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Anatomical and clinical study on effects of sonography with pulse inversion and microbubble contrast in rabbit kidney

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SUMMARY

Aim of the present study was to evaluate by transmission electron microscope (TEM) modifications in rabbit kidney-parenchyma after submission to ultrasound contrast agent (UCA) with Pulse Inversion Harmonic Imaging (PIHI).

Seven inbred male albino rabbits were divided into three groups: 1) control group (n= 1 animal); 2) sonicated group (n= 3 animals); 3) sonicated group with UCA injection (CEUS) (n= 3 animals). The first group was not exposed to ultrasonography (US) and/or UCA. The second and third groups underwent baseline US and later to US with PIHI with a high mechanical index; in the third group UCA was simultaneously administered. Ultrastructural studies and image analysis were blindly performed on 50 samples ($2mm^3$), including cortex and medulla, by two experienced pathologists with TEM.

By TEM observations of the first and second groups showed no structural modifications of renal cortex and medulla. TEM observations of the third group showed ultrastructural changes of renal corpuscle, proximal and distal convoluted tubules and collecting tubules; further in the most of observed sections the filtration membrane had an alteration of typical trilaminar pattern and vacuolisation of glomerular endothelial cells with irregular edges. Therefore in rabbit kidney submitted to CEUS some ultrastructural modifications were observed.

INTRODUCTION

It is known that the human parenchymas, in the diagnostic imaging, are submitted to ultrasonic investigations to evidence their alterations.

To verify if after the ultrasound exposure the parenchymas show ultrastructural modifications some animal parenchymas (rabbit, rats) were submitted to sonication with /without contrast agent.

Recent introduction of contrast agents permits to improve the quality of images and allows dynamic evaluation of vascular patterns of normal organs and their lesions (Leen et al., 2004).

Gas microbubbles determine decrease of the threshold for cavitational events caused by ultrasound (Holland et al., 1990). At high acoustic pressures, the microbubbles oscillation may become violent giving to their rupture, production of shock waves, microjets and microstreaming, and free radical formation (Leen et al., 2004). Multiple experiments in laboratory animals have showed that the combination of microbubble contrast agent and Ultrasound exposure can cause some bioeffects, such as rupture of the vessels in which the microbubbles are located (Skyba et al., 1998), myocardial damage (Chen et al., 2002; Ay T et al., 2001).

Our previous studies on the rabbit liver after exposure to ultrasound with UCA showed ultrastructural modifications in the hepatocytes and in the sinusoidal endothelium (Caruso et al., 2005). Biological effects after ultrasound exposure (Fowlkes et al., 2000) may be caused by thermal mechanisms and nonthermal mechanisms with cavitational and non cavitational effects.

On the basis of studies carried out in different organs (Wible et al., 2002; Skyba et a., 1998 Caruso et al., 2005), we supposed that microbubble contrast agents may be associated with ultrastructural damage in kidney parenchyma.

The purpose of our study was is to evaluate with transmission electron microscope (TEM) ultrastructural effects of ultrasound contrast agent (UCA) with Pulse Inversion Harmonic Imaging (PIHI) in the rabbit kidney parenchyma.

MATERIALS AND METHODS

This prospective experimental study was performed according to a protocol approved by our institutional animal care and use committee and in accordance with the guidelines issued by the National Research Council (1996). Seven inbred 8-month-old male albino rabbits (weight 1.500 – 1.700 g) were housed in a pathogenfree, temperature-controlled wire cages with free access to food and water. The rabbits were divided into three groups: 1) control group (one animal); 2) sonicated group (three animals); 3) sonicated group with UCA injection (CEUS) (three animals).

In all study groups a 24-gauge cannula, filled with heparinized saline (20 U/mL), was inserted into a vein of right auricle ear, used for anaesthetic administration and later for UCA injection. Other two cannula were inserted into left femoral artery, used for glutaraldehyde perfusion, and another into right femoral vein for the exanguination. A phial of 0.25 mg of atropine and 60 mg of barbiturate was administered i.v. for anaesthesia. Following induction of anaesthesia, animal's skin was shaved.

Sonographic Examination Technique

Sonography was performed using a HDI 5000 ultrasound scanner (Philips-ATL, Bothell, WA) equipped with PIHI software and a C5-2 multifrequency con-

vex-array probe (2-4 MHz). The transducer was coupled to the abdominal wall using a clinical scanning gel without the use of any standoff pad. We used a scan depth of 6.7 cm and a single focus of 2.5 cm. Right kidney was scanned on longitudinal axis and scanned field has about 5 mm width. All examinations were performed by the same radiologist.

The first group was no sonicated nor exposed to UCA; second and third study groups underwent fundamental grey-scale ultrasonography with optimized scanner settings for about 30 seconds, providing a baseline for identifying the right kidney on longitudinal scan plane.

Sonicated group. After fundamental gray-scale ultrasonography, performed to identify the longitudinal axis of right kidney, PIHI with a high mechanical index (MI 1.2) with a frame rate of 25 Hz was activated and administered in the same scan plane for 30 seconds.

Sonicated group with UCA injection or CEUS group. As for sonicated group, fundamental gray-scale ultrasonography was performed to identify the longitudinal axis of right kidney, than UCA injection was performed and contemporary PIHI with persistence switched off, maximal acoustic power (MI 1.2) and a single focal zone, was administered. CEUS was started 5 seconds after injection and 4 time-triggered images were recorded at a rate of 1 frame every 2 seconds, so whole examination lasted about 8 seconds and yielded a total of 4 frames.

Contrast agent used in the study was a suspension of galactose microparticles (99.9%) and palmitic acid (0.1%) in sterile water (Levovist, Schering, Berlin, Germany). Before CEUS examination, the contrast agent was prepared by shaking it vigorously with 11 ml of sterile water for 5–10 seconds. When mixed with saline, this suspension produces microbubbles of air covered by a thin stabilizing layer of palmitic acid with an average diameter of 2–8 μm, which can pass the pulmonary capillary bed. After leaving the contrast agent to stand for 2 minutes for equilibration, a bolus of 0.1 ml of Levovist (300 mg/ml) was manually injected. The injected volume of 0.1 ml if approximately equivalent to the manufacturer's recommended range for adult human use. After UCA administration no saline flush was injected. Heart rate and blood pressure were not monitored in this study.

For the investigations to TEM all animals, control, sonicated and sonicated with UCA injection, were sacrified by glutaraldehyde (1%) perfusion.

For ultrastructural study the right kidney of all groups was removed and sampled on its poles, because considering probe width of 5mm they were surely exposed to ultrasound. All samples included kidney cortex and medulla. Ultrastructural studies and image analysis were performed on 50 samples ($2mm^3$) fixed in glutaraldehyde (1%) in Millonig buffer, pH 7.2, for 24 hours and postfixed in osmium tetroxide (1%) in Millonig buffer, pH 7.2, for 2 hours. Fragments were then dehydrated in an ascending series of alcohols and embedded in Epon 812. Ultrathin sections were obtained using a Reichert ultramicrotome and examined using a JEM-1220 transmission electron microscope (TEM) (JEOL, Peabody, MA).

RESULTS

Control Group

The observations to TEM of rabbit kidney of this group showed normal ultrastructural organization of both cortex and medulla (Figs 1a,1b).

Sonicated Group

The observations to TEM of rabbit kidney exposed to fundamental gray-scale ultrasonography and later to PIHI without UCA administration showed in cortex: glomerular capillary endothelial cells and podocytes with their typical ultrastructural morphology; filtration diaphragms between adjacent foot processes (pedicels) well recognisable; filtration membrane with typical trilaminar organisation (Fig. 2a); epithelial cells of proximal (Fig. 2b) and distal (Fig. 2c) convoluted tubules; in medulla the collector tubules (Fig. 2d) without ultrastructural alterations.

CEUS group

In rabbit kidneys exposed to fundamental gray-scale ultrasonography and later to PIHI with UCA administration, were observed ultrastructural changes of cortex and medulla.

In all cortical sections observed, the filtration membrane in many tracts had an alteration of typical trilaminar pattern, vacuolisation of glomerular endothelial cells with irregular edges (Fig. 3a), fragmented podocyte foot processes and filtration diaphragm absence (Fig. 3b).

Ultrastructural changes of proximal and distal convoluted tubules were characterized by mitochondrial cristae degeneration (Fig. 3c) and organules-absence and disorganized cytoskeleton (Fig. 3d).

Fig. 1 — Transmission electron microscopy scan of rabbit kidney in control group. a) kidney cortex (6000x): normal podocytes and pedicles of visceral layer of Bowman's capsule (arrow); mesangium (*). b) kidney medulla: normal appearance of basal pole of distal tubule of the nephron; mitochondria (arrows) (5000x).

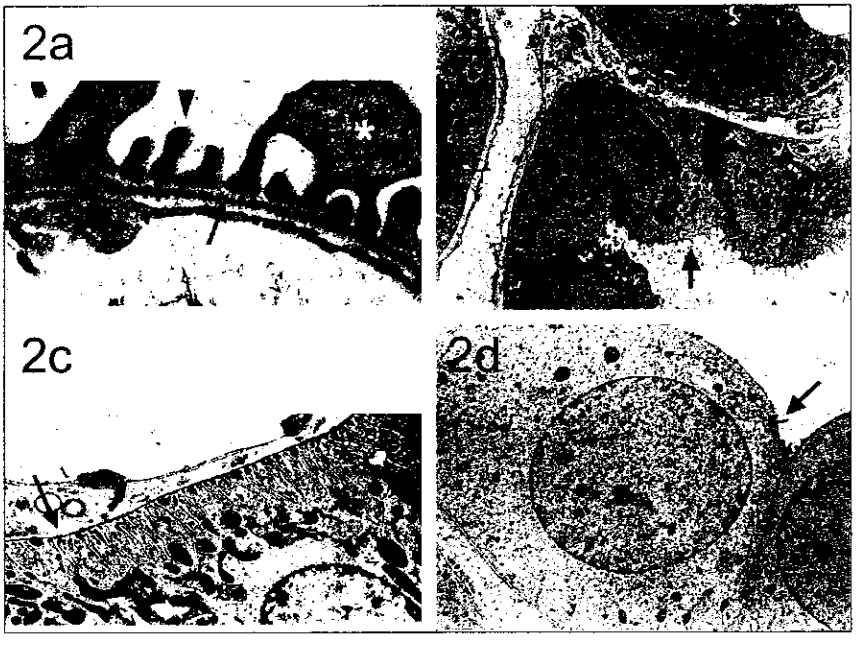

Fig. 2 — Transmission electron microscopy scan of rabbit kidney in sonicated group after ultrasound exposure with pulse inversion harmonic imaging (PIHI). a) kidney cortex: basal glomerular membrane (black arrows), endothelial cell (white arrow), podocytes (asterisk) with its foot processes (arrowhead) are normal (6000x). b) kidney cortex: normal epithelial cells of the distal convoluted tubule (arrow) (2500x). c) kidney cortex: normal epithelial cells of the proximal convoluted tubule (arrow) (6000x). d) kidney medulla: normal epithelial cell of the collecting tubule (arrow) (5000x).

In the renal medulla ultrastructural changes of collecting tubules were characterized by epithelial cells with few organules and electrondense cytoskeletal filaments and liquefied cytoplasm (Fig. 3e).

DISCUSSION

Biological effects after ultrasound exposure were previously described (Fowlkes et al., 2000). These may be caused by thermal and non thermal mechanism; there are two general classes of non thermal mechanism, cavitational and non cavitational, that may arise during exposure of the parenchymas to ultrasounds (Ay et al., 2001). Gas microbubbles determine decrease of the threshold for cavitational events caused by ultrasound exposure (Holland and Apfel, 1990). The mechanism by which a bubble may affect on the neighbour tissues is dependent on the magni-

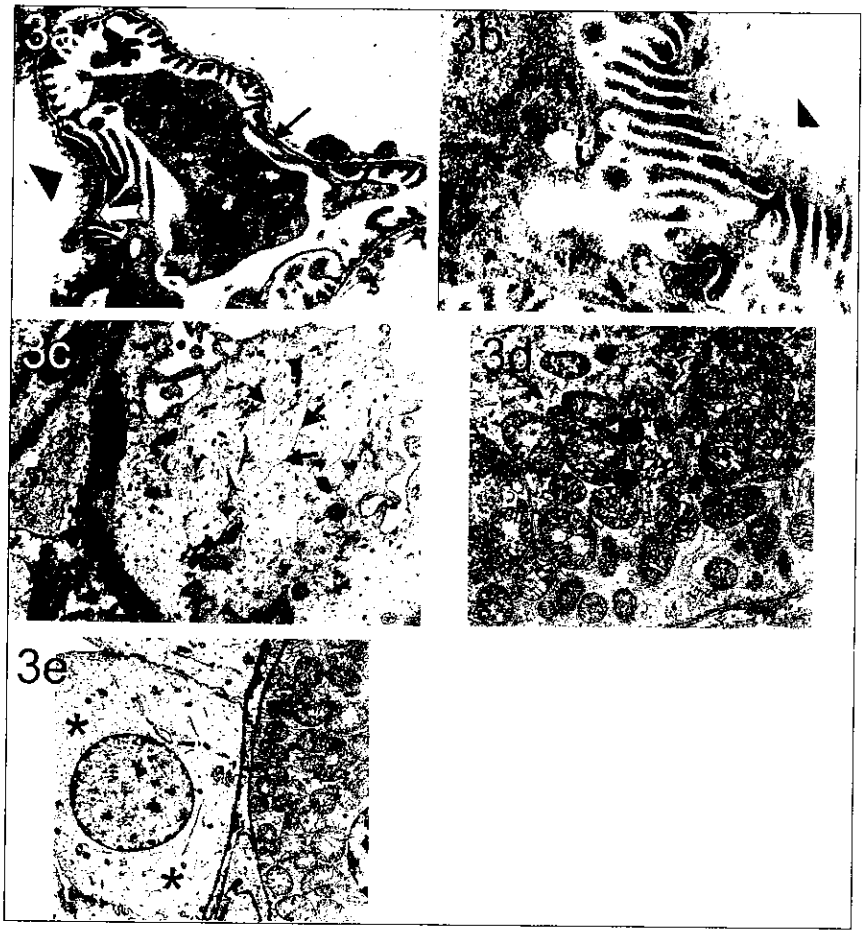

Fig 3 — Transmission electron microscopy scan of rabbit kidney in sonicated group after ultrasound exposure with pulse inversion harmonic imaging (PIHI) and ultrasound contrast agent (UCA). a) kidney cortex: preservation (thin arrow) and alteration of trilaminar pattern of vassal glomerular membrane (thick arrow), and vacuolisation of glomerular endothelial cells (arrowhead) area shown (8000x). b) kidney cortex: vacuolisation of glomerular endothelial cells with irregular edges (arrowhead) and filtration diaphragm absence (arrow) are shown (10000x). c) kidney cortex: mitochondrial cristae degeneration of distal convoluted tubules (arrow) are shown (6000x). d) kidney medulla: distal convoluted tubule cells show organules absence and disorganized cytoskeleton (arrows) that appears as assembled filaments (1000x) e) kidney medulla: collecting tubule cells show liquefied cytoplasm (asterisks) (4000x).

tude of its response to the acoustic field. Ultrasounds, interacting with microbubbles, cause the bubble diameter oscillation, with alternate contraction and expansion. Essentially, all bubbles produce acoustic radiation forces and microstreaming, while only the more strongly affected will exhibit the violent responses, such as shock wave generation, free radical production and microjets, characteristic of in-

ertial cavitation (Ay et al., 2001). Non cavitational mechanisms act in the form of radiation force, torque and acoustic streaming (Ay et al., 2001).

To date this study is the first on rabbit kidney showing the ultrastructural effects of CEUS with PIHI by TEM since no modifications have been evidenced on rabbit kidney surface. We observed in kidney simultaneously exposed to microbubbles and PIHI some ultrastructural changes as fragmented podocyte foot processes with filtration diaphragm absence and an alteration of typical trilaminar organisation of the filtration membrane. In only sonicated group, while, not exposed to microbubble contrast agent injection, normal ultrastructural appearance of renal parenchyma without any pathological changes was observed.

These findings suggest that CEUS with PIHI induces some ultrastructural alterations and we speculate that these alterations are induced by microbubble rupture with immediate release of mechanical and thermal energy. Ay et al. (2001) showed that isolated rabbit hearts exposed to ultrasound and UCA showed capillary rupture and left-ventricular dysfunction, while Chen et al. (2002) reported only mild troponin T elevations that were not associated with kinetic alterations or histopathological evidence of myocardial damage. In this study we reported some ultrastructural effects on rabbit kidney but we not observed any evidence that they are functional and permanent effects.

It is undefined if microbubbles exposure could cause some damages in human kidney and although there are interactions between microbubbles and human tissues, we cannot surely affirm that the exposure to microbubbles could produce some bioeffects on subcellular organules of human kidney. In clinical practice microbubbles are used with low mechanical index; instead in our speculative experience we used microbubbles with high mechanical index. Than considering adverse bioeffects that could be improved by high mechanical index, we think that this knowledge could be useful for the operators addressing avoid or to reduce as far as possible the source used for microbubbles breaking for refilling evaluation.

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