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ULTRASONICALLY INDUCED MORPHOLOGICAL DAMAGE TO MOUSE OVARIES

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Abstract—Mouse ovaries were exposed *in vivo* to 1 MHz continuous wave ultrasonic energy at spatial peak intensities ranging from 5 to 100 W/cm^2 for times varying from 300 to 15 s depending on the intensity. Following exposure the ovaries were surgically removed at times ranging from immediately (within 60 s) to 7 days and prepared histologically for light microscopic analysis. The observed tissue alterations varied from severe, at the higher intensities to subtle, at the lower intensities. Lesions were manifested by pyknosis of cells, vacuolization of cells and tissue, eosinophilic cytoplasm, and general cellular disruption. Subtle alterations showed large numbers of polyovular follicles and increases in the amount of PAS positive material in the interstitial tissue. Various ovarian structures showed differing sensitivities to the insult with luteinized structures being preferentially altered.

INTRODUCTION

A previously reported study (O'Brien *et al.*, 1979) from this laboratory examined the morphological changes to mouse testicular tissue from *in vivo* ultrasonic irradiation. Utilizing a continuous wave ultrasonic intensity of 25 W/cm^2 (spatial peak) for 30 sec at a frequency of 1 MHz, this study suggested that ultrasound at these levels was capable of markedly disrupting testicular tissue by affecting both spermatocytogenesis and spermiogenesis and by disrupting the interstitial tissue. The spatial distribution of damage over any single preparation was highly variable and in some cases did not correspond to the free field intensity distribution, which was uniform within 5% across the breadth of the testis specimens. The damage at the cellular level varied, depending upon the type of cell examined. Because of these findings and because female gonadal tissue receives ultrasonic exposure in clinical procedures of humans, we undertook a preliminary investigation of the effects of ultrasonic energy on the ovary. It was the focus of this preliminary study to determine whether morphological alterations of the female gonads are caused by ultrasound at intensities approaching therapeutic levels and if so, how these alterations are manifested in the ovary. Spatial peak intensities (25–100 W/cm^2) much in excess of therapeutic levels were used initially to produce lesions in order to demonstrate unequivocal effects by ultrasound. Lesser intensities and longer exposures were then used to approach therapeutic conditions.

MATERIALS AND METHODS

Two hundred nonpregnant females LAF_1/J (Jackson Memorial Laboratory, Bar Harbor, ME) and Hap: (ICR)BR (Harland Sprague–Dawley, Indianapolis, IN) mice between the ages of 2 1/2 to 10 months were used for this *in vivo* study. All animals were maintained on a 12 hr light/dark cycle and housed in close proximity to males.

The experimental animals were anesthetized with methoxyflurane after which an area approximately 6 × 3 cm over the back and sides was shaved, depilitorized, and bathed in a mild detergent solution to improve wetting by the aqueous coupling media. A routine vaginal smear was then taken to determine the animals phase of estrous. Following this the animals were mounted in a specially designed holder (Fry *et al.*, 1978) such that the animals' backs would face the transducer when the assembly was placed in the sound irradiation chamber. A short incision of approx. 1 cm was then made in the skin only, opposite each ovary, in order to view the ovaries through the abdominal wall and was sutured closed post-exposure. A small dot of India ink was placed on the abdominal wall over each ovary to serve as a target. The holder and animal assembly was then placed in the Lucite exposure tank containing 37°C degassed mammalian Ringer's solution such that the specimen's muzzle protruded slightly above the liquid surface and its back faced the acoustic transducer. Exposures of both ovaries were made using a 1 MHz, 1 in. focused quartz transducer operating at spatial peak intensities of 100, 50, 25, 10 and 5 W/cm^2, respectively, with maximum exposure times of 15, 30, 60, 250 and 300 sec.

Intensity calibration is performed using a suspended steel sphere radiation pressure measuring device as a primary standard in a calibration tank (Dunn *et al.*, 1977). Secondary standards are then used on a monthly basis to verify the ultrasound intensity output of the transducer. The calibration accuracy is ±5%. The lateral and axial half power beam widths were determined with the transient

thermoelectric technique to be 4.5–5 mm and 20–30 mm, respectively.

The animals were exposed in groups of five, one of which was a sham irradiated animal wherein all steps of the procedure were completed except for the acoustical exposure. Irradiations were blind in that the identity of the exposed and sham exposed animals was unknown until after the histological analyses were completed.

Following exposure, the animals were sacrificed at various times ranging from immediately after the last exposure (i.e. within 60 sec) to 7 days. A second vaginal smear was taken at sacrifice for all groups except the immediately sacrificed specimens, in order to check for cycle interruption or alteration. The ovaries, together with the oviducts and a small portion of the uterine tube, were removed and placed in Bouin's fixative, keeping the left and right specimens separate. After fixation for 18–20 hr, the tissue was routinely dehydrated, embedded in paraffin and sectioned in 6 μm serial sections. Every fifth section was mounted on glass slides and the slides alternately stained with periodic-acid–Schiff reaction and hematoxylin and eosin. The sections were then examined and analyzed for presence and extent of damage using light microscopy.

OBSERVATIONS

The terms light, moderate, and severe are used to describe the observed ultrasonically induced alterations to the ovarian structure. Although these descriptive terms are not precisely defined and are somewhat subjective, the following characterizations were employed: Areas of light damage are composed of primarily normal tissue but with some damaged cells interspersed throughout. Moderate damage indicates a great deal of cellular damage but with a substantial amount of normal components also present. Severe damaged areas are those where few, or no, normal components are seen.

(a) *Pan-ovarian observations*

The ovaries in the mouse are approx. 2–4 mm in diameter, located lateral to the kidneys at the end of the uterine horn, and are completely surrounded by a bursa. They are covered with a one-cell layer, viz., the germinal epithelium, under which lies a tissue layer, viz., the tunica albuginea. The tissue of the ovary can be roughly divided into two parts, viz., the inner medullary region which contains the stroma and vasculature, and an outer cortical region which contains the follicles and their developing germ cells. Also present are corpora lutea, corpora albicantia, and interstitial glands all of which result from atresia or ovulation of the oocyte.

The follicle consists of an outer thecal layer, a thin basement membrane, a mass of granulosa cells, and the oocyte which is surrounded by a mucoprotein layer, the zona pellucida. Large follicles contain an antrum which is filled with follicular fluid. Figures 1–4 show portions of normal mouse ovary.

Morphological alteration of cells, i.e. a lesion, was seen in ovaries exposed to intensities of 25 W/cm^2 and above, and sacrificed at 24 and 72 hr. For exposures at 50 and 100 W/cm^2, high animal mortality rates occurred within 24 hr along with extensive abdominal involvement. For those animals sacrificed immediately after exposure, no lesions were detected. It is not known if this is due to the requirement that a sufficient amount of time must elapse before the lesion is developed. This observation has been shown for neural tissue (Borrelli *et al.*, 1981). No lesions appeared in response to 5 and 10 W/cm^2 but abnormalities present suggest a more subtle form of damage, probably at the subcellular level. For all the exposures, the damage ranged from involvement of a small area to destruction of the entire ovary depending upon exposure intensity and duration. Also, some animals exhibited no morphological damage to the ovaries themselves, but did show damage or abnormalities to the oviducts and/or the uterus.

The lesions are characterized by several factors, though the presence of cells with pycnotic nuclei is the most predominate of these factors (Fig. 5). The luteinized granulosa cells of the corpus luteum and corpus albicans, as well as advanced atretic follicles, appear to be preferentially affected, with the stroma following in sensitivity. Healthy follicles of type 5a (Pedersen, 1970) and below, thecal cells, and the germinal epithelium appear to be quite resistant in all but the most severely damaged areas. The healthy oocyte, i.e. not atretic, appears to be most resistant. Condensation of cytoplasm often accompanies pycnosis leaving large gaps around the nuclei. In addition, the cytoplasm is often eosinophilic.

A second prominent characteristic of ultrasonically induced alterations is the presence of debris from disrupted cells and hematoxylin dye accumulation in degenerating areas (Fig. 6). Dye accumulation is most frequently associated with the granulosa cells of both healthy and atretic follicles in light and moderate damage but may be

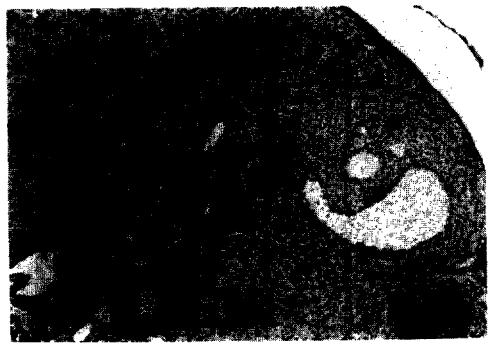

Fig. 1. Portion of a normal mouse ovary from a diestrus animal age 5 months showing antral follicle (F), corpus luteum (CL), interstitial gland (I), and atretic follicle (AF). 100×.

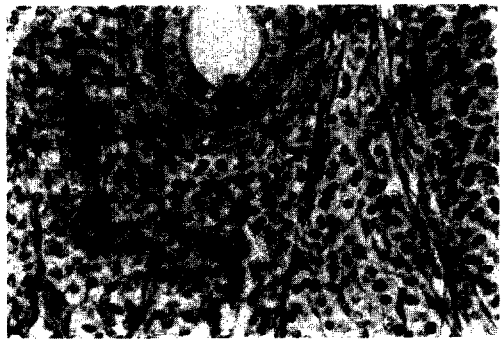

Fig. 2. Normal mouse ovarian tissue demonstrating a small healthy follicle (F) with a normal theca layer (T) and normal stroma (St). Diestrus animal which was 5 months of age. 400×.

Fig. 3. Nonatretic antral follicle from a mouse ovary demonstrating a normal oocyte (O), zona pellucida (Z), and granulosa cell mass (G). Same animal as in Fig. 2. 400×.

Fig. 4. Normal antral follicle from the same animal as Fig. 2 and 3 showing a normal oocyte (O), granulosa cell mass (G), theca cell layer (T) and a portion of a corpus luteum (CL). 250×.

Fig. 5. Portion of a mouse ovary (in early estrus) exposed to ultrasound at a spatial peak intensity of 100 W/cm^2 for 15 secs showing pycnotic nuclei (P), gapping of granulosa cells (Ga), oocyte alteration (O), and zona pellucida peeling (Z). 250×.

Fig. 6. Example of hematoxylin dye accumulation (Da) in a damaged follicle. Ovary was exposed to a spatial peak intensity of 25 W/cm^2 for 60 sec. Animal was in diestrus. 400×.

Fig. 7. Example of deposition of periodic-acid–Schiff positive material (PAS) in the stroma of an ovary exposed to a spatial peak intensity of 10 W/cm^2 for 250 sec. Animal was in post-estrus. 250×.

Fig. 10. Antral follicle demonstrating abnormalities in follicular fluid compared to normal fluid amounts shown in Fig. 1 and 4. Also noted is the spatial distribution of damage following exposure such as granulosa cells in a large portion of the follicle which are pycnotic (P). The surrounding tissue appears normal. Animal is in diestrus and was exposed to a spatial peak intensity of 50 W/cm^2 for 30 sec. 250×.

Fig. 8. Portion of an exposed ovary demonstrating vacuoles in the tissue (Vt), vacuoles in the cell nuclei (VC), and gapping (Ga). Also noted here is the apparently normal germinal epithelium (E). The ovary was exposed to a spatial peak intensity of 100 W/cm^2 for 15 sec and was in early estrus. 250×.

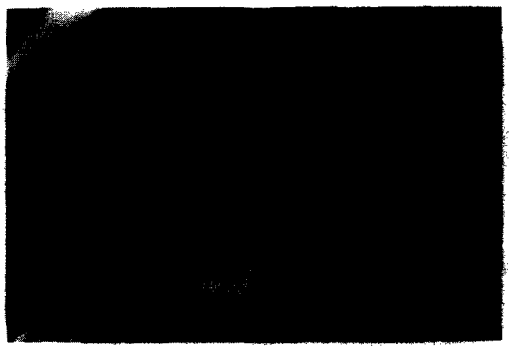

Fig. 11. Atretic polyovular follicle containing three oocytes (O). Ovary was in estrus and was exposed to a spatial peak intensity of 5 W/cm^2 for 300 sec. 250×.

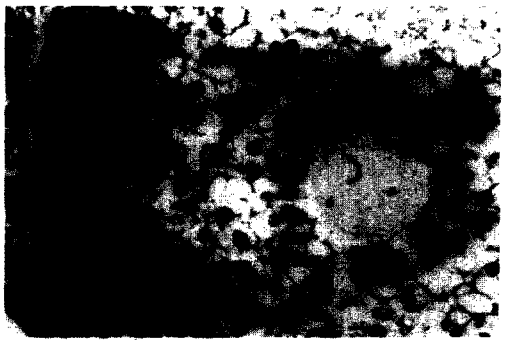

Fig. 9. Antral follicle demonstrating the loss of integrity of the granulosa cell mass (G) as compared to a normal follicle shown in Fig. 3 and 4. Also shown are polymorphonuclear leukocytes (PMN) lined up along the edge of the follicle. Animal was exposed to a spatial peak intensity of 100 W/cm^2 for 15 sec. and was in post-estrus. 400×.

seen in other structures, especially in severely damaged areas. While dye accumulation is seen in atretic follicles of normal and sham irradiated animals, in damaged ovaries it is seen in nonatretic follicles. Debris is seen in open areas such as the antrum and vascular areas and is most often seen in conjunction with severe damage.

One of the abnormalities noted at the lower intensities, especially at 10 W/cm^2, was the presence of a red-colored PAS positive material of undetermined origin (Fig. 7). This material is seen scattered in small amounts between interstitial cells in normal and sham animals, but is seen in large deposits in irradiated animals, and seems especially to be associated with degenerating areas such as corpus albicans, old interstitial glands and areas of the stroma. This material is not to be confused with other normal PAS positive material present or with lipofuchsin pigment, which is also occasionally present in normal ovaries.

Vacuolization of the tissue, as well as that of cell nuclei, is also present in damaged areas and in areas away from the central lesion area. Vacuoles in the tissue are most frequently seen in the corpus luteum and the stroma but occur in other structures also (Fig. 8). Vacuolated nuclei are seen in all cell types, but less often in granulosa cells. Vacuolization along with pycnosis is often seen in areas of light damage and is frequently an early indication of moderate or severe damage.

Disruption of the integrity of the follicular structure, i.e., thecal layers and granulosa cells, is also often seen. In severely damaged areas the thecal layers separate and gaps appear between them. A more subtle form of alteration (at spatial peak intensities of 5 and 10 W/cm^2) is a slight separation of the thecal layer from the surrounding interstitial cells which can often be seen involving follicles of type 4 and below. Granulosa cells in healthy and atretic follicles of all types show separation and loss of integrity as well as loss of normal orientation (Fig. 9). The corona radiata and cumulus oophorus are affected most often, whereas cells along the basement membrane in types 6 to 8 follicles often appear normal. In some cases of severely damaged follicles the pycnotic granulosa cells clump together leaving a large gap between them and the basal lamina (Figs. 5, 8 and 11). Large gaps are also often seen between the granulosa cells and the oocyte even in large preovulatory follicles.

Presence of abnormal fluids and coagulation of follicular fluid is another effect encountered after exposure to ultrasonic energy. PAS positive fluid, large numbers of red blood cells (RBCs), and lymphocytes have appeared in the peri-ovarian space in both ovaries which exhibit lesions, as well as exposed ovaries which do not. Cycle phase does not appear to be a factor as abnormal blood and fluid have been noted in ovaries of animals in all phases of the estrous cycle. Coagulation of liquor folliculi into clumps, leaving clear areas in the antrum, is present in healthy antral follicles of light and moderately damaged ovaries (Fig. 10). This is in contrast to the normal appearance of healthy, antral, follicular fluid which uniformly fills the entire antrum (Kang *et al.* 1979).

(b) *Germ cell observations*

The oocyte itself shows a variety of effects which depend on the severity of the insult, as well as the state of health of the follicle. The germinal vesicle frequently swells and vacuolizes, accompanied by dissolution of the nucleolis; on the other hand, pycnosis can also be seen. The cytoplasm can become highly eosinophilic and have a nonuniform appearance or may break-up completely as in the late states of atresia. The shape of the oocyte is often altered and in cases of severe damage (Fig. 5), appears completely dessicated and shrunken to a fraction of its original size . Many of the effects exhibited by the oocyte are similar to those exhibited at atresia but, when coupled with surrounding damaged tissue, it becomes clear that these effects are due to abnormal tissue damage, as the figures show.

Effects on the zona pellucida are also seen and are variable ranging from slight vacuolization to complete dissolution, again depending on the degree of insult. One frequent observation is that the zona spreads out around the oocyte often fusing with the liquor folliculi or spreading amongst the granulosa cells and/or into the antrum. In some cases the zona is broken and may be seen peeling off the oocyte surface (Fig. 5). Vacuolization of the zona occurs in normal and sham animals and may be due to the histological processing. In damaged ovaries, however, the vacuoles are much larger and tend to distort the zona.

A phenomenon seen particularly in 5 W/cm^2 and some 10 W/cm^2 (for exposure times of 300 sec and 250 sec respectively) irradiated animals is the occurrence of abnormally large numbers of polyovular follicles (Fig. 11). The occasional occurrence of bi- and triovular follicles in mice is not rare (Mossman and Duke, 1973, Rugh, 1968) and

has been observed in normal and sham animals in this study. However, as many as 30 polyovular follicles in one ovary have been counted in exposed animals of these low-intensity groups. As many as six ova per follicle have been recorded in exposed animals although two to three oocytes per follicle appear to be the most common number. The oocytes involved are variable in size and within a single follicle may range from small to fully enlarged. Also, while many of these oocytes appear atretic, a large number appear nonatretic and it is not unusual to see both types in the same follicle. It has been stated (Mossman and Duke, 1973) that the regular occurrence of polyovular follicles is always in juvenile animals and that these follicles are located in the medulla. The youngest animal in this experiment to exhibit such a follicle was 77 days old, and therefore cannot be considered a juvenile. In addition, the majority of polyovular follicles were seen in the cortex. These deviations suggest a subtle form of damage at the subcellular level with possible involvement of biochemical and/or endocrine processes.

(c) *Extra-ovarian observations*

Involvement of the fimbria, oviducts, and uterine tubes is much the same as the ovary with pycnosis, cell disruption, vacuolization, and presence of lymphocytes and RBC's characterizing the lesions. The bursa of damaged ovaries is sometimes thickened and may be attached to the ovary.

Polymorphonuclear leukocytes (PML) were seen at 24 hr but not at 72 hr in damaged ovaries. This is normal as PML are the immune system's first line of defense, are short lived and do not divide once they have left the bone marrow where they are produced (Youmans, 1975). PML are most frequently seen lined up between the basal lamina and thecal layer of type 6 follicles and above, but may also invade other areas of the damaged ovary (Fig. 9). They were occasionally seen in normal and sham animals in the antrum of advanced atretic follicles, but never in the configuration seen in damaged ovaries. Like the lymphocytes previously mentioned, they are occasionally seen in the periovarian space and around the oviducts and/or uterine tube in exposed ovaries which show no damage.

While no serious interruptions in the estrous cycle were apparent in this study, a definitive statement on whether or not ultrasound affects the estrous cycle cannot be made, in part due to variability in the length of cycle phases in individuals and because no long term survival of specimens was included in this study. Secondly it has been shown that despite the loss of oocytes, follicles and corpora lutea following exposure to X-rays, mice continue to demonstrate normal estrous cycles due to proliferation of chords of germinal epithelium which are estrogen producing (Baker *et al.*, 1977). The possibility exists that ultrasound exposure could trigger the same type of reaction in mice and thus mask cycle interruptions which may be apparent in other mammalian species.

Morphological alteration due to ultrasound is much the same as that resulting from ionizing radiation. However, as was the case with testicular tissue (O'Brien, *et al.*, 1979), the sensitivity of structures varies considerably. In the adult mouse, the primordial germ cells are the most sensitive to ionizing radiation followed by follicular oocytes and granulosa cells, with copora lutea and interstitial glands being virtually refractory even at high doses (Baker, *et al.*, 1977). As stated earlier, findings in this study suggest that luteinized cells (i.e. corpora lutea, interstitial glands, and corpora albicantia) are most sensitive to ultrasonic insult while healthy oocytes of all types are the most resistant. The germinal epithelium is the one cell structure which shares resistance to both types of exposure.

Both ionizing and ultrasonic insults result in cell pycnosis, eosinophilic cytoplasm of the oocyte, nuclear condensation and the presence of debris. Other alterations such as hyperemia, ovarian weight increase, and anovular follicles seen after X-ray exposure were not assessed in this study.

CONCLUSION

It has been shown that relatively short exposures (15–60 sec) to ultrasound at intensities of 25 W/cm^2 and greater can be highly destructive to the ovary and to surrounding structures, while longer exposures at lower intensities exhibit more subtle effects. It is unknown at the present time whether these alterations are permanent or reversible and what affect they may have on the fecundity of the specimen. It is felt that further investigation and additional studies are needed at the lower intensity range, below 5 W/cm^2, particularly at the electron microscopy level which may reveal suspected subcellular alterations of morphology and function. It should be noted that the intensities employed in this study are greater than those characteristic of clinical therapeutic ultrasound.

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