INVESTIGATION OF THROMBOCYTE FUNCTION AND OXIDATIVE STRESS ON PATIENTS WITH PERIPHERAL ARTERIAL DISEASES

PhD Thesis

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ABBREVIATIONS:

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CABG	coronary artery bypass grafting
CAT	catalase
COX	cyclooxygenase
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
GP	glycoprotein
GPx	**g**lutathione peroxidase
GSH	reduced glutathione
HDL	high-density lipoprotein
5-HT	5-hidroxi-triptamin
ICAM-1	intercellular adhesion molecule-1
IP_3	Inositol triphosphate or inositol 1,4,5-trisphosphate
IR	insulin resistance
LDL	low-density lipoprotein
MLC	myosin light chain kinase
mRNA	messenger RNA
NO	nitric oxide or nitrogen monoxide
NSAID	non-steroidal anti-inflammatory drug
OCS	open canalicular system
P47	pleckstrin
PAD	peripheral arterial disease
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet derived growth factor
PGG_2	prostaglandin G2
PGH_2	prosztaglandin H2
PGI_2	prosztaglandin I_2,
PIP_2	phosphatidylinositol biphosphate
PLA_2	phospholipases A2
TCT	thrombocyte
PMA	phorbol 12-myristate 13-acetate
PPP	platelet poor plasma
PRP	platelet rich plasma
RNS	**r**eactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
T_1DM	type-1 diabetes mellitus
T_2DM	**t**ype-2 diabetes mellitus
TGF	transforming growth factor
TIA	transient ischemic attack
TRIS	tris(hydroxymethyl)aminomethane
TS	thromboxane synthase
TXA_2	thromboxane A_2
Xox	Xanthine oxidase
vWF	Von Willebrand factor

1. Introduction:

1. 1. Peripheral arterial disease and ischemia reperfusion injury

The frequency of peripheral arterial disease (PAD) has been increased parallel with the elevation of the expected lifespan of human population. The frequency of PAD in adults above 50 years is 20 %.

There are common complex pathological backgrounds of peripheral obliterative arterial sclerosis, the coronary sclerosis, and the disease of carotis system, though PAD patients have been exposed to increased risc of cardiovascular and cerebrovascular events.

The incidence of PAD among diabetic patients is higher than in the metabolically healthy subjects. The complications of PAD accompanied by diabetes are more serious, than in non diabetic people. In spite of this comparative data of thrombocyte function and antioxidant / prooxidant status of type-1 and type-2 diabetic patients (T_1DM, T_2DM) with PAD are hardly found in the scientific literature.

1. 2. The special feature of thrombocyte among physiological and pathological circumstances

Thrombocytes have important roles in the maintenance of haemostasis, in the modulation of inflammation and immunity, they have bactericidal effect, they participate in wound healing, and tissue regeneration. At the same time platelets play a major role in acute ischaemic syndromes and in peripheral vascular disease. They are involved in the development and progression of atherosclerosis, native vessel and graft thrombosis. They have a central role in the development of restenosis and reocclusion after peripheral percutaneous transluminal angioplasty.

The most important inducers of platelet aggregation are thromboxaneA_2, adenosine diphosphate (ADP), collagen, thrombin, 5-hidroxi-triptamin (5-HT) and adrenaline.

Several author described the increase of TXA synthesis in instable angina, which is indicative of the direct effect of spontaneous ischemia on platelet aggregation.

ADP dependent platelet aggregation is increased in stable angina while the antiaggregating effects of nitrogen monoxide (NO) donors are reduced. Smoking, coronary artery diseases, diabetes, artheriosclerosis, hypercholesterolemia has been resulting in increased aggregability of platelet.

Coronary artery diseases are more serious, if accompanied by diabetes. The endothelial layer maintains the equilibrium among prostanoid synthetising sysem, cyclooxigenases and prostaglandins. Inflammed endothelium induces the adhesion of platelet into the cell surface, by the increases of the expression of intercellular adhesion molecule (ICAM-1), which accompanied by the increased expression of $\alpha_2\beta_3$ cell surface integrin in the platelet side. Several kind of inflammatory and mitogenic factors are secreated by thrombocyte into the microenvironment in the course of adhesion processes, which are able to influence the chemotactic, adhesive and proteolytic nature of endothelial cells. In pathological situations platelets can respond quickly to the changes of the endothelial cells (plack rupture, fatty streaks) and the exposition of subendothelial layers.

Thrombocytes of familiar hypercholesterolemic patients are more sensitive to platelet agonists than it can be observed in healty people. Hypertensive and hypercholesterolemic patients have higher thrombomodulin level and their tombin productions are increased. The proaggregatory effect of oxydised LDL cholesterol was also revealed. Summarising the literary data, it can be concluded that several diseases accompanied by hyperaggregability of platelets.

Studiing the clinical picture of different vascular diseases may results in the better undersending the diseases, and can provide new opportunities to find new therapeutic targets

for the treatmen. This was the reason why we begun to study the trombocyte function and antioxidant/prooxidant status of patients with peripheral arterial disease.

1. 3. The biology of ischemia reperfusion injury.

Ischemia/reperfusion injury is a relevant problem in case of thrombosis, embolisation, myocardial infarction, stroke, coronary bypass surgery, balloon angioplasty, thrombolysis, revascularization surgery of lower extremity and in every case when one segment of the vessels are excluded from the circulation, and than reopened. The vessel closure and the consecutive ischemia can be caused by arterial thrombosis (which originated from embolisation, stenotic arteriopathy or trauma), artérial spasmus, external compression, or anatomic alteration.

The tolerance of tissues to ischemia varies with the nature of the tissues and depends on the presence or absence of the collateral flow. Ischemia/reperfusion injury of skeletal muscles leads to an acute inflammation, which not only affects the nutritional territory of affected vessel, but also causes remote organ injury.

The acidosis, which occurs in the ischemic area, causes tissue injury or cell death. The remaining tissues adapted to the oxygen poor environment by changing their metabolic state form aerobic to anaerobic, but finaly this strategy can lead to further tissue injury and cell death. The masure of tissue injuries are depend on the duration of hypoxia, the amount of the tissues are involved, and the systemic arterial peressure.

The restoration of the blood flow is the only way to salvage the tissue from the devastation, but it can not be made without risk, because of the accompanied volume, pressure, and metabolic stress. The main component of the pathophysiological molecular cascade is the neutrophyl activation, and the rsulted free radical production (reactive oxygen and nitrogen species (ROS, NOS respectively), and the elevated intracellular Ca^{2+} levels. In the early phase of reperfusion a sharp increase in the proinflammatory cytokines occurs. Tese factors all together threat the integrity of the organism, due to the damage of the key macromolecules (proteins, lipids and nucleic acids). The final results are the disintegration of the membranes which are responsible for the intracellular compartementalisation, rhe injured ion transport, the injury of the contractile elements and the insufficient mitochondrial energy production.

2. The aims of the dissertation:

1. ***In the first part of our study we aimed to monitor the function of thrombocytes, the endogenous antioxidants*** (SOD, GSH, plasma total thiol group concentrations) ***and prooxidants*** (free radical production of whithe blood cells, MDA levels in red blood cell haemolysates and plasma, myeloperoxidase) ***levels in the perioperative period of emergency*** (12 patients; Acute group) ***and elective*** (10 patients; Elective group) ***revascularization surgery of lower limb.*** Similar values of 10 healthy blood donors (control group) were also considered.

In this prospective randomysed open study the trombocyte function was measured by two different techniques, in whole blood and in platelet rich plasma.

We investigated the time dependent changes of thrombocyte function and prooxidant/antioxidant status in the perioperative phase of revascularization surgery of lower limb.The interaction of the thrombocyte and the other circulating cells were also investigated.

2. PAD is frequently accompanied by diabetes, hypertension, and smooking caused lung disorders, as we observed in the first row of experiments and in the scientific literature. In the second part of our studies thrombocyte function, prooxidant/ antioxidant status of type 1

and type 2 diabetic patients with PAD (T_1DM; T_2DM, respectively) were compared to each other (altogether 46 patients) and to the parameters of 11 healthy blood donors. The reason of our investigation was, that only a few systematic, comparative data can be found in the literature about the thrombocyte function, and oxidative stress staus of T_1DM; T_2DM patients with PAD.

3. Monitoring thrombocyte function and oxidative stress markers in the course of revascularization surgery of lower limb

3.1. Patients

Patients in the **Acute** group suffered from serious lower limb ischemia for several hours (4–6 hours) before surgery. In 8 patients the cause of ischemia was clear embolism, which was solved by Fogarty's embolectomy. The other patients had an acute arterial thrombosis at the level of femoral artery and one rupture of infrarenal aortic aneurism was also involved.
Patients of the **Elective** group were scheduled for revascu larization surgery because of obliterative arterial disease with consequential ischemia at the level of superficial femoral artery, which was improved by angiographic and Doppler measurements. The surgical solutions were similar in all of these cases, with 42,8±16,3 min exclusion time.
All patients received antiplatelet therapy (at least 75 mg Aspirin) before the recruitment. Low molecular weight heparin was prescribed in the perioperative period.
All patients were taking part in the study had been operated in the Department of General and Vascular Surgery, Baranya County Hospital in Pecs. Laboratory measurements were carried out in Department of Surgical Research and Techniques of Pecs University. The average age was 58,1 ± 7,3 years. Peripheral blood samples had been taken before, 2 and 24 hours after the surgery, as well as one week after operation.

3. 2. Measurement of platelet aggregation and endogenous antioxidants and prooxidants.

-Measurement of platelet aggregation:
Platelet aggregation in platelet rich plasma (PRP) was measured by the turbidimetric method of Born, by a four-channel aggregometer (Carat TX4 instrument, Carat Diagnostics Ltd. Budapest, Hungary). ADP (5 and 10 μM) and collagen (2 μg/ml) were used as aggregation inductors. The results were expressed as percentage of 100 % aggregation.
Platelet aggregation in whole blood was measured by two-channel impedance aggregometer (Chrono-log aggregométer, USA), according to the user's manual of the instrument. ADP (5 μM) or collagen (2 μg/ml) were used as inductors. The development of aggregation was recorded for six minutes and was expressed in Ohm.

- Measurement of prooxidants:
Free radical production of leucocytes was induced by phorbol miristate acetate (PMA) in whole blood, and was followed by luminometric method by Chrono-Log luminoaggregometer.

Malondialdehyde (MDA) levels in red blood cell hemolysate and in plasma, and myeloperoxidase (MPO) levels in plasma, were measured by standard photometric methods.

-Measurement of antioxidants:
Measurement of reduced glutathione (GSH) and plasma thiol (SH) groups:
GSH and plasma SH level were determined in anticoagulated whole blood (EDTA) by Ellman's reagent according to the photometric method of Sedlak and Linsday.

Measurement of superoxide dismutase (SOD) activity in washed red blood cells haemolysate (RBC): The main principle of this measurement was that adrenaline is able to spontaneously transform to adrenochrome (a detectable colourful complex). This transformation can be blocked by SOD and SOD containing cells or tissues. The difference in the rate of rise of control and samples curves (obtained at 415 nm) were proportional to the SOD activity.

3.4. Statistics:

The results were expressed as mean ± SD or in percentage. The differences were calculated by paired and unpaired Student's t-test, and by one way analyses of variance. The alterations were considered significant when p values were less than 0,05.

3. 5. Results:

3. 5. 1. Aggregation in platelet rich plasma

Aggregation measurement made possible to study thrombocyte function independently from the effects of other circulating cells.

Before surgery ADP induced maximal aggregation in PRP was reduced in both **Acute** and **Elective** groups compared to the **Control** group ($p<0.05$). This trend prevailed in the course of the study. The same pattern occurred in case of collagen induced aggregation, as well. This reduced agreeability at the level of thrombocytes indicated a satisfactory antiplatelet therapy.

3. 5. 2. Aggregation measurement in whole blood:

The reduced aggregation, was observed in patients groups in response to ADP and collagen compared to healthy people's PRP, had not been observed in whole blood. ADP and collagen induced aggregation in patients groups were comparable to Control group before the surgery and 2 and 24 hours after it, in whole blood. A statistically highly significant elevation appeared in Acute group in response to ADP and collagen induced aggregation, one week after the surgery, compared to elective and control groups, and to its own baseline values, as well.

***3.5.* 3. Prooxidants:**

-PMA-induced ROS production in whole blood:

Addition of PMA to several cell types (among others circulating blood cells, such as granulocytes, neutrophil cells, and platelets), resulted in increased ROS production due to activation of NADPH oxidase enzyme. The main sources of ROS in the blood stream are the leucocytes. White blood cell counts were moderately elevated in the two patient groups in the whole period of the study ($p<0.05$). Before surgery, ROS production was disproportionately high in the **Acute** group considering the mild elevation of leucocyte numbers, signed an extreme increase in the free radical producing capacity of the indivividual leucocytes in this case. This elevated amount of free radicals increased further in the course of the study, reaching a twenty- to fortyfold elevation compared to healthy **Controls** or the **Elective** group ($p<0.01$). Significant elevation was also observed in maximum free radical production in the **Elective** group at the end of the week, but it did not exceed the twofold elevation. The lag time of ROS production in the **Acute** group

was significantly shorter before surgery compared to the other two groups, and shortened further in the early reperfusion. In the **Acute** group, the slope of the free radical generation curve was steeper at each time point than in the other two groups. Significant elevation in the slope of free radical production was observedin the **Elective** group as well, one week after the surgery.

- MDA levels inred blood cell hemolysate and in plasma

Reactive oxygen species degrade polyunsaturated lipids; forming malondialdehyde The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism. A significant elevation in MDA level was observed in the **Elective** group in the early phase of reperfusion compared to baseline level and to the **Control ($p<0.05$)**. In the **Acute** group a standard low level of MDA was measured in the perioperative phase. The value was low before the surgery as well, signed the lack of unsaturated fatty acids in the membrane. In our study plasma MDA levels increased significantly in patients groups.In the **Acute** group plasma MDA levels were higher than it was measured in **Elective** and **Control** groups($p<0.05$). Plasma MDA level of **Elektív** was higher than in Control, but it returned into the Control level one week after the surgery.

- ***Myeloperoxidase (***MPO) is a hydrogen peroxide oxidoreductase, specifically found on mammalian granulocytic leucocytes, including polymorphonuclear leucocytes (PMN), basophils and eosinophils, responsible for the bacteriocidal capability of these cells. PMN activation and mediator release are partially responsible for the morbidity and mortality of revascularization of ischemic lower limb, regardless of the mode of intervention, surgical or thrombolytic.

In our cases plasma myeloperoxidase levels were higher in both patient groups at each time point compared to control. In a very new study was improved that myeloperoxidase, but not C-reactive protein, predicts cardiovascular risk in peripheral arterial disease. In our study MPO level in both patients groups were higher, than in **Control** group. After a transient reduction MPO level it returned to the levels were measured before the surgery in the surgery .

***3. 5.* 4. Changes in endogenous antioxidants:**

- Superoxyde dismutase (SOD):

SOD activity was lower in patients groups compared to **Control**, even before the surgery, and these low levels decreased further in the early reperfusion. In Acute group SOD level were lower than it was measured in **Elective** group, as well.

- Reduced glutathione (GSH) levels and the concentration of plasma sulphydril groups

GSH levels in the three investigared groups were similar before the surgery in both patient groups, and decreased transiently during the early reperfusion, but it returned to the baseline by the end of the week. The concentrations of plasma –SH groups followed similar pattern.

3. 6. Summary and conclusion:

Restoration of the blood flow in the formerly ischemic tissues is initiates several complication, involving local and systemic responses. Ischemia/reperfusion injury was

described almost 50 years before, but the correct mediation, the way of prevention or treatmen is under investigation in nowdays, as well.
In the course of the ischemia reperfusion injury of lower limb, muscle changes is accompanied by a progressive microvascular damage.The inflammatory response following reperfusion vary greatly and depends on the time and severity of ischemia, as it was measured in our cases too.
To study the ischemia/reperfusion induced injury of revascularization surgery of patients with peripheral arterial diseases after acute critical limb ischemia and in the course of elective revascularization surgery were in the focus of the present study. According to our results in accordance with several other studies, the duration and severity of ischemia is proportional to the damage occurred after it. Studiing thrombocyte function and antioxidant prooxidant status of our unique patient groups, several new aspects of ischemia reperfusion injury were revealed. Peripheral artery disease is a common progressive disorder that attaches the circulation of the legs, particularly in people over 55 years, strengthening in these patients the greatly increased risk of heart attack or stroke, and of dying within a decade. Several aspects of the problem were intensively studied, but platelet function during the restoration of the circulation of ischemic lower limb was hardly invesbefore.

4. MONITORING THROMBOCYTE FUNCTION AND ANTIOXIDANT PROOXIDANT STATUS IN DIABETIC PERIPHERAL ARTERIAL DISEASES.

4. 1. Introduction

Diabetes mellitus is a potent risk factor for the development of a wide spectrum of cardiovascular (CV) complications. The complex metabolic milieu accompanying diabetes alters blood rheology, the structure of arteries and disrupts the homeostatic functions of the endothelium.
Diabetes and PAD are frequently accompanied by each other. The duration of diabetes correlate well with the seriousness of the PAD. Among diabetic people oftener the infrapopliteral arterial occlusion than in the nondiabetic patients.
The presence of diabetes increases the frequency of intermittent claudication.The relative risk of amputation in diabetic population is 12,4-fold higher compared to the nondiabetic patients (95%, 10,9-14,9), and this value doubles above 65 years.

4. 2. Aim of study.

The aim of the second part of our study was to compare the thrombocyte function and antioxidant/prooxidant status of type 1, and type 2 diabetic patients with PAD.

4.3. Materials and methods:

4. 3. 1. Patients

Diabetic patients involved in this study were selected randomly from the diabetic outpatients with peripheral arterial diseas of Department of General and Vascular Surgery, Baranya County Hospital in Number of patients was 24 in T_1DM, and 22 in T_2DM groups. Ten healthy blood donors were involved as controls.
T_1DM patients received insulin and T_2DM patients received oral antidiabteic agents as antidiabetic terapy, with the exeption of two patients who received insulin, as well.. All of the patients received antiplatelet agents. In T_1DM group 4 patients received Syncumar as anticoagulant, and other 14 received low molecular weight heparine. In T_2DM group all patients were received antiplatelet therapy, 4 patients were treated by Syncumar and 10 with low molecular weight heparine, as anticoagulant.

4.4. Methods:

The same methods were used as in 3.2.

4.4. *Statistics:*

The results were expressed as mean ± SD or in percentage. The differences were calculated by paired and unpaired Student's t-test, and by one way analyses of variance. The alterations were considered significant when p values were less than 0,05.
The areas under the platelet aggregation curves were calculated in whole blood by Microcal/Origin 6.0 professional program. By means of this program linear regression analyses were made and correlation was determined the serum glucose levels of the patients and area under aggregation curves.

4.5. Results

4.5.1. Clinical chemistry data

Fasted serum glucose and triglyceride levels were higher than the normal values. The mean value of cholesterol level was within the normal range, but individually high values were measure, as well. The elevated fibrinogen levels were measured in both groups were considered as a sign of increased aggregability. The highest white blood cell counts were measured in T_1DM (11,2 ± 1,3 x 10^4 cell/μl^3), which was significantly higher than in the control group (6,71 ± 0,32 x 10^4 sejt/μl^3), ($p<0.01$). The white blood cell count in the T_2DM group was 8,92 ± 0,99 x 10^4 cell/μl^3.

4.5.2. Thrombocyte function in PRP:

ADP and collagen induced aggregation was significantly reduced in isolated platelets of both diabetic patients groups, compared to control group. The reduction was less pronounced in T_2DM diabetic patients.

4.5.3. Investigation of aggregation in whole blood

ADP induced aggregation was higher in both patients groups,and Collagen induced aggregation was increased in T_1DM patients compared to Control.
Similarly to our previous data, platelet aggregation measure in PRP reflected a satisfactory antiplatelet therapy, but in whole blood these differences can not be observed.
Our data revealed that measurement of aggregation in platelet rich plasma and in whole blood parallel is usefull for the exploration of therapeutic defect. In spite of the effectivity of antiplatelet therapy, was observed in isolated thrombocytes, the other circulating cells, such as white blood cells, can bypass in a paracrine manner the antiplated therapy, and my be induce platelet aggregation by free radicals or by their own resynthetised COX enzymes.
Significant linear correlation was improved in T_1DM patients fasted serum glucose level and AUC of ADP-induced ($p<0,005$, $R^2 = 0,6551$, $y = 3,338x-11,378$).

4. 5. 4. ***Results of the prooxidant measurement***

- ***PMA-induced free radical production:***

In the control group the PMA induced ROS production corrected to white blood cell counts were minimal (9,072 ± 2,36 AU/10^3 , but In T_1DM group a twentyfold elevation was measured in ROS production compared to control. ROS production in T_2DM group was five times higher than it was observed in Control.

4. 5. 5. Results of the antioxidant measurements

SOD and GSH levels were reduced in both diabetic groups, which supposed to be an important determinant of the diabetes caused systemic oxidative stress. The reduction of SOD activity was independent from the type of diabetes.
GSH level was reduced only in T_1DM group.

5. NOVEL FINDINGS:

1. Our study primarily monitored the effect of ischemia reperfusion injury on thrombocyte function and on prooxydant status in the whole hospitalization phase of emergency and elective revascularization surgery of lower limb. In the course of our study we revealed that satisfactory inhibition in the level of isolated thrombocita dos not means definite prevention of thrombus formation, because of the modulatory role of cellular and non cellular components of the blood.

2. We demonstrated a robust significant elevation in ADP and Collagen induced aggregation in whole blood one week after revascularization surgery of lower limb, in spite of the inhibited platelet aggregation was measured in PRP.This was accompanied by the low level of antioxidant enzyme and a permanent increase in ROS production. Similar phenomenon was not observed in elective patients..

3. We demonstared that the restoration of the circulation in PAD patients with serious limb ischemia did not followed by the restoration of antioxidant /prooxidant satus.

4. Our measures reveal significant differences in thrombocyte function of T_1DM and T_2DM, patients with PAD and improved that area under curves of T_1DM correlate well with the serum glucose levels of these patients.

5. SOD deficiency was demonstrated in our study was independent of the type of diabetes.

6. PMA – induced ROS production was significantly higher in diabetic patients. It can be considered as novel finding, that free radical production of T_1DM group was significantly higher than in T_2DM patients.

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