

HEMORHEOLOGICAL PARAMETERS DURING HEART SURGERIES AND A NEW INDICATION OF METAMIZOLE IN THE INHIBITION OF PLATELET AGGREGATION

Ph.D. dissertation

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I. Introduction

Cardiovascular diseases are the most common cause of morbidity and the leading cause of mortality in the most developed countries. In the last few decades morbidity and mortality rates have shown a gradual improvement due to successful primary prevention and the development of medication therapies and invasive revascularization strategies. The discovery of the so-called classical risk factors - based on the Framingham Heart Study - and the newly discovered risk factors were of great importance in successful primary prevention. Numerous multicentre clinical studies confirmed that altered hemorheological parameters are independent risk factors of cardiovascular and cerebrovascular diseases. The most important hemorheological parameters are hematocrit (Hct), fibrinogen, plasma and whole blood viscosity (PV, WBV), red blood cell (RBC) aggregation and deformability. Hemorheology also focuses on the properties of white blood cells and platelets (eg. platelet adhesion and aggregation, white blood cell adhesion and deformability).

Revascularization strategies tending to open or bypass the occluded vessels were big steps in the treatment of coronary artery disease (CAD). Due to the development of percutaneous coronary intervention and coronary artery bypass grafting (CABG) the life expectancy of CAD patients has significantly increased in the last few decades.

Besides invasive procedures appropriate pharmacological treatment also plays an important role. Antiplatelet therapy is one of the major pharmacological methods in the prevention and treatment of acute cardiovascular diseases and in the treatment of stable coronary artery disease. For that purpose aspirin (acetylsalicylic acid, ASA) is the most widely used antiplatelet drug that inhibits the cyclooxygenase (COX) enzyme. Metamizole (MET, dipyrone) has a strong analgesic and antipyretic effect, inhibiting the COX enzyme as well, but the antiplatelet effect of MET and its interactions with ASA are hardly known.

II. Aims

The aim of our first study was to examine the changes in hemorheological parameters during coronary artery bypass grafting and in the 6 months postoperative period. We also compared the two main CABG surgery methods from a hemorheological aspect. Changes in platelet aggregation were also examined.

In the second study we aimed to compare the inhibition of platelet aggregation of MET to ASA, and to investigate the possible interactions between them *in vitro*. Then an *in vivo* crossover study was conducted to elicit the antiplatelet effect of intravenously administered MET and orally administered ASA and their co-administration in healthy subjects.

III. The influence of on-pump and off-pump coronary artery bypass grafting on hemorheological parameters

1. Introduction

Coronary artery bypass grafting plays an important role in the treatment of coronary artery disease, its effectiveness was confirmed by a number of clinical studies. The traditional so-called on-pump CABG is performed by using a cardiopulmonary bypass (CPB), where the circulation is maintained by the heart-lung machine during the cardioplegia-induced cardiac arrest. Extracorporeal circulation creates non-physiological conditions. The contact of blood with non-biological surfaces are potent stimuli for the immune system that leads to the activation of leukocytes, cytokines, adhesion molecules and the complement cascade that creates a systemic inflammatory response. Mechanical shear stress on blood cells and hemodilution can cause impaired hemostasis with embolization, hemolysis and hypoperfusion of different organs. To avoid the complications of extracorporeal circulation a new technique has been developed. Off-pump surgeries are performed on beating heart without the use of CPB and cardioplegia. The part of the heart where the surgical manipulation takes place is stabilized by a special heart positioner.

Numerous prospective and retrospective trials have been conducted to compare the two methods. Although several important outcomes are in favour of off-pump CABG, it is difficult to show the superiority of off-pump CABG in clear-cut clinical end-points such as mortality and morbidity. There are several reasons for this: first of all the mortality and morbidity rate of CABG is very low, which requires a huge number of patients in randomized trials to show statistically significant differences. Moreover off-pump CABG requires a more difficult surgical technique, so there is a steep learning curve, and different patients' characteristics can also play a role. However, further analysis of prospective and retrospective data revealed that the off-pump technique reduces mortality in high-risk patients.

2. Patients and methods

A total of 47 patients undergoing primary, isolated, elective CABG surgery in the Heart Institute of Pécs between October 2009 and May 2011 were included in our study. In 25 cases on-pump and in 22 cases off-pump procedures were performed. Blood samples were obtained on 9 occasions: **(1)** upon arrival to the operating theatre, **(2)** after the induction of anesthesia, **(3)** 20 and **(4)** 40 minutes after performing cardiopulmonary bypass (in case of off-pump surgeries we calculated with the mean time needed to perform a cardiopulmonary bypass), **(5)** after closing the thorax, **(6)** on the 1st and **(7)** 2nd postoperative days and **(8)** during the 2nd and **(9)** 6th month check-ups.

Hemorheological measurements were performed within 2 hours after blood sampling. Plasma and whole blood viscosity measurements, platelet aggregation measurements and measurements with LORCA were carried out at 37°C, other measurements were performed at room temperature (22 ± 1°C).

Hematocrit was determined by a microhematocrit centrifuge.

Plasma viscosity and whole blood viscosity were measured with Hevimet 40 capillary viscometer. Whole blood viscosity at 40% hematocrit was calculated by a mathematical formula according to Matrai et al.: $WBV_{40\%}/PV=(WBV_{Hct}/PV)^{40\%/Hct}$, where $WBV_{40\%}$ stands for the blood viscosity of the calculated and WBV_{Hct} for the original sample.

Red blood cell aggregation was determined by both Myrenne and LORCA (Laser-assisted Optical Rotational Cell Analyzer) aggregometers. Myrenne aggregometer employs

the light transmission method of Schmid-Schönbein et al. Red blood cells are disaggregated at high shear rate, then the shear rate decreased rapidly to zero (M mode) or to low shear (M1 mode). The extent of aggregation is characterized by the aggregation indices (AI_M , AI_{M1}), calculated from the surface area below the light intensity curve in a 10 s period of time. To measure RBC aggregation with LORCA 1 ml of oxygenated blood is injected into the gap between a static bob and a rotating cylinder that creates a simple shear flow. Erythrocytes are first disaggregated at high shear rate then the shear rate decreases rapidly to zero. After that the intensity of backscattered laser light is plotted on a syllectogram. The aggregation behaviour of blood sample is characterized by the aggregation index (AI) calculated from the first 10 seconds of the syllectogram after the shape recovery period. That smallest shear rate required for complete disaggregation is called threshold shear rate (γ).

Erythrocyte deformability was also measured by two different methods: ektacytometry (LORCA) and filtrometry (Carat FT-1 filtrometer). For the deformability measurement with LORCA, 25 μ l blood was suspended in 5 ml high viscosity (32.6 mPas) polyvinylpyrrolidone solution. RBCs are deformed by shear stresses from 0.3 Pa to 30 Pa that can be visualized by laser-diffraction. Deformation is characterized by the elongation index calculated from the elliptic diffraction pattern. During filtrometry the hematocrit of red blood cell suspension was adjusted to 10%. The suspension flows through a 5 μ m pore-diameter Nucleopore filter and relative cell transit time (RCTT) is calculated.

Platelet aggregation was measured from plasma by Carat TX4 optical aggregometer. Blood samples were centrifuged at 150 g for 10 minutes to separate platelet-rich plasma (PRP), then at 2500 g for 10 minutes to gain platelet poor plasma (PPP). 30 μ l adenosine-diphosphate (ADP, 5 μ M final concentration) and epinephrine (10 μ M final concentration) was added to 270 μ l PRP to induce platelet aggregation. Change in light transmission intensity was displayed on the aggregation curve taking the optical density of PRP as 0% and the one of PPP as 100% aggregation. Platelet aggregation was evaluated considering the maximal percentage of platelet aggregation.

The morphology of red blood cells was visualized by scanning electron microscopy in baseline, 40 minutes, 1st and 2nd postoperative day samples in the Central Electron Microscopy Laboratory, Faculty of Medicine, University of Pecs.

Data were compared as means \pm SD using paired and un-paired Student's t-test. Differences were considered significant at $p < 0.05$.

3. Results

Patients' preoperative characteristics were well matched between the two groups in terms of mean age, risk factors and history of cardiovascular diseases. There was no difference between the two techniques in the mean number of grafts, however mean surgery time was longer in case of off-pump technique. Patients undergoing on-pump surgery received more volume of sodium heparin and fluid.

There was a significant decrease in hematocrit and plasma viscosity values during the early phase of surgery in both groups. At the end of the operation and in the early postoperative period hematocrit and plasma viscosity started to recover and at 2nd and 6th months it reached the baseline values. In case of on-pump technique Hct was significantly lower in samples taken after 20 and 40 minutes and 48 hours than in case of off-pump. Plasma viscosity showed lower values in the on-pump group also in the 20 and 40 minutes samples and after closing the thorax. The change in whole blood viscosity followed the change of Hct and plasma viscosity, whole blood viscosity of the two groups differed significantly in the 20 and 40 minutes samples, at the end of the operation and on the 2nd postoperative day. Calculated whole blood viscosity at 40% hematocrit significantly lower during the first 48

hours in both groups when compared to the baseline, and a significant difference was also found in the 20 and 40 minutes samples between the two groups of patients, with a notable decrease in the on-pump group.

Myrenne aggregation indices showed a similar trend to that of hematocrit. A significantly greater decrease could be noticed in the 20 and 40 minutes and 48 hours samples in case of on-pump surgery. Aggregation indices measured by the LORCA decreased substantially in the early phase of reperfusion as well; thereafter it elevated and reached the baseline by 48 hours. Differences between the two techniques could be clearly seen here as well. The threshold shear rate showed a decreasing tendency in the off-pump group, but elevated markedly in the on-pump group during the surgery. There was a marked rise on the 2nd postoperative day in both groups then it returned to baseline during the late postoperative period.

The deformability of RBCs was observed both with ektacytometry and filtrometry. No significant difference was detected during the surgeries, nor between the two groups in elongation indices measured with ektacytometry by the LORCA. For filtrometry, relative cell transit time increased significantly during on-pump surgeries compared to baseline. RCTT was significantly lower on the 1st and 2nd postoperative days then it returned to the baseline during the following months. In the off-pump group RCTT did not change significantly except from a slight increase in samples taken after 20 minutes. There was a significant difference between the two groups in the samples taken after 20 and 40 minutes.

Scanning electron microscopy showed various deformed erythrocytes with some echinocytes among them in the 40 minutes samples during CPB. These changes were reversible and disappeared after 24 and 48 hours. No malformations of erythrocytes were visible when the surgery was performed on beating heart.

In the on-pump group both ADP- and epinephrine-induced platelet aggregation decreased slowly during surgery and showed a great fall by the end of the surgery. In the off-pump group platelet aggregation did not change during the operation but decreased significantly by the end of it. A significantly higher platelet aggregation was observed with both inducers in the on-pump group in samples after closing the thorax. Due to restarted antiplatelet therapy on the first postoperative day, comparing samples taken after 48 hours, 2 months and 6 months has no particular relevancy.

Data were compared as *means* \pm *S.E.M.* using paired and 2-sample Student's t -test. Differences were considered significant at $p < 0.05$.

4. Discussion

In our study hematocrit decreased significantly in the early phase of the surgery due to hemodilution and bleeding. During on-pump CABG patients received more fluid than off-pump patients due to the priming of the CPB, therefore Hct values were significantly lower in the 20 and 40 minutes samples during on-pump CABG. Values varied between 20-30% in the on-pump, while between 30-40% in the off-pump group, which significant difference may lead to hypoxia and hypoperfusion of different organs (e.g. brain, kidneys) or different complications during on-pump surgeries. According to other authors intraoperative blood loss is higher in on-pump patients, which can explain the difference between the two groups as well. At the end of the surgery and during the following days Hct started to recover, partially because of the administered transfusions.

The drop in plasma and whole blood viscosity and the differences between the two groups might be explained by the different extent of hemodilution. In order to compare viscosities with different hematocrits, a simple mathematical correction of whole blood viscosity was done. Still significantly lower corrected WBV values were calculated in the first

48 hours compared to the baseline that might be explained by the decrease in erythrocyte aggregation and the decrease in the level of plasma proteins (especially fibrinogen) due to hemodilution.

Aggregation indices were significantly reduced during CABG surgery and it was more pronounced in the on-pump group. RBC aggregation is known to be influenced by the Hct and the concentration of various plasma proteins, therefore the reduction can be explained by the low Hct levels and the hemodilution of plasma proteins. The analysis of the threshold shear rate revealed a remarkable result. In the off-pump group a slight decrease in both γ and AI was observed. However, in the CPB group γ elevated markedly during the surgery, while the aggregation index decreased. We hypothesized that due to the mechanical damage of erythrocytes caused by CPB aggregation forces increased and more shear stress was needed to disaggregate the deformed RBCs. Another unexpected result was the highly elevated γ on the 2nd postoperative day in both groups. These changes may be explained by an inflammatory acute phase response predominantly caused by the surgical trauma that leads to oxidative stress.

The two different methods to characterize RBC deformability provided conflicting results. There was no change in deformability when measured by ektacytometry, however RCTT measured by filtrometry increased significantly during on-pump surgeries that indicates the impairment in RBC deformability due to the mechanical trauma caused by the heart-lung machine. In case of off-pump CABG there was also a slight increase in RCTT in the 20 minutes samples that was statistically significant but probably irrelevant, and might vanish with increasing patients' number. Filtrometry seems to be more sensitive to detect the changes in RBC deformability caused by mechanical damage. The results of filtrometry are confirmed by scanning electron microscopic photos. Cells are exposed to high shear stress, turbulence, extreme hemodilution and hypothermia during CPB. In the 40 minutes samples erythrocytes seem to be rather damaged and malformed due to this mechanical trauma. These changes are reversible though, and can not be visible on the following days. These results were concordant with others. Erythrocytes seem not to be altered when surgery is performed on beating heart.

We examined the effect of different types of CABG on platelets as well. Platelet aggregation decreased significantly by the end of the surgery with both inducers and it was more pronounced in the on-pump group. It could also be due to the mechanical trauma caused by the CPB.

5. Conclusion

During CABG surgery most rheological parameters changed. Changes were more obvious in case of on-pump surgery, and there were significant differences in most measured parameters in the 20 and 40 minutes samples between the two methods. The differences ceased during long term follow-ups. Cells seem to be mechanically damaged by the heart-lung machine as suggested by electromicroscopy, filtrometry and threshold shear rate. We showed a significantly lower platelet aggregation at the end of the on-pump CABG that is concordant with the results of another group. Off-pump technique seems to be more favorable from a hemorheological point of view.

IV. Antiplatelet effect of acetylsalicylic acid, metamizole and their combination - *in vitro* and *in vivo* comparisons

1. Introduction

Platelets play an important role in the pathogenesis of thrombus formation and the progression of atherosclerosis. A number of multicenter, randomized clinical trials have shown that antiplatelet therapy decreases the risk of cardiovascular death, thus antiplatelet therapy is one of the major pharmacological methods in the treatment and prevention of ischemic vascular diseases.

Aspirin is the most widely used antiplatelet drug that was shown to reduce the risk of cardiovascular events and death. ASA is a non-steroid anti-inflammatory drug (NSAID), a non-selective cyclooxygenase inhibitor acting both on COX-1 and 2 isozymes, but it is less potent to inhibit the COX-2 enzyme. ASA irreversibly acetylates the serine residue of the COX enzyme, thus prevents the synthesis of thromboxane A₂ (TXA₂), therefore effectively inhibiting platelet aggregation for the lifespan of the platelet. In case of primary and secondary prevention ASA should be given in a low dose (75-150 mg), because higher doses do not increase effectiveness, but increase the occurrence of gastrointestinal bleeding. In acute coronary syndrome (ACS) aspirin is given as soon as possible after the diagnosis, with a combination of a P2Y₁₂ inhibitor (clopidogrel, prasugrel, ticagrelor). The recommended dose of ASA in ACS is 150-325 mg in a chewable form which absorbs rapidly in the stomach and upper intestine and inhibits platelet aggregation effectively, however therapy resistance occurs in some patients.

The antiplatelet effect of other nonaspirin NSAIDs (NANSAIDs) has also been analyzed. However epidemiologic studies have failed to demonstrate any risk reduction of NANSAIDs on cardiovascular events, NANSAIDs reversibly inhibit the COX-1 enzyme, and cause an intermittent inhibition of platelet aggregation. Different clinical trials revealed that NANSAIDs can negatively influence the antiplatelet effect of ASA due to possible interactions on the COX-enzyme. According to the European Heart Association using NSAIDs is not recommended in patients with long-term ASA therapy, because it can increase the incidence of ischemic vascular events (III C recommendation, 2011).

Metamizole is a pyrazolone derivate, with a relatively low gastrointestinal toxicity and a hypothetical COX-independent mechanism of analgesia, thus considered as a non-typical NSAID. It has a strong analgesic and antipyretic but only a weak anti-inflammatory effect, available in both oral and parenteral forms. MET is a prodrug hydrolyzing rapidly to its pharmacologically most potent metabolite, 4-methylaminoantipyrine that is converted into a variety of other metabolites, such as 4-formylaminoantipyrine, 4-aminoantipyrine and 4-acetylaminoantipyrine. MET and its metabolites inhibit both COX isozymes that has been shown both *in vitro* and *in vivo*. Although the antiplatelet effect of MET has already been observed by others, it is not used in this field, remaining only an antipyretic, analgesic drug in clinical use.

2. Methods

2.1. *In vitro* investigations

ASA and MET were obtained from Sigma-Aldrich, and were diluted in 0.9% sodium chloride. MET hydrolyzes when dissolved in water, therefore the tested MET solutions consisted of several pyrazolone derivatives.

Blood was taken for 10 healthy donors into Vacutainer tubes containing sodium citrate.

In the first part of the *in vitro* investigations we compared the antiplatelet effect of the two drugs by adding different concentrations (6, 12 and 25 µg/ml final concentrations) of ASA or MET solutions to the blood.

In the second part we examined the possible interactions between them by adding lower concentration of MET (1 µg/ml final concentration) and 15 minutes later lower concentration of ASA (2 µg/ml final concentration) to the blood. To see whether the interaction is competitive we added higher concentration of ASA (6 µg/ml final concentration) to MET and we administered ASA 15 minutes earlier than MET. All samples were incubated at 37 °C for 1 hour. Platelet aggregometry was performed as detailed above.

Data were compared as *means* ± *S.E.M.* using paired and 2-sample Student's t -test. Differences were considered significant at $p < 0.05$.

2.2. *In vivo* study

Twenty healthy, non-smoking male volunteers were selected, mean age was 24.2 ± 2.6 years, and average body mass index was in normal range ($24.6 \pm 3.5 \text{ kg/m}^2$). Medical history was obtained, routine physical and laboratory examinations were performed. Subjects with a bleeding disorder, anaemia, hypotension (systolic blood pressure $< 90 \text{ Hgmm}$) and allergy to aspirin or metamizole were excluded. The volunteers had not taken any medication which interferes with platelet functions for 10 days prior the examination.

In our crossover study the volunteers were divided into three groups. A peripheral 21-gauge venous catheter was applied into the forearm from which baseline samples were collected. In the *first group* one ampoule of 1 g/2 ml MET (metamizole sodium) was administered (usual intravenous or intramuscular dose) through the forearm catheter, in the *second group* half tablet of 500 mg (250 mg) chewable form of ASA was chewed and swallowed with 1 dl of water, while in the *third group* per os ASA and intravenous MET was administered at the same time. Blood samples were taken 7, 15 and 30 minutes, 1 and 4 hours after drug administration then the line was removed. After 24 and 72 hours, samples were taken from cubital vein. The groups were swapped twice 18 ± 4 days after first drug administration – sufficient time to eliminate previous drug effects – and the same protocol was applied. Platelet aggregometry was performed as detailed above.

Data were compared as *means* ± *S.E.M.* using paired and 2-sample Student's t -test. Differences were considered significant at $p < 0.05$.

To examine the onset and offset of the antiplatelet effect of the drugs given to healthy subjects IPA (inhibition of platelet aggregation) was calculated from maximal percentage of platelet aggregation indices measured by optical aggregometry as follows: $(\text{aggregation before treatment} - \text{aggregation after treatment}) / \text{aggregation before treatment} \times 100$ [30-31]. IPA was calculated at every time point in each subject then means were calculated.

To describe the time-dependence of IPA we assumed, that the onset of the effect can be represented by a bell-shaped Gaussian function and the offset of the effect can be described by an exponentially decreasing function. The parameters of the convolution (joint integrated appearance) of the two functions were determined by a self-developed approximation program. The program minimalizes the residual mean error between the model and the measured data by simultaneously changing the graph parameters and by gradually decreasing the degree of changes. The parameters of this graph can be well defined by the Gaussian function's 3 independent parameters: the amplitude of the curve's peak (**A**), the width of the bell (σ), the position of the centre of the peak (t_0), and by the time constant representing velocity of exponential elimination (τ).

3. Results

3.1. *In vitro* investigations

In the first part of the *in vitro* investigations a complete inhibition of epinephrine-induced platelet aggregation was observed by all concentrations of both ASA and MET. There was no significant difference between the antiplatelet effect of the two drugs when using 25 and 12 $\mu\text{g/ml}$ concentrations, however 6 $\mu\text{g/ml}$ ASA inhibited platelet aggregation significantly more than 6 $\mu\text{g/ml}$ MET.

ADP-induced platelet aggregation was significantly lower compared to untreated control and there was no difference between ASA and MET.

In the second part of the *in vitro* study the possible interactions of ASA and MET were studied in another group of healthy controls. Initial measurements showed that 2 $\mu\text{g/ml}$ ASA completely inhibited epinephrine-induced platelet aggregation, and 1 $\mu\text{g/ml}$ MET had no antiplatelet effect. When MET was added to blood 15 minutes before ASA, epinephrine-induced platelet aggregation did not differ from untreated control. Conversely when ASA was added first, significantly lower platelet aggregation was observed. When an increased concentration of ASA was added after MET, epinephrine-induced platelet aggregation was inhibited again.

When ADP was used as a platelet agonist aggregation was also significantly lower compared to untreated control when ASA was administered before MET or when the concentration of ASA was increased. The antiplatelet effect of ASA was also attenuated when ineffective concentration of MET was added first.

3.2. *In vivo* study

Baseline platelet aggregation induced by epinephrine did not differ between the groups. In the MET group, where single-dose MET was administered to subjects intravenously, a complete platelet inhibition already developed in the 7-minute samples. Aggregation indices then started to increase slightly, but after 4 hours it was still significantly lower than baseline. After 24 hours platelet aggregation returned to baseline. When volunteers received single-dose ASA orally, platelet aggregation decreased slowly and it reached its maximum inhibition after 4 hours. When compared to MET platelet aggregation was significantly higher at 7 and 15 minutes. ASA still had an antiplatelet effect after 24 hours, and after 72 hours aggregation was still significantly lower than at baseline. Combined therapy caused a rapid inhibition, same as in the MET group. Aggregation indices remained very low in the first hour. Inhibition was still effective in the 4-hour samples, it was more effective than the single-dose MET, but not as effective as in the ASA group. After 24 hours no antiplatelet effect was observed.

ADP-induced platelet aggregation showed similar kinetics as epinephrine-induced platelet aggregation, but the changes were moderate. The maximal inhibition could be seen in the MET group at 15 minutes. There was no difference between the groups at 30 minutes, 1 hour and 4 hours. At 24 and 72 hours aggregation indices showed a significant decrease only in the ASA group when compared to baseline.

The time-dependence of IPA was similar in case of both inductors, but it was more representative in the case of the epinephrine-inductor, therefore we do not discuss the ADP inductor. In the MET group the data of the graph indicate a rapid development of the onset of IPA ($\sigma = 0.01$ hours), and the elimination was also very quick ($\tau = 5.49$ hours). When ASA was administered orally, due to absorption data representing the onset were an order of magnitude higher ($\sigma = 0.50$ hours), and the offset of IPA was also slower ($\tau = 158.31$ hours).

In case of combined therapy data ($\sigma = 0.03$ hours, $\tau = 11.21$ hours) were closer to the MET group.

4. Discussion

In the first part of our *in vitro* investigations we demonstrated that MET can inhibit platelet aggregation effectively, as it has already been observed by others. We previously tested higher, analgesic doses and there was no difference between the antiplatelet effect of ASA and MET (data are not shown), likewise at concentrations of 25 and 12 $\mu\text{g/ml}$. Although 6 $\mu\text{g/ml}$ ASA is significantly more effective, epinephrine-induced platelet aggregation was still completely inhibited by both drugs.

It is interesting to speculate regarding the two main signal transduction pathways responsible for the physiological platelet activation: (1) activation of COX-1 enzyme by epinephrine and/or other inductors (arachidonic acid, collagen), inhibited by ASA and other NSAIDs; (2) activation through P2Y₁₂ receptors which can be blocked by thienopyridines on a receptorial level. During degranulation of activated platelets the inductors for both pathways are released causing the amplification of platelet activation and aggregation. *In vivo* both pathways play an important role. During *ex vivo* induced platelet aggregation one inductor is used in supernormal concentration, making its pathway dominant over the other. Effect of the inhibitor of this pathway is thus more pronounced. Other amplification processes contribute to aggregation in a smaller magnitude, hence inhibition of the other pathway causes a lesser – but still detectable – diminish in aggregation. Therefore it is not surprising that ADP-induced platelet aggregation (that is used to test the effectiveness of thienopyridines) was inhibited as well.

In the second part of our *in vitro* investigation the interactions between the two drugs were examined in 3 steps using effective ASA and ineffective MET concentrations: (1) when MET was added before ASA, the ASA inhibited platelet aggregation was attenuated; which was completely restored (2) when higher concentration of ASA was administered; (3) or when ASA was added before MET to the blood. These observations may indicate a competitive interaction between the two drugs.

Concordantly with our results, Hohfeld et al. have shown that 4-methylaminoantipyrine and other metabolites of MET as well were unable to inhibit the COX-1 enzyme at very low concentrations leading to a concentration-dependent attenuation or a total inhibition of the antiplatelet effect of aspirin. Their molecular explanation for the phenomenon was the following: ASA-induced irreversible blocking of COX-1 is attenuated by 4-methylaminoantipyrine occupying the binding place of ASA via forming bonds with 3 amino acids of the enzyme.

Our *in vivo* results indicate that MET has an antiplatelet effect when used intravenously in a dose of 1 g/2 ml. The inhibition of platelet aggregation has already been noticed in patients with various conditions, such as after subarachnoid hemorrhage and meniscectomy. The onset was rapid in our study presumably due to the intravenous administration. Maximal inhibition of the epinephrine-induced platelet aggregation was reached after 7 minutes and it was equal to the maximal inhibition by ASA that was reached only after 4 hours. As it has already been shown by others MET and its metabolites inhibit the COX-1 enzyme inhibiting platelets in TXA₂ synthesis and platelet aggregation. The effect is reversible, as it is indicated by the rapid offset of IPA, and the diminished antiplatelet effect after 24 hours. In the ASA group, the oral intake caused an order of magnitude slower onset like the offset due to the irreversible inhibition of platelets. When MET and ASA were co-administered, the time-dependence of IPA was similar to the MET group, because MET can bound to COX enzymes first due to the intravenous administration causing a reversible

inhibition. Since the half life of ASA is short (cca. 20-30 minutes), essentially the drug is totally eliminated by the 4th hour. The inhibitory effect of ASA depends on the extent of acetylation of COX-1 enzyme in the early period after its absorption which may be the reason why epinephrine-induced platelet aggregation was significantly lower at 30 minutes, 1 hour and 4 hours than in the MET group. After 24 hours there was no antiplatelet effect. It is assumed that due to its rapid onset MET could occupy the COX-1 enzyme, thus it weakened the effect of ASA. Nevertheless this becomes apparent only when the antiplatelet effect of MET has gone. Therefore MET creates a double effect: own antiplatelet effect + competition with ASA. Due to theoretical consideration the antiplatelet effect of the combination depends on the resultant of these two effects in every moment.

Antiplatelet therapy has a pivotal role in the management of acute cardiovascular events. Acute coronary syndrome is a life threatening condition; its treatment requires the rapid administration of ASA, which is known to reduce mortality. In acute coronary syndrome ASA is recommended to be administered in a dose of 150-325 mg in a chewable form chewed and swallowed to increase the rate of absorption. There are some contraindications in the use of ASA: it must not be given to patients with known hypersensitivity, active gastrointestinal bleeding, known clotting disorders, or severe hepatic diseases. In some cases ASA cannot be given in an oral form (e.g. unconsciousness, vomiting, risk of aspiration), and aspirin resistance has to be considered as well. We hypothesized that in case ASA cannot be given orally to patients with ACS intravenous MET can be an alternative treatment.

NANSAIDs are often prescribed as analgesics and anti-inflammatory drugs, and since many patients take ASA concomitantly, several *in vitro* and *in vivo* studies have been performed to examine the possible interactions between the drugs. However the results of clinical studies are controversial, caution is recommended in case of ASA and NANSAID co-administration. No multicenter, randomized clinical trials were conducted to examine the co-administration of ASA and MET. Our *in vitro* examinations suggest that if MET is added in a low and ineffective concentration it could inhibit the effect of ASA. This interaction was only seen after 24 hours *in vivo*. Further studies are required to elucidate the possible clinically significant interactions between long-term ASA therapy and MET.

5. Conclusion

MET significantly inhibits platelet aggregation. The effect is reversible and the offset is rapid therefore it cannot be used as a long-term antiplatelet drug. When administered intravenously complete platelet aggregation develops quickly, that can be an advantage in the treatment of acute cardiovascular events. Our *in vivo* experiments indicate that intravenously administered MET can be considered as a therapeutic alternative in acute coronary syndrome, if ASA cannot be used in oral form. The importance of the “golden hour” in revascularization therapy of acute coronary syndrome is well known. Since intravenous MET has a prompt antiplatelet effect in contrast to ASA, MET may increase the success of revascularization. Combination of the two drugs in acute coronary syndrome could also be considered, but further examinations are needed to investigate the interactions of the two drugs. Certainly randomized clinical studies are required to prove the clinical importance of our hypothesis.

V. Novel findings

Our experiments led to the following novel findings:

1. During CABG surgery hemorheological parameters notably changed.
2. We showed that hematocrit, plasma and whole blood viscosity and red blood cell aggregation values were significantly lower in the 20 and 40 minutes samples during on-pump surgeries, and red blood cell filterability was significantly impaired when compared to off-pump surgeries.
3. Red blood cells were mechanically damaged by cardiopulmonary bypass that was confirmed by scanning electron microscopy, red blood cell deformability and red blood cell aggregation results.
4. Platelet aggregometry results suggest that platelets are also mechanically damaged by CPB.
5. The differences in hemorheological parameters between the two types of CABG ceased during long term follow-ups.
6. During our *in vitro* examination we demonstrated that the antiplatelet effect of metamizole did not differ significantly from the antiplatelet effect of acetylsalicylic acid.
7. We showed that low, ineffective doses of MET inhibits the antiplatelet effect of ASA. We confirmed that it is a competitive interaction.
8. In our *in vivo* study we demonstrated that intravenously administered MET has a prompt antiplatelet effect therefore it can be considered as a therapeutic alternative in the treatment of acute coronary syndrome, when ASA is contraindicated
9. When intravenous MET and oral ASA were added together significant interaction was observed after 24 hours.

VI. Abbreviations

Hct	hematocrit
PV	plasma viscosity
WBV	whole blood viscosity
RBC	red blood cell
CAD	coronary artery disease
CABG	coronary artery bypass grafting
ASA	acetylsalicylic acid, aspirin
COX	cyclooxygenase
MET	metamizole
CPB	cardiopulmonary bypass
AI	aggregation index
γ	threshold shear rate
RCTT	relative cell transit time
PRP	platelet rich plasma
PPP	platelet poor plasma
ADP	adenosine-diphosphate
NSAID	non-steroid anti-inflammatory drug
ACS	acute coronary syndrome
TXA ₂	thromboxane A ₂
NANSAID	nonaspirin non-steroid anti-inflammatory drug
IPA	inhibition of platelet aggregation

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