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Effect of Red Blood Cell Aging In Vivo on Their Aggregation Properties In Vitro: Measurements with Laser Tweezers

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Abstract: Red blood cell (RBC) aggregation highly influences hemorheology and blood microcirculation in the human body. The aggregation properties of RBCs can vary due to numerous factors, including RBC age. The aim of this work was to estimate in vitro the differences in the RBC aggregation properties of different RBC age populations in single-cell experiments using laser tweezers. RBCs from five healthy volunteers were separated into four subpopulations by Percoll density gradient centrifugation. Each subpopulation of the RBC was separately resuspended in autologous plasma or dextran 70 kDa (50 mg/mL). The aggregation force between the single cells was measured with holographic laser tweezers. The obtained data demonstrated an enhancement of RBC aggregation force in doublets with age: the older the cells, the higher the aggregation force. The obtained data revealed the differences between the aggregation and aggregability of RBC in dependence of the RBC in vivo age.

Keywords: red blood cells; optical tweezers; RBC aggregation; RBC aging; aggregation force; Percoll

1. Introduction

Red blood cell (RBC) spontaneous aggregation and forced disaggregation processes strongly influence the microcirculation of blood and impact human health in general [1,2]. The understanding of RBC aggregation mechanisms is still not clear and contradictory at some points [2]. Since the middle of last century, two main hypotheses of RBC aggregation mechanism were coexisting: the "depletion" theory and the "bridging" model [2–4]. In the "depletion" theory, the interaction between RBCs is described by osmotic forces that arise in the solution of macromolecules (e.g., proteins or synthetic macromolecules) surrounding the cells. In the "bridging" model, the interaction is described by the forces that arise due to the adsorption of macromolecules at the surface of RBC membrane and producing the bridges of these macromolecules between RBC membranes. To this day, there are strong arguments in favor of both models and there are some assumptions that both mechanisms influence the RBC aggregation [5].

Aggregation properties of blood can change due to many factors: alterations of blood plasma composition, changes in blood temperature, pathological cell properties, RBC age, and many others [2,6–8]. To date, the influences of these factors on RBC aggregation are not fully understood. Here, we focused on the investigation of RBC in vivo age.

In a healthy individual, after being released from the bone marrow, RBCs remain in the circulation for approximately 115 days [9,10] and their degradation is best described by a Weibull distribution [11]. Among individuals, the mean lifespan of 115 days varies by approximately ±15% [9]. Old RBCs are removed from the blood circulatory system by the reticuloendothelial system. During aging, RBCs undergo intrinsic changes, such as the loss of membrane surface and increase in density, impaired deformation of membrane and other changes [12–14]. There are some in vitro separation techniques based on these changes. To fraction young and old RBCs from blood for in vitro studies, the separation of RBCs by cell density is usually performed using, e.g., high-speed centrifugation [15] or making use of density differences (self-forming gradient or layering) in Percoll (solution of colloidal silica particles of 15–30 nm diameter which have been coated with polyvinylpyrrolidone) in combination with centrifugation [16]. In addition, fractioning on the basis of differences in RBC volume using counterflow centrifugation or the combination of counterflow centrifugation and Percoll is used in many studies [17]. The use of Percoll has proven to be an efficient separation technique [13,17,18], however, it must be mentioned that there is no strict correlation between the RBC density and age [19]. In most studies, RBC separation can be described only in terms of older and younger cells without determining their exact age.

The aim of this work was to investigate the in vitro differences in the RBC aggregation properties of RBCs of different ages at the cellular level using laser tweezers (LTs). LTs are a powerful laser technique allowing to trap living cells to manipulate them without mechanical contact [7,20]. Recently, they proved to be a very useful tool for studying the characteristics of RBC interaction [21,22], to quantify aggregation forces [23] as well as for the complementation of other methods [24,25]. In this work, LTs were used to study the interaction of pair-aggregating RBCs in autologous plasma and in the dextran 70 kDa (50 mg/mL) solution.

2. Materials and Methods

The study was performed on the fresh blood of five non-smoking healthy 22–26 year-old volunteers who gave informed consent (approval number 51/18, Ärztekammer des Saarlandes). Blood was drawn by venipuncture and was collected into heparin-containing tubes. Then, the blood was centrifuged (200× *g*; 10 min), and the plasma and buffy coat were removed. Then, the RBCs were washed in PBS (Phosphate Buffered Saline, Gibco, pH 7.4) 3 times (650× *g*, 3 min).

2.1. RBC Fractionation

RBC age separation was carried out according to the following protocol: washed RBCs diluted with buffer 1:1 were centrifuged (4000 × *g*, 30 min, 4 °C) over Percoll gradients (five Percoll solutions of different densities) [13,16]. To obtain different densities, the Percoll solution, distilled water and 1.5 M of NaCl solution were mixed. The following densities were used: 1.122, 1.107, 1.101, 1.092 and 1.085 g/mL. Centrifugation provided 4 fractions (Figure 1): the youngest RBC to the top, and the oldest RBC to the bottom. The RBCs of different fractions were washed in PBS 3 times (650 × *g*, 3 min) and were then resuspended in autologous plasma or in the dextran 70 kDa solution for the following measurements. Dextran 70 kDa was used at the concentration of 50 mg/mL [26].

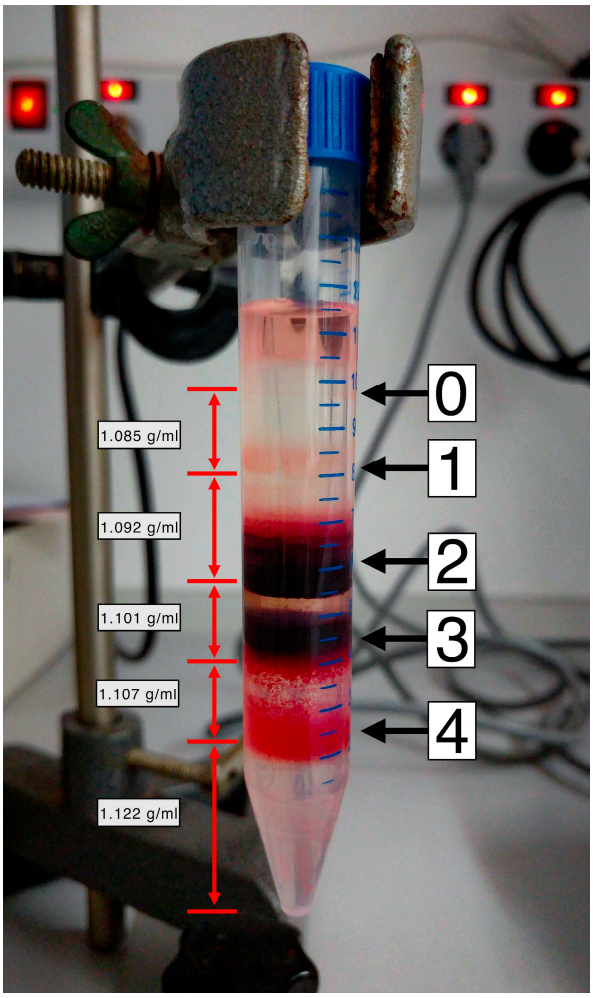

Figure 1. Red blood cells (RBCs) fractionated by Percoll. Four (1–4) different fractions of RBCs of different ages are presented. Fraction #0 for the blood of some volunteers had a negligible amount of RBCs, making it impossible to perform the experiment.

It is thoroughly important to mention that the amount of blood for different fractions varies from individual to individual. Additionally, layer #0 (the fraction between the layer 1.085 g/mL and PBS) mainly containing the reticulocytes and leukocytes can be determined. For two blood samples, even 5 different fractions were obtained and for another three blood samples the amount of RBCs in the layer #0 was negligible. Therefore, layer #0 was not considered for analysis. The total amount of reticulocytes in Fraction #1 was negligible. During measurements, reticulocytes were visually selected and such reticulocytes excluded.

2.2. Laser Tweezers

Holographic LTs were used to study the RBC aggregation properties of different RBC ages at the individual cell level. The procedure was similar as in our previous works [24,27,28]. The schematic layout of the setup is presented in Figure 2. The setup was based on an inverted microscope (TE 2000, Nikon, Tokyo, Japan). Independently controlled optical traps were formed using a laser beam from a single-mode Nd:YAG laser (1064 nm, 3 W) reflected by the parallel aligned nematic liquid crystal spatial light modulator (PAL-SLM, PPM X8267-15, Hamamatsu Photonics, Hamamatsu City, Japan) and focused with a large numerical aperture oil immersion objective (NA = 1.40, 60×, Nikon, Japan). A CMOS camera (ORCA Flash 4.0 V3, Hamamatsu Photonics, Hamamatsu City, Japan) was used for the visual control of the trapped RBC.

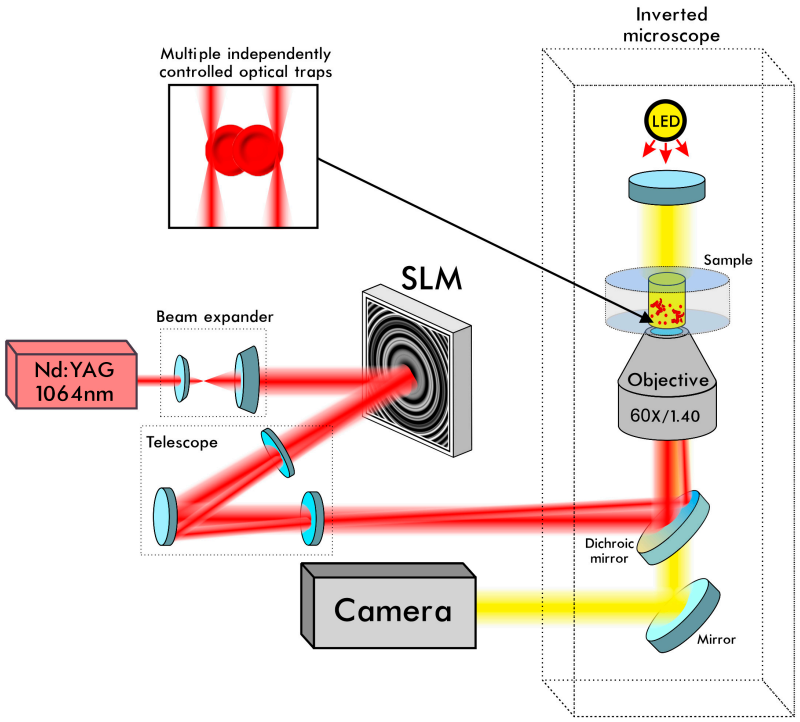

Figure 2. Schematic layout of the holographic laser tweezers for the RBC interaction force measurements. LED—light-emitting diode; SLM—spatial light modulator.

To prepare a sample for an experiment, a small amount of washed RBC of a particular layer (0.5 µL) was suspended in 0.5 mL of autologous plasma or the dextran 70 kDa solution. Approximately 50 µL of this solution was transferred to the measuring chamber. The chamber consisted of a through hole in an aluminum plate with two coverslips from both sides. The bottom glass was coated with a 1% water solution of human serum albumin and dried to avoid the adhesion and morphological changes in the cells caused by their interaction with the glass surface.

2.3. Measurements of the Interaction Forces between Individual RBCs

After placing the measuring chamber with RBCs over the objective, two single RBCs were trapped and brought together until they touched each other. The aggregation force (AF) as an aggregation parameter characterizing the spontaneous aggregation of two RBCs was measured. AF is the minimum force required to prevent the spontaneous aggregation of two interacting RBCs. The protocol of the AF measurements was described in detail in our previous papers [22,27,28]. The step-by-step protocol of the measurement of the AF is presented in Figure 3 and an example of the AF measurement is presented in Supplementary Video S1.

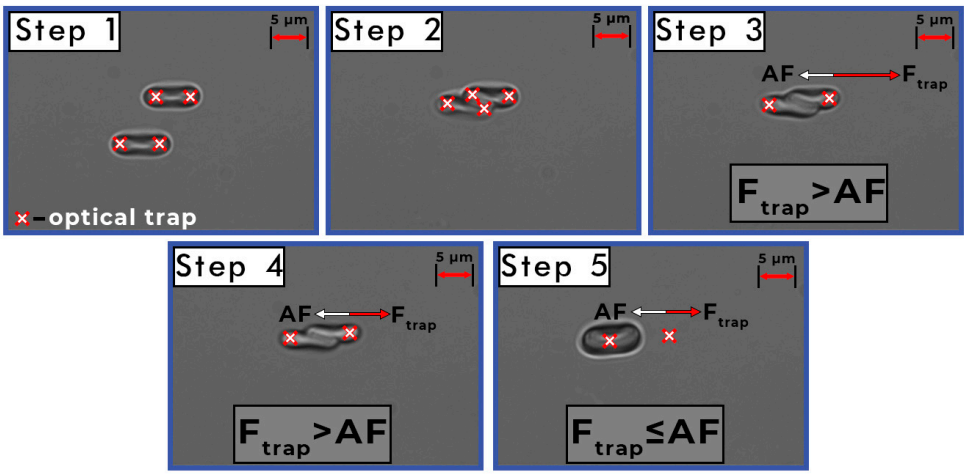

Figure 3. The step-by-step protocol of the measurement of the aggregation force (AF). Step (1): Two RBCs are trapped with four laser traps (red crosses). Step (2): RBCs are brought into contact. Step (3): the middle traps are switched off. Step (4): Process of decreasing the laser beam power (decrease in the trapping force F_{trap}). F_{trap} prevents the aggregation (F_{trap} > AF). Step (5): F_{trap} is not enough to prevent the aggregation of RBCs ($F_{trap} \leq$ AF).

Two single RBCs were initially trapped using four optical traps (red crosses on Figure 3). After trapping, two RBCs were brought to overlap by moving the optical traps and the two middle traps are switched off. As far as the trapping force (F_{trap}) exceeded the AF, trapping prevented the spontaneous aggregation process. Then, the laser beam power (i.e., F_{trap}) started to decrease. The distance between the two peripheral optical traps (the cross-section of RBC overlapping) remained unchanged during the measurement whilst F_{trap} exceeded the AF (F_{trap} > AF). At some point in time, the F_{trap} was not sufficient to prevent aggregation and the RBCs started to aggregate. The laser beam power and corresponding F_{trap} was measured at this particular time-point and it equaled the AF as the minimum force required to prevent the spontaneous aggregation of two interacting RBCs.

In order to compare the laser beam power and F_{trap}, the calibration procedure based on the comparison of the F_{trap} with the viscous Stokes force was performed. The calibration procedure is fully presented in our previous paper [24].

All experiments were performed within 6 h after blood withdrawal. During this time, the AF may undergo some changes, however, we performed the experiments (data not presented) on one healthy volunteer measuring the AF of fractioned RBCs in different orders of these fractions (e.g., 1–2–3–4, 4–3–2–1 etc.). No significant differences of the AF of the same fractions measured at different times were found.

All experiments were performed at the controlled room temperature (22 °C). As it is known, RBC aggregation is a temperature-dependent process [6,29]. The influence of temperature on RBC aggregation is related to the increase in plasma (or dextran) viscosity with decreased temperature and to the RBC membrane changes [30,31]. These changes (the membrane shear modulus and membrane viscosity) mainly lead to the membrane rigidity alterations. It was found that below 25 °C, the RBC deformability decreased as the temperature decreased, whilst there was no decrement in RBC deformability between 25 and 37 °C [30]. In this work, the RBCs were studied under 22 °C, as far as the heating effect of optical trapping for RBCs was negligible [32]. Additionally, it was shown that the temperature-dependent change of the AF is nearly absent for the temperatures of 20 and 38 °C [28].

2.4. Statistical Analysis

For each fraction of blood, the AFs were measured seven times for autologous plasma and 3–4 times for the dextran 70 kDa solution. Statistical difference was calculated with a two-tailed Student

t-test with unequal variance. Two samplings were considered statistically significant if the *p*-value * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$ according to the *t* test.

3. Results

The results of the AF measurements are presented in Figure 4a,b. The number of the density fraction corresponds to the cells age (the higher the layer number the older the cells). The enhancement of AF with the increase in RBC age for RBCs in autologous plasma was found (Figure 4a). It is important to mention that these data are from five healthy volunteers and for each volunteer the same pattern was observed. Furthermore, for each donor, the same number of cells were measured/analyzed to avoid bias towards a particular donor. Statistically significant differences were found between all density fractions. Interestingly, the older the cells, the less alteration of AF was observed. For example, the standard deviation of the first fraction was 3.5 times higher than that of the fourth fraction (Figure 4a). The wide distribution of the first fraction was mostly related to the different means of the AF of different donors. It seems that the older the cells of different donors, the more similar they become in terms of AF. In addition, we performed a donor-based statistic and plotted the AF against the cell density (as a surrogate for the cell age; Supplemental Figure S1) and found a significant correlation ($p = 0.0035$).

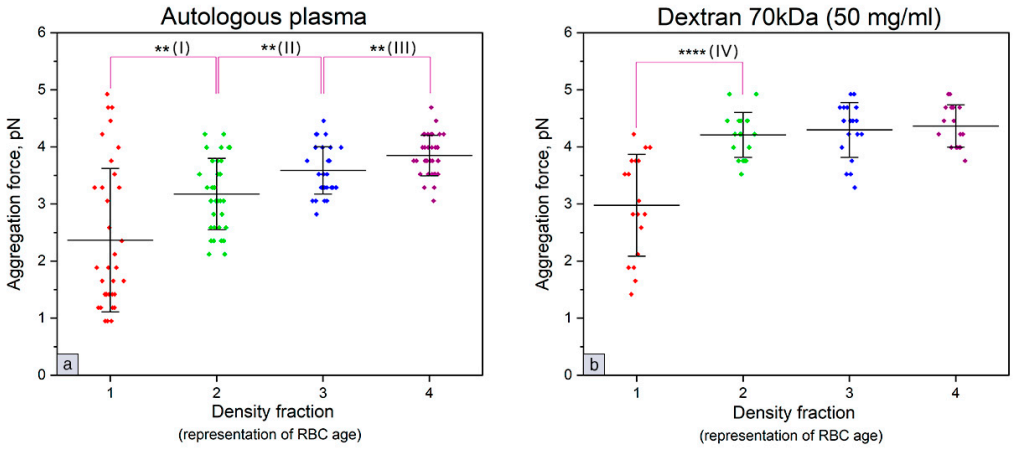

Figure 4. The dependence of the AF on the cells' age (the higher the layer number the older the cells) for (**a**) RBCs in autologous plasma and (**b**) RBCs in the dextran 70 kDa solution at the concentration 50 mg/mL. Data from 5 healthy volunteers are presented. Bars here represent standard deviations. (I) $p = 0.003$, (II) $p = 0.002$, (III) $p = 0.004$, (IV) $p = 2 \times 10^{-5}$.

The dependence of AF on the RBC age for RBCs in the dextran 70 kDa solution at the concentration of 50 mg/mL is presented in Figure 4b. Statistically significant differences were found between the first fraction and the others. No statistically significant differences were found between the second, third and fourth fractions (as well as there no significant correlation of the AF against the cell density (Supplemental Figure S1)). However, an increase in the AF was observed here for the oldest RBC compared to the youngest RBC.

4. Discussion

The results (Figure 4a,b) show an enhancement of AF in the process of RBC doublet formation with the RBC age both in autologous plasma and in the dextran 70 kDa solution at the concentration 50 mg/mL. For RBCs in autologous plasma, the youngest cells exhibit AF = 2.4 ± 0.2 pN (M ± SE), while the oldest ones—AF = 3.8 ± 0.1 pN (M ± SE); for RBCs in the dextran 70 kDa solution, the youngest cells exhibited AF = 3.0 ± 0.2 pN (M ± SE), while the oldest ones—AF = 4.4 ± 0.1 pN (M ± SE). AF monotonously increases with the density fraction for RBCs in autologous plasma, while for RBCs in dextran solution, the AF differs only between the first and all other fractions.

Previously, using a high-speed centrifugation technique and a Myrenne aggregometer, an increase in RBC aggregation (increase in aggregation indexes) with cell age was shown both in autologous plasma and dextran solution [33]. Other studies also confirmed the higher aggregation with cell age [2]. When older RBCs have diminished deformability [13,34], it may contribute to changes of RBC aggregation properties. Decrease in aggregation is expected with the decrease in RBC deformability. However, the inverse pattern was observed. It seems that other important cellular factors [35–37], changing throughout the life span of RBC, are relevant to the higher aggregation of older cells. Previously, the higher aggregation of old RBCs was related to decreased levels of membrane sialic acid [38], which is the main factor contributing to the RBC surface charge. As far as the surface charge is one the main factors of RBC aggregation in the "depletion" theory [2,4], the change of membrane sialic acid with RBC aging is one the key factors responsible for the aggregation differences of RBCs of different ages. Certainly, there are other age-related cellular factors that influence the aggregation, since the difference between younger and older cells still exists after removing sialic acid from the RBC surface [39].

Furthermore, it was shown that the old RBCs have a larger depletion layer compared to the young ones [40]. It could be explained either by a decrease in the RBC membrane glycocalyx thickness or a similar decrease in the polymer penetration into their glycocalyx considering the "depletion" theory [2,4]. A larger depletion layer leads to the enhancement of old RBC aggregation. The "bridging" model does not satisfy the obtained results, to the extent that the older the cells, the less receptors on RBC membrane [13] and the less "bridges" can be produced. As for the "bridging" model, receptor(s) responsible for the bridging is (are) not identified. It is known that the older the cells, the less functional receptors are on a RBC membrane [13]. However, "bridging" receptors could be lipids or non-functional receptors. For example, the putative increase in phosphatidylserine in the outer membrane leaflet with increasing RBC age could be an appealing concept in favor of the "bridging" model [36].

Based on the results, the ratio of RBCs of different ages can influence the rheology of blood, as far as the RBC aggregation highly influences the blood flow rate of capillaries and veins [2]. In numerous diseases, such as diabetes mellitus and many others, the lifespan of RBCs is decreased, and the ratio between RBCs of different ages varies compared to the healthy donors [41]. This means that senescent RBCs in case of pathology may not achieve maximum AF. Additionally, it means that the ratio of senescent and young RBCs could be one of the factors that determine the rheology of blood. In future, the study of the AF of pathology blood is planned.

In this work, we studied RBCs in autologous plasma and in the dextran solution. The aggregation of RBCs in autologous plasma addresses RBC aggregation, while the aggregation of RBCs in the dextran solution reflects the RBC aggregability [2]. RBC aggregability is a cellular property and describes the intrinsic tendency of RBCs to form aggregates. In this case, RBC aggregability shows the cellular factor for aggregation compared to the RBC aggregation in autologous plasma. From this point of view, the differences of RBC aggregability are observed only for the youngest cells (Figure 4b), whilst the differences of RBC aggregation are observed between all RBC ages (Figure 4a). This may correspond to the complex effect of cellular and plasma protein factors on the aggregation of RBCs of different ages.

5. Conclusions

In this work, the aggregation of RBCs of different ages was studied at the cellular level using LTs. The enhancement of aggregation with RBC age was found both in autologous plasma and in the dextran 70 kDa solution. It was shown that the LTs can be used to measure the changes of the RBC aggregation properties due to the RBCs in vivo aging. The obtained data reveal the differences between the aggregation and aggregability of RBCs depending on the RBC in vivo age. This is important for the development of future clinical applications.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/21/7581/s1. Video S1: Demonstration of the approach of RBC aggregation force (AF) measurement using laser tweezers. Figure S1: AF against the cell density.

Author Contributions: All authors participated in the conceptualization of the experiments; conceptualization, P.E., A.L., F.Y., K.L., L.K., C.W. and A.P.; Formal analysis, P.E.; Investigation, P.E. and F.Y.; Visualization, P.E.; Writing—Original draft, P.E., A.L. and A.P.; Writing—Review & editing, P.E., A.L., F.Y., K.L., L.K., C.W. and A.P. All authors participated in the general discussion of the obtained results, reviewed and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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