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ORIGINAL PAPER

Ovarian apoptosis after shock wave lithotripsy for distal ureteral stones

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Abstract The objective of this study is to identify any apoptotic effect of shock wave lithotripsy (SWL) for distal ureteral stones on ovarian tissue. Twenty-one female New Zealand White rabbits were divided into three groups of seven rabbits each: I (control), and II, III (treated and killed 14 and 28 days after SWL, respectively). The left distal ureteral segment of the anesthetized (ketamine HCl, 20 mg/kg) animals in groups II and III was exposed to 1,500 shock waves at 17 kV. Localization of the distal ureteral segments was achieved following contrast medium (Iohexol 300 mg of I/ml) injection. The animals were killed on day 14 or 28 after SWL, and the ovaries were removed. The follicle number with apoptotic changes in ovarian tissue was compared with control group. Apoptotic changes were determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method. No increased apoptosis was detected in all groups. The mean number of TUNEL-positive follicle in groups I, II and III was 9.3 ± 2.9, 8.1 ± 2.6 and 8.7 ± 2.9, respectively. There were no statistically significant differences among all groups regarding the number of TUNEL-positive follicle ($P = 0.647$). Also, no histomorphological change other than apoptosis was detected in the study groups. In conclusion, SWL treatment for distal ureteral stones does not induce apoptotic changes on ovarian tissue.

Keywords Shock wave lithotripsy · Apoptosis · Ovary

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Introduction

Extracorporeal shock wave lithotripsy (SWL) is generally accepted as a standard method for the treatment of most renal and ureteral stones. Despite initial clinical and experimental reports indicating that high-energy shock waves (HESW) are highly safe and effective, various studies in animals and patients show dose-dependent short-term and long-term adverse effects on the kidney, ureter and even adjacent tissues [1–4]. There are several experimental studies that investigated the cellular effects of HESW on ovary cells in the literature [5–7], whereas there is no prospective clinical study regarding this issue. Moreover, female patients less than 40 years old had been excluded because of the theoretical possibility of harm to the ovary by shock

waves in the initial clinical studies [8]. In a few retrospective clinical studies, it has also been reported that SWL of lower ureteral calculi did not adversely affect fertility or increase teratogenic risk in women of reproductive age [9, 10].

Apoptosis (programmed cell death) is an energy-dependent, orderly, gene-regulated process that is now recognized to play an important role in maintaining cell number homeostasis both in health and in disease [11]. Bcl-2-related proteins comprise a family of positive and negative regulators of apoptosis. Among them, Bax is central to apoptotic pathways that implicate the mitochondria [12]. The biochemical basis of the morphologic changes associated with apoptosis can be traced directly or indirectly to the actions of caspases, a family of intracellular cysteine proteases that behave as activators and effectors of apoptosis and play a central role in the process [13].

Apoptosis is involved in the regulation and selection of human ova from the primordial follicle pool [14]. Apoptosis also plays a major role in the depletion of oocytes from ovarian tissue during aging [15]. Ovarian follicular and lutheal-cell apoptosis also represents a pathogenetically fundamental process in various experimental models of ovarial disorders, including warm ischemia, cryopreservation, radiation and dexamethasone-induced ischemic necrosis of lutheal tissue [16–18].

In a recent study, it has been showed that SWL has an apoptotic effect on renal tubular cells that can be detected 4 weeks after the procedures, but no apoptotic effect on glomerular cells [19].

In the present study, our main goal was to evaluate whether any increased apoptotic changes in ovarian cells as in renal tubular cells, induced by HESW, focused prevesical ureteral segment in a rabbit model. To our knowledge, our investigation is the first of the apoptotic effect of HESW on ovarian tissue.

Materials and methods

The present study was carried out with a Multimed 2001™ lithotripter (Elmed Co., Turkey) located at the Uromed Urology Center, Ankara. This lithotripter has an 80-nF capacitor, a 135-mm focal distance, and a focal zone (F2) about 22 mm in diameter × 7.5 mm in length. Refurbished spark plugs were used for all experiments and were discarded after 1,500 shots. Twenty-one adult female New Zealand White rabbits, each weighing 3–5 kg, were included in the study program. All animals underwent an adaptation period of 1 week in cages under normal conditions prior to the SWL procedure and were fed standard rabbit chow and water. During the adaptation period, the female rabbits were kept with male ones in their cages in order to maintain their ovarian cycle. The animals were randomly assigned to one of three groups, each containing seven animals: group I (control), groups II and III (treated and then killed 14 and 28 days after SWL, respectively). In the SWL groups, localization of the distal ureteral segments was achieved following contrast medium (Iohexol 300 mg of I/ml) injection through an ear vein under fluoroscopic control. The left distal ureteral segment of the anesthetized (ketamine HCl, 20 mg/kg) animals was exposed to 1,500 shock waves at 17 kV.

The animals were killed under ketamine anesthesia on days 14 and 28 after the SWL procedures. The left ovaries were removed through a midline incision.

Histopathological examination

Ovaries from control and treated rabbits were fixed in 10% neutral-buffered formalin and then embedded in paraffin. Four-micrometer step sections were mounted at 50 μm intervals onto microscope slides to prevent counting the same follicle twice. One set of slides was stained with hematoxylin and eosin to count the number of follicles per ovary section and the others were immunostained with the TUNEL technique. Tissues were deparaffinized in xylene, rehydrated through a graded series of ethanols, washed in PBS and then stained with hematoxylin and eosin. Ovarian structures were grouped and counted according to the following classifications—*primary*: a single layer of cuboidal-shaped granulosa cells; *secondary*: two to three layers of granulosa cells surrounded by a thecal tissue, but no evidence of an antrum; and *tertiary* (*antral*): where an antrum was present within the granulosa cell layer. *Atretic follicle*: presence of at least five pyknotic granulosa cell nuclei and/or degrading oocyte. *Advanced atretic follicle*: pockets of highly vascularized, luteinized cells surrounded by pseudotheca layer. Granulosa cells classified as apoptotic on the basis of their morphologic features contained a single condensed nucleus, multiple nuclear fragments, or apoptotic bodies. To study ovarian morphology, five randomly selected fields were analyzed from each ovarian section (5 sections/ovary).

TUNEL assay

Apoptotic activity was assessed by the in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) technique with the The ApopTag Plus Peroxidase in situ apoptosis detection kit (Chemicon, S7101). The experiment was conducted in accordance with the protocol supplied by the manufacturer. The sections were dewaxed and treated with proteinase K (20 μg/ml) for 15 min at room temperature, then incubated with equilibration buffer for 10 min, followed by incubation with

working-strength TdT enzyme solution at 37°C for 60 min. The reaction was terminated by incubation in working-strength stop/wash buffer for 10 min at room temperature. Sections were then incubated with antidigoxigenin conjugate for 30 min at room temperature and then incubated with diaminobenzidine (DAB) for 5 min at room temperature. The sections were counterstained with hematoxylin and examined by light microscopy. Germinal centers of hyperplastic lymph nodes served as a positive control. Negative controls were processed in the absence of the TdT enzyme and showed no staining. The apoptotic signal was recorded as positive when either a diffuse type or a granular type dark Brown staining of the nucleus was apparent. Follicles with at least five-labeled cells were considered TUNEL positive. For each ovary, the average number of TUNEL-positive follicles per section was determined from the six sections.

Presentation of data and statistics

Data are presented as mean ± SD. Significance was determined by comparison of the data for groups using the Kruskal–Wallis test with SPSS 11.5 for Windows. Significant levels were set at $P < 0.05$.

Results

Histopathologic findings

Ovaries from control and shocked rabbits were typical of normal folliculogenesis and were characterized by the presence of follicles in all stages of development, atretic follicles and corpora lutea (Fig. 1). We did not detect any unusual histopathologic features in the ovarian tissue of animals in the study groups. The numbers of primary, secondary, tertiary (antral), atretic and advanced atretic follicles and corpora lutea were similar in all groups. No statistically significant difference was identified between the shocked and unshocked ovaries regarding all kinds of follicle numbers, corpora lutea and TUNEL-positive follicle numbers (Table 1).

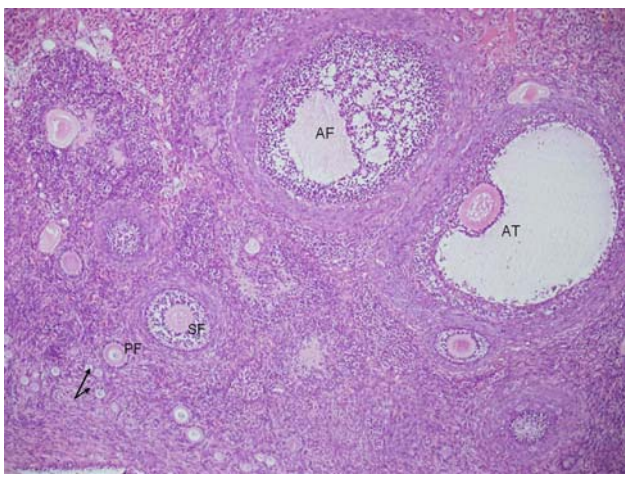

Fig. 1 Ovarian section with normal folliculogenesis. *Arrows* primordial follicles, *PF* primary follicle, *SF* secondary follicle, *AnF* antral follicle, *AtF* atretic follicle. H&E ×100

Evaluation of apoptotic changes

In all animals, TUNEL was completely absent in primary, secondary, and healthy antral follicles (Fig. 2). In contrast, apoptosis in granulosa cells of atretic follicles was obvious (Fig. 3). Thecal layer, corpora lutea and stromal cells were also contained scarce apoptotic cells. In other words, apoptotic changes were detected beginning from the atretic follicles in all groups. The average number of TUNEL-positive follicles was similar in all groups ($P = 0.647$).

Discussion

The effects of HESW on female reproductive tract have been a subject of interest after SWL takes a place in the treatment algorithm of urinary stone disease. In the beginning of SWL era, physicians were reluctant to application of SWL for women in reproductive ages, because of the possible harm effect of HESW on ovarian tissue [8]. Indeed, HESW are not completely safe and free of side effects. It is possible to speculate that SWL is one of the most frequent causes of iatrogenic urinary injury.

Since the ovary is adjacent to the pelvic ureter and HESW effects are detected several centimeters around F2 of ellipsoid reflector, the ovarian tissue may be exposed to shock wave energy during the SWL application for lower

Table 1 The mean numbers of TUNEL-positive follicles in the groups

	Group I	Group II	Group III	*P* value[a]
Primary follicle	12.1 ± 4.4	14.3 ± 3.9	13.3 ± 4.9	0.579
Secondary follicle	9.9 ± 2.9	9.1 ± 2.4	11.2 ± 5.1	0.662
Tertiary (antral) follicle	8.3 ± 3.3	8.4 ± 2.9	8.8 ± 2.3	0.688
Atretic follicle	4.3 ± 1.4	4.7 ± 2.1	4.9 ± 1.6	0.788
Advanced atretic follicle	5.6 ± 1.6	3.4 ± 1.5	4.4 ± 2.2	0.106
Corpus luteum	2.4 ± 1.6	2.4 ± 1.3	2.4 ± 1.9	0.941
TUNEL-positive follicle	9.3 ± 2.9	8.1 ± 2.6	8.7 ± 2.9	0.647

Values are mean ± SD

[a] Kruskal–Wallis test

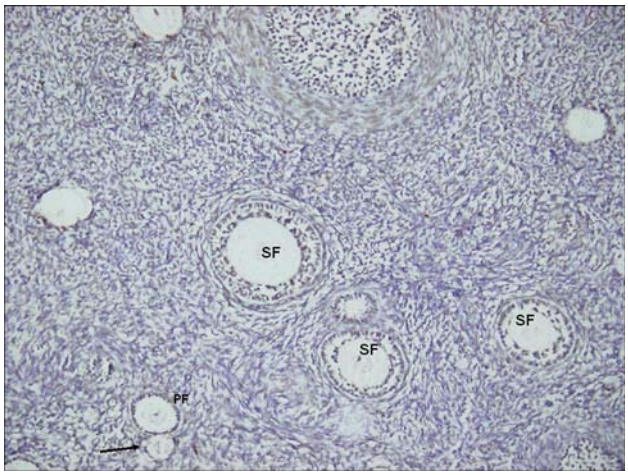

Fig. 2 Primordial (*arrow*), primary (*PF*), and secondary (*SF*) follicles showing no apoptotic signal. TUNEL ×100

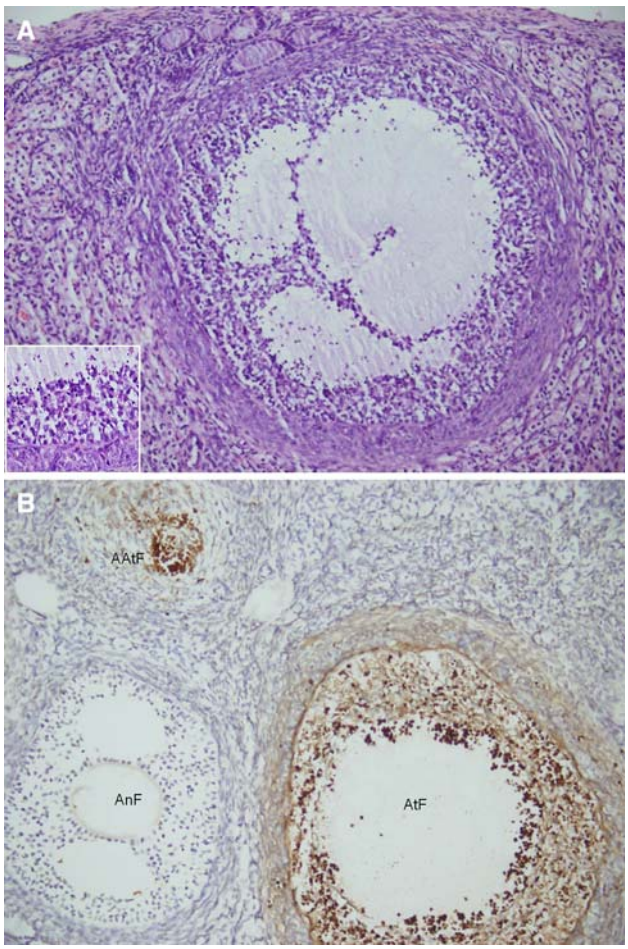

Fig. 3 **a** Atretic follicle with numerous apoptotic bodies (*inset*) in the granulosa cell layer. H&E ×200 (*inset*: H&E ×1,000). **b** Atretic follicle (*AtF*) with TUNEL-positive granulosa cells, TUNEL-negative healthy antral follicle (*AnF*) and advanced atretic follicle (*AAtF*). TUNEL ×200

ureteral stones. Indeed, Recker and co-workers [7] identified minimal subcapsular bleeding, desquamation of superficial cells and loss of microvilli in ovarian tissue, acute after SWL. But, they reported that no histomorphological changes were found in long-term follow-up. Moreover, it has been demonstrated that HESW has not adversely affected on fertility or increased teratogenic risk on fetus, therefore, SWL application for distal ureteral stones is a safe option in women of reproductive age, in both human and animal study [9, 20].

Most of the studies regarding the detrimental effects of SWL have been carried out on renal tissue. However, the mechanism of SWL-induced cellular damage is still controversial. Several possible mechanisms, such as a transient decrease in effective blood flow, oxygen free-radical formation, and thermal effect have been proposed. Ischemia, secondary to SWL application, may occur as a result of a decrease in renal parenchymal perfusion. It has been shown that ischemia is a stimulus for the beginning of apoptotic changes in renal tissue model of experimental unilateral ureteral obstruction [21]. In their experimental study, Malik et al. [22] also showed that stretching of the renal tubular cells by transmitted high hydrostatic pressure and the accompanying ischemia can provide a powerful stimulus to apoptosis in obstructed neonatal rat kidneys. Moreover, reactive oxygen species, which increase after HESW application [23], are known to reduce the threshold of tissues to undergo apoptosis [24]. Recently, the apoptotic effect of HESW on renal tubular cells has been showed in a rabbit model [19].

In this study, we prefer rabbit models, since many experimental studies regarding the effects of HESW on tissues have been carried out using rats or rabbits, up-to-date. Actually, the ovarian cycle of rabbits does not process similar with human ovarian cycle. A female rabbit caged alone shows little evidence of a cycle; she is always on heat and ready to mate, but ovulation cannot be detected. If she is mated with a male rabbit or if her cervix is stimulated mechanically, she ovulates 10–12 h later. This phenomenon is known as induced ovulation [25]. Thereof, the female rabbits were kept with male ones in their cages in order to maintain their ovarian cycle, during the adaptation period.

In fact, it has been showed that apoptosis in granulosa cells and cumulus cells is associated with follicular atresia during natural ovarian cycles [26]. In this experimental study, we aimed to clarify whether any increased apoptotic changes in ovarian tissue resulted from HESW applied for lower ureteral stones. The apoptotic changes were investigated 14 and 28 days after SWL application, in accordance with the course of programmed cell death, since the upregulation of cell death genes requires time. Actually, in their preliminary study, Cimentepe and co-workers [19] showed that the apoptotic effect of HESW on renal tubular cells

thoroughly appears 28 days after SWL, but not demonstrated in acute period. Because cell turnover in ovarian tissue is faster than that of renal tubular cells, we detected the apoptotic changes additionally 14 days after SWL in this study.

In the present study, there was no histomorphological change in ovarian tissue of the study groups that might be due to the animals that were killed in late period. Also, the mean number of all kind of follicles, corpora lutea and TUNEL-positive follicle numbers in the study groups were similar with the control group. We did not find any evidence of increased apoptotic changes in ovarian tissue of animals killed 14 and 28 days after SWL application. Apoptotic changes were detected beginning from atretic follicle. This finding is in accordance with previous studies that investigated ovarian apoptosis during folliculogenesis [27, 28].

The results of this preliminary study demonstrate that HESW for distal ureteral stones does not induce apoptosis in ovarian tissue. Indeed, teratogenic effects of HESW to the female or productive tract are also significant potential concern. In their retrospective study, Vieweg et al. analyzed treatment data and radiation exposure of 84 women in the reproductive period, and surveyed the patients by questionnaire, to which, 67 responded and 10 childless women attempted to become pregnant. They reported that seven children with no malformation or chromosomal anomalies were born to six patients. Miscarriages were noted in three patients, but they occurred at least 1 year after ESWL.

Our results from the present study focused on apoptotic changes supposed these previous studies. However, because ultimate test of infertility is pregnancy, to determine no increased apoptotic changes in ovarian tissue does not guaranty a normal pregnancy and childbearing. Therefore, more studies regarding the effect of HESW on female reproductive tract are necessary to reach a definitive judgment.

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