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Histological Changes in Rat Ovaries Exposed in utero to 5MHz Ultrasound Waves

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SUMMARY

In antenatal clinics of obstetric departments ultrasound is frequently used for various diagnostic reasons. Practically it has become one of the routine investigations, thus exposing the developing embryo and fetus to ultrasound waves many times during entire gestation. Many biological effects of ultrasound are known and various studies have been conducted to confirm these. This study was carried out to see the histological changes in the rat ovaries who were exposed to 5 MHz frequency ultrasound waves. Two experimental groups B1 and C1 received 6 and 9 exposures respectively, of 5 minutes duration each time during their intrauterine developmental period. After parturition the female offsprings were grown to puberty and then dissected to obtain their ovaries for histological study and comparison with control. The results showed degenerative changes in secondary follicles. The cortex and medulla of ovaries showed moderate congestion and dilatation of blood vessels and lymphatics. Fibrotic and oedematous changes were also observed in the medulla. Morphometric observations revealed the mean diameter of graafian follicles was 490.6±112.1 μm in control while the values in experimental groups B1 and C1 were 436.56±63.3 μm and 405.62±44.2 μm respectively. The mean diameter of Graffian follicles was reduced significantly in group C1 ($p<0.05$). The mean diameter of secondary oocytes in control was 47.34±11.2 and in experimental groups B1 and C1 were 47.3±4.9 μm and 38.3±12.7 μm respectively. These values were statistically non significant ($p > 0.05$).

INTRODUCTION

The early medical application of ultrasound waves was introduced by Dussik prior to World War II.[1] After world war II Douglass Howry and John Wild, used commercial or Navy Sonar equipment to demonstrate echoes reflected from tissues deep within the body. Its use in obstetrics was started in 1966.[2] The procedure once used in high risk pregnancies is now used for estimation of fetal age, placental localisation, amount of amniotic fluid, presentation, twining, determination of fetal sex and gross fetal abnormalities.

Ultrasound is basically any sound with a frequency higher than that of audible sound. Since audible sound ranges from 16,000 to 20,000 cycles per second, sound frequencies above 20,000 cycles per second are in the ultrasound range.[3] In clinical practice it is limited to frequencies in the range from 1 to 10 million cycles per second, that is, 1 to 10 megahertz (MHz).[4]

Many biological effects of ultrasound are known, for instance, it can elevate the temperature of tissue, can disrupt membranes and cells in the region of interface.[5] It can also produce cavitation and may catalyse certain noxious chemical reactions.[6,7]

Various investigations have been done to establish the safety/harmful effects of ultrasound on various organs of experimental animals. In a study on mice using 1.5 MHz frequency transducer, the adult male and female gonads were exposed to ultrasound waves of both continuous and pulsed types for 15 minutes. The results were compared with those from control and from mice exposed to 100 rad x-rays. There was no evidence of induction by ultrasound of dominant lethal mutation or sterility in males, no reduction in testis weight or sperm count was observed. Similarly, no dominant lethal induction was detected in females. By

contrast, clear genetic damage was observed in the X-ray treated animals.[8]

In another study the neonate rats who were exposed to ultrasound of 5 MHz in utero were examined for postnatal growth and neonatal development. Analyses of results indicated that there were no significant alterations in exposed and control groups.[9] Thermal bioeffects showed significant heating of rats skull at 2.5 MHz frequency.[10] Similarly in vitro heating of fetal bones exposed to 1 MHz, continuous wave ultrasound have also been reported.[11] Significant morphological changes such as pyknosis of nuclei, vacuolization of cytoplasm and general cellular disruption were observed when adult mouse ovaries were exposed in vivo to 1 MHz continuous wave ultrasonic energy for times varying from 15 to 300 seconds.[12]

An experimental study has also been performed on human embryos. The fetuses of 10 women at 9-12 weeks gestation were irradiated with ultrasound under diagnostic exposure conditions immediately prior to abortion. Electron microscopy of liver fragments revealed no morphological or structural changes.[13]

Indirect sonochemical effects of ultrasound have not yet been demonstrated in mammals, but concern persists that latent, infrequent effects might be overlooked and might present some risk of harm in critical situations such as the developing fetus. The present study was thus designed to observe the histological changes in the ovaries of rats exposed to 5 MHz, frequency ultrasound waves during their intrauterine developmental period.

MATERIALS AND METHODS

Adults albino rats of wistar strain were used in the present study. Forty (70-75 days old) female rats (200-300 grams) and fifteen male rats (400-450 gram) were obtained from department of animal nutrition, Agricultural University, Faisalabad. The facility of animal house was provided by the Zoology Department, University of the Punjab, Lahore.

The rats were provided with commercial chick feed No.1.

Every 1 kg of chick feed No: 1 contained following ingredients.

1.	Maize	150 gm
2.	Rice broken	280 gm
3.	Wheat	250 gm
4.	Cotton meal	20 gm
5.	Corn G meal	20 gm
6.	Canola meal	40 gm
7.	Guar meal	30 gm
8.	Soya Bean meal	100 gm
9.	Fish meal	60 gm
10.	Molasses	30 gm
11.	Lime stone	10 gm
12.	Di cal phos	7 gm
13.	L-Lysine	0.8 gm
14.	DL-Meth	0.7 gm
15.	Premix	1.5 gm

The following ingredients were added for every 05 kilograms of the chick feed.

1	Wheat flour	= 2½ kg
2.	Molasses	= 1 kg
3.	Fish meal	= 100 gm

After two weeks of acclimatization, the female rats were randomly separated into three groups:

1. Group A (control): This contained 10 female rats.
2. Group B (Experimental): This contained 15 female rats.
3. Group C (Experimental): This contained 15 female rats.

The rats were properly marked and the same marks were pasted on their cages. The female albino rats in three groups were conceived with the help of male rats. Their pregnancies were confirmed by examining vaginal plug. The indication of vaginal plug was counted as day first of gestation. The average gestational duration in albino rats is 21 days[14] and in present study it was divided into three equal trimesters; each of 07 days. The subsequent experimental procedure was as follows.

1. Ten pregnant rats in group A (control) completed their gestation without any exposure to ultrasound waves.

2. Fifteen pregnant rats in group B (experimental) were exposed to ultrasound waves, of 5 MHz frequency, twice weekly i.e 6 exposures during their entire gestation. The time for each exposure was five minutes in each rat.
3. Fifteen pregnant rats in group C (experimental) were exposed to 5 MHz frequency ultrasound waves, thrice weekly i.e 9 exposures during their entire gestation. The time for each exposure was five minutes in each rat.

Sonography machine, Toshiba, model SAL 32A linear array probe with 5MHz frequency was used for exposure. The facility of ultrasound machine was provided by the Department of Surgery, Shaikh Zayed Hospital, Lahore.

For exposure, skin infront of abdomen and pelvis of each experimental rat was shaved. After applying liquid paraffin, the transducer was placed on the abdomen and pelvis and rotated slowly for five minutes. In this manner the whole developing embryos and fetuses of rats were exposed to ultrasound waves.

After parturition of group A, B and C only female offsprings were selected in this study for further procedures. The female offsprings from Group A, B and C were than subgrouped as A1, B1 and C1, respectively. They were allowed to grow and all were dissected at 70 days of age. Ten ovaries from subgroup A1 and 15 ovaries from each subgroups B1 and C1 were selected, at random, for histological study and were fixed in 10% formaldehyde solution. The ovaries were processed in an autoprocesser of department of Anatomy, Allama Iqbal Medical College, Lahore. Blocks were made, cut, stained with haemtoxylin and eosin stain and mounted. The following histological parameters were considered in this study.

1. Morphometric study of graafian follicles and secondary oocytes: This was done with the help of an ocular micrometer, calibrated with a stage micrometer, by using a light microscope.
2. Morphology of Ovaries: The developing follicles, cortical stroma, the medulla including blood vessels and the corpora lutea were studied.

Statistical analysis

The diameter of graafian follicles and diameter of secondary oocytes were analysed statistically by one way ANOVA. p value < 0.05 being significant for all analysis.

RESULTS

Microscopic observations of ovarian cortex

Simple cuboidal lining epithelium of ovaries was evident in control and experimental groups. Follicles in various stages of development were present in all groups. The primordial follicles in control and in experimental groups were located just below the thin tunica albuginea. These were lined by single layer of flattened follicular cells and contained primary oocytes. No differences were observed in histological features of primordial follicles in control and experimental groups (Figs.1-3). The primary follicles, lined by cuboidal cells, containing primary occytes, in control and experimental groups showed no differences (Figs. 4-5 & 12).

In control the secondary follicles were normal in structure (Fig.4). Secondary follicles in experimental group B1 showed dehiscence of cells from the zona granulosa. The thickness of granulosa cells was variable at different areas around the follicle, whereas in control the thickness was uniform all around. Some of the granulosa cells showed degenerative changes in the form of pyknotic nuclei and karryorrhexis and were lying in the follicular antrum. Vacuolization was observed in the zona granulosa. Apart from these differences; the basement membrane, theca interna and externa appeared like normal (Fig.6). The secondary follicles in experimental group C1 also showed similar changes as in group B1. There was more vacuolization in the zona granulosa. In some cases the granulosa cells were clumped together resulting in gaps between them and the basement membrane (Fig.7).

The morphometric observations regarding graafian follicles indicated that the mean diameter of graafian follicles in control group A1 was

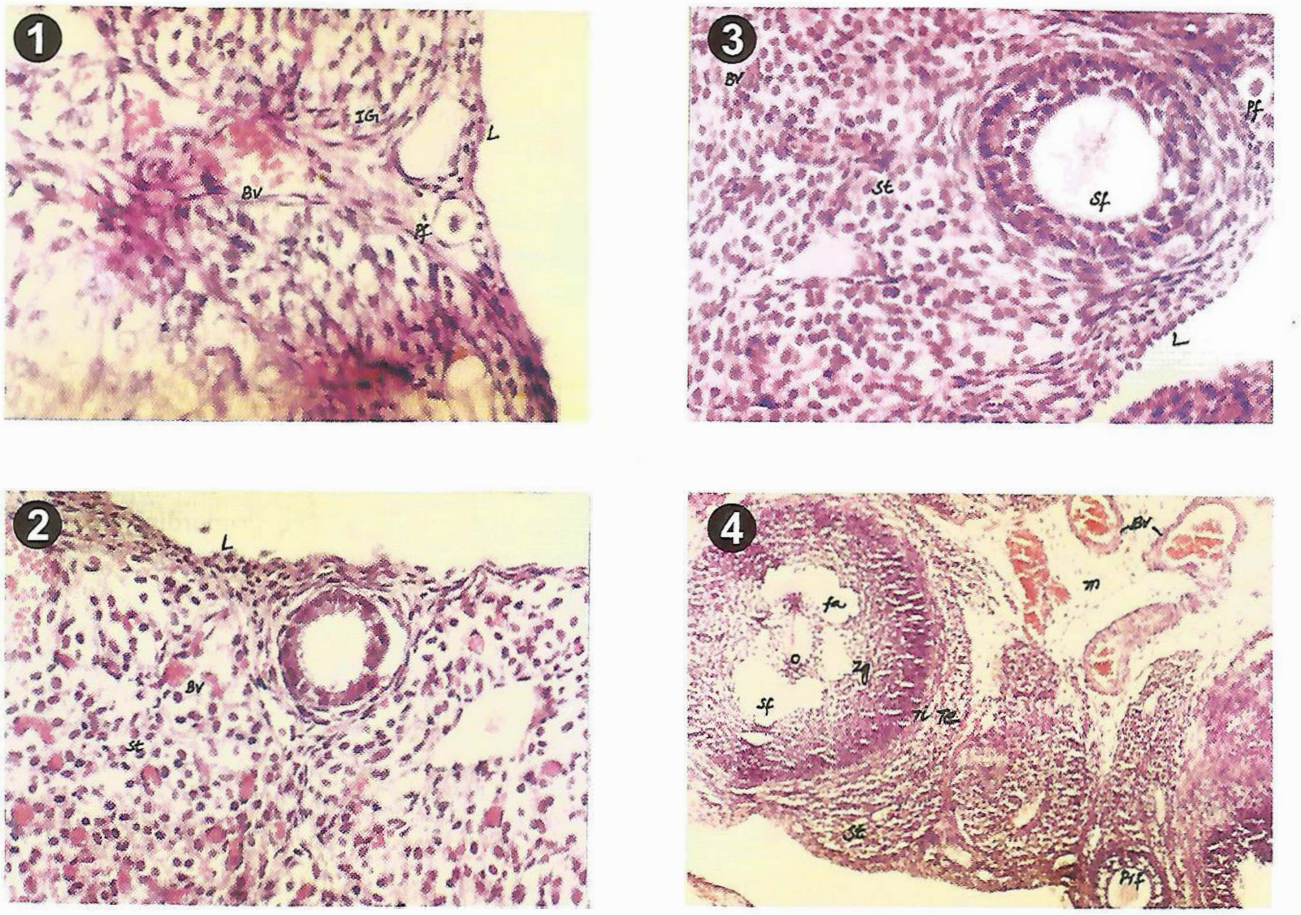


Fig. 1. **Photomicrograph of ovarian cortex of control.**

Fig. 2. **Photomicrograph of ovarian cortex of experimental group B1.**

Fig. 3. **Photomicrograph of ovarian cortex of experimental group C1 showing: (L) Cuboidal lining epithelium (pf) Primordial follicle (sf) Secondary follicle (st) Stroma (Bv) Blood vessel, (IG) interstitial gland H&Ex400.**

Fig. 4. **Photomicrograph of ovary of control showing: (m) medulla (Bv) blood vessels (sf) secondary follicle (zg) zona granulosa, (fa) follicular antrum (0) ovum, (Ti) theca interna (Te) Theca externa (st) stroma H&Ex100.**

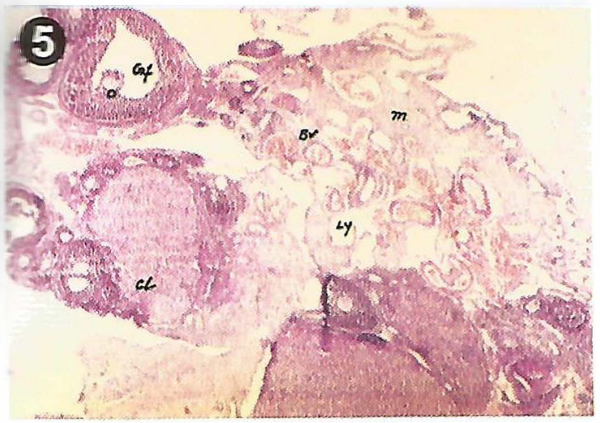
Table 1: **Diameter of graafian follicles of control and experimental animals**

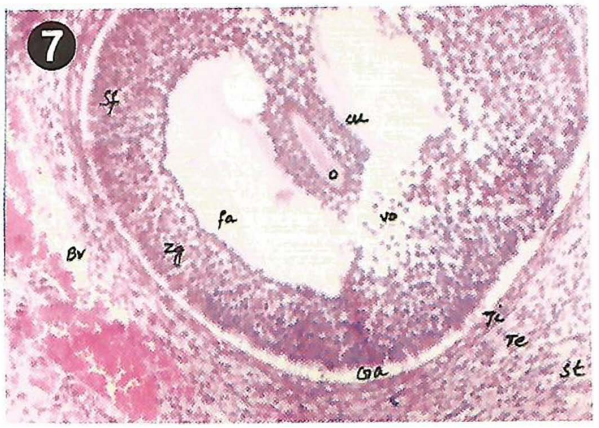
Group	Number	Diameter of Graffian follicles (µm)
A1 (Control)	8	490.62±112.1
B1 (Experimental)	10	436.56±63.33
C1 (Experimental)	15	405.62±44.2

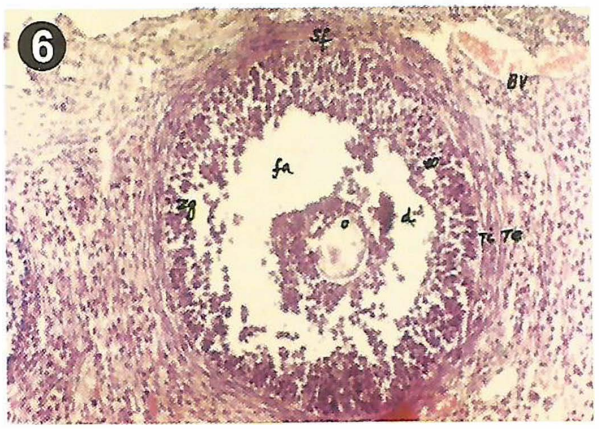
Values given are mean±SE

490.62±112.1 µm, while the values in experimental groups B1 and C1 were found to be 436.56±63.33µm and 405.62±44.2 µm respectively (Table 1). The mean diameter of graafian follicles in experimental group C1 reduced significantly ($p<0.05$) as compared with the control (Table 2).

The structure of graafian follicle in control was found normal (Fig.8). No differences from the control was observed in the graffian follicles of experimental group B1 (Fig.9), and in majority of graffain follicles in group C1 (Fig.10). However, in some cases of group C1, vacuolization was observed in the zona granulosa. The gaps between the zona granulosa and the basement membrane were also evident (Fig.11).







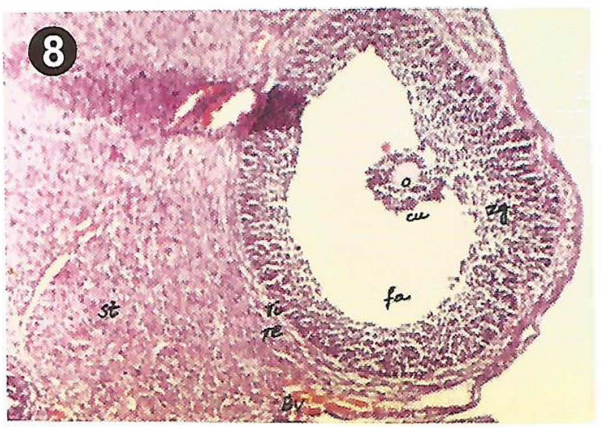


Fig. 5. **Photomicrograph of ovary of experimental group B1 showing: (m) Fibrotic medulla (Bv) Dilated blood vessels (Ly) Dilated lymphatic vessel (Prf) Primary follicle (Gf) Graafian follicle (O) Ovum (CL) Corpus luteum H&E x 40.**

Fig. 6. **Photomicrograph of ovary of experimental group B1 showing (sf) secondary follicle (fa) follicular antrum (zg) zona granulosa showing dehisence of cells from the wall (O) ovum (d) degnerating cells (vo) vacuolization (Ti) Theca interna (Te) Theca externa (Bv) Dilated blood vessels H&Ex200.**

Fig. 7. **Photomicrograph of ovary of experimental group C1 showing: (sf) Secondary follicle (zg) Zona granulosa (fa) Follicular antrum (Vo) Vacuolization (O) Ovum (cu) Cumulus oophorus (Ga) Gaping between the zona granulosa and basement membrane (Ti) Theca interna (Te) Theca externa (st) Stroma (Bv) Dilated stromal blood vessels H&E x 200.**

Fig. 8. **Photomicrograph of graafian follicle in control showing: (O) Ovum (cu) Cumulus oophorus (fa) Follicular antrum (zg) Zona granulosa (Ti) Theca interna (Te) Theca externa (st) Stroma (Bv) blood vessels H&Ex100.**

The morphometric studies of secondary oocytes indicated that the mean diameter of secondary oocytes in control group A1 was 47.34±11.2µm, while the values in experimental groups B1 and C1 were found to be 47.3±4.9 µm and 38.33±12.7 µm, respectively (Table 3). These figures were found statistically non-significant ($p > 0.05$) (Table 4).

Cortical Stroma

Blood vessels were present all around the cellular stroma of control (Fig.1). Congestion of stromal blood vessels was observed in experimental group B1 (Fig.5) while in experimental group C1 these were moderately congested (Fig.12). The stroma in the experimental groups looked loose and oedematous as compared to control.

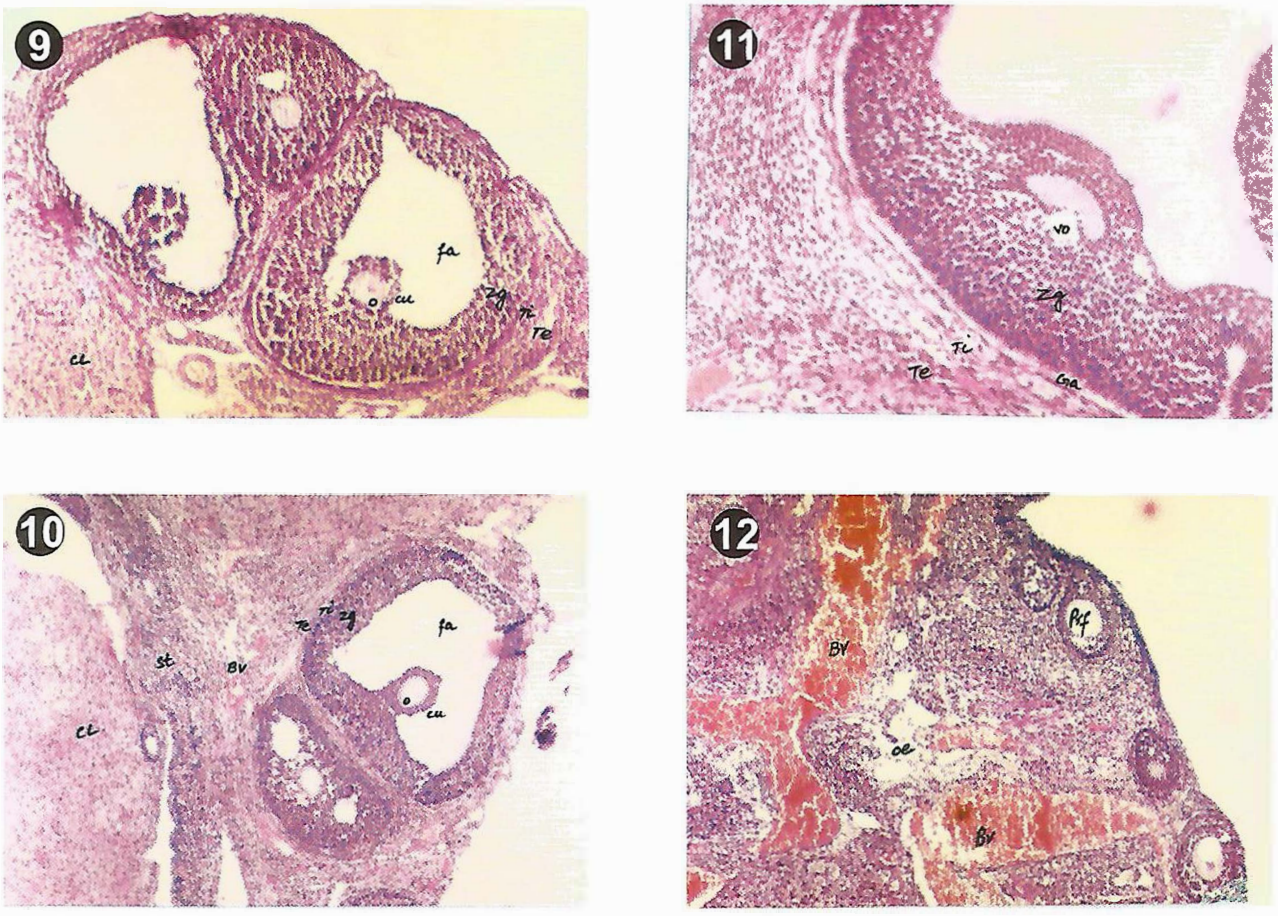


Fig. 9. **Photomicrograph of graafian follicles in experimental group B1 showing: (O) Ovum (Cu) Cumulus oophorus (fa) Follicular antrum (zg) Zona granulosa (Ti) Theca interna (Te) Theca externa (CL) Corpus luteum H&E x 100.**

Fig. 10. **Photomicrograph of graafian follicles in experimental group C1 showing: (O) Ovum (cu) Cumulus oophorus (fa) Follicular antrum (zg) Zona granulosa (Ti) Theca interna (Te) Theca externa (st) Stroma (Bv) Blood vessel (CL) Corpus luteum H&Ex100.**

Fig. 11. **Photomicrograph of wall of graafian follicle in experimental group C1 showing (Vo) Vacuolization in zona granulosa (Ga) Gaping between zona granulosa (zg) and basement membrane (Ti) Theca interna (Te) Theca externa H&Ex200.**

Fig. 12. **Photomicrograph of ovary of experimental group C1 showing (Bv) Moderately dilated blood vessels (oe) Stromal oedema (Prf) Primary follicle H&Ex100.**

Corpus Luteum

Polygonal shaped granulosa lutein cells contained eosinophilic cytoplasm. Theca lutein cells surrounded the granulosa lutein cells and were smaller in size with intensely stained cytoplasm. The vascularity was normal. Regarding the structure of corpus luteum no differences were observed in both the experimental groups as compared to control (Figs.13-15).

Microscopic observation of Ovarian Medulla

In the control group, the medulla contained loose connective tissue with fibroblasts and few smooth muscle cells. The blood vessels and lymphatics were in abundance and normal looking (Fig.4). Moderate congestion and dilatation of blood vessels and lymphatics was evident in the medulla of experimental group B1 (Fig.5) while in group C1, there was more congestion and dilatation of blood

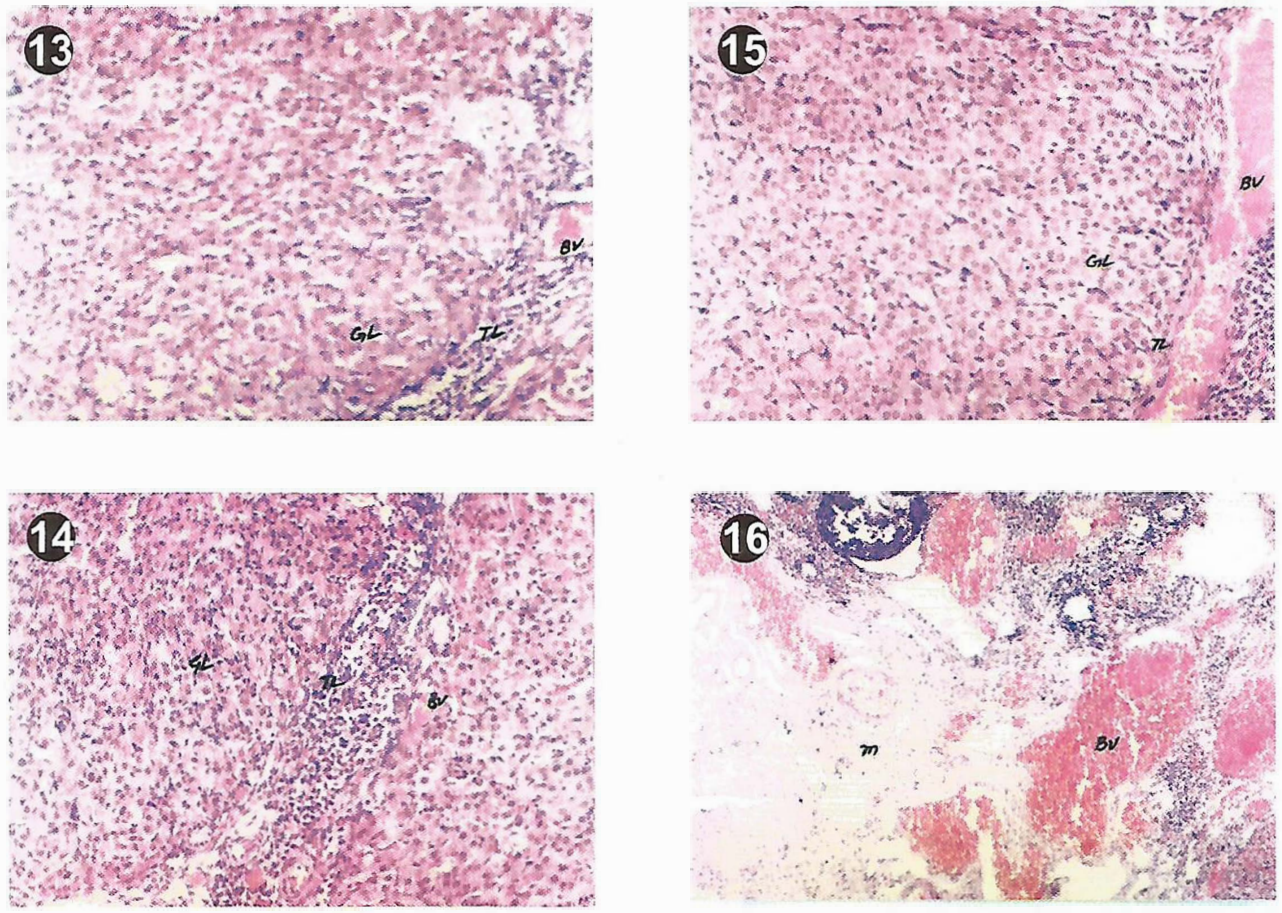


Fig. 13. **Photomicrograph of corpus luteum of control showing (GL) Granulosa lutein cells (TL) Theca lutein cells (Bv) Blood vessels H&E x 200.**

Fig. 14. **Photomicrograph of corpus luteum of experimental group B1 showing: (GL) Granulosa lutein cells (TL) Theca lutein cells (Bv) Blood vessel H&Ex200.**

Fig. 15. **Photomicrograph of corpus luteum of experimental group C1 showing: (GL) Granulosa lutein cells (TL) Theca lutein cells (Bv) Blood vessel H&E x 200.**

Fig. 16. **Photomicrograph of medulla of ovary in experimental group C1 showing: (Bv) Moderately dilated blood vessel (m) Fibrosis in medulla H&Ex100.**

vessels and lymphatics as compared to group B1 (Fig.16). The fibrotic changes were also observed in both the experimental groups but was more marked in group C1 (Fig.16).

DISCUSSION

In this study various histological derangements were observed in the ovaries, which were exposed to 5 MHz frequency ultrasound waves during intra uterine development. A total of six exposures were given during gestational period in group B1 and nine exposures in group C1. Since the post natal oogenesis starts at puberty, so the ovaries of offspring's were studied at 70 days of age (puberty in rats).

The secondary follicles of both the experimental groups showed degenerative changes. The graffian follicles of experimental group B1 and majority of experimental group C1 remained uneffected, while some of the graafian follicles of group C1 showed vacuolization in the zona

Table 2: Effect of Diagnostic Ultrasound (5 MHz) on diameter of Graafian follicles

Source of variation	Sum of Squares (S.S.)	Degree of Freedom (D.F.)	Mean Square (M.S.)	Variation Ratio (F)
Between levels	3770.8777	2	18854.3885	3.736 ++
Residual	151372.071	30	5045.7357	
Total:	189080.848	32		
A1 V B1	12990.01	1	12990.01	2.57 ++
A1 V C1	37695.652	1	37695.652	7.47*
B1 V C1	5742.7734	1	5742.7734	1.138 ++

A1 = Control group
B1 = Experimental group received 6 exposures of ultrasound inutero
C1 = Experimental group received 9 exposures of ultrasound in utero.
Significant differences are indicated by esterics
* $p < 0.05$
++ indicate non significant difference $p > 0.05$
Based on one way ANOVA

Table 4: Effect of Diagnostic Ultrasound (5 MHz) on Diameter of secondary oocytes

Source of variation	Sum of Squares (S.S.)	Degree of Freedom (D.F.)	Mean Square (M.S.)	Variation Ratio (F)
Between levels	302.351	2	151.175	1.5 ++
Residual	1307.388	13	100.568	
Total:	1609.739	15		
A1 V B1	0.00651	1	0.00651	0.000064 ++
A1 V C1	194.85	1	194.85	1.93 ++
B1 V C1	240.75	1	240.75	2.39 ++

A1 = Control group
B1 = Experimental group received 6 exposures of ultrasound inutero
C1 = Experimental group received 9 exposures of ultrasound in utero.
++ indicate non significant difference $p > 0.05$
Based on one way ANOVA

Table 3: Diameter of secondary oocytes of control and experimental animals

Group	Number	Diameter of Graffian follicles (μm)
A1 (Control)	4	47.34±11.2
B1 (Experimental)	6	47.3±4.9
C1 (Experimental)	6	38.33±12.7

Values given are Mean±SE

granulosa . The mean diameter of Graffian follicles reduced significantly in group C1 as compared to control. The cortex and medulla of ovaries in both the experimental groups showed moderate congestion and dilatation of blood vessels and lymphatics. Fibrotic and oedematous changes were also observed in the medulla of ovaries in both the experimental groups.

Bioeffects of ultrasound on various adult organs are known.[10,15] Deleterious effects on embryonic tissues have also been observed.[16]

Response of the adult ovarian tissue to ultrasound exposure has been monitored by Bailey et al.[12] Some results of the present study such as vacuolization of zona granulosa and the uneffected lining epithelium of ovaries support the findings of Bailey et al. Regarding other observations, such as uneffected corona radiata, cumulus oophorus, secondary oocytes and corpora lutea after ultrasound exposure in the present study are in contrast to the observations of Bailey et al, who noted the pyknotic nuclei and degenerative changes in the corpora lutea, corona radiata, cumulus oophorus and the secondary oocytes. The different results of these two studies may be explained due to the varied nature of the experimental designs and also due to different (adult/embryonic) nature of tissues. A peculiar feature of the ultrasound exposure during intrauterine period in this study was the marked congestion and dilatation of blood vessels and lymphatics in both the cortex and medulla of ovaries. This feature is directly related to the number of exposure of ultrasound waves as there was more congestion and dilatation of blood vessels and lymphatics in group C1, receiving 9 exposures compared with group B1, receiving 6 exposures. Such observations have not been reported earlier.

CONCLUSION

Although the secondary oocytes remained unaffected in the experimental groups, the graafian follicles in group C1 showed degenerative changes. The medullary region of the ovaries showed marked congestion and dilatation of blood vessels and lymphatics indicating some sort of inflammatory response related to ultrasound exposure. It is therefore suggested that the frequent use of ultrasound during pregnancy should be avoided.

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