Establishing Plasma Volume and Osmotic Fragility Reference Intervals For Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas*) Sea Turtles

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Introduction

Current recommendations for the safe withdrawal of blood for blood transfusions and diagnostic medical procedures in sea turtles[1] are extrapolated from studies in mammals[2] and a single study on the red-eared slider *Pseudemys scripta elegans*.[3] An accurate measure of the total red blood cell (RBC) number (also known as RBC volume or RBC mass) and total plasma volume in sea turtles would permit the development of evidence-based guidelines and protocols for blood and plasma transfusions in sea turtles. Currently, there are no reference intervals for circulating RBC volume or total plasma volume in healthy sea turtles of any species or life stage.

Key elements in establishing transfusion protocols includes assessing for red blood cell lysis in stored blood products or examining sored RBCs for increased osmotic fragility. A study comparing osmotic fragilities among reptiles, birds, and mammals has been performed in which amphibians were the most resistant to osmotic stress followed by reptiles and then mammals. In reptiles, there was no significant difference is fragilities for aquatic or semi-aquatic/terrestrial reptiles. This study used terrestrial and freshwater turtles, and getting complete data for sea turtles would prove beneficial in completing the information available in sea turtle hematology. This is especially important considering that osmotic fragility is a valuable measurement in determining whether or not cells are becoming more fragile in commonly used blood bank storage solutions over time[4].

The accurate measurement of plasma volume and circulating RBC volume in healthy sea turtles is critical in understanding basic physiologic principles[5] underlying blood transfusion and drug therapy as well as a patient's response to dehydration and disease. The total plasma volume is necessary for determining how much replacement volume might need to be given to a sea turtle undergoing a surgical procedure. Circulating RBC volume is an important measure of total body oxygen carrying and delivering capacity and is critical for determining the required RBC replacement volume for a patient requiring transfusion therapy. Plasma volume, in part, also determines how drugs move from blood into body tissues.[6] Together, accurate plasma and RBC volume data will provide the basis for understanding sea turtle pathophysiology and for implementing rapid and appropriate transfusion therapy and drug treatments in sick and debilitated sea turtles with the goal of reducing morbidity and mortality.

Blood transfusions are often necessary in sea turtles undergoing surgery for fibropapillomatosis (FP) tumor removal or are part of treatment of sea turtles with other conditions (e.g., emaciation, trauma). FP is a debilitating disease that affects sea turtles in Florida and other parts of the world. These lesions have been reported in all sea turtle species except in leatherbacks (*Dermochelys coriacea*). For unknown reasons, the frequency of FP is much higher in green turtles (*Chelonia mydas*) than in other species.[7] Based on information from the Florida Sea Turtle Stranding and Salvage Network (STSSN) database[8], 22.2 percent of dead or debilitated (i.e. stranded) green turtles (sample size=6027) found in Florida between 1980-2005 had FP tumors. Turtles with FP have external tumors that can grow so large as to hamper swimming, vision, feeding, and escape from predators. During surgical removal of FP tumors or other surgical procedures, plasma may be administered to maintain blood pressure and to restore fluid volume, and whole blood may be administered to provide oxygen-carrying RBCs following unanticipated surgical blood loss.

In our experiment we used Evans Blue binding to albumin to determine plasma volume. Evans Blue has largely been replaced by radio-labeled human albumin for estimating plasma volume. By using Evans Blue we avoid the regulatory complications associated with the use of radioactive tracers[9,10] Evans Blue gives the same results for estimating plasma volume as radiolabeling provided that the serum albumin levels and bond kinetics between albumin and the dye remain stable.[11]

Hypothesis

We hypothesized that the Evans Blue dye dilution method will be successful in determining circulating plasma volume in Green (*Chelonia mydas*) and Loggerhead (*Caretta caretta*) Sea Turtles, and that the circulating plasma volume is similar between Greens and Loggerheads when adjusted for life stage, size, and weight. Furthermore, we hypothesize that osmotic fragility results of Greens and Loggerheads will be similar.

Materials and Methods

To prepare a 5 mg/ml Evans Blue solution for injection, 400 ml of 0.9% sterile saline was mixed with 2g of Evans Blue and filtered through a .22 micron Nalgene Vacuum Filter via surgical suction. The solution was aliquoted into sterile vials via hemonate filters. For each turtle, a standard curve was create in reference to El Sayed et al (1995). In brief, 16 ml of whole blood was collected from the dorsal cervical sinus into red top tubes. The microhematocrit was measured in triplicate,

Figure 1: Injection of 15 mg Evans Blue dye into dorsal cervical vessel.

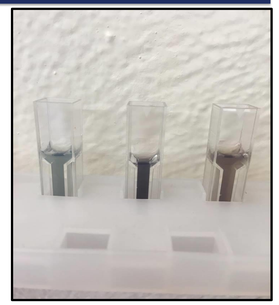

Figure 2: Serum samples 10, 20, and 30 minutes post Evans Blue injection.

and the sample was left to clot. Once clotted, the sample was centrifuged for 15 minutes at 3400 rpm on a Hamilton Vanguard V6000 centrifuge. Serum was aliquoted off and Evans Blue dye was added to create 5 and 10 mg/L concentrations. Absorbances were measured in quadruplicate via spectrophotometer and averaged for plain serum (0 mg/L), 5 mg/L, and 10 mg/L.

To gather data for plasma volume, an empty 6cc syringe with needle and cap were weighed in quadruplicate. 3 ml of 5mg/ml Evans Blue was withdrawn into this syringe. The syringe was then reweighed with needle and cap on. Using a 17 cm .26 ml male Leuer Lock adapter catheter extension set, the 3 ml of Evans Blue was injected into the dorsal cervical sinus. Three 5cc syringes of heparinized saline (5 IU/ml) were used to ensure dye was flushed into the vein. At 10, 20, and 30 minutes post injection, 3 cc of whole blood was collected from the dorsal cervical sinus into red top tubes. The blood was allowed to clot, spun for 10 minutes, and serum was aliquoted. Absorbance was measured in quadruplicate for each sample and averaged.

For Osmotic Fragility, 1.5 ml of blood was collected from the dorsal cervical vessel of loggerheads and greens and stored in lithium heparin green top tubes. 1.5 ml of varying concentrations of NaCl from 0.9% to 0% were added to 14 tubes. 100 ul of whole blood was added to each tube and inverted to mix. Tubes were allowed to incubate for one hour. After incubation, tubes were spun in a serofuge for 3 minutes. The supernatant was analyzed for absorbance via spectrophotometer. Percent hemolysis was calculated and graphed with the varying concentrations to determine a fragility curve.

Results: Evans Blue

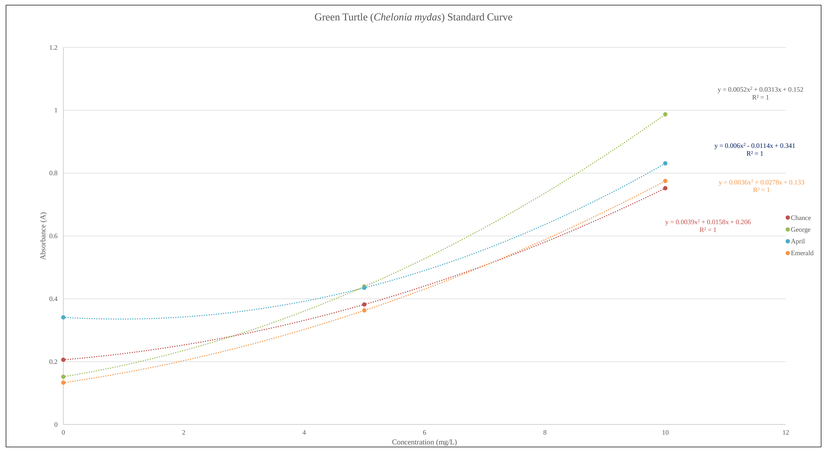

Figure 3: Standard Curves created for Green (*Chelonia mydas*) Sea Turtles based upon 0, 5, and 10 mg/L concentrations of Evans Blue dye in serum. These curves were used to determine concentration of Evans Blue dye in each sample taken at 10, 20, and 30 minutes post-injection of 15 mg of Evans Blue.

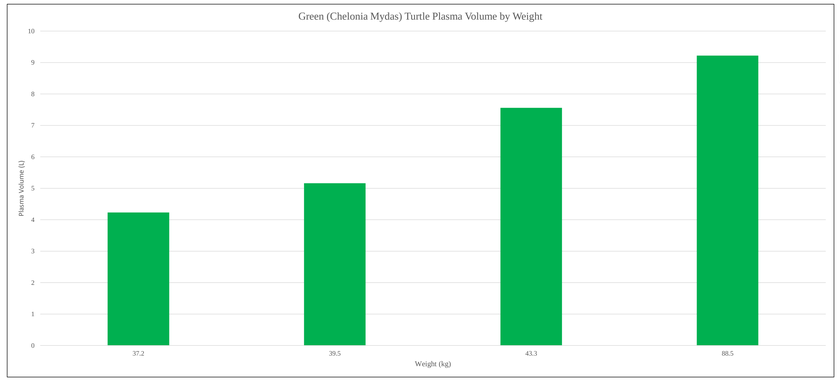

Figure 4: Bar graph of plasma volume (l) by weight (kg) of Green Sea Turtles as calculated using each turtle's individual standard curve and linear regression analysis on 10, 20, and 30 minute serial blood draws. Bar graph indicates that as weight increases, so does plasma volume and plasma volume is roughly 10-17% of bodyweight.

Results: Osmotic Fragility

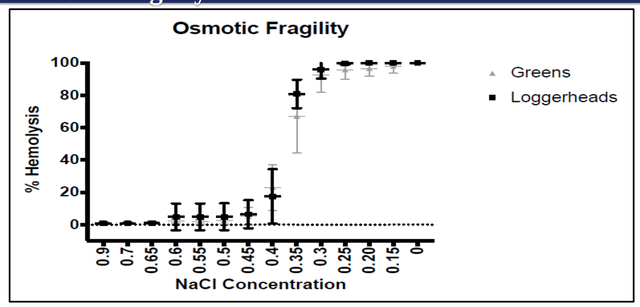

Figure 5: Osmotic Fragility Curve for Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas*) sea turtles.

Discussion

We had hypothesized that Green and Loggerhead Sea Turtles would have similar plasma volumes based on life stage, size, and weight and that Evans Blue dye would be a successful means of uncovering reference ranges. This hypothesis was proven untrue. As seen in Figures 3 and 4, only Green Sea Turtles were successfully analyzed using the Evans Blue dye dilution method. However, the amount of plasma volume calculated for these animals was quite beyond the range expected. Most estimates put blood volume as a whole (red blood cells plus plasma) at 7-10% the bodyweight of the animal[2]. Our values range from roughly 10-17% of the green sea turtle's bodyweight.

There are a few reasons why this may have occurred. First, there could possibly be storage depots in sea turtle vasculature for the dye to accumulate in. For instance, the dye was injected into the dorsal cervical sinus of the turtle. There is the possibility that, despite our efforts to flush the site, the sinus is much larger than 15cc worth of saline and dye was accumulating. Second, our standard curve may need to be adjusted. Our absorbance readings for our 10, 20, and 30 minute blood withdrawals primarily matched to the bottom end of the curve. In order to get more accurate estimates of concentration and therefore calculations of plasma volume, we may need to focus our standard curve on smaller known concentrations of Evans Blue in serum. Third, it is possible that the shear size of the sea turtles contributed to inaccurate measures of plasma. Loggerheads in particular are much bigger than green sea turtles, so it is possible that this came into play with their measures not being of value. Finally, it has been noted that the Evans Blue method requires albumin to remain intravascularly. Therefore, overestimation in plasma volume could occur in cases such as traumatic injuries, malignancies, and renal and cardiopulmonary disorders[11]. Considering the our samples came from a rehabilitation setting in which fibropapillomatosis and other injuries have occurred, it is possible that albumin may not have been purely intravascular in these turtles.

Regarding Osmotic Fragility, we hypothesized that the osmotic fragilities of greens and loggerheads would be similar. This was proven based on Figure 5 above, in which Loggerheads and Greens each had 50% hemolysis occur at .34-.40% NaCl. In mammalian species this commonly occurs at .45% NaCl[12]. However, considering that research shows that reptiles and amphibians are generally less fragile than mammalian species, it is not surprising that salt-water dwelling species such as sea turtles would have better methods for withstanding salt concentrations.[4] Based on this information, in developing storage solutions for blood banking, commercially available anticoagulants for mammals should not have to be adjusted for salt concentrations for sea turtles.

Conclusion

From this experiment, we conclude that Sea Turtles require a lower standard curve concentrations of Evans Blue in order to estimate their plasma volume. Furthermore, we conclude that Greens and Loggerheads have similar Osmotic Fragilities, in which 50% hemolysis occurs at a concentration of .34-.40% NaCl.

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