Cell Membrane Permeabilization of Human Erythrocytes by Athermal 2450-MHz Microwave Radiation

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***Abstract*—The effects of low-level microwaves (2.45 GHz) on the membrane of human erythrocytes were studied measuring the hemoglobin loss and osmotic resistance of erythrocytes exposed to different power densities (0.025–10.0 mW/cm^2) at different irradiation times. A significant increase of the hemoglobin loss by exposed erythrocytes as well as a strong dependence of the rate of the increase of hemoglobin loss on the initial level of spontaneous hemolysis were observed. It was found that at low power densities (0.84 and 1.36 mW/cm^2), the hemolysis degree increases quasi-linearily with the exposure time, while at higher density (5 mW/cm^2), this tendency is reversed after first 10 h of irradiation. It appears like long-term irradiation exerts a protective effect against spontaneous hemolysis caused by blood ageing. The osmotic fragility test performed on samples exposed to 5 mW/cm^2 at different irradiation times showed that the osmotic resistance of exposed erythrocytes increases in time, reaching a maximum at the end of irradiation (60 h), while the osmotic resistance of the controls is constant.**

***Index Terms*—Biological cells, effects of electromagnetic radiations, membrane.**

I. Introduction

THE purpose of this paper is to examine the effects of long-term exposure of human blood to 2.45-GHz continuos wave (CW) radiation at athermal power densities. This microwave frequency was chosen due to its predominance in industrial, scientific, medical, and domestic use. The power densities used were less than 10.0 mW/cm^2 according to Keillman [1] who theoretically demonstrated that up to this power density, the temperature raise in biological 1-μm-sized entities (cells) is less than 10^{-5} °C. The irradiation effects were characterized by measuring: 1) the hemoglobin loss (expressed as hemolysis degree $\propto$) during and after irradiation at different power levels (0.025–10.0 mW/cm^2); 2) Coulter counter control of irradiated blood; 3) kinetics of hemoglobin loss; and 4) kinetics of osmotic fragility of erythrocyte membrane during irradiation. The reversibility of the membrane changes after the microwave irradiation was also checked by washing irradiated samples and testing their hemoglobin loss comparatively to controls.

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II. Experiments and Results

A. Experimental Microwave Setup

The experimental setup (Fig. 1) allows simultaneous irradiation of blood samples with different power levels, cf. Sajin *et al.* [2]. In this figure, the microwave power generator (1) provides 2.45-GHz CW in a power range up to 5 W. This power is applied via the insolator (2) to 10-dB directional couplers (3) that allow simultaneous irradiation of up to three sets of blood samples. The variable attenuator (5) allows power adjustment. The group built by the dual directional coupler (6), the two synchronized microwave switches (7), and the power meter (9) with its detector (8) allows the measurement of incident and reflected powers from the applicator (11). The effective power level irradiated by the applicator (11) is obtained as the difference between incident and reflected powers.

The irradiation chamber consists of an $R32$ waveguide (12) containing the aliquots ensemble (13) made in a polystyrene block ($\varepsilon \cong 1$) with the dimensions $72 \times 34 \times 8$ mm^3, in which ten holes with 5-mm diameter and 32-mm depth were made so that these holes fill the whole cross section of waveguide (12). The holes were filled with human blood up to 25 mm (approx. 3/4 of their height) so that the blood quantity contained in each aliquot is approx. 0.5 mL (approx. 5 mL of blood in all aliquots). The microwave radiation is applied to the blood samples through an applicator made of a coaxial to $R32$ waveguide adapter (11). A second $R32$ waveguide to coaxial adapter (14) close this irradiation chamber. The emergent power from the irradiation chamber is measured by another power meter (15) with detector (16). The difference with the incident power gives an indication on the power effectively absorbed by the blood in aliquots. First measurement was performed for the maximum incident power density (10 mW/cm^2) in order to see if the microwave power effectively absorbed by the blood does or does not modify its temperature. In this respect, the input power level was adjusted for a value $P_{\text{in}} = 245.6$ mW, which means an incident power density of 10 mW/cm^2 on the surface of an $R32$ waveguide aperture. The output power level from the irradiating chamber was measured as $P_{\text{out}} = 118.4$ mW. The power absorbed by the polystyrene volume is approx. 10 mW. It follows that the ten aliquots containing blood samples absorb a power $P_{\text{abs}} = 117.2$ mW, which corresponds to an athermal microwave power density of approx. 5.5 mW/cm^2 calculated for the effective surface of blood samples. Moreover, during the irradiation, the aliquots were maintained in a temperature controlled water bath (4 °C).

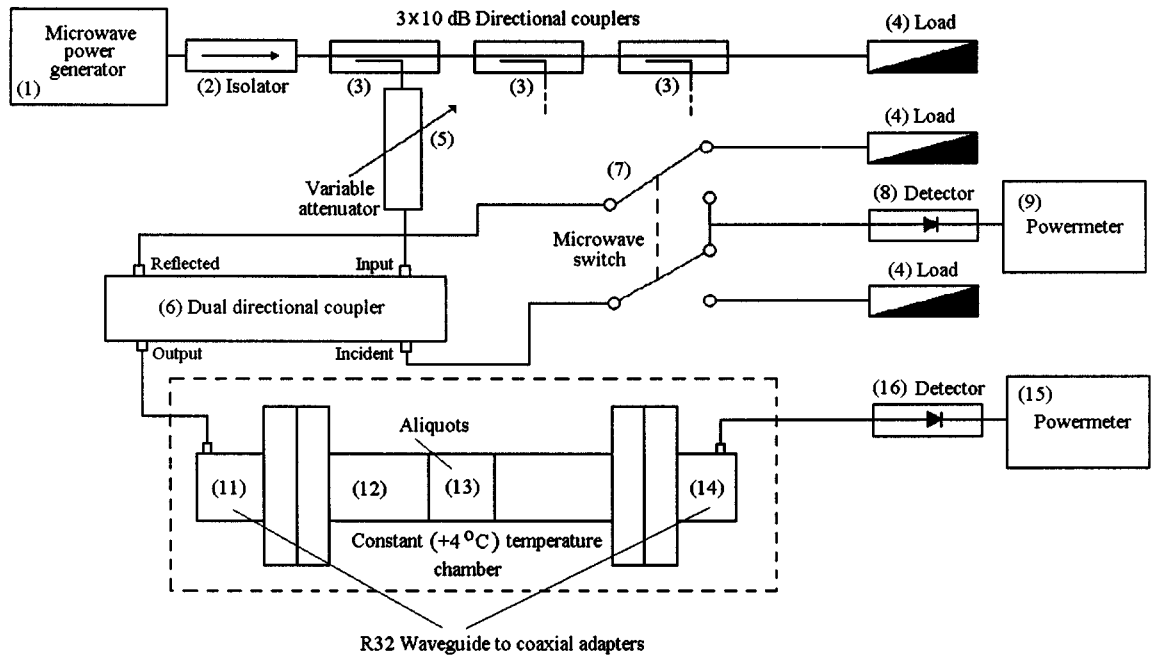

Fig. 1. Microwave experimental setup.

B. Hemoglobin Release Measurements

Blood samples from six different donors (men between 25–40 years, blood group O1) were collected in isotonic ACD (52-mmol/L citric acid, 119-mmol/L sodium citrate, 136-mmol/L glucose) at 1 : 5 v/v dilution. The samples were divided into 1-mL aliquots. In each experiment, ten of these aliquots were exposed to the microwave radiation and ten were used as controls.

Irradiation with a 2.45-GHz electromagnetic field was performed using the previously described experimental setup at different power densities, between 0.025 mW/cm^2 and 10.0 mW/cm^2, during 60 h (except the experiments for kinetic measurements, when exposure time was up to 84 h). During the irradiation, the aliquots were maintained in a temperature-controlled water bath (4 °C).

After irradiation, the free hemoglobin of both control and irradiated samples was measured. For this purpose, the suspension from each aliquot was washed in phosphate buffered saline (PBS) (0.122-M NaCl, 0.030-M $KH_2PO_4 + Na_2HPO_4$, pH 7.4, 2-g/L glucose, 310 mOsmol/kg) by diluting it in 3-mL PBS. The suspension was then homogenized and centrifugated for 30 min at 500 × g/4 °C. The samples were divided into 1-mL aliquots. The hemolysis degree α is defined as the ratio of the supernatant optical absorbances at 420 nm of irradiated sample to the optical absorbance of a blood sample belonging to the same donor, totally hemolyzed by osmotic shock. Optical absorbances were determined by spectrophotometry.

The main results are indicated in Table I where α_0 is the hemolysis degree of the fresh prelevated blood unit (initial level of spontaneous hemolysis) for each donor. One can see that obvious discrepancies between irradiated and control samples appear already at 0.025 mW/cm^2 60-h irradiation.

It was found that at the mentioned power level densities, irradiation induces a significant hemoglobin loss with a linear dependence of the hemolysis degree α on the logarithm of the incident power density. The increase rate of the hemoglobin loss with logarithm of power density was found to be highly dependent on the initial level of spontaneous hemolysis α_0 for all donors.

TABLE I
PERCENTUAL INCREASE OF HEMOLYSIS DEGREE (α) OF IRRADIATED BLOOD SAMPLES COMPARED TO CONTROLS (α_0): $[\alpha(\%) = (\alpha - \alpha_0)/\alpha_0 \times 100]$. EACH VALUE (IS THE AVERAGE OF EIGHT VALUES OBTAINED FROM DIFFERENT EXPERIMENTS PERFORMED IN THE SAME CONDITIONS. IRRADIATION TIME: 60 h

Blood unit	Microwave power density (mW/cm^2)								
	.0250	0.050	0.100	0.250	0.500	1.000	2.500	5.00	10.00
1	---	18.27	---	---	46.19	---	---	75.12	---
2	---	11.2	---	---	30.4	---	---	65.6	---
3	2.6	---	---	8.85	---	---	24.5	---	---
4	29.8	---	---	49.25	---	---	129.3	---	---
5	---	8.2	---	---	27	---	---	57.1	---
6	---	---	14.5	---	---	38.5	---	---	85.5

The blood count provided by a Coulter counter for buffer-washed samples indicate that the number of red blood cells (RBCs) is essentially the same in irradiated and control samples (Table II).

The hemoglobin content value indicated by the mean cellular hemoglobin concentration (MCHC—Table II) was, however, lower in irradiated than in control samples. This suggest that hemoglobin release induced by microwave irradiation is due to membrane permeabilization rather than to the lysis of the RBCs.

C. Reversibility of Irradiation Effect

By "reversibility," we mean the postirradiation recovery of the erythrocyte membrane permeabilized for hemoglobin during microwave irradiation. In order to check for possible reversibility of radiation effects, the pellets were washed by

TABLE II
BLOOD PARAMETERS OF TEN IRRADIATED AND TEN CONTROL SAMPLES BELONGING TO THE SAME DONOR (BLOOD UNIT 1). POWER DENSITY: 5 mW/cm^2; TIME OF IRRADIATION: 60 h

Probes	RBC*$\times 10^{12}$(l^{-1})	Hematocrit (l/l)	MCHC (mmol/l)
Irradiated	1.08 ± 0.02	0.91 ± 0.02	28,4 ± 0.03
Non-irradiated	1.04 ± 0.03	0.87 ±0.02	33.4 ± 0.30

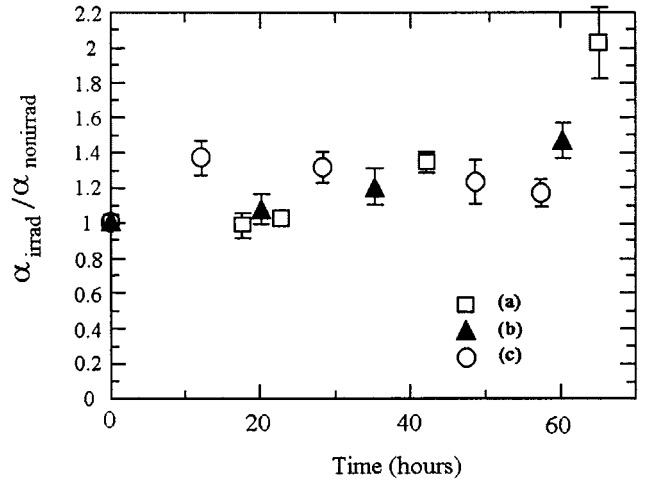

Fig. 2. Kinetics of hemolysis of the irradiated blood samples on exposure to: (a) 0.8 mW/cm^2, (b) 1.36 mW/cm^2, and (c) 5.00 mW/cm^2. Each plotted value is the average of ten values obtained from different samples belonging to the same donor. These plots are performed only for one donor (one blood unit).

centrifugation, resuspended in 3-mL PBS and left for 24 h at 4 °C. After this time, the hemolysis degrees of all samples were determined again. The measurements showed that irradiated samples are losing the same amount of hemoglobin as controls so that the membrane permeabilization caused by irradiation seems to be reversible. The only way to explain this result is that microwave-induced hemoglobin leakage of the irradiated erythrocytes occurs via transient permeabilization of their membranes. This is in agreement with other studies (i.e., [3], [4]) describing that, in the microwave irradiated liposomes, the lipid membrane becomes transiently leaky for entrapped carboxyfluorescein.

D. Kinetics of Hemoglobin Loss

For kinetic measurements, the hemoglobin loss of the irradiated and control samples belonging to the same donor was measured at different exposure times, up to 84 h, using three constant power densities: 0.84, 1.36, and 5.00 mW/cm^2 [see Fig. 2(a)–(c)].

The ratio $\alpha_{\rm irr}/\alpha_{\rm control}$ versus exposure time was plotted and errors were calculated according to limited error method, cf. [6].

While at low power densities (0.8 and 1.36 mW/cm^2), there is a quasi-linear increase of the hemolysis degree with the time of irradiation [see Fig. 2(a) and (b)] at higher power density (5 mW/cm^2 or more) this tendency seems to reverse after first 10 h of irradiation [see Fig. 2(c)]; one can see that hemolysis decreases for the irradiation times between 10–60 h. It appears like long-term irradiation would exert a protective action against spontaneous hemolysis caused by blood cells aging. The only reasonable explanation for this seems to be that the spontaneous hemoglobin loss of controls increases faster than that of the exposed samples.

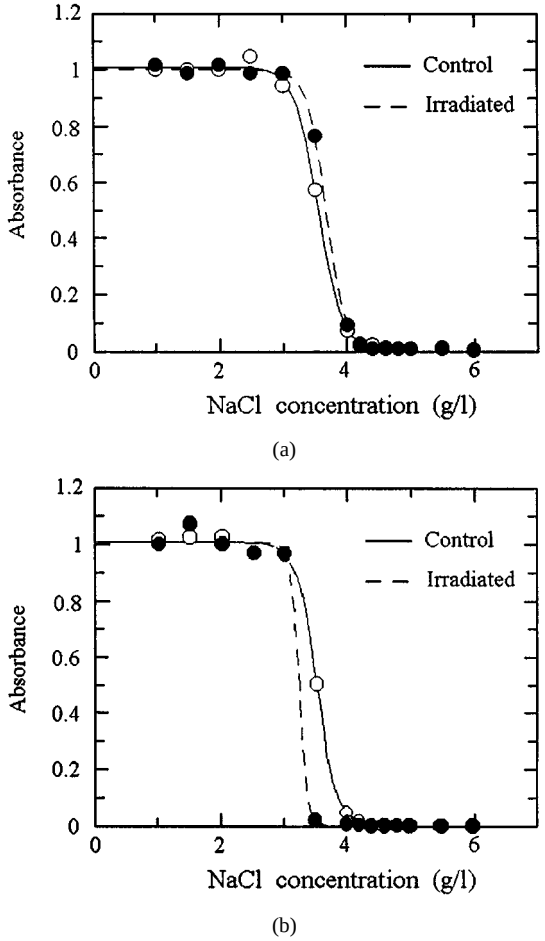

Fig. 3. Osmotic resistance of erythrocytes irradiated with 2.45-GHz 5.00-mW/cm^2 for: (a) 17 and (b) 84 h. Each point is an average of data from ten different aliquots belonging to the same donor. Error bars (corresponding to standard deviations) are not visible, being less than dots diameter.

E. Osmotic Fragility Measurements

In order to understand the apparent protective effect observed at prolonged irradiations with 5.00 mW/cm^2, kinetic records of hemoglobin loss were paralleled by the study of time dependence of the osmotic resistance of the irradiated red cells [see Fig. 3(a) and (b)]. The osmotic fragility test was performed on samples exposed to 5.00 mW/cm^2 at different irradiation times.

After collection of the supernatant for the spectrophotometric measurement of Hb release, the remaining pellets were washed twice by dilution with 3-mL PBS and 20-min centrifugation at 500×g in order to remove any extracellular hemoglobin. The pellets of the last centrifugation were resuspended in 4-mL PBS and used for the osmotic fragility test. In view of this, ten sets of 14 test tubes containing 5-mL NaCl buffered solutions of gradually decreasing concentration (from 0.60% to 0.1%) were prepared. An aliquot of 50 μL of the erythrocyte suspension was added to each of the first five sets and 50 μL of control erythrocyte suspension to the other five sets. The suspension were gently stirred and left for 30 min at room temperature, followed by 30-min centrifugation at 500×g/4 °C; the optical absorbance of the supernatant was measured at 420 nm.

Measurements show a time increasing of the osmotic resistance of exposed erythrocytes, reaching a maximum at the end of irradiation time (84 h) while the osmotic resistance of controls stay constant [see Fig. 3(b)].

Correlating the variation of hemoglobin leakage with the variation of osmotic resistance after 10 h of irradiating time [see Fig. 3(a) and (b)], one may conclude that, in irradiated cells, there is a kind of protecting mechanism that tends to limit hemoglobin loss and seems also to increase the membrane osmotic resistance.

III. Discussion and Conclusions

Summarizing our observations, the following may be said.

1) The microwave-induced increase of hemoglobin loss by irradiated erythrocytes is up to 80% of the spontaneous hemoglobin loss by the controls.
2) This increase reaches a saturation below 10 h for power density levels of 5.00 mW/cm^2. For lower power levels, hemoglobin loss reaches saturation in longer time intervals (about 30 h for 1.36 mW/cm^2 and more than 60 h for 0.84 mW/cm^2).
3) The rate of increase of hemoglobin loss versus the logarithm of the increasing power density is highly dependent on the initial level of spontaneous hemolysis. It looks like the membrane is as much sensitive to the irradiation power as it was leakier at the start.
4) The apparent "protective" effect of long term exposure at 5.00 mW/cm^2 seem to belong to the category of the nonlinear effects of electromagnetic fields reported earlier [5].

   Of primary interest is the mechanism by which microwave radiation causes an increase in hemoglobin loss. As a result of these experiments, one may consider that the hemoglobin is lost via permeabilization of the cell membrane and not due to cell lysis. The main argument for this is the drastic decrease of MCHC, while the average RBC is essentially the same for the irradiated and control samples (Table II).
5) It must be noted that the microwave-induced hemolysis is negligible at the power levels used in the described experiments. However, it may become important if the initial hemolysis is high, so that the erythrocyte membrane is already destabilized and leaky.

Probably, *in situ*, the natural protection mechanisms would defend/repair the red-cell membrane injured by prolonged interaction with high-frequency radiation. This may be the reason why a reliable reproducible effect of the microwave irradiation *in vivo* could be hardly observed.

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