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American Journal of Life Science Researches
www.worldofresearches.com

Am. J. Life. Sci. Res.
Vol. 3, Issue 4, 293-303, 2015
ISSN: 2332-0206 (Online) ISSN: 2375-7485 (Print)

Fertility and Reproductive Outcome in Mice Following Trichloroethane (TCE) Exposure

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Abstract: Exposure to trichloroethane (TCE), an industrial solvent, has been shown to be negatively associated with reproductive performance. The present study was performed to assess the effects of TCE exposure on the reproductive performance and outcome in mice during a critical developmental window of later reproductive life. A group of female mice were injected intraperitoneally twice weekly for three weeks with TCE (100 and 400 µg/kg). Mice were followed up for signs of toxicity and death. Changes in uterine tissues have also been investigated by histopathology. The results showed that TCE exposure has reduced the number of F0 fertile females comparing to controls. Moreover, TCE exposure resulted in a decreased pups number and changed sex ratio in the litter of F0 TCE-treated dams. Histopathological examination revealed a TCE-induced uterine toxicity appeared as a severe endometrial hyperplasia with squamous cell metaplasia and adenomyosis. These results indicate that TCE exposure during a critical reproductive developmental window could affect the fertility and interfere with the reproductive outcome in mice.

Keyword: TCE; fertility; reproductive outcome; uterus; mice.

INTRODUCTION

Infertility is a major worldwide public health concern because it affects approximately 10% of all reproductive-aged couples[1]. Exposure to chemicals during reproductive developmental windows may predispose individuals to disease and/or dysfunction later in life. 1,1,1-trichloroethan (TCE), an ambiguous environmental pollutant, is widely used as an industrial solvent and a degreasing agent [2-4]. It has been shown that TCE is well absorbed by all routes of exposure. At least by inhalation, the rate of uptake is driven initially by tissue loading and then by metabolism once steady state conditions have been reached[1]. Numerous studies with developmental exposure to lower doses than the "safe" dose suggest that TCE exposure causes various detrimental defects, such as low fetal weight, birth defects, and developmental disorders. Recent epidemiological studies have shown that chemical exposure environmentally or occupationally on a daily basis is associated with increase a woman's risk of spontaneous abortions, infertility, low fetal weights, birth defects[1].

However, to the best of the author's knowledge, it has not been examined whether TCE exposure, during a critical uterine developmental window, has consequences on reproductive performance (e.g. fertility, gestation and litter life) in later reproductive life. Thus, this study was performed to investigate the long-term effects of environmentally relevant low and high dose of TCE on adult reproductive functions, such as fertility and reproductive outcome.

MATERIAL AND METHODS

Animals and housing

A total of thirty six female Swiss Albino mice (F0 generation), with an age range of 4-6 weeks and weight range of 21–24 g, were used in this study. Mice were kept under a constant light-dark cycle (dark period from 7:00 pm to 7:00 am) at 24 ± 1ºC and 55 ± 5% relative humidity. Food and drinking water were available ad libitum. All efforts were made to minimize the pain during animal handling and experimentation and to reduce the number of animals used.

Study design and treatment regimen

TCE (Baxter International) was suspended in corn oil. Female mice (F0) were divided into four groups of six mice each. These groups were: the low and high dose TCE-treated (100 or 400 µg/kg) groups and the vehicle and sham control groups. For TCE-treated groups, the doses were calculated and delivered in 80-100 µl of corn oil based on their body weight[5-6] . TCE doses were selected as they considered safe by Environmental Protection Agency (EPA)[7] . Vehicle controls were received an equal volume of corn oil only. The sham controls were not received any exposure. TCE or vehicle were administered intraperitoneally at a defined time (10:00 am) every 3rd day. The exposure window was selected because this is the critical development window in the mouse[1].

After treatment, F0 female mice were mated with fertility confirmed control males (2 females:1 male ratio). Mating was confirmed by the presence of vaginal plug. Once the plug was observed, females were separated from males and individually caged. The day the vaginal plug detected was defined as the first gestation day (GD1). Dams (F0 generation) were observed daily and body weight gain was measured daily to further confirm pregnancy and for any adverse clinical signs or abnormal behavior that may result from toxicity. The dams were allowed to deliver naturally and the delivery day was designed as postnatal day 0 (PND0).

The study comprehensive teratological parameters for F0 females included body weight, reproductive performance (fertility index; gestation index, mortality), and gross pathology. The study parameters for F1 offspring included pup size, average pup weight, sex ratio, pup mortality and stillbirths as recorded on PND0.

After the two-week mating period, unmated females were singly housed, observed for estrous cycle for another 10 days, and body weight was monitored for another two weeks. The females were considered infertile if they did not cycle and/or did not have significant body weight gain during the entire testing period.

Clinical assessment

The clinical assessment included animal survival, body weight gain and histopathology.

Animal survival

During the course of the exposure period, mice were observed twice per day for any abnormal clinical signs or behavior that may result from toxicity. Mice were assessed for morbidity and mortality twice daily, midmorning and late afternoon. Night deaths were recorded the next morning. Two independent observers confirmed the cause of death to exclude TCE-nonrelated mortality.

Body weight

Mouse body weight in control and TCE-treated groups was assessed on a weekly basis to monitor the effect of TCE exposure on body weight.

Histopathological examination

After dissection, 10%-formalin-fixed uteri were processed in a series of graded ethanol solutions and embedded in paraffin wax. Paraffin sections were cut at 6-8 µm thickness, deparaffinized, rehydrated, stained with hematoxylin and eosin (H&E) and examined under a light microscope (Leica, Germany) for histopathology.

Statistics

Data were expressed as means ± SEM (standard error of the means) from 6 female mice of each group using SPSS software, version 20. A computerized Kolmogorov-Smirnov test was used to determine whether the data fitted a normal distribution. One-way ANOVA test followed by Tukey's post hoc comparisons was used to make multiple comparisons between treatment groups. Student's t-tests were used to make comparisons between two groups. Mann-Whitney U-test was used for nonparametric samples. Statistical significance was assigned at $P \leq 0.05$.

RESULTS

Effect of TCE on animal survival

No mortality has been recorded among mice in all groups along the course of the experiment except for one death case out of the six females in 400 µg/kg TCE-treated group recorded four weeks post TCE exposure.

Effect of TCE on body weight

Previous animal and epidemiological studies have linked developmental chemical/ toxicant exposure to metabolic disorder and obesity[1]. Therefore, in this study we decided to investigate the effect of TCE exposure on body weight of F0 female mice by monitoring the body weight either pre- and post-treatment.

In this study, TCE exposure have not shown an effect on overall body weight in F0 females as statistical analysis indicated no significant between treated and non-treated groups (data not shown). However weight gain in F0 females in 400 µg/kg TCEt-reated group, but not 100 µg/kg TCE-treated group ($P = 0.332$) was significantly higher ($P = 0.024$) comparing to controls (Figure1). This concludes that TCE exposure at the dose of 100µg/kg is a No Observed Adverse Effective Level (NOAEL) for F0 female mice in this study.

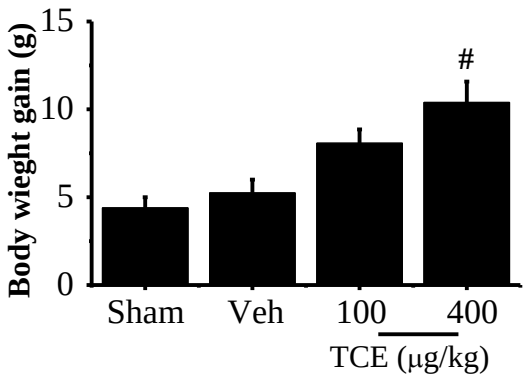

Figure 1. Body weight gain of the F0 females. TCE exposure significantly increased weight gain at a dose of 400 µg/kg. Data represent mean ± SEM of n = 6 animals per groups. # Significantly different from the vehicle controls ($P \leq 0.05$).

Effect of TCE on fertility

To determine the effect of TCE exposure on fertility in later reproductive life and whether the effects would change with age, a number of fertility and reproductive indicators including percentage of fertile female has been investigated.

The results showed an effect of TCE exposure on the fertility of F0 females. The analysis showed a reduction in the percent of fertile females in TCE-treated groups as in 100 µg/kg TCE-treated group, five females out of six females (~83%) gave birth while in 400 µg/kg TCE-treated group, only one female out of six females (~17%) gave birth (Figure 2).

Statistically, the fertility was significantly reduced in 400 µg/kg TCE-treated F0 females comparing to controls (P = 0.047; Figure 2).

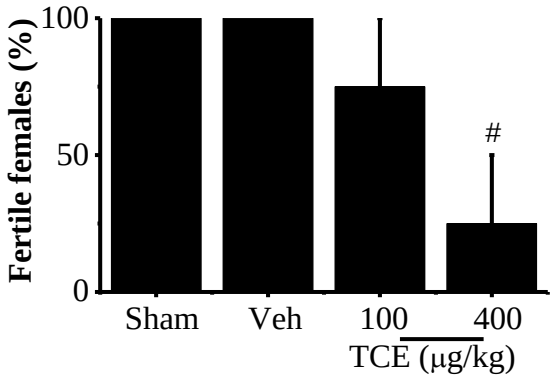

Figure 2. Effect of TCE exposure on fertility of F0 females. The fertility was significantly reduced in 400 µg/kg TCE-treated F0 females. Data represent mean ± SEM of n = 6 animals per groups. # Significantly different from the vehicle controls (P ≤ 0.05).

Effect of TCE on reproductive outcome

To determine the effect of TCE exposure on reproductive outcome, a number of reproductive indicators including litter number and mortality, pup size and weight and sex ratio have been investigated. The results showed also that TCE exposure at a dose of 100 or 400 µg/kg significantly affected the litter number and decreased the average number of pups, especially for the TCE 400 µg/kg TCE-treated group compared to controls (Figure 3A). However, TCE exposure at a dose of 100 or 400µg/kg had no effect on pup size comparing to controls (data not shown).

For the effect of TCE exposure on average live pup weight, TCE exposure at a dose of 100 µg/kg had no effect on the pup weight (P = 0.388) comparing to controls. However, TCE exposure at a dose of 400 µg/kg has significantly (P = 0.049) increased the average of live pups weight comparing to controls (Figure 3B). Furthermore, the results of this study showed that TCE exposure at either a dose of 100 or 400 µg/kg had no effect on litter mortality when compared with controls.

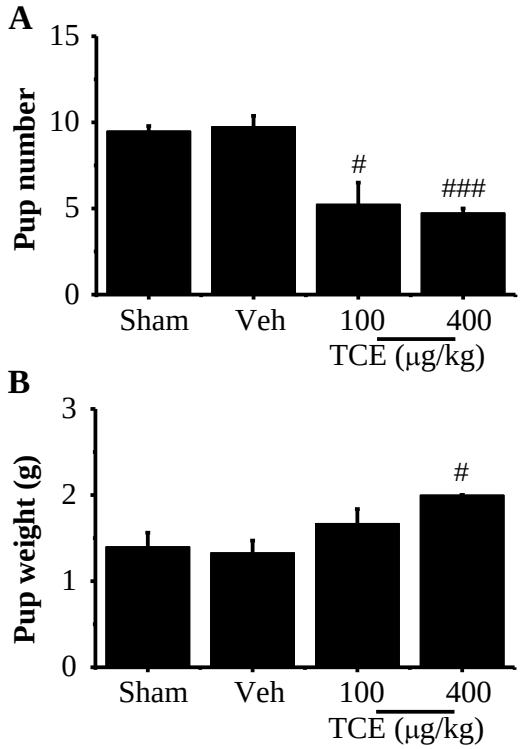

Figure 3. Effect of TCE exposure on reproductive life/ outcome. (A) Quantification of pup number. (B) Measurement of pub weight. Data represent mean ± SEM of n = 6 animals per groups. # Significantly different from the vehicle controls ($P \leq 0.05$).

Regarding the effect of TCE exposure on the litter sex ratios, TCE exposure had a significant effect on the male/female ratio at a dose of 400 µg/kg. This dose has significantly changed the litter sex ratio in favor of F1 males comparing to controls ($P < 0.05$) (Figure 4). However, neither 100 µg/kg TCE dose exposure nor the vehicle had effect on the litter sex ratio (Figure 4).

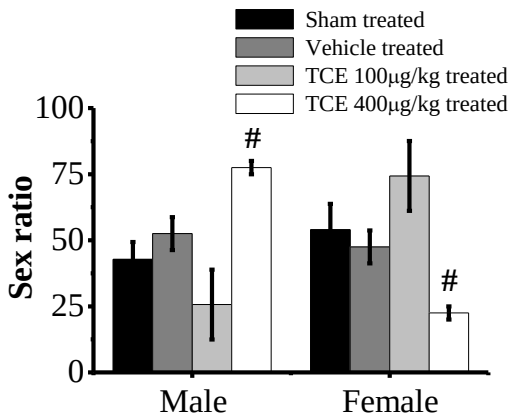

Figure 4. Effect of TCE exposure on reproductive outcome. TCE exposure at a dose of 400 µg/kg has significantly changed the litter sex ratio in favor of F1 males comparing to controls. Data represent mean ± SEM of n = 6 animals per groups. # Significantly different from the vehicle controls ($P \leq 0.05$).

Gross pathological findings and histopathological changes of uterine tissues

At necropsy, no gross pathological changes have been noticed on control mice. In addition, no histopathological changes were observed in their uterus (Figure 5A) or the other parts of reproductive tract. However, abilateral asymmetrical enlargement was seen in the uterine body of several TCE-treated mice at a dose of 100 and 400 µg/kg. Sections from different parts of uterus were submitted to histopathological examination.

The histopathological examination of uterine tissues of TCE-treated mice at a dose of100 µg/kg showed a marked increase in the endometrial and myometrial thickness (Figure 5B). This thickening is revealed as diffuse or focal proliferative reactions with characteristic hyperplasia of uterine glands with prominent stromal elements and/or formation of intra-luminal polyp. The glands were large, irregular, highly branched and/or cystic lined by single or double layer of non-ciliated secretary epithelium (Figure 5C). In some cases, polyps were appeared as evaginated circumscribed mass covered by columnar epithelium with underlying tissue formed from endometrial stromal elements (Figure 5D). The myometrium showed normal smooth muscles fiber and vascular constituent, however, some cases showed invaginated uterine glands in-between (Adenomyosis) (Figure 5E). These histopathological changes caused by TCE were remarkably increased in mice which were treated with 400 µg/kg TCE; the endometrial glands were severely ectatic, lined by flattened epithelial cells and contain few degenerative heterophils and necrotic cellular debris (Figure 5F) and most endometrial polyps were composed of abundant amounts of loosely or compactly arranged spindle-shaped or satellite endometrial glands (Figure 5G). Moreover, there was sever hyperplastic changes with squamous cell metaplasia in endometrial epithelium (Figure 5H). In conclusion, the uteri of TCE-treated mice showed a severe endometrial hyperplasia with squamous cell metaplasia and adenomyosis.

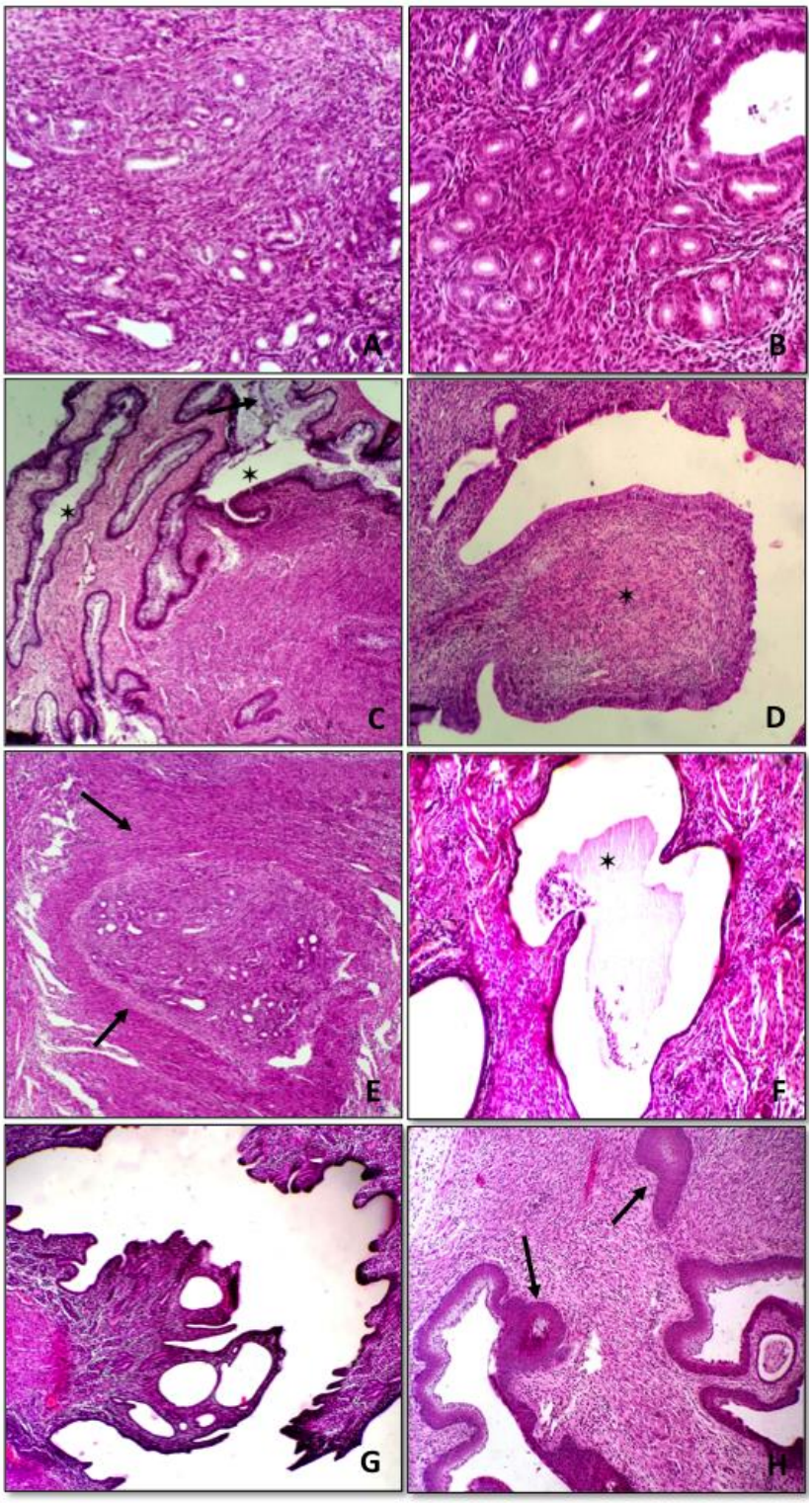

Figure 5. Representative uterine sections of control and TCE-treated mice (H&E staining). A. Uterine sections of control mice showing normal tissue architecture at magnification X200. B. An endometrial thickening with more µg/kg TCE-treated mice. C. TCE-treated mice١٠٠prominent hyperplasic uterine glands and stromal elements in endometrium showing hyperplasic glands (cystic endometrial hyperplasia) (asterisk). D. Pedunculated endometrial polyp with an epithelial covering and stromal hyperplasia in 100 µg/kg TCE-treated mice (asterisk). E. Endometrial tissue includes glands and stromal elements within the myometrium (arrows) in 100 µg/kg TCE-treated mice. F. The endometrial glands are severly ectatic, lined by flattened epithelial cells and contain few degenerative heterophils, necrotic cellular debris and secretory material (asterisk). G. Papillary fibroepithelial polyp arising from the endometrium with cystic change of uterine glands in 400 µg/kg TCE-treated mice

(asterisk). H. Hyperplasia and squamous metaplasia of epithelium of uterine glands in 400 µg/kg TCE-treated mice (arrows).

DISSCUSSION

The exposure to environmental toxic chemicals causes many harm effects in biological cell systems[2-3,5,8-10]. TCE is a non-carcinogenic (group 3) because there is inadequate evidence for carcinogenicity in both human and animals according to the last update of U.S Environmental Protection Agency (EPA) and National Toxicology Program (NTP) technical report[11-12]. In addition, according to WHO toxicological report, TCE is not considered as toxic, and not necessary to drive a health based guideline standard[13]. However, the Agency for Toxic Substances and Disease Registry (ATSDR) indicated that TCE affects many internal organs, such as cardiovascular and nervous system[14].

Despite the TCE safe profile claimed in the aforementioned reports, TCE reproductive toxicity was reported by several studies in many animal models by oral, inhalation and dermal exposure[15-18]. Moreover, prior animal studies have shown that perinatal exposure to TCE affects the development of the brain, liver, adipose tissue and reproductive tract and adversely affects their functions[2-3,5,8-10]. However, based on the best authors knowledge, no study had focused on the long-term effect of TCE exposure on the fertility in later reproductive life.

Thus, this study was designed to explore the possible hazard effects of the exposure to environmentally relevant levels of TCE during an embryonic ovarian developmental window has long lasting effects on reproductive performance in later reproductive life. The administration of TCE safe considered doses to female mice was through intra-peritoneal injection. This mode of exposure has never been reported previously but the advantage of such exposure is to put the toxic chemical in close contact with the target cells and avoid rapid bio-elimination of TCE, which is two hours[19].

In the present study, TCE exposure impaired the fertility and increased body weight gain in F0 females. In addition, most interestingly, TCE exposure also affected the reproductive outcome of F0 females.

The current data showed also that TCE had not resulted in a change in the body weight of F0 female mice. This is consistent with a previous study on rats which reported that no body weight changes were observed following intermittent or continuous exposure to trichloroethylene vapors at exposure levels in a range of 400–2,500 ppm for 2–13 weeks[20]. On the other hand, another study reported > 20% decreased body weight in male rats exposed to trichloroethylene vapors at 376 ppm for 4 hours per day, 5 days per week and a period of 12 or 24 weeks[21]. These findings are not consistent with other studies, which have shown that developmental TCE exposure disturbs metabolism, interferes with adipocyte proliferation and differentiation and may be linked to obesity in later life[20-21]. However, as no significant differences in body weight have been observed between TCE-treated mice and controls, we speculate in TCE exposure might reprogram the progenitor adipocytes and increase body weight significantly later in life.

Although some studies indicated that the exposure to TCE decreases body weight gain[17], the results of this study showed a dose-associated increase in the body weight gain of TCE-treated F0 females and their neonates. Many studies have found that certain toxic chemicals such as pesticides, the most environmental pollutants, and plastic products can cause weight gain by disrupting endocrine system[22-26]. These environmental chemicals have different mechanism of action such as increasing the activity of estrogen[23,25], abnormal adipogenesis[25], increasing inflammatory cytokine activity, causing oxidative stress, inducing abnormal thyroid function and impacting energy metabolism[27]. In addition, inspite of rare information of TCE endocrine toxicity, one study recorded that acute inhalation of TCE disrupts the concentration of corticosterone and adrenocorticotropic hormones[28]. It was also noted that TCE has increased the weight of internal organs of rats[29] and their pups[30-31]. In this study, it is possible that the

increase in body weight gain due to TCE exposure has been caused by one or more of above mentioned mechanisms.

Many toxicological and histopathological examinations in different animal models indicated that exposure to TCE through inhalation has not adverse effects on female reproductive system following wide range doses (acute, intermediate and chronic) [15,29,32-35]. Similar to TCE inhalation, oral exposure of 100, 300 and 1000 mg/kg/day in mice [36] and 3 mg/kg in rats[37-38] did not show any hazard effects on female body weight, survival, fertility, reproductive performance and gestation period. In this study, our model (with low doses) showed a significant decrease in the pub number in TCE-treated female litter. This might be attributed to the effect of TCE on oogenesis and oocyte quality, fertilization processes, early embryo death or the changes noticed in endometrium which could affect the proper formation and implantation of placenta.

Abnormal sex ratio may be induced in human and animals by the exposure to chemicals[39-40] but changes in sex ratio related to exposure to TCE have not been documented. This study is providing the first observation about changed litter sex ratio in mice due to exposure to high doses of TCE. This could be attributed to an increased sensitivity of sperms and/or embryos of certain genotype to the exposure to a particular chemical resulting in low fertilization rate or early embryonic death[41].

The uterus is one of the major organs of reproductive activity of mammals as it provides the place and the environment for gestation from the embryonic implantation and placentation till parturition. Therefore, the pathological conditions interfering with the normal uterine physiology might result in impaired reproductive performance. This study showed that TCE exposure induced a bilateral asymmetrical enlargement in the uterine body. Histpathologically, TCE exposure was associated with endometrial tissue changes appeared as severe endometrial hyperplasia and adenomyosis. This might be the responsible, at least in part, to the reduced fertility in TCE-treated F0 females and their reduced litter number.

In conclusion, the current study indicates that TCE exposure during a critical ovarian developmental window impairs female reproduction life. Further studies focused on examining the hormone profiles, ovarian morphology at later reproductive life, and pregnancy status during mid- and late gestation will be helpful in enhancing our understanding of the mechanism by which TCE affects female reproduction.

Acknowledgements

This investigation was supported in part by the Division of Developmental Biology, Faculty of Science, University of Tripoli, Tripoli-Libya. The authors zould like to thank Turkia Aduma, the technical staff at the Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, University of Tripoli, Tripoli-Libya, for her technical assistance.

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