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Original Contribution

OXYGEN RADICAL SCAVENGERS INHIBIT CLASTOGENIC ACTIVITY INDUCED BY SONICATION OF HUMAN SERUM

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Abstract—Clastogenic factors (CF) are diffusible molecules that damage DNA. They are generated within biological media by a variety of physical and chemical stimuli. Their nature and mechanism of action remain largely unknown. Clastogenic activity can be experimentally generated by pulsed ultrasound treatment of human serum. To investigate whether oxygen radicals are involved in the clastogenic activity induced by sonication of human serum, we examined the effects on such clastogenic activity of different oxygen radical scavengers added to human serum before and after sonication. Human serum was sonicated for 50 min at 24 $\mu W/cm^2$ by pulsed ultrasound. The clastogenic activity of sonicated human serum was examined in the presence or absence of oxygen radical scavengers by measuring the amount of DNA damage induced in autologous human lymphocytes, assessed with the fluorometric analysis of DNA unwinding (FADU). Sonication of human serum generated significant DNA damage in autologous lymphocytes (DNA unwinding averaged 31.79% ± 2.1 after sonication vs. 12.82% ± 2.6 in the controls, $p < 0.005$). Superoxide dismutase (SOD; 500 I.U./ml), catalase (500 I.U./ml), mannitol (50 mM), and glutathione (50 mM) completely prevented DNA damage when added before serum sonication, whereas only mannitol (86%) and glutathione (90%) almost completely inhibited DNA damage when added after sonication. SOD and catalase had only a partial inhibitory effect when added after sonication (49% and 63%, respectively). The prevention of DNA damage was also obtained by an association of subliminal amounts of glutathione (20 mM) and vitamin E (1 I.U./ml). These results suggest that the clastogenic activity generated by sonication of human serum is mediated by oxygen radicals.

Keywords—Free radicals, Human lymphocytes, Ultrasound, DNA damage, FADU, Clastogenic activity, Radical scavengers, Glutathione, Vitamin E

INTRODUCTION

Applications of ultrasound techniques for diagnostic purposes are widespread, therefore recent studies have been aimed at establishing whether ultrasound shows any kind of bioeffect or damage to tissues and cells.[1–6] Ultrasound may generate free radicals in aqueous solutions, as recently reviewed by Riesz and Kondo.[7] Chemical or physical generation of oxygen radicals has been found to increase chromosome breakage and sister chromatid exchanges (SCE) in cultured lymphocytes through the production of clastogenic factors (CF).[8] Activity of CF appears relevant in syndromes connected to hereditary chromosome breakings,[9,10] in chronic inflammations with spontaneous chromosomal instability, or after exposure of cell cultures to chemicals.[11]

Clastogenic activity can be induced in human serum by treatment with pulsed ultrasound.[12] The mechanism of this clastogenic activity is unknown. To determine whether oxygen radicals are involved in this clastogenic activity, we investigated whether it was inhibited by different oxygen radical scavengers,[13] i.e., dioxide (1−) dismutase (superoxide dismutase or SOD), catalase, D-mannitol, glutathione (γ-glutamylcisteinyl-glycine), and vitamin E (d,1-α-tocopheryl acetate).

MATERIALS AND METHODS

Free radical production by ultrasound

Serum samples (pH 7.2) were exposed to ultrasound for 50 min in a 7-ml stainless steel tank with an

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ophthalmological Kretztechnik probe, nonfocused, with frequency of 8 MHz and intensity 0.22×10^{-3} W/cm^2. The source of pulsed ultrasound was a Panametrics 5052 pulser (with 4 KHz pulse repetition frequency and 50 Ohm damping), whose intensity parameters have been reported elsewhere.[12] The amount of superoxide radicals in vitro induced by means of ultrasound was measured through cytochrome *c* reduction kinetics, as previously reported.[14]

Cytochrome *c* kinetics were monitored with a Kontron Uvikon 860 spectrophotometer (Kontron Instruments, Inc., Everett, MA), software version 8613.

Experimental protocol

To determine whether the clastogenic activity induced by ultrasound treatment of human serum was inhibited by oxygen radical scavengers (ORS), we performed series of different experiments. In the first series, human serum was divided into four 3-ml fractions: we used the first as a control, we treated the second with ultrasound, we added ORS to the third, and we added ORS to the fourth, as well as treating it with ultrasound. The four fractions were then incubated with autologous lymphocytes (from the same donor) in a thermostatic bath at 37°C for 1 h. Damage on lymphocyte DNA was then assessed by fluorometric analysis of DNA unwinding (FADU)[15] as described below. Concentrations of scavengers in the serum were the following: SOD = 500 I.U./ml; catalase = 500 I.U./ml; mannitol = 50 mM; and reduced glutathione (GSH) = 50 mM.

In the second series of experiments, we examined the time course of the clastogenic activity induced by ultrasound treatment of serum by adding ORS to the serum immediately after ultrasound treatment, and before incubation with autologous lymphocytes. Moreover, to test possible effects of inactive enzymes, SOD and catalase were inactivated by heat treatment at 100°C for 30 min and added to the serum after ultrasound exposure. DNA damage was then assessed by FADU.

Further experiments were performed to test whether the addition of iron or metal chelators could interfere with the results recorded for clastogenic activity of ultrasound-treated serum. The iron chelator desferrioxamine B methanesulfonate (Desferal, Ciba Geigy, Basel, Switzerland) was added to the serum before ultrasound exposure under the conditions described previously. Desferal concentration was 0.357 μM, sufficient to bind up to 20 μg/ml of iron ions. The clastogenic activity of ultrasound-treated serum was then assessed by FADU.

Finally, a series of experiments was performed to establish whether lymphocytes could increase the clastogenic activity of ultrasound-treated serum. Sterile isotonic saline solution (0.9% NaCl) was exposed to pulsed ultrasound in the presence of lymphocytes and in the absence of serum. The solution was then centrifuged at 1000 g for 30 min and the supernatant filtered with 0.22 μm Millipore filters. Clastogenic activity of the filtered supernatant was then assessed by FADU.

DNA damage assessment

Freshly drawn blood samples from healthy donors (courtesy of Transfusion Centre of St. Anna Hospital, Ferrara, Italy), containing 3.6 mM ethylene diaminetetraacetic acid disodium salt-2-hydrate (EDTA) (Idranal III Riedel-DeHaën) as an anticoagulant, were centrifuged at 1000 g for 30 min in sterile test tubes (Becton Dickinson, Oxnard, CA) to separate serum. The serum pH was 7.2. Lymphocytes were then isolated by layering on Histopaque 1077 (Sigma Diagnostics) and incubated at 37°C for 1 h in the presence of either normal or ultrasound-treated serum, with or without ORS such as superoxide dismutase (SOD) and catalase (both from Sigma Chemicals, St. Louis, MO), D-mannitol (Fluka, Buchs, Switzerland), glutathione (Tationil Boehringer Mannheim, Germany), vitamin E (Evion Bracco, Milan, Italy). The number of cells in each sample was estimated as 10^6 cells/ml by turbidimetric methods (absorbance at 280 nm measured by a Kontron Uvikon 860 spectrophotometer). DNA damage was assessed with a fluorometric method (Fluorometric Analysis of DNA Unwinding or FADU)[15] in which the lysis solution was modified as follows: 0.01 M Trizma Base (Sigma), 0.01 M EDTA, 0.01 M NaCl, 0.2% sodium dodecylsulfate (BDH), pH 7.2.

The FADU method involves cell lysis, alkalinization of lysates, and assessment of DNA damage by measuring the percentage of DNA unwinding. The fluorescent dye ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide), a DNA intercalating agent, selectively binds to double-stranded (ds) DNA when regions with single-strand breaks are destabilized by alkaline solutions; the fluorescence of each sample is read at room temperature in a Kontron SFM 25 spectrofluorimeter (520 nm excitation, 590 nm emission). Each value is obtained by comparison of three samples, each one in quadruplicate: T (for "total"), B (for "blank") and P (the experiment).

T samples give the values of total fluorescence (including possible contaminants), B samples those of complete DNA unwinding after sonication for 20 seconds in a Branson (Danbury, CT) B-10 sonifier (75 W/cm^2); and P samples give the DNA unwinding of each experiment. After alkalinization treatment, fluorescence of T minus the blank gives the amount of ds DNA in the extract, while P minus the blank gives the amount of ds DNA remaining. The percent of ds (intact) DNA is given by $(P - B)/(T - B) \times 100$. Data are also expressed as percentage of DNA protection by scavengers.

Statistical analysis

Each one of our data is the result of at least two experiments on different blood donors. Values of p for Student's *t*-test between control and treated serum are based on standard errors (SE) with 6 degrees of freedom. Standard error was evaluated by the following expression,[16] developed by Professor Italo Barrai, University of Ferrara:

$$E_s = \left[f'_P \left(\frac{P - B}{T - B}\right)^2 * \sigma_P^2 + f'_T \left(\frac{P - B}{T - B}\right)^2 * \sigma_T^2 + f'_B \left(\frac{P - B}{T - B}\right)^2 * \sigma_B^2 \right]^{1/2} * 100$$

RESULTS

Cytochrome c reduction by pulsed ultrasound

In aqueous solution, cytochrome *c* acts as a weak base according to the following reaction:

$$\text{cyt } c\ (Fe^{+++}) + O_2^{\cdot -} \rightarrow O_2 + \text{cyt } c\ (Fe^{++})$$

The concentration of cytochrome *c* in phosphate buffer (pH 7.8–8) was 1×10^{-4}. The kinetics of cytochrome *c* reduction by pulsed ultrasound is shown in Figure 1. Because the reduction rate of cytochrome *c* amounted to 22 nmoles/min, the concentration of oxygen radicals ($O_2^{\cdot -}$) induced by ultrasound in the solution during the 50 min of ultrasound treatment was 1.1 μM.

Induction of clastogenic activity in human serum

Table 1 shows the results of the first series of experiments in which ORS were added simultaneously to ultrasound treatment before incubation with autologous lymphocytes. All scavengers individually provide complete protection of DNA, supporting the assumption that at least three radical species (namely $O_2^{\cdot -}$, $HO^{\cdot}$ and H_2O_2)[17,18] are involved. Because radical life is known to be extremely short (between 10^{-10} and 10^{-16} seconds),[19] it is unlikely that radicals reach the nucleus before being inactivated by cytosolic and nuclear scavengers. Furthermore, given the total concentration of oxygen radicals (1.1 μM) in our experiments and the number of cells in the solution (about 1×10^6 cells/ml), the amount of radicals should be 1.1 femtomoles/cell, which is insufficient to account for the DNA damage. It is therefore likely for DNA damage to be caused by clastogenic activity of more stable intermediates produced in the serum after ultrasound treatment. These unknown intermediates could be produced by the radical chain reaction triggered by ultrasound and should be identified with previously described "clastogenic factors."[9,10,11]

When 0.357 μM Desferal was added to serum before ultrasound treatment, the percentage of DNA damage was 15.31 ± 1.7 against 14.42 ± 2.26 for controls (difference = 0.89). As previously mentioned, 0.3 mM EDTA was initially added to all blood samples as an anticoagulant.

Table 2 reports the results of experiments in which oxygen radical scavengers were added after ultrasound treatment: clastogenic activity is evident because radical species are still active 1 h after ultrasound treatment; that is after incubation with autologous lymphocytes.

Comparing Table 2 with Table 1, it appears that clastogenic activity induced in serum by ultrasound exhibits properties similar to $HO^{\cdot}$, because mannitol and glutathione ($HO^{\cdot}$ scavengers) completely quench clastogenic activity and prevent DNA damage, whereas SOD and catalase, scavengers of early radical species in the sequential one-electron reduction of O_2 to H_2O ($O_2^{\cdot -}$ and H_2O_2), are only partially effective.

Figure 2 shows the results of the experiments with heat-inactivated SOD and catalase compared to controls (i.e., absence of scavenger enzymes) and active enzymes. The difference in the percentage of DNA damage between control and inactivated enzyme is 1.9 (nonsignificant) for SOD, and 4.0 (almost significant, $p < 0.05$) for catalase. The difference in the percentages of DNA damage between active and heat-inactivated enzyme is largely significant for both scavenger enzymes. As previously seen in Table 1, neither SOD nor catalase completely protect DNA from damage.

Because clastogenic activity in serum persists beyond the duration of ultrasound treatment (50 min), it follows that this activity must be mediated by molecules more stable than oxygen radicals.[11] These

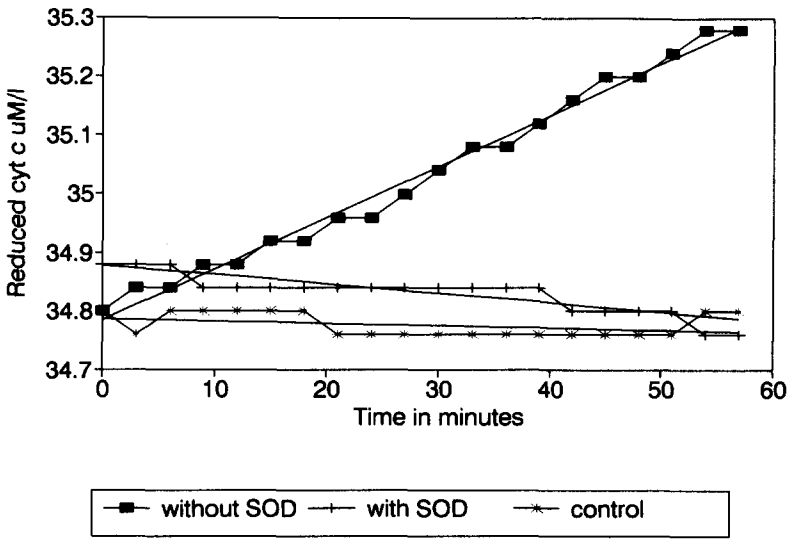

Fig. 1. Kinetics of cytochrome *c* reduction by pulsed ultrasound in the presence or absence of SOD. Concentration of cytochrome $c = 1 \times 10^{-4}$ M in phosphate buffer (pH 7.8–8). SOD concentration = 500 I.U./ml. Parameters of ultrasound treatment as described in the text. * = control; + = with SOD; ■ = without SOD. Reduction rate of cytochrome = 22 nmoles/min. Oxygen radical ($O_2^{\cdot -}$) concentration over 50 min of ultrasound treatment = 1.1 μM.

molecules are likely to cross the cell membrane by locally dissolving its lipid bilayer.

The results of experiments with sterile saline solution exposed to ultrasound in the presence of lymphocytes and in the absence of serum are shown in Table 3. No difference is found in clastogenic activity between controls and filtered saline solution.

Glutathione as radical scavenger in serum

Glutathione is indeed a ubiquitous component of nuclei, cytosol, and generally of biological fluids.[20] We therefore tried to establish the lowest glutathione concentration at which a complete protection of DNA could be obtained in serum. The methods for ultrasound treatment and DNA damage assessment were the same as in the previous experiments. Results (Fig. 3) show that in order to protect DNA completely from clastogenic activity, a concentration of 30 mM glutathione is required and sufficient, while a concentration of 20 mM or lower is ineffective.

Vitamin E as radical scavenger in serum

Vitamin E is well known as a chain-breaking antioxidant,[21,22] so we resolved to use this vitamin as a

Table 1. DNA Damage Expressed as a Percentage of Intact (Double-Stranded) DNA in Human Lymphocytes Incubated With Ultrasound-Treated Autologous Serum With or Without Oxygen Radical Scavengers

	Alkaline Denaturation		
	Control Serum % ds DNA ± SE	Ultrasound-Treated Serum % ds DNA ± SE	C-Tr
No SOD	81.90 ± 3.1	68.73 ± 2.0	13.17 $p < 0.01$
With SOD	90.36 ± 2.0	92.69 ± 2.7	n.s.
No Cat	92.31 ± 2.3	69.55 ± 3.3	22.76 $p < 0.001$
With Cat	88.17 ± 3.7	85.92 ± 3.5	2.25 n.s.
No Mann	82.65 ± 1.6	66.03 ± 2.8	16.62 $p < 0.001$
With Mann	89.57 ± 2.9	88.12 ± 2.0	1.65 n.s.
No GSH	91.84 ± 1.5	68.56 ± 2.4	23.28 $p < 0.001$
With GSH	89.84 ± 2.7	86.41 ± 2.0	3.43 n.s.

Note. Each experiment was repeated three times on different donors. C-Tr = control minus treated; SE = standard error; n.s. = nonsignificant; SOD = superoxide dismutase, 500 U/ml; Cat = catalase, 500 U/ml; Mann = D-mannitol, 50 mM; GSH = reduced glutathione, 50 mM.

Table 2. DNA Damage Expressed as a Percentage of Intact (Double-Stranded) DNA in Human Lymphocytes Incubated With Ultrasound-Treated Autologous Serum, With or Without Scavengers Added to the Serum After Ultrasound Treatment

	Alkaline Denaturation			
	Control Serum % ds DNA ± SE	Ultrasound-Treated Serum % ds DNA ± SE	C-Tr	% prot
No SOD	88.96 ± 2.6	70.19 ± 2.8	18.77 $p < 0.01$	
With SOD	87.41 ± 1.9	77.95 ± 2.1	9.45 $p < 0.05$	49.9
No Cat	97.99 ± 1.9	81.13 ± 2.8	16.86 $p < 0.01$	
With Cat	92.16 ± 1.6	86.04 ± 1.4	6.1 $p < 0.05$	63.2
No Mann	93.06 ± 2.1	79.26 ± 2.0	13.8 $p < 0.01$	
With Mann	88.53 ± 1.3	84.43 ± 2.7	4.06 n.s.	100
No GSH	88.35 ± 2.2	73.93 ± 2.6	14.42 $p < 0.01$	
With GSH	98.61 ± 3.1	94.63 ± 3.9	3.98 n.s.	100

Note. Each experiment was repeated three times. % prot = percentage of DNA protection. Other abbreviations as in Table 1.

scavenger of oxygen radicals and clastogenic activity, alone or combined with glutathione. The results of experiments with different concentrations of vitamin E in the same experimental conditions are shown in Figure 4. High concentrations of vitamin E exhibit efficient oxidant scavenger properties, but concentrations as low as 1.59 mM (= 1 I.U./ml) and 5 mM (= 3.1 I.U./ml) in our experimental conditions have no effect on the system.

There are reports of synergistic interaction between vitamin E and vitamin C as oxygen radical scavengers.[23] We tried associations of different glutathione concentrations with an extremely low amount of vitamin E (1 I.U./ml), insufficient to provide scavenging activity by itself. Figure 5 shows the data concerning the glutathione threshold of effectiveness when it is associated to 1 I.U./ml of vitamin E. It is clear that the minimal concentration of glutathione to work as an efficient scavenger in these conditions is as low as 20 mM.

Persistence of clastogenic activity in human serum

The final series of experiments was aimed at establishing the persistence of clastogenic activity in human serum several hours after ultrasound treatment. In the same conditions as for all the other ultrasound experiments, clastogenic activity was challenged with the minimal scavenger concentration (20 mM glutathione and 1 I.U./ml vitamin E) capable of com-

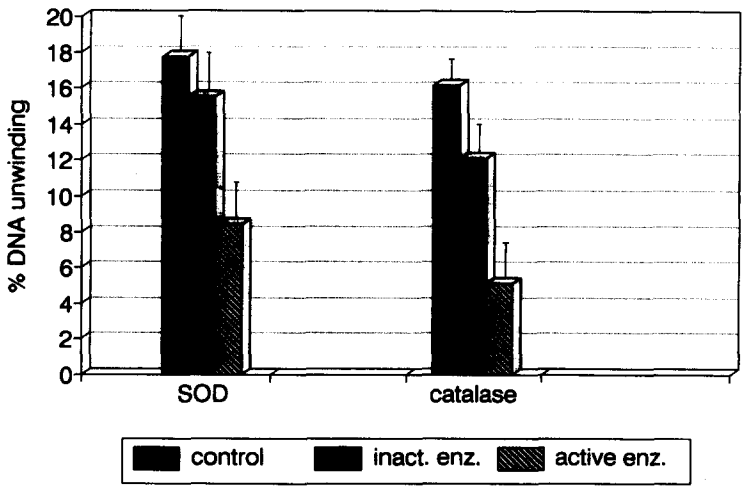

Fig. 2. Protective effects of heat-inactivated SOD and catalase on DNA damage induced on lymphocytes by ultrasound treated human serum. DNA damage is expressed as a percentage of DNA unwinding measured by FADU. Control = absence of enzymes. Inact. enz. = enzymes inactivated by heat treatment (100°C for 30 min). Other abbreviations as in Tables 1 and 2. Bars = standard deviations.

Table 3. DNA Damage Expressed as a Percentage of Intact (Double-Stranded) DNA in Human Lymphocytes Incubated With Ultrasound-Treated Sterile Saline Solution in the Presence of Lymphocytes

	Control	Ultrasound-Treated
Saline sol.	80.00 ± 1.7	81.33 ± 2.3
Saline sol. + lymphocytes	85.43 ± 2.1	84.71 ± 1.9

Data are expressed as percentages of intact DNA. Saline sol. = 0.9 M NaCl.

pletely protecting DNA from damage. Results are reported in Figure 6. The data clearly show that clastogenic activity persists up to 24 h after ultrasound treatment.

DISCUSSION

The present study shows that ultrasound treatment of human serum generates significant DNA damage in autologous lymphocytes and that oxygen radical scavengers such as dioxide (1-) dismutase or superoxide dismutase, catalase, D-mannitol, and glutathione completely prevent DNA damage when administered before ultrasound treatment. On the contrary, only mannitol and glutathione prevent DNA damage when administered after ultrasound treatment.

The limited scavenger effect observed for heat-inactivated catalase may be due to a mild, nonspecific scavenger activity exhibited by most heat-inactivated proteins (Fig. 2).

No influence on DNA damage was observed in our experimental conditions when serum was exposed to ultrasound in the presence of Desferal.

Antioxidants can be classified in two general categories: those that prevent oxidation by blocking newly formed oxygen radicals, and those that interrupt the peroxidative chain by preferentially interfering with intermediate steps. It is known that the lipid-soluble vitamin E (α-tocopherol) may interrupt the lipid peroxidative reaction chain[21,22] and is normally found in cell membranes. Recently, alterations were shown in the cell membrane of human lymphocytes treated with pulsed ultrasound.[24] We therefore resolved to use this vitamin as a scavenger of oxygen radicals and clastogenic activity, alone or combined with glutathione. Among all natural tocopherols, the fully methylated form of vitamin E (d,l-α-tocopheryl acetate) is the most active biologically as a chain-breaking antioxidant,[25] and represents a good choice as a scavenger of clastogenic factors for the following reasons: (a) it is a ubiquitous molecule in biomembranes; (b) as mentioned before, in human lymphocytes oxygen radicals induced by ultrasound damage the membrane bilayer[24] and increase its permeability (unpublished observations); and (c) in human plasma, vitamin E is carried by the lipoprotein fraction to target organs and acts as membrane-regenerating factor.[22,23]

The results of our experiments employing the

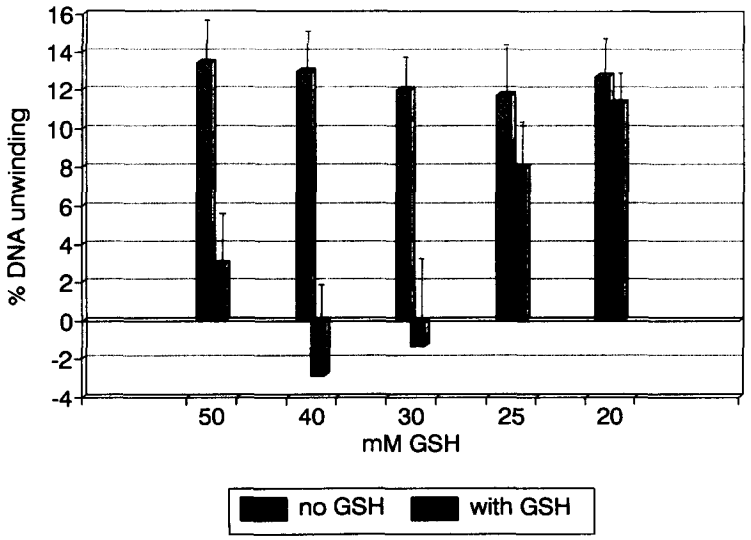

Fig. 3. Protective effects of decreasing concentrations of reduced glutathione against DNA damage caused by clastogenic factors induced by pulsed ultrasound in human serum. DNA damage is expressed as a percentage of DNA unwinding measured by FADU. Abbreviations as in Tables 1 and 2. Bars represent significant standard deviation (SD).

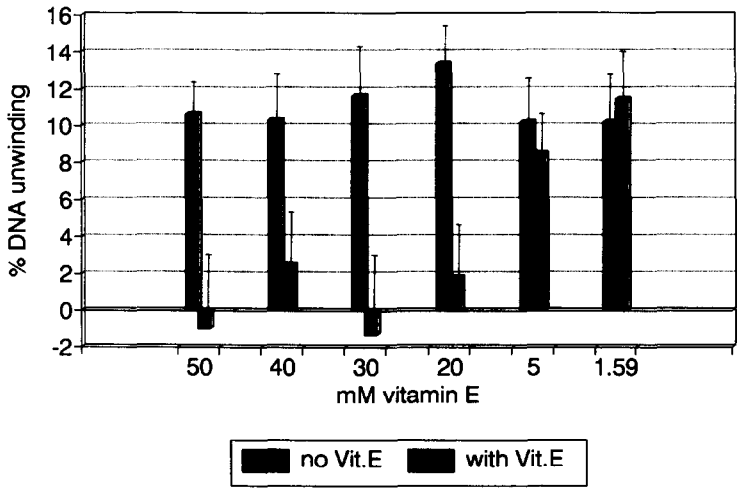

Fig. 4. Protective effect of decreasing concentrations of vitamin E (d,l-α-tocopheryl acetate) against DNA damage caused by clastogenic factors induced by pulsed ultrasound in human serum. DNA damage is expressed as in Figure 3. Vit E = vitamin E. Other abbreviations as in Tables 1 and 2.

above-mentioned scavengers and vitamin E (d,l-α-tocopheryl acetate) also support the fact that pulsed ultrasound induces oxygen radical species in human blood serum in a similar way to ionizing radiations. These radical species are able to damage the DNA of human lymphocytes through the production of molecules with clastogenic activity that persist in serum up to 24 h after ultrasound treatment. We think that these molecules, more stable than oxygen radicals and responsible for clastogenic activity, should be identified with the "clastogenic factors" described by Emerit.[8,11] Clastogenic activity appears to be responsible for several kinds of damage in human lymphocytes besides DNA strand breaks, e.g., lesions to cell membrane, microtubules, endoplasmic reticulum, and mitochondria.[18,25]

It is possible for lymphocytes to be involved in the production of clastogenic factors.[6] However, the results of our experiments with sterile saline solution, treated with pulsed ultrasound in the presence of lymphocytes and filtered before incubation, seem to exclude any involvement of these cells in clastogenic activity. Clastogenic activity appears, therefore, to be produced only by ultrasound-treated serum.

According to our results, some conclusions concerning the clastogenic activity and the nature of clastogenic factors may be drawn. First of all, our experiments with scavengers confirm that clastogenic activ-

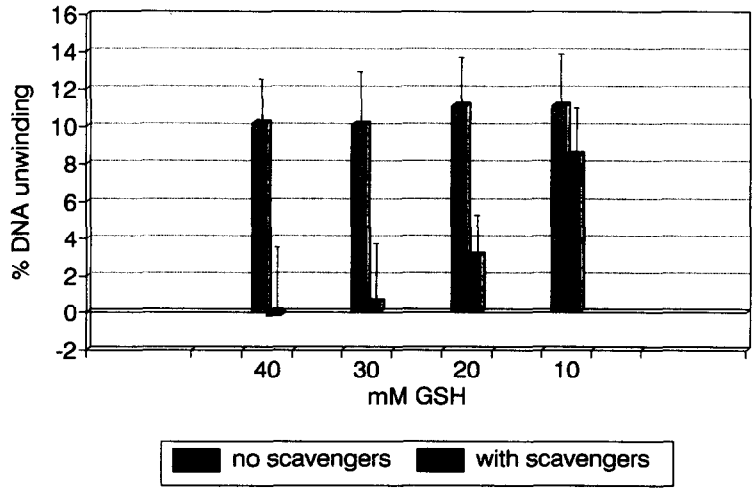

Fig. 5. Threshold of radical scavenging activity of glutathione in association with subliminal amounts of vitamin E (1 I.U./ml). GSH = reduced glutathione. Other abbreviations as in previous tables and figures.

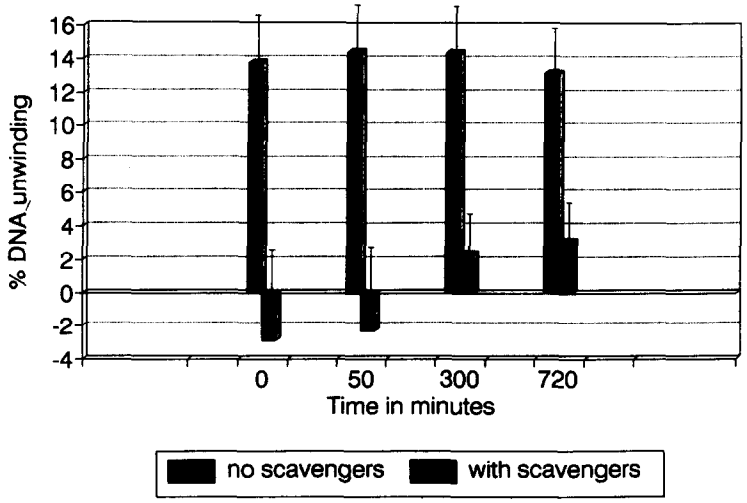

Fig. 6. Persistence of clastogenic activity induced by ultrasound treatment on human serum, in the presence or absence of oxygen radical scavengers (1 I.U./ml vitamin E and 20 mM glutathione).

ity involves oxygen radicals and elicits formation of clastogenic factors that are able to cross the cell membrane. Since HO$^{\bullet}$ scavengers (such as mannitol and reduced glutathione) are most effective in protecting lymphocyte DNA in our experimental conditions, we speculate that HO$^{\bullet}$ radicals may induce the production of clastogenic factors that can then cross the cell membrane and become a source of hydroxyl radicals at the nuclear level.

Glutathione and vitamin E provide full protection of DNA at a concentration, respectively, of 30 mM and 20 mM (that is 12.6 I.U./ml). In addition, the combination of subliminal amounts of glutathione and vitamin E provide complete DNA protection, confirming that the two scavengers act synergistically. Synergistic effects between vitamin E and vitamin C have been reported.[23] Vitamin C and reduced glutathione have also been reported as effective recyclers of vitamin E in rat liver cells undergoing lipid peroxidation.[26]

In conclusion, the results of our study suggest that oxygen radicals may be directly and indirectly involved in the clastogenic activity induced in human serum by pulsed ultrasound.

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ABBREVIATIONS

cyt *c*—cytochrome *c*
ds DNA—double-stranded DNA
EDTA—ethylene diaminetetraacetic acid disodium salt-2-hydrate
FADU—Fluorometric Analysis of DNA Unwinding
I.U.—International Units
ORS—oxygen radical scavengers
SOD—superoxide dismutase
vitamin E—d,l-α-tocopheryl acetate