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DISTRIBUTION OF DOG ERYTHROCYTE DENSITY AND RELATIONSHIP TO CELL SUSPENSION VISCOSITY

A thesis
Presented for the
Master of Science
Degree
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Seung Jin Baik

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DEDICATION

This thesis is dedicated to my parents, Jun-Kee Baik and Jeong-Bun Shon, for their great contribution, and my advisor, Dr. Richard J. Jendrucko, my committee members, Dr. Jack F. Wasserman and Dr. Joe S. Iannelli, and my friends and relatives, for having trust in me, and for encouraging me to reach higher in order to achieve my goals during my entire school life.

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ABSTRACT

An experimental investigation was undertaken to determine the density of young and old dog erythrocytes and the viscosity of suspensions prepared from these erythrocyte fractions.

The densities of young and old RBC from three dog donors were determined from direct measurements of post-centrifugation cell fraction weight and volume.

The density distribution range of young erythrocytes was found to vary from 1.035 g/ml to 1.088 g/ml. Meanwhile, the density of distribution range of old erythrocytes varied from 1.073 g/ml to 1.105 g/ml. The average density of old RBC was found to be about 1.0-1.5 % higher than that of young RBC in agreement with earlier studies. The average density of RBC obtained from the three donor dogs was 1.074 g/ml.

For young and old erythrocyte suspensions, viscosity was measured in a cone and plate viscometer at two shear rates (150 sec^{-1} and 225 sec^{-1}) and for measured Ht values ranging from 20% to 50%.

Dog erythrocyte suspension viscosity determinations with measured hematocrit (Ht) and shear rate parameters yielded a non-linear relationship for a hematocrit range of 20% to 50%. The viscosity values of suspensions of both young and old erythrocytes ranged from 2.30 cP to 5.53 cP at 25-27 °C. When these results were compared with available data for human blood, it was found that dog erythrocyte suspensions were slightly more viscous than those of human blood erythrocyte suspensions at the same Ht and shear rate values and temperature.

The viscosity of old erythrocyte suspensions was found to be slightly higher than that of young erythrocyte suspensions at shear rates of 150 sec^{-1} and of 225 sec^{-1}. This

result suggests that old erythrocytes are less flexible than young erythrocytes. The average blood viscosity value for a shear rate of 150 sec^{-1} was 5-9% higher than for a shear rate of 225 sec^{-1}.

The research performed using dog erythrocytes agrees with the earlier work with human blood and demonstrates that suspensions of older RBC have viscosities greater than those of younger RBC at the same hematocrit value. This result suggests that there may be significant effects on blood flow in normal and disease states when the average age of RBC is altered from a normal distribution. Additional research will be needed to improve the quantification of RBC age effects on blood flow properties and to establish the possible relevance of these effects on conditions of health and disease in humans and animals.

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LIST OF SYMBOLS

Y	Young erythrocyte suspensions
O	Old erythrocyte suspensions
M	Male
F	Female
N/A	Not available
Ave	Average Viscosity
Exp.	Experimental
Run #	Run Number
vs.	Versus

CHAPTER 1

INTRODUCTION

There has been a long history of study of the mammalian cardiovascular (CV) system that is central in material transport in humans and animals. An understanding of cardiovascular system function requires the study of the underlying mechanics that regulate blood flow behavior in the blood vessels. The development of a basic knowledge base of CV system function is an important prerequisite to the development of effective diagnoses and treatments for cardiovascular system pathologies.

Of particular interest has been the relationship of pressure generated by the heart to the resulting flow of blood to body tissues. In a simple electrical analogy, the flow is proportional to the pressure gradient divided by the resistance. The flow resistance in turn is a function of vessel geometry and blood viscosity.

Blood viscosity is known to depend on the composition of the plasma and the concentration and mechanical properties of the suspended erythrocytes, leukocytes and platelets. Since the normal volume concentration of erythrocytes (~ 40%) is much higher than that of white cells or platelets (< 1%), they have the major role in determining viscosity.

Many studies have been undertaken to quantify the relationship of whole blood viscosity to the red blood cell (RBC) concentration, or hematocrit, and typical relationships are well known (1, 2, 3). In contrast, data reflecting the effects of RBC geometry and flexibility on viscosity are more limited. Several prior works have focused on the measurement of the mechanical properties of individual RBC membranes with the

use of micro-pipettes (4, 5). On the macroscopic level, several studies have addressed the effect of RBC abnormalities on blood viscosity and flow.

In addition to the study of normal and pathological RBC suspensions, some limited attention has been directed to the fact that normal RBC may become more rigid during their normal lifespan. This effect may have importance in understanding the resistance to blood flow in conditions where the average age of circulating RBC is less (e.g. leukemia) or greater (e.g. transfusion) than normal. In light of the fact that relatively little quantitative data on blood viscosity related to RBC age is available, this study was undertaken to validate and further extend the small body of currently available data and to add to the understanding of the role of RBC mechanical properties in determining whole blood viscosity.

CHAPTER 2

LITERATURE SURVEY

Studies of RBC Mechanical Properties

Studies on Individual Erythrocytes

The various properties of erythrocytes, leukocytes and other formal elements in blood have been widely studied.

In humans, erythrocytes (RBC) are flattened, biconcave discs about 8 μm in average diameter and 2.2 μm thick at the outer edge. Human RBC have a circulating life span of only about 120 days before they are destroyed by phagocytic cells in the liver, spleen, and bone marrow. Blood flow in the microcirculation is determined to a large extent by red blood cell (RBC) geometry, deformability, concentration (hematocrit, Ht) and plasma viscosity. In larger microvessels (arterioles and venules 10 to 25 μm ID), the RBC take on random shapes in capillaries (2-10 μm ID), the RBC deform into a "bullet shape" while passing through individual vessels of a cross-section smaller than the largest diameter of the RBC. Thus, the study of blood flow in small vessels requires attention to the mechanical properties of cells and cell membranes, which may be substantially altered in disease states (8).

Photographs of individual red blood cells have been taken by many investigators (9, 10) with an interference microscope. Since earlier work research on RBC geometry (9, 10), the extreme-value distribution (11, 12), deformability of RBC (13, 14), and elasticity of the red cell membrane (15) have been studied. Extreme value distributions are the limiting distributions for the minimum or maximum of a very large collection of

random observations from the same arbitrary distribution. In other words, it generally refers to the distribution of the minimum of a large number of unbounded random observations. In the research on RBC extreme-value distribution, Chen et al. (12) studied the distribution of RBC diameter, area, volume, maximum thickness, and sphericity as measured with the largest diameter in RBC. For RBC, these researchers defined a sphericity index defined by the following formula:

$$\textbf{Sphericity index} = 4.84(\textbf{cell volume})^{2/3} \times (\textbf{cell surface area})^{-1} \quad (1)$$

Shinozuka et al. (23) investigated distributions of RBC density and showed that the post-centrifugation bottom layer ("old erythrocyte fraction") has a higher density value and a greater mean corpuscular average thickness (MCAT) than the top layer ("young" erythrocytes). The MCAT was calculated from the following equation:

$$\textbf{MCAT} = 4 \times \textbf{MCV}/(\textbf{MCD})^2 \times \pi \quad (2)$$

where MCV (Mean Corpuscular Volume)

MCD (Mean Corpuscular Diameter)

Shinozuka et al. (23) postulated that the MCAT of erythrocytes might be directly related to RBC density and age.

In a theoretical analysis of the elasticity of RBC, Fung demonstrated that the biconcave shape state is a zero-stress state (15). In the biconcave state, the RBC trans-membrane pressure difference is zero. There exist an infinite number of large deformations of RBC which preserve the volume of the cell, and surface area without stretch and tearing of the membrane. Several types of cell membrane experiments have been performed: osmotic swelling in hypotonic suspending media, compression between two flat plates, and aspiration with a micropipet (4, 5).

RBC Suspension Flow Properties

1) Normal RBC in Suspension

Human blood is a non-Newtonian fluid. The inverse of RBC fluidity mathematically termed RBC viscosity. Viscosity is that property of fluid by virtue of which it offers resistance to flow or shear. The dependence of blood viscosity on the shear rate has been widely reported. In particular, the viscosity of blood increases with a decrease in shear rates (42). The viscosity of RBC suspensions varies with hematocrit (Ht), the percentage of the total volume of blood occupied by the cells. It varies also with temperature. Cokelet et al. (43) postulated that blood has a finite yield stress. In normal blood, the yield stress is a strong function of concentration of the protein, fibrinogen. Cokelet et al. (43) stated that below a vanishing shear rate blood behaves like an elastic solid.

2) Abnormal RBC in Suspension

Erythrocytes with either or both abnormal shape and mechanical properties are found in a number of disease states. Among these are the following:

i) Sickle cell disease: This is an inherited disease caused by the presence of abnormal hemoglobin (hemoglobin S) in red blood cells (20). Red blood cells containing mostly hemoglobin S do not live as long as normal red blood cells (normally about 16 days). They become stiff, distorted in shape, and have difficulty passing through microvessels. When sickle-shaped cells occlude small blood vessels, the blood flow to affected vascular beds is reduced. In addition, sickling increases the viscosity (17-19 cP) of whole blood, impeding flow in larger vessels, and contributes to the deoxygenation of tissues. Tissues that do not receive a normal quantity of blood flow eventually become

damaged. Sickle cells are destroyed rapidly in the bodies of people with the disease, causing anemia, jaundice, and the formation of gallstones.

ii) Microangiopathic anemia: This anemia in renal failure was characterized by hemolysis, red-cell fragmentation, and thrombopenia, and fibrin and platelet deposition in arterioles and capillaries in the kidney. It is of particular importance to state that a decrease in size of the red cell is accompanied by a rapid increase in the red-cell rigidity (20).

iii) Spherocytosis: This condition is characterized by abnormal red blood cell membrane proteins, which cause the cell to assume a spherical shape rather than the usual bi-discoidal disk shape. The abnormal cells that result pass with greater difficulty through the spleen (a blood-filtering organ) and are sequestered in spleen tissue and destroyed rapidly. Murphy observed that blood from patients with hereditary spherocytosis showed an increase in whole blood viscosity and a marked decrease in its filterability at lower pH levels compared with normal blood (21).

iv) Heinz-body anemia: This condition is characterized by denaturation of proteins and especially hemoglobin in RBC; the red cells are much more rigid than normal cells. The viscosity of packed cells containing Heinz bodies can be twice that of packed normal cells (22).

v) Artificially hardened cells: Erythrocytes hardened by treatment with aldehyde (for study of hardening effects on flow properties) show viscosity-concentration behavior similar to that of rigid spheres (7). In the case of rigid spheres, viscosity is seen to rise more steeply with concentration than for normal RBC.

3) Age-Separated Normal RBC Suspensions

i) Separation of RBC of varying ages: Studies on the in-vivo aging of RBC have consisted of comparison of the mechanical properties of erythrocytes separated by density, based on the finding that increasing red cell density is a function of their age (23). Shinozuka et al. (23, 24) separated human erythrocytes by centrifugation into three fractions, the top (18% of volume, <1.092 g/ml density), middle (65% of volume, 1.092-1.100 g/ml density) and bottom (17% of volume, >1.100 g/ml) layers, by the density gradient centrifugation method. In this method washed RBC were suspended in a set of phthalate diester solutions of varying density. Erythrocytes' density fractions were identified by neutral buoyancy in one of the solutions. The top layer was considered a "young erythrocyte" layer, and the bottom layer was referred to as an "old erythrocyte" layer. Here, the middle layer was discarded, as the maximum difference in cell density was desired for study (23). The viscosity of suspensions of RBC fractions of different ages was then studied at 37 °C with a cone-plate viscometer. It was found that suspensions of older, denser red cells had a higher viscosity than suspensions of younger RBC for an identical hematocrit level (29).

Tillmann et al. (29) performed research with both human packed erythrocyte suspensions and RBC ghost suspensions. First of all, for packed erythrocyte viscosity measurements, suspensions of washed erythrocytes in autologous plasma were adjusted to contain $8.0 \pm 0.2 \times 10^9$ red cells/ml (approximately 72% Ht). Hematocrit was adjusted to the value of 72% (substantially higher than the normal physiological level (37%-54%)) to accentuate the viscosity variation being studied. The study revealed that the viscosity

of packed erythrocyte suspensions from old erythrocytes was found to increase versus that of young ones.

Meanwhile, the viscosity of packed ghost suspensions from aged erythrocytes was increased moderately compared to that of young ones. Here, the ghost suspensions are not normal functioning cells, but hemoglobin-free human erythrocyte membranes. That is, ghosts are RBC membranes devoid of leaked hemoglobin contents. For preparation of ghost suspensions, isolated red cell membranes were prepared from both young and old washed erythrocytes. Tillmann et al. (29) used a hypotonic phosphate buffer solution (pH 7.4) of 15-80 imOsm in order to swell the RBC and cause lysis. The results of this study showed that younger RBC ghosts appeared to be more flexible than older cells, which may result in lower viscosity for cell suspensions of such cells as compared to suspensions of older RBC ghosts.

Summary and Justification for Present Study

The mechanical properties of human erythrocytes of different ages have been studied in several laboratories. In these studies, the relationship between the erythrocytes age and cell density was investigated (24, 25, 32). In addition, the relationship between erythrocyte age and suspension viscosity has been studied (28). Some of the previous studies of human blood found that erythrocyte suspension viscosity correlates with both cell density and age (23, 29). However, currently available data are relatively limited. Further study of the viscosity of young and old erythrocyte suspensions and their relationship to cell age and density can also contribute to the basic understanding the rheological properties of erythrocyte suspensions.

In addition, RBC density and suspension viscosity may be potential clinical indicators of some blood diseases of patients, additional study is needed to validate and extend the earlier results.

CHAPTER 3

MATERIALS & METHODS

Owing both to the difficulty of obtaining human blood for experimentation and the ready availability of animal blood in the clinics of the University of Tennessee College of Veterinary Medicine in Knoxville, it was decided to use dog blood in this thesis project. The use of animal blood has the further advantage that various mammals have red blood cells (RBC) of different size (31). Such differences may facilitate the future study of RBC suspension property (e.g. viscosity) differences associated with species differences in cell geometry and physical properties (e.g. density, flexibility). Dog blood RBC are of the same shape as human RBC but are somewhat smaller with a mean diameter of 7.1 μm.

Healthy dog blood samples (Canis Familiaris with reference names: Elvis (male), Madge (female) and Jackson (male)) were acquired from the University of Tennessee Veterinary Hospital where blood was drawn by left jugular vein puncture. The date and time that each blood sample was obtained over a period of several months was recorded. Based on the relatively small size of the donor dogs (50 to 60 lbs), the volume of blood supplied at the times of each donation was limited to 12-15 ml and all experimental procedures used were designed around this volume limitation. The anticoagulant used was lithium heparin (8 I.U./ml blood) or EDTA (containing sufficient K_2 EDTA to anticoagulate 500 μL of skin puncture whole blood). When blood was not used immediately, it was stored in a refrigerator at 3-4 °C and used within 24 hours. The starting date and time for each experimental session and room temperature were

recorded. The RBC were observed under microscope at 1500x magnification for appearance assessment. The immersion oil was used when the RBC appearance was observed under a light microscope at this magnification. Any abnormalities were recorded. If a substantial fraction of the RBC (> 10 %) had visible abnormalities, a sample was discarded. The hematocrit (Ht) value of the whole blood sample as obtained from donor dogs was measured using an Autocrit Ultra 3 micro-centrifuge (Becton Dickinson, Parsippany, NJ) operating at 11,700 RPM (13,000 g). The whole blood sample was transferred with a syringe in 5 ml portions into two test tubes; of the 12-15 ml donated, therefore, about 2-5 ml was considered the discarded volume. A schematic diagram depicting the procedure used to prepare RBC suspensions for study is shown in Figure 1 below.

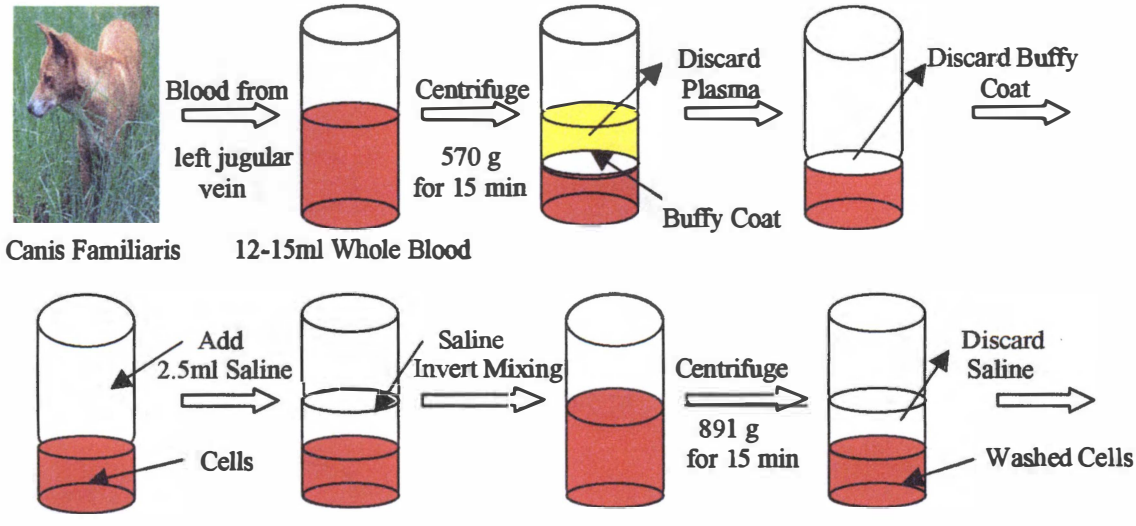


Figure 1. Schematic diagram of erythrocyte processing procedures used

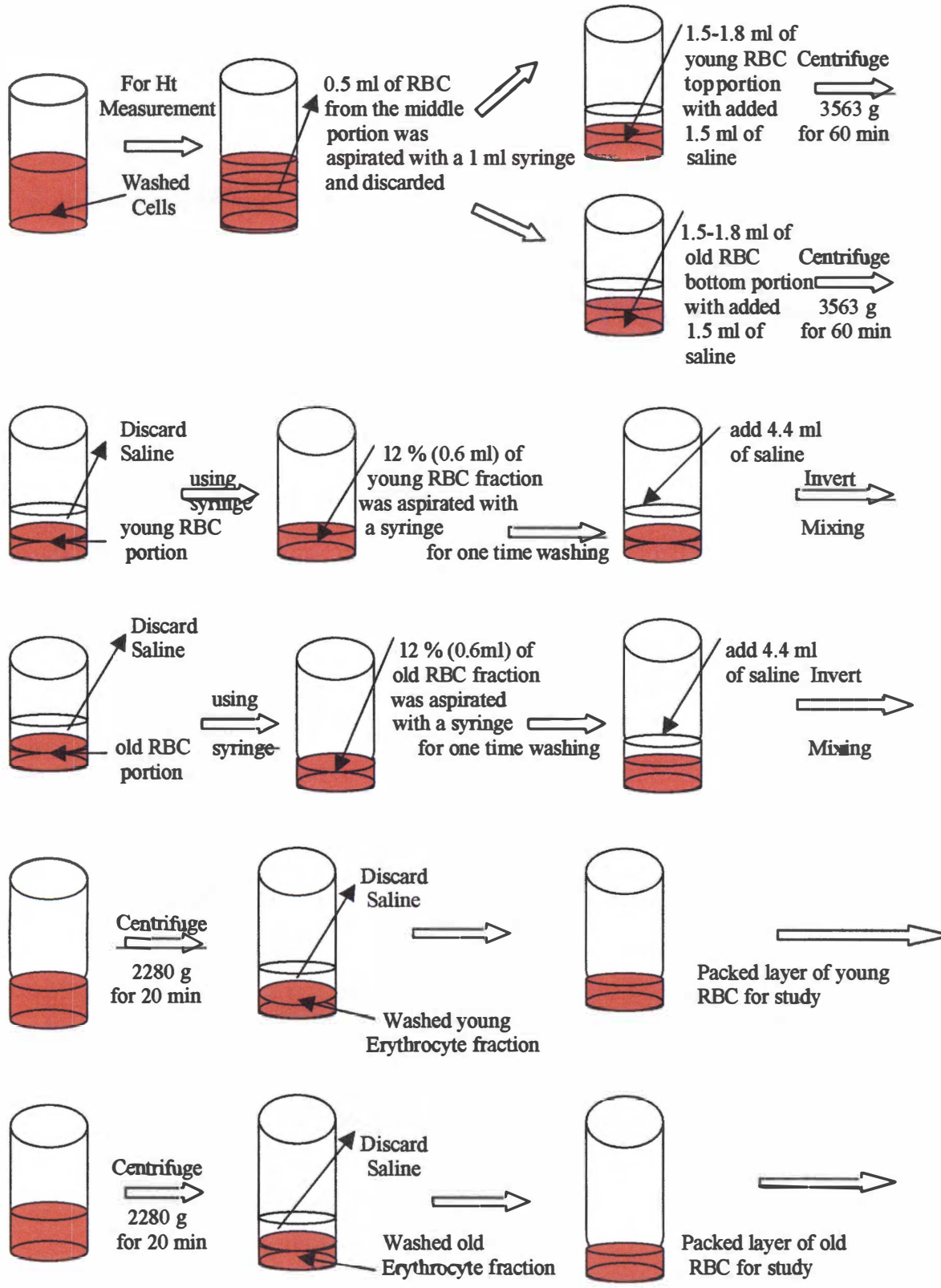


Figure 1. Continued.

Initially, the whole blood samples were centrifuged for 15 min at 2000 RPM (570 g) with an IEC HN-SII centrifuge (International Equipment Company, Needham Heights MA). This centrifuge could be operated at speeds from 0 RPM (0 g) to 5000 RPM (3563 g, full speed). Here, the reason that the author initially chose at 2000 RPM (570 g) is for consistency with the method of Tillmann et al. (29). The plasma and leukocyte "buffy coat" were removed by careful aspiration with a 1 ml syringe by the method of Tillmann et al. (29). In a slight modification, the packed RBC were washed by adding 2.5 ml of isotonic saline solution (0.9% w/v pH 6.0-7.5, Blood Bank saline, Fisherbrand Swedesboro NJ) into each test tube. The filled tubes were next centrifuged for 15 min at 2500 RPM (891 g) after inversion mixing with 2.5 ml of saline solution. A higher centrifugation speed than before, 2500 RPM (891 g) was used instead of 2000 RPM (570 g) to minimize the residual trapped saline without causing excessive lysis of RBC at this stage of the procedure. The top layer of saline solution was removed by careful aspiration with a 1 ml syringe. A 0.5 ml RBC sample was drawn from the middle portion with a 1ml syringe and was discarded. A volume of 0.5 ml of middle portion of RBC was added to a volume of 0.5 ml saline (Ht = 0) and was aspirated in a micropipette for Ht measurement. At this step, the microscopic appearance of the RBC was observed and documented. After discarding the "buffy coat," middle cell layer and old saline solutions, the "young RBC" fraction (1.5-1.8 ml from the top portion) was separated with a syringe from the "old RBC" fraction (1.5-1.8 ml from the bottom portion) and these fractions were put into two different tubes. Then, the fresh saline solution was added to the two test tubes for washing. Each of the test tubes containing the young and old RBC fractions was centrifuged for 1 hr at 5000 RPM (3563 g, full speed). Here, regardless of the occurrence

of slight cell lysis, the author chose 5000 RPM (3563 g, full speed) as the centrifuge speed in order to separate each portion of cells distinctly. After 1 hr centrifugation, the RBC were observed under 1500x magnification for normalcy.

Due to higher speed than before, (2500 RPM (891 g)) better cell compaction was achieved and, a layer of saline solution was separated. This saline solution was also discarded before recovering young and old erythrocyte fractions. From this, a volume of 0.6 ml of both the top and the bottom fraction of the packed cells was carefully aspirated with a 1 ml syringe. Each erythrocyte fraction containing relatively young and old RBC was again distributed to different tubes. Each erythrocyte fraction containing young and old RBC was washed once again with 4.4 ml of isotonic saline solution by tube inversion and centrifuged for 20 min at 4000 RPM (2280 g) to obtain the final suspensions used for cell density and suspension viscosity measurement. At this step, the reason that the author chose 20 minutes instead of 15 minutes such as previous step for cell washing was to reduce or minimize the existence of trapped saline. When the centrifuge speed was set over 4000 RPM (2280 g), and was observed with naked eye, there occurred substantial RBC lysis caused by centrifuge heat and vibration (see Figure 3 on page 23). Therefore, the author used 4000 RPM (2280 g) as the centrifuge speed in order to minimize the occurrence of RBC lysis.

For measurement of RBC fraction cell density, two different methods described in the literature were considered. The method used by Danon et al. (32) was to separate RBC according to their relative density by suspending cells in phthalate esters to assess neutral buoyancy. This method of separation determines the frequency distribution of

density of the RBC present in a sample. The precision of the measurement depends on the difference in the specific gravity among the set of separating liquids used (32).

The second method uses the most simple approach of the direct measurement of cell fraction mass and volume from which RBC density can be calculated as:

$$\textbf{Density} = \textbf{Mass (grams)/Volume (ml)} \quad (3)$$

A top-loading balance (XE series Model 300, Denver Instrument Company, Arvada Col) was used and displayed units of mass in grams so no calculation relating weight and mass were required.

Owing to the local unavailability of the required phthalate esters for the preparation of separating liquids and the ready availability of a centrifuge, and a top-loading precision measurement scale, the author decided to use the basic direct calculation method to determine the average density of young and old erythrocytes in the separated cell fractions. As the initial step for a cell fraction average density measurement, the weight of an empty tube was determined with the top-loading balance. This was repeated five times with the same tubes to obtain an average value for each tube used. After dispensing 0.20±0.01 ml of each erythrocyte fraction into two different tubes of determined empty weight, the RBC suspension-filled tubes were weighed. As for the empty tubes, all measurements on suspension-filled were repeated five times with the same blood contained in the same tubes for the determination of an average weight. The density of the erythrocyte volume was calculated by the following formula:

Density = (Average mass of suspension-filled tube-Average mass empty tube) / Erythrocyte suspension volume (0.20±0.01ml)

where erythrocyte suspension volume is the post-centrifugation packed cells with saline layer removed. For actual RBC density and measurement, the correction for the trapped plasma was required.

The hematocrit was measured for each erythrocyte suspension containing young and old RBC by mixing different ratios of added saline solution to a volume of post-centrifugation packed cells. The difference between the "anticipated" or "calculated" Ht value determined by mixing different ratios of saline solution to a volume of packed erythrocyte sample and Ht value measured using the microcentrifuge is reflected in the data listed in Table 1 below. As can be seen from Table 1, the saline trapping is a significant factor which had to be considered in specifying true hematocrit values of the suspension used for viscosity determination.

Table 1 The comparison between "calculated" and "measured" Ht values for prepared RBC suspensions

Sample	Calculated Ht /Measured Ht For Young RBC	Calculated/Measured Ht For Old RBC
Canis Familiaris (Madge, F)	30.0%/29.0%, 40.0%/35.1%, 50.0 %/45.3 %	30.0%/27.0%, 40.0%/34.5%, 50.0%/43.5%
Canis Familiaris (Madge, F)	30.0%/24.0%, 40.0%/37.9%, 50.0 %/45.5 %	30.0%/23.5%, 40.0%/34.2%, 50.0%/42.3%
Canis Familiaris (Jackson, M)	30.0%/22.5%, 40.0%/38.0%, 50.0 %/46.3 %	30.0%/21.5%, 40.0%/33.3%, 50.0%/42.0%
Canis Familiaris (Madge, F)	30.0%/28.0%, 40.0%/38.5%, 50.0 %/47.9 %	30.0%/27.1%, 40.0%/32.6%, 50.0%/46.4%

(Room Temp 25°C-27°C)

transferred via a 1 ml gas tight syringe into a viscometer sample cup for the measurement of viscosity. The viscosity values were measured at shear rates of 150 sec^{-1} and 225 sec^{-1}. These values were selected based on work by Andrew et al. (30) in which measured RBC suspension viscosity values were found to be relatively stable at shear rates of 150 sec^{-1} and 225 sec^{-1}. The relationship between the measured viscosity value and Ht value was recorded and graphed. After viscosity measurement, microscopic examination of erythrocytes in the young and old RBC suspensions was done to assure that RBC lysis was minimal. The appearance of the RBC were checked initially (the first day when the dog blood sample was acquired), after RBC suspension preparation was complete and immediately after measuring the viscosity values. The ending time of the experimental session, and any variations on experimental protocol used were recorded. All procedures were carried out at room temperature (measured to range from 25-27 °C).

CHAPTER 4

RESULTS & DISCUSSION

Preliminary Findings

Factors Affecting RBC Integrity

It was desired to conduct the present research with "normal blood" and RBC suspensions. Thus, it was necessary to ascertain which factors affecting RBC integrity, reflected in microscopy and in "red tint" in the plasma or saline decant layers, would have to be controlled during the experimental protocol used. For this research, fresh heparinized or EDTA-treated whole blood was used within 24 hours of collection from animal donors. In previous studies, it was noted that RBC become apparently more dense at the approximate rate of SG 0.008 every 5 hours (32). Thus, it was imperative to conduct all experiments in an expedient manner.

During the experiments conducted, blood and RBC in suspension were periodically examined microscopically at 1500x. The RBC were always found to be biconcave or elliptical-shaped on the day when they were received from the Small Animal Clinics of the University of Tennessee College of Veterinary Medicine. As time elapsed during refrigerated storage, the color of plasma started to gradually turn red, indicating RBC lysis and release of hemoglobin. The microscopic appearance of RBC also revealed the clotted shape of RBC due to cell lysis. The measured Ht value of the dog blood samples was examined over a period of four days and found to have decreased each day giving further indication of progressive lysis (Figure 2). As can see be seen from the plot of Figure 2 for dog Elvis, the measured Ht value decreased each day at about 1.0-1.5 % due to progressive cell hemolysis.

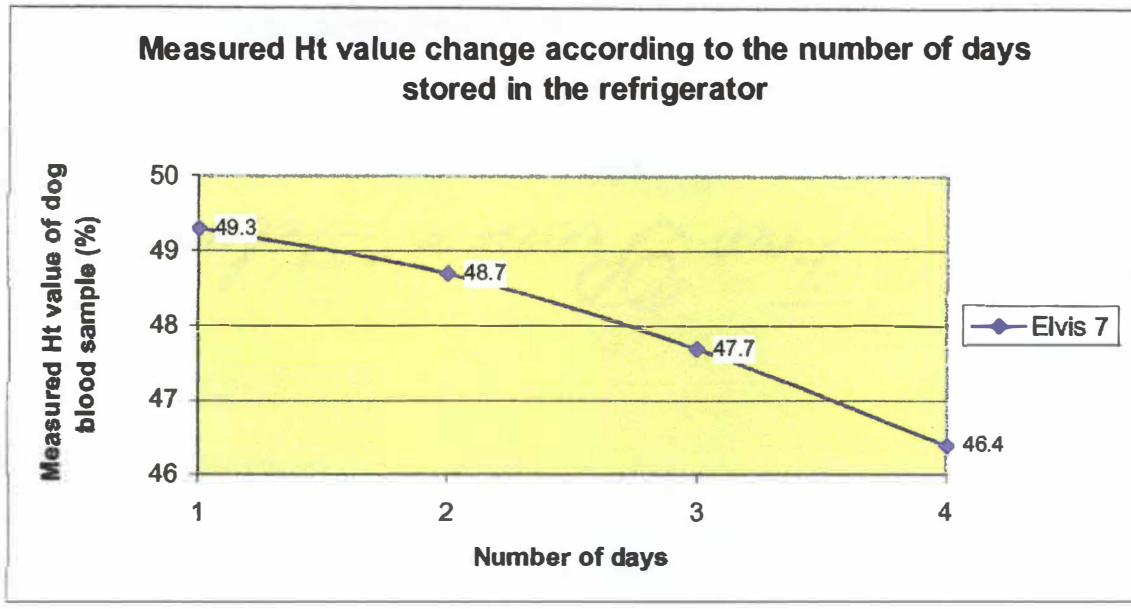


Figure 2. Plot of measured Ht value as a function of refrigerated storage time

Blood Handling

It is well known (18) that erythrocyte form and properties can be altered by laboratory manipulation including:

- Surface tension effects distorting RBC membrane at air interfaces
- Lysis due to excessive shear rates during centrifugation, syringe transfer and viscosity measurement especially for older, more fragile cells
- Contact of RBC with glass surface distorting cell shape and possibly contributing to lysis

The results of microscopic examination of RBC appearance during the experimentation conducted are listed in Table 2 on the next page.

Table 2 Observed conditions of cells for all experimental runs

Period	Appearance of RBC (1500x)
The first day when the dog blood sample was acquired	100 % normal with biconcave or donut shape
After centrifuging at 570 g for 15 min and removal of WBC layer	About >98 % normal with biconcave or donut shape
After adding saline solution for 1st washing and separating of 1.5-1.8 ml of young and old RBC portion into two test tubes	About 95% normal with biconcave or donut shape for both young and old erythrocyte suspensions
After adding saline solution for 2nd washing and centrifuging at 3563 g for 60 min	About 80-85% normal for both young and old erythrocyte suspensions; cell lysis begins
After adding saline solution for 3rd washing and centrifuging at 2280 g for 20 min	About 75-80% normal; significant cell lysis had occurred
After measuring density and viscosity values	About 70-75% normal; significant cell lysis had occurred

Selection of Centrifugation Speeds

A set of preliminary studies was conducted to ascertain how the apparent hematocrit and RBC integrity varied with centrifuge speed and time. The results are presented in Table 3 and the data of Table 3 are plotted in Figure 3 respectively.

As can be seen in the data of Figure 3, initially, when the Ht_r value was measured at 1283 g, 2280 g and 3563 g respectively in 20 min, Ht_r value at 1283 g was relatively higher than the values obtained at 2280 g and 3563 g. Meanwhile, as centrifugation time passed, the Ht_r value was remarkably decreased due to RBC hemolysis due to the heat and mechanical vibration produced during use of the IEC HN-SII centrifuge. Here, Ht_r values represent the apparent saline trapping factor, and the Ht_r value has specific meaning. After the cells were centrifuged for 90 min at 3563 g, the test tube itself was warm due to the heat emitted from the centrifuge, and relatively severe (>30%) RBC hemolysis was indicated by the "redness" of the saline layer. Microscopic examination revealed less than 60% normal cells as determined by performing a Ht measurement using the micro-centrifuge operating at 11,700 RPM (13,000 g).

Accuracy and Precision in the Measurement of RBC and Saline Volumes; Source of Error

i) Accuracy: Accuracy is defined as the extent that a measured values reflects the "true" value. There exist two different error sources; one is a random error and a systemic error. For example, the random error can be occurred in scale reading errors, and

Table 3 The relationship between Ht_r (measured Ht/apparent Ht) and RBC appearance to centrifuge speed used

RPM (g value)	Time (min)	Ht_r = Measured Ht/ Apparent Ht	Cell Appearance
3000 (1283 g)	20	0.914	> 90% normal
3000 (1283 g)	90	0.808	About 80% normal
4000 (2280 g)	20	0.840	About 85% normal
4000 (2280 g)	90	0.708	About 70% normal, moderate cell lysis
5000 (3563 g)	20	0.828	About 70-75% normal, slight cell lysis
5000 (3563 g)	90	0.656	< 60% normal, severe cell lysis

Room Temperature: 25-27 °C

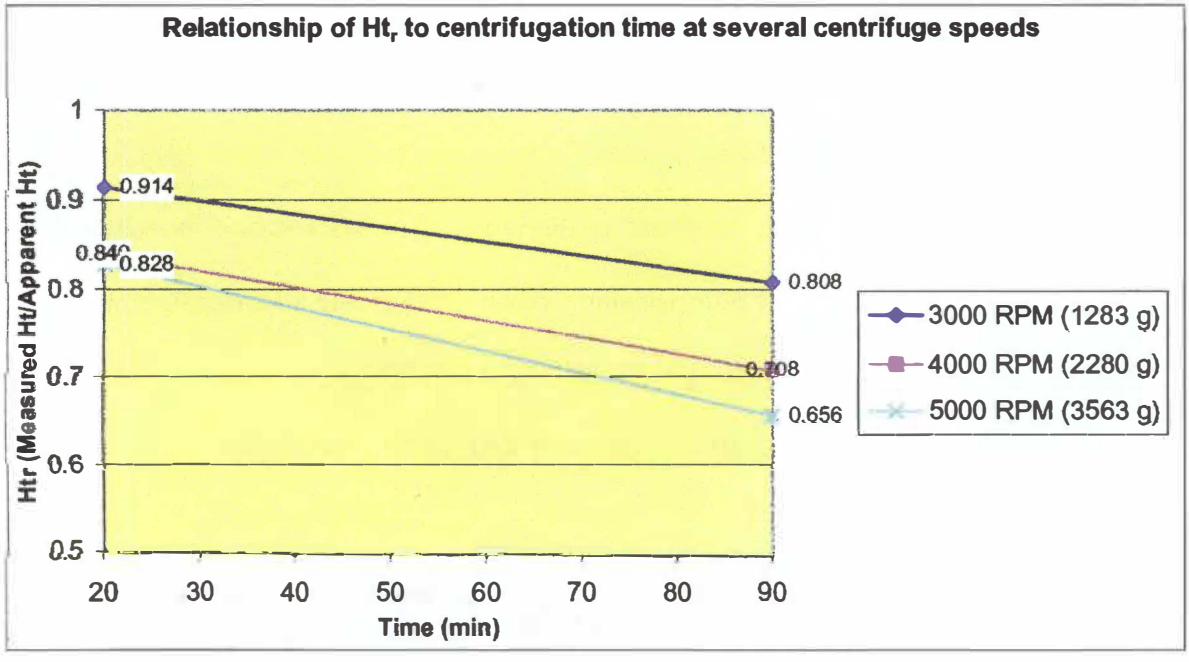


Figure 3. Relationship between Ht_r (measured Ht/apparent Ht) and centrifugation time

systematic errors can account for net + or – quantities due to presence of bubbles in syringe. In my experiment, in order to prevent the occurrence of an air bubble, a 1 ml gas-tight syringe was used for the transfer of young or old RBC into test tubes. However, as compared with Parker's dog density data (35), the present study yielded density values about 3% lower than those previously reported. This proves that there was still residual plasma/saline remaining in the syringe when the young or old RBC were aspirated. Also, some cells still remained in the syringe barrel attached to the syringe needle. Therefore, it appears likely that a systemic error in the procedure used in this research resulted in somewhat lower than actual density values.

ii) Precision: Precision is defined as the extent to which a measured value is specified in terms of a greater or lesser number of significant figures. In this research, the gas-tight syringe can measure from 0.00 ml to 1.00 ml, and the top-loading balance can be used from 0.000 g to 100.000 g. The scale markings in gas-tight syringe consist of the intervals of 0.01 ml. The appliances (both syringe and scale) had good capability of fine measurement. Thus, the author did not have to interpolate between marks. As discussed later, the average density variance of young and old erythrocytes in the experimental dogs (Madge, Elvis and Jackson) was 1.059-1.086 g/ml, 1.058-1.063 g/ml and 1.092-1.096 g/ml respectively. This proves that a high degree of precision was shown in this study.

iii) Reproducibility: Reproducibility reflects the extent to which when a procedure is repeated more than once the same numerical result is obtained. Lack of reproducibility may be due to some uncontrolled conditions during experimentation. In this study, meeting these conditions (e.g. individual measurements of the suspension volumes, weight and viscosity values) is based on the author's ability to read accurately

such things as the markings on the syringe barrel, or the mass of suspension tube using a balance under the same condition. For example, the measurement of the blood volume in the syringe barrel depends on the temperature and the observation angle of the scale barrel. Based on the procedures developed for use in this study, estimated error in reproducibility was1.0% or less.

Relative Difficulty of Separating "Old" from "Young" RBC Fractions

In the process of separating erythrocyte density fractions after centrifugation, the separation of old RBC at the tube bottom was relatively straightforward since the syringe needle could be accurately positioned near the bottom of the RBC suspension-filled tubes, where aspiration-needle motion did not overly disturb the packed cell layer. However, the collection of the top fraction of young RBC was performed with more difficulty. The young cell fractions could be aspirated, with a 1 ml syringe, from various portions, since a volume of about 0.6 ml was aspirated from the upper 1/3 portion of the cell layer. The author used manual positioning with no mechanical guide to position the needle tip. Thus, the measured values of density and viscosity could be slightly different for each experimental run in accordance with which part of the top fraction of packed cells was aspirated.

Since the WBC layer at the top of the "erythrocyte pack" was of a jelly-like consistency, it was difficult to remove only the WBC layer before RBC washing. As the WBC were aspirated from the top fraction of the packed cells, they could have been mixed with some young RBC from the upper portion of the RBC layer due to the lack of precise positioning of the syringe needle. Due to the difficulties in the aspiration of the

WBC layer only, substantial numbers of young RBC were discarded with the jelly-like WBC in most cases.

Besides, owing to the difficulty of complete removal of the leukocyte "buffy coat," when the young erythrocytes were aspirated with a 1 ml syringe, the RBC removed contained a small number of leukocytes. However, it was found that upon microscopic examination less than 0.1% of WBC were present, and it was assumed that these residual WBC had negligible influence on subsequent measurements of RBC density and suspension viscosity.

Measurement of Dog Blood RBC Density

Raw Data Table

As specified earlier, whole blood was obtained from three different dogs (Canis Familiaris breed dog identification names: Elvis/male, Madge/female, and Jackson/male). Following preliminary experimentation fifteen successive experimental runs were performed over a period of several weeks to collect data for calculation of RBC density and suspension viscosity at several Ht levels. The raw data table of measurement of dog blood RBC density is presented in Appendix Table 4 (page 59).

Distribution of Calculated Density Values

The density values of young and old erythrocytes of the three different dog blood donors were calculated from measurements of packed cell mass and volume. The calculated density values are listed in Appendix Table 4 (page 59). The distribution of these values is represented in the data plots of Figures 4-8 on the next pages.

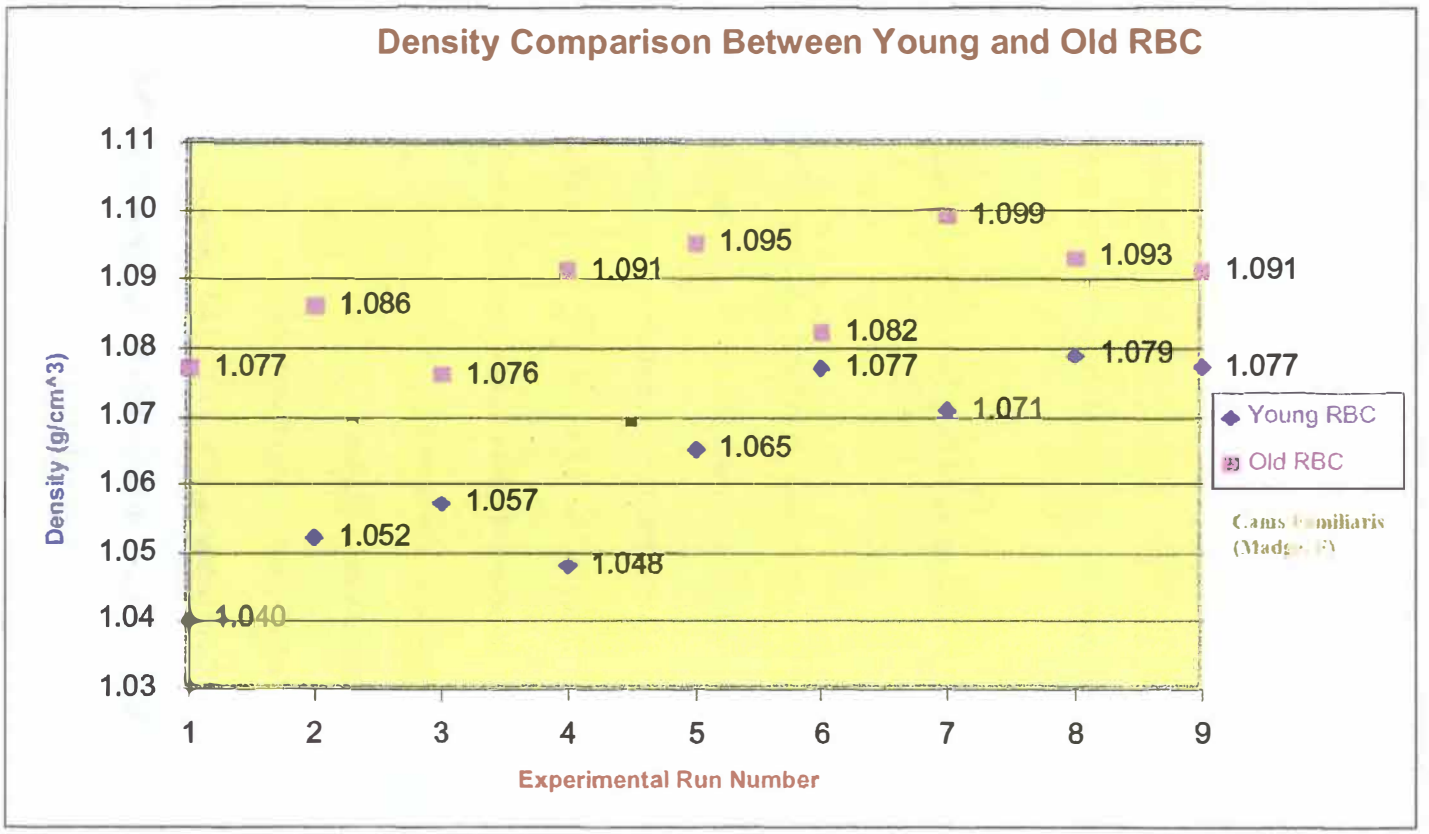


Figure 4. Distribution of density values between young and old RBC in dog named Madge

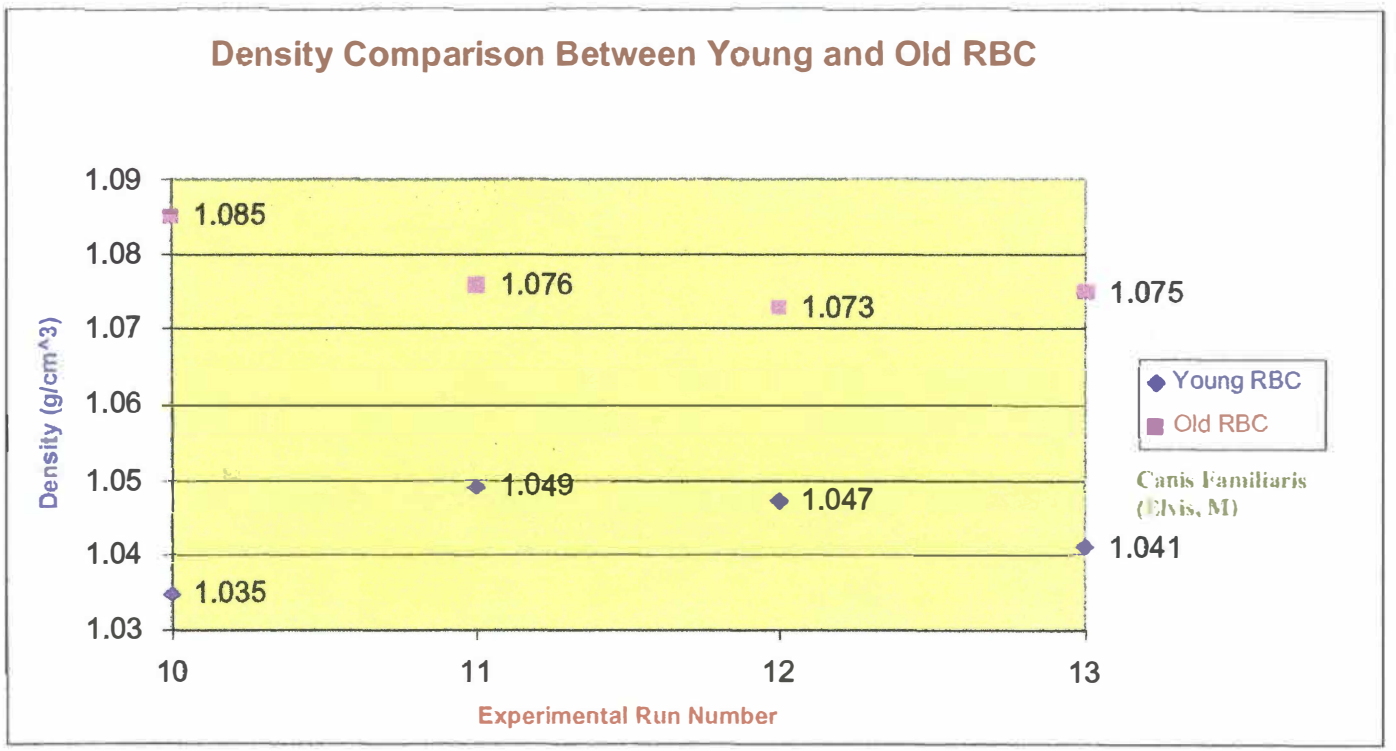


Figure 5. Distribution of density values between young and old RBC in dog named Elvis

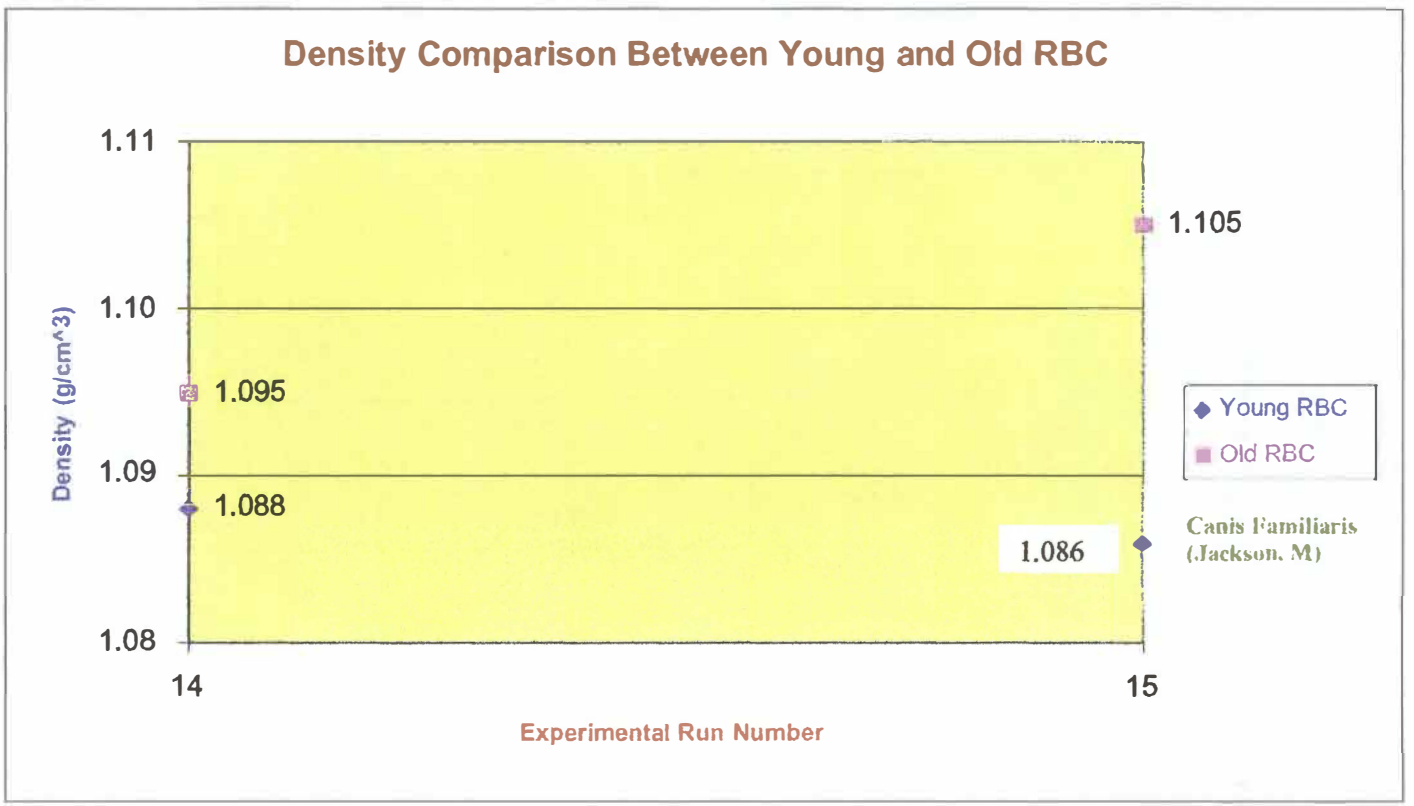


Figure 6. Distribution of density values between young and old RBC in dog named Jackson

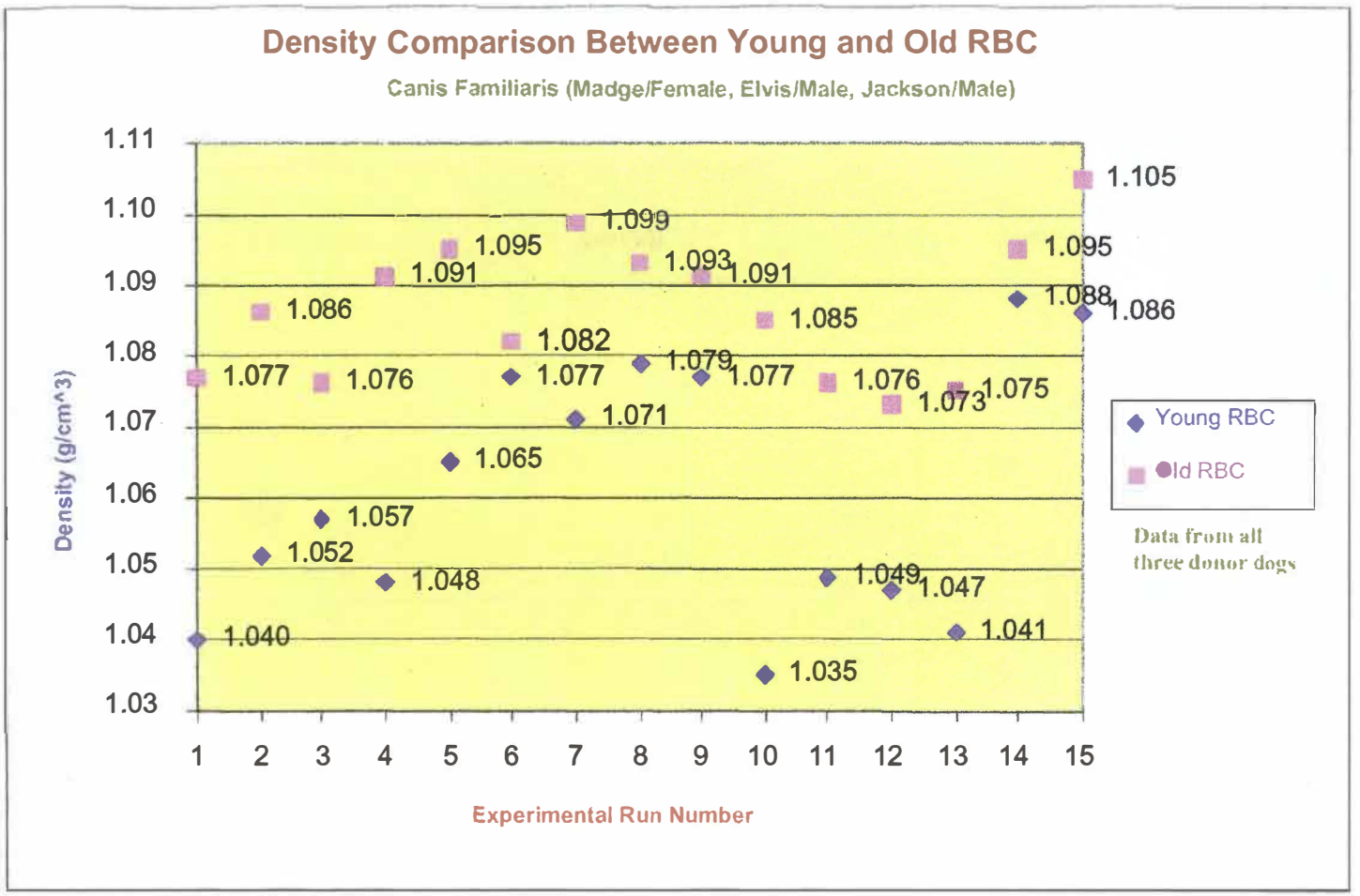


Figure 7. Distribution of density values between young and old RBC in all three donor dogs

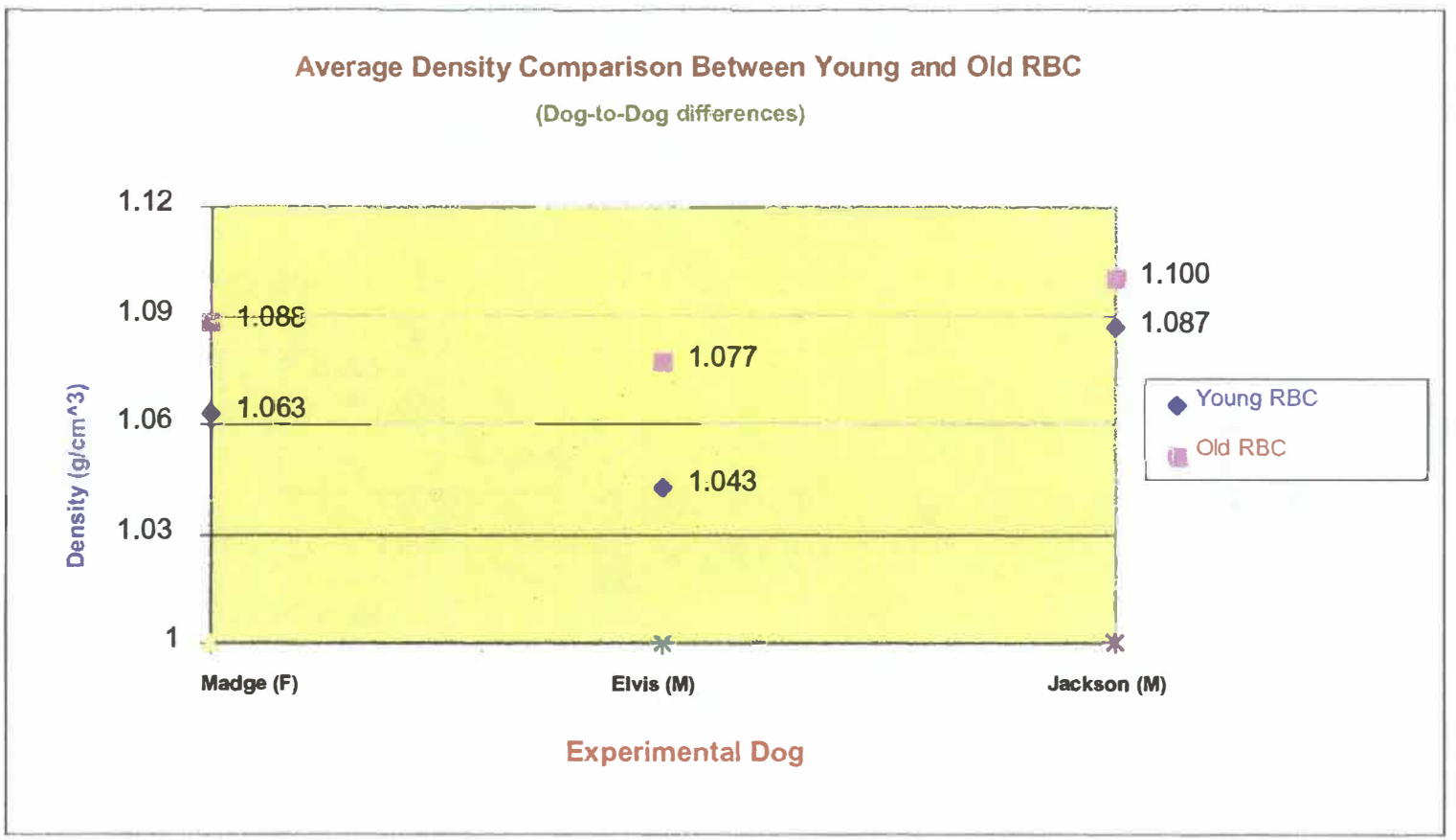


Figure 8. Average density for young and old RBC suspensions for the three donor dogs

In this research, the experimental dog Jackson yielded slightly higher RBC density values than the other two donor dogs (Madge and Elvis) for both young and old RBC. The average density variation of young and old erythrocytes in each experimental dog (Madge, Elvis, Jackson) was 1.075±0.017 g/ml, 1.060±0.002 g/ml and 1.094±0.002 g/ml respectively, where the +/– quantities represent the maximum (not standard) deviations from the mean value.

As the data of Figures 4-8 show, there was relatively few data for Elvis and Jackson since the Veterinary clinic selected the donor dogs based on their availability. The densities appear to be increased possible explanations as following reasons. One is an actual change in animal RBC density over time for unknown reasons. The other is some gradual change in the author's procedures, as the author became more proficient, and female dog menstrual cycle effects. Generally, the density of old erythrocyte fractions had a substantially higher value than the density of young erythrocyte fractions in all cases. This is in agreement with prior studies (23, 24, 25) as discussed further below. Generally, the average density of old RBC fractions was found to be 1.0-1.5% higher than that of young RBC.

As the data of Figure 6 (page 30) shows, the density value of young RBC was nearly same in both experimental run number 14 and experimental run number 15. However, the density value of old RBC of experimental run number 15 was relatively higher than the density of old RBC of experimental run number 14. The main reasons for the differences may be either difference in donor dog blood properties or experimental error in measuring old RBC density, or both.

In this research, for the pooled data from all 15 experimental runs, the average density value of young and old erythrocytes for RBC obtained from three different dogs was 1.074 g/ml. The average density value of only young erythrocytes of the same dogs was 1.061 g/ml. Meanwhile, the average density of the more dense old erythrocytes of the same dogs was found to be 1.087 g/ml. From these data, it is clear that there was a significant difference in the measured average density value in young and old erythrocytes.

RBC Density Data Comparison with Literature Values for Humans and Animals

Danon et al. (32) determined normal human RBC density distributions based on the measurement of neutral density for cell suspension in mixtures of methyl phthalate (SG (specific gravity) 1.189) and di-n-butyl phthalate (SG (specific gravity) 1.042). Different proportions of these agents were mixed to yield a battery of fluids with increments of specific gravity of 0.004. Here, specific gravity (SG) is defined as the density of a substance divided by the density of water at 1 atm pressure and 25°C. Danon et al. did not separate old from young cells but utilized all RBC from whole blood for the measurement of SG. The average specific gravity value they obtained in their studies was SG 1.104. The SG of water at 25 °C is 0.997. The RBC density data obtained in this research were of somewhat lower magnitude than the data of Danon et al (32). This difference is discussed further below.

Parker (35) used twelve different proportions of mixtures of dimethyl and dibutyl phthalate for density distribution determination of the distribution of dog RBC. The result of this experiment was an average SG of 1.104. This technique of separating cells by density fraction using mixtures of dimethyl and dibutyl phthalate with increments of

specific gravity of 0.002 was a modification of the method described earlier by Danon et al. (32). A pycnometer was used to measure the SG of the erythrocytes at 25 °C. When these two different average density values from the same dog species were compared, there was an approximately 3% difference with the average value obtained in this research, 1.074 g/ml (SG 1.077), as compared to Parker's average density value of 1.101 g/ml (SG 1.104).

In another survey dealing with density measurement, Shinozuka et al. (23) used centrifugation as a basis for separating old from young cells. Separated washed leukocyte-depleted human erythrocytes into three fractions by volume, top (18%), middle (65%), and bottom (17%) layers, by the density gradient centrifugation method using phthalate diesters as the separating fluid. The distribution of the density of the young and old erythrocyte fraction suspensions they obtained is given in Table 5 below. The average density of erythrocytes has been determined for several mammalian species in addition to humans and dogs, including rabbits, oxen, and sheep (35, 37, 38, 39), using a slightly modified version of the method used by Danon et al. (32). The calculated average erythrocyte densities for several studies on a variety of species are listed in Table 6 on the following page.

Table 5. The density comparison between young and old RBC in humans

Population fraction	Density (g/ml)
Top layer ("young" erythrocytes)	< 1.092 g/ml
Bottom layer ("old" erythrocytes)	> 1.100 g/ml

Table 6 Average RBC density values in different animal species

Species	Study	Average Density (g/ml)
Dog	Present[1]	1.074
Dog	Parker[2]	1.101
Human	Ponder[3]	1.096
Human	MacLeod[4]	1.099
Human	Lindeboom[5]	1.103
Human	Danon et al.[6]	1.101
Rabbit	MacLeod	1.098
Ox	MacLeod	1.084
Sheep	MacLeod	1.084

[1] Present study

[2] Flotation method: Dimethyl and dibutyl phthalate were mixed in different proportions.

[3] The weight of the sample of packed cells being known, and the weight of the same volume of water having been found by a calibration of the pipet the density of the cells can be calculated.

[4] Density of cells=[100*(density-packed cells)-S*(density plasma)]/C, where C is the percentage of cells, S is the percentage of plasma present in the thick suspension of packed cells.

[5] Se=[100*Sb-(100-V)*Sp]/V, where V is the percentage of cells, Sb is density-packed cells, Sp is density plasma, Se is density of erythrocytes.

[6] Methyl phthalate and di-n-butyl phthalate are mixed in different proportions to yield a battery of fluids with increments of specific gravity of 0.004.

Although most authors agree that erythrocyte size decreases somewhat during aging, except for the unknown behavior of the oldest cells (just prior to their removal from the circulation), the changes in red cell shape are a matter of controversy. Based on observations by light microscopy, some authors (40, 41) report that erythrocytes become more spherical as they age. However, in the present study, it could not be determined whether there was shape difference and thickness difference between young and old erythrocytes even under a light microscope at 1500x magnification.

As reflected in Table 6 data, generally, the present study yielded about 2-3% lower average density values than the data of other studies for five different species. This result could have two possible explanations: one is an actual density difference for unknown reasons, the other is a systematic error in the experimental procedure used.

For this study, there is no known reason why the dog RBC used would be of lower density than reported previously. This difference in density value could reflect experimental errors in measuring the average density value. In particular, as one of the main factors possibly leading to measurement errors, after centrifugation, more saline was trapped than the author expected when the young or old RBC were transferred with a 1 ml syringe between test tubes. This could have happened due to the small-bore size of the needle attached to the syringe barrel used to aspirate the RBC or due to the insufficient removal of WBC. Other factors causing this result include a slight dead volume of RBC that still cling at the end of the syringe barrel, slight RBC hemolysis, or the researcher's technical error in the process of measuring the density. Of course, it is possible that both actual density differences and procedural errors were involved.

Measurement of the Viscosity of RBC Suspensions Prepared from "Old" and "Young" Cell Fractions

Findings

In this phase of the thesis work, whole blood obtained from two dogs (Madge/F and Jackson/M) was used for the measurement of the viscosity of suspensions of young and old erythrocytes.

The shear rate is calculated from the equation:

$$\textbf{Shear rate (sec}^{-1}\textbf{) = SRC x RPM} \qquad (4)$$

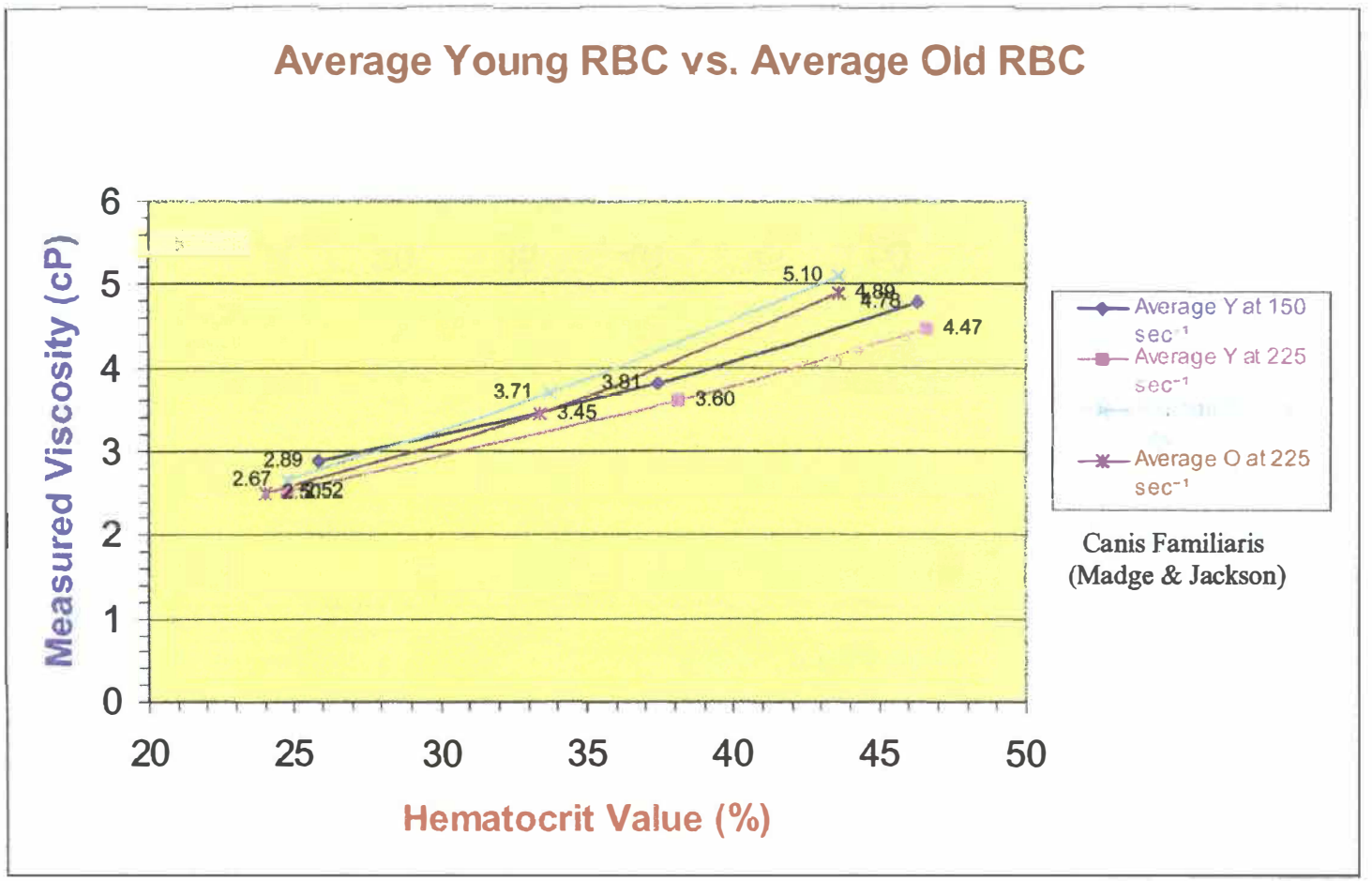
where SRC = viscometer shear rate constant

(when using spindle type CP 40, SRC = 7.5)

The raw data of measurement of dog blood RBC suspension viscosity at 150 sec^{-1} and 225 sec^{-1} is given in Appendix Table 7 and Appendix Table 8. As expected based on prior studies, blood viscosity was found to increase in direct proportion to the Ht values of both young and old erythrocyte fractions.

For blood from the two donor dogs (Madge/F and Jackson/M), the viscosity of young and old erythrocyte suspensions was measured at shear rates of 150 sec^{-1} and of 225 sec^{-1} for measured Ht values ranging from 20% to 50%. The distribution of measured viscosity based on Appendix Table 7 and Appendix Table 8 data is graphed in Figure 10 and Figure 11 on the following pages. These graphs represent the relationship of measured viscosity to hematocrit with the shear rates as the parameter.

As the plots of Figures 9-11 show, the viscosity of old erythrocyte suspensions are, in general, slightly higher than that of young erythrocyte suspensions for both values of shear rate used (150 sec^{-1} and of 225 sec^{-1}).



Canis Familiaris
(Madge & Jackson)

Figure 9. Viscosity versus measured Ht values for studied young and old RBC suspensions to show shear rate effect

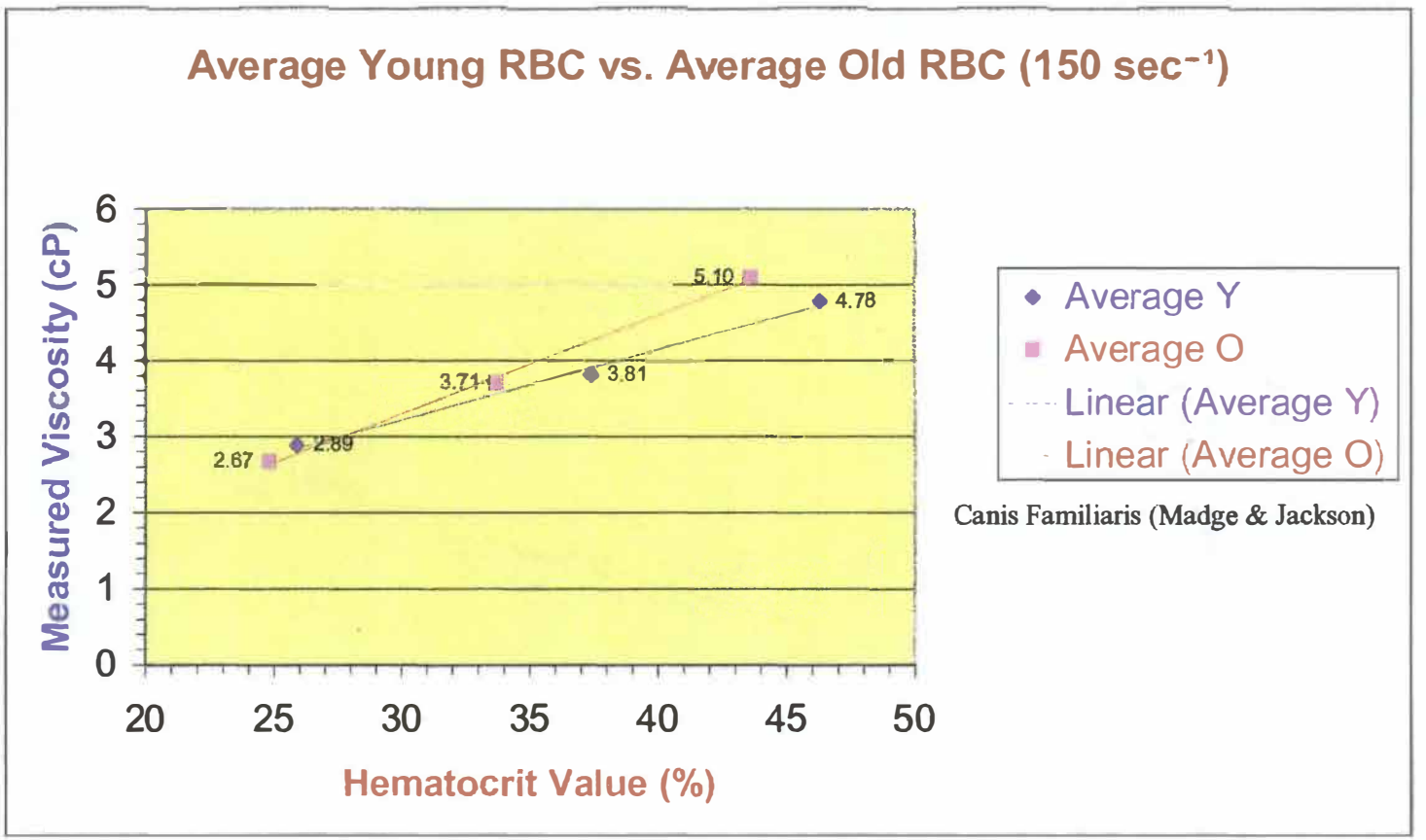


Figure 10. Viscosity versus measured Ht values for studied young and old RBC suspensions at shear rate 150 sec^{-1}

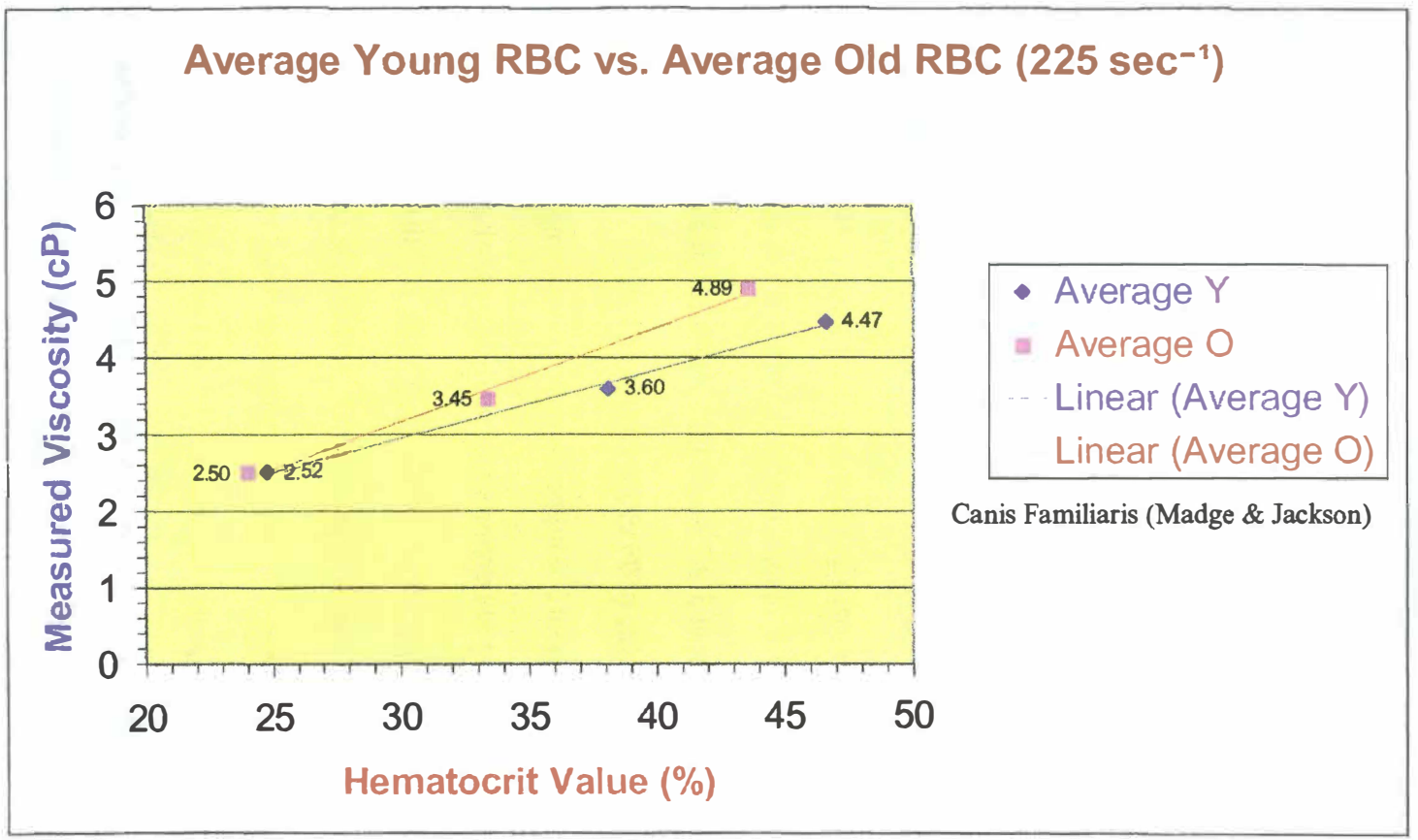


Figure 11. Viscosity versus measured Ht values for studied young and old RBC suspensions at shear rate 225 sec^{-1}

The average blood viscosity value for a shear rate of 225 sec^{-1} was about 5-9% lower than that measured at a shear rate of 150 sec^{-1}.

Comparison of Results Obtained to Literature Values for Human and Animal Bloods

In this study of the RBC suspensions of two healthy dogs, the average viscosity values of young and old erythrocyte suspensions obtained for the measured Ht value and at both shear rates (150 sec^{-1} and 225 sec^{-1}) used were as follows:

As figure 9 shows, in all the other cases, the average viscosity of young and old erythrocyte suspensions increased notably with decreasing shear rate. Also, figure 9 plots demonstrate that average young and old RBC suspensions viscosity values for shear rates of 150 sec^{-1} and 225 sec^{-1} were not significantly different in the Ht value range studied (21.5-29.0%), but in the Ht value range (35.1%-47.9%), the average viscosity values at a shear rate of 225 sec^{-1} were about 5-9% lower than that measured at a shear rate of 150 sec^{-1}. The average viscosity value (2.89 cP) for a Ht range of 22.5-29.0% of young erythrocyte suspensions was about 3% higher than that the average viscosity value (2.67 cP) of Ht value (21.5-27.1%) of old erythrocyte suspensions at shear rate 150 sec^{-1} (see figure 10, page 40). The viscosity values of young erythrocyte suspensions were found to be somewhat higher than the viscosity values for old erythrocyte suspensions. This could have been caused by several factors. One factor could have been cell properties other than age affecting mechanical properties. Another could have been an error in the measurement of the test suspension hematocrit used as a comparative parameter. The summary of viscosity measurement results is shown in Table 9 and Table 10 on the following page.

Table 9. The viscosity range for measured Ht values range at a shear rate of 150 sec^{-1}

Sample	Erythrocyte suspensions	Shear Rate (sec^{-1})	Measured Ht value range (%)	Viscosity range (cP) at 25-27 °C
Canis Familiaris (Madge&Jackson)	Young	150	22.5%-29.0%	2.76cP-3.63cP Ave: 2.89cP
Canis Familiaris (Madge&Jackson)	Old	150	21.5%-27.1%	2.71cP-2.94cP Ave: 2.67cP
Canis Familiaris (Madge&Jackson)	Young	150	35.1%-38.5%	3.61cP-4.00cP Ave: 3.81cP
Canis Familiaris (Madge&Jackson)	Old	150	32.6%-34.5%	3.42cP-3.98cP Ave: 3.71cP
Canis Familiaris (Madge&Jackson)	Young	150	45.3%-47.9%	4.47cP-5.12cP Ave: 4.78cP
Canis Familiaris (Madge&Jackson)	Old	150	42.0%-46.4%	4.75cP-5.53cP Ave: 5.10cP

Table 10. The viscosity range for measured Ht values range at a shear rate of 225 sec^{-1}

Sample	Erythrocyte suspensions	Shear Rate (sec^{-1})	Measured Ht value range (%)	Viscosity range (cP) at 25-27 °C
Canis Familiaris (Madge&Jackson)	Young	225	22.5%-28.0%	2.46cP-2.63cP Ave: 2.52cP
Canis Familiaris (Madge&Jackson)	Old	225	21.5%-27.1%	2.32cP-2.61cP Ave: 2.50cP
Canis Familiaris (Madge&Jackson)	Young	225	37.9%-38.5%	3.56cP-3.66cP Ave: 3.60cP
Canis Familiaris (Madge&Jackson)	Old	225	32.6%-34.2%	3.24cP-3.65cP Ave: 3.45cP
Canis Familiaris (Madge&Jackson)	Young	225	45.5%-47.9%	4.16cP-4.63cP Ave: 4.47cP
Canis Familiaris (Madge&Jackson)	Old	225	42.0%-46.4%	4.62cP-5.15cP Ave: 4.89cP

As cited above it is known that blood handling can affect RBC status. In particular, surface tension effects distorting RBC membranes at the air interface and the contact of RBC with the glass surface, distorting cell shape may affect measured properties. Factors related to age include the fact that excessive shear rates during centrifugation, syringe transfer, and viscosity measurement especially for older cells may promote cell damage. Another reason may be that blood proteins coating the spindle decreased the drag, and led to machine error. Also, technical errors must be considered: rounding of quantity values for the determination of accurate measured Ht values. On the other hand, it is possible that no error occurred but that the viscosity values simply reflected the actual conditions of the RBC for the suspensions studied.

However, as Figure 10 also shows, the average viscosity values of Ht value range (32.6-46.4%) of old erythrocyte suspensions increased dramatically compared to the average viscosity values of the similar Ht value range (35.1-47.9%) of young erythrocyte suspensions at shear rate 150 sec^{-1}. It is likely that this difference reflects a difference in the mechanical properties of the RBC since earlier data demonstrated that more rigid RBC yield more viscous suspensions.

Meanwhile, in the study of average viscosity value measurement of young and old erythrocyte suspensions at shear rate 225 sec^{-1}, the average viscosity value (2.52 cP) for Ht value (22.5-28.0%) of young erythrocyte suspensions was quite similar to the average viscosity value (2.50 cP) for Ht value in the range of 21.5-27.1% of old erythrocyte suspensions (see figure 11). However, the average viscosity values for Ht value in the range of 32.6-46.4% of old erythrocyte suspensions was found to increase rapidly

compared to the average viscosity values of the similar Ht value (37.9-47.9%) of young erythrocyte suspensions at the same shear rate, 225 sec^{-1}.

Overall, the viscosity data obtained in this study were comparable to data reported previously in the literature. Andrew et al. (30) determined viscosity of blood from seven horses using a digital cone-and-plate micro-viscometer at 37 °C. Blood samples were either diluted or concentrated by adding or removing plasma so that the final Ht was 20, 40 or 60%. Blood viscosity was measured three times each at these Ht levels, and at six spindle shear rates (230, 115, 46, 23, 11.5, and 5.75 sec^{-1}). Using this method, the viscosity data that they obtained is listed in Appendix Table 11. As the data of this table relate, when compared with this research, Andrew et al. obtained different results. When comparing the three different tables (Table 9-10, Appendix Table 11 and Appendix Figure 12), dog blood appears more viscous than horse blood at higher Ht value range (35-45%). However, from only this information, it is difficult to draw a conclusion, since the Ht value, shear rate and temperature were not the same for all runs. If the viscosity were to be measured at the same range of Ht value as in this author's study, horse blood might appear more viscous than dog blood at higher Ht values.

Rand et al. (26) used a micro cone-plate viscometer to measure the viscosity of venous blood from 60 healthy humans (aged 18-40) for which the hematocrits were adjusted to 20% and 40%, and the viscosity-shear rate relationships measured at 27.0 °C. Using this method, the viscosity result that they obtained is listed in Appendix Table 12.

For the composite data, the trend was very similar to data reported by Andrew et al. (30), demonstrating that with increasing shear rate, the RBC suspension viscosity decreases whereas viscosity increases with increasing Ht. According to work reported by

Andrew et al. (30) the order of blood viscosity by species for a given Ht value, from higher to lower viscosity, is goats, camels, sheep, horses, dogs, and humans.

Usami, et al. (28) determined canine jugular vein blood viscosity at shear rates varying from 0.0104 to 52 sec^{-1} using a modified G.D.M. [P. J. Gilinson, C. R. Dauwalter, and E. W. Merrill] coaxial cylinder viscometer at 37 °C. These investigators used post-centrifugation packed cells in volumes of 10% of the top, 10% of the bottom, and three equal portions of the center section. By the addition of autologous plasma, the hematocrit of each fraction was adjusted to 90%. The reason that they adjusted the Ht to this abnormal high value (higher than physiological level [37%-54%]) was to enhance any differences detectable between young and old erythrocyte suspension. Using these methods, Usami et al. showed that suspensions of erythrocytes of top and bottom fractions exhibit a shear-thinning behavior, i.e., the viscosity increases with a reduction in shear rate, as was found in this work. The viscosity of suspensions prepared with old RBC from the bottom fraction, however, was approximately 10% higher than that of the top fraction at every shear rate used in the work of Usami et al. The viscosity data that these authors reported is shown in Table 13 on the next page.

As in the case of previously referenced research results (26, 30), this data (28) also shows that the bottom fraction (old erythrocytes) has a higher viscosity than the top fraction (young erythrocytes) for all shear rates studied. In large vessels, viscosity value has approximately 3-5 cP at a normal Ht value of 40%. However, in the studies of Usami et al. (28), the Ht value is considerably greater than the physiologically normal Ht value (37%-54%).

Table 13 Comparison of measured viscosity for suspensions of the top and bottom fractions of centrifuged erythrocytes from the work of Usami et al. (28)

		Viscosity (cP) at 37 °C		
	H, %	Shear rate (sec^{-1})		
		50	0.5	0.01
Young Erythrocytes (n=6)	88.0±0.7	41±9 cP	240±59 cP	2,999±577 cP
Old Erythrocytes (n=6)	87.9±1.1	48±9 cP	264±65 cP	3,280±929 cP
Difference 100*(B-T)/T	0. 1%	17.1%	10.1%	9.4%
	Not significant	P< 0.05	P< 0.01	P< 0.025

The reason for the high viscosity values is that more the hematocrit increases, the higher the viscosity. Also, at very low shear rate blood has a measurable yield stress (45). Usami et al.(28) concluded that one of the factors contributing to the higher viscosity of the bottom fraction is a decrease of membrane flexibility caused by cell aging.

Tillman et al. (29) measured viscosity of different age fraction human blood samples obtained from 30 healthy adults. The methods that these researchers used compared to those of present study for the determination of young and old erythrocyte suspensions' viscosity. The crucial differences are briefly identified as follows: In Tillman et al.'s study, young erythrocytes were separated from old cells by re-centrifugation for 30 minutes at 30,000 g. In this research, centrifuge speed and time were only 3,563 g (maximum speed of IEC HN-SII centrifuge) and 60 minutes respectively. Therefore, there was a considerable difference between two values. In the research of Tillman et al (29), a volume of 10% of the top and bottom fractions of the

packed cells was carefully aspirated. In this present research, a volume of 12% of the young and old RBC fraction was used to obtain a great enough amount of the sample in order to measure density and viscosity. In the research of Tillman et al, erythrocytes were washed three times for 10 minutes at 2,000 g in 310 imOsm phosphate buffer (pH=7.4). In this study, 0.9% isotonic saline solution was used for washing the young and old RBC. In Tillman et al.'s research, for viscosity measurements suspensions of washed erythrocytes in autologous plasma were adjusted to contain $8.0 \pm 0.2 \times 10^9$ red cells/ml (Ht value: about 72%). In the current study, the measured Ht value for prepared RBC suspensions (see page 16), instead of an adjusted Ht value obtained by utilizing autologous plasma, was used.

Using a cone-plate viscometer methods, Tillman et al. found that the viscosity of the young cells was lower at all shear rates (0.39 to 78.65 sec^{-1}) used than for suspension of old cells. These researchers concluded the flexibility of young erythrocytes was markedly increased over that of old cells at the lowest shear rate ($P < 0.001$). However, the difference was less pronounced at the highest shear rate ($P < 0.05$). In their research, they confirmed by viscosity measurements of packed erythrocyte suspensions that old erythrocytes are less flexible than young cells, as was the main finding of the present research.

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS FOR FURTHER STUDY

Based on the research results reported above, the following conclusions were made.

- The measured Ht value of normal dog blood samples was found to decrease at a rate of 1.0-1.5% per day, indicating the progressive hemolysis of blood during refrigerated storage.
- The calculated Ht value of test samples was 3-25% higher than the measured Ht value due mainly to saline trapping associated with the modest centrifugation speed used.
- The density of the RBC from three donor dogs was calculated from direct measurements of cell fraction mass and volume. The overall average cell density was found to range between 1.058-1.096 g/ml with an average value of 1.074 g/ml.
- The average density value of young erythrocytes of the same three dogs was 1.061 g/ml. Meanwhile, the average density of the more dense old erythrocytes of the dogs was 1.087 g/ml. This result showed that the density of the old RBC was 1.0-1.5% higher than that of the young RBC, a finding which is in agreement with prior studies of cell density related to cell age.
- The average RBC density in the dog samples studied was about 3% lower than the values reported in the literature. After centrifugation, more trapped saline than the

author expected was found when the young and old RBC were transferred between test tubes with a precision syringe. Dog RBC suspension viscosity was found to vary non-linearly with measured Ht value (20%-50%) at shear rates of 150 sec^{-1} and 225 sec^{-1}. In this research, the viscosity values of both young and old erythrocyte suspensions for Ht value in the range of 20%- 50% varied from 2.30 cP to 5.53 cP at 25-27 °C.

- The viscosity of old erythrocyte suspensions was slightly higher than that of young erythrocyte suspensions at shear rates of both 150 sec^{-1} and 225 sec^{-1}. This finding suggests that old erythrocytes are less flexible than young erythrocytes.
- The average blood viscosity value for a shear rate of 225 sec^{-1} was about 5-9% lower than that measured at a shear rate of 150 sec^{-1}.

Based on the results obtained in this investigation, recommendations for future research include the following:

- Utilize fresh blood to minimize hemolysis effects.
- Measure RBC density by both the flotation as well as direct mass and volume measurements to improve the assessment of accuracy and precision in the latter technique.
- Use a "depth gauge" (attachment) to assure repeatable positioning of the syringe needle for aspiration of the top young RBC layer following suspension centrifugation, to prevent it from going further into the tube.

- More comprehensively quantify RBC age effects on blood flow properties and establish the possible relevance of these effects on conditions of health and disease in humans and animals.
- Study RBC density and viscosity measurement in mammalian species other than humans and dogs to clarify the RBC effects of geometric parameters on the results obtained.
- Measure density and viscosity from young and old donor dogs to ascertain animal age effects on results, if any.
- Measure density and viscosity from hemolytic, microangiopathic, and sickle-cell disease patients (including mammalian animals) and compare with data for normal human blood.

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APPENDIX

Table 4. Density comparisons between young RBC and old RBC for the set of experimental runs conducted

Exp.	(Elvis, M)	Unit: (g/ml)		(Madge, F)	Unit: (g/ml)
Run #	Young RBC	Old RBC		Young RBC	Old RBC
1	1.035	1.085	7	1.040	1.077
2	1.049	1.076	8	1.052	1.086
3	1.047	1.073	9	1.057	1.076
4	1.041	1.075	10	1.048	1.091
			11	1.065	1.095
	(Jackson, M)	Unit: (g/ml)	12	1.077	1.082
	Young RBC	Old RBC	13	1.071	1.099
5	1.088	1.095	14	1.079	1.093
6	1.086	1.105	15	1.077	1.091

Table 7. Raw data of measured dog blood RBC fraction suspension density and viscosity for a viscometer shear rate of 150 sec^{-1} (present study)

Date	Sample	Experimental run	Density	Viscosity at 25-27 °C (Hematocrit level)
Sep. 7, 2001	Canis Familiaris (Elvis, M)	1	Y: 1.035 O: 1.085	N/A
Sep. 7, 2001	Canis Familiaris (Elvis, M)	2	Y: 1.049 O: 1.076	N/A
Sep. 8, 2001	Canis Familiaris (Madge, F)	3	Y: 1.040 O: 1.077	N/A
Sep. 8, 2001	Canis Familiaris (Madge, F)	4	Y: 1.052 O: 1.086	N/A
Sep. 9, 2001	Canis Familiaris (Elvis, M)	5	Y: 1.047 O: 1.073	N/A
Sep. 10, 2001	Canis Familiaris (Madge, F)	6	Y: 1.057 O: 1.076	N/A
Sep. 28, 2001	Canis Familiaris (Elvis, M)	7	Y: 1.041 O: 1.075	N/A
Oct. 5, 2001	Canis Familiaris (Madge, F)	8	Y: 1.048 O: 1.091	3.63cP(29.0%),4.00cP(35.1%),4.64cP(45.3%) 2.94cP(27.0%),3.98cP(34.5%),4.92cP(43.5%) For 20 RPM (150 sec^{-1})
Oct. 5, 2001	Canis Familiaris (Madge, F)	9	Y: 1.065 O: 1.095	N/A
Oct. 19, 2001	Canis Familiaris (Madge, F)	10	Y: 1.077 O: 1.082	2.76cP(24.0%),3.82cP(37.9%),4.47cP(45.5%) 2.30cP(23.5%),3.66cP(34.2%),4.75cP(42.3%) For 20 RPM (150 sec^{-1})
Oct. 19, 2001	Canis Familiaris (Madge, F)	11	Y: 1.071 O: 1.099	N/A
Oct. 26, 2001	Canis Familiaris (Jackson, M)	12	Y: 1.088 O: 1.095	2.56cP(22.5%),3.61cP(38.0%),5.12cP(46.3%) 2.74cP(21.5%),3.77cP(33.3%),5.53cP(42.0%) For 20 RPM (150 sec^{-1})
Oct. 26, 2001	Canis Familiaris (Jackson, M)	13	Y: 1.086 O: 1.105	N/A
Nov. 2, 2001	Canis Familiaris (Madge, F)	14	Y: 1.079 O: 1.093	2.59cP(28.0%),3.80cP(38.5%),4.89cP(47.9%) 2.71cP(27.1%),3.42cP(32.6%),5.20cP(46.4%) For 20 RPM (150 sec^{-1})
Nov. 2, 2001	Canis Familiaris (Madge, F)	15	Y: 1.077 O: 1.091	N/A

Table 8. Raw data of measured of dog blood RBC fraction suspension density and viscosity for a viscometer shear rate of 225 sec^{-1} (present study)

Date	Sample	Experimental run	Density	Viscosity at 25-27 °C (Hematocrit level)
Oct. 19, 2001	Canis Familiaris (Madge, F)	10	Y: 1.077 O: 1.082	2.63cP(24.0%),3.66cP(37.9%),4.16cP(45.5%) 2.32cP(23.5%),3.46cP(34.2%),4.62cP(42.3%) For 30 RPM (225 sec^{-1})
Oct. 26, 2001	Canis Familiaris (Jackson, M)	12	Y: 1.088 O: 1.095	2.46cP(22.5%),3.56cP(38.0%),4.63cP(46.3%) 2.61cP(21.5%),3.65cP(33.3%),5.15cP(42.0%) For 30 RPM (225 sec^{-1})
Nov. 2, 2001	Canis Familiaris (Madge, F)	14	Y: 1.079 O: 1.093	2.48cP(28.0%),3.59cP(38.5%),4.63cP(47.9%) 2.58cP(27.1%),3.24cP(32.6%),4.89cP(46.4%) For 30 RPM (225 sec^{-1})

Table 11. Blood viscosity, spindle speed, and shear rates at each PCV (packed cell volume) value (Andrew et al.(30))

PCV (%)	Speed (RPM)	Shear rate (sec^{-1})	Viscosity (cP) at 37 °C
20	60	230	2.39±0.33
20	30	115	2.50±0.35
20	12	46	2.80±0.37
20	6	23	2.96±0.48
20	3	11.5	3.04±0.62
20	1.5	5.75	2.93±0.96
40	60	230	3.98±0.29
40	30	115	4.40±0.38
40	12	46	5.26±0.59
40	6	23	6.36±0.93
40	3	11.5	7.34±1.46
40	1.5	5.75	8.33±2.61

where the ± x reflects the "standard deviation" for the data sets

Table 12. Viscosity data for reconstituted normal human blood measured at various levels of hematocrit, temperature, and shear rate (Rand et al.(26))

Hematocrit	Speed (RPM)	Shear rate (sec^{-1})	Viscosity (cP) at 27 °C
20	60	212	3.1
20	30	106	3.3
20	12	42	3.7
20	6	21	3.9
40	60	212	4.8
40	30	106	5.5
40	12	42	6.7
40	6	21	7.9
40	3	11	9.6

where SD references the "standard deviation" for data sets

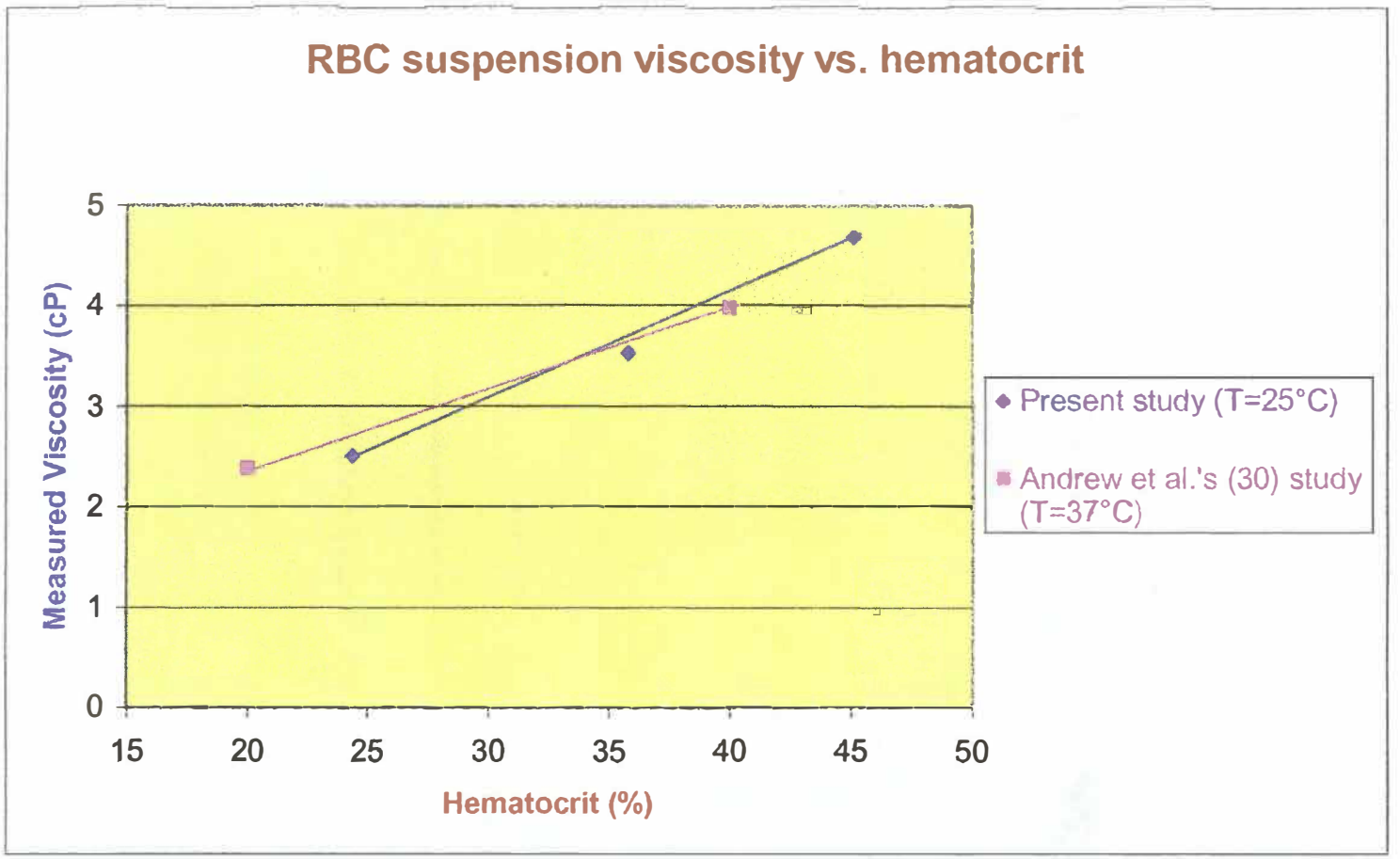

Figure 12. Viscosity versus measured Ht values for studied RBC suspensions from present study and work of Andrew et al.(30)

VITA

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