MMP-2 inhibition prevents platelet activation in ischemia/reoxygenation conditions

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Conflict of interest
None declared

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Abstract

Background. Platelets play a fundamental role in myocardial infarction and the pathogenesis of ischemia/reoxygenation (I/R) injuries. They contain matrix metalloproteinases (MMPs) that are involved in arterial thrombosis. The MMP inhibitor doxycycline has been shown to exert protective effects in I/R injuries involving various organs and mechanisms.

Objectives. To explore the influence of doxycycline on platelet activation and MMP-2 activity during I/R.

Materials and methods. Platelets isolated from the blood of healthy human volunteers were subjected to chemical I/R conditions. The study included aerobic controls (AERO), I/R platelets and I/R platelets pretreated with doxycycline (I/R+D). The concentration of doxycycline used was standardized to 10 μM. The analysis of platelet activation markers and platelet microvesicles (PMVs) was performed using flow cytometry. Adenosine diphosphate (ADP)-induced and collagen-induced aggregation, as well as MMP-2 activity and its concentration in platelets were evaluated.

Results. Doxycycline decreased the expression of activated glycoprotein IIb/IIIa on platelets ($p = 0.043$). Additionally, an increased expression of CD63 was observed in buffers containing PMVs after doxycycline administration ($p = 0.043$). The ADP-dependent aggregation of I/R platelets was significantly lower in comparison to AERO ($p = 0.022$). Furthermore, there was a stronger tendency of enhanced ADP-dependent aggregation in I/R platelets pretreated with doxycycline compared to platelets that underwent I/R without doxycycline. Higher MMP-2 activity was observed in I/R+D platelets compared to I/R platelets ($p < 0.01$).

Conclusions. The inhibition of platelet MMP-2 by doxycycline attenuated platelet activation and protected platelets by preserving their aggregation ability.

Key words: ischemia, reperfusion, platelets, metalloproteinases, doxycycline

Background

In myocardial infarction (MI), platelets participate in thrombus formation and the microembolization from unstable plaque rupture and intravascular intervention. Moreover, in acute MI, the triggered inflammation, in addition to platelet–leukocyte and platelet–endothelium interactions, leads to the release of vasoconstrictor and pro-inflammatory molecules from platelet microvesicles (PMVs) and exosomes.[1] Changes in the shape of platelet and an enhancement in mean platelet volume have been observed in acute MIs.[2] Several studies have revealed that platelets activate and contribute to cardiac ischemia/reoxygenation injury (IRI),[1] critical limb ischemia[3] and ischemic strokes.[4] The impact of platelets on the reperfused myocardium depends on their activation status.[5] The activation of platelets leads to the release of PMVs and granules containing growth factors as well as pro-inflammatory and proapoptotic molecules.[1,6] Platelet microvesicles are membrane fragments from 0.1 μm to 1.0 μm in diameter that form from platelets and other cells due to cell activation or apoptosis. Platelet microvesicles express surface proteins characteristic of platelets, modulate intercellular interactions and have prothrombotic properties.[6] It is well known that platelet activation, aggregation and subsequent thrombus formation play a major role in the pathophysiological process that underlies MIs.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteolytic enzymes involved in vascular remodeling.[7] Platelets contain MMP-2, which is localized in the cytosol and is translocated to the extracellular space during platelet activation.[8] Platelet-derived MMP-2 amplifies the response of platelets to low concentrations of agonists such as collagen, arachidonic acid and adenosine diphosphate (ADP).[9–11] The MMP-2 is involved in the formation of an atherosclerotic plaque and is released from platelets in vivo after the exposure to a damaged vessel wall at the site of vascular injury.[12] The inactivation of MMP-2 genes in mice has been shown to protect against arterial thrombosis, indicating that MMP-2 plays a promoting role in arterial thrombosis and can lead to the formation of an occlusive thrombus.[11]

Regulation of platelet activation and agonist-induced aggregation by intracellular MMP-2 has been associated with glycoprotein (GP) IIb/IIIa receptors that bind to fibrinogen and von Willebrand factor.[13] Moreover, by binding MMP-2, GP IIb/IIIa receptors facilitate the MMP-2-dependent cleavage of platelet PAR-1 and subsequent platelet activation.[14]

Antiplatelet therapy is widely used with good efficiency in the treatment of MIs and ischemic strokes. Current antiplatelet therapy aimed at preventing cardiac IRI includes a cyclooxygenase (COX) inhibitor – aspirin. However, there is a group of patients resistant to aspirin. This resistance has been correlated with an increased level of plasma oxidative stress markers in patients with coronary artery disease (CAD).[15] Cangrelor and ticagrelor are inhibitors of the P2Y12 receptor and have shown to have protective effects independently of their anti-aggregatory effect.[16,17] The interaction with platelets is required for the cardiac protection of cangrelor.[16] Cardioprotection induced by ticagrelor was related to a ticagrelor-mediated release of sphingosine-1-phosphate and adenosine from platelets.[17] As antiplatelet therapy may have unexpected mechanisms of action and aspirin resistance, there is a need to search for new treatment options against IRI and to learn about new mechanisms of action of currently available drugs.

Doxycycline is a well-documented inhibitor of MMPs due to its ability to bind zinc and calcium ions. It is presumed that doxycycline can inhibit pro-MMP activation by suppressing oxidative stresses.[18] A protective effect of doxycycline was shown in the hearts and kidneys of rats subjected to IRI.[19,20] In addition, the protective role of doxycycline used at subthreshold doses (1 μM) in the combination with subthreshold doses of a myosin light chain 1 (MLC1) phosphorylation inhibitor has been described.[19]

Objectives

Doxycycline is a well-documented inhibitor of MMPs, and platelets contain and release MMP-2 upon activation.[1,2] Since MMP-2 may reinforce a platelet response to agonists such as collagen and ADP in atherosclerotic plaque formation, we aimed to evaluate if the inhibition of MMP-2 in platelets by doxycycline can reduce platelet activation and aggregation. In addition, we evaluated whether doxycycline could potentially reduce arterial thrombus formation during coronary vessel obstruction.

We examined the effects of doxycycline in doses ranging from 5 μM to 30 μM on platelet activation in ischemia/reoxygenation (I/R) conditions in order to establish the lowest effective drug concentration. We explored changes in activation, the release of PMVs, and aggregation of platelets subjected to doxycycline and I/R. We measured MMP-2 concentrations and activity in isolated platelets subjected to I/R to assess the impact of doxycycline on MMP-2 release from platelets.

Materials and methods

Materials

Blood was obtained from 15 healthy human volunteers who had not used any medications for at least 14 days. Blood was collected using the S-Monovette® 10 mL blood collection system containing 106 mM of sodium citrate (Sarstedt, Nümbrecht, Germany). The study was approved by the Ethics Committee of the Wroclaw Medical University, Poland (approval No. KB-165/2020).

Platelet isolation

The isolation of platelets was performed using the method described by Wrzyszcz et al.[21] For platelet isolation, a continuous density gradient medium (OptiPrep™; Sigma-Aldrich, St. Louis, USA) was used. In brief, to prevent platelet activation, a prostacyclin (PGI2; Sigma-Aldrich) was added to the blood (final concentration: 2 µg/mL). To obtain platelet-rich plasma (PRP), the blood was centrifuged at 200 g for 20 min at 22°C. The PRP was then diluted (1 part of PRP per 1 part of HEPES buffer (140 mM NaCl, 20 mM HEPES, pH 7.4)), layered onto the surface of the gradient (3 mL of PRP over 5 mL of density gradient) and centrifuged at 300 g for 20 min at 22°C. We allowed the rotor to decelerate without braking. The suspension was extracted except for the last 0.5 mL just above the pellet of cells (leukocytes, erythrocytes). After collection, the suspension was washed in a HEPES buffer with the addition of PGI2 (final concentration: 0.3 µg/mL). All samples were prepared in the same way. Isolated platelets were resuspended in a stabilizing buffer (5.5 mmol/L HEPES, 63.7 mmol/L $CaCl_2$, 5 mmol/L KCl, 2.1 mmol/L $MgCl_2$, 5.5 mmol/L glucose, and 10 mmol/L taurine enriched with 55 µmol/L $CaCl_2$ and 0.75 mg/mL bovine serum albumin (BSA)). The platelet count of the suspensions was quantified using a KX-21N (Sysmex, Kobe, Japan) hematology analyzer.

Chemical I/R protocol

The I/R procedure was performed on 12-well culture plates with inserts (with 1.0-µm pores and 0.4-µm pores; ThinCert™; Greiner Bio-One, Kremsmünster, Austria) dedicated for multi-well plates, in order to allow or prevent the PMVs from entering through the porous membrane. Additionally, the use of inserts made it possible to not have to centrifuge the platelets after each step of the procedure, which could affect their activation. Freshly isolated blood platelets were suspended in stabilizing buffer and placed in the insert. The buffer was added to the wells. The I/R procedure consisted of 3 stages: stabilization, ischemia and reoxygenation. The study included platelets untreated with I/R (AERO), platelets that underwent I/R, and platelets that underwent I/R with doxycycline (I/R+D) (Fig. 1). The concentration of doxycycline (selected from the range of 5–30 µM) was used to determine the lowest concentration that produced a reduction in the expression of platelet activation markers. The I/R+D platelets were pretreated with doxycycline 5 min before the I/R protocol. Appropriate buffers were added to the wells during the subsequent stages of the experiment, and the inserts with platelets were not removed from the wells for the duration of the experiment. During the first phase, a stabilizing buffer was added to the wells. After 15 min, the buffer was removed and the platelets were exposed to ischemia

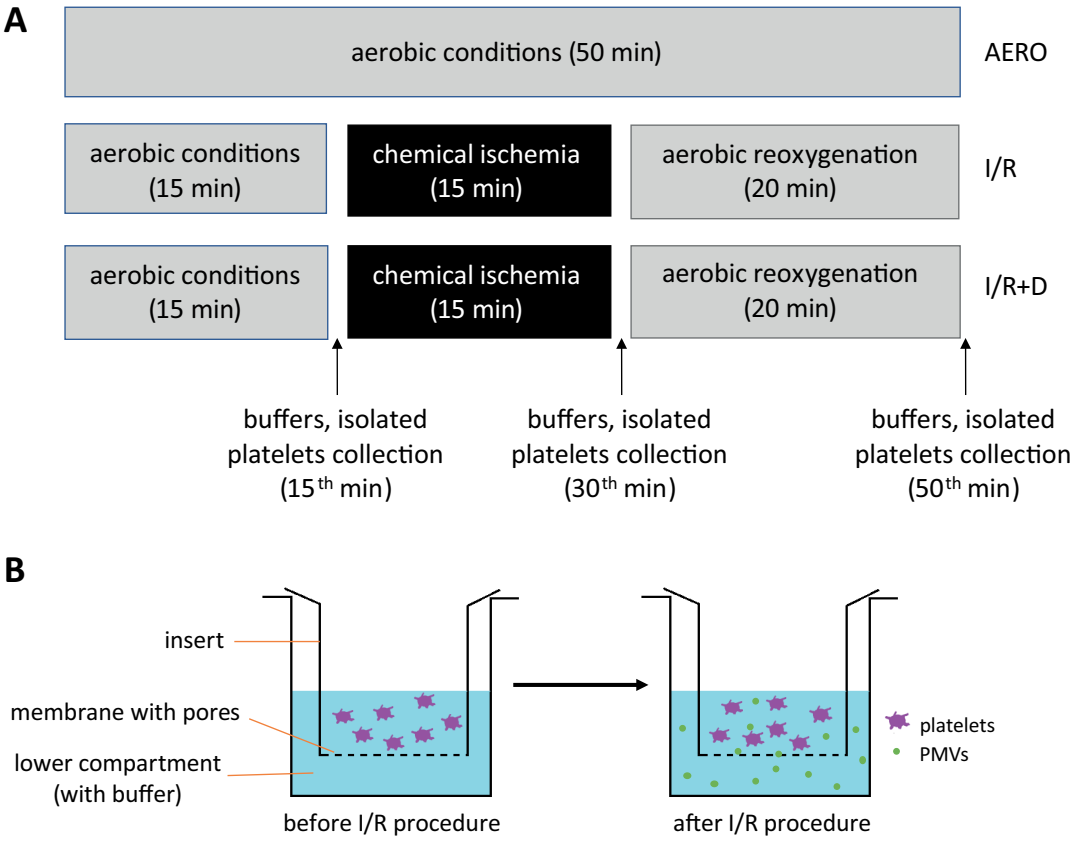

Fig. 1. A. Experimental protocol for aerobic controls (AERO), ischemia/reoxygenation (I/R), and I/R platelets pretreated with doxycycline (I/R+D) groups; B. Platelet microvesicles (PMVs) migrating through the insert with a porous membrane

for 15 min by using a fresh portion of stabilizing buffer with 4.4 mmol/L of 2-deoxyglucose to prevent glycolysis and 4.0 mmol/L of sodium cyanide as a cell respiratory inhibitor. After 15 min, the buffer used for ischemia was removed and a fresh portion of stabilizing buffer was added for 20 min to simulate a reoxygenation process. The buffers and isolated platelets were collected for cytometric analysis before and after ischemia, as well as after reoxygenation. An aggregation of isolated platelets was performed immediately after reoxygenation. The rest of the buffers and isolated platelets were stored at –80°C until further analysis.

Flow cytometry analysis of platelet activation markers

The state of platelet activation was determined by measuring the surface expression of P-selectin (CD62P), activated GP IIb/IIIa (PAC-1) and CD63 antigen. The platelets and the buffers (potentially containing PMVs) were fixed immediately after the collection with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 4°C, centrifuged (1200 g for 5 min), washed in PBS, and then resuspended in Stain Buffer (BD Biosciences, Franklin Lakes, USA). The fixed samples were incubated with fluorescently-labeled monoclonal antibodies: fluorescein isothiocyanate conjugated anti-human CD41/CD61 (clone PAC-1), phycoerythrin conjugated anti-human CD62P (clone AK-4), and phycoerythrin-Cy7 conjugated anti-human CD63 antigen (clone H5C6) or a fluorescently labeled nonspecific mouse IgG antibody (isotype control) for 20 min at room temperature (all antibodies were from BioLegend, San Diego, USA). Finally, the samples were washed, centrifuged, resuspended in PBS, and analyzed within 3 h. The PMVs were analyzed by setting gates based on 0.5-µm calibration beads. The analysis was performed using CyFlow Space (Sysmex) and 20,000 events were collected per sample.

Platelet aggregation

Platelet aggregation was carried out on each collected platelet suspension using light transmission aggregometry (Chrono-Log Corporation, Havertown, USA). Aggregation was performed according to the Platelet Physiology Subcommittee of the International Society on Thrombosis and Haemostasis (SSC/ISTH) recommendations for the standardization of light transmission aggregometry at 37°C, with constant stirring of platelet samples at 1000 rpm using a disposable stirrer.[22] Suspensions of platelets were adjusted to a count of 250×10^3/µL. After 1 min of incubation with fibrinogen (Sigma-Aldrich; final concentration: 200 µg/mL) at 37°C, the aggregation was induced by adding the appropriate agonist. The following agonists were used: 5.02 µg/mL collagen and 10.26 µM ADP (all from HYPHEN BioMed, Neuville-sur-Oise, France). The aggregation curve and the maximum platelet aggregation were recorded over 6 min after the agonist addition.

Gelatin zymography

The activity of MMP-2 was evaluated with gelatin zymography of the platelets after the I/R procedure. The samples were thawed just before use and protein concentrations were determined using the Bradford Protein Assay (Bio-Rad, Hercules, USA). Gelatin zymography was performed with the modification, as previously described.[23] After electrophoresis (Mini-Protean II; Bio-Rad) in 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with copolymerized gelatin (1 mg/mL; Sigma-Aldrich), the gels were washed 3 times in 2.5% Triton X-100 for 20 min to remove SDS and then incubated in an enzyme assay buffer (0.05 M Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.2 M NaCl, 0.05% NaN_3) at 37°C for 18 h. After incubation, the gels were stained with a mixture of 0.3% Coomassie brilliant blue (CBB) R-250 and 0.2% CBB G-250. The gels were scanned using a densitometer (GS-800; Bio-Rad) and analyzed based on standard activity using Quantity One software (Bio-Rad). The relative activity of MMP-2 was expressed in arbitrary units (AU) as an activity per µg of total protein.

Platelet homogenization

Platelet pellets were suspended in a homogenization buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Triton® X-100, Protease Inhibitor Cocktail without ethylenediaminetetraacetic acid (EDTA) set III (Sigma-Aldrich)) on ice. The cells underwent 3 cycles of freezing in liquid nitrogen and thawing at 37°C, and then were homogenized mechanically on ice (3 × 10 s) using a hand-held homogenizer. Cell homogenates were centrifuged for 5 min (14,000 g at 4°C) to collect the supernatant. The supernatants were stored at –80°C and thawed prior to analysis.

Analysis of MMP-2 concentrations in platelets

The MMP-2 content of platelet homogenates was measured using the quantitative Quantikine enzyme-linked immunosorbent assay (ELISA) Assay for Total MMP-2 (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Before ELISA, total protein concentrations were measured using the Bradford Protein Assay (Bio-Rad). The MMP-2 concentrations in platelets were expressed as ng per µg of total protein.

Statistical analyses

Statistical analyses of the results were performed in Statistica 13 (StatSoft Inc., Tulsa, USA). Only the zymography results showed a normal distribution (K-S test $p > 0.2$; Lilliefors correction $p > 0.2$). The F test to check the variance homogeneity was performed, the variance of the zymography results was homogeneous ($p = 0.893$, $F = 1.155$).

The results were then analyzed using the appropriate test: parametric t-test (normal data distribution) and nonparametric (non-normal data distribution) Friedman's analysis of variance (ANOVA) by ranks test with the Dunn's post hoc test or paired Wilcoxon test. The correlation was assessed using the Spearman's test. The results were expressed as mean ± standard error of the mean (M ±SEM). A value of $p < 0.05$ was considered statistically significant.

Results

Working doxycycline concentrations

The surface expression of activated GP IIb/IIIa was significantly lower in I/R+D platelets compared to I/R platelets in the following concentrations of doxycycline: 30 μM, 20 μM and 10 μM (paired Wilcoxon test: $p = 0.046$, $T = 1$; $p = 0.025$, $T = 2$; and $p = 0.036$, $T = 3$, respectively; Fig. 2). Moreover, the surface expression of P-selectin was significantly lower in I/R+D platelets compared to I/R platelets in a concentration of 30 μM (Friedman's ANOVA by ranks test: $p = 0.011$, $\chi^2 = 9$, degrees of freedom (df) = 2) and 20 μM (Friedman's ANOVA by ranks test: $p = 0.030$, $\chi^2 = 7$, df = 2), as was CD63 in concentration of 30 μM (Friedman ANOVA by ranks test: $p = 0.042$, $\chi^2 = 6.333$, df = 2, data not shown). A decreased expression of the above markers was not observed for platelets pretreated with 5 μM of doxycycline. Therefore, the lowest active drug concentration used in the study was 10 μM, based on the reduction of the expression of GP IIb/IIIa receptor on the platelet's surface.

An influence of doxycycline on surface expression of platelet activation markers

The decrease in activated GP IIb/IIIa surface expression in I/R+D platelets compared to I/R platelets of approx. 7.5% (paired Wilcoxon test: $p = 0.043$, $T = 0$) was

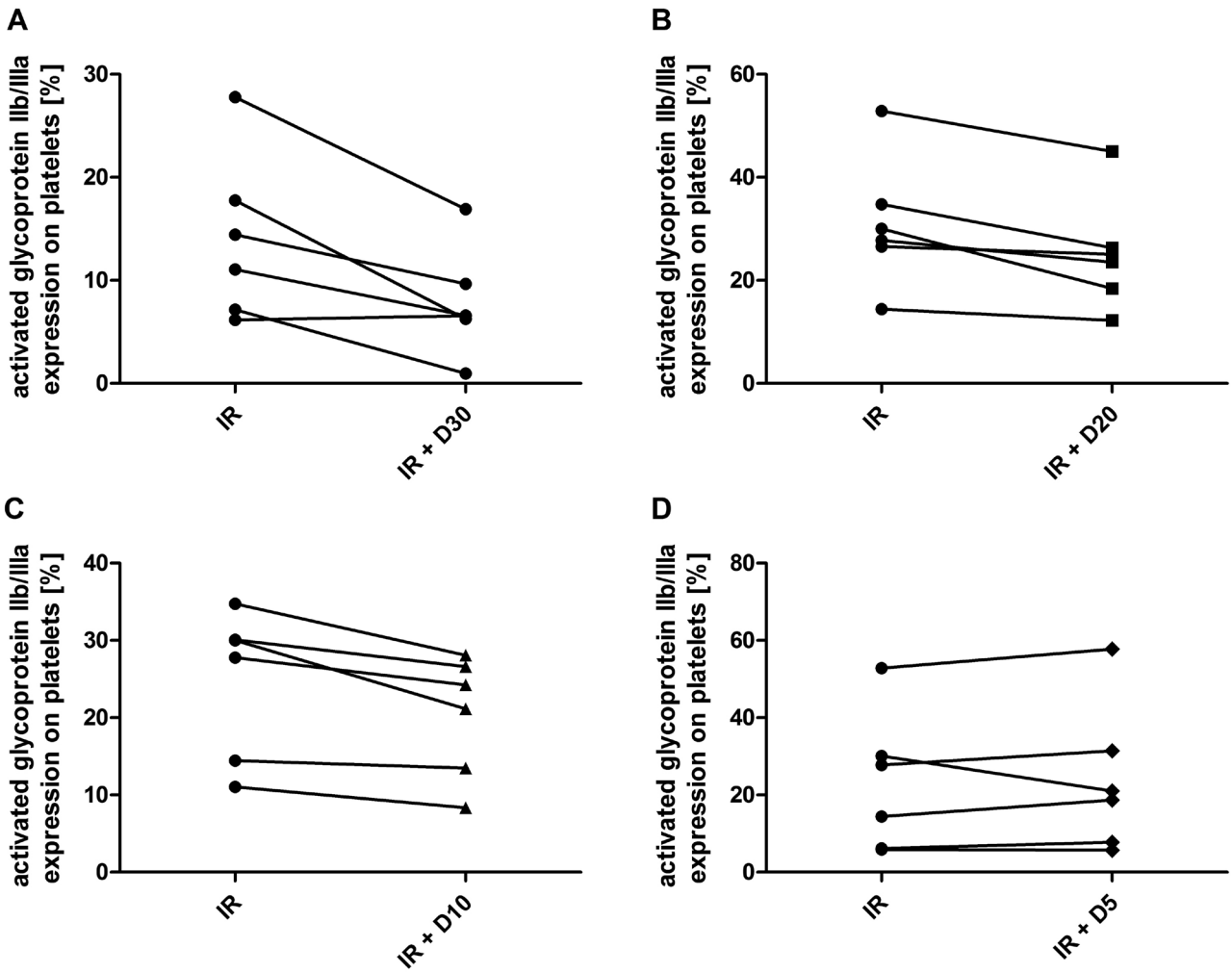

Fig. 2. Expression of activated glycoprotein IIb/IIIa on platelets pretreated with different concentrations of doxycycline: 30 μM (A), 20 μM (B), 10 μM (C), and 5 μM (D) after the ischemia/reoxygenation (I/R) procedure. The differences between I/R and 30 μM, 20 μM, and 10 μM of doxycycline (paired Wilcoxon test): A. 30 μM: $p = 0.046$, $T = 1$; B. 20 μM: $p = 0.025$, $T = 2$; C. 10 μM: $p = 0.036$, $T = 3$; n = 6/group

AERO – aerobic control; I/R – platelets underwent ischemia/reoxygenation; I/R+D30/20/10 – platelets underwent I/R following pretreatment with 30 μM, 20 μM and 10 μM of doxycycline, respectively.

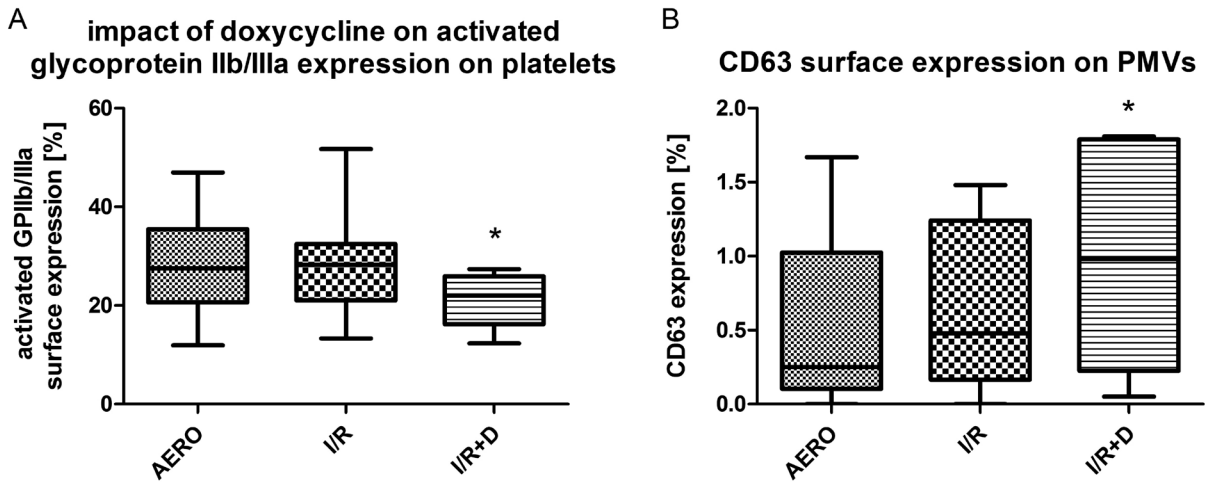

Fig. 3. Expression of activated glycoprotein (GP) IIb/IIIa on platelets after the ischemia/reoxygenation (I/R) procedure (A) and of CD63 on platelet microvesicles (PMVs) in the buffers after I/R procedure using pores with a diameter of 1.0 μm (B)

AERO – aerobic control; I/R – platelets underwent ischemia/reoxygenation; I/R+D – platelets underwent I/R following pretreatment with 10 μM of doxycycline; * $p < 0.05$. Data are expressed as median (middle line), box as 25th–75th percentiles, whiskers indicate minimum and maximum values (paired Wilcoxon test): A. $p = 0.043$, $T = 0$, $n = 7$/group; B. $p = 0.043$, $T = 0$, $n = 5$/group.

observed (Fig. 3A). Changes in the binding of P-selectin (paired Wilcoxon test: $p = 0.075$, $T = 2$) and CD63 (paired Wilcoxon test: $p = 0.463$, $T = 7$) based on the platelets exposure to doxycycline was not observed.

Interesting results were obtained after the last step of the procedure. Cytometric analysis of the buffers showed an increased surface expression of CD63 on PMVs after using inserts with a pore diameter of 1.0 μm in the I/R+D series compared to the I/R series (paired Wilcoxon test: $p = 0.043$, $T = 0$; Fig. 3B). No significant changes in CD63 surface expression were observed in buffers after using inserts with a pore diameter of 0.4 μm, which significantly reduced the transfer of PMVs into the buffer. In addition, there was a trend towards a higher expression of activated GP IIb/IIIa and P-selectin in buffers analyzed after the last step of the procedure in the I/R+D series compared to the I/R series, regardless of the diameter of insert used.

An influence of I/R and doxycycline on platelet aggregation

The aggregation was performed in platelets taken from inserts with a pore diameter of 1.0 μm, which significantly reduced the presence of PMVs in the inserts. Adenosine diphosphate (ADP)-dependent aggregation of platelets that underwent I/R and I/R with doxycycline was significantly lower in comparison to aerobic controls (Friedman's ANOVA by ranks test: $p = 0.022$, $\chi^2 = 7.684$, $df = 2$; Dunn's multiple comparisons post hoc tests: I/R compared to AERO: $p = 0.0003$, I/R+D compared to AERO: $p = 0.0417$). Additionally, there was a strong tendency for an enhanced ADP-dependent aggregation of I/R+D platelets compared to I/R platelets. The collagen-dependent aggregation showed no significant changes after the use of doxycycline (Fig. 4).

An impact of doxycycline on MMP-2 activity and concentration in platelets

Zymogram analysis revealed a 36% higher MMP-2 activity in I/R+D platelets compared to I/R platelets (t-test: $p = 0.007$, $t = 6.771$, $df = 3$) when 1.0-μm diameter inserts were used (potential MMP-2 in PMVs was eliminated; Fig. 5A). The examination of MMP-2 concentrations in platelets revealed a strong tendency (Friedman's ANOVA by ranks test: $p = 0.091$, $\chi^2 = 4.8$, $df = 2$) for higher MMP-2 levels in platelets treated with doxycycline (Fig. 5B). Additionally, a correlation between MMP-2 activity and the expression of activated GP IIb/IIIa on I/R+D platelets was observed but was not significant due to the small sample size ($p = 0.233$, Fig. 6). Interestingly, the use of a 0.4-μm pore size insert did not change the MMP-2 activity in platelets between series. Additionally, no differences in MMP-2 activity were noticed between the studied groups after the ischemia stage. The activity and concentration of MMP-2 were undetectable in buffers.

Discussion

The protective effects of doxycycline against ischemia have been shown to involve various organs and mechanisms. Doxycycline displays a protective effect by inhibiting MMP-2 in myocardial IRI studies.[19] In cerebral ischemia, the protection of doxycycline was associated with MMP-9, MMP-2 and PKCδ inhibition and upregulation of tight junction proteins.[24,25] Another protective mechanism of doxycycline was presented in a model using rat hepatocytes, where the protection against hypoxia and cell death was associated with the inhibition of mitochondrial calcium uniporters.[26] Tetracyclines have therapeutic

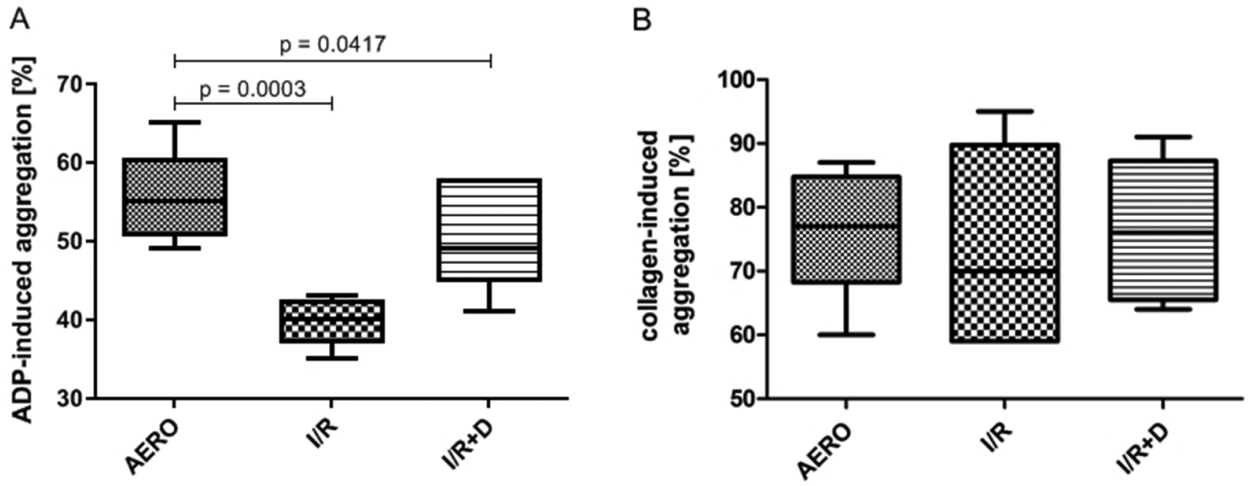

Fig. 4. Platelet aggregation after the ischemia/reoxygenation (I/R) procedure induced by adenosine diphosphate (ADP) (A) and collagen (B)

AERO – aerobic control; I/R – platelets underwent ischemia/reoxygenation; I/R+D – platelets underwent I/R following pretreatment with 10 μM of doxycycline. Data are expressed as median (middle line), box as 25th–75th percentiles, whiskers indicate minimum and maximum values; Friedman analysis of variance (ANOVA) by ranks test (A): $\chi^2 = 7.684$, df = 2, p = 0.022; Dunn's multiple comparisons post hoc test: I/R compared to AERO: p = 0.0003; I/R+D compared to AERO: p = 0.0417; n = 6/group (A); n = 6/group (B).

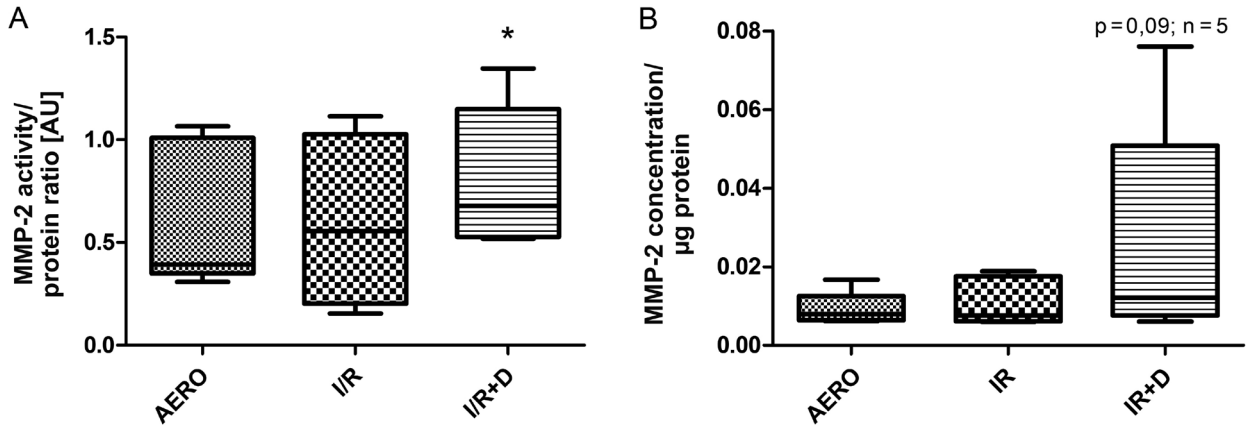

Fig. 5. The MMP-2 activity (A) and concentration (B) in platelets normalized to protein concentrations

AERO – aerobic control; I/R – platelets underwent ischemia/reoxygenation; I/R+D – platelets underwent I/R following pretreatment with 10 μM of doxycycline; * p < 0.05. Data are expressed as median (middle line), box as 25th–75th percentiles, whiskers indicate minimum and maximum values; A. t-test: p = 0.007, t = 6.771, df = 3; B. Friedman analysis of variance (ANOVA) by ranks test: p = 0.091, $\chi^2 = 4.8$, df = 2; I/R+D compared to AERO and I/R; n = 5/group (A); n = 5/group (B).

potential in cardiovascular diseases, especially in cardiac IRI, due to the involvement of MMP-2 in the degradation of cardiac contractile proteins, including troponin I.[27] The MMP-2 inhibition by doxycycline improved the recovery of cardiac mechanical function and led to a reduced endothelial permeability in cardiac IRI.[27] Additionally, doxycycline was able to protect cardiac tissue using a much lower concentration when combined with myosin light chain kinase inhibitors than doxycycline alone.[19] In addition to cardiac IRI, the protective effects of doxycycline against IRI have also been demonstrated in the kidneys and the nervous system.[24,28] In a rat stroke model, doxycycline preserved the function of blood–brain barrier and attenuated cerebral infarction.[24] The numerous in vitro and animal model studies suggesting that doxycycline can protect various organs against IRI and cell death have resulted

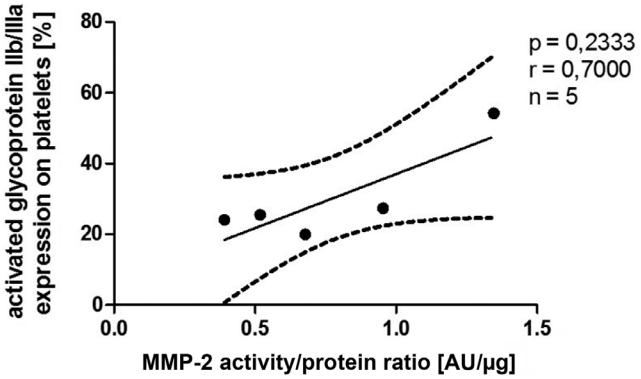

Fig. 6. Correlation between MMP-2 activity in platelets and activated glycoprotein IIb/IIIa expression on platelets pretreated with doxycycline after the ischemia/reoxygenation (I/R) procedure

Spearman's correlation: p = 0.233, r = 0.7, n = 5.

in clinical trials evaluating doxycycline in the treatment of I/R. Patients with ST-elevation myocardial infarction (STEMI) and left ventricular (LV) dysfunction treated with timely primary percutaneous intervention and short-term treatment with doxycycline were shown to have decreased infarct sizes and severities and a reduction in LV dysfunction and remodeling.[29] These changes were associated with plasma levels of tissue inhibitors of metalloproteinase 2 (TIMP-2), since doxycycline therapy resulted in higher plasma levels of TIMP-2.[29] In patients with active CAD, subantimicrobial doses of doxycycline reduced the levels of C-reative protein (CRP), interleukin 6 (IL-6) and pro-MMP-9. The authors suggested that doxycycline may potentially exert beneficial effects on inflammation and prevent coronary plaque rupture events.[30]

In this study, we demonstrated that doxycycline decreased platelet activation in I/R conditions. Doxycycline reduces platelet activation by decreasing the expression of GP IIb/IIIa on platelets. At the moment, we do not know the mechanism of action of doxycycline. In our study, we demonstrated that I/R platelets pretreated with doxycycline presented higher activity and concentration of MMP-2 than I/R platelets without doxycycline. The MMP-2 is released from platelets as a result of their activation.[8] Moreover, MMP-2 was shown to regulate platelet activation and aggregation by the interaction between MMP-2 and GP IIb/IIIa.[13] Our findings confirmed that the mechanism of doxycycline underlying platelet activity is based on the interaction of MMP-2 and platelet GP IIb/IIIa in I/R conditions. Furthermore, the released MMP-2 mediates the activation of consecutive platelets by increasing the expression of P-selectin, one of the platelet activation markers.[31] The increased activity and concentration of MMP-2 in platelets subjected to I/R+D, as well as the lack of increase in the concentration and activation of MMP-2 in the extracellular space and the decreased activity of platelets, prove the mechanism of action of doxycycline in our model. Similar results were demonstrated in the in vitro model of knockout mice, MMP-2$^{-/-}$, where the inactivation of the *MMP-2* gene impaired P-selectin expression and decreased the formation of platelet/leukocyte aggregates upon in vivo activation.[11] We showed that doxycycline's mechanism of action on platelets arises from the blocking of MMP-2 release from platelets, leading to a decrease in the expression of activated GP IIb/IIIa on platelets, a key player in thrombus formation contributing to MI. The intracellular-active MMP-2 was shown to affect the activation and aggregation of platelets through the hydrolytic activation of talin, which is associated with the activation of GP IIb/IIIa.[13] Platelets release pro-MMP-2 and the active form of MMP-2 at the site of vascular damage in vivo. The amount of MMP-2 released locally is capable of enhancing the response of platelets to a stimulus.[12] Additionally, platelets are a major source of MMP-2 in acute coronary syndrome (ACS) patients.[31] The MMP-2 contributed to cardiac contractile dysfunction following I/R through the degradation of cardiac contractile proteins. We have shown that doxycycline prevents platelet activation by inhibiting MMP-2 release. Given the participation of MMP-2 in the degradation of cardiac contractile proteins and the fact that platelets were the source of MMP-2, it seems that doxycycline may prevent cardiac injury through the regulation of platelet MMP-2.

Previous data have shown that MMP-2 released from platelets was able to facilitate ADP-, collagen- and thrombin-induced aggregation, although MMP-2 alone did not induce resting platelet aggregation.[10] On the other hand, high concentrations of MMP-2 inhibited collagen-induced aggregation of platelets.[9] We demonstrated that doxycycline did not affect collagen-induced platelet aggregation. Our results are partially consistent with a study using healthy rats, where intravenous administration of doxycycline resulted in no changes in ADP and collagen-induced platelet aggregation after 15 min.[32] Our results also revealed that doxycycline had a strong tendency to enhance ADP-induced platelet aggregation in I/R. This finding is opposite to another study using healthy dogs, in which doxycycline administered at therapeutic antimicrobial concentrations had no effect on platelet aggregation using 2 agonists: ADP and platelet-activating factor (PAF).[33] Another study reported a positive correlation between levels of ADP-induced aggregation and GP IIb/IIIa content in healthy volunteers and patients with ACS within the first hour upon admission to the hospital. However, after a few days, there was no correlation between ADP-induced aggregation and GP IIb/IIIa content in ACS patients.[34] Discrepancies in the results obtained by us and other researchers may be both due to the dose of doxycycline and the final concentration of agonist. Additionally, in our research, in addition to the use of doxycycline, platelets were also exposed to I/R, which undoubtedly affected them in a different way than after the use of doxycycline alone. Also, collagen is a strong agonist. Therefore, it is possible that slight changes in platelet aggregation caused by doxycycline may not have significantly affected their response to this agonist. We showed that ADP-induced platelet aggregation capacity decreased when platelets were subjected to I/R and I/R with doxycycline compared to platelets in aerobic conditions. Interestingly, I/R platelets pretreated with doxycycline presented a strong tendency to increase ADP-induced platelet aggregation capacity compared to I/R platelets without the drug. It seems that as a result of I/R, the aggregation capacity of platelets is reduced and their function is impaired, similar to the case of, for example, the storage of platelets.[35] At the same time, the use of doxycycline had a protective effect on the maintenance of platelet function in I/R.

Platelets microvesicles, as mentioned, are released from platelets during their activation in response to stimulants such as oxidative stress.[6] Vélez et al. showed that in STEMI, the PMV fibrinogen is upregulated; therefore, PMVs may be involved in the excessive platelet aggregation responsible

for atherothrombosis.[36] Jung et al. showed that in STEMI patients, circulating PMV levels are correlated with the degree of ischemia.[37] In this research, we used inserts with 2 different pore diameters: 1.0 μm to allow the PMVs enter the buffers and 0.4 μm to prevent penetration by most of the PMVs. For this reason, the use of the appropriate pore size allowed us to study the changes resulting from the presence or absence of PMVs, since MI triggers PMVs, which contain pro-inflammatory, proapoptotic and prothrombotic properties.[6] By using inserts allowing the separation of platelets from PMVs, we confirmed the release of PMVs in I/R conditions. We demonstrated that platelets pretreated with doxycycline showed enhanced CD63 expression on PMVs after I/R in comparison to PMVs from platelets without doxycycline when 1.0-μm inserts were used. According to our knowledge, there is no evidence explaining the interaction between CD63 and doxycycline. Tetraspanin CD63, present on lysosomes and membranes of dense granules in resting platelets, is expressed on platelet membranes as a result of their activation. The CD63 is a common platelet-derived exosomal marker that is also present on PMVs.[6,38] However, pharmacotherapy, especially antiplatelet drugs, have been shown to reduce the level of PMVs released.[6] This effect may be dose-dependent since low-dose acetylsalicylic acid therapy did not reduce the release of PMVs into the microcirculation.[39] Additionally, PMVs may be a potential biomarker for the response to antiplatelet therapy. As in the case of clopidogrel, increased levels of circulating PMVs were observed in ACS patients with a high on-treatment platelet reactivity compared to patients with a low on-treatment reactivity on clopidogrel.[40] In our model, we observed an increase in the expression of CD63 on PMVs, which may be related to concentrations of doxycycline being not sufficient to reduce the release of PMVs.

Enhanced activation and aggregation of platelets is a well-established component of the pathophysiology of MIs. This study revealed a new possibility for the application of doxycycline in antiplatelet therapy.

Limitations

A limitation of our study is the small sample size, which could have affected the results. Therefore, further research on this topic should be based on a larger number of cases.

Conclusions

In our research, we showed that the inhibition of platelet MMP-2 by doxycycline attenuated platelet activation and protected platelets by preserving their aggregation ability. The use of doxycycline in cardiac IRI may indicate a two-fold benefit: first, protection of cardiac contractile proteins, and second, prevention of platelet activation and unwanted aggregation. Doxycycline should be taken into consideration for antiplatelet therapy and MI prevention.

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