A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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U. Kraliez, B. Kotelba-Witkowska, T. Pietrucha

THE STUDY ON THE EFFECT OF DMSO ON THE THROMBIN-INDUCED PLATELET AGGREGATION

Thrombin-induced aggregation have been employed to study the effect of commonly used cryoprotectant, DMSO, on platelet function. This cryoprotectant caused pronounced decrease in aggregation response of both human and porcine platelets. This harmful effect was dependent on the concentration of DMSO and incubation time. The inhibitory effect was nearly fully reversible. Spin-labelling experiments indicate that DMSO inhibition of platelet aggregation may be mediated by structural changes in membrane proteins.

Introduction

Platelet transfusion has become a common practice. Several factors including availability of donors and preparation delay the prompt provision of platelets. The problem were, in part, resolved when it was shown that platelets could be stored for up to 72 hours at 4°C or 22°C. However, the short shelf-life of platelets makes inevitable the loss of unused platelets [18]. The extreme polymorphism of HLA system presents also a significant obstacle in providing compatible platelets [3]. Only the long-term cryopreservation of single-donor platelet concentrate would offer a mechanism to prevent immunization as well as provide compatible platelets to a previously sensitized recipient [7].

According to current cryobiological theory of cell preservation by freezing, there is an optimal concentration for cryoprotective agents. Below this concentration the additional effect will be offset by the agent inherent toxity or by osmotic dama-

ge occuring while the cryoprotective agent is being removed [10]. A variety of extracellular cryoprotectants can enhance the recovery of cells following freezing. These include sugars, sugar alcohols and polymers such as polyvinylpyrrolidone and hydroxyethyl starch. However, these extracellular cryoprotectants are not efficient in reducing freezing damage to platelets [13, 15]. Numerous studies have shown that dimethyl sulfoxide (DMSO), possesses the best cryoprotective properties with respect to platelets [2, 8, 11]. However, DMSO may be harmful by itself [4, 16] and it is important to determine the amount of damage caused by this cryoprotectant. The aim of this study was to examine the influence of DMSO on platelet aggregation induced by thrombin.

Materials and methods

Preparation of platelets. Platelets were isolated from ACD human or porcine blood and they were washed in the modified Tyrode buffer (135 mM NaCl, 1 mM $MgCl_2$, 3 mM KCl, 5 mM glucose and 10 mM Tris-HCl pH 7.4). All preparation steps were run at room temperature.

Platelet aggregation measurements. Platelet aggregation induced by thrombin was studied photometrically using a conventional aggregometer equiped with stirring device. 0.1 ml of thrombin solution in saline was added to 1.4 ml of platelet suspension. 0.08 NIH of thrombin per ml of platelets suspension was used to induce human platelet aggregation. In the case of porcine platelets, thrombin concentration ranged between 0.1- 3 NIH per ml of platelet suspension was used. Platelet count was adjusted to 0.3×10^9 per ml. DMSO was dissolved in Tyrode buffer and it was added to platelets in 1 : 3 ratio. Incubation of platelets with DMSO was carried out at room temperature and aggregation measurements were performed at $37^{\circ}C$.

Spin-labeling of platelet membrane proteins. Spin label 4--(N-maleimido)-2,2,6,6-tetramethylpiperidine-1-oxyl, (MSL), synthetized by Dr. K. Gwoździński, was used in ESR study. The method of spin-labeling of membrane proteins was based on the procedure used in our laboratory in the case of erythrocyte membra-

nes [1, 5, 6]. Platelets were spin-labeled with MSL by adding appropriate amount of the label into the cell suspension. The final label concentration amounted to 2 mM. Incubation with MSL was carried out for 4 h at room temperature with a constant stirring. Unbound label was removed by repetitive washing with Tyrode buffer. No ESR signal could be found in supernatant after the third wash, indicating that all spin label present was platelet-bound. Spin-labeled platelets were treated with DMSO for 20 min. at room temperature. ESR spectra were recorded using a SE/X-28 ESR spectrometer (Wroclaw, Technical University).

Results and discusion

Typical time-course of thrombin-induced aggregation response of porcine platelet is presented in Fig. 1. With control cells aggregation reached its maximum about 9 minutes after the addition of thrombin, as reflected in light transmitance changes. Platelets incubated for 20 min. with DMSO exhibited markedly smaller aggregation response. 35% inhibition was observed for platelets treated with 2.5% DMSO. At 5% DMSO inhibition of platelet aggregation reached about 61%. Besides porcine platelets, the effect of DMSO on aggregation response to thrombin has been also studied with human platelets. The general pattern of DMSO effect on human platelets closely resembles that observed for porcine platelets. Figure 2 shows typical aggregation curves obtained for human platelets preincubated for 15 min. with increasing concentration of DMSO. Like with porcine platelets there is a pronounced decrease in aggregation response upon addition of DMSO.

The inhibitory effect of DMSO on platelet aggregation response to thrombin depends on the incubation time. Figure 3 shows typical data obtained for human platelets incubated with 1% DMSO. The inhibitory effect of the cryoprotectant increases markedly with increasing incubation time. The data obtained for human platelets are summarized in Fig. 4. This Figure shows both the effect of DMSO concentration and the effect of incubation time on thrombin-induced platelet aggregation. The inhibitory effect of DMSO increases with increasing cryoprotectant concen-

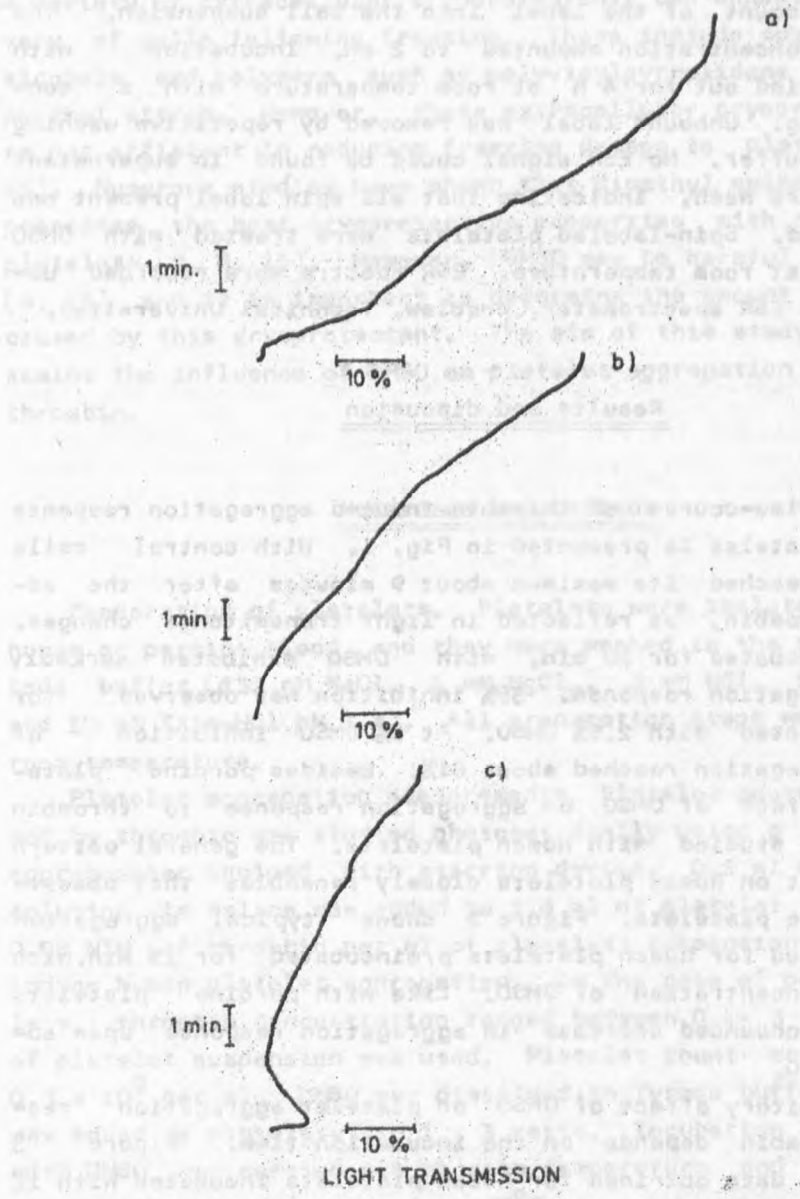

Fig. 1. Time-course of thrombin-induced aggregation response of porcine platelets. Platelets were incubated for 20 min. with DMSO
a) control (without DMSO); b) 2.5% DMSO; c) 5% DMSO

Agregacja krwinek płytkowych świni domowej. Krwinki inkubowano 20 min. z DMSO
a) kontrola (bez DMSO); b) 2,5% DMSO; c) 5% DMSO

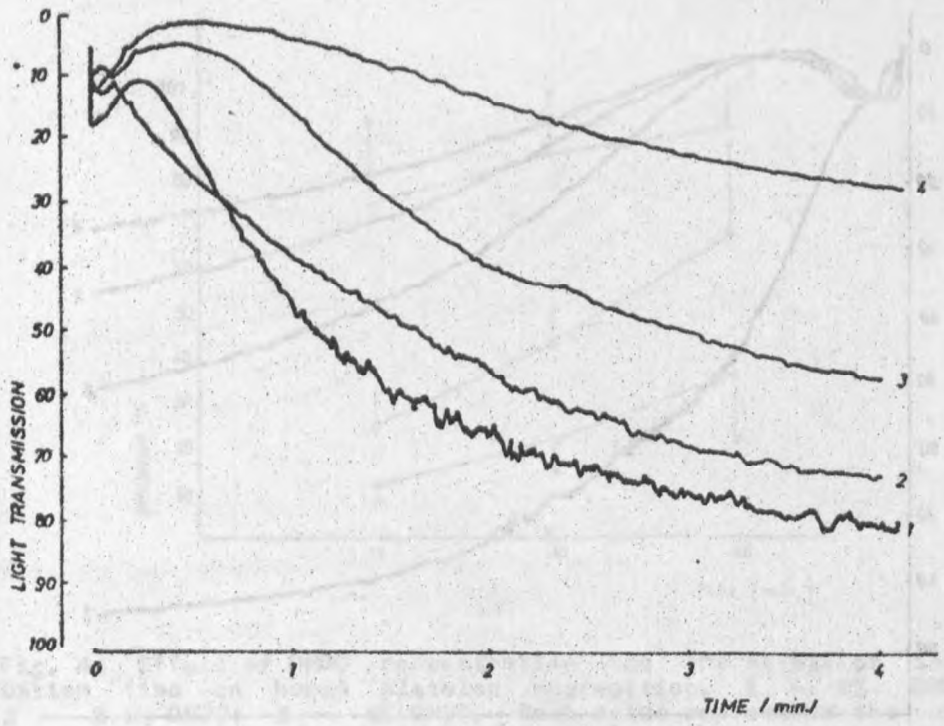

Fig. 2. Aggregation response of human platelets incubated for 15 min. with DMSO. 1 - control (without DMSO); 2 - 1% DMSO; 3 - 2.5% DMSO; 4 - 5% DMSO

Agregacja krwinek płytkowych człowieka. Krwinki inkubowano 15 min. z DMSO. 1 - kontrola (bez DMSO); 2 - 1% DMSO; 3 - 2,5% DMSO

tration. For a given DMSO concentration the effect increases with increasing incubation time. DMSO induced inhibition of the platelet aggregation response appears to be almost fully reversible. Figure 5 shows that after washing of 5% DMSO, aggregation response to thrombin was nearly the same as in the case of untreated platelets. On washing off DMSO aggregation reached about 94% of its control value in the case of porcine platelets and about 90% in the case of human platelets. The obtained in this study results are compatible with those of C h i f f e r et al. [17]. Our data confirm the observation that DMSO in the concentration range of 1-5% perturbs the

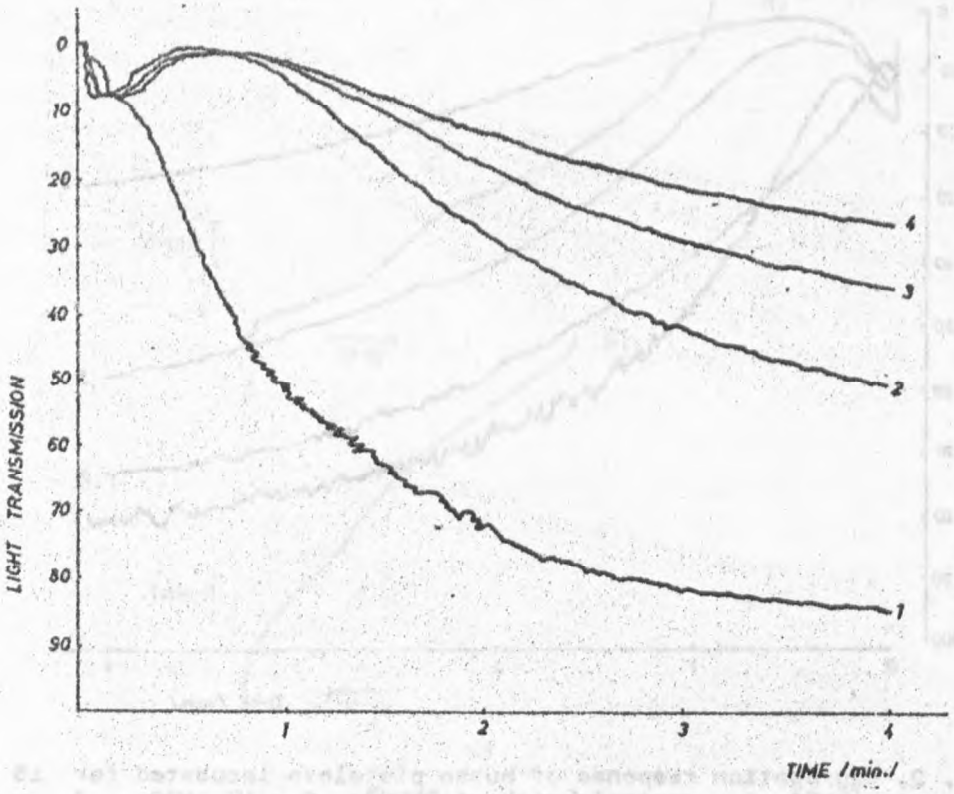

Fig. 3. Aggregation response of humen platelets incubated with 1% DMSO. 1 - control (without DMSO); 2 - 15 min.; 3 - 30 min.; 4 - 45 min.

Agregacja krwinek płytkowych człowieka. Krwinki inkubowano z 1% DMSO. 1 - kontrola (bez DMSO); 2 - 15 min.; 3 - 30 min.; 4 - 45 min.

functional properties of platelets and they point to the reversibility of this effect.

Several observations indicate that DMSO may act at a membrane level. This interaction may occur either by changing the interfacial water structure at cell interface or by forming hydrogen bonds between DMSO molecules and various membrane components. Both types of interactions mentioned are likely to induce diverse perturbation in membrane structure [9, 19]. Structural changes induced in platelet membrane by DMSO have been observed by spin-labeling method. A typical ESR spectrum of ma-

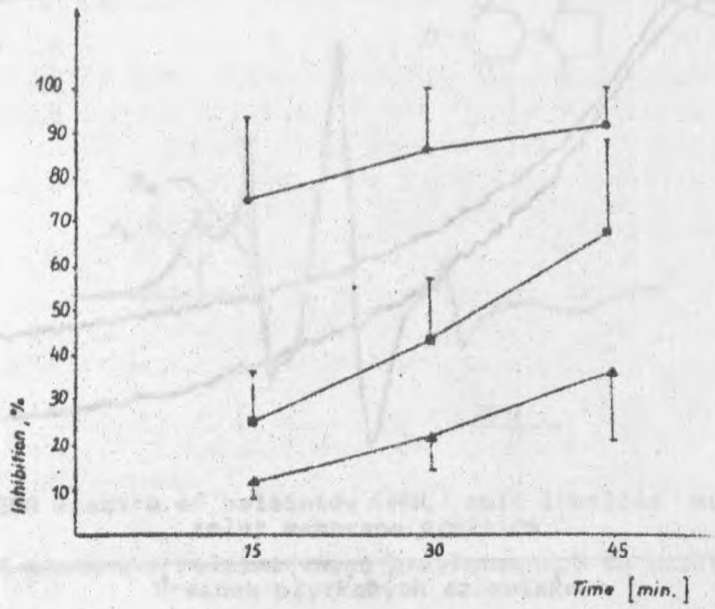

Fig. 4. Effect of DMSO concentration and the effect of incubation time on human platelet aggregation. 1 - 1% DMSO; 2 - 2.5% DMSO; 3 - 5% DMSO. Each value represents the mean ±standard deviation of seven experiments

Wpływ stężenia DMSO i czasu inkubacji na agregację krwinek płytkowych człowieka. Każdy punkt przedstawia wartość średnią ±odchylenie standardowe z 7 pomiarów

leimide spin-labeled platelets membrane proteins is shown in Fig. 6. Similar spectra have been described as reflective of at least two classes of spin-label binding sites: one strongly immobilized (S) and one weakly immobilized (W). The h_w/h_s ratio of signal height of MSL attached to weakly immobilized sites to those attached to strongly immobilized sites may be used as a convenient and sensitive monitor of protein organizational changes in the membrane [1, 11, 13]. Figure 7 shows the effect of DMSO on the above defined structural parameter. The concentration-dependent decrease in h_w/h_s ratio in DMSO treated samples indicate that this cryoprotectant produce significant structural perturbation in platelet membrane proteins. These perturbation manifest itself in a conversion of weakly im-

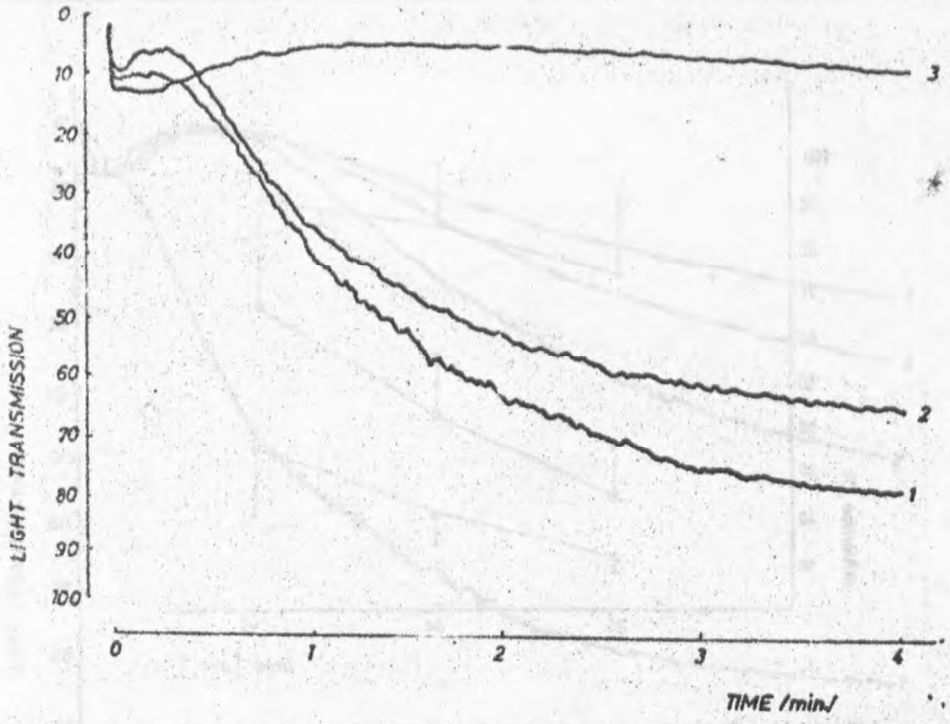

Fig. 5. Reversibility of DMSO-inhibition of human platelets aggregation. 1 - control (without DMSO); 2 - after washing of DMSO; 3 - 5% DMSO

Odwracalność hamowania agregacji krwinek płytkowych człowieka. 1 - kontrola (bez DMSO); 2 - po przemyciu krwinek i usunięciu DMSO

mobilized spin-label binding sites to strongly immobilized ones. In addition, the structural changes produced by DMSO appear to be fully reversible. On washing off DMSO the original spectra have been restored.

The data presented in this paper indicate that DMSO in the concentration range routinelly employed at platelet cryopreservation exerts the inhibitory effect on platelet aggregation. This effect is almost fully reversible and it is probably medieted by structural changes in membrane proteins. It may be suggested that the decrease susceptibility of thawed and washed platelets to aggregation is not due to the toxic effect of the cryoprotectant. The depressed response to aggregation stimuli of thawed platelets should be attributed rather to the cell injury during the freezing-thawing cycle.

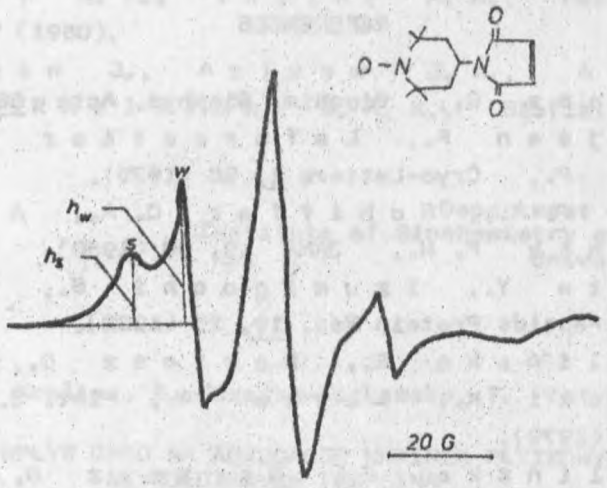

Fig. 6. ESR spectra of maleimide (MSL) spin labelled human platelet membrane proteins

Widmo EPR znacznika maleimidowego przyłączonego do białek błony krwinek płytkowych człowieka

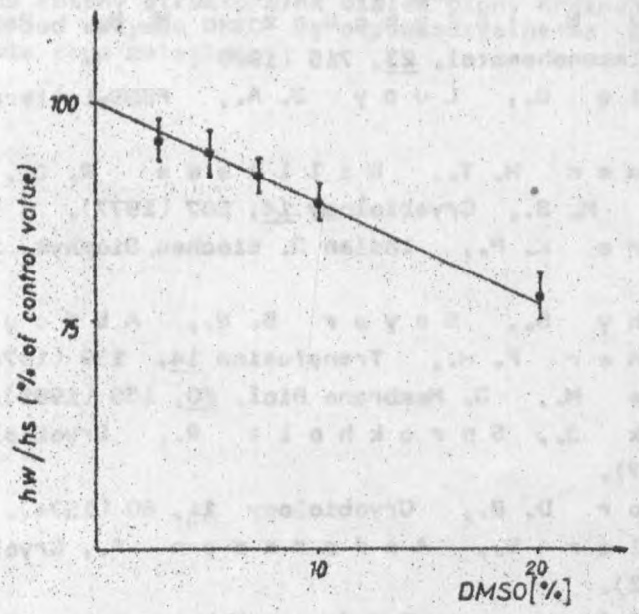

Fig. 7. Effect of DMSO on the structural parameter h_w/h_s. Data are expressed as means 5± standard errors of the mean of five experiments

Wpływ DMSO na parametr strukturalny h_w/h_s. Każdy punkt przedstawia wartość średnią ± odchylenie standardowe z 5 pomiarów

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Department of Biophysics
Institute of Biochemistry and Biophysics
University of Lodz

U. Kralisz, B. Kotelba-Witkowska, T. Pietrucha

WPŁYW DMSO NA AGREGACJĘ KRWINEK PŁYTKOWYCH STYMULOWANYCH TROMBINĄ

W pracy wykazano, że DMSO hamuje agregację krwinek płytkowych w sposób zależny od jego stężenia i czasu działania. Stwierdzono, że hamowanie agregacji ma charakter odwracalny. Wyniki uzyskane dzięki metodzie znakowania spinowego pozwalają przypuszczać, że zmiany strukturalne białek błony krwinek płytkowych zachodzące pod wpływem DMSO są odpowiedzialne za antyagregacyjne działanie tego związku.