Increased eryptosis in smokers is associated with the antioxidant status and C-reactive protein levels

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ABSTRACT

Cigarette smoking has been linked with oxidative stress and inflammation. In turn, eryptosis, the suicidal erythrocyte death similar to apoptosis that can be triggered by oxidative stress, has been associated with chronic inflammatory diseases including atherosclerosis. However, the link between smoking and eryptosis has not been explored so far. The aim of the present study was to determine the level of eryptotic erythrocytes in healthy male smokers (n = 21) compared to non-smokers (n = 21) and assess its relationship with systemic inflammation (CRP) as well as with antioxidant defense (GSH) and their resistance to ex-vivo induced hemolysis. Smoking caused an increase in phosphatidylserine translocation outside the erythrocyte membrane (hallmark of eryptosis), significantly correlated to the plasma level of CRP (r = 0.546) and GSH concentration in erythrocytes (r = −0.475). With respect to non-smokers, smokers show a marginal increase of total leucocytes and erythrocyte volume, no modifications of the RBC resistance to oxidative stress-induced hemolysis and hematological and lipid parameters unvaried. We conclude that the inflammatory status (high CRP levels) and RBC oxidative stress (low GSH levels) caused by cigarette smoking are associated with an increase of eryptotic erythrocytes, a yet unknown relationship potentially involved with atherosclerosis and cardiovascular disease in smokers.

1. Introduction

Cigarette smoking is an important preventable cause of mortality and a modifiable risk factor showing direct causal relationship with different cancers (lung, head, neck, liver, bladder, cervix, esophagus, colon and rectum), and chronic diseases such as cardiovascular (coronary heart disease, stroke, aortic aneurysm and peripheral arterial disease), respiratory (chronic obstruction pulmonary disease, asthma and pneumonia), reproductive and others such as cataracts (Asgary et al., 2005; Pannuru et al., 2011; Onor et al., 2017).

Active and passive smoking creates an oxidative stress environment largely attributed to the activity of a multitude of molecules such as carcinogens, mutagens, free radicals and heavy metals, all capable of generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) that readily react with biomolecules favoring DNA injury and lipid peroxidation (Asgary et al., 2005; Pannuru et al., 2011; Seet et al., 2011; Lymperaki et al., 2015; Masilamani et al., 2016). Accordingly, various studies have reported that increased levels of smoking-derived oxidants in association with reduced levels of serum antioxidants (vitamin E), total antioxidant capacity, -SH groups and increased anti-oxidant enzymes (SOD, CAT, GSH-Px) in circulating blood of smokers can be regarded as evidence that smoking-derived toxins spread out the circulation resulting in cell damage, including RBC hemolysis and physico-chemical alterations in erythrocyte membrane, as well as increased protein carbonyls, lipid peroxidation and inflammation (Asgary et al., 2005; Padmavathi et al., 2010; Pannuru et al., 2011; Miri et al., 2012; Lymperaki et al., 2015; Masilamani et al., 2016). In addition, among men, smoking habits have shown a strong association with levels of C-reactive protein (CRP), a sensitive non-specific serum marker of inflammation (Madsen et al., 2007). Moreover, cigarette smoking has also been linked with modifications in hematological and lipid parameters such as increase in hemoglobin, hematocrit, total leucocyte

Abbreviations: CAT, catalase; CRP, C-reactive protein; CXCL16, transmembrane CXC chemokine ligand 16; CXCR6, C-X-C chemokine receptor type 6; FSC, forward scattering; GSH, glutathione; GSH-Px, glutathione peroxidase; HDL, high density lipoprotein; LDL, low density lipoprotein; PCV, packed cell volume (hematocrit); PGE-2, prostaglandin E-2; RBC, red blood cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TG, triglycerides; VLDL, very low density lipoprotein; WBC, white blood cells

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count, total cholesterol, triglycerides (TG), LDL, VLDL and reduction on HDL that may cause fatal cardiac diseases among the smokers (Flouris et al., 2012; Lakshmi et al., 2014; Shahabinejad et al., 2016).

Eryptosis is a form of erythrocyte death characterized by cell shrinkage, membrane blebbing and breakdown of the membrane phospholipid asymmetry with phosphatidylserine exposure in the outer leaflet (Pagano and Faggio, 2015; Repsold and Joubert, 2018). Excessive eryptosis is associated with several pathophysiological conditions such as anemia, atherothrombosis, atherosclerosis, cardiac and renal failure, obesity, diabetes, chronic inflammatory disease and malignancies, among other ailments (Qadri et al., 2017). This type of cell death can be triggered by oxidative stress, hyperosmolarity, energy depletion, hyperthermia, heavy metal exposure, and a wide variety of xenobiotics (Lang and Qadri, 2012; Lang et al., 2014). Oxidant attack and/or impairment of antioxidant defenses can activate Ca²⁺-permeable cation channels stimulating Ca²⁺ entry, and activate aspartyl- and cysteinyl-proteases (caspases) that promote phosphatidylserine exposure, whereas Cl⁻ channels contribute to eryptotic cell shrinkage (Lang et al., 2014). These events can be linked with atherosclerosis and cardiovascular disease since eryptotic erythrocytes adhere to endothelial cells of the vascular wall by interaction of phosphatidylserine exposed at the membrane surface with endothelial CXCL16 (Borst et al., 2012; Silva-Herdade et al., 2016), triggering blood clotting and thrombosis (Andrews and Low, 1999), thus interfering with microcirculation (Close et al., 1999; Lang et al., 2013; Briglia et al., 2017). In addition, increase in ROS, hemolysis, lipid peroxidation, PGE-2 release and decrease in GSH are common features in oxidative stress-induced eryptosis (Bissinger et al., 2014; Mischitelli et al., 2016a, 2016b; Tesoriere et al., 2014; Álvarez-Sala et al., 2018).

To the authors’ knowledge, no studies to date have reported a direct association between level of eryptosis and smoking and attributed an increase in eryptosis to smoking. In this context, an association between level of eryptosis and smoking and attributed an increase in erythroid Hb concentrations and an increase in eryptosis to smoking (Tesoriere et al., 2014; Álvarez-Sala et al., 2018). Oxidative stress hemolysis were measured to assess associations between eryptotic erythrocytes and in smokers versus non-smokers (Sikdar et al., 2017). Although several markers have been established for smokers compared with never-smokers, ex smokers and subjects with BMI ≥ 25 Kg/m² or who underwent recent hospitalization, were also excluded.

2.2. Hemachemical tests

The recruited participants underwent venipuncture, after a fasting period of 10–12 h. A blood sample was used for hemachemical tests and a blood sample, collected in EDTA, was used to immediately isolate RBC by a 20 min centrifugation at 2000g, 4 °C, over Ficoll (Biochrom KG, Berlin, Germany).

Complete blood counts were estimated using cell counter. Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured by standard clinical chemistry methods (GlucoChek HK UV; Cholesterol gen 3 Mod P/D; HDL- Cholesterol gen 4 mod P/917; triglycerides (Roche diagnostics, Monza, Italy). Low-density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) cholesterol concentration were calculated by means of Friedewald’s formula (Friedewald, 1972).

\[ \text{LDL (mg %) = Total cholesterol – (HDL cholesterol + TG/5)} \]
\[ \text{VLDL Cholesterol (mg %) = TG/5}. \]

CRP was measured using a high-sensitive latex-enhanced CRP assay, detection limit below 0.1 mg/L, with reagents from Roche adapted for analysis in a Hitachi 917 automatic analyzer (Hitachi, Tokyo).

2.3. Measurement of phosphatidylserine externalization and forward Scatter (FSC)

Isolated RBC were immediately washed twice in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH, 5 glucose, 1 CaCl₂, pH 7.4. RBCs samples were adjusted at 1.0 × 10⁶ cells/mL with binding buffer following the instructions of the protocol’s manufacturer (Annexin V Apoptosis detection Kit FITC; ebioscience Inc., San Diego, CA, USA). Cell suspension (100 μL) was added to a new tube and incubated either in the absence (blank) or in the presence of 5 μL Annexin V-FITC, at room temperature in the dark for 15 min. Then samples of at least 1 × 10⁴ cells were subjected to fluorescence-acti- vated cell sorting (FACS) analysis by Epics XL® flow cytometer, using Expo32 software (Beckman Coulter, Fullerton, CA). Cells were analyzed by forward scatter, and annexinV-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Blank sample was used to check the appropriate parameters of fluorescence analysis.

2.4. Intracellular glutathione (GSH)

GSH content was determined with 1 μM (final concentration) of 5-chloromethylfluorescein diacetate (Green CMFDA) (Abcam, Cambridge, UK) according to Álvarez-Sala et al. (2018). The CMFDA was added to a RBCs sample adjusted at 2.0 × 10⁶ cells/mL with PBS. Then, the mixture was incubated during 40 min (37 °C, 5% CO₂, 90% humidity) and erythrocytes were collected by centrifugation (800 rpm, 24 °C, 5 min). Cells were suspended in 500 μL of PBS and analyzed by flow cytometry (Epics XL™, Beckman Coulter, Fullerton, CA) measuring fluorescence intensity in the FL-1 fluorescence channel at an excitation wavelength of 488 nm and emission wavelength of 530 nm. At least 10,000 events per sample were evaluated.

2.5. Oxidative hemolysis

A suspension of RBCs in PBS (HT 1%; HbO₂ = 170 ± 2 μM per heme group) was incubated at 37 °C in the presence of a 300 μM cumene hydroperoxide (cumOOH) ethanol solution. The volume added never exceeded 0.5% of the total incubation volume. The extent of
hemolysis at any given incubation time was determined as follows: A volume (0.2 mL) of the incubation mixture was diluted with 10 volumes of PBS and centrifuged at 1000g for 10 min to precipitate the cells. The absorbance of the supernatant was then evaluated at 540 nm. Similarly, a volume of the incubation mixture was treated with 10 volumes of 5 mM sodium phosphate buffer, pH 7.4 (hypotonic PBS), and briefly exposed to an ultrasonic bath to yield complete hemolysis. The supernatant after a centrifugation at 1000g for 10 min was evaluated spectrophotometrically at 540 nm. The percentage of hemolysis was calculated from the ratio of the absorbances.

2.6. Statistical analysis

Data were subjected to statistical analysis using Instat-3 statistical software (GraphPad Software Inc., San Diego, CA, USA). Values are the means ± standard deviation (SD) of 21 subjects in each group. The data were normally distributed and comparison between smokers and non-smokers was performed by the unpaired Student t-test. In all cases, significance was accepted if the null hypothesis was rejected at the p < 0.05 level. Correlations between variables were assessed with Pearson’s correlation coefficient (r).

3. Results

3.1. Health status of the subjects

Various biochemical analysis including hematological (blood and leucocytes count) and lipid (total cholesterol, LDL, HDL, VLDL and TG) parameters and an inflammation biomarker (CRP) were measured to monitor the healthy status of the participants. They are commonly ordered tests in health care to provide an overview of an individual’s general health status (Flouris et al., 2012).

Biochemical parameters for smokers and non-smokers and the significance of the differences in the comparison between the groups are shown in Table 1. None of the hematological parameters showed statistically significant differences (p > 0.05), although the total leucocyte count (WBC) displayed an upward trend (t-value: 1.7630; p-value: 0.0857) in smokers versus non-smokers. Similarly, no statistically significant differences in the lipid profile and the total cholesterol/HDL and LDL/HDL ratios between smokers and non-smokers were observed. On the contrary, the level of CRP, a suitable biomarker of the inflammatory status including low-grade systemic inflammation (Karadag et al., 2008), was significantly higher (t-value: 2.0430; p-value: 0.0479) in smokers.

3.2. Eryptotic status of circulating erythrocytes

Externalization of phosphatidylserine to the outer leaflet of the erythrocyte membrane (determined by Annexin V-binding cells) and decrease in cell volume (determined by FSC) are two main hallmarks of eryptosis (Lang et al., 2014; Repsold and Joubert, 2018) which were analyzed by flow cytometry. As indicated in Fig. 1A, smokers had significantly higher % of Annexin V-binding cells versus non-smokers (12.09 ± 1.69 vs. 9.03 ± 1.84 t-value: 5.5470; p-value: < 0.0001). On the other hand, according to the geo mean values derived from FSC (Fig. 1B), a growing trend of cell volume was observed in smokers compared to non-smokers (554.30 ± 32.31 vs. 536.30 ± 30.76 t-value: 1.8260; p-value: 0.0756), possibly due to changes in morphology associated with preliminary steps of hemolysis (Álvarez-Sala et al., 2011). Representative histograms of Annexin V-binding and FSC of erythrocytes from one non-smoker and one smoker volunteer are also shown (Fig. 1C and D, respectively).

3.3. GSH levels

Smoking causes serious oxidative damage to cell components (Seet et al., 2011), implying alterations in the cell antioxidant status. Then, the level of GSH, one of the main non enzymatic antioxidant of the erythrocytes (Cimen, 2008) was determined by flow cytometry (Fig. 2A). We observed a significant decrease in the GSH content in smokers compared to non-smokers (78.80 ± 21.02 vs. 98.28 ± 15.19 t-value: 3.3860; p-value: 0.0016).

3.4. Resistance to oxidative stress-induced hemolysis

Ex vivo experiments were carried out to assess the resistance to oxidant-induced hemolysis (lag time in minutes) of erythrocytes from smokers compared to non-smokers. Cells were exposed to cumO2H that triggers radical production and oxidation of cell components, finally causing hemolysis (Tesoriere et al., 1999). No differences were observed in the resistance to oxidative stress-induced hemolysis between both groups of participants (smokers: 96.24 ± 20.24 vs. non-smokers: 99.00 ± 23.43 p-value: 0.4046; t-value: 0.6880) (Fig. 2B).

3.5. Correlations of eryptotic, antioxidant and inflammatory parameters in smokers

Fig. 3 illustrates the correlation analysis (r) between Annexin V-binding cells (eryptosis) vs. CRP levels (A), and GSH (B), as well as between GSH vs. CRP (C) in smokers. A clear positive moderate correlation between eryptotic RBC and CRP (r = 0.5465, p = 0.0104) and negative moderate correlation between eryptotic RBC and GSH (r = −0.4749, p = 0.0296) was observed, with no statistical correlation between GSH and CRP (r = −0.05291, p = 0.8198), that then appear independently connected with eryptosis in smokers.

4. Discussion

Mounting evidence indicates that smoking may bring about significant alterations in hematological and lipid parameters; however, literature shows controversial results. Based on the smoking history of the subjects of the present study (8 ± 3 cigarettes/day during three years) one could expect a lower detrimental effect on lipid and/or
hematological parameters compared with other studies such as Haswell et al. (2014) (≥20 cigarettes/day), Flouris et al. (2012) (≥15 cigarettes/day) or Pannuru et al. (2011) (4–6 cigarettes/day during 5–7 years) where hard smokers are enrolled. Nevertheless, this is not always certain since other studies involving subjects with similar smoking habits as in the present study such as Lakshmi et al. (2014) (≥1 cigarettes/day during ≥1 years) or Shahabinejad et al. (2016) (≥5 cigarettes/day during ≥1 years) display significant deleterious effects. In this context, it has been reported a worsening of the lipid profile in smokers with increases in total cholesterol, TG, LDL, VLDL (Lakshmi et al., 2014), total cholesterol/HDL and LDL/HDL ratios (Pannuru et al., 2011) and a decrease in HDL (Haswell et al., 2014; Lakshmi et al., 2014). These dyslipidemic levels have been implicated with greater risk of developing atherosclerotic plaques and progression of cardiovascular disease (Pannuru et al., 2011; Haswell et al., 2014; Lakshmi et al., 2014). Nevertheless, in agreement with other studies (Seet et al., 2011; Miri et al., 2012; Haswell et al., 2014), our results showed that lipid parameters were comparable between smokers and non-smokers.

Fig. 1. Comparison of percentage of Annexin V-binding cells (A) and Forward scattering (B), in RBCs between smokers (n = 21) and non-smokers (n = 21) and original representative histograms of Annexin V-binding (C) and FSC (D) of erythrocytes from one non-smoker and one smoker volunteer.

Fig. 2. Comparison of GSH levels (A) and resistance to oxidative stress-induced hemolysis (B) in RBCs between smokers (n = 21) and non-smokers (n = 21).
Concerning the hematological parameters, it has generally been reported an increase in total white blood cells (WBC) in smokers as a circulatory inflammation biomarker associated with bronchial tract tissue damage, a condition associated with an increased risk for developing atherosclerosis (Flouris et al., 2012; Haswell et al., 2014; Lakshmi et al., 2014; Shahabinejad et al., 2016). Although in the present study there was no significant modification in hematological parameters due to smoking in concordance with other studies (Miri et al., 2012; Lymperaki et al., 2015), a marginal increase (non significant trend) of total leukocytes observed in smokers suggests a condition of systemic inflammation. Accordingly, the level of the inflammatory biomarker CRP was significantly higher in the group of smokers compared to non-smokers.

Various studies provide either direct or indirect evidence that systemic inflammation of smokers may be linked to RBC damage and eryptosis. Smokers are at high risk for chronic obstructive pulmonary disease, a condition characterized by a systemic inflammatory response associated with elevated levels of several inflammatory markers such as CRP, leukocytes, IL-6, IL-8 and fibrinogen (Su et al., 2016). Interestingly, the inflammatory IL-8 induces morphological changes in erythrocyte structure (membrane blebbing) ascribed to the initiation of eryptosis (Bester and Pretorius, 2016). In addition, present findings and other results show that individuals daily exposed to tobacco cigarette smoke exhibit high levels of CRP (Panagiotakos et al., 2004). Accordingly, it has been reported ex vivo that CRP at concentrations prevailing in plasma of patients with inflammation (appendicitis) induce eryptosis through an increase in the percentage of phosphatidylserine-exposing erythrocytes, intracellular calcium, ceramide and caspase activity. In the same study, a significant correlation was observed between CRP and the percentage of phosphatidylserine-exposing erythrocytes (Abed et al., 2017). In this context, however, our study is the first one that shows an in vivo correlation between CRP plasma levels and the percentage of Annexin V-binding cells, thus linking systemic inflammation with eryptosis in smokers.

It is well-known that cigarette smoking induces systemic oxidative stress through a decline of circulating endogenous antioxidants, in tune with the drop of GSH in RBCs described in the present study. Decreased levels of GSH in RBC have also been observed in moderate smokers (Pannuru et al., 2011) and in erythrocytes exposed to nicotine and cotinine ex vivo (Asgary et al., 2005). Recently, it has been reported that the increased oxidative stress in young smokers with endothelial dysfunction may have a causal role in the repression of the Nrf2/ARE antioxidant defense pathway and in the GSH depletion of the endothelial cells (Fratta Pasini et al., 2012). On the other hand, it is established that oxidants, and the consequent RBC redox distress, may elicit eryptosis (Lang et al., 2014). In this context, various inducers such as oxysterols (Tesoriere et al., 2014) and tert-butylhydroperoxide (Álvarez-Sala et al., 2019) have been reported to induce eryptosis with a fall in GSH levels. According to these data, the negative correlation observed between the erythrocyte GSH and the extent of eryptosis (percentage of Annexin V-binding cells) appears reasonable.

To date, data connecting the induction of eryptosis with smoking are inferential. In this sense, the highly reactive aldehyde acrolein present in huge quantities in tobacco smoke was reported to induce eryptosis ex vivo (Ahmed et al., 2013). This compound is metabolized by conjugation with GSH and excreted in urine as mercapturic acid metabolites (Stevens and Maier, 2008). Thus, a decline in GSH is expected, which is possibly associated with higher circulating eryptotic red blood cells. Another study has associated the oxidative milieu produced by smoking with a rise in ROS and osmotic fragility together with a decline in GSH levels in erythrocytes (Sikdar et al., 2017). Furthermore, these authors indicated the alteration of erythrocytes morphology with an increase in stomatocytes (increased volume potentially linked with hemolysis) and spherocytes (associated with reduced pliability and osmotic fragility) (Sikdar et al., 2017). Moreover, we have previously reported ex vivo that tert-butylhydroperoxide induced oxidative stress in erythrocytes resulted in two cell populations, with low size (FSC < 20, associated with eryptosis) and big size (FSC > 80, associated with a preliminary step of hemolysis) (Álvarez-Sala et al., 2018). In line with this, we have shown in the present study a marginal increase (non significant trend) of FSC (higher cell volume) in erythrocytes from smokers to be possibly ascribed to the presence of high quantities of stomatocytes. However, and contrary to the expected, we have failed to see a lower resistance to hemolysis in smokers, as previously reported (Asgary et al., 2005; Padmavathi et al., 2010; Masilamani et al., 2016; Sikdar et al., 2017). Instead, our results are in agreement with Pannuru et al. (2011) who indicated no significant changes in erythrocyte hemolysis in moderate smokers versus controls, attributed to the increase in nitric oxide and antioxidant enzymes (CAT, SOD and GSH-Px) that may quench free radicals produced by smoking and protect cells from hemolysis. The link of smoking with eryptosis and potential adverse consequences in cardiovascular disease development can be inferred from other studies. Manzur-Jattin et al. (2016) have indicated that erythrocyte eryptotic forms such as stomatocytes and spherocytes, exposing phosphatidylserine out of the membrane have higher chance to adhere to endothelium through the transmembrane chemokine CXCL16 (Borst et al., 2012), eventually promoting thrombus formation. In this context, it has recently been reported that cigarette smoke increases endothelial CXCL16-leukocyte CXCR6 adhesion in vitro and in vivo (Marques et al., 2017).

Finally, though the extent of eryptotic RBC of smokers has appeared correlated with both the inflammatory status (increased CRP levels) and the RBC oxidation (decreased GSH), CRP and GSH measurements did not show any correlation, that is systemic inflammation and RBC oxidative stress appear powerful independent conditions that may induce eryptosis through different mechanisms. Therefore, the extent of
5. Conclusions

The core findings in our study in healthy men have shown, for the first time, that inflammatory status and oxidative stress environment caused by cigarette smoking are associated with an increase in eryptotic erythrocytes, a non yet described relationship potentially involved with atherosclerosis and cardiovascular disease in smokers. Based on these results, a future prospective work should consider a larger sample of subjects, to unravel if a rise in eryptotic erythrocytes due to smoking-derived oxidative stress and/or inflammation as driving forces can be regarded as a new early biomarker of oxidative stress and potential cardiovascular disease development in smokers.

Conflict of interest

The authors declare that there is no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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