Reversible Suppression of Hemostasis in Hibernation and Hypothermia

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CHAPTER 1

General introduction

Part of this chapter is based on:

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THROMBOSIS: A MAJOR CAUSE OF DEATH AND GLOBAL DISEASE BURDEN

Thrombosis is the intravascular formation of a blood clot (thrombus) by a process of platelet activation and blood coagulation, which leads to occlusion of the blood vessel causing a disruption of the blood flow. Thrombotic processes can occur both in arteries and veins and are the leading cause of death worldwide. In the United States and Europe, over 2,200 and 10,000 patients die each day respectively due to cardiovascular disease, with the vast majority suffering from thrombus formation in coronary or cerebral arteries leading to myocardial infarction and stroke, respectively [1, 2]. Besides arterial thrombosis, venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), is a major cause of morbidity and mortality worldwide. PE is a life-threatening consequence of DVT that occurs when the thrombus dislodges from deep veins and migrates into the lungs. VTE affects approximately 1 per 1000 adults of European ancestry annually and accounts for approximately 12% of all deaths in European countries and the United States [3-6]. Additionally, despite novel antithrombotic and prophylactic strategies, the annual incidence of VTE has increased during the past decades and is predicted to increase further due to the aging population [7]. Therefore, thrombosis is an important healthcare issue. Furthermore, (microvascular) thrombosis can occur secondary to many other conditions, such as sepsis [8], organ and tissue transplantation [9-12], major surgery [13], trauma [14], and hypothermia [15, 16].

Unfortunately, antithrombotic treatments affect both pathological thrombosis as well as physiological hemostasis, which is the biological process that prevents bleeding after vessel injury. Consequently, current therapies reduce the risk of thrombosis while increasing the risk of unwanted bleeding as exemplified by the majority of emergency hospitalization due to adverse drug events being from anticoagulant and antiplatelet therapies (6.4-17.3% and 8.7-10.4% of cases respectively) [17, 18]. Improving our understanding of natural hemostatic and antithrombotic mechanisms may identify novel ways or improve current strategies to enable specific inhibition of arterial and/or venous thrombosis while maintaining normal hemostasis.

NORMAL HEMOSTASIS

When damage occurs to a blood vessel, either accidentally or surgically, its endothelial barrier is disrupted, enabling contact of blood components with the subendothelial and extracellular matrix (Figure 1). Blood now passes through the vessel wall until the bleeding is stopped physiologically (by hemostasis) or artificially (e.g. by a tourniquet

or surgical clamp). The word hemostasis is derived from the Greek αίμα/hema (=blood) and στάσις/stasis (=halt), literally the stopping of blood. Although a myriad of processes occurs simultaneously, hemostasis is generally divided into several phases, of which each phase can be divided in sequential steps (Figure 1).

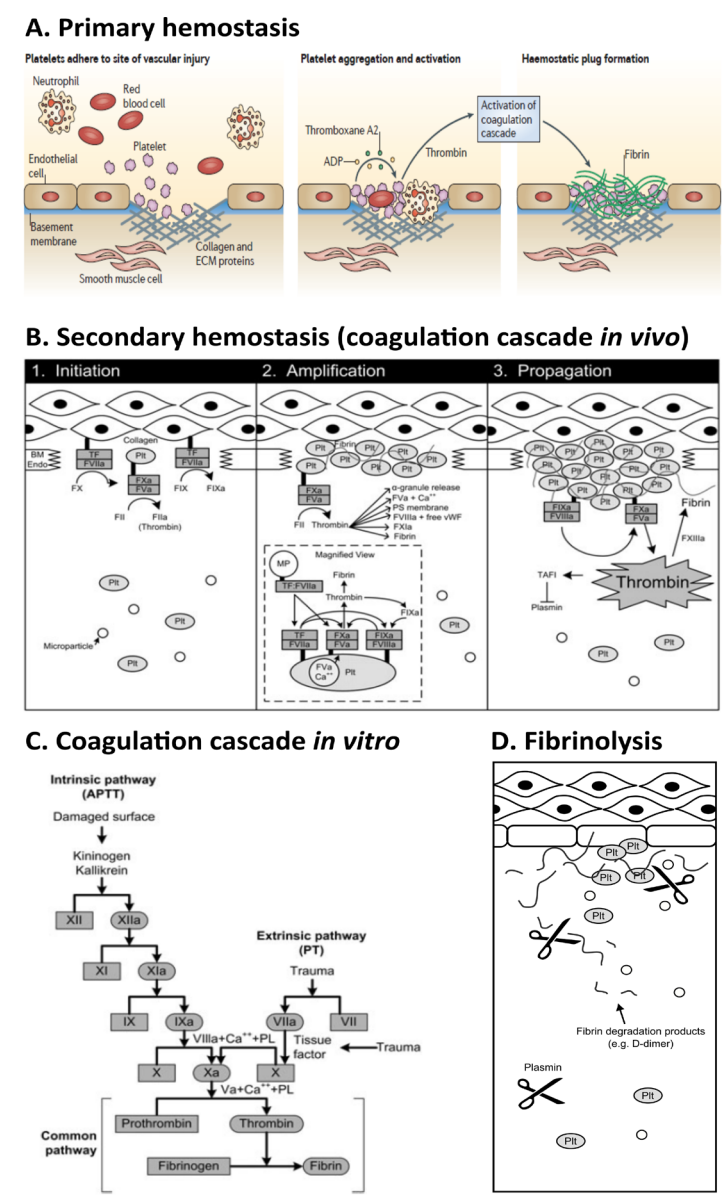

FIGURE 1. Schematic overview of hemostasis. *Legend on the next page.*

FIGURE 1. Schematic overview of hemostasis. (A) Primary hemostasis is initiated by vessel wall injury exposing the extracellular matrix (ECM) to blood inducing platelet (plt) adherence, activation and aggregation, forming a hemostatic plug. Activated and degranulated platelets initiate platelet and white blood cell recruitment, which furthers platelet aggregation, vasocontriction and wound regeneration, and activate the coagulation cascade (secondary hemostasis). (B) Secondary hemostasis occurs via activation of the plasmatic coagulation system due to exposure of plasma factors to tissue factor (TF) and platelet activation products, forming a fibrin rich network that traps red and white blood cells and further strengthens the hemostatic plug. (C) Laboratory measurement of the coagulation cascade can be divided into intrinsic pathway, measured by activated partial thromboplastin time (APTT) dependent on factors XII, XI, IX, and VIII, and extrinsic pathway, measured by prothrombin time (PT) dependent on factor VII. Both APTT and PT also depend on the common pathway factors II (prothrombin), V, X, and fibrinogen. (D) Finally, fibrinolysis gradually degrades the fibrin network and hemostatic plug, allowing full recovery of blood flow when the endothelium has recovered. Figure A reprinted by permission from Nature, Nature Reviews Immunology, Semple et al. Platelets and the immune continuum, 2011© [19], Figure B and C reprinted by permission from John Wiley & Sons, Nephrology, Adams et al. Review article: Coagulation cascade and therapeutics update: Relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants, 2009 © [20].

Primary and secondary hemostasis

Primary hemostasis starts the moment circulating platelets make contact with damaged endothelium or subendothelial tissue (Figure 1A). Platelets are the smallest of blood cells; they are anucleated cells budded off from megakaryocytes, their large multinuclear mother cells mainly residing in bone marrow. Human platelets average 2-5 µm in size, but despite their small size they play a major role in hemostasis, inflammation, bacterial defense, wound regeneration and cancer metastasis [19, 21-23]. Platelets are activated by a whole range of molecules present at the site of a damaged blood vessel, e.g. extracellular matrix (ECM) proteins, such as Von Willebrand Factor (VWF) or collagen, and soluble factors such as thrombin, adenosine di-phosphate (ADP) and adrenaline [24, 25]. Damaged endothelial cells release the contents of Weibel-Palade bodies, which are granules filled with coagulation and inflammation enhancing and modulating compounds, such as VWF and various cytokines [26], while subendothelial smooth muscle cells and fibroblasts express tissue factor (TF), a potent activator of the plasmatic coagulation system (initiating secondary hemostasis) [27]. Activated platelets express several membrane (glyco)proteins, amongst others GPIb-IX-V and P-selectin that bind to activated endothelium or subendothelial collagen directly or via

intermediate adhesion factors, such as VWF [24]. Similar to endothelial cells, platelets degranulate upon activation, thus releasing a myriad of molecules enhancing platelet activation, plasmatic coagulation, inflammation, tissue regeneration and bacterial killing [19, 23]. Upon activation, platelets change shape by increasing surface area with membrane extensions thus enabling quicker adherence to other platelets and cells. Consequently, more platelets are now recruited to the site of injury (Figure 1B). Platelets stick to the subendothelial ECM and to each other, forming the hemostatic plug, while platelet thromboxane A_2 released from granules induces vasoconstriction to prevent further blood loss. Once the platelet hemostatic plug covers the damaged site completely, it is further strengthened via secondary hemostasis by the plasmatic coagulation system.

Secondary hemostasis, occurring simultaneously with primary hemostasis, creates a fish-net like fibrin network to trap red and white blood cells and further strengthen the hemostatic platelet plug, reducing the bleeding risk. Secondary hemostasis occurs via plasmatic coagulation cascades which are classically divided into either two pathways, the intrinsic and extrinsic pathway of coagulation, or three phases according to a widely used current model [28] (initiation, amplification, and propagation phase, Figure 1B). During the **initiation phase**, low amounts of active coagulant factors are generated. This starts with exposure and binding of TF to plasma coagulation factor VII, which forms a TF/VIIa complex. The TF/VIIa complex proteolytically activates factor IX and X, creating a prothrombinase complex with Va that converts prothrombin (factor II) into thrombin [25]. Thrombin slowly accumulates during the **amplification phase**, activating platelets and platelet derived factor V, amplifying the prothrombinase activity. Thrombin also activates factor XI and VIII, the latter acting as cofactor to IXa on the surface of activated platelets, generating more factor Xa. Thus, the amplification phase boosts the level of active coagulation factors (Va, VIIIa, IXa and XIa) [25]. Factor XIa initiates the **propagation phase**, by activating factor IX that associates with VIIIa. Factor VIII and IX are crucial in the coagulation cascade, since their (near-)absence leads to severe bleeding disorders with hemorrhagic complications (hemophilia A and B, respectively). On procoagulant membranes of activated platelets, the IXa/VIIIa complex stimulates Xa and Xa/Va complex formation, subsequently propagating thrombin formation. The increase in thrombin generates gross amounts of fibrin fibers from fibrinogen, which are cross-linked yielding an elastic, polymerized fibrin network and clot that strengthens the hemostatic plug [29]. The initiation phase is classically referred to as **extrinsic pathway** (Figure 1C), which can be assessed *in vitro* by measuring the prothrombin time (PT). The **intrinsic pathway** of coagulation overlaps with the amplification and propagation phase, but can also be triggered independently by collagen, polyphosphates secreted by platelets, neutrophil extracellular traps (NETs), and artificial material such as glass,

leading to activation of factor XII, XI and kallikrein and the subsequent downstream coagulation factors (Figure 1C) [25, 30]. The intrinsic pathway can be assessed *in vitro* by measuring the activated partial thromboplastin time (APTT). Both PT and APTT determine the time it takes to form a fibrin clot, partially depending on the common pathway of coagulation and either extrinsic or intrinsic pathway of coagulation, respectively.

Counterbalancing clot formation

Under normal conditions, endothelium constantly prevents unwanted thrombus formation by actively producing and excreting anticoagulant compounds, preventing platelet adhesion and coagulation cascade activation. Such anticoagulants are supported by plasmatic anticoagulant factors produced by the liver, such as protein C, protein S and antithrombin, which inhibit specific procoagulant factors. An important physiological process following hemostasis is the recovery of blood flow due to degradation of the formed clot by a process called **fibrinolysis** (Figure 1D). The cross-linked fibrin network is enzymatically degraded by plasmin, which is formed from plasminogen by tissue plasminogen activator (t-PA). t-PA is slowly released by damaged endothelium enabling a gradual degradation of fibrin after the bleeding has stopped and tissue regeneration has started. Fibrin is cleaved into fibrin degradation products, of which amongst others D-dimer can be detected in plasma and is commonly used in the diagnosis of venous or arterial thrombosis.

PATHOLOGICAL HEMOSTASIS

In pathological thrombotic conditions, the balance between thrombus formation on one hand and the inhibition of clotting with clot lysis on the other hand tips towards clot formation, leading to thrombi and/or emboli and subsequent organ damage (briefly outlined in Figure 2). Contrarily, if the balance tips towards less clotting, bleeding may be the result. To date, many patient characteristics for an increased risk of thrombosis are known. Although the etiology of arterial and venous thrombosis is somewhat different, several shared risk factors are: age, overweight/obesity, smoking and thrombophilia (inherited or acquired procoagulant disorders) [31-34], although age and body mass index are not consistently associated with increased VTE risk in literature [35]. Moreover, there are many conditions that can provoke both arterial and venous thrombosis, such as hyperhomocysteinemia, antiphospholipid antibodies, malignancies, infections and the use of hormonal therapy [36]. Classical risk factors for arterial thrombosis include smoking, overweight, hypertension, diabetes and hypercholesterolemia, these are also

characteristics that can be found in patients with atrial fibrillation [37]. Specific risk factors for VTE are deficiencies in anticoagulant factors (antithrombin, protein S, protein C), increased level or activity of procoagulant factors (e.g. factor V, VIII, IX, fibrinogen, prothrombin), hospitalization, cancer and surgery [7, 38], but also >4 hours of travel [39, 40], immobility [41], oral contraceptive use and pregnancy [7, 38].

Arterial and venous thromboembolism have long been considered as distinct pathophysiological conditions with arterial thrombosis due to platelet activation on atherosclerotic plaques on one hand and coagulation cascade activation in VTE on the other hand. However, an overlap in pathophysiology also exists, for instance coagulation cascade activation resulting in fibrin-rich thrombi also occurs in arterial thrombosis, specifically in atrial fibrillation and myocardial infarction [36]. The key role of coagulation in the formation of arterial thromboembolism, hence beyond VTE, is supported by anticoagulant drugs which are also highly effective in preventing arterial embolism in atrial fibrillation [42] and can be used in addition to antiplatelet drugs to increase the effectiveness in treatment of established coronary artery disease [43]. Furthermore, patients with hemophilia (less functional coagulation cascade) have an 80% reduced risk of myocardial infarction [44]. Thus, arterial thromboembolism is not only due to platelet activation but also due to coagulation cascade activation. Similarly VTE comprises both coagulation activation and platelet activation, several examples support this notion. For example, during early venous thrombus formation aggregated platelets attach to endothelium [45] and excrete granular content [46]. Inhibiting platelet adhesion to endothelium by blocking P-selectin reduces venous thrombus formation [47] and inhibiting platelet function by clopidogrel or aspirin reduces experimental venous thrombus formation and PE mortality, respectively [48, 49]. Thus, these studies demonstrate the role of platelets, besides the already known role of coagulation cascade, in the development and consequences of VTE. Moreover, inhibiting platelets by aspirin after a first unprovoked VTE can reduce the recurrence of VTE in patients by 42% [50]. Antiplatelet drugs are therefore effective in the prevention of VTE although to a lesser extent than anticoagulant drugs [51, 52].

Consequently, platelets play a role in both arterial and venous thromboembolism [53-56] and both primary and secondary hemostasis are crucial in the development of diseases such as myocardial infarction, stroke, deep vein thrombosis and pulmonary embolism. Additionally, pathological hemostasis is implicated in other conditions like sepsis and accidental hypothermia [8, 15, 16, 57, 58].

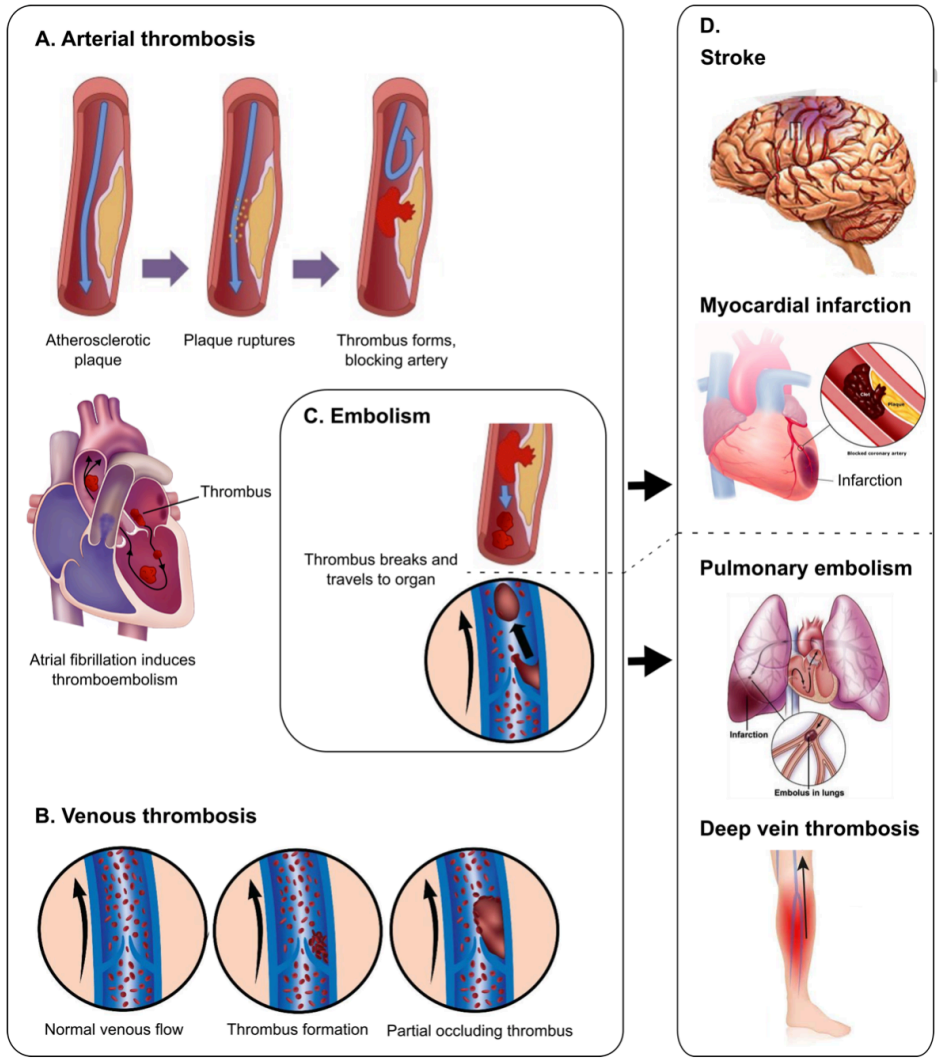

FIGURE 2. Pathological hemostasis. A) Atherosclerotic plaques expose procoagulant contents activating platelets and subsequently the coagulation cascade, inducing arterial thrombosis, which leads to complete or partial occlusion of the artery and stops blood supply to the tissue (infarction) potentially inducing necrosis. Atrial fibrillation allows relative stasis of blood in atria of the heart by improper contractions, inducing thrombus formation of which emboli can break off and travel through the ventricles out of the heart, e.g. into the aorta and other organs. B) Stasis of blood flow near venous valves creates relative hypoxia and induces endothelial cell activation. Stasis may occur due to (fracture cast) limb immobilization or long travel, whereas the concomitant hypercoagulability of blood is often present due to trauma, surgery or altered blood constituents (e.g. thrombophilia or oral anticonceptive use), priming the hemostatic and

inflammatory system. Subsequent activation of coagulation cascade and platelets occur and the ensuing thrombus formation leads to complete or partial occlusion of the vein critically reducing blood flow. C) When part of a thrombus breaks, an embolus is formed which travels from either the venous or arterial thrombus with the remaining blood flow until it occludes a subsequent blood vessel in an organ. D) Potentially lethal occlusive diseases in different organs. Arterial thromboembolism can induce occlusion of blood supply to for instance the brain or heart, leading to stroke or myocardial infarction. Venous thrombosis may lead to deep vein thrombosis of legs and arms and emboli moving through the venous system into the right heart subsequently occluding pulmonary arteries leading to pulmonary embolism.

HEMOSTASIS IN HYPOTHERMIA

Hypothermia is a condition wherein the body has lost heat faster than it can produce, resulting in a lower than normal (~37°C) body temperature. Accidental hypothermia can lead amongst others to arrhythmias, central nervous system depression and respiratory failure, eventually leading to death [16, 59, 60]. Moreover, hypothermia may induce pathological activation of the hemostastic system. The ensuing disseminated intravascular coagulation (DIC) of hypothermic patients may result in ischemia and necrosis of organs and eventually result in death [16, 57, 59]. Besides these thrombotic complications, DIC may provoke hemorrhage due to consumption of clotting factors and platelets, thereby leaving a hypocoagulopathy to favor bleeding [61]. Consequently, hypothermia is associated with a hypocoagulated state with prolonged PT and APTT already when temperatures drop below 35 °C [62, 63]. Low temperature *in vivo* has also been shown to increase activation, aggregation, and sequestration of platelets [64, 65]. Low temperatures of the extremities have been implicated to 'prime' platelets for activation at these sites most susceptible to bleeding throughout evolutionary history, which also leads to increased clearance of these platelets from circulation [66]. Furthermore, both accidental and therapeutic hypothermia are associated with a reduction in platelet count (thrombocytopenia) [60, 67-72]. Whether this thrombocytopenia can be reversed quickly by rewarming is still not clear.

Ex vivo cooling of platelets induces platelet shape changes similar to activation of platelets [73-76] and several studies described low temperature to increase degranulation of activated platelets and activation products of platelets in plasma [65, 77]. Moreover, cooled platelets demonstrate an increased tendency to aggregate [78]. Furthermore, cold (4°C) stored platelets are rapidly cleared from circulation after transfusion [65, 79, 80]. Therefore, platelets are stored at 22-24°C room temperature before transfusion which increases risk of bacterial contamination and thus limits shelf-life to only 5-7

days, compared to 40 days for cold stored red blood cells [81]. Enabling cold storage of platelets without their activation may reduce monetary losses from discarded and expired platelet concentrates, improve logistics and limit bacterial contamination. Besides provoking prothrombotic effects, low temperature has also been described to induce anticoagulant mechanisms, for instance lowering the enzymatic coagulation reactions [63], prolonging bleeding time in cold skin and [82] and diminishing thromboxane A_2 release from platelets [83]. Taken together, the effects of temperature on hemostasis are still incompletely understood, specifically since hypothermia is associated with both prothrombotic and hypocoagulant effects, of which the latter can be secondary to the consumption of coagulation factors and platelets in cases of DIC, however this cannot explain all anticoagulant effects studied so far. Further unraveling the temperature effects on hemostasis may yield improved knowledge on hemostasis and potentially new pathways for drug development focused on novel antithrombotic strategies.

MAMMALIAN HIBERNATION: A UNIQUE NATURAL MODEL OF SUPPRESSING HEMOSTASIS

Hibernation is an energy conserving behavior, which in small rodents consists of repetitive cycles of torpor and arousal. During torpor, metabolism, body temperature, heart and respiratory rate as well as other physiological processes reduce to a minimum and revert during each short period of arousal (Figure 3). Torpor lasts several days to weeks, whereas arousals last several hours to a day. Contrarily to this 'deep torpor', some mammals perform 'daily torpor' to save energy, entering torpor for a few hours while remaining normothermic the rest of the day.

Hibernators also embody several risk factors for thrombosis compliant with the triad of Virchow for thrombotic risk - by stasis of blood, hypercoagulability, and endothelial activation/injury. Specifically, hibernation entails periods of prolonged immobility [84-86] with low blood flow (stasis) in veins and atria [87], increased blood viscosity (hypercoagulability) [88-90], cycles of hypoxia-reoxygenation and cooling-rewarming with signs of endothelial activation [84, 91]. Additionally, at entrance of the hibernation season, hibernators are generally grossly overweighed/obese [92]. Remarkably, despite these risk factors for thrombosis, hibernators do not demonstrate signs of organ damage due to thrombosis during hibernation or upon arousal in spring. Moreover, despite the frequent periods of dramatically reduced body temperatures, hibernators emerge apparently unharmed from hibernation and seem to escape from the potential fatal consequences of hypothermia as well.

Therefore, it seems that hibernators developed mechanisms to prevent amongst others pathological activation of the hemostatic system. Alterations in components of primary hemostasis, secondary hemostasis and fibrinolysis may play a crucial role to diminish the risk of thrombosis during hibernation as some of these alterations have been disclosed in different hibernating species, amongst others in brown bear, ground squirrel species, hedgehog, and Syrian hamster [93-97]. However, mechanisms governing the opposite, i.e. the rapid normalization of hemostasis in arousal to prevent bleeding, is less well documented. Additionally, platelets from hibernators resist exposure to low temperatures for a prolonged time and *ex vivo* cold storage of hibernator platelets still allows transfusion in summer animals without rapid platelet clearance [98]. This in sharp contrast to the cold exposed human platelets which are rapidly cleared from circulation after transfusion [79, 80].

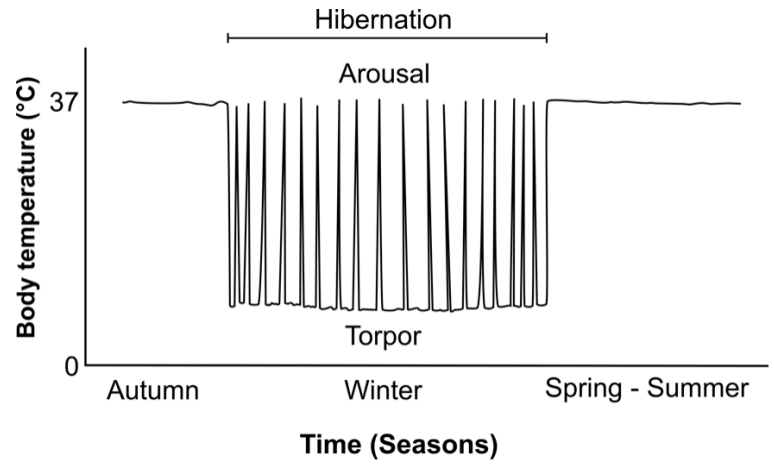

FIGURE 3. Schematic overview of hibernation. In autumn environment, hibernators prepare for hibernation by increasing body weight and/or harvesting food. For most hibernators their endogenous circannual clock dictates the entrance of and exit from hibernation. During phases of torpor metabolism and subsequent body temperature reduce to near ambient temperature and recover swiftly during phases of arousal. For other hibernators during winter the day length shortening and drop in ambient temperature trigger the start of torpor-arousal cycles, together called hibernation, until springtime when day length and ambient temperature increase giving a cue to emerge from hibernation and prepare for breeding and summer time.

Thus, hibernation features a potentially lifesaving natural anticoagulant mechanism to prevent thrombosis in times of increased risk. Unlocking this mechanism for

humans may allow improved anticoagulant strategies for thromboembolic conditions. To elucidate this mechanism, one has to determine whether hibernators develop thrombosis followed by rapid resolution or whether they prevent thrombosis as might be accomplished by preventing activation of platelets and the coagulation system at low temperature. Understanding the basic processes and players involved in hemostasis and thrombosis, as visualized in Figures 1 and 2, and investigating them in hibernators is pivotal in addressing this topic and will help us disclose the hibernator's solution to thrombosis.

AIM OF THIS THESIS

The main goal of this thesis is to elucidate the regulation of key modulators of hemostasis in hibernation and establish to what extent these are present in non-hibernating mammals.

Therefore, **Chapter 2** describes the effects of hibernation and hypothermia on circulating platelet dynamics in hibernating and non-hibernating mammals. **Chapter 3** presents an overview of alterations in the components of primary and secondary hemostasis as well as in the fibronolytic pathway in the hibernating Syrian hamster. The underlying mechanism of platelet dynamics in hibernating hamsters is further assessed and described in **Chapter 4**. Since the findings in Chapter 2 demonstrated that the platelet dynamics are temperature dependent and applicable in non-hibernating mammals, the study in **Chapter 5** further assessed the application of hypothermia induced suppression of hemostasis via a thrombocytopenia in non-hibernating mammals, amongst others by (intravital) imaging studies to elucidate the underlying mechanism in rat and mouse. Additionally, in **Chapter 6** the role of cytoskeletal rearrangements was explored in underlying shape changes of platelets associated with hibernation and a comparison was made with shape changes of platelets from human and other non-hibernating species. Lastly, in **Chapter 7** we summarize and discuss the obtained data in our experimental chapters, review the literature on hemostasis in hibernation and provide future perspectives.

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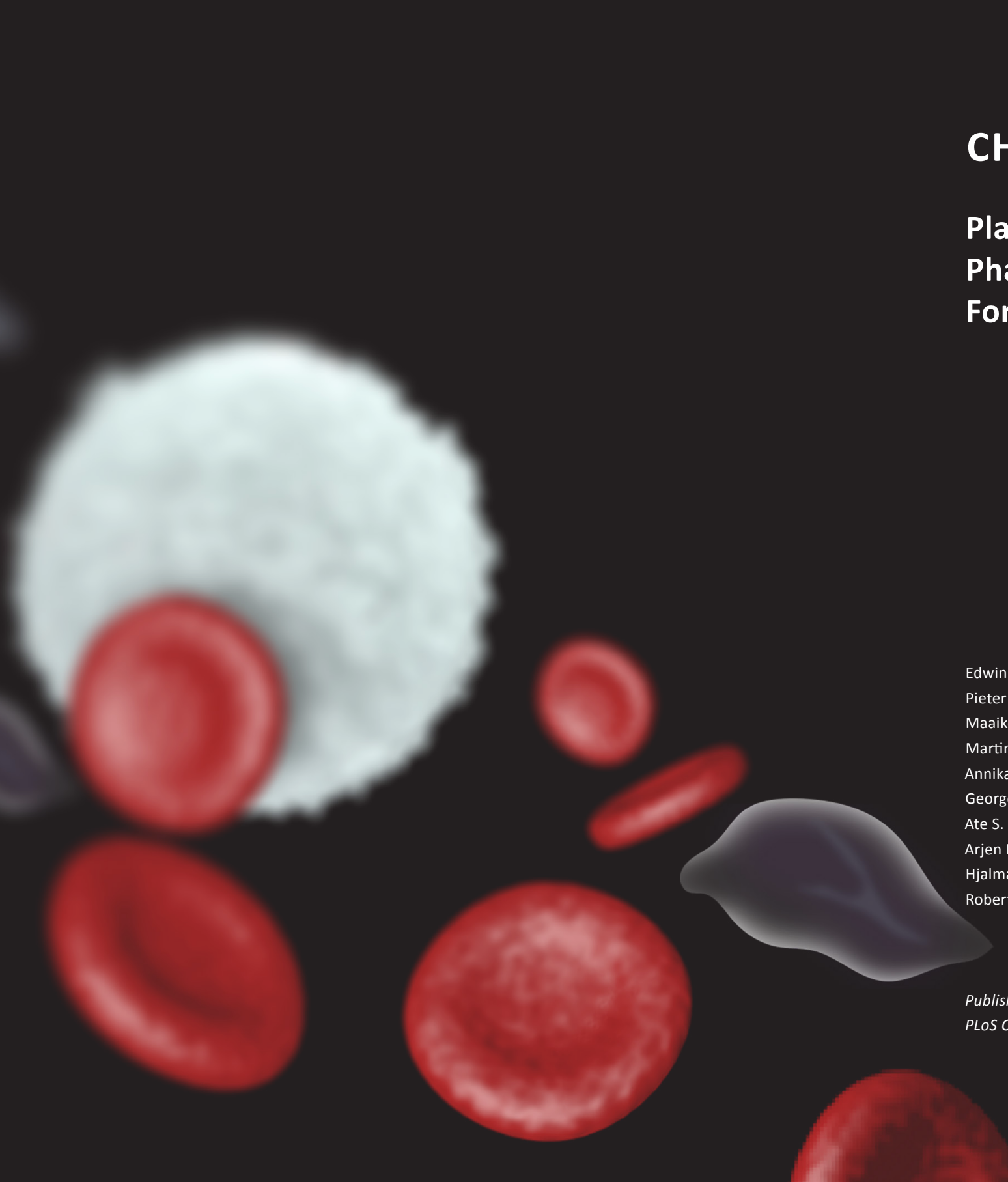

CHAPTER 2

Platelet Dynamics During Natural and Pharmacologically Induced Torpor and Forced Hypothermia

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ABSTRACT

Hibernation is an energy-conserving behavior in winter characterized by two phases: torpor and arousal. During torpor, markedly reduced metabolic activity results in inactivity and decreased body temperature. Arousal periods intersperse the torpor bouts and feature increased metabolism and euthermic body temperature. Alterations in physiological parameters, such as suppression of hemostasis, are thought to allow hibernators to survive periods of torpor and arousal without organ injury. While the state of torpor is potentially procoagulant, due to low blood flow, increased viscosity, immobility, hypoxia, and low body temperature, organ injury due to thromboembolism is absent. To investigate platelet dynamics during hibernation, we measured platelet count and function during and after natural torpor, pharmacologically induced torpor and forced hypothermia. Splenectomies were performed to unravel potential storage sites of platelets during torpor. Here we show that decreasing body temperature drives thrombocytopenia during torpor in hamster with maintained functionality of circulating platelets. Interestingly, hamster platelets during torpor do not show surface expression of P-selectin, but surface expression is induced by treatment with ADP. Platelet count rapidly restores during arousal and rewarming. Platelet dynamics in hibernation are not affected by splenectomy before or during torpor. Reversible thrombocytopenia was also induced by forced hypothermia in both hibernating (hamster) and non-hibernating (rat and mouse) species without changing platelet function. Pharmacological torpor induced by injection of 5'-AMP in mice did not induce thrombocytopenia, possibly because 5'-AMP inhibits platelet function. The rapidness of changes in the numbers of circulating platelets, as well as marginal changes in immature platelet fractions upon arousal, strongly suggest that storage-and-release underlies the reversible thrombocytopenia during natural torpor. Possibly, margination of platelets, dependent on intrinsic platelet functionality, governs clearance of circulating platelets during torpor.

INTRODUCTION

Hibernation is an energy conserving behavior in animals during winter that is characterized by two phases: torpor and arousal. During torpor, metabolic activity is markedly reduced resulting in inactivity and a drop in body temperature, meanwhile various physiological parameters change including a steep decline in heart rate and ventilation rate [1-5]. Bouts of torpor are interspersed by short arousal periods, during which metabolism increases and body temperature returns to euthermia [2, 6, 7]. Key changes in physiological parameters are thought to lead to an increased resistance to ischemia/reperfusion [8, 9] allowing hibernating mammals to survive periods of torpor and arousal without signs of organ injury. Therefore, hibernating animals have been used in various studies as a model to investigate the effects of low body temperature and hypoxia on organs, in attempts to unravel the adaptations that allow these animals to cope with the physiological extreme conditions of torpor [5]. These studies mainly focused on identifying mechanisms employed by these animals to protect their internal organs from injury during hypothermia and rewarming [10-14]. The torpid phase embodies several potentially procoagulant conditions, including low blood flow [15], increased blood viscosity [16, 17], immobility, chronic hypoxia, and low body temperature [5]. Although low body temperature has not been described by Virchow in his "triad of risk factors for thrombosis", it is well established that low temperature leads to platelet activation and aggregation in mammals [18-20]. In addition to aggregation, platelet activation also leads to inflammatory reactions and potential organ injury, e.g. via platelet-leukocyte complex formation [21]. Although aggregation of platelets generally lead to thrombus formation, organ injury resulting from thrombotic complications has not been observed in hibernating animals during torpor [5]. We speculate that suppression of hemostasis, as observed by a hypocoagulative state in the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) [22], might play an important role in the prevention of organ injury as well.

Circulating platelet numbers are decreased during torpor in hibernating ground squirrels as compared to summer euthermic animals [23]. Consequently, the blood clotting is reduced during torpor [22, 24]. Upon arousal, platelet numbers are rapidly restored, i.e. within 2 hours upon rewarming to 37°C in ground squirrels [22, 25, 26] and its coagulative function returns to normal [22]. This rapid restoration of platelet count and coagulative function is unlikely to be due to increased platelet production from the bone marrow, because platelet synthesis from megakaryocytes takes 24-48 hours to restore circulating platelet counts after an induced thrombocytopenia [22, 27]. Therefore, the rapid dynamic of restoration of platelet numbers upon arousal suggests

a storage-and-release mechanism to underlie thrombocytopenia during torpor rather than clearance-and-reproduction. However, to date, the mechanism(s) that underlie thrombocytopenia during torpor and the full restoration during early arousal are still unclear.

Similarly to platelets, specific classes of leukocytes also disappear from the circulation during torpor [23]. We previously showed the importance of the decrease in body temperature in the mechanism governing the decline in leukocytes, which constitutes of a temperature driven drop in plasma S1P levels [28]. Thus, we hypothesized that body temperature is critical in the initiation of a decrease in circulating platelets. To examine this, we investigated changes in the number of circulating platelets in different stages of natural hibernation in hamster species that undergo either deep multiday torpor bouts or shallow daily torpor. Effects were compared with those found in hamsters, rats and mice that were cooled under anesthesia or in which torpor was induced pharmacologically by 5'-AMP. In order to examine the origin of platelet number decrease and restoration, splenectomy was performed and immature platelet fraction determined. To investigate the coagulative function of the remaining circulating platelets, we performed platelet function measurements by aggregometry and by measurement of platelet activation marker expression by flow cytometry analysis.

Understanding the mechanism of thrombocytopenia and the effect on platelet function in torpor and its subsequent restoration in arousal might lead to new insights to inhibit platelet function or extend platelet shelf life, e.g. under hypothermic conditions.

MATERIALS AND METHODS

Ethics statement

All animal work has been conducted according to relevant national and international guidelines, and was approved by the Institutional Animal Ethical Committees of the University Medical Center Groningen and University of Aberdeen.

Hibernation

Prior to experiments, hamsters were kept at summer conditions (L:D cycle of 12 h:12 h) and fed *ad libitum* using standard animal lab chow. To induce hibernation in Syrian hamsters (*Mesocricetus auratus*), the light:dark (L:D) cycle was shortened to 8 h:16 h for ~10 wk followed by continuous dim light (< 5 lux) at an ambient temperature of 5 °C. Movement detectors connected to a computer were used to determine the animals' hibernation pattern. In the Djungarian hamsters (*Phodophus sungorus*), hibernation was induced by shortening the L:D cycle to 8 h:16 h for ~14 wk at an ambient temperature of 21 ± 1 °C. Daily torpor was determined by observation in the middle of the light phase (usual torpor phase) and a single body temperature measurement at the time of euthanization. Animals were sampled related to the time of entry into torpor (at lights on; t=0 h). Blood was collected from animals at 4 h (torpor), 8 h (arousal) and normothermic animals at 12 h. Blood was collected by cardiac puncture and body temperature was measured i.p. just prior to euthanization.

Forced Hypothermia

Summer-euthermic Syrian hamsters, Wistar rats, and C57Bl/6 mice were housed at an L:D cycle of 12 h:12 h. The Syrian hamster and Wistar rat were anesthetized by injecting 200 mg/kg ketamine and 1.5 mg/kg diazepam i.p. C57Bl/6 mice were anesthetized by brief isoflurane 2.5% inhalation before ketamine infusion in the jugular vein of 7mg/hr. Prior to experiments, animals were fed *ad libitum* using standard animal lab chow. Spontaneously breathing hamsters were cooled and rewarmed. In contrast to the hamsters, rats and mice had to be intubated and ventilated to maintain adequate oxygenation. Animals were cooled by applying ice-cold water to their fur and were rewarmed using a water-based or electrical heating mattress and evaporation by airflow. Procedures were adjusted to change body temperature at a rate of ~1 °C per 3 min. Upon reaching 20 degrees body temperature (mouse), 15 degrees (rat), or 8 degrees (hamster), application of ice-cold water was reduced to sustain a stable body temperature for 3 hours in rat and hamster, and for 1 hour in mouse. In the hamster, a catheter was inserted into the jugular vein for blood sampling, while in the rat and mouse a catheter was inserted into the carotid artery to monitor heart

rate, blood pressure and draw blood. In hamster, samples were taken on the cooling and rewarming curve. Hence body temperature reflects the time of sampling; e.g. a body temperature of 30 °C (coming from 37°C) was reached 3x7 = 21 min after start of cooling. Forced-cooled rats and mice were sampled during euthermia while under anesthesia, 3 hours after cooling the rat and 1 hour after cooling the mouse, and after reaching 37 degrees body temperature upon rewarming. Due to low sample volume, mice were either sampled 1 hour after cooling or after reaching 37 degrees body temperature. Rectal temperature was measured continuously, and heart rate (ECG) was monitored (Cardiocap S/5, Datex Ohmeda).

Pharmacological induction of torpor

C57Bl/6 mice were housed under standard L:D-conditions (L:D cycle of 12 h:12 h) in the animal facilities of the University of Groningen, The Netherlands. Prior to experiments, animals were fed *ad libitum* using standard animal lab chow. Torpor was induced pharmacologically by injecting 7.5 mmol/kg of 5'-AMP (Sigma Aldrich) in 0.9% saline (pH 7.2-7.5) intra-peritoneally. To record body temperature during experiments, we measured the body temperature using a rectal probe (Physitemp Instruments). Mice were euthanized at different times after injection of 5'-AMP or saline. The minimum body temperature during torpor was reached at 4-5 hours following 5'-AMP injection and full arousal with normalization of body temperature occurred by 10 hours after 5'-AMP administration. At euthanization, animals were anesthetized using 3% isoflurane/oxygen and up to ~800 µl blood was drawn immediately by abdominal aortic puncture into 3.2% sodium citrate and small EDTA-coated tubes. Automated hematological analysis was performed within 5 hours using a Sysmex XE-2100 [29]. The platelets were discriminated from other cells by Forward and Sideward Scatter characteristics. Mature and immature platelets were separated on the basis of Side Scatter, by virtue of the increased amount of granular (i.e. scattering) organelles in immature platelets.

Splenectomies

Splenectomies were performed on summer-euthermic and torpid Syrian hamsters. Immediately after induction of anesthesia (2–2.5% isofluorane/O_2), a blood sample was drawn by cardiac puncture, and 4 mg/kg flunixin-meglumin (Finadyne; Schering-Plough) was given s.c. as analgesic. The abdomen was shaved and disinfected by chlorhexidine. The abdominal cavity was opened by a midline incision and the spleen was exposed by careful manipulation of the internal organs using a pair of blunt tweezers. Next, the splenic artery and vein were ligated and the spleen was removed. The abdominal cavity was closed in two layers using ligations. Summer-

euthermic animals that underwent splenectomy recovered in a warm room (L:D cycle 8 h:16 h). Once animals started to hibernate, animals were sacrificed during their third torpor bout, which was 60.3 ± 8.1 d following splenectomy. Torpid animals underwent splenectomy during their third torpor bout while being kept at < 10 °C body temperature using ice-packs. Subsequently, they were allowed to recovered at an ambient temperature of 5 °C during which period all animals developed surgery induced arousal. Animals were euthanized upon reaching euthermia.

Platelet preparation for platelet function measurements

Rodent blood samples were drawn into 3.2% sodium citrate tubes and stored at room temperature under gentle continuous rotation after being used for flow cytometry preparation. Within 24 hours, platelets were prepared as previously described [30] with small adaptations. Rat blood was centrifuged for 8 minutes at 180 x *g* while mouse blood was centrifuged for 11 minutes at 100 x *g*. Platelets were then resuspended in buffer A (6 mM dextrose, 3 mM KCl, 0.81 mM KH_2PO_4, 9 mM $MgCl_2$, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM sodium citrate, 10 mM tris (hydroxymethyl)aminomethane, pH 7.4) as previously described [31] and platelet concentrations were determined on a Horiba ABX Micros 45 hematology analyzer. If needed, platelet suspensions were further diluted in buffer A in order to match with the lowest platelet yield among all samples on that day. These platelet suspensions were then allowed to rest for at least 15 minutes.

Microtiter plate platelet aggregation (MTP)

Platelet aggregation was determined as previously described [31]. Aliquots (90 µL) of platelet suspension were dispensed on a clear flat bottom 96-wells plate and baseline optical density was measured on BioTek ELx808 absorbance microplate reader every minute. After 6 minutes, 10 µL of ADP and $CaCl_2$ in buffer A was added to each well to final concentrations of 20 µM and 1,8 mM respectively. During the remaining 12 minutes run time, the plate was vigorously shaken, not stirred, in between measurements. Separate experiments were corrected by subtraction of baseline absorption. Finally, platelet aggregation was normalized by dividing by the optical densities of an internal standard included in each experiment. To display platelet aggregation, data were transformed to show the increase in light transmission instead of a decrease in optical density.

Flow cytometry analysis for P-selectin

Surface expression of P-selectin (CD62P), as platelet activation marker, and platelet glycoprotein IIIa (integrin β3 or CD61), as platelet marker, on platelets from rat and

mouse whole blood samples was analyzed by double label flow cytometry. In hamster, only the P-selectin antibody could be used. One microliter of whole blood was 1:25 diluted in phosphate buffered saline (PBS), and incubated with anti-CD61-FITC and/or anti-CD62P-PE with or without 10uM ADP platelet agonist for 30 min in the dark. The activation was stopped by addition of PBS and fixation by 2% formaldehyde in 300uL end volume. Samples were stored at 4 degrees in the dark until measurement the next day. Samples were acquired with low flow rate on a FACS Calibur flow cytometer equipped with CellQuest software (BD Biosciences). Samples were analyzed using Kaluza 1.2 software (Beckman Coulter). Platelet populations were gated on cell size using forward scatter (FSC) and side scatter (SSC) and CD61 positivity, or by FSC and SSC alone in hamster. Light scatter and fluorescence channels were set at logarithmic gain and measurement of the platelet population gate was stopped after 20.000 events per sample or after 180 seconds in case of low platelet counts (thrombocytopenia).

Statistical Analysis and Data Presentation

Data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA with post hoc Tukey, Wilcoxon signed rank test, one-way ANOVA with post hoc least significant difference, One-Sample T-test, or by ANOVA for repeated measures (SPSS 20.0 for Windows), with $P < 0.05$ considered significantly different. Correlations were calculated using Pearson's correlation. Sigmaplot 12.0 and SPSS 20 were used to produce the graphs shown in this article.

RESULTS

Platelet dynamics during natural torpor

Platelet count and body temperature were measured during the different phases of hibernation. Body temperature of the Syrian hamster entering torpor decreases from 35 °C to 8 °C in 12 hours (Figure 1A). In torpor, the number of circulating platelets decreases by 96 % from the normal euthermic level of 198 x 10^9/L (Figure 1C) to 8 x 10^9/L (Figure 1D). The state of torpor lasts for 6-7 days in the Syrian hamster. At the end of torpor, the body temperature increases from 8 °C to 35 °C during arousal within 180 minutes (Figure 1B). The number of circulating platelets increases in this 3 hour timeframe from 12 x 10^9/L to 187 x 10^9/L (Figure 1E) approximating the normal euthermic resting rate of 198 x 10^9/L (Figure 1C). The platelet count correlates well with body temperature during torpor (Pearson's $R = 0.825$; $P < 0.01$, $n=31$) and arousal (Pearson's $R = 0.757$; $P < 0.01$, $n=42$) (Figure 1D-E). Thus, the drop in body temperature during deep torpor in the Syrian hamster is associated with the concurrent thrombocytopenia, and the rise in temperature during arousal associates with a restoration of platelet count.

To assess platelet function throughout hibernation, CD62P surface expression was determined on platelets in whole blood from Syrian hamsters in euthermia, torpor and arousal (Figure 1F-I). While P-selectin positive platelets are absent in the hamsters in torpor, they are present at normal levels in aroused and euthermic hamster (One-Sample T-test, test value = 0; $P < 0.05$, Figure 1F). In contrast, the percentage of P-selectin positive platelets following activation with ADP of torpid hamster was similar to those of aroused and euthermic animals (Figure 1G). Likewise, the P-selectin surface expression level of unstimulated platelets was significantly lower in torpid hamster compared to aroused and euthermic groups (One-Sample T-test, test value = 0; $P < 0.01$, Figure 1H). Upon activation with ADP, however, P-selectin expression reaches similar levels in euthermia, torpor and arousal (Figure 1). Together, these data imply that P-selectin surface expression on circulating platelets is significantly decreased in torpid hamster, but restores to normal euthermic levels shortly after arousal.

During daily torpor, the body temperature of the Djungarian hamster decreases from 35 °C to 25 °C. As seen in Figure 1J, the number of circulating platelets is reduced by 52% from euthermic 797 x 10^9/L to 381 x 10^9/L ($P < 0.01$) during this torpor bout and is restored to 739 x 10^9/L (93% of euthermic condition) during arousal with 35 °C body temperature ($P < 0.05$; compared to torpor). Thus, daily torpor in the Djungarian hamster also leads to thrombocytopenia, but to a lesser extent than the deep torpor in Syrian hamster, and platelet count also rapidly restores towards euthermic level upon arousal.

Forced hypothermia induces thrombocytopenia in hibernating and non-hibernating animals, but maintains platelet function

In order to determine the effect of body temperature on the platelet count irrespective of metabolic suppression during natural torpor, forced hypothermia was induced in anesthetized euthermic (summer-active) Syrian hamsters until a body temperature of 8.7 ± 2.2 °C was reached (Figure 1C-E, open dots). Platelet numbers were measured during the process of cooling and rewarming similar to measurements in hibernating Syrian hamster. Platelet count diminishes by forced hypothermia to 78 x 10^9/L (Figure 1D, n=5), a drop of 53% compared to euthermic platelet counts of 166 x 10^9/L (Figure 1C, n=5; Wilcoxon signed rank test, $P < 0.05$), and restores upon rewarming to 149 x 10^9/L (Figure 1E, n=5; Wilcoxon signed rank test, $P < 0.05$) in a similar fashion as during torpor.

Additionally, the number of circulating platelets correlated with body temperature during cooling (Figure 1D; Pearson's $R = 0.727$; $P < 0.01$, n=29) and during rewarming following forced hypothermia (Figure 1E; Pearson's $R = 0.660$; $P < 0.01$, n=16). Curves in Figure 1D and 1E have been fitted to a polynomial quadratic curve ($y=y_0+ax+bx^2$) with constraints of $y_0 > 0$ and $y_0 \leq$ lowest platelet count for the data points of torpor ($y=4.9e^{-16}+0.81x+0.15x^2$), hypothermia ($y=1.3e^{-16}+5.48x+0.04x^2$), arousal ($y=3.8e^{-15}+8.21x-0.06x^2$), and rewarming ($y=20-3.04x+0.17x^2$). The curves show a steady decline during torpor and forced hypothermia, and steady incline upon arousal, whereas the rewarming curve shows a delayed but progressive incline towards reaching euthermia.

To examine the role of body temperature in a non-hibernator, platelet count and function was assessed in anesthetized rats in which forced hypothermia was induced to reach a body temperature of 15 °C. Considering the euthermic number of platelets in rats (793 x 10^9/L), circulating platelet count decreases by 35% in the hypothermic condition (513 x 10^9/L, $P < 0.01$) and restores upon rewarming to 85 % (671 x 10^9/L, $P < 0.05$) of euthermic condition (Figure 2A).

To assess platelet function, CD62P surface expression level and platelet aggregometry was measured on platelets from the forced-cooled rats (Figure 2B-D). The fraction of P-selectin positive platelets does not differ between anesthetized, cooled or rewarmed rats, both in non-activated and ADP activated blood samples (Figure 2B). Furthermore, the P-selectin surface expression level is similar in platelets from all groups both in non-activated and ADP activated blood samples (Figure 2C). Further, aggregation of rat platelets is unaffected during anesthesia, cooling and subsequent rewarming (Figure 2D). However, while maximum aggregation is similar in all groups, the velocity of aggregation in cooled rats appears to be increased in comparison to anesthetized and rewarmed rats, albeit not reaching a significant difference (Figure 2E and Table 1).

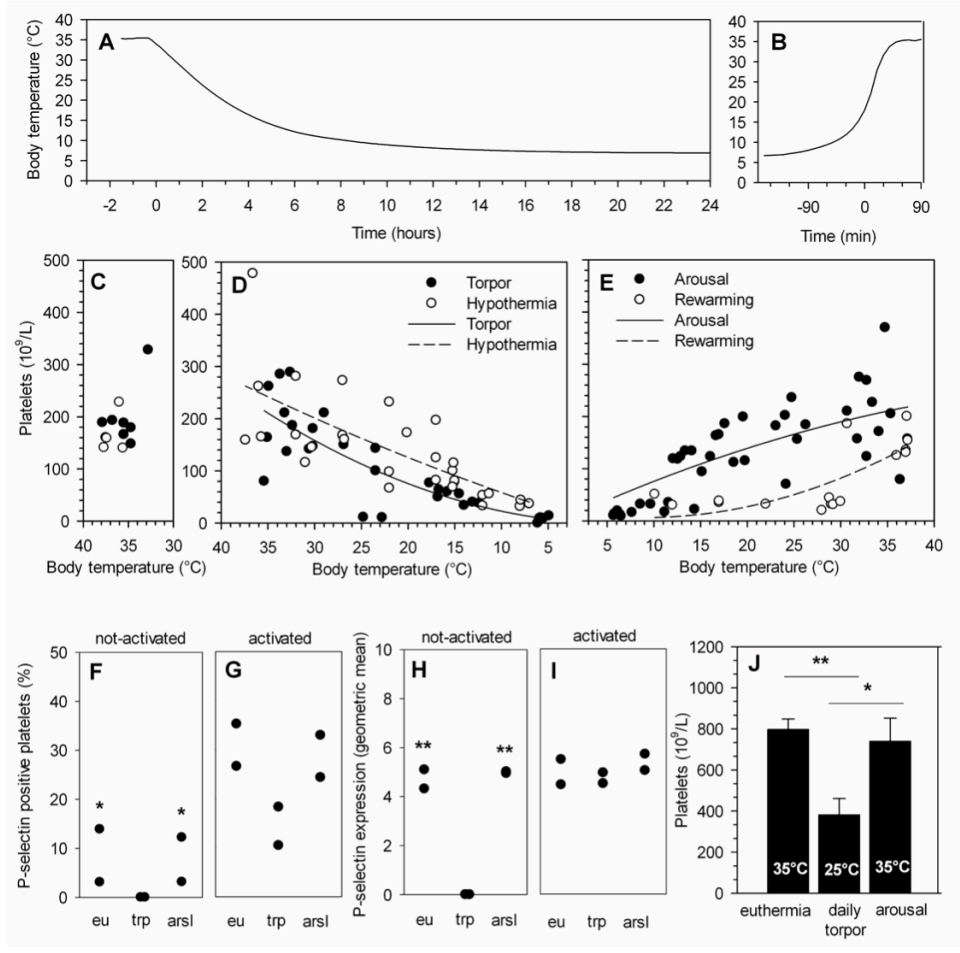

FIGURE 1. Body temperature dependent platelet count of functional platelets during torpor and arousal in natural hibernating Syrian hamster at 5 °C ambient temperature.

A) During spontaneous entrance into torpor body temperature gradually declines from 35 °C to 8 °C in a matter of hours. B) Increase in body temperature during a spontaneous arousal, demonstrating the rapid increase to euthermic level. Line represents one of thirty-one Syrian hamsters, measured with an intraperitoneal implanted Thermochron iButton. C) Normal platelet count in summer-euthermic Syrian hamster (n=5, open dots; n=7, black dots). D) Platelet count decreases with lower body temperature from euthermic stage to deep torpor in the Syrian hamster (n=31), both during natural hibernation as well as during forced hypothermia (n=8, multiple sampling). Curves from D) and E) are fitted to a polynomial quadratic curve with equation $y = y_0 + ax + bx^2$ and constraints of $y_0 > 0$ and $y_0 \leq$ lowest platelet count for torpor. Black dots (•) are natural hibernating hamsters, open dots (◦) are forced-cooled hamsters. E)

Platelet number increases rapidly to a normal level during arousal (n=42) or rewarming from forced hypothermia (n=7, multiple sampling). F) P-selectin positive platelets are absent in torpid hamsters. G) The platelets are activatible following addition of ADP and the subsequent percentage of P-selectin positive platelets is similar to euthermic and aroused animals. H) The P-selectin surface expression level per platelet was significantly decreased in non-activated platelets from torpor compared to euthermia and arousal hamsters. I) Upon activation with ADP, P-selectin surface expression reaches similar levels in euthermia (eu), torpor (trp) and arousal (arsl). Please note that F-I are n=2 per group. J) Circulating platelet count is reduced during daily torpor in the Djungarian hamster, and restored upon arousal. Bars represent mean ± SEM of 5 to 9 animals per group. *P < 0.05, **P < 0.01.

To further corroborate the finding of decreased platelet count upon decreased body temperature, we forced-cooled anesthetized mice (37.2 ± 0.7 °C) to a body temperature of 20.1 °C ± 0.3 °C for 1 hour and subsequently rewarmed them to 37.5 ± 0.8 °C. Platelet count decreases from 1,036 x 10^9/L in euthermia to 777 x 10^9/L during cooling (28 +/- 0.02 % decrease, P < 0.01), and partially restored to 817 x 10^9/L (P < 0.01, 12 +/- 0.01% lower than euthermia) upon rewarming (Figure 2F). Thus, platelet counts were significantly lower in 20 °C animals compared to rewarmed animals (P < 0.01). Forced cooling did not appear of influence on platelet activation, as ADP induced P-selectin surface expression, was not significantly different between the groups (Figure 2G-H).

Taken together, the reduction in platelet count by forced hypothermia in the rat and mice is less substantial than in the Syrian hamster (35% and 28% versus 53% reduction), while all are less than the reduction during natural deep torpor in the hamster (96%). However, the minimum body temperature reached during forced cooling and natural deep torpor in the Syrian hamster correlates well with platelet numbers, emphasizing the relevance of body temperature in the reduction of platelet numbers (Pearson's R = 0.727; P < 0.01, n=29 for forced hypothermia hamster and Pearson's R = 0.825; P < 0.01, n=31 for natural deep torpor hamster).

Further, during forced cooling of rat and mice, platelet function is not altered. This is demonstrated by similar percentages of P-selectin positive platelets from both not-activated or activated blood samples during all timepoints sampled during the cooling/rewarming procedure (Figure 2B). Moreover, the platelets express similar amounts of P-selectin (Figure 2C). Additionally, platelet aggregometry in rat (both velocity and maximum of aggregation) shows no difference between groups (Figure 2D-E, Table 1).

TABLE 1. Aggregation of platelets from forced-cooled rats.

Rat	Velocity (%Light transmission min^{-1})	Max aggregation
Anesthetized	12.6 ± 1.82	57.0 ± 8.57
Cooled	21.2 ± 5.77	60.5 ± 10.2
Rewarmed	12.6 ± 3.70	45.7 ± 11.6

Velocity and max amplitude of aggregation of rat platelets in response to 20 µM of ADP is not significantly different between anesthetized, cooled and rewarmed rats. Values are mean ± SEM of 10 rats per group.

Platelet dynamics during pharmacologically induced torpor

5'-AMP can induce a torpor-like state in non-hibernators. This torpor-like state is characterized by a.o. a leukopenia (predominantly lymphopenia) dependent on the decrease in body temperature [32]. To investigate the effect of hypothermia induced by metabolic suppression on platelet count in a non-hibernator, pharmacologic torpor was induced by administrating 5'-AMP to normothermic mice (36.4 ± 0.8°C). Subsequent hypothermia reaches a minimum body temperature of 20.5 ± 0.5°C at 5 hours after injection. At this point the platelet count is similar in 5'-AMP and sham injected animals, amounting 537 x 10^9/L versus 523 x 10^9/L, respectively (Figure 3A). Platelet count increases when body temperature returns to euthermic value (35.4 ± 0.5°C) after 10 hours and shows a clear elevation, amounting 795 x 10^9/L (Figure 3A, P < 0.01). Finally, platelet function, as assessed by aggregometry was not changed throughout 5'-AMP induced torpor and arousal in mice compared to sham injected animals. Full irreversible aggregation was observed in all groups (supplementary Figure S1 and Table S1). P-selectin positive platelets were present in the same amount in blood samples from torpor and arousal mice compared to euthermia with a similar expression level between the groups (supplementary Figure S2-3).

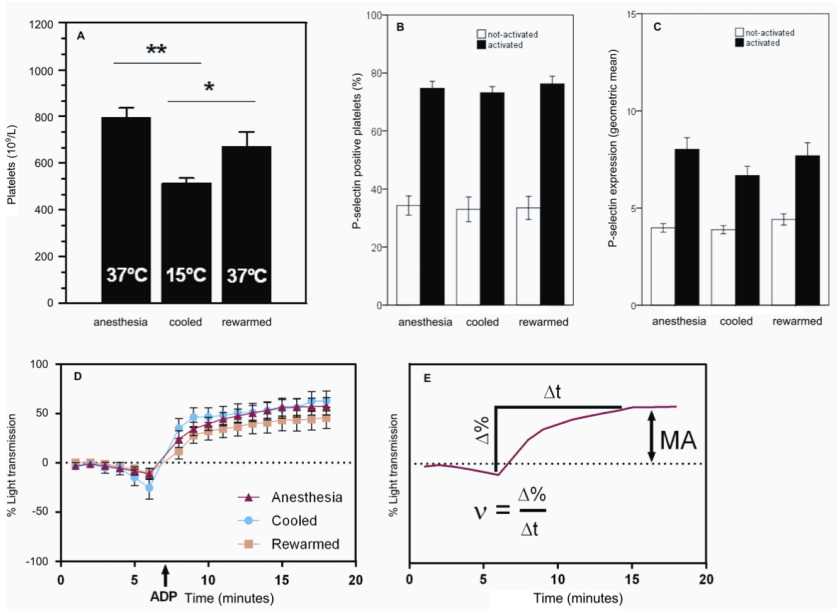

FIGURE 2. Decreased platelet count with preserved function during forced hypothermia.

Platelet count and function during hypothermia in rats (A-E) and mice (F-H). A) Rats forced to hypothermia of 15 °C have a decreased amount of platelets, which partially restores during rewarming. B) No difference in amount of activatable platelets from anestethized euthermic, cooled or rewarmed rats. C) Unchanged P-selectin surface expression at all time points in both non-activated and activated whole blood samples. D) Unchanged aggregometry at all time points upon addition of ADP. E) Mathematical approach for velocity and max amplitude of platelet aggregation. v, velocity of aggregation; Δ%, change in percentage light transmission; Δt, timespan over which velocity is determined; MA, maximum aggregation in % light transmission. F) Mice forced to hypothermia of 20 °C have a decreased amount of platelets, which partially restores during rewarming. Panels G) and H) show unchanged platelet P-selectin surface expression between time points in non-activated and activated whole blood samples. Bars represent mean ± SEM of 7 to 27 rats per group and 3 to 9 mice per group. *P < 0.05, **P < 0.01.

To assess whether torpor induction by 5'-AMP was successful, body temperature and leukocyte count were measured, both decreased during torpor [28, 32]. As found previously, body temperature and leukocyte level dropped from 36.4 °C and 5.8 x 10^9/L in euthermia to 20.5 °C (P < 0.01) and 0.4 x 10^9/L (P < 0.01) in torpor, and restored to 35.4 °C (P < 0.01) and 3.6 x 10^9/L (P < 0.05) upon arousal respectively (Figure 3B). Thus, while 5'-AMP induced torpor, it does not decrease platelet count or function in mice during torpor, whereas platelet counts are increased upon arousal with increasing body temperature. To further investigate the role of body temperature in the decrease in circulating platelets, all data from the animal experiments were plotted (Figure 3C). The reduced platelet count (expressed as percentage of euthermia platelet count) during cooling or torpor correlates well with decreased body temperature in all animals, except for torpor in mice induced by 5'-AMP administration.

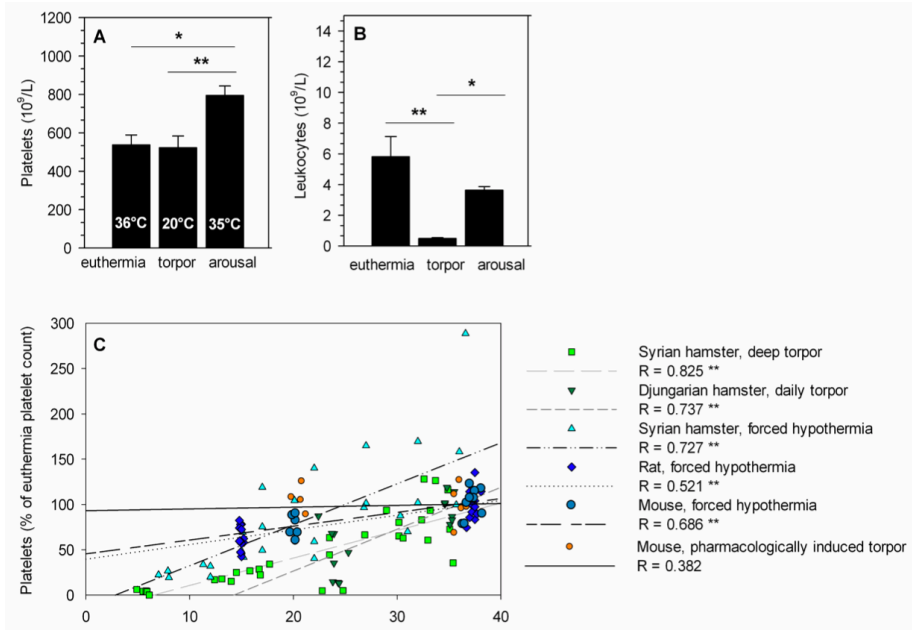

FIGURE 3. Pharmacologically induced torpor by 5'-AMP does not decrease platelet count despite decreased body temperature.

A) Pharmacologically induced torpor by 5'-AMP in mice does not decrease platelet count during torpor and shows an increase upon arousal. Body temperature drops during torpor and restores during arousal. B) Leukocyte level decreases with falling body temperature. C) The correlation of decreased body temperature and reduced platelet count is prominent in deep hibernating hamster (n=31), daily hibernating hamster (n=15), forced-cooled hamster (n=8, multiple sampling), forced-cooled rat (n=25), and forced-cooled mouse (n=15), but absent in 5'-AMP induced torpor in mice

(n=10). Bars represent mean ± SEM of 5 to 6 animals per group. *P < 0.05, **P < 0.01.

The graph shows good correlations for Syrian hamster in deep torpor (Pearson's R = 0.825; P < 0.01, n=31), Djungarian hamster in daily torpor (Pearson's R = 0.737; P < 0.01, n=15), forced-cooled Syrian hamster (Pearson's R = 0.727; P < 0.01, n=29), forced-cooled rat (Pearson's R = 0.521; P < 0.01, n=26), and forced-cooled mouse (Pearson's R = 0.686; P < 0.01, n=9). However, there is no good correlation between platelet count and body temperature in 5'-AMP induced torpor in mice (Pearson's R = 0.382; P > 0.05, n=16).

Storage and release as mechanism of thrombocytopenia

Given the rapid restoration of platelet counts upon arousal or rewarming, our data suggest that thrombocytopenia occurs due to storage-and-release, rather than clearance-and-reproduction. To further establish whether bone marrow massively releases fresh platelets upon arousal or rewarming, we determined the immature platelet fraction (IPF) in peripheral blood of Syrian hamsters after arousal, in forced-cooled rats after rewarming, and in mice after arousal of pharmacological induction of torpor (Figure 4A-C). The IPF increases from 0.7% in euthermic Syrian hamsters to 3.1% during torpor (P < 0.01), followed by a decrease to 1.7% upon arousal (P < 0.05, Figure 4A). In rats, the IPF reduces from 1.8% during anesthesia to 0.9% during cooling (P < 0.01) and 0.8% after rewarming (P < 0.01; Figure 4B). The IPF in mice did not change from euthermia to torpor and arousal (0.9%, 1.1%, 1.2% respectively; Figure 4C). Consequently, a massive increase in IPF is absent upon arousal and rewarming, which strongly substantiates the hypothesis that restoration of platelets is caused by release from a storage site.

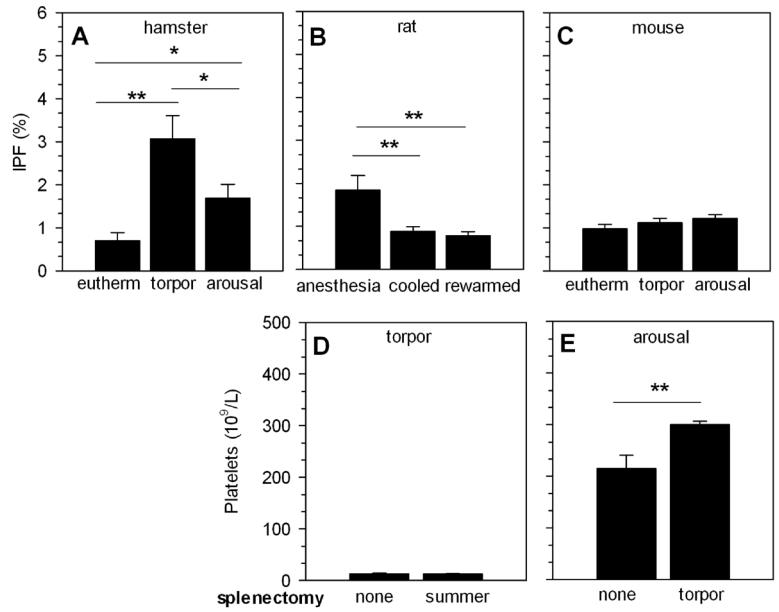

FIGURE 4. Restoration of circulating platelet numbers during arousal and rewarming does not originate from spleen or bone marrow.

A) Immature platelet fraction (IPF) is increased in torpor, but decreases in arousal toward normal euthermic percentage in Syrian hamster. B) In rat, IPF decreases during cooling and rewarming. C) In mice IPF only increases during arousal. D) Splenectomy in hamster prior to hibernation does not inhibit induction of thrombocytopenia in torpor. E) Splenectomy during torpor does not prevent restoration of platelet count during the subsequent arousal in hamster. Bars represent mean ± SEM of 4 to 12 animals per group. *P < 0.05, **P < 0.01.

The spleen is a platelet sequestering organ [33] and can function as a platelet reservoir [34]. To investigate a potential role of spleen in the regulation of circulating platelet numbers during hibernation, splenectomies were performed either before hibernation or during torpor in the Syrian hamster. Splenectomy before the hibernating season did not preclude the induction of thrombocytopenia during torpor (Figure 4D), suggesting the spleen is not needed to sequester platelets in this phase. To investigate the opposite, i.e. whether the spleen is involved in restoration of platelet counts during arousal, splenectomy was performed during torpor. In this case, splenectomy did not impede the restoration of platelet count during arousal compared to arousal with native spleen (300 x 10^9/L vs. 215 x 10^9/L, P < 0.01, respectively; Figure 4E). Thus, these experiments demonstrate that the spleen is neither essential for platelet storage during torpor, nor for restoration of platelet count upon arousal.

DISCUSSION

In the current study we demonstrate that thrombocytopenia as observed in deep and daily torpor is not confined to hibernating animals. Also non-hibernators decrease their platelet count during forced hypothermia. The thrombocytopenia in both hibernators and non-hibernators is reversible upon arousal and rewarming, respectively. Thus, this study suggests that body temperature is a main driving factor for thrombocytopenia during hibernation. Moreover, this study suggests that platelet intrinsic function is maintained throughout torpor/arousal in hibernators as well as throughout cooling/rewarming and pharmacological induced torpor, as demonstrated by P-selectin surface expression and platelet aggregometry. Importantly, however, in natural torpor, circulating platelets were found not to express P-selectin in contrast to force-cooled rat, mouse, and 5'-AMP injected mouse. Finally, aggregometry indicates that neither the velocity nor the maximum aggregation show any changes among groups of forced-cooled rats and pharmacological torpor in mice.

Further, the decrease in body temperature during the initiation and continuation of deep torpor and during forced hypothermia in the Syrian hamster correlates well with the reduction in platelet count in peripheral blood. Moreover, thrombocytopenia is present in both deep and daily torpor, which demonstrates that this phenomenon is not confined to deep torpor only. Furthermore, forced hypothermia induces thrombocytopenia both in hibernators and non-hibernators, i.e. hamster, rat and mouse. The decrease in body temperature correlates well with the decrease in platelet count in deep and daily torpor, and forced hypothermia in both hamster, rat and mouse. These correlations suggest a similar underlying mechanism of temperature dependent platelet dynamics in both hibernating and non-hibernating mammals. Likewise, in both deep and daily hibernating hamsters and in forced-cooled rats and mice, platelet count increased rapidly to euthermic level upon arousal and rewarming. The more rapid recovery of the platelet count in hamsters aroused from natural torpor as compared to forced-cooled hamster may be caused by a hysteresis effect of core body temperature increase. Whereas, during arousal the body temperature is increased from the inside out, the body temperature following forced-cooling increases from the outside in. Ultimately, this may result in a slower warming of platelet storage sites in forced-cooled animals. Interestingly, while *ex vivo* cooling initiates the rapid clearance of platelets by the liver upon reinfusion in mice and humans [35, 36], such effect may be absent following *in vivo* cooling, as in our study hamster, mouse and rat platelet numbers were restored upon rewarming. However, it is difficult to compare *in vivo* observations to *ex vivo* experiments, especially when accounting for the fact that the extend of cooling of mice and rats to 20°C and 15° respectively is markedly less

profound than 4°C *ex vivo* storage.

In contrast, the platelet count is unaffected during pharmacological induced torpor in mice by 5'-AMP despite the decrease in body temperature. Moreover, upon arousal the platelet count surpasses the initial euthermic level when body temperature returns to normal, suggesting a release of already stored platelets, compensating the platelet reduction during torpor induced by decreasing body temperature. Most likely, the different pattern in change of platelet count in 5'-AMP induced torpor compared to natural hibernation and forced cooling is attributable to the effect of the compound on platelet function (see also below) [37].

Contrarily to immature platelet levels in hibernating ground squirrel [22], there was a relative, but marginal, increase in immature platelet fraction (IPF) during torpor of Syrian hamster, but not in cooled rat or induced torpor in mouse. The small increase in IPF, however, cannot account for the massive increase of platelet count upon arousal. Increased IPF in torpid hamster may result from a decreased clearance of immature platelets during torpor compared to mature platelets.. Further, the difference in IPF between torpid squirrel and hamster may reflect a species difference. Alternatively, the method used to determine IPF in these studies differs, which may well result in the difference in IPF count between species. In addition, whether hamster platelets change their shape upon cooling, as described in ground squirrel (22), is not yet known. Possibly, the shape change influences the flow cytometric measurement of platelets . The latter seems less likely an explanation, as samples were stored at room temperature before processing, allowing for reversal of the potential shape change (22), thus granting normal platelet counts during flow cytometry. Further, in all animal species, the rapid restoration of platelet count in face of the marginal changes in the IPF support a storage-and-release mechanism over a clearance-and-reproduction mechanism to underlie thrombocytopenia of torpor and forced cooling. Finally, by splenectomizing animals we revealed that the spleen is not crucial to either induce or restore thrombocytopenia during natural hibernation. Taken together, while in 5'-AMP induced torpor thrombocytopenia is not present, inhibition of coagulation during natural torpor and forced cooling is instituted by a body temperature dependent reduction in the number of circulating platelets, rather than on the inhibition of their function.

Our data suggest that low body temperature induces clearance of free circulating cells, likely by storage-and-release. Release of newly formed platelets from the bone marrow is unlikely to play a significant role in the restoration of normal platelet counts upon rewarming, even though the steady state megakaryocytopoiesis supplies 10^{11} platelets daily and can increase 10-fold on demand [27]. Supporting this, the immature IPF was not significantly increased upon arousal in our study. Therefore, the rapid

and full restoration of platelet count during arousal is unlikely to result from release of newly formed platelets from the bone marrow. Likely, storage of platelets governs thrombocytopenia during torpor. A potential storage location might be the spleen [26], which has a relatively large capacity to sequester and destroy (abnormal) platelets as compared to other organs [33]. Moreover, the spleen can release platelets into the circulation after sequestration [34]. In hibernating ground squirrel, potential platelet storage sites include spleen, but also lungs and liver as all three appeared to sequester platelets [26]. However, by splenectomizing animals before torpor, we revealed that the spleen does not play an essential role in the induction of thrombocytopenia. Further, by splenectomizing animals during torpor we demonstrate that the spleen neither plays a key role in the restoration of normal platelet counts, as splenectomy did not prevent the restoration of normal platelet counts upon arousal. Although these results suggest no essential role for the spleen in the induction of thrombocytopenia or restoration of normal platelet counts, a potential role cannot be ruled out. In a study from the 1970's, Reddick et al. reported that thrombocytopenia was precluded by splenectomy prior to hibernation in the 13-lined ground squirrel [26]. While details of splenectomy are not included and splenectomy did not increase platelet count in non-hibernating squirrels, it is difficult to comment on possible causes for the apparent different observations. As this is possibly due to species differences, future studies taking a similar approach as ours with respect to timing of splenectomy are needed to confirm this assumption. Notably, splenectomy during torpor increased the amount of platelets upon arousal. Thus, our findings imply a role for spleen in the sequestration of platelets during arousal rather than in their release. The alternative would be that spleen is essential to sequester and release platelets in hibernation, but that the effects of splenectomy are masked by the effects of abdominal surgery. This would imply that surgery in summer induces sequestration of platelets in organs other than spleen months later, which appears less likely. Further, surgery during torpor may induce release of platelets from other sources than spleen (reactive thrombocytosis). However, as these platelets are mostly bone-marrow derived, this normally leads to a large increase in IPF, which is absent in our animals. Thus, the most likely explanation is that spleen does not play an essential role in platelet dynamics during torpor."

In euthermic conditions part of the platelet count is sequestered in the spleen [33, 38]. After splenectomy, this sequestering capacity will be decreased. Thus, the reversible hypothermia induced thrombocytopenia and low IPF during torpor and arousal advocate a storage-and-release mechanism of platelets. The spleen, as natural platelet sequestering organ, is not essential for potential platelet storage during torpor, nor for restoration of platelet count upon arousal, but might play a role in platelet sequestration during arousal.

By splenectomizing animals, we demonstrated that the spleen does not play a key role in the induction of thrombocytopenia during torpor. Platelets can reversibly adhere to arterioles, venules, and capillaries by means of margination. Therefore, a potential storage mechanism of platelets during torpor might be platelet margination. By computational and experimental methods several factors promoting platelet margination are revealed, including increased hematocrit [39], platelet shape (spherical particles marginate more quickly) [40], lower flow rate [41], and augmented expression of adhesion molecules [42]. Of these factors, hematocrit is increased [24], flow rate is decreased [15], and platelet shape changed to spherical at a body temperature < 25 °C during torpor in 13-lined ground squirrels [26]. Changes in platelet shape are mediated by intracellular cytoskeletal microtubule rearrangements and are reversible upon rewarming in 13-lined ground squirrel platelets [22], and partly reversible in mice and humans [35, 43, 44]. Therefore, reversible platelet shape change might contribute to the storage-and-release mechanism mediated by platelet margination.

Besides changes in hemodynamics and platelet shape, increased adhesion molecule expression might promote platelet margination during torpor as well. In this perspective, our observation that P-selectin expression is absent on circulating platelets during torpor in the Syrian hamster is intriguing. One of the attractive hypotheses is that hibernating animals indeed increase P-selectin expression on platelets upon entrance into torpor to induce margination. Consequently, only a small fraction of platelets not expressing P-selectin remains in the circulation. Nevertheless, the remainder of circulating platelets from torpid animals can still be activated, possibly to ensure appropriate coagulation during arousal should this be needed. However, future studies should address P-selectin expression and of other adhesion molecules, such as ICAM-1, alphavbeta3 integrin and GPIbalpha [45, 46], in hibernating animals in more detail.

One of the factors that might add to increased margination of platelets in torpor or cooling is hypoxia. Hypoxia during torpor might lead to exocytosis of endothelial cell Weibel-Palade bodies and subsequent release of von Willebrand factor and P-selectin expression [47], both stimulating platelet binding to the endothelial cell. Also, during torpor, the endothelial adhesion molecules VCAM-1 and ICAM-1 are modestly upregulated in the lungs, followed by normalization during arousal [14]. Further, *in vitro* experiments reveal that plasma from hibernating, but not summer euthermic ground squirrels, stimulates the expression of ICAM-1, VCAM and E-selectin on rat endothelial cells [48]. Together, endothelial activation leading to the expression of adhesion molecules may stimulate platelet margination in torpid animals.

Despite the procoagulant state of torpor due to low blood flow [15], increased blood

viscosity [16, 17], immobility, chronic hypoxia, and low body temperature [5], no organ injury has been demonstrated after arousal [5], advocating absence of thromboembolism during hibernation. We speculate that margination of platelets might prevent thromboembolism formation during torpor. Taken together, margination-promoting factors during torpor might well underlie the clearance of free circulating functional platelets shown in this study upon lowering of body temperature.

While a decrease in platelet count was observed in hibernators and forced-cooled animals, pharmacological induction of torpor by 5'-AMP did not induce thrombocytopenia, despite clear reductions in body temperature and leukocyte count. The most likely explanation for this discrepancy is that 5'-AMP interferes with the temperature dependent regulation of platelet counts. Interestingly, very recently it has been shown that 5'-AMP inhibits platelet function, including the inhibition of P-selectin expression upon platelet activation [37]. In the same study the adenosine A_{2A} receptor is activated by 5'-AMP and inhibits platelet function. A_{2A} receptor is not the main target of 5'-AMP for the induction of torpor. Induced torpor acts via A1 receptors in Syrian hamster, ground squirrel and rat [49-51]. 5'-AMP has been shown to be a true A1 receptor agonist [52]. These studies, however, did not measure platelet count. Likely, stimulation of A1 receptor alone is not sufficient to induce thrombocytopenia in mice. Our finding that P-selectin is absent in circulating platelets of torpid animals and that 5'-AMP inhibits platelet function may thus implicate that platelet functionality, particularly the surface expression of adhesion molecules, is essential for the temperature dependent decrease in platelet count in torpor and cooling.

While our data demonstrate platelet aggregation not to be affected largely by torpor or cooling, some technical limitations may apply. For flow cytometry analyses 10uM ADP was used as platelet agonist, effective for hamster and rat platelets, but elicited only minimal activation in mouse platelets. Studies are ambiguous if ADP sensitivity is sex dependent, potentially C57BL/6J male mice are less sensitive to platelet agonists than female littermates [53]. Due to limitations in sample volume of rodent blood, platelet aggregation was determined with a microtiterplate assay (MTP) rather than the classical light transmittance aggregometry (LTA). While optimal platelet concentrations of 600 x 10^9 /L for MTP have been reported [31], platelet yields did not allow for an equal platelet concentration among each experiment. To compensate for these differences in platelet concentrations, aggregation of mouse and rat platelets was compared to an internal standard which was matched in platelet concentrations, which allowed representation of the data as a percentage of the internal standard. Moreover, the MTP method has been reported to have a lower sensitivity than LTA

when low concentrations of agonist are used [54]. However, this difference in sensitivity is deemed absent at higher agonist concentrations, motivating the use of 20 µM ADP to induce a full irreversible aggregation. Finally, since aggregation of rodent platelets is measured in a buffer instead of in plasma, the effect of any plasma factor that influences platelet aggregation may be lost, e.g. a decrease in coagulation factors as seen in the 13-lined ground squirrel [22].

Up to now, human platelets intended for transfusion are stored up to 5 days on room temperature, risking bacterial contamination [54], because cold storage on the other hand leads to aggregation upon rewarming and other detrimental effects that change platelet function [55, 56]. Finding ways for cold storage of platelets, a.o. to prevent bacterial growth, while preserving platelet function, would reduce transfusion associated infections and lead to increased use before expiration date. This study introduces the possibility of a shared mechanism between non-hibernating and hibernating mammals for reversible hypothermia induced hypocoagulability, via platelet storage in the cold with preserved platelet function.

Conclusion

During torpor, free circulating platelets are cleared from the blood. The resulting thrombocytopenia is reversible and due to a lowering in body temperature. The hypothermia induced thrombocytopenia is not confined to deep torpor or hibernating animals, as it was also observed in daily torpor and upon forced hypothermia in non-hibernators. Decreased platelet count does not coincide with decreased platelet function, and recovers rapidly upon arousal and rewarming due to release of retained platelets. Platelet storage and release in hibernators are not mediated by the spleen. Understanding the underlying mechanisms that govern the reversible hypothermia induced thrombocytopenia, with preservation of platelet function, might yield improved uses for therapeutic hypothermia, as well as potential cold storage of human platelets, extending their shelf life.

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SUPPLEMENTAL DATA

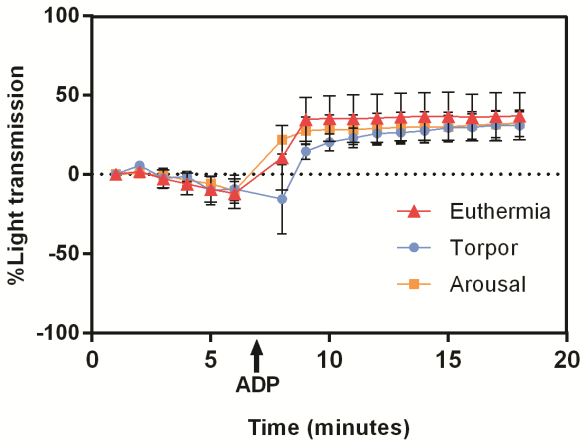


FIGURE S1. Platelet aggregation of mouse platelet suspensions does not differ between euthermia and pharmacologically induced torpor and arousal.

Velocity is the slope of % light transmission per minute in the first 5 minutes after addition of agonist. Max amplitude is the mean light transmission of the last three measurements when a stable plateau is observed. No differences between groups. Data is shown as mean (n=6 euthermia, n=5 torpor, n=7 arousal) ± SEM.

TABLE S1. Maintenance of velocity and maximum amplitude of platelet aggregation in pharmacologically induced torpor in mice

Mouse	Velocity (%Light transmission min^{-1})	Max amplitude
Euthermia	11.7 ± 4.86	32.6 ± 15.7
Torpor	9.56 ± 4.40	30.4 ± 8.9
Arousal	12.2 ± 3.49	31.6 ± 8.4

Velocity and maximum amplitude of aggregation of mouse platelets in response to 20 µM of ADP is similar in all euthermic, torpid and aroused mice. Values are mean ± SEM.

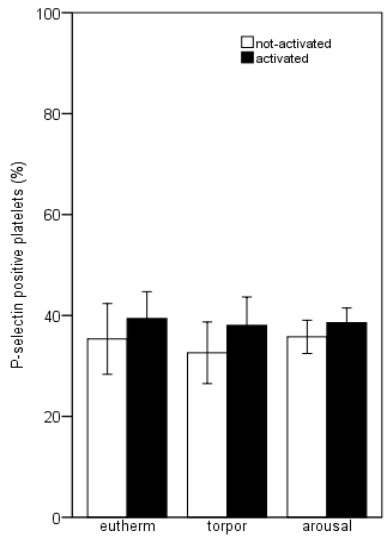


FIGURE S2. Normal platelet activation during pharmacologically induced torpor and arousal in mice.

No difference in amount of ADP activatable platelets from euthermic, torpid or aroused mice. Bars represent the mean (n=6 euthermia, n=5 torpor, n=7 arousal) ±SEM.

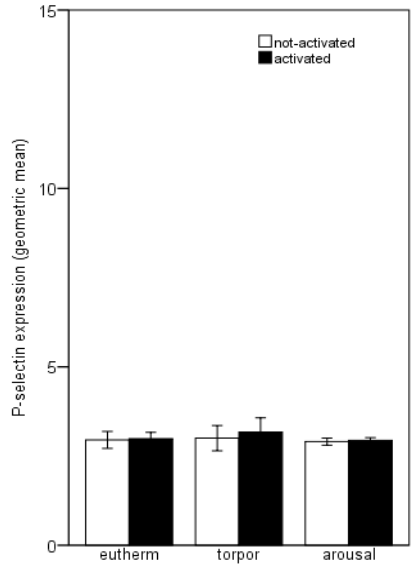
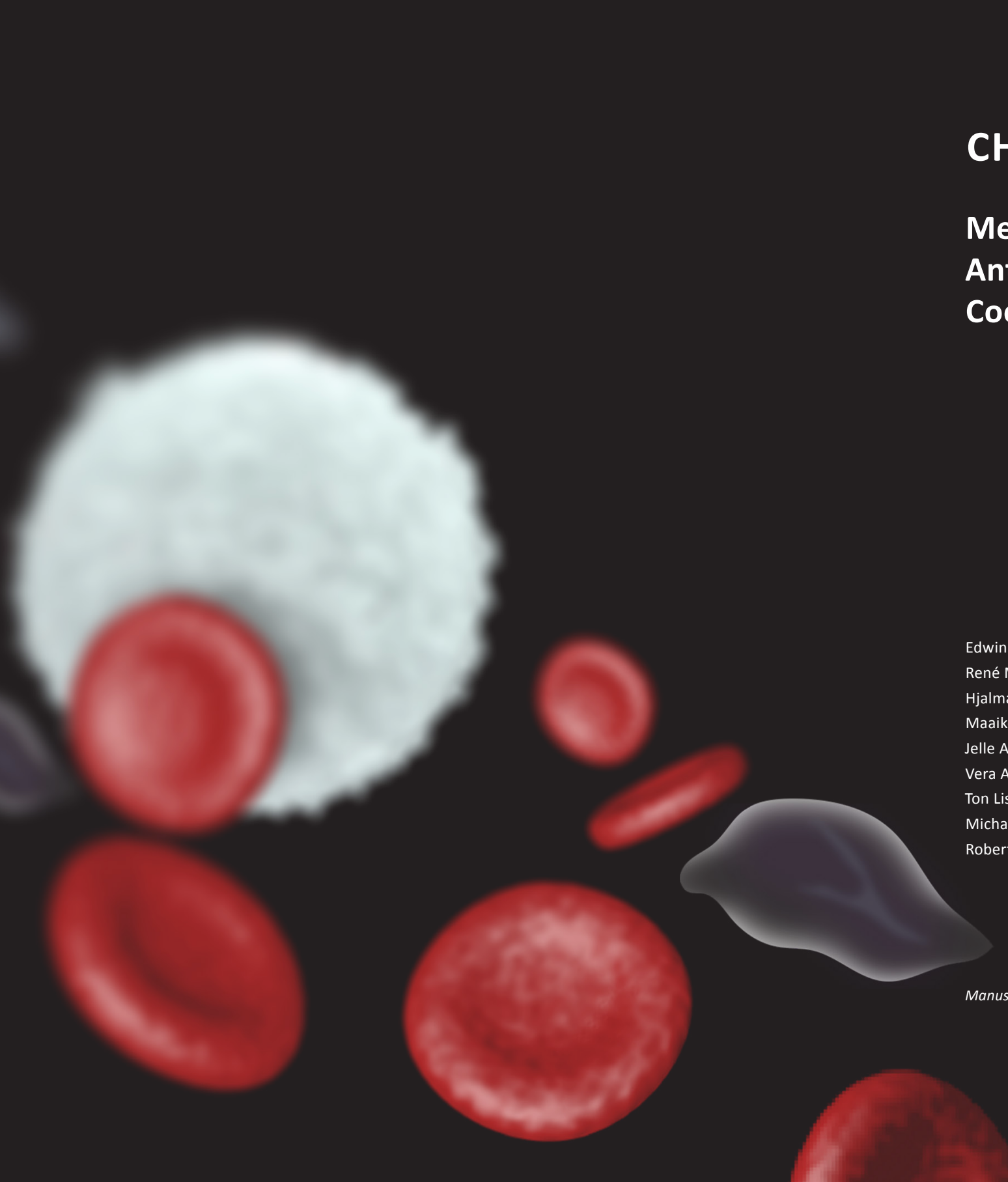


FIGURE S3. Similar P-selectin expression on platelets during euthermia and pharmacologically induced torpor and arousal in mice.

Unchanged P-selectin expression at all time points in both non-activated and ADP activated whole blood samples. Bars represent the mean (n=6 euthermia, n=5 torpor, n=7 arousal) ±SEM.



CHAPTER 3

Mechanisms and Dynamics of Anticoagulation in Hibernation - a Cool Way to Suppress Hemostasis

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ABSTRACT

Background

Venous thromboembolism (VTE) impairs quality of life, causes early mortality and occurs even in subjects receiving thromboprophylaxis. Immobilization is one of the most important risk factors and induces thrombus formation via stasis of blood. Hibernating animals are immobile for prolonged periods with greatly reduced blood flow and increased blood viscosity without the occurrence of VTE. The mechanisms by which hibernators alleviate immobility-related thrombosis are incompletely understood.

Objectives

To explore the anticoagulant strategies used by hibernating hamsters.

Methods

Elements of primary hemostasis, secondary hemostasis and fibrinolysis were characterized in hibernating hamsters, with non-hibernating hamsters serving as controls.

Results

During the immobile torpor phase, platelet count and VWF level reduced by 90%. PT and APTT increased 2- and 10-fold during torpor, while thrombin generation was reduced. Extrinsic pathway factor VII did not change, whereas common pathway factors decreased by 80% and 53% (factor V and fibrinogen) as well as intrinsic pathway factors VIII, IX and XI with 89%, 66% and 67% respectively. Further, levels of antithrombin, protein C, plasminogen and plasmin inhibitor demonstrated that anticoagulant and fibrinolysis pathways were sustained throughout hibernation. D-dimer level was low in all hibernating and non-hibernating hamsters, minimizing likelihood of concurrent VTE.

Conclusions

Hibernation features extensive adaptations in primary and secondary hemostasis resulting in a rapidly reversible anticoagulant state during torpor. The anticoagulant profile likely reduces risk of venous thromboembolism during periods of increased immobility, and seems preserved among hibernating species, inviting translational research to non-hibernating mammals.

INTRODUCTION

Venous thromboembolism (VTE) affects annually approximately 1 per 1000 adults in the United States, causing impairment of quality of life and early mortality resulting in nearly 300,000 deaths [1-3]. Nearly 60% of idiopathic VTE - i.e. not due to e.g. genetic predisposition, cancer or trauma - is attributed to immobility or nursing home residency [1, 4]. Immobility may occur, amongst others, during hospitalization, spinal cord injuries, after orthopedic surgery and prolonged travel, the latter increasing venous thrombosis risk already 2- to 4-fold [5, 6]. Despite thromboprophylactic treatment, VTE still often occurs in patients at risk, as exemplified by an incidence of 37% in patients with lower leg immobilization using prophylactic dosages of low molecular weight heparin [7]. Despite ongoing research and improved understanding of Virchow's triad (i.e. the mechanisms involved in stasis of blood flow, endothelial activation and hypercoagulability), failure of the hemostatic system during immobility is still incompletely understood [8].

New insights and potential solutions may be derived from an ancient source, wherein nature has solved the problem of unwanted thrombus formation during immobility: hibernation [9]. Hibernation is an energy conserving behaviour adopted by many mammalian and other species and is characterized by repetitive phases of torpor and arousal [10]. During torpor, hibernating animals have a substantial decrease in metabolism, body temperature, heart rate, blood flow and respiration. Torpor bouts last from several days up to weeks and are alternated with many short phases of arousal lasting less than 24 hours wherein metabolism, temperature, heart and respiration rate rapidly recover to euthermic levels within two hours [11-13]. Hibernation is a potential procoagulant state due to the presence of several risk factors of VTE, namely obesity, immobility, reduced blood flow and increased blood viscosity. Prior to hibernation, many species fatten up to a level of gross overweight or even obesity [14]. During hibernation, some species are immobile for months throughout torpor and arousal cycles, whereas others are mainly immobile and only show some motor activity during each brief arousal period [15]. Furthermore, torpor is characterized by a decreased blood flow [16] and increased blood viscosity [17, 18]. Despite these risk factors, organ injury as a result of thrombosis has not been observed during torpor or after arousal in springtime [15]. Therefore, hibernators seem to have found a solution for immobility-related activation of hemostasis.

The different hibernating species show alterations in components of primary and secondary hemostasis, which are reversed during arousal. Specifically, platelet count and von Willebrand factor level are reduced in torpor [11, 12, 19, 20]. Additionally, several procoagulant factors are reduced in torpor [13, 21, 22] and clotting times are prolonged [13, 21-

[23]. Elucidating the reversible anticoagulant mechanism(s) in hibernators may yield new anticoagulant tools for VTE risk reduction in humans. Because there is a scarceness in data arising from a single hibernating species, we aimed to obtain a complete overview of the anticoagulant strategies adopted by hibernating golden hamster during its prolonged periods of immobility. To this end, we assessed components from primary and secondary hemostasis as well as from the fibrinolysis pathway both during torpor and arousal stages and compared them to non-hibernating, summer euthermic animals. We hypothesized that hamsters show a reversible anticoagulation profile during torpor by reducing procoagulant components of both primary and secondary hemostasis and by maintaining anticoagulant and fibrinolytic properties.

MATERIAL AND METHODS

Animals

Golden (Syrian) hamsters (*Mesocricetus auratus, age 3 months*) were obtained from Envigo USA and kept at 'summer' photoperiod light:dark cycle (L:D) of 14h:10h at 20-22°C with free access to standard laboratory chow and water until induction of hibernation. Animal work was approved by the Institutional Animal Ethical Committee of the University Medical Center Groningen.

Hibernation in hamsters

After 7 weeks at 'summer' photoperiod, hamsters were housed at 'autumn' photoperiod: L:D of 8h:16h for 7 weeks, followed by reduction of ambient temperature to 5°C and housing under constant darkness ('winter' period) [11]. Passive infrared sensors coupled to a computer system monitored individual movements. Hamsters were euthanized at different stages of euthermia or hibernation: summer euthermia (SE), winter euthermia (WE), early torpor (TE), late torpor (TL), early arousal (AE) and late arousal (AL). Summer and winter euthermia were defined as a euthermic body temperature (approximately 37˚C) during 'summer' and 'winter' photoperiods in absence of any torpor bouts. Early and late torpor were defined as 12-48 and >48 hours of immobility respectively and confirmed in all animals by oral temperatures below 10°C. Early and late arousal were defined as 1.5 hours and >8 hours after induced or natural arousal, and a body temperature of ≥35°C.

Blood samples

Blood was obtained under isoflurane 2% in O_2 anaesthesia by cardiac punction into one-tenth volume of 3.2% sodium citrate or in lithium heparin coated tubes. Plasma was prepared by whole blood centrifugation at 3,000 g x 15 minutes at 22°C and subsequently aliquoted and stored at -80°C. All assays were performed according to manufactures instructions and calibrated with normal human plasma.

Thrombin generation

The thrombin generation test was performed using plasma with the fluorimetric method described by Hemker et al. [24]. Calibrated Automated Thrombography® (CAT) Coagulation was activated using commercially available reagents containing recombinant tissue factor (TF, final concentration 5 pM) and phospholipids (final concentration 4 µM). Thrombin Calibrator was added to calibrate the thrombin generation curves. A fluorogenic substrate with $CaCl_2$ (FluCa-kit) was dispensed in each well to allow a continuous registration of thrombin generation. Fluorescence was

read in time by a fluorometer, Fluoroskan Ascent® (ThermoFisher Scientific, Helsinki, Finland). All materials and procedures were according to the protocol suggested by the manufacturer (Thrombinoscope, Maastricht, The Netherlands).

Clotting and activity assays

A Sysmex S-2100i analyzer was used to perform coagulation tests as well as chromogenic and immunologic assays. Protein C activity levels were measured with Berichrom protein C test from Siemens (Marburg, Germany). Factors II, V, VII, VIII, IX, X, and XI were measured by a one-stage clotting assay with reagents from Siemens. The PT was measured with Innovin Reagent and the APTT with Actin FS and fibrinogen with Thrombin Reagent, antithrombin activity with INNOVANCE Antithrombin, all from Siemens (Marburg, Germany). For Plasmin Inhibitor and Plasminogen, the Berichrom α2-Antiplasmin Kit and Plasminogen Kit were used (Siemens). D-dimer was measured with a Modular analyzer (Roche Diagnostics) with reagents from Roche.

Enzyme-linked immunosorbent assays (ELISA's)

VWF antigen levels were measured with an ELISA with reagents obtained from DAKO. VWF:CBA was measured using Technozym VWF:CBA ELISA (Diagnostica Stago, Paris, France).

Statistics

Data are presented as mean with standard deviation. Statistical analysis was performed by one-way ANOVA and post-hoc Tukey analysis or Kruskal Wallis test with post hoc Dunn analysis for non-parametric data, and by linear regression and Pearson's correlation (Graphpad Prism v7.01, GraphPad Software, USA) with $P<0.05$ considered significantly different. The same software was used to produce the graphs.

RESULTS

No signs of venous thromboembolism during hibernation, despite low body temperature, prolonged immobility and increased blood viscosity

After entering stable torpor-arousal cycles, hibernating hamsters were immobile for 76.3 ± 15.2 hours during the low metabolic torpor phase and active for 22.4 ± 12.9 hours in arousal phase ($P<0.05$, n=32). Body temperature decreased from 36.3 ± 0.9°C before hibernation to 8.8 ± 0.7°C during torpor, which reversed in arousal (Figure 1A). Blood increased in viscosity during torpor, as demonstrated by increased haematocrit, which reversed during arousal to pre-hibernation level (Figure 1B). Thrombin-anti-thrombin complex and tPA:PAI1 complexes could not be determined with commercially available tests, likely because of lack of crossreactivity of the antibodies with hamster protein. Therefore, plasma D-dimer level was determined as a measure of activation of the coagulation and fibrinolysis system and was low in summer and winter euthermic hamsters and remained low throughout torpor and arousal (Figure 1C). Thus, despite prolonged immobility, stasis and increased viscosity of blood as risk factors for venous thromboembolism (VTE), the lack of D-dimers is consistent with a lack of VTE, although it does not fully prove that VTE is not occurring.

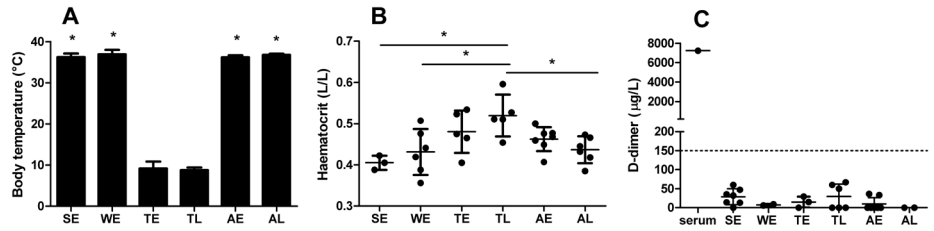

FIGURE 1. No plasmatic signs of thromboembolism during hibernation despite low body temperature, prolonged immobility and increased blood viscosity. A) Hamster body temperature was measured orally at euthanization in euthermic hamsters (37°C) in summer (SE) and winter (WE) conditions, and during hibernation in hamsters in early and late torpor (9°C, TE and TL) and in early and late arousal (37°C, AE and AL). B) Blood viscosity increases during torpor as measured by haematocrit and reverts to euthermic level upon arousal. C) Low D-dimer levels in summer and hibernating animals. Dotted line represents the human threshold demonstrating plasmatic signs of thrombosis; hamster serum was used as positive control. Data are mean ± SD, sample sizes between n=2 and n=7, * $P<0.05$.

Primary hemostasis components are reduced during torpor

To demonstrate whether VTE risk is reduced in hibernating hamsters through changes in components of primary hemostasis, we measured platelet count and von Willebrand factor (VWF) antigen and activity. Platelet count from summer euthermic hamsters reduced by 90% during torpor (Figure 2A), in line with our previous findings in hibernating hamsters [11]. This thrombocytopenia recovered swiftly within the 1.5 hours of early arousal. Compared to summer euthermia, plasma VWF antigen level reduced fourfold in winter euthermic hamsters, although not significantly, and decreased more than 13 fold in late torpor (Figure 2B). Although measured in a low range of the assay, VWF collagen binding showed an overall 3-fold decrease in hibernating animals compared to non-hibernating (Figure S1). Moreover, during hibernation, VWF collagen binding showed a gradual decrease from TE to AL. Thus, during torpor there are less circulating platelets available and less plasma VWF to contribute to thrombus formation.

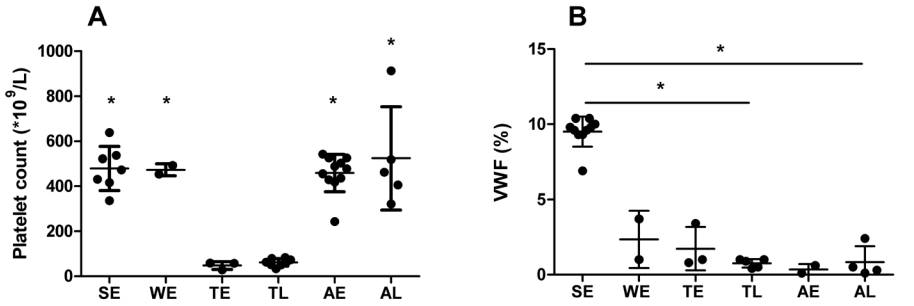

FIGURE 2. Elements of primary hemostasis are reduced during hibernation. Circulating platelet count and plasma von Willebrand factor (VWF) from non-hibernating and hibernating hamsters. A) Platelet count reduces with 90% during torpor (TE, TL) compared to summer and winter euthermia level (SE, WE) and recovers to euthermic level during arousal (AE, AL). B) VWF plasma level relative to human pooled normal plasma reduces during winter in hibernating and non-hibernating hamsters. Sample sizes between n=2 and n=11, * P<0.05.

Measures of secondary hemostasis show reduced thrombin generation and prolonged PT and APTT in torpor.

To assess whether the potential of the secondary haemostatic system is reduced during torpor, we measured thrombin generation. During torpor, the maximum thrombin production was reduced more than 12-fold compared to summer euthermia

(Figure 3A). Thrombin generation recovered during arousal, although not significantly. Next, to elucidate which coagulation factors are altered during hibernation, we first measured prothrombin time (PT, Figure 3B) and activated partial thromboplastin time (APTT, Figure 3C). During late torpor, clotting times were prolonged almost 2- and 10-fold, for PT and APTT respectively, increasing from ~ 10 and 30 seconds in summer to 19 and 102 seconds in late torpor (Figure 3B-C). During arousal, PT and APTT recovered to euthermic levels.

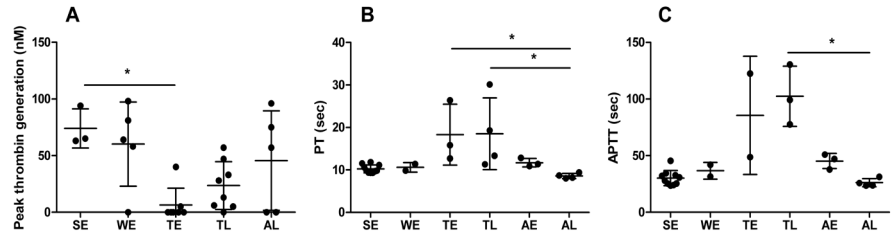

FIGURE 3. Thrombin generation and coagulation times throughout hamster hibernation. Thrombin generation and clotting times (PT and APTT) were measured in plasma from hamsters in summer (SE) and winter (hibernating (TE, TL, AE, AL) and non-hibernating (WE)). A) Less thrombin generation and (B-C) prolonged clotting times (PT and APTT) during torpor phase of hibernation, which normalize with progression of arousal. Sample sizes between n=2 and n=10, * P<0.05.

Changes within common coagulation pathway proteins during phases of hibernation

To determine whether prolonged clotting times were the result of reduction in coagulation factors of the common pathway rather than within specific determinants of PT or APTT pathway, fibrinogen and factor II activity was measured (Figure 4). During hibernation, fibrinogen levels were reduced in late torpor and early arousal and recovered to summer euthermic level during late arousal (Figure 4A). Factor II activity was similar in hibernating and non-hibernating hamsters, but increased almost two fold during late arousal (Figure 4B). During torpor, factor V reduced by 80%, which recovered to summer level during late arousal (Figure 4C). Contrarily to the reductions in fibrinogen and factor V, factor X showed similar levels between non-hibernating hamsters in summer and those in hibernation, whereas non-hibernating animals in winter increased in factor X activity (Figure 4D). Thus, during torpor the common coagulation pathway determinants were either similar to euthermic level or reduced.

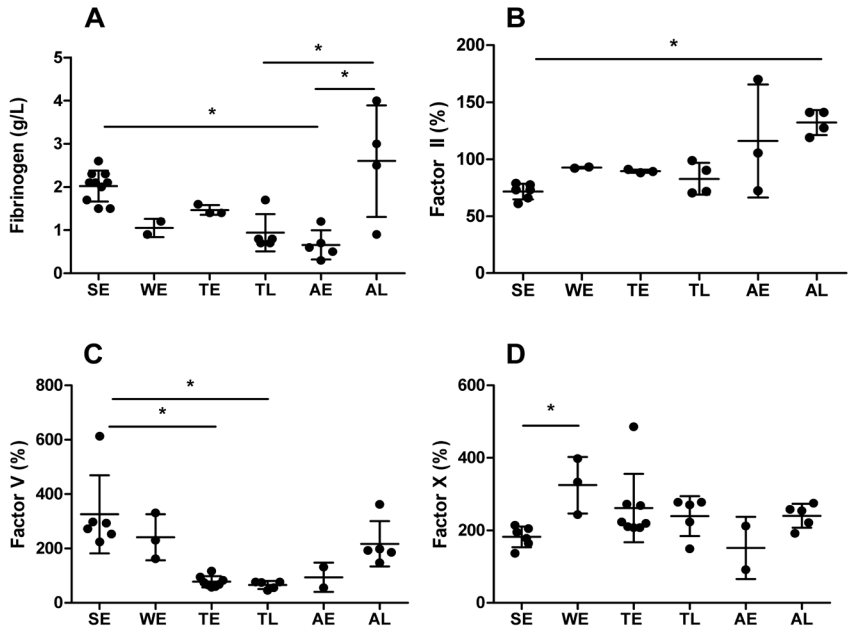

FIGURE 4. Elements involved in the common pathway throughout hibernation. Fibrinogen level and factor II, V and X activity relative to human pooled normal plasma were measured in plasma from hamsters in summer (SE) and winter (hibernating (TE, TL, AE, AL) and non-hibernating (WE)). A) During hibernation, fibrinogen is reduced in late torpor and early arousal and recovers to summer level in late arousal. B) Factor II does not alter during torpor but increases during late arousal compared to summer level. C) Factor V is reduced in torpor and recovers to summer level in arousal. D) Factor X activity is similar in hibernating and non-hibernating summer animals, whereas higher in non-hibernating hamsters in winter environment. Sample sizes between n=2 and n=10, * P<0.05.

Procoagulant factors reduce and anticoagulant factors remain stable during torpor

To determine whether the prolonged clotting times during torpor were the result of alterations in PT or APTT pathway, specific determinants of PT and APTT were measured, namely factor VII for PT and VIII, IX, XI for APTT (Figure 5A-D). Factor VII activity was high compared to human standard (100%) and not altered during hibernation. Coagulation factors VIII, IX and XI activity decreased substantially during torpor (by 89%, 66% and 67% respectively), although only the decrease in VIII and XI reached significance. Factor IX and XI activity level recovered quickly during early arousal, whereas VIII had recovered by late arousal (Figure 5B-D). Thus, PT elongation

during torpor does not coincide with a decrease in factor VII activity, whereas APTT elongation coincides with a decrease in factor VIII, IX and XI activity.

In order to demonstrate a potential role of anticoagulants in reducing the risk of VTE throughout torpor, we measured antithrombin and protein C activity (Figure 6A-B), our assays however failed to detect protein S. Although antithrombin seemed different between groups, the data remains inconclusive due to large variances and low sample size (Figure 6A). Protein C activity did not change throughout torpor and early arousal, but demonstrated an increase during late arousal (Figure 6B). So far, most procoagulant factors decreased more than half during torpor, whereas anticoagulant factors demonstrated stable plasma levels during torpor and only protein C increased in late arousal.

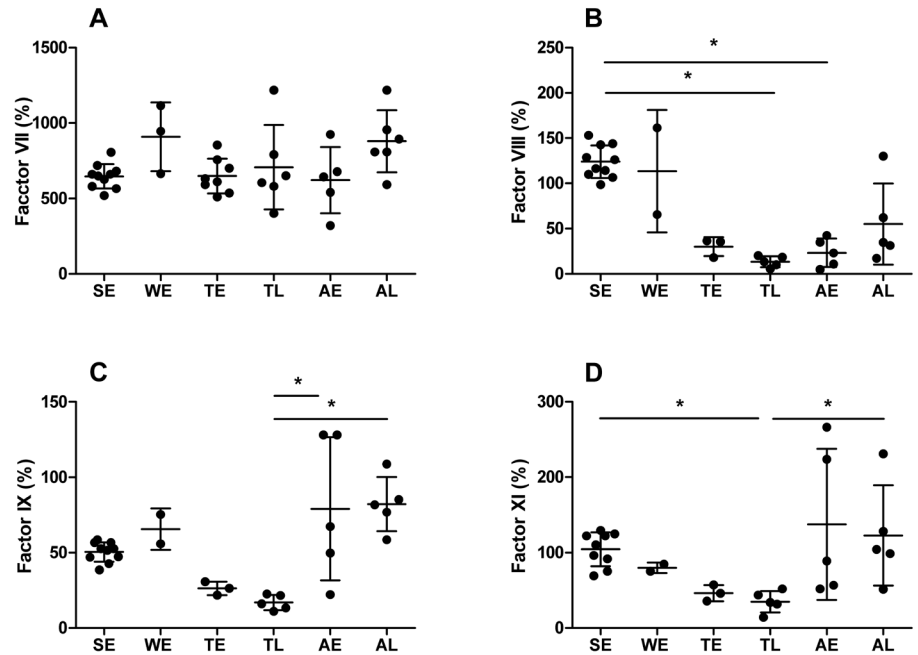

FIGURE 5. Procoagulant factors VIII, IX and XI decrease during torpor and normalize during arousal. Factor VII, VIII, IX and XI activity relative to human pooled normal plasma was measured in plasma from hamsters in summer (SE) and winter (hibernating (TE, TL, AE, AL) and non-hibernating (WE)). A) Factor VII does not alter throughout phases of hibernation. B-D) Factor VIII, IX and XI demonstrated a pattern of reduction during torpor and recovery during arousal. Sample sizes between n=2 and n=10, * P<0.05.

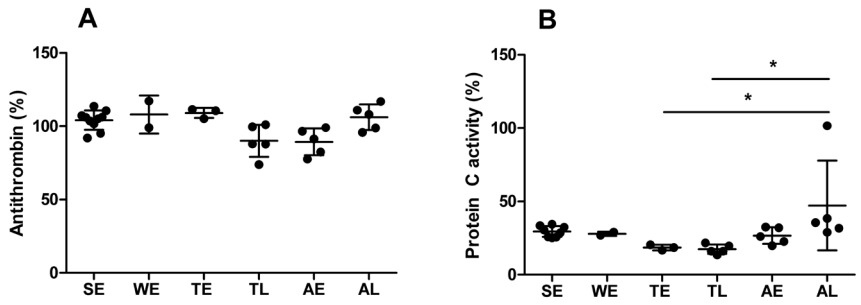

FIGURE 6. Anticoagulant factors throughout hibernation. Antithrombin and Protein C activity were measured relative to human pooled normal plasma in plasma from hamsters in summer (SE) and winter (hibernating (TE, TL, AE, AL) and non-hibernating (WE)). A) Antithrombin activity changed throughout samples (P<0.05), but could not be specified with Dunn's multiple comparisons. Protein C activity only increased somewhat in late arousal. Sample sizes between n=2 and n=10, * P<0.05.

Differential effects of hibernation on fibrinolytic factors

To determine if increased fibrinolysis may contribute to an anticoagulant state during hibernation, we measured activity of plasminogen and plasmin inhibitor (Figure 7A-B). Rather than a reduction, as observed in levels of procoagulant factors, plasminogen level increased about 2.5-fold during late arousal compared to summer and was similar to levels in torpor (Figure 7A). Plasmin inhibitor level remained the same throughout all time points (Figure 7B). Thus, factors involved in fibrinolysis were stable or increased during hibernation.

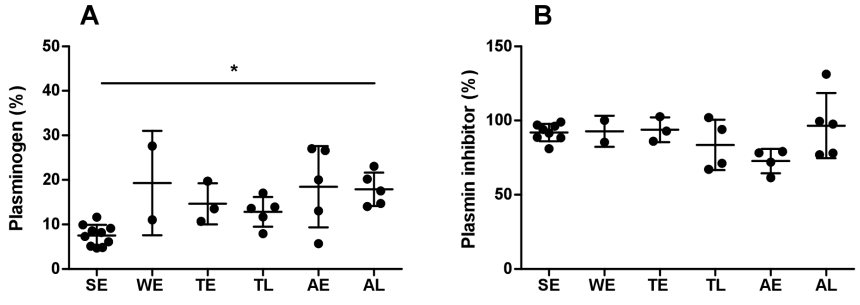

FIGURE 7. Fibrinolysis pathway proteins are stable or increased during hibernation. Plasminogen and plasmin inhibitor were measured relative to human pooled normal plasma in plasma from hamsters in summer (SE) and winter (hibernating (TE, TL, AE, AL) and non-hibernating (WE)). Plasminogen was increased during late arousal compared to summer level and similar to torpid level. Plasminogen remained stable in summer and winter, regardless of hibernation. Sample sizes between n=2 and n=10, * P<0.05.

Changes of coagulation factors in torpor favour risk reduction of venous thromboembolism

Finally, to grant an overview of all changes per coagulation factor favouring or disfavouring an anticoagulant state during torpor, we summarized our findings (Figure 8). The effect of torpor on the level of each coagulation factor is compared to each summer reference value and demonstrates the overall effect towards an anticoagulated state during this cold phase of hibernation.

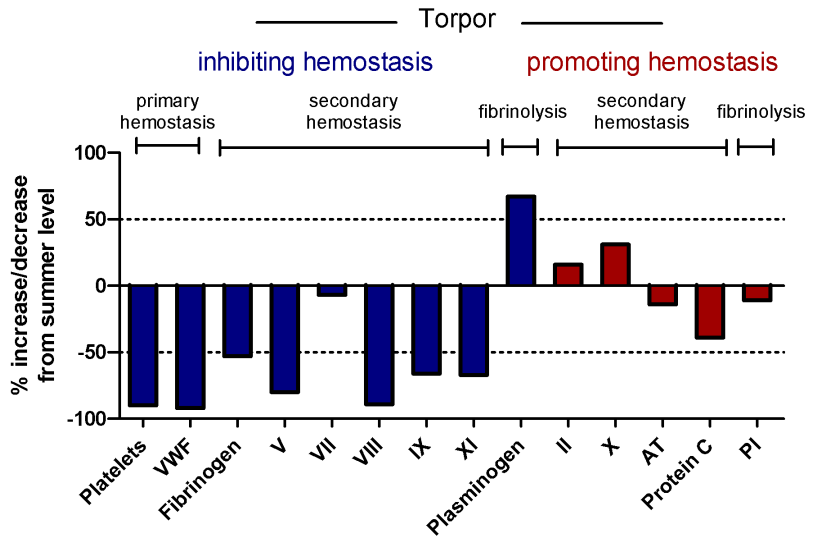

FIGURE 8. Overview of changes during torpor in elements of primary and secondary hemostasis and fibrinolysis reducing the risk of venous thromboembolism during hibernation. Parameters involved in hemostasis were measured in whole blood or plasma obtained from golden hamsters during the late torpor phase of hibernation. Each factor is shown as % increase or decrease from its average reference value in summer (non-hibernating hamsters). Dotted lines demonstrate a ≥ 50% change from summer level, implicating a potential inhibiting (blue) or promoting effect (red) on hemostasis and risk of venous thromboembolism. Above the graph, each parameter is grouped according to its role in either primary hemostasis, secondary hemostasis or fibrinolysis. Torpor sample sizes between n=5 and n=8; summer euthermia between n=5 and n=10. VWF: Von Willebrand Factor, AT: antithrombin, PI: plasmin inhibitor.

DISCUSSION

During hibernation, torpor is associated with a reduction in several determinants of primary and secondary hemostasis, which likely reduces the risk of venous thromboembolism. During the immobile torpor phase, there is a 90% decrease in platelet count and von Willebrand factor (VWF) level, and VWF activity reduces about 3-fold. During arousal, platelet count recovers swiftly to pre-hibernating levels, whereas VWF level and activity remain low throughout hibernating season. Determinants of secondary hemostasis are also reduced during torpor. Procoagulant factors V, VIII, IX, XI and fibrinogen are reduced during torpor, as well as the anticoagulant factor antithrombin. The reduction in procoagulant factors coincides with the 2-fold and 10-fold prolongation in PT and APTT, hinting at a reduced secondary haemostatic capacity. In agreement, thrombin generation is reduced during torpor and recovers to summer levels during arousal. Thus, hibernation features an anticoagulant state during torpor, which is rapidly reversed upon arousal. Likely, anticoagulation during torpor serves to compensate for the procoagulant factors of immobility, stasis and increased blood viscosity of the torpor phase.

Factors involved in suppressing primary and secondary hemostasis and enhancing fibrinolysis during hibernation

Determinants of primary hemostasis are suppressed in torpor since both platelet count and VWF level and activity reduced. During arousal, platelet count recovered swiftly whereas VWF level and activity remained low. Common pathway determinants of secondary hemostasis are partially suppressed, because coagulation factor V and fibrinogen reduced with 80% and 53% during torpor and recovered slowly during late arousal. Contrarily, factors II and X were similar in hibernating and non-hibernating hamsters. Within the extrinsic pathway, factor VII was unaltered during hibernation. The intrinsic pathway is strongly suppressed during torpor by reducing factors VIII, IX and XI with 89%, 66% and 67% respectively, which essentially mimics haemophilia type A, B and C altogether. Contrarily, anticoagulant factors remained generally similar to summer levels, with the exception of antithrombin decreasing significantly by 14%, unlikely to cause a prothrombotic effect. The 2-fold prolonged PT during torpor is caused by suppression of the common pathway through factor V and fibrinogen rather than by suppression of factor VII in extrinsic pathway. Additionally, suppression of both common and intrinsic pathway during torpor causes a 10-fold prolongation of APTT, specifically by decreasing factor VIII, IX and XI. When assessing the influence of all factors combined, we demonstrate that the hamsters' capacity for thrombin generation is reduced in torpor and recovers to summer level in arousal. Additionally,

throughout hibernation the fibrinolysis pathway seems sustained, since plasminogen increased 0.5 to 1.5-fold and plasmin inhibitor remained unchanged from summer level. Finally, the lack of D-dimers in torpor and arousal is consistent with a lack of VTE, although it does not fully prove that VTE is not occurring. Taken together, we demonstrate that hamsters induce a reversible antithrombotic state during the torpor phase of hibernation, by suppressing components of both primary and secondary hemostasis and by maintaining anticoagulant and fibrinolytic properties.

The antithrombotic profile during hibernation is consistent among species

Primary hemostasis seems suppressed in all hibernating species investigated so far. Specifically, platelet count reduction during torpor is a common trait, including in ground squirrels, hamsters and bears [11-13, 19, 21, 25, 26]. Also platelet sensitivity to activators is reduced in torpor in hamster and brown bear [11, 27]. Additionally, torpor reduces VWF level also in squirrels and bears [12, 26, 28], and diminishes squirrel VWF activity and mRNA expression as well as whole blood thromboelastography, which normalizes during arousal [12, 22]. Thus, primary hemostasis seems suppressed by hibernators via two mechanisms: 1) reducing platelet count and activatibility, and 2) reducing VWF level and activity, which reverse rapidly during arousal as demonstrated in different hibernating species. Further, secondary hemostasis seems suppressed in several other hibernating species. Although previous studies did not find PT prolongation during torpor in squirrels, bears or hedgehogs [13, 21, 26, 29], factor V is reduced by 45% in torpid Franklin's ground squirrel [13] and by 43.6% in hibernating bears [26]. Contrarily, factors X, V and fibrinogen were unaltered in 13-lined ground squirrel and hedgehog in torpor [21, 29], whereas fibrinogen was either unaltered or decreased in hibernating bears [26, 28]. Therefore, suppressing the common pathway of coagulation during torpor seems to occur only in a few hibernating species, of which our study in torpid hamsters detected a minor PT elongation, likely due to the large reduction in factor V and fibrinogen. Factor VII has not been studied often but showed stable levels in hibernating ground squirrels [21], in line with our findings, and decreased level in hibernating brown bear [26]. Remarkably, factor VII levels of summer hamsters were 7-fold higher than the reference level in humans, which may emphasize the importance of this factor in the hamster coagulation system. Of all factors analysed, only baseline (summer euthermic) levels of VWF and protein C were less than 50% from human baseline level, which may be due to issues with quantification of these analytes in hamster plasma or actual lower plasma level, the latter being in line with lower VWF level in non-hibernating ground squirrel (approximately 25%) compared to humans [22]. A principal finding of the current study is the greatly prolonged APTT, which was rapidly reversed early in arousal. The rapid reversal is likely due to a similarly rapid reversal of factor

IX and XI early in arousal, whereas VIII recovered later in arousal. The slow recovery of factor VIII may partially be due to the low level of VWF in arousal, which normally carries factor VIII, prolonging its half-life. APTT prolongation was previously reported in different ground squirrel species and American black bears during hibernation [13, 21, 23]. Similar to our data, factor VIII is reduced by 71-79% in torpid 13-lined ground squirrel and Scandinavian brown bear [21, 22, 26] and factor IX by 50-67% in torpid 13-lined ground squirrels [21, 22]. In contrast to the changes in procoagulant factors, we did not find an increased contribution from anticoagulants, which is in line with unaltered anticoagulants assessed in Franklin's ground squirrel [13] and even slightly decreased anticoagulants in brown bears [26, 26]. The focus in torpor on suppressing intrinsic rather than extrinsic pathway of coagulation likely occurs due to the immobile nature of hibernation and therefore the small risk of trauma and subendothelial tissue factor exposure (the major activator of the extrinsic pathway), making an extrinsic pathway induced VTE less likely during torpor.

Finally, during torpor and arousal we found an increased level of fibrinolysis. Contrarily, in hibernating brown bears plasminogen is reduced as well as plasmin inhibitor [26, 28], which may be further compensated by an increased level of nonspecific protease inhibitor α2-macroglobulin [26]. Taken together, secondary hemostasis is likely suppressed during torpor due to impaired common pathway and in some species by reduced extrinsic pathway, and in all species due to suppressed intrinsic pathway of coagulation and reverts to summer level during arousal.

Potential mechanisms underlying the reversible anticoagulant state in torpor

We proposed previously a temperature driven storage-and-release mechanism of platelets during the cycles of torpor-arousal [11], which is corroborated by findings in hibernating ground squirrel [30]. Likely, margination of platelets to endothelium underlies this reversible thrombocytopenia [11]. The reduction in plasma levels of (anti) coagulation factors in torpor could be due to decreased production, increased break down, storage of factors or increased consumption. Although both procoagulant and anticoagulant factors are reduced in hamster plasma during torpor, not all factors produced by the liver are reduced. Factor II, VII, X, protein C, plasmin inhibitor and plasminogen are produced in liver and were not reduced during torpor. Furthermore, stable levels of these factors also indicate that the vitamin K driven gamma-carboxylation of coagulation factors is not affected. Together, this indicates that the underlying mechanism reducing VTE risk in torpor may not be dependent on hypometabolism of liver, but rather on clearance of specific coagulation factors from the circulation, either via increased elimination or via uptake/storage. Indeed, procoagulant factors with a long half life of several days, such as fibrinogen

and factor XI [31, 32], reduced faster and more than half in (early) torpor even when proteolytic activity is expected to be slower with the reduced body temperature. Contrarily, factor VII is known for its short half-life of several hours [33] but remained stable throughout hibernation, indicating that entry in torpor may not stimulate the breakdown or uptake of all coagulation factors. This further advocates a mechanism of increased elimination or storage during torpor for specific factors only. Cooper et al. demonstrated that mRNA level of factor IX and VWF reduced in torpor [22], indicating a contributory role of reduced production to the reduction in plasma level of specific factors during torpor. This involvement of synthesis should also be assessed in future studies, e.g. by measuring liver mRNA level of coagulation factors. The swift recovery of coagulation factors IX and XI early in arousal further signifies a role of storage and release of these factors, rather than elimination and resynthesis. Contrarily, factors VIII and fibrinogen do not return to summer level within 1.5 hour of arousal, therefore these factors might be resynthesized throughout arousal or stored and released via a separate slower mechanism, e.g. via endocytosis by megakaryocytes during torpor and slow release during arousal. Megakaryocytes are known to endocytose plasma fibrinogen, factor V, VIII and VWF and can even synthesize factor VIII [34-38]. Additionally, there are some indications that megakaryocyte number might reduce throughout hibernation [12], which should be further investigated as well and if this affects the recovery in coagulation factors during late arousal. Moreover, whether uptake and release of factors is dependent on body temperature is unknown, as is whether other cells are capable of endocytosing and releasing factors IX and XI.

Limitations

Although golden hamster specific assays were unavailable, we could measure hamster plasma samples in assays optimized for human plasma. Likely, this is due to the high level of sequence homology - on average more than 80% - with human coagulation factors (data not shown). Although both VWF and plasminogen were measured in a low range of the assay, only VWF demonstrated less than 80% similarity to the human sequence, namely 60.3% with 34.4% gaps, potentially explaining the low range of measurements. Due to the reduced cardiac output and increased viscosity of blood during torpor, we were limited in the volume to withdraw from torpid hamsters upholding proper anticoagulation. Therefore, we divided the animals accordingly over the assays to determine most parameters with limited sample size.

Though one might argue that decreased factors in torpor might be due to coagulation of blood, e.g. in response to lowered temperature, suggested to activate platelets and coagulation factors [39, 40], we did not find increased levels of D-dimer as a measure of coagulation and fibrinolysis activation and other studies did not find

histological signs of thromboembolism or subsequent signs of ischemia throughout hibernation [10, 22]. Moreover, we previously showed that platelets from torpid and aroused animals are not activated [11]. Unfortunately, a more sensitive measurement for coagulation activation such as thrombin-antithrombin complex was unavailable for golden hamsters. Contrarily, reduced temperature *in vivo* and *ex vivo* has been demonstrated to suppress coagulation. When coagulation factor levels are normal, hypothermia itself (as measured at 34, 31 and 28°C) reduces coagulation by increasing PT and APTT [41]. Our clotting assays were performed at 37°C, therefore an additional anticoagulant effect of low body temperature is expected on top of the reduction in coagulation factors during torpor. In addition, effects of hypothermia on kinetics of coagulation factors and cascades may even further substantiate the anticoagulant effect of low body temperature [42]. Therefore, the anticoagulant effects of hibernation demonstrated in the current study may underestimate the actual effect in torpor.

Comparison to human findings

VTE in humans may be demonstrated by clinical findings of swelling, pain and redness of limbs (for venous thrombosis) and dyspnoea, painful breathing or even death (for pulmonary embolism). Hibernators continue a healthy state after torpor [15], insinuating the absence of VTE. Also histological signs of thromboembolism or signs of ischemic damage are absent in different organs of hibernators throughout different phases of hibernation [15, 22]. In a setting of low clinical suspicion, measurement of D-dimer is very sensitive in excluding VTE in patients [43, 44]. Hamster D-dimer level remained low throughout hibernation, advocating the chance of VTE occurrence or the presence of a prethrombotic state to be small. Factors contributing most to VTE in humans – e.g. (acquired) deficiencies in antithrombin or protein C - were not observed in our hibernating hamsters. The maintenance of these factors in torpor may therefore also signify their importance in preventing thrombosis in hibernators. Moreover, elevated levels of factor VIII and VWF are independent risk factors for VTE in humans [45]. Therefore it may also be important for hibernators to reduce factor VIII and VWF, which occurs in several hibernating mammals studied so far [21, 22, 26].

Conclusion

Hibernation features an antithrombotic state during torpor that reverts upon arousal. The antithrombotic profile consists of both suppressed primary and secondary hemostasis and likely reduces risk of venous thromboembolism during periods of increased immobility, blood stasis and viscosity. The underlying mechanism remains to be disclosed. Our current study suggests coagulation factors to be either eliminated or stored during torpor and resynthesized or released during arousal with potentially

different mechanisms for different coagulation factors. Elucidating this natural phenomenon of reversible anticoagulation might yield new ways to limit venous thromboembolism and other thrombotic diseases, such as myocardial or cerebral infarction.

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SUPPLEMENTAL DATA

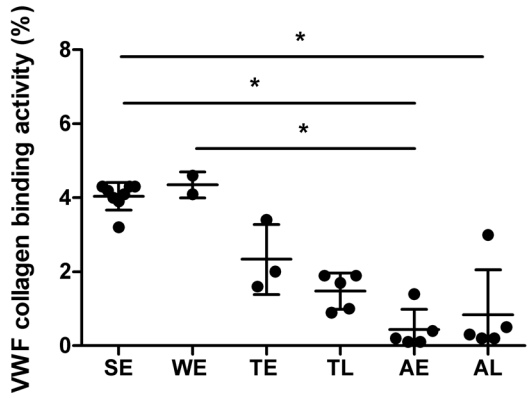

FIGURE S1. Von Willebrand factor collagen binding activity reduces during hibernation. Collagen binding activity of von Willebrand factor (VWF) measured in plasma from non-hibernating and hibernating hamsters relative to human pooled normal plasma. VWF remains as functional in winter euthermia (WE) as in summer euthermia (SE), whereas VWF activity reduces in hibernating hamsters (torpor and arousal (TE, TL, AE, AL)). Sample sizes between n=2 and n=8, * P<0.05.

CHAPTER 4

Reversible Thrombocytopenia during Hibernation Originates from Storage and Release of Platelets in Liver Sinusoids

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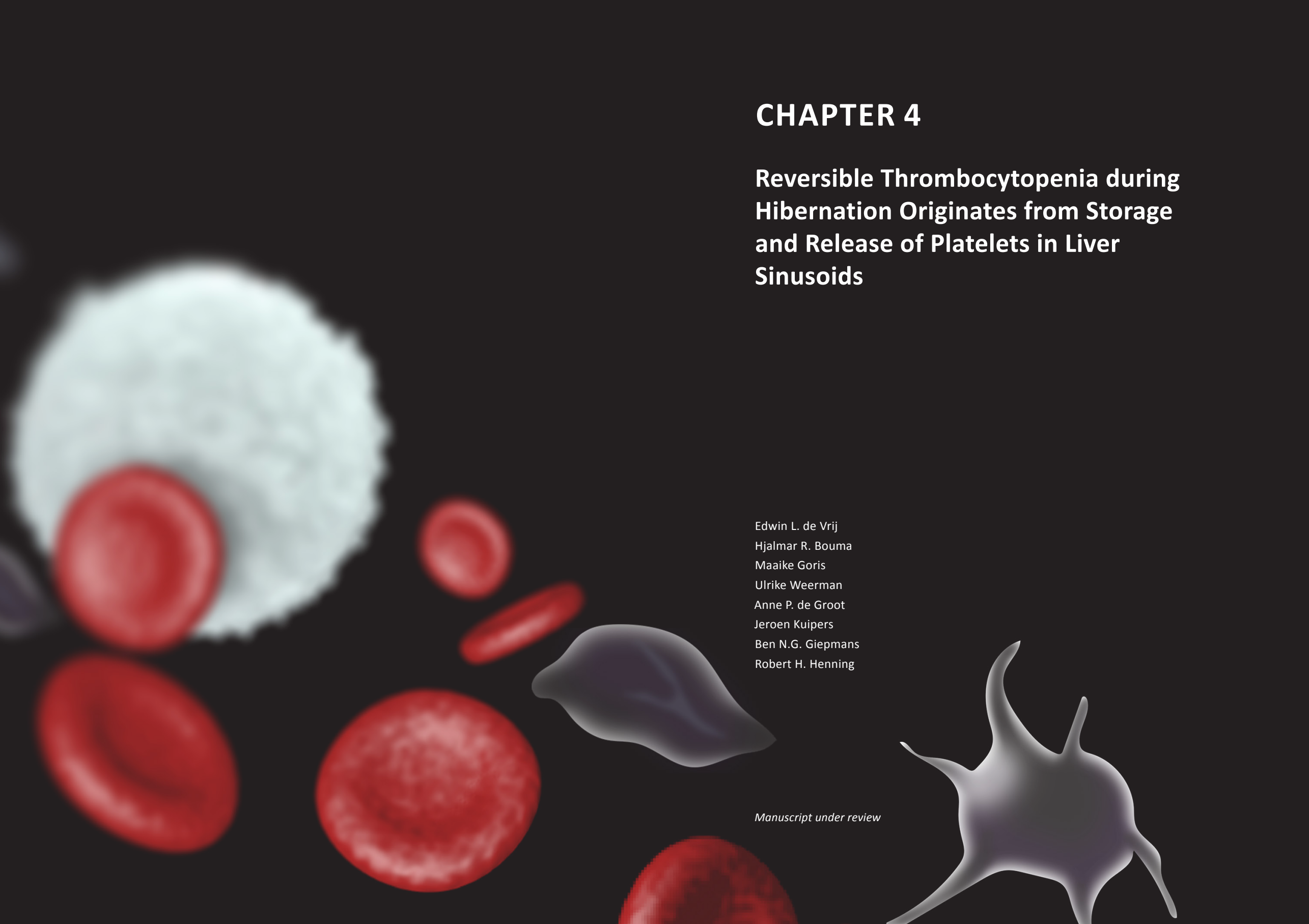


ABSTRACT

Immobility is a major risk factor for thrombosis due to low blood flow resulting in activation of the coagulation system and recruitment of platelets. Nevertheless, hibernating mammals - who endure lengthy periods of immobility - do not show signs of thrombosis throughout or after hibernation. One of the adaptations of hemostasis in hibernators consists of a rapidly reversible reduction of the number of circulating platelets during torpor, i.e. the phase of metabolic reduction with low blood flow and immobility. Whether these platelet dynamics originate from storage and release or breakdown and *de novo* synthesis is unknown. This study aimed to demonstrate platelet storage during torpor and hypothesized that its mechanism involves storage in central organs by margination to the vessel wall. CFMDA-labeled platelets were transfused in hibernating Syrian hamster (*Mesocricetus auratus*) and platelets were analyzed using flow cytometry and electron microscopy. Lifetime of labeled platelets was about 50% extended in hibernating animals compared to non-hibernating hamsters (half-life 30 h versus 20 h). Total and labeled platelet count was reduced more than 90% in circulation during torpor and recovered rapidly during arousal. Activatibility of circulating platelets was reduced in torpor. Aroused animals had baseline number of immature platelets, low plasma interleukin-1α concentration and normal numbers of megakaryocytes in bone marrow, thus excluding platelet synthesis and megakaryocyte rupture to account for recovery in platelet counts upon arousal. Large scale electron microscopy revealed that platelets accumulate in liver sinusoids during torpor, but not in spleen or lung, in line with previous splenectomy studies excluding a role of spleen. Additionally, hemostatic activation was absent during hibernation, demonstrated by low plasma D-dimer level and absence of degranulation of platelets. These results demonstrate unequivocally that platelet dynamics in hibernation are caused by storage and release of platelets, most likely via liver sinusoids. This antithrombotic mechanism of hibernation may aid in management of hemostasis during accidental hypothermia and in development of novel antithrombotic strategies.

INTRODUCTION

Immobility in humans bears an increased risk of thrombosis [1] - even in healthy subjects as exemplified by the 2 to 4-fold increase in relative risk of deep vein thrombosis (DVT) after 4 hours of immobility during travel [2]. Platelets are crucial in the development of both venous and arterial thrombosis. In humans, increased DVT risk by immobility is due to reduced venous flow, inducing hypoxia and subsequent activation of the endothelium, staging a scaffold for adherence of platelets and coagulation factors firing off the coagulation cascade and inducing thrombus formation [3, 4]. Dislodging of such a thrombus by restored blood flow may cause life-threatening pulmonary embolism (PE) [5]. In addition, platelets are involved in arterial thrombus formation during stasis of blood flow, specifically in the heart atria during atrial fibrillation [6, 7], thereby increasing the risk of arterial thromboembolic events such as cerebrovascular accidents and extracranial embolism with a 30 day mortality as high as 55% [8]. Furthermore, platelets can initiate thrombosis in vasculitides and in atherosclerotic bloodvessels by adhering to the inflamed or activated endothelium [9, 10].

Curiously, immobility induced thromboembolism is absent in hibernators, in spite of several risk factors being present throughout hibernation. Hibernation is used by many mammalian species to survive extreme environments [11]. Hibernation is characterized by torpor phases with extreme reduction of metabolism leading to a large decrease in amongst others heart and respiratory rate, as well as in body temperature [11]. Torpor bouts last several days to weeks and are interspersed by short phases of arousal wherein metabolism and other physiological parameters fully recover. All hibernators are immobile during the torpor phase and some species even remain immobile during arousal phases until springtime [11-13]. At face value, because of reduced blood flow and immobility of the animal, hibernators would suffer an increased risk of thrombosis, the more so because of an increased blood viscosity during torpor [14, 15]. However, hibernators induce crucial changes to their hemostatic system during torpor to prevent thrombosis, amongst others by reducing platelet count with more than 90% and reducing coagulation factors, such as factor VIII and IX, suppressing blood clotting [16-18]. Although hibernators suppress hemostasis during torpor, presumably to preclude inadvertent formation of thromboembolisms, the risk of bleeding lurks during arousal if changes are not reversed timely. Therefore, torpid squirrel and hamster for instance rapidly recover platelet count within 2 hours of arousal [16, 17, 19] and adequately recover (although not completely restore) whole blood clotting tendency, as measured by thromboelastography [18]. Reduction and reversal of circulating platelet count during hibernation is hypothesized to be caused by storage and release, rather than by

breakdown and *de novo* synthesis. Main arguments are that platelet count rapidly normalizes within a few hours of arousal, i.e. faster than accounted for by synthesis from megakaryocytes, and that the amount of newly synthesized platelets does not increase in arousal [17, 18]. Alternatively, megakaryocyte rupture, recently discovered as a rapid platelet producing process [20], might play a role in the swift recovery of platelet count during arousal.

In this study, we set out to identify platelet storage and release as the mechanism governing platelet dynamics in hibernation and to disclose the major locations involved. We hypothesized that platelets are stored during torpor in well vascularized organs by means of margination to the vessel wall and are released during arousal. By assessing platelet amount in circulation and in several organs in time by flow cytometry and electron microscopy in hibernating hamsters transfused with CMFDA-labeled platelets, we demonstrate unequivocally that platelet dynamics in hibernation is governed by storage and release of platelets, most likely via liver sinusoids. Examination of platelet activation markers, immature platelet amounts and bone marrow megakaryocytes reveals no signs of *de novo* synthesis of platelets to account for the rapid and major recovery in platelet count during arousal, while low D-dimer levels diminish the likeliness of thrombus formation during hibernation.

MATERIAL AND METHODS

Animals

Syrian hamsters (*Mesocricetus auratus*, age 3 months) were obtained from Envigo USA and individually housed at 'summer' photoperiod light:dark cycle (L:D) of 14h:10h at 20-22°C with free access to standard laboratory chow and water until induction of hibernation. Animal work was approved by the Institutional Animal Ethical Committee of the University Medical Center Groningen.

Hibernation in hamsters

After 7 weeks at 'summer' photoperiod, hamsters were housed at 'autumn' photoperiod (L:D of 8h:16h at 20 °C) for 7 weeks, followed by reduction of ambient temperature to 5°C and housing under constant darkness ('winter' period) [17]. Passive infrared sensors coupled to a computer system monitored individual movements. Hamsters were euthanized at different stages of euthermia or hibernation: summer euthermia (SE), winter euthermia (WE), early torpor (TE), late torpor (TL), early arousal (AE) and late arousal (AL). Summer and winter euthermia were defined as a euthermic body temperature (approximately 37˚C) during 'summer' and 'winter' photoperiods in absence of any torpor bouts. Early and late torpor were defined as 24-48 and >48 hours of immobility respectively and confirmed in all animals by oral temperature measurements. Early and late arousal were defined as 1.5 hours and >8 hours after induced arousal, and a body temperature of ≥35˚C.

Blood samples

Blood was obtained under isoflurane 2% in air/O_2 anesthesia from the abdominal aorta into one-tenth volume of 3.2% sodium citrate or in lithium heparin coated tubes. Cell count was performed on a Sysmex PoCH 100-iv analyzer, while immature platelet fraction was determined with a Sysmex XE-2100 by staining with a dye for reticulated cells[21]. Plasma was prepared by whole blood centrifugation at 3,000 g x 15 minutes at 22°C and stored at -80°C. D-dimer was measured with a Modular analyzer (Roche Diagnostics) with reagents from Roche.

Allogeneic labeled platelet transfusion

Donor blood from euthermic animals was diluted 1:1 (v/v) in Buffered Saline Glucose Citrate (116 mM NaCL, 13.6 mM Na_2Citrate*H_2O, 8.6 mM Na_2HPO_4*$2H_2O$, 1.6 mMKH_2PO_4, 11.1 D Glucose*H_2O, pH 6.8). The diluted donor blood was centrifuged at 160 x g for 20 minutes at room temperature to obtain platelet rich plasma (PRP), which was fluorescently labeled with 5-Chloromethylfluorescein Diacetate (CMFDA,

ThermoFischer C7025)[22-25]. Hereto, CMFDA dissolved in DMSO/PBS 1:5 (v/v) was added to the PRP at a final concentration of 100 µM and incubated for one hour at room temperature. Next, the fluorescent intensity of CMFDA per platelet was determined and platelets were transfused into recipient hamsters following cannulation of the superficial femoral artery under isoflurane anesthesia. Hibernating hamster were returned to their winter environment while remaining in darkness. CMFDA labeled platelet amount was determined prior to and 10 minutes after transfusion and at euthanization one or more days after transfusion.

Flow cytometry analysis

Expression of P-selectin (CD62P) and CMFDA levels in platelets were analyzed by flow cytometry. One microliter of whole blood was diluted 1:25 (v/v) in phosphate buffered saline (PBS), and incubated with PE-labeled anti-CD62P (GeneTex 43039) with or without 10 µM adenosine diphosphate (ADP) for 30 minutes in the dark. The activation was stopped by fixation with 2% formaldehyde in 300 µL PBS (v/v). Samples were acquired on a BD Biosciences Calibur flow cytometer equipped with CellQuest software (BD Biosciences). Platelet populations were gated on cell size using forward scatter (FSC) and side scatter (SSC). At least 50,000 platelets per sample were analyzed, or 180 seconds in case of low platelet counts (thrombocytopenia). Data was analyzed using Kaluza 1.2 software (Beckman Coulter). Not-activated samples were compared and activated samples were compared.

Assessment of megakaryocyte rupture

Femurs were collected at euthanization and immediately fixated and decalcified in DECAL (containing <15% formaldehyde, <5% methanol, <10% formic acid, Surgipath Leica microsystems) and stored at least 24 hours at 4°C. Bones were further decalcified (20% EDTA, 2% NaOH in PBS (w/v)) for 48 hours, then sagitally cut in half, paraffin embedded and sectioned longitudinally. Sections of 4µm were placed on poly-L-Lysine coated slides and incubated overnight at 60°C. After further deparaffinization, bone marrow sections were stained with Hematoxylin-Eosin (HE) and embedded with Dibutylphthalate Polystyrene Xylene (DPX). Quantification of megakaryocytes was performed in 20 field of views per femur section with light microscopy (Nikon Eclipse 50i) in a blinded fashion.

Plasma interleukin-1 alpha (IL-1α) concentration, as a marker of megakaryocyte rupture, was determined by ELISA according to the manufacturer's instructions (Hamster Interleukin 1A ELISA Kit, MBS006418 MyBiosource.com).

Electron microscopy (Nanotomy)

Liver, spleen and lung were harvested upon euthanization and small blocks of approximately a cubed millimeter were immediately fixated in 2% glutaraldehyde plus 2% formaldehyde (v/v) in 0.1M sodium cacodylate for at least 24 hours at 4°C. After post-fixaton in 1% osmium tetroxide/1.5% potassium ferrocyanide for 2 hours at 4°C, samples were dehydrated using ethanol and embedded in EPON epoxy resin. Sections of 60 nm were collected on single slot grids and contrasted using 5% uranyl acetate in water for 20 minutes, followed by Reynolds lead citrate for 2 minutes. Next, scanning transmission electron microscopy (STEM) was performed on ~ 70,000 µm^2 areas as described previously [26, 27] to generate a large field of view at high resolution, which is called 'nanotomy', for nano-anatomy. Data was acquired on a Supra 55 scanning EM (SEM; Zeiss, Oberkochen, Germany) using a STEM detector at 28kV with 2.5 nanometer pixel size using an external scan generator ATLAS 5 (Fibics, Ottawa, Canada) as previously described [26, 27]. After tile stitching, data were exported as an html file and uploaded to the online image database (www.nanotomy.org). Platelets were detected morphologically in fields of view of ~ 25 x 25 µm and confirmed by size and electron dense granular content on fields of view of ~ 8 x 8 µm. Next, representative images were processed similarly in opensource GIMP software (GNU Image Manipulation Program, The GIMP team, GIMP 2.8.10, www.gimp.org), as previously published for selecting areas of interest[28]. In short, a mask was created over every platelet in one separate layer over the original image, colored red and set to opacity 75%.

Statistics and data presentation

Data are presented as mean ± SD. Statistical differences between groups were calculated using repeated measures ANOVA, one-way ANOVA and post-hoc Tukey analysis (Graphpad Prism v6, GraphPad Software) with $P < 0.05$ considered significantly different. Sum of squares F test was used to compare coefficients of non-linear regression curves. The same software was used to make the graphs.

RESULTS

Platelets are stored during torpor and released upon arousal

Summer and winter euthermic hamsters had body temperatures of 35.7 ± 0.4 °C and 36.3 ± 1.1 °C (Figure 1A). During the torpor phase of hibernation, body temperature reduced to 8.2 ± 0.7 °C and recovered within 1.5 hours of arousal to values not different from summer and winter euthermic values. Torpor was associated with a > 90% reduction in platelet count, reducing from 430 ± 82 in summer to 36 ± 17 x 10^9/L early in torpor, which also recovered swiftly and fully upon arousal (Figure 1B). Next, we determined whether platelet dynamics result from breakdown and *de novo* synthesis or from storage and release of platelets. Hereto, fluorescent CMFDA-labeled allogeneic platelets were transfused in torpid hamster, which induces an arousal due to handling the animal. Subsequently, hibernating animals re-entered torpor 38 ± 19 hours following transfusion. Transfused non-hibernating winter euthermic hamsters served as controls. The number of circulating labeled platelets was assessed by flow cytometry 10 minutes after transfusion and at euthanization during torpor or arousal, at least one day after transfusion (Figure 1C). Serial sampling demonstrated an exponential decay of labeled platelets with half-lifes amounting 20.3 and 29.6 hours in non-hibernating and hibernating hamsters (P < 0.05, Figure 1C). Thus, labeled platelets of hibernating animals exit and return to the circulation similarly as non-labeled platelets, signifying that platelets were stored during torpor and subsequently released during arousal. Additionally, platelet survival is prolonged during hibernation.

Rapid platelet recovery in arousal is not due to platelet synthesis or megakaryocyte rupture

To further substantiate that platelet dynamics are governed by storage and release, rather than clearance and *de novo* synthesis of platelets, we determined the amount of *de novo* synthetized platelets by measuring the immature platelet fraction (IPF) and amount of IL-1α, which may induce megakaryocyte rupture, rapidly producing platelets while reducing megakaryocyte number [20]. IPF was low in euthermic animals and increased slightly in torpor and remained low during arousal (Figure 2A). IL-1α plasma levels (Figure 2B) as well as bone marrow megakaryocyte numbers were similar in non-hibernating and hibernating hamsters that had undergone 9.3 ± 2.2 torpor bouts (Figure 2C-I). Together, these results imply that *de novo* platelet synthesis, either by normal production or by megakaryocyte rupture, does not contribute to normalization of platelet amount during arousal.

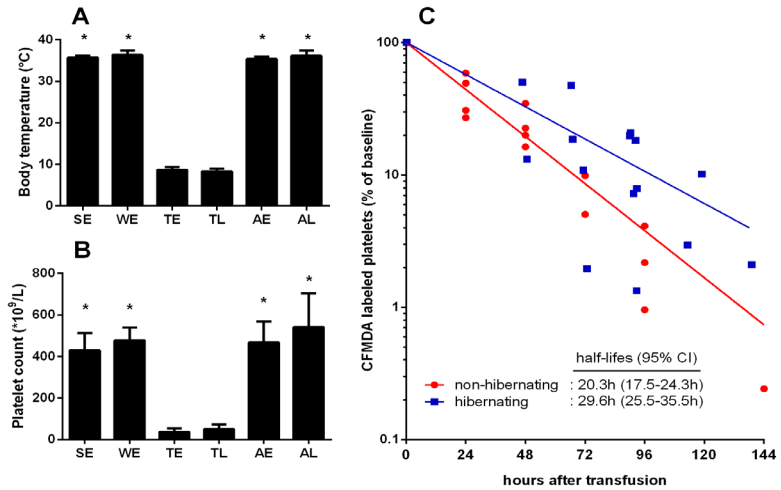


FIGURE 1. Reversible thrombocytopenia during torpor is via storage and release of platelets. A) Oral body temperature at blood sampling confirmed torpor and arousal states of hibernating animals. B) Platelet count reduces during torpor and rapidly recovers to euthermic level during 1.5 hour of early arousal. C) Decay of CMFDA labeled platelets expressed as % of baseline. Platelet survival is longer in hibernating (blue) than non-hibernating hamsters (red) (P < 0.05). Sample sizes between n=3 and n=12, * P < 0.05. SE = summer euthermia, WE = winter euthermia, TE = early torpor (12-48h), TL = late torpor (>48h), AE = early arousal (1.5h), AL = late arousal (>8h).

Platelet storage and release occurs in liver sinusoids, but not in spleen or lung

Since platelets are stored during torpor and released in arousal, we next set out to determine their storage location. Unfortunately, immunohistochemistry and Western blot analyses is precluded by absence of specific anti-platelet antibodies in Syrian hamster (i.e. CD61, CD41, CD49b and glycoprotein Ibα). As an alternative, we used morphological identification by scanning transmission electron microscopy (STEM) and used Nanotomy (for nano-anotomy) [26, 27], which allows to create a single large scale EM dataset that represents the equivalent of thousands of conventional transmission EM photos. Sections were made of liver, lung and spleen of torpid and aroused hamsters. Liver sections demonstrated a large increase in the number of platelets per area in torpor compared to arousal on the large scale EM scan (Figure 3A-B), while the number of platelets in aroused animals was similar to summer animals (Figure S1A). During torpor, platelets were localized primarily in liver sinusoids, often filling the entire sinusoid by forming platelet clusters and displacing erythrocytes (Figure 3C). Conversely, in aroused animals, sinusoids were filled mainly with erythrocytes with the presence of an occasional,

single platelet (Figure 3D), similar to summer animals (Figure S1B). In addition, although rare, we also found platelets in the process of being phagocytosed by Kupffer cells in torpor and arousal and in the subendothelial space of Disse during torpor (Figure S2A-B). In contrast to liver, no changes were found in lung and spleen. In lung, few platelets were found within capillaries, whereas red blood cells were abundantly present, which was similar for torpor and arousal hamster (Figure 4A-B). Red pulp of spleen contained both in torpor and arousal a high amount of red blood cells with platelets distributed homogeneously in between (Figure 5A-B). Together, the amount of platelets in liver increased strongly during torpor because of storage in sinusoids, which reversed rapidly during arousal, while numbers and distribution of platelets in lung and spleen was not affected by torpor or arousal.

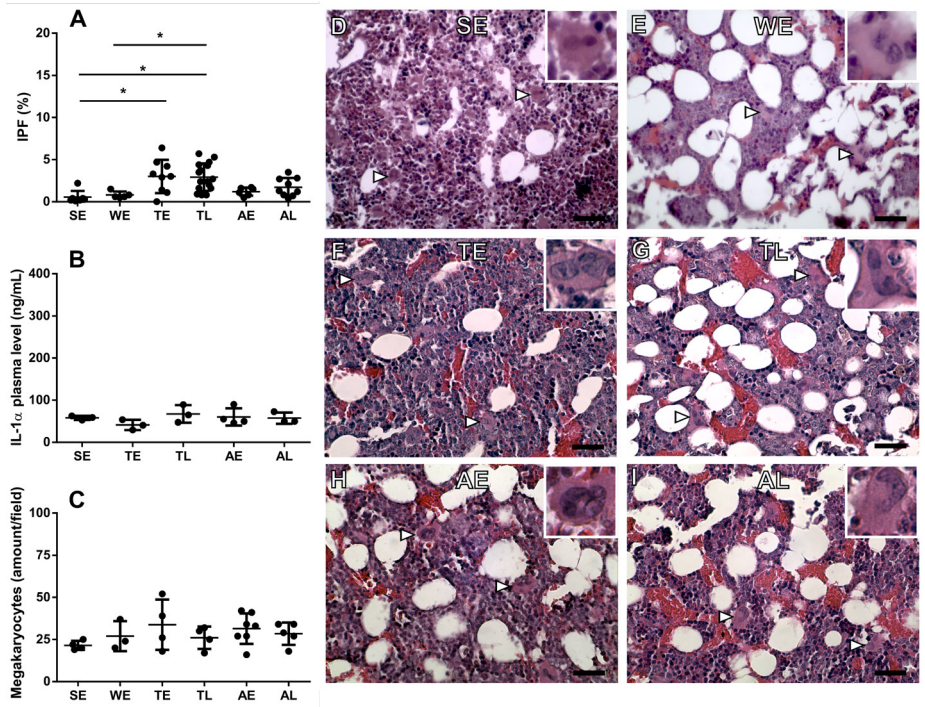


FIGURE 2. Absence of relevant *de novo* platelet synthesis in hibernating hamster.
A) Immature platelet fraction (IPF) as determined by flow cytometry, and B) plasma interleukin 1α (IL-1α) ELISA measurements during different phases of hibernation. C) Quantification of megakaryocyte numbers in sections of hamster femurs, expressed as average amount per 20 fields of view. D-I) Representative fields of view of femur bone marrow from euthermic hamsters in summer or winter condition (SE, WE) or hibernating hamsters early or late in torpor and arousal (TE, TL, AE, AL). Two megakaryocytes are pointed out per image (arrowheads), one of them 2.5x magnified in inset. HE staining, scale bars are 50 µm. Sample sizes between n=3 and n=16, * P < 0.05.

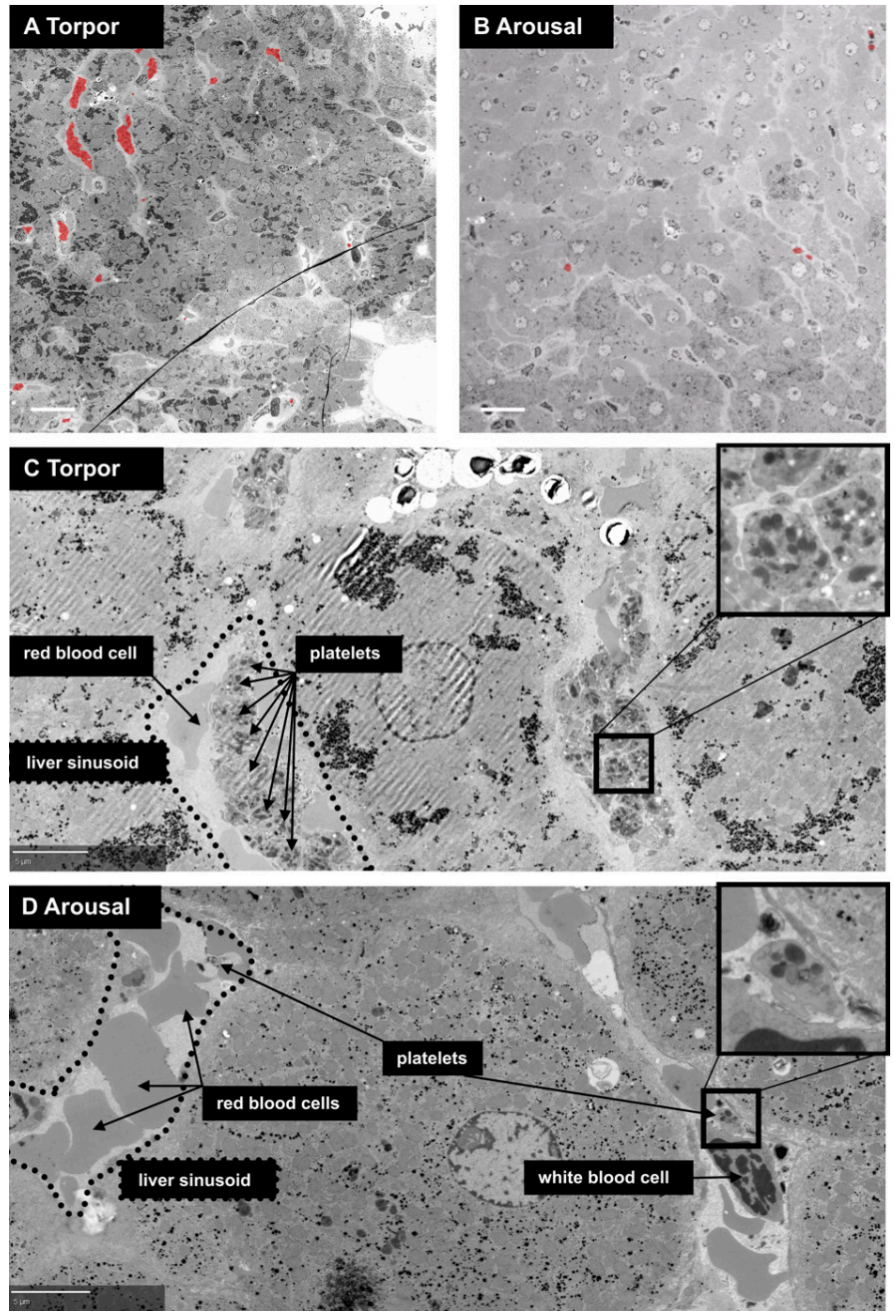


FIGURE 3. Platelets are stored in liver sinusoids during torpor and released during arousal.
Representative images from large scale transmission electron microscopy (TEM) of hamster liver, according to nanotomy protocol. A) Low magnification of the entire section of liver from a hamster in torpor imaged by TEM demonstrating high density of platelets (overlay in red)

in liver during torpor. Single and accumulated platelets are depicted in red overlay. B) Low magnification of entire section of liver from a hamster in arousal demonstrating low density of platelets. C) Liver sinusoids are filled with platelets during torpor. D) During arousal, red blood cells are the predominant cell type in liver sinusoids with very few platelets present. Examples of different cell types are identified. Insets are a 3x zoom on representative platelet. Scale bars represent 20 µm (A-B) and 5 µm (C-D), respectively.

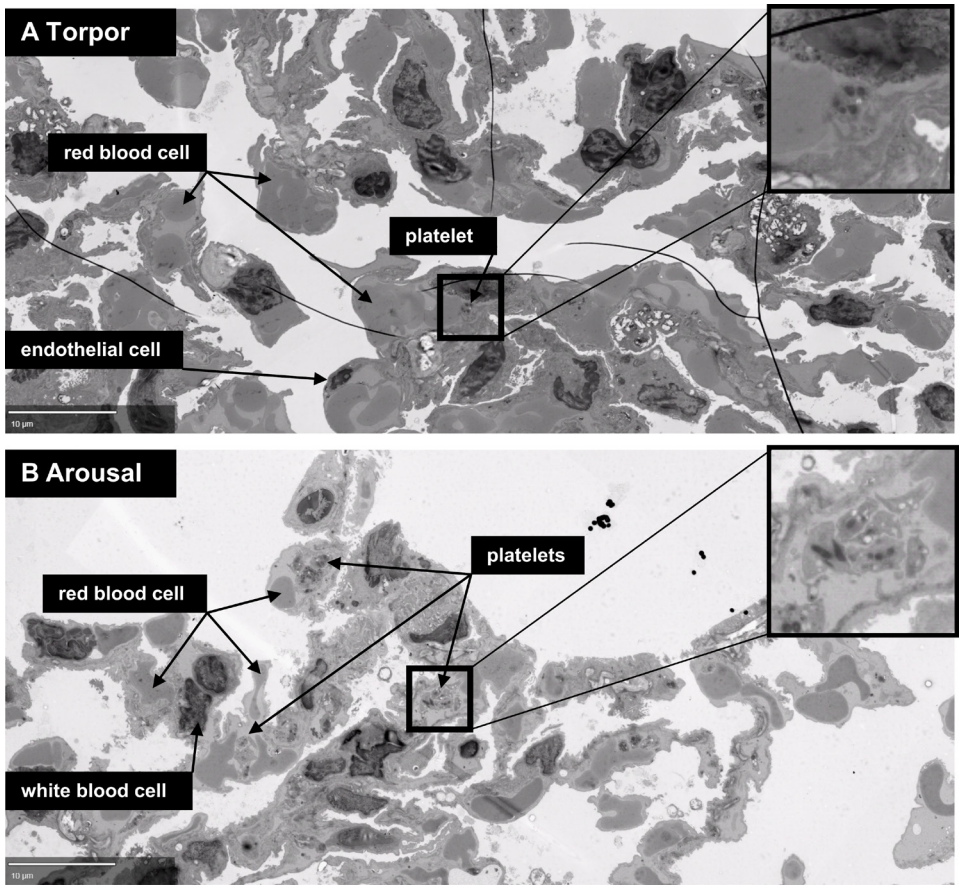


FIGURE 4. Platelet distribution in lung is similar in torpor and arousal.
A) Few platelets are seen in lung sections from torpid hamsters, whereas red blood cells are abundant. One platelet is pointed out within a capillary lumen (arrow). B) Similarly low amounts of platelets are present during arousal in capillaries of lung compared to abundant red blood cells. Examples of different cell types are identified. Insets are a 3x zoom on representative platelets. Scale bar is 10 µm.

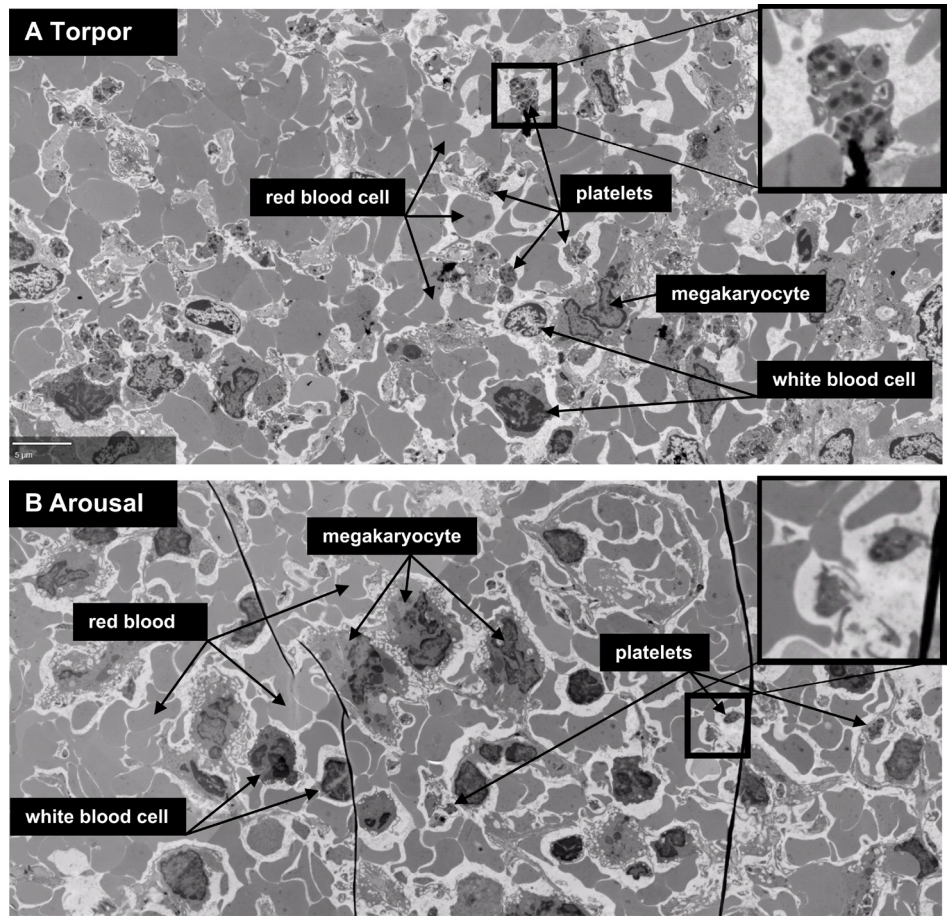
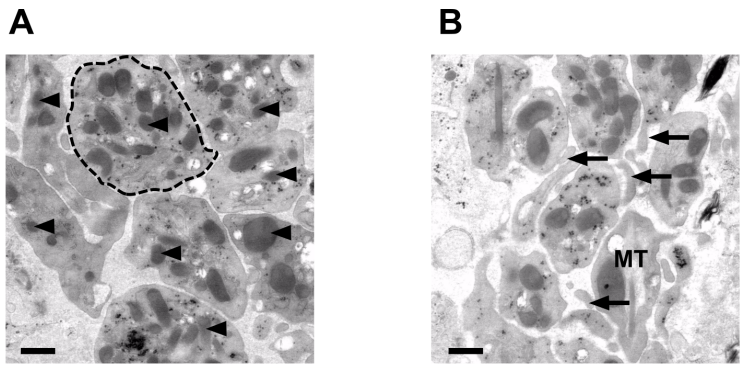


FIGURE 5. Similar platelet distribution in spleen during torpor and arousal. A) Representative zoomed image of large scale transmission electron microscopy map of spleen from a torpid hamster. Red pulp from the spleen is in view with many red blood cells and dispersed platelets and several nucleated white blood cells. B) Spleen from a hamster in arousal, with similarly high amount of red blood cells with dispersed platelets and white blood cells. Examples of different cell types are identified. Insets are a 3x zoom on representative platelets. Scale bar is 5 µm.

No signs of platelet activation or coagulation during torpor and arousal

Large scale EM analysis of torpid animals demonstrated that platelets stored in liver still contain granules (Figure 6A), arguing against degranulation of platelets during torpor. However, we observed occasional membrane folds in platelets (Figure 6B), mimicking filopodia, which may reflect platelet activation. To determine whether platelets and the coagulation system are activated during torpor, we determined

platelet P-selectin expression on circulating platelets and plasma D-dimer levels (Figure 6C-D). Circulating platelets in torpid and aroused hamsters had similar basal P-selectin expression, whereas activatibility gradually increased from torpor to early and late arousal, reaching levels similar to winter euthermia (Figure 6C). D-dimer levels remained low in hibernating and non-hibernating animals and below threshold used in diagnosing thrombosis in humans (500 µg/L, Figure 6D). Thus, thrombocytopenia during torpor is not associated with activation of platelets or the coagulation system. In addition, activatibility of circulating platelets seems reversibly suppressed during torpor.



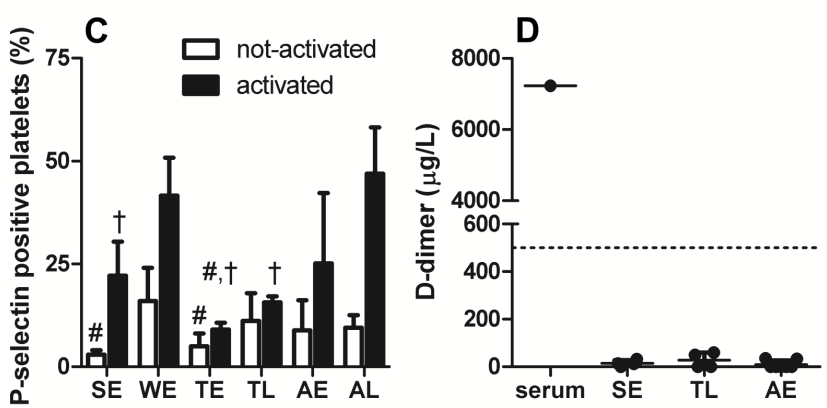


FIGURE 6. Thrombocytopenia in torpor is not linked to platelet activation or plasma coagulation activation. A) Representative transmission EM image of stored platelets in liver sinusoids during torpor with retained granules (arrowheads denote some example granules), platelets are not visibly degranulated. Dotted line encircles one platelet. B) Stored platelets in sinusoids during torpor generally show a rounded shape, and occasionally demonstrate extended membrane protrusions (filopodia, indicated by arrows) and centralized elongated microtubules ("MT"). Scale bars denote 50 µm. (C) P-selectin expression, as a measure of platelet degranulation, of circulating platelets ("not activated") and ADP-stimulated platelets ("activated") expressed as % of total platelets. Activatibility of platelets is reversibly reduced in torpor. D) Plasma D-dimer levels throughout hibernation. Hamster serum was used as positive control. Sample size n=2 to 11, '#' denotes difference from WE, '†' denotes difference from AL, $P < 0.05$. SE = summer euthermia, WE = winter euthermia, TE = early torpor (12-48h), TL = late torpor (>48h), AE = early arousal (1.5h), AL = late arousal (>8 hours).

DISCUSSION

Thrombocytopenia during torpor is governed by reversible storage and release in liver sinusoids

Here, we demonstrate unequivocally that thrombocytopenia during torpor is due to storage of platelets, followed by subsequent release of the same platelets upon arousal. Storage and release of platelets is principally evidenced by the observation that CMFDA labeled platelets injected prior to torpor, exit the circulation during torpor and recirculate upon arousal. To our knowledge, we are the first to demonstrate the platelet half-life in hamsters and its increase during hibernation. In addition, we show that platelets are mainly stored in liver sinusoids during torpor and released in arousal. The finding that storage and release governs platelet dynamics during hibernation is further supported by 1) absence of platelet activation or coagulation (i.e. no degranulation of platelets, low plasma D-dimer levels) and 2) no signs of *de novo* synthesis of platelets (i.e. low immature platelet fraction and plasma IL-1α and no signs of megakaryocyte rupture). Finally, we demonstrate that circulating platelets during hibernation are not activated, whereas platelets are suppressed in activatibility during torpor, which reverses during arousal. Together, these results demonstrate that hibernators may shield themselves from thrombosis induced by immobility and low body temperature by reversibly suppressing the number and functionality of circulating platelets.

Torpor induces platelet storage in liver with reduced activatibility of circulating platelets and absence of hemostatic activation

We demonstrate that liver sinusoids constitute the main compartment of platelet storage during torpor, from where platelets are released upon arousal. These findings match a recent study demonstrating increased amount of platelet glycoprotein Ib staining in liver of hibernating torpid ground squirrels, which reverses in arousal [29]. By large scale electron microscopy (nanotomy) analysis [26, 27] we determined that the platelet storage location in torpor was not in lung or spleen, since the number of platelets did not change in these organs. In accord, we previously excluded a role of spleen in platelet storage by demonstrating that splenectomy before or during torpor is without an effect on platelet dynamics in hibernating hamster [17], which was recently corroborated in splenectomized squirrels [29]. Finally, our results exclude thrombosis and trapping of platelets within immune complexes or rosette cell formation as a contributor to platelet storage, since (micro)thrombi were absent in liver and lung, levels of D-dimer remained low throughout torpor and arousal, and platelets in torpid liver sinusoids were not degranulated and did not form large activated aggregates,

but rather non-activated accumulations. Additionally, circulating platelets were not activated throughout hibernation, since circulating platelets of hibernating animals expressed similarly low levels of P-selectin. Moreover, the few platelets that circulated during torpor had a reduced activatibility in response to ADP, as implied previously [17], which is in line with reduced aggregation of platelets from hibernating bears in response to ADP and other agonists [30]. Thus the reversible platelet storage in liver sinusoids is due to platelet accumulation without activation and without thrombus formation or hemostatic activation.

Platelet spear shape is not linked to storage and release

Reddick *et al*. proposed that ground squirrel platelets would become trapped in the spleen during torpor due to a platelet shape change [31]. This was rooted in their observation that some platelets in spleen of torpid squirrel possessed an elongated spear-like shape associated with elongated rods of microtubules, similar to shape change of isolated squirrel platelets stored at 0°C. In contrast, we observed only a very small number of spear shaped platelets with centralized and elongated microtubules during torpor, both in spleen and in liver sinusoids, whereas the majority of platelets was round in shape. The maintenance of platelet microtubule patency at low body temperature, as previously also observed in ground squirrel [18, 29], sets aside hibernator from human platelets, as the latter are unable to maintain microtubular structure during cooling [32]. Although species differences may exist in temperature associated platelet shape changes, our findings do not support a relationship between spear shape change and platelet storage.

Reversible storage and release of platelets in liver sinusoids due to margination

Margination is dependent on platelet-endothelium interaction and reflects a balance between adhesion and detachment. Several factors during torpor likely increase the adhesive forces, while lowering detachment forces. First of all, rheological forces stimulate margination because of substantial reductions in cardiac output and blood flow in torpor [33] and increase of hematocrit [30], driving platelets to the vessel wall [34, 35]. Hematocrit also increased in our hamsters from 0.45 ± 0.04 L/L in summer to 0.51 ± 0.04 L/L in torpor, reversing to 0.42 ± 0.04 L/L in arousal ($P < 0.05$). Secondly, relative hypoxia during entrance in torpor [11] might lead to exocytosis of endothelial cell Weibel-Palade bodies, exposing P-selectin and releasing von Willebrand factor (VWF) [36], thereby stimulating platelet adhesion to endothelial cells. Thirdly, reduced temperature and blood flow may induce endothelium activation with increased expression of adhesion molecules to further increase the adhesive forces [37, 38].

Increased endothelial activation markers have also been found in hamsters during torpor [39], whether this results from reduced flow and/or temperature is not yet known. Besides effects of torpor on endothelium and blood rheology, one might hypothesize that platelets increase adhesiveness during torpor. Although expression of adhesive markers has not been studied on stored platelets, our finding of platelet storage in liver implies that stored platelets may be obtained simply by flushing the liver of torpid animals. This way it will be possible to compare circulating and stored platelets of torpid animals. However, the low P-selectin expression and suppressed activatibility of circulating torpid platelets argues against relevant pro-adhesive effects of torpor on platelets themselves. Since platelets re-appear swiftly in circulation upon arousal, platelets likely detach from endothelium solely due to increases in blood flow and temperature. Hence, low blood flow, increased hematocrit and potentially increased adhesion molecule expression and VWF levels due to relative hypoxia and low temperature, likely shift the balance towards platelet margination in torpor, which is rapidly reversed upon arousal.

We previously demonstrated that reversible thrombocytopenia in torpor is dependent on lowering of the body temperature [17]. Here, we reveal margination as mechanism underlying reversible storage and release of platelets in hibernation. Lowering body temperature in non-hibernators decreases cardiac output, blood flow and increases blood viscosity [40, 41], which favors platelet margination [34]. Importantly, we recently demonstrated that lowering the body temperature induces platelet storage and release via margination of platelets to liver sinusoidal endothelium leading to thrombocytopenia in rat and mouse (de Vrij et al., submitted). Hence, the effect of lowered body temperature on margination of platelets, which can lead to a profound drop in the number of circulating platelets, is a widely conserved phenomenon that is not specific for hibernating species. Since accidental and therapeutic hypothermia in humans are also associated with thrombocytopenia [42-47], knowledge of its underlying mechanism may aid in (hemostatic) management of hypothermia. Furthermore, the ability to pharmacologically induce reversible storage of platelets might be exploited for development of novel reversible antithrombotic strategies.

Acknowledgements

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Wang CH, Chen NC, Tsai MS, et al. Therapeutic Hypothermia and the Risk of Hemorrhage: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Medicine (Baltimore). 2015;94(47):e2152.

SUPPLEMENTAL DATA

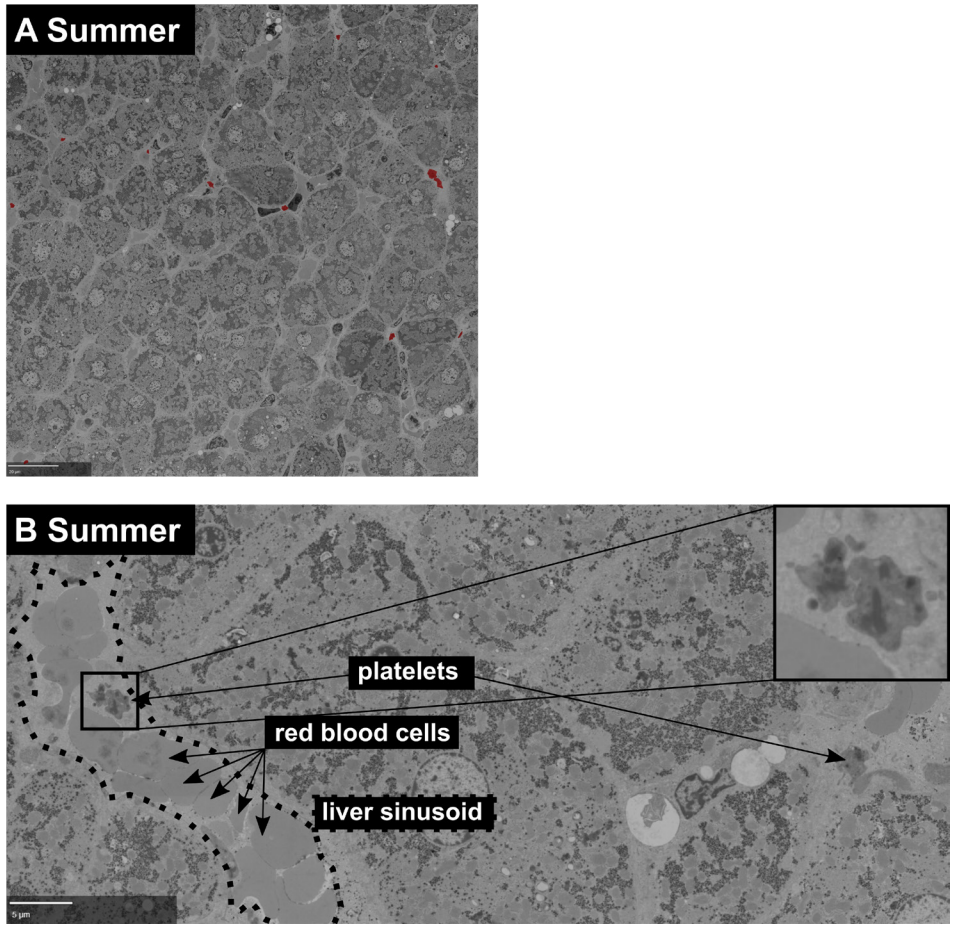


FIGURE S1. Platelet distribution in liver of hamsters in summer condition.

Representative images from large scale transmission electron microscopy (TEM) of hamster liver in, according to nanotomy protocol. A) Low magnification of the entire section of liver from a hamster in summer euthermia demonstrating low density of platelets (overlay in red). B) Liver sinusoids are mainly filled with red blood cells in summer, occasionally platelets can be found. Inset is 3x zoom on representative platelet. Scale bars are 20 µm (A) and 5 µm (B), respectively.

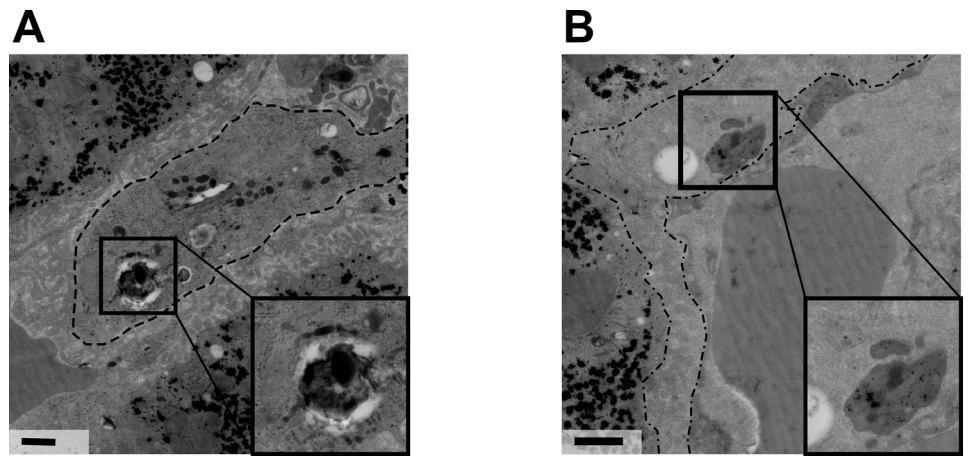
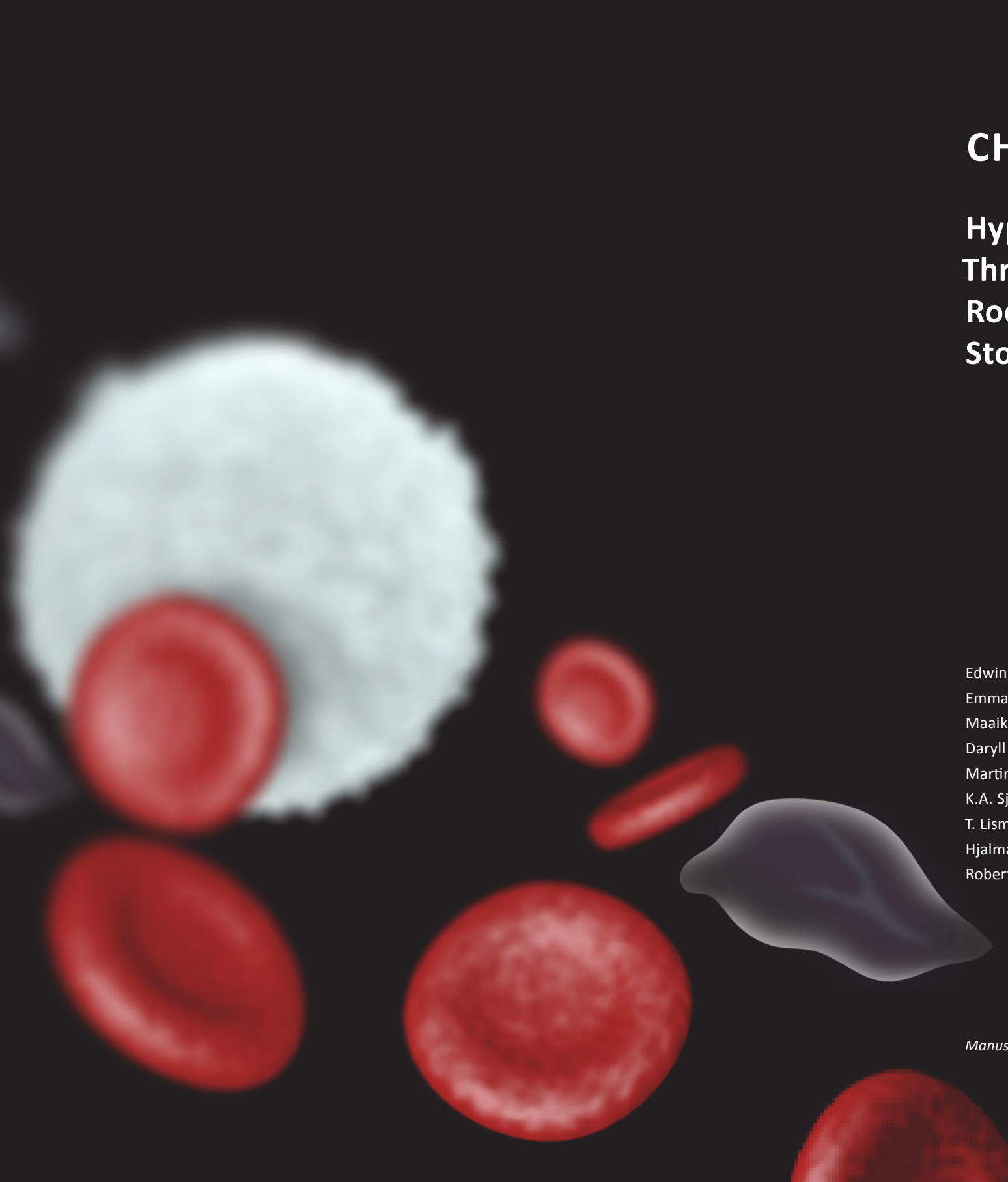


FIGURE S2. Platelet phagocytosis in Kupffer cells and platelets in space of Disse during hibernation.

A) Electron microscopy imaging of hibernating hamster liver demonstrated some Kupffer cells, liver macrophages, in the process of phagocytosing platelets in torpor. Dashed line encircles a Kupffer cell. B) In one instance we found a platelet in torpor in the space of Disse, the space between endothelial cells and hepatocytes, denoted by the space between dash-dotted lines. On the luminal side of the sinusoidal endothelium is an erythrocyte. Insets are a 2x zoom of platelets, scale bars represent 1µm.



CHAPTER 5

Hypothermia Associated Thrombocytopenia is Governed in Rodents by Reversible Platelet Storage in Liver Sinusoids

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ABSTRACT

Hypothermia, either accidental or therapeutic, is associated with a reduction of circulating platelets which generally is reversed by rewarming. Here, we determined the mechanism(s) that drive reversible hypothermia induced thrombocytopenia. Fluorescently labeled platelets of rat and mouse undergoing whole body hypothermia and rewarming were analyzed using flow cytometry, immunohistochemistry and (intravital) microscopy analysis to determine amount, activation status and location. During hypothermia in rat (15°C, 3h), circulating platelets decreased with 52.5 ± 4.3 %, which was fully restored during rewarming. Importantly, the fraction of labeled platelets remained constant during all phases, implying platelet storage and release. Hypothermia induced a major increase of platelet numbers in liver sinusoids, with a minor additional increase in lungs, but not in spleen. Intravital imaging during hypothermia in mouse (20°C, 1h) demonstrated platelet margination in liver sinusoids to constitute the storage mechanism, which was temperature and blood flow dependent. Storage was reversed by increased blood flow during rewarming. Further, P-selectin expression and activatability of platelets was unaltered by hypothermia and rewarming and D-dimer levels remained low, signifying absence of platelet activation or dysfunction and intravascular coagulation during *in vivo* hypothermia and rewarming. The sharp contrast with irreversible clearance of platelets following transfusion of *ex vivo* cold stored platelets signifies that systemic factors -possibly related to liver storage- shield platelets from activation, dysfunction and phagocytosis during *in vivo* hypothermia. Deciphering the shielding mechanism may enable transfusion of cold stored platelets, improve liver regeneration after transplantation, and contribute to improved management of hemostasis or antithrombotic therapies.

Keypoints:

- Reduction in circulating platelets during lowered body temperature is driven by margination and storage of platelets in liver sinusoids.
- Rewarming restores circulating platelet count by release from liver sinusoids without a significant contribution of *de novo* synthesis.

INTRODUCTION

Hypothermia is associated with a reduction in circulating platelet count (thrombocytopenia) which may occur in patients with accidental[1-5] or therapeutic hypothermia[6, 7]. Hypothermia induced thrombocytopenia is a widely conserved phenomenon among different mammalian species, including humans[8-10], advocating a common underlying (patho)physiological process reducing platelet count. The reduction of circulating platelets is associated with the drop in body temperature with the most dramatic reduction of 90-95% found during mammalian hibernation when body temperature drops below 8°C.[8, 11-13] Remarkably, circulating platelet count is rapidly and completely restored when hibernators rewarm.[8, 11] A similar recovery of platelet count is also observed in humans after rewarming from hypothermia[2, 4, 5], albeit at slower rate than in hibernators. Further, hypothermia may induce effects that coincide, induce or are the consequence of platelet clearance, including (1) platelet activation or increased activatibility[14], (2) trapping of platelets within immune-complexes (as observed in cyroglobulinemia[15], platelet-leukocyte aggregates or rosettes[16]), (3) reversible clearance by margination to the vessel walls[17], (4) bone marrow suppression[18], and (5) irreversible clearance/phagocytosis (without overt platelet activation)[19].

Although no large clinical studies have been performed to assess which of these effects of hypothermia underlies the associated thrombocytopenia, several case reports demonstrate disseminated intravascular coagulation (DIC) to occur[20, 21]. However, DIC may not occur during hypothermia, but rather during the subsequent rewarming phase[22] and may not be linked to thrombocytopenia at all[23]. Experimental evidence however favors hypothermia associated thrombocytopenia to originate from reversible storage of platelets with spleen, liver and lungs implicated as retention sites.[9, 10] Despite these findings, the mechanism that drives the hypothermia induced reduction of circulating platelets remains currently unknown. Given its apparent reversibility, both storage and release and breakdown and *de novo* synthesis of platelets may qualify. We hypothesized that hypothermia induces a reversible clearance of circulating platelets by storage and release of platelets via margination to the vessel wall in central organs, rather than by (irreversible) clearance and *de novo* synthesis of platelets. To address both mechanism and location, we fluorescently labeled platelets in rat and mouse to track platelets in time throughout hypothermia and rewarming. Flow cytometry, immunohistochemistry and (intravital) microscopy were used to determine amount and activation status of platelets as well as platelet location during hypothermia and after rewarming.

MATERIAL AND METHODS

Animals

Wistar rats (Charles River, the Netherlands, 387 ± 28g, n=24) and C57Bl/6 mice (Charles River, 31.3 ± 3.1 g, n=6) were housed at a light:dark cycle of 12h:12h. Animals were fed *ad libitum* using standard animal lab chow and drinking water. Experiments were approved by the Institutional Animal Ethical Committee of the University Medical Center Groningen.

Allogeneic labeled platelet transfusion

Donor blood was obtained by puncture of the abdominal aorta and diluted in one-tenth volume 3.2% sodium citrate. Next, a cell count was performed on a Sysmex PoCH 100-iv analyzer, while the immature platelet fraction was determined with a Sysmex XE-2100 by staining with a dye for reticulated cells[24]. Per mL of blood 0.4mL of Buffered Saline Glucose Citrate was added (116 mM NaCL, 13.6 mM $Na_2Citrate*H_2O$, 8.6 mM $Na_2HPO_4*2H_2O$, 1.6 mMKH_2PO_4, 11.1 D Glucose*H_2O, pH 6.8). The diluted donor blood was centrifuged at 160 x g for 20 minutes at room temperature to obtain platelet rich plasma (PRP), which was fluorescently labeled with 5-Chloromethylfluorescein Diacetate (CMFDA, ThermoFischer C7025)[25-28]. Hereto, CMFDA dissolved in DMSO/PBS 1:5 (v/v) was added to the PRP at a final concentration of 100 µM and left to incubate for one hour at room temperature. Next, the fluorescent intensity of CMFDA per platelet was determined and platelets were infused into recipients rats.

Forced hypothermia in rat

Anesthesia was induced by isoflurane 2.5% in O_2/air (1:1), followed by maintenance of anesthesia by ketamine infused through a tail vein catheter (18-27mg/kg/h). To maintain adequate oxygenation during cooling, rats were intubated and mechanically ventilated (Amsterdam Infant Ventilator; Hoekloos, Amsterdam, The Netherlands). Animals were placed on a water based heating mattress, while body temperature was measured rectally. A catheter inserted into the carotid artery was used to monitor heart rate, blood pressure and to draw blood samples. The CMFDA-labeled donor platelets were infused 30 minutes before onset of cooling. Next, rocuronium (20mg, i.v.) was administered and animals were cooled by applying ice-cold water to their fur and were rewarmed using a water-based heating mattress and drying of fur.[8, 29] Procedures were adjusted aiming at a body temperature change of ≈1°C per 3 minutes. At a body temperature of 26°C, ketamine infusion was paused. Animals were maintained at 15°C for 3 hours. While one group of rats was euthanized at hypothermia, another group was rewarmed. Ketamine infusion was restarted upon reaching a body temperature of 26°C. The body temperature was at 37°C for one hour prior to euthanization. A normothermic sham control group was kept at 37°C throughout the entire experiment with blood samples taken time-matched to the other groups.

Splenectomy

In a separate experiment, after cooling, rats were maintained at 15°C for 1 hour after which rewarming was initiated. Splenectomies were performed on euthermic rats prior to induction of hypothermia or at the end of 1 hour hypothermia. The abdomen was shaved and disinfected by chlorhexidine, the abdominal cavity was opened by a subcostal incision and the spleen was exposed by careful manipulation of the internal organs using a pair of blunt tweezers. Next, the splenic artery and vein were ligated and the spleen was removed. The abdominal cavity was closed in one layer with single sutures. Throughout cooling and rewarming and immediately after splenectomy, blood samples were drawn via the carotid artery catheter. Animals were euthanized after rewarming to 37°C for 1 hour.

Intravital imaging of mouse liver throughout forced hypothermia

Mice were subjected to forced hypothermia and rewarming as described previously.[8] Throughout this procedure platelets within the liver were imaged in by intravital microscopy as described previously.[30] Mice were anesthetized with isoflurane 2% in O_2/air (1:1). Prior to cooling, platelets were labeled *in vivo* by injecting PE-labeled hamster-anti-mouse CD49b (103506 clone HMa2, BioLegend) via the penile vein. Next, a partial upper abdominal midline incision was made followed by a lateral subcostal incision to the right midaxillary line, thereby exposing the liver. The falciform ligament was incised, releasing the liver from the diaphragm. The mouse was placed in right lateral position and the liver externalized onto a 24x60 mm glass coverslip, watertightly sealed in an object carrier that was placed on the stage of a Zeiss 780 (inverted) confocal microscope equipped with an incubator. The liver and abdominal tissue were covered with saline-soaked fiberless optics tissue and gauze. The trachea was cannulated and the mouse was ventilated (MiniVent model 845, Harvard Apparatus) after receiving a bolus of rocuronium (16mg/kg, s.c.). Body temperature was monitored rectally. Throughout the preparatory phase and the subsequent 30 minutes, animals were maintained at > 35°C via a heating mattress and infrared heat lamp. Next, the microscope heating system was switched off and animals were cooled by applying ice-cold saline to their fur to reach a body temperature of 20°C that was maintained for one hour. Mice were then rewarmed to 37°C and maintained at this temperature for 30 minutes. Procedures were adjusted aiming at a change in body temperature of ≈1°C per 3 minutes. During hypothermia, isoflurane

was reduced to 0.6%. The liver microcirculation was visualized using a 40x/1.2 W Korr-C-apochromat objective with glycerin immersion using the autofluorescence of liver (405 nm excitation, 499-552 nm emission) and fluorescently labeled platelets (488 nm excitation, 522-735 nm emission) throughout hypothermia and rewarming. Acquired images were analyzed using ImageJ software (Wayne Rasband National Institutes of Health, USA)[31, 32].

Flow cytometry analysis

Expression of P-selectin (CD62P) and CMFDA levels in platelets were analyzed by flow cytometry. One microliter of whole blood was diluted (1:25, v/v) in phosphate buffered saline (PBS), and incubated with PE-labeled anti-CD62P (GeneTex 43039) with or without 10 µM ADP for 30 minutes in the dark. The activation was stopped by fixation with 2% formaldehyde in 300 µL PBS (v/v). Samples were acquired on a BD Biosciences Calibur flow cytometer equipped with CellQuest software (BD Biosciences). Platelet populations were gated on cell size using forward scatter (FSC) and side scatter (SSC). At least 100.000 platelets per sample were analyzed, or 180 seconds in case of low platelet counts (thrombocytopenia). Data was analyzed using Kaluza 1.2 software (Beckman Coulter).

Immunohistochemistry

Frozen sections (4 µm) were cut from liver, spleen and lung using a CN1860 UV cryostat (Leica Microsystems), which were fixed in formaldehyde 0.2% (v/v in PBS) for 5 minutes. Sections were stained with a mouse anti-rat antibody to CD61 (GeneTex 75341) in a 1:500 dilution (v/v) for onehour, followed by incubation with a secondary TRITC labeled antibody (SouthernBiotech 1030-03) for 30 minutes (1:100, v/v) supplemented with 1% normal rat serum (v/v). Sections were washed with PBS three times for 5 minutes between staining steps. Scans of the whole tissue sections were made with a TissueFaxs (TissueGnostics) and analyzed using ImageJ v1.50g (Wayne Rasband National Institutes of Health, USA)[31, 32] to determine platelet to nuclei ratio, using a self-made macro. In short, background signal was subtracted from the red (TRITC) channel and a particle analysis was performed to count platelets and nuclei. Subsequent platelet to nuclei ratio was used to compare amount of platelets per organ between the groups. Because platelets in spleen in normal situation are abundant, separate platelets could not be counted, therefore area of platelets : area of nuclei was determined in spleen.

Measurement of D-dimer

Blood was centrifuged 2,500 x g for 15 minutes to obtain plasma. D-dimers were

measured using a Modular analyzer (Roche Diagnostics) in heparinized plasma samples. Rat serum samples served as positive control.

Statistics and data presentation

Data are presented as mean ± standard deviation (SD). Statistical differences between groups were calculated using a repeated measures ANOVA, one-way ANOVA and post-hoc Tukey analysis (Graphpad Prism v7.01, GraphPad Software) with $P < .05$ considered significantly different. The same software was used to make the graphs.

RESULTS

Platelets are stored during hypothermia and released during rewarming

Forced cooling of anesthetized rats from 37.7 ± 0.6°C to 15.0 ± 0.1°C body temperature substantially reduced total circulating platelets to 47.5 ± 4.3% of baseline (Figure 1A, $P < .05$; baseline platelet count 732 ± 80 x 10^9/L), which fully recovered after rewarming for 1 hour to 37°C (100.1 ± 20.3%). To study whether these dynamics are induced by cell death and *de novo* synthesis or due to reversible storage and release of platelets, CMFDA-labeled platelets were transfused prior to cooling. CMFDA labeling and transfusion resulted in 5.4 ± 1.4 % of platelets labeled in circulation (n=8). During the ensuing hypothermia and rewarming, absolute numbers of labeled platelets demonstrated a similar reduction and recovery as non-labeled platelets (Figure 1A, $P < .05$) resulting in a constant relative fraction of CMFDA labeled platelets (Figure 1B), unequivocally implicating reversible storage and release as the underlying mechanism. To evaluate if spleen plays a crucial role in the reversible storage and release of platelets, splenectomy was performed either at baseline (37°C) or at the end of 1 hour hypothermia (15°C). Baseline platelet count (750± 59 x 10^9/L) was reduced by 42% after 1 hour hypothermia and returned to baseline level by rewarming to 37°C. Removing spleen either before or after hypothermia did not influence the reduction and recovery of platelet count (Figure 1C). To further substantiate reversible storage and release, we measured the immature platelet fraction (IPF) in animals with spleen upon rewarming. The IPF remained low throughout the cooling and rewarming phase (Table 1), indicating that recovery of normal platelet count upon rewarming does not depend on *de novo* synthesis. Together, forced hypothermia to 15°C induces a profound and fully reversible thrombocytopenia, regardless of presence of spleen, which is governed by storage and release of platelets.

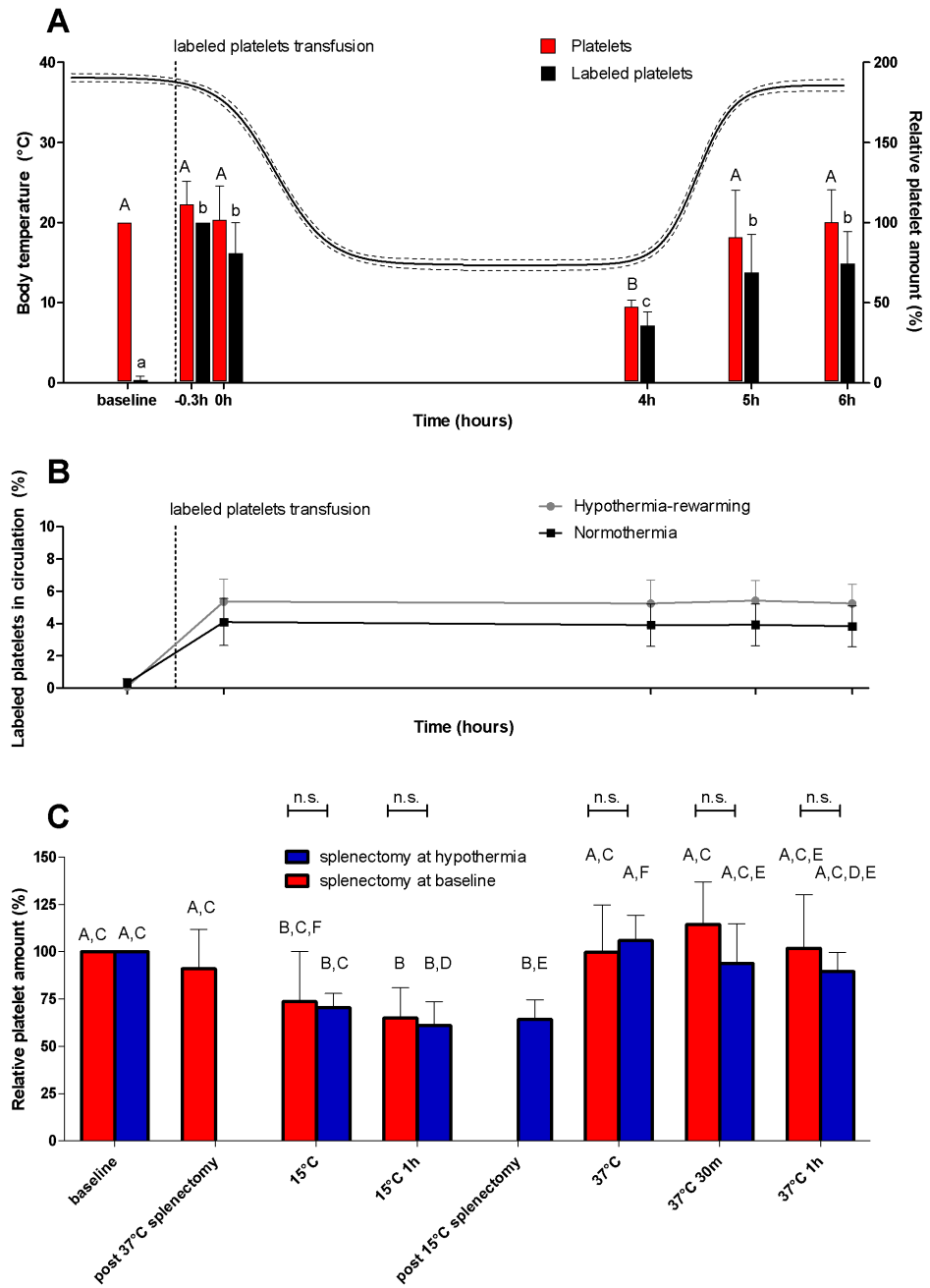

FIGURE 1. Hypothermia induced reversible thrombocytopenia is governed by storage and release, without an essential role of spleen. *Legend on next page.*

FIGURE 1. Hypothermia induced reversible thrombocytopenia is governed by storage and release, without an essential role of spleen. Amount of platelets and labeled platelets in circulation was determined in rats throughout hypothermia and rewarming. (A) Hypothermia in rat induces thrombocytopenia, which recovers completely during rewarming (red bars). Labeling of platelets does not affect clearance from the circulation during hypothermia. Moreover, labeled platelets (black bars) reappear upon rewarming as well, indicating storage and release as mechanism underlying thrombocytopenia, rather than clearance and de novo synthesis. (B) The relative number of transfused CMFDA-labeled platelets is unaffected by time during normothermia (black lines) or hypothermia/rewarming (grey lines). (C) Removing spleen before start of hypothermia at baseline or after 1h hypothermia does not affect the platelet dynamics throughout hypothermia and rewarming. Line in panel A represents a sigmoidal fitted curve for body temperature with 90% confidence interval, n=5; dots in panel B represent mean ± SD, n=3-8. All bars represent mean ± SD, in panel A n=5, panel C n=7 (splenectomy at hypothermia) and n=8 (splenectomy at baseline). Bars with different letters were significantly different, $P < .05$. *Figure on previous page.*

FIGURE 2. Platelet storage and release in organ sections from hypothermic and rewarmed rat. Representative images of liver, spleen and lung sections of control (A, E and I), normothermia (B, F and J), hypothermia (C, G and K) and rewarmed (D, H and L) rats. Platelets labeled anti CD61 (red) can be seen in the lumen of central veins in the liver (A,B). Hypothermia induces retention of large numbers of platelets in liver sinusoids (C), which is reversed upon rewarming (D). In spleen, platelets are clearly visible in the red pulp, while white pulp with nucleated cells (blue) is devoid of platelets (E-H). Platelets in lung are found within large vessels and capillaries (I-L). Hypothermia induces an increase in the number of platelets in lung (K), albeit to a lesser extent compared to liver, which is reversed upon rewarming (L). Nuclei are stained blue and autofluorescence of hepatocytes and elastic lamina of arteries is visible in green. Notably, autofluorescence of liver from hypothermic rat is increased during hypothermia. Original magnification 400x, insets are 1.5x zoom. Bars are 50 µm. *Figure on next page.*

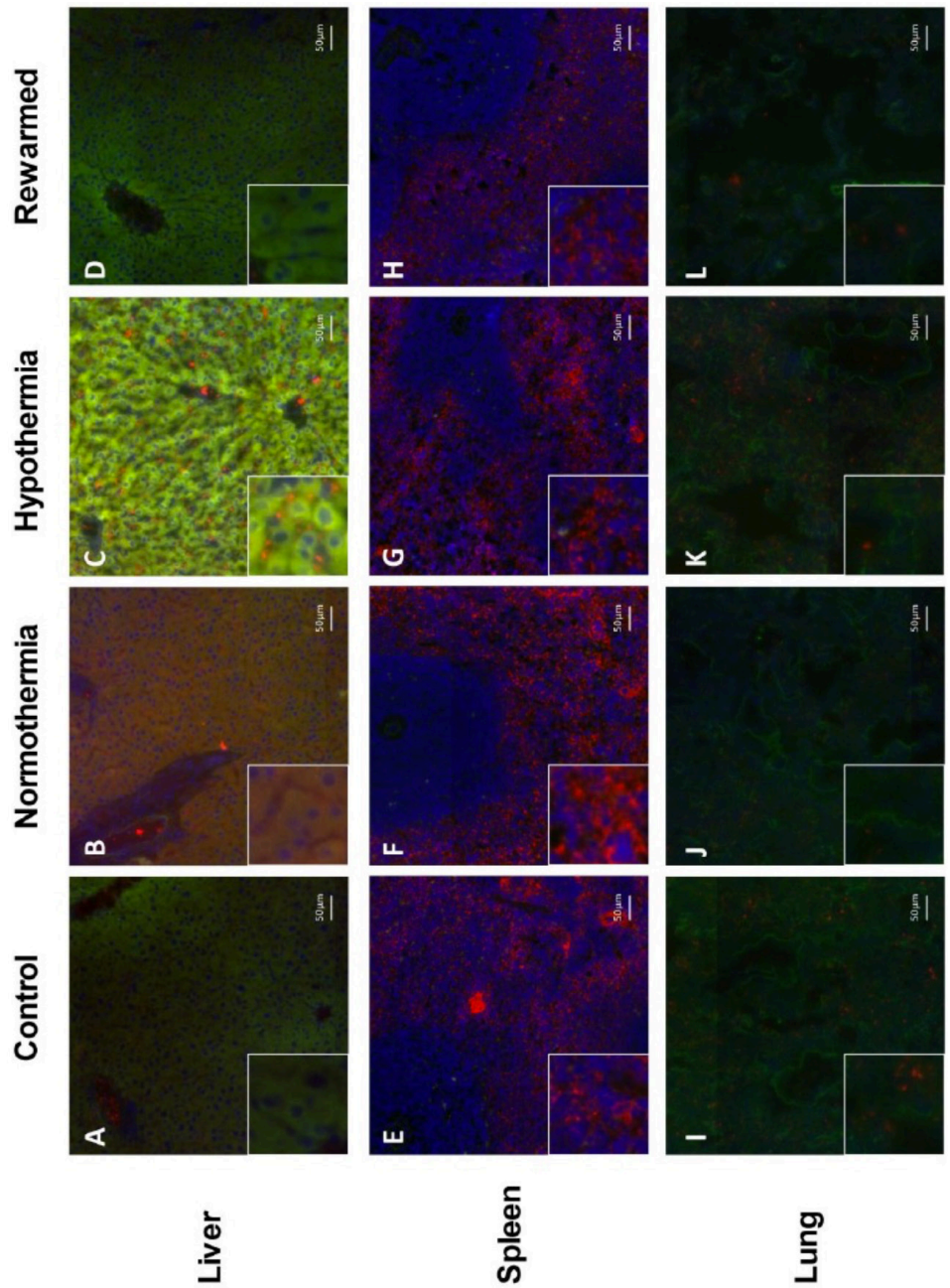

FIGURE 2. Platelet storage and release in organ sections from hypothermic and rewarmed rat.
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TABLE 1. Immature platelet fraction (IPF) does not increase after rewarming from hypothermia. Rats were subjected to hypothermia and rewarming and blood samples were obtained at different timepoints. IPF remains low throughout hypothermia and rewarming, indicating low amounts of newly synthetized platelets throughout the process of body cooling and rewarming. After 30 min rewarming a reduced IPF is seen, demonstrating a relatively fast increased recovery of mature platelets, which normalized after prolonged rewarming. Data are mean ± SD, * P < .05 compared to t= -0.3h.

	Normothermia (37°C)			Hypothermia (15°C)	Rewarming (37°C)	
Time points	Baseline	-0.3h	0h	4h	4.5h	5.5h
% IPF	1.12 ± 0.40	1.14 ± 0.24	1.00 ± 0.70	0.84 ± 0.27	0.56 ± 0.21*	1.12 ± 0.52

Storage of platelets occurs principally in liver sinusoids

To identify the storage site of platelets during hypothermia, we investigated spleen, liver and lung. In control rat liver, platelets were mainly located in the lumen of larger blood vessels at all time-points (Figure 2A-D), specifically within the lumen of central veins, while sinusoids were largely devoid of platelets. Low body temperature, however, led to an accumulation of marginated, non-aggregated platelets in liver sinusoids as compared to non-hypothermic control livers (Figure 2C), whereas the distribution of platelets in central veins remained unaffected. When quantifying the amount of platelets per whole section of liver, hypothermia increased platelet count in liver almost 20-fold (Figure 3A, *P* < .05). Rewarming was associated with a reduction of platelets in the liver (Figures 2D and 3A, *P* < .05) and an almost complete absence of platelets in sinusoids (Figure 2D).

In contrast to the liver, neither lowering the body temperature nor rewarming affected the amount of platelets in spleen (Figure 2E-H and 3B). However, hypothermia increased the platelet count by approximately 2-fold in lung, where platelets mainly accumulated in interstitial capillaries (Figure 2I-L, and 3C; *P* < .05). Similar to platelet retention in the liver, rewarming from hypothermia reduced the platelet count in lung to similar values as control animals. Thus, hypothermia leads to a reversible and profound increase of marginated, non-aggregated platelets in liver sinusoids and in addition induces a reversible and minor rise in the number of platelets in lungs.

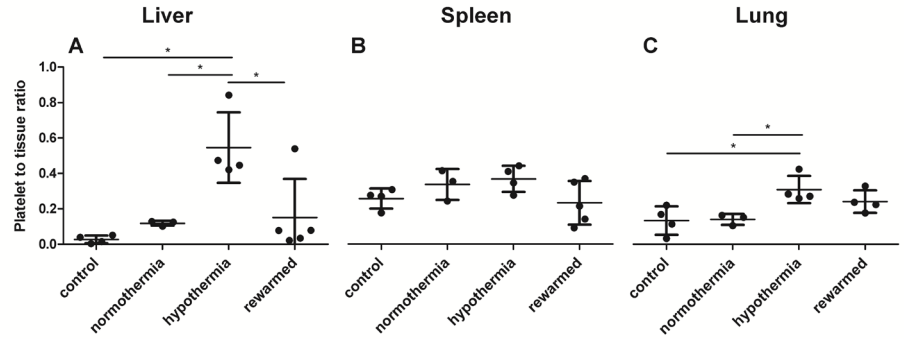

FIGURE 3. Hypothermia increases platelets mainly in liver. A) Platelet amount in liver sections increased during hypothermia, which reversed during rewarming. B) Platelet amount in spleen tissue did not change throughout hypothermia rewarming, compared to normothermia and control group. C) Platelet to lung tissue ratio increased slightly during hypothermia and seemed to reverse slowly during rewarming. Amount of platelets per liver and lung is expressed as ratio between CD61 positive count (platelets) and DAPI count (nuclei), whereas amount of platelets per spleen is expressed as ratio between CD61 positive area and total tissue area within the red pulp of spleen. Large scale sections were imaged and analyzed with TissueFAXS and ImageJ software. Groups: control (n=4), normothermia (n=3), hypothermia (n=4) and rewarmed (n=5),* P < .05.

As the largest increase in platelet accumulation was found in liver, we next quantified the number of platelets per volume of liver tissue to estimate the relative number of circulating platelets that might be stored in the liver during hypothermia (Table S1). The number of platelets per μm^3 of liver tissue was increased more than 15-fold during hypothermia, as compared to control animals; rewarming reduced this number by more than half. Given the reduction in the number of circulating platelets (Figure 1A), and an average total blood volume of a rat of 64 ml/kg[33], the estimated total number of platelets that is reversibly removed from the circulation during hypothermia is 5.9 ± 1.6 $x10^9$ platelets. To compare this to the number of platelets that was stored in liver during hypothermia, we calculated the liver volume from the body weight of the animal (Table S1). Thereby, we estimated that 3.5 x 10^9 platelets are stored per liver in hypothermia in addition to 0.2 ± 0.2 x 10^9 platelets already present in control liver. Thus, platelet storage in liver is the principle mechanism of the reversible hypothermia induced platelet reduction, with a modest additional contribution from lung, but not spleen.

Temperature dependent margination results in storage and release of platelets via liver sinusoids

To further substantiate that retention of platelets within the liver is due to margination or adherence within liver sinusoids during hypothermia, PE-labeled platelets were visualized by intravital imaging in mice throughout cooling and rewarming. At baseline, during normothermia (body temperature 36.9 ± 0.4°C), most platelets were rapidly circulating with occasional margination to the sinusoidal lining (Video 1), as known from patrolling "touch-and-go" platelets[37], while only few platelets marginated to sinusoids (Figure 4A). Cooling to a body temperature of 19.8 ± 0.3°C induced a gradual decrease in blood flow that was closely associated with higher numbers of platelets marginating in sinusoids (Figure 5, Video 2), as well as a higher amount of platelets adhering to liver sinusoidal cells (Figure 4B). Remarkably, during hypothermia some sinusoids were not perfused at all while being completely filled with platelets (Video 2). During rewarming, blood flow increased and platelets were released from the sinusoids within 30 minutes. Moreover, as compared to hypothermia, sinusoids were devoid of platelets, although some platelets remained adherent (Figure 4C, Video 3). Quantitative analysis demonstrates a 3.5-fold increase in the relative number of platelets retained within liver sinusoids during hypothermia in the mouse, as compared to normothermia (Figure 4E, $P < .05$). Furthermore, rewarming restored the number of retained platelets in the liver to values similar to baseline (Figure 4E, $P < .05$). The platelet margination demonstrates a high negative correlation with body temperature (Figure 4F, Pearson's r = -0.95, $P < .05$). Taken together, hypothermia induced a temperature and blood flow dependent margination of platelets to liver sinusoids which reversed rapidly during rewarming.

Hypothermia neither activates, nor affects functionality of platelets

Next, we analyzed whether *in vivo* cooling induced activation of platelets and the hemostatic system, by measuring P-selectin (CD62P) expression on rat platelets (Figure S1) and plasma levels of D-dimer. The relative amount of P-selectin positive platelets at baseline was 9.7 ± 2.7% (naïve) and 83.2 ± 6.5% (ADP stimulated, Figure 6A). In addition, the basal P-selectin expression (geometric mean) was 2.2 ± 0.2 and increased to 7.3 ± 1.8 after activation (Figure 6B). Hypothermia nor rewarming affected the number of P-selectin positive platelets and the expression of P-selectin per platelet with similar results obtained for transfused CMFDA-labeled platelets (Figure 6A-B). After transfusion (t = 10 min), the number of P-selectin positive CMFDA labeled platelets amounted 6.8 ± 2.1 % (naïve) and 79.4 ± 6.0% (ADP stimulated), while their P-selectin expression was 2.2 ± 0.2 (naïve) and 7.2 ± 1.4 (ADP stimulated). Both fluorescence intensities remained stable throughout subsequent hypothermia and rewarming. Together, hypothermia and rewarming did not activate platelets, as illustrated by the unaffected P-selectin expression and amount of P-selectin positive platelets throughout cooling and rewarming. In addition, platelet isolation, CMFDA-labeling and transfusion did not activate the CMFDA-labeled platelets, which remained functional in circulation as measured by ADP activation *ex vivo*. Plasma levels of D-dimer remained below threshold and detectable levels, i.e. less than 500 and 150 µg/L respectively, whereas serum control was high (data not shown). Taken together, we substantiate that during *in vivo* hypothermia and rewarming no signs of platelet activation or clotting and fibrinolysis were present as measured by platelet P-selectin expression and plasma D-dimer.

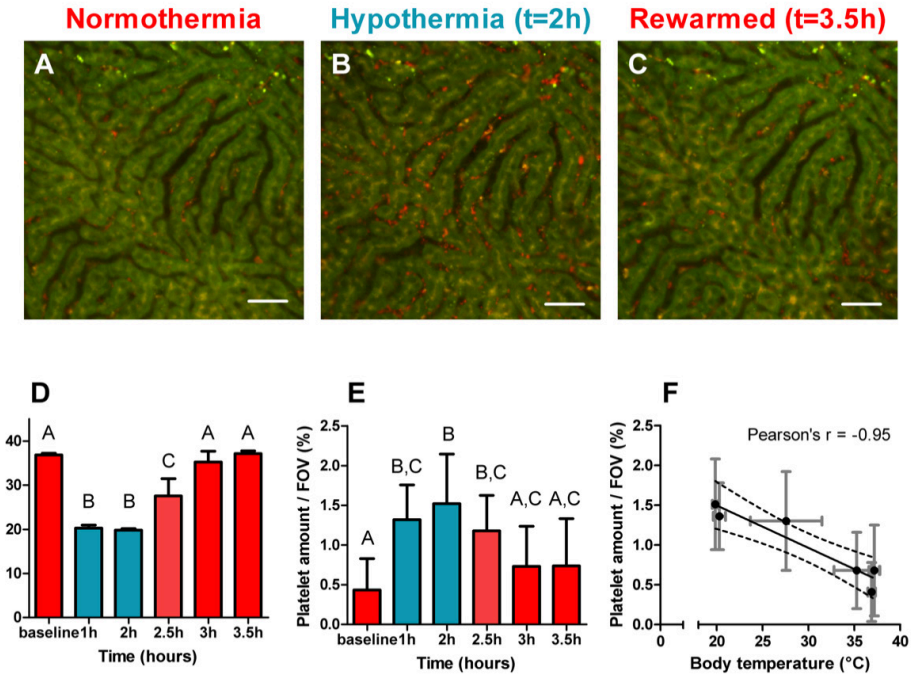

FIGURE 4. Platelet storage and release in mouse liver induced by hypothermia and rewarming. A-C) Representative field of view (FOV) during intravital microscopy imaging throughout normothermia (A), hypothermia (B) and at the end of rewarming (C). Anti-CD49b PE-labeled platelet accumulation (red) can be seen in several hypothermic liver sinusoids, which is reversed during rewarming. Liver autofluorescence (green) lines the sinusoids. Original magnification 400x, scale bars are 50µm. D) Rectal body temperature measurements throughout hypothermia and rewarming. Bars in blue represent hypothermia, bars in red normothermia and rewarming. E) The relative number of platelets per FOV increased during hypothermia, which was reversed

during rewarming. Data represent mean ± SD of n=6 mice. F) Platelet amount per FOV is negatively correlated with body temperature (Pearson's r = -0.95, P < .05), 95% confidence interval of linear fitted curve is depicted by dotted lines. Bars with different letters were significantly different from each other, P < .05.

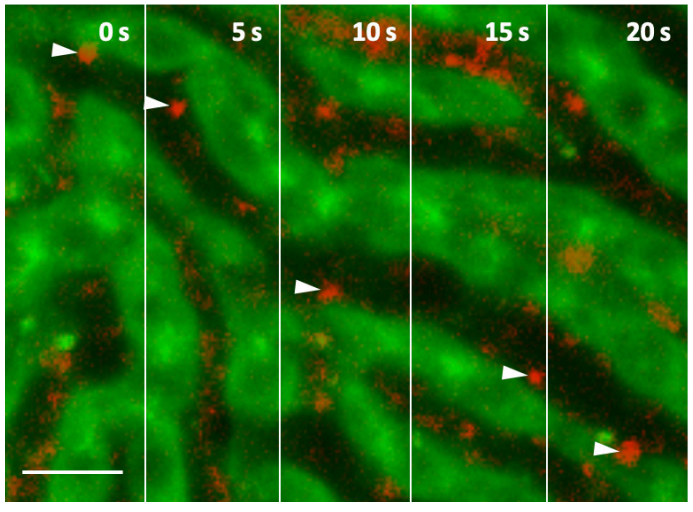

FIGURE 5. Platelet margination in liver sinusoids during hypothermia. Some platelets (red) were found marginating in the sinusoids during hypothermia; captured is the margination of a single platelet (white arrowhead) followed for 20 seconds. Blood flows from top left to right bottom. Images are a digital zoom and tilted 90° to the left from original timelapse (Video 2). Body temperature in this mouse was 19.5°C (t=2h). Scale bar 50µm, original magnification 400x.

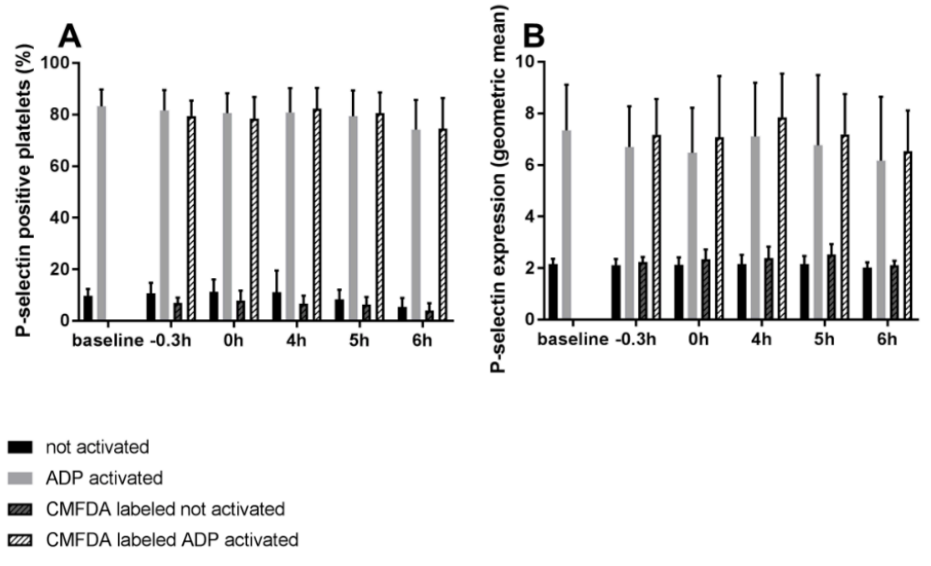

FIGURE 6. Platelets in circulation are not activated by hypothermia and rewarming. A) Percentage of P-selectin positive platelets (black bars) increases after stimulation with 10µM ADP (light grey and dashed bars). B) P-selectin expression increases on platelets stimulated with ADP. Blood samples were taken at all time-points: t= baseline normothermia before transfusion of CMFDA-labeled platelets, t=-0.3h normothermia 10min after infusion of platelets, t=0h normothermia 30min after infusion prior to cooling, t=4h hypothermia (15°C 3h), t=5h rewarming to 37°C, t=6h maintaining 37°C for 1 hour. Note that at baseline CMFDA-labeled platelets are absent. Bars are mean ± SD.

DISCUSSION

Thrombocytopenia during hypothermia is governed by reversible storage and release, mainly in liver sinusoids

This study demonstrates unequivocally that thrombocytopenia during hypothermia results from storage of platelets, followed by subsequent release of the same platelets upon rewarming. This is principally evidenced by the recovery of the same percentage of circulating CMFDA labeled fluorescent platelets following rewarming of hypothermic rat as was present during baseline. Moreover, the rapid restoration of total platelet count upon rewarming excludes destruction of platelets during hypothermia and regular *de novo* synthesis upon rewarming as a main mechanism, as platelet synthesis from bone marrow takes 24-48 hours to reverse thrombocytopenia[11, 38]. Absence of *de novo* synthesis is further substantiated by a stable low number of immature platelets throughout hypothermia and subsequent rewarming. Consequently, it is unlikely that megakaryocyte rupture[39] may serve as a lead mechanism restoring platelet count upon rewarming. In addition, given that IPF was stable and platelets recovered rapidly, hypothermia induced bone marrow failure is also excluded. Importantly, we exclude a crucial role of spleen in platelet storage and demonstrate that liver sinusoids constitute the main compartment of platelet storage during hypothermia, which release platelets rapidly upon rewarming. In addition, a smaller proportion of platelets is stored in lung capillaries, showing a much slower release profile following rewarming. Further, we excluded platelet activation, DIC and trapping of platelets within immune-complexes as a contributor to platelet storage, since platelets did not increase P-selectin expression, (micro)thrombi were absent in liver and lung, levels of D-dimer remained low throughout hypothermia and rewarming, and platelets in hypothermic liver sinusoids did not form large aggregates. Thus, *in vivo* hypothermia may be safely used to lower platelet amounts temporarily while preserving their functionality in healthy subjects.

Margination in liver sinusoids as mechanism governing reversible platelet storage and release

We showed that platelet margination to liver sinusoids is a temperature dependent, rapid and reversible phenomenon. By intravital imaging of mouse liver microcirculation we demonstrated blood flow to reduce and platelet margination to occur during one hour of hypothermia, which both rapidly reversed during 30 minutes to one hour of rewarming. Mouse hypothermia to 20°C and rat hypothermia to 15°C both increased platelet amount in liver several fold and the amount in rat liver accounted for the majority of platelets that had exit the circulation. We demonstrated that platelet margination was highly correlated with lowering body temperature and when animals were rewarmed, platelet amount in liver and circulating platelet count recovered to normothermic level. Our data therefore identify margination of platelets as the mechanism conferring reversible platelet storage in liver during hypothermia. Nevertheless, the activators of hypothermia induced margination remain to be identified. Several factors may contribute to margination by influencing either attaching forces (i.e. membrane glycoproteins on endothelial cells and platelets, and the distribution of platelets towards the vessel wall) or detaching forces (i.e. shear stress). Rheological parameters such as reduced blood flow and increased blood viscosity constitute one of the most likely candidates. Arai et al. and Zarins and Skinner showed that lowering body temperature leads to a reduced cardiac output, which results in lower shear stress.[40, 41] Thereby, the reduced blood flow that occurs increases the contact-time of platelets with endothelium and shifts the balance towards more attaching forces favoring margination to liver sinusoids.[42] Additionally, an increased hematocrit, as likely occurs as a consequence of hemoconcentration at low temperature[43], increases platelet distribution near the vessel wall stimulating platelet margination[44, 45]. Indeed, hematocrit in our rats increased from 0.40 ± 0.08 L/L at baseline to 0.50 ± 0.04 L/L during hypothermia and recovered to 0.42 ± 0.02 L/L after rewarming. Moreover, platelet shape changes from disc to sphere during hypothermia[46], further stimulating platelet margination[44, 47, 48]. Finally, hypothermia and reduced flow or flow cessation may induce endothelium activation with increased expression of adhesion molecules to further increase the attaching forces.[49, 50] Reduced body temperature, blood flow and increased hematocrit are also observed in hibernating mammals and recently is has been shown that platelet storage in liver also occurs in hibernating ground squirrels [66]. Hence, storage of platelets during hypothermia is likely mediated by margination to sinusoidal endothelium secondary to a reduced blood flow, combined with increases in hematocrit. Conversely, the rise in cardiac output upon rewarming, and hence blood flow, likely stimulates detachment of the platelets and governs their reappearance in the circulating pool.

Cooling/rewarming does not induce platelet activation

Our data demonstrate that *in vivo* cooling and rewarming does not induce platelet activation, dysfunction or hyperactivity. Like us, previous exploration of platelet activation or altered function from both *ex vivo* and *in vivo* cooled platelets also measured P-selectin expression following stimulation with ADP or other agonists.[14, 51-53] These studies generally cool from 20°C down to 0-4°C. However, these studies report both inhibition of basal platelet function as well as (hyper)activation upon addition of agonists[14, 52, 53] with effects being reversed by rewarming either *in vivo* or

ex vivo[51, 52]. Therefore, in our study, a possible effect of *in vivo* 15°C cooling on platelet activation or function may have been offset by *ex vivo* rewarming to 20-22°C room temperature prior to flow cytometry measurements. If so, this would merely signify that hypothermia effects on platelet activation are reversible, implying that activation does not contribute to platelet storage during hypothermia as this storage is also observed at 20°C.

Biomedical relevance

Our data may bear considerable relevance to platelet preservation. *Ex vivo* cooled platelets are rapidly and irreversibly cleared by the liver from the circulation after transfusion[54, 55], whereas we demonstrated in this study that *in vivo* cooled platelets are stored reversibly. Our models thus offer tools to disclose the mechanism of (reversible) platelet clearance by comparing both. Possibly, effects of *ex vivo* cooling on platelets (e.g. deglycosylation of glycoproteins or clustering of glycoprotein GPIbα[19, 56-59]) do not occur during *in vivo* cooling. Alternatively, the *in vivo* hypothermia and rewarming of the liver may affect the mechanisms normally employed by normothermic liver to clear cooled platelets, such as recognition and phagocytosis by Kupffer cells and hepatocytes[19]. In addition, the difference between clearance of *in vivo* and *ex vivo* cooled platelets may originate from various other sources, including blood itself.

It may be that liver hypothermia, and subsequent rewarming, is crucial in the reversibility of storing either *in vivo* or *ex vivo* cold exposed platelets. This strategy might therefore be utilized to transfuse cold stored platelets to patients who, or whose liver, can briefly be subjected to therapeutic hypothermia, e.g. peroperatively or in the intensive care unit. Another use of platelet storage in hypothermic liver may be in transplantation. Cooling of the donor prior to harvesting the liver would induce a much higher amount of functional donor platelets in the graft, which may subsequently boost or sustain their documented liver regenerating effect in the recipient following transplantation[60].

The management of coagulation in patients with accidental or therapeutic hypothermia may improve by a better understanding of the effects of temperature on the hemostatic system and which effects are reversible by rewarming. This knowledge may help in correcting the increased bleeding and mortality risk for trauma patients with hypothermia[61, 62].

Future studies may identify targets for novel anticoagulant drugs, inducing a reversible suspended coagulation. Mimicking the mechanisms of hypothermia induced thrombocytopenia may rapidly induce a state of profound and reversible thrombocytopenia without the need to reduce body temperature. Such a strategy may be utilized during defined periods of increased thrombotic risk, such as in hospitalized

patients, specifically in the critically ill who are at great risk of deep vein thrombosis despite the use of thromboprophylaxis[63]. Additionally, patients with myocardial infarction and those at risk of cerebrovascular disorders may benefit from this strategy since a suspended coagulation is essential in limiting thrombus propagation and preventing ischemic events[64, 65].

Taken together, our data contribute to an improved understanding of the hemostatic system under influence of body temperature. We demonstrated that thrombocytopenia during hypothermia occurs via platelet storage mainly in liver sinusoids. Platelets marginate to the sinusoidal endothelium during hypothermia and are released during rewarming due to the temperature dependent change of blood flow. No signs of platelet activation, platelet dysfunction or intravascular coagulation were found, such in sharp contrast with studies in *ex vivo* cold stored platelets. Deciphering the molecular mechanisms governing reversible cold storage of platelets in liver sinusoids without their activation, while maintaining platelet functionality, may enable transfusion of cold stored donor platelets, improve liver regeneration after transplantation, contribute to management of hemostasis in trauma patients and may contribute to antithrombotic therapies in the critically ill.

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E.L.d.V is PhD candidate at University of Groningen and this work is submitted in partial fulfillment of the requirement for the PhD.

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SUPPLEMENTAL DATA

TABLE S1. Platelet count reduction accounted for storage in liver during hypothermia.

	Rat weight (g)	Liver mass (g)[a]	Liver volume (10^{12} µm^3) [b]	Platelet count per µm^3 liver (10^{-5}) [c]	Platelet count per whole liver (10^9) [d]
Control	364 ± 28	13.9 ± 1.1	13.1 ± 1.1	1.84 ± 1.59	0.2 ± 0.2
Hypothermia	364 ± 13	13.9 ± 0.5	13.1 ± 0.5	28.2 ± 7.92	3.7 ± 1.0

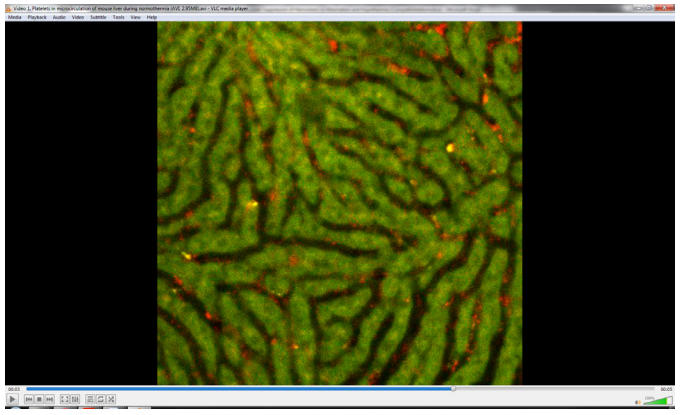
Assuming liver mass [34] = (40.5 x rat weight – 824.4)/1000

Assuming liver density [35, 36] = 1.06 mg/µL

Platelet count per 4 µm liver section x area of section

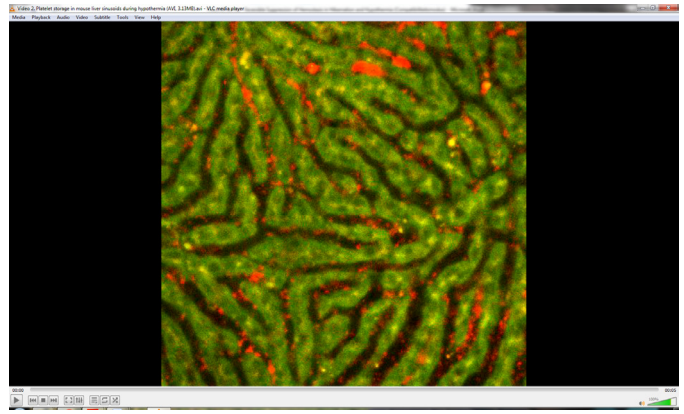
Platelet count per µm^3 liver * liver volume

With calculations and some assumptions the amount of platelets in whole livers was estimated. Platelet amount in liver during hypothermia increases approximately 20-fold compared to control. Data are mean ± SD, n=3-5.



VIDEO 1. Platelets in microcirculation of mouse liver during normothermia. During mouse normothermia (body temperature of 36.4°C), anti CD49b labeled platelets (red) pass rapidly through liver sinusoids with few marginated platelets. Liver autofluorescence (green) is visualized as average intensity of all frames to enhance contrast with microcirculation. Original magnification 400x, timelapse acquisition speed 1Hz, videoclip framerate 10 frames per second.
A snapshot of the video is demonstrated, video accessible via QR-code or tinyurl.com/EdV-chapter5video1 or hibernation.nl/storporage/thesisDeVrij/

All videos from this chapter are online available at www.hibernation.nl/storporage/thesisDeVrij/



VIDEO 2. Platelet storage in mouse liver sinusoids during hypothermia. During mouse hypothermia (body temperature of 19.5°C), anti CD49b labeled platelets (red) pass slowly through liver sinusoids with many marginated platelets. In several sinusoids platelet margination can be observed. Liver autofluorescence (green) is visualized as average intensity of all frames to enhance contrast with microcirculation. Original magnification 400x, timelapse acquisition speed 1Hz, videoclip framerate 10 frames per second.
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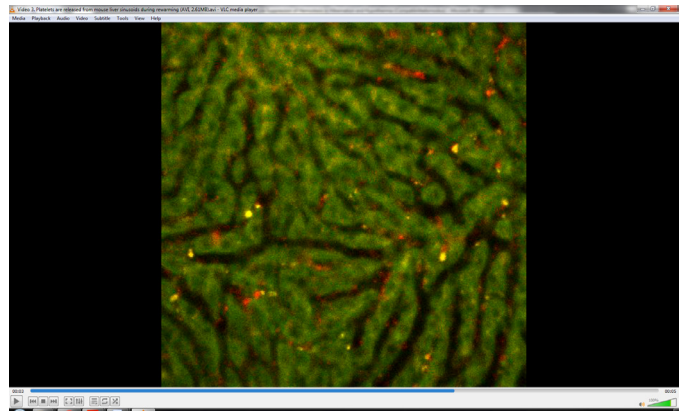

VIDEO 3. Platelets are released from mouse liver sinusoids during rewarming. During mouse rewarming (body temperature of 36.7°C), anti CD49b labeled platelets (red) pass again rapidly through liver sinusoids with less marginated platelets than during hypothermia. Liver autofluorescence (green) is visualized as average intensity of all frames to enhance contrast with microcirculation. Original magnification 400x, timelapse acquisition speed 1Hz, videoclip framerate 10 frames per second.
A snapshot of the video is demonstrated, video accessible via QR-code or tinyurl.com/EdV-chapter5video3 or hibernation.nl/storporage/thesisDeVrij/

FIGURE S1. Flow cytometry of rat blood samples during hypothermia and rewarming. Schematic overview of gating strategy. Identification of the platelet population (red) by a relative small forward and side scatter (A), confirmed by analysis of CMFDA labeled and transfused platelets (green) (B). Absence of CMFDA labeled platelets prior to their transfusion both in naïve (C) and ADP stimulated platelets (E). Persistent presence of CMFDA labeled platelets following hypothermia and rewarming (D, t=5h), which remain as functional as recipient platelets following ADP stimulation (F).

CHAPTER 6

Temperature Dependent Platelet Shape Changes through Tubulin Polymerization in Hibernating and Non-Hibernating Mammals

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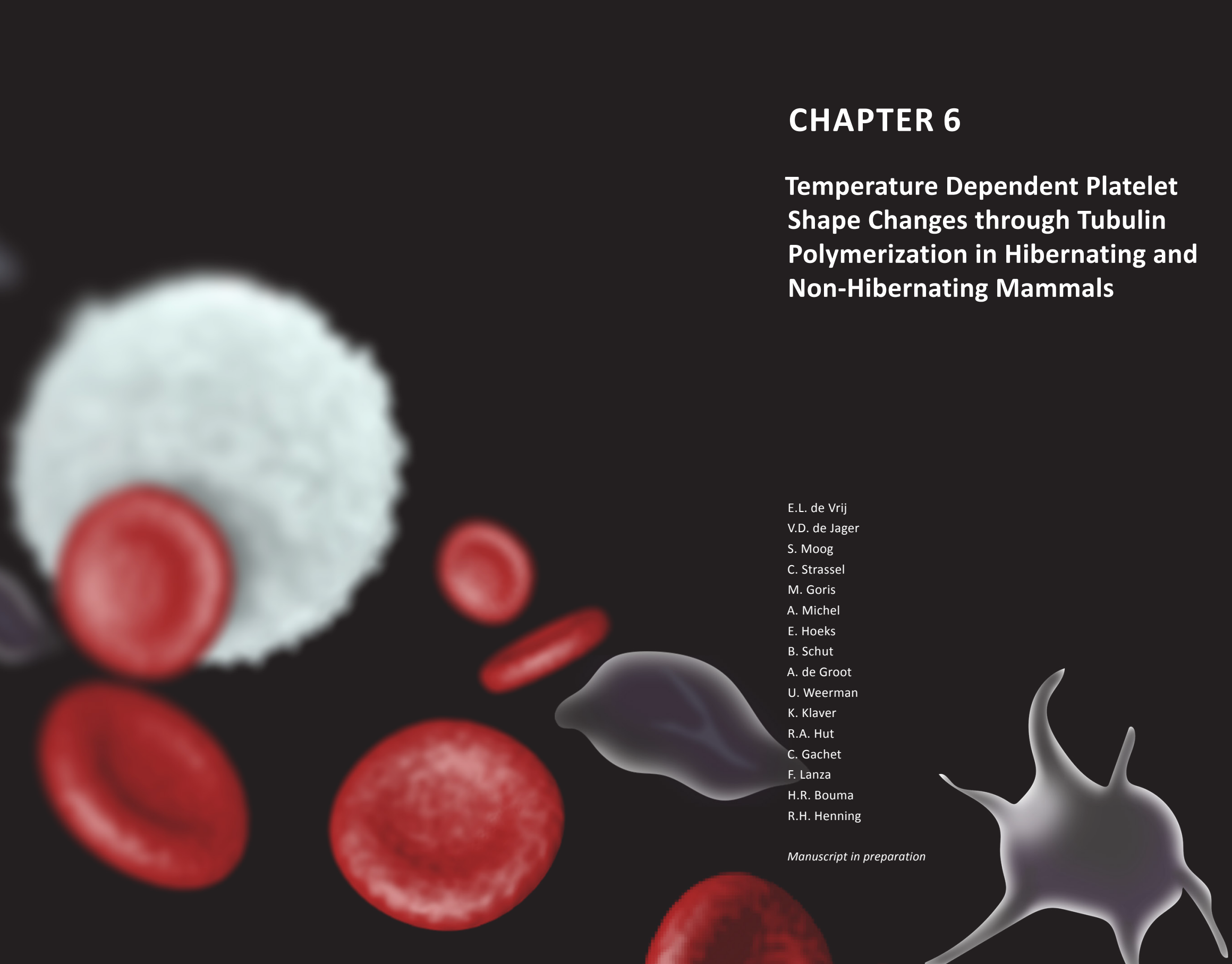


ABSTRACT

Background

Current room temperature storage limits platelet shelf-life to 5-7 days due to storage lesions and bacterial growth. *Ex vivo* cooling of human platelets induces shape changes and rapid clearance after transfusion. Hibernator platelets change shape differently with cooling and are not cleared after transfusion. Unraveling the cold resilience of hibernator platelets may unlock cold storage of human platelets. Possibly, differences in cold induced platelet shape change are involved.

Objectives

To compare temperature dependent shape changes of platelets from hibernating and non-hibernating mammals and to reveal the underlying molecular mechanism governing the reversibility in shape.

Methods

Shape and cytoskeletal rearrangements of platelets from hibernating and non-hibernating hamster and torpid mouse were compared with non-hibernating human, rat and mouse throughout in vivo and in vitro cooling and rewarming.

Results

Cooling of hamster platelets, either during torpor in hibernation (body temperature ~9°C) or *in vitro* (4°C), induced formation of spear shaped platelets that maintained polymerized tubulin, with full reversal upon arousal or rewarming. Daily torpor in mouse (body temperature ~25°C) did not affect platelet shape. In contrast, cooling of non-hibernator platelets induced spherical platelet shapes with depolymerized tubulin and filopodia formation, which occurred without degranulation. Rewarming of non-hibernator platelets re-polymerized tubulin, thus reverting platelet's shapes from spherical to discs via an intermediate spear shape, which occurred independently of plasma factors.

Conclusions

Hibernator platelets possess cold-stable tubulin that remains polymerized within disc and spear shaped platelets under cold conditions, potentially contributing to their cold resilience. Cooling of non-hibernator platelets depolymerizes tubulin, but allows a platelet autonomous normalization to disc shape during rewarming via an intermediate spear shape. Elucidating the mechanism that stabilizes tubulin in hibernators may enable cold-resilience of human platelets and subsequent cold storage for transfusion.

INTRODUCTION

Platelet concentrates can be life-saving products in conditions of thrombocytopenia or platelet dysfunction [1]. In contrast to other blood cells [2], platelets must be stored at 22-24°C because refrigeration induces rapid platelet clearance by liver macrophages and hepatocytes after transfusion [3-5]. Enabling cold storage, however, limits bacterial growth and may increase shelf-life [6, 7], thus reducing expiration of platelet units and the ensuing yearly economic loss [8-10].

Nature's solution to enable cooling of platelets without inducing subsequent clearance may be found in hibernating mammals, specifically in species with body temperatures below 10 °C during hibernation [11, 12]. Hibernation consists of 2 phases: torpor and arousal. Torpor bouts with low body temperature last from several days to weeks and are alternated by short phases of interbout arousal wherein metabolism and temperature recover to euthermic level within 1.5-2h. The hypothesis that hibernators may hold the key to safe cooling of platelets is underscored by the observation that cold storage (4°C) of platelets from 13-lined ground squirrel, a hibernator, does not induce their rapid clearance upon transfusion in summer animals [13] as opposed to cold stored and transfused human platelets. During torpor, hibernators remove up to 90% of their platelets from circulation and their amounts rapidly recover during arousals [11, 13, 14]. Likewise, reversible thrombocytopenia is also observed in non-hibernating species during periods of hypothermia [11, 15, 16]. Moreover, we demonstrated previously that it is the decrease in body temperature that drives removal of platelets from the circulation of both hibernating and non-hibernating animals [11]. Recently, we demonstrated cooling induced thrombocytopenia to depend on accumulation of platelets in liver via margination to liver sinusoids during torpor in hamster (de Vrij et al., submitted) and in hypothermic rat and mouse (de Vrij et al., submitted), thus extending the previously reported increase in platelets in liver of torpid squirrel [17].

In addition to storage in liver, platelets also undergo shape changes in response to cooling, a feature that has been proposed crucial to their organ storage during torpor [18]. In hibernators, both during torpor and *ex vivo* cooling, platelets change shape from disc to spear-like with elongated and centralized rods of tubulin [13, 18]. *Ex vivo* cooling of platelets from non-hibernating mammals, including humans, induces different shape changes – generally from a smooth-surfaced disc into a sphere with membrane protrusions, resembling activated platelets [19-21]. Despite these observed differences in shapes and potential of platelets to remain in circulation after transfusion, still much is unclear about the underlying mechanisms governing platelet shape change

in hibernators and non-hibernators. Therefore, we first assessed platelet shape during deep torpor in the Syrian hamster and daily torpor in the mouse. Next, by light-, fluorescence and electron microscopy studies we set out to determine the temperature dependency of shape changes of platelets from hibernators and non-hibernators by *ex vivo* cooling and rewarming. Finally, we determined whether shape change is platelet autonomous, by comparing *in vivo* shape change with *ex vivo* shape change in the absence of humoral factors and determination of the underlying cytoskeletal determinants.

METHODS

Animals

Syrian hamsters (*Mesocricetus auratus*, age 3 months*)* were ordered from Envigo USA and kept at 'summer' photoperiod light:dark cycle (L:D) of 14h:10h at 20-22°C until induction of hibernation according to the protocol described below. Wistar rats (*Rattus norvegicus albinus*, 300 grams) and C57Bl/6J mice (age 6 months) were ordered from Envigo Netherlands and housed at 20-22°C with standard L:D cycle of 12h:12h. All animals were fed *ad libitum* with standard lab chow and water. All animal work was according to relevant national and international guidelines, and was approved by the Institutional Animal Ethical Committee of the University Medical Center Groningen.

Hibernation in hamsters

After 7 weeks at 'summer' photoperiod, Syrian hamsters were housed at 'fall' photoperiod: L:D of 8h:16h during 7 weeks, followed by constant darkness at and ambient temperature of 5°C ('winter' period) [11]. Passive infrared sensors coupled to a computer system monitored individual movements. Summer and winter euthermia (SE and WE, respectively) were defined as a euthermic body temperature (~37˚C) during 'summer' and 'winter' photoperiods in absence of any torpor bouts. Torpor (T) was defined as >24 hours of inactivity. Arousal (A) was induced by handling the animals, and was defined as a body temperature of ≥35˚C at >1.5 hours after induction. Torpor was confirmed in all animals by oral temperature measurements.

Daily torpor in mice

A working for food protocol was applied to induce serial daily torpor in mice. Briefly, a small food pellet was delivered in the mouse cage after a set number of running wheel revolutions using computer controlled pellet dispensers. During a reward reduction phase, workload was increased daily resulting in less food per revolution. Ultimately, the high workload relative to low food reward results in daily torpor in these mice [22]. Throughout the experiment, body mass was closely monitored and workload levels were individually titrated to maintain body mass above 75% of its initial value. A euthermia control group received food *ad libitum*.

Blood sampling and platelet isolation

Animal blood was obtained under anesthesia (2% isoflurane in air/O_2) from the abdominal aorta into one-tenth volume 3.2% sodium citrate. Rat and mouse platelet rich plasma (PRP) was prepared by adding 0.4 mL Buffered Saline Glucose Citrate (BSGC: NaCl (116 mM), trisodium citrate (13.6 mM), Na_2HPO_4 (10.8 mM), KH_2PO_4 (1.6

mM), D-glucose (11.0 mM), pH = 7.38) per mL of blood and centrifugation at 160g x 10 minutes at 24°C without brake. Citrated human venous blood was obtained with informed consent by the donors and approval by the local Ethical Committee based on the Helsinki Declaration of 1975, as revised in 2013. Human PRP was collected from blood after centrifugation (250g x 15 minutes), and left to rest at 37°C for 10 minutes. Washed platelets were prepared according to a published protocol [23] by centrifugation of PRP containing 10 U/mL heparin and 0.5 µM PGI_2 at 2,200g x 15 minutes, followed by resuspension of the platelet pellet in Tyrode's albumin solution. After 10 minutes incubation at 37°C, 0.5 µM PGI_2 was added and centrifuged again (1900g x 8 minutes). The platelet pellet was then resuspended in Tyrode's albumin solution (containing 0.5 µM PGI_2), incubated for 10 minutes at 37°C and centrifuged again (1,900g x 8 minutes) after adding 0.5 µM PGI_2. The pellet was resuspended again in Tyrode's albumin solution (containing 0.02 U/mL apyrase) and the platelet count adjusted to 300,000/µL.

Ex vivo cooling and rewarming of platelets

Blood samples from hamster, rat, mouse and human were subjected to *ex vivo* cooling and rewarming, as well as human PRP and washed platelets. Bloodsmears were obtained at baseline/euthermia (37°C), after 1 hour of cooling (4°C), and after 30 minutes and 2 hours rewarming (37°C). After each incubation moment, human PRP and washed platelets were fixated in 9 volumes 4% phosphate buffered formaldehyde and centrifuged onto poly-L-lysin coated slides (Thermo Scientific) by 300 rpm for 5 minutes (Cytospin 4 Cytofuge, Thermo Fisher Scientific). To examine cytoskeletal involvement in shape change, colcemid (10 µM), cytochalasin D (10 µM), and nocodazole (10 µM) were added to the mouse blood samples 10 minutes prior to rewarming.

Light microscopy morphological analysis

Hamster blood smears were fixated in methanol for 5 minutes and air-dried afterwards. Slides were stained with Giemsa stain (1:20 v/v in deionized water) for 15 minutes at room temperature, rinsed in deionized water, air-dried and embedded in dibutylphthalate polystyrene xylene. Quantification of platelet shapes was performed by light microscopy (Nikon Eclipse 50i) in a blinded fashion. The shapes of one hundred platelets were determined per smear and divided into two categories: discoid/spherical and spear-shaped. Albeit semantics, given its pointy endings we propose 'spear' shape, an umbrella term also entailing javelin and *pilum*, to be more in place than 'elongated' or 'spindle' shaped, as a well-balanced athletic counterpart to the disc shape.

Fluorescence microscopy cytoskeletal analysis

Blood smears were air-dried and stored overnight at room temperature or fixated for 2 minutes in ice cold acetone and stored -20°C. The next day, smears were fixated for 5 minutes with 4% phosphate buffered formaldehyde and rinsed with PBS, followed by permeabilization with 0.1% Triton X-100 (T8787, Sigma) for 5 minutes at room temperature and rinsing with PBS. Smears and cytospin spots were incubated for 1 hour with mouse IgG1 anti α-tubulin (T9026, Sigma) and Texas Red phalloidin to label F-actin (T7471, Thermo Fisher) for hamster, rat and mouse; phalloidin–TRITC (P1951, Sigma) for human. Secondary antibodies used were goat anti-mouse Ig FITC for hamster and rat (554001, BD Pharmingen), goat anti-mouse IgG1 Alexa 488 (A-21121, Thermo Fisher) for mouse, and goat anti-mouse IgG Alexa 488 (A-11001, Thermo Fisher) for human, 1% normal rat serum was added to rat samples to prevent non-specific binding. Slides were rinsed with PBS and embedded with Vectashield mounting medium, a coverslip was added and sealed off with nail polish. Blood smears were kept at 4°C in darkness until quantification. Quantification of platelet shapes (discoid, sphere, single activated, aggregated and spear-shaped) was performed with fluorescence microscopy (Leica DM 2000 LED) in a blinded fashion. Images were taken with a Leica DFC3000 G camera, using Leica Application Suite Advanced Fluorescence (LAD AF6000). At least one hundred platelets were analyzed per condition.

Scanning and transmission electron microscopy (SEM and TEM) ultrastructural analysis of platelets

For SEM analysis, platelets in suspension were fixated with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and allowed to adhere to coverslips coated before by incubating with 10% poly-L-lysine in deionized water for 15 minutes on room temperature and drying for 1 hour at 60°C. Samples were dehydrated, air-dried, sputtered with platinum palladium, and examined at 10 kV under a PHENOM scanning electron microscope (PHENOM World).

For TEM analysis, platelets in suspension were fixated in 2.5% glutaraldehyde and 0.25% formaldehyde of either 4°C or 37°C, depending on prior platelet incubation temperature, and then incubated for 1 hour at room temperature, followed by storage at 4°C and subsequent embedding in epon. Thin sections were stained with uranyl acetate and lead citrate and examined under a CM120 transmission electron microscope (FEI, The Netherlands).

Live differential interference contrast (DIC) and fluorescence imaging of morphological dynamics

PRP was diluted in BSGC with 100 nM SiR-tubulin to $4x10^9$/L (Spirochrome, CY-SC006,

Switzerland) and incubated in darkness at 37°C for 2 hours and incubated at 37°C for 30 minutes in single wells with coverslip bottom (MatTek Corporation, P35G-1.0-20-C) coated with 1% albumin (A9418, Sigma), and subsequently cooled for 1 hour on ice. Thereafter, continuous live cell imaging was performed with Deltavision Elite™ Imaging System at 60x and 100x magnification, frame rate of 1 sec^{-1}, DIC exposure time 0.169 second, fluorescence exposure time 0.025 second and laser power at 10%. Platelets were rewarmed from 4°C to 37°C in 15 minutes. Overlay video and single platelet cropping was performed using ImageJ software [24, 25].

Flow cytometry analysis of platelet activation

One microliter of whole blood was diluted 1:25 in PBS and incubated with PE-labeled anti-CD62P (anti-P-selectin, GeneTex 43039) with or without 10 µM ADP for 30 minutes in darkness. The activation was stopped by addition of formaldehyde/PBS (2% v/v) and stored at 4°C in darkness until measurement. Samples were acquired on a Calibur flow cytometer equipped with CellQuest software (BD Biosciences). Platelet populations were gated on cell size using forward and side scatter measuring 20.000 events per sample. Light scatter and fluorescence channels were set at logarithmic gain. Data was analyzed using Kaluza 1.2 software (Beckman Coulter).

Statistical analysis and representation of data

Values are reported as mean ± standard deviation. Differences between groups were analyzed using one-way ANOVA followed by Tukey test with GraphPad Prism software version 6.01 for Windows, GraphPad Software, La Jolla California USA. P-values lower than 0.05 were considered statistically significant.

RESULTS

Platelet shape in deep and daily torpor

During torpor in hamster, body temperature decreased from ~37°C to values close to ambient temperature (~9°C), which reversed upon arousal (Figure 1A). The number of circulating platelets dropped more than 90% in torpor and was fully reversed during arousal (Figure 1B). Next, the platelet shapes were assessed throughout hibernation by Giemsa staining (Figure S1A-B) and fluorescent cytoskeletal staining (Figure 1C-H). Euthermic platelets mainly had a flat round shape (referred to as 'disc' or 'discoid') and contained granules (Figure S1A). Contrarily, only half of the circulating platelets in torpor, i.e. the few that remained in circulation during the thrombocytopenia, were discoid (28 ± 10 $\times 10^9$/L); the other majority was mainly elongated in shape (referred to as 'spear shaped'), and still contained centralized granules (Figure S1B), and a minority became spherical (Figure 1C-H). In arousal, both the relative and absolute amount of disc shaped platelets increased to euthermic level, leading to a reduction in the fraction of spear shaped platelets similar to euthermic level (Figure 1C-D). During summer and winter euthermia nearly all platelets were shaped as discs with a continuous marginal band of tubulin, as demonstrated by immunofluorescent staining of the cytoskeleton (Figure 1D).

Hibernation did not increase the amount of activated or aggregated platelets (as determined microscopically by the presence of membrane protrusions (Figure 1F), clusters of platelets (Figure 1G) and due to the presence of granules (Figure S1A-B)). Thus, the reversible thrombocytopenia during torpor is accompanied by an enrichment of the circulating spear shaped platelet fraction that does not show morphological signs of activation (Figure 1H).

To investigate whether changes in platelet count and shape also occur in daily torpor, featuring less extreme body temperatures, we induced serial daily torpor in mouse by the working for food paradigm[22]. The animals had a daily 4.3 ± 2.5 h reduction in metabolism and body temperature. Oral temperature decreased to 25.3 ± 3.7 °C, which reversed in arousal (Figure 2A). Euthermic platelet count decreased with 35% in torpor (Figure 2B), which reversed in arousal. Nearly all platelets were disc shaped, which remained unaffected during torpor and arousal (Figure 2C). Thus, as opposed to deep torpor in hamster, daily torpor in mouse reduced platelet numbers without initiating shape changes.

Platelet shape throughout ex vivo cooling and rewarming

Since deep and daily torpor differed in extent of body temperature reduction and only

deep torpor demonstrated platelet spear shapes, we next determined whether further reducing platelet temperature *ex vivo* to 4°C would induce similar platelet shapes in hamster and mouse (Figure 3A-E and F-J, respectively), which we then compared with non-hibernators, i.e. rat and human (Figure 3K-O and P-T, respectively). In blood from summer and arousal hamsters, nearly all platelets were disc shapes (Figure 3A). Subsequent *ex vivo* cooling reduced the relative amount of disc shapes and induced formation of spear shapes (Figure 3E). 3D rendering of fluorescent tubulin staining confirmed that hamster spear platelets were not disc shapes viewed from aside (Video S1). The amount of spear shaped platelets already present in torpor blood (~9°C) did not further increase after e*x vivo* cooling to 4°C (Figure S2). Subsequent rewarming led to a time dependent reversal of the spear shaped fraction to discoid platelets (Figure 3A,E). Almost no spherical platelets were observed in all phases (Figure 3B).

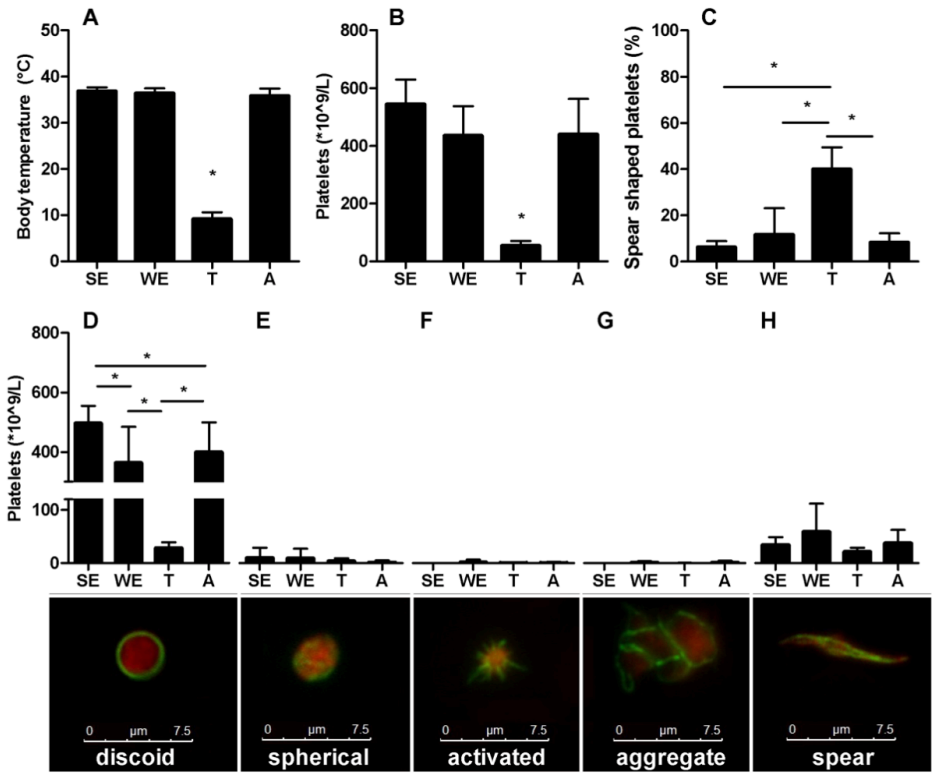


FIGURE 1. Reversible thrombocytopenia during hibernation is characterized by a reduction in discoid platelets and enhancement of spear shaped fraction. *Legend on the next page.*

FIGURE 1. Reversible thrombocytopenia during hibernation is characterized by a reduction in discoid platelets and enhancement of spear shaped fraction. Automated cell count was performed on whole blood and platelet shapes were counted manually on immunofluorescent staining of cytoskeleton of blood smears. (A) Torpor is associated with low body temperature. (B) Platelet count of summer and winter euthermic hamsters ("SE" n=3 and "WE" n=4) cycles from low counts in torpor ("T" n=9) to counts not different from euthermic values during arousal ("A" n=6). (C) Spear shaped platelet fraction increases in torpor and reverts to euthermic level during arousal. (D) Amount of discoid platelets in circulation reduces during torpor and is reversed in arousal. Discoid platelets are characterized by heterogenous actin staining (red) and a circular marginal band of tubulin (green). (E-G) The amount of platelets with interrupted tubulin, of activated platelets (with visible filopodia) and of platelet aggregates (several adhering platelets) were consistently low or absent in bloodsmears from all hamsters. (H) A small amount of spear shaped platelets, with elongated tubulin rods and actin, was present in all bloodsmears - specifically in torpor ("T"). Typical example images with 1000x magnification. Bars are means ± SD, * $P < 0.05$.

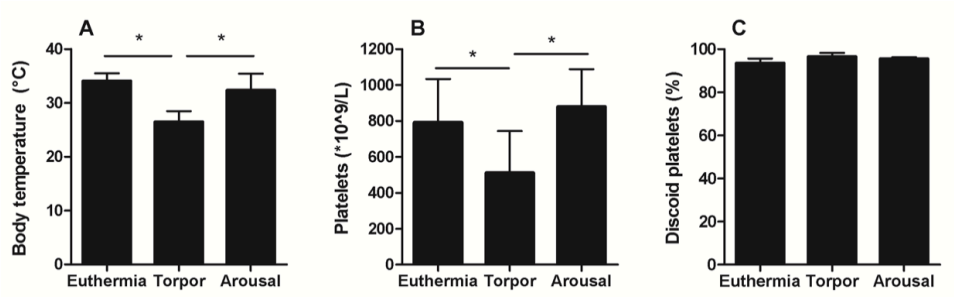


FIGURE 2. Daily torpor in mouse (*in vivo* cooling) induces a platelet count reduction but no platelet shape change. (A) Daily torpor is associated with low body temperature. (B) Platelet count reduced in torpor and recovered to euthermic level during arousal. (C) Platelets remained disc shaped despite body temperature changing from 35°C in euthermia to 25°C in torpor and 34°C in arousal. Bars are means ± SD, * $P < 0.05$.

In contrast, platelets from mouse and non-hibernators (rat and human) responded quite differently, as their *ex vivo* cooling induced a spherical shape in virtually all platelets (Figure 3G,L,Q). Subsequent rewarming partly reversed platelet shape to discs (Figure 3F, K, P). Additionally, a relatively large amount of spear-shaped platelets was formed upon rewarming in mouse (23.9 ± 15.0%, Figure 3J) and rat (28.6 ± 3.0%, Figure 3O), whereas human platelets also formed spear shaped platelets, but to

a much lower amount (Figure 3T, 10.6 ± 4.5%). Thus, in hamster similar reversible platelet shape changes occurred in hibernation and in *ex vivo* cooling/rewarming with formation of spear shapes in torpor and during cooling, which is not restricted to the hibernation season. In contrast, platelets from mouse, rat and human changed into spheres during cooling, which reversed during subsequent rewarming with early formation of spear shaped platelets and ultimately reversion to discs.

To demonstrate that the same platelet undergoes the serial changes in shape, we performed time-lapse imaging of mouse platelets by fluorescent labeling of tubulin. Spherical platelets that were formed during cooling transformed to discs through an intermediate spear shape, albeit with different lag times and rates between platelets (Video S2). The lag time is evidenced clearly after 15 minutes rewarming when platelets have partly recovered to spear shapes with elongated rods of tubulin and partly to disc shapes with a complete marginal band (Video S3). These results further demonstrate that platelets of mouse reversibly changed shape via a spear shape throughout cooling/rewarming. Notably, the spear shape occurs in platelets during cooling in hamster and ground squirrel[13], but during rewarming in non-hibernators.

FIGURE 3. *Ex vivo* cooling does not induce tubulin depolymerization and sphere formation in platelets from hibernating and summer euthermic hamsters, but induces spear shapes unlike non-hibernating mammals (mouse, rat, human). (A, E) Platelets from hibernating (grey bars, arousal) and non-hibernating hamsters (black bars, summer euthermic) shift from disc to spear shape during cooling, which reverts during rewarming. No tubulin depolymerization is seen in hamster platelets. (F) Disc shaped platelets from mouse depolymerize the tubulin marginal band (green) and become spherical during cooling (G) and disc and spear shape during rewarming (F, J). Similar to mouse, rat platelets (K-O) and human platelets (P-T) change shape during cooling, from discoid to spheres (L, Q). Almost no platelet showed signs of activation by filopodia formation or aggregation during cooling or rewarming. Different than hamster, in mouse, rat and human, spear shaped platelets were not shaped during cooling, but during rewarming. Further rewarming returned most platelets to their original discoid shape. Typical example images with 1000x magnification. Bars are means ± SD, hamster n=1-5, mouse n=3, rat n=3, human n=4, * P < 0.05. *Figure on next page.*

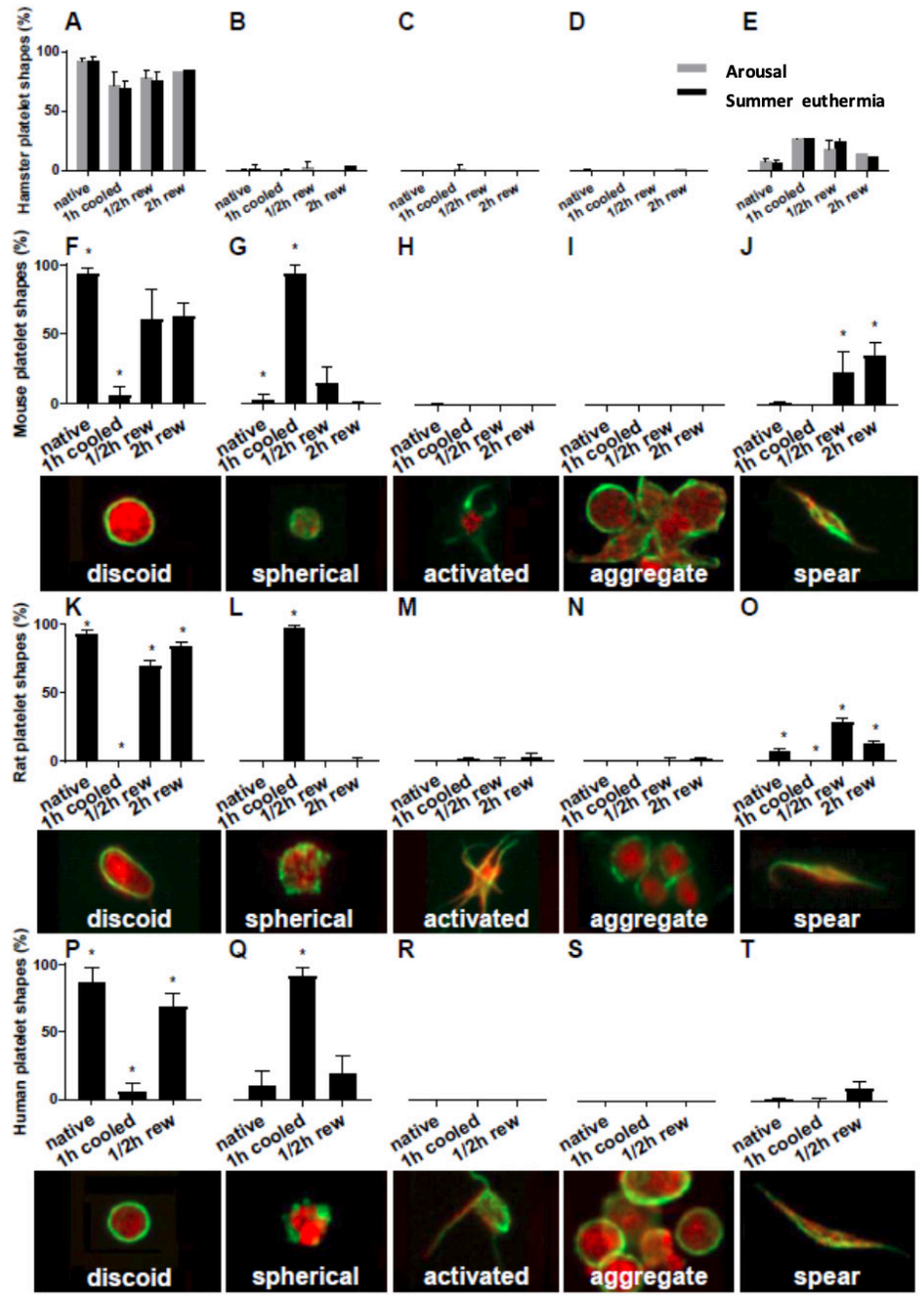


FIGURE 3. *Ex vivo* cooling does not induce tubulin depolymerization and sphere formation in platelets from hibernating and summer euthermic hamsters, but induces spear shapes unlike non-hibernating mammals (mouse, rat, human). *Legend on the previous page.*

Hibernator and non-hibernator platelets are not activated by ex vivo cooling

To investigate whether platelets are activated by *ex vivo* cooling, P-selectin (CD62P) expression was measured in blood samples from hamster (hibernator) and rat (non-hibernator). Native blood of euthermic hamsters had 3.9 ± 2.4 % P-selectin positive platelets (Figure 4A), which remained similar after cooling, 30 minutes rewarming and 2 hours rewarming, while addition of ADP increased the amount of activated platelets. *Ex vivo* cooling/rewarming did not affect the amount of activated platelets, as might be suggested by morphological changes, or of aggregated platelets (Figure 3C-D), in line with the unaltered P-selectin expression. Rat blood had 18.8 ± 4.2 % of platelets positive for P-selectin, which did not change during cooling or short rewarming and decreased to 11.8 ± 1.1 % after 2 hours rewarming (Figure 4B). Similar to hamster, activation of rat platelets by ADP increased the P-selectin positive fraction (Figure 4B) and average level per platelet (Figure 4D). Additionally, throughout cooling and rewarming platelets from mouse, rat and human did not appear activated or aggregated in the smears (Figure 3 H-I, M-N, R-S). Together, we found that *ex vivo* cooling of hibernator and non-hibernator platelets induced different shape changes without signs of platelet activation, while they remained activatable by ADP.

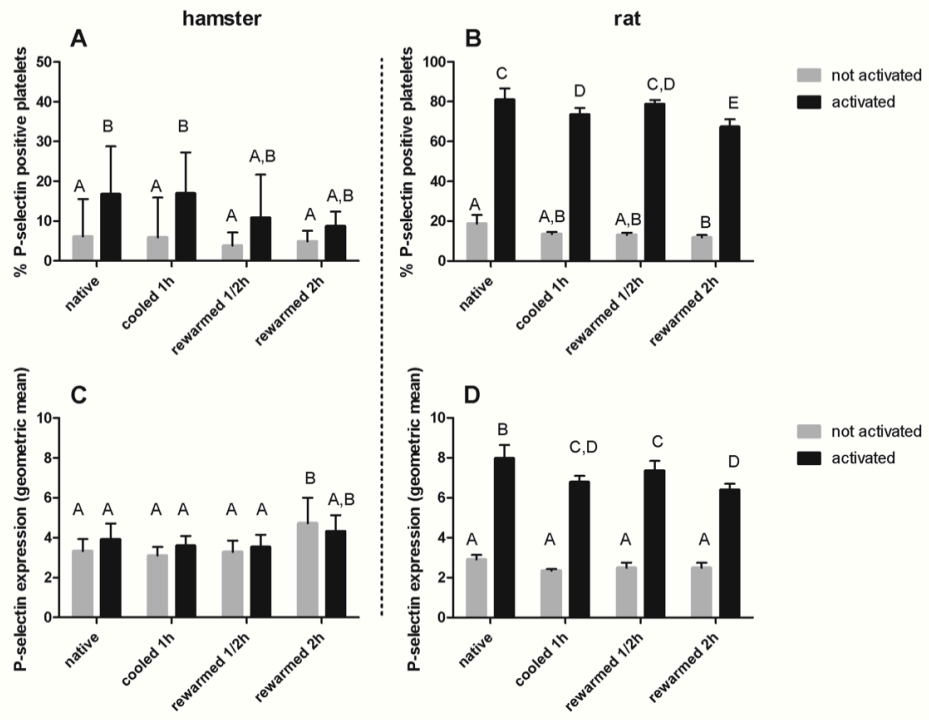


FIGURE 4. Platelets of hibernator (hamster) and non-hibernator (rat) are not activated by *ex vivo* cooling. *Legend on the next page.*

FIGURE 4. Platelets of hibernator (hamster) and non-hibernator (rat) are not activated by *ex vivo* cooling. (A,B) Amount of P-selectin positive hamster (n = 6 to 11) and rat (n = 3) platelets does not increase due to cooling/rewarming. Activatibility by ADP stimulation decreases in hamster platelets after 2 hours rewarming. (C) P-selectin expression of hamster platelets remains similar during cooling/rewarming. (D) P-selectin expression of rat platelets is not altered by cooling/rewarming, whereas ADP activated platelets demonstrate minute differences in activatibility. Different letters above bars denote significant difference, $P < 0.05$. *Figure on previous page.*

Temperature associated dynamics of shape change are platelet autonomous

To study platelet shape changes in humans in greater detail, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. SEM analysis demonstrated the native platelets were shaped as flat discs (Figure 5A) with invaginations of the open canalicular system (OCS), the export system of alpha-granule content [26]. Disc shaped platelets contained a marginal band consisting of several tubulin multimers, clearly seen in cross-section with TEM (Figure 5E top and bottom panel and insets). *Ex vivo* cooling induced a platelet shape change from disc to sphere with formation of filopodia and lamellipodia (Figure 5B), resembling activated platelets. These cooled platelets were devoid of tubulin microtubules (Figure 5F). Rewarming (15 minutes at 37°C) led to the formation of spear shapes (Figure 5C), characterized by a smooth membrane, without evident invaginations of the OCS, and by recovery of tubulin microtubules protruding into the spikey ends of the spear shape (Figure 5G and inset). Prolonged rewarming resulted in disc shapes and the reappearance of OCS invagination sites on the membrane (Figure 5D) and complete recovery of the circular marginal band (Figure 5H), similar to native discs. Throughout cooling and rewarming, platelets remained with granules.

Next, we examined whether platelet shape changes were dependent on humoral factors by exposing human platelets to cooling (1 hour at 4°C) and rewarming (15 minutes, 30 minutes or 2 hours at 37°C), both in the presence or absence of plasma. Similar shape changes from discs to spheres and back to discs via spears were found during cooling and rewarming both in the presence or absence of plasma (Figure 6A-K), indicating that these shape changes are platelet autonomous.

Tubulin polymerization confers platelet spear formation

To gain insight into the underlying cytoskeletal rearrangements that govern changes in platelet morphology, mouse platelets were cooled to induce spherical shapes and subsequently rewarmed to induce spears. Cooling induced spherical platelets with completely depolymerized tubulin, which transformed into spear shapes

while concurrently repolymerizing tubulin (Video S4). In a separate experiment, pharmacological agents were added prior to rewarming to influence the platelet cytoskeleton (Figure 7A-L). To block actin polymerization, cytochalasin D was added, which did not affect spear and disc formation upon rewarming (Figure 7). To inhibit tubulin polymerization, colcemid was added, which effectively blocked the transition from sphere to spear or discs (Figure 7), indicating that tubulin polymerization is essential for platelet spear and disc shape formation. To assess whether inhibition of tubulin polymerization by colcemid is specific for its colchicine-binding site to tubulin we added nocodazole to inhibit tubulin at a different binding site [27]. Nocodazole was unable to block spear formation and yielded spear platelets in high amounts after 30 minutes and even higher after 2 hours rewarming (Figure 7). Thus, repolymerization of tubulin, rather than actin, was essential in spear and disc formation and was susceptible to inhibition via its colchicine-binding site by colcemid.

FIGURE 5. Scanning and Transmission Electron Microscopy imaging of ultrastructural changes in human platelets throughout cooling and rewarming. (A-B) Cooling of platelets changes discs to spheres with filopodia protrusions from the platelet membrane (B, F). The marginal band of tubulin in native discs (E, white arrowhead and inset) is absent after cooling (F, inset). (C) During rewarming platelets become elongated and assume a spear like shape with reformed tubulin microtubules from one pointy ending of the platelet to the other (G, white arrowhead and inset). Spear shaped platelets demonstrated a smooth membrane (C), almost absent of signs of the open canalicular system (OCS), which is present in both native and rewarmed disc shapes (A,D). The spear shape returns to disc shape after prolonged rewarming (D) with recovery of the circular marginal band (H, inset demonstrating transection of several tubulin microtubules). Throughout cooling and rewarming, platelets remained with granules (black arrowheads E-H). Original magnification A-H 12.500x, scale bars top panel 2µm and lower panel 1um. *Figure on next page.*

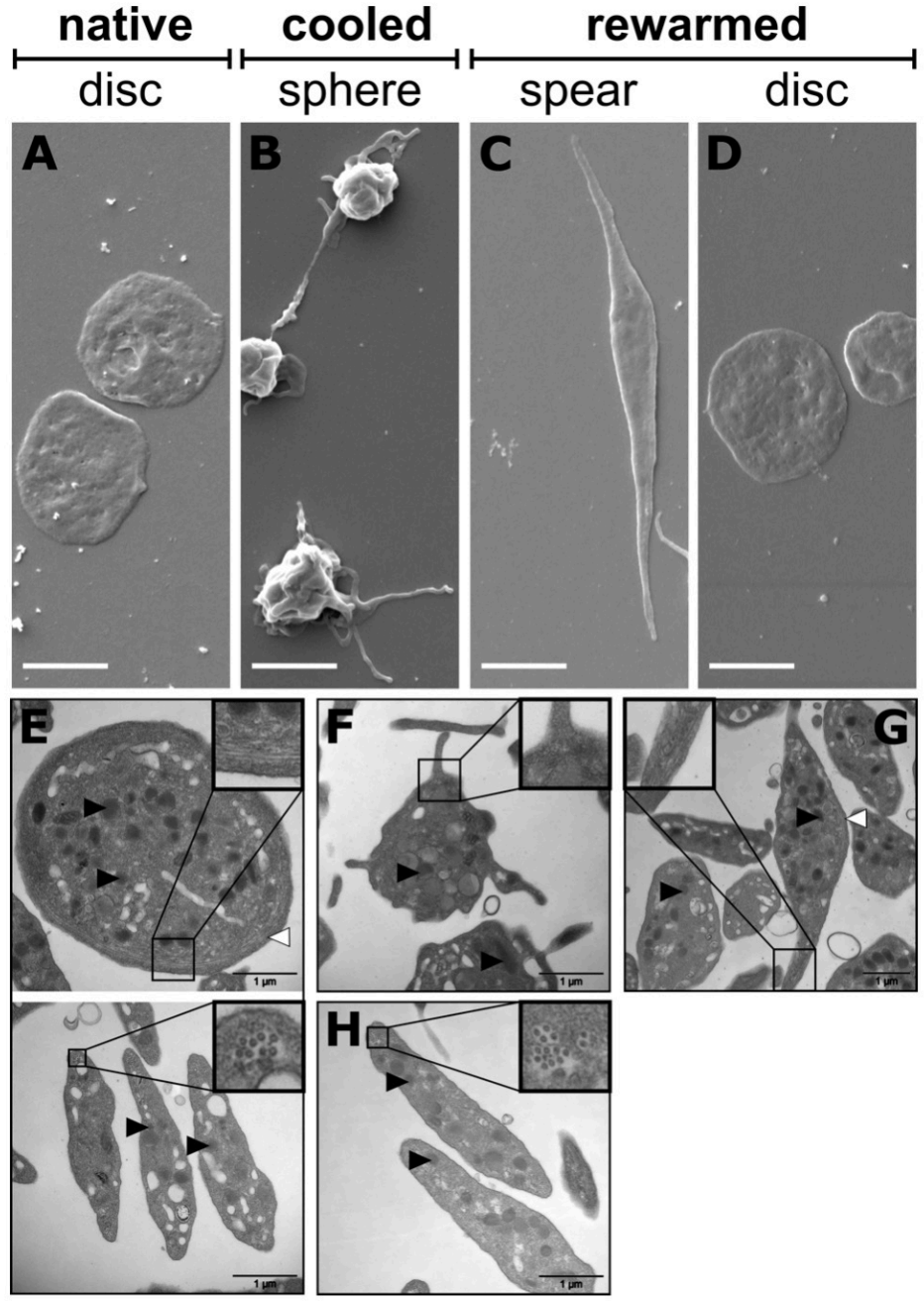
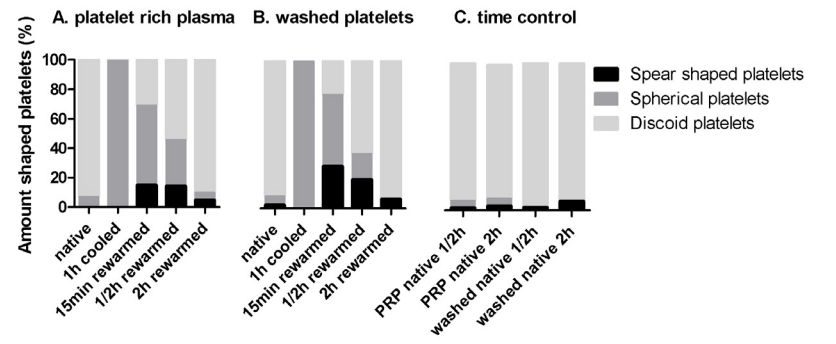


FIGURE 5. Scanning and Transmission Electron Microscopy imaging of ultrastructural changes in human platelets throughout cooling and rewarming. *Legend on previous page.*



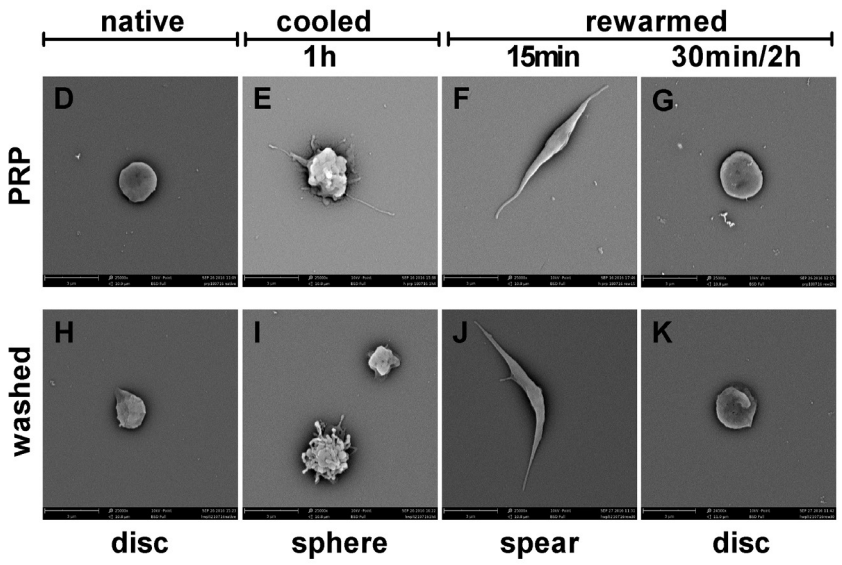


FIGURE 6. Plasma is not a necessary factor for platelets to become spear shapes. (A, and D-G) Human platelets with plasma (platelet rich plasma (PRP)) and (B, and H-K) washed platelets, without plasma, were cooled and rewarmed and spear shaped platelets were present in rewarmed samples (F,J). Continuous rewarming up to 2 hours reversed spear shapes back to discoid shapes both in platelets with and without plasma (G and K). (C) Keeping platelets warm up to 2 hours did not show an increase in spear shaped platelets in either PRP or washed platelets. Bars are n=1. Original magnification D-K 25,000x, scale bars 3µm.

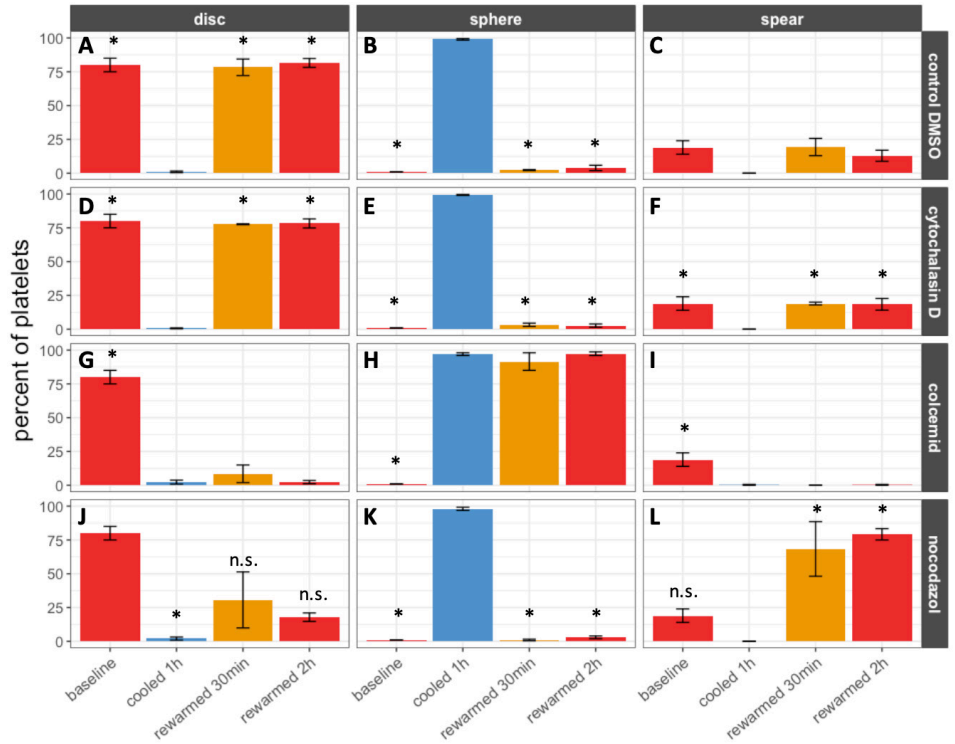


FIGURE 7. Tubulin polymerization is essential for mouse platelet shape maintenance and spear shape formation, whereas actin polymerization is not essential for spear shape formation. (A-C) *Ex vivo* cooling/rewarming of platelets in presence of DMSO as control for the interventions does not affect tubulin depolymerization (spheres) and repolymerization (discs and spears). (D-F) Inhibiting actin polymerization by Cytochalasin D does not influence sphere formation during cooling or spear formation during rewarming. (G-I) Colcemid prevents tubulin marginal band polymerization and spear and disc shape formation during rewarming. (J-L) Nocodazole does not prevent spear shape formation. Contrarily, nocodazole increases amount of spear shapes during rewarming. Bars are means ± SD, mouse n = 2-3, n.s. non-significant, * P < 0.05.

DISCUSSION

In this study we demonstrate reversible temperature dependent platelet shape changes in both hibernating and non-hibernating mammals. In hamsters, during the torpor phase of hibernation with low body temperature, there is a major reduction in amount of discoid platelets, leaving mainly spear shaped platelets in circulation. Similarly, *ex vivo* cooling of hamster platelets induces a fully reversible shape change from disc to spear, characterized by elongated rods of cold-stable tubulin, which is independent from the hibernation season. Contrarily, cooling of platelets from mouse, rat and human depolymerizes the tubulin marginal band and induces the transformation from discs into spheres with filopodia. Subsequent rewarming induces repolymerization of tubulin and reversal to discoid platelets via an intermediate spear shape.

Platelet shape change in hamster torpor and cooling

Reducing hamster platelet temperature, either *in vivo* by hibernation (9°C) or *ex vivo* by cooling (4°C), induces a shape change from disc to spear. In torpor (>24hours of low body temperature), the relative amount of spear shaped platelets in circulation was higher than after one hour *ex vivo* cooling. This difference could be explained by the rate and/or duration of cooling, as documented for the latter in torpid 13-lined ground squirrels and in *ex vivo* cooling of their platelets [13, 17]. Cooling induced a similar disc to spear shape change both in platelets from hibernating and non-hibernating hamsters in summer conditions. Similarly, hamster platelets reversed to a discoid shape both during arousal and *ex vivo* rewarming. Collectively, these data demonstrate that the platelet shape change in hamster is dependent on temperature, is platelet autonomous, independent of the hibernation season and fully reversible.

During torpor, a gross reduction in circulating platelet number due to storage in liver [17] (de Vrij et al., submitted), coincides with an increase in the relative amount of circulating spear shaped platelets. Whether the spear shape is essential to platelet storage in liver is unlikely, since platelet storage in liver occurs both in hibernating hamsters and in non-hibernating rats and mice forced to hypothermia (de Vrij et al, submitted) and we demonstrated in this study that rat and mouse platelets do not form spear shapes when temperature is reduced. This issue may be further addressed by isolating hibernator platelets, and subsequent re-infusion after fluorescent labeling and inhibition of cytoskeleton rearrangements with paclitaxel and cytochalasin, and quantification of circulating platelets throughout torpor/arousal cycling.

Platelet shape change in mouse torpor and mouse, rat and human ex vivo cooling

Mice displaying serial daily torpor induced by balancing workload to food intake also decrease metabolism and body temperature. Platelet count reduced with 35% during torpor, which reversed to euthermic level during arousal, in line with mammalian deep torpor [11, 13, 14]. However, platelets from torpid mice did not form spear shapes *in vivo.* Although their body temperature decreased to only 25°C, further *ex vivo* cooling of these platelets to 4°C did not induce spear shape formation either, but instead rendered them spherical with complete depolymerization of the tubulin marginal band. *Ex vivo* cooling of rat and human platelets also induced sphere formation, with SEM analysis demonstrating filopodia on human platelets and TEM and fluorescence analysis demonstrating loss of the tubulin marginal band. Interestingly, during rewarming the tubulin of mouse, rat and human platelets repolymerized and platelet shapes shifted to discs, but also to spears with formation of elongated rods of tubulin. Prolonged rewarming stimulated a further formation of discs. By time-lapse imaging of *ex vivo* cooled and rewarmed mouse platelets we found a large variety in rate of shape change, while spears appeared an indispensable intermediate shape change to reverse spheres to discs. Few previous studies have observed the presence of spear shaped platelets, describing rewarmed platelets as spindle shaped [28] or elongated [19]. Human washed platelets devoid of plasma also formed spear shapes and discs during rewarming, signifying that the reversible shape change is platelet autonomous. This is in line with the findings of *ex vivo* cooled squirrel platelets that were washed, maintaining the ability to change shape [13].

Although cooling changed the shape of platelets of non-hibernators to spherical with filopodia, often denoted as signs of activation, there were no signs of platelet degranulation in rat and hamster platelets, as demonstrated by low expression of P-selectin, consistent with the presence of Giemsa-stained granules in hamster platelets. While we only cooled for one hour, cooling for several days may neither activate platelets [29-31]. These results add to the controversy whether cooling and room temperature storage lead to platelet activation, since for both storage conditions there is evidence that platelets do become activated, be it by P-selectin expression or excretion of alpha-granule contents [32]. Taken together, it seems that platelet shape change from disc to spear during cooling is a feature only of seasonal hibernators, and not of 'emergency hibernators' (mice in daily torpor), warranting further research comparing species with seasonal daily torpor such as Djungarian hamsters, bats and other species.

Reversible platelet shape change is tubulin-dependent

It remains to be studied which intrinsic factor in platelets drives the shape change from disc or sphere to spear. In general, factors contributing to platelet shape change are for instance during activation: cytosolic Ca^{2+} [33], de-acetylation and polymerization of tubulin [34, 35] and polymerization of actin filaments [36]. Several studies in non-hibernators explored the role of actin in platelet sphere shape formation during cooling by incubating platelets before cooling with cytochalasin B, an inhibitor of actin polymerization, and EGTA or Quin2-AM, chelators of Ca^{2+}, resulting in maintenance of disc shapes during cooling without filopodia formation [21, 37]. However, neither tubulin nor the role of Ca^{2+} on tubulin polymerization were explored. Given that Ca^{2+} influx promotes tubulin depolymarization [38, 39], it is conceivable that chelation of Ca^{2+} maintains the marginal band and platelet disc shape. Indeed, only adding cytochalasin B before cooling does not maintain disc shapes during cooling, whereas only adding taxol (a tubulin stabilizer) does [21, 40, 41]. Treating platelets with taxol and cytochalasin B maintains disc shapes completely during cooling and rewarming [40]. By inhibiting cytoskeletal repolymerization with colcemid via the colchicine-binding site of tubulin we demonstrated that the reversibility of spherical platelets to spear and disc shape is dependent on repolymerization of tubulin, rather than actin. Given our results, tubulin depolymerization likely occurs in non-hibernators during cold induced sphere formation, whereas tubulin polymerization likely is maintained in hibernators to induce spear shapes during cooling, and tubulin repolymerization seems crucial for spear and disc formation after rewarming.

Post translational modification may trigger tubulin stabilization

Different than colcemid, nocodazole treatment did not prevent repolymerization of tubulin, which could be due to its different binding site to tubulin and/or due to the stability of tubulin, which can be increased by posttranslational modifications such as acetylation, detyrosination or binding of STOP's (stable tubule only polypeptides) [42, 43]. STOP's can deliver cold-resistance to microtubules with or without nocodazole-resistance, depending on its molecular composition [42]. Such resistance might explain why our cold treated mouse platelets seemed resistant to nocodazole treatment prior to rewarming, but not to colcemid treatment. Moreover, hibernators may be able to form and maintain spear and disc platelets in the cold due to differently composed STOP's resulting in cold resistant tubulin already during cooling down.

Reversible shape changes may govern cold-resilience of platelets

We demonstrate hibernator platelets to have stable tubulin during cold exposure (both in spear and disc form) *in vivo* and *ex vivo*, in line with data from hibernating ground squirrels [17]. Moreover, Cooper et al. showed that further stabilizing hibernator tubulin with taxol enhanced platelet spear shape formation [17]. To date, hibernator platelets are the only mammalian platelets able to resist cold storage and allow transfusion afterwards without rapid clearance [13]. Changes to platelets associated with its cold-stable tubulin are therefore likely involved in its cold resilience. To our knowledge, no previous study has yet attempted to maintain tubulin microtubule structure to assess its effect on platelet clearance after cold storage. It should therefore be assessed whether the cold-stable tubulin is linked to changes in sialylation of membrane receptors or clustering of GPIbα on the platelet membrane, which govern clearance of non-hibernator platelets after short cold storage [5, 44, 45], and whether it allows preservation of functionality after prolonged storage. Spear shape formation is not essential in the reversible retention of platelets during hibernation, since reversible retention also occurs in non-hibernators throughout hypothermia-and-rewarming [11, 15, 16] and we now demonstrated that non-hibernators do not form spear shapes during cold exposure. Storage lesions and bacterial contamination are still the main reasons for the 5-7 days storage limit for platelet concentrates and the yearly losses due to outdated, dysfunctional and discarded units. Elucidating the molecular mechanism that stabilizes tubulin in hibernators may give a new hope to enable cold-resilience of human platelets and allow platelet cold storage, improving shelf life of platelet concentrates, decreasing bacterial contamination and reducing monetary losses.

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SUPPLEMENTAL DATA

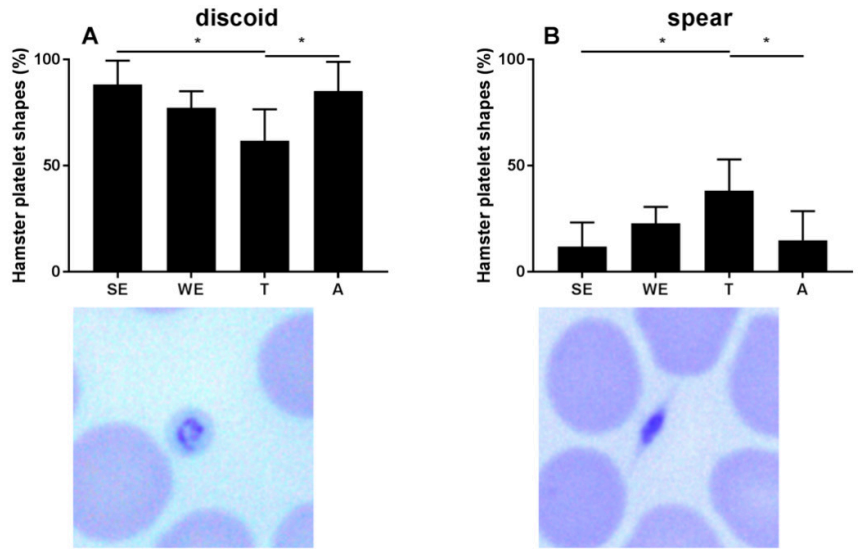


FIGURE S1. Giemsa stain of bloodsmears demonstrating platelet spear shape during thrombocytopenia of torpor in hamster. (A-B) When the hibernating hamster decreases metabolism and body temperature during torpor ("T"), relative amount of discoid/spherical platelets is reduced and spear shaped platelets increased. Discoid platelets are characterized by their round shape and purple stained granules. Spear shaped platelets are characterized by a clear elongated shape and centralization of their granules. When the hamster increases body temperature during arousal ("A") platelets are reversed from spear back into discoid shape. Surrounding the platelets are agranular and anuclear erythrocytes in pink. Typical example images below the graphs with 1000x magnification, bars are means ± SD, hamster n= 3 to 10, * $P < 0.05$.

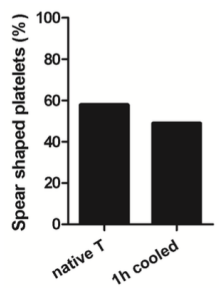
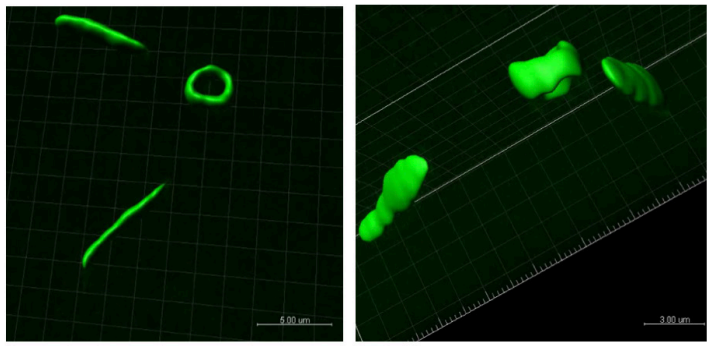
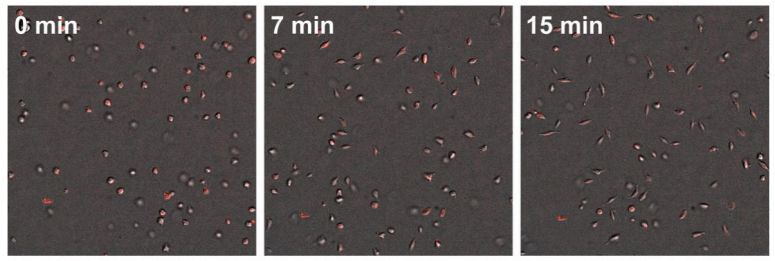


FIGURE S2. *Ex vivo* cooling of torpor blood does not further increase spear formation. Platelets from a hibernating hamster in torpor (8°C, 'native T') have already spear shapes and do not form more spear shaped platelets by *ex vivo* cooling for 1 hour (4°C). Bars are n= 1.

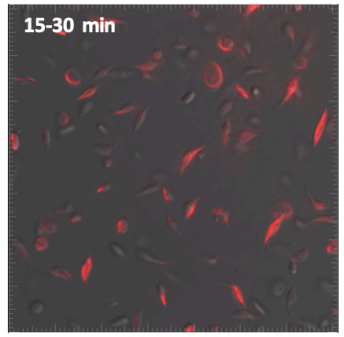


VIDEO S1. 3D rendered representation of tubulin in hamster spear and disc shaped platelets. Surface rendering of fluorescent tubulin staining (green) in *ex vivo* cooled hamster platelets demonstrates spear shapes are distinct from disc shapes in all 3 dimensions. Original magnification 600x, scale bar 3-5µm according to zoom in video. Video accessible via QR-code or tinyurl.com/EdV-chapter6video1 or hibernation.nl/storporage/thesisDeVrij/

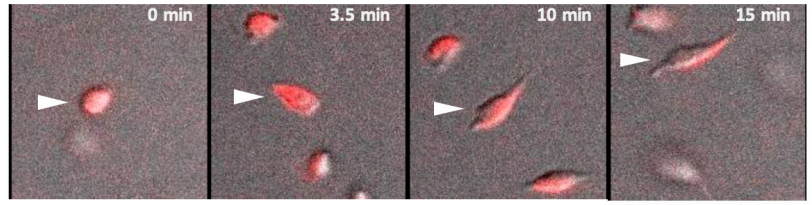


VIDEO S2. Timelapse of cooled mouse platelets (4°C 1h) who have turned from discs to spheres and are rewarmed (37°C 15min) to reform into discs through intermediary spear shapes. Fluorescently labeled tubulin (red) can be seen dispersed in the cytoplasm of platelets due to depolymerization after cooling. Throughout rewarming the majority of platelets starts reforming elongated microtubules of tubulin inducing spear shapes of the platelets before changing into disc shapes. Differential interference contrast (DIC) plus fluorescent imaging of SiR Alexa 647 labeled tubulin, fluorescent signal bleaches during prolonged live imaging. Original oil (N=1.250) magnification 600x, frequency 1Hz, duration 15min, video frame rate 20 frames per second. Video accessible via QR-code or tinyurl.com/EdV-chapter6video1 or hibernation.nl/storporage/thesisDeVrij/

All videos from this chapter are online available at www.hibernation.nl/storporage/thesisDeVrij/



VIDEO S3. Timelapse of cooled and rewarmed platelets. Due to a lag time in shape change, after 15min rewarming from cooling platelet shapes are divided between spear shapes with elongated rods of tubulin (red) and disc shapes with complete recovered marginal band. DIC imaging (grey scale) plus fluorescent imaging of SiR Alexa 647 labeled tubulin. Original magnification 1000x, frequency 1Hz, duration 15min, video frame rate 20 frames per second. Video accessible via QR-code or: tinyurl.com/EdVchapter6video3 or hibernation.nl/storporage/thesisDeVrij/



VIDEO S4. Time lapse of a cooled mouse platelet (4°C 1h) that is being rewarmed (37°C 15min). Rewarming induces reformation of tubulin (red) into an elongated microtubule allowing platelets their intermediate spear shape before transforming in a disc after prolonged rewarming. White arrowhead indicates the same individual platelet. Differential interference contrast (DIC) plus fluorescent imaging. Single platelet is followed and cut out per frame with post processing macro in ImageJ. Original oil (N=1.250) magnification 600x, imaging frequency 1Hz, duration 15min, video frame rate 20 frames per second. Video accessible via QR-code or tinyurl.com/EdV-chapter6video4 or hibernation.nl/storporage/thesisDeVrij/

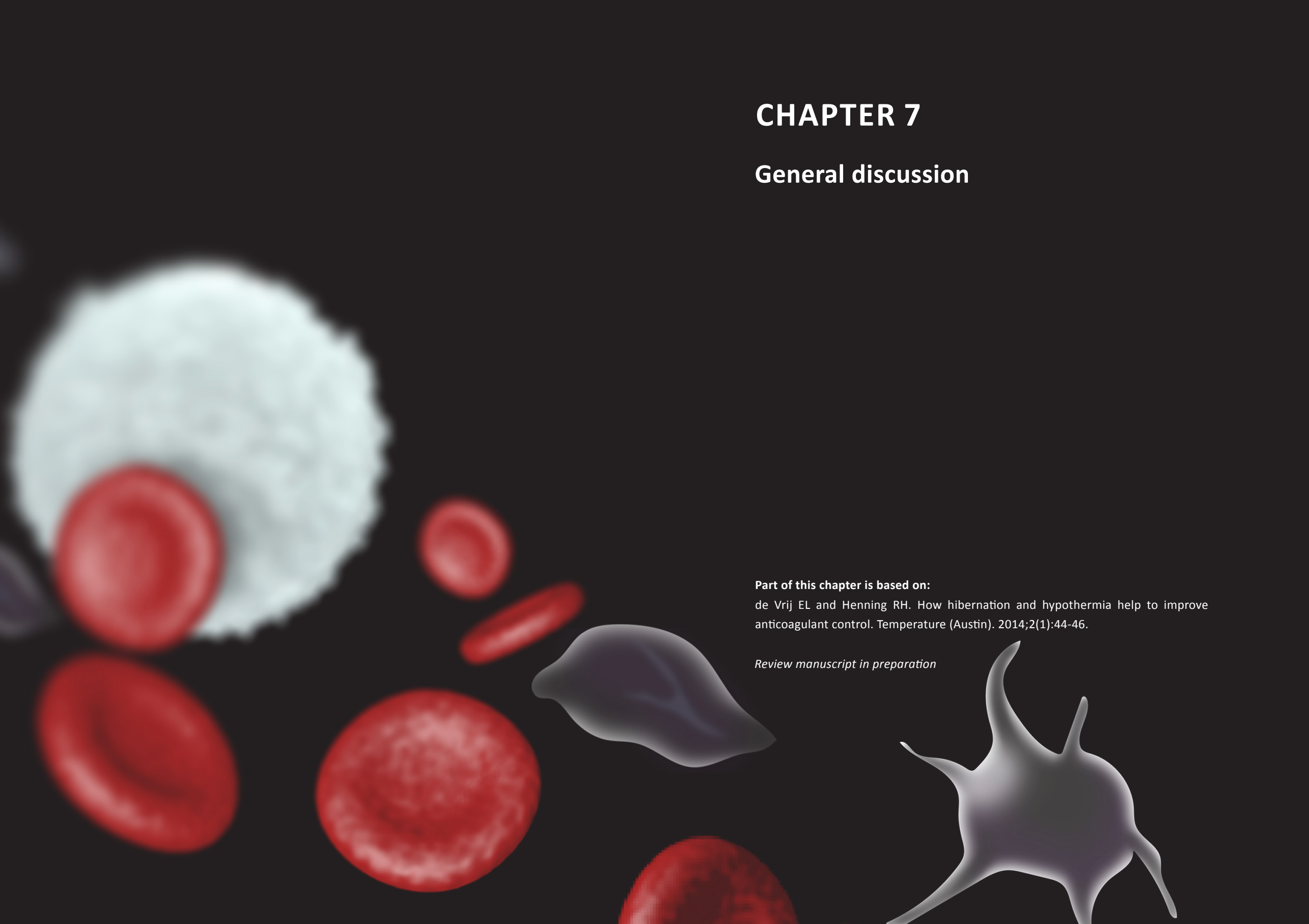
CHAPTER 7

General discussion

Part of this chapter is based on:

de Vrij EL and Henning RH. How hibernation and hypothermia help to improve anticoagulant control. Temperature (Austin). 2014;2(1):44-46.

Review manuscript in preparation



SUMMARY

Thrombosis is a major cause of death and global disease burden. Both primary and secondary hemostasis are involved in venous and arterial thrombosis. Thrombosis might be expected to occur during hibernation due to presence of several factors known in man to increase thrombotic risk: prolonged immobility [1-3], blood stasis in veins and atria [4], increased blood viscosity [5-7], cycles of cooling-rewarming with relative hypoxia and reoxygenation and signs of endothelial injury [1, 8], and gross overweight at entrance of hibernation [9]. Despite these risk factors, hibernators show no signs of thrombosis or embolism, likely due to alterations in key modulators of hemostasis during hibernation.

The aim of this thesis was to provide an overview of alterations in key modulators of hemostasis during hibernation in one species, namely the Syrian hamster, and to determine whether these changes can be mimicked in non-hibernating mammals through forced hypothermia. This thesis also aims at providing insight into the underlying mechanism of the torpor associated reversible thrombocytopenia, and of the morphological changes of platelets including the relative cold resistance of the cytoskeleton of hibernator platelets, with the ultimate aim to identify potential therapeutic targets for antithrombotic drugs and for long term platelet storage for transfusion.

General features of hemostatic suppression in hibernation

We set out to determine the components for hemostasis that are altered during torpor and likely prevent thrombosis. In **Chapter 2** we investigated the effects of hibernation and hypothermia on circulating platelet dynamics in hibernating and non-hibernating mammals and assessed the effect on platelet dynamics using a pharmacological tool (5'-AMP) to induce torpor. Likely, lowering of the body temperature is one of the main driving factors in reversibly reducing circulating platelet count in hibernating hamster as well as non-hibernating species when exposed to forced hypothermia. The thrombocytopenia that ensues at low body temperatures recovered rapidly in all analyzed species upon returning to euthermia, either by natural arousal or by forced rewarming. The quick recovery led us to hypothesize that platelet storage and release underlies the thrombocytopenia at low temperature, rather than (irreversible) clearance and subsequent reproduction. Further, platelet integrity during hibernation or hypothermia seemed maintained in both hibernating and non-hibernating species, in view of absence of signs of platelet activation throughout the experiments and full restoration of platelet functionality when reaching euthermia. In addition, the spleen does not contribute in the temperature associated storage and release of platelets,

as removal of the spleen before or during hibernation did not affect the lowering of the number of circulating platelets in hibernation. Interestingly, pharmacological induction of a torpor-like state by 5'-AMP injection did not induce thrombocytopenia despite the substantial lowering of body temperature and may therefore interfere with the underlying mechanism of temperature associated platelet dynamics. Thus, temperature dependent platelet count reduction is a major alteration within the primary hemostatic system during torpor.

In addition, in **Chapter 3**, we analyzed the main determinants of the hemostatic system throughout hibernation by interrogating components of primary and secondary hemostasis as well as the fibrinolytic pathway in the hibernating Syrian hamster. Hemostasis is likely inhibited in torpor, as demonstrated by reduced thrombin generation with prolongation of clotting times (PT and APTT), which recovered in arousal. Activation of secondary hemostasis with fibrinolysis is unlikely since plasma D-dimer levels remained low throughout hibernation. Suppression of hemostasis during torpor is likely achieved by the reduction of the number of platelets, and of levels of von Willebrand Factor (VWF), fibrinogen, coagulation factor V, VIII, IX, XI and by increasing levels of plasminogen. The reduced hemostasis was slightly counterbalanced by minor increases in factor II and X and a reduction of the anticoagulant factors antithrombin, protein C and plasmin inhibitor. Nevertheless, our data demonstrate that during torpor, the hemostatic balance tips clearly towards inhibition, which reverses during arousal.

Mechanisms of suppressing hemostasis in hibernation

The underlying mechanism of platelet dynamics in hibernating hamsters was further assessed in **Chapter 4**. In this study we demonstrated platelet storage and release to underlie the reversible thrombocytopenia in torpor in hamster. Fluorescent platelets transfused in hibernation followed the same platelet dynamics through torpor-arousal cycles as native platelets. Virtually all transfused platelets fully recovered in circulation upon arousal, thus not being phagocytosed or irreversibly cleared from circulation. Platelets also did not show signs of activation. We further demonstrated a 50% increase in platelet life-span in hibernation compared to non-hibernating hamsters. Finally, we demonstrated that liver sinusoids rather than spleen or lung represent the most likely platelet storage and release location, as found in electron microscopy analysis of platelet accumulation in these organs. Accumulated platelets in liver sinusoids were not degranulated. Thus, low body temperature induces thrombocytopenia during torpor via reversible storage of platelets, probably in liver sinusoids, which reverses by rewarming during arousal and occurs seemingly without activation and degranulation of platelets. Given the location of platelet accumulations

adjacent to liver sinusoidal endothelium, the low flow and increased blood viscosity during torpor and given the reversibility of the platelet accumulation during arousal, the likely underlying mechanism of platelet count reduction constitutes margination of platelets to endothelium.

Since the findings in **Chapter 2** demonstrated that the platelet dynamics are temperature dependent and applicable in non-hibernating mammals, in **Chapter 5** we further assessed the storage site and mechanism for reversible thrombocytopenia in non-hibernating mammals. By (intravital) imaging studies in rat and mouse we revealed that margination of platelets to liver sinusoidal endothelium during hypothermia represents the underlying mechanism of the reversible thrombocytopenia. Moreover, a role of the spleen was excluded in hypothermia induced thrombocytopenia by performing splenectomy before and during cooling, which was without effects on temperature dependent platelet dynamics. In **Chapter 4**, platelets stored in liver sinusoids during torpor were occasionally observed as spear shaped with elongated microtubules, in line with previous findings of cooled squirrel platelets [10, 11]. Reddick et al. proposed that these spear shapes in ground squirrel platelets may lead to trapping in spleen and to the consequent thrombocytopenia [10]. Although we demonstrated that spleen is not involved in temperature dependent platelet dynamics in hamster (**Chapter 2**), which has recently been confirmed in ground squirrel [12], platelet trapping and subsequent retention in liver due to shape change might still occur during torpor. Whether this shape change of platelets also occurs during hypothermia in non-hibernators and may thus be a prerequisite to storage in liver sinusoids was not yet studied.

Therefore, in **Chapter 6** the role of cytoskeletal rearrangements in shape changes of platelets during hibernation was explored and a comparison was made with shape changes of human platelets and of other non-hibernating species during *ex vivo* cooling. We showed that in torpor with low body temperature, the remaining circulating hamster platelets are either spear shaped or disc shaped with maintenance of tubulin cytoskeleton structure. Contrarily, low temperature *ex vivo* in platelets of mice, rat and human depolymerized tubulin, thus rendering a sphere shape with formation of filopodia, mimicking activated platelets. However, activation marker expression was neither increased in hibernating nor non-hibernating platelets after *ex vivo* cooling and rewarming. We were able to induce spear-shape in platelets of mice, rat and human platelets after rewarming from cooling, which mechanism was dependent on tubulin polymerization via the colchicine binding site of tubulin. Thus, lowering temperature induces spear shapes only in hibernator platelets, whereas rewarming induces spear shapes in non-hibernator platelets. Therefore, the induction

of platelet spear shape by low temperature might not be a prerequisite for platelet storage in liver sinusoids during hypothermia since non-hibernators do not form spear shapes when cooled. Additionally, hibernator platelets seem resilient to cooling induced activation and to degradation of cytoskeletal tubulin.

Collectively, this thesis identified several key modulators of primary hemostasis, secondary hemostasis and fibrinolysis that prevent activation of the hemostatic system of Syrian hamster during hibernation. We focused on primary hemostasis and elucidated the mechanism underlying the reversible thrombocytopenia to be temperature dependent in both hibernators and non-hibernators. We demonstrated a major role of liver in the storage and release of platelets, resulting in a 50% increase in platelet half-life. Further, we found that low temperatures did not activate platelets of hibernators and non-hibernators despite striking - albeit reversible - changes in morphology. Together these findings help us understand why hibernating mammals such as Syrian hamster do not suffer from thromboembolic complications during hibernation. Furthermore, temperature dependent suppression of hemostasis also exists in non-hibernating mammals as well as the ability to reversibly alter platelet morphology without activating platelets. These results may lead to new antithrombotic strategies and provide new strategies for long term platelet cold storage for transfusion.

SUPPRESSION OF HEMOSTASIS IN HIBERNATING HAMSTER COMPARED WITH OTHER HIBERNATORS

In this thesis, Syrian hamster was studied as a model organism for hibernation. The suppression of hemostasis during hibernation has been demonstrated in several other hibernating species, albeit less extensively. In torpid ground squirrel, hemostasis suppression is exemplified by reduced thromboelastography [11]. Further support for hemostasis suppression originates from the lowered impedance aggregometry in hibernating bears [7] and prolongation of whole blood clotting time in torpid hedgehog and ground squirrels [13-15] as well as in hibernating black bears [16]. Thus, suppression of hemostasis in hibernation, specifically during torpor, seems a preserved phenotype throughout several hibernating species. In general, the suppressed hemostasis recovers swiftly towards pre-hibernating level upon arousal (Table 1). Overall hemostasis depends on the effects of primary and secondary hemostasis together with fibrinolysis. Therefore, suppressing hemostasis in hibernation may require adaptations in one or more of these pathways.

Primary hemostasis in hibernators

Suppression of primary hemostasis has been described in several squirrel, hamster and bear species and in hedgehog (Table 1). Similar to hamster, these hibernators demonstrate reductions in platelet count, von Willebrand factor (VWF) level and activity, as well as reduced thrombin elastography, *ex vivo* platelet aggregation, and platelet degranulation. In **Chapter 2** we showed platelet count reduction to be associated with lowering of the body temperature. In Table 1 we review the literature on hibernation and primary hemostasis, including platelet count, and demonstrate in Figure 1 that the temperature association with platelet count reduction is consistent for all hibernating mammals studied so far (Pearson's r=0.95, P<0.05). Also forced hypothermia in mammals that can enter torpor reduces platelet count (Table 1, **Chapter 2**).

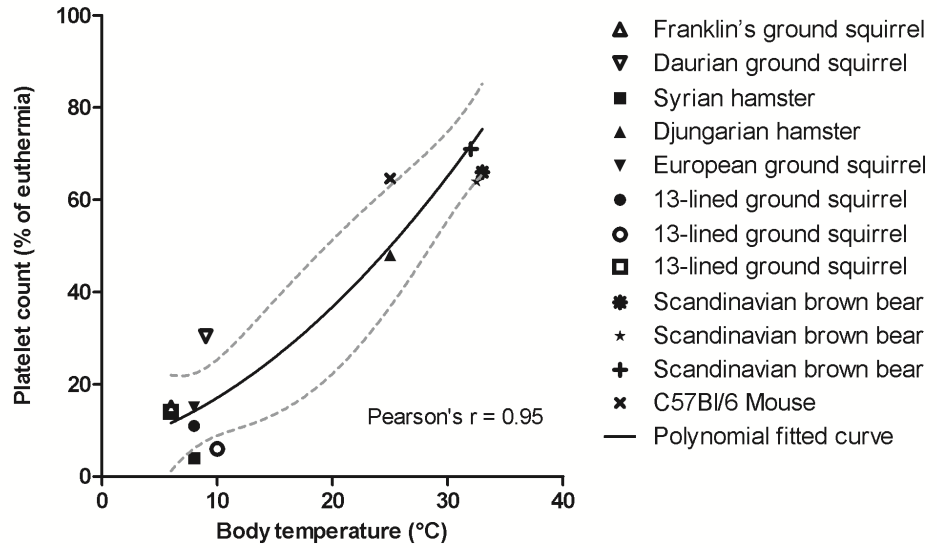


FIGURE 1. Temperature association with platelet count reduction is consistent for all hibernating mammals studied so far. Platelet count in torpor as percentage of euthermia platelet count was calculated from values of studies summarized in Table 1. If a study did not report euthermic platelet count, literature data was used. Fitted polynomial quadratic curve with constraint 100% at 37°C (black line) with 95% confidence interval (dotted gray line). Pearson's r=0.95, P<0.05.

In contrast to circulating platelet numbers, platelet functionality during hibernation is less well studied. Platelet binding to VWF and activation by endogenous agonists, such as ADP, thrombin and VWF, are important in the initial steps of primary hemostasis. In our hamster studies, platelets from torpid animals were activated by ADP to a lesser extent than those from aroused and summer euthermic animals (**Chapter 2 and 4**). Platelets from hibernating squirrels in interbout arousal bind less VWF *ex vivo* than platelets from non-hibernating squirrels in summer [12]. Whether torpor also reduces platelet activation by VWF and binding to VWF has yet to be studied. A partial or total deficiency of VWF or VWF function in humans is known as Von Willebrand Disease [17], the most common inherited bleeding disorder. The ten-fold reductions in VWF during hibernation (**Chapter 3** and Table 1) might suggest a similar pro-hemorrhagic phenotype. Together these results imply a reduced primary hemostasis with reduced platelet function (i.e. activation and adherence) and reduced availability of VWF during hibernation. However, a key question remains whether the altered functionality of platelets from hibernators originates during the preparation phase ('late fall') or

reflects specific adaptations during hibernation. Collectively, our data demonstrate hamster to be a good model organism to study hemostasis in hibernation since they share common alterations in primary hemostasis of other hibernators.

Mechanisms suppressing primary hemostasis

The suppression of primary hemostasis during torpor is chiefly conferred by a reversible thrombocytopenia and suppression of plasma VWF level and activity. In this thesis, we identified that the reversible thrombocytopenia in torpor is governed by platelet storage in and release from liver sinusoids. The storage of platelets in liver occurs also in hibernating ground squirrel [12]. The underlying mechanism likely entails margination of platelets to the endothelium [18]. Margination in turn is largely dependent on the expression of adhesion factors on both the platelet and endothelium, and on blood flow velocity. The reduction in metabolism, body temperature and subsequent cardiac output (~97 % reduction in ground squirrels for example [19]) induces a substantial reduction of blood flow velocity ([20], increasing platelet-endothelium contact time and platelet distribution near the vessel wall. Increased expression of endothelial adhesion molecules during torpor has been demonstrated in hamster lungs [8], but whether this is secondary to low blood flow and occurs in more than one organ is unknown. Moreover, several other factors besides blood flow velocity and adhesion molecule expression may modulate margination, such as plasmatic and rheological factors and platelet morphology [21-24]. Increased viscosity may increase margination by distributing platelets from the central part of laminar flow towards the vessel wall [21], thus increasing platelet-endothelium contact time. Platelets favor margination when shaped as spheres rather than discs [21]. However, circulating platelets in torpor are mainly spear shaped (**Chapter 6**), with the predominant shape of platelets stored in liver sinusoids being spherical or discoid (**Chapter 4**). However, the role of spear shaped platelets remains unclear and needs to be addressed in future studies. On the one hand, circulating spear shaped platelets during torpor may have evaded margination, and may thus represent 'patrolling' platelets allowed to circulate. Alternatively, the platelet spear shape induced by low temperature during torpor may promote or initiate platelet margination to liver sinusoids, after which platelet shape changes to disk or sphere while being stored.

To marginate reversibly to intact endothelium, platelets have to adhere in a non-permanent fashion. Important factors involved in platelet-endothelium adhesion are endothelial VWF and its ligands glycoprotein Ibα (GPIbα) and GPIIb/IIIa on platelets. A suppression of this part of hemostasis may contribute to preventing irreversible platelet-endothelial binding and thrombus formation and allow reversible margination. VWF

level and activity are decreased in torpor in hamster, squirrel and brown bear (Table 1), which is likely due to decreased VWF production and multimer-to-oligomer ratio [25]. Other factors that influence VWF function, although not yet studied in hibernators, are: reduced signal response with lower temperature [26], reduced binding affinity of platelet GPIbα to the A1 domain of VWF during lowered shear force [27], and reduced kinetics and levels of endothelial VWF release into the bloodstream by Weibel Palade Body exocytosis, which is a strongly temperature dependent process [28]. Together these factors likely contribute to lower VWF level and activity in torpor, in line with the reduced circulating plasma level of VWF studied in torpid hamster, squirrel and bear (Table 1). However, also kinetics of VWF proteolysis by the cleaving metalloprotease ADAMTS-13 are temperature-dependent, with reduced activity down to 4°C [29], which conversely may contribute to increased (multimer) plasma level of VWF. In addition, endothelium may also upregulate the expression of adhesion molecules under low flow [30, 31], subsequently increasing platelet adherence; whether this also occurs in hibernators is unknown. To date, increased adhesion molecule expression (ICAM-1 and VCAM) during torpor has been shown in lung tissue [8], which reverses during arousal, but has not been investigated in liver yet. Further, plasmatic factors might contribute to platelet margination as well, for instance by inducing upregulation of specific adhesion molecules. Hibernating squirrel plasma has been shown to increase ICAM-1 expression in rat cerebral endothelium and subsequently increase monocyte margination [32]. Whether this upregulation occurs in liver endothelium cells and also affects platelet margination is still unknown.

Taken together, suppression of primary hemostasis in torpor encompasses a number of mechanisms common in hibernators, including a reversible thrombocytopenia by storage and release of platelets in liver sinusoids and a reduction in VWF level and activity. The storage and release of platelets in liver sinusoids is due to margination. In torpor, the reduced body temperature increases blood viscosity subsequently distributing platelets towards the vessel wall while the reduced blood flow prolongs platelet-endothelium contact time, increasing likelihood of adhering. Margination may further be stimulated by potential increases in adhesion molecule expression and platelet shape change. VWF level and activity reduce in torpor because of decreased VWF production, reduced multimer to oligomer ratio and due to lowering of shear stress which decreases affinity of platelet receptors to VWF. All these reductions, and a reduced platelet activatibility by mechanisms still unknown, likely prevent an irreversible adhesion of platelets to endothelium and thus allow the reversibility of platelet margination. Oppositely, chances of bleeding should remain low since the animals in torpor do not move and thus have neglectable risk of trauma and

subsequent bleeding.

Importantly, the lowering of body temperature constitutes one of the driving factors conferring changes in primary hemostasis in hibernation. Similar to torpor, low body temperature stages key factors promoting reversible platelet margination, such as the reduction in blood flow velocity and inhibiting VWF signal response and enzymatic kinetics. Whether all effects on hemostasis in torpor are due to lowering body temperature is not yet clear. For example, circulating platelet reduction is far greater in torpid hamster compared to hypothermic hamster with the same body temperature (**Chapter 2**). The difference in extent of thrombocytopenia may be dependent on 'cold time', as torpor lasted several days and hypothermia minutes to hours. However, an additional effect specific for torpor, such as the preparation phase to hibernation, cannot be excluded.

Why platelets sequester specifically in the liver is still unresolved. Possibly, liver sinusoidal endothelium reacts differently to low temperatures and flow than endothelium in other vascular beds, e.g. by abundant expression of adhesion factors. Alternatively, liver sinusoid platelet sequestering may be flow related, as euthermic flow rate in liver is already low but the perfusion of the liver is maintained during periods of reduced body temperature [20], albeit at even lower flow rates [33, 34]. This may generate ideal docking conditions for platelets, as explained above. Yet another reason for platelet storage in liver may be the presence of a set of specific receptors in liver sinusoids, able to attach platelets under cold and low flow circumstances. For example, desialylation of platelet glycoproteins by cold or ageing induces irreversible platelet clearance in liver sinusoids in non-hibernators via hepatic Ashwell-Morell receptors and Kupffer cell $\alpha_M\beta 2$ integrins [35], inducing irreversible phagocytosis of platelets. Perhaps these receptors attach marginating platelets in torpor without inducing phagocytosis, and hence increase platelet half-life. Given that *ex vivo* cooled hibernator ground squirrel platelets can be transfused without irreversible clearance [11], as opposed to for instance human platelets, exploration of the sialylation state of hibernators' platelet glycoproteins following cold exposure is warranted, as is exploration of the expression of the associated liver sinusoid receptors during the hibernation cycle. Depending on the mechanism of sequestering, the reversibility of adhesion upon arousal and rewarming of the animal may depend on specific, yet unexplored, mechanisms. Irrespectively, the large increase in cardiac output and blood flow upon arousal, will anyhow stimulate detachment of platelets from endothelium and govern their reappearance in the circulating pool [36].

TABLE 1. Primary hemostasis in hibernation

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
Whole-blood clotting time (sec)	210 ± 76 (n=18)	315 ± 66 (n=9) *		13-lined ground squirrel	Lechler et al. 1963 [15]
(sec)	81 ± 12 (n=3) summer	217 ± 30 (n=10) "early denning"	164 ± 33 (n=11) "late denning"	American black bear	Iles et al. 2017 [16]
(min)	2.2 ± 0.3 (n=9)	48.0 ± 5.4 (n=24) *	11.5 ± 1.5 (n=12) *,#	Franklin's ground squirrel	Pivorun et al. 1981 [14]
(min)	4 [3-7.5] (n=6)	11 [9-14.5] (n=5)	5 [3-7] (n=12)	Hedgehog	Biorck et al. 1962 [13]
Thromboelastography (n=7-8)					
R(min)	1.6 ± 0.4	16.2 ± 12.3 *	4.7 ± 1.2 *,#	13-lined ground squirrel	Cooper et al. 2012 [11]
Alfa (°)	58.8 ± 11.3	6.6 ± 6.2 *	17.1 ± 7.4 *,#	13-lined ground squirrel	Cooper et al. 2012 [11]
Maximum amplitude (mm)	47.2 ± 5.2	6.0 ± 6.8 *	17.2 ± 10.4 *,#	13-lined ground squirrel	Cooper et al. 2012 [11]
G (K d/sec)	4.6 ± 1.0	0.3 ± 0.4 *	1.1 ± 0.8 *,#	13-lined ground squirrel	Cooper et al. 2012 [11]
Platelet aggregation (arbitrary aggregation units)					
ADP	70.0 ± 26.6 (n=6)	29.2 ± 8 (n=6) *		Scandinavian brown bear	Arinell et al. 2011 [37]
	66 ± 23 (n=12)	33 ± 10 (n=12) *		Scandinavian brown bear	Arinell et al. 2017 [7]
Arachidonic acid	73 ± 16 (n=6)	28 ± 9 (n=6) *		Scandinavian brown bear	Arinell et al. 2011 [37]
	68 ± 20 (n=12)	33 ± 10 (n=12) *		Scandinavian brown bear	Arinell et al. 2017 [7]
Collagen	68.3 ± 17 (n=6)	30.7 ± 10 (n=6)		Scandinavian brown bear	Arinell et al. 2011 [37]
	63 ± 22 (n=12)	30 ± 7 (n=12) *		Scandinavian brown bear	Arinell et al. 2017 [7]
TRAP	18.5 ± 10.0 (n=11)	9.2 ± 6.9 (n=11) *		Scandinavian brown bear	Arinell et al. 2017 [7]
PAR-4	22.5 ± 7.1 (n=6)	12.7 ± 7.1 (n=6) *		Scandinavian brown bear	Arinell et al. 2017 [7]
Platelet count					
x 10^9/L	445.15 ± 123.4 (n=19) 36.3°C	47.94 ± 22.26 (n=17)* 7.9°C (11% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
	303.6 ± 10.6 (n=10) 37°C	45 ± 3.4 (n=37) * 6°C (15% of EU)	232.4 ± 19.7 (n=14) 37°C *,# (77% of EU)	Franklin's ground squirrel	Pivorun et al. 1981 [14]

TABLE 1 CONTINUED.

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
	375.33 ± 40.79 (n=6) 37°C	114.17 ± 36.01 (n=6) * 9°C (30% of EU)	217.00 ± 35.88 (n=6) *,# 37°C	Daurian ground squirrel	Hu et al. 2017 [38]
	198 ± 59 (n=7) 35°C	8 (8°C) * (4% of EU)	187 (35°C) # (94% of EU)	Syrian hamster	De Vrij et al. 2014 [39]
	797 ± 124 (n=6) 35°C	381 ± 239 (n=9) * 25°C 48%	739 ± 253 (n=5) # 35°C 93%	Djungarian hamster	De Vrij et al. 2014 [39]
	293 ± 81 (n=4) 36°C	44 ± 30.87 (n=5) * 8°C (15% of EU)	194 ± 54 (n=5) *,# 35°C (66% of EU)	European ground squirrel	Bouma et al. 2010 [40]
		23.3 ± 1.3(n=14) * 9.8 ± 2.1°C (6% of EU)	410.9 ± 59.2 (n=14) 36.4 ± 0.8°C (100% of EU)	13-lined ground squirrel	Cooper et al. 2012 [11]
	394 ± 157 (n=8)	55 ± 30 (n=8) * 6°C (14% of EU)		13-lined ground squirrel	Reddick et al. 1973 [10]
	207 ± 24 (n=6)			Scandinavian brown bear	Fröbert et al. 2010 [41]
	262 ± 61 (n=13) 39.8 ± 0.8°C	174 ± 51 (n=13) * 33.4 ± 1.1°C (66% of EU)	262 ± 61 0.69 (100% of EU)	Scandinavian brown bear	Arinell et al. 2017 [7]
	229 ± 39 (n=6)	146 ± 47 (n=6) * (during hibernation) 64%		Scandinavian brown bear	Arinell et al. 2011 [37]
	228 ± 36 (n=7) (summer) 37°C	149 ± 43 (n=7) * (winter) 32°C (71% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
	791 ± 242 (n=19) 37°C	511 ± 232 (n=12) * 25.3 ± 3.7 °C (64.6% of EU)	879 ± 209 (n=13) (111.1% of EU)	Daily torpor C57Bl/6 Mouse	De Vrij et al. unpublished
$10^2/mm^3$ Mean ± SE	5.60 ± 0.61 (n=9)		6.02 ± 1.14 (n=6) (spring arousal)	Common yellow bat	Rashid et al. 2016 [43]
$10^2/mm^3$ Mean ± SE	7.54 ± 1.001 (n=7)		7.38 ± 1.15 (n=9) (spring arousal)	Common pipistrelle bat	Rashid et al. 2016 [43]
P-selectin expressing platelets (%)	8 ± 7 (n=2)	0 (n=2)	7 ± 6 (n=2)	Syrian hamster	De Vrij et al. 2014 [39]
	3 ± 1 (n=11)	11 ± 7 (n=2)	10 ± 3 (n=4)	Syrian hamster	De Vrij et al. unpublished
% platelets activated by 10uM ADP	16 ± 14 (n=2)	16 ± 6 (n=2)	29 ± 6 (n=2)	Syrian hamster	De Vrij et al. 2014 [39]
	22 ± 8 (n=10)	16 ± 2 (n=2)	47 ± 11 (n=4) *,#	Syrian hamster	De Vrij et al. unpublished

TABLE 1 CONTINUED.

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
VWF (von Willebrand Factor) % relative to human plasma	24.9 ± 3.7 (n=10)	2.4 ± 0.01 (n=10) * (10% of EU)		13-lined ground squirrel	Cooper et al. 2016 [25]
	9.5 ± 1.0 (n=10)	0.7 ± 0.3 (n=4) * (7% of EU)	0.8 ± 1.1 (n=4) *	Syrian hamster	De Vrij et al. unpublished
IU/mL	1.72 ± 0.18 (n=10)	1.30 ± 0.24 (n=11) * (76% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
VWF:collagen binding activity (% relative to human plasma)	4.0 ± 0.4 (n=7)	1.4 ± 0.6 (n=4)	1.0 ± 1.3 (n=4) *	Syrian hamster	De Vrij et al. unpublished
Platelet count x 10^9/L	166 ± 36 (n=5) 37 ± 0.9°C	78 ± 25 (n=5) * 8.7 ± 2.2°C (47% of EU)	149 (n=5) 37 ± 0.1°C (88% of EU)	Syrian hamster	De Vrij et al. 2014 [39]
	1036 ± 165 (n=9) 37.2 ± 0.7°C	777 ± 122 (n=6) * 20.1 ± 0.3°C (72% of EU)	817 ± 117 (n=3) 37.5 ± 0.8°C (88% of EU)	C57Bl/6 mouse	De Vrij et al. 2014 [39]
	800 (n=7) 37°C	600 (n=7) * 28°C (75% of EU)		C57Bl/6 mouse	Straub et al. 2011 [44]

Data are represented as mean ± SD, or in one study as mean ± SE [14] and with corresponding body temperature. Two studies only provided a range with or without an average [13, 45]. For some parameters, only an average could be retrieved without range, SD or SE, or only individual data was given and a mean and SD had to be calculated. Sample sizes are given when this was retrievable per parameter. Some parameters are also represented as percentage of euthermia (% of EU) for use in Figure 1 and 2.

* $P < 0.05$ from (summer) euthermic values

# $P < 0.05$ from hibernating/torpid values

Secondary hemostasis and fibrinolysis in hibernators

Secondary hemostasis is likely reduced in torpor as well, as indicated by an increased whole blood clotting time (Table 1), which assesses both primary and secondary hemostasis. Hitherto no comprehensive overview of coagulative changes in hibernators has been published, therefore the most important parameters in secondary hemostasis and fibrinolysis throughout torpor and arousal are listed in Table 2 and compared in this section. Hibernating squirrel and bear species, hedgehog` hamster and even turtle all demonstrate suppression of secondary hemostasis (Table 2). In general, hibernating animals in torpor reduce the level of coagulation factors VIII, IX and XI (Table 2 and **Chapter 3**), resembling human bleeding diseases called Hemophilia A, B and C respectively [46, 47]. Some animals also reduce factor V and fibrinogen (Table 2 and **Chapter 3**). Together, the overall effect results in suppression of the coagulation cascade in torpor, as we exemplified in hamsters by reduced thrombin generation and

prolonged PT and APTT (**Chapter 3**). While reduction of pro-coagulant factors may be paramount, anticoagulant factors (protein S, protein C and antithrombin) likely need to be maintained in hibernation, as their deficiency in humans is a major risk factor for thrombosis [48]. Correspondingly, protein C and antithrombin levels are not reduced in torpid hamster (**Chapter 3**). In hibernating bears, however, antithrombin as well as plasminogen levels are reduced (Table 2). This potential reduction in anticoagulation and fibrinolysis in bear is however compensated by the decrease in plasmin inhibitor, maintained levels of other protease inhibitors and increased level of the nonspecific protease inhibitor α2-macroglobulin, which may collectively maintain anticoagulation and fibrinolysis [42, 49]. Together, these data demonstrate that the prolonged clotting time in torpor is indeed due to suppression of both primary and secondary hemostasis, with generally intact fibrinolysis as demonstrated in Figure 2, depicting a summary of all hibernating studies on hemostasis to date from the review of Table 1 and 2.

TABLE 2. Secondary hemostasis in hibernation

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
Thrombin time (sec)	13.2 ± 0.7 (n=7) 37°C	15.8 ± 0.7 (n=8) * 6°C	14.7 ± 1.3 (n=5) 37°C	Franklin's ground squirrel	Pivorun et al. 1981 [14]
PT (sec)	9.8 ± 1.6 (n=10) 36.3°C	10.6 ± 1.5 (n=6) 7.9°C		13-lined ground squirrel	Lechler et al. 1963 [15]
	8.1 ± 0.3 (n=10) 37°C	8.3 ± 0.2 (n=13) 6°C	14.0 ± 2.1 (n=5) *,# 37°C	Franklin's ground squirrel	Pivorun et al. 1981 [14]
	Summer: 42.6 (n=15)	Winter: 60.5 (n=9)	Spring: 51.2 (n=10)	European Hedgehog	De Wit et al. 1985 [50]
	8.75 ± 0.5 (n=6)			Golden hamster	Deveci et al. 2001 [51]
	41-48 (n=16) (summer months)	38-62 (n=16) (winter months)		Red-eared slider (turtle); Painted turtle	Barone et al. 1975 [45]
	8.6 ± 0.3 (n=2)	7.6 ± 1.5 (n=9) "early denning"	8.1 ± 2.2 (n=11) "late denning"	American black bear	Iles et al. 2017 [16]
	22 [18.3-26.9] (n=12)	23 [16-43] (n=16)	22 [19.4-28] (n=7)	Hedgehog	Biorck et al. 1962 [13]
	10.1 ± 0.3 (n=5) 36.6 ± 0.9°C	13.0 ± 1.6 (n=5) * 20.5 ± 0.5°C	11.1 ± 0.4 (n=5) # 35.5 ± 1.1°C	C57Bl/6 pharmacological torpor	De Vrij et al. unpublished
	10.2 ± 0.9 (n=10) 36.3 ± 0.9°C	18.8 ± 8.4 (n=3) 8.8 ± 0.7 °C	8.6 ± 0.6 (n=4) # 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
APTT (sec)	45.5 ± 8.7 (n=13) 36.3°C	109.3 ± 42 (n=13) * 7.9°C		13-lined ground squirrel	Lechler et al. 1963 [15]

TABLE 2 CONTINUED.

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
	25.0 ± 1.0 (n=16) 6°C	51.0 ± 2.4 (n=13) * 6°C	30.1 ± 1.4 (n=8) # 37°C	Franklin's ground squirrel	Pivorun et al. 1981 [14]
	29.8 ± 0.27 (n=6)			Golden hamster	Deveci et al. 2001
	17.6 ± 0.8 (n=2)	29.7 ± 7.9 (n=8) * "early denning"	24.5 ± 2.1 (n=11) * "late denning"	American black bear	Iles et al. 2017 [16]
	30.2 ± 6.6 (n=10) 36.3 ± 0.9°C	102.4 ± 26.5 (n=3) 8.8 ± 0.7 °C	26.1 ± 3.6 (n=4) # 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Thrombin generation (peak nM)	74 ± 17 (n=3) 36.3 ± 0.9°C	6 ± 14(n=7) * 8.8 ± 0.7 °C	45.6 ± 43 (n=5) 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Prothrombin U/mL	443 ± 132 (n=10) 36.3°C	698 ± 143 (n=12) * 7.9°C (158% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
IU/mL	1.10 ± 0.19 (n=17)	1.29 ± 0.31 (n=15) * (117% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
sec	11.5 ± 0.3 (n=9) 37°C	11.9 ± 0.3 (n=6) 6°C (90% of EU)	10.5 ± 0.3 (n=8) *,# 37°C (208% of EU)	Franklin's ground squirrel	Pivorun et al. 1981 [14]
U/mL	20 [18-21] n=4	55 [46-70] (275% of EU) n=4	90 [60-120] n=4	Hedgehog	Biorck et al. 1962 [13]
% relative to human plasma	71.6 ± 6.9 36.3 ± 0.9°C	82.8 ± 14.0 (116% of EU) 8.8 ± 0.7 °C	132.1 ± 10.9 * 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Residual prothrombin in serum U/mL	27 ± 38 (n=15) 36.3°C	449 ± 157 (n=9)* 7.9°C		13-lined ground squirrel	Lechler et al. 1963 [15]
Factor II, VII, X (combined assay) sec	12.8 ± 0.4 (n=3) 37°C	14.3 ± 0.6 (n=4) 6°C (90% of EU)		Franklin's ground squirrel	Pivorun et al. 1981 [14]
Factor V	639 ± 212% (n=8) 36.3°C	570 ± 143% (n=6) 7.9°C (89% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
sec	16.2 ± 0.3 (n=3) 37°C	20.1 ± 0.3 (n=4) * 6°C (55% of EU)	17.8 ± 0.4 (n=5) *,# 37°C (76%)	Franklin's ground squirrel	Pivorun et al. 1981 [14]
%	450 [250-500] (n=5)	542 [300-1000] (n=15) (120% of EU)	354 [250-450] (n=10)	Hedgehog	Biorck et al. 1962 [13]
	325 ± 143 36.3 ± 0.9°C	66 ± 15 * 8.8 ± 0.7 °C (20% of EU)	235 ± 85 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished

TABLE 2 CONTINUED.

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
Factor VII	369 ± 138% (n=8) 36.3°C	536% (n=2) 7.9°C (145% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
	1.01 ± 0.6 (n=15)	0.57 ± 0.14 (n=15) * (56% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
	649 ± 98 36.3 ± 0.9°C	605 ± 140 8.8 ± 0.7 °C (93% of EU)	866 ± 72 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Factor VIII	165 ± 73% (n=5) 36.3°C	35 ± 11% (n=6) * 7.9°C (=21% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
% relative to human plasma	232 ± 2.0 (n=6)	68 ± 0.1 (n=6) (=29% of EU)	230% (n=6)	13-lined ground squirrel	Cooper et al. 2016 [25]
IU/mL	2.92 ± 1.03	0.86 ± 0.35 * (29% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
% relative to human plasma	124 ± 18 36.3 ± 0.9°C	13.5 ± 6.0 * 8.8 ± 0.7 °C (=11% of EU)	64.5 ± 45.8 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Factor IX % relative to human plasma	378 ± 157 (n=11) 36.3°C	188 ± 65 (n=6)* 7.9°C (50% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
	425 ± 20 (n=6)	140 ± 4.0 (n=6) (33% of EU)	380% (spring arousal)	13-lined ground squirrel	Cooper et al. 2016 [25]
	50.4 ± 6.4 36.3 ± 0.9°C	16.9 ± 5.1 8.8 ± 0.7 °C (34% of EU)	75.6 ± 11.8 # 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Factor X	867 ± 126% (n=8) 36.3°C	805 ± 269% (n=6) 7.9°C (93% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
sec	18.9 ± 0.4 (n=6) 37°C	18.7 ± 0.7 (n=6) 6°C (96% of EU)	19.4 ± 0.3 (n=5) 37°C (102% of EU)	Franklin's ground squirrel	Pivorun et al. 1981 [14]
% relative to human plasma	182 ± 29 36.3 ± 0.9°C	239 ± 55 8.8 ± 0.7 °C (131% of EU)	252 ± 23 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Factor XI %	111 (n=1) 36.3°C	72 (n=2) 7.9°C (65% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
	104.4 ± 22.5 36.3 ± 0.9°C	35.0 ± 14.2 * 8.8 ± 0.7 °C (33% of EU)	95.6 ± 32.1 # 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Factor XII	291 ± 71% (n=9) 36.3°C	222 ± 70% (n=6) 7.9°C (76% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]
Fibrinogen (mg%)	189 ± 49 (n=10) 36.3°C	145 ± 34 (n=5) 7.9°C (77% of EU)		13-lined ground squirrel	Lechler et al. 1963 [15]

TABLE 2 CONTINUED.

Measurement	Euthermia (EU)	Torpor	Arousal	Species	Reference
g/L	2.09 ± 0.94 (n=16)	2.26 ± 0.46 (n=14) (108% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
%	0.54 [0.32-0.90] (n=3)	0.5 [0.07-1.04] (n=7) (93% of EU)	0.29 [0.06-0.42] (n=7)	Hedgehog	Biorck et al. 1962 [13]
	2.0 ± 0.4 36.3 ± 0.9°C	0.9 ± 0.4 8.8 ± 0.7 °C (47% of EU)	2.6 ± 1.3 # 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Plasminogen (%)	7.5 ± 2.3 (n=10)	12.6 ± 3.8 (n=5) (168% of EU)	18.7 ± 3.8 (n=5) *		De Vrij et al. unpublished
Plasmin inhibitor % relative to human plasma	93.5 ± 4.5 36.3 ± 0.9°C	83.6 ± 17.0 8.8 ± 0.7 °C (89% of EU)	101.5 ± 22 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Protein C IU/mL	0.44 ± 0.08 (n=17)	0.33 ± 0.08 (n=14) * (75% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
	30.0 ± 3.6 36.3 ± 0.9°C	18.4 ± 2.8 8.8 ± 0.7 °C (61% of EU)	33.7 ± 4.2 # 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Antithrombin IU/mL	0.98 ± 0.09 (n=17)	0.47 ± 0.04 (n=14) * (48% of EU)		Scandinavian brown bear	Welinder et al. 2016 [42]
	104.1 ± 6.6 36.3 ± 0.9°C	90.0 ± 11.0 8.8 ± 0.7 °C (87% of EU)	108.7 ± 7.5 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished
Heparin (sec)	36.6 ± 0.3 (n=9) 37°C	37.7 ± 0.4 (n=7) 6°C (103% of EU)		Franklin's ground squirrel	Pivorun et al. 1981 [14]
D-dimer		152 (n=8) "early denning"	124 (n=8) "late denning"	American black bear	Iles et al. 2017 [16]
	33.5 ± 0.1 36.3 ± 0.9°C	59.0 ± 8.5 8.8 ± 0.7 °C	9.9 ± 16.9 36.7 ± 1.1°C	Syrian hamster	De Vrij et al. unpublished

Data are represented as mean ± SD, or in one study as mean ± SE [14] and with corresponding body temperature. Two studies only provided a range with or without an average [13, 45]. For some parameters, only an average could be retrieved without range, SD or SE, or only individual data was given and a mean and SD had to be calculated. Sample sizes are given when this was retrievable per parameter. Some parameters are also represented as percentage of euthermia (% of EU) for use in Figure 1 and 2.

* $P < 0.05$ from (summer) euthermic values

# $P < 0.05$ from hibernating/torpid values

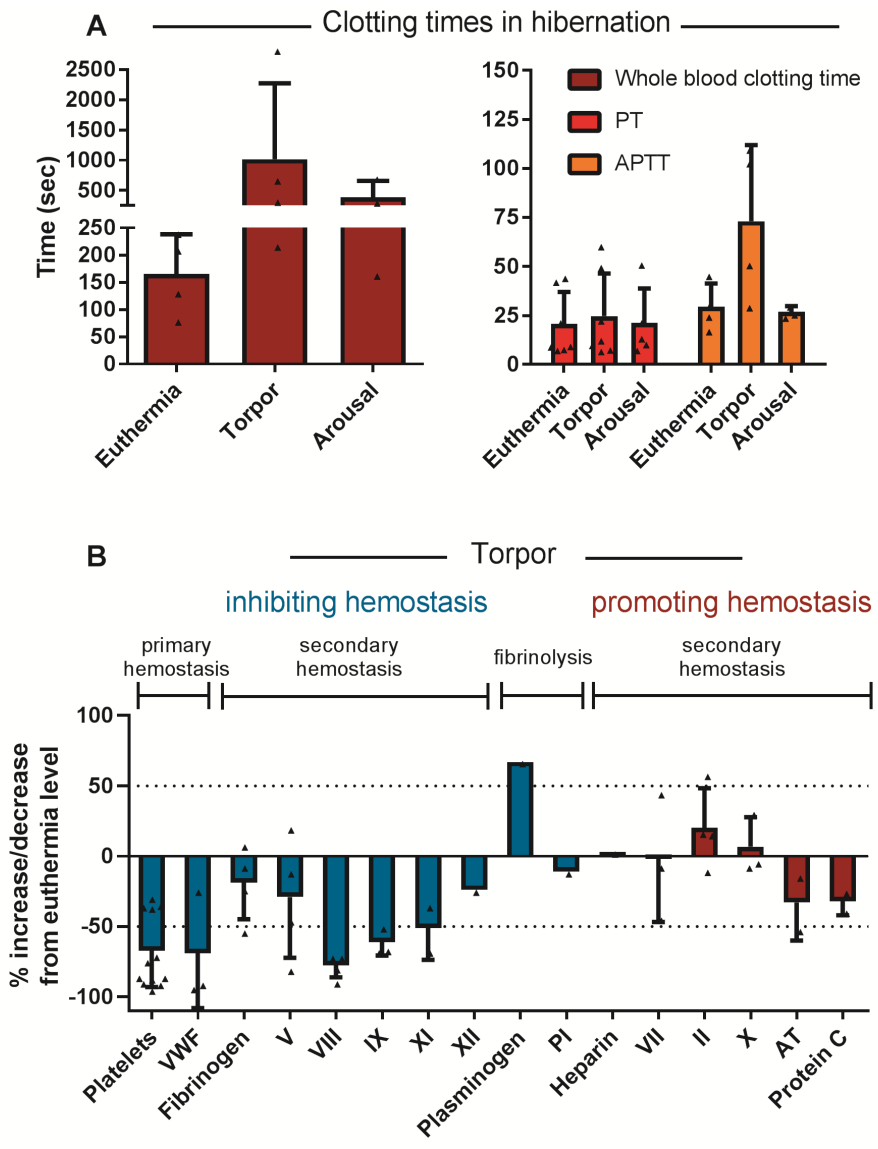


FIGURE 2. Regulation of components of primary hemostasis, secondary hemostasis and fibrinolysis tilts towards inhibition of hemostasis during torpor. A) Whole blood clotting time and APTT prolong during torpor in several studied species reviewed in Table 1 and 2, whereas PT is not consistently prolonged. In arousal APTT is recovered to euthermic level. B) All factor levels as percentage of euthermia level were calculated from studies summarized in Table 1 and 2. If a study did not report euthermic level, literature data was used. Data is represented as mean and standard deviation, with each triangle representing the data from each individual study.

SUPPRESSION OF HEMOSTASIS IN HYPOTHERMIA IN NON-HIBERNATORS

Non-hibernating animals (rat and mouse) subjected to hypothermia demonstrated the same principle of temperature and cold-time dependent platelet count reduction as hypothermia and torpor in hamster (**Chapters 2 and 5)**. Here we discuss whether this hypothermia induced, reversible thrombocytopenia is present in other non-hibernating species and which other hemostatic alterations occur due to hypothermia.

General hemostasis in hypothermia

The effect of hypothermia on hemostasis has mostly been assessed *ex vivo*. *Ex vivo* hypothermia from 35°C downwards prolongs clot initiation and total clotting time, and decreases clot propagation speed, but does not alter maximum clot firmness as measured in human blood by thromboelastography in both adults and neonates [52-54]. Effects of hypothermia *in vivo* are less clear. For instance, hypothermia in cardiac arrest patients undergoing targeted temperature management to 33°C did not change thromboelastography measurements (clotting time, maximum velocity, time to maximum velocity and clot firmness) compared to normothermic patients in one study [55], whereas 32°C hypothermia resulted in prolonged clot initiation time and reduced clotting speed in another recent study [56]. Hence, hypothermia *in vivo* in non-hibernating mammals may induce suppression of general hemostasis but requires further investigation. Since thromboelastography assesses whole blood rather than platelets or plasma alone, the effects of hypothermia on clot formation may reflect both primary and secondary hemostasis. To better understand the effects of hypothermia on hemostasis, we need to differentiate between effects of temperature *in vivo* and *ex vivo* on different components of the hemostatic system, specifically exploring determinants of primary hemostasis, secondary hemostasis and fibrinolysis.

Primary hemostasis in hypothermia

Of the factors involved in primary hemostasis, platelets seem the most affected by temperature effects. Thrombocytopenia during *in vivo* hypothermia has been observed in human, dog, hamster, rat and mouse and is generally reversible by rewarming [39, 57-62]. In humans, deep *in vivo* hypothermia (22 °C) of the forearm induces an anti-thrombotic response with increased bleeding time, because of reduced platelet activation and aggregation, and decreased platelet thromboxane A2 generation, which all reverted to normal after rewarming [63, 64]. This selective limb cooling may reflect the effects of temperature on primary hemostasis in patients suffering whole body hypothermia. Thrombocytopenia is reported in patients suffering accidental

hypothermia [58-60, 65, 66] or in adults and neonates treated with therapeutic hypothermia [67, 68]. Platelet dysfunction and count reduction may already occur from 35°C downwards [69]. Whereas these studies implicate that hypothermia suppresses primary hemostasis both *in vitro* and *in vivo*, other studies show enhanced activation and aggregation of human and mouse platelets during mild and moderate hypothermia [44, 70-72]. Enhanced activation of platelets by cooling has led to the hypothesis of platelet 'priming'. Platelet priming is believed to be an evolutionary process during which platelets acquired thermosensitive capacity, initiating their priming toward enhanced activation in colder extremities, i.e. at locations more prone to injury [73]. Moreover, primed platelets may be more subjected to clearance from the circulation by liver macrophages and hepatocytes to prevent unwanted activation and thrombosis [73]. Such rapid clearance of hypothermia primed platelets corresponds with the rapid clearance of cold stored platelets after transfusion [74-76]. Contrarily, deep hypothermia (20°C) in dogs actually prolonged platelet lifespan from 4.2 to 4.9 days [62]. Very little is known about hypothermia effects on the other players in primary hemostasis, including VWF. One study implies an increase in plasma VWF in hypothermic pediatric patients undergoing cardiopulmonary bypass surgery [77]. Additionally, this rise in plasma VWF was linked to the depth of hypothermia and not the duration of surgery. Taken together, by assessing the current literature it seems there are controversies on the consequences of hypothermia on primary hemostasis, specifically on the effects of *in vivo* cooling. Differences are likely due to the difference in analyzed species (e.g. humans versus rodents), as well as pre-analytical and analytical variation between studies [70], such as rate and duration of cooling or rewarming, extremity cooling versus whole body cooling, presence or absence of anesthesia, time between sampling and measurement and/or sample temperature during measurement. Therefore, by large, that temperature can affect factors involved in primary hemostasis is clear but it remains incompletely understood exactly how and under which circumstances it can inhibit versus promote.

Secondary hemostasis and fibrinolysis in hypothermia

The effects of hypothermia on secondary hemostasis and fibrinolysis are even less well understood. Hypothermia (<33°C) reduces the kinetics of clotting enzymes and plasminogen activator inhibitors, overall leading to prolonged PT and APTT and a mild bleeding diathesis [78]. Nevertheless, despite the inhibitory effects on the hemostatic system, the use of mild or moderate therapeutic hypothermia (down to 32°C) does not increase the risk of hemorrhage in patients irrespective of the indications of therapeutic hypothermia [68]. In pigs, 33°C hypothermia exerted an anticoagulant effect by increasing antithrombin III and protein C [79], whereas reductions of these enzymes

were found in 32°C hypothermic rabbits along with increased plasminogen level and reduced α-2-antiplasmin level [80]. Thus, hypothermia suppresses secondary hemostasis and fibrinolysis, but due to fragmented data the specific changes in determinants of either secondary hemostasis or fibrinolysis and its underlying mechanism remain unclear.

Does low temperature activate coagulation factors and platelets?

Data from patients with accidental hypothermia have been used to advocate that hypothermia or cooling activates the hemostatic system. Coagulopathy upon accidental hypothermia is described during admission to the emergency room, as demonstrated by patients displaying a prolonged PT and APTT [65, 81]. It is generally believed that this coagulopathy is due to reduced kinetics and increased usage of coagulation factors and platelets, which is most strikingly seen in hypothermic cases with disseminated intravascular coagulation (DIC) [65, 82]. However, these observations do not demonstrate that hypothermia activates the hemostatic system. DIC is not observed in all cases of hypothermia and may be induced during rewarming rather than during cooling [83]. Possibly, cases of hypothermia induced coagulopathy are precipitated by the concomitant acidosis, the lowering in blood pH, known to induce coagulopathy by a different mechanism than hypothermia [84]. Thus, hypothermia itself causing directly a usage or activation of coagulation factors has yet to be demonstrated.

Data on platelet activation due to hypothermia are mainly based on findings of *ex vivo* cooled platelets, for instance inducing shape changes that mimic activated platelets [85, 86], which may already occur at 20°C [87]. Additionally, platelet aggregation may be enhanced after *in vivo* or *ex vivo* cooling [44, 70-72], in line with the hypothesis of 'priming' platelets in colder extremities to prepare for potential injury. However, cooling for several days seems not to activate platelets but rather retain platelet characteristics *in vitro* better than storage at 22°C [70, 88, 89], one may consider these cold stored platelets as 'primed', unfortunately transfusing cold stored platelets causes their rapid irreversible clearance by phagocytosis in the liver [74-76]. Whether cooling and room temperature storage lead to platelet activation remains a controversy, since for both storage conditions there is evidence that platelets do get activated, be it by P-selectin expression or by excretion of alpha-granule contents [90]. Besides data on platelet activation, cooling at 4°C may also activate plasmatic factors, as e.g. demonstrated by the increase in activated factor VII [91]. Therefore, it remains unclear whether (*in vivo* and/or *ex vivo*) hypothermia activates platelets and coagulation factors.

Mechanisms suppressing secondary hemostasis

The mechanism of secondary hemostasis suppression during torpor is still unknown. Several hypotheses coexist, which may each be true for different coagulation factors. The mechanisms may comprise 1) increased breakdown, 2) reduced synthesis, 3) reversible inhibition of functionality, and/or 4) reversible storage of factors.

Plasma levels of coagulation factor are governed by their synthesis and elimination. Increased breakdown during torpor may occur for some coagulation factors. During torpor in squirrel, liver mRNA of factor VIII increases, whereas factor VIII level and activity in plasma decrease, because of loss of factor VIII stability due to decreased level of VWF, normally protecting it from degradation [25]. By increasing VWF plasma level during arousal, factor VIII stability and plasma level will likely recover concurrently.

Synthesis of coagulation factors may decrease, for instance liver mRNA of factor IX decreases threefold in torpid squirrel [25]. The mRNA levels have not been studied in arousal however, likely these levels will increase to recover plasma levels of for instance factor IX. The effect of hibernation on vitamin K dependent factors (II, VII, IX) is different per factor (Table 1), for example factor VII hardly changes in torpor whereas factor IX is one of the most suppressed factors, demonstrating that production of vitamin K dependent coagulation factors is not uniformly altered in torpor.

Generally all enzymatic processes are affected by temperature lowering, which may also account for changes in hemostasis. For example, enzymatic activities of thrombin and Xa generation are temperature dependent and reduced from 33°C downwards [92]. Activity of coagulation factors is temperature dependent as well, as demonstrated by increasingly longer PT and APTT measurements when temperature falls below 35°C [92, 93]. The inhibiting effect of temperature on the kinetics of the coagulation cascade reverts when temperature is increased.

Endocytosis of coagulation factors has been demonstrated for fibrinogen, factor V, VII, VIII, X, and VWF [94-101]. Although the endocytosis occurs in different cell types, it may still contribute to suppressing secondary hemostasis. Exocytosis has been documented less, but has been shown for fibrinogen and factor V from megakaryocytes [101] and factor VIII and VWF from endothelial cells [102]. The trafficking of coagulation factors involves various cell types, including platelets, megakaryocytes, macrophages, dendritic cells and endothelial cells. The role of temperature in endocytosis and exocytosis has not been studied well. However, it has been shown that Weibel Palade body exocytosis is temperature dependent [28]. Furthermore, it has been demonstrated that endocytosis of factor VIII-VWF complex by macrophages is promoted by shear stress [94]. This mechanism is however less likely to contribute to reducing factor VIII and VWF during torpor and hypothermia, since blood flow and shear stress are reduced in this phase.

FUTURE PERSPECTIVES

Factors determining margination

It should be assessed which factor is crucial in the margination of platelets to liver sinusoidal endothelium. Hereto, the hypothermia model can be used to systematically analyze the effect of platelet and endothelial adhesion molecules (e.g. GPIba, GPIIbIIIa, VWF, and P-selectin) and of platelet activation (e.g. via cyclooxygenase, ADP-receptors, and protease activated receptors). One study demonstrated that during hypothermia of 28°C in mice, administration of soluble CD39 (NTPDase1, the main ADP metabolizing enzyme) reversed thrombocytopenia [44]. Thus, slight platelet activation, potentially via ADP, may be needed for platelet margination under cold conditions. This would match our data in 5'-AMP injected mice which demonstrated a drop in body temperature but no change in platelet count (**Chapter 2**), potentially due to 5'AMP being metabolized to adenosine by CD39 and CD73, thereby inhibiting platelet activation. The plasma of hypothermic animals may be used to determine *ex vivo* whether a plasmatic factor, such as cytokines or interleukins, increases platelet-endothelial adhesion under conditions of low flow and/or low temperature. Preliminary data from our group demonstrates that GPIIb/IIIa (i.e. integrin $\alpha_{IIb}\beta_3$) may be involved in the reversible platelet-endothelial adhesion, since blockade of GPIIb/IIIa by Tirofiban precluded the temperature and flow dependent adhesion of platelets to human umbilical vein endothelial cells. Future studies should determine the role of platelet and endothelial adhesion molecules in margination in torpor and hypothermia and specify the main required ligand, for instance VWF, fibrinogen or fibronectin. Once the crucial factor in platelet margination has been found, blocking of platelet margination by inhibiting this factor should be investigated in hibernators to substantiate that thrombocytopenia during torpor prevents initiation of thrombosis. Additionally, hibernating hamsters can be infused with recombinant coagulation factors to compensate for the relative decrease in factor VIII, IX and XI in torpor to study whether reduced clotting factors are essential to preclude thrombosis in torpor.

We pointed out that despite a same low body temperature, torpid hamster reduced platelet count more than hypothermic hamster (**Chapter 2**). The platelet count reduction is temperature dependent, but the extent of this reduction is likely cold-time dependent as torpor lasts several days whereas our hypothermia experiment minutes to hours. This time dependency can be studied by measuring platelet count in hypothermia over a longer time period. In **Chapter 5** we corroborate this time-dependency since cooling of rat for 1 hour at 15°C decreased platelet count by 42%, whereas cooling for 3 hours at 15°C reduced platelet count by 52%. Although the

effect of time on extent of the temperature dependent thrombocytopenia is small, it seems to be a contributing factor. An additional effect, albeit it small, of preparation to hibernation on the extent of thrombocytopenia during periods of low body temperature can be ruled out by comparing effects of forced hypothermia in summer hamsters and hamsters that are fully prepared for hibernation after a fattening period with shortened light:dark cycle and lowered ambient temperature. If the hibernation-prepared hamsters reach a lower platelet count than summer hamsters, likely hibernation preparatory processes increase the animals susceptibility to hemostatic suppression, which may depend on (epi)genetic regulation, transcriptional changes or protein modification (e.g. glycosylation).

Improving platelet storage for transfusion

Storage lesions and bacterial contamination are still the main reasons for the 5-7 days storage limit for platelet concentrates stored at 22-24°C and the yearly losses due to outdated, dysfunctional and discarded units. Storage at lower temperatures reduces bacterial growth [88] and may retain platelet functionality longer [89], but induces rapid platelet clearance from circulation after transfusion [74-76]. Changes in sialylation of membrane receptors and clustering of GPIbα on the platelet membrane govern the clearance of transfused non-hibernator platelets after short cold storage [76, 103, 104]. Whether these changes occur or are prevented in hibernator platelets remains to be studied. The unique model of hibernation demonstrates that prolonged storage of platelets is possible at low temperatures *in vivo.* The mechanism in hibernators that prevents platelet clearance after cold storage may be applicable *ex vivo* as well, since hibernator platelets can be cooled *ex vivo* - without platelet activation (**Chapter 6**) - and transfused without rapid clearance [11]. All these data inspire reflection on improvement of *ex vivo* cold storage of human platelets and acquiring cold resilient human platelets, improving shelf life of platelet concentrates, decreasing bacterial contamination and reducing monetary losses. It may be that hypothermic liver and its subsequent rewarming is crucial in the reversibility of retaining either *in vivo* or *ex vivo* cold exposed platelets. Future studies should therefore investigate whether hypothermic liver allows transfusion of cold stored platelets. Hereto, platelets may be labeled and transfused after either cold storage or after standard 22-24°C storage with subsequent assessment of platelet lifespan in circulation of animals undergoing hypothermia and rewarming or those that are already hypothermic. If hypothermic liver cannot phagocytose or clear cold stored platelets, the platelets will likely start margination during hypothermia and be released in circulation during rewarming just like native non-stored platelets. It should be assessed whether during rewarming with warm liver the cold stored platelets are rapidly cleared or remain in circulation like

22-24°C stored platelets. This strategy might then be utilized to transfuse cold stored platelets to patients who, or whose liver, can briefly be subjected to therapeutic hypothermia, e.g. peroperatively or in the intensive care unit. Thus if the patient or its liver can be cooled, it may also receive cooled platelets.

Liver cooling and transplantation

The induction of platelet margination to liver sinusoidal endothelium might not be dependent on total body cooling but instead rely more on cooling of only liver. This would suggest that temperature effects on liver and its sinusoidal endothelium are crucial, rather than temperature effects on non-liver components, such as heart rate, blood flow and - importantly - platelets. To discern between the systemic effects of cooling versus effects due to local liver cooling two different approaches may be used. Firstly, in a systemically cooled animal heart rate and blood flow may be pharmacologically increased or via cardiopulmonary bypass (CPB), although the latter may require use of anticoagulants to prevent platelet activation. Secondly, liver may be cooled locally without increasing core body temperature, for instance by surgical exposure and insulation of the liver. Subsequently, platelet retention in hypothermic liver may be used in transplantation. Cooling of the donor prior to harvesting the liver, or slowly perfusing the liver with cooled donor or recipient blood, would induce a much higher amount of functional donor platelets in the graft, due to platelet retention in sinusoids via margination, which may subsequently boost or sustain their documented liver regenerating effect in the recipient following transplantation [105]. Thus, investigating the effects of hypothermia on platelets and the liver may therefore yield several clinical benefits, including the use of cold stored platelets in patients whose liver can be cooled and improving regeneration of donated livers by increasing retained platelet content in the donated liver.

Auxilliary platelet functions

We studied hibernator platelet activation, degranulation and shape change (**Chapter 2, 4 and 6**). Future studies should determine if other aspects of platelets also remain functional, such as aggregation and adhesion to surfaces and how this contributes to primary hemostasis. Furthermore, platelets are involved in more than hemostasis, amongst others in wound healing, microbial defense and cancer metastasis. Some of these auxiliary functions of platelets should be assessed throughout hibernation. Especially platelet function in immunology may be relevant to hibernating mammals such as bats in North America affected by White Nose Syndrome (WNS). WNS is a fungal infection by the European fungus *Pseudogymnoascus destructans* which grows well at low temperatures and induces more frequent arousals in hibernating bats,

thus depleting their energy reserve. WNS has already resulted in death of around six million bats [106]. Platelets act directly, and indirectly via complement activation and phagocyte recruitment, to kill fungi, but may also interact and lead to thrombosis [107], how this is affected by hibernation and influences the course of WNS infection has not yet been studied. Since platelets are generally believed to become activated due to lowering of temperature, platelet-fungi induced thrombosis may be involved in WNS as well. Indeed, there are histological signs of thrombosis in some of the bats affected by WNS (dr. Meteyer, wildlife pathologist, personal communication)

Standardization of analyses

However, it remains unclear whether hypothermia indeed activates platelets and coagulation factors. In this thesis we demonstrated that platelets are not activated by cooling *in vivo* (daily torpor mice 25°C, **Chapter 6**; hypothermic rat 15°C, **Chapter 4**; deep torpor hamster 9°C, **Chapter 2, 4 and 6**) or *ex vivo* (4°C platelets from human, rat, mouse, hamster, **Chapter 6**). Some studies demonstrate a reduction in platelet function during hypothermia and *ex vivo* cooling, which is reversible by rewarming [63, 108], this matches our data in torpid hamster (**Chapter4**), but not our data in hypothermic rat where functionality was unchanged throughout cooling and rewarming (**Chapter 5**). The effects of hypothermia on platelet function and coagulation depend on factors reviewed by Van Poucke et al. [70]:

- the actual body temperature during sampling
- the pre-analytical and analytical temperature
- sample type (*in vivo*, *ex vivo*, *in vitro*; whole blood, washed platelet preparation)
- temperature changes during the sampling time (induction, maintenance, and rewarming)
- the moment of sampling in relation to agonist stimulation
- the duration of hypothermia
- the cause of hypothermia (spontaneous, whether induced externally or internally)
- coexisting factors (extracorporeal circulation, comorbidity, drugs)
- the modality of induced hypothermia (local, regional, or general)

Applying golden standard techniques and standardizing pre-analytical and analytical variables to assess platelet activation and functionality is important in determining the effects of temperature on platelets. These factors should be taken into account in any future study determining the effect of temperature on coagulation and platelet function, including the auxilliary functions of platelets such as those in the immune system.

Improved understanding of hemostasis in hypothermia and new antithrombotic therapies

Since the majority of changes in the hemostatic system are present throughout the hibernating species investigated so far, it is likely that a species-overlapping antithrombotic mechanism is present throughout mammalian evolution. This is further corroborated by the fact that coagulation factors are well preserved throughout mammalian evolution, e.g. factor XI and XII already made their first appearance with the evolution of amphibians [109]. Since all hibernating animals differ widely in phylogenetic heritage - e.g. brown bear, hamster and turtle - it is likely that the natural antithrombotic strategy is also present and inducible in non-hibernating mammals. In this thesis we demonstrate for primary hemostasis that the temperature inducible thrombocytopenia indeed is present in non-hibernating mammals. To elucidate the anticoagulant (secondary hemostatic) strategy, first the driving force in the hibernation induced suspended coagulation needs to be determined. This may be achieved by performing intervention studies on the synthesis and breakdown of coagulation factors or by (radio)labeling of factors and subsequent dynamic/kinetic studies, and to assess its effect on anticoagulation in torpor and in hypothermia of hibernators. Furthermore, the coagulation cascade should be investigated in hypothermia of non-hibernating mammals to determine if a similar inhibitory effect on secondary hemostasis occurs when body temperature decreases as in torpor. The speed and extent of recovery of hemostatic components, such as platelet count, VWF and coagulation factor level, and hemostatic function should be assessed in hypothermic patients throughout rewarming. This allows better understanding of what is needed in which timeframe to recover hemostasis to euthermic situation and to determine which factors recover completely by rewarming.

By increasing our knowledge on the effects of hypothermia on hemostasis and the consequences of rewarming, physicians may evaluate better the benefits and disadvantages of (therapeutic) hypothermia. For example, cardiac arrest patients treated with mild hypothermia (32-36°C) do not have an increased incidence of bleeding compared to normothermic treatment but have better neurological outcomes [110, 111]. They receive similar amount of transfusion compared to normothermic patients, potentially to compensate for low platelet counts. Knowing that rewarming will generally recover the key components of hemostasis including platelet count may reduce the amount of transfusion required in these patients treated with hypothermia.

The knowledge on speed and extent of recovery of specific hemostatic components by rewarming will also be helpful in trauma patients, often subjected to hypothermia, acidosis and coagulopathy, also known as the Triad of Death [112]. Different than in accidental or therapeutic hypothermia, trauma often adds systemic activation of

the hemostatic system due to tissue injury and subsequent activation of platelets and coagulation factors or even disseminated intravascular coagulation (DIC). As a consequence, the concurrent coagulopathy is not only due to hypothermia induced hypocoagulation and suppression of primary hemostasis due to platelet margination. Acidosis, a lowering in blood pH, induces a coagulopathy by a different mechanism than hypothermia [84]. Knowing to what extent the hypocoagulopathy may recover due to rewarming alone may guide to which extent additional plasma and platelets will be transfused.

Therapeutic hypothermia and targeted temperature management are already applied in cases of cardiac arrest survivors, head injury, neonatal encephalopathy, and in patients undergoing cardiopulmonary bypass surgery [67, 78, 113]. The beneficial effect of low temperature and reduced metabolism on neurological outcome and survival has been demonstrated [110, 114, 115], and in neonates and cardiac arrest patients this beneficial effect is demonstrated without increases in hemorrhagic or thrombotic lesions [68, 116]. However, for out of hospital cardiac arrest patients targeted temperature management below 36°C does not seem to add more survival benefit compared to targeting 36°C [117].

More applications for targeted temperature management or therapeutic hypothermia may arise, for instance mild intraoperative hypothermia may be beneficial in plastic surgery by reducing thrombosis in free tissue transfer, hence improving free flap survival [118]. Therapeutic hypothermia may also improve the coagulopathy as measured by thromboelastography in patients with sepsis or septic shock [119], and even decrease mortality and end-organ damage in sepsis as demonstrated experimentally [120], although clinical trials in elderly did not show this decrease in mortality [121]. Additionally, therapeutic hypothermia might suppress hypercoagulative states such as in DIC with organ failure and in heparin-induced thrombocytopenia with prothrombotic immunecomplexes and a mortality up to 30% of cases [121]. If a pharmacological tool becomes available to mimic the torpor and temperature induced suppression of thrombosis, it may add the benefit of a rapid reversal strategy, since arousal and rewarming rapidly reverse antithrombotic effects within minutes to hours. A rapid reversal of anticoagulation is useful since bleeding is the most common adverse drug event bringing patients to emergency wards [122, 123], and requires correction of anticoagulation as quickly as possible. However, most reversal techniques require several hours to reverse anticoagulation, i.e. by administering fresh frozen plasma, Vitamin K or prothrombin complex concentrate [124, 125]. To date, only one registered monoclonal antibody fragment against direct oral anticoagulant (DOAC) dabigatran is faster and reverses anticoagulation within minutes [126], but is highly expensive. Therefore, the development of new treatment and reversal strategies may help

physicians to effectively manage the life-threatening emergency of bleeding in anticoagulated patients [127].

CONCLUSIONS

In summary, despite the expected risk of thrombosis during hibernation due to prolonged immobility with reduced blood flow and increased blood viscosity, hamsters do not show signs of thrombotic complications during or after hibernation. We revealed that during the immobile torpor phase there is an anti-thrombotic shift of hemostasis by regulating the amount of platelets, VWF and coagulation factors, resulting in a thrombocytopenia and a phenotype resembling Von Willebrand Disease with Hemophilia A, B and C, while maintaining factors required for fibrinolysis. Low body temperature during torpor induces the thrombocytopenia in hibernators via reversible storage of platelets, most likely via margination of platelets in liver sinusoids, reversed by rewarming during arousal and seems to occur without activation of platelets. Platelet counts are associated with body temperature in all hibernating species studied to date. Margination of platelets to liver sinusoidal endothelium also represents the underlying mechanism of reversible thrombocytopenia during hypothermia in non-hibernators. Also patients with accidental or therapeutic hypothermia demonstrate temperature dependent platelet dynamics and often signs of coagulopathy. However, concurrent morbidities in (trauma) patients can cause coagulopathy with thrombosis and conclusions that low temperature alone activates platelets and the coagulation system are therefore arguable. The underlying pathways, key receptors and ligands for platelet margination and suppression of the coagulation system remain to be determined. Platelets from both hibernators and non-hibernators reversibly change shape throughout cooling and rewarming without activation advocating that low temperatures may not activate platelets, adding to the controversy of temperature effects on platelets. Awareness of (pre)analytical variables in studying such temperature effects on hemostasis is crucial. Future studies investigating hemostasis in hibernation and (therapeutic) hypothermia may yield several clinical benefits for instance by suppressing hemostasis reversibly in patients suffering prothrombotic episodes.

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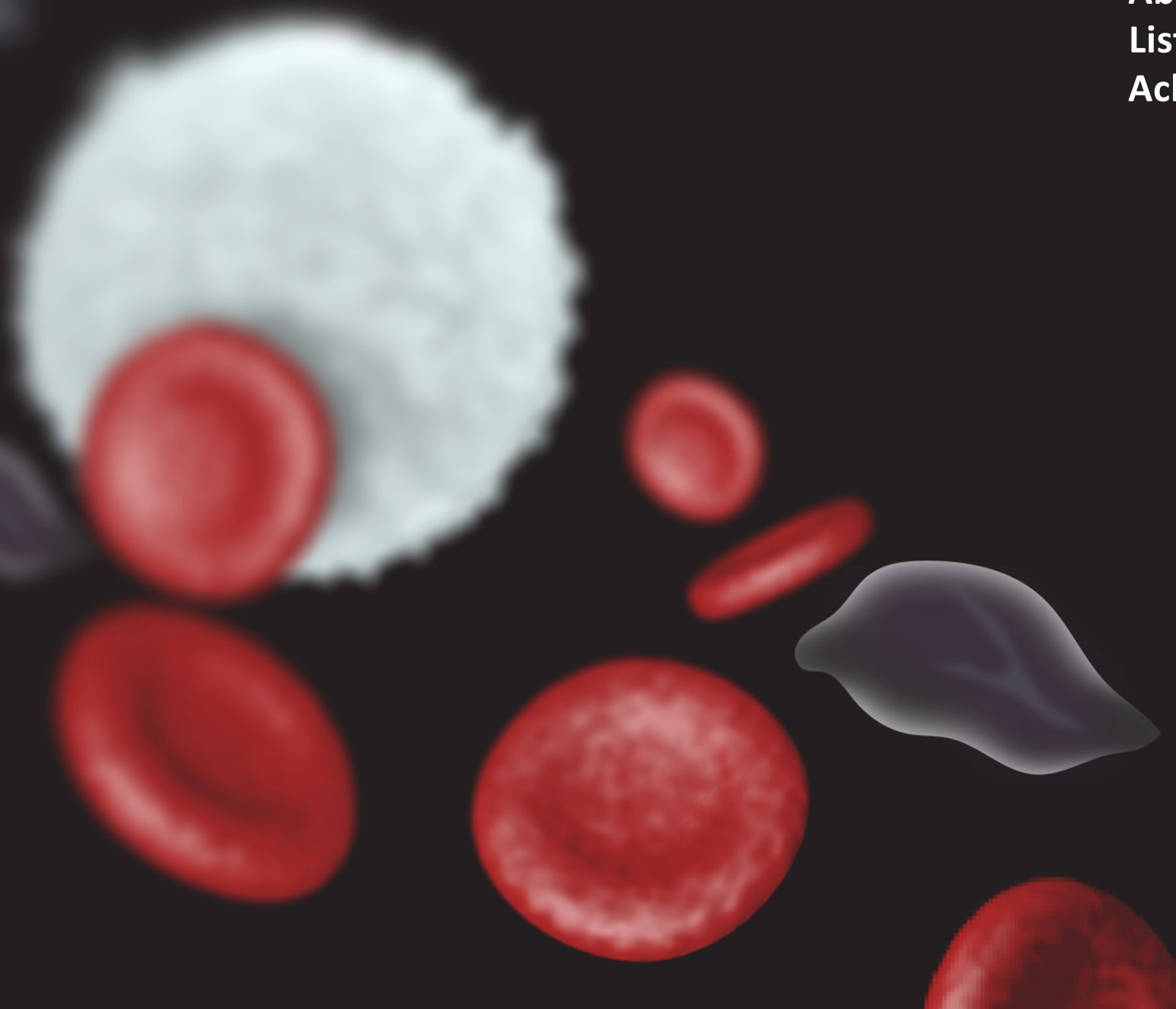
APPENDICES



NEDERLANDSE SAMENVATTING VOOR NIET-INGEWIJDEN

Inleidend

In dit hoofdstuk is het proefschrift in het Nederlands samengevat. Het proefschrift is tot stand gekomen uit een MD/PhD-traject, waarin de opleiding geneeskunde gecombineerd is met een promotietraject. Allereerst wordt nu de achtergrond van het onderzoek kort besproken, waarna één voor één de belangrijkste bevindingen per hoofdstuk worden samengevat. Biomedische terminologie wordt op enkele plekken tussenhaakjes uitgelegd. Dit hoofdstuk eindigt met een conclusie op basis van de bevindingen en interpretaties vanuit dit proefschrift.

Achtergrond en doel van het proefschrift

Trombose is de vorming van bloedstolsels door bloedplaatjes activatie en bloedstolling leidend tot afsluiting van een bloedvat en onderbreking van de bloeddoorstroming naar een weefsel of orgaan. Trombose is een belangrijke doodsoorzaak en wereldwijde ziektelast, denk hierbij aan het hartinfarct, herseninfarct, trombosebeen en longembolieën [1-6]. Zowel primaire als secundaire hemostase (het voorkomen van bloedingen door o.a. bloedstolling) zijn betrokken in veneuze (aderlijke) en arteriële (slagaderlijke) trombose. Het is te verwachten dat trombose ontstaat tijdens winterslaap vanwege verschillende factoren die in mens het trombose risico vergroten: langdurige onbeweeglijkheid [7-9], stilstand van bloed in aders en hartboezems [10], verhoogde bloed viscositeit (stroperigheid) [11-13], cycli van koeling-opwarming met relatieve hypoxie (zuurstoftekort) en herstel van zuurstof aanvoer met tekenen van endotheelschade [7, 14], en overgewicht tijdens het begin van winterslaap [15]. Ondanks deze risicofactoren tonen winterslapers geen tekenen van trombose of embolisatie (losschieten van een bloedstolsel welke vervolgens elders een bloedvat afsluit), waarschijnlijk door veranderingen aan essentiële onderdelen van hemostase tijdens de winterslaap.

Het doel van dit proefschrift was om een overzicht te genereren van veranderingen aan belangrijke componenten van hemostase tijdens winterslaap in een enkele diersoort, namelijk de Syrische (goud)hamster. Een ander doel was om aan te tonen of deze veranderingen in niet-winterslapende zoogdieren kunnen worden nagebootst via geforceerde hypothermie (onderkoeling). Dit proefschrift focusde op het krijgen van inzicht in het onderliggende mechanisme van de torpor (laag metabole fase van winterslaap) geassocieerde reversibele trombopenie (laag aantal bloedplaatjes), en van de morfologische veranderingen van bloedplaatjes, inclusief de relatieve koude resistentie van het cytoskelet (moleculaire cel-skelet) van winterslaper bloedplaatjes. Het uiteindelijke doel omhelst het identificeren van mogelijke therapeutische aangrijpingspunten voor antitrombotische medicijnen en voor lange termijn opslag van bloedplaatjes voor transfusie.

Algemene uitingen van hemostase onderdrukking in winterslaap

We bepaalden de compenten van hemostase die veranderd zijn tijdens torpor en waarschijnlijk trombose voorkomen. In **Hoofdstuk 2** onderzochten we de effecten van winterslaap en hypothermie op de dynamiek van circulerende bloedplaatjes in zowel winterslapende als niet-winterslapende zoogdieren. Ook onderzochten we het effect van een farmacologisch middel (5'-AMP) om torpor te induceren. Het verlagen van de lichaamstemperatuur is waarschijnlijk een van de belangrijkste aansturende krachten voor de reversibele daling in bloedplaatjes aantal in zowel winterslapende als niet-winterslapende diersoorten wanneer zij blootgesteld zijn aan geforceerde hypothermie. De hieropvolgende trombopenie (lage plaatjes aantal) tijdens de lage lichaamstemperatuur herstelde snel tijdens opwarming in alle onderzochte diersoorten wanneer zij weer eutherm werden, hetzij door natuurlijke arousal hetzij door geforceerd opwarmen. Door de snelheid van herstel hypothetiseerden wij dat een opslag en vrijlaat mechanisme ten grondslag ligt aan de trombopenie bij lage lichaamstemperatuur, in plaats van het onherstelbaar afbreken en vervolgen weer aanmaken van bloedplaatjes. We vonden verder dat plaatjes integriteit behouden bleef tijdens winterslaap en hypothermie in zowel winterslapende als niet-winterslapende diersoorten, omdat er geen tekenen waren van bloedplaatjes activatie gedurende de experimenten en functionaliteit van bloedplaatjes herstelde bij het bereiken van euthermie. Bovendien speelt de milt geen grote rol in de temperatuur gestuurde opslag en vrijlating van bloedplaatjes, aangezien het verwijderen van de milt alvorens de winterslaap geen effect had op de daling in bloedplaatjes aantal tijdens winterslaap. Interessant genoeg leidde farmacologische inductie van torpor door 5'-AMP injectie niet tot daling van bloedplaatjes aantal, ondanks dat de lichaamstemperatuur wel daalde. 5'-AMP interfereert daarom mogelijk met het onderliggende mechanisme van de temperatuur gestuurde bloedplaatjes dynamiek. Samenvattend is de temperatuur afhankelijke bloedplaatjes daling een grote aanpassing in het primaire hemostase systeem tijdens torpor.

In aanvulling hierop hebben we in **Hoofdstuk 3** belangrijke determinanten onderzocht van het bloedstolling systeem tijdens winterslaap in de Syrische hamster, namelijk die van primaire en secundaire hemostase als ook die van het fibrinolytische systeem. De hemostase is zeer waarschijnlijk geremd in torpor, zoals gesymboliseerd door verminderde trombine generatie met verlengde stollingstijden (PT en APTT), welke herstelden in arousal. Activatie secundaire hemostase en fibrinolyse is onwaarschijnlijk tijdens torpor gezien het plasma niveau van D-dimer laag blijft gedurende de winterslaap.

Het onderdrukken van hemostase gebeurt ogenschijnlijk door vermindering van aantal bloedplaatjes en het niveau van von Willebrand Factor (VWF), fibrinogeen, stollingsfactor V, VIII, IX, XI en door het toenemen van het plasminogeen niveau. De verminderde hemo-

stase werd gedeeltelijk tegengewerkt door matige stijging in factor II en X en een afname in antistolling factoren antithrombine, proteïne C en plasmine inhibitor. Hoe dan ook laat laat onze data zien dat tijdens torpor de hemostastische balans duidelijk helt naar remming wat gecorrigeerd wordt tijdens arousal.

Mechanismen van hemostase onderdrukking in winterslaap en hypothermie

Het onderliggende mechanisme van de bloedplaatjes dynamiek in winterslapende hamsters werd verder onderzocht in **Hoofdstuk 4**. In deze studie toonden we aan dat plaatjes opslag en vrijlating ten grondslag ligt aan de reversibele trombopenie in torpor in de hamster. Fluorescente bloedplaatjes volgden na transfusie dezelfde dynamiek in de bloedsomloop gedurende torpor-arousal cycli als de bloedplaatjes van de ontvanger. Vrijwel alle getransfundeerde bloedplaatjes herstelden zich in de bloedsomloop tijdens arousal en waren dus niet irreversibel verwijderd van de bloedsomloop. Bloedplaatjes lieten daarnaast geen tekenen zien van activatie. Verder toonden we aan dat de levensduur van bloedplaatjes met 50% is verlengd tijdens winterslaap vergeleken met niet-winterslapende hamsters. Tot slot toonden we met electronen microscopy analyse van bloedplaatjes in organen aan, dat lever sinusoiden in plaats van de milt of long de meest waarschijnlijke opslag en vrijlaat locatie is voor bloedplaatjes. Opgeslagen bloedplaatjes in lever sinusoiden waren niet gedegranuleerd. Dus, lage lichaamstemperatuur leidt tot trombopenie tijdens torpor via reversibele opslag, aannemelijk in de lever sinusoiden, wat zich herstelt tijdens opwarming tijdens arousal en gebeurt ogenschijnlijk zonder activatie en degranulatie van bloedplaatjes. Gezien de locatie van bloedplaatjes ophoping aan liver sinusoidaal endotheel, de lage bloedstroomsnelheid en de verhoogde bloed viscositeit tijdens torpor en gezien de reversibele aard van plaatjes opslag tijdens arousal, is marginatie van bloedplaatjes aan het endotheel het meest waarschijnlijke onderliggende mechanisme aan de plaatjes aantal daling.

De bevindingen in **Hoofdstuk 2** toonden aan dat de bloedplaatjes dynamiek temperatuursafhankelijk is en toepasbaar in niet-winterslapende zoogdieren. Derhalve onderzochten we verder in **Hoofdstuk 5** de opslag locatie en het mechanisme voor reversibele trombopenie in niet-winterslapende zoogdieren. Met behulp van (intravitaal) beeldvormende studies in rat en muis toonden we dat marginatie van bloedplaatjes aan lever sinusoidaal endotheel tijdens hypotherie het onderliggende mechanisme behelst van de reversibele trombopenie. Bovendien sloten we een rol uit van de milt in hypothermie geïnduceerde trombopenie door het uitvoeren van miltextirpatie alvorens en tijdens de onderkoeling, waarvan er geen effect was op de temperatuursafhankelijke bloedplaatjes dynamiek. In **Hoofdstuk 4** waren de opgeslagen bloedplaatjes in lever sinusoiden soms gezien in een speer vorm met verlengde microtubuli, wat in lijn is met eerdere bevindingen van gekoelde grondeekhoorn bloedplaatjes [16, 17]. Reddick et al. stelden voor dat

deze speer vorm in eekhoorn bloedplaatjes zou kunnen leiden tot vastlopen in de milt en dientengevolge tot de trombopenie [16]. Wij lieten echter zien dat de milt niet betrokken is bij de temperatuursafhankelijke bloedplaatjes dynamiek in hamster (**Hoofdstuk 2**), wat recentelijk ook is bevestigd in grondeekhoorn [18]. Echter, bloedplaatjes zouden alsnog kunnen vastlopen in de lever door vormverandering tijdens torpor. Of deze vormverandering van bloedplaatjes ook tijdens hypothermie gebeurt in niet-winterslapers en dus een vereiste zou kunnen zijn voor de opslag in lever sinusoiden is nog niet onderzocht. Daarom werd in **Hoofdstuk 6** de rol van veranderingen aan het cytoskelet onderzocht in vormveranderingen van bloedplaatjes tijdens winterslaap en vergeleken met vormveranderingen van bloedplaatjes van mens en andere niet-winterslapende diersoorten tijdens *ex vivo* koeling. Wij lieten zien dat in torpor met lage lichaamstemperatuur de circulerende hamster bloedplaatjes ofwel speer vormig danwel discus vormig zijn met behoud van cytoskelet structuur van tubuline. Daarentegen depolymerizeerde de tubuline in bloedplaatjes van muis, rat en mens tijdens lage *ex vivo* temperatuur, met als gevolg een bolvorm met het ontstaan van filopodia, lijkend op geactiveerde bloedplaatjes. Ondanks deze ogenschijnlijke activatie, na *ex vivo* koeling en opwarming was er geen stijging in expressie van activatie markers op bloedplaatjes uit winterslapende en niet-winterslapende dieren. We waren succesvol in het induceren van de speervorm in bloedplaatjes van muis, rat en mens na opwarming van de kou, welk mechanisme afhankelijk was van tubuline polymerizatie via de colchicine bindingsplaats op tubuline. Dus verlaging van temperatuur induceert een speervorm in bloedplaatjes van winterslapers, terwijl het weer opwarmen juist speervorming uitlokt in bloedplaatjes van niet-winterslapers. Daarom is het onwaarschijnlijk dat de speervorm een vereiste is voor bloedplaatjesopslag in lever sinusoiden tijdens hypothermie, aangezien niet-winterslapers geen speervorm maken in de kou. Winterslaper bloedplaatjes lijken ook nog eens beschermd tegen activatie door de kou en tegen het afbreken van het tubuline cytoskelet door de kou.

Conclusies

Samenvattend hebben we in dit proefschrift een aantal belangrijke elementen van primaire hemostase, secundaire hemostase en fibrinolyse geïdentificeerd die door hun aanpassing de activatie van het bloedstollingsysteem van de Syrische hamster voorkomt tijdens de winterslaap. We hebben ons gefocust op de primaire hemostase en ontraadselden de temperatuursafhankelijkheid van het mechanisme dat ten grondslag ligt aan de reversibele trombopenie in winterslapers en niet-winterslapers. We verklaarden de daling in bloedplaatjes tijdens torpor met een belangrijke rol voor de lever door opslag en later vrijlating van bloedplaatjes, resulterend in een 50% verlenging van de levensduur van winterslaper bloedplaatjes. Verder maakten we duidelijk dat lage temperaturen de

bloedplaatjes van winterslapers en niet-winterslapers niet activeerden ondanks de opvallende - doch omkeerbare - vormveranderingen. Gezamenlijk helpen deze bevindingen van dit proefschrift ons om te begrijpen waarom winterslapende zoogdieren zoals de Syrische hamster niet lijden aan tromboembolische complicaties tijdens en na de winterslaap. Bovendien bestaat de temperatuursafhankelijke onderdrukking van bloedstolling ook in niet-winterslapende zoogdieren alsook de eigenschap om omkeerbaar de vorm van bloedplaatjes te veranderen zonder hen te activeren. Deze resultaten kunnen in de toekomst leiden tot nieuwe antitrombotische strategieën en de langdurige koude opslag van bloedplaatjes voor transfusie.

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ABOUT THE AUTHOR

On 28th November 1988 I was born and hereafter raised in Stiens, Friesland, the Netherlands. At the age of 18 I finished secondary school at the Christelijk Gymnasium Beyers Naudé in Leeuwarden in 2007 and started studying Biology at the University of Groningen, since I didn't draw a place by lot for the study Medicine. This lottery for Medicine was unsuccessful the year after as well, so I continued and finished the Bachelor Degree in Biology in 2010. During my bachelor my major focused on Biomedical Sciences, e.g. via several research projects, and my minor focused on Medicine. One of my bachelor research projects studied the role of platelets in kidney transplantation, which sparked my fascination for platelets. My interest in science started to grow. By passing an entrance and selection exam I finally started studying Medicine in the same year via a fast-track (zij-instroom) program and qualified for the Bachelor of Medicine in one year to commence the master program afterwards. I realized I wanted to continue both research and Medicine and my MD/PhD proposal got accepted at the lab of prof. Henning in the Department of Clinical Pharmacy and Pharmacology. During the next years I alternated the master program with PhD research. I experienced medical internships in Groningen, Assen, Zwolle, Meppel, Harderwijk, Emmeloord and in Kumi - Uganda. My final half year of internships of choice were in Plastic, Reconstructive and Hand Surgery in the hospitals Medical Center Leeuwarden and University Medical Center Groningen. I obtained a grant to perform additional experiments which extended my PhD research, and part of the research I performed in Strasbourg, France. In 2017 I obtained my degree as Medical doctor and I started working in the Surgery department of Martini Hospital in Groningen, where I finished writing the PhD thesis.

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