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Assessment of platelet function in endogenous hypercortisolism

Ocena funkcji płytek krwi u pacjentów z endogenną hiperkortyzolemią

Renata Świątkowska-Stodulska[1], Andrzej Mital[2], Piotr Wiśniewski[1], Anna Babińska[1], Anna Skibowska-Bielińska[3], Krzysztof Sworczak[1]

[1]Department of Endocrinology and Internal Medicine, Medical University of Gdańsk, Poland
[2]Department of Haematology and Transplantology, Medical University of Gdańsk, Poland
[3]General Clinical Laboratory, Clinical University Centre, Gdańsk, Poland

Abstract

Introduction: It is commonly known that glucocorticoids exert a significant effect on haemostasis. Studies that have analysed the plasmatic coagulation system and fibrinolysis parameters in hypercortisolaemic patients are abundant. Platelet function, which plays a vital role in primary haemostasis, is much less clear in this context.
We aimed at assessing platelet function in endogenous hypercortisolaemic patients.
Material and methods: Twenty-five hypercortisolaemic patients were included in the study. Twelve of them were diagnosed with overt Cushing's syndrome (OCS) and 13 had subclinical Cushing's syndrome (SCS). Thirty healthy volunteers comprised the control group. In all subjects platelet function parameters were examined: ADP- and collagen-induced platelet aggregation (ADP-IPA and Col-IPA, respectively), IMPACT R (expressed as percentage of surface covered (SC) by platelets and average size (AS) of the adhering particles in μm^2), as well as closure time (CT) after platelet activation with agonists: ADP and Col or Col and epinephrine (EPI). The statistical significance level was set at 0.05.
Results: There was no significant difference in mean values of ADP-IPA, Col-IPA, Col/EPI CT, Col/ADP CT, SC, and AS between hypercortisolaemic subjects and controls. No statistically significant differences in means of examined parameters were found between overt and subclinical Cushing's syndrome patients. Furthermore, no statistically significant relationships were found between these parameters and hormonal indicators of hypercortisolism: 24-hour urinary cortisol excretion, morning and evening serum cortisol level, and overnight-test cortisol concentration.
Conclusions: In hypercortisolaemic patients no primary haemostasis disorders are present, as reflected by platelet adhesion and ADP- and collagen-induced aggregation measurements. **(Endokrynol Pol 2015; 66 (3): 207–213)**

Key words: *primary haemostasis; platelet function; Cushing's syndrome; subclinical Cushing's syndrome*

Streszczenie

Wstęp: Powszechnie wiadomo, że glikokortykoidy to grupa hormonów wykazująca szczególny wpływ na układ hemostazy. Badania oceniające osoczowy układ krzepnięcia i fibrynolizy u pacjentów z hipekortyzolemią są dość liczne. Zdecydowanie więcej niejaności pozostawia wpływ hiperkortyzolemii na funkcję płytek krwi, odgrywających kluczową rolę w hemostazie pierwotnej.
Celem pracy była ocena funkcji płytek krwi u pacjentów z endogenną hiperkortyzolemią.
Materiał i metody: Badaniami objęto 25 chorych z endogenną hiperkoryzolemią. U 12 chorych rozpoznano pełnoobjawowy, a u 13 — subkliniczy zespół Cushinga. Trzydziestu zdrowych ochotników dobranych odpowiedno pod względem płci i wieku stanowiło grupę kontrolną. U wszystkich pacjentów oznaczono następujące parametry: agregację płytek krwi po stymulacji ADP i kolagenem (odpowiednio: ADP-IPA i Col-IPA), IMPACT R (wyrażony jako odsetek powierzchni płyty testowej pokryty płytkami krwi (SC) oraz średnia wielkość (AS) agregatów płytkowych w μm^2), będący miarą adhezji płytek krwi; jak również czas okluzji (CT) po aktywacji płytek ADP i Col lub Col i epinefryną (EPI). Poziom istotności statystycznej wynosił 0,05
Wyniki: Nie wykazano znamiennych różnic pomiędzy grupą osób z hiperkortyzolemią a grupą kontrolną w średnich wartościach ADP--IPA, Col-IPA, Col/EPI CT, Col/ADP CT, SC i AS. Nie stwierdzono również znamiennych różnic w badanych parametrach pomiędzy grupą pacjentów z pełnoobjawową hiperkortyzolemią a subklinicznym zespołem Cushinga. Co więcej nie stwierdzono znamiennych zależności pomiędzy badanymi parametrami a hormonalnymi wykładnikami stopnia nasilenia hiperkortyzolemii, takimi jak: dobowym wydalaniem kortyzolu z moczem, porannym i wieczornym stężeniem kortyzolu w surowicy oraz w teście *„over night"*.
Wnioski: U pacjentów z endogenną hiperkortyzolemią nie stwierdza się zaburzeń hemostazy pierwotnej wyrażonej adhezją oraz agregagacją płytek krwi po ADP i kolagenie. **(Endokrynol Pol 2015; 66 (3): 207–213)**

Słowa kluczowe: *hemostaza pierwotna; czynniki krzepnięcia; zespół Cushinga; subkliniczny zespół Cushinga*

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Renata Świątkowska-Stodulska M.D., Ph.D., Department of Endocrinology and Internal Medicine, Medical University of Gdańsk, tel.: +48 58 349 28 40, fax. +48 58 349 28 41, e-mail: rensto@gumed.edu.pl

Introduction

It is commonly known that glucocorticosteroids exert a significant effect on haemostasis [1–3]. A plethora of studies confirms these hormones' influence on haemostasis and fibrinolysis, which leads to increased prevalence of thromboembolic events [4–5]. On the other hand, in Cushing's syndrome patients, skin diathesis is often observed, which appears to be connected with the steroid's damaging effect on the blood vessel walls [3].

Haemostasis disorders observed in overt hypercortisolaemic patients are multifaceted. Currently, no doubt remains concerning the elevated activity of coagulation pathway as well as fibrinolysis inhibition [6–15]. The effect of hypercortisolaemia on platelet function is much less clear.

Platelets have two major roles in haemostasis. First, they form the haemostatic platelet plug. Second, they participate in the activation of the coagulation pathway by delivering negatively charged phospholipids from the inner to the outer layer of the thrombocyte membrane. In the process of coagulation three steps of thrombocyte action can be listed: adhesion, secretion, and aggregation. Collagen exposition from a damaged vessel wall leads to platelet adhesion through specific collagen receptors in the platelet cellular membrane and von Willebrand factor (vWF); during this step platelet activation takes place [16, 17]. This process, in turn, leads to the secretion of biologically active substances such as thrombin, collagen, platelet activating factor (PAF), adenosine diphosphate (ADP), thromboxane A2, and serotonin, which promote platelet aggregation and inhibit natural anticoagulation factors of endothelial cells. Optimal platelet aggregation also requires fibrinogen and calcium ions. Apart from the platelet-activating agonists mentioned, there are other factors with a minor aggregative ability, which increase the effect of other agonists [17]. One instance of such a compound is epinephrine. Under the influence of activating factors, negatively charged phospholipids phosphatidylserine and phosphatidylethanolamine are exposed, which bind with calcium ions and carbonyl groups of coagulation factors.

So far, few reports have been published on the effect of corticosteroids on platelet function, most of them were veterinary medicine studies [16, 18], and their results indicate a suppressive effect of exogenous glucocorticosteroids on blood platelet aggregation in animals [16, 19]. These observations contrast with reports on megakaryocyte function in Cushing's disease (CD) patients. It was shown, namely, that hypercortisolism may lead to vWF multimers overexpression, which can spontaneously trigger platelet aggregation [6]. Since many ambiguities remain, platelet function in hypercortisolaemic patients should be investigated more closely.

We aimed at assessing platelet function in endogenous hypercortisolaemic patients as well as verifying associations between possible primary haemostasis disorders and hormonal parameters.

Material and methods

The study was approved by the ethics committee of the Medical University of Gdańsk, Poland (NKBBN/43/2013).

In this prospective study 25 endogenous hypercortisolaemic patients were included, 21 women and 4 men, mean age 56.7 ± 11.9 years, who were hospitalised in the Department of Endocrinology and Internal Medicine of the Medical University of Gdańsk or monitored at the department's outpatient centre. Exclusion criteria encompassed patients with an acute or chronic symptomatic infection, connective tissue diseases, thrombocytopaenia, decreased activity of coagulation factors VIII and von Willebrand, women treated with oral contraceptives and hormonal replacement therapy, and individuals who used non-steroid anti-inflammatory or anti-platelet drugs in the two-week period preceding the examinations. Endogenous hypercortisolism was diagnosed in all patients. ACTH-dependent Cushing's syndrome (CS) was diagnosed in 10 patients: 6 had a corticotropinoma, in 2 patients CD recurred after surgery, and in 2 patients further ectopic ACTH secretion was present. In 15 subjects a hormonally active adrenal tumour was found: 2 subjects displayed overt hypercortisolism due an adrenocortical carcinoma, while the remaining patients had an adrenal adenoma and met the criteria of subclinical Cushing's syndrome (SCS). In all subjects physical examination and hormonal tests were performed. CS was diagnosed in patients with clinical hypercortisolaemic features: plethora, moon face, buffalo hump, central obesity, proximal muscle atrophy, and abnormalities in hormonal examinations: lack of cortisol suppression in the overnight, 1-mg-dexamethasone test, as well as two-day dexamethasone suppression test, lack of circadian cortisol secretion rhythm, and elevated 24-hour free urinary cortisol excretion. In adrenal-dependent CS, ACTH levels were suppressed (≤ 10 pg/mL), while pituitary-dependent CS had inadequately elevated morning ACTH levels, no circadian ACTH secretion rhythm, and a typical response in the corticotrophin-releasing hormone (CRH).

As defined, SCS was diagnosed in patients with an incidental adrenal mass revealed in a CT abdominal scan, who did not have typical physical features of CS, and who exhibited endogenous hypercortisolism

in hormonal tests [20–22]. Cortisol serum level of 140 nmol/L and above in the overnight 1-mg-dexamethasone suppression test was a sufficient criterion for the diagnosis of SCS. Patients with cortisol concentrations between 50 and 140 nmol/L in this test also had to have met at least one of the following criteria: abnormal serum circadian cortisol secretion, decreased morning ACTH concentration (≤ 10 pg/mL), and/or increased 24-hour free cortisol urinary excretion. These diagnostic criteria were applied to our patients [23]. Moreover, in all SCS patients cortisol was not fully suppressed (> 50 nmol/L) in the two-day low-dose dexamethasone test (0.5 mg every 6 hours).

In all adrenal-dependent hypercortisolaemic patients a mass of the gland was found in a targeted adrenal CT scan, while in all CD subjects MR of the pituitary revealed a microadenoma. All hormonal assays were performed in the same laboratory with freely available kits. ACTH serum level was determined using a solid-phase, two-site sequential chemiluminescent immunometric assay Immulite®1000ACTH by Siemens. Serum and urinary cortisol levels were tested with a Chemiluminescent Microparticle Immunoassay (CMIA), specifically — a Cortisol Reagent Kit on an Abbott's Architect device.

Our control group comprised 30 healthy individuals who were matched for age, sex, and BMI (24 women and 6 men, mean age 49.2 ± 11.6 years). In all controls full cortisol suppression in the overnight 1-mg-dexamethasone was recorded (*i.e.* serum level < 50 nmol/L), which excluded subclinical hypercortisolism. No study participant received drugs altering platelet function.

Fasting blood samples were acquired in the morning from a cubital vein.

In all subjects the following parameters were examined immediately: ADP- and collagen-stimulated platelet aggregation, IMPACT R, and PFA 100.

A multiplate electron aggregometry system, which uses impedance aggregation, was applied to assess ADP- and collagen-induced platelet aggregation (ADP-IPA and Col-IPA, respectively). Roche Diagnostics (Germany) Multiplate system and designated kits were used.

The PFA 100 (Platelet Function Analyser) is a microprocessor-controlled device that provides a measure of whole blood platelet related haemostasis at high shear stress. In this method, the closure time (CT) of an aperture containing a nitrocellulose membrane coated with platelet agonists is measured. ADP and collagen or collagen and epinephrine (EPI) are agonists that activate platelets in this method [24]. The measure of platelet function in the PFA system reflects their adhesion and aggregation in full blood. In this study the PFA system and test assays Col/EPI and Col/ADP by Siemens Healthcare Diagnostics (Germany) were used.

Impact R measures platelet adhesion in whole blood in arterial flow conditions (1800 s^{-1};for 2 min.). Laminar flow over polystyrene surface of the wall is achieved according to the Cone and Plate principle [24, 25]. Results are expressed as percentage of surface covered (SC) by platelets and average size (AS) of the adherent particles in μm^2 [24]. Tests here were performed using DiaHem (Switzerland) kit and reagents (DiaMed Impact-R test kit).

In all representatives, the estimation of basic coagulation parameters, such as international normalised ratio (INR), activated partial thromboplastin time (APTT), fibrinogen concentration, and complete blood count, was also determined with routinely used methods.

All statistical calculations were performed with the use of STATA 13.1 software (StataCorp, Texas, USA). Prior to analysis, data were screened for potential errors. Standard descriptive statistics were computed. Some variables were transformed before parametric analyses. Comparisons between groups were carried out using ANOVA test. Scheffe post-hoc test was used when indicated. Correlations were evaluated using Spearman rank correlation method. The level of statistical significance was set at 0.05.

Results

Hormonal results are presented in Table I. Clearly, the highest serum cortisol concentrations and the hormone's highest urine excretion, plainly diverging from those of other subjects, were stated for ectopic ACTH synthesis and adrenal cortex cancer patients.

A comparison of studied parameters between hypercortisolaemic patients and controls is shown in Table II. Mean values of ADP-IPA and Col-IPA in hypercortisolaemic subjects were not significantly different from those of controls ($P = 0.127$ and $P = 0.630$, respectively). Also, no statistically significant differences in mean Col/EPI CT ($P = 0.359$), Col/ADP CT ($P = 0.863$), SC ($P = 0.405$), and AS ($P = 0.209$) were found between patients and controls.

Taking into account the possible influence of neoplastic disease on thrombocyte function, a separate analysis was carried out for patients with adrenal cortex cancer and ectopic ATCH-secreting patients, who had the greatest abnormalities in hormonal examinations. No differences in mean values of studied parameters were found in patients with OCS due to malignancies and in the remaining hypercortisolaemic patients (Table II).

Table I. *Hormonal characteristics of overt and subclinical hypercortisolaemic patients*

Tabela I. *Charakterystyka hormonalna pacjentów z pełnoobjawową i subkliniczną hiperkortyzolemią*

Laboratory examination	OCS		SCS		Normal ranges
	Mean	SD	Mean	SD	
Serum cortisol [nmol/L]: morning	810.8	848.5	302.5	98.1	101–536
Serum cortisol [nmol/L]: night	654.4	701.7	203.5	93.4	–
Serum cortisol in an overnight suppression test (1 mg dexamethasone) [nmol/L]	662.3	980.3	149.2	103.9	< 50
24-hour urinary free cortisol excretion [nmol/24 h]	3699.5	7442.4	218.3	115.9	12–486
Plasma ACTH [pg/mL]: morning	70.5	69.9	13.8	5.9	15–46
Serum DHEA-S [μg/dL]	243.4	252.8	41.0	40.2	80–560

Table II. *Parameters of platelet aggregation and adhesion in hypercortisolaemic patients (whole group and after exclusion of malignant neoplasm) and healthy controls*

Tabela II. *Parametry agregacji i adhezji płytek krwi u pacjentów z hiperkortyzolemią (u wszystkich chorych oraz po wyłączeniu pacjentów z chorobą nowotworową) i w grupie kontrolnej*

	CS (OCS and SCS)		CS after exclusion of neoplasm		Control group	
	Mean	SD	Mean	SD	Mean	SD
ADP-IPA [U]	83.5	32.9	86.4	32.2	70.1	31.3
Col-IPA [U]	65.4	27.3	68.3	27.0	68.7	23.2
CT-Col/EPI [s]	145.5	93.2	148.4	94.3	127.0	52.6
CT-Col/ADP [s]	106.3	75.4	102.6	70.1	103.3	49.4
SC (%)	12.8	4.1	12.7	3.8	11.9	3.8
AS [μm^2]	53.4	23.6	49.3	19.7	45.5	22.4

Further analysis was performed, in which OCS and SCS patient subgroups were compared to healthy controls. No statistically significant differences of studied parameters were recorded (in the OCS versus controls comparisons: ADP-IPA: $P = 0.642$, Col-IPA: $P = 0.946$, CT-Col/EPI: $P = 0.509$, CT-Col/ADP: $P = 0.850$, SC: $P = 0.913$, AS: $P = 0.733$; for SCS versus controls: ADP-IPA: $P = 0.325$, Col-IPA: $P = 0.562$ CT-Col/EPI: $P = 0.945$, CT-Col/ADP: $P = 0.964$, SC: $P = 0.670$, AS: $P = 0.476$) (Table III).

Moreover, no statistically significant differences in means of examined parameters were found between overt and subclinical CS patients: ADP-IPA: $P = 0.904$, Col-IPA: $P = 0.503$, CT-Col/EPI: $P = 0.773$, CT-Col/ADP: $P = 0.777$, SC: $P = 0.903$, AS: $P = 0.943$ (Table III).

Next, we analysed the relation between ADP-IPA, Col-IPA, CT-Col/EPI, CT-Col/ADP, SC, AS values, and hormonal test results illustrating the degree of hypercortisolaemia: 24-hour urinary cortisol excretion, morning and late evening serum cortisol level, and overnight-test cortisol concentration. No statistically significant relationships were found between these parameters (Table IV).

Discussion

Studies that have analysed the plasmatic coagulation system and/or fibrinolysis parameters in endogenous hypercortisolaemic patients are abundant. Platelet function, which plays a vital role in primary haemostasis, is much less clear in this context. Most reports so far have investigated the effect of synthetic steroids on platelet count, while there are far fewer in which platelet function was analysed, and these were predominantly performed in veterinary research [16, 18]. Moreover, until now, the majority of studies have investigated the influence of exogenous steroids on platelet aggregation. In our research the effect of endogenous hypercortisolaemia on platelet adhesion as well as aggregation was tested. Furthermore, in the current study platelet function was examined in whole blood, *i.e.* in the presence of blood cells, which can modulate platelet response to aggregation stimuli.

Table III. *Parameters of platelet aggregation and adhesion in OCS and SCS patients versus controls*
Tabela III. *Parametry agregacji i adhezji płytek krwi u pacjentów z OCS i SCS w porównaniu z grupą kontrolną*

	OCS		SCS		Controls	
	Mean	SD	Mean	SD	Mean	SD
ADP-IPA [U]	80.5	33.1	86.3	33.8	70.1	31.3
Col-IPA [U]	71.6	32.2	59.8	21.6	68.7	23.2
CT-Col/EPI [s]	156.6	90.0	135.2	98.6	127.0	52.6
CT-Col/ADP [s]	115.6	88.3	97.7	63.8	103.3	49.4
SC (%)	12.5	4.9	13.1	3.3	11.9	3.8
AS [μm^2]	51.8	26.1	54.9	22.0	45.5	22.4

Table IV. *Coefficients and p values of correlations between examined platelet function and hormonal parameters (Spearman rank correlation method)*
Tabela IV. *Współczynniki korelacji pomiędzy badanymi parametrami, oceniającymi funkcję płytek krwi a parametrami hormonalnymi wg Spermana oraz poziomy istotności statystycznej p*

	Serum cortisol morning	Serum cortisol night	Serum cortisol in "overnight" test	Urinary cortisol excretion
ADP-IPA	–0.26	–0.26	–0.09	–0.15
	p = 0.204	p = 0.216	p = 0.659	p = 0.463
Col-IPA	0.02	–0.17	–0.05	0.06
	p = 0.937	p = 0.415	p = 0.819	p = 0.784
CT-Col/EPI	0.21	0.16	0.02	0.34
	p = 0.312	p = 0.459	p = 0.919	p = 0.100
CT-Col/ADP	–0.08	–0.01	–0.03	0.05
	p = 0.710	p = 0.971	p = 0.892	p = 0.805
SC	–0.08	0.07	–0.10	–0.14
	p = 0.706	p = 0.728	p = 0.624	p = 0.515
AS	–0.12	0.05	–0.04	0.10
	p = 0.581	p = 0.802	p = 0.864	p = 0.649

We found no statistically significant differences in platelet function parameters between hypercortisolaemic patients and healthy controls. This result was found both for ADP, collagen-stimulated aggregation, Impact R, and occlusion time with Col/ADP and Col/EPI assays. It is difficult to compare our results with those of other groups due to the scarcity of such research. Casonato et al. examined 20 CS patients and found elevated spontaneous platelet aggregation as well as hyper-reactivity in ristocetin-induced aggregation. They demonstrated that in active hypercortisolaemic patients over-expression of abnormally high molecular weight multimers takes place, which are capable of inducing spontaneous platelet aggregation that is found physiologically only in the cellular compartments and not in the plasma [6, 26]. This explains the elevated ristocetin aggregation. Studies where ADP- and collagen-induced aggregation were examined mainly concern the influence of exogenous steroids on these parameters; the results are discordant and thus inconclusive. Liverani et al. demonstrated *in vitro* the presence of the glucocorticoid receptor on blood platelets, and an inhibitory effect of prednisolone on their adhesion and aggregation, and therefore on the formation of the platelet plug as well as on the interaction with monocytes [27]. On the other hand, Rosenfeld et al. stated elevated collagen-induced platelet aggregation following infusion of stress hormones, among them hydrocortisone [28]. A direct effect of exogenous glucocorticosteroids on platelet activation was recorded in studies by Jilma et al., who found a statistically significant increase in P-selectin, a marker of blood platelet activation,

in healthy men after short-term administration of dexamethasone in high doses [29].

Animal models, however, indicate impaired platelet ADP-stimulated aggregation after hydrocortisone infusion. Casella et al. suggest that hydrocortisone modulates the interaction between ADP and the appropriate receptor, as well as phospholipase A2 and phospholipase C activation by thromboxane, PAF, and/or ADP [16]. These results were not confirmed by Schuerholz et al., who found no alteration of platelet receptor expression by hydrocortisone in septic patients [30]. Taking into account the results of the above-mentioned reports, the sort of glucocorticosteroid and its dose should be investigated as the reason for these contrasting findings. [16, 19].

It is understandable, therefore, that our results on the effect of endogenous hypercortisolism on blood platelet function should not be set on equal footing with reports on exogenous corticoids. The only report on ADP-, collagen-, and adrenaline-induced aggregation in CD patients was published in 1985 [31]. Ikkala et al. assessed primary haemostasis parameters in 12 endogenous hypercortisolaemic patients. Similarly to our results, no statistically significant disorders of the above parameters were found. ADP- or adrenaline-induced aggregation was borderline or subnormal in five patients, while only in one was impaired collagen-induced aggregation recorded. Our study is the first to assess platelet adhesion in vascular flow conditions (Impact R) as well as occlusion time in the PFA system, which analyses adhesion and aggregation in whole blood in endogenous hypercortisolaemic patients. It should be stressed that ADP- and collagen-stimulated platelet aggregation was tested using an impedance method, which reflects physiological conditions.

In our study no significant differences in platelet function were found between overt and subclinical CS patients, and neither did we find any relation between platelet function parameters and hormonal hypercortisolaemic parameters (*i.e.* 24-hour urinary cortisol excretion, morning and evening serum cortisol level, overnight-test cortisol concentration). This supports the result that hypercortisolism does not influence the studied parameters.

The authors recognise that the patient sample studied here is small and heterogeneous. The low incidence of endogenous Cushing's syndrome, the fact that patients treated with platelet function-altering medication had to be excluded, and the necessity of performing platelet function tests directly after drawing blood were the main reasons for the size of our sample. These difficulties probably explain the scarce body of research on primary haemostasis, particularly platelet function, in hypercortisolaemic patients.

Taking into account the possible effect of malignant neoplasm on platelet function, patients with adrenocortical cancer and ectopic ACTH-secreting ones were analysed separately. No differences in platelet function were found for them compared to other hypercortisolaemic subjects.

The results of the previous reports and our study suggest that there are no disorders in ADP- and collagen-stimulated platelet aggregation or platelet adhesion tested in the PFA and Impact R systems in endogenous hypercortisolism.

Conclusions

In Cushing's syndrome patients, no primary haemostasis disorders are present as reflected by platelet adhesion and ADP- and collagen-induced aggregation measurements.

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