Metformin-suppressed platelet's function in vitro: Possible relation to delayed or failure of platelet-rich fibrin preparation

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ABSTRACT

Platelet-rich fibrin (PRF) is a popular autologous blood-derived biomaterial that is used in regenerative therapy. Owing to its simple preparation without additional factors, the PRF quality directly reflects the characteristics of individual blood samples. Antiplatelet or anticoagulant drugs can hamper the successful preparation of PRF. We recently observed similar phenomena in metformin-taking type-2 diabetics (T2DM). Thus, we hypothesized that metformin interferes with platelet function, thereby suppressing coagulation. For practical reasons, leukocyte- and platelet-rich plasma was prepared from healthy male donors ($n = 9$–15, age: 26–80 years) and treated with metformin (1–10 mM) for 24–72 h. Intrinsic and extrinsic coagulation activities were evaluated using pro-thrombin time (PT) and activated partial thromboplastin time (ATPP). Platelet adhesion and aggregation assays were performed using ADP stimulation. Among the parameters tested, APTT was the most sensitive and was significantly prolonged in the concentration range of 1–10 mM in a time- and concentration-dependent manner. Although obtained from healthy platelets and relatively higher concentrations of metformin, these findings suggest that metformin may induce further dysfunction of platelets to suppress intrinsic coagulation activity in T2DM patients, leading to failure of PRF preparation. This phenomenon may not have a severe impact on clinical diabetology or hematology. However, clinicians using PRF are recommended to be more sensitive to such information to avoid unexpected events in clinical settings.

1. Introduction

Platelet-rich fibrin (PRF) was developed by modifying a conventional protocol of platelet-rich plasma (PRP) preparation (Dohan et al., 2006). The major advantages of PRF are that its preparation protocol does not require anticoagulants or coagulation factors and is rarely sensitive to operator skill (Kawase, 2015). The major disadvantage is that several known diseases and medications related to platelet and coagulation pathways may negatively affect PRF preparation during centrifugation (approximately 12 min). In most cases, such failure can

be predicted by carefully examining the patient's medical history. However, in cases involving unknown factors, it is almost impossible to avoid unexpected events. Regardless of these factors, clinicians may need to quickly modify their therapeutic plans when unexpected events occur.

To date, we have observed this type of PRF preparation failure in two out of approximately 500 cases. Careful medical history analysis revealed a possible factor in these patients: they commonly suffered from type 2 diabetes mellitus (T2DM) and long-term treatment with metformin. Metformin has been the preferred first-line treatment for

T2DM in many countries and regions for decades (Good and Pogach, 2018). Unlike in the US and Europe, metformin is not chosen for the treatment of patients with T2DM, preferably in Japan. The current Japanese T2DM management guidelines leave medication selection at the discretion of the physician in charge, considering the patient characteristics and pathophysiology of the disease. Recent statistics show that dipeptidyl peptidase-4 inhibitors were the most prescribed (65.1%) compared to biguanides (15.9%), and biguanides were not prescribed at all in 38.2% of non-Japan Diabetes Society-certified facilities (Bouchi et al., 2022). In addition, the number of patients with DM is <20% of that in the US (American Diabetes Association, 2023; Kohsaka et al., 2021). Considering these numerical disadvantages, our findings cannot be ignored for PRF users in western countries.

Metformin has recently been repurposed or repositioned as a promising drug for cancer prevention and treatment (Andrzejewski et al., 2018; Tossetta, 2022) and as an anti-aging therapy (Mohammed et al., 2021). This trend appears to be consistent with the discovery of the molecular mechanisms of metformin. Metformin enters the cytoplasm through organic cation transporter member 1 (OCT1) (Wu et al., 2018) and directly acts on the mitochondria to decrease respiration instead of increasing aerobic glycolysis (Andrzejewski et al., 2018; Protti et al., 2012). This finding raises the possibility that platelet function and subsequent coagulation can be suppressed if a similar mechanism is observed in the platelets. In support of this possibility, a few studies have reported that metformin reduced platelet aggregation and adhesion in vitro (Markowicz-Piasecka et al., 2019).

To determine the role of metformin in failed or delayed PRF preparation, we evaluated the effects of metformin on platelet physiological function and coagulation in vitro using platelets from non-diabetic healthy donors rather than from patients with T2DM receiving no medication.

2. Materials and methods

2.1. Blood collection

The study design and consent forms for all procedures (project identification code: 2021–0126) were approved by the Ethics Committee for Human Participants at Niigata University (Niigata, Japan) and complied with the Helsinki Declaration of 1964, as revised in 2013. Informed consent was obtained from all subjects involved in the study.

Blood samples were collected from male volunteers ($n = 9$–15, age: 26–80 years). Approximately 7.5 mL of peripheral blood was collected in glass vacuum blood collection tubes (Vacutainer®; BD Biosciences, Franklin Lakes, NJ, USA) containing 1.5 mL A-formulation of acid-citrate-dextrose solution (ACD-A) (Terumo, Tokyo, Japan). The inclusion criteria were male, healthy, and non-smoking. The participants provided written agreement for informed consent but did not receive continuous medical treatment. Donors who had smoking habits and severe lifestyle-related diseases, especially blood diseases, and were taking medications, especially anti-coagulant or anti-platelet drugs, were excluded, regardless of the degree of limitation in their daily activities. Donors, who were positive for HIV, HBV, HCV, or syphilis were also excluded by pre-checking their medical history. In addition, blood samples with milky plasma were excluded.

Blood cell counts were determined using an automated hematology analyzer (pocHi V-diff, Sysmex Corporation, Kobe, Japan). If the histograms of the platelet distribution did not display a smooth curve, the samples were discarded and not subjected to further experiments. Following the ethics committee guidelines, three blood collection tubes (~20 mL) were used for each donor, and each sample was randomly allocated to one of the treatment groups, depending on platelet counts.

2.2. Leukocyte-rich-PRP preparation and treatment with metformin

The blood samples were stored or transported to the laboratory and subjected to the leukocyte-rich PRP (L-PRP) preparation within ~20 h to reduce the volume of the samples. The samples were centrifuged horizontally at 415 *g* for 10 min (soft spin) (Kubota, Tokyo, Japan). The upper plasma fraction, which was ~2 mm beyond the interface of the plasma and red blood cell fractions, was transferred into 2 mL sample tubes and centrifuged at 664 ×*g* for 4 min (hard spin) using an angle-type centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany) to collect the blood cell pellets. Platelets were then resuspended in platelet-poor plasma. The original blood cell counts (mean ± SD) are shown in Table 1.

Each L-PRP preparation was treated with metformin in sample tubes and incubated for 24, 48, or 72 h by intermittent stirring with a tube roller mixer at room temperature (20–23 °C). Platelets are very sensitive to external stimuli and tend to easily adhere to various materials at 37 °C. Thus, to preserve platelets for therapeutic use, that is, platelet transfusion, and to minimize the possible loss of their functional activity for several days, platelets are generally incubated at room temperature with gentle agitation. Based on such an established procedure (Aubron et al., 2018), we chose to incubate the sample at room temperature instead of incubation 37 °C.

2.3. Determination of modified prothrombin time

Prothrombin time (PT) is a routine laboratory test that evaluates coagulation status, more specifically, the status of the extrinsic and common pathways of coagulation (Yang and Moosavi, 2023).

According to the manufacturer's instructions, PT should be determined at the point of care using freshly collected blood without anti-coagulants in the case of CoaguChek (Roche Diagnostics Japan, Tokyo, Japan). Therefore, to examine stored citrated blood samples, appropriate amounts of CaCl2 should be added to restore coagulation activity. In our modification protocol, immediately before measurement, each L-PRP sample (15 μL) was mixed well with the same volume of pre-warmed 0.02 M CaCl$_2$ solution (Sysmex, Kobe, Japan) and applied to the strip.

This device maintains the incubation chamber at 37 °C (fixed) and provides data on PT, prothrombin time percentage activity (PTPA), which was expressed as %Q in this device, and international normalized ratio (INR). Both PTPA and INR also represent PT; however, because usefulness and accuracy may depend on patient conditions (Takikawa et al., 2014), in this study, we expressed PT data using both PT and PTPA styles.

2.4. Determination of modified activated partially thromboplastin time

The activated partial thromboplastin time (APTT) is a commonly used coagulation assay that measures the function of intrinsic and common coagulation pathways (Ignjatovic, 2013).

The APTT was determined manually using an aPTT-SLA kit (Sysmex). This kit was also designed for use with stored citrated blood samples. However, the original protocol was slightly modified to amplify the possible differences by decreasing the temperature from 37 °C to 30 °C. Briefly, 100 μL of the plasma fraction prepared from each L-PRP sample was mixed with 10 μL of APTT reagent, incubated for 3 min at 30 °C, and subsequently mixed with 90 μL of 0.02 M pre-warmed CaCl$_2$ solutions, and incubated at 30 °C. The time required for fibrin clot formation was determined.

Table 1
Blood cell counts in L-PRP.

	n	Counts (mean ± SD)	
Age	15	49.2 ± 15.3	
White blood cells	15	62.8 ± 23.7	($\times 10^2$/μL)
Red blood cells	15	89.9 ± 37.6	($\times 10^4$/μL)
Platelets	15	53.4 ± 22.7	($\times 10^4$/μL)

2.5. Pooled normal plasma used as a standard

To test the possible direct effects of metformin on plasma without blood cells, pooled normal human plasma (citrated plasma) was obtained from George King Bio-Medical, Inc. (Overland Park, KS, USA). The modified PT and APTT values were determined as previously described.

2.6. Determination of platelet ATP levels

The platelet ATP assay is not used in clinical practice. However, because most platelet functions require energy (i.e., ATP), monitoring platelet ATP levels is expected to indirectly evaluate platelet functional activities.

An aliquot of L-PRP samples treated with metformin was centrifuged at 664 g for 4 min to prepare a platelet suspension in PBS. The platelet suspension was subjected to cell counting and was diluted 10-fold with pure water. Platelet ATP levels were determined using a luminescence ATP assay kit (Dojin, Kumamoto, Japan) and luminescence kit (AB-2200, Atto Corp., Tokyo, Japan). The data were normalized to the platelet counts.

2.7. Platelet functional assay (1): Adhesion assay

The platelet adhesion assay is popular but rarely routine in clinical practice, but can be used for monitoring platelet function regarding adhesion and the effect of antiplatelet drugs (Lopez-Alonso et al., 2013).

Platelets in 200 μL of L-PRP were stimulated with 0.2 mM ADP (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) and incubated for 10 min at room temperature to test platelet adhesion ability. In the second half (5 min) of this incubation period, 100 μL of glass microbeads (BZ-04, φ0.350–0.500 mm) (AS ONE, Tokyo, Japan) was added to the L-PRP samples. At the end of the incubation, the number of platelets in the supernatant was counted.

2.8. Platelet functional assay (2): Aggregation assay

Platelet aggregation (aggregometry) assays are also used to analyze platelet aggregation (Tsoupras et al., 2019) and activity.

L-PRP samples were quickly centrifuged, and the upper plasma fractions were used as pure PRP samples. When platelet counts were > 20–40 × 10^4/μL, pure PRP samples were appropriately diluted with platelet-poor plasma. Pure-PRP samples were pre-incubated for 1 min at 37 °C, treated with ADP (0.1 mM), and aggregation was monitored for 5 min using a spectrophotometer-based aggregometer (PRP3000S, TAIYO, Osaka, Japan). The levels of maximal aggregation and the aggregation area under the curve (AUC) were determined.

2.9. Statistical analysis

Because platelet activation levels and responsiveness vary broadly among individuals, and platelet and coagulation activities decline with incubation time, all data regarding platelets and coagulation were expressed as fold-change (ratio to the control). The data for age and blood cell count are expressed as mean ± standard deviation in tables, and the other data are presented as dot plots in the figures. The plots were drawn using KaleidaGraph version 5 (Synergy Software, Reading, PA, USA). For multi-group comparisons, the Kruskal-Wallis test by ranks was performed, followed by Tukey's post-hoc test (SigmaPlot 13.0; Systat Software, Inc., San Jose, CA, USA). A *P*-value <0.05 was considered to be indicative of a statistically significant difference.

3. Results

The effects of metformin on the modified PT and PTPA of the L-PRP samples at 24, 48, and 72 h are shown in Fig. 1. Metformin prolonged PT and decreased PTPA levels in a concentration-dependent manner. Significant changes were not observed with 1 mM metformin at any time point, while metformin at 5–10 mM significantly influenced both parameters at 48 h.

The effects of metformin on modified PT and PTPA of the pooled plasma at 24, 48, and 72 h are shown in Fig. 1S. In contrast to L-PRP samples, either parameter of acellular pooled plasma was not significantly influenced by metformin (1–10 mM) at any time.

The effects of metformin on modified APTT of L-PRP samples at 24, 48, and 72 h are shown in Fig. 2. Metformin (1–10 mM) significantly prolonged the APTT in a concentration- and time-dependent manner.

The effects of metformin on the modified APTT of pooled plasma at 24, 48, and 72 h are shown in Fig. 2S. As observed for PT, this parameter of acellular pooled plasma was not significantly influenced by metformin (1–10 mM) at any time.

The effects of metformin on ADP-induced adhesion of platelets contained in L-PRP samples at 24, 48, and 72 h are shown in Fig. 3. Metformin (5–10 mM) significantly and substantially suppressed adhesion activity in a concentration- and time-dependent manner.

The effects of metformin on the ADP-induced platelet aggregation activity in pure-PRP samples at 24, 48, and 72 h are shown in Fig. 4. Similar to platelet adhesion, metformin (5–10 mM) significantly and substantially decreased both the maximal aggregation and aggregation AUC of aggregation activity in a concentration- and time-dependent manner.

The effects of metformin on ATP levels in platelets that were washed and suspended in PBS are shown in Fig. 5. Significant changes were not observed at 24 h; however, at 48 h and later, metformin (5–10 mM) reduced platelet ATP levels in a concentration- and time-dependent manner.

4. Discussion

To address our clinical question, we performed in vitro experiments and found that metformin inhibited coagulation by reducing platelet activity. To our knowledge, only a few studies have examined the inhibitory effects of metformin on platelets (Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2017; Xin et al., 2020). Previous studies have reported that in vitro treatment with metformin does not affect either the extrinsic or intrinsic coagulation pathways, regardless of the varied concentrations and incubation times (Markowicz-Piasecka et al., 2017). In contrast, clinical studies have shown that metformin prolongs APTT and PT in a dose-dependent manner (Ghatak et al., 2011; Krysiak et al., 2013). The discrepancy between these findings raises the possibility that metformin suppresses coagulation activity in animals and patients, probably through condensation or intermediation.
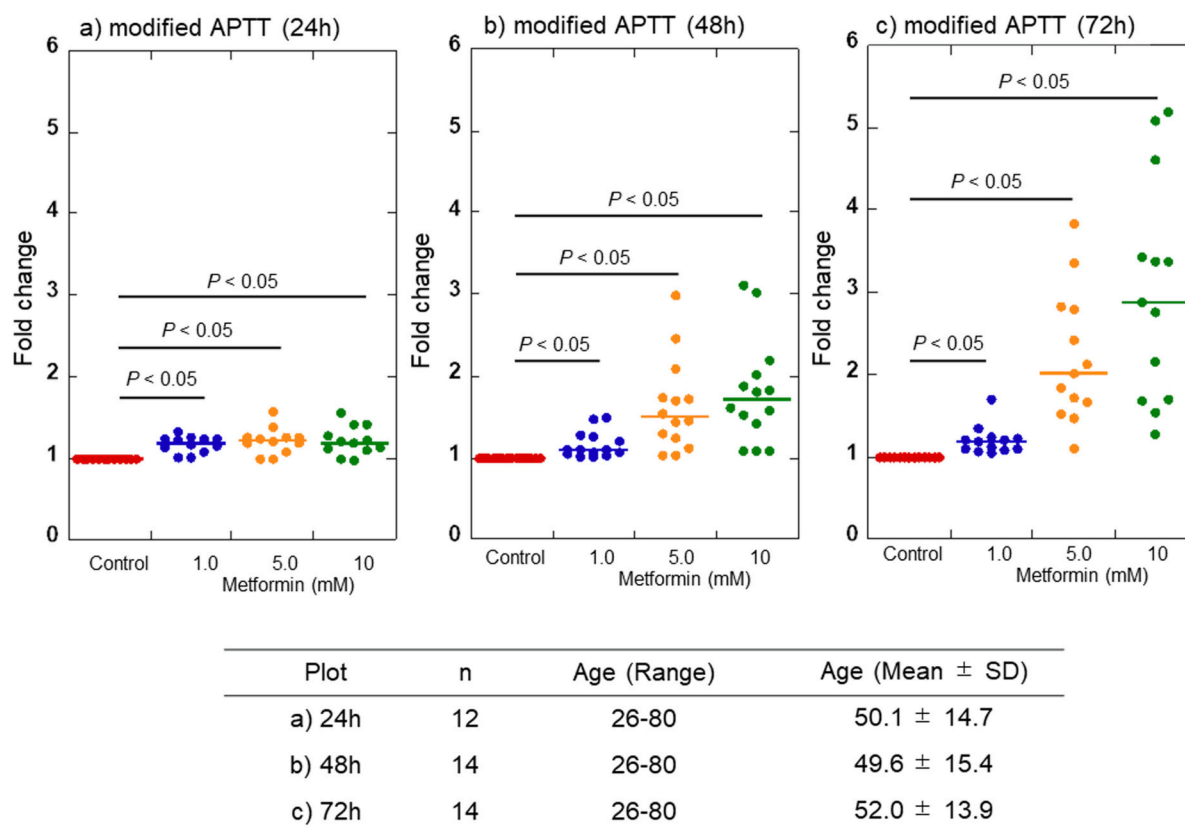
4.1. Multidirectional effects of metformin on thrombosis and hemostasis

Metformin is the safest and most widely prescribed first-line therapy for T2DM, with over 120 million prescriptions worldwide annually (Xin et al., 2020). However, its effects are not limited to its glucose-lowering activity. Metformin has recently been recognized by its lipid-lowering, cardioprotective, and anti-inflammatory properties (Markowicz-Piasecka et al., 2020). In addition, interesting findings have been reported regarding the effects of metformin on platelet and plasma hemostasis. Although the available data are limited, fragmented, and unstructured, recent studies have demonstrated that metformin reduces the severity of ischemic stroke in T2DM patients and inhibits platelet activation and thrombosis in mouse models (Xin et al., 2020). Another study demonstrated that metformin improved endothelial function, reduced smooth muscle proliferation, and decreased reactive oxygen species production (Markowicz-Piasecka et al., 2020). Taken together, metformin may hamper or delay in vitro PRF preparation in particular types of patients without clear subjective symptoms such as prolonged bleeding time (Xin et al., 2020).
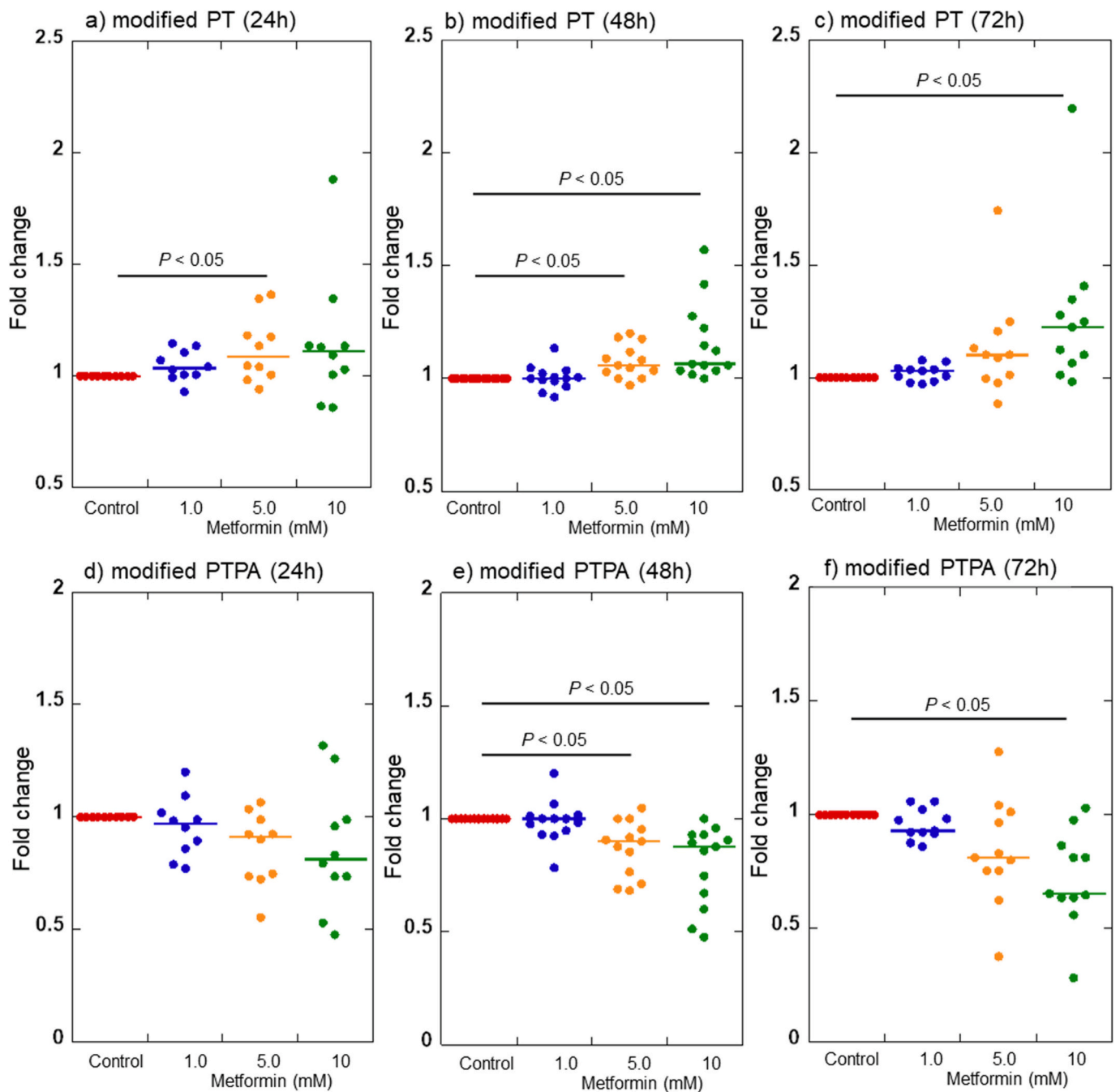
Plot	n	Age (Range)	Age (Mean $\pm$ SD)
a) 24h	10	31-80	53.2 $\pm$ 14.3
b) 48h	13	26-80	53.3 $\pm$ 17.3
c) 72h	11	26-80	53.2 $\pm$ 17.3

Fig. 1. The effects of metformin on (a − c) modified PT and (d − f) PTPA of L-PRP samples at (a, d) 24, (b, e) 48, and (c, f) 72 h. $P < 0.05$ represents a significant difference from the respective controls. The sample size and donors' age of each group are summarized in the table at the bottom of the figure. The raw mPT data of controls were 14.8 $\pm$ 1.7 s (24 h), 15.3 $\pm$ 2.6 s (48 h), and 16.6 $\pm$ 3.3 s (72 h). The raw PTPA data of the control were 69.7 $\pm$ 10.7% (24 h), 70.8 $\pm$ 10.8% (48 h), and 64.4 $\pm$ 14.4% (72 h).

Plot	n	Age (Range)	Age (Mean ± SD)
a) 24h	12	26-80	50.1 ± 14.7
b) 48h	14	26-80	49.6 ± 15.4
c) 72h	14	26-80	52.0 ± 13.9

Fig. 2. The effects of metformin on modified APTT of pooled plasma at (a) 24, (b) 48, and (c) 72 h. $P < 0.05$ represents a significant difference from the respective controls. The sample size and donors' age of each group are summarized in the table at the bottom of the figure. The raw mAPTT data of controls were 121.1 ± 30.8 s (24 h), 138.7 ± 42.6 s (48 h), and 148.9 ± 41.6 s (72 h).

4.2. Metformin action in coagulation pathways

To the best of our knowledge, no previous study has reported the effects of metformin on any component of the coagulation pathway. In this in vitro study, we demonstrated that metformin inhibited coagulation only in the presence of platelets. Based on comparisons of the effective concentrations, platelets were more sensitive to metformin than to the coagulation pathway. Considering the generally accepted mechanism by which the intrinsic coagulation pathway requires activated platelets at two reaction sites, prothrombin and factor X (Rosing et al., 1985), it is possible that metformin primarily suppresses platelet activation, thereby reducing coagulation (Fig. 6).

However, it is possible that metformin acts on red blood cells to release factors, such as lactic acid exhausted from the glycolytic ATP generation system, and impaired coagulation cannot be ruled out (DeFronzo et al., 2016; Engstrom et al., 2006; Piel et al., 2015). This cycle can be repeated and prolonged by metformin accumulation in major blood cells (Lalau and Lacroix, 2003). Further studies are required to elucidate the mechanisms underlying this phenomenon.

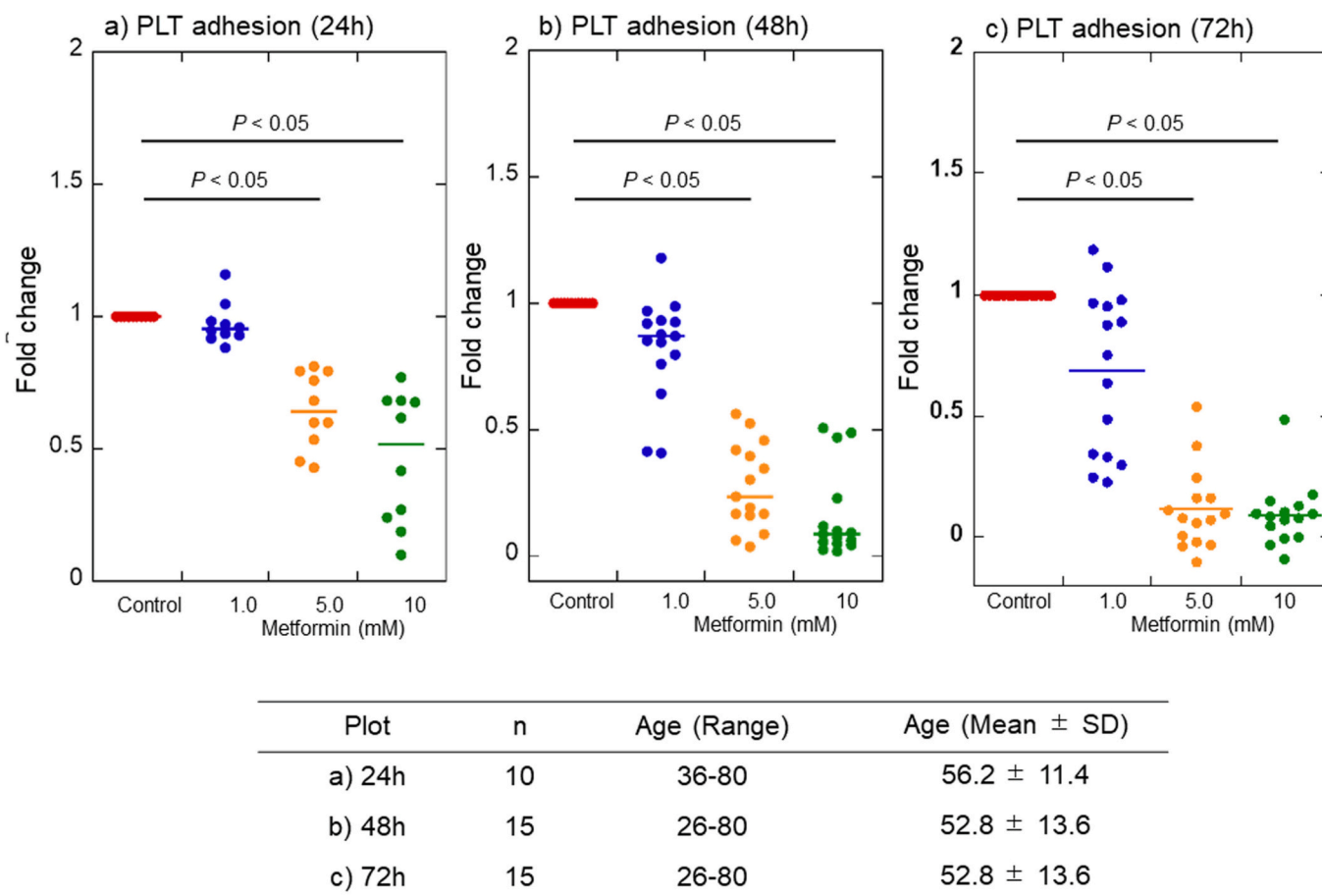### 4.3. Pharmacokinetics and incorporation of metformin into platelets

Metformin is not metabolized, but is excreted unchanged in the urine, with a half-life of 4–8.7 h (Gong et al., 2012; Scheen, 1996). Therefore, patients with T2DM should take this drug at appropriate intervals. After oral administration, metformin is absorbed from the entire intestine with its main absorption site in the duodenum (Song et al., 2006), transported via the bloodstream, and delivered to organs and peripheral tissues (Szymczak-Pajor et al., 2022). However, metformin is a highly hydrophilic cation molecule (logP octanol: water = −2.6) under physiological conditions, only a limited amount of metformin can

passively diffuse into the cytoplasm through the cell membrane (Kawoosa et al., 2022). Instead, organic cation transporters, such as OCT1 in the plasma membrane are thought to act as carriers for the transport of metformin into the cytoplasm (Kawoosa et al., 2022; Wu et al., 2018; Zajda et al., 2022). Metformin has multidirectional activity (Markowicz-Piasecka et al., 2020), and its effects may extend beyond the previously identified sites. Nevertheless, to further understand the reduction in platelet activity, research must focus on platelet energy metabolism.

4.4. Effects of metformin on platelet mitochondria

It is generally thought that metformin incorporated into platelets (and other common cell types) inhibits complex I of the mitochondrial electron transport chain, thereby reducing ATP production in platelets (Andrzejewski et al., 2018; Siewiera et al., 2022). However, the mechanism by which metformin reduces and depletes intracellular ATP levels has not been elucidated. This effect may depend on cell type. In this study, we observed no reduction in the total amount of platelet ATP did not occur within the initial 24 h, although platelet function was inhibited during the early phase. The balance between mitochondrial ATP generation and glycolysis might have influenced this onset. There are two possible explanations for the delayed reduction in platelet ATP levels. As demonstrated in intestinal cells (Rittig et al., 2021; Yang et al., 2021), glycolysis can be upregulated to compensate for the reduced platelet ATP levels. Alternatively, prolonged exposure of blood platelets to high concentrations of glucose provided by the anticoagulant used in this study could elevate the basal activity of platelet mitochondrial respiration (Siewiera et al., 2022) before treatment with metformin.

Whether metformin on target molecules or intracellular organelles exerts direct or indirect action on the mitochondria remains to be

Plot	n	Age (Range)	Age (Mean ± SD)
a) 24h	10	36-80	56.2 ± 11.4
b) 48h	15	26-80	52.8 ± 13.6
c) 72h	15	26-80	52.8 ± 13.6

Fig. 3. The effects of metformin on the ADP-induced adhesion activity of platelets contained in L-PRP samples at (a) 24, (b) 48, and (c) 72 h. $P < 0.05$ represents a significant difference from the respective controls. The sample size and donors' age of each group are summarized in the table at the bottom of the figure. The raw adhesion data of controls were $49.2 \pm 12.8 \times 10^4/\mu L$ (24 h), $45.1 \pm 10.5 \times 10^4/\mu L$ (48 h), and $36.8 \pm 10.6 \times 10^4/\mu L$ (72 h). The platelet count in the original L-PRP preparations was $48.4 \pm 12.5 \times 10^4/\mu L$.

elucidated (Fontaine, 2018). However, owing to its inhibitory action, metformin has attracted increasing attention in the field of applied science because of its applicability as an anticancer drug (Chen et al., 2022; Zhao et al., 2020). Thus, regardless of the cell type, it is worth exploring the balance between glycolysis and oxidative phosphorylation and further investigating the regulatory mechanism of cellular energy metabolism in metformin-treated cells.

4.5. Limitations

Diabetes is characterized by elevated blood glucose levels and impaired physiological balance between coagulation and fibrinolysis due to platelet hyperreactivity and increased coagulation factor activity (diabetic thrombophilia) (Zajda et al., 2022). Furthermore, this study demonstrated that metformin reduced coagulation activity by inhibiting platelet function in L-PRP preparations in vitro. If metformin had the potential to suppress platelet activity and reduce coagulation activity in patients with T2DM, it would be beneficial to pursue this possibility if it is used as a first-line antidiabetic drug.

In this study, blood samples obtained from healthy non-diabetic volunteers were used to examine the in vitro effects of metformin on platelets. In patients with T2DM who are constantly taking metformin, it should be noted that metformin is accumulated in platelets, as well as in red blood cells. Metformin concentration in the steady state is approximately 10-fold higher in the peripheral organs than in the plasma (Piel et al., 2015). Standard therapeutic plasma concentrations of metformin are controlled in the range of 0.6 and 6.0 μM, and toxic concentrations

lie between 60 μM and 1 mM (Protti et al., 2012). Nevertheless, to simulate the chronic effects of metformin in vivo, metformin concentrations are inevitable, exceeding those observed at toxic levels (≥ 1 mM) in in vitro studies (Andrzejewski et al., 2018; Piel et al., 2015; Protti et al., 2012; Siewiera et al., 2022).

Furthermore, to directly link this observation to clinical concerns, such as coagulation disorders, an appropriate number of patients with T2DM who receive no medications and are constantly taking metformin should be gathered. Under restricted conditions in Japan, as described in the Introduction, it is difficult to collect blood from either metformin-treated patients or untreated T2DM patients. Nevertheless, further in-depth studies using blood obtained from T2DM patients are needed to directly address our clinical questions and reach a definitive conclusion.
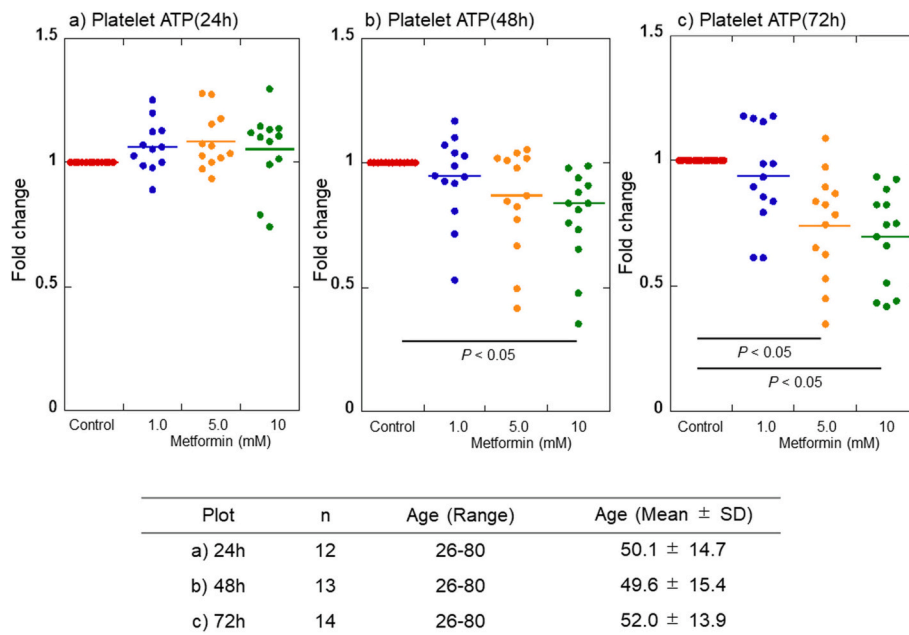
4.6. Clinical relevance

These findings may not have a severe impact on clinical diabetology or hematology but instead imply a rare but possible failure or delay of PRF preparation in patients receiving metformin treatment in regenerative therapy. Some antiplatelet and anticoagulation drugs have often been indicated to influence PRF preparation in clinical settings; thus, patients taking these drugs are often excluded from clinical studies depending on their purpose. Our findings add metformin to the list of "undesirable" drugs for PRF regenerative therapy.
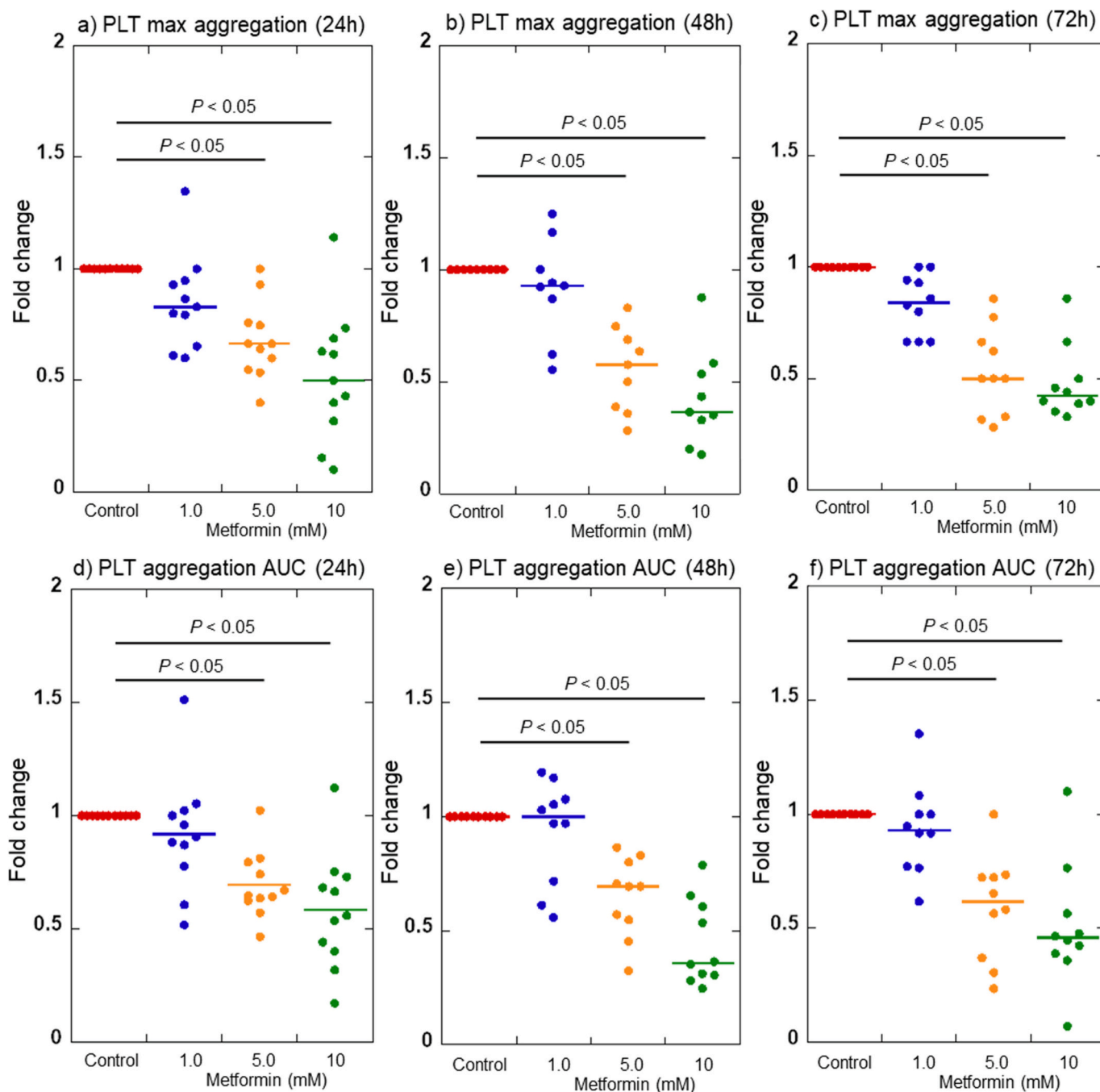
Fig. 4. The effects of metformin on the ADP-induced aggregation activity, (a − c) maximal aggregation and (d − f) aggregation area under curve (AUC), of platelets contained in pure-PRP samples at (a, d) 24, (b, e) 48, and (c, f) 72 h. $P < 0.05$ represents a significant difference from the respective controls. The sample size and donors' age of each group are summarized in the table at the bottom of the figure. The raw max aggregation data of controls were $18.0 \pm 12.9\%$ (24 h), $15.0 \pm 7.6\%$ (48 h), and $10.5 \pm 5.3\%$ (72 h). The raw aggregation AUC data of controls were 122.0 ± 62.6 unit (24 h), 110.2 ± 52.7 unit (48 h), and 84.1 ± 41.1 unit (72 h).

Plot	n	Age (Range)	Age (Mean ± SD)
a) 24h	12	26-80	50.1 ± 14.7
b) 48h	13	26-80	49.6 ± 15.4
c) 72h	14	26-80	52.0 ± 13.9

Fig. 5. The effects of metformin on ATP levels of platelets washed and suspended in PBS at (a) 24, (b) 48, and (c) 72 h. $P < 0.05$ represents a significant difference from the respective controls. The sample size and donors' age of each group are summarized in the table at the bottom of the figure. The raw ATP data of controls were 65.1 ± 10.8 pM/10^7 platelet (24 h), 71.4 ± 14.6 pM/10^7 platelet (48 h), and 64.6 ± 9.5 pM/10^7 platelet (72 h).
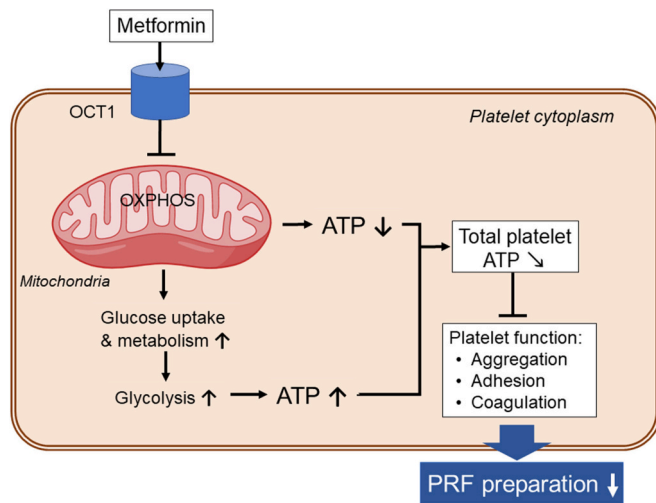
Fig. 6. Scheme of the mechanism of action of metformin in platelets. Our data did not demonstrate that the reduction of ATP levels precedes the impairment of platelet function; however, unless metformin directly inhibits platelet locomotor systems and/or related signaling systems, it can be thought that ATP reduction may cause the suppression of platelet adhesion, aggregation, and secretion. Metformin may delay or inhibit clot formation during PRF preparation. OCT1: organic cation transporter member 1, OXPHOS: oxidative phosphorylation.

5. Conclusions

At relatively high concentrations, metformin primarily acts on platelets and subsequently hampers the intrinsic coagulation activity in vitro. Although these findings were obtained from healthy non-diabetic subjects, a similar phenomenon may occur in T2DM patients. Thus, regardless of its clinical impact, clinicians using PRF should be encouraged to be more sensitive to this information to avoid undesirable events during regenerative therapy.

Supplementary data to this article can be found online at https://doi. org/10.1016/j.tiv.2023.105692.

Institutional review board statement

The study design and consent forms for all procedures (project identification code: 2019–0423) were approved by the Ethics Committee for Human Participants at Niigata University (Niigata, Japan) and complied with the Helsinki Declaration of 1964, as revised in 2013.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Takashi Uematsu: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Hideo Masuki:** Investigation. **Masayuki Nakamura:** Investigation. **Hideo Kawabata:** Investigation. **Yutaka Kitamura:** Investigation. **Taisuke Watanabe:** Investigation, Project administration. **Takao Watanabe:** Investigation. **Tomoharu Mochizuki:** Formal analysis, Writing – original draft. **Takashi Ushiki:** Conceptualization, Validation, Writing – original draft, Writing – review & editing, Funding acquisition. **Tomoyuki Kawase:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Data availability

The data are available from the corresponding author on reasonable request.

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