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COMPARISON AND SIMULATION OF DIFFERENT LEVELS OF ERYTHROCYTE AGGREGATION WITH PIG, HORSE, SHEEP, CALF, AND NORMAL HUMAN BLOOD

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ABSTRACT **Erythrocyte aggregation levels in pig, horse, sheep, and calf blood samples were investigated and compared to that of normal human blood. The aggregation kinetics and adhesive forces between red cells, and an index of structure of the aggregates were determined with an erythroaggregameter (Regulest, France) at constant hematocrit (0.40 l/l) and temperature (37° C). The adhesive forces and the index of structure in pig blood were close to those of normal human blood. The results for horse blood showed a very high level of aggregation kinetics and adhesive forces between red cells. For sheep and calf blood, little erythrocyte aggregation was found. To simulate different levels of red cell hyperaggregation in humans, a volume of horse plasma was replaced by isotonic NaCl in different proportions (5 to 40% V/V). The kinetics of rouleaux formation and especially the adhesive forces between erythrocytes were systematically decreased, while the index of structure was raised with increasing concentrations of isotonic NaCl. By replacing the porcine plasma with isotonic NaCl, normal and hypoaggregating levels of human red cells were simulated. The aggregation kinetics and the adhesive forces were reduced and the index of structure was raised when the concentration of isotonic NaCl was increased. In summary, large differences in the aggregation parameters were found between mammals. This study also showed that different human erythrocyte aggregation levels can be simulated by diluting the concentration of plasma proteins in equine and porcine bloods.**

Introduction

Many studies reported quantitative results on red blood cell aggregation kinetics and adhesive forces between red cells in normal and pathological human conditions. However, relatively little information is available on other

KEYWORDS: **Erythrocyte aggregation; animal blood models; laser light erythroaggregameter; aggregation kinetics; adhesive forces; index of structure of the aggregates.**

mammalian species. By calculating the erythrocyte sedimentation rate, Dintenfass and Liao (1982) found a high level of erythrocyte aggregation in racehorses. The whole blood apparent viscosity at low shear rates was also investigated as an index of red cell aggregation in several species of mammals (*e.g.*: horse, leopard, rhinoceros, lion, zebras, bison, tiger, goat, sheep, cattle; McMillan *et al.* 1988). The largest values of blood viscosity were observed in horse and leopard, and the smallest levels were found in cattle, sheep and goat. Using red cell sedimentation rate and apparent blood viscosity at low shear rates, the degree of red cell aggregation in athletic animal species and nonathletic species was characterized by Popel *et al.* (1994). Their data showed that the athletic species (antelope, horse, and dog) exhibited a higher degree of red cell aggregation than the nonathletic species (sheep and cow).

Laser light transmission and reflection methods were also used to characterize erythrocyte aggregation in mammals. Statistically significant differences in the erythrocyte aggregation rate between cat, dog, rat, rabbit, gerbil and fowl were measured (Ohta *et al.* 1992) using the light transmission aggregameter developed by Tomita *et al.* (1986). The minimal values of the aggregation rate were found in fowl while the largest values were observed in cats. Recently, parameters related to the aggregation of red cells were evaluated in buffalo, cow, horse, goat, rabbit, and human using a light transmission method (Kumaravel & Singh, 1995). It was found that the process of aggregate formation was faster in horse than in human and buffalo blood samples. In cow, goat, and rabbit blood, no aggregation was observed. Using a light reflection method developed in the sixties (syllectometry), Brinkman *et al.* (1963) also found excessive rouleaux formation in horse blood.

Objectives

The purpose of the present study was to provide more quantitative data on red cell aggregability in pig, horse, sheep, and calf blood. A laser light erythroaggregameter was used to quantify the aggregation kinetics and adhesive forces between red cells, and an index of structure of the aggregates. These parameters obtained for each animal species were compared to those measured in normal human subjects.

Following the recent development of ultrasound backscattering methods to study red cell aggregation, there has been an increased requirement for large volumes of human blood substitutes (up to 2 liters / experiment) characterized by different levels of red cell aggregation. In the process of developing ultrasound methods for studying erythrocyte aggregation in large vessels, *in vitro* hemodynamic flow loop models are often used. Ultrasound studies using porcine (Yuan & Shung, 1988a, 1988b; Cloutier & Shung, 1993; Shehada *et al.* 1994; Cloutier *et al.* 1996; Allard *et al.* 1996) or canine (Mahony *et al.* 1992; Yamada *et al.* 1992) blood flowing at different shear rates were reported. To study ultrasound backscattering over a wider range of red cell aggregation levels, other alternatives must be developed. Dextran solutions (Brooks *et al.* 1974; Chien, 1976; Knox *et al.* 1977) can provide different levels of red cell aggregation. However, this approach is limited by the cost of these bridging macromolecules and by the associated viscosity increase of the suspending medium. In the present study, a simple and inexpensive alternative is proposed. Another objective of the study was thus to create blood models simulating normal, hypo and hyperaggregating human erythrocytes from horse, pig, calf, and sheep blood.

Materials and Methods

Blood collection

Blood samples from pigs (n = 22) and calves (n = 10) were collected from local abattoirs. Horse (n = 12) and sheep (n = 10) blood samples were drawn from the jugular vein of healthy animals located at the Faculty of Veterinary Medicine of the University of Montreal. Human blood was obtained from 19 non-smoking healthy adult volunteers (31 ± 5 years old) by venepuncture. The blood samples were anticoagulated with EDTA (1.5 g/l) in standard Vacutainer tubes.

Preparation of blood samples

All experiments were performed within 6 hours of blood collection. The blood samples were centrifuged (IEC PR-6000) at 1300 g for 10 min and the hematocrit was adjusted at 0.40 l/l by adding or eliminating autologous plasma. The hematocrit of the whole blood was measured with a microcentrifuge (Haemofuge, Heraeus Instruments) operating at 14980 g for 10 min.

To simulate different levels of human red blood cell aggregation using porcine or equine blood, a volume of plasma was replaced by the same volume of isotonic NaCl. The proportions of isotonic NaCl used ranged between 5 and 40% of the total volume of the sample in 5% increments. The hematocrit was maintained at 0.40 l/l in all blood preparations.

Measurements of erythrocyte aggregation

Erythrocyte aggregation measurements were performed with an erythroaggregameter based on a Couette flow arrangement (Regulest, Florange, France). A laser diode (Hitachi, HL7801G) providing a radiation at 780 nm was used as the light source. Quantitative parameters of erythrocyte aggregation were derived from an analysis of the variation in light intensity of the signal scattered by blood. Each blood sample (1.5 ml) was put into a chamber between two co-axial cylinders and the outside cylinder was rotated to provide a range of shear rates. All measurements were performed at a constant hematocrit (0.40 l/l) and 37° C by controlling the temperature in the blood chamber with circulating water.

Two series of measurements were performed. In the first series, the blood sample was sheared for 10 secs at 550 s^{-1} to provide rouleaux disruption and red blood cell orientation with the flow. After abrupt cessation of flow rotation, the variation of the scattered light intensity was recorded during the rouleaux formation process. The instrument provided the following parameters on red blood cell aggregation kinetics: tA, tF, and S10 that correspond to the primary aggregation time, the final aggregation time, and a mean kinetic index at 10 secs, respectively. An index of structure (IS) of the aggregates was also measured (a large index meaning little aggregation). Further detail on the determination of these indices related to the aggregation kinetics can be found in the publication of Donner *et al.* (1988). For the second series of measurements, the blood sample was sheared at 96 different levels varying from 6 to 720 s^{-1}. Using a method similar to those described by Donner *et al.* (1988) and Pignon *et al.* (1994), the partial (γD) and total (γS) dissociation thresholds providing information on the adhesive forces between red blood cells were determined.

Microscopic photography of red cells under static conditions

To appreciate the morphology of the red blood cell aggregates under static conditions, photographs of equine, porcine, sheep, calf, and normal human red cells were taken using a Zeiss Axiophot microscope equipped with a 35 mm camera. The hematocrit of blood on the glass slide was adjusted to 0.20 l/l.

Statistical analysis

All results were expressed in terms of mean ± one standard deviation (SD). One-way analyses of variance for repeated measures, using the Bonferroni's method for multiple comparisons (SigmaStat statistical software, version 1.0, Jandel Scientific), were used to assess differences in erythrocyte aggregation between mammals and differences among blood preparations. A level of statistical significance of 5% was used in all analyses.

Results

Erythrocyte aggregability in mammalian species

The erythrocyte aggregation indices and the original (non adjusted) hematocrit in horses, pigs, sheep, calves, and humans are shown in Table I. When compared to the human blood, statistically significant lower values of the aggregation times tA and tF, and higher values of the aggregation index S10 were found in horse and pig blood. These differences are attributable to the rapid red blood cell aggregate formation in these mammals. The small value of IS ($p < 0.05$) in horse blood is related to the larger size of the aggregates compared to those in human blood. The index of structure for pig blood and the adhesive forces between red cells (γD, γS) were very similar to normal human blood. As noted in Table I, it was impossible to measure γD and γS in horse blood because the adhesive forces between red cells were very strong and exceeded the limit of the instrument (maximum 720 s^{-1} for γS).

Table 1

Erythrocyte aggregation indices and non-adjusted hematocrits in different mammalian species

	Horse (n = 12)	Pig (n = 22)	Human (n = 19)	Sheep (n = 10)	Calf (n = 10)
tA (s)	1.5 ±0.1*	1.8 ±0.2*	2.9 ±0.8	N/A‡	N/A‡
tF (s)	7 ±1*	13 ±3*	25 ±5	58±15*	64 ±10*
S10	39 ±1‡	29 ±3*	23 ±3	N/A[u]	N/A‡
γD (s^{-1})	N/A‡	46 ±14	49 ±4	18 ±6*	23 ±13*
γS (s^{-1})	N/A‡	135 ±30	119 ±18	30 ±24*	27 ±16*
IS	0.52 ±0.01*	0.58 ±0.02	0.58 ±0.02	0.93 ±0.06*	0.95 ±0.01*
Ht (l/l)	0.35 ±0.05*	0.41 ±0.02	0.44 ±0.05	0.37 ±0.04*	0.34 ±0.02*

N.B.: The erythrocyte aggregation was measured from blood adjusted to a hematocrit of 0.4 l/l. The results are expressed in terms of mean ± one standard deviation. * $p < 0.05$ (the indices are compared to the values measured in human). [u] Parameter not measured.

The final aggregation times (tF) in sheep and calf bloods were significantly higher than the values measured in human. The parameters tA and S10 were not measured because of the absence of decaying scattered light intensity after the stoppage of the rotation of the cylinder. Therefore, it can be concluded that there was very little rouleaux formation in calf and sheep blood samples. The dissociation thresholds γD and γS in these mammals were significantly lower than the values in human indicating that little shearing completely dispersed red cells. The large values of IS are another indication of the absence of significant aggregation in sheep and calf blood samples.

Fig. 1 shows the morphology of red cells and rouleaux under static conditions for horse, pig, human, sheep, and calf bloods. As expected, horse blood formed very long chains of rouleaux linked together to form clusters. In pig blood, clusters characterized by smaller rouleaux were observed. Human blood also forms small clusters with rouleaux of red cells. In sheep and calf bloods, almost no aggregation was found.

Blood models simulating human erythrocyte aggregation based on porcine blood preparations

To simulate different human erythrocyte aggregation levels, a volume of the porcine plasma was replaced by isotonic NaCl. The results from 8 experiments (mean ± SD) obtained with different blood samples are shown in Fig. 2. The value of each index for different concentrations of isotonic NaCl was compared to that found in human blood using the Bonferroni's method. The aggregation times tA and tF were systematically prolonged with increasing concentrations of isotonic saline solution. For saline concentrations between 15 and 30% V/V, both tA and tF were within the range of values found in normal human blood. The aggregation index S10 was reduced as the concentrations of isotonic NaCl increased. The dissociation thresholds γD and γS were also reduced, while the index of structure IS increased. Using different concentrations of isotonic NaCl, blood models simulating normal and hypoaggregating human red cells were obtained. In general, a concentration ranging between 10–15% V/V was appropriate to obtain blood models approaching the aggregation characteristics of normal human blood, and concentrations between 35–40% V/V simulated human severe hypoaggregation levels.

Blood models simulating human erythrocyte aggregation based on equine blood preparations

Eight experiments were performed at isotonic NaCl concentrations varying from 0 to 30% V/V and four at concentrations of 35 and 40% V/V. The results (mean ± SD) are presented in Fig. 3. By increasing the concentration of isotonic NaCl, tA and tF were increased and S10 was reduced, corresponding to a decrease in the aggregation kinetics. The adhesive forces between red cells were very strong and no measurement of γD and γS was possible at concentrations below 10%. As the concentration of saline increased from 15 to 40% V/V, both γD and γS decreased. Values higher and smaller than those observed in normal human blood were obtained for γD, while the total dissociation threshold γS was always higher than the values found in normal human blood. The structure of the aggregates changed (IS increased meaning less aggregation) as the concentration was increased. With the exception of IS, the concentrations of isotonic NaCl that best simulated hyperaggregating human red cells were between 10–25% V/V.

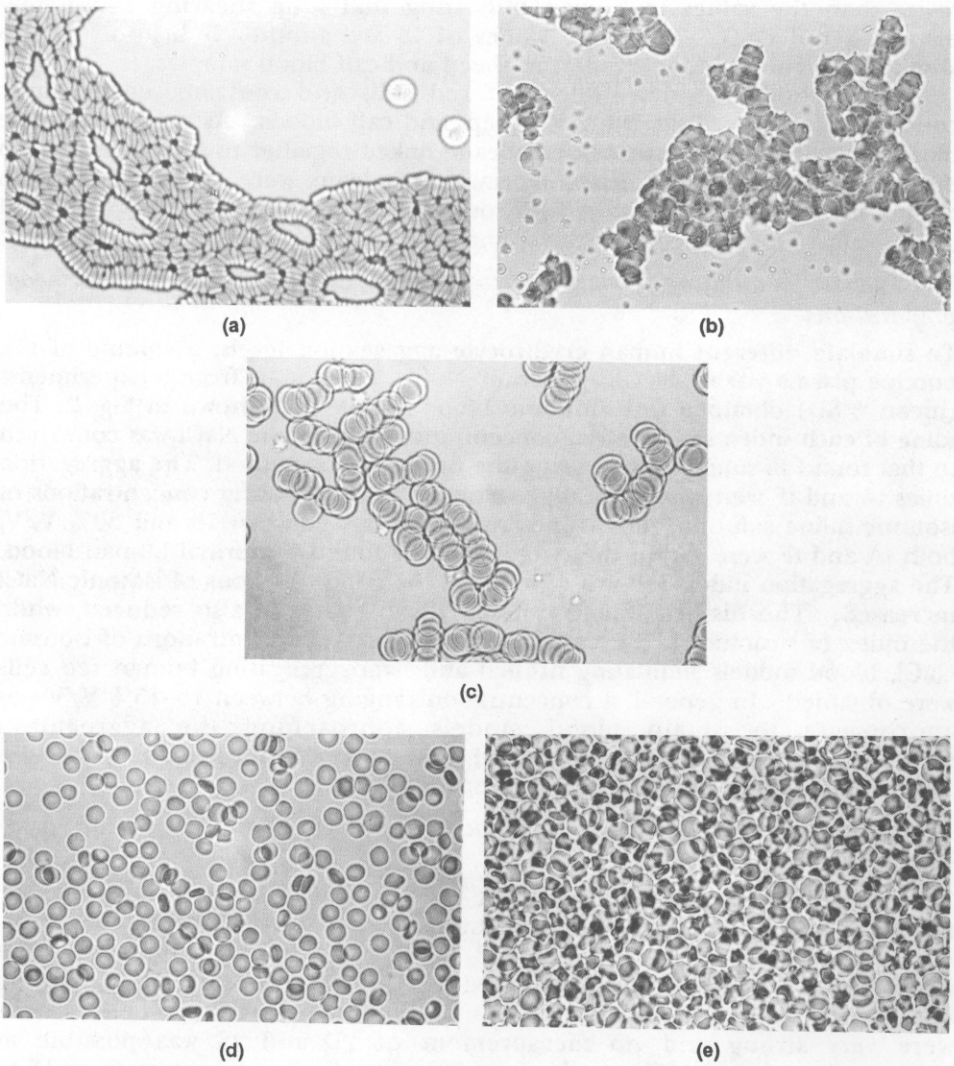


Fig. 1. Photographs of red blood cells in horse (A), pig (B), human (C), sheep (D), and calf (E). A microscope magnification of 100 X was used for each sample.

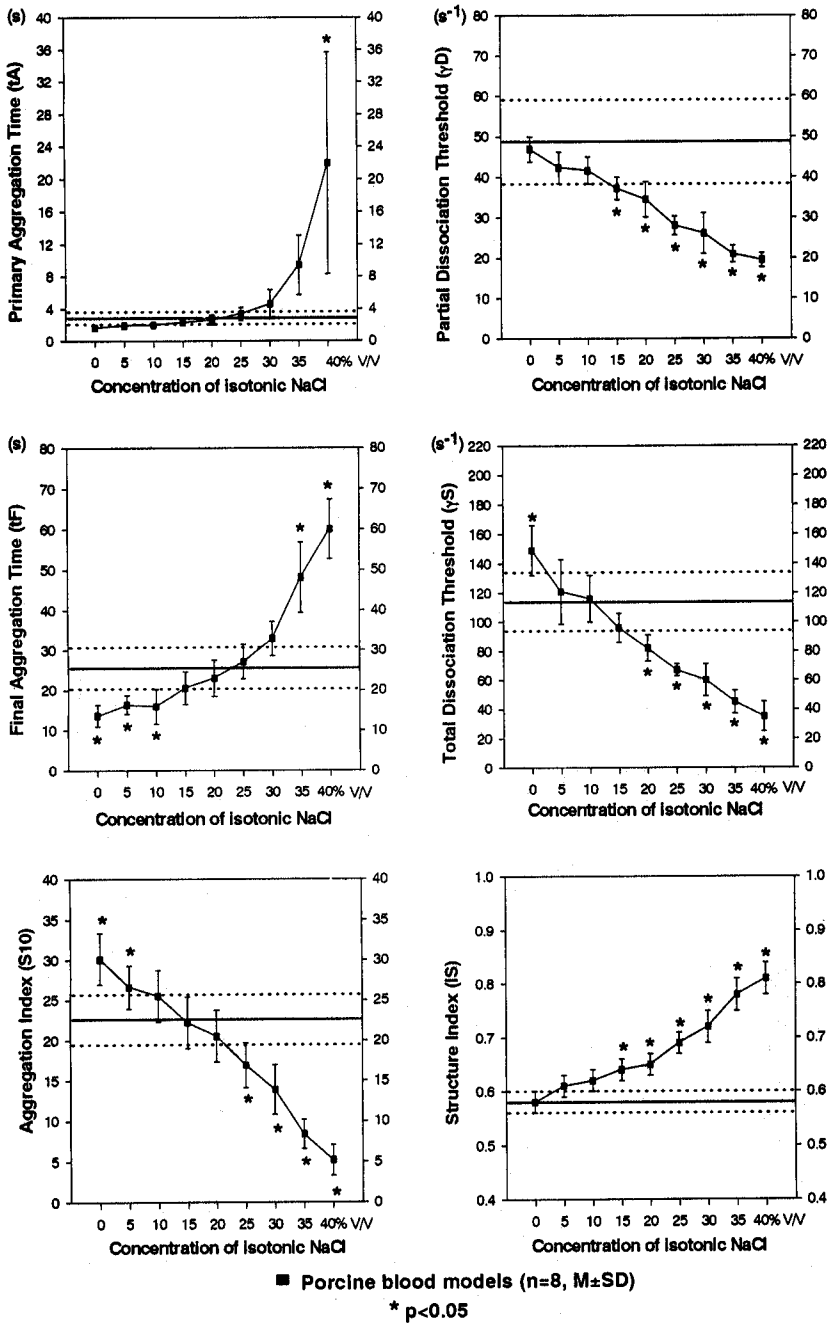
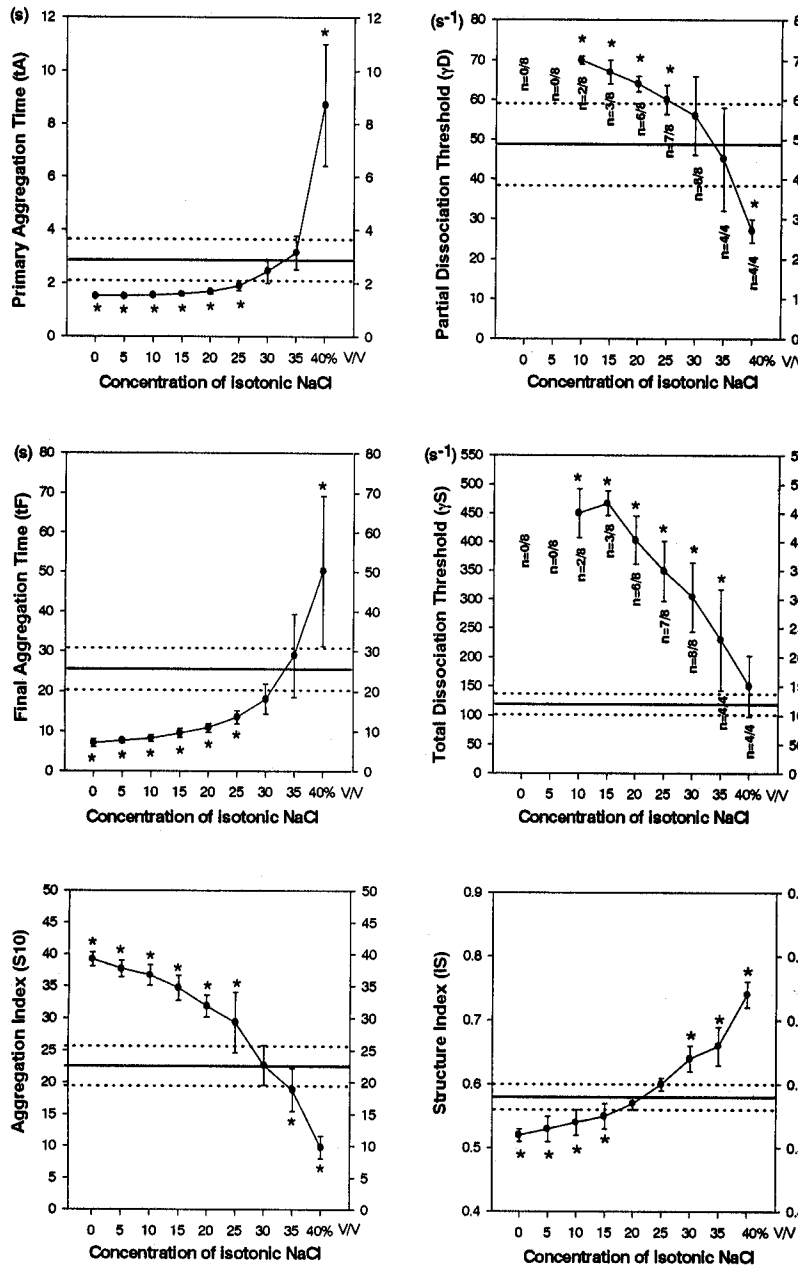


Fig. 2. Primary (tA) and final (tF) aggregation times, aggregation index at 10 sec (S10), partial (γD) and total (γS) dissociation thresholds, and index of structure of the aggregates (IS) in porcine blood models obtained by replacing a volume of plasma with different proportions of isotonic NaCl. The results for normal human blood are given for comparison (mean = full horizontal line, standard deviation = dashed horizontal lines, n = 19).



• Horse blood models (M±SD)

* p<0.05

Fig. 3. Primary (tA) and final (tF) aggregation times, aggregation index at 10 secs (S10), partial (γD) and total (γS) dissociation thresholds, and index of structure of the aggregates (IS) in equine blood models obtained by replacing a volume of plasma with different proportions of isotonic NaCl. The results for normal human blood are given for comparison (mean = full horizontal line, standard deviation = dashed horizontal lines, n = 19).

Discussion

Using the erythroaggregameter, quantitative data on the kinetics of rouleaux formation, the adhesive forces between red cells, and the structure of the rouleaux were obtained for pig, horse, sheep, calf, and human blood samples. The experimental data showed considerable mammalian species differences in erythrocyte aggregation which is in agreement with the results reported by Brinkman *et al.* (1963). In that study, the erythrocyte aggregation levels were classified into four categories: Type a (no rouleaux formation) was observed for ox, sheep and goat, Type b (slight rouleaux formation) for rabbit, Type c1 (moderate rouleaux formation) for pig, dog, cat, rat, and human, and Type c2 (excessive rouleaux formation) for horse blood. Our data on the aggregation kinetics of equine red cells are also in agreement with the results of Kumaravel & Singh (1995) who reported a significantly faster aggregate formation in horse blood at a hematocrit of 5 % compared to that of human blood. Using other indirect methods (*e.g.*; viscosity and sedimentation rate measurements), higher levels of erythrocyte aggregation were also observed in horse blood compared to other blood species (Dintenfass & Liao, 1982; McMillan *et al.* 1988; Popel *et al.* 1994). Based on our knowledge, no data are available to compare with our results on the aggregation kinetics and especially the adhesive forces between red cells in pig, horse, sheep and calf. Our results on blood from young human subjects (10 males and 9 females) are in agreement with data reported by Pignon *et al.* (1994).

Mechanisms of erythrocyte aggregation in mammals

The mechanisms of rouleaux formation and dissociation in human red cells are very complex. In other mammalian species they are also complex and not well understood. Erythrocyte aggregation is dependent on many factors such as the plasma protein content, the shape and deformability of red cells, the membrane surface electrical charges, the hematocrit, the shear rate, etc. (Chien, 1975; Maeda *et al.* 1988). Fibrinogen is known to be the major erythrocyte aggregating factor in human blood (Rampling *et al.* 1984; Lowe, 1993; Potron *et al.* 1994). However, in animals, the levels of fibrinogen were not always well correlated with both the whole blood viscosity and the degree of red cell aggregation (Schneck, 1988; Johnn *et al.* 1992; Ohta *et al.* 1992). Therefore, animal species differences in fibrinogen configuration or differences in the surface affinity of red cells for that protein may be considered as possible causes for the variations observed in erythrocyte aggregation.

Another cause that may explain the differences in erythrocyte aggregation between mammals may be the level of other plasma proteins. For instance, a level of ceruloplasmin (6.06 ±0.74 g/l) ten times higher than that observed in normal human blood was found in horse blood (Okumura *et al.* 1991). In a recent study (Weng *et al.* 1996), the addition of ceruloplasmin to human blood to levels of 4.40 and 7.72 g/l significantly affected the aggregation kinetics and adhesive forces between red cells, respectively. A high concentration of α2-macroglobulin (4.9 ±1.5 g/l) was also reported in normal horse blood (Milne *et al.* 1991). In humans, this globulin was identified as a protein responsible for erythrocyte aggregation in patients with myeloma (Schmid-Schönbein *et al.* 1973). The high level of ceruloplasmin and α2-macroglobulin may contribute to the higher erythrocyte aggregation level in horse blood samples. In cat, dog, human, rat, rabbit, gerbil and fowl, a correlation ($r = 0.57$) was found between the level of globulin and the erythrocyte aggregation rate (Ohta *et al.* 1992).

As suggested by Chien (1975), the deformation of red cells is also important in the process of rouleaux formation to provide parallel alignment between adjacent red cells. It is known that red cell deformability is mainly dependent on the red cell surface area to volume ratio, the internal viscosity of the cell, the membrane viscoelasticity, the shear stress, and the hematocrit (Stoltz & Donner, 1987). A low surface area to volume ratio and small red cell sizes result in a high membrane bending rigidity and therefore a low level of erythrocyte aggregation. Remarkable animal species variations in the red cell surface area to volume ratio, mean cell volume (MCV), and diameter were reported (Chien *et al.* 1971; Schneck, 1988; Waugh, 1992; Johnn *et al.* 1992; Ohta *et al.* 1992). These differences may contribute to the animal species differences in erythrocyte aggregation. For instance, sheep red cells forming very little aggregation were shown to have a small surface area to volume ratio compared to human (Chien *et al.* 1971).

Animal blood models of human erythrocyte aggregation

Since our results showed important differences in red cell aggregation between mammals, the possibility of modeling different levels of human red cell aggregation was obvious. Porcine red cells exhibited aggregability close to that of normal human red cells which allowed the simulation of human normal and hypoaggregation levels by isotonic NaCl dilution. Due to the very high level of aggregation in equine blood, the simulation of pathological human hyperaggregating red cells was also possible by replacing the plasma with isotonic NaCl, thus providing a simple, economic, and effective way of creating models of red cell aggregation. Another important advantage in using animal models of red cell aggregation instead of human blood is the reduction of possible infectious contamination. The reduction of the concentration of the plasma proteins responsible for the erythrocyte aggregation can explain the reduction of the kinetics and adhesive forces when increasing the concentration of isotonic NaCl. The formation of some echinocytes mainly due to changes in the oncotic pressure after the decrease of the albumin concentration may have also induced a decrease in the deformability of the red cell membrane and consequently a reduction of the erythrocyte aggregation.

Summary

It has been demonstrated that pig, horse, sheep, calf, and normal human bloods have specific erythrocyte aggregation characteristics. Horse red cells are characterized by very strong adhesive forces as well as rapid aggregate formation. The dissociation thresholds in porcine red cells (*i.e.*; the shear rate needed to disrupt the aggregates) were very close to that of normal human blood. Little aggregation was found in calf and sheep blood samples.

Considering species differences in erythrocyte aggregation levels, animal blood models simulating normal, hypo- and hyperaggregating human red cells were created. The replacement of plasma by isotonic saline solution provided an effective, economic, and simple way of simulating different levels of human red cell aggregation. Using around 10 to 15% V/V of isotonic saline solution, the porcine blood models simulated normal human red cell aggregation, while replacing the porcine plasma by 35 to 40% V/V, hypoaggregating human red cell were obtained. Concentrations varying from 10 to 25% V/V of isotonic saline solution in equine blood was shown to simulate pathological hyperaggregating human red cells.

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ERRATUM

In the last issue of *Biorheology*, Volume 33, Numbers 4 & 5, the author affiliation for Dr. Guy Cloutier was mistakenly listed as the Faculty of Veterinary Medicine, University of Montreal. The actual affiliation is the Laboratory of Biomedical Engineering, Clinical Research Institute of Montreal. Additionally, we are reprinting Table 1 from Dr. Cloutier's paper, with corrections, below. We regret any inconvenience these oversights may have caused.

Table 1

Erythrocyte aggregation indices and non-adjusted hematocrits in different mammalian species.

	Horse (n = 12)	Pig (n = 22)	Human (n = 19)	Sheep (n = 10)	Calf (n = 10)
tA (s)	1.5 ±0.1*	1.8 ±0.2*	2.9 ±0.8	N/A‡	N/A‡
tF (s)	7 ±1*	13 ±3*	25 ±5	58±15*	64 ±10*
S10	39 ±1‡	29 ±3*	23 ±3	N/A‡	N/A‡
gD (s^{-1})	N/A‡	46 ±14	49 ±4	18 ±6*	23 ±13*
gS (s^{-1})	N/A‡	135 ±30	119 ±18	30 ±24*	27 ±16*
IS	0.52 ±0.01*	0.58 ±0.02	0.58 ±0.02	0.93 ±0.06*	0.95 ±0.01*
Ht (l/l)	0.35 ±0.05*	0.41 ±0.02	0.44 ±0.05	0.37 ±0.04*	0.34 ±0.02*

N.B.: The erythrocyte aggregation was measured from blood adjusted to a hematocrit of 0.4 l/l. The results are expressed in terms of mean ± one standard deviation. * $p < 0.05$ (the indices are compared to the values measured in human). ‡Parameter not measured.