

Effects of low-frequency ultrasound combined with microbubbles on benign prostate hyperplasia

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Abstract

Introduction: Our objective is to assess the effects of low-frequency ultrasound combined with microbubbles on benign prostate hyperplasia (BPH).

Methods: Sixteen Beagle dogs with BPH were randomly assigned into 4 groups (n = 4): control group (without treatment), G1 group (injection with 2 mL of microbubble contrast agent); G2 group (21 kHz ultrasound); and G3 group (injection with 2 mL of microbubble contrast agent +21 kHz ultrasound). The histopathological damage to prostate cells was assessed via transmission electron microscopy and optical microscopy. The protein expressions of prostate-specific antigen (PSA), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD) of vessels were detected by enzyme-linked immunosorbent assay (ELISA).

Results: Histopathologically, the prostate cells exhibited nuclear chromatin contraction, mitochondrial swelling, degranulation of rough endoplasmic reticulum, basement membrane rupture and cell apoptosis in the G2 and G3 groups; it was especially obvious in the G3 group, while no changes were observed in the control and G1 groups. Although prostate volume using imaging was not significantly changed in all groups after treatment, PSA was significantly reduced in the G2 and G3 groups, and especially obvious in the G3 group ($p < 0.05$). The iNOS and SOD, which are important oxidative stress factors, significantly increased after treatment in the G2 and G3 groups, but not in the control and G1 groups ($p < 0.05$).

Conclusions: Low-frequency ultrasound is effective in treating BPH; low-frequency ultrasound combined with microbubbles improves the treatment efficacy.

Introduction

Benign prostatic hyperplasia (BPH) is a non-malignant enlargement of the prostate, ranking among the 10 most common diseases in aging men.¹ BPH is characterized by

smooth muscle and epithelial proliferation primarily within the prostatic transition zone, which results in a variety of problems for patients. The most frequent problem is lower urinary tract symptoms (LUTS)² and 80% of men in their 70s suffer from BPH-related LUTS.^{3,4}

Currently, the management of BPH involves non-surgical and surgical techniques. Alpha-blockers and 5-alpha reductase inhibitors are common medical options due to their excellent efficacy and convenience of administering without severe adverse effects.^{5,6} Although transurethral resection of the prostate (TURP) is the gold standard for BPH-related LUTS, it has disadvantages. Its potential disadvantages include significant blood loss and operative hyponatraemia, need for hospitalization and possible prolonged catheterization, and low, but real, risks of urinary incontinence, erectile dysfunction, bladder neck contractures, and urethral stricture disease.⁷ Laser surgery as a substitute has also been attempted and has a satisfactory short-term efficacy.⁸⁻¹⁰ However, elderly and more infirm patients are least likely to undergo it; this raises concern about the underutilization of the technique in this population.¹¹ Therefore, a more effective, safe, and easy treatment strategy for BPH is urgently needed.

Ultrasound is generally used for diagnosis,^{12,13} and it is also being developed as a therapeutic tool related to the direct mechanical energy effects of low-frequency ultrasound. A microbubble ultrasound contrast agent is widely used as contrast media in ultrasonography, yet it acts as a cavitation nuclei or enhancer. Low-frequency ultrasound and microbubbles have been shown to accelerate thrombolysis in vitro and in vivo.^{14,15} In addition, low frequency ultrasound has also been effective for suppressing tumour proliferation and for promoting tumour apoptosis.¹⁶⁻¹⁸ However, there is currently little knowledge on the role of microbubble-mediated ultrasound in treatment of BPH. The present study was designed, in a canine model, to demonstrate the effect of low-frequency ultrasound combined with microbubbles on BPH.

Methods

Ultrasound system and microbubble

The FS-450 low-frequency ultrasonic processing system (Shanghai Institute of Ultrasound in Medicine, Shanghai, China) was used in all experiments. This device is equipped with a built-in digital timer, intensity regulator and duty factor controller; settings were as follows: duty cycle, 10%–90%; probe frequency, 21 kHz. A sulfur hexafluoride (SF_6)-filled microbubble ultrasound contrast agent (Sonovue, Bracco SpA, Milan, Italy) was used in this study, which consisted of 59 mg of SF_6 gas and 25 mg of freeze-dried white powder. After adding 5 mL of 0.9% saline into the vial and shaking for several seconds, phospholipid-stabilized microbubbles filled with SF_6 with a diameter of $<8 \mu\text{m}$ (mean $2.5 \mu\text{m}$) were generated at a concentration of $(2\text{--}5) \times 10^8$ microbubbles/mL.¹⁹ MyLab 90 ultrasound imaging system equipped with TRT33 Bi-plane electronic linear and microconvex array and transrectal transducer (4–13 MHz) (Esaote, Genoa-Florence, Italy) was used to measure the prostate diameter and volume, and the system equipped with CA431 electronic curvex array transducer (3.5–5 MHz) was used to identify the position for displaying the prostate image.

Animals and grouping

We used 16 7-year-old male Beagle dogs diagnosed with hyperplasia nodule in the prostate by ultrasound, each weighing 17.5 to 25.6 kg, in this study. All procedures were done in accordance with guidelines of the Chinese Council on Animal Care. Protocols were approved by the local experimental ethics committee. All animals were intravenously anesthetized with 35 mg/kg pentobarbital sodium. The lower abdomen and suprapubic region were shaved and the penis was retracted laterally to the right to provide clear acoustic access through the abdominal wall to the prostate. Transrectal ultrasound imaging and volume measurement (ellipsoid approximation) was performed using the ultrasound imaging system. The low-frequency probe was placed on lower abdomen skin where prostate image could be displayed by the 3.5 MHz convex array transducer near the root of the penis.

The animals were randomly divided into 4 groups: G1, G2, G3 and control; there were 4 dogs in each group. Each animal in the G1 group was injected with 2 mL of microbubble contrast agent without exposure to ultrasound; each animal in the G2 group was exposed to 21 kHz ultrasound without microbubble contrast agent injection; each animal in the G3 group was injected with 2 mL of microbubble contrast agent combined with exposure to 21 kHz ultrasound; while the other animals without ultrasound exposure and microbubble therapy served as controls.

Ultrasound exposure

The 21-kHz transducer with 20 mm in diameter was positioned on the lower abdomen skin near the penis. The cold acoustic coupling gel was placed between the skin and the probe surface to prevent reflections and standing waves. The peak acoustic amplitude in degassed water was measured using a calibrated poly(vinylidene difluoride-trifluoroethylene) needle-type hydrophone (Toray Techno Co., Ltd., Japan), 0.5 mm in diameter, connected to a personal or compatible computer and a digitizing oscilloscope (TDS3034, Tektronix Japan, Ltd., Japan). The spatial-average temporal average intensity (ISATA) was 318 mW/cm^2 and the peak acoustic pressure was 0.95 MPa, respectively. The duty ratio was 67% (the length of pulse was 20 s and the duty of pulse was 30 s). The ultrasound exposure was repeated for 3 times in 3 days with a 1-day interval, of 30 minutes each time.

Detection of blood samples

Blood samples (4 mL) were collected from the femoral vein before treatment and after the last ultrasound exposure. The prostate-specific antigen (PSA) was detected using double antibody sandwich immunoassay, and inducible nitric oxide synthase (iNOS) and super oxidase dimutase (SOD) were measured using enzyme-linked immunosorbent assay (ELISA).

Following the last ultrasound exposure treatment, all animals were sacrificed immediately, and the prostate was surgically removed. The prostate harvested was fixed in formalin for 1 week, and then cut into pieces (measuring $10 \times 10 \times 10 \text{ mm}^3$), which were subsequently dehydrated, paraffin embedded, cut into sections of 5- μm in thickness with a microtome and stained with hematoxylin and eosin (H&E). All sections were scanned with a flatbed scanner with resolution of 2400×2400 dots per inch (dpi).

TUNEL

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (TUNEL) was performed with a TACS 2 TdT-DAB kit (Trevigen, Gaithersburg, MD) following the manufacturer's instructions. Briefly, after deparaffinization and hydration, sections were digested with proteinase K at a concentration of 20 g/mL for 15 minutes. Endogenous peroxidase activity was quenched with 2% H_2O_2 for 5 minutes. The slides were immersed in terminal deoxynucleotidyl transferase (TdT) buffer. After, TdT, 1 mmol/L Mn^{2+} , and biotinylated deoxynucleoside triphosphates (dNTPs) in TdT buffer were added to cover the sections and incubated in a humid atmosphere at 37°C for 60 minutes. The slides were washed with phosphate buffered saline (PBS) and incubated with streptavidin-horseradish peroxidase (HRP) for 10 min-

utes. After being rinsed with PBS, the slides were immersed in a diaminobenzidine (DAB) solution. All slides were counterstained with 1% methyl green for 3 minutes.

Statistical analysis

All data were expressed as mean \pm standard deviation (SD), and all statistical analyses were performed using the statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL). The statistical significance before and after treatment was tested using paired Student's *t* test, and analysis of variance (ANOVA) with Greenhouse-Geisser correction was used to compare between groups. A *p* < 0.05 was considered statistically significant.

Our experiments were performed with the understanding and consent of each subject, with the approval of China ethics committee.

Results

Changes of prostate volume

The mean volumes of the dog prostate in the 4 groups (control, G1, G2 and G3) were 17.16 ± 2.07 ; 17.93 ± 2.76 ; 16.76 ± 2.93 ; and 22.62 ± 8.89 before treatment, respectively; after treatment the volumes were 17.26 ± 2.19 ; 17.52 ± 2.54 ; 16.83 ± 3.00 ; and 22.38 ± 8.93 after treatment, respectively. No significant difference was observed in the prostate volume before and after treatment in all 4 groups (all *p* > 0.05) (Table 1).

Changes of PSA, iNOS and SOD levels

We tallied the PSA, iNOS and SOD levels in the blood of dogs from the 4 groups before and after treatment (Table 2). No significant differences in the PSA, iNOS and SOD levels were observed in the control and G1 groups before and after treatment (all *p* > 0.05), while the PSA level was significantly reduced, iNOS and SOD levels were significantly increased in the G2 and G3 groups after treatment compared with pre-treatment (all *p* < 0.05). Apart from the lack of statistical difference in blood PSA, iNOS, and SOD level after treatment between the control and G1 groups

Table 1. Prostate volume of dogs before and after treatment in the four groups

Group	Prostate volume pre-treatment (mean \pm SD, mm ³)	Prostate volume post-treatment (mean \pm SD, mm ³)	<i>p</i> value
Control	22.62 ± 8.89	22.38 ± 8.93	0.488
G1	17.93 ± 2.76	17.52 ± 2.54	0.115
G2	16.76 ± 2.93	16.83 ± 3.00	0.817
G3	22.62 ± 8.89	22.38 ± 8.93	0.245

SD: standard deviation; G: group.

(*p* > 0.05), there were significant differences between the G2 group and the control group, G3 group and the control groups, the G2 and the G1 group, the G3 and the G1 group (both *p* < 0.05); a statistically significant difference was also observed between the G2 and G3 groups (*p* < 0.05).

Histopathological analysis

Under a transmission electron microscope, the prostate cells from the dogs in the G3 group exhibited nuclear chromatin contraction and margination, nuclear shrinkage (Fig. 1, part A), mitochondrial swelling, degranulation of rough endoplasmic reticulum (Fig. 1, part B), basement membrane rupture (Fig. 1, parts C and D) and vacuolization. All of these changes were observed in the G2 group, but the degree was minor than in the changes in the G3 group.

Under an optical microscope, we observed infiltration of a large number of eosinophil granulocytes (Fig. 2, part A), tissue congestion, prostatic vesicle collapse (Fig. 2, part B), microthrombus formation and presence apoptotic body in the G2 and G3 groups, and more apoptotic bodies were found in the G3 group than in the G2 group. However, none of these changes were shown in the control and G1 groups.

Apoptosis of prostate cells

The TUNEL assay was performed to detect apoptosis of prostate cells. TUNEL positive cells were detected in the G2 and G3 groups, and more apoptosis was observed in the G3 group (Fig. 3, part D) than in the G2 group (Fig. 3, part C), while no positive cells were detected in the G1 (Fig. 3, part B) and control groups (Fig. 3, part A).

Table 2. Blood PSA, iNOS and SOD levels before and after treatment in the 4 groups (mean \pm SD, ng/L)

Group	PSA level pre-treatment	PSA level post-treatment	iNOS level pre-treatment	iNOS level post-treatment	SOD level pre-treatment	SOD level post-treatment
Control	1486.74 ± 47.99	1497.05 ± 57.23	38.71 ± 0.87	37.64 ± 2.12	139.83 ± 6.12	140.63 ± 3.97
G1	1492.30 ± 53.34	1509.35 ± 54.95	38.89 ± 0.69	39.04 ± 0.27	140.20 ± 3.50	139.95 ± 1.16
G2	1697.04 ± 148.36	875.83 ± 30.75	40.37 ± 1.75	50.21 ± 1.73	164.51 ± 2.70	201.48 ± 5.39
G3	1699.39 ± 129.71	708.49 ± 20.62	37.26 ± 4.08	56.91 ± 3.21	164.87 ± 648	220.93 ± 10.55

SD: standard deviation; G: group.

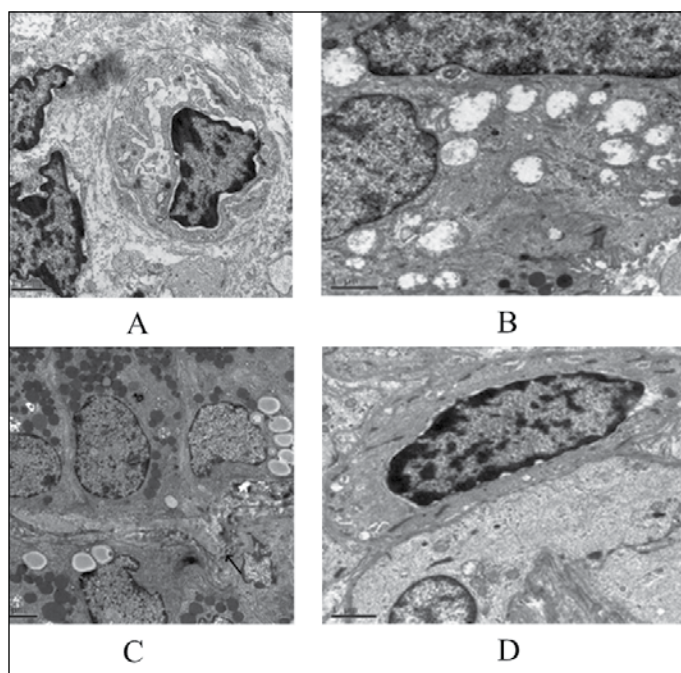


Fig. 1. Transmission electron microscope analysis of the prostate cells. A: The prostate cells from the dogs in the G3 group exhibit nuclear shrinkage, nuclear chromatin contraction near the nuclear membrane and margination (arrow, $\times 10000$). B: Mitochondrial swelling and degranulation of rough endoplasmic reticulum in the prostate cells from the dogs in the G3 group (arrow, $\times 10000$). C: Basement membrane rupture in the prostate of the dogs in the G3 group (arrow, $\times 5000$). D: Normal basement membrane ($\times 10000$).

Discussion

Several studies have investigated the targeted treatment using low-frequency ultrasound.¹⁷ Microbubbles are cavitated by ultrasound, leading to permeabilization of prostatic capillary or small vessels followed by inducing a serial of pathological alterations such as thrombosis, micro-circulation blockage, prostatic necrosis and atrophy. Theoretically, microbubble-mediated ultrasound cavitation may be effective to treat BPH.²⁰ In the present study, an in vivo experiment was designed to further confirm our hypothesis. As expected, low-frequency ultrasound in the presence of microbubbles significantly increased the pathological damages of prostate tissues, such as nuclear chromatin condensation, swelling of mitochondria, rough endoplasmic reticulum degranulation, basement membrane rupture and cell apoptosis, compared with other groups.

Although the pathogenesis of BPH is complex, it is reported that both tissue damage and oxidative stress may lead to compensatory cellular proliferation with resulting hyperplastic growth.²¹ The cause of enhanced oxidative stress could be overproduction of free radicals or decrease in the activities of free radical scavenging enzymes like SOD.²² Peroxidation-antioxidant balance may be used to treat BPH to alter the overproduction of free radicals. In the present

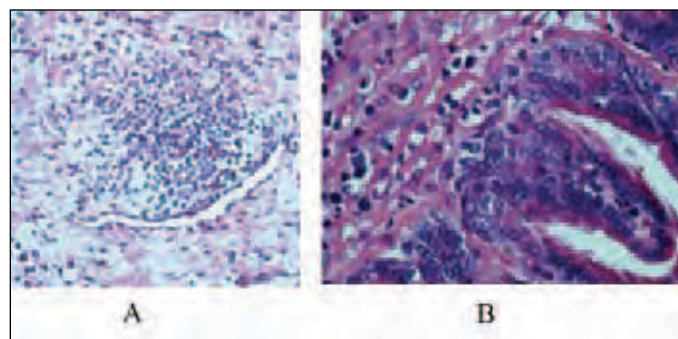


Fig. 2. Hematoxylin and eosin staining of the prostate cells. A: Infiltration of a large number of eosinophil granulocytes in the prostate of the dogs in the G3 group (arrow, $\times 200$). B: Increased prostatic vesicle nuclei and prostatic vesicle collapse in the G3 group (arrow, $\times 400$).

study, the SOD significantly increased in the G2 and G3 groups after treatment; this was especially obvious in the G3 group. No significant difference was observed in the G1 and control groups compared with pre-treatment. This suggests that treatment with low-frequency ultrasound and microbubbles significantly improves BPH.

Recently, increasing evidence indicates that nitric oxide is involved in modulating the prostatic smooth muscle relaxation; it is also involved in the control of the urethral outlet activity and in the nonadrenergic, non-cholinergic (NANC)-mediated cascades that control lower urinary tract storage and emptying.²³ This suggests that an increase in the nitric oxide activity and/or its availability may alleviate BPH.²⁴⁻²⁶ Both macrophages and neutrophils are the source

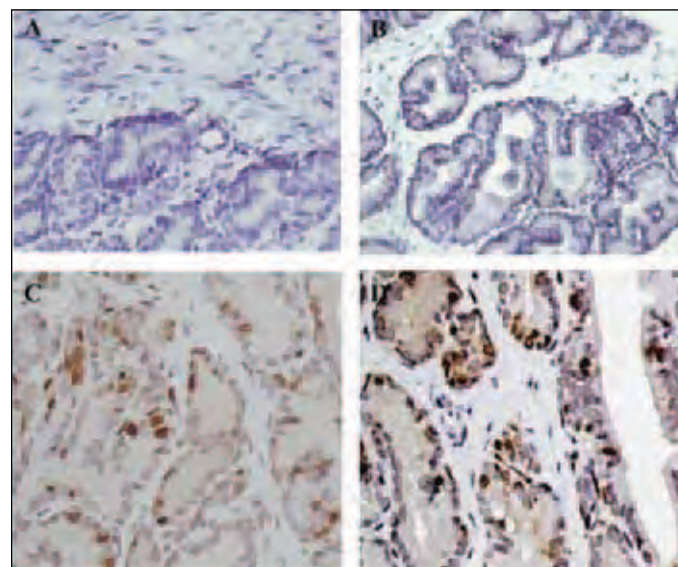


Fig. 3. Terminal uridine deoxynucleotidyl transferase nick end labelling (TUNEL) assay was performed to detect apoptosis of prostate cells. A: Control group, ($\times 400$); B: Sonovue group (G1, $\times 400$); C: 21kHz group (G2, $\times 400$); D: 21kHz+sonovue (G3, $\times 400$). TUNEL positive cells were detected in the G2 and G3 groups (arrow), and more apoptosis was observed in the G3 group than that in the G2 group (arrow), while no positive cells were detected in the G1 and control groups.

of inducible nitric oxide synthase (iNOS) which can catalyze nitric oxide generation in a reaction where the amino acid L-arginine is converted into L-citrulline.²⁷ Thus, iNOS is shown to be up-regulated after BPH treatment. As expected, our results also showed that iNOS was also significantly increased after treatment in the G2 and G3 groups. In addition, it is also suggested that high expression of iNOS in BPH may promote nitric oxide release, exert cytotoxic and cytostatic effects on BPH cells, and induce cells apoptosis, necrosis or DNA injury.²⁸ This was also in line with our results of the TUNEL assay.

Furthermore, prostate volume predicts treatment outcome of BPH, which was assessed by either transrectal ultrasound or magnetic resonance imaging and baseline serum PSA.^{29,30} A recent study also demonstrated that serum PSA is a stronger predictor of growth of the prostate in placebo-treated patients than baseline prostate volume.³¹ As expected, our results also demonstrated that there were no significant differences in prostate volume between pre-treatment and post-treatment in all groups by using imaging. However, the PSA was shown to be significantly reduced in the G2 and G3 groups; it was especially obvious in the G3 group. This result concurs with previous studies.³²

Conclusion

Low-frequency ultrasound is effective in treating BPH; combined low-frequency ultrasound and microbubbles further improves the treatment efficacy by increasing iNOS and SOD, but decreasing PSA. However, there are still some limitations in this study. We applied low-frequency ultrasound (20 kHz) to explore its treatment effect for BPH. However, whether low frequency ultrasound (1 MHz) is also effective or whether the therapeutic effect of 20 kHz ultrasound is superior to 1 MHz remains unclear. In addition, how the apoptosis factors (such as caspase-3, Bcl-2 and survivin) change remains under-investigated. Further studies are still needed.

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Competing interests: Dr. Yang Shao-ling, Dr. Tang Ke-qiang, Dr. Bai Wen-kun, Dr. Shen, Dr. Zhao Yi-wen, Dr. Lin Yan-duan, Dr. Nan Shu-liang, and Dr. Hu Bing all declare no competing financial or personal interests.

This paper has been peer-reviewed.

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