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Styrene-Mediated Oxidative Stresses in Rat Sperm Cells

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ABSTRACT. Styrene is a commercially important chemical used mainly in the production of plastics. A toxic effect exerted by styrene exposure may cause infertility, congenital anomalies or death in offspring. Treatment with styrene for 0, 50, 100, and 500 mg/kg for 5 days in Sprague-Dawley rats significantly decreased sperm motilities and sperm counts while sperm abnormalities were significantly increased. To determine the relationship between changes in sperm motilities and roles of reactive oxygen species (ROS), we determined the effect of styrene on ROS production and mRNA expression of antioxidant enzymes in rats. ROS production was enhanced by styrene treatment in a dose-dependent manner. The mRNA expression of catalase and superoxide dismutase (SOD) 2 was strongly suppressed by styrene treatment although SOD1 or glutathione peroxidase (GPX) 4 expressions were not significantly changed. Taken together, these results indicate that styrene may cause toxic effect in rat sperm cells by enhancing oxidative stresses.

Keywords: Styrene, Reactive oxygen species, Catalase, Rat sperm.

INTRODUCTION

Styrene is one of the most important chemicals used for reinforced plastics, rubber products, latex paints, coatings, and polyester resins fabrications. The effects of styrene have been extensively studied and the genotoxicity, hepatotoxicity, immunotoxicity, pneumotoxicity, or neurotoxicity were previously reported (Bond, 1989; Gadberry *et al.*, 1996; Sumner *et al.*, 1997).

The toxic potential of styrene has been postulated due to its metabolite, styrene-7,8-oxide (SO). *In vivo*, styrene is metabolized to SO by the cytochrome P450 enzymes, particularly by CYP 2B6, CYP 2E1, CYP 1A2, CYP 2F1, CYP 2C8 and CYP 3A4 (Nakajima *et al.*, 1993). SO is efficiently inactivated to phenylethylene glycol by hepatic epoxide hydrolase (EH) or, to a minor extent, by conjugation with glutathione by glutathione *S*-transferases (GST) (Csanady *et al.*, 1994). SO induces high incidences of both benign and malignant tumors of the forestomach in both male and female rats and mice.

In addition, styrene has shown the ability to induce an increase in the number of lung tumors in CD-1 mice (Csanady *et al.*, 1994). Based on the available animal and human data, the International Agency for Research on Cancer (IARC) classified styrene as a possible human carcinogen (group 2B) and its principal metabolite, SO, as a probable human carcinogen (group 2A).

Moreover, the reproductive and developmental toxicity of styrene has been mainly studied in various experimental animals, reporting effects such as teratogenicity and postnatal development toxicity following exposures to high doses (Brown *et al.*, 2000). It also has been reported that a single intraperitoneal injection at a dose level of 1000 mg/kg of a compound containing low molecular weight styrene oligomers resulted in a significant decrease in the number of days required for opening of the vagina, as an indication of estrogenic activity, in immature female rats. In spite of the large number of studies on reproductive toxicity for animals, there is still a lack of data for the detailed mechanism of toxicity (Brown *et al.*, 2000; Naccarati *et al.*, 2003).

Excessive generation of reactive oxygen species has been shown to cause various damages to the plasma membrane and thought to be associated with sperm abnormalities (Kobayashi *et al.*, 1991). In the present study, we examined the effect of styrene on the counts and motilities of rat sperm cells and further characterized the relationship between toxic effects of styrene and oxidative stresses.

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MATERIALS AND METHODS

Animals and treatment

Specific pathogen-free 8-week old male Sprague-Dawley (SD) rats were purchased from Orient, Inc (Seoul, Korea). Following 1 week of acclimatization, 12 males showing normal weight gain and behavior were selected and randomly assigned to four groups consisting of three rats. The animal facility and the exposure chamber were maintained at 23 ± 3°C with 55 ± 15% humidity and light from 08:00 to 20:00 h. The air in the room was changed continuously. The animals were housed three per cage in stainless steel wire mesh cages and were fed commercial rodent chow (Harlan Co., USA) with tap water *ad libitum*. Clinical signs and viability of administered rats were observed once or twice daily during the course of the study. Individual body weights of animals were measured shortly before the administration.

Styrene (>99% purity), a liquid at room temperature was obtained from Sigma (Saint Louis, MO, USA). Styrene was resuspended in 5% (v/v) tween 80 solution and the four groups of rats were administered to 0, 50, 100, or 500 mg/kg/day styrene for 5 days.

Cell culture

RAW 264.7 murine macrophage cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2.

Evaluation of sperm motility

Animals were killed by inhalation of carbon dioxide and their blood was collected via the inferior vena cava and subjected to autopsy. The testes and epididymides were removed and weighed. Subsequently, the right caudal epididymis was minced in 10 ml Hank's balanced salt solution with 5 mg/ml of bovine serum albumin (pH 7.2) and the numbers of motile sperm cells were determined under a microscope. The sperm samples were mixed well with 1% Eosin Y and the morphological changes for 200 sperm cells were examined. The sperm cells were classified by normal, small head type, amorphous head type, two heads or two tails, excessive hook type, blunt hook type, detached tail type, folded tail type, or short tail type.

Measurement of ROS generation

The sperm cells were stained with 5 μM of 2,7-dichlorofluorescein diacetate (DCF-DA) for 20 min. Cells were washed twice with PBS, and were lysed by the addition of cold lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 100 ng/ml leupeptin, and 1 mM PMSF. The cell lysates were centrifuged at 15,800 ×*g* at 4°C for 3 min. The supernatant was collected and analyzed for determination of ROS generation. The intensity of DCF fluorescence was measured with excitation at 485 nm and emission at 535 nm by GENios Pro fluorometer (Maennedorf, Switzerland).

RT-PCR

Total RNA was extracted from rat sperm cells using a Trizol reagent (Gibco BRL, Gaithersburg, MD). The RNA (2 μg) were transcribed at 42°C for 1 h in a volume of 30 ml containing 5× RT-PCR buffer, 10 mM dNTP, 40 U RNase inhibitor, 200 U Maloney murine leukemia virus reverse transcriptase and 100 pmole of oligo-dT primer. One μl of reaction mixture from each samples was amplified with 10 pmole of each oligonucleotide primers (Table 1), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1.25 U of *Taq* DNA polymerase (Promega) in a final volume of 20 μl. PCR was performed as follows: One cycle of denaturing at 95°C for 5 min, followed by 25~35 cycles of denaturation at 95°C for 30 s, annealing at 58~64°C for 45 s, extension at 72°C for 1 min, and the final extension at 72°C for 10 min. The amplified PCR products were analyzed by 1.5% agarose gel

Table 1. RT-PCR primers and conditions used for this study

PCR primer		Sequence	Annealing temp (°C)	PCR cycle	Product size (bp)
SOD1	Sense	5'-AGG ATT AAC TGA AGG CGA GCA TG-3'	60	25	273
	Antisense	5'-GCC CAA GTC ATC TTG TTT CTC GT-3'			
SOD2	Sense	5'-GAT GTG TGG AGC ACG CTT ACT-3'	60	35	780
	Antisense	5'-CAC AAT GTC ACT CCT CTC CGA ATT A-3'			
Catalase	Sense	5'-TTA CTT TCT TGT TCA GCG ACC GA-3'	60	35	765
	Antisense	5'-CAC CTT CGT ATA GAA TGT CCG CA-3'			
GPX4	Sense	5'-TTA TTG AAG CCA GCA CTG CTG T-3'	58	30	113
	Antisense	5'-GAA TTC GTG CAT GGA GCG C-3'			
β-Actin	Sense	5'-CCT GAC CCT GAA GTA CCC CA-3'	60	25	273
	Antisense	5'-CGT CAT GCA GCT CAT AGC TC-3'			

electrophoresis and ethidium bromide staining.

Statistical analysis

All experimental data were represented as the mean ± SEM. Differences between the control group and the treated group were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's Pairwise Multiple Comparison *t*-test. The difference was considered statistically significant at $P<0.05$.

RESULTS

Gross observation

The present studies were undertaken to characterize whether styrene suppressed rat sperm motility via oxidative stresses. Administration of styrene at concentration of 50, 100, or 500 mg/kg into SD rats for 5 days did not cause any significant change in clinical signs and all rats survived until their scheduled necropsy. The total body weights and weights of testis and epididymides also remained at the control level (Table 2).

Effects of styrene on the spermatogenesis within the testis and epididymis

The numbers of sperm cells in testes and epididymides were counted and motility and quality of sperm were evaluated in each group of rats after 5 days of styrene administration (Table 3). Sperm counts were significantly lower in the 500 mg/kg group than the control group. The results of the sperm motility analysis were shown that the motility of sperm was significantly reduced in the 500 mg/kg group. In the 100 mg/kg group, sperm motility was slightly suppressed but not statistically significant. We also determined the abnormal phenotypes of sperm cells in each treated groups. Sperm abnormalities were significantly increased in the 500 mg/kg group compared to the control group. The major types of sperm abnormalities were the detached tail, excessive hook, amorphous head, blunt hook, and folded tail.

Effect of styrene on ROS generation and antioxidant enzyme expression

To evaluate whether styrene increases sperm abnormalities through ROS generation, the production of ROS was determined with isolated rat sperm cells. Fig. 1 showed that styrene treatment significantly enhanced ROS generation in RAW 264.7 murine macrophage cells and rat sperm cells. Because expression levels of various antioxidant enzymes such as SOD, catalase, glutathione peroxidase can modulate the intracellular ROS level, we further determined the effect of styrene on the mRNA expression of SOD1, SOD2, catalase, or GPX4 to elucidate the mechanism of induction by styrene on the intracellular ROS level. Fig. 2 showed that styrene significantly suppressed the mRNA expression of catalase and SOD2 although the levels of SOD1 or GPX4 were not strongly changed by styrene.

DISCUSSION

Styrene is one of the most extensively used chemical

Table 2. Organ weights of Sprague-Dawley male rats exposed to styrene for 5 days

Dose (mg/kg)	0	50	100	500
Absolute				
Body (g)	289.1 ± 6.3[a]	294.7 ± 7.6	287.1 ± 9.0	287.6 ± 5.2
Testis(g)	3.072 ± 0.022	3.133 ± 0.146	2.959 ± 0.149	3.041 ± 0.125
Epididymis (g)	0.687 ± 0.022	0.764 ± 0.028	0.742 ± 0.036	0.726 ± 0.003
Relative				
Testis (%)	1.063 ± 0.008	1.063 ± 0.050	1.031 ± 0.052	1.057 ± 0.044
Epididymis (%)	0.238 ± 0.008	0.259 ± 0.010	0.258 ± 0.013	0.252 ± 0.001

[a]Values represent mean ± S.E.M. (n=3).

Table 3. Examinations of rat testicular and epididymal sperm cells exposed to styrene for 5 days

Dose (mg/kg)	Sperm head count (× 10^6)		Percent motile sperm (%)	Percent sperm abnormality (%)
	testis	epididymis		
0	241.4 ± 13.2 (100%)	83.6 ± 10.6 (100%)	82.7 ± 1.5 (100%)	4.0 ± 1.3
50	203.5 ± 14.9 (84.3)	76.0 ± 2.6 (90.9)	80.3 ± 7.3 (97.1%)	6.8 ± 1.7
100	186.4 ± 3.4* (77.2)	63.2 ± 2.8 (75.6)	71.7 ± 11.5 (86.7%)	5.3 ± 2.7
500	185.1 ± 15.5* (76.7)	47.3 ± 3.7* (56.6)	19.5 ± 0.5* (23.6%)	38.3 ± 14.8*

[a]Values represent mean ± S.E.M. (n=3).
*Significant difference from the control ($p<0.05$).

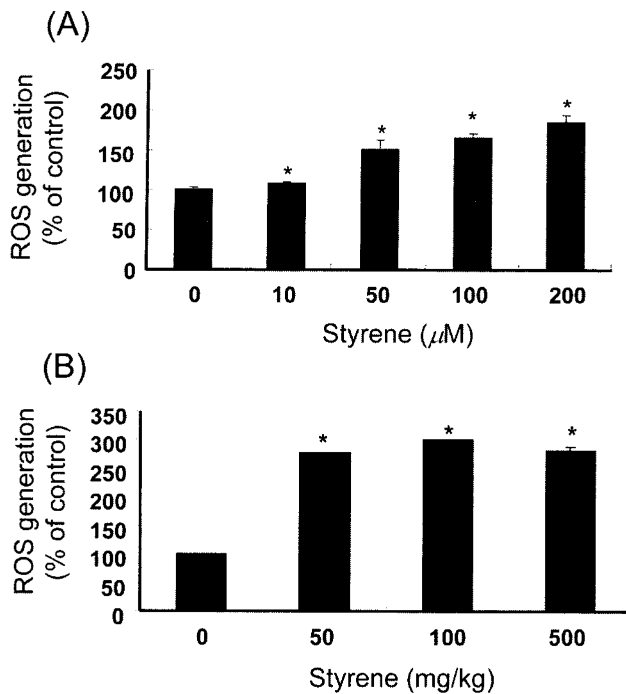

Fig. 1. Effect of styrene on ROS generation in the RAW 264.7 murine macrophage cells and epididymal sperm of adult rats. (A) RAW 264.7 cells were treated with 0, 10, 50, 100, or 200 mM styrene for 24 h. After staining with 5 μM DCF-DA for 20 min, the fluorescence was measured with excitation at 485 nm and emission at 535 nm. (B) Adult SD rats were administered with styrene (0, 50, 100, or 500 mg/kg) for 5 days. The epididymal sperm cells were isolated and stained with 5 μM DCF-DA for 20 min. The values represent the mean ± SEM of three independent determinations. *Significant different from the control ($p<0.05$).

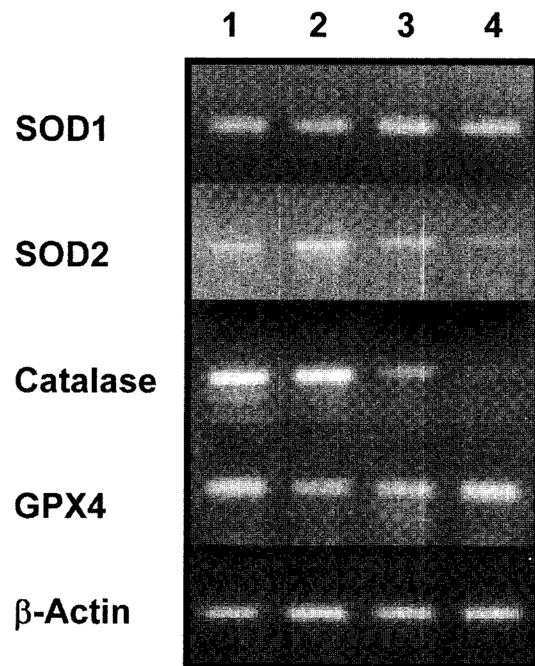

Fig. 2. Effect of styrene on the mRNA expression of antioxidant enzymes. The expression levels of mRNA of SOD1, SOD2, catalase, or GPX4 were determined by RT-PCR with total RNA from the epididymal sperm cells of adult SD rats, which were treated with 5% tween 80 (lane 1), styrene 50 mg/kg (lane 2), styrene 100 mg/kg (lane 3), or styrene 500 mg/kg (lane 4) for 5 days. Expression of β-actin mRNA was determined as a loading control.

to which thousands of workers worldwide are exposed. Occupational exposure in hand lamination work in the reinforced plastics industry may entail a daily intake of grams of styrene via inhalation. Styrene is also contaminated in small amounts in food products, tobacco smoke and engine exhausts. Therefore, it may be very important to study the possible toxic effects of this chemical. Although styrene is regarded as one of the endocrine disruptors, the experimental evidences were still not enough. To evaluate the effects of high concentration of styrene exposure on male reproductive functions, sperm production and sperm functions were evaluated. Although styrene did not cause any significant changes in body and male reproductive organ weights, decreased numbers of sperm cells and sperm motilities were observed significantly in high concentration of styrene. Sperm abnormalities were also observed in 500 mg/kg-treated groups.

Reactive oxygen species (ROS) such as hydroxyl radicals (•OH), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) are generally produced during the cellular function. They are very unstable due to their high chemical reactivity that leads to lipid peroxidation and oxidation of some enzymes, and a massive protein oxidation and degradation (Mates *et al.*, 1999). The role of oxygen-derived species in causing cell injury or death is increasingly studied. The generated epoxides may spontaneously react with nucleophiles in the cell and thereby covalently bind to DNA, RNA and protein (Mates and Sanchez-Jimenez, 1999). Such a reaction may lead to cellular cytotoxicity, mutagenicity, and carcinogenicity.

The detrimental effects of ROS on mammalian sperm cells were suggested more than 50 years ago. The susceptibility of sperm cells to oxidative stress stems from the abundance of unsaturated fatty acids in the sperm plasma membrane. These unsaturated fatty acids provide fluidity that is necessary for sperm motility, and membrane fusion for fertilization. However, under oxidizing conditions, lipid peroxidation is increased and leads to rapid loss of sperm motility and eventual sperm cell death (Alvarez *et al.*, 1987; Aitken *et al.*, 1989).

In most cells, there exist multiple defense mechanisms designed to protect ROS-induced injury. In sperm cells, the endogenous antioxidant enzymes including superoxide dismutase (Mennella and Jones, 1980), catalase (Jeulin *et al.*, 1989), and glutathione peroxidase (Alvarez and Storey, 1989) may be involved in protecting cells from oxidative stress. Superoxide dismutase destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by catalase or GPX. Of three classes of SOD enzymes in mammalian cells, cytosolic Cu, Zn-SOD (SOD-1) are believed to play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals, to form hydrogen peroxide and molecular oxygen. These enzymes are conserved throughout evolution, which usually have two identical subunits of about 32 kDa, each containing a metal cluster, the active site, constituted by a copper and a zinc atom bridged by a common ligand, His 61 (Banci *et al.*, 1998). Mn-SOD (SOD2) is a mitochondrial homotetramer (96 kDa) containing one manganese atom per subunit that cycles from Mn(III)–Mn(II) and back to Mn(III) during the two step dismutation of superoxide. The respiratory chain in mitochondria is a major source of oxygen radicals (Guan *et al.*, 1998).

Catalase is a heme (ferriprotoporphyrin) protein consisting of four identical tetrahedrally arranged subunits of 60 kDa. This enzyme has been identified in most mammalian tissues including testis, generally detectable in peroxisomes. Catalase reacts with hydrogen peroxide (H_2O_2) to form water and molecular oxygen and is able to protect the cell from the effects of hydrogen peroxide.

Glutathione peroxidase is a selenium-containing peroxidase which catalyzes the reduction of a variety of hydroperoxides such as ROOH and H_2O_2 using GSH, thereby protecting mammalian cells against oxidative damage (Imai *et al.*, 1998). There are at least five GPX isozymes including GPX1, GPX2, GPX3, GPX4, and GPX5 found in mammals. Among them, GPX4 is highly expressed in testes.

In this study, we have determined four antioxidant enzymes mRNA in sperm cells from rats treated with styrene up to 500 mg/kg for 5 days. The results presented in Fig. 2 showed that treatment with styrene strongly suppressed catalase and SOD2 expression in a dose-dependent manner while the expression of SOD1 or GPX4 was not significantly changed. It has been reported that SOD activity in human spermatozoa showed a significant correlation to the number of motile spermatozoa (Kobayashi *et al.*, 1991) and significant catalase activities were found in migrated, motile spermatozoa (Jeulin *et al.*, 1989). The superoxide anion would be to undergo dismutation to H_2O_2 at the near neutral pH values (pH 6.6~6.7) typical of sperm cytosol by SOD and excess H_2O_2 may be degraded by catalase (Babcock *et al.*, 1983). Thus, SOD or catalase depletion results in an immediate rise of hydroperoxyl radical (HCOO•) which is potentially pernicious.

From the present findings it appears that suppression of catalase and SOD2 expression could be primary cause for styrene-mediated changes of sperm motility and abnormalities. Down-regulation of catalase or SOD2 mRNA expression by styrene may enhance oxidative stresses and lipid peroxidation in sperm cells. These findings suggest that antioxidant enzymes may play an important role in styrene-mediated reproductive toxicity.

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REFERENCES

Aitken, R.J., Clarkson, J.S. and Fishel, S. (1989): Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol. Reprod.*, **41**, 183-197.

Alvarez, J.G. and Storey, B.T. (1989): Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.*, **23**, 77-90.

Alvarez, J.G., Touchstone, J.C., Blasco, L. and Storey, B.T. (1987): Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J. Androl.*, **8**, 338-348.

Babcock, D.F., Rufo, G.A. Jr. and Lardy, H.A. (1983): Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. *Proc. Natl. Acad. Sci. USA*, **80**, 1327-1331.

Banci, L., Benedetto, M., Bertini, I., Del Conte, R., Piccioli, M. and Viezzoli, M.S. (1998): Solution structure of reduced monomeric Q133M2 copper, zinc superoxide dismutase (SOD). Why is SOD a dimeric enzyme? *Biochemistry*, **37**, 11780-11791.

Bond, J.A. (1989): Review of the toxicology of styrene. *Crit. Rev. Toxicol.*, **19**, 227-249.

Brown, N.A., Lamb, J.C., Brown, S.M. and Neal, B.H. (2000): A review of the developmental and reproductive toxicity of styrene. *Regul. Toxicol. Pharmacol.*, **32**, 228-247.

Csanady, G.A., Mendrala, A.L., Nolan, R.J. and Filser, J.G. (1994): A physiologic pharmacokinetic model for styrene and styrene-7,8-oxide in mouse, rat and man. *Arch. Toxicol.*, **68**, 143-157.

Gadberry, M.G., DeNicola, D.B. and Carlson, G.P. (1996):

Pneumotoxicity and hepatotoxicity of styrene and styrene oxide. *J. Toxicol. Environ. Health*, **48**, 273-294.

Guan, Y., Hickey, M.J., Borgstahl, G.E., Hallewell, R.A., Lepock, J.R., O'Connor, D., Hsieh, Y., Nick, H.S., Silverman, D.N. and Tainer, J.A. (1998): Crystal structure of Y34F mutant human mitochondrial manganese superoxide dismutase and the functional role of tyrosine 34. *Biochemistry*, **37**, 4722-4730.

Imai, H., Narashima, K., Arai, M., Sakamoto, H., Chiba, N. and Nakagawa, Y. (1998): Suppression of leukotriene formation in RBL-2H3 cells that overexpressed phospholipid hydroperoxide glutathione peroxidase. *J. Biol. Chem.*, **273**, 1990-1997.

Jeulin, C., Soufir, J.C., Weber, P., Laval-Martin, D. and Calvayrac, R. (1989): Catalase activity in human spermatozoa and seminal plasma. *Gamete Res.*, **24**, 185-196.

Kobayashi, T., Miyazaki, T., Natori, M. and Nozawa, S. (1991): Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Hum. Reprod.*, **6**, 987-991.

Mates, J.M. and Sanchez-Jimenez, F. (1999): Antioxidant enzymes and their implications in pathophysiologic processes. *Front. Biosci.*, **4**, D339-345.

Mates, J.M., Perez-Gomez, C. and Nunez de Castro, I. (1999): Antioxidant enzymes and human diseases. *Clin. Biochem.*, **32**, 595-603.

Mennella, M.R. and Jones, R. (1980): Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal-ion-catalysed lipid-peroxidation and reactions in semen. *Biochem. J.*, **191**, 289-297.

Naccarati, A., Zanello, A., Landi, S., Consigli, R. and Migliore, L. (2003): Sperm-FISH analysis and human monitoring: a study on workers occupationally exposed to styrene. *Mutat. Res.*, **537**, 131-140.

Nakajima, T., Elovaara, E., Gonzalez, F.J., Gelboin, H.V., Vainio, H. and Aoyama, T. (1993): Characterization of the human cytochrome P450 isozymes responsible for styrene metabolism. *IARC Sci. Publ.*, 101-108.

Sumner, S.C., Cattley, R.C., Asgharian, B., Janszen, D.B. and Fennell, T.R. (1997): Evaluation of the metabolism and hepatotoxicity of styrene in F344 rats, B6C3F1 mice, and CD-1 mice following single and repeated inhalation exposures. *Chem. Biol. Interact.*, **106**, 47-65.