Zagazig University Medical Journal
www.zumj.journals.ekb.eg
https://dx.doi.org/10.21608/zumj.2020.25193.1764 Volume 28, Issue 6, November 2022(132-142) Supplement Issue

Manuscript ID ZUMJ-2003-1764 (R2)
DOI **10.21608/zumj.2020.25193.1764**

ORIGINAL ARTICLE

Effect of maternal exposure to triclocarban on the postnatal development of the ovary in the albino rat offspring: A histological and immunohistochemical study

soad mohamed saad email [1]; Abd Al-Mawla Al Sayed Aidaros[2]; Dalia abdelhameed mandour[3]
1anatomy and embryology, faculty of medicine,zagazig university zagazig,egypt
2anatomy and embryology, faculty of medicine,zagazig university zagazig,egypt
3anatomy department faculty of medicine zagazig university

Corresponding author
Soad Mohamed Saad
Demonstrator in Human Anatomy and Embryology department.
E.mail :
Soadsaad.dr@gmail.com

Submit Date 2020-03-14
Revise Date 2020-04-26
Accept Date 2020-04-28

ABSTRACT

Background: Triclocarban (3,4,4 '-trichlorocarbanilide; TCC), an antimicrobial compound largely used in personal and household products, designated as one of the endocrine-disrupting substances(EDS),that adversely affect the endocrine function in vitro and in vivo.

Aim: The objective of this study was to investigate whether maternal exposure to TCC may pose a serious risk to the development of the ovary in their offspring.

Materials and Methods: Ten pregnant female rats were equally divided into control dam group and TCC-treated dam group were supplemented with TCC 0.5 mg/L in drinking water from the 5th day of gestation till the 14th post natal day (PND14). Then, ten female offspring of the control dams were divided into 2 subgroups: Neonatal and Infantile control offspring. Also, 10 female offspring of TCC-treated dams were divided into: Neonatal and Infantile TCC offspring. At the end of the experimental period, the female offspring were anesthetized and the ovaries were excised and weighed then processed for histological examination and immunohistochemical staining of both Ki67 and Caspse-3. Serum estradiol, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), C-reactive protein (CRP), malondialdehyde (MDA) and total antioxidant capacity (TAC) were measured.

Results: TCC impaired normal folliculogenesis and impeded the normal primordial transition to other types of more developed follicles. Also, it decreased the mean area% o f Ki67 and increased caspase-3 immunoexpression reflecting a decrease of proliferation and a provocation of apoptosis of follicular cells.TCC disturbed the feminine hormonal profile with increasing oxidative stress of the neonatal and infantile offspring.

Conclusion: This work highlighted the deleterious effect of maternal exposure to TCC on the folliculogenesis in the rat ovary offspring that could have implications on fetal outcome.

Keywords: Triclocarban, maternal exposure, postnatal development, ovary, offspring, rats

INTRODUCTION

Increased exposure to the endocrine-disrupting substances (EDS) in daily life has raised public concern relating to their potential human health impacts. Efforts to identify and characterize EDS have revealed that a relatively large number of them have estrogenic, antiestrogenic or antiandrogenic activity **[1].** However, few EDS have been associated with androgenic activity **[2, 3].**

Triclocarban (TCC; 3,4,4'-trichlorocarbanilide) is one of these EDS. TCC is a topical antimicrobial agent used in a wide range of personal hygiene

products including many brands of soap, toothpaste, and shampoo **[4].**

It was reported that direct human exposure to TCC led to a significant body burden, where three hours after a single 15-minute whole-body shower with soap containing 0.6% TCC, the peak circulating level of TCC reach up to 530 nmol/L **[5].** Also, indirect exposure to TCC is documented because TCC has a robust propensity to accumulate to the sludge due to its hydrophobic nature allowing for potential transfer to the environment and to the plants, particularly when this sludge is applied as a fertilizer in agriculture use **[6].** Also, TCC was detected in all samples of downstream water collected from a wastewater treatment **[7].**

The widespread use of TCC, the direct human exposure and high environmental contamination have raised the concerns regarding its reproductive effects in females. In particular, it was reported that the development of the ovaries that continues until the postnatal pubertal period is very sensitive to different environmental contaminants **[8].** To date, there are no data on the effects of TCC exposure on the ovarian development in offspring. This study was warranted to determine whether TCC at the environmentally relevant concentrations could be transferred from the mother to the offspring during gestational and lactational periods and whether this transfer has an impact on the postnatal development of the offspring ovary.

MATERIALS AND METHODS

Chemicals: Triclocarban (TCC) powder of 98.8% purity (Sigma-Aldrich Company Cat. No. 105937, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) prior to its addition to the drinking water. The percent of DMSO in drinking water was less than 0.001%. Because of TCC demonstrated high adsorption in the traditional animal water bottles synthesized of rubber or plastic materials; 40±7% of TCC stock was lost within hours. Therefore, glass bottles were used for keeping TCC **[9].**

Animals: A total of 10 adult female and 5 male Sprague Dawley albino rats, weighing 180-220 gm. The animals were housed in well ventilated cages with a temperature maintained at 23±2 °C and in a 12 h light/12 h dark cycle. The animals were maintained on a standard chow diet and water ad libitum all over the study period. Before the start of the experiment, the rats were left to acclimatize for a period of one week. All the experiments were performed following the standard guide of the Institutional Animal Care and Use Committee of Zagazig University (IACUC) in accordance with the international guidelines.

Sample size and sampling frame: Assuming mean ± standard deviation of different parameters during ovarian development in offspring of gestational control rats versus offspring of gestational rats exposed to triclocarban (60 ± 2 vs 63 ± 3.1). Sample was calculated to be 10 rats, 5 in each group using Open Epi program with test power 80%, confidence level 95%.

Experimental protocol: The female rats were overnight housed with males at a ratio of 2:1 per cage. Serial morning vaginal smears were taken from the females to detect the onset of pregnancy. The day at which sperms were detected in the vaginal plug smear was considered as the day zero of gestation (G0). Ten pregnant rats were equally divided into two equal groups; First, is the control dam group (was supplied with water enriched with 0.001% DMSO). Second, is the TCC-treated dam group (was supplemented with TCC in drinking water in a dose of (0.5 mg/L) starting from the 5th day of gestation (G5) until PND14 **[9].** After delivery, the offspring were kept with their mothers for breast feeding.

Offspring subgroups: Ten female offspring of the control dam group were further subdivided according to the developmental age period into 2 subgroups:

- control neonatal (PND7) offspring.
- control infantile (PND14) offspring.

Ten of the TCC-treated dam group were further subdivided according to the developmental age period into 2 subgroups:

- TCC neonatal (PND7) offspring.
- TCC infantile (PND14) offspring.

Experimental study: At the end of the experimental period, the body weight (BW) of the female offspring was determined then they were anesthetized by intraperitoneal (i.p.) injection of sodium thiopental (40mg/kg). Blood samples from all offspring were left to clot then centrifuged for ten minutes at 2500 rpm. Serum was separated and stored at –20 °C until further biochemical analysis. Thereafter, the female offspring were sacrificed by cervical dislocation then the ovaries were excised and weighed.

Hematoxylin and Eosin staining: The excised ovaries were fixed in a neutral-buffered formalin for 24 hours. The samples were dehydrated through ascending graded series of alcohol, cleared in xylene, and then embedded in paraffin wax. Thereafter, a serial paraffin ovarian sections were sliced at 4-5µm and stained with hematoxylin and eosin (H&E) **[10].** All sections were examined and imaged at ×100 and ×400

magnification under a light microscope, Leica DM500 (German) in the Department of Anatomy, Faculty of Medicine, Zagazig University, Egypt.

Immunohistochemistry: Paraffin sections of the ovaries of 4 µm thickness were processed for immunohistochemistry **[11].**

The sections were deparaffinized, rehydrated. For antigen retrieval,exposure of the antigenic sites was performed thermally by incubation of the sections in 0.01 M citrate buffer solution (pH 6.0) in a microwave oven at 800 W, for three cycles lasting 5 min each. To inhibit endogenous peroxidase activity, 0.3% H2O2 in methanol was used. The slides were incubated overnight at 4 °C with antibodies. First is mouse monoclonal anti-Ki-67 antibodies (clone MIB-5; Dako, Carpiteria, CA, USA) at dilution of 1/50. The second is polyclonal anti-caspase 3 antibodies (Thermo Fisher Scientific Inc., USA, Cat, No. PAI-29157) at dilution of 1/100. The sections were incubated with biotinylated anti-rabbit secondary antibody (Amersham) 1:200 in PBS for 30 min. followed by addition of streptavidin-peroxidase solution. Afterwards, diaminobenzidine (DAB) chromogen solution was added. Finally, the sections were counterstained with Mayer's hematoxylin. The slides were examined and imaged with the use of a light microscope at × 400 magnification.

Morphometric study: Area % of Ki-67 and caspase-3 immunoexpression (the percentage of Ki-67- and caspase-3 positive cells) was calculated in five non-overlapped high power fields (X400) in the immunohistochemical stained-slides of each pup, using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA).

Biochemical study: Serum level of estradiol, progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Uscn Life Science Inc., Houston, TX, USA)**[12].** Serum malondialdehyde (MDA) **[13]**, total antioxidant capacity (TAC) **[14]** and c-reactive protein (CRP) **[15]** were measured.

Statistical analysis: The data were statistically analyzed using SPSS version 21 for windows (SPSS Inc., Chicago, IL, USA).The results were expressed as the Mean± SD. Data were checked for normality by using Shapiro Wilk test. In the independent samples, Student's t-test was used to compare between two groups of normally distributed variables, while Mann Whitney-U test was used for non-normally distributed variables. P-value <0.05 was considered statistically significant and ≥ 0.05 was considered statistically insignificant.

RESULTS

ONE WEEK RESULTS

Hematoxylin and Eosin results: Light microscopic examination of ovarian sections from the control neonatal (PND7) offspring showed the primordial follicles which were the most prominent feature of this age group. Large number of primordial follicles was present and occupied the thick peripheral cortex of the ovary **(Fig.1A).** Each primordial follicle was formed of a single layer of flattened follicular cells surrounding an immature ovum (primary oocyte) that had a large eccentric vesicular nucleus. Also, primary follicles and some immature secondary follicles were laying in the central core of the ovary. The primary follicles were formed of a single layer of cuboidal follicular cells. While, the immature secondary follicles were formed of two or three layers of cuboidal follicular cells with no fluid filled cavities. The follicular cells were discrete and not adherent to the primary oocyte with absence of zona pellucida. The follicles situated within loose mesenchymal stromal cells. The surface of the ovary was covered by a single layer of squamous cells with ill-developed tunica albuginea beneath it **(Fig.1B).** Light microscopic examination of sections from the ovaries of TCC neonatal (PND7) offspring exhibited a discernible change of the normal ovarian architecture in the form of an apparent decrease of the ovarian size that appeared as a solid parenchymal sheet of primordial follicles. There was a relative decrease of the number of primary follicles with absence of the secondary follicles compared to the control neonatal offspring **(Fig.1C).** The oocytes of primordial and primary follicles had vacuolated cytoplasm with deeply stained shrunken nuclei. Atretic follicles with involution of their oocytes and degenerated ovarian surface epithelium were obvious **(Fig.1D).**

Immunohistochemical results: The ovary of the control neonatal (PND7) rat offspring showed positive nuclear immunoreactions for Ki-67 in some granulosa cells of primordial, primary and immature secondary follicles **(Fig.1E).** The ovarian sections of the neonatal (PND7) TCC offspring revealed negative nuclear immunoreaction for Ki-67 in the follicular cells of primordial and primary follicles **(Fig.1F).** The ovary of the control neonatal rat offspring showed negative cytoplasmic immunoreaction for caspase-3 in the follicular cells of the primordial, primary, immature secondary follicles **(Fig.1G).**

The ovarian sections of the neonatal (PND7) TCC offspring revealed positive nuclear and cytoplasmic immunoreaction for caspase 3 in some follicular cells of the primordial and primary follicles **(Fig.1H)**.

TWO WEEK RESULTS

Hematoxylin and Eosin results: Light microscopic examination of ovarian sections from the control infantile (PND14) offspring showed persistence of some primordial follicles limited to the thin peripheral rim of the ovary while the primary and the immature secondary follicles situated in the core. Interestingly, the ovarian sections showed the appearance of a third type of more developed growing follicles; the mature secondary (early antral) follicles that were settled in the central core of the ovary and were comparatively larger in size than the other follicles (**Fig.2A**). Both types of secondary follicles were surrounded with three or more layers of granulosa cells that showed large basophilic nuclei and became tightly adherent to the primary oocytes. The mature secondary follicles had discrete vesicular spaces among stratum granulosa. The zona pellucida surrounding the primary oocyte became apparent. Some follicles started to be rounded by a stromal capsule of thecal cells (theca folliculi). The stroma of the ovary at that age was mainly concentrated in the centre of the ovary with notable absence of the interstitial glands **(Fig.2B).** Light microscopic examination of ovarian sections from TCC infantile (PND14) offspring revealed an apparent decrease of the ovarian size compared to the control infantile offspring. A thin peripheral rim of primordial follicles and a central core of shrunken primary, immature secondary and mature secondary (early antral) follicles **(Fig.2C).** Some secondary follicles had primary oocytes with vacuolated cytoplasm and deeply stained shrunken nuclei. Other follicles revealed involuted oocytes. The granulosa and stromal cells appeared disarranged and vacuolated with pyknotic nuclei. Dilated congested blood vessels were obvious (**Fig.2D**).

Immunohistochemical results: Ovary of the control infantile (PND14) rat offspring showed positive nuclear immunoreactions for Ki-67 in some granulosa cells of secondary follicles **(Fig.2E).** The ovarian sections of the infantile (PND14) TCC offspring revealed negative nuclear immunoreaction for Ki-67 in the granulosa and stromal cells **(Fig.2F).** The ovary of the control infantile rat offspring showed negative cytoplasmic immunoreaction for caspase-3 in the granulosa cells of the secondary follicles **(Fig.2G).** The ovarian sections of the infantile (PND14) TCC offspring exhibited positive immunoreaction for caspase 3 in the granulosa cells of the degenerated follicles and early antral follicles. Also, positive immunoreaction for caspase 3 was observed in the stromal cells **(Fig.2H)**.

Morphometric results: In the control offspring, the mean area % of Ki-67 immunoexpression was significantly decreased (P=0.04) in the control infantile than the control neonatal offspring. In TCC neonatal offspring, the mean area % of Ki-67 immunoexpression was significantly decreased (P =0.0001) compared to the age-matched control neonatal offspring. Also, this mean area was significantly decreased (P=0.0001) in TCC infantile compared to control infantile offspring **(table 1).**

In the control offspring, the mean area % of Caspase-3 immunoexpression was insignificantly changed (P=1.06) between the control infantile and the control neonatal offspring. In the TCC neonatal offspring, the mean area % of caspase-3 immunoexpression was significantly increased (P=0.0001) compared to the control neonatal offspring. Also, this mean area was significantly increased (P=0.0001) in TCC infantile offspring compared to the control infantile offspring (**table 2**).

Biochemical results: In the control offspring, Estradiol was insignificantly changed (P=0.41) between the infantile and the neonatal offspring. In the TCC neonatal offspring, estradiol was insignificantly changed (P=0.967) compared to the control neonatal offspring. Also, Estradiol was insignificantly changed (P=0.483) between TCC infantile and the age-matched control infantile offspring **(table 3).**

In the control offspring, Progesterone was significantly increased (P=0.021) in the infantile than the neonatal offspring. In the TCC neonatal offspring, Progesterone was significantly increased (P= 0.001) compared to the control neonatal offspring. While, Progesterone insignificantly decreased (P=0.115) in TCC infantile compared to the control infantile offspring **(table 3).**

In the control offspring, FSH was significantly increased (P=0.0001) in the infantile than the neonatal offspring. In the TCC neonatal offspring, FSH was significantly decreased (P= 0.032) compared to the control neonatal offspring. While, FSH significantly decreased (P=0.015) in TCC infantile compared to the control infantile offspring **(table 3).**

In the control offspring, LH was significantly increased (P=0.01) in the infantile than the neonatal offspring. In TCC neonatal offspring, LH was significantly increased (P= 0.038) compared to the control neonatal offspring. While, LH significantly decreased (P=0.025) in TCC infantile compared to the control infantile offspring **(table 3).**

In the control offspring, MDA was insignificantly changed (P=0.74) between the infantile and the neonatal offspring. In TCC neonatal offspring, MDA was significantly increased (P=0.033) compared to the control neonatal offspring. Also, MDA was significantly increased (P=0.001) in TCC infantile compared to the control infantile offspring **(table 4).**

In the control offspring, TAC was insignificantly changed (P=0.820) between the infantile and the neonatal offspring. In the TCC neonatal offspring, TAC was significantly decreased (P=0.002) compared to the control neonatal offspring. Also, TAC was significantly decreased (P=0.0001) in TCC infantile compared to control infantile offspring **(table 4).**

In the control offspring, CRP was significantly increased (P=0.041) between the infantile and the neonatal offspring. In the TCC neonatal offspring, CRP was significantly increased (P=0.021) compared to the control neonatal offspring. Also, CRP was significantly increased (P=0.025) in the TCC infantile compared to the control infantile offspring **(table 4).**

Results of BW, ovary weight and ovary weight/BW ratio

In the control offspring, BW was significantly increased (P=0.001) in the infantile than the neonatal offspring. BW was insignificantly changed (P= 0.401) between the TCC neonatal and the control neonatal offspring. Also, BW was insignificantly changed (P=0.521) between the TCC infantile and the control infantile offspring **(table 5).**

In the control offspring, the ovary weight was significantly increased (P=0.04) in the infantile than the neonatal offspring. In the TCC neonatal offspring, ovary weight was insignificantly changed (P= 0.337) compared with the control neonatal offspring. Also, the ovary weight was insignificantly changed (P=0.839) in TCC infantile compared with the control infantile offspring **(table 5).**

In the control offspring, ovary weight/BW ratio was insignificantly changed (P=0.822) between the infantile and the neonatal offspring. In the TCC neonatal offspring, the ratio was insignificantly changed (P=0.523) compared with the control neonatal offspring. Also, the ratio was insignificantly changed (P=0.556) in the TCC infantile compared with the control infantile offspring **(table 5).**

Table (1): Area % of Ki-67 at the neonatal and infantile periods of the control and TCC offspring (Mean± SD)

	Control offspring (N=5)	TCC offspring (N=5)	P2
Neonatal Ki-67	30.09±4.58	0.153±0.034	0.0001
Infantile Ki-67	25.86±6.4	5.84±0.786	0.0001
P1	0.04	0.001	

Table (2): Area % of Caspase-3 at the neonatal and infantile periods of the Control and TCC offspring (Mean± SD)

	Control offspring (N=5)	TCC offspring (N=5)	P2
Neonatal Caspase-3	0.0014±0.00052	0.682±0.036	0.0001
Infantile Caspase-3	0.0016±0.0007	21.28±1.8	0.0001
P1	1.06	0.0001	

Table (3): Serum Estradiol, Progesterone, FSH and LH at the neonatal and infantile periods of the Control and TCC offspring (Mean± SD)

	Control offspring (N=5)	TCC offspring (N=5)	P2-value
Estradiol (pg/ml)			
Neonatal	45.25±9.32	45±6.78	0.967
Infantile	48.5±4.79	55.75±17.82	0.483
P1	0.41	0.33	

	Control offspring (N=5)	TCC offspring (N=5)	P2-value
Progesterone (ng/ml)			
Neonatal	0.62±0.07	0.99±0.09	0.001
Infantile	1.14±0.29	0.83±0.18	0.115
P1	0.021	0.240	
FSH (ng/ml)			
Neonatal	2.78±0.23	1.25±11.5	0.032
Infantile	59.25±7.54	41.75±7.14	0.015
P1	0.0001	0.0001	
LH (mIU/ml)			
Neonatal	1.72±0.17	3.55±1.05	0.038
Infantile	4.77±1.89	2.45±.49	0.025
P1	0.010	0.041	

Table (4): Serum MDA, TAC and CRP at the neonatal and infantile periods of the control and TCC offspring (Mean± SD)

	Control offspring N=5	TCC offspring N=5	P2
MDA(nmol/ml)			
Neonatal	10.99±0.04	16.94±3.16	0.033
Infantile	11.02±0.36	19.55±1.68	0.001
P1	0.74	0.65	
TAC (mM/L)			
Neonatal	1.66±0.05	1.38±0.097	0.002
Infantile	1.72±0.09	1.03±0.04	0.0001
P1	0.820	0.001	
CRP (ng/ml)			
Neonatal	0.27±0.02	2.53±1.48	0.021
Infantile	0.47±0.04	4.61±0.97	0.025
P1	0.041	0.035	

Table (5): Body weight (BW) , Ovary weight and Ovary weight/BW ratio at the neonatal and infantile periods of the control and TCC offspring (Mean± SD)

	Control offspring N=5	TCC offspring N=5	P2
BW (gm)			
Neonatal	12.18±0.81	12.6±0.68	0.401
Infantile	28.2±6.81	30.7±4.79	0.521
P1	0.001	0.001	
Ovary weight (gm)			
Neonatal	0.0032±0.24	0.0031±0.19	0.337
Infantile	0.0075±1.79	0.0077±1.15	0.839
P1	0.04	0.03	
Ovary weight/BW ratio			
Neonatal	0.0264±0.0028	0.0244±0.0030	0.523
Infantile	0.0265±0.0020	0.0251±0.0039	0.556
P1	0.822	0.711	

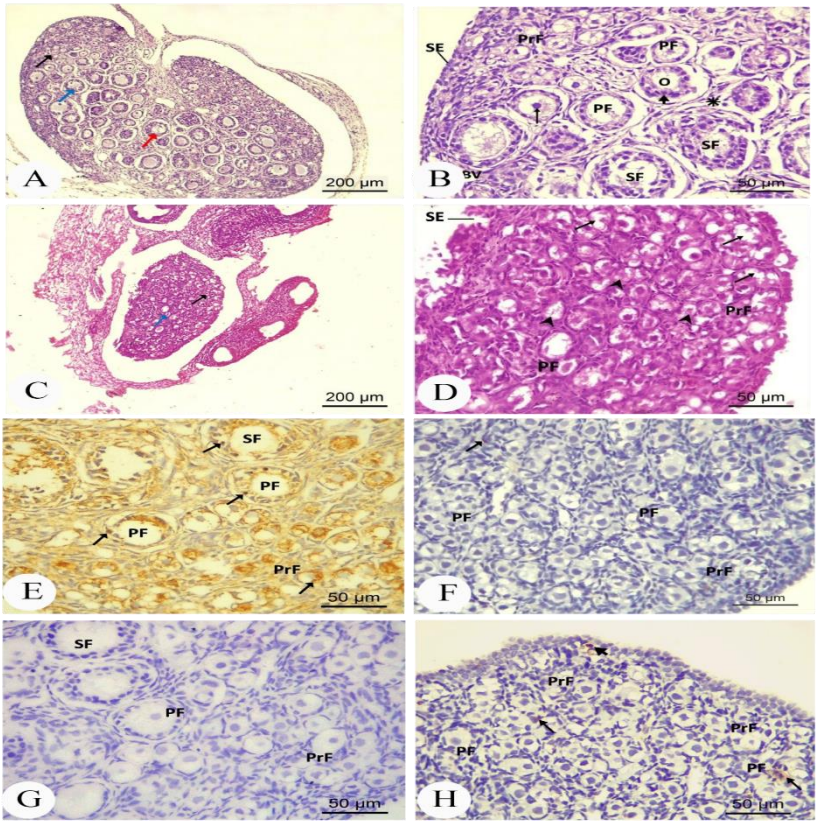

Fig. (1) histopathological and immunohistochemical ovarian sections at 1st week:

A: control group showing predominance of primordial follicles (black arrow) in the cortex, while the primary follicles (blue arrow) and immature secondary follicles (red arrow) are laying in the core of the ovary (H & E X100); **B:** showing the ovarian surface epithelium (SE) as a single layer of squamous cells, primordial (Prf), primary (PF) and immature secondary follicles (SF).These follicles contain primary oocytes (O) with large vesicular nuclei (thin arrow). The cuboidal follicular cells (thick arrow) are discrete and not adherent to the primary oocyte. Loose mesenchymal spindle-shaped stromal cells (star) with blood vessel (BV) inbetween the follicles are seen (H&E X 400); **C:** TCC treated group showing an apparent decrease in ovarian size that appeared as a solid parenchymal sheet of primordial follicles (black arrow) and primary follicles (blue arrow). (H&E x100); **D**: showing small-sized primordial (PrF) and primary follicles (PF) with immature follicular cells. Most of the primary oocytes have vacuolated cytoplasm (arrow). Atretic follicles with involution of their oocytes (arrow head) and a degenerated ovarian surface epithelium (SE) are noticed. (H&E x400); **E**: control group showing positive nuclear immunoreaction in some follicular cells (arrow) of primordial (PrF), primary (PF) and immature secondary follicles (SF). (Ki-67 immunostaining x400); **F**: TCC treated group showing negative nuclear immunoreaction in the follicular cells of primordial (PrF) and primary follicles (PF). Few follicular cells exhibit positive immune reaction (arrow). (Ki-67 immunostaining x400); **G**: control group showing negative immunoreaction for caspase 3 in the follicular cells of the primordial (Prf), primary (PF) and immature secondary (SF) follicles. (Caspase 3 immunostaining X 400); **H**: TCC treated groupshowing positive nuclear (thick arrow) and cytoplasmic (thin arrow) immunoreaction for caspase 3 in some follicular cells of the primordial (Prf) and primary (PF) follicles. (Caspase 3 immunostaining X 400).

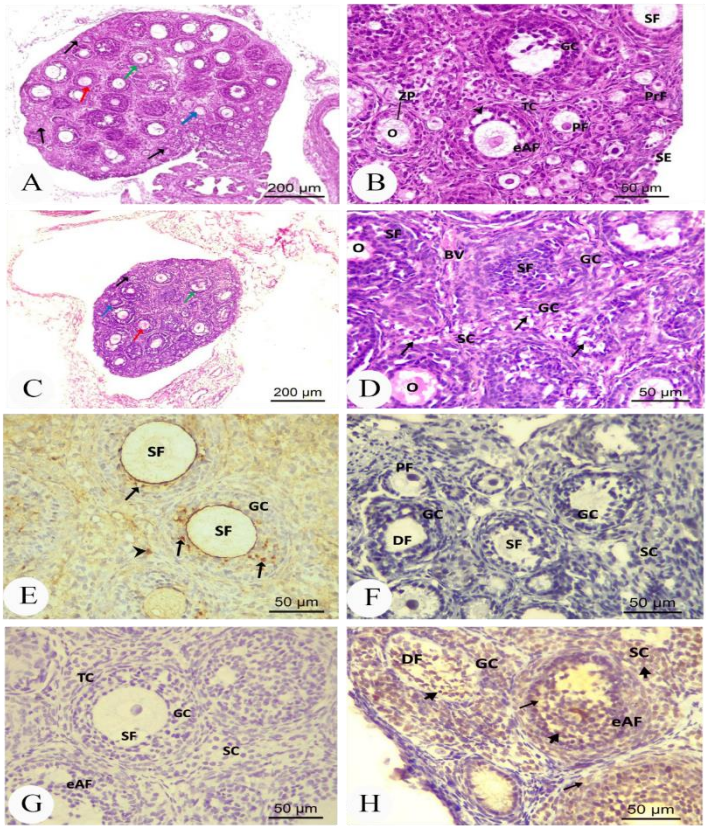

Fig. (2) histopathological and immunohistochemical ovarian sections at 2nd week:

A: control group showing a thin peripheral rim of primordial follicles (black arrow) and a central core of primary follicles (blue arrow), immature secondary follicles (red arrow) and early antral (green arrow) follicles. (H & E X 100); **B**: showing cuboidal surface epithelium (SE) and primordial follicles (PrF) in the periphery of the ovary. The core of the ovary reveals primary follicles (PF), immature secondary (SF) and early antral follicles (eAF) with their granulosa cells (GC). eAF is surrounded with fluid filled cavities (arrow head) and thecal cells (TC). Zona pellucida (ZP) surrounds the oocyte (O). (H & E X 400); **C**: TCC treated group showing an apparent decrease in ovarian size. A thin peripheral rim of primordial follicles (black arrow) and a central core of shrunken primary (blue arrow), immature secondary (red arrow) and early antral follicles (green arrow). (H&E x100); **D**: showing secondary follicles (SF) with vacuolated involuted oocytes (O). The granulosa cells (GC) and stromal cells (SC) appear disarranged and vacuolated with pyknotic nuclei (arrow). A dilated congested blood vessel (BV) is noticed. (H&E x400); **E**: control group showing positive nuclear immunoreaction (arrow) in the granulosa cells (GC) of secondary follicles(SF) and in the stromal cells (arrow head). (Ki-67 immunostaining x400); **F**: TCC treated group showing negative nuclear immunoreaction in the granulosa cells (GC) of degenerated follicles (DF), primary follicles (PF), secondary follicles(SF) and stromal cells (SC). (Ki-67 immunostaining x400); **G**: control group showing negative immunoreaction for caspase 3 in the granulosa cells (GC) and thecal cells (TC) of the Immature secondary follicles (SF) and early antral follicles (eAF).Also, negative immunoreaction in the stromal cells (SC). (Caspase 3 immunostaining X 400); **H**: TCC treated group showing positive nuclear (thick arrow) and cytoplasmic (thin arrow) immunoreaction for caspase 3 in the granulosa cells (GC) of the degenerated follicle (DF) and early antral follicle (eAF). Also, positive immunoreaction for caspase 3 in the stromal cells (SC) is observed. (Caspase 3 immunostaining X 400).

DISCUSSION

In this study, during the control neonatal period, the main feature of the newborn rat offspring ovary was the predominance of primordial follicles that were settled mainly at the periphery of the ovary. Also, few primary and immature secondary follicles were lying in the core of the ovary. The follicles were separated by little intervening flattened stromal interstitial cells.

In this work, hormonal analysis during the neonatal period revealed that the control

offspring had a basal normal serum levels of Estradiol, Progesterone, FSH and LH.

Physiologically, during the neonatal period, there is a correlation of the ovarian morphology to the neuroendocrine parameters, where the ovary, for the most part, is independent of the hypothalamus and pituitary, and the development of primordial to immature secondary follicles is controlled by paracrine and autocrine growth factors produced locally by the oocyte, the granulosa cells, or the stromal cells that work in complex synchrony **[16].**

Factors required for primordial follicles to develop into primary follicles include members of growth factors and cytokines including transforming growth factor beta (TGFβ), fibroblast growth factor–2 (FGF-2), bone morphogenetic protein-4 (BMP-4), leukemia inhibitory factor (LIF), and keratinocyte growth factor (KGF) **[17].** Also, the circulating insulin is needed to activate recruitment of primordial follicles **[18].** Factors required for the development of primary to immature secondary follicles include growth differentiation factor 9 (GDF-9; produced by the oocyte), and neurotransmitters, such as nerve growth factor (NGF**) [19].**

Despite, the ovary is a pituitary independent organ during this neonatal stage, there is a relatively high level of LH production by the anterior pituitary. The production of LH hormone is controlled by a gonad-independent mechanism via gamma aminobutyric acid (GABA) that exerts a stimulatory effect on luteinizing hormone–releasing hormone (LHRH) at the level of the hypothalamus. Also, a gonad-dependent mechanism via absence of an operative E2 negative feedback loops because serum estrogen level is low at the neonatal period **[19].**

This minimum serum estrogen level might be for two reasons: First, the aromatase activity of granulosa cells (which is required to convert androgens into estrogens) is insufficient. Second, there is circulating alpha fetoprotein (AFP) that binds any small amount of estrogen produced and prevents it from triggering the negative feedback at the hypothalamus **[16].**

Little pituitary dependence in the neonatal period is reported by its role in the recruitment of the primordial follicles. Also, the pituitary gland plays a role in the transition from primary to immature secondary follicles, where the granulosa cells of primary follicles begin to express very little amounts of LH and FSH receptors by the late neonatal period (PND 5–PND 7), and this low complement of receptors along with FSH is required for complete development of secondary follicles **[19].** It is reported that the developing granulosa cells of primary follicles regulate recruitment of additional primordial follicles by secreting antimullerian hormone (AMH) that turns off recruitment, so an "internal" negative feedback loop within the ovarian parenchyma is operative **[18]**.

In this study, during the normal infantile period there was characteristic appearance of a third type of more developed growing type of follicles; the mature secondary (early antral) follicles that settled in the central core of the ovary. The primary oocytes and their nuclei increased in size and became surrounded by zona pellucida. The stroma of the ovary at that age was mainly concentrated in the medullary region and become arranged around the growing follicles forming the theca cells (theca folliculi).

During the normal infantile period, particularly at PND 10, early antral development occurs, and small antrum formation is the hallmark morphologic feature. This feature indicates the switch of the ovary from pituitary independence (neonatal period) to pituitary-dependence (early infantile period) **[20].**

In the present study, the normal transition of primordial into secondary follicles could be explained by an increase in the mean area % of Ki67 which provokes proliferation of the granulosa cells of the follicles together with a decrease in the mean area % of caspase-3 that indicated a decrease of apoptosis in the follicular cells.

Moreover, during the early infantile period there is an increase in serum LH and FSH levels **[19]**. Serum LH and FSH levels increase due to the continued excitatory effect of GABA on the hypothalamus. LH and FSH receptor levels on the theca and granulosa cells increased throughout this period **[20].**

Antral follicle development proceeds, despite waning FSH levels during the late infantile period. This apparent paradox is attributed first to increased density of LH/FSH receptors on granulosa cells with resulting high sensitivity of the follicles to the waning levels of LH/FSH and second to prolactin production by the pituitary that facilitates effects of LH/FSH on the ovary **[21]**.

In this study, the ovaries of TCC neonatal offspring showed a discernible decrease of the ovarian size and a relative decrease in the number of primary follicles with absence of the secondary

follicles compared to the control neonatal offspring. Atretic follicles with involution of their oocytes and degenerated ovarian surface epithelium were obvious. Also, the ovaries of TCC infantile offspring showed an apparent decrease of the ovarian size, shrunken primary, immature secondary and mature secondary (early antral) follicles compared to the control infantile offspring. The follicles revealed involuted oocytes with pyknotic nuclei. Also, the ovary revealed disarranged and apoptotic granulosa cells with pyknotic nuclei, vacuolated stromal cells and congested blood vessels.

In the present study, the effect of TCC on the neonatal and infantile ovary could be explained by decreasing the mean area % of Ki67 which means that TCC decreased proliferation of the granulosa cells of the follicles. Also, TCC increased the mean area % of caspase-3 that indicated that TCC induced apoptosis in the follicular cells.

Also, the atresia and involution of the primordial and primary follicles under the effect of TCC might be attributed to its ability to increase oxidative stress via increasing the serum levels of MDA and decreased TAC. In addition, TCC increased CRP in the serum of the neonatal and infantile offspring, a mechanism that indicate its pro-inflammatory effect.

Moreover, the mechanism of TCC-induced atresia of primordial and primary follicles could be explained by the androgen-augmenting activity of TCC. In vitro, TCC effect on androgen receptor (AR) was evaluated in a cell-based human bioassay system and it was found that TCC alone had no effect on AR-mediated transcriptional activity; however, it augmented the testosterone-induced effects on AR. In vivo, androgenic effect of TCC was also observed, where it augmented androgen induced weight gain of accessory sex organs in peripubertal male rats **[22].** Hyperplasia of primary sex organs was also found in juvenile animals after they had been treated with TCC **[4].** Exposure to high TCC concentration during lactation induced deleterious reproductive effects and endocrine disruption in rodents **[23].**

In this work, during the infantile period, TCC decreased the serum Progesterone, FSH and LH. It was reported that TCC exposure caused altered adrenal steroidogenesis by affecting an early step in steroid biosynthesis. Pregnenolone, progesterone, 11-Deoxycorticosterone, 17α-hydroxyprogesterone and Dehydroepiandrosterone were the most sensitive to the increasing TCC concentrations **[24].**

REFERENCES

1- Louis J, Guillette Jr. Endocrine disrupting contaminants—beyond the dogma. Environ Health Perspect 2006;114 (1):9-12.

2- Dallinga JW, Moonen EJ, Dumoulin JC, Evers JL, Geraedts JP, Kleinjans JC. Decreased human semen quality and organochlorine compounds in blood. Hum Reprod 2002; 17:1973-1979.

3- Korner W, Vinggaard AM, Terouanne B, Ma R, Wieloch C, Schlumpf M, et al. Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals. Environ Health Perspect 2004; 112:695-702.

4- Duleba AJ, Ahmed MI, Sun M, Gao AC, Villanueva J, Conley AJ, et al. Effects of triclocarban on intact immature male rat: augmentation of androgen action. Reprod Sci 2011; 18:119–127.

5- Schebb NH, Ahn KC, Dong H, Gee SJ, Hammock BD. Whole blood is the sample matrix of choice for monitoring systemic triclocarban levels. Chemosphere 2012;87(7):825-827.

6- Wu C, Spongberg AL, Witter JD, Fang M, Czajkowski KP. Uptake of pharmaceutical and personal care products by soybean plants from soils applied with biosolids and irrigated with contaminated water. Environ Sci Technol 2010;44(16):6157-6161.

7- Sapkota A, Heidler J, Halden RU. Detection of triclocarban and two contaminating chlorocarbanilides in U.S. aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry. Environ Res 2007; 103:21–29.

8- Bojanic V, Bojanic Z, Najman S, Savic T, Jakovl JV, Najman, et al. Diltiazem prevention of toxic effects of monosodium glutamate on ovaries in rats. Gen Physiol Biophys 2009; 28:149-154.

9- Enright HA, Falso MJ, Malfatti MA, Lao V, Kuhn EA, Hum N. Maternal exposure to an environmentally relevant dose of triclocarban results in perinatal exposure and potential alterations in offspring development in the mouse model. PLOS ONE 2017; 12(8): 81-96.

10- Bancroft JD, Gamble M. Theory and Practice of Histological Techniques.6th edition. Churchill Livingstone, New York, Edinburgh, London 2008;p: 83-92.

11- Ramos-Vara JA, Kiupel M, Baszler T, Bliven L, Brodersen B, Chelack B, et al . Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. J Vet Diagn Invest 2008; 20:393-413.

12- Cao XY, Hua X, Xiong JW, Zhu WT, Zhang J, Chen L. Impact ofTriclosan on Female Reproduction through Reducing Thyroid Hormones to Suppress Hypothalamic Kisspeptin Neurons in Mice. Frontiers in Molecular Neuroscience 2018; 6-11.

13- Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Annals of Biochemistry 1979; 95:351-358.

14- Koracevic D, Koracevic G, Djordjevic V. Method for the measurement of antioxidant activity in human fluids. J Clin Pathol 2001 ;54:356-361.

15- Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation.
Nature 2008; 454:436-444.

16- Picut CA, Dixon D, Simons ML, Stump DG, Parker GA, Remick AK. Postnatal ovary development in the rat: morphologic study and correlation of morphology to neuroendocrine parameters. Toxicologic pathology 2015; 43(3): 343-353.

17- Skinner MK. Regulation of primordial follicle assembly and development. Hum Reprod Update 2005; 11:461-471.

18- Williams CJ, Erickson GF. Morphology and physiology of the ovary. Endocrinology of Female Reproduction. 2012. from Endotext website: [Accessed January 14, 2014].

19- Ojeda SR, Skinner MK. Puberty in the rat. Physiology. Academic Press, San Diego, CA; of Reproduction 2006; 3 (2): 2061-2126.

20- Hirshfield AN, Desanti AM. Patterns of ovarian cell proliferation in rats during the embryonic period and the first three weeks postpartum. Biol Reprod 1995; 53:1208-1221.

21- Fortune JE. Ovarian follicular growth and development in mammals. Biol Reprod 1994; 50:225.

22- Chen J, Ahn KC, Gee NA. Triclocarban enhances testosterone action: a new type of endocrine disruptor? Endocrinology 2008;149(3):1173-1179.

23- Kennedy RC, Menn FM, Healy L, Fecteau KA, Hu P, Bae J, et al. Early Life Triclocarban Exposure During Lactation Affects Neonate Rat Survival. Reproductive Sciences 2015; 22(1):75-89.

24- Tonoli DC, F¨urstenberger J, Boccard . "Steroidomic Footprinting Based on Ultra-High Performance Liquid Chromatography Coupled with Qualitative and Quantitative High-Resolution Mass Spectrometry for the Evaluation of Endocrine Disrupting Chemicals in H295R Cells." Chemical Research in Toxicology 2015; 28(5):955-966.

How to cite

Saad, S., Aidaros, A., mandour, D. Effect of maternal exposure to triclocarban on the postnatal development of the ovary in the albino rat offspring: A histological and immunohistochemical study. Zagazig University Medical Journal, 2022; (132-142): -. doi: 10.21608/zumj.2020.25193.1764