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Ultrasound Backscattering from Erythrocyte Aggregation of Human, Horse and Rat Blood under Rotational Flow in a Cylindrical Chamber

Kweon-Ho Nam§, Dong-Guk Paeng§,*, Min Joo Choi§,**
§Interdisciplinary Postgraduate Program in Biomedical Engineering, Cheju National University
*Marine Industrial Engineering, Cheju National University
**Department of Biomedical Engineering, College of Medicine, Cheju National University
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Abstract

Human, horse and rat bloods in a cylindrical chamber where flow was controlled by a stirring magnet were used for studying red blood cell aggregation. Ultrasound backscattered powers from blood were obtained from the backscattered signals measured by a 5 MHz focused transducer in a pulse-echo setup. The experimental results showed the differences in red blood cell (RBC) aggregation tendency among the three mammalian species with an order of horse > human > rat. The ultrasound backscattered power decreased with stirring speed in human and horse blood, but no variations were observed in rat blood. Sudden flow stoppage led to the slow increase of the backscattered power for human and horse blood. There was no self-aggregation tendency in rat blood. The enveloped echo images showed the spatial and temporal variations of RBC aggregations in the cylindrical chamber. These observations from the different mammalian species may give a better understanding of the mechanism of RBC aggregation.

Keywords: *Ultrasound, Backscatter, Red blood cell, Aggregation, Human, Horse, Rat.*

I. Introduction

Red blood cell (RBC) aggregation and disaggregation are dynamic and reversible process in flowing blood. RBCs aggregate to form a stack of coins referred to as a rouleau, which is mediated by the macromolecules including fibrinogen in plasma. It was known that excessive RBC aggregation can decrease tissue perfusion, resulting in ischemic cardiovascular diseases, and can disturb flow by the increased blood viscosity, particularly in microcirculatory flow [1]. In various diseases and physiological states, such as diabetes [2], hypertension [3], hypercholesterolemia [4], sepsis [5], inflammation [6], myocardial infarction [7], thrombosis [8], obesity [9] and malignancies [10], greater RBC aggregation has been observed. It has thus been assumed that the increased RBC aggregation may play an important role in those diseases. However, despite a lot of the previous investigations, a complete understanding of its physiological and pathological mechanism of the RBC aggregation has not yet been fully established.

Although many studies have reported the RBC aggregation characteristics both in normal and in pathological human blood, relatively little information is available on other mammalian species. In spite of being similar in basic structure and in physiological functions in all mammals, RBC aggregabilities differ extensively among

Corresponding author: Dong-Guk Paeng (paeng@cheju.ac.kr)
66 Jejudaehakno, Jeju-si, Jeju special self-governing province, Korea 690-756

various species [11-14]. The physiological significance of these variations among mammalian species has not yet been established. Because the hematological factors such as plasma proteins, cell shape, deformability, and surface electrical charge differ among mammalian species, the comparative studies of RBC aggregation with hematology from the different mammals can give us useful information regarding the mechanisms of rouleaux formation and its physiological roles. Furthermore, these researches can be applicable to studying human hematological diseases regarding RBC aggregation. Therefore, the accurate measurement system for RBC aggregation is of critical importance.

Several methods such as a microscopic examination including image analysis in a flow chamber [15], erythrocytes sedimentation rate (ESR) [16], viscometry [17], photometry [12-14] and ultrasound backscattering [18], have been used to quantify the degree of RBC aggregation. Measuring the intensity of the transmitted and reflected light under the defined shearing conditions has been widely used to assess the different aspects of RBC aggregation. The optical methods provide aggregation indices (aggregation time and aggregation index at 10 seconds after flow stoppage), and shear rate values necessary to break rouleaux (dissociation threshold). However, there are some limitations in its applications because of the light opacity in blood [18]. The gap between the two coaxial cylinders that contain the blood sample should be within a few millimeter to obtain an efficient light transmission. The narrow gap, however, does not provide the spatial information of RBC aggregation and is not freely applicable to various blood flow systems in laboratory as well as in vivo applications. ESR cannot apply to flowing or shearing blood but only to static blood, and it is also influenced by the plasma viscosity and several other factors. Viscometry is an indirect method so that it does not always agree with aggregation tendency. Microscopic methods are not quantitative in nature.

It is well known that ultrasound backscattering of blood is caused by the presence of RBCs. Because of their small size (approximately 6~8 microns) compared to the ultrasound wavelength for up to 15 MHz, the scattering process is primarily Rayleigh scattering in nature. Sigel et al. [19] first showed that the ultrasonic echogenicity was dependent on shear rate in flowing blood. They suggested that RBC aggregation was a major cause of the increased echogenicity from blood. Since then, many studies on ultrasonic scattering from blood have been carried out and confirmed the observation [20-22]. Due to its ability to penetrate the tissue including blood compared to opacity of photometric methods, ultrasound appears to be a promising non-invasive tool for hemodynamic measurements including RBC aggregation.

The interest of the present study is to propose an ultrasound method using a cylindrical chamber that is simple and requires smaller volume of blood samples than the conventional loop flow system. Shear rate to affect RBC rouleaux was varied in a cylindrical chamber where flow was controlled by a stirring magnet. Using A-mode ultrasound from rotational blood flow in a cylindrical chamber, the comparative tendency of RBC aggregabilities among human, horse and rat blood was investigated. These three mammalian species were chosen based on the distinct differences in their RBC aggregation characteristics. Horse RBCs have a strong aggregation tendency but rat RBCs very weak. Human blood is intermediate between these two extremes [13].

The measured ultrasound backscattering power showed the differences of RBC aggregability among the three mammals. This ultrasound setup using a cylindrical chamber was suggested as an alternative tool that may elucidate physiological and pathological roles of RBC aggregation, especially for small animals such as rat and guinea pig with artificial enhancement of RBC aggregation.

II. Materials and Methods

2.1. Blood collection

Horse blood was withdrawn by jugular vein puncture from 2-year-old males of *Equus Caballus*. Rat blood was obtained from 9~12-week-old Sprague-Dawley rats (Charles River, Korea) by abdominal aorta puncture after laparotomy under intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. Human blood samples were obtained from the healthy male volunteers with their age range of 26 to 39 years by venipuncture. All blood samples were anticoagulated with EDTA dipotassium salt

(1.5 mg/ml blood as EDTA). The hematocrit was determined by reading RBC portions of capillary tubes after 5 minutes of centrifuging at 10000 revolution per minute with a microcentrifuge (Microspin, Hanil scientific industrial Co., Korea). The hematocrits in human, horse and rat blood were 45.2 ± 2.3, 38.2 ± 3.1 and 44.0 ± 1.3 %, respectively. All experiments were completed within 4 hours after collecting the blood.

2.2. Mixing chamber

To obtain the backscattered ultrasonic signals from the blood samples both at stasis and under shear forces, a cylindrical chamber with 0.1 mm thick polyester membrane and 2 cm diameter was designed, which contains a small teflon-coated stirring magnet (diameter of 1.8 cm round type with a crucial projection on the top) at the bottom. The magnetic stirrer (PC-420, Corning, USA) was used to spin the magnet. The ultrasound transducer was mounted so as its focal zone to be placed at 0.5 cm above the top of the magnetic stirrer.

2.3. Experimental system

The experimental setup is shown in Fig. 1. A focused transducer (V326, Panametrics, Waltham, MA, USA) with a center frequency of 5 MHz and 9.5 mm aperture diameter was used in the measurements. The beam profile of the transducer was measured by a planar scanning system (NTR, USA) containing a PVDF needle hydrophone (TNU001A, NTR) with a 30 dB preamplifier (HPA30, NTR) and an electric motor drive system. The relative acoustic intensity map in Fig. 2 shows −6 dB beam width of 2.5 mm at 60.5 mm focal point. The cylindrical blood container was positioned at 6.5 cm in front of the transducer. Both the ultrasound transducer and the blood container were immersed in distilled and degassed water.

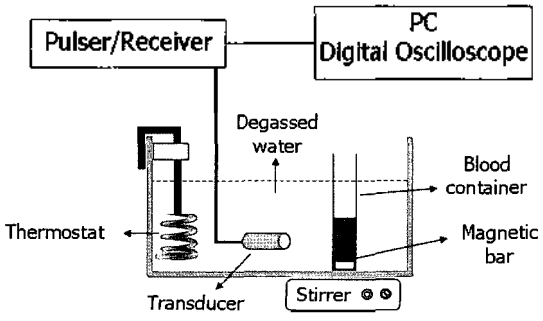

Fig. 1. Experimental block diagram for ultrasound measurement.

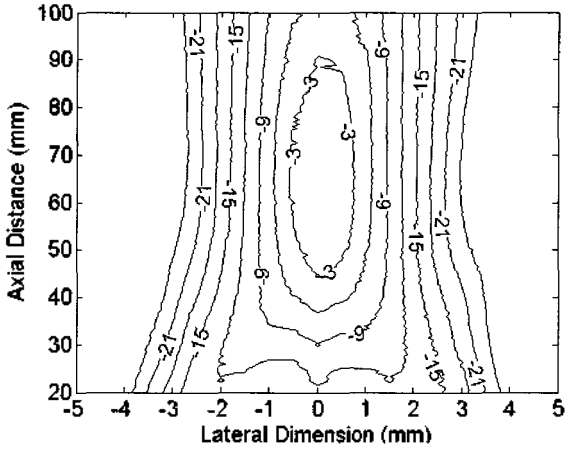

Fig. 2. A contour plot of the relative acoustic intensity for the transducer with 5 MHz center frequency and 9.5 mm aperture diameter. The numbers are in dB.

The temperature was constantly controlled to be 37℃ using thermostat (EH4, IKA, Germany). A pulser/receiver (5800, Panametrics, Waltham, MA, USA) was used to drive the transducer for transmitting and receiving ultrasonic signal. A National Instruments 100 MHz digitizer (NI PCI-5122, Austin, TX, USA) with the LABVIEW® system was used to collect the backscattered signal.

2.4. Measurement protocol and data analysis

To investigate RBC aggregation tendency, 9 ml of fresh whole blood was placed into the cylindrical chamber. The blood was stirred in the container for at least 15 minutes before making any measurements to remove the bubbles inside the chamber and to allow the blood to reach 37 ℃ temperature. Stirring speed was altered in six steps of revolutions per second (rps) from high to low (4, 3, 2,

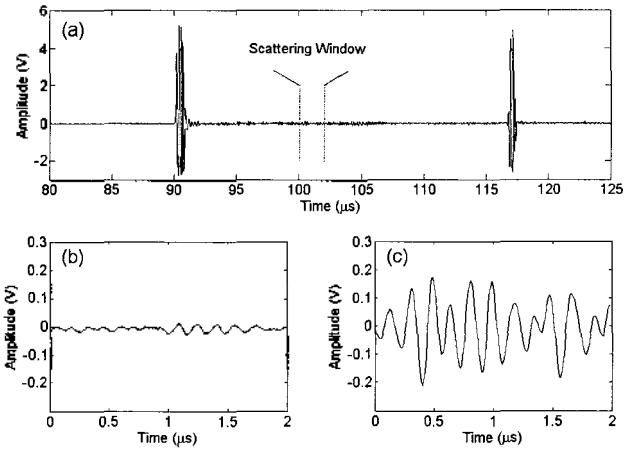

Fig. 3. Illustration of time characteristics of the backscattered signal (a), zoom images of the selected scattering window at stirring speed of 4 rps (b) and stasis (c) in horse whole blood.

1.5, 1 and 0 rps). Blood samples were sheared for 30 seconds at high speed of 4 rps for the purpose of total dissociation of RBC aggregates. Then the flow was suddenly reduced or stopped to each stirring speed of 4, 3, 2, 1.5, 1 and 0 rps. The backscattered signal was digitized with a sampling frequency of 100 MHz. For 5 minutes, a total of 600 recordings of time domain ultrasound signals backscattered from the blood sample were collected. Each signal in the scattering window was consisted of 200 data points which corresponded to the 1.4 mm width of the blood sample for calculating backscattered power (Fig. 3. (a)). The backscattered power was computed in the time domain by summing the squared values of the signal over the time window:

$$\text{Backscattered Power} = 20 \times \log\left(\frac{V_{rms2}}{V_{rms1}}\right), \quad V_{rms} = \sqrt{\frac{1}{N}\sum_{i=1}^{N} V_i^2}$$

where N is the data points in scattering window and V is voltage. V_{rms1} and V_{rms2} represent the root mean square of voltage from the signals in water as a reference and from the blood sample, respectively. To reduce fluctuations in the mean ultrasound backscattered power curves, a 5 point-smoothing filter was used.

The enveloped echo images for monitoring the entire process of spatial and temporal RBC aggregation in the cylindrical tube were obtained from A-lines of the backscattered signals using the Hilbert transform. For a better visualization of these images, the brightness was converted into darkness. All data were processed on a personal computer using MATLAB® software. Measurements were performed on the blood samples from six different humans, horses and rats and the results were expressed as mean ± standard error (SE).

III. Results

Fig. 3 (b) and (c) show the signals in the scattering window in Fig. 3 (a) from horse whole blood at 5 minutes after onset of the stirring with the rates of 4 rps and 1 rps, respectively. A remarkable increase in amplitude of the backscattered signal was seen at low shear rate of 1 rps condition. Fig. 4 shows the typical echo images that represent the spatial and temporal variations of RBC aggregations for horse blood, at six different stirring rates including stasis. All x-axes represent the time for 5 minutes, where the zero time represents the switching point from 4 rps to a reduced stirring rate. The y-axes represent the normalized diameter of the cylindrical chamber. The darkness of each pixel means the amplitudes of the backscattered signal envelope by Hilbert transform. At 3 rps, a dark zone is clearly seen along the beam center. It was expanded toward the tube wall with lowering the stirring rate. This dark zone was started to appear at 10 to 20 seconds after sudden flow reduction and lasted for 5 minutes. The flow velocity in a cylindrical chamber varies from center to the tube wall along the radial direction. The shear force acting on a RBC aggregation at the far point from the center is higher than at the center, so that low shear rate around the tube center may cause higher RBC aggregation. The echogenic contrast between the dark zone and the surrounding region may be explained by the distribution of RBC rouleaux caused by radial variation of shear rate. As seen in this figure, the overall echogenicity increased with lowering stirring rate from 4 to 1 rps. However, this tendency was not applied to the stasis condition of zero rps. The sudden flow stoppage did not induce the remarkable increase of echogenicity.

Fig. 5 presents the backscattered power variations at the different stirring rates for human, horse and rat whole blood as a function of time during the process of RBC aggregation. As seen in horse and human blood, the powers increased rapidly during the first 1 minute and

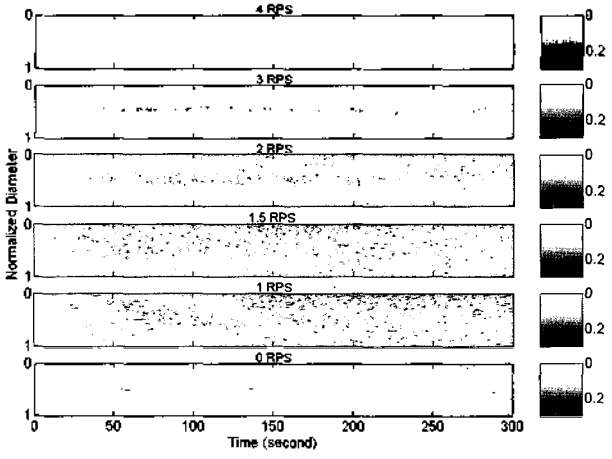

Fig. 4. Typical echo images from horse blood with various stirring rates for 5 minutes. The gray scale corresponds to the enveloped amplitude of backscattered signal.

reached a plateau. The time to reach a plateau was decreased by increasing the stirring rate. For all flow speeds, decreasing the stirring rate resulted in power increases. The backscattered power was markedly greater for horse blood under shearing condition compared with that for human blood. In contrast, rat blood exhibited no remarkable variations of the backscattered power for all stirring rate conditions. At the highest stirring rate of 4 rps, the backscattered powers from human, horse and rat blood were approximately 5, 7 and 1 dB, respectively. It is considered that the power differences at 4 rps may be due to the different hematocrits and RBC sizes, and the incomplete disaggregation of RBC rouleax among three mammals because the shear rate for total dissociation of RBC rouleaux depends on mammalian species. Fig. 6 shows the mean backscattered power for the last 1 minute of Fig. 5 as a function of stirring rate. In this figure, it was clearly indicated that the RBC aggregability depends on the mammalian species among human, horse and rat blood. It also decreased with stirring rate except in rat blood. The shear rate dependency in horse blood was higher than that in human blood.

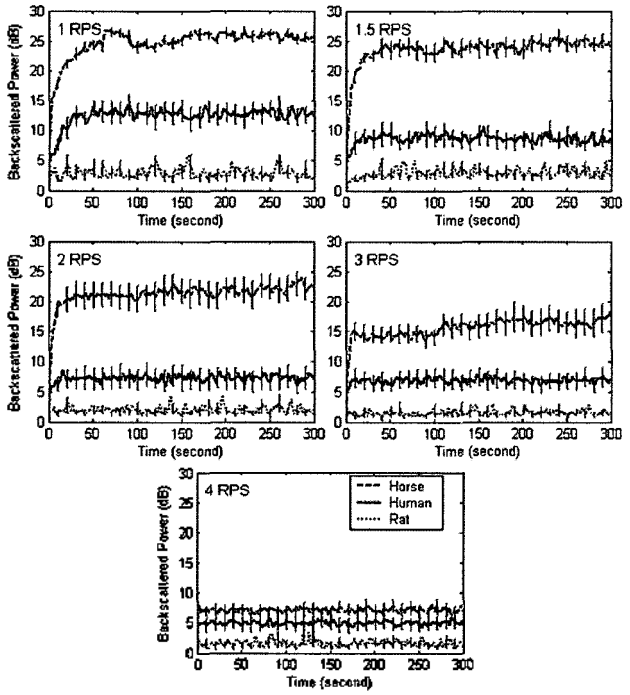

Fig. 5. The variation of ultrasound backscattered power following a sudden flow reduction from 4 rps to 1, 1.5, 2, 3 and 4 rps in human, horse and rat blood. Each plot represents the mean ± SE of six individuals. The SEs are only displayed for selected times in order to enhance the visualization of the graphs.

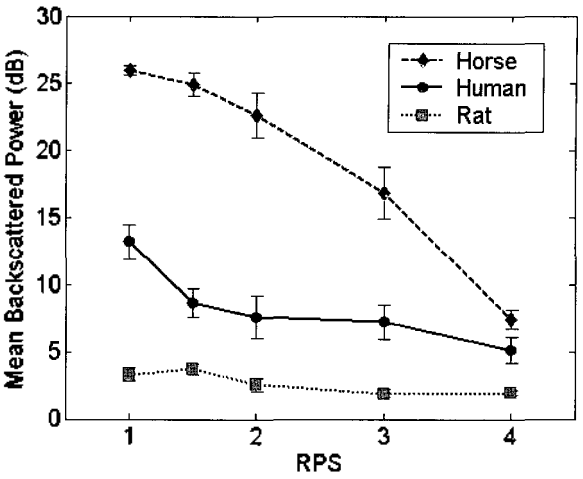

Fig. 6. Mean backscattered power as a function of stirring rate obtained by averaging of last 1 minute data in Fig. 5

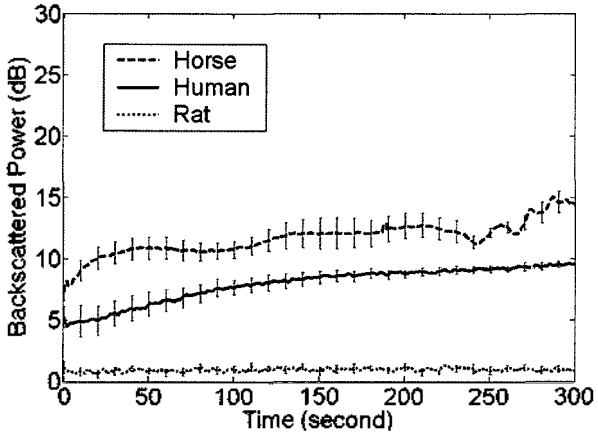

Fig. 7. The variation of ultrasound backscattered power following a sudden flow stoppage from 4 rps in human, horse and rat blood. Each plot represents the mean ± SE of six individuals. The SEs are only displayed for selected times in order to enhance the visualization of the graphs.

The sudden flow stoppage in Fig. 7 did not induce a remarkable increase of the backscattered power in the three mammals. The powers were increased slowly for 5 minutes in human and horse blood, but not in rat blood. The backscattered powers from human and horse blood for zero rps at 5 minutes were smaller than those from human for 1.5 rps and from horse blood for 3 rps, respectively. Therefore, the inverse proportion of aggregation tendency with stirring rate was not applicable to stasis condition.

IV. Discussion

Using the 5 MHz focused transducer under rotational

flow in a cylindrical chamber, the quantitative data of the backscattered power were obtained from human, horse and rat whole blood. The levels of ultrasonic backscattered power represented the differences of RBC aggregation of the mammalian species. The enveloped echo images showed the spatial and temporal variations of rouleaux distribution for whole blood in a cylindrical chamber.

It has been well known that shear rate is the most important hemodynamic parameter that affects RBC aggregation. For that reason, shearing systems are critical to investigate RBC aggregation phenomenon. Many researchers investigated the influence of shear rate on the ultrasonic backscattered power from blood [21-23]. In those studies, the backscatter was examined in a mock flow loop by varying the average flow velocity. The loop flow system is similar to the physiological blood vessels and useful to study the effects of various blood flow on RBC aggregation. However, it needs large volume of blood to fill the flow loop, so has some difficulties to investigate the blood of small animals. Therefore, a cylindrical chamber for smaller blood volume was applied in the present study. Although the rotational flow system in a cylindrical chamber does not exactly represent physiological blood flow, the present experimental results of ultrasound backscattered power and echo images show that it is possible to compare the relative RBC aggregability among the various blood samples at both flowing and static conditions. Moreover, the circular flow pattern can be produced by the atrioventricular blood flow which is similar to the present shearing system. The previous studies using B-mode images from human blood also demonstrated the usefulness of a cylindrical mixing chamber in the measurement of blood echogenicity [24, 25].

The mechanisms of rouleaux formation and its physiological and pathological roles in human blood are not well understood. Although, fibrinogen level in plasma is known to be the major RBC aggregating factor in human blood, other macromolecules in plasma such as α2-macroglobulin, haptoglobulin, ceruloplasmin, C-reactive protein and serum amyloid A are considered as possible factors for influencing RBC aggregation [1, 26]. Especially, the ceruloplasmin concentration in horse blood was ten times higher than that in normal human blood [27]. The addition of ceruloplasmin to human blood also showed the significant increase in RBC aggregation [26]. Andrews et al. [28] reported that the higher RBC aggregation in horse blood is partially ascribed to the higher molecular weight of horse fibrinogen. The cellular factors such as deformability, morphology, cell aging and surface charge can affect RBC aggregation [29]. The previous reports revealed that the three mammalian species have difference in RBC surface charge with the order of rat > human > horse [13]. A higher negative charge on rat RBC membrane would be associated with low aggregation tendency by inducing electrostatic repulsive force. These results support the present experimental measurements of ultrasonic backscattered power from the three mammalian species.

Although our results of RBC aggregabilities among the three mammalian species agree with the previous reports measured by Myrenne aggregometer [12-14], Ohta et al. [11] reported that similar RBC aggregation for rat and human blood was measured by photometry. It is assumed that this discrepancy may be related to the methodological differences in shearing blood and the optical detecting system. Therefore a better measurement system is essential to study RBC aggregation phenomenon.

V. Conclusions

Using ultrasound backscatter measurement, the highest level of RBC aggregation was observed from horse blood followed by human and rat blood both at stasis and under shear forces. The present investigation of RBC aggregability from the various mammalian species can provide insight into the mechanisms of RBC aggregation. This measurement system of A-mode ultrasound in a cylindrical chamber with a stirring magnet is simple and requires relatively small amount of blood. It will also be useful to evaluate rat RBC aggregation in polymer solution which simulates hyper-aggregation in pathological states.

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[Profile]

• **Kweon-Ho Nam**

1990 : College of Pharmacy, Chonnam Natl. Univ. (B.S.)
1992 : College of Pharmacy, Chonnam Natl. Univ. (M.S.; Biopharmaceutics)
1992~1997 : Research Scientist in Daewoong Pharm. Co. R & D Center
1997~1998 : Research Scientist in LG Life Science R & D Park
2004~present : IPPBME, Cheju Natl. Univ. (Ph.D. Candidate; Biomedical Ultrasound)

• **Dong-Guk Paeng**
The Journal of the Acoustical Society of Korea, 24 (4E), 2005

• **Min Joo Choi**
The Journal of the Acoustical Society of Korea, 24 (4E), 2005