Effect of direct ultrasonic exposure on histopathology of ovaries, inflammatory and oxidative responses in dogs

Arian Rajabi
Shiraz University
Asghar Mogheiseh (mogheiseh@yahoo.com)
Shiraz University
Saeed Nazifi
Shiraz University
MohammadSaeed Ahrari-Khafi
Shiraz University
AmirReza Dehghanian
Shiraz University of Medical Sciences
Naser Vesal
Shiraz University
Amin Bigham-Sadegh
Shiraz University

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Abstract

Background: The harmful effect of ultrasonic waves on sex gonads may be a non-surgical spays method in dogs. This research was designed to evaluate the effects of ultrasound waves on ovarian germinal tissue and inflammatory cytokines (interleukin-6 (IL-6), IL1β, tumor necrosis factor-α (TNF-α)), acute phase proteins (serum amyloid A (SAA), C reactive protein (CRP)) and oxidative stress (total antioxidant capacity (TAC), and malondialdehyde (MDA)) in dogs. Twenty six clinically healthy adult mix-breed female dogs were aligned into three groups. Laparotomy was performed in control (n=6) and treatment (T5, n=10; T10, n=10) groups. The therapeutic ultrasonic transducer was used by round motions on the ovaries (1 MHz frequency, 1.5 W/cm^2) for 5 min in T5 group and for 10 min in T10 group. Blood samples were collected from the jugular vein into plain glass tube on days 0 (before laparotomy), 3, 6 and 9 after surgery. Ovariectomy was conducted for histological evaluation on day 60.

Results: Direct exposure of ovaries with ultrasound waves induced inflammation and oxidative stress in compared with control group. Histopathological evaluation of treated ovaries with ultrasound waves indicated decrease number of primordial follicles (ovarian reserve) and oocyte preservation score in compared with controls.

Conclusions: These changes may be able to cause infertility and sterility in long term and it seems that inflammatory response and oxidative stress is a factor in the permanent damage of ovarian tissue.

Background

The population of dogs is increasing in an uncontrolled manner, and this issue has caused many problems for human and dog communities. The increase in the possibility of transmission of zoonosis diseases and non-human encounters is significant and worrisome (1). Therefore, population and fertility control methods are very important. The goal is to provide a method that completely sterilizes the dog with a single use, minimal damage and complications. Different methods of fertility control and sterilization in dogs have their own advantages and disadvantages. Surgical, chemical, physical and immunological methods are used to control fertility in dogs (2). Surgical procedures in female dogs include ovariectomy, ovariohysterectomy, and tubectomy. It has been shown in the physical methods of fertility control that ultrasound waves with different intensities and frequencies can cause damage and necrosis in the target tissues. These findings have been proven in male rat (3), ram (4), monkeys (5), and dogs (6–9). Local testicular thermal treatment has the potential to induce reversible oligospermia or azoospermia in monkeys as a result of germ cell apoptosis. Only one exposure of the testicles of monkey or rat at 43 *°C* caused reversible harm to seminiferous epithelium. Regional heating of monkey testicles to 43 *°C* water for 2 consecutive days (30 minutes per day) has shown that the quantity of sperm in the seminal fluid has dropped up to 80% at 28 days and is totally revocable (10). Ultrasound affects the tissue, mainly due to its heating effect as a result of tissue absorption of ultrasonic waves. In fact, the real mechanism of destructive ability of the applied ultrasonic waves is unknown. Ultrasound waves have caused damage to sperms in the *In vitro* conditions, and exposure to these waves in the *in vivo* has

caused a decrease in the quality of sperms and irreversible histopathological changes in the sperm-producing tissues of the testicles. This method does not have the disadvantages of surgical methods, including bleeding and side effects after surgery and anesthesia, and has a permanent effect on sterilization. Therefore, many researches are being conducted today and this method can sterilized permanently the dogs as a cheap and accessible tool.

After any tissue injury, acute phase response occurs and characterized by a number of different systemic effects including changes in the concentrations of blood proteins, called acute phase proteins (APPs). Some of APPs decrease in concentration (negative APPs; albumin or transferring) and others of which increase in concentration (positive APPs; CRP, SAA, haptoglobin (Hp), alpha-1-acid glycoprotein (AGP), ceruloplasmin (Cp), and fibrinogen). Production and response of APPs varies depending on the species. For example, in the dog, a strong response occurs with CRP. In dogs, major APPs (CRP and SAA) usually have an early and high rise in concentration and a very rapid decline. Most positive APPs are glycoproteins synthesized mainly by hepatocytes upon stimulation by proinflammatory cytokines and released into the bloodstream (11). The main proinflammatory cytokines are interleukin (IL)-6, IL-1, and TNF-α. Serum levels of IL-6 markedly increase during an acute phase response in dogs. Cytokine assays could be used for quantifying the induced systemic response to infection or inflammation (12). The acute phase response only last a few days, however increasing in APPs also have been described in chronic inflammation. In these cases, an aberrant continuation of some aspects of the acute phase response may contribute to the underlying tissue damage (13). Exposure of dog testes with ultrasonic waves induces systemic acute phase response and increases significantly the concentrations of CRP, SAA, and AGP on days 3, 5, and 7 compared to those of the control group (14).

Ultrasonic waves can induce oxidative stress using the thermal effects in dog’s testes (14). Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of reactive oxygen species (ROSs). The result of the reactions of ROSs with biomolecules is the formation of substances that can be used as markers of oxidative damage such as MDA (15). The antioxidants are the first line of choice to take care of stress. Endogenous antioxidant defenses include a network of compartmentalized antioxidant enzymatic and non-enzymatic molecules that are usually distributed within the cytoplasm and various cell organelles (16). Total antioxidant power considers the cumulative effect of all antioxidants present in blood and body fluids. It is considered as a useful indicator of the body’s antioxidant status to counteract the oxidative damage due to ROS (17).

In our previous study, we showed that exposing the ovaries to ultrasound waves from the abdominal skin may have effects on the ovarian tissue. Although we did not reach a definitive conclusion (18). The female dog's systemic response to ultrasonic waves and the straight impact of ultrasound waves on the ovarian tissue has not been investigated. Therefore, the goals of the current study were to investigate the impact of ultrasonic radiation on inflammatory responses and oxidative stress in the first week and ovarian tissue changes during two months after direct exposure to ultrasonic waves.

Results

IL6

Time, group and interaction of group and time factors were significantly affected the concentration of IL-6 during this study ($p < 0.0001$). IL6 serum concentration was not significantly different between T5, T10 and control groups prior treatment and on day 9 after treatment. In days 3 and 6 after treatment, IL6 serum concentration in T5 and T10 groups was significantly increased in compared with control group ($p < 0.0001$). Also increase of IL6 serum concentration in treated group T10 was significant in comparison with treated group T5 ($p < 0.002$). There was no significant difference for IL6 serum concentration between day 0 and day 9 in all groups. There was significant difference in increase of IL6 serum concentration on days 3 and 6 vs. day 0 ($p < 0.02$). There was significant decrease of IL6 serum concentration on days 6 and 9 vs. day 3 and between day 9 vs. day 6 ($P < 0.01$; Table 1).

Table 1

The results (mean ± standard deviation) of measuring different factors including inflammatory cytokines, acute phase proteins and total antioxidant capacity and lipid peroxidation index in the serum of dogs in the control group (only laparotomy, n = 6), the 5 minutes treatment group, laparotomy and exposure of the ovaries with ultrasonic waves for 5 minutes (n = 10) and 10 minutes treatment group, laparotomy and exposure of the ovaries with ultrasonic waves for 10 minutes (n = 7) in the days before surgery (day 0), and the days after surgery and exposing the ovaries to ultrasonic waves (days 3, 6 and 9)

Factor	Group	Day			
		0	3	6	9
IL6 (ng/ml)	**Control**	0.3 ± 0.04^{A}	0.66 ± 0.07^{aB}	0.37 ± 0.06^{aC}	0.29 ± 0.04^{A}
	T5	0.31 ± 0.04^{A}	1.55 ± 0.1^{bB}	1.01 ± 0.06^{bC}	0.31 ± 0.04^{A}
	T10	0.29 ± 0.05^{A}	1.63 ± 0.11^{cB}	1.13 ± 0.06^{bC}	0.29 ± 0.04^{A}
TNFα (ng/ml)	**Control**	1.04 ± 0.46^{B}	2.32 ± 0.85^{aA}	1.37 ± 0.54^{aB}	1.03 ± 0.42^{B}
	T5	0.97 ± 0.48^{A}	3.49 ± 0.44^{bB}	2.18 ± 0.15^{bC}	1.10 ± 0.47^{A}
	T10	1.08 ± 0.47^{A}	3.86 ± 0.41^{bB}	2.29 ± 0.14^{bC}	1.10 ± 0.43^{A}
IL1β (pg/ml)	**Control**	0.1 ± 0.02^{B}	0.27 ± 0.06^{aA}	0.14 ± 0.02^{aB}	0.09 ± 0.03^{B}
	T5	0.1 ± 0.02^{A}	0.54 ± 0.1^{bB}	0.28 ± 0.08^{bC}	0.1 ± 0.03^{A}
	T10	0.09 ± 0.02^{A}	0.57 ± 0.11^{bB}	0.29 ± 0.08^{bC}	0.11 ± 0.03^{A}
SAA (mg/ml)	**Control**	6.49 ± 0.77^{B}	15.99 ± 1.85^{aA}	8.22 ± 1.06^{aB}	6.35 ± 0.77^{B}
	T5	6.50 ± 0.74^{A}	41.01 ± 4.83^{bB}	28.5 ± 4.40^{bC}	6.54 ± 0.77^{A}
	T10	6.52 ± 0.67^{A}	45.38 ± 3.41^{cB}	29.20 ± 3.60^{bC}	6.59 ± 0.76^{A}
CRP (µg/ml)	**Control**	3.27 ± 0.45^{B}	9.11 ± 1.32^{aA}	4.07 ± 0.32^{aB}	3.25 ± 0.47^{B}
	T5	3.33 ± 0.43^{A}	30.56 ± 6.53^{bB}	20.26 ± 3.55^{bC}	5.29 ± 0.85^{A}
	T10	3.34 ± 0.37^{A}	36.66 ± 5.05^{cB}	20.74 ± 4.39^{bC}	5.35 ± 1.04^{A}
TAC (mmol/l)	**Control**	3.18 ± 1.05	2.79 ± 0.86	3.12 ± 0.85	3.18 ± 1.05
	T5	3.22 ± 1	2.70 ± 0.61	3.13 ± 0.82	3.20 ± 1.02
	T10	3.19 ± 0.85	2.54 ± 0.54	2.96 ± 0.70	3.19 ± 0.82

^{ABC} superscript letters indicate significant difference between days of study in each row.

^{abc} superscript letter indicate significant difference between groups in each column.

MDA (µmol/l)	Control	0.57 ± 0.03^{B}	0.62 ± 0.05^{aA}	0.57 ± 0.03^{aB}	0.55 ± 0.02^{B}
	T5	0.55 ± 0.03^{A}	0.77 ± 0.05^{bB}	0.64 ± 0.05^{bC}	0.55 ± 0.03^{A}
	T10	0.54 ± 0.02^{A}	0.81 ± 0.04^{cB}	0.65 ± 0.04^{bC}	0.54 ± 0.03^{A}
^{ABC} superscript letters indicate significant difference between days of study in each row.					
^{abc} superscript letter indicate significant difference between groups in each column.					

TNFα

Time, group and interaction of group and time factors were significantly affected the concentration of TNFα during this study ($p < 0.0001$). TNFα serum concentration was not significantly different between treatment and control groups on day 0 and 9. In days 3 and 6 after treatment, increase of TNFα serum concentration in T5 and T10 groups was statically significant in comparison to control group ($p < 0.0002$). In control group, increase of TNFα serum concentration in day 6 and 9 in compared with day 0 and decrease of TNFα serum concentration between days 9 and 6 was not significant. TNFα serum concentration was increased significantly on day 3 vs. day 0 in control group ($p < 0.0001$). Also, TNFa serum concentration was significantly increased on days 6 and 9 vs. day 3 in control group ($p < 0.0004$). There was no significant difference in TNFa serum concentration on day 0 vs. day 9 in T5 and T10 groups. There was significant increase of TNFa serum concentration on days 3 and 6 vs. day 0 and decrease of TNFa serum concentration on day 6 and 9 vs. day 3 in T5 and T10 groups ($p < 0.0001$; Table 1).

IL1β

Time, group and interaction of group and time factors were significantly affected the concentration of IL1β during this study ($p < 0.0001$). IL1β serum concentration was not significantly different between T5, T10 and control groups on day 0 and 9. IL1β serum concentration was significantly increased in T5 and T10 groups in comparison to control group on days 3 and 6 ($p < 0.0001$). IL1β serum concentration was significantly increased on day 3 vs. day 0 in control group ($p < 0.0001$). Also, IL1β serum concentration was significantly decreased on days 6 and 9 vs. day 3 in control group ($p < 0.0001$). There was no significant difference in IL1β serum concentration on day 0 vs. 9 in T5 and T10 groups. There was significant increase of IL1β serum concentration on day 3 and 6 vs. day 0 and decrease of IL1β serum concentration on days 6 and 9 vs. days 3 in T5 and T10 groups ($p < 0.0001$; Table 1).

SAA

Time, group and interaction of group and time factors were significantly affected the concentration of SAA during this study ($p < 0.0001$). SAA serum concentration was not significantly different between T5,

T10 and control groups on day 0 and 9. SAA serum concentration was significantly increased in T5 and T10 groups in comparison to control group on days 3 and 6 ($p < 0.0001$). SAA serum concentration in control group was significantly increased on day 3 vs. day 0 ($p < 0.0001$). Also, SAA serum concentration was significantly decreased on days 6 and 9 vs. day 3 in control group ($p < 0.0001$). There was no significant difference in SAA serum concentration on day 0 vs. day 9 in T5 and T10 groups. There was significant increase of SAA serum concentration on days 3 and 6 vs. day 0 and decrease of SAA serum concentration on days 6 and 9 vs. day 3 in T5 and T10 groups ($p < 0.0001$; Table 1).

CRP

Time, group and interaction of group and time factors were significantly affected the concentration of CRP during this study ($p < 0.0001$). CRP serum concentration was not significantly different between T5, T10 and control groups on day 0 and 9. CRP serum concentration was significantly increased in T5 and T10 groups in comparison to control group on days 3 and 6 and in treated group T10 in compared with treated group T5 on day 3 ($p < 0.0001$). CRP serum concentration was significantly increased on day 3 vs. day 0 in control group ($p < 0.0001$). Also, CRP serum concentration was significantly decreased on days 6 and 9 vs. day 3 in control group ($p < 0.0001$). There was no different difference in CRP serum concentration on day 0 vs. day 9 in T5 and T10 groups. There was significant increase of CRP serum concentration on days 3 and 6 vs. day 0 and decrease of CRP serum concentration on days 6 and 9 vs. day 3 in T5 and T10 groups ($p < 0.0001$; Table 1).

TAC

Group and interaction of group and time factors were not significantly affected the concentration of TAC, but time factor was significantly affected the concentration of TAC during this study ($p < 0.04$). TAC serum concentration was not significantly different between T5, T10 and control groups on days 0, 3, 6 and 9. There was no significant difference in TAC serum concentration between days of sampling in each group (T5 and T10 treatments and control; Table 1).

MDA

Time, group and interaction of group and time factors were significantly affected the concentration of MDA during this study ($p < 0.0001$). MDA serum concentration was not significantly different between T5, T10 and control groups on day 0 and 9. MDA serum concentration was significantly increased in T5 and T10 groups in comparison to control group on day 3 and 6 ($p < 0.0001$). Also, MDA serum concentration was significantly increased in treated group T10 in comparison to treated group T5 on day 3 ($p < 0.02$). MDA serum concentration was significantly increased on day 3 vs. day 0 in control group ($p < 0.0001$). Also, MDA serum concentration was decreased on days 6 and 9 in comparison to day 3 in control group ($p < 0.0001$). There was significant increase of MDA serum concentration on days 3 and 6 vs. day 0 and decrease of MDA serum concentration on days 6 and 9 vs. days 3 in T5 and T10 groups ($p < 0.0001$; Table 1).

Histopathological evaluation

The OPS, TNS, cortical thickness, and the number of primordial, primary, and secondary follicles of ovaries were compared between the treatment and control groups. The cortical thickness, mean number of primary and secondary follicles and TNS were not significantly different between the treated (T5 and T10 groups) and control groups. However, there were fewer primordial follicles in the treated (T5 and T10 groups) than control and in T10 group than T5 group ($p < 0.0001$). The OPS was fewer in treated group T5 than control ($p < 0.03$) and also was fewer in treated group T10 than control ($p < 0.0004$). The value of OPS was not different between the treated groups (T5 and T10 groups; Table 2).

Table 2
The results (mean ± standard deviation) and comparison of the histopathology evaluation of dog ovaries during two months after laparotomy of the control group (n = 6), the laparotomy group and exposure of the ovaries for 5 minutes with ultrasonic waves (n = 10) and the laparotomy group and exposure 10 Minutes of ovaries with ultrasonic waves (n = 7)

Group	Average Cortical Thickness (mm)	Primary follicles/HPF	Secondary Follicles/HPF	Primordial follicles/HPF	Oocyte preservation score (OPS)	Tissue necrosis score (TNS)
Control	0.9 ± 0.19	2.83 ± 1.47	1.33 ± 0.65	9.83 ± 3.27^{a}	9.67 ± 0.78^{a}	0 ± 0
T5	0.78 ± 0.30	1.37 ± 0.76	1.37 ± 0.76	7.68 ± 3.15^{b}	8.32 ± 1.20^{b}	0.53 ± 2.29
T10	0.88 ± 0.25	1.67 ± 0.49	1.42 ± 0.51	4.92 ± 2.02^{c}	7.5 ± 0.90^{b}	0.83 ± 2.89

^{abc} superscript letter indicate significant difference between groups in each column.

Discussion

In our study, direct exposure of ovaries with ultrasound waves induced inflammation and oxidative stress in compared with control (laparotomy) group. Histopathological evaluation of treated ovaries with ultrasound waves indicated decrease number of primordial follicles (ovarian reserve) and oocyte preservation score in compared with controls (laparotomy).

In this study, laparotomy increased inflammatory cytokines and acute phase proteins in the first three days after surgery. These factors decreased during the 6th and 9th days after surgery and returned to the level of the day before surgery. Surgeries performed in dogs, including spaying surgeries, increase inflammatory cytokines and phase proteins during the first week after surgery (19, 20). The duration of surgery and the intra-operative manipulations and the drugs are effective on the duration and intensity of the inflammatory response (21). Both ovariohysterectomy and ovariectomy caused major post surgical changes in iron serum levels, CRP and glucose. CRP is a significant acute phase protein in dogs and a mild inflammatory response happens after both ovariectomy and ovariohysterectomy (22). Ovariohysterectomy is a spaying method commonly employed in female dogs. A surgical technique that

interrupts tissue integrity, it induces a temporary local inflammatory reaction accompanied by a systemic response called an acute phase response (22, 23). Inflammatory processes are subject to underlying ovarian pathology and if pushed, pro-inflammatory conditions may have a negative impact on ovarian follicular dynamics. Aberrant inflammation can alter normal ovarian follicular dynamics resulting in impaired oocyte quality, anovulation and associated infertility. C-reactive protein is an acute phase reactant produced by hepatocytes and is a marker for systemic inflammation. Serum CRP levels rise in response to increased production of TNF-α and IL-6 from macrophages and adipocytes, which then activates an inflammatory response through the complement system (24). Low-grade, chronic inflammation impaired folliculogenesis (25). ROS are important to ovulation, but in excess they are cytotoxic to the cell and organelles, and without a responsive increase in antioxidants, oocytes are subsequently damaged or of poorer quality through the phenomenon known as lipotoxicity (26) early expression of these inflammatory markers may induce a premature influx of leukocytes and as a result, impair maturation and subsequent ovulation (27). Additionally, inflammation has been associated with oxidative stress, and oxidative stress has been shown to induce inflammation, thus suggesting a perpetuating cycle.

In the present study, exposing the ovaries to ultrasonic waves for 5 or 10 minutes increased the intensity of the inflammatory response, especially in the first 3 days. Exposure of dogs' testicles to ultrasonic waves induced an inflammatory response, and it seems that this inflammatory response plays a role in the permanent damage of testicular tissue (14).

Oxidative stress index (MDA concentration) increased in laparotomy group. Increasing of MDA level in T5 and T10 treated groups was greater than laparotomy group. Oxidative stress is destructive to tissue and can cause permanent damage and infertility in sensitive tissue such as testis and ovary. Exposure of dogs' testicles to ultrasonic waves has induced oxidative stress in the tissues and probably plays a role in their permanent damage and sterility (14). The use of ultrasonic radiation is one of the safe methods to control fertility in male dogs. It was Fahim et al. who introduced ultrasound as a method of male fertility control for the first time in 1975. Leoci and co-workers (2015) exposed every dog testicle to a therapeutical ultrasonography (1.5 W/cm^2, 1 MHz) for 5 minutes every two days and for 3 therapies. Following two weeks, a notable decline was seen in the sperm count and testicle size of the dogs (9). High-intensity ultrasonic radiation could trigger the oxidative stress and acute phase response of inflammatory and create more injury to the testicle tissue in dogs. This was seen mostly in the first week following the testicles have been exposed to ultrasonic radiation (14). It has been stated that radiofrequency electromagnetic wave expose from cell phones can motive oxidative stress, decline the action of antioxidant enzymes (GPx and SOD), and rise the density of MDA in the sperm of male Wistar rats. It may be related to sterility and a change in the reproductive function (28). Tang et al 2015 summarized the non-thermal effect of ultrasound (focused, pulsed ultrasound frequency 20 KHz-3 MHz) to sonomechanical including acoustic radiation force, shockwave and micro-jets induced by cavitation and sonochemical including inertial cavitation generated reactive oxygen species (ROS) and intracellular ROS release from mitochondrial damage. Their biological effects are cytoskeletal remodeling, modulation

of cellular proliferation and protein synthesis, enhanced gene transfection, induction of oxidative stress and up-regulation of apoptotic genes (29).

Histopathological changes after direct exposing the ovaries to ultrasonic waves explain that the count of primordial follicles and the egg preservation score decreased. These changes can cause infertility and sterility in the long-term. The lower temperature of the follicular fluid prior to ovulation may be essential for normal oocyte development and changes in temperature within the ovary at the ovulatory phase may be important for optimal nuclear cytoplasmic and membraneous maturation (30). Local cooling of the female reproductive tract, especially of specific ovarian tissues, occurs close to ovulation favoring male and female gamete maturation (31). A 1°C increase in average maximum temperature during the 90 days before ovarian reserve testing was associated with a -1.6% lower antral follicle counting (32). In our preliminary study, the ovaries were exposed to ultrasonic waves through the skin, and limited and variable histopathological changes have been found in the ovarian tissues of dogs (18). Various protocols were tested for exposure of the testicles to ultrasonic waves, which gave relatively various outcomes. Indeed, the frequency and strength of waves, the time of exposure, frequency and interval among exposures can affect the results (14). Three treatments within 48 hours interval at 1 MHz, 1.5 W/cm2 for 5 min, lead to permanent testicular injury and azoospermia in the dog. No significant adverse effect was reported and sterility was occurred with testosterone concentration in physiological range (9).

In ram, as the time and repeats of ultrasonication increase the affected spermatozoa level increased although the mitochondrial activity reduced. The most effectiveness of ultrasonication related to the damaged spermatozoa rate and mitochondrial activity rate was seen in the protocol with 8-repeating, 10-s duration group in Merino ram spermatozoa (4). The combination of thermal and non-thermal (mechanical) impact of the ultrasonic waves cause an ion exchange between the fluid in the seminiferous tubules and the rete testis, create an unsuitable condition for spermatogenesis, and disrupt the spermatogenesis (8).

Ultrasound treatment seems to be most efficient for males with smaller testes, suggesting that higher levels of exposure may be necessary to obtain fertility control in individuals and animals with larger testicular mass. It was verify in a non-human primate species with testes that compare in size with those of men that ultrasound treatment results in decrease sperm count and quality. This study gives an evidence of theory that testicular ultrasound exposure has the ability to be a practical tool for fertility control in humans (5). Therapeutic ultrasound depleted growing germ cells from the testicle. They also noted that combining of raised temperature, high power and high frequency is essential to decreasing sperm number. The correct combining of these features are the hardest part of neutralization by ultrasound (3).

Ultrasound therapy was found to be related with congenital deformities and exposure to short waves was found to be associated with reduced birth weight and with an enhanced danger for male infants. Women physiotherapists' exposure to shortwave radiation during pregnancy could have negative effects on pregnancy results and that short-wave use in pregnancy may be considered a potential risk for

reproduction (33). Exposure to short waves and ultrasound was associated with medically diagnosed spontaneous abortions happening after the tenth week of pregnancy but not with those happening before (34).

Controversially, trans-abdominal exposure of transplanted ovarian tissue with low intensity pulsed ultrasound (5 minutes daily, 0.3 W/cm2, frequency of 3 MHz) in mice (35), and treatment of cases with premature ovarian failure in rats (frequency of 900KHz, 90 W/cm^2 for 10 days) and mice (200 mW/cm2, frequency of 0.3 MHz) for 20 min, 15 consecutive days) (36, 37) could improve increase re-angiogenesis and promote ovarian follicular growth, and some functions of ovaries.

Conclusion

It seems that direct exposure of ovaries with the therapeutic ultrasound (1 MHz frequency, 1.5 W/cm^2) has a limited effect on the ovarian tissue, especially that this treatment was not repeated as the same as previous studies that were conducted on the testes of dogs and other species. It is suggested the ultrasound direct exposure of ovaries studied using different protocols with higher frequency and power than this study.

Methods

Experimental treatment regimens have been accomplished in compliance with the Iran's Animal Ethics system under the oversight of the Iran's community for the avoidance of harshness to Animals and Shiraz University study board (IACUC no: 4687/63). The recommendations of the EU Committee Directive (2010/63/EU) of September 22, 2010, on animal welfare standards for trial basis, were monitored as well.

Animals

Twenty six clinically healthful mixed-breeds reproductively adult anestrous female dogs, aged approximately 1.96 ± 0.64 years old and weighing 17.55 ± 1.5 kg, were used for this research. The dogs were owned and maintained in the School of Veterinary Medicine Shiraz University, ovariectomised upon completion of the study, and retain in a non-government shelter. Every dog has been fed with commercial food dog (300 g/dog/day; Nutripet™; Behintash Co., Karaj, Iran) and was given ad libitum access to water. They were adapted to the new conditions during the first 2 weeks. All dogs were treated with anti-parasitic tablets (Fenbendazole, 150 mg; Pyrantel embonate, 144 mg; Praziquantel, 50 mg; Caniverm®, 0.7 mg/10 kg, PO). Overall dog health was assessed everyday following the examination of their body temperature, heart rate, respiratory rate, appetite, and attitude when feeding or cleaning of their house. The state of pregnancy of dogs was checked with a trans-abdominal ultrasound before treatments. The phase of the estrous cycle was assessed with vaginal cytology.

Experimental Design

The dogs were aligned into three groups, a control (laparotomy) group (n = 6) and two treatment groups: 5 min treatment of ovaries with ultrasound wave during laparotomy (T5, n = 10), and 10 min treatment of ovaries with ultrasound wave during laparotomy (T10, n = 10) (Fig. 1). Exposing of both ovaries without ultrasound treatment was performed in control group. In treatment groups after exposing the ovaries, the therapeutic ultrasound (Unix U500, Iran) transducer was used by round motions on the ovaries (1 MHz frequency, 1.5 W/cm^2) for 5 min in T5 group and for 10 min in T10 group. Blood samples were obtained from the jugular vein on days 0 (before laparotomy), 3, 6 and 9 after surgery for evaluating inflammatory cytokines, acute phase proteins and oxidative stress (IL-6, α-TNF, 1β-I ,SAA ,CRP and TAC, MDA)

Laparotomy And Ovariecomy

Laparotomy was performed under general anesthesia in control and treatment groups. Animals were fasted for about12 hours before the surgery. The anesthesia protocol was chosen and applied under supervision of the anaesthetist. The dogs were pre-medicated via acepromazine (0.05 mg/kg, intra-muscular) and xylazine (0.5 mg/kg, intra-muscular). Anaesthesia was generated through using ketamine (5 mg/kg, Intra-Venous) and diazepam (0.25 mg/kg, Intra-Venous). After tracheal intubation, overall anesthesia was preserved with isoflurane (1.2%) sprayed into oxygen using discontinuous positive pressure ventilation. Tramadol (2 mg/kg; IM) and cefazolin (20 – 30 mg/kg; intra-muscular) were given at the onset and end of surgery. Ovariectomy was conducted for histological evaluation of ovaries on day 60 following ultrasound therapy.

Histopathological Evaluation

After the ovariectomy, the ovarian tissues were fixed in 10 percent phosphate-buffered formalin. A fresh solution was then substituted for the formalin after one day, dried in an automated tissue transformer, and transformed in paraffin wax. The section (5–10 μm) was prepared with microtome and stained with hematoxylin-eosin. Light microscope was used to assess tissue structure and counting the number of various types of follicles in three sections from each ovary. The ovaries were evaluated by a reproductive pathologist who was blind to the research groups. The count of follicles (primordial, primary, and secondary), tissue necrosis score (TNS), and oocyte preservation score (OPS) were identified in the ovary segments using the scoring method that has been used in human ovary researches. The presence of total necrosis in each ovary was set by assessing the percent of necrosis using TNS per samples. The TNS is an observable assessment of necrosis and presents the percent of necrosis/infarction in ovary samples. TNS was rated from lowest (0) to highest (4) for characterizing necrotic transformation. The OPS was used for assess the level of histopathological injury. The OPS description was established to evaluate both the structural preserving of follicles and oocytes and the embedding tissue micro-environment related to these structures. The intensity and progress patterns of tissue lesions were determined according to the magnitude of inflammatory alterations and tissue necrosis from one to nine (38). Follicles have been divided into 4 classes: primordial follicles (a layer of squamous granulosa cells nearby an oocyte), primary follicles (one layer of cuboidal granulosa cells nearby an oocyte), secondary

follicles (a complete double layer of cuboidal granulosa cells nearby the oocyte), and antral follicles (multi laminar granulosa cells nearby the oocyte in the present of an antrum). Follicles classified into a structure comprised the oocyte to inhibit recounting of the same follicles (39).

Laboratory assays

Interlukin-6 (IL-6) was measured by a quantitative sandwich enzyme immunoassay using a commercial dog-specific competitive ELISA kit (CUSABIO, Shanghai, China, Code CSB-E11260c). The sensitivity of the kit was 0.39 pg/mL (the detection limit of the kit was 1.56 pg/mL-100 pg/mL). The intra-assay and inter-assay coefficient of variation of the interlukin-6 (IL-6) kit was CV < 8% and CV < 10%, respectively. C-reactive protein was measured using a canine solid phase sandwich ELISA method (Shanghai Crystal Day Biotech Company, China; Catalog Number: CRP E 0124ca). CRP ELISA kit was specified with Intra-Assay CV < 8%, Inter-Assay CV < 10%, and sensitivity 7.8 pg/ml. Serum amyloid-A (SAA) was measured using a canine solid phase sandwich ELISA method (Shanghai Crystal Day Biotech Company, China; Catalog Number: SAA E0125ca). SAA ELISA kit was specified with Intra-Assay CV < 8%, Inter-Assay CV < 10%, and sensitivity 0.156 pg/ ml). TNF-α was measured using a canine solid phase sandwich ELISA method (Shanghai Crystal Day Biotech Company, China; Catalog Number: TNF E0025 ca). TNF-α ELISA kit was specified with Intra-Assay CV < 8%, Inter-Assay CV < 10%, and sensitivity 0.01 ng/l). IL-1β was measured using a canine solid phase sandwich ELISA method (Shanghai Crystal Day Biotech Company, China; Catalog Number: IL1B E0002ca). IL-1β ELISA kit was specified with Interleukin-1β Intra-Assay CV < 8%, Inter-Assay CV < 10%, and sensitivity 7.8 pg/ml) (40).

A commercial kit (ZellBio GmbH kit, Germany) was used to determine the TAC level. The color product of the chromogenic substrate (tetramethylbenzidine) emerged at the ending phase. The difference in color was calculated calorimetrically using a spectrophotometer (Jenway 6300 Spectrophotometer, UK) at 450 nm and represented as mmol/L. This method can determine TAC with 0.1 mM sensitivity (100 µmol/L). The intra- and inter-assay CVs were below 3.4% and 4.2%, respectively. An assay kit purchased from ZellBio GmbH (Germany) was used to measure MDA (µmol/L; Cat. no. ZB-MDA96A). In this kit, MDA is measured based on its reaction with thiobarbituric acid in an acidic condition and high temperature. The color complex was measured colorimetrically at 535 nm. The assay kit sensitivity was 0.1 µM (inter-assay CV: 5.8%) for MDA (41).

Statistical analysis

The statistical analyzing was performed using the Graph-Pad Prism 6 program utilizing the Two-way ANOVA repeated measures and paired test for serum factors. The data were normally distribution. The One-way ANOVA repeated measures was used to assess statistical differentiation between treated and control groups. All results have been stated as mean ± SD. It was significant to be mean differences when there was a P-value of less than 0.05.

Abbreviations

Interleukin-6 (IL-6); interleukin-1β (IL1β); tumor necrosis factor-α (TNF-α); serum amyloid A (SAA); C reactive protein (CRP); acute phase proteins (APPs); haptoglobin (Hp); alpha-1-acid glycoprotein (AGP); ceruloplasmin (Cp); reactive oxygen species (ROSs); tissue necrosis score (TNS); oocyte preservation score (OPS); catalase (CAT); malondialdehyde (MDA).

Declarations

Animal ethics approval and consent to participate

Our study was approved by the Iranian laboratory animal ethics framework under the supervision of the Iranian Society for the Prevention of Cruelty to Animals and Shiraz University Research Council (IACUC no: 4687/63). A written informed consent was obtained by the shelter. The recommendations of European Council Directive (2010/63/EU) of September 22, 2010, regarding the standards in the protection of animals used for experimental purposes, were also followed. The authors confirmed all methods in this study are reported in accordance with ARRIVE guidelines 2.0 (https://arriveguidelines.org) for the reporting of animal experiments. all methods were carried out in accordance with relevant guidelines and regulations.

Consent to publish

Not applicable.

Availability of data and material

The datasets generated and/or analysed during the current study are available from the corresponding author on request.

Competing interests

'The authors declare that they have no competing interests.

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Authors’ contributions

All authors contributed in all parts of study from designing study to writing and preparing of manuscript. AM, SN, MSAK, and AR contributed in study design, performing study, sampling, data collection and analysis and preparing manuscript. NV and AB aimed performing laparotomy and ovarietomy.

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Not applicable.

References

1. Smith LM, Hartmann S, Munteanu AM, Dalla Villa P, Quinnell RJ, Collins LM. The effectiveness of dog population management: a systematic review. Animals. 2019;9(12):1020.
2. Urfer SR, Kaeberlein M. Desexing dogs: a review of the current literature. Animals. 2019;9(12):1086.
3. Tsuruta JK, Dayton PA, Gallippi CM, O'Rand MG, Streicker MA, Gessner RC, et al. Therapeutic ultrasound as a potential male contraceptive: power, frequency and temperature required to deplete rat testes of meiotic cells and epididymides of sperm determined using a commercially available system. Reprod Biol Endocrinol. 2012;10(1):1-15.
4. Akalin P, Baspinar N, Coyan K, Bucak MN, Gugor S, Özturk C. Effects of ultrasonication on damaged spermatozoa and mitochondrial activity rate. Turk J Vet Anim Sci. 2016;40(2):195-9.
5. VandeVoort CA, Tollner TL. The efficacy of ultrasound treatment as a reversible male contraceptive in the rhesus monkey. Reprod Biol Endocrinol. 2012;10(1):1-7.
6. Fahim M, Fahim Z, Der R, Hall D, Harman J. Heat in male contraception (hot water 60° C, infrared, microwave, and ultrasound). Contraception. 1975;11(5):549-62.
7. Fahim M, Fahim Z, Harman J, Thompson I, Montie J, Hall D. Ultrasound as a new method of male contraception. Fertil Steril. 1977;28(8):823-31.
8. Leoci R, Aiudi G, De Sandro Salvati A, Silvestre F, Binetti F, Lacalandra G. Ultrasound as a mechanical method for male dog contraception. Reprod Domest Anim. 2009;44:326-8.
9. Leoci R, Aiudi G, Silvestre F, Lissner E, Marino F, Lacalandra G. Therapeutic ultrasound as a potential male dog contraceptive: Determination of the most effective application protocol. Reprod Domest Anim. 2015;50(5):712-8.
10. Liu Y-X. Temperature control of spermatogenesis and prospect of male contraception. Front Biosci (Schol Ed). 2010;2(2):730-55.
11. Ceron JJ, Eckersall PD, Martýnez-Subiela S. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet Clin Pathol. 2005;34(2):85-99.
12. Eckersall PD, Duthie S, Toussaint MJ, Gruys E, Heegaard P, Alava M, et al. Standardization of diagnostic assays for animal acute phase proteins. Adv Vet Med. 1999;41:643-55.
13. Ceciliani F, Giordano A, Spagnolo V. The systemic reaction during inflammation: the acute-phase proteins. Protein Pept Lett. 2002;9(3):211-23.
14. Khanbazi MH, Mogheiseh A, Khafi MSA, Nazifi S, Ahmadi N, Khazaei M. The effects of therapeutic ultrasound waves on testicular tissue, echogenicity, semen quality, oxidative stress, and acute-phase proteins in dogs. Theriogenology. 2020;153:39-47.
15. Russo C, Bracarense APFRL. Oxidative stress in dogs. Semina: Ciências Agrárias. 2016;37(3):1431-9.

16. Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, et al. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed research international. 2014;2014.
17. Suresh D, Annam V, Pratibha K, Prasad B. Total antioxidant capacity–a novel early bio-chemical marker of oxidative stress in HIV infected individuals. Journal of biomedical science. 2009;16(1):1-4.
18. Khazaei M, Mogheiseh A, Ahrari-Khafi MS, Dehghanian A, Khanbazi MH. A preliminary study of transabdominal ultrasonic wave treatment on the germinal tissues of dog ovaries as a contraceptive approach. Anim Reprod Sci. 2020;221:106586.
19. Kumari A, Tiwary R, Kumar R, Guha SK. Oxidative stress and antioxidant activity in female dogs undergoing laparoscopic and open elective ovariectomy. Indian J Anim Sci. 2022;92(7):825-9.
20. Lee JY, Kim MC. Comparison of oxidative stress status in dogs undergoing laparoscopic and open ovariectomy. J Vet Med Sci. 2014;76(2):273-6.
21. Sakundech K, Chompoosan C, Tuchpramuk P, Boonsorn T, Aengwanich W. The influence of duration on pain stress, oxidative stress, and total antioxidant power status in female dogs undergoing ovariohysterectomy. Vet World. 2020;13(1):160.
22. Moldal ER, Kjelgaard-Hansen MJ, Peeters ME, Nødtvedt A, Kirpensteijn J. C-reactive protein, glucose and iron concentrations are significantly altered in dogs undergoing open ovariohysterectomy or ovariectomy. Acta Vet Scand. 2018;60(1):1-8.
23. Dabrowski R, Wawron W. Acute-phase response in monitoring postoperative recovery in bitches after ovariohysterectomy. Ann Anim Sci. 2014;14(2):287.
24. Boots CE, Jungheim ES, editors. Inflammation and human ovarian follicular dynamics. Seminars in reproductive medicine; 2015: Thieme Medical Publishers.
25. Robker RL, Wu LL, Yang X. Inflammatory pathways linking obesity and ovarian dysfunction. J Reprod Immunol. 2011;88(2):142-8.
26. Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N. Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A. 2011;108(4):1462-7.
27. Schmidt J, Weijdegård B, Mikkelsen AL, Lindenberg S, Nilsson L, Brännström M. Differential expression of inflammation-related genes in the ovarian stroma and granulosa cells of PCOS women. Mol Hum Reprod. 2014;20(1):49-58.
28. Kesari KK, Kumar S, Behari J. Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male Wistar rats. Appl Biochem Biotechnol. 2011;164(4):546-59.
29. Tang J, Guha C, Tomé WA. Biological Effects Induced by Non-thermal Ultrasound and Implications for Cancer Therapy: A Review of the Current Literature. Technol Cancer Res Treat. 2015;14(2):221-35.
30. Wang JZ, Sui HS, Miao DQ, Liu N, Zhou P, Ge L, et al. Effects of heat stress during in vitro maturation on cytoplasmic versus nuclear components of mouse oocytes. Reproduction. 2009;137(2):181-9.
31. Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review.

Human reproduction update. 2018;24(1):15-34.
32. Gaskins AJ, Mínguez-Alarcón L, VoPham T, Hart JE, Chavarro JE, Schwartz J, et al. Impact of ambient temperature on ovarian reserve. Fertil Steril. 2021;116(4):1052-60.
33. Lerman Y, Jacubovich R, Green MS. Pregnancy outcome following exposure to shortwaves among female physiotherapists in Israel. Am J Ind Med. 2001;39(5):499-504.
34. Taskinen H, Kyyrönen P, Hemminki K. Effects of ultrasound, shortwaves, and physical exertion on pregnancy outcome in physiotherapists. J Epidemiol Community Health. 1990;44(3):196-201.
35. Abtahi NS, Eimani H, Vosough A, Shahverdi A, Fathi R, Hayati N, et al. Effect of therapeutic ultrasound on folliculogenesis, angiogenesis and apoptosis after heterotopic mouse ovarian transplantation. Ultrasound Med Biol. 2014;40(7):1535-44.
36. Tang H, Liu Y, Fan Y, Li C. Therapeutic Effects of Low-Intensity Pulsed Ultrasound on Premature Ovarian Insufficiency. Ultrasound Med Biol. 2021;47(8):2377-87.
37. Xu H, Xia Y, Qin J, Xu J, Li C, Wang Y. Effects of low intensity pulsed ultrasound on expression of B-cell lymphoma-2 and BCL2-Associated X in premature ovarian failure mice induced by 4-vinylcyclohexene diepoxide. Reprod Biol Endocrinol. 2021;19(1):1-11.
38. Asfour V, Polson A, Varma R, Menon P. Evaluating ovarian preservation after ovarian torsion using the ovarian preservation score and tissue necrosis score. Clin Obstet Gynecol Reprod Med. 2015;1(2):34-9.
39. Liu J, Van der Elst J, Van den Broecke R, Dhont M. Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. Hum Reprod. 2002;17(3):605-11.
40. Dearakhshandeh N, Mogheiseh A, Nazifi S, Ahrari Khafi MS, Abbaszadeh Hasiri M, Golchin-Rad K. Changes in the oxidative stress factors and inflammatory proteins following the treatment of BPH-induced dogs with an anti-proliferative agent called tadalafil. Journal of Veterinary Pharmacology and Therapeutics. 2019;42(6):665-72.
41. Mogheiseh A, Koohi F, Nazifi S, Shojaee Tabrizi A, Taheri P, Salavati S. Oxidative-antioxidative status and hepatic and renal factors following melatonin administration in castrated and intact dogs. Basic Clin Androl. 2019;29:14.

Figures

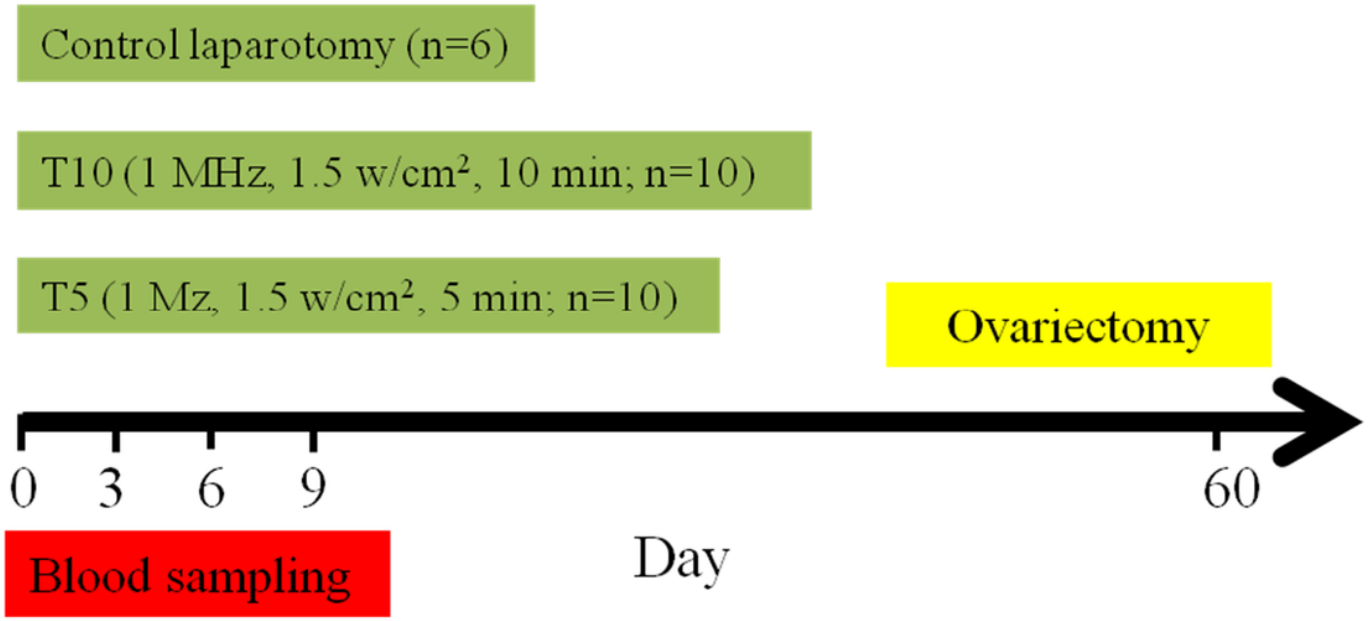

Figure 1

The dogs were aligned into three groups, a control (laparotomy) group (n=6) and two treatment groups: 5 min treatment of ovaries with ultrasound wave during laparotomy (T5, n=10), and 10 min treatment of ovaries with ultrasound wave during laparotomy (T10, n=10).