and oxidizing compounds. Depletion of GSH levels in testis can explain a decreased concentration of ascorbic acid, which enters the cells mainly in oxidized form, where it is reduced by GSH. The diminution of ascorbic acid levels has serious implications, since, in addition to its antioxidant function, it also participates in the regeneration of other antioxidants. Significant decrease in both ascorbic acid and tocopherol clearly suggests that testis is indeed subjected to significant oxidative stress after 2-week HP exposure.

Further evidence of HP-induced oxidative damage in testis was evident from the elevated levels of

protein carbonyls at higher doses. Among the various oxidative modifications of amino acids in proteins, protein carbonyl formation may be an early biomarker of ROS-mediated protein oxidation (Ong et al, 2002). Accumulation of high amounts of carbonyls in testis after 2-week HP exposure reflects a high rate of protein oxidation, consistent with a high degree of LPO in the organ. Further, it also reflects a very low rate of oxidized protein degradation and/or low repair activity, since oxidized forms of some proteins and proteins modified by LPO products not only are resistant to proteolysis but can also inhibit the ability of proteases to degrade the oxidized forms of other proteins.

In the present model, HP-induced oxidative damage was not confined only to the testicular milieu, as we found significant elevation in both MDA and ROS levels in epididymal sperm after 1 and 2 weeks. Although the sperm produced in the testis are well protected by the microenvironment of Sertoli cells, they are relatively less protected against oxidant environment in the epididymis, due to the slow transit time and prolonged storage of 10 to 12 days (Cummins et al, 1994). More importantly, spermatozoa are extremely susceptible to damage by ROS as well as ROS-generated products, due to the preponderance of oxidation-prone unsaturated fatty acids in their plasma membrane (Lenzi et al, 2000). Further, numerous in vitro studies have documented that oxidative damage to sperm can subsequently lead to DNA damage, alter membrane functions, impair motility characteristics, and alter capacity to undergo acrosomal reaction and fertilization (Vernet et al, 2004). Thus enhanced LPO and ROS levels associated with DNA damage in epididymal sperm clearly indicate their vulnerability to HP-induced oxidative damage.

In this model, the induction of LPO was concurrent with significant oxidative damage to testicular DNA. This data corroborates earlier findings in cell models (Latour et al, 1995; Sestili et al, 1998) and in testis of mice (Rajeshkumar et al, 2002) and rats (Li et al, 2006). Earlier studies in rodent hepatocytes have shown that exposure to tbHP results in reduced GSH, peroxidation of cellular lipids, formation of GSSG, oxidation of pyridine nucleotides, and altered calcium homeostasis. While the mechanisms of HP-induced LPO and DNA damage are not clear, involvement of metal catalyzed Haber-Weiss reaction have been implicated (Sestili et al, 1998). The increased level of total iron in testis in this study supports such a possibility. The potential of iron to induce DNA lesions in mammalian cell lines (Menghini, 1997), testis (Lucesoli et al, 1999), and genotoxic implications (Doreswamy and Muralidhara, 2005) have been adequately demonstrated.

The pattern of oxidative stress induction in testis and associated biochemical implications under iron overload and zinc deficiency conditions in rodent models have been reported earlier (Oteiza et al, 1995; Lucesoli and Fraga, 1999; Lucesoli et al, 1999). While it was shown that oxidative damage is associated with altered testicular physiology in the above models, the high incidence of pathological lesions precluded understanding of critical oxidative events. In this regard, the present short-term HP exposure model is likely to offer certain advantages to obtaining insights into the biochemical as well as mutagenic consequences in the male reproductive system, since the oxidative damage in testis was moderate and consistent and the doses of HP used were not cytotoxic to germ cells. However, it is not clear how chronic oxidative stress induction in testis affects the status of cytochrome P450 enzymes and steroidogenesis, since we did not measure the status of androgenesis in the HP model. However, such studies are currently underway in our laboratory.

In summary, these findings show that repeated sublethal doses of organic HP induce varying degrees of oxidative stress in both the testicular and epididymal milieu of adult rats, which is associated with significant oxidative damage to proteins and DNA and altered testicular physiology. Further investigations are under progress to characterize the degree and nature of DNA lesions in postmeiotic germ cells, which would have direct relevance in understanding the impact of oxidative

damage in testis and its contribution to development of subfertility/infertility in males.

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Footnotes

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