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Induction of Oxidative Stress in the Epididymis of Rats After Subchronic Exposure to Epichlorohydrin

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Abstract This study investigated the effects of epichlorohydrin (ECH) on spermatogenesis and antioxidant system in rats. An increase in the incidence of clinical signs, gross pathology and histopathology findings in the epididymidis, and sperm abnormalities and a decrease in the testicular spermatid counts, epididymal sperm counts, and sperm motility were observed at 30 mg/kg/day. Oxidative stress in the epididymal tissue was detected at $\geq$3.3 mg/kg/day. The results show that graded doses of ECH elicit depletion of antioxidant defense system and that the adverse effects on male reproductive function in ECH-treated rats may be due to the induction of oxidative stress.

Keywords Epichlorohydrin · Reproductive dysfunction · Sperm · Oxidative stress

Epichlorohydrin (ECH) is one of the industrial chemicals used in the manufacture of glycerol, epoxy resins, and other products (SRI 2007). Previous studies demonstrated that ECH is an anti-fertility agent that acts both as an epididymal toxicant and an agent capable of directly affecting sperm motility (Cooper et al. 1974; Hahn 1970; Toth et al. 1989). According to the report of Jones et al. (1981), in vitro incubation of mature sperm with alpha-chlorohydrin, a potential metabolite of ECH, resulted in the inhibition of sperm glyceraldehydes-3-phosphate dehydrogenase (GAPDH) activity. Jelks et al. (2001) and Jelks and Miller (2001) demonstrated that in vivo administration of alpha-chlorohydrin directly affects spermatozoa by the depletion of ATP levels and the inhibition of GAPDH activity in rat sperm and epididymis. However, the exact cause of the inhibition of GAPDH activity is unknown at present. Moreover, whether inhibition of the enzyme is causally related to epidydimal toxicity is unclear.

It is well known that reactive oxygen species (ROS) play a critical role in the pathogenesis of reproductive disorders and especially in pathological mechanism of male infertility (Sharma and Agarwal 1996). Because GAPDH is a cytosolic protein whose active center contains an SH group that is sensitive to oxidation (Nakajima et al. 2007), this enzyme is known as a major target protein in oxidative stresses. Therefore, we hypothesized that the inhibition of GAPDH activity may result from induction of oxidative stress in rat sperm and epididymidis. To test this hypothesis, we examined the spermatotoxicity and epididymal oxidative damage of ECH after repeated oral administration in male rats to better understand a possible mechanism for the spermatotoxicity of ECH.

Materials and Methods

Male Sprague–Dawley rats aged 5 weeks were purchased from Orient-Bio Inc. (Seoul, Korea) and used after 1 week of quarantine and acclimatization. ECH (Aldrich Co. Milwaukee, WI, USA) was dissolved in corn oil and was administered orally to rats for 70 days. The vehicle control rats received an equivalent volume of corn oil alone. Healthy

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males were assigned randomly into four experimental groups of 12 rats each: three treatment groups receiving 3.3, 10, and 30 mg/kg/day ECH and a vehicle control group.

All rats were observed daily for clinical signs and body weight and food consumption were measured twice a week during the treatment period. The animals were sacrificed 24 h after the last administration using anesthetic ether. The gross weights of the testis, epididymis, prostate gland, and seminal vesicle were measured and then their weights relative to body weight calculated. The weighed left testis (n = 6 per group) was homogenized with 12 mL of distilled water for the sperm head counts. The sperm suspension was placed into a hemacytometer and the number of spermatids was counted using a light microscope (Leica, Germany). The weighed left cauda epididymis was homogenized with 10 mL physiological saline to determine the sperm counts. The number of sperm was counted as in the testis. For the motility measurements, the sperm was obtained from the ductus deferens, placed in Hanks' balanced salt solution (pH 7.2) containing 5 mg/mL bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) and maintained at 37°C. Motility was observed using a microscope with a stage warmer. The sperm morphology was also examined using optical microscopy of the sperm smears (sperm suspension containing 1% Eosin Y) collected from the ductus deferens. The right testis and right epididymis were taken and fixed with 10% neutral buffered formalin solution. The tissues were routinely processed, embedded in paraffin and sectioned at 3–5 μm thickness, deparaffinized, and rehydrated using standard techniques. These sections were stained with Hematoxylin–Eosin for microscopic examination.

The weighed frozen epididymal tissue (right, n = 6 per group) was homogenized in a glass-Teflon homogenizer with 50 mM phosphate buffer (pH 7.4) to obtain 1:9 (w/v) whole homogenate. The homogenates were then centrifuged at 11,000*g* for 15 min at 4°C to discard any cell debris, and the supernatant was used for the measurement of malondialdehyde (MDA), reduced glutathione (GSH), catalase, superoxide dismutase (SOD), and glutathione-S-transferase (GST). The concentration of MDA was assayed by monitoring thiobarbituric acid reactive substance formation by the method of Berton et al. (1998). Glutathione was measured by the method of Moron et al. (1979). The activities of antioxidant enzymes including catalase (Aebi 1984), SOD (McCord 1994) and GST (Habig et al. 1984) were also determined. Total protein contents were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

The results are expressed as mean ± SD, and all statistical comparisons were made by one-way ANOVA followed by Tukey–Kramer multiple comparison test.

Results and Discussion

The male rats treated with 30 mg/kg/day of ECH showed an increase in the incidence of clinical signs such as nasal discharge (n = 4), soft feces (n = 1), depression (n = 2), and piloerection (n = 3) in a dose-dependent manner. These findings may be related to mucus and gastrointestinal irritation effects of ECH because ECH is a chemical that acts as eye, nose and skin irritant (Gage 1959). Although the oral administration of ECH to male rats produced some clinical signs at 30 mg/kg/day, body weight, food intake, and reproductive organ weights were not affected at any dose tested.

At the scheduled necropsy, cystic pustule of the epididymidis was observed in five cases in the high dose group. Although the difference was not statistically significant between the groups, the incidence of cystic pustule observed in the 30 mg/kg group was considerably higher (41.7%) than controls. The increased incidence of cystic pustule was considered to be treatment-related effect, since this finding is uncommon in normal control rats (Boorman et al. 1990; Haschek and Rousseaux 1998) and is consistent with the significantly increased incidence of histopathological alterations.

On histopathologic examination, spermatic granuloma (n = 6), cell debris in the ducts (n = 12), epithelial cell desquamation (n = 6), epithelial cell vacuolization (n = 9), and oligospermia (n = 3) were observed in epididymis of the 30 mg/kg group (Fig. 1). Cell debris in the ducts (n = 3) and epithelial cell vacuolization (n = 6) were also found in the 10 mg/kg group. The incidence of histopathological alterations observed in the 30 mg/kg group was significantly higher than the control. The gross and histopathological findings observed in this study are in agreement with the results of previous studies (Cooper et al. 1974; Jelks et al. 2001).

Sperm head count in testis, sperm count in caudal epididymis, and sperm motility in the high dose group were significantly decreased in a dose-dependent manner compared with those of the control group (Table 1). On the contrary, sperm morphological abnormalities in the high dose group were significantly increased compared to the control group. These findings were also considered to be related to the ECH administration because these changes were remarkable and showed a clear-cut dose–response relationship. According to previous studies, single or repeated exposure to ECH results in decreased sperm motility and reduced fertility or complete sterility in male rats (Cooper et al. 1974; Toth et al. 1989). Cassidy et al. (1983) reported that a single oral dose of ECH at 50 mg/kg causes an increase in the incidence of abnormal sperm in male rats. Jelks et al. (2001) and Jelks and Miller (2001) demonstrated that administration of alpha-chlorohydrin to

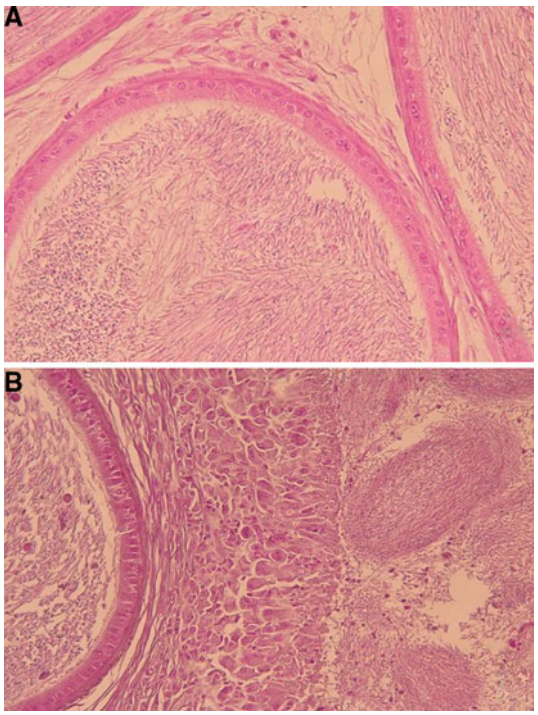

Fig. 1 Representative photographs of the epididymis from the control and high dose groups. **a** A control rat, showing normal appearance. **b** A high dose group rat, with cell debris in ducts, spermatic granuloma, and vacuolization of epithelial cells

rats at ≥25 mg/kg produces signs of edema, disruption of spermatogenesis in the testis, and few sperm and epithelial disruption in the epididymal tubules. The results of previous studies and the present study clearly show that ECH and their metabolite have adverse effects on spermatogenesis and sperm function in rats.

The concentration of MDA, an end product of lipid peroxidation, in the 10 and 30 mg/kg groups were significantly increased in a dose-dependent manner when compared with the control (Table 2). However, the concentration of GSH was significantly decreased in all of treatment groups compared to the control group. The catalase, GST, and SOD activities in the 10 and 30 mg/kg groups were also significantly decreased when compared with the control group. Oxidative stress is generally defined as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (Valko et al. 2007). Spermatozoa are particularly susceptible to the peroxidation in the presence of elevated seminal ROS levels because their plasma membranes contain high content of polyunsaturated fatty acids (Alvarez and Strey 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Jones et al. 1979). Previous studies reported that ROS induce lipid peroxidation and the toxicity of lipid peroxides play a key role in the sperm dysfunction and male infertility (Alvarez et al. 1987; Sharma and Agarwal 1996). It was also reported that high concentrations of ROS cause sperm pathology such as ATP depletion leading to insufficient axonemal phosphorylation, lipid peroxidation and loss of motility and viability (de Lamirande et al. 1997; Dokmeci 2005). In the present study, the animals treated with ECH showed decreased activities of antioxidant enzymes catalase, GST, and SOD and GSH concentration, while increased concentrations of MDA in the epididymis in a dose-related manner. An increase in the lipid peroxidation indicated that ECH induced oxidative stress in the epididymis by decreasing the activity of antioxidant enzymes, thereby leading to an excessive generation of ROS. In addition, reduction in catalase, GST, SOD, and GSH can be the cause of increased hydrogen peroxide levels in the epididymis because these antioxidants are unable to scavenge hydrogen peroxide generated in the epididymis as a result of excessive ROS formation. Hydrogen peroxide, through the production of hydroxyl radicals, can initiate lipid peroxidation, which can cause structural damage to the epididymal cell membrane (Selvanial and Ursini 2000). Therefore, increased lipid peroxidation and reduced levels of antioxidants of epididymis in rats treated with ECH may indicate an increased ROS generation and could be closely linked to its effect on the epididymal and sperm indices. Previous studies have demonstrated that alpha-chlorohydrin directly affects spermatozoa by the depletion of ATP levels and the inhibition of GAPDH activity in rat sperm and epididymis

Table 1 Sperm analysis of male rats treated with ECH for 10 weeks (mean ± SD)

Items	Epichlorohydrin (mg/kg/day)			
	0	3.3	10	30
No. of rats	6	6	6	6
Sperm heads in testis ($\times 10^6$)	237.1 ± 22.62	226.8 ± 19.86	224.3 ± 19.97	189.9 ± 33.66*
Sperm in caudal epididymis ($\times 10^6$)	230.2 ± 12.16	226.7 ± 19.53	204.0 ± 19.45	158.8 ± 44.37**
Sperm motility (%)	84.1 ± 7.76	80.6 ± 6.08	68.4 ± 6.93	50.1 ± 29.64**
Sperm abnormalities (%)	4.5 ± 1.52	6.8 ± 3.35	10.6 ± 5.53	19.6 ± 15.35*

*, ** Significant difference at $p < 0.05$ and $p < 0.01$ levels compared with the control group, respectively

Table 2 Determination of lipid peroxidation, antioxidant, and antioxidant enzymes in the epididymis of rats treated with ECH for 10 weeks (mean ± SD)

Items	Epichlorohydrin (mg/kg/day)			
	0	3.3	10	30
No. of rats	6	6	6	6
MDA (umol/mg protein)	264.3 ± 33.28	271.8 ± 25.21	323.9 ± 23.29**	450.0 ± 65.62**
GSH (umol/mg protein)	0.71 ± 0.066	0.66 ± 0.036*	0.64 ± 0.018**	0.61 ± 0.024**
CAT (unit/mg protein)	28.58 ± 2.336	25.12 ± 3.088	21.53 ± 3.930**	20.34 ± 4.639**
GST (umol/mg protein)	1.66 ± 0.086	1.55 ± 0.143	1.45 ± 0.059**	1.37 ± 0.123**
SOD (unit/mg protein)	7.50 ± 0.344	7.32 ± 0.269	6.62 ± 0.275**	6.93 ± 0.303**

*, ** Significant difference at $p < 0.05$ and $p < 0.01$ levels compared with the control group, respectively

(Jones et al. 1981; Jelks et al. 2001; Jelks and Miller 2001). Because GAPDH is known as a major target protein in oxidative stresses (Nakajima et al. 2007), we considered that oxidative damage induced by ECH administration can cause an inhibition of GAPDH activity in rat sperm and epididymis, followed by sperm ATP depletion.

Based on these results, it can be concluded that the 10-week repeated oral dose of ECH to male rats at ≥3.3 mg/kg/day elicits oxidative damage and spermatotoxicity in the epididymis, and that the adverse effects of ECH on epididymal sperm and histology may be at least partially due to the induction of oxidative stress in rats.

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