

Full Length Research Paper

# Impact of organic hydroperoxides on rat testicular tissue and epididymal sperm

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Organic hydroperoxides such as t-butyl hydroperoxide and cumene hydroperoxide have been implicated to cause oxidative stress leading to damage in membrane lipids, proteins, carbohydrates and DNA. This study was aimed to develop an *in vivo* animal model. The effects of hydroperoxides on testicular tissue and epididymal sperm were investigated. Male Wistar rats aged 10 - 12 weeks were randomly placed in groups and received standard rat chow and water *ad libitum*. Animals were injected intraperitoneally with saline (0.5 ml), t-butyl hydroperoxide (5, 10, 20 and 40  $\mu$ M; 0.5 ml) or cumene hydroperoxide cHP (2.5, 5, 10 and 20  $\mu$ M; 0.5 ml) over a 60 day period. It was found that cumene hydroperoxide cHP (10 and 20  $\mu$ M) and t-butyl hydroperoxide tbHP (20 and 40  $\mu$ M) led to significantly lower epididymal sperm concentrations and motility. Superoxide dismutase and glutathione activities were also higher with an accompanying increase in lipid peroxidation in both testicular tissue and epididymal sperm. It can be concluded that *in vivo* intraperitoneal administration of organic hydroperoxides negatively affect the male reproductive system. We have therefore successfully created an animal model to test the adverse effects of oxidative stress on male reproductive parameters, thereby, enabling us to study possible *in vivo* treatments.

**Key words:** Hydroperoxide, sperm, motility, lipid peroxidation, superoxide dismutase, glutathione.

## INTRODUCTION

Many environmental, physiological and genetic factors have been implicated in poor sperm function and infertility (Kovacic and Jacintho, 2001). Free radical-induced oxidative damage to sperm is one such condition and it is gaining considerable attention due to its contribution to sperm damage (Agarwal et al., 2003). A better understanding of how these conditions affect sperm

function will be beneficial as it might help in the design of new and effective treatment strategies to combat the problem of increasing male subfertility.

In mammals, the epididymis is known to play an important role in the maturation and storage of sperm. During epididymal transit, sperm metabolism increases, accompanied by the threat of oxidative stress (OS) (Dacheux et al., 2003). OS is a cellular condition associated with an imbalance between the production of free radicals, mainly reactive oxygen species (ROS) and their scavenging capacity by antioxidants. When the production of ROS exceeds the available antioxidant defence, significant oxidative damage occurs to many cellular organelles due to damage to lipids, proteins, DNA and carbohydrates. These processes can ultimately lead to cell death. Sperm is particularly susceptible to oxidative damage due to its unique structural composition

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**Abbreviations:** cHP, cumene hydroperoxide; GSH, glutathione; LPO, lipid peroxidation; MDA, malondialdehyde; OS, oxidative stress; RLU, relative luminescence units; ROS, reactive oxygen species; SOD, superoxide dismutase; tbHP, t-butyl hydroperoxide

of high polyunsaturated fatty acid content in its plasma membrane (Garg et al., 2000; Lenzi et al., 2000; Sanocka and Kurpisz, 2004).

Some chemical, physical, or biological agents that alter physiological control processes and affect the normal functioning of the gonads will cause gonadal toxicity (Kelce et al., 1994; Schrader and Kanitz, 1994). Any potential gonadotoxic agent can interrupt the normal functioning of the male reproductive system in the following ways:

(a) At the hypothalamic pituitary- gonadal axis level, (b) Directly at the gonadal level, or (c) By altering post-testicular events, such as sperm motility or function or both (Sokol, 1987). Disruption of such physiological events may lead to hypogonadism, infertility, decreased libido and/or sexual dysfunction (Sokol, 1987).

Organic hydroperoxides such as t-butyl hydroperoxide (tbHP) and cumene hydroperoxide (cHP) are widely used in the chemical industry as initiators of oxidation for the production of polymers and fibre-reinforced plastics, in the manufacturing of polyester resin coatings and pharmaceuticals. Short term studies have shown that exposure to hydroperoxides (intraperitoneal injection) dramatically increases the level of lipid peroxidation (LPO) and enhances ROS generation in the testes of rats (Kaur et al., 2006; Kumar et al., 2002; Kumar and Muralidhara, 2007). Organic hydroperoxides have been extensively used as model pro-oxidants to induce OS in various *in vitro* systems (Chen et al., 2000; Kumar et al., 2002; Kumar and Muralidhara, 2007). However, these pro-oxidants have not been used in long-term *in vivo* animal model studies to investigate the underlying biochemical mechanisms by which organic hydroperoxides induce oxidative damage in the testis and spermatozoa. This study aimed at developing an *in vivo* animal model to investigate the effect of OS on male reproduction by studying the *in vivo* effects of intraperitoneal administration of tbHP and cHP over a 60 day period on epididymal sperm and testicular tissue in order for these hydroperoxides to manifest itself during the process of complete spermatogenesis.

## MATERIALS AND METHODS

### Animal treatment and research design

This study received institutional review board clearance and rats were housed in an ethically approved animal facility. Male Wistar rats aged between 10 to 12 weeks were randomly placed in 3 groups. Animals were fed *ad libitum* with standard rat chow (SRC) and water while their beddings were changed 3 times per week. Rats (n = 54) were randomly allocated to either a placebo group receiving an intraperitoneal injection of saline (Control) or cHP (2.5, 5, 10 and 20  $\mu$ M; 80% aqueous, Sigma Chemical Co, South Africa) or tbHP (5, 10, 20 and 40  $\mu$ M; 70% aqueous, Sigma Chemical Co, South Africa). 6 rats were included in each individual treatment respectively. Injections (0.5 ml) were administered on 5 consecutive days per week up to 60 days in order to target, at least one complete cycle of spermatogenesis as it takes 58 days in rats

(Franca et al., 1998). The concentrations of cHP and tbHP were adopted and modified from the study of Kumar and Muralidhara (2007).

During the experiments, maximum care was taken to minimize animal suffering. Body weights were recorded at both the onset and completion of the study period. Immediately after euthanization, the testes and epididymis were excised and their weights recorded. One epididymis was rinsed and gently minced in 1.5 ml of phosphate buffered saline (PBS, Sigma Chemical Co, South Africa). The fragments were allowed to sediment for 5 minutes and 1 ml of the supernatant containing the sperm was filtered and collected for further analysis. One of the testes was snap frozen in liquid nitrogen (-196°C) and stored at -80°C.

### Sperm parameters

One drop of sperm suspended in PBS was placed on a glass slide and 10 random fields were manually scored for the number of motile and non-motile sperm. Motility was expressed as a percentage of motile sperm compared to total cells. Epididymal sperm concentration was determined as per the method described in the WHO Manual (WHO, 1999). Briefly, a 50  $\mu$ l aliquot of epididymal sperm was diluted with 950  $\mu$ l of diluents (50 g sodium bicarbonate, 10 ml formalin (35%) and 0.25 g trypan blue were added and made up to a final volume of 1 L with distilled water). A cover slip was secured to the counting chambers of a Neubauer type hemocytometer. Approximately 10  $\mu$ l of the thoroughly mixed diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 min in a humid chamber in order to prevent drying. The cells settled during this time and were subsequently counted with a light microscope at X 40 magnification.

### Assessment of lipid peroxidation

Lipid peroxidation (LPO) was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) and expressed as nmol malondialdehyde (MDA) formed / mg testicular tissue or  $2 \times 10^6$  sperm (Draper et al., 1993). In short, 50  $\mu$ l of epididymal sperm ( $2 \times 10^9$ /ml) or 50  $\mu$ l of homogenized testicular tissue (50 mg frozen testis homogenized in 10 volumes of 1.15% KCl, tris-HCl, 10 mM, pH 7.4, at 4°C, protease inhibitor: P8340 Sigma Chemical Co, South Africa) were added to 6.25  $\mu$ l cold butylated hydroxyl toluene /ethanol (4 mM) and 50  $\mu$ l of ortho-phosphoric acid (0.2 M) in an eppendorf tube. After mixing for 10 s, 6.25  $\mu$ l of thiobarbituric acid reagent (0.11 M), was added and then heated to 90°C (45 min). Samples were subsequently first cooled on ice (2 min) and thereafter at room temperature (5 min) before the addition of n-butanol (500  $\mu$ l) and saturated NaCl (50  $\mu$ l). Eppendorfs were centrifuged (12000 rpm, 2 min, 4°C) and 300  $\mu$ l of the supernatants (top butanol) was transferred to a 96 well plate. Absorbance was measured (532 and 572 nm) by a GloMax® Multi Detection System (Promega, UK).

### Assessment of antioxidant activities

50 mg of frozen testis were homogenized (15000 rpm, 20 min) in 10 volumes of 1.15% KCl, tris-HCl (10 mM, pH 7.4) at 4°C. The activity of the antioxidant enzymes glutathione (GSH) and superoxide dismutase (SOD) concentration were assayed in both epididymal sperm and testicular homogenates using a plate reader GloMax® Multi Detection System (Promega, UK).

The GSH assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalyzed by glutathione S-transferase. The signal generated in a coupled reaction

**Table 1.** Mean ( $\pm$  SEM) body, testicular and epididymal weights as well as epididymal sperm concentration of rats treated (n = 6 per treatment) with different concentrations of cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP).

| Parameter                                 | Control           | cHP (2.5 $\mu$ M) | cHP (5 $\mu$ M)   | cHP (10 $\mu$ M)    | cHP (20 $\mu$ M)    | tbHP (5 $\mu$ M)  | tbHP (10 $\mu$ M) | tbHP (20 $\mu$ M)   | tbHP (40 $\mu$ M)   |
|---|-------------------|-------------------|-------------------|---------------------|---------------------|-------------------|-------------------|---------------------|---------------------|
| Initial body weight (g)                   | 284.5 $\pm$ 18.85 | 260.8 $\pm$ 8.48  | 282.8 $\pm$ 18.09 | 279.8 $\pm$ 17.16   | 307.7 $\pm$ 4.04    | 276.5 $\pm$ 12.80 | 295 $\pm$ 5.20    | 298 $\pm$ 15.64     | 299 $\pm$ 6.99      |
| Final body weight (g)                     | 420.6 $\pm$ 26.09 | 408.2 $\pm$ 14.72 | 412.5 $\pm$ 6.26  | 394.7 $\pm$ 12.78   | 414.7 $\pm$ 12.58   | 416.5 $\pm$ 6.14  | 434.3 $\pm$ 13.43 | 416.7 $\pm$ 8.25    | 381.8 $\pm$ 9.93    |
| Weight gain (%)                           | 30.6 $\pm$ 22.47  | 35.6 $\pm$ 11.16  | 31.29 $\pm$ 12.17 | 29.35 $\pm$ 14.97   | 25.55 $\pm$ 8.31    | 33.43 $\pm$ 9.47  | 31.73 $\pm$ 9.31  | 28.41 $\pm$ 11.94   | 21.44 $\pm$ 8.46    |
| Testes weight (g)                         | 4.09 $\pm$ 0.15   | 3.72 $\pm$ 0.05   | 3.61 $\pm$ 0.09   | 3.75 $\pm$ 0.16     | 3.78 $\pm$ 0.23     | 3.69 $\pm$ 0.06   | 3.79 $\pm$ 0.10   | 3.85 $\pm$ 0.17     | 3.55 $\pm$ 0.07     |
| Epididymis weight (g)                     | 0.54 $\pm$ 0.03   | 0.45 $\pm$ 0.01   | 0.49 $\pm$ 0.02   | 0.48 $\pm$ 0.03     | 0.50 $\pm$ 0.01     | 0.48 $\pm$ 0.02   | 0.52 $\pm$ 0.03   | 0.50 $\pm$ 0.04     | 0.49 $\pm$ 0.01     |
| Sperm concentration (10 <sup>6</sup> /ml) | 109.0 $\pm$ 10.88 | 104.8 $\pm$ 7.68  | 93.00 $\pm$ 7.53  | 67.00 $\pm$ 5.73*** | 58.67 $\pm$ 4.80*** | 113.3 $\pm$ 11.26 | 101.2 $\pm$ 7.89  | 65.50 $\pm$ 4.05*** | 59.17 $\pm$ 4.10*** |

\*\*\* P < 0.001 vs. control.

with firefly luciferase is proportional to the amount of glutathione present in the sample. The assay was conducted according to the protocol provided by the manufacturer (Promega, UK). 50  $\mu$ l of prepared GSH-Glo™ Reagent 2X was added to 50  $\mu$ l of 20000 epididymal sperm cells or 50  $\mu$ l supernatant of testicular tissue homogenate on a 96-well plate and incubated at room temperature (30 min). Subsequently, 100  $\mu$ l of reconstituted luciferin detection reagent was added to each well, mixed and the luminescence was read after incubation (15 min).

SOD activity was determined from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase to form superoxide anion. The superoxide anion then converts WST-1 to WST-1 formazan, a colored product that absorbs light at 450 nm. The relative SOD activity of the experimental sample is determined from the percentage inhibition of the rate of formation of WST-1 formazan. The assay was conducted according to the protocol provided by the manufacturer (Assay Designs, USA) using 20000 sperm cells or supernatant of testicular tissue homogenate.

#### Statistical analyses

GraphPad™ PRISM 4 was used for all statistical evaluations and graphical representations. Data are expressed as mean  $\pm$  S.E.M. A one-way analysis of variance (ANOVA) test (with Bonferroni post test if P < 0.05) and Pearson correlation were used for statistical analyses. Differences were regarded as statistically

significant if P < 0.05 and highly significant if P < 0.001.

## RESULTS

### Body, testicular and epididymal weights

From Table 1, it can be seen that the weight of the animals did not differ significantly at the onset or at the end of the experiment. All animals gained weight during the 60 day treatment period. Both testicular and epididymal weights of the treated animals did not differ from the control values at the end of the experimental period (Table 1).

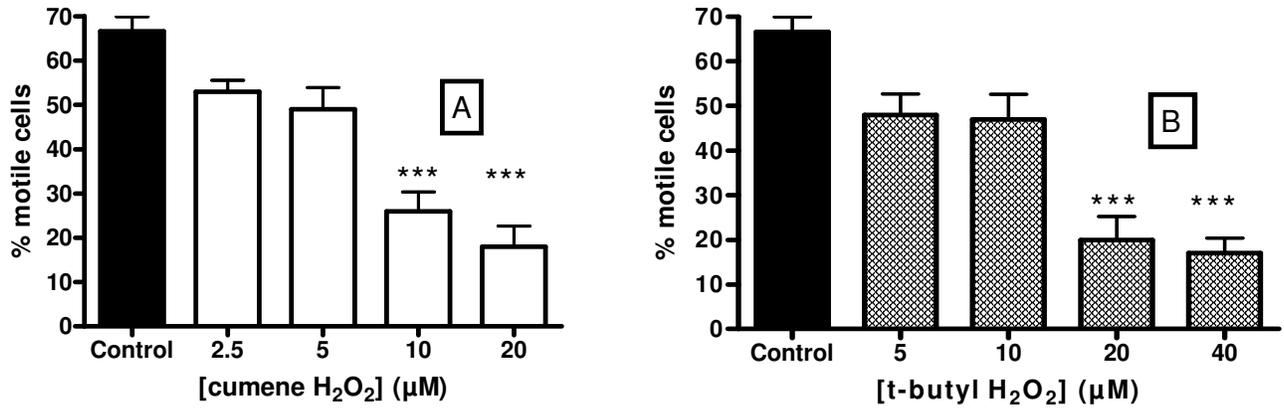
### Sperm count and motility

A significantly (p < 0.001) lower epididymal sperm concentration was observed in animals injected with 10  $\mu$ M (67.00  $\pm$  5.73  $\times$  10<sup>6</sup>/ml) and 20  $\mu$ M (58.67  $\pm$  4.80  $\times$  10<sup>6</sup>/ml) of cHP and 20  $\mu$ M (65.50  $\pm$  4.05  $\times$  10<sup>6</sup>/ml) and 40  $\mu$ M (59.17  $\pm$  4.10  $\times$  10<sup>6</sup>/ml) of tbHP when compared to the control group (109  $\pm$  10.88  $\times$  10<sup>6</sup>/ml) (Table 1). Furthermore, sperm from animals injected with cHP (10  $\mu$ M: 26  $\pm$  4.30%; 20  $\mu$ M: 18  $\pm$  4.63%) and tbHP (20  $\mu$ M: 20  $\pm$  5.24%; 40  $\mu$ M: 17  $\pm$  3.39%) showed a significant (p < 0.001) lower motility when compared

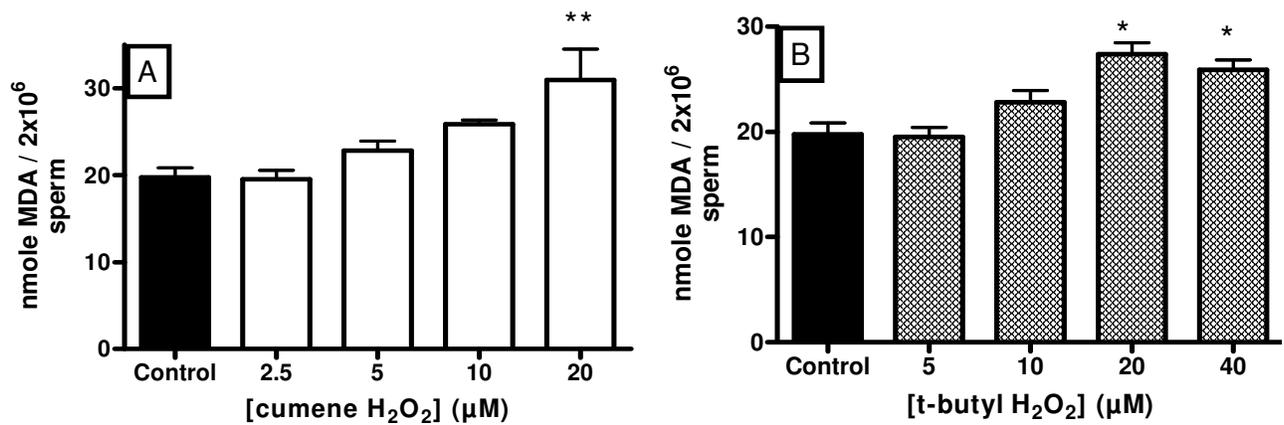
to sperm from control animals (66.60  $\pm$  3.36%) (Figures 1A and B).

### Lipid peroxidation

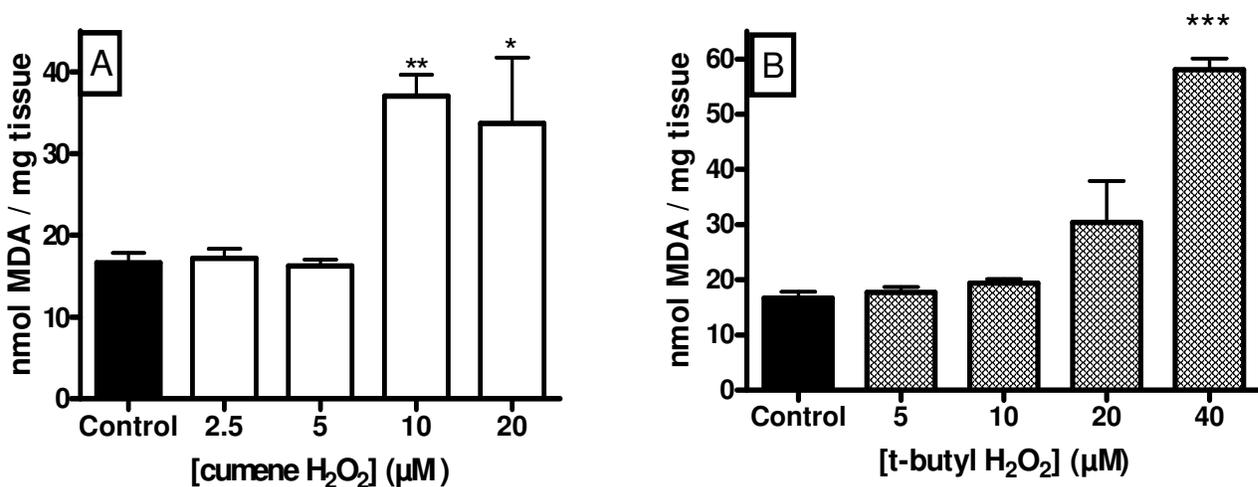
The amount of LPO in epididymal sperm and testicular tissue were significantly higher when exposed to higher dosages of hydroperoxide. Figures 2A and B show that the production of MDA after administration of 20  $\mu$ M cHP (30.94  $\pm$  3.55 nmol MDA / 2 $\times$ 10<sup>6</sup> sperm, p < 0.01) or 20  $\mu$ M and 40  $\mu$ M tbHP (27.38  $\pm$  1.07 and 25.92  $\pm$  0.91 nmol MDA / 2  $\times$  10<sup>6</sup> sperm, p < 0.05) were significantly elevated above control values (19.77  $\pm$  1.07 nmol MDA / 2  $\times$  10<sup>6</sup> sperm) in epididymal sperm. Moreover, there was significantly higher formation of MDA in the testicular tissue of animals receiving 10 and 20  $\mu$ M cHP (37.05  $\pm$  2.59 and 33.70  $\pm$  8.05 nmol MDA / mg tissue, p < 0.01 and p < 0.05 respectively) or 40  $\mu$ M tbHP (58.09  $\pm$  1.98 nmol MDA / mg tissue, p < 0.001) compared to control (16.63  $\pm$  1.20 nmol MDA / mg tissue) (Figures 3A and B). Interestingly very strong negative/inverse correlations were found between epididymal sperm motility and LPO in: animals treated with cHP (r = -0.9640, p = 0.0082) or tbHP (r = -0.9725, p = 0.0055) as well as



**Figure 1.** Epididymal sperm motility of rats treated (n = 6 per treatment) with (A) cumene hydroperoxide and (B) t-butyl hydroperoxide (\*\*\*) p < 0.001 vs. control).



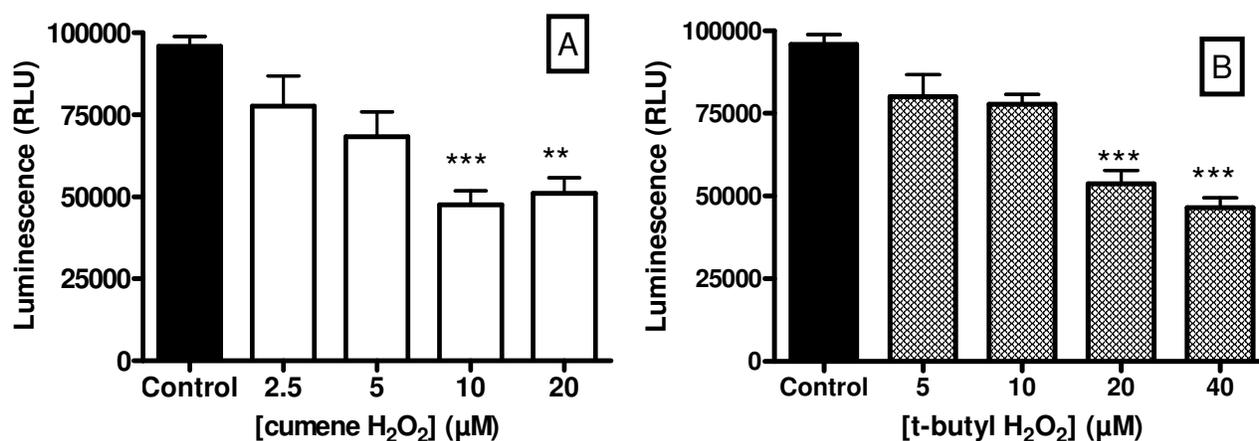
**Figure 2.** Lipid peroxidation in epididymal sperm of rats treated (n = 6 per treatment) with (A) cumene hydroperoxide and (B) t-butyl hydroperoxide (\* p < 0.05 vs. control, \*\* p < 0.01 vs. control).



**Figure 3.** Lipid peroxidation in testicular tissue of rats treated (n = 6 per treatment) with (A) cumene hydroperoxide and (B) t-butyl hydroperoxide (\* p < 0.05 vs. control, \*\* p < 0.01 vs. control, \*\*\* p < 0.001 vs. control).

**Table 2.** Correlation between lipid peroxidation (LPO) in epididymal sperm and testicular tissue and sperm motility after cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP) exposure.

| Treatment group |          | Lipid peroxidation (LPO) |         |                   |         |
|-----------------|----------|--------------------------|---------|-------------------|---------|
|                 |          | Epididymal sperm         |         | Testicular tissue |         |
|                 |          | cHP                      | tbHP    | cHP               | tbHP    |
| Motility        | <i>r</i> | -0.9640                  | -0.9725 | -0.9682           | -0.9254 |
|                 | <i>p</i> | 0.0082                   | 0.0055  | 0.0068            | 0.0242  |



**Figure 4.** Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on epididymal rat sperm glutathione (GSH) expressed as Relative Luminescence Units RLU (n = 6 per treatment, \*\* p < 0.01 vs. control, \*\*\* p < 0.001 vs. control).

epididymal sperm motility and testicular tissue LPO (cHP:  $r = -0.9682$ ,  $p = 0.0068$  and tbHP:  $r = -0.9254$ ,  $p = 0.0242$ ) (Table 2).

## Antioxidant activities

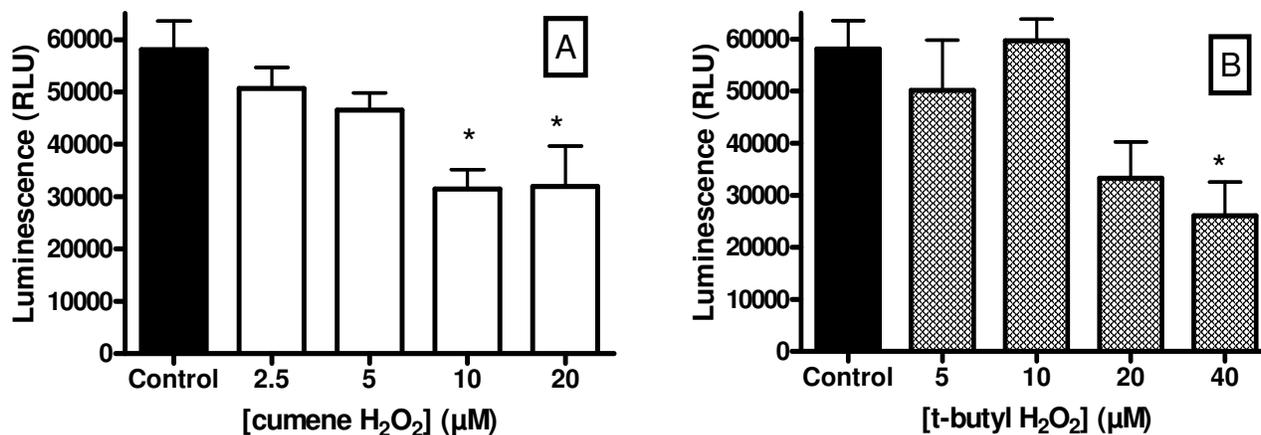
### GSH

The activity of GSH is expressed in Relative Luminescence Units (RLU). From Figures 4A and B, it can be seen that the formation of luciferase in the epididymal sperm of animals injected with cHP (10 μM:  $47662 \pm 4243$  RLU,  $p < 0.001$  and 20 μM:  $51118 \pm 4776$  RLU,  $p < 0.01$ ) or tbHP (20 μM:  $53671 \pm 4112$  RLU and 40 μM:  $46548 \pm 2949$  RLU,  $p < 0.001$ ) was significantly lower compared to the control group ( $9589 \pm 2982$  RLU). A similar trend was observed in testicular tissue with significantly lowered luciferase fluorescence at 10 μM ( $31456 \pm 3742$ ,  $p < 0.05$ ) and 20 μM ( $31922 \pm 7776$  RLU,  $p < 0.05$ ) cHP and 40 μM tbHP ( $26094 \pm 6409$  RLU,  $p < 0.05$ ) compared to control ( $58077 \pm 5482$  RLU) (Figures 5A and B). A negative/inverse correlation was found between epididymal sperm LPO and GSH activity in animals treated with cHP ( $r = -0.9542$ ,  $p = 0.0117$ ) or tbHP ( $r = -0.9749$ ,  $p =$

$0.0047$ ) as well as testicular tissue LPO and GSH activity (cHP:  $r = -0.9631$ ,  $p = 0.0085$  and tbHP:  $r = -0.8483$ ,  $p = 0.0693$ : not significant) (Table 3). However, a positive correlation was found between epididymal sperm motility and GSH activity in animals treated with cHP ( $r = 0.9543$ ,  $p = 0.0116$ ) or tbHP ( $r = 0.9750$ ,  $p = 0.0047$ ) as well as testicular tissue and GSH activity (cHP:  $r = 0.9631$ ,  $p = 0.0085$  and tbHP:  $r = -0.8488$ ,  $p = 0.0689$ : not significant) (Table 3).

### SOD

The concentrations of SOD were significantly lower ( $p < 0.001$ ) in the epididymal sperm of animals treated with cHP (10 μM:  $0.20 \pm 0.01$  U/μl; 20 μM:  $0.18 \pm 0.02$  U/μl) or tbHP (20 μM:  $0.21 \pm 0.01$  U/μl; 40 μM:  $0.17 \pm 0.01$  U/μl) when compared to the control ( $0.51 \pm 0.21$  U/μl) (Figures 6A and B). A similar trend was observed in SOD concentration of testicular tissue ( $p < 0.001$ ) in animals treated with cHP (10 μM:  $0.21 \pm 0.08$  U/μl; 20 μM:  $0.18 \pm 0.02$  U/μl) or tbHP (20 μM:  $0.21 \pm 0.02$  U/μl; 40 μM:  $0.16 \pm 0.03$  U/μl) when compared to control ( $0.56 \pm 0.21$  U/μl) (Figures 7A and B). Table 3 shows that a negative/inverse correlation exist between epididymal sperm LPO



**Figure 5.** Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on rat testicular glutathione (GSH expressed as Relative Luminescence Units RLU) (n = 6 per treatment, \* p < 0.05 vs. control)

**Table 3.** Correlation of glutathione (GSH) activity as well as superoxide dismutase (SOD) concentration in epididymal sperm and testicular tissue with sperm motility and lipid peroxidation (LPO) after cumene hydroperoxide (cHP) and t-butyl hydroperoxide (tbHP) exposure.

| Treatment group |   | Glutathione (GSH) |         |                   |         | Superoxide dismutase (SOD) |         |                   |         |
|-----------------|---|-------------------|---------|-------------------|---------|----------------------------|---------|-------------------|---------|
|                 |   | Epididymal sperm  |         | Testicular tissue |         | Epididymal sperm           |         | Testicular tissue |         |
|                 |   | cHP               | tbHP    | cHP               | tbHP    | cHP                        | tbHP    | cHP               | tbHP    |
| Motility        | r | 0.9543            | 0.9750  | 0.9631            | 0.8488  | 0.9800                     | 0.9619  | 0.9805            | 0.9619  |
|                 | p | 0.0116            | 0.0047  | 0.0085            | 0.0689  | 0.0034                     | 0.0089  | 0.0033            | 0.0089  |
| LPO             | r | -0.9542           | -0.9749 | -0.9631           | -0.8483 | -0.9502                    | -0.8930 | -0.8236           | -0.7994 |
|                 | p | 0.0117            | 0.0047  | 0.0085            | 0.0693  | 0.0132                     | 0.0413  | 0.0865            | 0.1045  |

and SOD concentration in animals treated with cHP (r = - 0.9502, p = 0.0132) or tbHP (r = - 0.8930, p = 0.0413) as well as testicular tissue LPO and SOD concentration (cHP: r = -0.8236, p = 0.0865: not significant and tbHP: r = - 0.7994, p = 0.1045: not significant). However, a positive correlation was found between sperm motility and epididymal sperm SOD concentration in animals treated with cHP (r = 0.9800, p = 0.0034) or tbHP (r = 0.9619, p = 0.0089) as well as testicular tissue and SOD concentration (cHP: r = 0.9805, p = 0.0033 and tbHP: r = 0.9619, p = 0.0089) (Table 3).

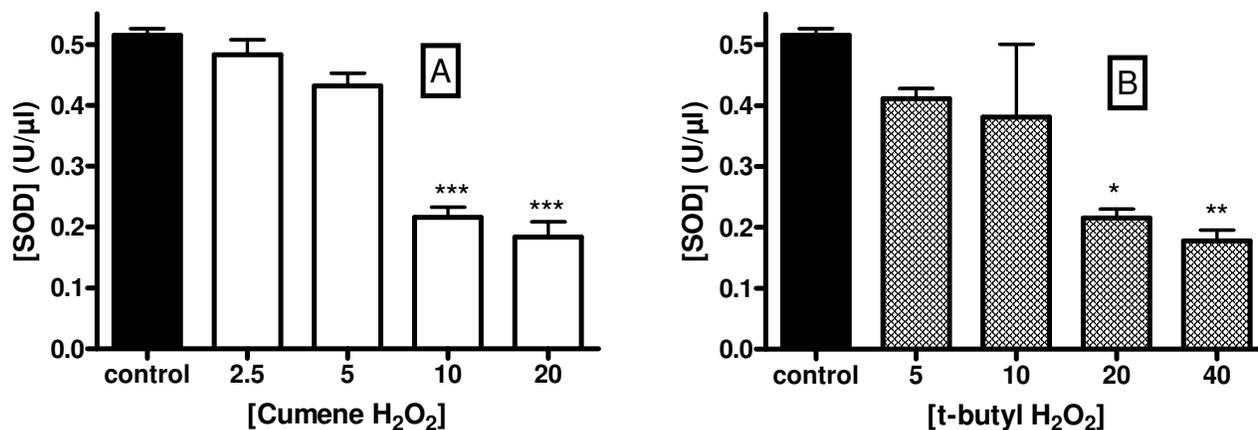
**DISCUSSION**

The process of spermatogenesis, from germ cell recruitment to spermiation, takes a couple of weeks and renders the developing male gametes extremely vulnerable to any form of oxidative insult (Dacheux et al., 2003). In mammals, the epididymis is known to play an important role in the final development of motility and fertilizing ability as well as storage of sperm. During the period of epididymal transit, sperm concentration can increase up to 10<sup>10</sup> cells/ml. Sperm metabolism also

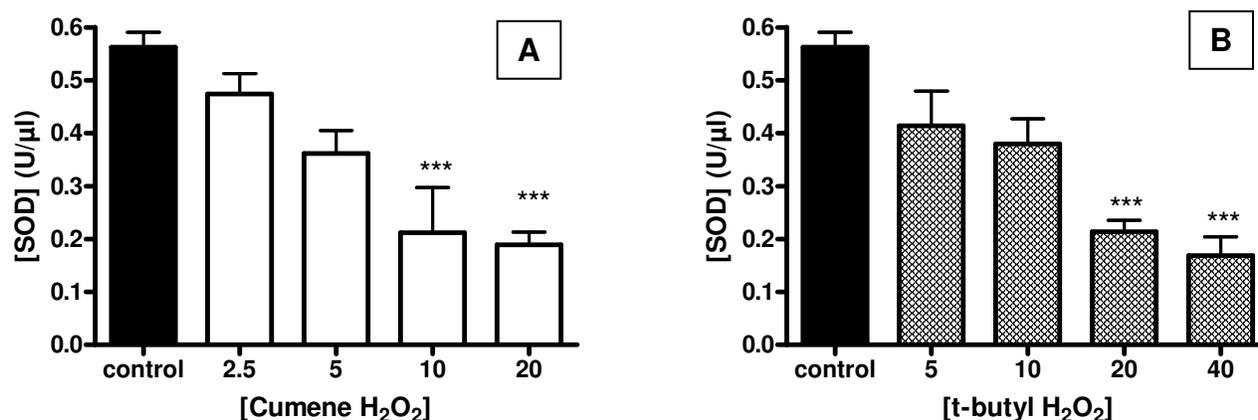
increases simultaneously and the possibility of OS generations threatens the survival of these male gametes (Dacheux et al., 2003).

In this study, the long-term exposure of male rats to organic hydroperoxides (cHP and tbHP) via intraperitoneal injection did not lead to mortality or any clinical signs of general toxicity. However, it negatively affected sperm parameters by decreasing both sperm concentration and sperm motility as measured at the end of the study. Furthermore, both cHP and tbHP treatments significantly increased LPO while simultaneously lowered GSH activity and SOD concentration. These effects were observed in epididymal sperm and testicular tissue. The higher doses of cHP (10 and 20 μM) and tbHP (20 and 40 μM) might affect the spermatogenesis process and particularly sperm transition through the epididymis not only by decreasing the number of sperm available but also by compromising the quality through the process of epididymal sperm maturation (Dacheux et al., 2003).

Previous studies have shown a correlation between high levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxytrifluoromethyl) and sperm motility (Agarwal et al., 2003; Armstrong et al., 1999; Bilodeau et al., 2002; Lenzi et al., 1993). De Lamirande and Gagnon



**Figure 6.** Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on superoxide dismutase (SOD) concentration of epididymal rat sperm (n = 6 per treatment, \* p < 0.05 vs. control, \*\* p < 0.01 vs. control, \*\*\* p < 0.001 vs. control).



**Figure 7.** Effects of (A) cumene hydroperoxide and (B) t-butyl hydroperoxide on superoxide dismutase (SOD) concentration of rat testicular tissue (n = 6, \*\*\* p < 0.001 vs. control).

1992) also reported that ROS causes sperm immotility within 5 - 30 min, depending on the concentration. The H<sub>2</sub>O<sub>2</sub> might diffuse across the membranes into the cells and inhibit the activity of enzymes such as glucose-6-phosphate dehydrogenase (G6PD), which led to a decrease in the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). In turn this decreased the formation of ATP, which is an important metabolite for sperm motility. This was confirmed by our findings and could explain the decrease in sperm motility observed. The peroxidative process initiated by the high doses of cHP and tbHP may lead to ROS-mediated protein oxidation (Ong et al., 2002) which reduced the sperm counts (Agarwal et al., 2003) possibly due to cell death. H<sub>2</sub>O<sub>2</sub> can penetrate the plasma membrane, cause protein oxidation and increase LPO production. In the light of the membrane permeability of H<sub>2</sub>O<sub>2</sub>, the external production or addition of this oxidant must have a negative effect on sperm motility, LPO accumulation,

antioxidant (SOD, GSH) activities and DNA integrity. Ramos and Wetzels found DNA fragmentation in human sperm after addition of H<sub>2</sub>O<sub>2</sub> (Ramos and Wetzels, 2001). These authors as well as Giwercman and co-workers emphasized the correlation between sperm motility and DNA integrity (Giwercman et al., 2003). Negative correlations were also observed between DNA fragmentation and semen quality as reflected by sperm motility, morphology, and concentration (Sun et al., 1997). Furthermore, a strong correlation was found between DNA strand breaks and the susceptibility of sperm to low pH-induced DNA denaturation (Aravindan et al., 1997).

Mammalian sperm membranes are rich in polyunsaturated fatty acids (PUFA), which make them very fluid but at the same time very susceptible to free radicals and ROS. Griveau and co-workers have shown that reactive oxygen species cause a decrease in sperm motility, an increase in lipid peroxidation and a loss of membrane

PUFA (Griveau et al., 1995). In the sperm plasma membrane, PUFAs are required to give the plasma membrane the fluidity needed for sperm motility. Spontaneous lipid peroxidation was also shown in rabbit and mouse sperm and a close linear correlation existed between the extent of peroxidation and the loss of sperm motility (Alvarez and Storey, 1992). Our results show that the amounts of MDA in the epididymal sperm and in the testicular tissue negatively correlate with the percentages of sperm motility in rats, which is in agreement with the literature.

The principal antioxidant defenses in sperm are SOD and GSH peroxidase (Storey, 1997). The physiological role of GSH is an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. GSH protects cells against oxidative stress and other types of damage, which may arise from compounds of endogenous and exogenous sources. The continued activity of glutathione peroxidase depends on the regeneration of reduced glutathione by glutathione reductase, which in turn relies on NADPH, the principal source of which in sperm is the pentose phosphate shunt. The activity of G6PD, which is the first enzyme in the pentose phosphate pathway, may limit the rate of NADPH production and hence, the ability of the glutathione peroxidase system to detoxify peroxides (Storey et al., 1998). The high levels of cHP (10 and 20  $\mu\text{M}$ ) and tbHP (20 and 40  $\mu\text{M}$ ) overwhelmed the antioxidant capacity of both SOD and GSH. The lower SOD concentration in sperm cells could be attributed to the assault of the high doses of cHP and tbHP. SOD presents the first line of defense against superoxide, as it dismutates the superoxide anion to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  (Nehru and Anand, 2005). Organic hydroperoxides (cHP and tbHP) might affect the GSH synthesis by decreasing the activity of glutathione-synthase thus leading to a reduced GSH content. On the other hand, the decreased GSH level could be also ascribed to insufficient supply of NADPH. This could explain the correlations found, in this study, between epididymal sperm motility, epididymal sperm LPO, SOD concentration and GSH activity as well as in the testicular tissue.

## Conclusion

Intraperitoneal injection of organic hydroperoxides (cHP and tbHP) lowered sperm concentration and sperm motility. It furthermore, impaired antioxidant activities in both epididymal sperm and testicular tissue. We can therefore conclude that we have successfully created an animal model to test the adverse effects of OS on male reproductive parameters, which will also allow us to study possible treatments *in vivo*. For future reference, 60 days of 10  $\mu\text{M}$  cHP and 20  $\mu\text{M}$  tbHP treatment can be used as doses of organic hydroperoxides to successfully induce OS in the rat model in order to target the complete process of spermatogenesis.

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