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Seasonal Variation of Hemostatic and Hemolytic Factors in Hibernating and Non Hibernating Mammals

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Variation in Thrombotic and Thombolytic Factors in the Cold-exposed Hibernator

(Mesocricetus auratus)

ABSTRACT

Variation in thrombotic and thrombolytic factors has long been suspected in the process of hibernation. Animals with reduced blood flow and heart rate should be a significant risk for thrombosis, yet this is not observed. Little work has been done on hibernating or cold-exposed mammals with respect to clotting factors. Srere, et.al., 1995 have investigated changes in a_2-macroglobulin and heparin in hibernating ground squirrels, but little has been done on other species. What is known is that the process of hibernation in mammals varies greatly especially between obligate and non-obligate hibernators. Golden Syrian Hamsters represent one model for non-obligate hibernation but little work has been done on this species. In 1961, Svihda, et. al. demonstrated increased clotting times and measured increased heparin levels by crude extraction. Recent techniques have not been applied to this question and no additional, more current, literature has been found on Golden Syrian Hamsters. Our experiment sought to determine if currently available antibodies, made to closely related species, could be used to investigate variation in seasonal clotting factors. We additionally sought to use any cross-reacting antibodies to thrombotic and thrombolytic factors to assess relative variation by season. Our results, using gene alignments and accession numbers of current antibodies, suggested a probability that antibodies to fibrinogenα (Fα), antithrombin III (ATIII), and Factor X might be successful. All three antibodies successfully cross-reacted using reduced western blotting. Our results showed significant decreases in ATIII in both plasma and serum of cold-exposed animals (23% and 30% respectively). a-fibrinogen showed no significant difference in control vs. cold-exposed animals, however we did find a significant difference in a-fibrinogen in male vs. female animals irrespective of experimental conditions when using a small number of pooled samples. The significance of sex differences remains to be investigated. Although we found binding of the Factor X antibody to plasma in reduced samples via western blotting techniques, the quantity appears to be too low for measurement by ELISA. Future experiments with chemiluminescent western blotting are planned.

Background information:

Mammalian hibernation is an adaption marked by a controlled decrease in metabolic, heart, and respiratory rates to allow the conservation of energy that results from food shortages during hibernation. During hibernation a mammal's heart rate and blood flow slow to a near standstill. Hibernating ground squirrels have a heart rate of only 5-10 beats per minute and a core temperature of 5^{o} C compared to a normal heart

rate of 350-400 beats per minute (Storey 2009).This stagnation in blood flow would cause clotting and death if there were not a decrease in clotting factors or an increase in anti-clotting factors. In 2009, Storey discusses his thoughts on mammalian hibernation in his Mini-Review.

> Mammalian hibernation is perhaps the most complex form of natural hypometabolism because it involves not just a torpid state but also the inhibition of thermogenesis so that core body temperature (Tb) can fall to near 0^{o} C. However, being mammals, the metabolic challenges encountered by hibernators and their solutions to them are probably the most relevant to a goal of developing inducible torpor as treatment strategy for humans and their organs.

A long-term viability in the hypometabolic state could be useful in the preservation of organs before a different organ can be transplanted and during surgery. Hibernators activate strategies that reduce the clotting response, preventing thrombosis.

While mammalian hibernation has significance in understanding physiology of the hypometabolic state and applications to medicine, the earliest studies on seasonal variation and clottling were in turtles. Seasonal variation in clotting was first studied because researchers supposed that turtles in hibernation or cold torpor must make an anticoagulant to prevent clotting. A significant increase in the anticoagulant heparin was measured in plasma beginning in late October and continuing until metabolic increase began around late February. Few studies have addressed the environmental trigger, which stimulates the production of such thrombolytic agents. An environmental signal was never found which could trigger the decrease in clotting ability. Laboratory testing ruled out changes in temperature, light exposure, and solar radiation as causes for the alteration in clotting factors (Kupchella, 1970). Since these early studies were

done on cold-blooded animals, which have significant differences in metabolism, it stands to reason that comparable studies should be done on mammalian hibernation. Very little literature exists which examines either true hibernation or the reduced state of activity found in cold-torpor. (Johansson 1972, Srere 1995) While one study was done on hibernating ground squirrels, other more accessible models have not been studied. The Golden Syrian Hamster (*Mesocricetus auratus*) is a facultative hibernator and when, in cold-exposed environment, will either enter a state of cold torpor or true hibernation. Because facultative hibernators do not put on a pre-hibernation layer of fat or increase metabolic stores, they often hibernate with short periods of arousal to feed intermittently. (Srere 1995). The purpose of this study was two-fold: First, to determine if known clotting and anti-clotting factors could be measured with current techniques in the Golden Syrian Hamster. Second: to determine if cold-induced torpor or hibernation could be induced in the summer by environmental light reduction followed by reducing ambient temperature, over time, to a constant final temperature of 4^{o} C. for several weeks. If so, are there measurable changes in the clotting factors studies.

Method:

Hamsters arrived 5/31/2011. Food and water were given ad lib. Control hamsters (4) were caged and exposed to natural day-night cycle and exercised 20 minutes a day to prevent estivation. No exercise was given for the 6 cold induced hamsters. Day-night cycle control was initiated on 6/1/2011. Temperature decrease to 18^{o} C began 6/6/2011. Hamsters were moved to a cold room (12^{o} C) 6/15/2011, daylight hours decreased to 6 hours. On 6/21/2011 hamsters were moved to larger cages to reduce the stimulation to

the animals through cage changes, and the temperature was decreased to 11° C. Temperature decrease 1° C everyday until 5° C was reached on 6/27/2011. Day night cycle was stopped and the hamsters were kept in total darkness to simulate winter hibernation in a burrow on 7/6/2011. Water levels were checked 6/25/2011, 7/5/2011, 7/12/2011 and 7/18/2011.

Significant obstacles existed to examining clotting factors in Syrian Hamsters. Primarily there were no antibodies to clotting factors in hamsters that are currently available. Through gene blasts, using accession numbers of antibodies made to closely related species, and using gene alignments, we were able to find epitopes of similarity that had a chance of successful binding to our clotting factors. The three factors chosen for study based on homology and the likelihood of adequate binding were Fibrinogen Alpha (F α), Anti-thrombin III (ATIII), and Factor X (FX). Fibrinogen alpha (F α) is a cleavage product of fibrinogen and is found in plasma both circulating and in greater quantity during clot formation. ATIII is an anti-clotting factor that has been implicated in some human seasonal studies (Bull, et al., 1979), and seemed a reasonable choice for examination given the similarity of the mouse gene sequence and the hamster sequence. Factor X was chosen because of reported homology in the human gene sequence and because it is the common factor in both the intrinsic and extrinsic pathways of the clotting mechanism.

Antithrombin III antibody was purchased from abcam (ab79934). Goat serum was purchased from Santa Cruz (sc2043), and stored in aliquots in approximately -50° C freezer. Rabbit serum was purchased (R4505), and stored in aliquots in -50° C

freezer. Donkey anti goat (biotinylated) was purchased from Santa Cruz, and stored at 4^{o} C. A number of failures with biotinylated secondary antibodies suggested that a direct HRP conjugated antibody would be a better chcice. Donkey anti- rabbit (biotinylated) was purchased from Santa Cruz (sc4042), and stored in 4^{o} C. Streptavidin (HRP) was ordered from Invitrogen (434323) and stored at 4^{o} C. TMB was ordered from Invitrogen (00-2023). Donkey serum was ordered from Sigma (delivery #838978101), and stored at less than 50^{o} C. Continued levels of background noise due to non-specific binding led us to try yet another secondary antibody. New secondary antibody was ordered and arrived (chicken anti -rabbit and goat anti rabbit Fab^{2}).

Initially, antibody binding to plasma was determined through Western Blotting using the reduced protein with significant binding of all three antibodies to GS Hamster plasma and serum. It is not certain that this binding would be similar in the unreduced state in fresh-frozen plasma and serum. Standard ELISA techniques were used by coating 96 well plates with 100 μl plasma or serum in specified dilutions (which were optimized by beginning with known values for humans). The plates were left at 4^{o} C overnight. Wells were blocked in Pierce Non-protein blocker for 2 hours. Plates were washes 3 x with PBST and appropriate concentrations of 100μl of primary antibody were incubated on the plates with shaking for two hours. The plates were washed in PBST 4 times (300μl each) with a final 10 minute rest period before the final wash. Appropriate concentrations of secondary antibodies conjugated with HRP were incubated on the plate with shaking for 1.5 hours. Plates were rinsed as previously described and color was developed with TMB for 10 minutes followed by a stop solution

of 50μl of 2N H_2SO_4. Plates were read at 495nm on a Bio-Rad Benchmark Plate reader. Standards were not always available for quantification of protein but non-specific protein standards were run on each plate for internal control. The peptide sequence for Hamster ATIII is available. (Mak, 1996) We were able to have the ATIII (25 mer peptide) synthesized for use as a standard, so ATIII results are converted into μg/mL. Fibrinogen α results shown are qualitative for relative difference between male and female as well as winter and summer samples.

Western Blotting techniques were used to determine primary and secondary antibody binding. 17 well NuPAGE BisTris gels were washed 3 times with runner buffer then loaded with plasma and serum. The inner chamber was filled with 200 mL running buffer and 500 mL antioxidant. The outer chamber was filled with running buffer. The gel ran at a voltage of 200 for 35 minutes. The gel was removed and sandwiched between filter paper, nitrocellulose and sponges. The inner chamber was then filled with transfer buffer until the tops of the sponges were covered and the outer chamber was filled with deionized water. This ran for 1 hour at a constant voltage of 30mV. The nitrocellulose was then left at 4^o C overnight with secondary antibody. The gel was then rinsed with PBST X2 and re-stained with TMB. Bands appeared at expected frequencies at expected concentrations.

Results:

When examining ATIII in cold exposed vs. control animals, the controls showed more ATIII in plasma and serum than the cold-exposed group. (Graph 1) We looked at male vs female in pooled plasma and serum and found no significant difference

between males and females in terms of levels of ATIII. (Graph 2) More tests should be done to verify this because there were large variations among individual animals leading to large standard deviations. There is, however, a statistically significant difference between ATIII levels in plasma of control hamsters vs cold-induced hamsters (Graph 1) with the cold-induced hamsters showing less ATIII than the controls (23% change).There is a statistical difference between ATIII levels in the serum of control-treated and cold-induced hamsters with the control hamsters producing more ATIII (30% change). Female serum showed a higher amount of ATIII in comparison to males. There was no statistical difference in ATIII levels between male and female plasma (Graph 2).

Fibrinogen α levels between control hamsters and cold-induced hamsters showed no variation (Graph 3). There was, however, a statistically significant difference between fibrinogen α levels in males and females in both serum and plasma (Graph 3).

Discussion:

A hypometabolic state, occurring during hibernation, allows an organism to maximize survival time in unfavorable environmental conditions. The entry into such state increases clotting time. This is suggested to be caused by an upregulation of the production of α_2-macroglobulin in the liver, which inhibits activated Factor X and thus reduces clotting. (Srere et al., 1995 and Story, 2009) Inducing cold conditions can bring about a hypometabolic state, increasing the preservation of energy and potentially increase lifespan of organs and organism. Other potential causes for a hypocoagulating

state include decreased circulating levels of platelets, suppressed production in the liver of other clotting factors, and the upregulation of several types of serine protease inhibitors (serpins) during hibernation.

Our results indicate a reduced production in ATIII in cold-induced animals, with much higher difference in experimental subject seen in plasma. It remains unexplained why ATIII, which is a thrombolytic factor, would be elevated in controls when compared to cold-exposed animals or why the difference should be greater in plasma than serum. Perhaps once clot formation begins there is little variation in the final concentration, but there may be some value to having different circulating levels in plasma prior to clot formation. There were higher serum levels of ATIII when compared to plasma in all groups, likely representing the release during clot formation. Although this is only seen when experimental groups are averaged together. This is expected because ATIII is produced primarily during a clotting phase, and is released into the serum as the clot forms. There were no significant differences in Male vs. Female pooled plasma or serum when measuring ATIII. These results do not further the explanation for lengthened clotting times in the winter. It is likely that ATIII does not play a role in the reduced clotting of hibernation. ATIII has a rather narrow specificity, and works by inhibiting proteinases of the clotting cascade, but does not influence trypsin, plasmin, or kallikrein. We would suggest that the reduced clotting of hibernation and cold torpor most likely works through a different pathway than the one controlled by ATIII.

With respect to Fibrinogen α in plasma, there was no difference between control and cold-exposed animals. There was however a significant difference between male and female animals. Male pooled plasma had a greater than 60% increase in the levels

of Fα when compared to female pooled plasma. Serum levels of Fα were very low in both control and hibernating animals with no significant difference between groups.

Little has been done on clotting factors in hibernation and even less in rodents which have some rodent specific clotting proteins such as murinoglobulin (Story, 1996) While the pathways are similar, there are also differences that may be part of the mechanism involved in prevention of thrombosis in the cold-induced state. Our results suggest that ATIII is likely not involved in the clotting pathway that is altered in cold exposure and reduced metabolic rate. But at least one paper suggests that when circulating heparin increases, ATIII is thought to change in such a way that it produces less inhibition of clotting.

Fibrinogen alpha is also not a likely candidate for seasonal variation. However, fibrinogen as a whole circulating molecule may be involved since there are several subunits, which are part of the clotting cascade, that we did not look at because there were no closely related antibodies.

If ATIII is inhibited by heparin, then perhaps heparin, just as in the turtle will prove to be the anti-coagulant responsible for reduced clotting in cold torpor and hibernation of mammals. Future work will focus on completing the work of examining Factor X with more sensitive methods, and additional experiments will be performed to examine circulating heparin levels.

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