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●*Original Contribution*

EFFECTS OF ULTRASOUND ON AGGLUTINATION AND AGGREGATION OF HUMAN ERYTHROCYTES *IN VITRO*

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Abstract—A new experimental approach has shown that human erythrocytes of different blood groups were induced to form more agglutinates at a sound pressure of 70–240 kPa *in vitro* than the control erythrocytes. Similar effects were observed for alcian blue and dextran stimulated aggregation and for spontaneous aggregation. The increase of agglutination or aggregation was reversible. Heating and acoustic cavitation were shown not to be responsible for this effect. Bulk fluid movement produced by ultrasound irradiation appeared to cause the described phenomenon. Possible underlying mechanisms connecting the acoustic streaming and agglutination or aggregation behaviour of the cells are proposed.

Key Words: **Erythrocyte membrane, Bulk fluid movement, Unstirred layer, Blood group antigens, Ultrasound bioeffects, Dextran.**

INTRODUCTION

Despite the large number of investigations devoted to low dose ultrasound bioeffects on cells and cell surface, the experimental endpoints are different (Wells 1987). For example, Pinamonti et al. (1982) observed a decrease of erythrocyte agglutination after ultrasound treatment, which they attribute to removal of blood group antigens from the cell membrane. These results were not confirmed by Miller et al. (1986) and Rosenfeld et al. (1990) but they were similar to those of Piruzyan et al. (1986). Many authors have remarked that the ultrasound induced effects appeared to be reversible (Hrazdira and Adler 1983; Pinamonti et al. 1982; Repacholi et al. 1971). Thus it could have been supposed that irreparable damage to cells, or rather to their membranes, did not occur. The interpretation of the results was based on measurements performed after, and not during, sonication, and this might be a source of artefact as the transient character of the effects found required a registration technique providing for measurements simultaneous with ultrasound exposure.

In the present study, ultrasound treatment and aggregation or agglutination measurements were undertaken without time delay and independently from each other. Therefore, it was possible to investigate the underlying mechanism of ultrasound-induced alterations of cell behaviour. It is commonly accepted that the dominant interacting mechanism of ultrasound with biological material is either heating or some form of cavitation activity. Since it is not always possible to explain observed biological effects by these two mechanisms (e.g., Piruzyan et al. 1986; Repacholi et al. 1971), nonthermal, noncavitational mechanisms by which ultrasound may interact with tissue were postulated (Nyborg 1989; ter Haar 1987). Recently, Starritt et al. (1989) demonstrated that acoustic streaming with a maximum velocity of 14 cm s^{-1} can be induced in water within milliseconds by a number of clinical commercially available pulse–echo devices. The peak positive pressure amplitude, responsible for the high streaming velocity, achieved values greater than 1 MPa, whereas the time-average intensities remained less than 100 mW cm^{-2}. The problem of acoustic streaming becomes more important for the clinical use of ultrasound as it may be produced *in vivo* (*e.g.*, in amniotic fluid, blood, urine within the bladder) (Starritt et al. 1989).

Although the movement of fluid has been discussed as one of the possible mechanisms of cell-surface bioeffects (Dunn 1985; Lota and Darling 1955; Mortimer and Dyson 1988; Taylor and Newman 1972) the ability of quartz wind to alter cell behaviour remained questionable. Dunn (1985) found a cellular inactivation by heat and shear, but not revealed in his studies was the nature of interaction between these

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agents. Mortimer and Dyson (1988) did not distinguish between effects due to macrostreaming or streaming around oscillating bubbles. Shear stresses induced by these two mechanisms differ by orders of magnitude. Nevertheless, Taylor and Newman (1972) discussed the shear forces generated by quartz wind in terms of alterations of the carbohydrate-rich cell coats. Experiments carried out on model membranes have shown that the increase in permeability due to acoustic macrostreaming did not require any membrane alterations. It was mediated by alterations of near-membrane concentration gradients; that is, by shifts of the concentration in the immediate membrane vicinity, whereas the bulk concentration of a permeating substance remained constant (Pohl et al. 1993a, 1993b). Similar effects were expected adjacent to cell membranes. In this case it should be possible to affect the cell behaviour in a completely reversible manner. The present study was undertaken to examine the effect of ultrasound irradiation on the ability of red blood cells to form aggregates or agglutinates.

MATERIALS AND METHODS

Blood samples

Blood was obtained from human donors by venipuncture in EDTA-Monovette® and was used within 6 h. The erythrocytes were separated from the plasma by centrifugation (3000*g* for 5 min) and washed three times in phosphate-buffered saline (pH 7.4). The cells were resuspended in buffered saline to a predetermined haematocrit. To examine spontaneous aggregation the cells were resuspended in plasma. All suspensions were divided in two parts: control samples and samples for ultrasound exposure. Unless otherwise indicated, the final haematocrit was 3%. The experiments were carried out at room temperature. Following insonation, both the control and exposed blood samples were centrifuged at 3000*g* for 5 min. The extent of haemolysis was compared to determine the extent of ultrasound-induced haemolysis. For the estimation of free haemoglobin the optical absorbance of the supernatant at 410 nm and 540 nm was measured, using a UV/VIS spectrophotometer (Perkin Elmer).

Preparation of blood samples for measurements

Agglutination and two different types of aggregation (rouleaux formation, agglutinationlike aggregation) of erythrocytes were studied.

The agglutination was induced by human (for ABO system) or mouse (for MNSs system) antibodies to blood group antigens. Before the experiments began the blood groups (ABO, MNSs systems) had been determined. All sera were obtained from Sifin (Berlin, Germany) and Biotest (Frankfurt/Main, Germany).

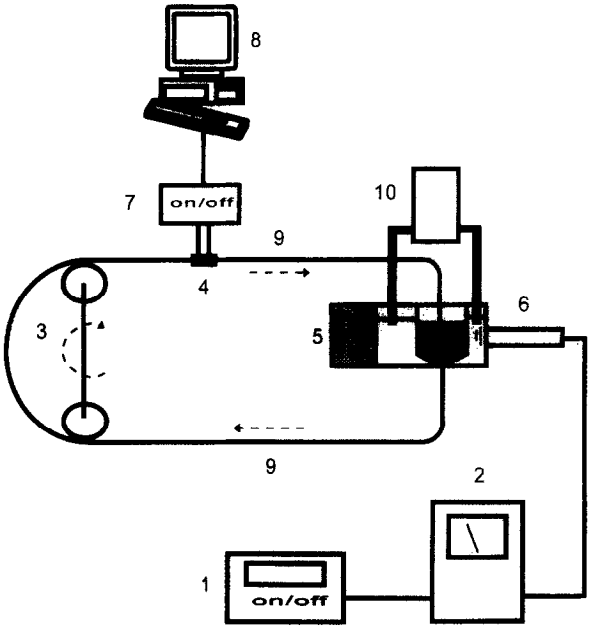

Fig. 1. Scheme of the CW Doppler experimental arrangement. 1—Wave generator, 2—amplifier, 3—pump, 4—measurement chamber, 5—container, 6—ultrasound transducer, 7—CW Doppler device, 8—personal computer, 9—tubes, 10—thermostat.

The titre of the antisera used was 1:64 for anti-A, anti-B and 1:16 for anti-N, anti-M. The final concentration of antisera (0.64% for anti-A, anti-B; 1.6% for anti-M, anti-N) and blood haematocrit were chosen for each blood group to achieve the optimal conditions for antigen–antibody reaction.

The rouleaux formation was induced by dextran-100 (Fluka) or blood plasma (spontaneous aggregation). Aggregation of an agglutination nature was induced by alcian blue (Fluka) (O'Brien 1970). The final concentration of alcian blue was 0.0075% and dextran 3.5 mg mL^{-1}.

The antibodies, dextran or alcian blue were added after 4-min circulation of blood in the system. The sonication of the treated samples began immediately thereafter.

Sonication

A container was designed, which allowed the sonication of blood during circulation in a closed system of tubes (Fig. 1). The inner diameter of the tubes was 1 mm. The velocity of the blood suspension in the system was 0.11 m s^{-1}. Both of two windows of the container were covered with an acoustically transparent film. The container was placed in a water tank. Standing waves were avoided by the incorporation of an oil absorber at the far end of the water tank (Fig. 2).

To vary both frequency and geometry of the con-

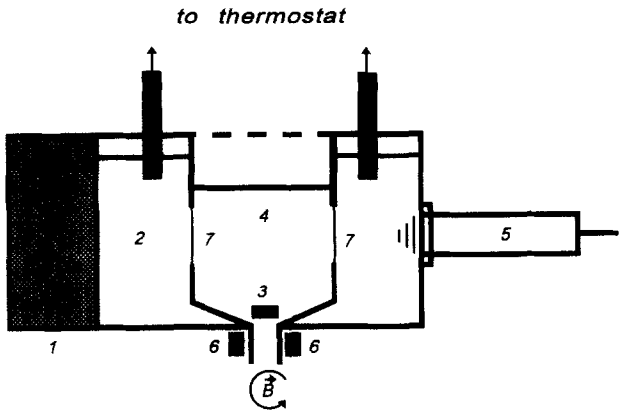

Fig. 2. Scheme of the container for blood samples sonication or stirring. 1—Oil absorber, 2—water bath, 3—magnetic stirrer bar, 4—erythrocyte suspension, 5—transducer, 6—magnetic coils inducing a rotating magnetic field, 7—acoustic windows.

tinuous sound field different transducers were used: (a) two focused transducers at the frequencies of 2 MHz and 10 MHz obtained from Panametrics; or (b) a plane transducer (6 mm diameter) at the frequency of 1.55 MHz.

The focal point of sound beam (a) was positioned at the centre of the blood sample container. The 6-dB beamwidths at focus were r_s = 0.7 and r_s = 1.9 mm for the 10- and 2-MHz transducer, respectively. The sonication of samples were undertaken in the far field for transducer (b). The system consisted further of a signal generator (Wavetek) and a broadband amplifier (ENI) (Fig. 1).

The transducers were calibrated using a hydrophone obtained from NTR Systems. The blood samples were exposed to spatial peak intensities of ultrasound ranging from 0.1 to 2.0 W cm^{-2} (continuous wave) during a 10–30-min period. The temperature of the blood samples was measured before and after sonication.

To exclude cavitation during cell exposure a hydrophone was used (Morton et al. 1983). Since acoustic signals subharmonic to the fundamental frequency above the detection threshold of the hydrophone (p = 250 Pa) were not observable cavitational events seemed to be unlikely.

Measurements of erythrocyte agglutination and aggregation

The erythrocyte agglutination was measured by means of two independent methods, using: a cell counter (Coulter® Multisizer II) (a); and a continuous-wave (CW) Doppler device (b). For registration of aggregation only the second method was used.

The cell counter (a) is a particle size analyzer, which employs the Coulter electrical impedance method to provide a particle size distribution analysis within the overall range 0.4–1200 μm. The registrations with this method required the fixation of the agglutinates with glutaraldehyde (1.5%), which was carried out immediately after exposure to ultrasound in the blood container (Fig. 1; 1—on, 7—off).

The second method (b) used for the detection of cell aggregates or agglutinates was based on ultrasound backscattering combined with the Doppler effect and was described in detail by Cobet (1990). The transducers were positioned in a very small flow chamber incorporated in the circulation system (Fig. 1, 4). The Doppler shifted backscatter signal was recorded in terms of its amplitude (U) and it was related to amount and size of erythrocyte aggregates. The intensity of ultrasound in the measurement chamber was estimated from the electrical power applied to the transducer. It was much smaller (less than 2 mW cm^{-2}) than the intensity used for ultrasound treatment. Any possible influence of the measuring device on the object under study was therefore excluded. The experimental arrangement (Fig. 1) allowed the carrying out of the measurements at any instant of time, that is, simultaneously with sonication, at a constant flow velocity.

RESULTS

Erythrocytes exposed to continuous-wave ultrasound were induced to form more agglutinates than the control erythrocytes. This result was measured using both the cell counter (Fig. 3) and the CW Doppler

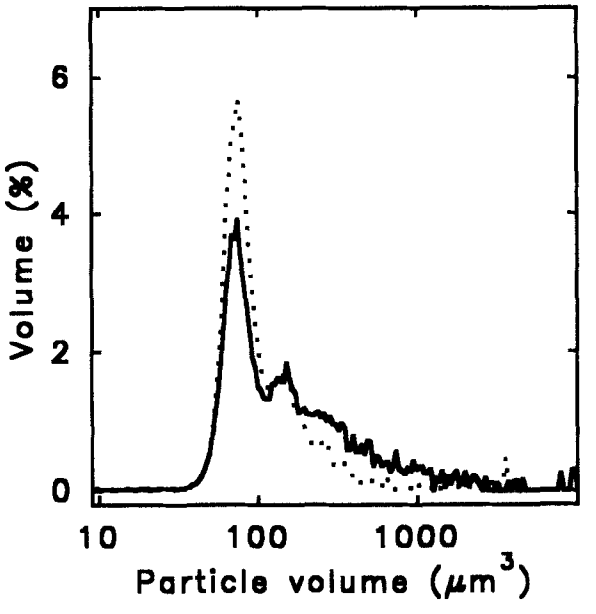

Fig. 3. Volume fractions of erythrocyte agglutinates by antibody-induced agglutination (anti-A) under reference conditions (dotted line) and in the ultrasound field (spatial peak pressure p = 106 kPa, f = 1.55 MHz, continuous wave; solid line).

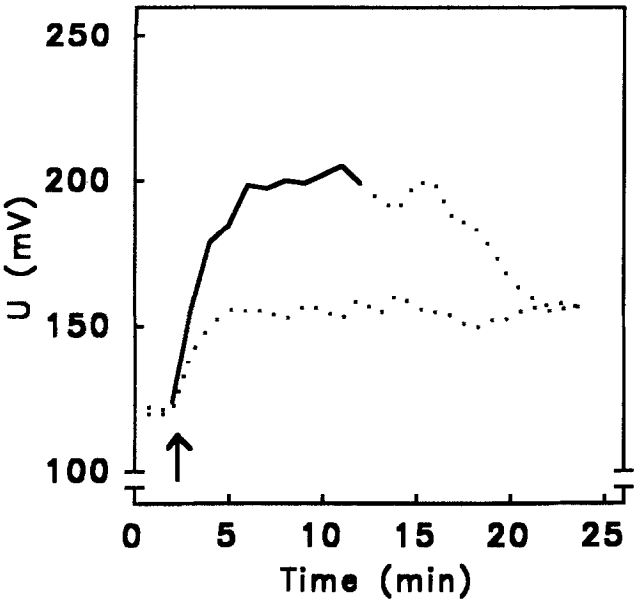

Fig. 4. Time dependence of erythrocyte agglutination in the ultrasound field (spatial peak pressure p = 106 kPa, f = 1.55 MHz, continuous wave; solid line) and without sonication (dotted line). ↑—Anti-N antiserum addition (MN blood). U is a Doppler shifted backscattered signal recorded in terms of its amplitude and related to amount and size of erythrocyte aggregates.

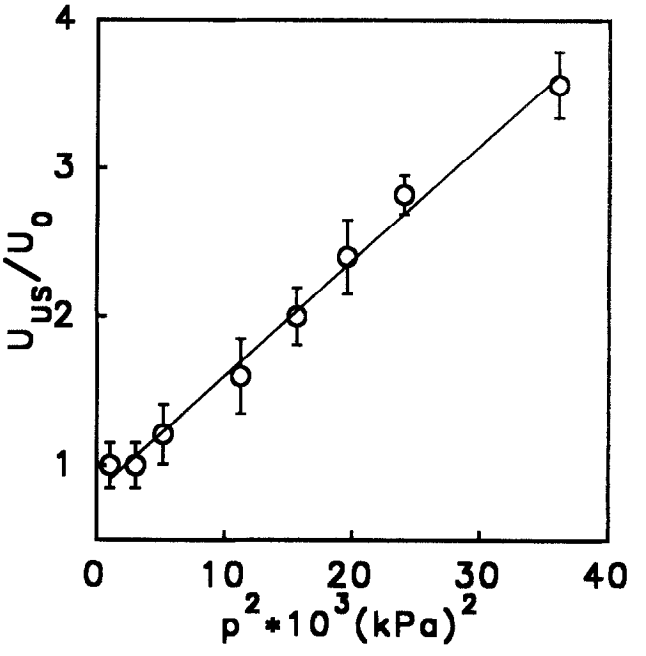

Fig. 5. Dependence of erythrocyte agglutination (U_{US}/U_0) from sound pressure for NN blood. The ultrasound field (f = 1.55 MHz, continuous wave) was produced by a nonfocused ultrasound beam. The time of ultrasound exposure of each sample was t = 10 min. The quotient U_{US}/U_0 describes the ratio of the erythrocyte agglutination level after ultrasound U_{US}, and at control conditions U_0. Data represent mean ± SD for four to six blood samples from different donors.

device (Fig. 4). Onset was usually observed 2–3 min after the initiation of sonication. An increase of the sound pressure amplitude was accompanied by an increase of agglutination at a definite frequency of ultrasound (Fig. 5). An example (Fig. 6) shows that the observed effect has an optimum at a sound pressure of ca. 190 kPa.

The quotient used for the presentation of the results describes the ratio of the erythrocyte agglutination level after ultrasound exposure (U_{US}) and of the control samples (U_0). The effect of ultrasound was reversible. The agglutination level was the same for sonicated and control erythrocytes some minutes after the end of the ultrasound treatment (Fig. 4). It was found that the increase of ultrasound frequency from 2 to 10 MHz at the same sound pressure (continuous wave) produced an increase in agglutination (Fig. 7). This phenomenon was observed for cells of A-, B-, AB-, NN-, MM- and MN blood groups (Table 1).

Aggregation was produced by dextran and alcian blue. Both were enhanced due to sonication. An increase of aggregation induced by blood plasma due to ultrasound exposure was also observed (Table 2).

It was possible to produce the same results by means of magnetic stirrer bars as were obtained with ultrasound exposure (Fig. 2). The level of agglutination strongly depended on the stirring conditions. Vigorous stirring decreased the amount of agglutinated

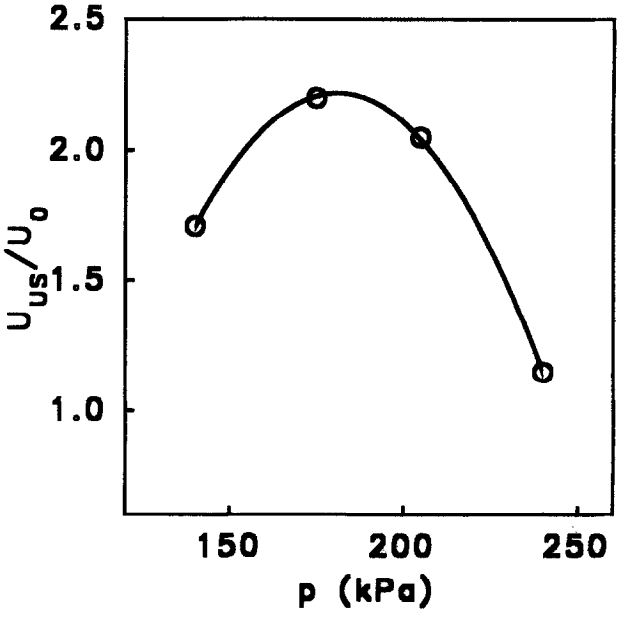

Fig. 6. An example of erythrocyte agglutination (U_{US}/U_0) in an ultrasound field at high sound pressure values for AA blood. The ultrasound field (f = 1.55 MHz, continuous wave) was produced by a nonfocused ultrasound beam. The time of ultrasound exposure of each sample was t = 7 min.

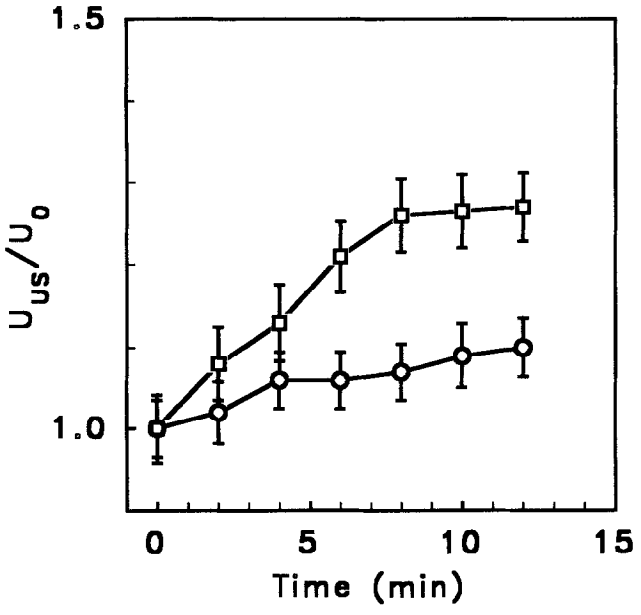

Fig. 7. Time dependence of erythrocyte agglutination (U_{US}/U_0) by two frequencies of ultrasound (f_1 = 2 MHz [○], and f_2 = 10 MHz [□]) for AA blood. The ultrasound field (spatial peak pressure p = 160 kPa, continuous wave) was produced by focused ultrasound beams. The quotient U_{US}/U_0 describes the ratio of the erythrocyte agglutination level after ultrasound U_{US}, and at control conditions U_0.

cells (Fig. 8). When the suspension was stirred gently an increase of the stirring rate was accompanied by an enhancement of agglutination (Fig. 8).

A system consisting of two pumps (Fig. 9) was arranged to simulate the ultrasound streaming. Again, an increase of the agglutination level was found for small fluid velocities. The quotient of the reference agglutination level U_0 to the agglutination level U_P after 8 min of initiation of the second pump was $U_P/U_0 = 1.24 \pm 0.15$ for AA blood. The flow velocity in the second pump

Table 1. Increase of erythrocyte agglutination U_{US}/U_0 for different blood groups of ABO and MN systems due to ultrasound irradiation

Blood group	Antibody	U_{US}/U_0	SD
AA	anti-A	2.33	±0.13
AB	anti-A	1.2	±0.03
BB	anti-B	2.93	±0.2
NN	anti-N	2.4	±0.18
MN	anti-N	1.38	±0.15

The erythrocytes were exposed to ultrasound (spatial peak pressure p = 140 kPa, f = 1.55 MHz, continuous wave) during 10 min at room temperature. The quotient U_{US}/U_0 describes the ratio of the erythrocyte agglutination level after ultrasound treatment U_{US}, and at control conditions U_0. Data represent mean ± SD for three to six blood samples from different donors. An exception is the result obtained for the AB blood group (three samples from one single donor).

Table 2. The increase of erythrocyte agglutination induced by alcian blue (ALB), aggregation induced by dextran (D) and spontaneous aggregation (SA) in an ultrasound field

	U_{US}/U_0	SD
ALB	1.18	±0.1
D	1.55	±0.1
SA	1.23	±0.15

The erythrocytes were exposed to ultrasound (spatial peak pressure p = 140 kPa, f = 1.55 MHz, continuous wave) during 10 min at room temperature. The quotient U_{US}/U_0 describes the ratio of the erythrocyte agglutination level after ultrasound treatment U_{US} and at control conditions U_0. Data represent mean ± SD for three to five blood samples from different donors.

system was 0.084 m s^{-1}. A high shear rate led to agglutination levels lower than the control ones.

The absorbance of the free haemoglobin in supernatant was measured to detect the degree of cell haemolysis due to sonication. No significant difference was found between ultrasound exposed (1.33% ± 0.29%) and control (1.38% ± 0.32%) samples. The difference of suspension temperature before and after sonication was minimal (1–2°C). Nevertheless, the erythrocytes were incubated in a water bath for 30 min at 24°, 27°, 30° and 37°C using the above arrangement (Fig. 1, 7—off). No significant increase of erythrocyte agglutination was observed (Table 3).

DISCUSSION

An increase in number and size of erythrocyte agglutinates and aggregates in the ultrasound field was

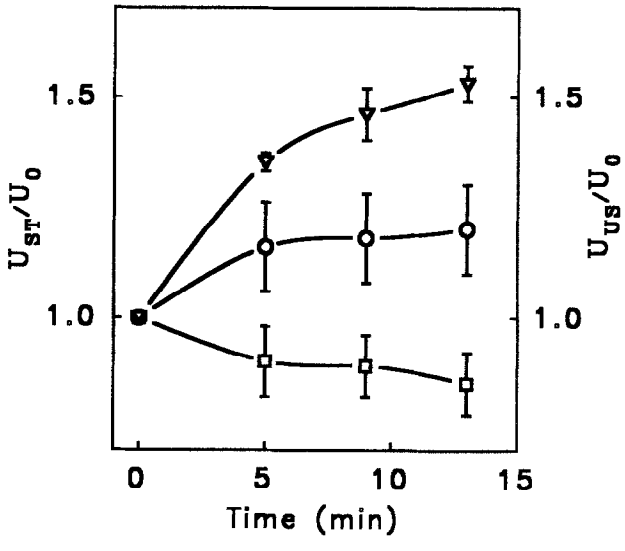

Fig. 8. Time dependence of erythrocyte agglutination during vigorous (□) and gentle (○) stirring, and during stirring in an ultrasound field (spatial peak pressure p = 106 kPa, f = 1.55 MHz, continuous wave; [▽]). Quotients U_{ST}/U_0 and U_{US}/U_0 describe the ratio of the erythrocyte agglutination level by stirring with magnet stirrer U_{ST} or by ultrasound exposure U_{US}, and at control conditions U_0.

Table 3. No effect on erythrocyte agglutination (NN blood group) due to incubation at different temperature

T	U_T/U_0	SD
24°C	1.03	±0.05
27°C	1.01	±0.05
30°C	0.9	±0.07
37°C	1.04	±0.08

The erythrocytes were incubated at different temperatures for 15 min. The quotient U_T/U_0 describes the ratio of the erythrocyte agglutination level after incubation U_T and at control conditions U_0.

observed using the cell counter setup (Fig. 3). Aggregation tendencies in blood cell suspensions were predicted theoretically in any ultrasound field, if the velocity amplitude was sufficiently high (Nyborg 1989). However, the experimental observations were different. It has been reported that agglutination (Pinamonti et al. 1982) or aggregation (Hrazdira and Adler 1983) of human erythrocytes was reduced after ultrasound treatment. On the contrary, no effects were observed by other authors employing similar conditions (Miller et al. 1986; Rosenfeld et al. 1990).

An increase in the aggregation ability of platelets after sonication has been shown more than once (Miller et al. 1979; Williams et al. 1976; Williams 1983). It was supposed that stable cavitation may have been responsible for this effect.

In the present study measurements with the cell counter were carried out after the ultrasound irradiation. This is the familiar sequence of treatment and registration (Pinamonti et al. 1982, and others). Cells were fixed with glutaraldehyde before being measured. However, the results were strongly influenced by the fixation procedure and the time period between fixation and measurement. Additionally, the standard deviation of the results obtained with blood from one single donor was very high. To overcome these disadvantages another experimental setup was arranged (Fig. 1), which allowed registrations of the agglutination parameter not only without cell fixation, but simultaneously with ultrasound exposure, to follow the changes dynamically. The increase of agglutination during ultrasound exposure was also observed, using the CW Doppler device (Fig. 4). Unlike the first method, the second one allowed the registration of small increases of agglutination. Changes in structure and amount of antigens on the cell surface (Buts and Skibenko 1991; Pinamonti et al. 1982) seemed not to be primarily involved, because aggregation induced by dextran, alcian blue or blood plasma showed the same tendency (Table 2).

To explain the above results some of the possible ultrasound interaction mechanisms such as heating, cavitation phenomena and mechanical forces should be discussed.

Heating can be excluded, because of the minimal temperature elevation observed and the absence of agglutination or aggregation enhancement due to incubation at different temperatures (Table 3). These results are in accordance with the reports of other authors (Barnett et al. 1994; Booth et al. 1970; Thomenius and Lewin 1991). Thomenius and Lewin (1991) showed that the ultrasound absorption of the blood suspension is not significant and thus specific heating effects on the erythrocyte membrane should be improbable. Of interest are the observations of Booth et al. (1970), who showed a reduction of A1, B and H antigen activity of erythrocytes after heat treatment.

Cavitation seems to be unlikely also since subharmonic signals had not been detected. Cavitation threshold increases with an increase in frequency, contrary to the findings shown in Fig. 7. Furthermore, the absence of haemolysis showed that ultrasound at the frequencies and intensities used caused no strong membrane damage. The reversible nature of the response does not support the hypothesis of transient cavitation as a probable underlying mechanism too.

Since heating and transient cavitation appear not to be mechanisms involved, one is led to consider that forces of nonthermal, noncavitational origin, and particularly bulk fluid movement, may be important for explaining the bioeffects found.

Nyborg (1989) has shown theoretically that aggregation of blood cells may occur due to radiation force. According to his theory, an ultrasound field of an arbitrary nature forces particles to form dimers when a critical intensity is achieved and the wavelength is larger than the cell diameter. The calculated energy

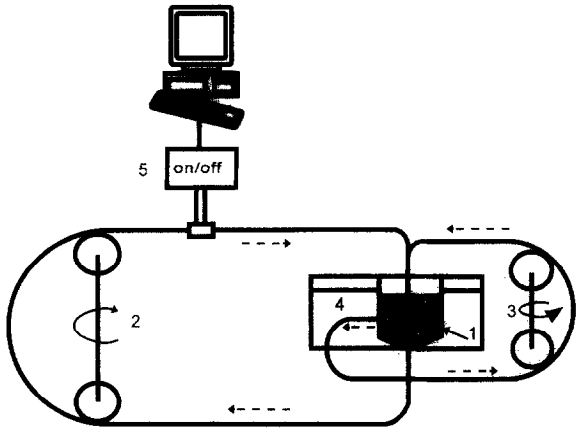

Fig. 9. Scheme of experimental arrangement with the second pump to simulate ultrasound streaming. 1—Container with blood suspension, 2—first pump, 3—second pump, 4—water bath, 5—CW Doppler detection device with computer.

density required for pearl chain formation of erythrocytes is equal to 130 mW cm^{-2}. According to the theory, the magnitude of the interparticular radiation forces increases with the square of the pressure amplitude. Although the pressure dependencies of aggregation and agglutination coincide with the predicted one, the frequency dependence does not. Moreover, the experimental results do not support the hypothesis that the origin of the ultrasound effect differs from the one, leading to an augmentation of aggregation due to a nonacoustic fluid flow generated by means of a stirrer (Fig. 8) or a pump (Fig. 9). It is not possible to deny that the processes described by Nyborg (1989) take place but their contribution to the enhancement of aggregation and agglutination seems to be small compared to the contribution of acoustic streaming.

Liebermann (1949) showed that the streaming velocity v is related to the beam intensity I and frequency f:

$$v = (2 + b)\frac{(2\pi f)^2 IG}{\rho u^4} \qquad (1)$$

where u is the sound velocity, ρ is the density of water and b is a constant factor depending on the medium viscosity and for water equals 2.4 (Liebermann 1949). The geometrical factor G can be calculated as (Liebermann 1949):

$$G = \frac{r_s^2}{2}\left(\frac{r_s^2}{r_0^2} - 2\ln\frac{r_s}{r_0} - 1\right) \qquad (2)$$

where r_s is the radius of the sound beam, and r_0 the radius of the tube containing the ultrasound-exposed solution. Although the geometry of our container deviates from a cylindrical one the resulting error of estimation of v will be small, because $r_s \ll r_0$.

As the streaming velocity depends on the ultrasound frequency and intensity [eqn (1)], it may be expected that agglutination and aggregation are also functions of these parameters. Indeed, an increase of ultrasound frequency and pressure leads to higher agglutination levels in our experiments (Figs. 5 and 7). Additionally, the velocity of the streaming and the aggregation level, respectively, were enhanced by substituting a focused transducer with a small effective radius of the sound beam (1.9 mm for the 2-MHz transducer) by a plane transducer with a threefold larger one, which is in agreement with eqn (1) as well.

A further argument in favour of the streaming hypothesis is the stimulation of agglutination by gentle stirring (Fig. 8). The observed dependence of aggregation level on the shear gradient is in qualitative agreement with the results obtained by Schmid-Schönbein et al. (1968), Shiga et al. (1990) and Murata and Secomb (1988). Shiga et al. (1990) reported that an increase of the shear rate dispersed the aggregates, whereas at low shear rates the aggregation was accelerated with an increase in the shear rate.

The calculated velocity of the streaming [eqn (1)] in the ultrasound field produced by the 10-MHz transducer (p = 160 kPa) was v = 0.043 m s^{-1} for our experimental arrangement. The coefficient U_{US}/U_0 = 1.26 for 10-MHz ultrasound exposed cells is in good accordance with the coefficient U_P/U_0, obtained using the second pump (Fig. 9), generating a flow velocity of the same order of magnitude as the calculated velocity of the streaming produced by the 10-MHz transducer. It should be mentioned that, although the direction of the streaming in both of these cases was similar, simulated fluid flow lacked the backward-directed streaming occurring near the container wall. Therefore, a qualitative comparison only may be made. The qualitative agreement between these two experiments indicates that the underlying mechanism for the effect observed does not depend on the origin of the streaming.

Since the stirring rate is known to determine the thickness of the unstirred layer (Barry and Diamond 1984; Mierle 1985) it is possible to assume that the alteration of the unstirred layer of red blood cells is responsible for the increase of the cell aggregation. The following arguments support this hypothesis. As can be seen (Fig. 5), the magnitude of the observed effect increased as a function of the square of the sound pressure. A similar dependence was obtained for the decrease of the transmembrane potential of planar bilipid membranes in an ultrasound field (Pohl et al. 1993b). It was shown that this behaviour was based on bulk fluid movement due to ultrasound irradiation. However, streaming was treated as a one-dimensional problem (Pohl et al. 1993a). A model system much closer to the three-dimensional geometry of erythrocytes was studied by Schmidt et al. (1987), who found an increase of activity of enzymes immobilized on spheres as a function of the square of the sound pressure. Acoustic streaming was supposed to be involved. Both of the ultrasound effects on planar membranes and enzymes were mediated by changes in the unstirred layer thickness. It is reasonable to consider that the alterations in cell behaviour occurred due to a reduction in thickness of the unstirred layers near the cell as well. Thereby it can be expected that the fluid flow due to ultrasound irradiation alters the transmembrane potentials and ion gradients within the unstirred layers adjacent to cells, analogous to those of model membranes. If ultrasound indeed decreases the unstirred layer thickness, an increase of cell permeability should be observed. The permeability of cell membranes is comprised of the permeability of the bilayer

itself and the permeability, P, of the surrounding unstirred layers. The latter is a function of the thickness, Δx, of the unstirred layer:

$$P = \frac{D}{\Delta x} \quad (3)$$

where D is the diffusion coefficient.

Indeed Lota and Darling (1955) reported that the permeability of erythrocyte membranes can be changed due to sonication. Chapman et al. (1979) demonstrated the decrease of potassium content in rat thymocytes due to ultrasound exposure without cell lysis or gross membrane damage. Mortimer and Dyson (1988) observed that ultrasound treatment could increase calcium uptake of fibroblasts for intensities of 0.5–1.0 W cm^{-2} SPPA, with a maximum increase after 5-min exposure. On the other hand, alterations of membrane potentials and near-membrane ion concentrations should influence the aggregation and agglutination ability of cells. The latter was found to be affected by ultrasound in our experiments.

According to Shiga (1990) the collision frequency among cells increases with an increase in shear rate. At low shear rates, the erythrocyte aggregation may be accelerated with an increase in shear rate. A further increase of the shear rate disperses the aggregates. This mechanism must be discussed as an alternative hypothesis. It is, however, uncertain whether aggregation behaviour results from an increase in the collision frequency alone or if the reduction of unstirred layers is also required.

The fact that the agglutination behaviour of the cells has an optimum at a definite peak pressure (Fig. 6) may explain the contradictory results obtained by different authors (Miller et al. 1986; Pinamonti et al. 1982; Piruzyan et al. 1986; Rosenfeld et al. 1990). It is reasonable to believe that high values of sound pressure will lead to a decrease of the agglutination level in ultrasound fields.

The reported results demonstrate the possibility of ultrasound bioeffects on erythrocytes being due to mechanical forces. The enhancement of the agglutination level should cause a risk for patients, which seems to be tolerable due to its transient character.

The fact that the sound pressure in modern diagnostic devices is even higher (Starritt and Duck 1992) than in the experiments described herein has the consequence that the presence of biological effects due to acoustic streaming during clinical exposures cannot be excluded. It is obvious that, along with the temporal average intensity, the positive peak pressure has to be indicated to evaluate the risk associated with ultrasonic exposure in medicine.

In the light of the fact that the aggregation and agglutination behaviour of the erythrocytes has to be regarded as an integral parameter of cell-surface properties the acoustical macrostreaming becomes significant for therapeutic and pharmacological applications of ultrasound. Thus, it was reported by Supersaxo et al. (1993) that the release of dextran, selected as a macromolecular model drug, from microspheres was enhanced in a ultrasound field. Recent experiments on vascular endothelial cells (Shen et al. 1993) have shown that shear stress has a profound influence on their function. An increased release of ATP due to acoustic streaming should enhance, for example, the process of wound healing.

The results show that the fluid streaming induced by ultrasound can be used for the generation of functional alterations of cells in therapeutic applications. Further studies are now required to demonstrate that streaming effects on endothelial and immunocompetent cells occur *in vivo*.

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