Testicular changes in rat exposed to Trichloroacetic Acid (TCA) during organogenesis

Author(s): Royana Singh

Vol. 16, No. 1 (2005-01 - 2005-03)

Biomedical Research 2005; 16 (1): 45-52

Royana Singh

Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Key words: Trichloroacetic acid, Humic acid, fulvic acid, disinfection byproducts, Apoptosis

Accepted September 29 2004

Abstract

Halogenated hydrocarbons such as Trichloroacetic acid (TCA) are among the most common water supply contaminants in the world. The study was, therefore undertaken to examine the effect of Trichloroacetic acid on the developing testis of Charles foster rat. The rats were randomly placed in the test groups and exposed to various concentrations of TCA i.e. 1000,1200,1400,1600 and 1800 mg/Kg body weight by oral gavage throughout the period of organogenesis, from day 6 to 15 of gestation. TCA was administered in the form of sodium trichloroacetate, which in the body is reduced to trichloroacetic acid by P450 enzyme in the liver. TCA administration led to dose related reduction in the fetal weight and the testicular weight, when they were collected on day 19 of ges-tation. Control mothers were administered equal volume of distilled water. Histological studies of the testis when compared to the controls revealed decrease in the length and diameter of the seminiferous tubules with 1400 mg/kg and higher doses. The rapid assault on the cellular components with the increase in the TCA concentration causing enhanced apoptosis of the gonocytes were well evident, with the sub- sequel lying in the total reduction in the testicular size.

Introduction

Halogenated hydrocarbon such as Trichloroacetic acid (TCA) and the other related compounds are among the most common water supply contaminants in the world. Purification of drinking water by the process of Chlorination had become vitally important in order to eliminate the bacteria and thus, to decreases the mortality in the early 1900s caused by these pathogens. Chlorine in the water reacts with the naturally occurring organic substances such as humic and fulvic acid to form a wide range of

Disinfection by–products (DPBs). The most commonly formed disinfection byproducts are Tri-chloroacetic acid (TCA) and Chloroform [1,2,3,4,].

In 1993 W.H.O. declared TCA, a major contaminant of the environment and set a provisional guideline value of 100µg/ L in drinking water. These manmade pollutants have distributed itself throughout the atmosphere in different concentrations. Exposure to such contaminants before and after conception may adversely effect reproduction both in man and animals by inducing cell death or dysfunction which may lead to infertility [5,6,7].

A detailed survey of the international literature on sperm and sperm levels published in between 1930 and 1991 revealed that men are producing only ½ as much sperm per ejaculation as they did in the 1940sI. In the 1940 the average sperm count was 113 millions/ml and that 50 years later it had fallen to 66 million/ ml [8,9]. Further more, there is evidence that the male reproductive function seems to have deteriorated considerably during the past 4-5 decades which is attributed to environmental or genetic factors or combination of both. The environ-mental chemicals pollutants –interact with the individual's genetic susceptibility to cause a range of increasingly common problems, Testicular Dysgenesis Syndrome (TDS)- which include poor semen quality, testicular cancer and abnormality in male sex organs [10]. Such a remarkable increment in the occurrence of gonadal abnormalities over a relatively short period of time is more likely to be due to the environmental factors rather than genetic factor. These environmental factors playing a role in this increase in the gonadal abnormality are pollutants, smoking, alcohol and sexually transmited diseases. The factors responsible for wild life fertility problems are chlo-rinated chemicals [11].

Drinking water from the Thames water supply in the united kingdom lead to lower sperm count and increase in abnormally shaped sperm [12].

TCA may be responsible for the decrease in fertility in males, therefore, the present study was undertaken to evaluate the role of TCA in the gonadal development during the embryonic period.

Material and Methods

Rat

Inbreded Charles Foster Strain of male and virgin females of 85 -120 days, weighing to an average of 225 ± 60 g was in-cluded in the study group. They were obtained from animal house, Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University.

Macro environment

The Charles Foster rats, were maintained in environmentally controlled rooms at temperature of 28-32 °C, 40-60 % humidity, in a noise free environment, and 12 hours

light – dark cycle. The females were housed in the pens of three, while the males were placed individually in a Solid bottom plastic cages having a wire top with locking clips, which were packed with absorbent material. A total of 27 rats were chosen at a time for the experimental study. All the rats had access to water and animal rat diet (Hindustan lever Animal feed) ad libitum.

Reagent

Trichloroacetic acid was purchased from LOBA CHEMIE PVT. LTD, (Mumbai). The Sodium hydroxide pellets, used to neutralize Trichloroacetic acid solution (Ka =0.3) to required pH 7.0 – 7.5, was obtained from GLAXO CO. LTD. The purity of Trichloroacetic acid and sodium hydroxide were 99.0%+.

Trichloroacetic acid (TCA) is stable in neutral solution and is classified as “non biodegradable” with a “low bioaccumulation potential” for fish and a “high biochemical potential” for terres-tial plants.

Confirmation of Pregnancy

Females in pro estrous were selected and placed in cages with male in ratio of 1 Female: 1 Male, overnight. Females were checked, for the presence of the spermatozoa in the vaginal smear and /or the vaginal plug the following morning at 8.00 A.M. The day of observation of sperm or a plug was desig-nated as day zero of pregnancy. The pregnant female was thereafter, kept in separate cage, labeling it with an ear notch code.

Drug Administration to Animals

Trichloroacetic acid (99.0%+ pure) was dissolved in distilled water. The solution was adjusted to the pH 7.0- 7.5, by titration with sodium hydroxide. Dosing solution was prepared daily. Each pregnant rat was identified by an ear notch code and they were subsequently divided into six groups, according to the dose of TCA to be administered, i.e., 1000, 1200, 1400, 1600 or 1800 mg/kg /day, from day 6 to day 15 of gestation. The pregnant rats were administered TCA by oral intubations, using a No.16 infant feeding tube. Normal saline is used as lubricant during intubations. Control animals for this study re-ceived distilled water through oral gavage through out the pe-riod of organogenesis.

Quantity Administered

Depending upon the weight of the rat, the quantity of fluid (TCA + Vehicle) administered ranged from 0.5ml to 1.0ml.

Each rat from the experimental sets of study, was carefully observed through out the pregnancy for signs of toxicity. Their weights, were monitored and recorded daily.

All the rats were handled with utmost humane care.

Collection of Pups

On the day 19 of gestation, in order to avoid premature birth, each pregnant rat, from all the experimental groups was weighed and then euthanatized using over dose of ether anesthesia. An examination of the mother was conducted for any overt sign and symptoms of toxic effect. The gravid uterine horns were opened through laparatomy, by a low midline incision in the lower abdomen, exposing all fetuses, and the resorption sites.

Rat Fetuses.

Each fetus and placenta was examined in situ, then removed, sexed, and examined externally for any morphological abnor-malities. The fetuses were separated of their placenta, mopped dry on blotting paper and weighed using a weighing balance. Each fetal crown rump (C/R) lengths were recorded meticu-lously. Thereafter, they were subjected to evaluation for external gross abnormalities using a dissecting microscope or a hand lens for magnification. The fetuses and their placentae were preserved in 10% buffered Neutral formalin.

Collection and Examination of Testis

The two sides testes could be seen in the lower part of the cavity of the abdomen near the posterior abdominal wall. The gonads could be easily identified with the prominent bulging of the epididymis near its upper pole. The size of the epididymis was found to be comparatively larger. The vas deferens could be seen arising from the lower pole, converging from either size to the external genitalia. The testes of each pup of different groups, collected on day 19 of gestation, were dissected out, using a dissecting microscope. They were mopped dry on a blotting paper, weighed on a Sartorius electronic balance (least count 0.001mg), and preserved in 10% Neutral buffered formalin.

Testicular changes in rat exposed to TCA

Histological Techniques

The testis of all the experimental series and control were sub-jected to histological examination.

Statistical Analysis

Statistics for the individual fetal data were analyzed by using Fischer's exact test. Difference between the dose group and the control groups were found using pair wise 't' test.

Observations and Results

The total resorptions were significantly different statistically fromthoseof the control groups (Table 1). However, no external abnormalities were noted.

Table 2 and Fig. 1 and Fig. 2, show that the weight of the testes collected on the 19 day of gestation. The weight of the control testis were in the range of 0.89- 1.74 mg (mean 1.5 ± 0.26mg). The 1000 mg/Kg body weight group exhibited an average weight of 1.3 mg which showed no significant difference when compared to the control. However, at 1200 mg and 1400mg/ kg dose the testis had an average weight of 1.1 and 1.05 mg respectively, which were significantly reduced when compared to the controls ($P<0.05$).The average weight of the testis with 1600 and 1800 mg/kg dose group of TCA was 0.65 and 0.62 mg, respectively, which were significantly reduced as compared to control ($P< 0.001$). Fig. 1 and Fig. 2 shows tendency of gradual reduction in weight of the testis as a direct relationship with the dose treatment.

Table 1: Showing percentage of fetal post-implantation loss following TCA exposure during organogenesis, collected on day 19 of gestation.

Group	TCA mg/kg body weight					
	Control	**1000**	**1200**	**1400**	**1600**	**1800**
No. of females inseminated	6	6	6	8	8	12
No. of pregnant females	6	6	6	8	8	12
No. of dams with fetuses	6	6	5	5	5	5
No. of dams with total resorption	0	1	1	3	3	7
Average no. of implants at pregnancy	11.5±0.54	DIFFICULT TO OBSERVE Therefore, average number of implants as that in control group (11.5±0.54) was taken as the number for these groups also.				
Average no. of living fetuses at collection ±S.D.	11.2±0.75	9.0±4.43	0.78±3.92	5.75±4.83	4.4±3.70	0.91±1.17
Percentage of Post	3.03±4.69	22.22±38.61*	32.20±34.52*	52.27±39.84**	62.21±32.05**	92.15±9.94***

implantation lost ± S.D.						
Total No. of fetuses recovered	67	54	47	46	35	11

 Post implantation loss % = No. of implants – live fetuses / No. of implants.
.lortnoc eht morf tnereffid yltnacifingis ,50.0>p *
cifingis ,10.0 >p **ant different from the control.
lortnoc eht morf tnereffid tnacifingis 100.0>p ***

Table 2: Showing weight of the fetal testis (mg) following TCA exposure during the period of organogenesis collected on day 19 of gestation

Dose mg/kg	No. of fetuses	No. of Testes	Weight of the testis in mg		
	Male	Female		Range	Mean ± SD
Control	38	19	76	0.89-1.74	1.5±0.26
1000	35	19	70	0.96-1.43	1.3±0.14
1200	27	20	54	0.96-1.26	1.1±0.15*
1400	27	19	54	0.58-1.56	1.05±0.28*
1600	20	15	40	047-0.84	0.65±0.16**
1800	6	5	12	0.49-0.74	62±0.15**

derapmoc nehw ,tnacifingis ,10.0 > p * lortnoc ot derapmoc nehw tnacifingis ,50.0 > p * to control

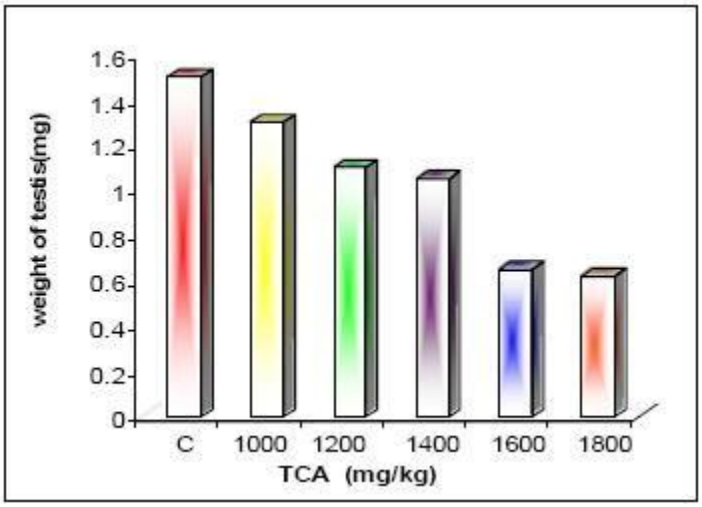


Fig. 1: Fetal testis weight changes following TCA exposure

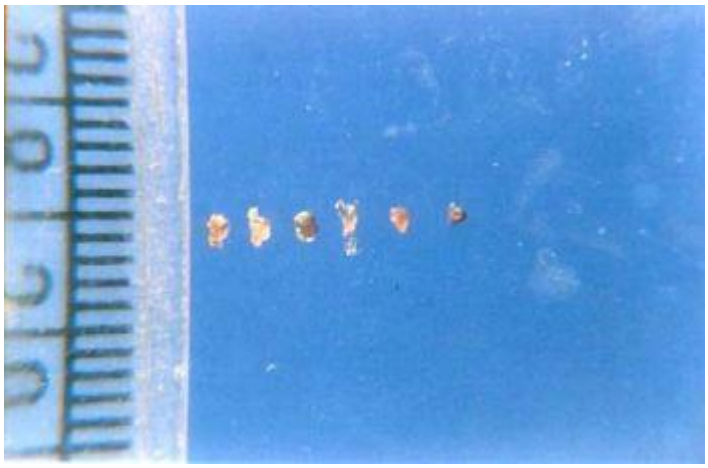
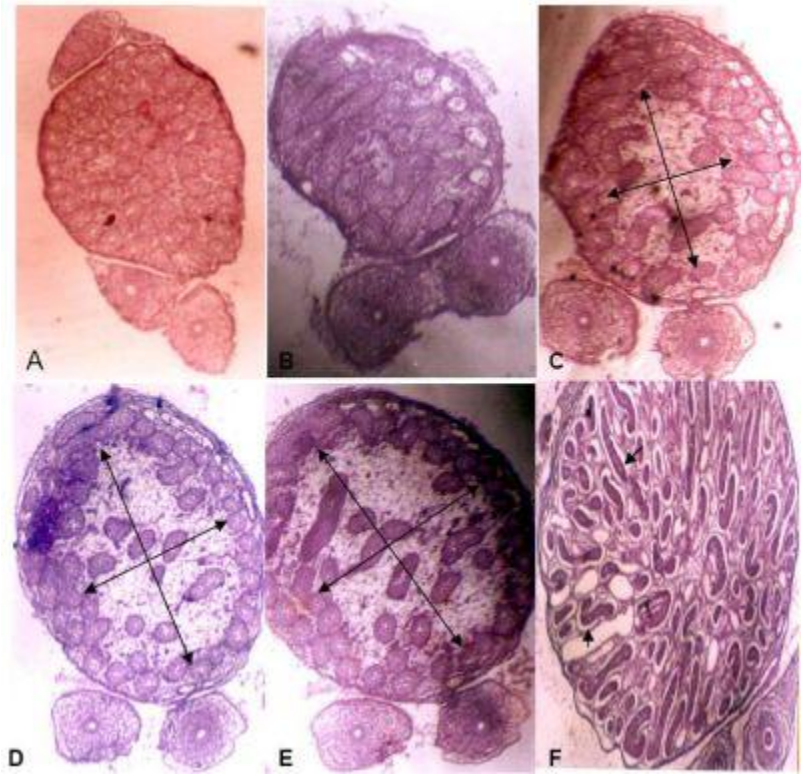
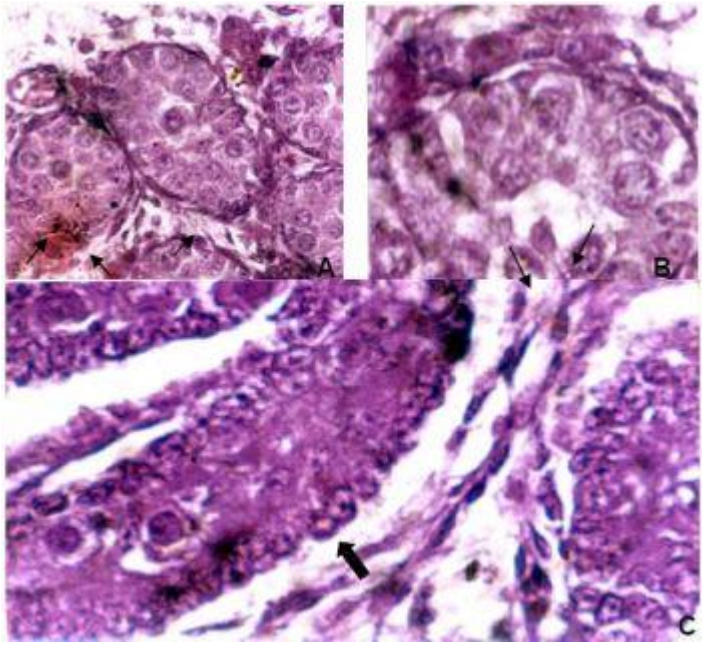


Fig. 2: Photograph of fetal rat testes collected on day 19 of gestation showing control rat testis (A) and treated testes 1000mg/Kg (B), 1200 mg/kg©, 1400 mg/kg (D), 1600 mg/kg (E)and 1800 mg/ kg (F). The treated rat testes show gradual reduction in size with increase in dose of TCA



(For larger image, click here)

Fig. 3: Photomicrographs of transverse sections of fetal rat testes from control (A) and treated groups collected on day 19 of gesta-tion. Transverse section of 1200©,1400(D), 1600(E)mg/kg treated TCA fetal rat testis showing seminiferous tubules present mainly in the peripheral region of the testis, while their distribution being sparse in the central region with intervening mesenchymal tissue. T.S. of 1000 mg/kg (B) showing similar distribution of the seminiferous tubules as that of control. T.S. of a 1800 mg/kg (F)treated rat testis reveal thinned out and folded tunica albugenia and the cellular constituents of the seminiferous tubules concen-trated at the centre detached from the basement membrane (↑). X40.



(For larger image, click here)

Fig. 4: Photomicrographs of the transverse section of testes from treated rat pups collected on day 19 of gestation. The gonocytes undergoing apoptosis possessing pyknotic nuclei (A, ↑, x 200)with 1600 mg/kg treated TCA dose are well evident. The same could be seen at higher magnification i.e., x 400.(B). T.S. of the rat testes treated with 1800 mg/kg show clear vacuolar spaces(bold arrow) be-tween the basement membrane and the seminiferous tubules constituents and dark stained degenerating gonocytes, x 400.

Histological changes as evident in Fig. 3, shows control fetal rat testis with a well defined tunica albuginea, surrounding tunica vasculosa. Tunica vasculosa contains plexus of blood vessels. The blood vessels are seen extending to the inner aspect of the tunica albuginea and the septa in the medullary region of the testis. The testicular cytoarchitecture was dominated by the compactly placed seminiferous tubules, present within the mesenchymal tissue , homogenously distributed throughout stroma , i.e., both the central and peripheral region.

The 1000 mg/kg (Fig. 3 B), of TCA dose group treated fetal testis revealed similar cytoarchitecture to that of the controls. However 1200mg/kg dose group fetal testis revealed a reduc-tion in the diameter of the seminiferous tubules (Fig. 3 C). The seminiferous tubules did not occupy the testicular stroma to its full extent. They were limited to the peripheral (cortical region) region. The central region of the testis was devoid of seminiferous tubules. This very effect at 1200mg/kg dose of TCA was more pronounced with 1400 and 1600mg/kg dose (Fig. 3D and Fig 3E). The length as well as the diameter of the seminiferous tubule had failed to attain its normal size resulting in the non occupation of the central (medulla) region of the testis. This residual region was

found to be directly proportional to the dose of TCA, i.e., the area increasing with the increase in the dose of TCA.

With 1800 mg/kg dose of TCA, the testis revealed the following changes; the tunica albuginea had thinned out and showed several folding with detachment from the underlying stroma. The diameter of each of the seminiferous tubules was reduced in comparison with the control and was associated with an increase in the mesenchymal tissue interposed in between the two seminiferous tubules. The most conspicuous feature observed was the detachment of the cellular constituents of the seminiferous tubules from the underlying basement membrane. The cellular constituents were concentrated in the cen-tral region of the seminiferous tubule leaving a fluid filled clear vacuolar space all around it (Fig. 3 F and Fig. 4 C arrow bold).

Detailed study at higher magnification of the seminiferous tubule revealed that at lower dose with 1200 mg/kg dose of TCA, there was increased apoptosis of the gonocytes within the seminiferous tubules in comparison to the controls. The apoptotic cells had a shrunken size, irregular shape and a pyknotic i.e., dense chromatic material attached to the nuclear membrane. With 1400 mg/kg dose of TCA the gonocytes undergoing apoptosis had increased more in comparison to control and several cystic spaces could be seen (Figs. 4 A, 4 B). At higher dose i.e., with 1600 and 1800 mg/kg TCA dose the degenera-tion of the gonocytes had increased to several folds and appeared as a constant and regular feature.

Discussion

In in-vivo experimental studies, TCE (Trichloroethylene) and its metabolites including trichloroacetic acid (TCA), and dichloroacetic acid (DCA) were determined in epididymis and testis of mice exposed to TCE (1000 parts per million) by inha-lation for 1 to 4 weeks. In other studies, incubations of monkey epididymal microsomes were performed in the presence of TCE and NADPH. Human seminal fluid and urine samples evaluation, results showed that seminal fluid from all eight subjects contained TCA. Chloral and TCA were identified in microsomal incubations with TCE in monkey epididymis [13].

A P450 enzyme that metabolizes TCE, was localized in human and monkey epididymal epithelium and testicular Leydig cells. These results indicated that TCE is metabolized in the repro-ductive tract of the mouse and monkey. Furthermore, TCE and its metabolites accumulated in seminal fluid, and suggested associations between production of TCE metabolites, reproductive toxicity, and impaired fertility [14].

Evaluation of the exact mechanism by which TCA brings about the changes in the developing gonads needs to be further studied; however, depending upon the findings, the observations of the present study can be discussed as follows:

The germ cells called gonocytes in the testicular cord were randomly distributed within the seminiferous tubules among many sertoli cell precursor. The gonocytes are large cells

with a less eosinophilic cytoplasm, a large vescicular nucleus placed centrally, which has a spherical nuclear membrane, with an evenly distributed chromatin and multiple nucleoli [15]. These germ cells are the precursor for the sperm cell line [16]. The germ cells undergo programmed cell death in the fetal life. In the present study, TCA exposure has led to enhancement of programmed cell death of the gonocytes as well as of the ser-toli cells (Fig. 4).The cell death rate increased with increase in dose of TCA.

The development of the seminiferous tubules has been studied in details in different mammalians species [17]. In the present study, the results indicate that the area occupied by the seminiferous tubules in a cross section of the testis has decreased with the increase in dose of the TCA. Maximum reduction was observed with 1600 mg/kg dose (Fig. 3 E).The reduction of the seminiferous tubules in the experimental group had led to consequential increase in the interstitial spaces in the testicular tissue. These findings were not observed with 1000 mg/kg dose (Fig 3 B). This may be due to the lesser assault by TCA at low dose. But with the increase in the TCA dose, the development of the seminiferous tubules in the testis has been hampered more severely resulting in the reduction of the size of the seminiferous tubules, both in its length as well as its diameter, and thus its non-occupation in the central zone of the testicular tissue, and sparsely distribu-tion in the peripheral region with increase in the interstitial space in between them.

The two cell type, i.e., germ cells and the sertoli cells, show active mitotic activity throughout the fetal life. The reduction in the seminiferous tubules diameter may be due to the reduction in the number of gonocytes as well as the sertoli cells. This has resulted in the overall reduction in the fetal testis with weight inversely proportional to the TCA dose.

The thinning of the tunica albuginea was observed with 1800 mg/kg dose of TCA as compared to the controls, associated with increased folding, and its detachment from the underlying stroma. These features suggest defective and poor development of the tunica albuginea as a result of TCA assault. The high dose assault of TCA resulted in clear vacuolar spaces in the seminiferous tubules where its constituent cells detach from the basement membrane and are concentrated as a mass in the centre, showing that TCA has somehow led to tissue injury, where the cells shrink in size as a result of the extravasations of the fluid. This fluid gets accumulated around as a clear vacuolar space.

Therefore, it can be concluded that TCA has led to the qualitative and quantitative reduction of these cellular elements of the seminiferous tubules with diminution in its size resulting subsequel lies in total reduction in the testicular size.

The testis after TCA assault at 1400, 1600 and 1800 mg/kg demonstrated few gonocytes interspersed in the seminiferous tubules. Such an appearance may be explained as follows:

- TCA might be interfering with the migration of the primordial germ cells from the yolk sac to the gonadal ridge. Those of them which escaped the TCA assault

reached the developing gonads but in an environment which was not conducive and healthy enough to keep them viable and led to their disintegration and disappearance.

- The second possibility is that those primordial germ cells (PGCs) which succeeded in reaching the gonadal ridge fail to interact with the supporting somatic cells due to TCA toxicity and undergo apoptosis. The mode of development of the somatic cell is critically dependent on the germ cells with which they interact [18,19].The lack of interaction of supporting cell with the PGC leads to their cell death.

In totality it appears, that the testis changes are due to the anoxia and the oxidative stress, caused by TCA exposure. TCA may act through receptors and induce increased Apoptosis in the testis. The decrease in weights of the testis found in the offspring is a propable result of acceleration of the nor-mal process of Apoptosis. TCA could be a direct gonadotoxic agent which needs to be further evaluated.

In Conclusion, the exact mechanism by which TCA may have resulted in its teratogenic effect is difficult to establish, but it can be suggested that. TCA results in the increase in the 8-hydroxy-2-guanosine, a marker for oxidative stress [20].The oxidative stress can induce apoptosis. Furthermore, anoxia that can cause necrosis may induce apoptosis at lower doses [21]. These two factors acting in collaboration with each other or acting independently may be responsible for the teratogenic effect of TCA.

In 1993, W.H.O. declared TCA as major contaminants of the environment and set a provisional (maximum permissible) guideline value of 100 μg /L in drinking water. The NOAEL (No observable adverse effect level) in a 90- day study in dogs- the most sensitive species tested- was determined as 30mg/kg body weight /day. The NOAEL in a 2 year feeding study in rats was 80mg/kg body weight /day and the NOAEL for repeated dose toxicity in a 4 month feeding study with rats was 365 mg/kg body weight/ day, however NOAEL was difficult to establish in study of the reproductive toxicology [22]. As such extrapolation in relation to human is difficult. The frank terato-genicity observed, following the direct assault with high doses of TCA may not be applicable to the community but, the continuous exposure of TCA through drinking water for a long duration might lead to subtle fertility changes, which may not be apparent at birth. Furthermore some of the factors that need to be considered are the spatial and temporal variability in individual the different exposure routes; inhalation, ingestion, dermal absorption and daily activities including showering, bathing, and swimming. For the better understanding of these factors and in depth assessment of exposure specific study models design are needed.

Acknowledgments

The author is deeply indebted to Professor Gajendra Singh for his useful suggestion and encouragement.

References

1. Environment Protection Agency,U.S.A. 2000.
2. Morris ED, and Bost JC..Acetic acid: Halogenated Derivatives pp 169-171 of vol 1 of Kirkothmer Encyclopedia of chemical technology, 4ed. JL Krostwitz(ed) Wiley N.Y. 1991.
3. Morris JC. Aqueous chlorination in the treatment of water supplies.Ch 2 of organic Carcinogens in Drinking water.eds Ram NM, EJ. Calabrese and RF. Christmas, Wiley New York 1986.
4. McCulloch A. Trichloroacetic acid in the environment. Chemosphere 2002; 47: 667-686.
5. Guidelines for drinking water quality, 2nd ed. Vol. Recommendations. Geneva, World Health Organization, 1993; pp 101-103.
6. Bove FJ, Fulcomer MC, Klotz JB, Esmart J, Dufficy EM, Savrin JE. Public drinkig water contamination and birth outcomes. Am J Epidemiol 1995; 141: 850-862.
7. Nieuwenhuijsen MJ, Toledano MB, Elliott P. Uptake of chlorination disinfection byproducts; A review and a discussion of its implications for exposure assessment in epidemi-ological studies. J Expo Anal Environ Epidemiol 2000; 10: 586-599.
8. Skakkebaek NE, Grigor KM, Giwercmann A, Rorth M. Management and Biology of Carcinoma in situ and Cancer of testis. Karger Basal (eds) 1993; pp1-256.
9. Sharpe RM. Falling sperm counts in men- is there on endocrine cause? J Endocrinol 1993; 137: 357-360.
10. BBC. Chemical query over testicular problem (06/25/2001)..
11. Goldstein M and Sinclair W. Male and Female infertility problems. Chlordane research Index 1998.
12. Grinsburg Jean.Male infertility and chemicals in drinking water. Lancet 2003; Jan 22.
13. Forkert PG, Lash L, Tardif R, Tanphaichitr N, Vandevoort C, Moussa M. Identification Of Trichloroethylene And Its Metabolites In Human Seminal Fluid Of Workers Exposed To Trichloroethylene. Drug Metab Dispos 2003; 31: 306-311.
14. Forkert PG, Lash LH, Nadeau V, Tardif R, Simmonds A. Metabolism And Toxicity Of Trichloroethylene In Epididymis And Testis. Toxicol Appl Pharmacol 2002; 182: 244-254.
15. Merchant H. The onset of testicular differentiation in the rat: an ultrastructural study. Am J Ant 1976; 145: 319-330.
16. Orwig KE, Buom- Yong Ryu, Mary R. Avarbock, and Ralph L. Brinster. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes Cell Biology 2002; 99: 11706-11711.
17. Steinberger E, and Steinberger A. Sprematogenic function of the testis. In: The handbook physiology 5. 1-9 (D.W. Hamilton and R.O. greep eds) Waunders press, Baltimore1975.
18. McLaren A.Bio. Essays 1991a; 13: 151.
19. Mclaren A.Nature 1991b; 351: 96.
20. Austin EW, Parrish JM, Kinder DH, Bull RJ. Lipid peroxidation and formation of 8-hydroxydeoguanosine from acute doses of halogenated acetic acids. Fundam Appl Toxicol 1996; 31: 77-82.

21. Kam PCA, and Ferch NI. Review Article, Apoptosis: mechanism and clinical implications. Anaesthesia 2000; 44:1081-1093.
22. UK Independent. British men are less fertile than Hamsters.
23. Pollution may be to blame for collapse in sperm counts in industriliased world. (03/17/2002).

Correspondence to:
Dr. Royana Singh
B-115 Brij Enclave
P.O. Sundarpur
Varanasi-221005, India
e-mail: singhroyana (at) rediffmail.com