BLOOD COMPONENTS AND REGENERATIVE MEDICINE

Original article

Cigarette smoking and antioxidant defences in packed red blood cells prior to storage

Renata E. Boehm[1,2], Sabrina N. Do Nascimento[3], Carolina R. Cohen[2], Solange Bandiera[1], Rianne R. Pulcinelli[1], Almeri M. Balsan[2], Nuryan S. Fao[3], Caroline Peruzzi[3], Solange C. Garcia[3], Leo Sekine[2], Tor G.H. Onsten[2], Rosane Gomez[1]

[1]*Post-graduate Progamme in Pharmacology and Therapeutics, Universidade Federal do Rio Grande do Sul (UFRGS);*
[2]*Haemotherapy Unit of the Hospital de Clínicas de Porto Alegre (HCPA);*
[3]*Toxicology Laboratory, Department of Analyses, (LATOX), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil*

Background - Red blood cells from smoking donors can have more lesions from oxidative stress, decreasing the benefits of blood transfusion. We aimed to explore the effect of cigarette smoking on the oxidative status of packed red blood cells (PRBCs) prior to storage.

Materials and methods - We compared serum vitamin C, plasmatic malondialdehyde (MDA), and non-protein thiol groups (GSH) levels in PRBCs, as well glutathione peroxidase (GPx) and glutathione s-transferase (GST) activity in PRBCs from smoking (n=36) and non-smoking (n=36) donors. We also correlated urinary cotinine levels with these parameters.

Results - Cigarette smoking was associated with decreased serum levels of vitamin C and GPx, and increased GST activity in PRBCs. We found negative correlations between cotinine, GPx activity and vitamin C levels, and a positive correlation between cotinine and GST activity.

Discussion - Cigarette smoking changed antioxidant defences of PRBCs prior to storage and these parameters are correlated with cotinine levels. Increased RBC antioxidants such as GST may reflect an exposure to oxidants during erythropoiesis. Because of the inability of mature RBCs to resynthesise antioxidants, PRBCs from smokers may have higher risk of storage lesions than those from non-smoker donors.

Keywords: *blood donation, cotinine, oxidative stress, tobacco, vitamins.*

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INTRODUCTION

Red blood cell (RBC) transfusion is one of the most common and valuable life-saving treatments in many areas of modern medicine[1,2]. The functional role of RBCs is to transport oxygen from the lungs to the tissues, providing all cells with the required oxygenation. Thus, RBC transfusion is used for treating acute anaemia due to massive haemorrhage or chronic anaemia secondary to bone marrow dysfunction[3,4]. Red cells are typically administered as a concentrate, called packed red blood cells (PRBCs), that maintain cell viability for up to 35-42 days under refrigerated storage, depending on the anticoagulant and preservative solution used[1,5].

It is well known that RBCs accumulate some metabolic, biochemical, and morphological lesions during storage[2,6,7]. *In vivo,* RBCs are continuously exposed to both endogenous

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Correspondence: Renata E. Boehm
e-mail: rboehm@hcpa.edu.br

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and exogenous sources of reactive oxygen species (ROS) that can damage them and impair their functionality. To minimise the effect of these ROS and the resultant oxidative stress, RBCs preserve an antioxidant system involving both non-enzymatic low molecular weight antioxidants like glutathione (GSH), the most abundant cellular component of non-protein thiols, and ascorbic acid (vitamin C)[3,8]. RBCs also have enzymatic antioxidant defences, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione transferase (GST), and peroxiredoxin-2 (PRDX-2)[3,8]. Because RBCs have a limited capacity to restore damaged elements (due to loss of protein expression and nuclei during erythropoietic maturation), they are completely dependent on antioxidant defensive components over their lifespan[9]. RBC storage lesions include ATP and 2,3-diphosphoglycerate (2,3-DPG) depletion, exhaustion of endogenous antioxidant defences, subsequent oxidation of haemoglobin, and leakage of lactate, lactate dehydrogenase (LDH), haemoglobin, and potassium/calcium ions into the suspending medium[2]. Moreover, membrane microvesiculation, as well as reversible and irreversible morphological changes occur[10-12]. These changes can lead to rapid clearance of RBCs from the patient's bloodstream, reducing the beneficial effect of the transfusion[6]. Damaged RBCs could also be associated with deleterious side effects, such as toxicity/inflammation induced by haemolysis and free iron, as well as other adverse events, including a higher infection risk and a higher transfusion-dependent mortality rate[6,13,14]. A major contributor to RBC storage lesions is oxidative stress, which progressively increases over time due to consumption of endogenous antioxidants, which promotes oxidative lesions to proteins (carbonylation, fragmentation, denatured haemoglobin) and lipids (peroxidation)[12]. Recent studies have shown that PRBCs that are at least 30 days or more old are associated with an increased risk of mortality compared to PRBCs of less than 10 days in hospitalised high-risk patients[15,16]. Besides storage time, donor-to-donor variations due to low serum uric acid levels, genetic factors, sex, and social habits, such as physical activity, sleeping, diet, and smoking, could increase oxidative lesions and affect RBC quality[6,7,17].

Cigarette smoke is rich in carcinogenic and mutagenic molecules, free radicals, and heavy metals, which generate ROS and reactive nitrogen species (RNS) that readily react with biomolecules, causing DNA injury and lipid peroxidation in tissue[18]. Although smoking has declined over the years in Brazil as a result of several initiatives to control the habit, the percentage of smokers in the general population is approximately 14.7%[19,20]. In a recent study, we showed that 5.9% of all blood donations were from smokers[21]. There are no current restrictions on smoking blood donors[22,23]. Moreover, the effects of cigarette exposure on the quality of donated blood have not been extensively explored. A study shows that serum acid uric levels, that are associated with an elevated RBC storage lesion and ROS accumulation, is lower in >50% of PRBCs donated by smokers[7].

Considering that oxidative stress leads to lesions in RBCs and cigarette smoking can lead to oxidative stress, we aimed to explore the influence of smoking on the oxidative status of PRBCs before storage by analysing some oxidative parameters and their association with cotinine, the main biomarker of cigarette exposure.

MATERIALS AND METHODS

Study population

This observational, paired case-control study was conducted from March to June 2017 at the Hospital de Clínicas de Porto Alegre (HCPA) Blood Bank in Porto Alegre, Rio Grande do Sul, Brazil. The study included donors of both sexes, >18 years of age, who admitted to having a smoking habit during compulsory clinical screening, and who were matched with non-smoker donors. Although tobacco smoking is not included in the Brazilian clinical screening protocol for blood donation[23], we added this question to our service's routine questions to donors based on the results of a previous study by our group[21]. Blood donors reporting an average consumption of at least 20 cigarettes per day were included as the exposed group. Informed consent was provided after the aims and benefits of this study had been explained to them. Non-smoking donors (n=36), matched for age and gender with the exposed group (n=36), were invited to participate as controls, following the same research protocol. After providing informed consent, an additional interview with the donors was conducted to investigate socioeconomic characteristics, health status, and lifestyle. The exposed group were asked additional questions about

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smoking, such as the number of cigarettes smoked per day and time of abstinence prior to donating blood. The average smoking load (pack years) in the exposed group was calculated as the number of cigarettes smoked per day, divided by 20 (number per pack) and multiplied by years smoking.

Samples from donors who reported exposure to an environment polluted by cigarette smoke (second-hand smokers) or smoke from wood, coal, automobiles, machinery, or other sources of trace elements were excluded. The sample size was calculated using data from our blood donor population based on changes in carboxyhaemoglobin concentration in smokers and non-smokers[21]. With a significance level of 5% and a statistical power of 90%, considering a 3.3% and 1.1% standard deviation of carboxyhaemoglobin concentrations between cases and controls, respectively, with a minimum assumed difference of 2%, 34 smokers and 34 non-smokers were required (WinPepi, v. 11.43, freeware computer programs). This study was approved by the Universidade Federal do Rio Grande do Sul Ethics Committee and the HCPA Ethics Committee and was registered on the Brazil Platform (CAAE 55786016.0.0000.5347).

Sample collection

Prior to blood donation, approximately 10 mL samples of urine were collected in polyethylene bottles and stored at –80 °C until cotinine and creatinine analysis had been completed. Total blood samples were collected into Vacutainer™ tubes (BD, Franklin Lakes, NJ, USA) from all participants by the established venipuncture technique during routine blood donation. Two tubes of 4 mL were collected, one without anticoagulant, which provided serum for determining vitamin C, and another containing an anticoagulant (ethylenediaminetetraacetic acid [EDTA]), providing plasma to determine MDA. Both tubes were centrifuged at 1,500 *g* for 10 min at room temperature, and aliquots were immediately analysed.

An additional sample was obtained from the original PRBC immediately after the whole blood bag was centrifuged (24 °C and 2,016 *g* for 5 min [KR4i; Thermo Scientific, Waltham, MA, USA]) to separate the components (Giotto; Delcon, Arcore, Italy) not receiving any treatment (irradiation or leucoreduction). A 50 mL sample was carefully removed from the original CPDA-1 bag (JP Farma, Ribeirão Preto, Brazil) through a sterile connection (TCD; Haemonetics, Braintree, MA, USA) and transferred to a paediatric transfusion bag with the same characteristics. The remaining original CPDA-1 bags were stored for transfusion purposes. Samples were taken from the paediatric transfusion unit by a single puncture and aliquoted in cryogenic vials (2 mL) for immediate determination of non-protein thiol groups. Additional aliquots were stored at –80 °C for GPx and GST analysis.

Urinary cotinine levels

Cotinine levels were determined in urine samples using high-performance liquid chromatography (Shimadzu®, Kyoto, Japan) and a UV detector (HPLC-UV), adapted from Cattaneo *et al.*[24]. The separation was achieved with a reversed phase C18 column. The mobile phase was a mixture of Milli-Q® (Merck KGaA, Darmstadt, Germany) water/methanol/sodium acetate 0.1 mol L^{-1}/acetonitrile (50:15:25:10, v/v), adding 1 mL of citric acid 0.034 mol L^{-1} and 5.0 mL of trimethylamine for each litre of solution, adjusting to pH 4.4. The flow rate was maintained isocratically at 0.5 mL min^{-1}. The absorbance of the eluent was monitored at 260 nm; the total run time was 10 min. 2-Phenylimidazole was used as internal standard. The detection limit of the method was 5.0 ng mL^{-1}. For the sample, 25 µL 10 M NaOH and 100 µL 2-phenylimidazole (1.0 µg mL^{-1}), used as internal standard, were added to 2 mL previously centrifuged urine. Extraction was then performed with 4.0 mL dichloromethane and a rotatory mixer for 40 min, followed by centrifugation at 300 *g* for 15 min. Two mL of organic phase were dried under compressed air at ambient temperature. Then, 200 µL of mobile phase was added, and 20 µL was injected into the HPLC. Cotinine assays were performed in duplicate, and cotinine levels were expressed as ng mL^{-1}. Cotinine levels were also adjusted according to urinary creatinine excretion and were expressed as µg g^{-1} creatinine.

Creatinine concentration

Urinary creatinine concentration was determined by spectrophotometry according to Jaffe (2009)[25] with commercial kits (Doles reagents, Goiânia, GO, Brazil). These results were used to adjust cotinine values.

Oxidative status of packed red blood cells

Serum vitamin C levels

Serum vitamin C concentration was determined according to Baierle *et al.*[26] by HPLC-UV, Knauer (Berlin, Germany)

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using tris[2-carboxy-ethyl]phosphine hydrochloride as a reducing agent. First, the sample was deproteinised with perchloric acid 10% (v/v) and the supernatant obtained after centrifugation was injected into the chromatographic system. The vitamin C concentration results were expressed as mg/L.

Plasma malondialdehyde levels

Lipid peroxidation was quantified by analysing plasma malondialdehyde (MDA) levels using HPLC-UV, according to a method previously developed by Grotto *et al.*[27]. The MDA levels were expressed as µM.

Non-protein thiol groups

An aliquot (1 mL) from the PRBCs was added to Triton X-100® (Union Carbide, Danbury, CT, USA) for haemolysis and then precipitated with 20% trichloroacetic acid (w/v). After centrifugation, the supernatant aliquots were reacted with 10 mM of 5,5-dithiobis(2-nitrobenzoic acid), and the non-protein thiol groups levels were determined in PRBCs spectrophotometrically at 412 nm, as described by Ellman[28]. The results were expressed as [mM]*(Ht/Hb).

Glutathione peroxidase activity

The enzymatic activity of GPx was determined in 500 mL of PRBCs. This method is based on the oxidation of NADPH, which can be measured as the decrease in absorbance at 340 nm[29]. The results were expressed as µmol NADPH/min/mg protein.

Glutathione S-transferase activity

Glutathione S-transferase activity was determined in 500 µL of PRBCs using CDNB (1-chloro-2,4-dinitrobenzene) as substrate and 0.15 M GSH, according to Habig *et al.*[30]. GST activity was expressed as U/mg protein.

Statistical analysis

The Shapiro-Wilk test was used to determine the normality of the variables. Normally distributed data were analysed using Student's *t*-test for independent samples. Non-parametric data were analysed using the Mann-Whitney test. The χ^2 test was used to compare categorical variables between groups. The Spearman correlation test was used to determine the degree of association between the different parameters. The results were shown as mean±standard error of the mean or as the median±interquartile intervals, depending on the test type. The significance level was set at 0.05. The statistical analyses were performed in SPSS 18.0 (IBM Company, Armonk, NY, USA).

RESULTS

General sample characteristics

The sample consisted of 36 smoking blood donors, who were matched by age and gender with non-smoking donors. The general characteristics are shown in **Table I**. Smoking donors were mainly male, aged 41.3±13.8 years, with a smoking history of 34.1±27.5 pack-year and regular blood donation. According to the demographic data, most donors were single (55%), employed (64%), sedentary (75%), overweight (58%), had a high school education (53%), and lived in Porto Alegre (78%). Differences between groups were found in education and marital status, as well as regular physical activity and body mass index. Although we found a higher frequency of regular physical activity

Table I - *Demographic characteristics of smoking (n=36) and non-smoking (n=36) donors at the blood bank of the Hospital de Clínicas de Porto Alegre, Brazil*

	Non-smoking % (n)	Smoking % (n)	p
Male	66.7 (24)	66.7 (24)	1.000
Age, years	41.3±13.8	41.3±13.8	0.993
Smoking history, packs/year	-	34.1±27.5	-
Blood donation frequency			
First time	13.9 (5)	30.5 (11)	0.216
>3 times	16.7 (6)	16.7 (6)	
>3 times	69.4 (25)	52.8 (19)	
Education level[b]			
Elementary school	5.6 (2)	33.3 (12)	**0.001**
High school	47.2 (17)	52.8 (19)	
College	47.2 (17)	13.9 (5)	
Work activity	75.0 (27)	63.9 (23)	0.443
Marital status			
Single[a]	27.8 (10)	55.6 (20)	**0.031**
Married	72.2 (26)	44.4 (16)	
Regular physical activity	58.3 (21)	25.0 (9)	**0.009**
Body mass index			
Normal	16.7 (6)	41.7 (15)	**0.038**
Overweight	83.3 (30)	58.3 (21)	
Vitamin supplementation	11.1 (4)	5.6 (2)	0.670
Place of origin			
Porto Alegre	86.1 (31)	77.8 (28)	0.540
Other cities	13.9 (5)	22.2 (8)	

[a]Considering complete and incomplete education. [b]Includes single, widowed and divorced donors. Data are presented as mean±standard deviation for continuous variables and absolute value and percentage for categorical variables. Bold font indicates significant differences.

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among the non-smokers than the smokers (58 *vs* 25%, respectively), overweight donors were more prevalent among non-smokers, and neither group regularly took vitamin supplements.

Urinary cotinine levels

Urinary cotinine levels were used as a biomarker of cigarette smoke exposure, and cotinine levels were significantly higher among smokers (p<0.001) (**Figure 1**). Additionally, when cotinine levels were adjusted by urinary creatinine excretion, the difference was still significant: 2.02 (1.14, 2.74) *vs* 0.08 (0.05, 0.13) ng g^{-1} creatinine (p<0.001) for smoking and non-smoking donors, respectively.

Exogenous and endogenous antioxidants and lipid peroxidation biomarkers

We quantified vitamin C, an exogenous non-enzymatic antioxidant, and the serum levels were significantly lower among smokers than non-smokers (p<0.001) (**Figure 2**). Additionally, endogenous antioxidant biomarkers (non-protein thiol groups, GST, and GPx activity) were analysed in PRBCs. There were no differences in levels of non-protein thiol groups between groups (p=0.068) (**Figure 3A**). On the other hand, enzymes that use non-protein thiol groups in their antioxidant mechanisms were significantly different. GST levels were higher (**Figure 3B**) and GPx levels were lower (**Figure 3C**) in smokers than non-smokers (p<0.001). Despite significant differences in endogenous (GST and GPx) and exogenous (vitamin C) antioxidant levels between groups, we found no significant difference in MDA, a biomarker of lipid peroxidation, between non-smoking (9.3 [5.35, 13.64] μM) and smoking (9.8 [5.52, 11.40] μM) donors (p=0.956). Additional statistical analysis showed a negative correlation between cotinine, a biomarker of nicotine exposure, and both GPx activity (r= −0.693; p<0.001) (**Figure 4A**) and vitamin C levels (r=0.338; p=0.004) (**Figure 4B**). In addition, a positive correlation was found between cotinine levels and both GST activity (r=0.327; p=0.005) (**Figure 4C**) and non-protein thiol groups levels (0.268; p=0.023) (**Figure**

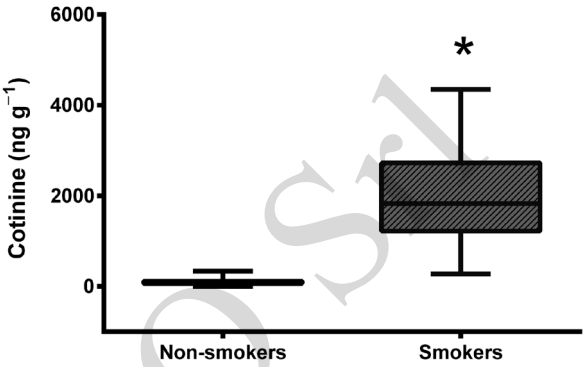


Figure 1 - Urinary cotinine levels of non-smoking (n= 36) and smoking (n=36) donors.
Data are expressed as median±interquartile intervals. Mann-Whitney test. *Different from non-smokers (p<0.001).

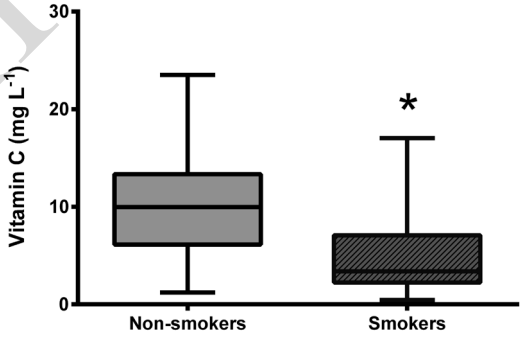


Figure 2 - Serum vitamin C levels of non-smoking (n=36) and smoking (n=36) donors.
Data are expressed as median±interquartile intervals. Mann-Whitney test. *Different from non-smokers (p<0.001).

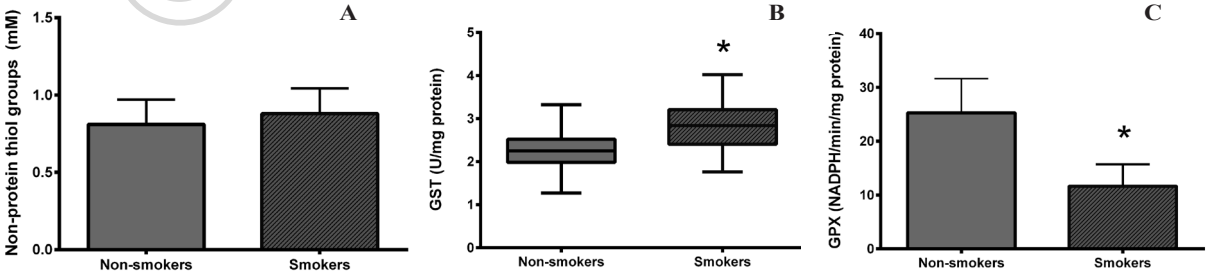


Figure 3 - Endogenous antioxidant biomarkers in packed red blood cells of non-smoking (n=36) and smoking (n=36) donors.
(A) Non-protein thiol group levels are shown as mean standard deviation/*t*-test. (B) Glutathione s-transferase (GST) levels are shown as median±interquartile intervals/Mann-Whitney test. (C) Glutathione peroxidase (GPX) levels are shown as mean±standard deviation/*t*-test. *Different from non-smokers (p<0.001).

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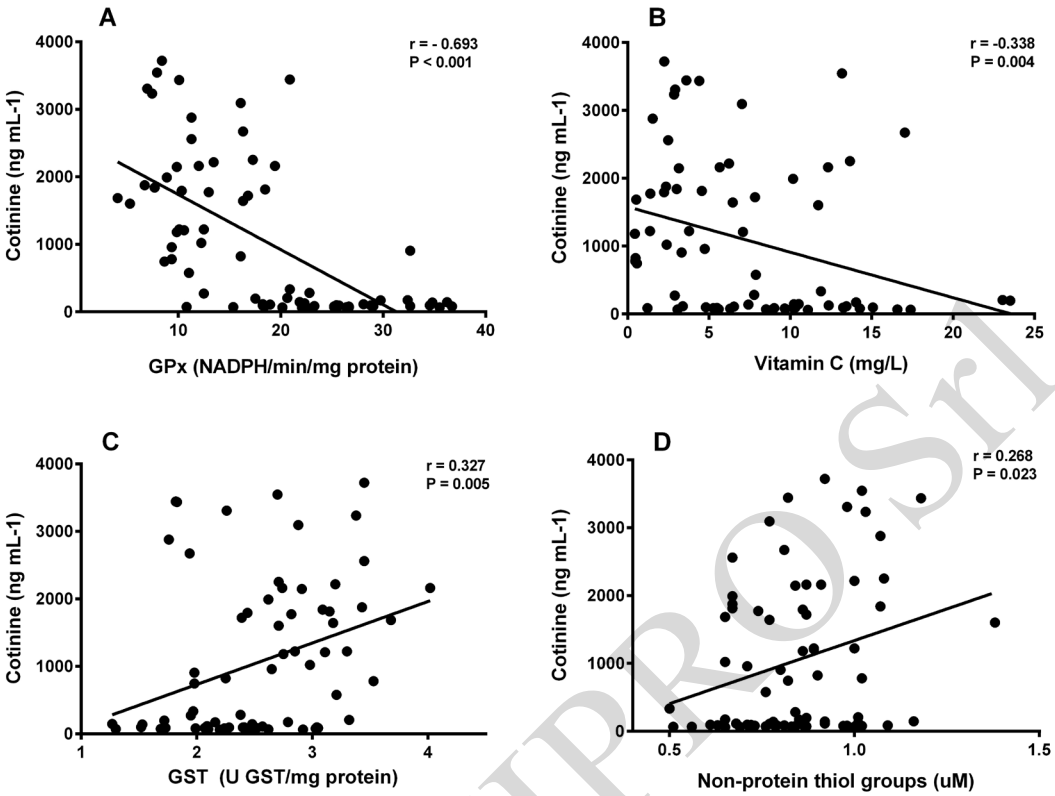


Figure 4 - Spearman's rank correlations between urinary cotinine levels (ng mL^{-1}).
(A) Glutathione peroxidase (GPx) activity in packed red blood cells (PRBCs) (µmol NADPH/min/mg protein). (B) Serum vitamin C levels (mg/L). (C) GST activity in PRBCs (U/mg protein) and (D) non-protein thiol groups PRBC levels ([mM]*(Ht/Hb) (n=72). Ht: haematocrit; Hb: haemoglobin.

4D), which corroborated the effects of cigarette smoking on PRBCs antioxidant behaviour.

DISCUSSION

In this study, we aimed to determine whether cigarette smoking could affect the oxidative status of PRBCs prior to storage. Our results showed that smoking was associated with lower serum levels of exogenous (vitamin C) antioxidants and changed RBCs endogenous antioxidant enzyme (GST and GPx) levels.

The demographic characteristics of smoking and non-smoking donors in this study match those of previous studies[21,31]. We also found that the vitamin supplementation is uncommon among blood donors. Despite the lower physical activity and lack of vitamin supplementation in the smoking group, smoking could be directly responsible for our findings, since antioxidants could be expended in response to oxidative stress from cigarette toxins and even vitamin C absorption could be impaired[32].

Storage lesions in PRBCs have been widely discussed in haemotherapy, but it is unclear whether and to what extent these lesions impair the safety and effectiveness of blood transfusions[33]. Variability in donated units arises from biological (donor) and technical factors[10,34]. The sustained production of reactive free radicals from the tar and gas phases of cigarette smoke causes oxidant stress on circulating erythrocytes[35]. Additionally, because storage lesions have been associated with increased risk of mortality[16], Tzounakas *et al.*[7] conducted studies to identify biomarkers that could predict susceptibility to storage lesions. They found an inverse correlation between serum uric acid levels, ROS accumulation, and storage lesions in PRBCs[7]. They also found that 50% of the PRBC from smoker donors had lower uric acid levels, making these bags more susceptible to storage lesions[7]. Although we did not measure the uric acid levels from our smoker donors, we may suppose that the lowest acid uric levels and the

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limited antioxidant reserves in RBCs may increase the risk of storage lesions and haemolysis in these bags.

Indeed, biological factors can affect not only a range of physiological properties in stored cells but are also the most significant contributing factors to in-bag haemolysis and RBC recovery after transfusion[2]. It was been demonstrated that chronic cigarette smoking reduces erythrocyte deformability, and this can contribute to impaired oxygen delivery, affecting the main purpose of the blood transfusion[36]. Moreover, the sustained production of reactive free radicals from the tar and gas phases of cigarette smoke causes oxidant stress on circulating erythrocytes[35]. It is well recognised that the main contributor to RBC lesions is oxidative stress and that the donor-dependent capacity of RBCs to cope with oxidative injury may be directly linked to the final quality and effectiveness of PRBC transfusions[2,6,12].

Cigarette smoke is a vast source of oxidants, and numerous oxidant compounds have been identified among the 4,000-7,000 constituents in cigarette smoke[37,38]. A single puff of cigarette smoke contains 10^{17} free radicals in the tar phase and 10^{15} in the gas phase[39]. Smoking produces a shift in the normal balance between oxidants and antioxidants, impacting the endogenous oxidative stress system[37,38]. Oxidative stress imbalance and inflammatory tissue response are related to disease, and more than 3 billion deaths per year are due to cigarettes[40]. Measuring urinary cotinine can provide a sensitive estimate of tobacco smoke exposure. The urinary cotinine levels found in our smoking group are compatible with reference values for heavy smokers[41,42].

It has been well documented that cigarette smoking also depletes antioxidants[43] and that vitamin C concentrations are lower among smokers[44]. Our findings agree with the literature. In the present study, smokers had an average 3 times less vitamin C than non-smokers, and the biomarker cotinine was negatively correlated with vitamin C concentration, which confirmed smoking as a causal factor[32]. These specific changes in ascorbate content appear to correspond to elevated oxidative stress from smoking rather than dietary intake, since neither group regularly took vitamins[32,45].

Despite lower plasmatic vitamin C levels in smoker donors, we found a higher GST activity and a lower GPx activity in the PRBCs from smokers when compared to non-smoker donors, without changes on PRBCs non-protein thiol groups or serum MDA levels. These findings, without considering storage conditions, may suggest that PRBCs from smoker donors are more prepared to deal with oxidative stress since GST is active in detoxifying components of tobacco smoke. Studies also showed lower GPx activity in the blood from individuals who smoked, associated to cigarette toxins[9,46,47]. Other studies also found elevated GSH, catalase, and nitrite/nitrate levels in the plasma and RBCs of moderate smokers[48,49]. They conclude that increased antioxidant status protect RBCs from free radical damage induced by cigarette smoke[48,49]. Antioxidant mechanisms are preserved in RBCs in a subcellular location and, despite the fact that RBCs from smoker donors carry a high concentration of antioxidants, they cannot be renewed and could make the PRBCs more susceptible to the environmental conditions and storage lesions. Although some authors suggest that chronic cigarette smoking increases erythrocyte production as a compensatory mechanism to maintain oxygen transportation[50], we suggest that lower vitamin C and limited antioxidant defences can contribute to this high RBC production.

It is known that smoking causes lipid peroxidation and the conversion of polyunsaturated fatty acids to hydroperoxides, endoperoxides, aldehydes (e.g., MDA) and alkanes (e.g., ethane and pentane)[51]. It is important to point out that we analysed oxidative stress parameters in fresh samples, before storage, from donors presenting good general health status. Thus, even if oxidative cell damage had been partially neutralised by antioxidant defenses, the glycolytic pathway and glutathione concentrations will decrease after storage[52]. Under such circumstances, superoxide radicals and water react with free iron (haeme), undergoing Fenton transformation and generating hydroxyl radicals, which readily damage lipids and proteins in RBCs[10].

CONCLUSIONS

Our results show that cigarette smoking was associated with lower serum vitamin C levels and altered endogenous antioxidant (GST and GPx) enzyme activity in PRBCs prior to storage. Increasing antioxidant defences such as GST in PRBCs from smoking donors is related to cell defences against the toxic compounds of cigarette smoke exposure

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during erythropoiesis. Because of the inability of mature RBCs to resynthesise antioxidants and its continuous exposure to xenobiotics from cigarette smoking, PRBCs from smoking donors may have higher risk of storage lesion than non-smoking donors. Further studies are needed to explore the combined impact of smoking and storage on PRBC oxidative status and lesions.

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AUTHORSHIP CONTRIBUTIONS

REB, SNdN, RG and SG designed the study. REB, SNN, RG, CRC, NSF and CP collected and analysed data. REB, SNN and RG drafted the manuscript. SB, RRP, AMB, SCG, LS and TGHO reviewed and revised the manuscript.

All Authors critically reviewed and revised the manuscript drafts, approved the final version, and take responsibility for the integrity of the data and accuracy of analysis.

The Authors declare no conflicts of interest.

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