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Cardiovascular Resistance to Thrombosis in 13-Lined Ground Squirrels

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Abstract

Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) enter hibernation as a survival strategy during extreme environmental conditions. Typical ground squirrel hibernation is characterized by prolonged periods of torpor with significantly reduced heart rate, blood pressure, and blood flow, interrupted every few weeks by brief interbout arousals (IBA) during which blood flow fluctuates dramatically. These physiological conditions should increase the risk of stasis-induced blood clots and myocardial ischemia. However, ground squirrels have adapted to survive repeated bouts of torpor and IBA without forming lethal blood clots or sustaining lethal ischemic myocardial damage. The purpose of this study was to determine if ground squirrels are resistant to thrombosis and myocardial ischemia during hibernation. Blood markers of coagulation, fibrinolysis, thrombosis, and ischemia, as well as histological markers of myocardial ischemia were measured throughout the annual hibernation cycle. Hibernating ground squirrels were also treated with isoprenaline to induce myocardial ischemia. Thrombin-antithrombin complex levels were significantly reduced ($p < 0.05$) during hibernation, while D-dimer level remained unchanged throughout the annual cycle both consistent with an antithrombotic state. During torpor the ground squirrels were in a hyperfibrinolytic state with an elevated ratio of tissue plasminogen activator complexed with plasminogen activator inhibitor to total plasminogen activator inhibitor ($p < 0.05$). Histological markers of myocardial ischemia were reversibly elevated during hibernation with no increase in markers of myocardial cell death in the blood. These data suggest that ground squirrels do not form major blood clots during hibernation through suppression of coagulation and a hyperfibrinolytic state. These animals also demonstrate myocardial resistance to ischemia.

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Author Contributions

S.C., A.B. and L.A. conceived the ideas and designed the experiments. G.T. and B.L. performed the fibrinolysis assays. L.A., A.B., K.C., J.U., J.J.A. and E.S. performed the myocardial infarction experiments. A.B., J.S., A.S. and A.K. assayed markers of coagulation and fibrinolysis.

Disclosures

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Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Keywords

Coagulation; fibrinolysis; hemostasis; hibernation; ischemia; TAT complex; torpor; tPA-PAI1

Endothermic animals living in environments with cold winters rely on several adaptations to survive. During torpor, hibernating mammals such as the 13-lined ground squirrel (*Ictidomys tridecemlineatus*, hereafter referred to as ground squirrels) can decrease body temperature from 35–38°C to 4–8°C (Lechler and Penick 1963, Reddick et al. 1973), heart rate from 200–300 to 3–5 beats/min (Zatzman 1984), and respiration 100–200 to 4–6 breaths/min (McArthur and Milsom 1991). As expected, blood pressure also drops from 140/100 mm Hg to 60/30 mm Hg, with values as low as 10 mm Hg reported (Lyman and O'Brien 1960). During torpor, ground squirrel hearts and brains show an 8-fold drop in blood flow from 2400 to 300 ml/100g/min (Bullard 1962) and from 62 to 7 ml/100g/min respectively (Frerichs 1995). At the same time, the heart is still capable of producing a strong QRS peak with broad spacing between peaks instead of a broader prolonged peak, causing more fluctuation in blood pressure and flow (Hampton et al. 2010). During hibernation, long periods of torpor are interrupted by periodic interbout arousals (IBAs) during which body temperature returns to normal within 1–2 hours. This aroused state is maintained for 12–18 hours, followed by return to torpor in 2–4 hours (Nelson et al. 2009). Each IBA can be thought of as an ischemic reperfusion, as hypoxic tissues are reoxygenated and warmed (Kurtz et al. 2006, Bogren et al. 2014, Otis et al. 2017). Because ground squirrels go through this reperfusion 10–20 times in each annual hibernation cycle, they must have adaptations to protect them from damage that would occur in humans going through similar challenges (Prendergast et al. 2002, Carey et al. 2003, Ma et al. 2005, Kurtz et al. 2006).

In humans, abnormal blood flow is one component of Virchow's triad leading to an increase in thrombosis (Byrnes and Wolberg 2017). Increased risk of DVT is associated with both immobilization and decreased blood flow including prolonged travel, wearing a cast, and bed rest (Chandra et al. 2009, Esmon 2009, van Adrichem et al. 2014). The most common animal model for DVT is ligation of the infrarenal vena cava (IVC) in mice causing either complete stasis, or stenosis leading to reduced blood flow (Diaz et al. 2012). Other animal models that have been used include pigs, dogs, rabbits and mice (Albadawi et al. 2017). Immobilization of non-human animals does induce arterial thrombosis, but is thought to occur primarily by the stress induced by the process and is not a good model for DVT (Stampfli et al. 2014). Repeated rounds of immobility followed by rapid arousal could create a potentially lethal state of ischemia and vascular reperfusion in non-hibernating mammals (Lindenblatt et al. 2005). For example, 60% of mice subjected to a similar state of decreased blood flow seen in hibernators formed deep vein thrombi (DVT) within 2 days (von Bruhl et al. 2012). Only recently have hibernating animals like ground squirrels been used for these studies (Quinones et al. 2016, Salzman et al. 2017).

Blood clots form following the activation of platelets which aggregate at a site of damage by binding to von Willebrand factor (vWF), and activation of serine proteases in the clotting cascade. In hibernating ground squirrels circulating platelets and vWF both drop 10-fold,

while Factors VIII and IX drop 3-fold, leading to prolonged clotting times (Suomalainen and Lehto 1952, Svihla et al. 1952, Svihla et al. 1952, Svihla et al. 1953, Smith et al. 1954, Lechler and Penick 1963, Pivorun and Sinnamon 1981, de Vrij et al. 2014). The duration of the periodic IBAs is important in primary hemostasis as the time frame is long enough for sequestered platelets to be released back into circulation, but too short to allow for new cell or protein synthesis based on measured post-arousal kinetics (Cooper et al. 2012, Cooper et al. 2016, Cooper et al. 2017). Because ground squirrel platelets can withstand repeated cycling between 4°C and 37°C for several months, they are an intriguing model for platelet storage in the cold.

In the last step of the cascade Factor Xa proteolytically activates thrombin which then cleaves fibrinogen to form a fibrin clot. Clots are broken down by fibrinolysis in which tissue plasminogen activator (tPA) proteolytically activates plasminogen into plasmin, which then cleaves fibrin in the clot releasing degradation products including D-dimer. The serine protease inhibitors (serpins) antithrombin (AT) and plasminogen activator inhibitor-1 (PAI-1) regulate clotting and fibrinolysis by inhibiting thrombin and tPA respectively producing thrombin-AT (TAT) and tPA-PAI-1 complexes (Figure 1). Both AT (Emerson et al. 1989, Jochum 1995, Harada et al. 1999, Wang et al. 2013) and PAI-1 (Pinsky et al. 1998, Lau et al. 2009) provide protection from repeated ischemic reperfusion in non-hibernating mammals, and could also do so during entrance and emergence from torpor in hibernators.

In addition to damage by blood clots, ischemia and reperfusion in the coronary circulation can damage cardiomyocytes causing the cells to enter a state called a “hibernating myocardium” with suppressed metabolism and long-term contractile dysfunction in cardiomyocytes (Buja 2005, Canty and Suzuki 2012). Within 60 seconds of oxygen depletion, cardiac cells begin to lose contractility and can become stretched by the surrounding adequately perfused cells causing the ischemic tissue to have a wavy appearance. If the ischemia lasts for more than 30 minutes the damage can become permanent. This irreversible damage is characterized histologically by coagulative necrosis (loss of striation and nuclei disruption), and hypereosinophilia (increase uptake of eosin through damaged cell membranes) (Buja 2005). A marker of reperfusion in injured cardiomyocytes is the appearance of contraction bands which form as sarcomeres and clump as a result of hyper-contraction and increased intracellular calcium (Ganote 1983). After cardiomyocyte death the tissue is infiltrated by neutrophils with phagocytosis of the dead tissue and release of cardiac enzymes into the blood such as troponin T and lactate dehydrogenase (LDH), followed by granulation, angiogenesis, and collagen deposition. Consecutive brief periods of ischemia known as ischemic pre-conditioning can render cardiomyocytes more resistant to subsequent ischemia (Sarkar et al. 2012). In both the hibernating and active state ground squirrel tissues are resistant to ischemic damage including; liver (Bogren et al. 2014), kidney (Jani et al. 2012), brain (Bhowmick et al. 2017) and intestines (Kurtz et al. 2006). *In vivo* induction of myocardial infarction by arterial ligation in hibernators led to reduced plasma levels of Troponin I, myocardial apoptosis, and left ventricular contractile dysfunction compared to rats (Quinones et al. 2016). Similarly, induction of ischemia *ex vivo* in perfused hearts and measurement of myocardial infarction by circulating ischemic markers reveals that ground squirrel hearts are more resistant to ischemia than rats (Salzman et al. 2017).

Because ground squirrels must survive repeated rounds of low perfusion during torpor followed by rapid warming and reperfusion during an IBA, it is plausible that they have protective adaptations to decrease blood clotting and natural resistance to heart ischemia. Here, we show that ground squirrels do not form large detectable blood clots during hibernation and offer evidence suggesting the mechanism behind this adaptation is in-part due to suppression of coagulation and induction of a hyperfibrinolytic state. We also tested the hypothesis that ground squirrel hearts are resistant to ischemia by using an *in vivo* experimental model of myocardial ischemia induced by isoprenaline.

MATERIALS AND METHODS

Animals.

Ground squirrels were collected from a local golf course or born in captivity and housed at the University of Wisconsin-La Crosse Laboratory Animal Care Facility. All animals in the study were at least one year old and had gone through one winter hibernation cycle in the lab. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed individually in controlled light-dark cycle environments mimicking a Wisconsin photoperiod (9 light hours in December gradually increasing to 15.5 light hours in June then decreasing again in December). Non-hibernating ground squirrels in the summer were in an active state during June-August, with body temperatures of 37 °C while upon entrance into hibernation in the fall, their temperature dropped to 25 °C. Animals were subsequently moved into a 4°C hibernaculum, and classified as torpid when inactive with body temperatures less than 10°C. Squirrels were checked daily for activity during hibernation, and IBA ground squirrels were classified as those aroused from the torpid state with euthermic rectal body temperatures (37 °C). In March, hibernators were aroused manually and moved out of the hibernaculum to cages in a room at 25 °C. Rats were maintained in standard laboratory conditions with free access to food and water. Blood samples were collected from the tail arteries of isoflurane (1.5-5%) anesthetized rats and ground squirrels, or by exsanguination after euthanized by CO₂ asphyxiation. Ground squirrels in torpor were sacrificed by cervical dislocation. Body temperatures were measured rectally at each blood collection event. Blood was collected from human volunteers using a 22 gauge butterfly needle and syringe. Blood was collected in 150 µl acid citrate dextrose per ml of blood and centrifuged for 5 minutes at 1000 × g to isolate plasma.

Plasma protein and complexes.

Plasma concentrations of proteins and complexes were determined by capture ELISA performed at 37 °C. All washes were performed five times with TBST (Tris Buffered Saline with 0.5% Tween 20) and blocking and dilutions were done in TBST containing 1% powdered milk. Flat bottom 96-well microplates were coated for one hour with capture antibody, washed, blocked for one hour, washed, and plasma samples diluted 1:10 in TBST-milk added for one hour. Plates were washed, incubated for one hour with the detection antibody, washed, an enzyme linked secondary antibody added for one hour, washed, and substrate added. TAT complexes were detected with 1µg/ml sheep anti-human thrombin antibody (PA1-43040; Thermo Fisher Scientific, Waltham, MA), and 1:1000 diluted rabbit anti-human antithrombin antibody (A9522; Sigma-Aldrich, St. Louis, MO). Secondary

antibody was a 1:10,000 dilution of goat anti-rabbit IgG antibody linked to alkaline phosphatase (AP) (Sigma-Aldrich, St. Louis, MO) and the substrate 1 mg/ml 4-nitrophenyl disodium salt hexahydrate (Alfa Aesar, Ward Hill, Massachusetts). PAI-1 was measured with 1:1000 dilution of rabbit anti-mouse PAI-1 antibody, 1:5000 dilution of rabbit anti-human PAI-1 antibody linked to biotin (Abcam, Cambridge, MA), 1:5000 dilution of HRP-avidin and detected by 1-Step™ Turbo TMB (Thermo Fisher Scientific, Waltham, MA). tPA-PAI1 complexes were measured using 1:1000 anti-mouse tPA capture antibody (ab62763; Abcam, Cambridge, MA), and the same detection steps as in the PAI-1 ELISA. All primary antibodies were tested for cross-reactivity with ground squirrel antigens by immunoblot. D-Dimer levels in ground squirrel plasma were measured with competitive rat D-Dimer ELISA Kit (Elabsience, Wuhan, China). Negative and positive controls were generated from ground squirrel whole blood allowed to clot at room temperature for 2 hours and then breakdown for an additional 20 hours respectively.

Fibrinolysis assay:

A fibrinolysis assay was performed by diluting 15 µl of plasma into 200 µl of buffer (3 µg/µl fibrinogen, 10 mM Tris, 5 mM EDTA, pH 7.4). Thrombin (2.5 nM) was added to initiate clot formation at 37 °C for 40 minutes and fibrinolysis measured for the next 3 hours by measuring A₄₀₅ on a plate reader. Plasma plasminogen was measured using a Actichrome PLG assay kit (American Diagnostica).

Troponin assays.

Troponin T levels were measured in plasma samples by immunoblot. Controls of ground squirrel heart, lung and liver were used to ensure that troponin T was specific to squirrel heart muscle. Approximately 0.1g of each organ was homogenized in 200 µl sample buffer with 20 µl β-mercaptoethanol and run on a 10 % SDS-PAGE gel. The proteins were immunoblotted and detected with a 1:1000 dilution of the primary mouse anti-troponin T antibody (MA124611, Invitrogen). Troponin I levels were measured with a commercial capture ELISA (G-Biosciences, Rat Tnni3/cTn-I) using a 1:1 dilution of plasma. Plasma LDH was measured using a kit from Pierce (Fisher Scientific).

RT-qPCR.

Total RNA was isolated from ground squirrel livers using an Absolutely RNA Miniprep Kit (Agilent Technologies, Cedar Creek, Texas). Total RNA (1 µg) was reverse transcribed into cDNA using the Affinity Script QPCR cDNA Synthesis Kit (Agilent Technologies, Cedar Creek, Texas). All real-time PCR reactions were performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and cDNA amplifications were performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) with 400 nM primers. Primers were generated using ground squirrel sequences from ENSEMBL for PAI-1 (*SERPINE1*), forward 5'-CCTTTCTGCCCTCACCATA-3', reverse 5'-GAGAACTTGGGCAGAACTAGG-3' and plasminogen forward 5'-TGTCTGTCAAAGGTGGAGTG-3', reverse 5'-CTGGGTTGCGACAGTAGTT-3' (Integrated DNA Technologies, Coralville Iowa). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize each sample (Otis et al. 2010).

Heart histological staining:

Hearts were collected at different stages in the ground squirrel annual hibernation cycle. Myocardial ischemia was also induced in ground squirrels and rats by intraperitoneal injection of 4 mg/kg isoprenaline and hearts collected 48 hours later (Bertinchant et al. 2000, Saleem et al. 2013, Wei et al. 2013). Hearts were removed and immediately flushed with a syringe containing PBS, preserved in formalin, and the entire heart sectioned longitudinally into four sections prior to tissue processing (VIP 3000 Tissue Processor by Tissue Tek), and embedded in paraffin blocks. Four μ m sections were prepared on a microtome (Thermo Shandon Finesse) and replicates stained with either hematoxylin and eosin stain (H&E) or trichrome stain. The entire hearts were scored to avoid bias of localized damage cause by isoprenaline (Siddiqui et al. 2016). Scoring was done blindly by three reviewers based on the percent of the heart showing markers of damage and quantified using Image J. The scoring scale went from 0-4, with 0 being no damage, 1 being damage in one field of the microscope, 2, 3 and 4 representing 5, 10 and 20% of the heart showing markers of ischemic damage including wavy fibers, hypereosinophilia, contraction bands, and collagen scars.

RESULTS

Suppression of coagulation in hibernating squirrels

Previous studies demonstrated a decrease in clotting factors and circulating platelet levels during torpor, but did not measure markers of clot formation such as TAT complexes or D-dimers (Figure 1) (Suomalainen and Lehto 1952, Svihla et al. 1952, Svihla et al. 1952, Svihla et al. 1953, Smith et al. 1954, Lechler and Penick 1963, Pivorun and Sinnamon 1981, de Vrij et al. 2014). A capture ELISA was performed to measure plasma TAT level in ground squirrels throughout the annual hibernation arousal cycle and normalized to non-hibernating animals (Figure 2). No significant difference in TAT was observed in entrance animals relative to non-hibernators (1.29 ± 0.18 and 1.00 ± 0.27 , respectively) while torpid and interbout aroused ground squirrels had significantly lower TAT levels (0.15 ± 0.15 and 0.30 ± 0.16 , respectively, t -test $p < 0.05$). A competitive D-dimer ELISA was performed to measure the level of fibrin degradation and normalized to the non-hibernating group. The D-dimer level of non-hibernators (1 ± 0.55) was not statistically different from either the torpid (0.76 ± 0.22) or IBA (0.80 ± 0.26) stages (Figure 4).

Hyperfibrinolysis in hibernating ground squirrels

The absence of blood clotting in hibernating animals may be explained by the suppression of clot formation and/or efficient clearance of a clot. Fibrinolysis was measured *in vitro* by forming a clot in a 96-well plate using human and ground squirrel plasmas and the dissolution of the clot measured by the change in A405 over time (Figure 5). Normalized to the rate of fibrinolysis in human plasma (1.00 ± 0.22) the rate of non-hibernating plasma fibrinolysis was not significantly different (1.74 ± 0.20) while that of plasma from torpid animals was 3-fold faster (3.19 ± 0.13).

During fibrinolysis, tPA cleaves inactive plasminogen into the fibrin-degrading enzyme plasmin. However, the serpin PAI-1 forms an irreversible tPA-PAI-1 complex that blocks fibrinolysis (Figure 1). If fibrinolysis is activated during hibernation, a decrease plasminogen

levels and/or an increase of tPA-PAI complex levels would be predicted. Plasma plasminogen levels dropped 1.9-fold during torpor from 37.6 ± 9.2 to 19.7 ± 12.3 measured as a percent of human pooled normal plasma. A capture ELISA was performed to detect the level of total PAI-1 protein in ground squirrel plasma (Table 1). Plasma level of PAI-1 in non-hibernators was 2.15 ± 1.04 ng/ml while all other stages of the annual hibernation arousal cycle had total PAI-1 levels significantly lower (Table 1). Specifically, upon entry into hibernation, normalized PAI-1 levels decreased approximately 2-fold (0.54 ± 0.20) and continued to drop throughout both the torpid (0.21 ± 0.16) and IBA (0.13 ± 0.10) stages.

A capture ELISA was performed to quantify plasma tPA-PAI-1 complexes at different hibernation stages. tPA-PAI-1 complex values were not significantly different between non-hibernators and entrance animals, while those in torpor and IBA showed 2 and 5-fold decreases respectively (Table 1). Compared to non-hibernators, the ratio of tPA-PAI-1 to total PAI-1 in both torpid and IBA animals was elevated 2.3 and 1.7-fold respectively, while entrance animals were not significantly different (Table 1).

Liver PAI-1 mRNA decreased in hibernating ground squirrels

To determine if fluctuations in PAI-1 plasma protein level throughout the annual hibernation arousal cycle may be due to differential production, we assessed the levels *SERPINE1* mRNA, encoding PAI-1 protein, in liver as a major source of plasma PAI-1. Quantitative PCR was performed on cDNA from non-hibernating, hibernation-entrance, torpid and IBA ground squirrel livers. Significant decreases in mRNA level relative to NH were observed in entrance (6-fold), torpor (5-fold) and IBA (4-fold) animals (Figure 3). In contrast, normalized plasminogen mRNA levels were not significantly different in either torpor (1.36 ± 0.52) or IBA (1.04 ± 0.17) relative to non-hibernators (1.00 ± 0.20).

Myocardial resistance to ischemia

In addition to blood clots, ischemic reperfusion may cause myocardial ischemia. Markers of myocardial ischemia (contraction bands, wavy fibers, and hypereosinophilia) were lowest in the summer and increased 4-fold during fall entrance into hibernation and IBA when animals were going through the most dramatic changes in blood flow. Scoring trichrome stained heart sections for markers of ischemic damage revealed a 2-fold increase during torpor and in spring post arousal (Figure 6), there was no correlation between the amount of time spent in hibernation and these scores (not shown). Ground squirrel hearts were resistant to induced ischemic damage by isoprenaline in both the non-hibernating and torpid states showing no significant change, while rats showed a dramatic increase in myocardial ischemia (Figure 7). Plasma LDH activity was measured on summer non-hibernating animals and winter torpor and IBA animals. Normalized to the non-hibernators (1 ± 0.30) there was a significant difference between the torpid (1.70 ± 0.76) and the IBA animals (0.77 ± 0.24) but neither were different from the non-hibernators. Troponin T levels were not detected in plasma from ground squirrels at any of the stages of the annual cycle or when treated with isoprenaline (data not shown). There was no significance difference in plasma Troponin I levels between animals treated with isoprenaline and controls treated with saline ($n=6$ per group). Compared to non-hibernators (23.4 ± 5.5 pg/ml), plasma Troponin I levels were increased in

IBA ground squirrels (47.0 ± 11.5 pg/ml) but not animals entering hibernation (26.3 ± 6.5 pg/ml) or in torpor (30.7 ± 8.5 pg/ml) as measured by t-test ($n=4-6$ per group).

DISCUSSION

Ground squirrels survive dramatic changes in blood flow during consecutive bouts of torpor and interbout arousals throughout their 6-7-month hibernation season. Ground squirrels, bears, and hamsters have developed antithrombotic adaptations including thrombocytopenia and decreases in vWF, Factor VIII and Factor IX to avoid lethal blood clots during hibernation (Lechler and Penick 1963, Pivorun and Sinnamon 1981, Cooper et al. 2012, de Vrij et al. 2014, Cooper et al. 2016, Friedrich et al. 2017). However, it is unknown if these adaptations completely block the formation of blood clots. In this study, we took two approaches to determine the presence of blood clots in squirrels. First, we used human clinical assays to measure markers of coagulation, fibrinolysis activation, and fibrin-degradation products. Second, we looked for myocardial ischemia by measurement of released heart proteins into the blood and histological analysis of the ground squirrel heart.

Ground squirrels enter a hypocoagulative state during hibernation. Elevated plasma TAT complexes are a marker of activation of the coagulation cascade (Lee et al. 2017, Weymann et al. 2017). Rather than being elevated, TAT was significantly decreased during hibernation consistent with previous studies showing a decrease in coagulation factors and platelets during hibernation (Lechler and Penick 1963, Pivorun and Sinnamon 1981, Cooper et al. 2012). D-dimers released during the degradation of a fibrin clot are a clinical marker of venous thromboembolism (Douketis et al. 2010, Pulivarthi and Gurram 2014). A steady low level D-dimer level throughout all stages of the annual cycle, along with an inactivated coagulation cascade during hibernation suggested that venous thrombosis is not occurring during hibernation in ground squirrels. This result is consistent with steady low level of D-dimers in active and hibernating black bears (Friedrich et al. 2017). However, it is possible that the D-dimers are cleared from circulation before they can be detected.

In contrast to suppression of coagulation, hibernating ground squirrels were in a hyperfibrinolytic state compared to non-hibernators. Plasminogen levels in ground squirrels had a similar fold decrease to that seen in hibernating black bears (1.9 and 1.6 fold respectively) (Friedrich et al. 2017). Plasminogen mRNA levels were constant throughout the year, suggesting increased consumption rather than decreased production of plasminogen. Although the level of tPA-PAI-1 complexes dropped significantly during hibernation, the ratio of tPA-PAI-1 to total PAI-1, a marker of fibrinolytic activity, was significantly elevated in the torpid and IBA state. Healthy humans have an average tPA-PAI-1: PAI-1 ratio of approximately 0.22, indicating that about one fifth of their PAI-1 is complexed with tPA (Watanabe et al. 2001) and a similar ratio was seen in non-hibernators. Patients in a hyper-fibrinolytic disease state such as disseminated intravascular coagulation (DIC) display an elevated ratio of tPA-PAI-1 complex to total PAI-1 of 0.38 (Watanabe et al. 2001). A similar tPA-PAI-1: PAI-1 ratio was seen in hibernating ground squirrels, consistent with a two-fold increase in PAI-1 complexed with tPA. These findings suggest that fibrinolysis is in a hyperactive state during hibernation, even in the absence of blood clots to break down. It is possible that there are small clots forming that are too small to see in the

heart sections observed in this study and in brain and lung samples examined previously (Cooper et al. 2016).

Decreased plasma protein levels of PAI-1 correlate with increased clot lysis, whereas increased PAI-1 is associated with resistance to clot lysis (Okafor, 2015; Zhu et al., 1999). In mice, adipocytes can produce PAI-1 and obese mice display elevated plasma PAI-1 (Ohkura et al. 2012). While ground squirrels in the fall entrance period and hibernation have elevated adipose they had significantly lower plasma PAI-1 levels compared to non-hibernators. RT-qPCR of ground squirrel liver total RNA suggests that the drop in plasma PAI-1 was in-part due to decreased production of the protein by the liver in entrance and post arousal animals. While a decreasing trend in IBA and torpid *SERPINE1* mRNA was seen, the difference compared to nonhibernating levels was not significant. A recently published study found that *SERPINE1* mRNA levels decreased during torpor 5.2-fold in bone marrow and 4-fold in brown fat compared to non-hibernators (Hampton et al. 2013, Cooper et al. 2016); a reduction similar to that observed in torpid liver *SERPINE* mRNA levels. PAI-1 and tPA levels are also known to fluctuate with circadian cycles in humans (Scheer et al. 2011, Scheer and Shea 2014), and it is possible that some of the fluctuations seen in hibernation are circadian in nature. Finally, ischemia and reperfusion could damage the liver, leading to decreased protein production, however ground squirrel livers appear to be resistant to such damage (Otis et al. 2017).

Circulating levels of common clinically diagnostic blood markers of clotting and fibrinolysis were consistent with decreased clot formation and increased fibrinolysis during hibernation. However, the levels of these markers could be influenced by many variables including the amount of time between the process being measured and when the sample was drawn, and the rates of blood flow, metabolism and enzyme activity in different states of hibernation. To determine if some clots may still have formed in coronary arteries, hearts were examined for histological markers of ischemia and heart proteins released into the blood were measured. While Troponin T did not change significantly in any of the groups studied, Troponin I increased in IBA animals, while LDH levels increased in torpor relative to IBA animals, consistent with some damage during torpor that is repaired during arousal. The differences between markers could be due to the earlier release and clearance of Troponin I relative to LDH (Mythili and Malathi 2015). In addition, histological markers of what would be irreversible myocardial ischemic damage in humans (wavy fibers, contraction bands and hypereosinophilia) increased during entrance into hibernation and remained elevated through one week post arousal (Figure 6). This observed histological pattern of myocardial damage could be due to either small or transient blood clots not detected histologically that formed in coronary circulation during torpor, or cardiac output not meeting the metabolic demand of heart tissue which continues to contract during torpor. When a ground squirrel enters an IBA it will go from a body temperature of 5-8°C to 37°C in a matter of hours, putting its organs at risk for ischemic reperfusion injury (Kurtz et al. 2006, Bogren et al. 2014, Otis et al. 2017). The heart and other organs active during torpor have a six-fold increase in metabolic rate during an IBA, and the rate of warming may be limited by the rate of oxygen delivery to these active organs (Hampton et al. 2010). No pulmonary or cerebral emboli are seen during hibernation (Cooper et al. 2016). These markers of cardiac ischemia are often irreversible in humans, yet the squirrels seem to be resistant to this damage and return to normal

morphology during the summer. Previous studies have shown that ground squirrel heart is more resistant to ischemia than rats using *in vitro* perfusion of hearts (Salzman et al. 2017) or *in vivo* coronary artery ligation (Quinones et al. 2016). In both of these experiments the ischemia was relatively short-term and required surgical manipulations. In this study, isoprenaline was used to induce myocardial damage as it could be administered by intraperitoneal injection in animals already in torpor and the damage measured days later with no surgical interventions. The results of isoprenaline treatment were consistent with the other *in vitro* and *in vivo* studies, showing permanent damage and leukocyte infiltration in the treated rat hearts, while both non-hibernating and torpid ground squirrels showed no signs of leukocyte infiltration or long term ischemic damage. It is possible that the reversible structural damage seen in the squirrels is reversible because they are also resistant to ischemia. In contrast in rats and other non-hibernating mammals, the ischemia and structural changes happen coincidentally making the latter irreversible.

The data in this study suggest that during hibernation blood clots are less likely to form due to suppression of coagulation and are more likely to be broken down during fibrinolysis. Furthermore, ground squirrel hearts are more resistant to ischemic damage caused by the formation of small clots or insufficient blood flow. In combination these adaptations allow ground squirrels to survive dramatic fluctuations in blood flow without forming thromboemboli or sustaining permanent myocardial damage. Continuing to further elucidate the mechanisms underlying these adaptations to avoid the risk of clot formation, and permanent ischemic damage during periods of both low, and drastically fluctuating blood flow may potentially translate to advances in prevention and therapy for ischemic injury, DVT, and stroke.

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Figure 1. Schematic diagram of the links between the formation of fibrin clots in coagulation and breakdown of fibrin clots in fibrinolysis to form fibrin degradation products such as D-dimers. Clinical markers of the activation of these pathways include monitoring plasma levels of thrombin-antithrombin complexes (TAT), tPA-PAI-1 complexes, and D-dimers.

Figure 2.

Thrombin-antithrombin Complex (TAT) level relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=8); Entrance into hibernation, ENT (n=4); Torpor, TOR (n=7); Interbout Arousal, IBA (n=7). Significance testing used the student t-test to compare average TAT complex of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

Figure 3.

SERPINE1 mRNA relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH; Entrance into hibernation, ENT; Torpor, TOR; Interbout Arousal, IBA. Significance testing used the student t-test to compare average PAI-1 mRNA level of each group to nonhibernators (n=3). *- indicates significant difference, $p < 0.05$.

Figure 4.

D-dimer level relative to nonhibernators throughout annual hibernation arousal cycle in thirteen lined ground squirrels. Nonhibernators, NH (n=8); Torpor, TOR (n=8); Interbout Arousal, IBA (n=6). Significance testing used the student t-test to compare average D-dimer level of each group to nonhibernators. *- indicates significant difference, $p < 0.05$.

Figure 5.

Rate of fibrinolysis in a clot formed with platelet poor plasma in a microtiter plate and relative absorbance measured at 405 nm. Plasma samples (n=5) were taken from humans, non-hibernating and torpid ground squirrels. *- indicates significant difference in the slope of the line by t-test, $p < 0.05$.

Figure 6.

Degree of cardiac ischemia based on histological markers throughout the annual cycle of the thirteen-lined ground squirrel (n=6-10 per time point). Hearts were sectioned, trichrome stained, and images taken with a final magnification of 100X. *- indicates significant difference from late summer non-hibernator, $P < 0.05$ t-test.

Figure 7.

Degree of cardiac ischemia 48 hours after intraperitoneal injection of isoprenalene (treated) or saline (control) in rats and non-hibernating (NH) or torpid thirteen-lined ground squirrel (n=6). Hearts were sectioned, trichrome stained, and images taken with a final magnification of 100X. *- indicates significant difference from control saline injection, $P < 0.05$ t-test.

Table 1.

Levels of PAI-1, tPA/PAI-1 complex and the ratio of complex:total PAI-1 at different stages.

<i>Annual stage</i>	<i>Total PAI-1 (ng/ml)</i>	<i>tPA/PAI-1 complex (ng/ml)</i>	<i>Ratio complex:total</i>
Summer, non-hibernator	2.15 ± 1.04	0.37 ± 0.11	0.188 ± .043
Fall, entrance	1.16 ± 0.44 [#]	0.54 ± 0.54	0.395 ± 0.275
Winter, torpor	0.45 ± 0.35 [#]	0.19 ± 0.13 [#]	0.432 ± 0.134 [#]
Winter, interbout arousal	0.29 ± 0.21 [#]	0.071 ± 0.041 [#]	0.324 ± 0.142 [#]

* n=4-6 individual samples measured in duplicate

[#] Significant difference from summer non-hibernating animals (t-test, p<0.05).