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Gender and chronological age affect erythrocyte membrane oxidative indices in citrate phosphate dextrose adenine-formula 1 (CPDA-1) blood bank storage condition

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Abstract. It is well known that *in vitro* storage lesions lead to membrane dysfunction and decreased number of functional erythrocytes. As erythrocytes get older, in storage media as well as in peripheral circulation, they undergo a variety of biochemical changes. In our study, the erythrocytes with different age groups in citrate phosphate dextrose adenine-formula 1 (CPDA-1) storage solution were used in order to investigate the possible effect of gender factor on oxidative damage. Oxidative damage biomarkers in erythrocyte membranes such as ferric reducing antioxidant power, prooxidant-antioxidant balance, protein-bound advance glycation end products, and sialic acid were analyzed. Current study reveals that change in membrane redox status during blood-bank storage condition also depends on both gender-depended homeostatic factors and the presence of CPDA-1. During the storage period in CPDA-1, erythrocytes from the male donors are mostly affected by free radical-mediated oxidative stress but erythrocytes obtained from females are severely affected by glyoxidative stress.

Key words: Aging — Free radicals — Erythrocyte — Blood bank storage — Percoll density gradient centrifugation

Abbreviations: CPDA-1, citrate phosphate dextrose adenine-formula 1; FRAP, ferric reducing antioxidant power; PAB, prooxidant-antioxidant balance; prb-AGE, protein-bound advanced glycation endproduct; ROS, reactive oxygen species; SA, sialic acid.

Introduction

Aging is associated with many physiological and cellular changes, many of which are related to oxidative modifications in plasma membrane (Pandey et al. 2013). Exposure to high level of reactive oxygen species (ROS), the absence of mitochondria and nucleus, incapability to synthesize their cellular proteins makes erythrocytes uniquely vulnerable to oxidative damage (Pandey and Rizvi 2011). Erythrocytes are capable of extreme morphological changes. Due to their membrane flexibility, erythrocytes can easily squeeze through capillaries much narrower than their diameter and can reform rapidly to their original shape (Pandey and Rizvi 2010). Oxidative damage of membrane macromolecules has a considerable effect on biomechanical properties of plasma

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membrane (Pandey and Rizvi 2010). An increasing body of experimental evidence point out to the importance of erythrocytes to act as model cells for the study of aging and age-related disorders. The erythrocyte provides an array of redox biomarkers which have been successfully used to assess age-related changes in redox status (Pandey and Rizvi 2010).

It is well known that *in vitro* storage lesions lead to membrane dysfunction and decreased number of functional erythrocytes (Latham et al. 1982; Fitzgerald et al. 1997; Donadee et al. 2011). Biochemical, biomechanical, and immunologic variations occur within erythrocytes and the associated storage media during the storage period. During aging/storage period, erythrocytes lose their water, 2,3-bisphosphoglycerate, ATP, proteins, hemoglobin and vesicles leading to decreasing cell volume, surface charge and increasing density (Huang et al. 2011). There is also a decrease of pH value and formation of cytokines and bioreactive substances in preserved blood (Huang et al. 2011). Storage lesions also interferes plasma membrane redox homeostasis and transport function (Latham et al. 1982; Fitzgerald et al. 1997; Donadee et al. 2011). Anticoagulant citrate phosphate dextrose adenine-formula 1 (CPDA-1) is a preservative solution of citric acid, sodium citrate, monobasic sodium phosphate, dextrose, and adenine in an aqueous milieu. Erythrocytes are routinely stored at 1–6°C in the anticoagulant/preservative solution CPDA-1 (Young and Olutoyin 2015).

As erythrocytes get older, in storage media as well as in peripheral circulation, they undergo a variety of biochemical and morphological changes. In the peripheral circulation, senescence cells are targeted for elimination before becoming harmful. However, upon transfusion process, millions of aged erythrocytes are introduced into a blood circulation of patient. Subsequently, the burden of acidic, hemolytic, and inefficient erythrocytes can create serious problems for the new host, such as increased susceptibility to infection and impaired end-organ perfusion (Hoehn et al. 2015).

We tried to eliminate the ability of various environmental agents (e.g. toxins, stress, diet and drug) to interfere the storage process as well as biological aging process of erythrocytes by using rat blood. If we have used human erythrocytes instead, we would not be able to eliminate the effect of exogenous factors. Little is known on the underlying molecular mechanisms of membrane redox homeostasis in aging erythrocytes. The erythrocytes provide a major component of the systemic antioxidant capacity of blood through the following: Cu, Zn-superoxide dismutase, catalase, glutathione system, low-molecular-weight antioxidants (Pandey and Rizvi 2010, 2011). However, no research is available in current literature about both the effect of storage period and chronological aging on membrane redox status at various erythrocyte subpopulations of different ages according to donor gender. To the best of our knowledge, the oxidant and antioxidant balance of the erythrocyte membrane as a function of the storage period and erythrocyte age groups remain unclear in the literature.

In our study, the erythrocytes with different ages in CPDA-1 storage solution were used in order to investigate the possible effect of donor gender on both radical-induced (e.g. ferric reducing antioxidant power (FRAP), prooxidant--antioxidant balance (PAB)) and non-radical-induced (protein-bound advance glycation endproduct (prb-AGE), sialic acid (SA)) oxidative damage.

Materials and Methods

Chemicals and apparatures

The chemicals were of the highest analytical grade available. All chemicals and reagents were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St Louis, MO, USA). CPDA-1 storage solution was procured from Fenwal (Deerfield, USA). Deionized water was used in the analytical procedures. All reagents were stored at +4°C. The reagents were brought to room temperature for twenty minutes before use. Centrifugation procedures for the analysis of various oxidative stress biomarkers were performed at +4°C with a Sigma 3-18 KS centrifuge (SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany). Oxidative damage biomarker profiles of erythrocyte membranes were analyzed by spectrophotometric and spectrofluorimetric manual methods with Biotek Synergy™ H1 Hybrid Multi-Mode Microplate Reader (BioTek US, Winooski, VT, USA).

Animal model and treatment protocol

The experiments were carried out with two different groups of Wistar rats: male (6 months, $n = 6$) and female (6 months, $n = 6$). Ethical protocol of the current research was approved by Ethics Committee of Bezmialem Vakif University, Istanbul, Turkey. Ethics Committee Issue Number: (2011/46 1374).

Animals were housed in a temperature-controlled room (20 ± 4°C) with 12-h light/dark cycles. All rats were fed with a rodent pellet containing standard nutrients and had free access to tap water.

Blood sampling

Animals were anaesthetized with ketamine (44 mg/kg) and blood was drawn from the heart into 10% EDTA-coated

tubes and rat blood was depleted of leukocyte-rich plasma by centrifugation at 1500 × *g* for 15 min at 4°C. After the removal of plasma, buffy coat, and the upper 15% of packed red blood cells, the resulting packed erythrocytes were washed three times in 154 mM NaCl and then used for fractionation.

Fractionation of erythrocytes according to density/age

Whole bloods collected from both genders were fractionated with "Percoll density gradient" method according to their age groups (Figure 1). Isolation of young and old red blood erythrocytes was carried out on Percoll/BSA (Bovine serum albumin) gradient as described by Rennie et al. (1979) with minor modifications. Two solutions of BSA were prepared. Solution 1 contained BSA in water, pH 7.4. Solution 2 contained BSA in Percoll. Solution A was prepared by adding one volume of Hepes buffer, pH 7.4, to 19 volumes of BSA Solution 1. Adding 19 volumes of BSA Solution 2 to one volume of Hepes buffer, pH 7,4, prepared solution B. Solutions A and B were mixed to obtain gradients of 2.0 ml each of 70, 68, 66, 64 and 62% (v/v) of Percoll. 0.5 ml of approximately 40% hematocrit washed red blood cells was layered on the top of the gradient and centrifugation was carried out at 1,000 × *g* for 10 min at 12°C (Sigma 3-18 KS centrifuge). The erythrocytes were collected at the respective Percoll interfaces. The erythrocytes separated in the 70% were referred as 'aged', erythrocytes found in the 68 and 66% were pooled as 'middle age cells' and erythrocytes found in the 62 and 64% were pooled as 'young cells' (Figure 2). The young, middle age and aged cells were collected separately and extensively rinsed with 154 mM NaCl and centrifuged thrice for 10 min each to remove Percoll. Aged, middle age and young erythrocytes fractions were divided

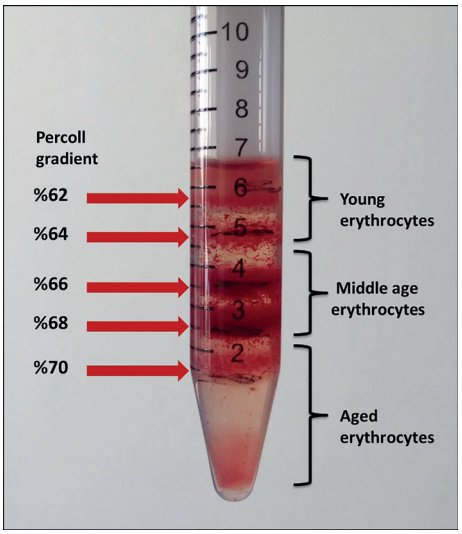

Figure 1. Whole blood samples were fractionated with "Percoll density gradient" method according to their age groups.

into two groups: One group of aged, middle age and young erythrocytes fractions were used as controls. Control group of erythrocytes rinsed with 154 mM NaCl and erythrocyte membranes were isolated. Other group of aged, middle age and young erythrocytes fractions which are also called as treated erythrocytes, were stored in CPDA-1 solution that includes 75% hematocrit for 7 days at +4°C. The young, middle age and aged erythrocytes were rinsed with 154 mM NaCl and centrifuged thrice of 10 min each to remove residual CPDA-1. CPDA-1 solution consists of citric acid, sodium citrate, monobasic sodium phosphate, dextrose, and adenine in an aqueous milieu.

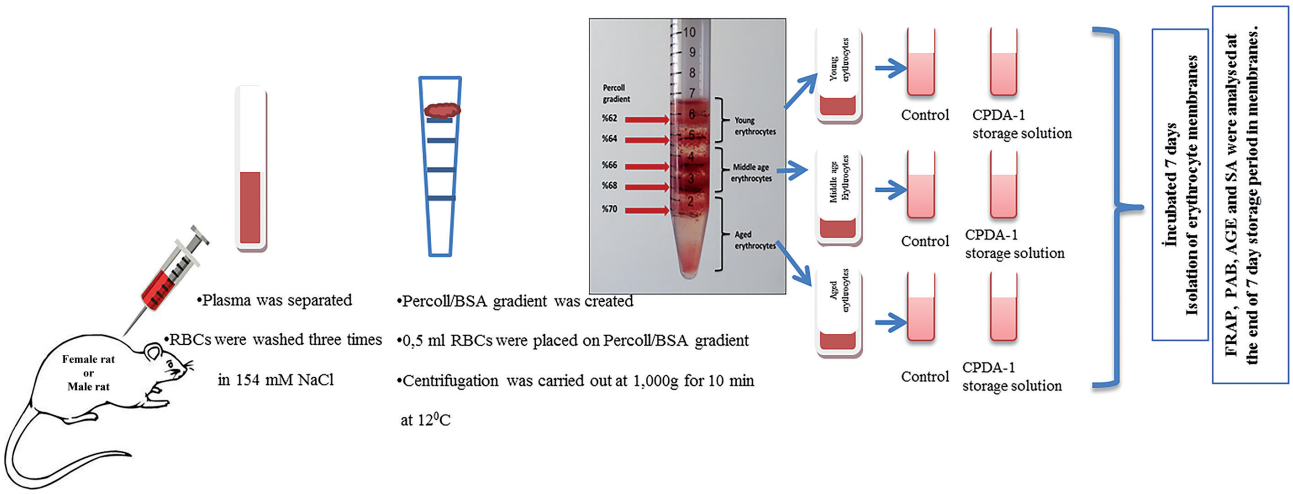

Figure 2. Experimental procedure and studied parameters. For more details see "Materials and Methods".

Erythrocyte membrane isolation

Erythrocytes fractions were hemolyzed by pipetting washed young, middle age and aged cell suspensions in tubes containing ice-cold hypotonic phosphate buffer (20 mOsm, pH 7.4). The contents were mixed gently and centrifuged at 20,000 × *g* for 40 min. The supernatant was decanted and the ghost (membrane) was re-suspended by adding the same strength buffer and ghosts were washed three times subsequent to hemolysis. During sample preparation erythrocyte membrane fractions were kept at +4°C in dim light. The membrane pellet was re-suspended in an isotonic buffer with 2% sodium dodecyl sulfate (SDS) and were divided into aliquots (one for each assay) and immediately stored at –80°C (eight-week maximum) for FRAP, PAB, prb-AGE and SA assays.

Analytical methods

Estimation of non-enzymatic antioxidant status: Ferric reducing antioxidant power assay

The FRAP assay was performed according to the protocol of Benzie and Strain (1999), with volumetric modifications. To prepare the FRAP solution at 37°C, 10 ml of acetate buffer (300 mM, pH = 3.6) was mixed with 1 ml of 20 mM $FeCl_3$ dissolved in distilled water and 1 ml of 10 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mM HCl. Next, 50 µl of membrane was added to 200 µl of a freshly prepared FRAP solution in each well of a 96-well plate and measured over a period of 4 min at 593 nm (BioTek US, Winooski, VT, USA). Different concentrations of uric acid (0.09–9.00 mM) were used to obtain a calibration curve on each day of the experiment. The coefficients of intra- and inter-assay variations were 4.1% ($n = 10$) and 5.3 % ($n = 10$), respectively.

Estimation of pro-oxidant-antioxidant status: Pro-oxidant-antioxidant balance assay

The PAB assay was performed according to the method of Alamdari et al. (2008) with volumetric modifications. The standard solutions were prepared by mixing varying proportions (0–100%) of 1 mM H_2O_2 with 3 mM uric acid (in 10 mM NaOH). Sixty milligrams of 3,3′,5,5′-tetramethylbenzidine (TMB) powder was dissolved in 10 ml absolute dimethyl sulfoxide (DMSO). To prepare the TMB cation, 400 µl of TMB/DMSO was added to 20 ml of acetate buffer (0.05 M buffer, pH 4.5). Subsequently, 70 µl of fresh chloramine-T (100 mM) solution was added to the above solution, mixed well and incubated for 2 hours at room temperature in the dark. Following this incubation, 25 U of peroxidase enzyme solution was added to the 20 ml TMB cation, dispensed in 1 ml and placed at –20°C. To prepare the TMB solution, 200 µl of TMB/DMSO was added to 10 ml of acetate buffer (0.05 M buffer, pH 5.8). The working solution was prepared by mixing 1 ml of the TMB cation with 10 ml of the TMB solution, followed by incubation for 2 minutes at room temperature in the dark. Working solutions were used immediately. A 10-µl aliquot of membrane solution, standard or blank (distilled water) was mixed with 200 µl of the working solution in each well of a 96-well plate, which was then incubated at 37°C for 12 min in the dark. Following the incubation time, 100 µl of 2 N HCl was added to each well, and the absorbance was measured using a microplate reader at 450 nm, with a reference wavelength of 620 or 570 nm. A standard curve was determined using the values obtained from the standard samples. PAB values are expressed in arbitrary units, which correspond to the percentage of H_2O_2 in the standard solution. The values of the unknown samples were then calculated based on the values obtained from the above standard curve. The coefficients of intra- and inter-assay variation were 5.0 % ($n = 20$) and 6.1% ($n = 20$), respectively.

Estimation of glycoxidation status: Protein-bound advanced glycation end products

Analyses of prb-AGEs were performed based on the modified spectrofluorimetric method according to Yanar et al. (2015). Fluorescent AGE-peptides present in membrane were measured by spectrofluorimetry (ex: 247 nm, em: 440 nm). Briefly, 20 µl membrane suspension was added to 480 µl of 0.15 mM trichloroacetic acid and 100 µl chloroform. Samples were shaken vigorously, centrifuged, and 200 µl of the aqueous layer was then transfer into the 96 well plate (BioTek US, Winooski, VT, USA). Samples were run in triplicate. The assay was calibrated against AGE-peptide obtained from enzymatic hydrolysis of AGE-BSA (10 g/l) based on to the method of Wrobel et al. (1997). The coefficients of intra- and inter-assay variations for protein-bound AGE assay were 4% ($n = 8$) and 5.3% ($n = 8$), respectively, and tested according to the assay protocol.

Sialic acid assay

Colorimetric quantitation of sialic acids in microscale was carried out using the resorcinol/Cu/HCl reagent as described by Spyridaki et al. (1996). Sialic acid level in membrane is reported in terms of µg/mg membrane protein.

Estimation of total protein content

Membrane protein levels were measured by a commercially available assay kit (Sigma, SL, USA, Cat no; TP0300). The

membrane pellet was re-suspended in an isotonic buffer with 2% SDS and were divided into aliquots (one for each assay) so the concentration of proteins in samples are the same.

Data processing

Data was analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, Calif) and presented as means ± SEM. Results obtained in different groups were compared using two-way analysis of variance and, when appropriate, post hoc analyses with Bonferroni tests. Differences were considered statistically significant at $p < 0.05$.

Results

FRAP activity of treated middle age erythrocytes were significantly higher in male rats compared to female rats ($p < 0.01$). FRAP activity of treated middle age erythrocytes in male rats were significantly higher than that of treated aged erythrocytes in male and female rats ($p < 0.01$ for both genders). Treated young erythrocytes have a metabolic tendency to show high FRAP activity when compared to aged erythrocytes. FRAP activity of the CPDA-1-treated middle age erythrocytes in male rats were significantly higher than non-treated control groups of both genders (comparisons of each gender were done exclusively within that gender; $p < 0.001$). Although CPDA-1-treated aged erythrocytes have more tendency for high FRAP activity when compared to non-treated aged control erythrocytes, FRAP activity of CPDA-1-treated young erythrocytes does not have any difference when compared to non-treated young control erythrocytes (comparisons of each gender were done exclusively within that gender) (Figure 3A).

Plasma membrane PAB levels of treated aged erythrocytes were significantly increased in male rats compared to female rats ($p < 0.01$). PAB levels of treated aged erythrocytes in male rats were significantly higher than both PAB levels of treated middle aged erythrocytes in male and female rats and also PAB levels of treated young erythrocytes in male and female rats ($p < 0.01$). PAB levels of CPDA-1-treated aged erythrocytes were significantly higher than PAB levels of non-treated aged control erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.01$). PAB levels of CPDA-1-treated middle aged erythrocytes were significantly higher than PAB levels of middle aged non-treated control erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.01$). PAB levels of CPDA-1-treated young and old erythrocytes were not significantly different than PAB levels of non-treated aged-matched control erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.01$) (Figure 3B).

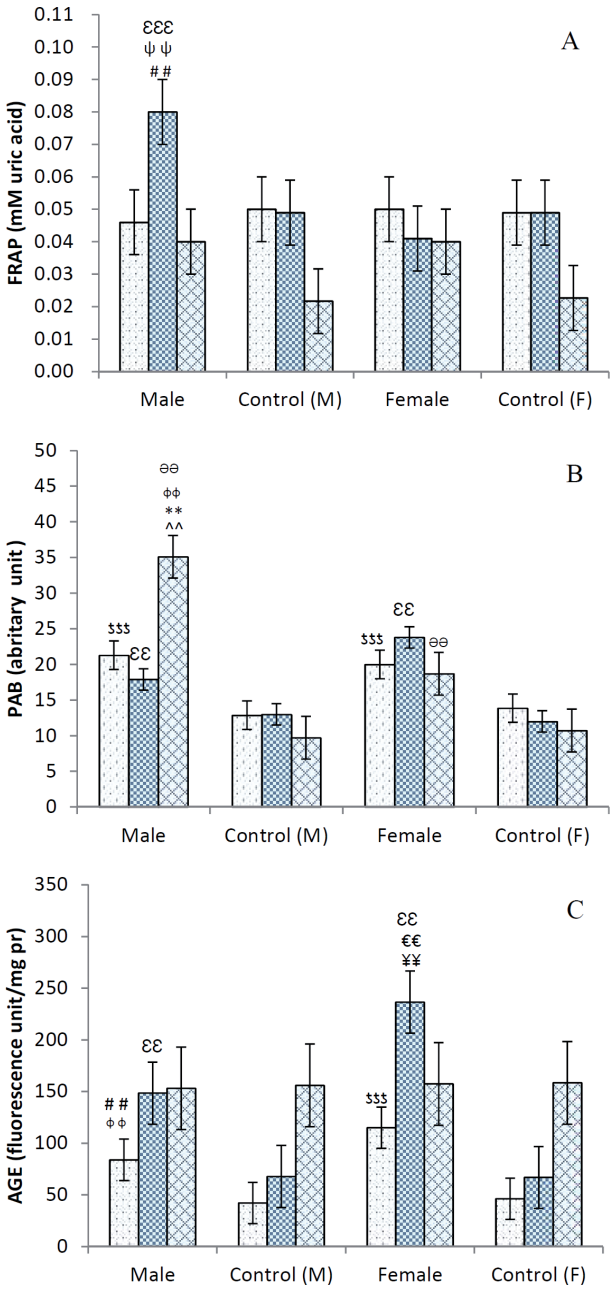

Figure 3. The result of erythrocyte membrane ferric reducing antioxidant power (FRAP, **A**), pro-oxidant-antioxidant balance (PAB, **B**) and advanced glycation end products (AGE, **C**) in experimental groups. $^{\Psi\Psi}$ $p < 0.01$ *vs.* treated middle aged erythrocytes of female group; $^{\#\#}$ $p < 0.01$ *vs.* treated aged erythrocytes of both gender; $^{\wedge\wedge}$ $p < 0.01$ *vs.* treated young erythrocytes of both gender; ** $p < 0.01$ *vs.* treated aged erythrocytes of female group; $^{\Phi\Phi}$ $p < 0.01$ *vs.* treated middle aged erythrocyte of both gender; $^{¥¥}$ $p < 0.01$ *vs.* treated middle aged erythrocytes of male group; $^{€€}$ $p < 0.01$ *vs.* treated young erythrocytes of both gender group; $^{\varepsilon\varepsilon\varepsilon}$ $p < 0.001$ *vs.* middle aged erythrocytes of non-treated control group; $^{\partial\partial}$ $p < 0.01$ *vs.* aged erythrocytes of non-treated control group; $^{\$\$\$}$ $p < 0.001$ *vs.* young erythrocytes of non-treated control group.

prb-AGEs levels of plasma membrane of treated middle age erythrocytes were higher in female rats compared to male rats ($p < 0.01$). prb-AGEs levels of treated middle aged erythrocytes in female rats were significantly higher than both prb-AGEs levels of treated aged erythrocytes in male and female rats and also prb-AGEs levels of treated young erythrocytes in male and female rats. prb-AGEs levels of treated young erythrocytes in male rats were significantly lower than both prb-AGE levels of treated middle aged erythrocytes in male and female rats and treated aged erythrocytes in male and female rats ($p < 0.01$). prb-AGEs levels of CPDA-1-treated middle aged erythrocytes were significantly higher than AGE levels of middle aged non-treated control erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.01$). prb-AGEs levels of CPDA-1-treated young erythrocytes in female rats were significantly higher than prb-AGEs levels of young non-treated control erythrocytes ($p < 0.001$). Although CPDA-1-treated young erythrocytes have more tendency for high prb-AGEs level when compared to non-treated young control erythrocytes, prb-AGEs level of CPDA-1 -treated aged erythrocytes does not have any difference when compared to non-treated aged control erythrocytes (comparisons of each gender were done exclusively within that gender) (Figure 3C).

Sialic acid levels of male and female rats were found to be decreased in treated aged erythrocytes fraction when compared to corresponding treated young fractions ($p < 0.05$ for both genders). Although there was a trend toward lower SA in treated middle aged erythrocytes which were not found to be significantly lower when compared with those in treated young erythrocytes ($p > 0.05$ for both genders). SA levels of aged non-treated control erythrocytes were significantly higher than SA levels of CPDA-1-treated aged erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.01$). Sialic acid levels of middle aged non-treated control erythrocytes were significantly higher than SA levels of CPDA-1-treated middle aged erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.001$). SA levels of young non-treated control erythrocytes were significantly higher than SA levels of CPDA-1-treated young erythrocytes (comparisons of each gender were done exclusively within that gender; $p < 0.001$) (Figure 4).

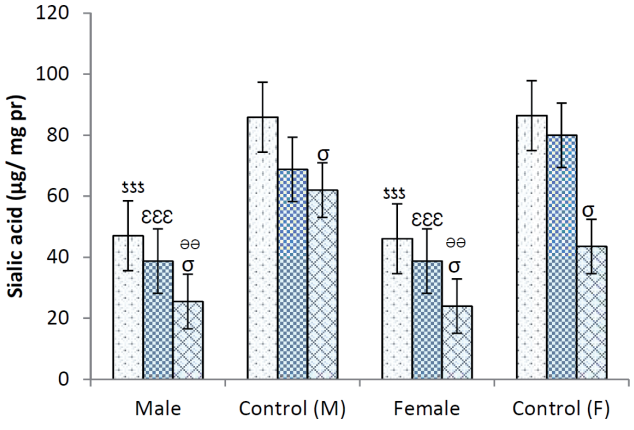

Figure 4. Erythrocyte membrane sialic acid levels of male and female rats. $^{\sigma}p < 0.05$ *vs.* treated young erythrocytes of both gender, $^{\varepsilon\varepsilon\varepsilon}p < 0.001$ *vs.* middle aged erythrocytes of non-treated control group, $^{\theta\theta}p < 0.01$ *vs.* aged erythrocytes of non-treated control group, $^{\$\$\$}p < 0.001$ *vs.* young erythrocytes of non-treated control group).

Discussion

Erythrocyte senescence in health, disease and blood-bank storage condition is of considerable interest for numerous applications. Erythrocyte membrane components undergo progressive oxidative modifications during aging process and blood-bank storage period. Lipid peroxidation, protein oxidation, oxidative modifications of plasma membrane ATPases, and glucose transporters are implicated in storage lesions and also aging process.

FRAP assay measures the ferric reducing ability of the biological sample which is using ferric iron as a redox sensor. Urate, ascorbate, tocopherol and proteins contribute significantly to the total free radical trapping capacity of biological samples (Benzie and Strain 1999; Cebe et al. 2014). FRAP activity of the CPDA-1-treated middle age erythrocytes in male rats were found to be significantly higher than non-treated control groups of both gender. We also found that membrane FRAP activity of treated middle age erythrocytes were significantly higher in male rats compared to treated female rats. Additionally, it was also found that FRAP activity of middle age erythrocytes in male rats were significantly higher than that of aged erythrocytes in male and female rats. Erythrocytes play a crucial role in recycling ascorbate in systemic circulation. The plasma membrane redox system (PMRS) is currently known to incorporate ascorbate free radical reductase which responsible to reduce extracellular ascorbate free radical to ascorbate using electrons derived from intracellular ascorbate through PMRS (Pandey and Rizvi 2010). The action of erythrocyte PMRS and ascorbate free radical reductase provides a mechanism for recycling of ascorbate between intracellular and extracellular compartments (Pandey and Rizvi 2010). Ascorbate has also been shown to protect erythrocyte plasma membrane and other hydrophobic compartments from oxidative damage by regenerating the antioxidant form of tocopherol (Pandey and Rizvi 2010). We are of the conviction that the increased FRAP activity determined in plasma membrane of middle age erythro-

cytes of male rats may be related to higher PMRS activity, as the non-radical ascorbate and tocopherol levels in their plasma membrane were increased.

Glucose derived from dextrose may cause protein glycation, glyoxidative damage, and the formation of prb-AGEs. Erythrocytes are incubated with dextrose containing CPDA-1: Lipid peroxidation and glyoxidative stress are expected to be increased. CPDA-1 application increases glyoxidative stress without modifying FRAP activity in membranes of young erythrocytes. On the other hand, CPDA-1 application does not cause higher glyoxidative stress but leads to increase in FRAP activity in aged erythrocytes. FRAP activity of young erythrocytes was higher than that of aged erythrocytes and glyoxidative stress levels of aged erythrocytes were higher than of young erythrocytes. This shows that young erythrocyte FRAP activity and aged erythrocyte glyoxidative stress have reached saturation plateau.

We found that PAB levels of CPDA-1-treated groups of erythrocytes of different ages were significantly higher than PAB levels of non-treated controls. CPDA-1 is known as citrate containing preservative (van de Wier et al. 2013; Young and Olutoyin 2015). Recently, it was concluded that citrate does not have an effect on oxidative stress directly, but citrate promotes and aggravates ROS formation caused by hydrogen peroxide formation in cell. Hydrogen peroxide is a non-radical ROS by itself and relatively non-reactive. However, in the iron–citrate complex, iron apparently is more susceptible to react with hydrogen peroxide and leads to the increased formation of the highly reactive hydroxyl radical in the Fenton reaction (van de Wier et al. 2013). It has been well known, that hydrogen peroxide, generated during the autoxidation of oxyhemoglobin, is the main source of hydroxyl radicals in senescence erythrocytes (Nagababu et al. 2003). Our current results showed that citrate containing CPDA-1 has a negative effect on redox status of the erythrocytes with different age groups and leads to higher oxidative stress. The role of impaired PAB during blood bank storage, and their relationship to erythrocyte injury, is an area that requires more attention. We propose the measurement of PAB as a reliable marker to estimate the degree of oxidant-mediated membrane damage in senescence erythrocytes and to predict the potential efficacy of storage strategies aimed at reducing such an oxidative stress. In blood banks, PAB can be used as a fast, easy, reliable and cost effective parameter for the estimation of biological/functional age of erythrocytes under oxidative stress.

PAB is a state of delicate balance established under conditions of homeostasis between free radicals that are formed and those scavenged. For the precise estimation of the PAB, the simultaneous determination of both the oxidant and the antioxidant status needs to be performed. Many analytical methods have been developed that can estimate separately the total prooxidants and antioxidant capacities, but no one is for PAB. Therefore, for estimation of prooxidant/antioxidant balance, the only way is to perform two separate redox assays. It was also reported that PAB values showed a linear variation against a changes in series of oxidants (hydrogen peroxide, tert-buthylhydroperoxide, chloramine-T and HClO) and antioxidants (ascorbic acid, trolox, glutathione, uric acid, bilirubin, albumin and ceruloplasmin) (Alamdari et al. 2007). PAB values are also known to be significantly and linearly associated with the levels of various oxidative stress parameters such as protein carbonyl groups, advanced glycation end products and advanced oxidative protein products (Mohammadi et al. 2014). Although there is no study on the link of PAB to plasma membrane redox status, our current results related to PAB are consistent with the results of other studies in which impaired redox homeostasis has been studied during erythrocyte senescence (Pandey and Rizvi 2013; Kumar and Rizvi 2014; Mohammadi et al. 2014). Persistent oxidative stress related to higher PAB and decreased antioxidant defense observed in plasma membrane of senescence erythrocytes obtained from male rats may contribute to enhanced susceptibility for oxidative damage. It has thus been found that gender indeed affects the age-related oxidation of plasma membrane constituents but the extent of the underlying mechanisms affecting membrane redox homeostasis and its etiology are still obscure.

prb-AGEs are reliable oxidation biomarkers for the accurate estimation of early glycation/glyoxidation steps of cellular proteins (Cebe et al. 2014; Yanar et al. 2015). Therefore, we preferred to assay prb-AGEs for the evaluation membrane-bound protein redox status of aging erythrocytes. prb-AGEs are the products of oxidatively modified proteins which are nonenzymatically reacting with aldose sugars (Cebe et al. 2014; Yanar et al. 2015). Early glycation and oxidation processes initiate with the formation of Schiff bases and Amadori products. Further glycation reaction steps of proteins cause intramolecular rearrangements that lead to the generation of prb-AGEs. During Amadori rearrangement process, reactive carbonyl compounds known as α-dicarbonyls or oxoaldehydes are also accumulated. Such build up is referred to as "carbonyl stress." The α-dicarbonyl compounds have the ability to react with amino, thiol, and guanidine functional groups in cellular proteins (Cebe et al. 2014; Yanar et al. 2015).

prb-AGE levels of CPDA-1-treated groups of erythrocytes of different ages were found to be significantly higher than prb-AGE levels of non-treated controls. There may be also an association between increased erythrocyte prb-AGE and dextrose content of CPDA-1 solution. It is clearly seen that the increase in prb-AGEs is sharper when compared to male groups. Estrogens play an important role within the antioxidant defense systems in systemic

circulation (Topçuoglu et al. 2009). Estrogens may also actively participate in the regulation of membrane function. In an *in vitro* study, Tsuda et al. (2001a) showed that 17β-estradiol, estrone, and estriol increased the membrane fluidity of erythrocytes and improved the rigidity of cell membranes *via* the NO- and cGMP-dependent mechanism not only in females but also in males. One hypothesis is that the membrane action of estrogens could be one of the mechanisms responsible for their beneficial effects on the rheologic properties of erythrocyte membranes (Tsuda et al. 2001a, 2001b). The tendency of prb-AGEs to increase in plasma membrane of middle aged erythrocytes of female rats may be related to adaptive response to cyclic variations in systemic estrogen levels.

SAs are substituted neuraminic acid derivatives which are mostly found at the outermost end of glycan chains on the plasma membrane in erythrocytes. The role of erythrocyte membrane SA during erythrocyte senescence has been clarified; however, the exact relationship between SA and oxidative damage is not fully understood (Mehdi et al. 2012). There is a significant biological relevance of erythrocyte surface SA, remaining intact for plasma membrane structure, shape, and erythrocyte survival (Huang et al. 2011). The loss of SA residues during erythrocyte senescence has already been widely reported and has been hypothesized to contribute to the removal of erythrocyte from the systemic circulation at the end of its life span (Mehdi et al. 2012). The increase in ROS formation and inflammatory mediators during aging process has been linked to the higher levels of advanced glycation end products (AGE's) (Tsuda et al. 2001b). The increase in ROS formation during aging process causes increased SA removal from erythrocyte membrane which may lead to altered biophysical property of the membrane affecting many enzymatic and transporter activities (Peppa et al. 2008; Mehdi et al. 2012). Prooxidant factors such as PAB and prb-AGEs may also contribute to our observed changes in SA level during aging. The SA results of the current study suggest that in oxidatively-induced erythrocyte aging, there is an association between desialylation process and increased oxidation of plasma membrane components that leads to impaired redox homeostasis in various age groups of erythrocyte subpopulations.

Conclusions

In 1978 a novel solution containing adenine (CPDA-1) was introduced to permit extension of erythrocyte shelf life from 21 to 35 days. The success of CPDA-1 and the high percentage of blood units processed into components (estimated 87% in 1983) have stimulated a burst of research and development activity to develop improved erythrocyte preservation systems (Moore 1987). The current study is novel and a nominee to be the first experimental study in the literature, implying the effect of CPDA-1 storage solution on redox status changes in plasma membrane of aging erythrocyte subpopulations obtained from different sex. This study clearly reveals that changes in erythrocyte membrane redox status during blood bank storage condition also depend on both gender-depended homeostatic factors and also the content of CPDA-1 storage solution. During the storage period, erythrocytes from the male donors are mostly affected by free radical-mediated oxidative stress but erythrocytes obtained from females are severely affected by glyoxidative stress. However, male donors could be better choice for CPDA-1.

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