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Ovarian toxicity of benzene in rat

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Abstract: *Present report describes ovarian toxicity of benzene in rat. Effects of benzene in ovary of rat have been attributed to increased lipid peroxidation, alterations in progesterone values and activity of $CYP_{450}2E1$. Histopathological observations confirm ovarian toxicity of benzene. Number of primodial follicles decreased after benzene treatment. Cortex, medulla and corpus luteum also exhibited pathological changes. It is concluded that female reproductive system is vulnerable to benzene. Since a number of female workers are exposed to organic solvents like benzene through their work environment, present observations are important from occupational health point of view.*

Key Words: *Benzene, Ovary, Lipid peroxidation, Progesterone, $CYP_{450}2E1$.*

Introduction

Several environmental xenobiotics are known to impair reproductive health in man and animals. During last few years, a number of studies have provided important insights into how toxicants alter ovarian function. Environmental chemicals may act directly through receptor mediated pathways mimicking endocrine signals to and from the ovary. Alternatively, the chemicals may disrupt the hypothalamus – pituitary cell functions resulting in secondary ovarian dysfunction. Classic example of compounds that manifest the receptor mediated ovarian toxicity include diethylstilboesterol (Gray *et al.,* 1996) methoxychlor (Martinez *and Swartz, 1991*), dioxins (IARC, 1987) and phthalates (Lovekamp-Swan and Davis, 2003).

The ovaries are target organs for injury caused by many chemicals (Mattison et al., 1990). Alkylating agents have been shown to destroy oocytes in both humans and experimental animals (Barber, 1981). Mercury and cadmium can also produce ovarian damage (Mattison, 1983). 1-bromopropane induces ovarian dysfunction in non-pregnant female rats associated with disruption in follicular growth (Yamada *et al.,* 2003). Dioxins also inhibit follicular development (Heiden *et al.,* 2006)

Benzene toxicity seriously compromises the immune and endocrine systems. Occupational exposure to benzene has led to a number of leukemias (Goldstein, 1988; Maltoni *et al.,* 1989). Benzene induces toxicity through free radical mediated mechanisms (Verma and Rana, 2004). Toxic effects of gasoline and additives range from induction of cancer to disruption of haematopoiesis and endocrine functions (Schlosser *et al.,* 1993; Orzechowski *et al.,* 1995). The leucotoxic action of benzene is potentiatied by administration of female hormones to male rabbits (Desoille *et al.,* 1962). Benzene depleted $CYP_{450}2E1$ in castrated rats, however, the enzyme was restored after testosterone treatment (Verma and Rana, 2008). Women smokers and those exposed to environmental tobacco smoke containing various organic vapours have reduced ovarian function as evidenced by an earlier menopause, reduced follicular numbers, decreased levels of circulating estradiol and decreased conception rates (Vidal *et al.,* 2006). However, female reproductive toxicity of benzene remains poorly known. Recently, benzene toxicity in testis of rat

was attributed to oxidative stress (Singh and Rana, 2009). Can oxidative stress induced by benzene contribute in ovarian toxicity too? To address this question, present investigations were undertaken in rat. We describe the effects of benzene on lipid peroxidation, reduced glutathione, cytochrome $P_{450}2E1$ and histopathology of ovary in this communication. Changes in serum progesterone values have also been described.

Materials and Methods

Healthy three months old female Wistar rats (130 ± 20g) were procured from animal facility of Jamia Hamdard, New Delhi. They were maintained under standard laboratory conditions (room temperature -25°C ± 5°C, relative humidity -60% ± 10%) in the animal house of Department of Zoology, Ch. Charan Singh University, Meerut. Each rat was housed in a separate polypropylene cage, offered commercial food pellets (Golden Feeds, New Delhi) and water ad libitum. These experiments were performed after the approval of institutional *animal ethical committee*. Benzene was procured from S. Merk (Mumbai) whereas olive oil was purchased from (Olemessa, New Delhi).

Treatments : Rats were divided at random into four groups each containing five rats. Rats of group A, intramuscularly injected with 0.2 ml olive oil/100 g body weight served as controls. Each rat of group B was treated with predetermined sublethal dose of benzene (0.2 ml/100 g body weight of 2% benzene prepared in olive oil) through intramuscular injection as described earlier on each alternate day for 30 days (Verma and Rana, 2004). Rats of group C received the similar treatment for 45 days whereas rats of group D were similarly treated for 60 days.

After respective treatment, rats were starved overnight and sacrificed next morning by light ether anesthesia. Ovaries were carefully removed, weighed and processed for following observations. Blood was collected through cardiac puncture. Serum was separated through centrifugation and processed for the estimation of progesterone.

Lipid peroxidation : Malondialdehyde, a lipid peroxidation product was estimated in ovary by the method described by Jordan and Schenkman (1982). The absorbance was recorded at 532 nm using a spectrophotometer (Systronics, India). 1,1,3-tetramethoxypropane (Sigma, USA) was used as standard. Total proteins in the ovary were determined following the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma, USA) as the standard.

Reduced glutathione (GSH) : Samples of ovary were homogenized in ice cold 1.15% KCl, (4 volumes) using a Potter Elevhzem Homogenizer and centrifuged. Sulfosalicylic acid was used for protein precipitation. GSH was estimated using Ellman's reagent. 5,5-dithiobis-2-nitrobenzoic acid (Sigma, USA). Absorbance was recorded at 412 nm. GSH values were calculated by comparing absorbance values with a standard curve generated from known GSH concentrations (Ellman, 1959).

$CYP_{450}2E1$: Microsomes were prepared using an ultracentrifuge (Sorvall, USA). Enzyme activity in microsomal preparations of the ovary were estimated according to the method of Koop (1986). Briefly the reaction mixture consisted of microsomal protein (0.2 mg mL^{-1}). 0.1 Mol L^{-1} potasium phosphate, pH 6.8, 1 M Mol L^{-1} p-nitrophenol. Sample were incubated at 37°C for 3 minute prior to the addition of NADPH to start the reaction. After 10 min., the reaction was stopped with 1.5 Mol L^{-1} perchloric acid. Formation of p-nitrocatechol was measured spectrophotometrically at 510 nm.

Progesterone : Serum samples were analysed for progesterone using an ELISA kit supplied by Equipar Diagnostics (Italy). The absorbance was recorded at 450 nm using microplate reader (Electronic Corporation, India).

Histopathological observations : Ovaries were fixed in 10% neutral formaline for 24 hrs. at room temperature. These samples were embedded in paraffin. Six micron thick sections,

stained with hematoxylin and eosin were examined under a research microscope (Nikon, Japan).

Statistics : The data were statistically analyzed through student's "t" test using SPSS software.

Results and Discussion

The biological observations showed that benzene exposure increased body weight of female rats, corresponding to the duration of treatment i.e. 30 days, 45 days and 60 days. A progressive increase in the body weight was observed reciprocal to the duration of exposure to benzene. Gonadosomatic indices were calculated in rats of all the groups. The highest gonadosomatic values were recorded after 30 days of benzene treatment. However, it decreased after 45 and 60 days of treatments with benzene (Table 1). Serum progesterone values increased after benzene treatment(s). The highest value was recorded after 45 days of treatment of benzene. The protein concentration in ovary decreased in all the experimental rats. The highest depletion was recorded after 60 days of benzene treatment.

Benzene is a potent inducer of lipid peroxidation. It induced LPO in ovary also. Maximum LPO was induced in the ovary after 60 days of treatment. It increased progressively with the duration of treatment. Result on GSH in ovary of benzene treated rats showed a biphasic response. Its value decreased after 30 days of treatment but increased after 45 days and 60 days of treatments (Table 1).

Significant histopathological changes were observed in the ovary of benzene treated rats. Number of primordial cells resting in the outer cortex was significantly reduced after 30 days of benzene treatment. Degeneration of primordial follicles increased with progesterone benzene treatments (Fig. 1). Most striking effects of benzene were observed in the cortex and medulla of ovary. Constriction of cortex and medulla both was recorded after 45 days of benzene treatment (Fig. 2). However, corpus luteum also showed pathological changes. Granulosa cells that line the primordial follicles were found damaged after 60 days of benzene treatment (Fig. 3). Primary follicles did not show pathological changes. However, granulose cells were injured (Fig. 4).

Injury to endocrine structures leading to dysfunction of glands is typical of many toxic conditions. Specific lesions of the female reproductive system can be induced by a direct or indirect action of the toxin. Benzene can be classified as an indirectly acting toxin as it requires metabolic activation for its toxicity (Rana and Verma, 2005). It has been shown that ovary has microsomal monoxygenases viz.: epoxide hydrases and transferases capable of metabolizing many xenobiotics (Mukhtar *et al.*

Table 1. Range of variation and average values along with standard error of total length, total weight, gut length, gut weight, stomach weight, liver weight of *Sperata seenghala, Xenentodon cancila* and *Labeo boggut.*

S. No.	Species name	Total length (cm)	Total weight (g)	Gut length (cm)	Gut weight (g)	Stomach weight (g)	Liver weight (g)
1.	*Sperata seenghala*	15.2 - 38.0 26.76 ± 1.15	25.0 - 315.0 112.63 ± 13.14	15.1 - 30.5 20.33 ± 0.90	1.5 - 8.25 3.51±0.37	1.01 - 6.12 2.54 ± 0.29	0.19 - 2.5 0.81 ± 0.11
2.	*Xenentodon cancila*	10.5 - 30.2 22.4 ± 0.95	10.0 - 70.0 35.5 ± 3.14	4.5 - 14.2 8.85 ± 0.48	0.19 - 3.41 1.23 ± 0.16	0.11 - 3.03 0.59 ± 0.16	0.11 - 2.02 0.65 ± 0.11
3.	*Labeo boggut*	14.0 - 20.1 17.53 ± 0.36	30.0 - 87.0 64.09 ± 3.11	131.0 - 238.0 165.31 ± 6.22	1.1 - 5.56 3.15 ± 0.30	0.11 - 0.35 0.21± 0.01	0.04- 0.81 0.37 ± 0.05

1978). Benzene can also be metabolized by ovary and the polar metabolites this formation many undergo conjugation or be exerted directly. Since metabolites of benzene i.e. phenol, hydroquinone, catechol and trans trans micronic acid are chemically reactive and are capable of interacting with cellular macromolecules, they may significantly contribute in the ovarian toxicity of benzene.

Present results show that benzene induces lipid peroxidation in the ovary. The rate of lipid peroxidation increased with the increase in duration of benzene treatment. No similar reports on benzene induced LPO in the mammalian ovary are available, however, several reports including our own observations that attribute benzene toxicity to lipid peroxidation in liver, kidney, lungs, testis support present observations (Arinc, *et al.,* 1991; Anders, 1988).

Direct effect of benzene on ovary was indicated by observations on progesterone. Its values decreased after 30 days of treatment then increased after 45 days of treatment and then again decreased after 60 days of treatment. These results indicate the differential effects of benzene on corpus luteum that secretes progesterone. We speculate that benzene might not work as an oestrogenic chemical unlike aldrin, DDT and PCB that interfere with mammalian reproduction through oestrogenic alterations (Mattison *et al.,* 1981; and Bulger and Kupfer, 1983).

The result shows that GSH values in ovary declined after 30 days of benzene treatment. Similar observations have been made earlier in liver and kidney also (Verma and Rana, 2003). However, its values increased after 45 and then 60 days of treatment. This adaptive function of GSH is known for many xenobiotics and can be confirmed during ovarian toxicity caused by benzene (Allen *et al.,* 2004).

$CYP_{450}2E1$ is the main enzyme participating in the metabolism of benzene (Snyder *et al.,* 1993). Hormonal regulation/expression of this enzyme has also been reported (Kato and Kamataki, 1982). Alterations in the status of ovarian hormones might have caused changes in the enzyme activity. This observation is due to relationship between progesterone and $CYP_{450}2E1$ enzyme. When progesterone values

Table 2. Effect of benzene on selected parameters in the ovary of rat

Parameters	Control (A)	Duration of benzene treatment		
		30 days (B)	45 days (C)	60 days (D)
Body weight (g)	188 ± 7.84	220.4 ± 11.08^{NS}	234.2 ± 9.04*	282 ± 16.39^{NS}
Ovary weight (g)	0.132 ± 0.009	0.218 ± 0.04	0.188 ± 0.007*	0.196 ± 0.01
Gonadosomatic index	0.0476 ± 0.002	0.084 ± 0.012^{NS}	0.052 ± 0.003*	0.052 ± 0.003*
Progesterone	0.582 ± 0.023	0.5994 ± 0.029*	0.713 ± 0.012*	0.613 ± 0.017*
Protein (g/dl)	11.99 ± 0.474	9.68 ± 0.293^{NS}	10.02 ± 0.148*	7.19 ± 0.670^{NS}
Lipid peroxidation (n mol malondialdehyde mg/protein)	0.023 ± 0.001	0.033 ± 0.007*	0.044 ± 0.005*	0.051 ± 0.005^{NS}
GSH (µmol/mg protein)	0.398 ± 0.022	0.0186 ± 0.057^{NS}	0.316 ± 0.035*	0.436 ± 0.022*
$CYP_{450}2E1$	9.500 ± 0.830	16.500 ± 1.670^{NS}	27.24 ± 2.240*	21.00 ± 1.140^{NS}

Results are expressed as mean ± SE (n = 5)

* Values are significantly different from controls ($p<0.05$)

NS- Non significant.

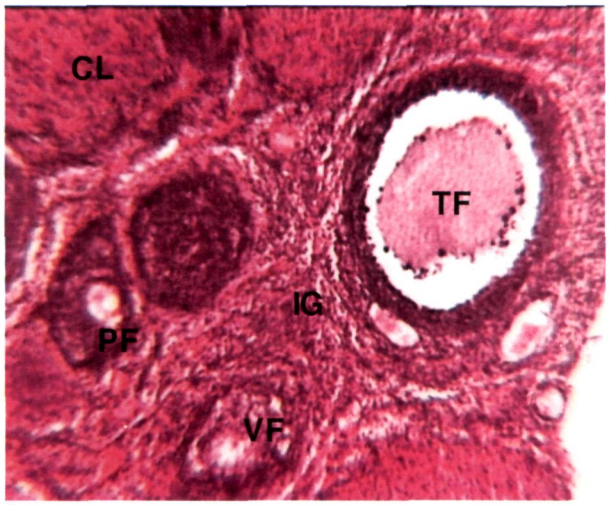

Fig. 1. T.S. of the ovary of a rat treated with olive oil only showed intact follicles and corpus luteum [CL: corpus lutem; PF: Primordial follicle; IG: Interstitial gland; TF: tursary follicle; VF: Vesicular follicle]. H/E ×400

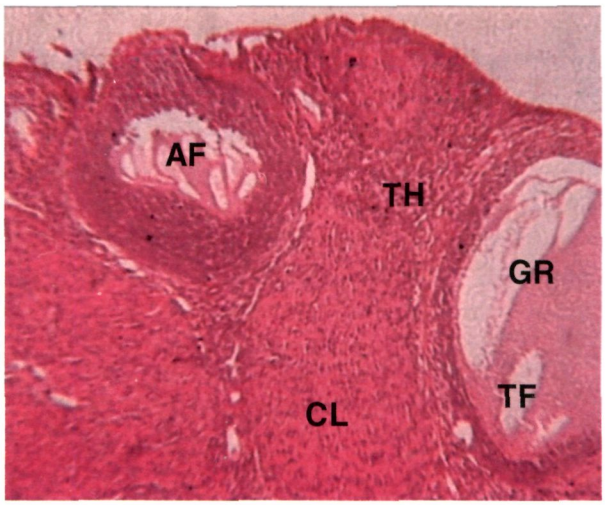

Fig. 2. T.S. of a ovary of a rat treated with benzene for 30 days showed reduced cortex and medulla. Moreover, atratic follicles (AF) and tersary follicles (TF) were also found injured. H/E ×400

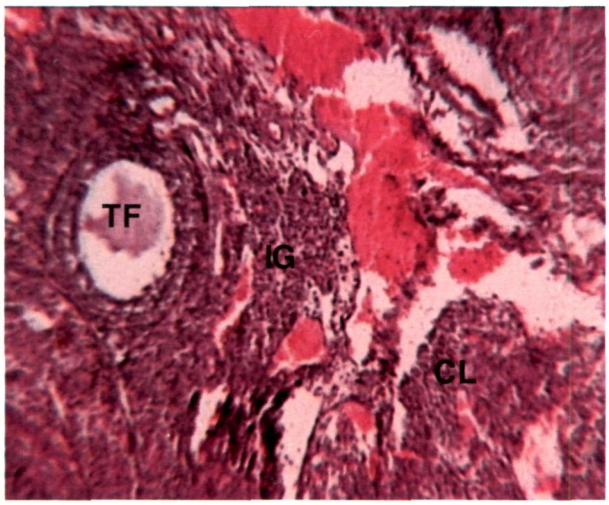

Fig. 3. T.S. of a ovary of a rat treated with benzene for 45 days showed increased degeneration of primodial follicles (PF). Corpus lutem (CL) and interstitial were also found damaged. H/E ×400

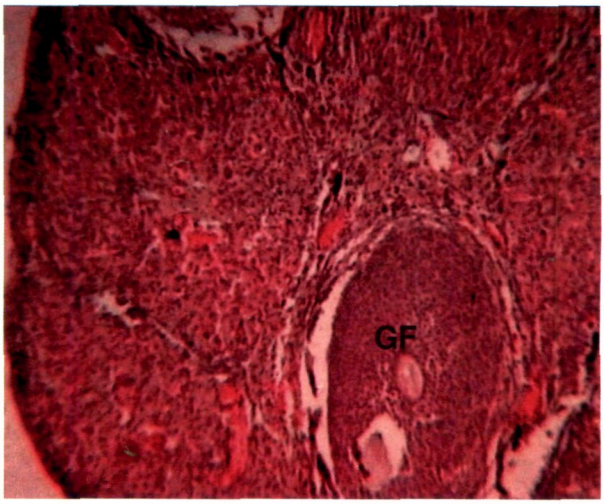

Fig. 4. T.S. of the ovary of a rat treated with benzene for 60 days shows changes in the graffian follicle (GF). H/E ×400

increased, the enzyme activity also increased. Although, only a few CYP_{450} isozymes associated with bio-transformation of drugs manifest under oestrogenic control, present results suggest that CYP_{450}2E1 in the ovary is influenced by progesterone. Further, investigations are needed to support this hypothesis.

Histopathological observations show that ovaries are also one of the target organ of benzene toxicity. Granulosa cells possess many sites of vulnerability to chemical insult. During present study also granulose cells were found injured. Similarly thecal cells might be the targets of chemical injury. Agents that impair cell proliferation, migration and communication may affect thecal cell function. Oocytes like granulose cells and thecal cells can also be injured by a wide variety of chemicals. We observed that

prolonged treatments with benzene caused constriction of both, cortex and medulla. Most of the PAH compounds have significant endocrine and reproductive effects and are known to cause ovarian tumors in mice and rats. It is possible that ability of these PAH compounds to induce ovarian tumors may correlate with their ability to bind with aryl hydrocarbon receptors (AhR).

Present observations confirm that benzene toxicity is ovary in mediated by oxidative stress. Further its effects on progesterone and consequent changes in $CYP_{450}2E1$ enzyme activity also contribute in its ovarian toxicity. However, knowledge about pathologic mechanisms of female reproductive damage is limited. Effects may be direct when environmental or occupational agents interact with specific reproductive target cells or indirect when they act on endocrine or other systems. The ovaries and ova are susceptible to direct damage for an extended period of time from meiosis through ovulation. The xenobiotics may interfere with the hypothalamic-pituitary-ovarian axis at different levels by modifying the secretion of prolactin, adrenocortical steroids, thyroid hormones and sex hormones. Current evidence indicates that female reproductive system is vulnerable to benzene. Since the number of working women are increasing word wide they are in the risk of being exposed to organic solvents. Some of these women are in the reproductive age and half of the women are pregnant and out of these 20 prevent are expose to chemicals which is of potential concern. (Foster 2003;Kumar, 2004). Therefore, a baseline information on organic solvents toxicity to female reproductive system will be important from occupational health point of view.

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References

Allen, T., Singhal, R. and Rana, S.V.S. (2004) Resistance to oxidative stress in a freshwater fish Channa punctatus after exposure to inorganic arsenic. *Biol. Trace Element Res.*, **98**, 63-72.

Anders, M.W. (1988) Bioactivation mechanisms and hepatocellular damage. In: Arias IM, Jakoby WB, Popper H, Schachter D and Shafritz DA (Eds). The Liver: Biology and Pathology. Second ed Raven. Press, New York. pp. 389-400.

Arinc, E., Adali, A., Iscan, M. and Guray, T. (1991) Stimulatory effects of benzene on rabbit liver and kidney microsomal cytochrome P450 dependent drug metabolizing enzymes. *Arch. Toxicol.*, **65**, 186-190.

Barber, H.R.K. (1981) The effect of cancer and its therapy upon fertility. *Int. J. Fertil.*, **26**, 250-259.

Bulger, W.H. and Kupfer, D. (1983) Estrogenic action of DDT analogs. *Am. J. Ind. Med.*, **4**, 163 -173.

Desoille, H., Philbert, M. and Albahary, C. (1962) Effects of *hormones on chronic benzene poisoning in rabbits.* II. Influence of sex, castration, homologous or heterologous sex hormone therapy on leukocytosis in rabbits exposed to short-term (6 weeks) benzene poisoning. *Arch. Mal. Path.*, **23**, 209-218.

Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem.*, **82**, 70-77.

Foster, W.G. (2003) Environmental toxicants and human fertility. Minerva. *Ginecol.*, **55**, 451-457.

Goldstein, B.D. (1988) Benzene toxicity. *Occup. Med. Rev.*, **3**, 541-554.

Gray, K., Bullock, B., Dickson, R., Raszmann, K., Walmer, D., McLachlan, J. and Merlino, G. (1996) Potentiation of diethylstilbestrol-induced alterations in the female mouse reproductive tract by transforming growth factor-alpha transgene expression. *Mol. Carcinog.*, **17**, 163-173.

Heiden, P.K., Cawen, M.J. and Hutz, R.J. (2006) Inhibition of *follicular development, vitellogenesis and serum* 17 beta-estradiol concentrations in zebafish following chronic sublethal dietary exposure to 2,3,7,8-tetra chlorodibenzo-p-dioxin. *Toxicol. Sci.*, **90**, 490-499.

IARC (1987) IARC monographs on the evaluation of carcinogenic risks to humans. Overall evaluations of carcinogenicity: An updating of IARC Monographs Volumes 1 to 42. Suppl 7. Lyons, France: World Health Organization, International Agency for Research on Cancer, 38-74.

Jordan, R.A. and Schenkman, J.B. (1982) Relationship between *malondialdehyde production and arachidonate* consumption during NADPH-supported microsomal lipid peroxidation. Biochem. *Pharmacol.*, **31**, 1393-1400.

Kato, R. and Kamataki, T. (1982) Cytochrome P-450 as a determinant of sex difference of drug metabolism in the *rat. Xenobiotica*, **12**, 787-800.

Koop, D.R. (1986) Hydroxylation of p-nitrophenol by rabbit ethanol inducible cytochrome P450 isozymes. *Mol. Pharmacol.*, **29**, 399-404.

Kumar, S. (2004) Occupational exposure associated with reproductive dysfunction. *J. Occup. Hlth.*, **46**, 1-19.

Lovekamp-Swan, T. and Davis, B.J. (2003) Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ. Hlth. Perspect.*, **111**, 139-145.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol *reagent. J. Biol. Chem.*, **193**, 265-275.

Maltoni, C., Ciliberti, A., Cotti, G., Conti, B. and Belpoggi, F. (1989)

Benzene an experimental multipotential carcinogen, results of the long-term bioassay performed at the Bologna Institute of Oncology. *Environ. Hlth. Perspect.*, **82**, 109-124.

Martinez, E.M. and Swartz, W.J. (1991) Effects of methoxychlor on the reproductive system of the adult female mouse. 1 Gross and histologic observations. *Reprod. Toxicol.*, **5**, 139-147.

Mattison, D.R. (1983) The mechanisms of action of reproductive toxins. *Am. J. Ind. Med.*, **4**, 65-79.

Mattison, D.R., Chang, L., Thorgeirsson, S.S. and Shiromizu, K. (1981) The effects of cyclophosphamide, azathioprine, and 6-mercaptopurine on oocyte and follicle number in C57BL/6N mice. *Res. Commun. Chem.* Pathol. *Pharmacol.*, **31**, 155-161.

Mattison, D.R., Plowchalk, D.R., Meadows, M.J., Al-Juburi, A.Z., Gardy, J. and Malek. A. (1990) Reproductive toxicity male and female reproductive systems as targets for chemical injury. *Med. Clin. N. Am.*, **74**, 391-411.

Mukhtar, H., Philpot, R.M. and Bend, J.R. (1978) Epoxide-metabolizing enzyme activities and cytochrome P-450 content of rat ovaries during pregnancy. *Biochem. Biophys. Res. Commun.*, **81**, 89-98.

Orzechowski, A., Schwarz Schwegler, U., Boak, W., Snyder, R. and Schrenk, D. (1995) Benzene metabolism in rodent hepatocytes, role of sulphate conjugation. *Xenobiotica*, **25**, 1093-1102.

Rana, S.V.S. and Verma, Y. (2005) Biochemical toxicity of benzene. *J. Env. Biol.*, **26**, 157-168.

Schlosser, P.M., Bond, J.A. and Medinsky, M.A. (1993) Benzene and phenol metabolism by mouse and rat liver microsomes. *Carcinogenesis* **14**, 2477-2486.

Singh, A. and Rana, S.V.S. (2009) Role of oxidative stress in testicular toxicity of benzene in rat. *J. Ecophysiol. Occup. Hlth.*, **9**, 11-16.

Snyder, R., Witz, G. and Goldstein, B.D. (1993) The toxicology of benzene. Environ. *Hlth. Perspect.*, **100**, 293-306.

Verma, Y. and Rana, S.V.S. (2003) Gender differences in the metabolism of benzene, toluene and trichloroethylene in rat with special reference to certain biochemical parameters. *J. Environ. Biol.*, **24**, 135-140.

Verma, Y. and Rana, S.V.S. (2004) Sex differences in oxidative stress induced by benzene *ind* rats. *Ind. J. Exp. Biol.*, **42**, 117-120.

Verma, Y. and Rana, S.V.S. (2008) Effects of progesterone on benzene toxicity in rats. Arch. Hig. Rada. Toksikol. 59, 1-9.

Vidal, J.D., Vonde Vort, C.A., Maucus, C.B., Lazarewicz, N.R. and Conley. A.J. (2006) In vitro exposure to environmental tobacco smoke induces CYP1B1 expression in human luteinized granulose cells. *Reprod. Toxicol.*, **22**, 731-737.

Yamada, I., Tehihara, G., Wang, H., Yu, X., Maeda, K., Tsukamura, H., Kamijima, M., Nakajima, T. and Takeuchi, Y. (2003) Exposure to 1-bromo-propane causes ovarian dysfunction in rats. *Toxicol. Sci.*, **71**, 96-103.