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Storage of laboratory animal blood samples causes hemorheological alterations : Inter-species differences and the effects of duration and temperature

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Abstract

Hemorheological results may be influenced by the time between blood sampling and measurement, and storage conditions (*e.g.*, temperature, time) during sample delivery between laboratories may further affect the resulting data. This study examined possible hemorheological alterations subsequent to storage of rat and dog blood at room temperature (22°C) or with cooling (4~10°C) for 2, 4, 6, 24, 48 and 72 hours. Measured hemorheological parameters included hematological indices, RBC aggregation and RBC deformability. Our results indicate that marked changes of RBC deformability and of RBC aggregation in whole blood can occur during storage, especially for samples stored at room temperature. The patterns of deformability and aggregation changes at room temperature are complex and species specific, whereas those for storage at the lower temperature range are much less complicated. For room temperature storage, it thus seems logical to suggest measuring rat and dog cell deformability within 6 hours; aggregation should be measured immediately for rat blood or within 6 hours for dog blood. Storage at lower temperatures allows measuring EI up to 72 hours after sampling, while aggregation must be measured immediately, or if willing to accept a constant decrease, over 24~72 hours.

Keywords **:** in vitro time, blood storage, hemorheology, rat, dog

1. Introduction

Standardization of hemorheological measurements is of great importance in both clinical and experimental research, with sampling, handling and storage of blood being areas of concern. Hemorheological results can be influenced by the time between blood sampling and measurement (Alexy *et al.*, 2005; Baskurt *et al.*, 2009; Bernat *et al.*, 2005; Forconi, 1985; Hardeman *et al.*, 2007; ICSH Expert Panel on Blood Rheology, 1986; Jung *et al.*, 1986; Kenyeres *et al.*, Uyuklu *et al.*, 2009; Zhang *et al.*, 2004), and it seems reasonable to assume that storage conditions (*e.g.*, temperature, vibration, shaking, mixing) during sample delivery between laboratories might further affect the results. For how long can the blood samples be stored without significant changes in hemorheological parameters? Can this safe storage time be prolonged with cooling the sample?

It is known that hemorheological parameters show inter-species differences (*e.g.*, Baskurt, 1996; Baskurt *et al.*, 2000; Chien *et al.*, 1971; Nemeth *et al.*, 2006; Plasenzotti *et al.*, 2004; Usami *et al.*, 1969; Windberger *et al.*, 2003; Windberger and Baskurt, 2007). Differences in red blood cell morphology, size and structure, deformability and aggregation are known, and may influence the effects of blood storage.

Unfortunately, there is currently very little information regarding storage of animal blood, especially the allowable period of storage prior to rheological alterations. Zhang *et al.* (2004) investigated the effects of storage time for mouse, rat, guinea pig, dog and human blood samples, and found a correlation between the threshold time and the weight of the animal. However, they only measured whole blood and plasma viscosity, then estimated RBC rigidity and aggregation using these data (Zhang *et al.*, 2004), neither erythrocyte deformability nor RBC aggregation were directly measured.

In the current study we evaluated possible hemorheo-

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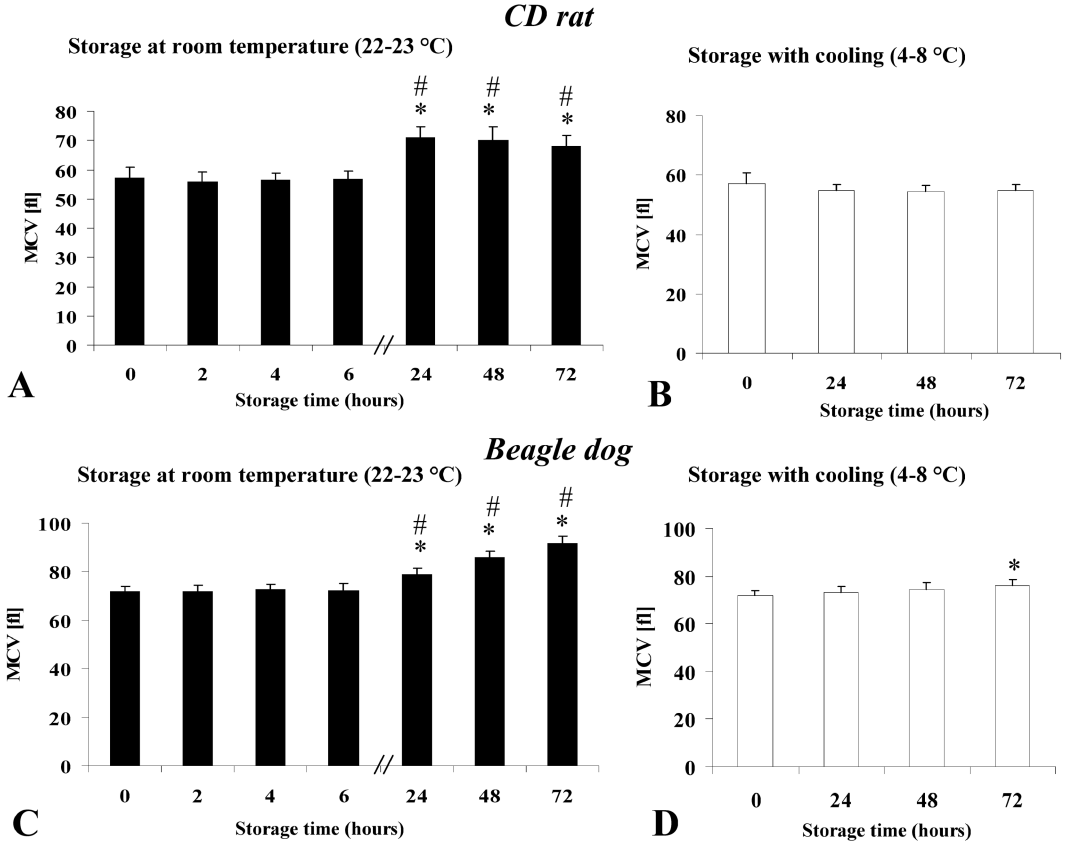

Fig. 1. Mean cell volume (MCV) with storage at room temperature or with cooling for blood samples from CD rats (A,B) or dogs (C,D). Means±S.D. * $p<0.05$ vs. base (0 hour); # $p<0.05$ vs. cooling

logical alterations subsequent to *in vitro* storage of blood obtained from rats and beagle dogs; samples were stored at room temperature or at 4~10°C and studied over a 72 hour period.

2. Materials and Methods

2.1. Animals and sampling

The experiments were approved by the Committee on Animal Research, University of Debrecen (Permission Nr.: 37/2007. UDCAR).

Beagle dogs: Eight ml of blood was drawn from five healthy female beagle dogs (bodyweight: 12.1±2.13 kg) between 08:00 to 09:00 via cephalic vein puncture; vacuum tubes containing K_3-EDTA (1.5 mg/ml) were used for sampling.

CD rats: Eight ml of blood was obtained from five healthy female CD rats (bodyweight: 329±83 g) between 08:00 to 09:00 via cardiac puncture under general anesthesia (sodium-pentobarbital, 35 mg/kg, i.p.), again using vacuum tubes containing K_3-EDTA (1.5 mg/ml).

2.2. Storage of sample aliquots

Blood samples from each animal were divided into 10 aliquots. Seven were kept at room temperature (22~23°C) in air-conditioned laboratory and tested at 0 hours (*i.e.*, 10~15 minutes after sampling) and at 2, 4, 6, 24, 48 and 72 hours.

Three aliquots were stored on "wet ice" (4~10°C) in a small insulated container and tested at 24, 48, and 72 hours. The ice was contained in plastic bags and the sample tubes were in a tray on the ice bags, and thus the tubes did not directly contact the ice. The temperature inside the container was continuously monitored: 4.4°C at the start, 6.1°C at 24 hours, 6.8°C at 48 hours and 9.8°C at 72 hours. Prior to testing, the cooled samples were removed and at room temperature for 20 minutes, then each blood sample was gently mixed and measured.

2.3. Laboratory tests

2.3.1. Hematological parameters

Mean cell volume (MCV) was determined by a microcell counter (Sysmex F-800 microcell-counter, TOA Medical Electronics Corp., Ltd., Japan).

2.3.2. Red blood cell deformability

Red blood cell deformability was measured by a slit flow ektacytometer based upon analysis of RBC laser diffrac-

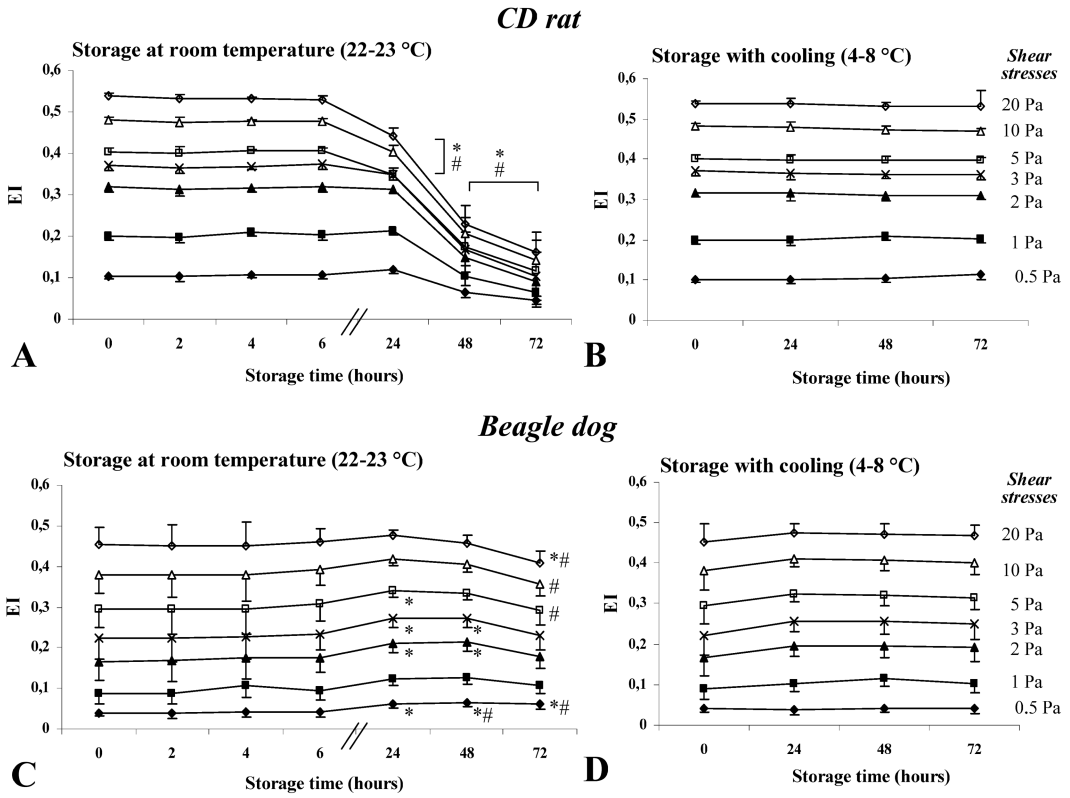

Fig. 2. EI values at shear stresses of 0.5, 1, 2, 3, 5, 10 and 20 Pa with storage at room temperature or with cooling for blood samples from rats (A,B) or dogs (C,D). Means±S.D. * $p<0.05$ vs. base (0 hour); # $p<0.05$ vs. cooling

tion images at various levels of shear stress (Rheoscan-D200 slit-flow ektacytometer, RheoMeditech Inc., South Korea) using cells suspended at about 1% hematocrit in a viscous, isotonic solution of 360 kDa polyvinylpyrrolidone. RBC deformation at shear stresses between 0.5 and 20 Pa was quantified by calculating an elongation index (EI) equal to (L−W)/(L+W) where L is the length and W is the width of the deformed cell; at a constant shear stress, EI increases with cell deformability (Shin *et al.*, 2005).

2.3.3. Erythrocyte aggregation

Two indices of RBC were determined using a Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany): 1) "M" indicating the extent of aggregation at stasis following an abrupt cessation of high shear to disperse pre-existing aggregates; 2) "M1" indicating the extent of aggregation at a low shear rate of 3 s^{-1}, again following an abrupt cessation of high shear (Schmid-Schönbeim *et al.*, 1990). Note that both M and M1 increase with enhanced aggregation.

2.4. Statistical analyses

Data are presented as mean and standard deviation (SD). Differences from fresh samples at 0 hour were evaluated by one way ANOVA tests (Dunn's and Bonferroni's method). Differences between room temperature and cooling were tested by Student's t-test or Manny-Whitney rank sum tests according to the data distribution. A p value <0.05 was considered as statistically significant.

3. Results

3.1. RBC mean cell volume (MCV)

Fig. 1 presents MCV-storage time results for blood stored at 4~8°C or room temperature. No effect of storage was observed during the first 6 hours at room temperature for either species, whereas both dog and rat MCV increased at 24, 48 and 72 hours: 1) rat MCV increased by 25% at 24 hours and remained at this level during the remaining storage period (Fig. 1A); 2) in dogs the increase of MCV was continuous over 24, 48 and 72 hours (*i.e.*, 9%, 19% and 28%, respectively, Fig. 1C). Cooling prevented these increases in both species, although in dogs MCV showed a 5% increase during storage at 72 hours (Fig. 1 B,D).

3.2. RBC deformability

Fig. 2 A-D shows elongation index (EI) values at shear stresses of 0.5, 1, 2, 3, 5, 10 and 20 Pa for both species. In rat blood stored at room temperature, cell deformability was unaltered during the first 6 hours, then markedly decreased during storage; changes at 24 hours were seen only at the two highest shear stresses while at 48 and 72

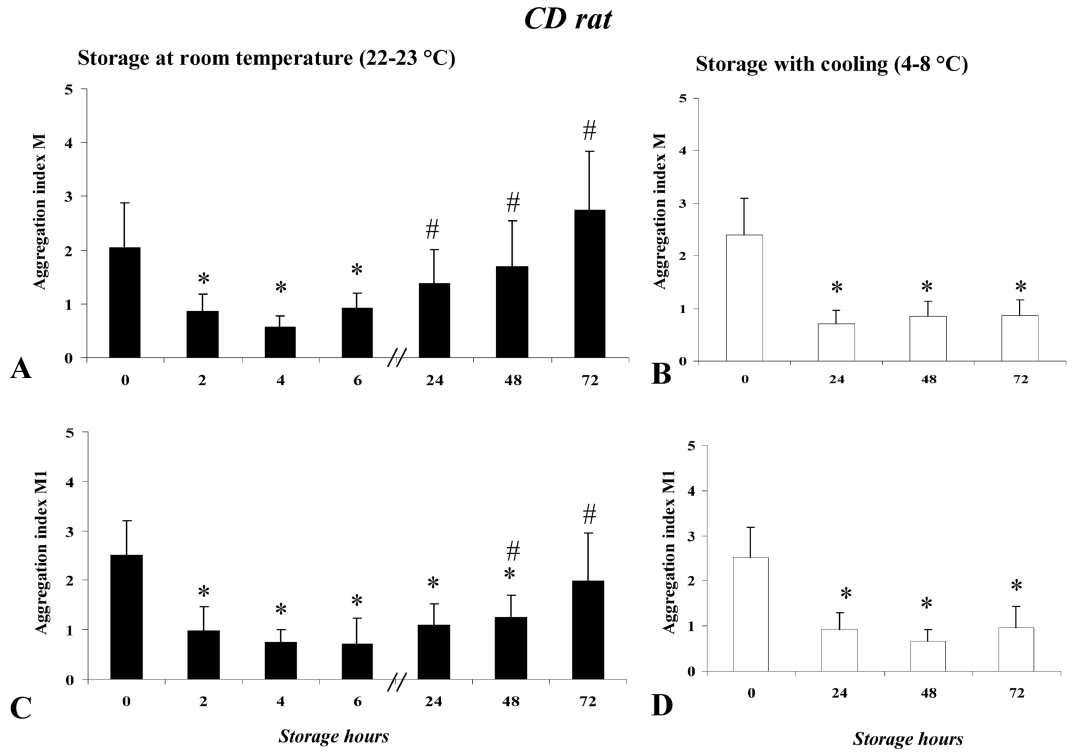

Fig. 3. RBC aggregation indices "M" (A,B) and M1 (C,D) with storage at room temperature or with cooling in blood samples from rats. Means ± S.D., * $p<0.05$ vs. base (0 hour); # $p<0.05$ vs. cooling

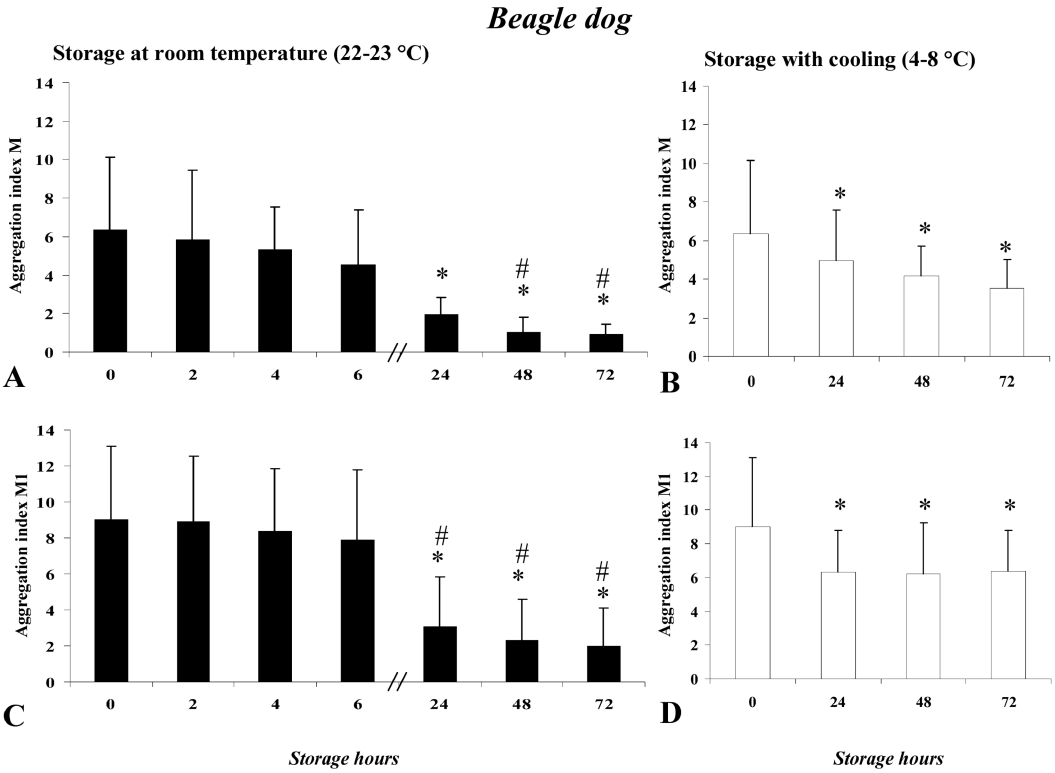

Fig. 4. RBC aggregation indices "M" (A,B) and "M1" (C,D) with storage at room temperature or with cooling in blood samples from dogs. Means ± S.D., * $p<0.05$ vs. base (0 hour); # $p<0.05$ vs. cooling

hours EI at all stress levels was affected (Fig. 2A). Storage at 4~10°C prevented any decrease of EI over the entire 72 hour period (Fig. 2B).

EI values for dog blood were also stable for 6 hours at room temperature, but unlike rat cells, dog RBC showed a slight increase of deformability at 24 and 48 hours; these increases were observed at low to intermediate stress levels were less evident at 72 hours (Fig. 2C). Again, storage at 4~8° prevented any decrease of EI over the entire 72 hour period (Fig. 2D).

3.4. RBC aggregation

RBC aggregation results, as M and M1 values, are shown in Fig. 3 and 4. Rat blood stored at room temperature exhibited a bi-phasic change of M and M1 with storage (Fig. 3A and 3C): significant decrease at 2 hours, continued decrease at 4 and 6 hours, and then a return toward control over the 24~72 hour period. The M index for rats at 24, 48 and 72 hours did not differ from the initial sample, whereas M1 remained decreased at 24 and 48 hours. Interestingly, storage at 4~10°C did *not* prevent a decrease of M or M1, with both remaining significantly below the initial value at 24~72 hours (Fig. 3B and 3D).

Aggregation-storage time relations for dog blood at room temperature differed markedly from that seen for rat blood (Fig. 4): 1) no significant changes of M or M1 for up to 6 hours; 2) marked decreases of both aggregation indices at 24 hours; 3) continuation of the 24 hour decrease at 48 and 72 hours. In a manner similar to rat blood (Fig. 3B and 3D), storage at 4~10°C did *not* prevent decreases of M or M1 (Fig. 4A and 4B) although the percentage decrease was smaller for dog blood.

4. Discussion

Although interspecies differences in hemorheological parameters have been reported (*e.g.*, Baskurt, 1996; Baskurt *et al.*, 2000; Chien *et al.*, 1971; Nemeth *et al.*, 2006; Plasenzotti *et al.*, 2004; Usami *et al.*, 1969; Windberger *et al.*, 2003; Windberger and Baskurt, 2007), only a few studies (*e.g.*, Zhang *et al.*, 2004) have explored the rheologic effects of in vitro storage of laboratory animal blood. Our study was thus aimed at investigating possible temporal changes of MCV, RBC deformability and RBC aggregation in rat and dog blood samples stored at room temperature and at 4-10 °C. Use of room temperature for storage is an obvious choice in that no special environmental system is needed unless, of course, room temperature varies widely from the "usual" range of about 20-25 °C. The range utilized for storage at lower temperature was an approximation of conditions during shipment of samples via air: blood is placed in an insulated container with wet ice (*i.e.*, ice plus water) and the temperature rises after the ice is completely melted.

Our results indicate MCV, RBC deformability and RBC aggregation can be significantly affected by temperature during storage, with the patterns of changes often being species specific.

1) At room temperature, MCV was unaltered up to 6 hours for rat and dog, followed by an increase that was stable over 24~72 hours. Storage at the lower temperature range abolished all changes of MCV for both species over the 72 hour period (Fig. 1). Medaille *et al.* (2006) found increased MCV of dog RBC in samples kept at room temperature over 24~48 hours, and Furlanello *et al.* (2006) demonstrated that sample storage with cooling prevents increases of MCV of dog erythrocytes. Similar changes of MCV in mini-pig blood have been reported by Olsen *et al.* (2001). The MCV results reported herein are thus consistent with these earlier studies.

2) At room temperature, RBC deformability (EI) was also unaltered up to 6 hours, and then progressively decreased for rat cells while dog cells showed slight increases at 24 and 48 hours. Again, storage at the lower temperature range abolished all changes of deformability for both animals over the 72 hour period (Fig. 2).

3) Temporal changes of the aggregation indices were more complicated. With rat blood at room temperature there was a biphasic decrease over 2~6 hours followed by a return to control at room temperature (Fig. 3A and 3C). However, under the same conditions, aggregation in dog blood was unaltered up to 6 hours then markedly decreased and remained stable over 24~72 hours (Fig. 4A and 4C). Storage at the lower temperature range did *not* eliminate changes of aggregation, with both rat and dog blood having lower indices at 24, 48 and 72 hours (Fig. 3B, 3D, 4B, 4D); decreases of aggregation at the lower temperature were greater for rat blood.

Red blood cell deformability is determined by numerous factors such as internal viscosity, membrane rheologic behavior, cellular membrane surface area to cell volume ratio, and cell shape (Stoltz *et al.*, 1999). Since a decreased area to volume ratio tends to reduce RBC deformability, the increases of MCV at 24~72 hours may be partially responsible for the decreased deformability of rat RBC stored at room temperature (Fig. 1A and 2A). However, this explanation is not tenable for dog RBC where comparable increases of MCV were associated with slight increases of EI (Fig. 1C and 2C). Other contributing factors to the altered EI values at room temperature include changes of RBC morphology from the normal biconcave shape (Meiselman, 1978 and 1981; Reinhart and Chien, 1980) and an altered metabolic state of the cells (Baskurt, 2007). Tozzi-Ciancarelli *et al.* (1992) also found increased deformation at low shear stress for stored human blood.

The observed changes of RBC aggregation for rat (Fig. 3) and dog blood (Fig. 4) during storage at room temperature do not lend themselves to an easy explanation. It

is well known that RBC aggregation at stasis (*i.e.*, the M index) and at low shear (*i.e.*, the M1 index) are affected by both plasmatic and cellular factors (Neu and Meiselman, 2007): plasma factors include fibrinogen and other protein levels, pH and osmolarity, while cellular factors include surface charge, physicochemical properties of the membrane glycocalyx, and cellular deformability. However, alterations of cell deformability do not appear to be involved (Fig. 2A, 2C, 3A, 3C): 1) Both rat and dog RBC have unaltered EI for up to 6 hours yet both aggregation indices are markedly lower during the same time period; 2) rat RBC become less deformable at 24~72 hours, yet over the same period aggregation returns to control. Plasma protein degradation, altered surface charge, hemolysis and glycocalyx changes are also possible, and rat RBC have been shown to be very sensitive to chemical challenges (*e.g.*, 2-butoxyethanol, butoxyacetic acid) (Udden, 2000 and 2002), but it is difficult to propose a specific factor responsible for the complex pattern of RBC aggregation. Interestingly, aggregation changes during storage at 4~10°C follow a simpler pattern (Fig. 3B, 3D, 4B, 4D): aggregation is decreased at 24 hours and remains at this lower level at the 48 and 72 hour time points; percentage changes of aggregation are greater for rat versus dog blood.

In overview, our results indicate that marked changes of RBC deformability and of RBC aggregation in whole blood can occur during storage, especially for samples stored at room temperature. The patterns of deformability and aggregation changes at room temperature are complex and species specific, whereas those for storage at the lower temperature range are much less complicated. For room temperature storage, it thus seems logical to suggest measuring rat and dog cell deformability within 6 hours; aggregation should be measured immediately for rat blood or within 6 hours for dog blood. Storage at lower temperatures allows measuring EI up to 72 hours after sampling, while aggregation should be measured immediately. A less-preferred but possible alternative to immediate measurement of RBC aggregation is to accept the reduced but then stable aggregation indices obtained over a 24~72 hour period; such an alternative could be of value when immediate testing is not possible.

5. Acknowledgements

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