Effects of oxidative stress on erythrocyte deformability

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ABSTRACT

Hemolysis as a consequence of open heart surgery is well investigated and explained by the oxidative and/or mechanical stress produced, e.g. by the heart lung machine. In Europe O_3 is widely used by physicians, dedicated to alternative medicine. They apply O_3 mostly by means of the Major Autohematotherapy (MAH, a process of removing 50-100 ml of blood, adding O_3 gas to it and returning it to the patient's body). No controlled studies on the efficacy of O_3 are available so far, but several anecdotal cases appear to confirm that MAH improves microcirculation, possibly due to increased RBC flexibility.
Most methods established to estimate RBC deformability are hard to standardise and include high error of measurement. For our present investigation we used the method of laser diffraction in combination with image analysis. The variation coefficient of the measurement is less than 1%.
Previous investigations of our group have shown, that mechanical stress decreases deformability, already at rather low levels of mechanical stress which do not induce hemolysis. On the other hand exposure to O_2 , H_2O_2 or O_3 does not alter the deformability of RBC and - except O_3 - does not induce considerable hemolysis. However this only holds true if deformability (shear rates 36/s - 2620/s) is determined in isotonic solutions. In hypertonic solutions O_3 decreases RBC deformability, but improves it in hypotonic solutions. The results indicate that peroxidative stress dehydrates RBC and reduces their size. To explain the positive effect of O_3 on the mechanical fragility of RBC we tentatively assume, that the reduction of RBC size facilitates the feed through small pore filters. In consequence, the size reduction in combination with undisturbed deformability at iso-osmolarity may have a beneficial effect on microcirculation.

2. INTRODUCTION

During its lifetime an erythrocyte circulates in the body about a quarter of a million cycles. Thereby it is repetitively loaded with O_2, has to pass narrow capillaries, is exposed to high shear rates in laminar as well as turbulent flow [1] , and, in addition, has to pass narrow capillaries.

The uptake of triplet Oxygen ($^3O_2$) and aerobic metabolism entails the generation of reactive oxygen species (the superoxide anion O_2^- , singlet molecular oxygen $^1O_2$, hydrogen peroxide H_2O_2, the hydroxyl radical • OH, the peroxyl radical ROO•) capable of damaging DNA, proteins, carbohydrates and lipids. Thus oxydative stress is an attribute of normal aerobic life, which however is balanced by a powerful enzymatic and nonenzymatic antioxidant defense system (i.e. carotinoids, vitamin E and C, flavonoids, GSH-peroxidases, superoxide-dismutases, catalase).

Thus on one hand, the human RBC must have some basic mechanical properties. It has to be stable enough to resist fragmentation and it must be able to undergo extensive deformations in order to pass through the capillaries, which have a much smaller diameter than do the RBC. On the other hand the RBC have to be armed with mechanisms to overcome the oxidative stress (e.g., GSH-peroxidase, superoxide-dismutase, catalase). If the RBC are unable to fulfill these conditions, they will probably disturb capillary perfusion and finally, be lysed or sequestered in the spleen [2]. In this context the question arises whether the lifetime of a single RBC is determined by the decay of energy metabolism or by small, but accumulating defects following mechanical and/or oxydative stress.

Reduced RBC flexibility is reported to occur during open heart surgery [3], after ozone exposition [4], in photosensitized RBC [5, 6] and to complicate the treatment of diabetes mellitus [7], sepsis [8], arteriosclerosis, myocardial or cerebrovascular infarction [9, 10]. In some diseases it is speculated that the loss of RBC deformability could be due to elevated mechanical or oxidative stress to which the cells are exposed.

Adverse hemodynamic situations as well as adherence to endothelial cells that are changed in surface may be taken into account as pathogenic factors.

In Europe O_3 is widely used by physicians, dedicated to alternative medicine. They apply O_3 mostly by means of the Major Autohematotherapy (MAH, a process of removing a 50-100 ml of blood, adding O_3 gas to it and returning it to the patient's body). No controlled studies on the efficacy of O_3 are available so far, but several anecdotal cases indicate that MAH may improve microcirculation, possibly via an increase of RBC deformability. However, this assumption is paradoxical, since the triatomic allotrope of oxygen is an extremely potent oxidant: It has been shown to posses antimicrobal and antiviral activity. In mice, exposed to filtered air with O_3 (1 ppm) it was reported to causes morphological and biochemical changes in RBC and to decrease their deformability [4, 11]. Therefore one may expect that also application of O_3 during MAH reduces the flexibility of RBC. However, it has to pointed out, that at only at pH $\geq$ 8 O_3 decays to the peroxyl radical $\bullet$ OH, whereas at physiological pH and acidosis it reacts „ionlike" forming hydroperoxides with polyunsaturated fatty acids.

While the mechanical properties of the RBC membrane are well known [12, 13, 14], the mechanical characteristics of the whole cells are less explored. This is partly due to the methodical difficulties and the lack of accuracy to measure the mechanical performance of the whole cell. The present paper describes a system to measure RBC deformability using laser diffraction. Introduction of image analysis (laser diffractoscopy[14,15,16]) has considerably improved the accuracy of the conventional laser diffraction method (ektacytometry [17, 18, 19]) considerably. In a series of experiments the effects of short lasting of mechanical stress, by O_2 and O_3 on deformability is studied by means of the new method.

3. MATERIAL AND METHODS

3.1. Blood preparation and solutions

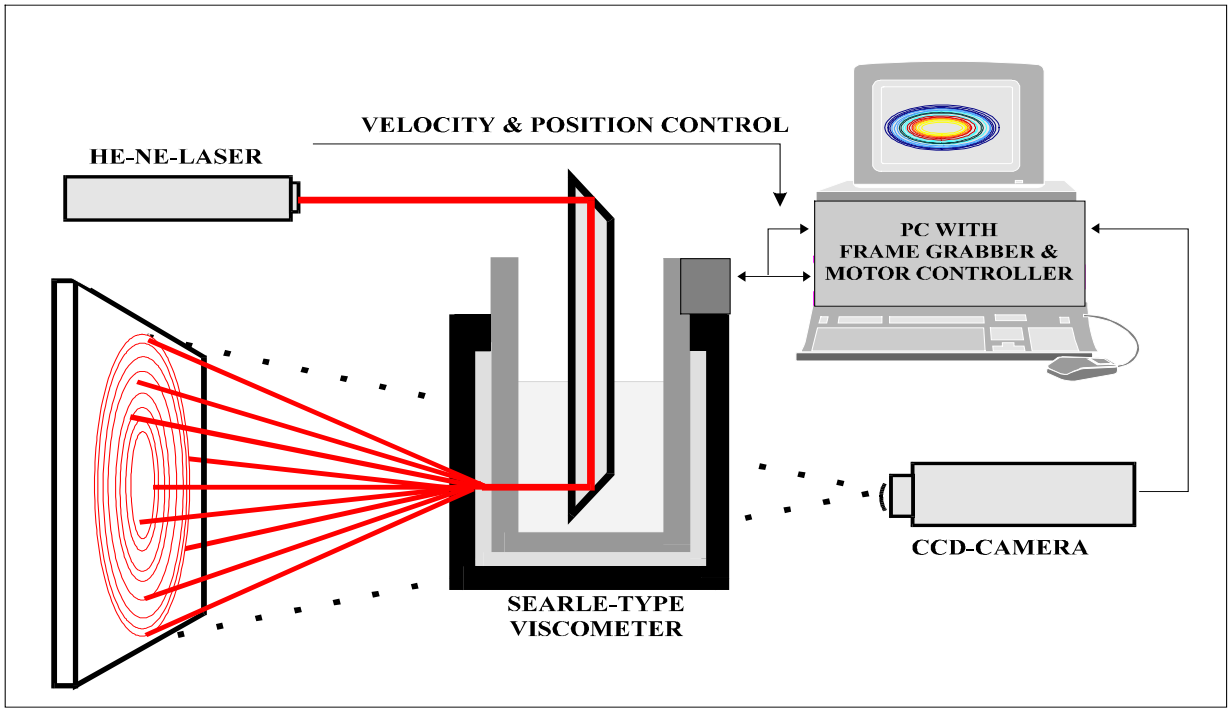
Blood was drawn from the cubital vein of healthy volunteers in heparinized tubes (Vacutainer, Becton & Dickinson). For the ozone experiments fresh (1-3 days) conserved ACD-blood was used. The suspending medium contained 210 g/l dextran (MW 60000, Schiwa) in a MOPS buffered saline (140 mM/l NaCl, 5 mM/l KCl, 5mM/l glucose, 3 mM/l MOPS buffer). The pH was adjusted to 7.4, the viscosity of the suspending medium (22-24 cpoise) controlled with a Hoeppler viscometer (Haake), the osmolarity (305 mOsm) by micro osmometry (Knauer). For measurement of RBC deformability 4 ml of the dextran containing solution and 0.2 ml of blood gently were mixed and filled in the laser diffractoscope.

Free hemoglobin (FHb) was determined as cyano methemoglobin at 546 nm (hemoglobin test combination, Boehringer, Mannheim, FRG). Ozone was generated by Ozonosan Lab I (Hänsler). All measurements were carried out at room temperature.

3.2. The apparatus

The method to measure elongation of RBC by means of laser diffraction has already been described previously [14, 15, 16]. Figure 1 shows schematically the experimental set-up. A viscometer is used to produce well defined shear stress within a gap of 0.5 mm between two transparent cylinders. The rotating inner cylinder (R_i = 24.5 mm) offers the advantage of effortless gap filling and emptying and, most importantly, the absence of lens effects of the outer (R_o = 25 mm), non-rotating cylinder (Searle-system) due to its plane front face. The inner cylinder can be driven with velocities in the range of 0 to 500 rpm, corresponding to shear rates between 0 and 2620 /s. A photo detector controlles the velocity and the position of the inner cylinder, in order to gain the current shear rate allowing to shoot the diffraction pattern at a defined position. The laser beam (He-Ne-Laser, 20 mW) passes into the rotating cylinder via an aluminized dove prism. Phase-matching achieves undisturbed transmission from the prism to the gap. The gap is filled with RBC, suspended in an isotonic solution of high viscosity. In this manner, the RBC are exposed to variable shear stress, depending on solution viscosity and shear rate. The diffracted laser beam is projected on a reflection screen and photographed with a CCD camera. The BAS video signal is digitized ("Fast screen machine II", 8 bit, real-time, maximum resolution 736 x 560 pixels) and transferred to a 486 PC for display and further analysis.

As in ektacytometry [17, 18, 19] the image analysis bases on light- intensity measurement. After A/D conversion discrete intensity values are attached to each pixel, which linearily relate to the intensity of incoming light. The main issue of laser diffractoscopy is to extract intensity information from up to 412.160 points. This offers the opportunity to compare light intensity at different loci and to evaluate areas of selected intensity forming circles or ellipsoids of equal light intensity. These isointensity lines represent the geometric form of RBC. Elongated RBC diffract collimated light according to their shape. Circular RBC yield a circular diffraction pattern, elliptical RBC yield an elliptical pattern with the same eccentricity, but rotated by 90°. The diffraction intensity distribution becomes wider as particles get smaller, the minor axis of an ellipsoid isointensity line corresponds to the major axis of the sheared RBC and vice versa. Since the distribution of points of equal intensity form circles or ellipses, a linear



correlation can be applied (using the square of loci of each pixel) to determine the parameters of ellipses.

Figure 1: Experimental set-up of the laser diffractoscope (for further explanation see text).

From each diffraction pattern (figure 2) a series of isointensity lines and the corresponding E values are calculated. Circular RBC yield a circular diffraction pattern, while elliptical RBC produce an elliptical pattern. The diffraction intensity distribution becomes wider as particles get smaller, the minor axis of an ellipsoid isointensity line corresponds to the mean major axis of the sheared RBC and vice versa.

For an elliptical pattern, the extent of elongation proves as

$$\text{elongation [E]} = \frac{\text{major axis - minor axis}}{\text{major axis + minor axis}}$$

Due to the noise of CCD chips, the elongation coefficient [E] calculated for the low intensity range includes rather high errors (correlation coefficients for isointensity lines < 0.9000). Close to the center of the diffraction image (high intensity range) the light of the non-diffracted laser beam adds a circular

intensity distribution to the elliptical one. Consequently, the inner and outer isointensity lines are not included to determine the average E (with standard deviation SD) for each diffraction picture. The method error for this determination of RBC elongation has been previously shown to be about 1% (variation coefficient) [14]

In laminar Searle- (or Couette-) flow the shear stress applied is a function of the viscosity of the suspending fluid, the rotation speed of the outer cylinder and the geometry of the cylinders. From the analysis of error of measurement it emerged that at low shear rates, which correspond to the steep part of the elongation curve, small changes of the gap width or unsteady cylinder rotation are the main cause of errors[15]. Therefore a computer assisted control of motor speed and , most importantly, a position control device were introduced to avoid small fluctuations of gap width and to monitor the rotation of the cylinder continuously. Such, the variation coefficient declines to less than 0.5%.

3.3. Application of tangential mechanical stress

The force produced within the gap of the viscometer can be given in terms of shear stress [dyne/cm^2] which is determined by the product of shear rate [1/s] and viscosity of the suspending medium [cpoise] [2]

In the following experiments the apparatus described here is also used for continuous application of tangential mechanical stress in order to cause a graded damage of RBC.

3.4. Measurement of RBC fragility

Mechanical fragility of RBC was studied by using a technique modified from Nordt [20]. The whole blood was diluted with an isotonic and buffered solution to give a hematocrit of 5%. Two ml of the RBC suspension was filtered through 3 µm pore sized filters (25 mm diameter, Nuclepore) under electronically controlled 200 mmHg constant pressure. The filtrate was centrifuged at 25000g for 5 min. The supernatant was removed and the free hemoglobin was analyzed as described above.

4. RESULTS AND DISCUSSION

In previous investigations we could demonstrate that mechanical stress, applied to RBC by shearing (2620/s ≈ 630 dyn/cm^2), reduces their flexibility. The decrease of RBC deformability was only detectable, if elongation was measured at lower rates of shearing (e.g. 260/s ≈ 60 dyn/cm^2) and a threshold of shear stress (1100/s ≈ 260 dyn/cm^2) was exceeded. The loss of deformability reported depends on the duration and extent of stress. It is irreversible and not accompanied by hemolysis [21, 22]. However, in those experiments the duration of mechanical stress (≥ 30 second) was considerably larger than that expected to occur in vivo.

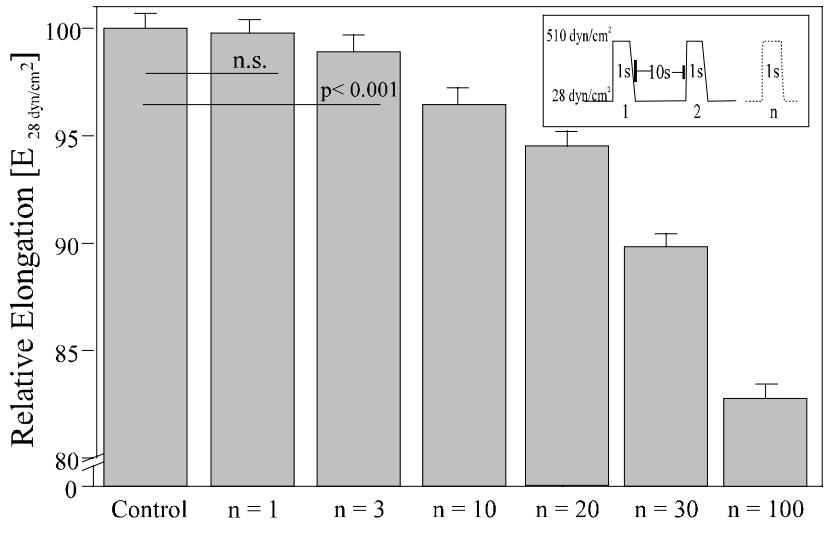


Figure 2: Effect of repetitive (n = 1 to 100) stress cycles (510 dyn/cm^2 , duration 1s). The intervals between step changes of shear stress was 10s, the effect on elongation was determined at 28 dyn/cm^2. Mean and SD of 20 measurements each.

Figure 2 shows the results of experiments exposing human RBC were to repetitive mechanical stress, lasting only 1s each. By increasing the rotation speed of the inner cylinder of the viscometer, the shear force was raised from 28 dyn/cm^2 to 510 dyn/cm^2 (rise-time 70 ms, duration 1000 ms decay-time 700 ms). After an interval of 10s this cycle was repeated up to 100 times. The elongation was determined during the stress free intervals at 28 dyn/cm^2 and related to the respective value measured prior to application of stress. The elongation decreases with increasing numbers of the short lasting strains, reaching after 100 cycles 82.8% (± 0.6%) of control. Already after 3 cycles (i.e. accumulated time of mechanical stress = 3s) the decrease of deformability is statistically significant as compared with control.

In vivo, especially under adverse hemodynamics (e.g. atherosclerosis, hypertension, stenosis, intense physical activity, artificial valves), the human RBC may be experience comparable mechanical stress. We therefore tentatively assume, that the survival time of erythrocytes is limited by loss of elasticity, at least in addition to the decay of their energy metabolism.

The results are in accordance with micro pipette experiments, showing a liquid-like behavior and permanent deformation of the cell membrane under the influence of large forces. Lower, short lasting forces lead to solid behavior of the cell and, consequently, complete recovery to the original shape [23].

To explain the loss of deformability one has to keep in mind the three major factors controlling RBC flexibility [24,25] : Cellular viscosity (i.e. mainly the intracellular hemoglobin concentration), the ratio of surface area to volume, and membrane properties. Loss of water leading to increased intracellular viscosity will alter the deformability at low as well as at high shear rates. But in our experiments maximum remained unchanged. Hence a substantial loss of water seems not reasonable to assume. Further, the unchanged maximum elongation of the cells proves that the observed reduction of deformability at low shear rates is not due to loss of surface area. At high shear rates the internal viscosity is the major determinant of the deformation, whereas at lower rates of shearing the membrane properties and cell geometry become more important. Consequently, the influence of internal viscosity is highly unlikely to contribute to the loss of deformability observed. Therefore, we suggest that the shear-induced rigidification of erythrocytes is at least partly due to alterations of membrane properties, which are mainly determined by cytoskeletal protein structure.

Alteration of RBC deformability by mechanical stress is also known from clinical trials. Thus, it is well known that the flexibility of erythrocytes in patients undergoing open-heart surgery is markedly reduced due to shearing and long time exposure to high oxygen concentrations in the heart-lung machine. In this context Hirayama et al. [3] have proposed another theory of how mechanical stress might reduce cellular deformability. They suppose that mechanical stretching of the erythrocytes causes leak formation in the membrane followed by a loss of potassium and a gain of sodium and calcium. The elevated concentrations of calcium would be responsible for alterations in the conformation of the spectrin molecules leading to a reduced deformability of the whole cell.

In previous papers [5, 25] we could show that neither O_2, nor O_3 (40 -80 µg O_3/ml blood) nor H_2O_2 (1-10µM) alter the deformability of human RBC. Ozone only induced small but dose-dependent hemolysis (2% free Hb of total at maximum therapeutic concentrations: 40 -80 µg O_3/ml blood) concentration. However, in mice, after inhalating ozone RBC flexibility has been reported to decrease [4, 11]. In this context it must be pointed out that the reactive ozone molecule unlikely passes the alveolar border. Therefore the effects of ozone inhalation must be attributed to secondary processes following ozone interaction with cells of the bronchial or alveolar system (e.g. macrophages). Our results indicated that a direct exposition of RBC (with an intact catalase and/or glutathion system) to O_3 leads to an all or nothing effect either leaving the RBC unaffected (no influence on elongation) or destroying it (hemolysis).

It is well known that RBC and other cells [27, 28] exposed to H_2O_2 loose water. Therefore we studied the action of O_3 on the deformability as depending on the osmolarity of the suspending medium. The solution given in the methods was modified by altering its NaCl concentrations. 200µl of blood was added to 4 ml of the suspension, and then gassed with O_3 (8µg O_3/ml suspension). The elongation measured was compared to samples, gassed with pure O_2.

As shown in figure 4 deformability increases with increasing osmolarity, reaching a maximum in a range slightly below iso-osmolarity and decreases again at hyper-osmolar conditions (for literature see [29]). In hypotonic solutions the RBC are transformed to spheres, at iso-osmolarity to biconcave discs and in

hypertonic solutions they shrink to echinocytes. At low shear stress the elongation coefficient [E] does´nt

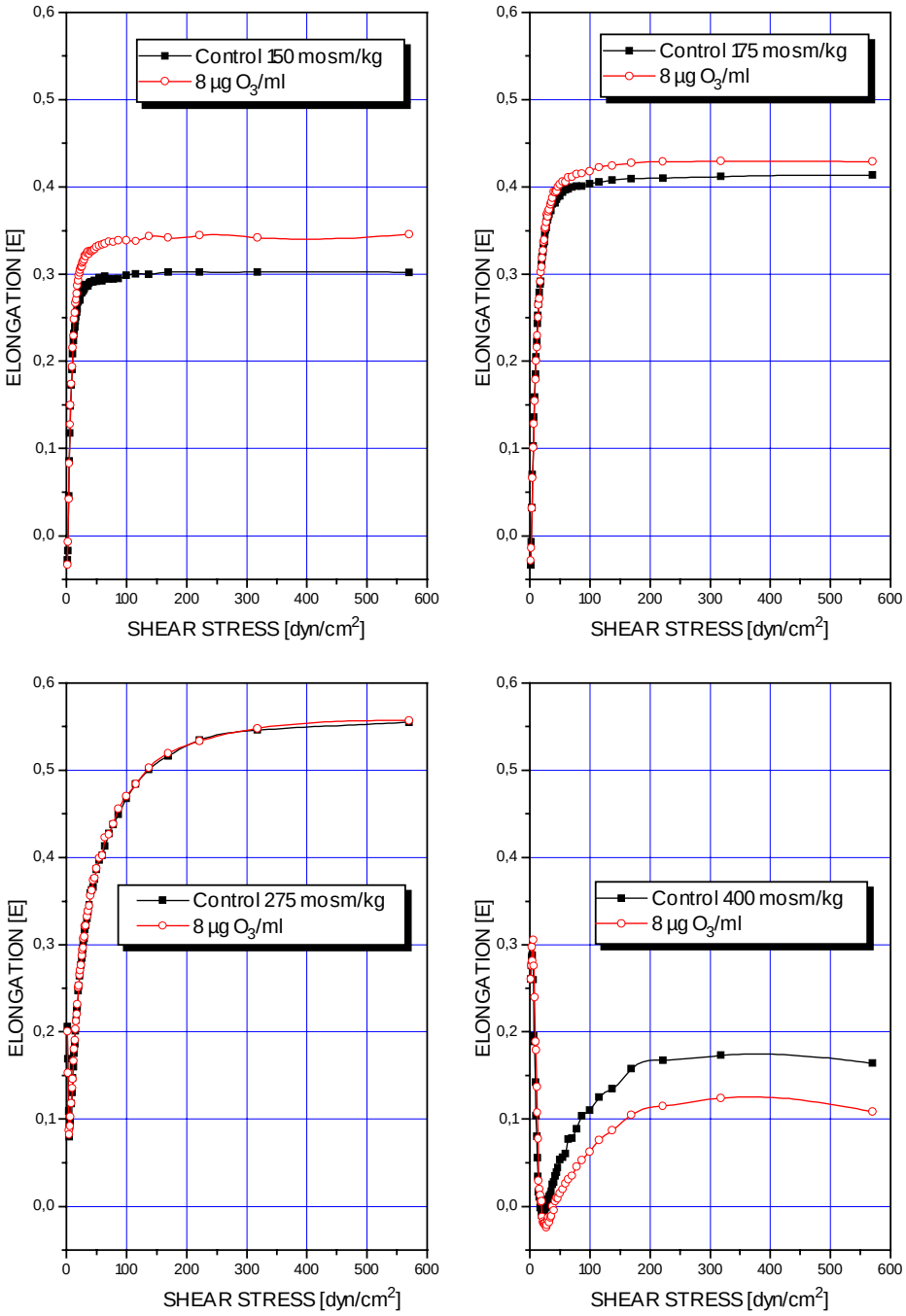


Figure 4:

Effect of ozone (8µg%ml RBC suspension, hematocrit ≈ 2%) on elongation [E] as depending on osmolarity. Note that in hypertonic solutions O_3 decreases RBC deformability, but improves it in hypotonic solutions.

reflect RBC elongation, but the orientation of the cells in the laminar flow. With increasing osmolarity more shear stress is needed to complete the orientation process. Thus, the initial slope of the elongation curve becomes flatter with increasing osmolarity or even biphasic due to a 90° rotation of a large part of the cells[15,16].

In an isotonic medium (not shown here) and in slightly hypotonic(≥ 275 mosm/kg) solutions O_3 does not alter the elongation curve, whereas in hypotonic solutions an increase, in hypertonic solutions a decrease occurs.

Mechanical fragility was studied using filtration of diluted blood through 3µm pores at a filtration pressure of 200 mmHg. This pressure was chosen, because control experiments showed, that in a pressure range of 200 ± 20 mmHg a hemolysis could be determined with reasonable accuracy (31 ± 2% free Hb). Since O_3 has an hemolytic potency (2% free Hb of total at 80 µg O_3/ml blood), the pre filtration FHb had to be subtracted to calculate the pure effect of the filtration procedure.

After exposure to O_2, fragility (FHb after filtration) did not change. Surprisingly treatment of RBC with O_3, reduced mechanical fragility (figure 3). If compared with control (= exposition to oxygen) this effect was statistically significant at O_3 - concentrations ≥ 7 µg O_3 / ml blood. Since O_3 has an hemolytic potency (2% free Hb of total at 80 µg O_3/ml blood), the pre filtration FHb had to be subtracted to calculate the pure effect of the filtration procedure.

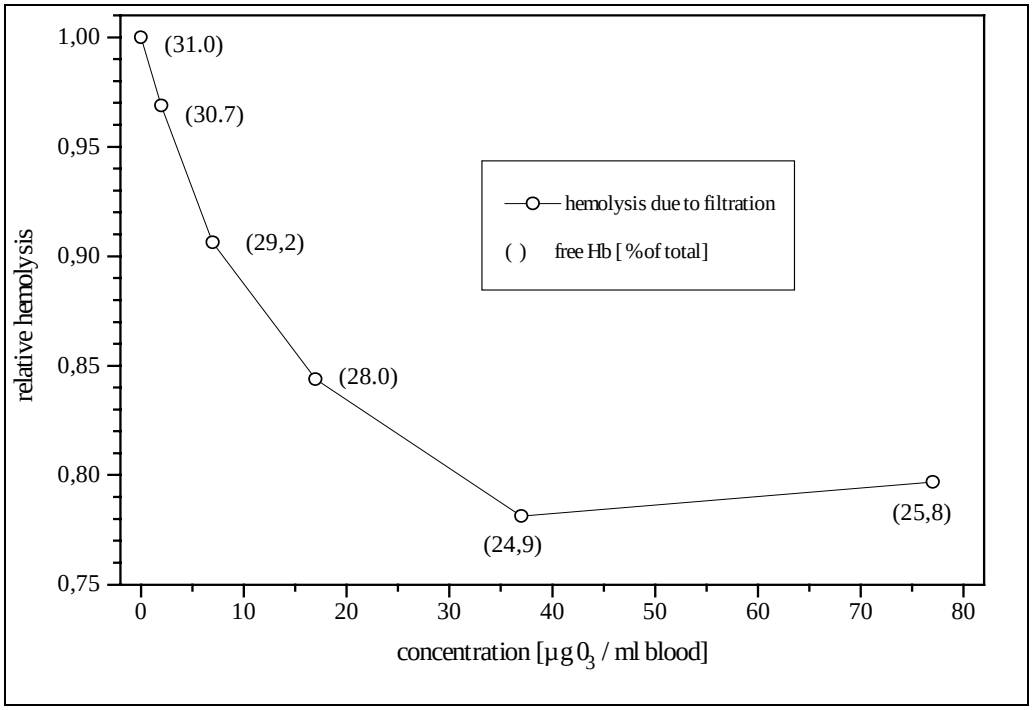


Figure 3: The effect of ozone on hemolysis due to filtration through 3 µm pore filters. Each point represents mean values of n= 6

In the highest O_2 concentrations used in our experiments (270 mgO_2/ ml blood = 0.054 µg/RBC) each erythrocyte was exposed to an amount of O_2 equal to that of 5-6 days in the body. The results show that oxygen in presence of intact oxidative defense mechanisms does neither influences RBC deformability and mechanical fragility nor induces hemolysis. Hirayama et al. [3] showed that in open heart surgery RBC filterability (as a measure of deformability) decreases and positively correlates with the amount of oxygen used in the heart lung machine. However, it can not excluded, that the oxidative defense system was blocked by some drugs given to the patients. On the other hand it seems more plausible that in heart lung machines the mechanical stress (see above) due to pumping plays the key role in RBC rigidification.

As shown by Kuypers et al. [27] H_2O_2 in presence of sodium azide (an inhibitor of catalase) shifts the relationship between elongation and osmolarity of the suspending medium to the right. At low osmolarities a slight increase at high osmolarities a decrease of elongation is described. In principal the same holds true

for the action of O_3 shown in this paper. But in contrast to the action of H_2O_2 this effect already appeared without blocking the catalase. To explain the improved deformability of O_3 in hypotonic suspension it seems likely to assume that O_3 triggers a process dehydrating the cells (as H_2O_2 + sodium azide does in RBC and pure H_2O_2 in hepatocytes) [27, 28] . Thus, in hypotonic solutions by taking up water, ozone treated RBC optimize their surface to volume ratio and become more flexible, whereas in non treated cells the surface to volume ratio becomes worse.

In presence of isotonic media dehydration will result in a shrinking of the cells. This in turn may explain that hemolysis due to forced filtering through small pores is reduced in O_3 - treated cells. Since (except in hypertonic media) O_3 does not reduce deformability the size reduction may have a beneficial effect on microcirculation.

In isolated rat hepatocytes Schreiber et al. [28] demostrated aniso-osmolarity affecting intracellular pH. Hypo-osmolarity decreases and hyper-osmolarity increases cytosolic pH and vice versa to the pH in vesicles. In context with the osmolarity dependent action of O_3, one also may assume that the pH-shift determines the type of hydroperoxides formed with polyunsaturated fatty acids or controls the amount of peroxyl radicals.

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