COAGULATION PARAMETER DIFFERENCES BETWEEN *MACACA MULATTA* AND *MACACA FASCICULARIS*. Waller, Donald; McGeehan, Elizabeth; Dubach, Jean; Hoppensteadt, D.A.; Fareed, J. Sponsor: Baneux, P.

Abstract:

Macaca mulatta (rhesus) is used extensively for anticoagulant therapy development. Availability, cost and size has led to an increased use of *M. fascicularis* (cynomolgus) for testing. We observed genetic diversity among macaques from different sources and wanted to compare coagulation responses to examine potential interspecific disparities. The coagulation cascade is evaluated by clotting tests such as prothrombin time (PT), activated partial thromboplastin time (aPTT), Anti-Xa clotting method (Heptest), and thrombin time (TT). Serine protease inhibitors (Anti-Xa, Anti-IIa, and ATIII) target trypsin-like serine proteases including thrombin and plasmin. Interactions with these inhibitors are also used to assess potency and efficacy. Blood was drawn, transferred to tubes with 3.2% sodium citrate, centrifuged to obtain plasma and stored at -70oC. Clotting tests performed included PT, aPTT, Heptest and TT assays using fibrometers. Commercially available chromogenic substrate assays for Anti-Xa and Anti-IIa were also performed in the presence of heparin (H) and Enoxaparin (E). Pooled samples were also serially diluted and percentage of antithrombin determined. The PT and TT activities were higher for cynomolgus vs rhesus whereas aPTT and Heptest were lower. Similar interspecies responses for aPTT were observed with increasing concentrations of H; with E differences were observed at higher concentrations. The rhesus demonstrated a stronger inhibition of Anti-IIa activity when supplemented with H, however with E, the differences in inhibition was reduced between species. Similar responses were observed for Anti-Xa for both species and drugs. ATIII activity was higher in cynomolgus at greater than 50% dilution. The data show striking differences for coagulation parameters and responses to heparins between the two species of macaques. A clear understanding of the differences in these species is essential when interpreting past data and assessing new anticoagulants for efficacy, safety and pharmacokinetics.

Introduction:

Macaca mulatta (Rhesus) has been used extensively in the development of new anticoagulant therapies. However the lack of availability, cost and size has led to an increasing use of the *Macaca fascicularis* (cynomolgus) for this testing. We have been evaluating the diversity of the genetic background in both the rhesus and the cynomolgus animal models which clearly demonstrate genetic disparities between various sources of these animals. We wanted to compare the rhesus coagulation responses to those of the cynomolgus to begin our investigations of potential disparities in the response between the two species to different anticoagulants.

The coagulation system is typically evaluated by the use of clotting tests such as prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen (Heptest), and thrombin time (TT), all of which screen the different pathways of the coagulation cascade. Serine protease

inhibitors are used for a variety of cardiovascular interventions and target chymotrypsin-like serine proteases including thrombin. Interactions with inhibitors, such as anti-Xa and anti-lla, are also used to assess potency and efficacy of these agents.

Previous studies have suggested that there are species differences in the use of both unfractionated heparins (UFH), ie heparin (H), and low molecular weight heparins (LMWH), ie enoxaparin (E). H is a unique pentasaccharide with a high affinity binding sequence to antithrombin (ATIII) as the mechanism of action, however due to the drugs unpredictable effect and poor bioavailability intensive monitoring through activated partial thromboplastin time (aPTT) is recommended. LMWH, although a more stable and predictable alternative to UFH, is however a shorter saccharide chain and thus can not bind to both AT and thrombin. Consequently the Anti-Xa chromogenic assay has become the standard in monitoring LMWH's activity.

It was hypothesized that interspecific differences may also play a role in the effects of certain drugs due to genetic variation between species.

This poster presents results from our coagulation analysis of rhesus and cynomolgus macaques to characterize the effects of the anticoagulants heparin and enoxaparin.

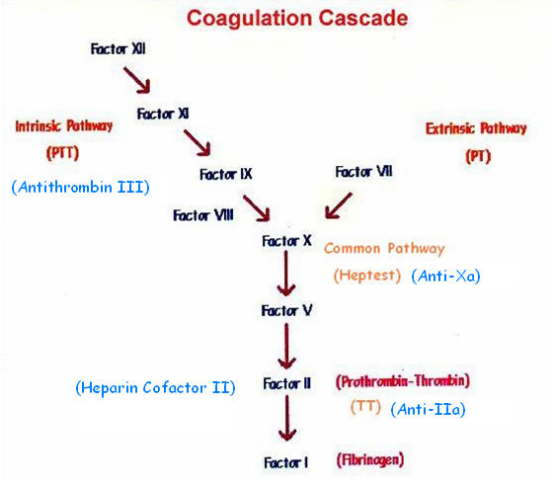
Methods:

Blood collection and sample preparation: The blood collection procedures were under the institution's IACUC approved protocols. A total of 5 mLs of whole blood was drawn from either the femoral or saphenous vein from normal healthy Chinese derived *M. mulatta* (n=16) and normal healthy *M. fascicularis* (n=12) derived from Indonesia (4), Vietnam (4) and China (4) sources. The samples were transferred to 3.2% sodium citrate tubes (9 parts blood to 1 part sodium citrate). Blood was centrifuged at 3000 rpm for 20 minutes to obtain plasma. Plasma was collected and aliquoted; pools were made from each group at the same time, and samples were stored at -70°C until tests were run. Pooled samples were thawed at room temperature and supplemented with either unfractionated heparin or enoxaparin at graded concentrations of 0-10 µg/ml.

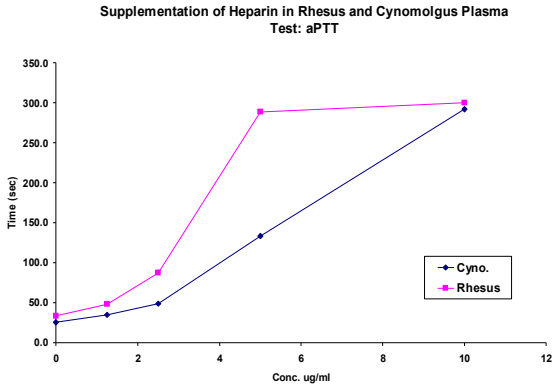
Coagulation analysis: All manual clotting tests were carried out by using procedures established for the use of human plasma. For each of the manual tests, measurement of the clotting time was stopped if the time exceeded 300 seconds as this time was outside the linear range of the instrument.

Coagulation testing was run on both individual baseline samples and pooled graded concentration samples. Tests run on individual baseline samples consisted 4 manual clotting test: prothrombin timeA (PT), activated partial thromboplastin timeB (aPTT), common pathway testC (Heptest), and thrombin timeD (TT 5 U/ml) run on manual fibrometersE and one chromogenic substrate assay for antithrombinF run according to the manufacturer's instructions: pooled samples from each group were serially diluted in 0.9% NaCl in a range consisting of 100%, 75%, 50%, 25% and 0. For each sample, 250µl was placed into a well of a 98-well microtiter plate and the percentage on antithrombin read. Tests run on pooled samples consisted of 2 manual tests: aPTT and Heptest, and 2 chromogenic substrate assays: Anti-Xa and Anti-llaG and were used according to the manufacturer's instructions. For all chromogenic assays the optical density (OD) at an endpoint setting of 405 nm was read using the SpectraMax PlusH with the SoftMax Pro softwareI. The OD values were then converted to percentage inhibition of factor Xa or lla relative to baseline values by using the following formula: % of Inhibition= 100% x [(ODbaseline –OD sample)/OD baseline).

Results:



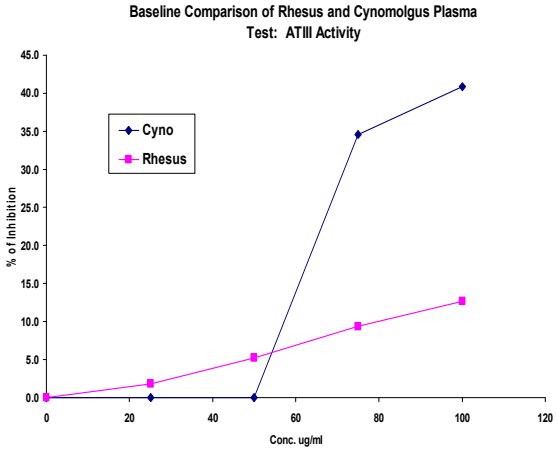
The aPTT results for heparin demonstrated a striking difference between species. The 5.0µg/ml and 2.5µg/ml concentrations with the rhesus produced a considerably greater anticoagulant response than the cynomolgus plasma. The graph plateaus at 10µg/ml rather than continuing upward for the Rhesus, because it has reached the upper limit of the testing range.



The Anti-Xa results for enoxaparin also displayed a difference between species starting at the 5.0µg/ml concentration. The cynomolgus plasma produces a greater response than the rhesus plasma.

When comparing the results of the Anti-IIa test for both heparin and enoxaparin the two species did not appear to differ in the presence of heparin, however with enoxaparin the cynomolgus plasma demonstrated a greater percentage of inhibition than the rhesus starting at the 5.0µg/ml concentration.

There was a large difference between species in the ATIII assay with the cynomolgus plasma showing considerably stronger ATIII activity at the baseline levels than observed in the rhesus plasma.



PT and the TT 5U/ml test (data not shown) were higher for the cynomolgus compared to the rhesus whereas the APTT and Heptest were lower for the cynomolgus compared to the rhesus. Increasing concentrations of heparin showed a similar response between the two species.

Further evaluations based on comparisons of geographic source provided some interesting results. All the samples from Vietnam and Indonesia groups were consistent in their responses within their group, whereas, one sample of Chinese origin was consistently different from the others in the group in most testing. Table 1 provides the group values for the effect of heparin on aPTT values by geographic origin. The first columns include all samples and the last columns provide the values without the one sample which appeared to be different from the others within the Chinese group. Initially with all samples, there were no significant differences between the groups, however once the one sample was removed significant differences between the Chinese

group and the other geographic origins were observed. These differences will be further evaluated based on genetic profiles of the animals.

Conclusions:

These results demonstrate striking differences between the two species of macaques when evaluating coagulation parameters and responses to anticoagulants. A clear understanding of the disparity in these species is essential when interpreting past data and assessing new anticoagulants for efficacy, safety and pharmacokinetics.

The variety of geographic sources of the cynomolgus monkey and the small number of animals often used in toxicology studies can lead to outliers which skew data and increase difficulty in making accurate conclusions. It is essential to carefully consider the source of the animals and their genetic background when beginning any studies to obtain valid and consistent data.

References:

A) Thromboplastin C+; Dade-Behring, Miami, FL
B) Trinity Biotech, Wicklow, Ireland
C) Purified Bovine Factor Xa and Recalmix; Hemochem, St. Louis, MO
D) Human α-thrombin (5U/ml); Enzyme Research, South Bend, IN
E) BBL Fibro Systems; Beckton Dickson, Franklin Lake NJ
F) Stachrom ATIII; Diagnostica Stago, Asnieres-sur-Seine; France
G) American Diagnostic; Greenwich, CT
H) SpectraMax Plus, Molecular Devices; Sunnyvale, CA
I) SoftMax Pro (version 5), Molecular Devices, Sunnyvale, CA