Divergent enhancement of in vitro induced coagulopathy by microparticle subtypes

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Abstract

Background

Aggressive fluid therapy of patients following a major trauma may result in hypothermia, acidosis and haemodilution that deteriorates even further towards a trauma-induced coagulopathy. The combination of these three factors is referred as lethal triad (LT) and a progressive course of LT is associated with a worsening or complete failure of haemodynamics and coagulative function. Within the process of coagulation, microparticles (MP) are crucial players at the interface of cellular and plasmatic coagulation. This study aimed at the characterization of coagulative effects originating from MP with different cellular origin and concentration under *in vitro* simulated traumatic conditions of haemodilution (HD) and LT.

Methods

HD was induced by replacing a blood volume of 33% by crystalloids and for LT, samples were further processed by reducing the temperature to 32 °C and lowering the pH to 6.8. MP were obtained either from platelet concentrates (platelet-derived MP, PDMP) or from cell culture (ECV304 cells for endothelial-derived MP, EDMP) by targeted stimulation. Effects of MP on coagulation depending on concentrations (1.000, 10.000 and 15.000 MP/µl blood) were characterized by flow cytometric platelet activation and by quantification of fibrin clot propagation and spontaneous clotting using Thrombodynamics® technology.

Results

MP originated from platelets and endothelial cell culture affected blood coagulation in a concentration-dependent manner. Particularly, high PDMP quantities significantly induced platelet activation and fibrin clot growth and size in HD conditions. In LT conditions, the highest PDMP concentration enhanced platelet activation, clot growth and size. In contrast, EDMP supplementation did not affect platelet activation, but resulted in enhanced formation of spontaneous clots, irrespective of simulated condition. With increasing EDMP concentration, the time until the onset of spontaneous clotting decreased in both HD and LT conditions.

Discussion

The study demonstrates the essential role of MP within the coagulation process in the simulated traumatic conditions. While PDMP affected platelets promoting clot formation likely by providing a surface enlargement, EDMP presumably affected clotting factors of the plasmatic coagulation resulting in an increased formation of spontaneous fibrin clots.

Conclusion

The diverse effects of *in vitro* generated MP from different cellular origin indicate a divergent mechanism of action exhibiting distinct functions within the coagulation process.

Introduction

Despite continuous improvements in trauma management, traumatic injuries still account for the leading cause of death and disability in adults aged under 40 years [1–3]. Particularly, uncontrolled bleeding contributes to more than 50% of all trauma-related death as a result of a coagulation breakdown within the first hours after injury [4, 5]. Approximately, one of four trauma patients arrive the emergency department with a hemodynamic depletion and signs of trauma-induced coagulopathy (TIC) [6–8]. The pathophysiology and collapsing of the entire coagulation system following traumatic injuries remains still unclear. Currently, the emergence of TIC is separated into the two types of trauma and/or traumatic shock-induced endogenous coagulopathy (including endotheliopathy) also described as acute traumatic coagulopathy (ATC) and iatrogenic coagulopathy (IC) [3, 9, 10]. In particular, IC is triggered by an aggressive trauma management leading to hypothermia, acidosis and dilution, which in combination are referred as "lethal triad" (LT) and worsen the outcomes of severely injured patients [10]. A progressive course of LT is associated with a deterioration of coagulation accompanied by disturbed clot formation and strength resulting at worst in a complete failure of coagulation function [11–13]. For primary hemostasis and in particular platelet function under clinical conditions of ATC, a significant decrease in platelet activation and aggregation leading to deterioration of clot formation could be shown [14, 15].

In contrast, small circulating microparticles (MP), with diverse cellular origin exhibit different function supporting coagulation at the boundary of cellular- and plasmatic-driven coagulation. Beside platelet-derived MP (PDMP), own investigations also revealed endothelial-derived MP (EDMP) as crucial players in the trauma setting as being associated with improved coagulation parameters [16, 17]. However, to date it is still less understood how different cell-specific MP physiologically functionalize under trauma-induced coagulation disturbances. In addition, the coherence of the hypocoagulative state of primary hemostasis under ATC conditions and the MP release after trauma is not understood. Therefore, we aimed to elucidate the role of PDMP and EDMP on functional coagulation under standardized simulated *in vitro* IC-conditions.

Methods

Targeted in vitro synthesis of microparticles

Cell line and cultivation for EDMP production

Human ECV304 cells (Sigma-Aldrich, Steinheim, Germany) were used to investigate endothelial MP generation properties. Cultivated in Dulbecco's Modified Eagle Medium (DMEM) and incubated under

constant conditions at 37 °C, 5 % CO_2 and 90 % humidity, cells were subcultured by trypsinization (0.25 % trypsin, Sigma-Aldrich, Steinheim, Germany).

For generating particles, ECV304 cells were incubated with 1 mM hydrogen peroxide (Sigma-Aldrich, Steinheim, Germany); platelets with 1.5 µg/ml bacterial lipopolysaccharide (LPS) (Sigma-Aldrich, Steinheim, Germany) – both for 22 hours at 37 °C maintaining established cultivation conditions. Subsequently, a first centrifugation step was performed to remove cells (10 min at 1.000 x g, 4 °C) followed by a second step to sediment particles from transferred supernatant (45 min at 10.000 x g, 4 °C). After removing residual supernatant, pellets were resuspended in 1x phosphate buffered saline (PBS), pooled and stored at -80 °C until analysis.

Platelet concentrates and PDMP production

Human platelets were obtained from platelet apheresis concentrates of the Institute of Transfusion Medicine (ITM) of the Cologne-Merheim Medical Centre that were not being authorised for transfusion.

For *in vitro* synthesis of PDMP, 40 mls of platelet concentrates were stimulated with 1.5 µg/ml LPS for 22 h at 37 °C in a humidified incubator with 5 % CO_2 (ThermoFisher, Marietta, USA). Subsequently, the platelet suspension was centrifuged at 1.000xg for 10 min at 4 °C (Heraeus, Hanau, Germany). Cell-free supernatant was centrifuged twice at 10.000xg for 45 min at 4 °C (Heraeus, Hanau, Germany) for PDMP pellet sedimentation, which was resuspended in the following in PBS obtaining pure PDMP. PDMP pellets were pooled, aliquoted and stored at -80 °C until analysis.

Blood donation and processing for inducing coagulopathic conditions

Study approval was given by the ethical committee of Witten/Herdecke University (#182/2016). Eleven healthy volunteers, fulfilling the ITM criteria for blood donation (age ≥18 years; no preexisting history of coagulation disorders, anticoagulant and/or platelet-inhibiting medication or viral infection), consented to participate and donated 60 mls blood, which was collected in citrated monovettes (Sarstedt, Nümbrecht, Germany)

Coagulopathic conditions such as haemodilution (HD) and lethal triad (LT) were simulated *in vitro* as previously described [18,19]. In brief, conditions were either induced by diluting whole blood (WB) by replacing by crystalloids (Sterofundin® ISO Infusionslösung, B. Braun Melsungen AG, Melsungen, Germany) only (hereafter be referred to as haemodilution, HD) or in combination with lowering the pH value to 6.8 using 2 M HCl and decreasing the temperature from 37 to 32 °C (hereafter be referred to as lethal triad, LT). Constant temperature was realized by continuous storage of samples in an appropriate tempered water bath (Thermolab1070, GFL, Burgwedel, Germany).

Application of PDMP and EDMP to the experimental HD and LT approaches

After introducing the conditions of HD and LT, either no MP (untreated controls) or EDMP/PDMP were supplemented in distinct concentrations of 1000 (1k), 10.000 (10k) or 15.000 (15k) MP/µl (Figure 1).

Exact microparticle quantities required for the experimental approaches had been determined shortly before flow cytometric analysis by using the BD Accuri™ C6 (BD, Heidelberg, Germany). For this purpose, particles were defined by size (0.5 to 0.9 µm) [21] and by typical surface marker originated from the parental cell such as CD42b for platelets and CD144 for endothelial cells, respectively (BD, Heidelberg, Germany). In addition, Annexin V dye was used as marker for the externalisation of phosphatidylserine (PS). After a microparticle incubation of 5 min, samples were processed for subsequent coagulation analysis.

Extended coagulation analysis

Detection of activated platelets

For measuring platelet activation, cells were fixed according to Cyfix III protocol [20] and P-selectin expression was flow cytometric measured by using CD42b and CD62p antibodies (BD. Heidelberg, Germany) and the BD Accuri™ C6 device (BD, Heidelberg, Germany). Results are presented as relative changes of $CD42b^+/CD62p^+$ stained platelets referring to the respective unstimulated approach of each experimental group (set as 100%).

Thrombodynamics® (TD)

Differential centrifugation was applied for TD analysis (1.600 x g for 15 minutes followed by a spin of plasma for 5 minutes at 10.000 x g) and platelet-poor plasma (PPP) was immediately shock-frozen in liquid nitrogen and stored at -80 °C until analysis.

For TD analysis, PPP samples of WB and HD were thawed at 37°C and those of LT were thawed accordingly at 32°C in a water bath. The corresponding temperatures for WB, HD and LT were maintained during the TD measurements.

For the determination of spatial clot growth, the TDX kit consisting of reagent I (lyophilized protein of FXIIa inhibitor) and reagent II ($CaCl_2$) was used according to the manufacturer's recommendation (HemaCore, Moscow, Russia). Briefly, 120 µl of PPP were supplemented with reagent I and incubated in the device thermostat for 15 min (HemaCore, Moscow, Russia). Subsequently, the PPP samples were treated with reagent II and directly placed into the micro chamber. By inserting a with immobilized tissue factor coated insert, the reaction had been initiated and clot growth and spontaneous clot formation was recorded over 45 min. The following parameters were measured: lag-time (Tlag, min), rate of clot growth (V, µm/min), initial rate of clot growth (Vi, µm/min), clot density (D, a.u.) and clot size (Cs, µm). In addition to these parameters, Cs was measured in five minutes intervals (0-20 min) to estimate the influence of MP on the dynamics of clot growth. In order to statistically record the changed Cs over time, the individual areas under the curve (AUC) of each donor was calculated.

Statistical analysis

Statistics and graphical data analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, USA). Non-parametric Friedman test and Dunn's post-hoc test to correct for multiple comparisons were applied to determine significant differences across the collected parameters in the groups of HD and LT.

In a first step, the MP-untreated samples in the groups of WB, HD and LT were pairwise compared as follows: WB vs. HD, WB vs. LT and HD vs. LT. Secondly, the effects of dose-specific application of PDMP or EDMP were tested in the HD and LT groups by comparing. Finally, untreated WB samples a non-parametric Wilcoxon test revealed potential effects between untreated and MP-supplemented samples (C vs. 1k). P-values with a significance level <0.05 were considered statistically significant.

Values with respect to platelet count and TD temporal course of clot growth (Cs) are presented as arithmetical mean and standard deviation.

Results

Demographics of healthy volunteers

Overall, 11 healthy donors were enrolled for analysis. Volunteers of which 54.5% were males had a median age of 42 years.

Effect of experimental conditions and MP supplementation on platelet count and activation

While the condition of HD led to a marginally decreased platelet quantification than WB (WB: 2.7 $x10^5$/µl vs. HD: 2.2 $x10^5$/µl), the LT caused a significant reduction of cell number than in WB samples (Figure 2 A).

The application of MP resulted in a varying platelet activation depending on the supplemented dose. Under HD conditions, high PDMP quantities (10k and 15k MP/µl blood) induced an activation of platelets, which was demonstrated by a significant increase of the cell surface marker $CD42b^+/CD62p^+$ expression compared to controls (Figure 2 B). Particularly the administration of 15k PDMP/µl blood further increased platelet activity in this group. Similarly, the highest PDMP concentration showed also the greatest effect on platelet activation within the LT group compared to untreated LT controls (Figure 2 B). In contrast to PDMP, EDMP supplementation did not affect platelet activation significantly either in HD or LT groups, but a trend of slight enhancement of activated platelets was observed after supplementation of 10k and 15k EDMP/µl blood (Figure 2 C). Irrespective of supplemented MP subtype, low concentration (1k) had no effect on platelet activation (Figure 2 B, C).

Kinetics of fibrin clot formation and density after PDMP application

The kinetics of clot formation measured by Tlag remained unaffected in the experimental controls of WB, HD and LT as well as after supplementation of PDMP (Table 1). Similarly, no kinetic changes were observed for the parameters rate of clot growth (V) and initial rate of clot growth (Vi) in the control groups

of WB and HD, but a significant enhancement was recorded in LT conditions compared to WB (Table 1). However, low quantities of PDMP (1k MP/µl blood) improved significantly V and Vi in WB; in hemodiluted blood the high PDMP concentrations (10k and 15k PDMP/µl blood) achieved an enhanced of both growth parameter (Table 1). Under LT conditions, the application of 10k and 15k PDMP/µl blood resulted in a further significant increase of fibrin growth rate (V) compared to untreated LT controls (Table 1). Furthermore, a tendential but not significant increase of initial clot growth could be observed after PDMP administration in LT samples (Table 1).

In contrast to WB, fibrin polymerization and the associated clot density (D) deteriorated under the traumatic *in vitro* HD and LT conditions (Table 1) whereby a significant reduction compared with untreated WB was detected for LT (Table 1). An improved clot density of fibrin polymerization was achieved by PDMP quantities of 1k MP/µl blood in WB and by high PDMP quantities (10k and 15k MP/µl blood) in HD samples (Table 1). A PDMP stimulating effect on clot density could not be detected for LT (Table 1).

Due to the formation of spontaneous fibrin clots following EDMP administration in all experimental groups, no kinetic parameters describing clot kinetic and development (Tlag, V, Vi, and D) could be recorded.

Fibrin clot formation following PDMP supplementation - growth and size

The concentration dependent PDMP application resulted in a significantly increased fibrin clot growth (Cs) over the measured time of 20 min in the WB and HD groups (Figure 3). While the administration of low PDMP concentration 1k PDMP/µl blood caused an enhanced clot growth in WB (Figure 3 A), similar effects had been achieved in the HD group after supplementing high level of PDMP (10k and 15k PDMP/µl blood, Figure 3 B). The improved fibrin formation in WB and HD was also mirrored in the respective AUCs over a time course of 15 min (Figure 3 D, E).

Noteworthy, already the condition of LT led to an increased fibrin growth 15 min after measure initiation compared to WB controls (Fig. 3 D, E). However, the supply of PDMP had no further impact except for the addition of 15k PDMP/µl blood at the time point of 10 min (Figure 3 C).

Spontaneous clotting after EDMP supplementation

Data collection for the parameters defining fibrin clot growth could not be collected in the EDMP groups due to the early formation of spontaneous clotting originating from EDMP that occurred irrespective of the experimental setting (Figures 5, 6). While no spontaneous clot formation was observed in the control groups of WB and HD, it occurred under LT control conditions (Figure 6).

Even low EDMP concentrations (1k EDMP/µl blood) caused a median spontaneous clot formation in WB (13.4 min), HD (10.1 min) and LT samples (7.7 min) and resulted in unrestricted formation of fibrin clots (Figures 5, 6 A, B, C). While the supplementation of high EDMP quantities (10k and 15k EDMP/µl blood) initiated spontaneous clotting in haemodiluted samples already after one minute (1.55 min vs. 1.7 min,

Figure 6 B), a complete coagulation was measured after two minutes (Figure 5). Within the LT group a similar effect could be observed when applying high EDMP concentrations (10k EDMP/µl blood: 1.35 min; 15k EDMP/µl blood: 1.75 min, Figure 6 C). In all experimental approaches, spontaneous clot formation led to the expansion of clot propagation up to complete coagulation in the measuring cuvette, which emanated from the spontaneous fibrin clot.

The event of spontaneous clot formation only occurred following EDMP but not PDMP supplementation.

Discussion

The present study aimed to elucidate the role of PDMP and EDMP on functional coagulation under *in vitro* simulated traumatic conditions. In line with previous findings, the applied experimental settings of HD and LT in this study were appropriate models for standardized *in vitro* simulations of coagulation disturbances that may occur after trauma [18, 19].

Special emphasis was given on the analysis of selected MP on blood coagulation with particular focus on PDMP and EDMP. Both MP subtypes had been reported to be released in high quantities in patients who sustained major traumatic injuries indicating that these particles might be involved in regulating blood coagulation after trauma [16, 17, 21, 22]. With this study we demonstrated for the first time a divergent mechanism of action on coagulation originating from MP with different parental cells. Supplemented PDMP mainly affected circulating platelets by inducing their activation, which was measured by enhanced P-selectin expression on platelet membranes ($CD42b^+/CD62p^+$), particularly, but not exclusively, in HD conditions. It is conceivable that an increased platelet activation likely compensates for reduced platelet counts in HD and LT conditions potentially via the ligand-receptor interaction between the P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) forming a PSGL-1/P-selectin complex that leads to exponential cell activation [23–26]. An increased cell activation is associated on the one hand with a promoted phosphatidylserine (PS) exposure resulting in procoagulant platelet formation and with an increased PDMP secretion and release of coagulation-promoting components from the platelet storage granules on the other [27]. Furthermore, not only platelets expose PS on their membrane, but also *in vitro* generated PDMP as determined in this study by flow cytometric measurements. It is well known that PS on platelet surfaces cause an assembly of coagulation factors promoting functional clotting [28, 29]. Related to our findings Lipets *et al.* also revealed that *in vitro* generated PDMP contributing to an enhanced coagulation propagation by expressing PS on their cell membrane [30].

PDMP together with activated platelets could result in a surface expansion for clotting factors, which would explain the locally increased formation of thrombin and fibrin polymerization under high PDMP treatment mainly in HD samples.

In contrast to PDMP, *in vitro* generated EDMP affected the impaired coagulation of HD and LT unexpectedly not by activating platelets, but by inducing spontaneous clotting originating from the

particles itself. Therefore, the expected EDMP-mediated platelet activation via membrane P-selectin seems to play a secondary role in coagulation stimulation, which is likely due to a lower P-selectin exposure present on *in vitro* generated EDMP, which seem to be insufficient to activate platelets in a way PDMP do.

Furthermore, the PDMP and EDMP supplementation only showed effects when particles were available in high concentrations (10 k or 15 k MP/µl blood) indicating a concentration-dependent mode of action. Regarding spontaneous clotting, the used EDMP quantity has been irrelevant for triggering this process as already low EDMP concentrations (1 k MP/µl blood) led to spontaneous fibrin formation, irrespective of the experimental group. However, higher MP level (10 k and 15 k MP/µl blood) further accelerated this process in HD (< 5 min) and LT (< 3 min) conditions resulting in unrestricted clotting. An underlying mechanism by which EDMP cause spontaneous clotting could be based on the presence of tissue factor (TF) on EDMP, which has been shown to be released after targeted stimulation from human endothelial cells *in vitro* [31–33]. TF in turn mediates a coagulation initiation via the coagulation factor VII/TF-dependent extrinsic pathway and likely results in spontaneous fibrin formation [34, 35]. The earlier formation of spontaneous fibrin clots following EDMP administration in LT in comparison to HD potentially resulted from the combination of both, the TF-bearing EDMP and the acidosis-related increased basal platelet activation under LT conditions.

As recently published by our group, there is a positive correlation between injury severity and MP concentration after trauma likely reaching high quantities inducing procoagulative effects [16]. Our *in vitro* experimental setup indicates that under LT conditions MP promote procoagulative function by different pathways based on their cellular origin. Therefore, at first sight, our data seems to be contrary to recent clinical observations of platelet dysfunction in TIC-related patients showing reduced platelet activity levels after trauma [14, 15]. These clinical data reflect the final common path of the multicausal pathophysiology of TIC resulting in the hypocoagulative state after trauma. The results from our experimental model should only be carefully extrapolated to a clinical picture but describes the procoagulative potential. It remains to be conclusively clarified and requires further investigation whether and how MP are involved in the pathophysiology of TIC after major trauma *in vivo* and whether those high quantities as used in this study can be physiologically achieved in a clinical setting.

Conclusion

The present study revealed a divergent mechanism of action originating from PDMP and EDMP indicating that MP subtypes have diverse function in stimulating coagulation under the investigated traumatic conditions of HD and LT. Particularly, high MP concentrations of both MP types showed effects on the coagulation process. Likely due to the platelet-mediated activation (increased P-selectin expression) and the presence of PS lipids on PDMP membranes, clotting factor assembly and thus clot formation could be promoted on PDMP surfaces. In association with high EDMP quantities, an improved coagulation with formation of spontaneous fibrin clots was determined, which could presumably be induced via the TF/FVIIa-dependent extrinsic pathway of coagulation.

Limitations

It would have been desirable to perform the analysis with isolated MP subtypes from real trauma patients. But since high MP quantities would have been required for this study and the extraction capacity from patient's blood is limited, we relied on *in vitro* synthesized MP. Additionally, we are aware of the fact that the current *in vitro* study reflects the pathophysiological mechanism present in trauma patients only in part. For this reason, findings should cautiously extrapolate to a clinical setting.

Abbreviations

ATC: Acute traumatic coagulopathy, C_S:Clot size, CMMC:Cologne-Merheim Medical Centre, EDMP:D:Clot density, Endothelial-derived microparticles, HD:Haemodilution, IC:Iatrogenic coagulopathy, ITM:Institute of Transfusion Medicine, LPS:Lipopolysaccharide LT:Lethal triad, MP:Microparticles; PDMP:Platelet-derived microparticles, PPP:platelet-poor plasma; PS:Phosphatidylserine, PSGL-1:P-Selectin gylcoprotein ligand-1, TD:Thrombodynamics, TIC:Trauma-induced coagulopathy; Tlag:lag-time, TF:Tissue factor, Tsp:Time of spontaneous clot formation, V:Rate of clot growth, Vi:initial rate of clot growth, WB:Whole blood

Declarations

Ethics approval and consent to participate

Study approval was given by the ethical committee of Witten/Herdecke University (#182/2016).

Consent for publication

All volunteers (blood donors) gave their written and informed consent for participating in this study.

Acknowledgement

We thank HemaCore (Moscow, Russia) for providing the Thrombodynamics Analyser System T2 and the corresponding TDX kit systems.

Availability of data and materials

All data that are relevant for the study are included in this published article. Further datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MC, NS and MM designed the study. JB, NS collected, analyzed and interpreted the data, wrote the manuscript, which has been critically reviewed by MM, MC, BS and UB. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

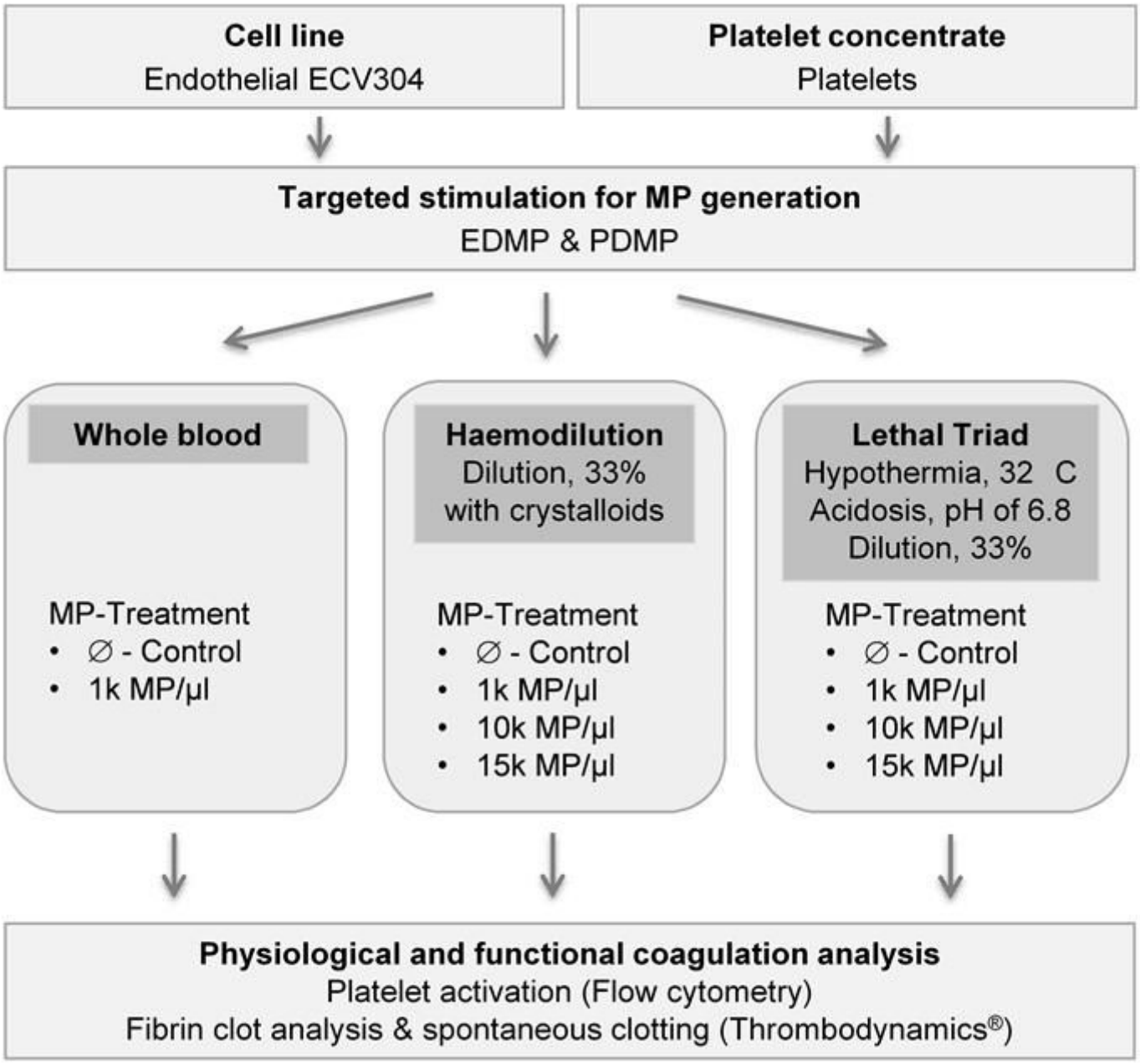

Figure 1

Schematic representation of experimental design.

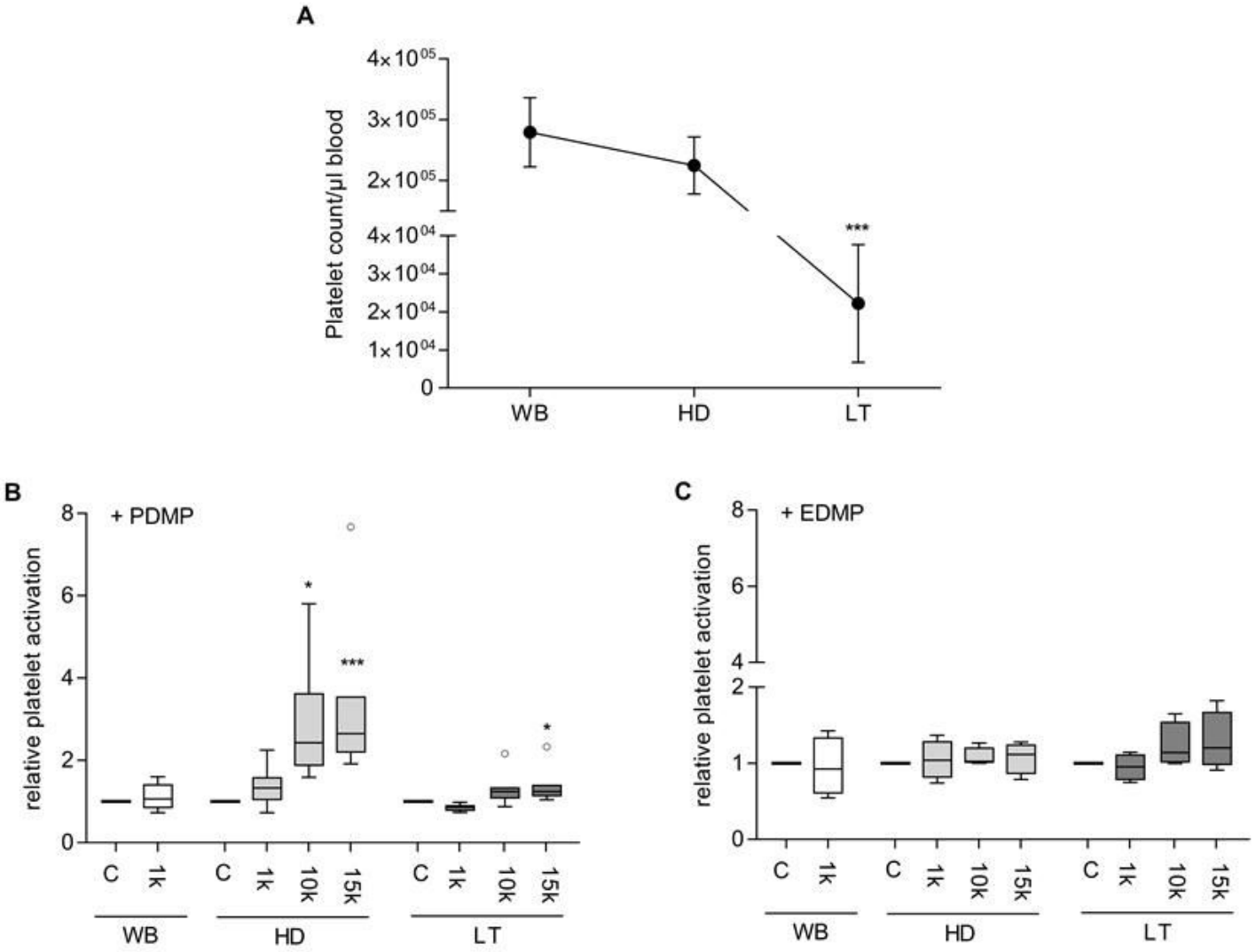

Figure 2

Platelet count (A) and platelet activation after concentration dependent PDMP (n=7) (B) and EDMP (n=4) (C) supplementation under the experimental conditions of whole blood (WB), haemodilution (HD) and Lethal Triad (LT). Platelet activation was measured by expression levels of CD42b+/CD62p+ on platelets surface. Results are presented as relative changes referring to the respective unstimulated approach of each experimental group. Values are presented as mean/median ± SD (A) or as Tukey boxplots with whiskers of length 1.5 x IQR (B, C). Statistical significances were marked with asterisks for concentration-dependent differences within one experimental group (*,+p ≤0.05, **,++p ≤0.01, ***,+++p ≤0.001), respectively. The abbreviation k signifies a thousand.

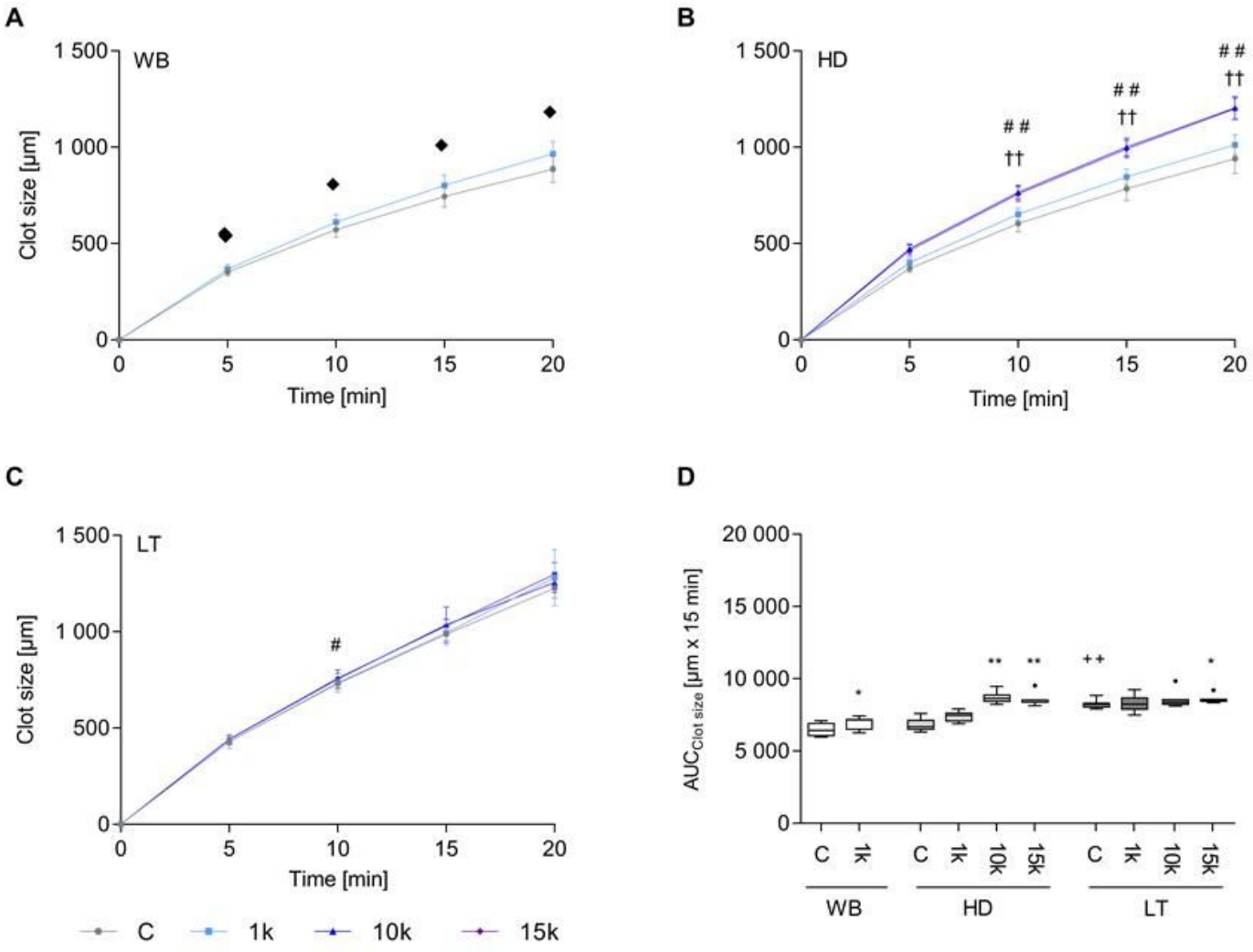

Figure 3

Dynamic fibrin increases over time measured by clot size in whole blood (WB), haemodilution (HD) and lethal triad (LT) after PDMP supplementation (A-C). Values are represented as mean ± standard deviation. The respective AUCs at 15 minutes (D) following measurement initiation are depicted as Tukey-boxes. Significances are indicated with the following symbol for the following comparisons: diamond - controls vs. 1k PDMP, cross – controls vs. 10k PDMP and hash - controls and 15k PDMP. Statistical significances regarding AUCs were marked with plus symbols for differences between unstimulated groups (WB vs. HD and WB vs. LT) or with asterisks for concentration-dependent differences within one experimental group. Irrespective the comparison one symbol indicates p ≤0.05, two symbols p≤0.01 and three symbols p ≤0.001). The abbreviation k signifies a thousand.

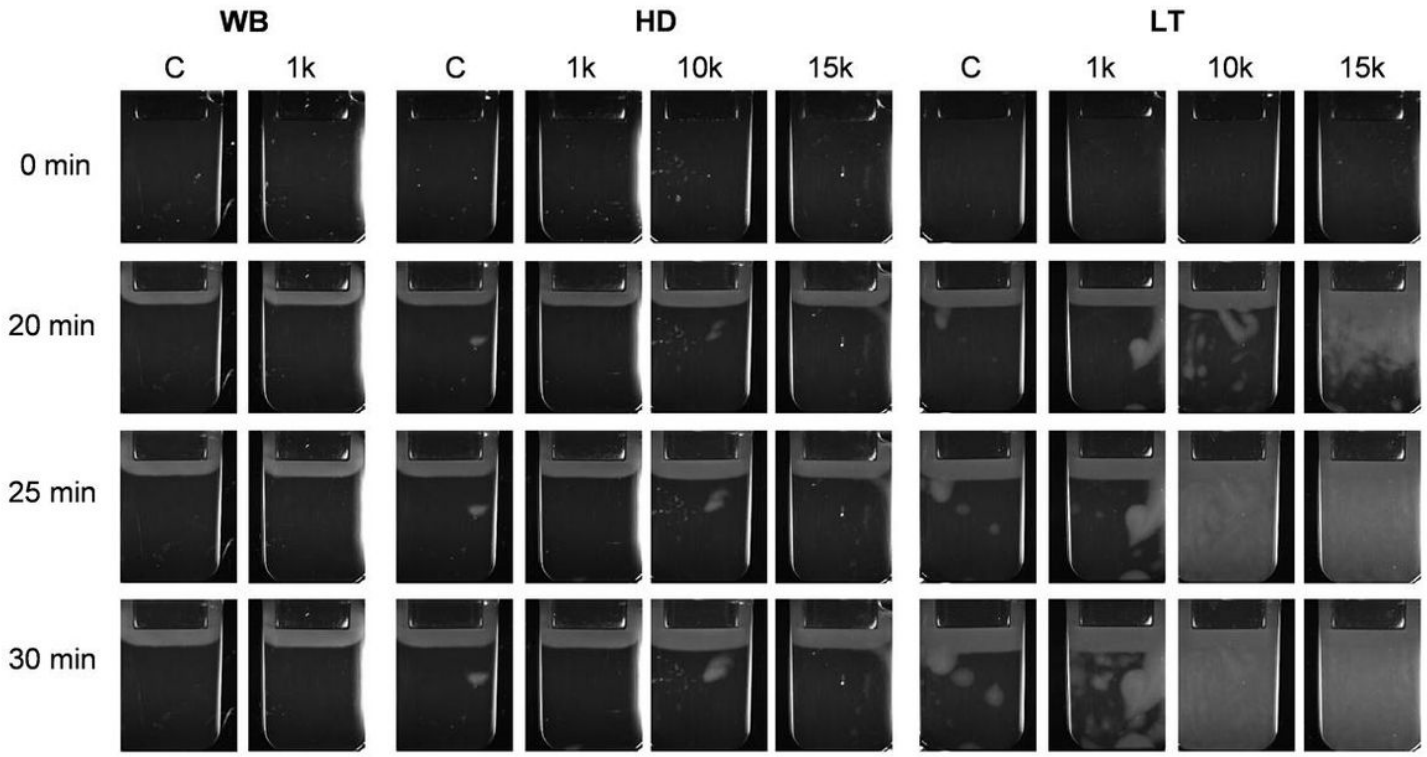

Figure 4

Fibrin growth and development over time after PDMP supplementation in increasing concentrations (1k, 10k and 15k) in whole blood (WB), haemodilution (HD) and lethal triad (LT) groups.

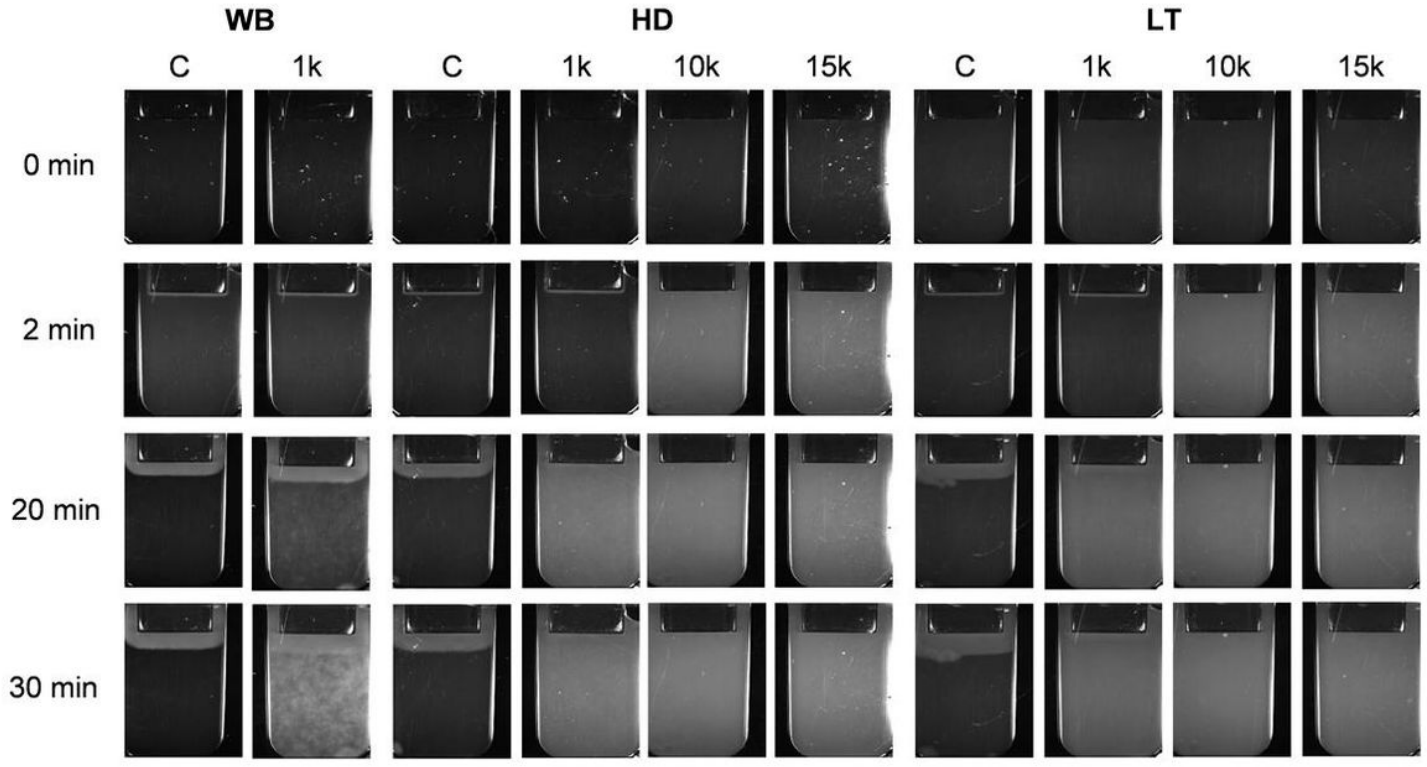

Figure 5

Fibrin growth and development over time after EDMP addition in increasing concentrations (1k, 10k and 15k) in whole blood (WB), haemodilution (HD) and lethal triad (LT) groups.

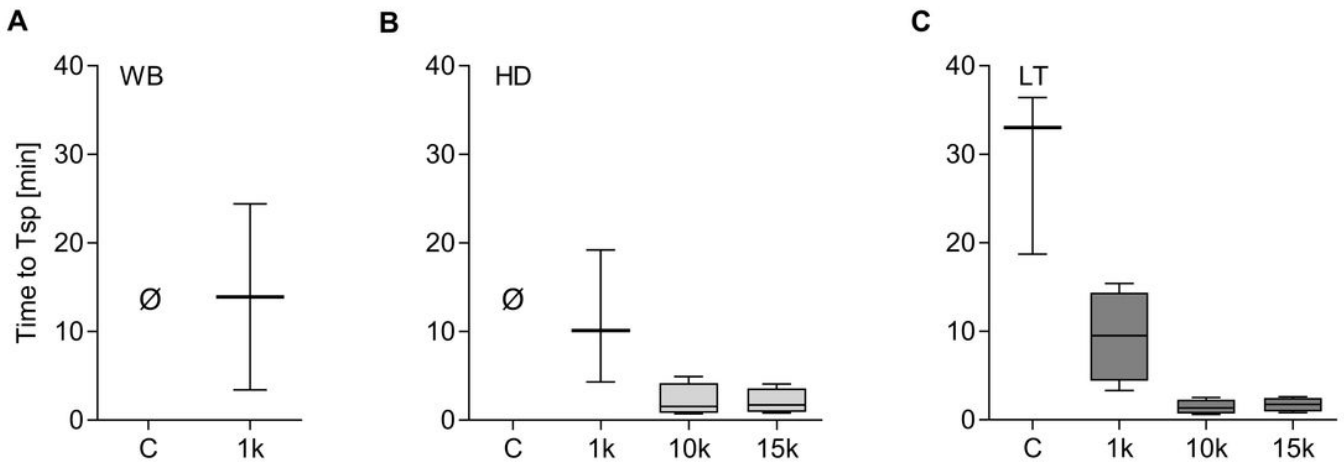

Figure 6

The time elapsed until spontaneous clotting (Tsp) after increasing EDMP supplementation under experimental condition of whole blood (WB), haemodilution (HD) and lethal triad (LT). The abbreviations k signifies a thousand and Ø no occurrence of spontaneous clotting.