Z. Baj, R. Olszański

FLOW CYTOMETRIC TESTING OF BLOOD PLATELETS ACTIVATION IN AIR DIVING

W artykule przedstawiono zastosowanie metod cytometrii przepływowej w badaniach płytek krwi u nurków.

INTRODUCTION:

Decompression sickness (DCS) is a life-threatening illness affecting divers. Inert gas supersaturation during decompression in divers results in gas bubbles formation leading to gas emboli and activation of coagulation cascade (5). Surface of the „silent bubbles" acts as an activator of the Hageman factor and of platelets (10, 11). Platelets are involved both in prothrombotic and in proinflammatory processes, therefore the increased activation of platelets is potentially hazardous and may form a part of DCS clinical picture. There is a very high variability in susceptibility to DCS among candidates for professional divers (5). We noted increased activation of platelets after divings in our earlier experiments employing credible but less modern laboratory tests and we suggested testing of the platelets activation markers as a tool in estimation of individual susceptibility to DCS (7, 8). Flow cytometry has been recognised the most useful and sensitive method in clinical testing and monitoring changes in the platelets system (1, 6). We hypothesise that the activation of platelets during decompression corresponds to the risk of DCS, therefore the aim of our studies was testing changes in platelets activation markers and in the white blood cells count in divers subjected to air diving.

1. METHOD

We have measured white blood cell count and activation molecules expression on whole blood platelets in 30 professional male divers (age 27.8 ±3.7 years) submitted to 0.6 MPa hyperbaric exposures corresponding to 60 m of depth for 35 min (the plateau phase). Total time of decompression was 3h 7 min (Polish Navy tables for air). Comparative group consisted of fifteen healthy male volunteers (age 29.3 ±2.9). Non of the tested divers and volunteers had taken Aspirin or any other drug affecting platelets function within 14 days before the test.

The 0.1 ml blood samples were taken from cubital vein to EDTA Vacutainers and to tubes with 1% paraformaldehyde solution immediately before and 30 min after the decompression.

We measured percentage of platelets bearing molecules using monoclonal antibodies CD42b (GPIbα), CD61 (GPIIa) (Dako A/S, Denmark), CD62p (GMP140) (Becton Dickinson, Mountain View, CA) and expression of these molecules on platelets in paraformaldehyde fixed blood samples. The molecule expression reflects a density of the molecules on the platelet surface and is expressed as a mean of fluorescence intensity (MFI). Employing flow cytometer we measured also the percentage of

microplatelets and the platelet and platelet-neutrophil aggregates according to our method described in details in Baj 1996 (1).

Mouse monoclonal antibodies (mAb) anti-human platelet glycoprotein (gp) IIIa (a part of the fibrinogen receptor - CD61) labelled with fluorescein isothiocyanate (CD61-FITC), anti-human gp Ibα (a part of the receptor for von Willebrand factor - CD42b) labelled with phycoerythrin (PE) from Dako A/S (Denmark) and anti-human platelet PADGEM (platelet activation-dependent granule external membrane protein - CD62p - activation marker for platelets active in release reaction) labelled with PE from Becton-Dickinson (Mountain View, CA) were used. Flow cytometer FACScan hardware and Lysis II software (Becton Dickinson) were employed in platelets examination and a double staining procedure for platelets examination in whole blood was applied according to the method proposed by Shattil at al. (9). Cell-Dyn 3500 apparatus (ABBOT) in platelets and leukocytes count testing was used.

The experimental protocols were approved by the Committee on the Ethics of Research in Human Experimentation at Military Medical Academy and were performed under the guidelines of the Helsinki Declaration for human research.

The exposures were carried out at the Department of Diving Gear and Underwater Work Technology, Naval Academy in Gdynia.

Statistical analysis of the tested parameters before and after the compression were done applying paired T-test. The Monna-Whitney test was used for the analysis of differences between divers and the comparative group. The probability value $p<0.05$ was considered significant.

2. RESULTS

We observed significant increase in the percentage of platelets bearing CD62P molecule which is a typical symptom of platelets activation and releasing reaction: increase from 3 ±2.3% before exposure to 5.9 ±2.4% after decompression compared to 0.9 ±1.2% in controls. Platelets aggregates were unexpectedly diminished from 12 ±2.1% to 7.2 ±2.3% after decompression. We noted marked increase in the percentage of platelets forming aggregates with neutrophils from 3.7 ±1.5% to 10.7 ±4.4% what is probably a result of simultaneous activation of neutrophils in blood (**fig.1**). Changes in other molecules expression were not significant. In the divers group the direction of changes in leukocyte count was the same, however the rise in white blood cell count from 5.4 ±1.0 k/µl to 6.4 ±2.1 k/µl and in neutrophils from 3.4 ±1.0 k/µl to 3.9 ±1.2 k/µl were not significant and the leukocyte count in divers did not differ from the values in the control group: 5.3 ±0.8 k/µl of white blood cells and 3.0 ±0.6 k/µl of neutrophils. No significant differences were noted in the examined parameters at baseline between divers and healthy volunteers except the percentage of CD62P positive platelets.

3. CONCLUSIONS

Obtained results prove activation of platelets in divers subjected to air diving. Increase in the number of platelets bearing CD62P molecules was the most significant change noted after the diving. CD62P expression on platelets is a morphological marker of release reaction, which results in the secretion of many prothrombotic substances to blood. Therefore, the more evident increase in the percentage of platelets bearing PADGEM is a direct sign of the platelets release reaction due to N_2 microbubble formation in blood (2).

Insignificant drop in the platelet count and a marked increase in the number of microplatelets suggests destruction of a small portion of platelets. Our results are similar to the results in animal model of DCS of another author, who noted fragmented

platelets circulating in blood after decompression (4). Increased number of micreplatelets accompanies activation of the coagulation system or directly results from the effect of the increased shear-stress forces on platelets (3). Increase in neutrophil count after diving seems to be a stress induced activation of marginal pool of leukocytes and the simultaneous rise in the percentage of platelet-neutrophil aggregates supports the suggestion about activation both leukocyte and platelet systems during air diving.
Flow cytometry testing of blood platelets activation markers can be a sensitive indicator of DCS threatening.

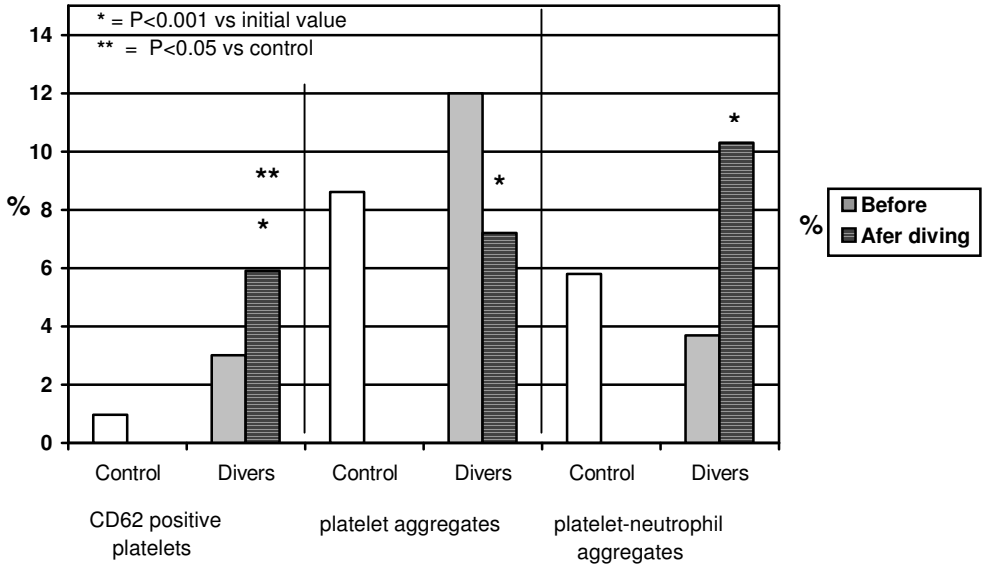

fig. 1. Changes in the percentage of CD62P positive platelets, platelet and platelet – neutrophil aggregates in blood after air diving.

Acknowledgement: The work was supported by research grant KBN Nr 4P05B 074 12

REFERENCES:

1. Baj Z. (1996). Application of flow cytometry in blood platelets testing. Centr. Europ. J. Immunol. 21, suppl. 3: 119-127
2. Berman C.L., E.L. Yeo, J.D. Wencel-Drake, B.C. Furie, M.H. Ginsberg, B. Furie (1986). A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterisation and subcellular localisation of platelet-activation-dependent granule-external membrane protein. J. Clin. Invest. 78: 130-137
3. Holme P.A., M. Roger, N.O. Solum, F. Brosstad, A.M. Larsen, T. Hovig (1996). Glycoprotein IIb-IIIa on platelet-derived microparticles, and microparticle structures studied by electron microscopy, confocal laser microscopy and crossed radio-immunoelectrophoresis. Platelets, 7207-7214

4. Kuroiwa K. (1984). The functional and biochemical changes of platelets in experimental decompression sickness of rabbits. Bull. Tokyo Med. Dent. Univ. 31: 73-84
5. Masurel G. (1989). The value of ultrasonic detection of circulating bubbles in animal and man. The contribution to physiopathogenesis of a decompression accident. Schweiz. Z. Sportmed. 37: 41-44
6. Michelson A.D. (1996). Flow cytometry: a clinical test of platelet function. Blood, 87: 4925-4936
7. Olszanski R., E. Czestochowska, I. Lipinska, Z. Sicko (1990). Effect of nitrox saturation diving upon platelet hemostasis. Bull. Inst. Marit. Trop. Med. Gdynia, 41: 59-62
8. Olszanski R., E. Czestochowska (1995). Evaluation of the risk of decompression sickness in saturated nitrox diving by examination of haemostasis. Bull. Inst. Marit. Trop. Med. Gdynia, 46: 27-30
9. Shattil S.J., M. Cunningham, J.A. Hoxie (1987). Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood, 70: 307-311
10. Softeland E., T. Framstad, A. Nordvik, I. Strand, T. Thorsen, H. Holmsen (1994). N_2 microbubbles induce a disappearance of single platelets (aggregation) with porcine platelets: a comparative study of the effects of anticoagulants and blood collection methods. Thromb. Res., 76: 61-70
11. Thorsen T., H. Dalen, R. Bjerkvig, H. Holmsen (1987). Transmission and scanning electron microscopy of N_2 microbubble-activated human platelets in vitro**.** Undersea Biomed. Res., 14: 45-58

Recenzent: prof. dr hab. n. med. K. Dęga

Autorzy:
prof. dr hab. med. Z. Baj – Uniwerstyet Medyczny w Łodzi,
dr hab. med. R. Olszański – Wojskowy Instytut Medyczny.