Journal of Biosciences and Medicines, 2020, 8, 125-130
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Morphofunctional Properties of Human Platelets, Treated with Low Doses of Hydrogen Peroxide

Maksim S. Makarov, Maya V. Storozheva

Laboratory of Biotechnologies and Transfusiology, N. V. Sklifosovsky Research Institute for Emergency Medicine of Moscow Healthcare Department, Moscow, Russia
Email: mcsimmc@yandex.ru

How to cite this paper: Makarov, M.S. and Storozheva, M.V. (2020) Morphofunctional Properties of Human Platelets, Treated with Low Doses of Hydrogen Peroxide. *Journal of Biosciences and Medicines*, **8**, 123-130.
https://doi.org/10.4236/jbm.2020.89010

Received: August 10, 2020
Accepted: September 12, 2020
Published: September 15, 2020

Copyright © 2020 by author(s) and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).
http://creativecommons.org/licenses/by/4.0/

Open Access

Abstract

We studied morphofunctional properties of human platelets, treated with low doses of hydrogen peroxide (H_2O_2). It was shown that platelet incubation with 20 - 75 µM H_2O_2 during 24 hours at 20˚C - 22˚C did not cause visible structural deficiencies in cells. Platelets maintained their adhesion activity and velocity of degranulation at 20 µM H_2O_2. 30 - 60 µM H_2O_2 dose-dependently reduced lamella's growth and allowed retaining granule bulk inside cytoplasm of spreading platelets. After 1 - 2 hours, the most effect of platelet granules' stabilization was registered at samples, previously incubated with 60 µM H_2O_2. Adrenalin activation led to rapid degranulation of stabilized platelets, whereas lamella's growth in activated platelets was low or medium.

Keywords

Platelets, Granules, Lamella, Degranulation, Stabilization

1. Introduction

Hydrogen peroxide (H_2O_2) not only provides pathological effects, but also takes part in cell signaling pathways [1] [2]. For example, H_2O_2 molecules activate cyclooxygenase and protein kinase C, uprising cytoplasmic concentration of Ca^{2+}-ions, which plays a dramatic role in human platelet activation [3] [4]. High doses of H_2O_2 (300 - 2000 µM) enhance platelet aggregation [5] [6] [7] and could even stimulate platelet activity without standard agonists [3] [6]. On the other hand, in certain studies, it was found that low doses of H_2O_2 (40 - 80 µM) reduced platelet aggregation *in vitro* [6] [8]. One could suppose, low doses of H_2O_2 stimulate stabilization of platelet granules, blocking their rapid exocytosis under

activation. All studies of H_2O_2-treated platelets were performed without morphofunctional analysis. Registration of morphofunctional platelets' properties allows valuing structure integrity of cells and their granules, and allows checking intensity of platelet interaction during cell and substrate contact.

The aim of this work was to study the influence of low concentrations on human platelets' morphofunctional properties.

2. Materials and Methods

We used venous donor blood, kept in EDTA. Platelets were extracted by double centrifugation at 300 g and 700 g. In platelet-rich plasma (PRP) total cell concentration estimated 1.0 - 1.6 × 10^9/ml. Initial clinical 3%-solution of hydrogen peroxide was diluted by phosphate buffer PBS (0.15 M, pH = 7.2) to 100 µM H_2O_2, that was measured with PRP in different proportions. In experimental samples final concentration H_2O_2 was 20 µM, 30 µM, 45 µM, 60 µM and 75 µM, control samples did not contain H_2O_2. Experimental and control PRP samples were incubated at 20˚C - 22˚C during 24 hours. To obtain the platelet adhesion, 0.1 - 0.2 ml of control and experimental PRP inflicted on the glass slide, placed in Petri dish and saturated at 37˚C for 1 - 2 hours to stimulate platelet massive adhesion. Then we washed slides with PBS buffer to remove the liquid fraction with non-active cells and studied platelets, spreading on glass. We also researched the possibility of platelet activation after H_2O_2-treatment. Platelets were exposed on glass with 45 - 60 µM H_2O_2 and activated with 1 - 5 mM adrenalin. After adrenalin activation we valued granule volume and intensity of lamella growth.

Biological integrity of platelets was studied with original morphofunctional method, based on vital fluorochrome dye (trypaflavin and acridine orange) staining [9]. This method allows valuing the number of granules and their attachment inside platelets before and during adhesion and value retaining of granules at different time. Microscopic study was managed with confocal microscope Nikon Eclipse 80i (Nikon, Japan) under objectives ×60 and ×100. In control and experimental samples we valued content of platelet with granules (%), percentage of cells with lamella during platelet adhesion, % (shows H_2O_2-dependent reduction of platelet spreading); granules retain index, % (GRI). Granules retaining analysis based on value of morphofunctional activity rate (MFAR) in control and experimental samples. We registered fluorescence intensity in normal platelets with granules, FIpl.gr. (in points), fluorescence intensity of non-granule cytoplasm in discoid platelets without membrane damage, FIcyt. (in points). In spreading platelets we valued mean $MFAR_{experiment}$ to one cell. According to our previous studies, MFAR reduction in normal cell is mainly accompanied with granules exocytosis [8] [9]. Granules retain index, GRI (%) calculated by formula GRI = ($MFAR_{experiment}$ − FIcyt): (FIpl.gr. − FIcyt) × 100%. At $MFAR_{experiment}$ = FIpl.gr. GRI estimates 100% (all cells retained all their granules), at $MFAR_{experiment}$ = FIcyt. GRI estimates 0% (all cells are totally degranulated). Received statistical

data processed using the variation statistical methods using the software package "Microsoft Excel 2000", calculating mean arithmetic values (M) and standard deviation (σ). Differences of values considered reliable in more than 95% significance level.

3. Results

In non-treated PRP level of FIpl.gr. varied from 65 to 100 points, value of FIcyt—from 25 to 35 points, and mean values were estimated 78.6 ± 6.5 and 30.6 ± 3.2 points consequently. Mean content of platelets with granules was estimated 52.5% ± 4.3%. PRP exposition with 20 - 75 μM H_2O_2 during 24 hours did not cause any visible changes in platelet structural integrity; in experimental samples FIpl.gr. and FIcyt were similar to control. Studying spreading platelets we found that 20 μM H_2O_2 had no influence on lamella's growth and platelet degranulation. After 1 hour adhesion on glass at 20 μM H_2O_2 platelet effluxed all their granules, formed wide lamellas, were tightly attached, as it took place in control samples (**Figure 1(a)**). At the opposite, at 30 μM and higher H_2O_2 concentrations, a large amount of spreading platelets reduced lamella forming ability and did not contact each other (**Figures 1(b)-(e)**). At the same time, part of granule bulk retained inside spread platelets (**Table 1**). The highest GRI rate was registered at samples, incubated with 60 μM H_2O_2, estimating 59%. 30 - 60 μM H_2O_2 provided dose-dependent decay of lamella's growth and platelet degranulation. At 75 μM H_2O_2 GRI was already lower than at 60 μM H_2O_2, and was similar to samples with 45 μM H_2O_2 (**Table 1**). Such effects depended on H_2O_2-incubation time, which ought to be 30 min or more. In samples, incubated during 5 - 10 min, spreading platelets did not maintain their granules and were able to lamella forming.

Table 1. Morphofunctional analysis of platelet adhesion after previous platelet incubation with low doses of H_2O_2 during 30 min.

Concentration of H_2O_2 in PRP	Morphofunctional parameters of spreading platelets after 1 hour adhesion at 37˚C			
	Percentage of cells with lamella during platelet adhesion, %	Percentage of adhesive cells, bearing 3 or more visible granules, %	MFAR of adhesive cells, points	Granules retain index, %
control (without H_2O_2)	97.0 ± 1.4	0	30.7 ± 1.2	0
20 μM	94.8 ± 3.2	0	31.1 ± 1.7	0
30 μM	40.0 ± 4.0*	39.5 ± 3.5*	49.3 ± 6.1*	39.1
45 μM	30.1 ± 5.3*	59.1 ± 4.2*	52.3 ± 7.9*	45.3
60 μM	20.1 ± 2.1*	67.0 ± 3.8*	59.2 ± 9.6*	59.6
75 μM	24.2 ± 3.1*	62.1 ± 3.3*	51.9 ± 6.7*	44.4

*$p < 0.05$ relative to control.

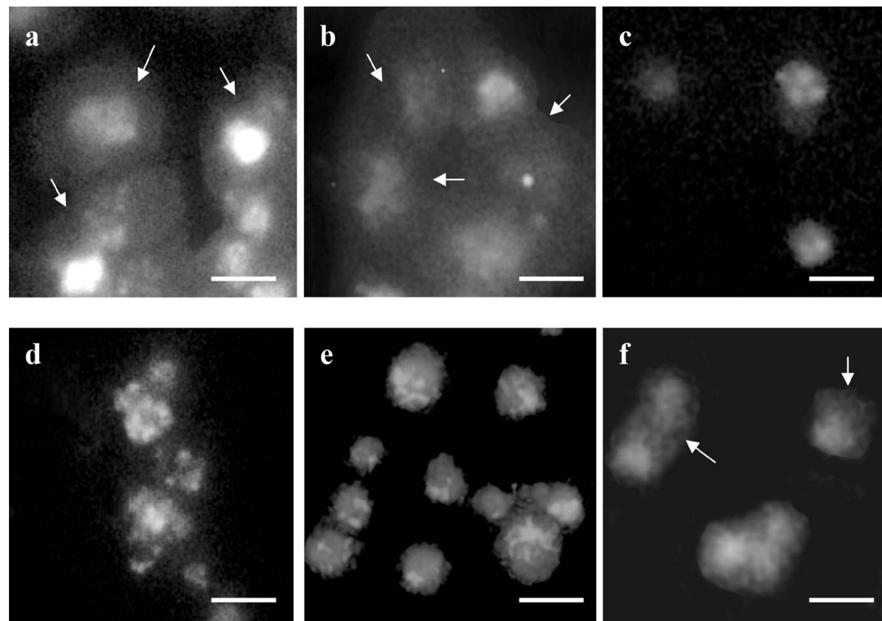

Figure 1. Influence of H_2O_2 on platelet adhesion after 1 hour contact to glass. Stained by trypaflavin and acridine orange. Magnification × 1500. Scale bar 5 μm. (a) Platelets without previous H_2O_2-incubation (control); (b) Platelets, incubated with 20 μM H_2O_2; (c) Platelets, incubated with 30 μM H_2O_2; (d) Platelets, incubated with 45 μM H_2O_2; (e) Platelets, incubated with 60 μM H_2O_2; (f) Platelets, incubated with 60 μM H_2O_2 and treated with 1 mM adrenalin. Arrows show platelet membrane invasions (lamella and lamellipodias).

Differences between platelets, incubated for 30 min and 1 - 2 hours, were statistically insignificant ($p > 0.05$). In samples, incubated during 24 hours, mean GRI was in average 1.6 times lower, comparing to samples, incubated for 30 min and 1 - 2 hours ($p < 0.05$). Obviously 30 min incubation with low concentrations of H_2O_2 is most effective for platelet granules stabilization. On the other hand, prolongation of platelet adhesion gradually reduced granule content in spreading cells. After 2 hours exposition GRI at 30 μM H_2O_2 was 22%, at 45 μM—30%, at 60 μM—51%, at 75 μM—32%. In all experimental samples, avoiding 60 μM H_2O_2, GRI in spread platelets after 2 hours was much lower than after 1 hour ($p < 0.05$). One could conclude that 60 μM H_2O_2 is most effective for platelet granule stabilization. This effect was reversible: treatment with 1 - 5 mM adrenalin caused total degranulation of previously stabilized platelets at 5 - 10 min. Noticeably, this process was followed by forming of lamellipodias, but their square was markedly lower than in control samples (Figure 1(f)).

Hydrogen peroxide significantly affects the balance of oxidizing and reduction processes (redox potential) in living cells and intracellular compartments. Molecules of H_2O_2 shift redox potential to the area of positive values, stimulating oxidized forms, which are observed during cell aging, apoptosis and other pathophysiological processes [1] [10] [11]. In platelets H_2O_2 is involved in activation cascade, so hydrogen peroxide may be used to initiate blood clotting in medical practice [12] [13]. The redox potential shifting to negative area turns

H_2O_2 into H_2O molecules and inhibits the active action of peroxide. However, it is worth emphasizing that high negative levels of redox potential intensively transit -S-S-links into -SH groups in platelet integrin proteins, stimulating platelet adhesion and aggregation [14]. One could suppose, activating of platelet adhesion receptors occurred in reduction medium, that could be delayed by low doses of H_2O_2 (30 - 75 μM) and blocks platelet rapid spreading. As a result, platelet granules retain inside spreading platelets for a long time. Nevertheless, decay of lamella's growth may prevent intensive platelet degranulation [4] [10], *i.e.* substrate-contacting platelets without lamella could be activated faster than platelets with wide lamella. Our study showed that 30 - 75 μM H_2O_2 inhibits growth of platelet lamella, the maximum preservation of platelet granules was similar to observed in experiments, where platelets were incubated with 2.5 μM nanosilver [15]. At the same time, adding of 2.5 - 5.0 μM nanosilver to platelets, which have already begun to spread, retained more than 70% of granules bulk, maintaining lamella's growth. Thus, suppression of the lamella forming does not mean complete inactivation of platelets. Moreover, the exposure of H_2O_2-stabilized platelets with activating doses of adrenaline causes rapid degranulation of platelets. Apparently, the described decay of platelet aggregation under low doses of H_2O_2 may be primarily due to decrease of platelets contact and inability to form close connections between many cells. Stabilization of platelets with low doses H_2O_2 is actualized for producing of biotransplants, enriched by platelet components, especially for wound covers in regenerative and emergency medicine.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Buettner, G.R., Wagner, B.A. and Rodgers, V.G.J. (2013) Quantitative Redox Biology: An Approach to Understanding the Role of Reactive Species in Defining the Cellular Redox Environment. *Cell Biochemistry and Biophysics*, **67**, 477-483. https://doi.org/10.1007/s12013-011-9320-3

[2] Jones, D.P. and Sies, H. (2015) The Redox Code. *Antioxidants & Redox Signaling*, **23**, 734-746. https://doi.org/10.1089/ars.2015.6247

[3] Loiko, E.N., Samal, A.B. and Shulyakovskaya, S.M. (2003) H_2O_2-Induced Platelet Aggregation and Increase in Intracellular Ca^{2+} Concentration Are Blocked by Inhibitors of Intracellular Signaling. *Biochemistry*, **68**, 1210-1216. https://doi.org/10.1023/B:BIRY.0000009135.85883.65

[4] Offermanns, S. (2006) Activation of Platelet Function through G Protein-Coupled Receptors. *Circulation Research*, **99**, 1293-1304. https://doi.org/10.1161/01.RES.0000251742.71301.16

[5] Loiko, E.N., Samal, A.B. and Lapsha, V.I. (2004) Disaggregation of Platelet Aggregates Formed by the Action of Thrombin in the Presence of Hydrogen Peroxide. *Biochemistry*, **69**, 602-606. https://doi.org/10.1023/B:BIRY.0000033732.00565.e7

[6] Vavaev, A.V., Buryachkovskaya, L.I., Uchitel, I.A., Tishchenko, E.G. and Maksi-

menko, A.V. (2012) Effect of Hydrogen Peroxide and Catalase Derivatives on Functional Activity of Platelets. *Bulletin of Experimental Biology and Medicine*, **152**, 307-312. https://doi.org/10.1007/s10517-012-1515-0

[7] Vavaev, A.V., Buriachkivskaia, L.I., Tishchenko, E.G., Uchitel, I.A. and Maksimenko, A.V. (2012) Antioxidant and Antiaggregant Effects of Covalent Bienzyme Superoxide Dismutase-Chondroitin Sulfate-Catalase Conjugate in Platelet Interactions. *Biomeditsinskaya Khimiya*, **58**, 300-309. (In Russian) https://doi.org/10.18097/pbmc20125803300

[8] Bulanova, K.J., Sydorenko, V.N., Lobanuk, L.M., Gerasimovich, N.V., Kobjashev, A.A., Zhiv, A.Yu. and Bokut, O.S. (2009) Role of Reactive Oxygen Species in Platelet Functional Activity Changes of Pregnant Women with Gestosis. *Medicinsky Zhurnal*, **28**, 25-29.

[9] Makarov, M.S., Kobzeva, E.N., Vysochin, I.V., Borovkova, N.V. and Khvatov, V.T. (2014) Morphofunctional Analysis of Human Platelets by Vital Staining. *Bulletin of Experimental Biology and Medicine*, **156**, 409-412. https://doi.org/10.1007/s10517-014-2360-0

[10] Dayal, S., Wilson, K.M., Motto, D.G., Miller, F.J.Jr., Chauhan, A.K. and Lentz, S.R. (2013) Hydrogen Peroxide Promotes Aging-Related Platelet Hyperactivation and Thrombosis. *Circulation*, **127**, 1308-1316. https://doi.org/10.1161/CIRCULATIONAHA.112.000966

[11] Pushkina, T.A., Tokayev, E.S., Popova, T.S. and Borodina, E.N. (2016) Superoxide Dismutase as a Component of Antioxidant Therapy: Current State of the Issue and Prospects. A Literature Review. *Sklifosovsky Journal of Emergency Medical Care*, **4**, 42-47. (In Russian)

[12] Korotkevich, A.G., Antonov, U.A., Serebrennikova, E.V., Leontiev, A.S. and Kuznetsov, V.V. (2012) Effectiveness of Infiltration Endoscopic Hemostasis in Patients with Upper Gastrointestinal Bleeding. *Neotlozhnaya Medicinskaya Pom*, **3**, 16-20. (In Russian)

[13] Medvedev, I.N., Plotnikov, A.V. and Kumova, T.A. (2008) Rapid Normalization of Platelet Hemostasis in Patients with Arterial Hypertension and Metabolic Syndrome. *Russian Journal of Cardiology*, **2**, 43-46. (In Russian)

[14] Murphy, D.D., Reddy, E.C., Moran, N. and O'Neill, S. (2014) Regulation of Platelet Activity in a Changing Redox Environment. *Antioxidants & Redox Signaling*, **20**, 2074-2089. https://doi.org/10.1089/ars.2013.5698

[15] Makarov, M.S., Borovkova, N.V. and Storozheva, M.V. (2017) Morphofunctional Properties of Human Platelets Treated with Silver Nanoparticles. *Bulletin of Experimental Biology and Medicine*, **164**, 241-246. https://doi.org/10.1007/s10517-017-3966-9