Ann Ist Super Sanità 2018 | Vol. 54, No. 4: 300-307
DOI: 10.4415/ANN_18_04_06

Comparative effects between electronic cigarette and tobacco cigarette smoke on oxidative markers in cultured immune cells isolated from rats

Antonella Di Biase[1], Lucilla Attorri[1], Rita Di Benedetto[1] and Massimo Sanchez[2]

[1]*Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Rome, Italy*
[2]*Servizio Grandi Strumentazioni e Core Facilities, Istituto Superiore di Sanità, Rome, Italy*

Abstract

Background. Tobacco cigarette smoke (TCS) was previously demonstrated to affect the innate and adaptive immune responses as a consequence of oxidant generation which play a pivotal role in neutrophilic airway inflammation. Aim of this paper was to investigate whether electronic cigarette smoke (ECS) generates reactive oxygen species (ROS) similarly to cigarette smoke.

Method. By means of a house made apparatus, ECS and TCS were collected in fetal bovine serum (FBS) which was used to grow immune cells isolated from rats. As index of oxidative products nitrite, superoxide, and thiobarbituric acid-reactive substances (TBARS) were determined in the medium before and after cell growth.

Results. The results showed that: i) ECS caused a remarkable increase of nitrites and TBARS although in lesser extension than TCS; ii) the spleen and lymph node cells grown in ECS and TCS-exposed medium were able to reduce TBARS but not nitrites present in the medium; iii) PBMC in TCS-exposed medium were able to reduce nitrites and TBARS more efficiently than spleen and lymph node cells, but released more superoxide anion; iv) TCS and ECS not influence the PBMC and spleen T cell subtype populations ($CD4^+$, $CD8^+$).

Conclusions. As ECS nicotine-free gave the same results of unexposed medium, we can support the hypothesis that the increase of ROS in ECS exposed medium was prevalently due to nicotine.

Key words

- electronic cigarette smoke
- tobacco cigarette smoke
- nicotine
- reactive oxygen species
- immune cells

INTRODUCTION

Recently, a new device to smoke, the e-cigarette (EC), was introduced and is gaining popularity, especially as a help to quit smoking. An EC is a nicotine-dispensing device in which a mixture of chemicals ("e-liquid") in a chamber is intermittently heated and aerosolized when a battery-powered heating unit is triggered by an inhalation ("puff"). Although ECs are not cigarettes at all (no tobacco, no smoke), they are similar to tobacco cigarettes (TCs) as a source of gases and PM2.5. Indeed, there is almost a complete overlap in PM2.5 size and concentration between TC smoke (TCS) and EC smoke (ECS), with the difference that TCS only is produced by the combustion of organic material, and it is the combusted organic PM2.5 that confers the greatest toxicity. The burning of a TC produces 4,000 chemicals, of which 100 have been identified as known carcinogens – cancer causing agents. ECS does neither require combustion to deliver TCS-comparable doses of nicotine, nor do they include many of the potentially carcinogenic additives that are found in TCS [1-3]. The principle component of e-liquid and e-vapor is propylene glycol, classified as "generally recognized as safe" by Food and Drug Administration (FDA) and present in many common FDA-approved injectable medications.

At present, there are few human data on the health effects of EC [4-7]. In the few available reports, mainstream e-vapor is void of carbon monoxide and other toxic gases and has trace to no detectable toxicants, including volatile organic compounds, carbonyls, tobacco specific nitrosamines, polycyclic aromatic hydrocarbons, and metals; the concentrations of these toxicants,

Address for correspondence: Antonella Di Biase, Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: antonella.dibiase@iss.it.

if detected, are 9 to 450 times lower in e-vapor than in mainstream smoke from tobacco cigarettes [8].

Cigarette smoking was previously demonstrated to affect the innate and adaptive immune responses, and it has been speculated that many of the health problems caused by cigarette smoking are due to its effects on the immune system [9, 10]. Accumulation of macrophages, which play a central role in the innate immune system, is observed in the lungs of smokers and patients with chronic obstructive pulmonary disease (COPD). COPD is a chronic inflammatory disease characterized by a specific pattern of inflammation involving neutrophils, macrophages and $CD8^+$ T lymphocytes and is caused by long-term exposure to noxious gases, such as cigarette smoke. Macrophages, activated by oxidants derived from cigarette smoke, are capable of releasing reactive oxygen species and inflammatory mediators and are believed to play a pivotal role in neutrophilic airway inflammation and progressive destruction of the alveolar walls, resulting in emphysema [11].

The presence or generation of reactive oxygen species (ROS) associated with ECS has yet to be evaluated and may pose a health risk that is under appreciated [12-14].

With the aim to investigate this field, we set up an apparatus to collect ECS and TCS in fetal bovine serum (FBS). Afterwards, FBS was added to culture medium in which peripheral blood mononuclear cells (PBMC) and cells isolated from spleen and lymph nodes of rats, were grown for one days. Nitrite, as index of nitric oxide (NO) production, superoxide release, and thiobarbituric acid-reactive substances (TBARS), as index of oxidative products, were determined. Further, to assess the role of nicotine in ROS generation, e-liquid nicotine free (nic-) was also used. As PBMC, splenocytes and lymphocytes comprise a mixture of immune cells and so they are a suitable model to mimic the whole immune system in the body, the effects of ECS and TCS on principal immune cell subtypes ($CD4^+$, $CD8^+$) was also investigated by flow cytometry analysis.

MATERIAL AND METHODS

Animals. Adult Wistar rat weighing 150-170 g were obtained from Envigo (San Pietro al Natisone, Udine, Italy). The animals were housed in stainless steel cages (two rats per cage) at a controlled room temperature of 24 °C, under a 12:12 light:dark cycle. Rats were anaesthetised by a mixture of medetomidine and ketamine (1:1, v/v) and fasting blood samples were collected in 0.2% EDTA from the left atrium of the heart. Spleen and mesenteric lymph nodes were quickly removed. All animal protocol were carried out according to the European Community Council Directive of 24 November 1986 (86/609/EEC) and Italian legislation (DL 116/92).

Cell isolation. PBMC were isolated by layering blood over an equal volume of Ficoll density gradient (Lympholytes-H, Cedarlane, Zierikzee, Netherlands) and centrifuged at 1500× g for 20 min. The cells collected at the interface were washed with PBS, and lymphocyte separation was repeated to achieve a lower degree of contamination by erythrocytes. Splenocytes were flushed out from the spleens gently using a syringe plunger with PBS and centrifuged (220× g, at 4 °C). The pellet was resuspended and left 5 min at 37 °C in 5 mL of red blood cells lysis buffer (17 mM Tris, 140 mM NH4Cl) to remove erythrocytes. Mesenteric lymph nodes were accurately cut and cells collected in PBS were filtered through filcons 70 µm and then centrifuged (220× g, at 4 °C).

Cell culture. After washing with PBS, the cells were resuspended in RPMI supplemented with 10% FBS and then cultured in 24-well plates at a working concentration of 2.5×10^5 cells/mL for oxidative parameters determination, and at the concentration of 5×10^5 cells/mL for flow cytometry analysis. After 24 h medium was removed and cells were grown for further 24 h in medium containing FBS exposed to TCS or ECS.

Smoke exposure device. Preliminary tests were performed to develop a device that allowed the reproducible incorporation of CS in a liquid put in a test tube. Briefly, two holes were made in the cap of a Falcon tube and two plastic pipes were inserted into them. A cigarette was secured on the top of one of them and the other was connected to a pump under controlled vacuum which drew the smoke from the lit cigarette into the Falcon tube. Thus, smoke from one MS cigarette, the most popular brand of Italian cigarettes, was bubbled through 5 ml of FBS. Vacuum was arranged in such a way that it took 4 minutes to consume a cigarette, the mean time utilized by smokers. To verify the consistency of the apparatus, smoke from 2 and 3 MS cigarettes were bubbled through FBS in 8 and 12 minutes, respectively and TBARS and nitrites were measured in smoke exposed FBS. The toxic effect of substances generated by smoke was tested in cells grown in the medium containing FBS-smoke exposed. Specifically rat PBMC were cultured for 24 h and cell viability was measured by BrdU assay.

With the same apparatus, vapor from e-cig (Kelvin, Guangdong, China -Mainland) containing e-liquid (propylenic glycol, glycerin, water, nicotine 0.6%, Apache BAL Atmos Lab) was aspirated in 5 mL FBS (1 puff/10 sec). To assess the role of nicotine, e-liquid nicotine free (Apache BAL 0 mg) was also used. E-cig and e-liquids were purchased from local retailers.

Nitrite assay. Synthesis of nitric oxide (NO) was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with oxygen. In brief, 200 µL of supernatants were allowed to react with 800 mL of Griess reagent (sulfanilamide 1% in phosphoric acid 5%; N-(1-naphthyl)ethylenediamine dihydrochloride 0.1% 1:1 v:v) and incubated at room temperature for 15 min. The optical density was measured spectrophotometrically at 541 nm (Beckman Coulter, Miami, FL, USA). The concentration was calculated using a calibration curve obtained with sodium nitrite ($NaNO_2$) and expressed as µmol/L.

Thiobarbituric acid-reactive substances assay. Peroxidation products were measured by the formation of thiobarbituric acid reacting substances (TBARS) according

to Fremont *et al.*, [15]. Briefly, 100 µL of 3mol/L trichloracetic acid and 150 µL of 90 mmol/L thiobarbituric acid in 75 mmol/L NaOH were added to 150 µL of cell culture supernatant. The tubes were incubated at 80° C for 40 min. After cooling, the tubes were centrifuged for 10 min (1500 g) at 4 °C. The supernatant absorbance was read at 535 nm. The concentration was calculated using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} and expressed as µmol of TBARS/L.

Superoxide release. Reduction of ferricytochrome c to ferrocytochrome c, in the presence and absence of 10 mg/ml SOD, was used to measure formation rates of superoxide anion (O_2^-). After supernatant removal, cells were washed with PBS containing 0.1 mM diethylentriaminopentacetic acid (DTPA) and then 1 ml of 10 mM cytochrome c in DTPA was added. The reaction was monitored spectrophotometrically at 550 nm, using the extinction coefficient of 2.1 x 10^4 $[mol/L]^{-1}$ cm^{-1} which corresponds to ΔE between ferricytochrome c and ferrocytochrome c.

Flow cytometry analyses. The T-lymphocyte subsets $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ were determined by flow cytometry analysis of cells isolated from spleens and PBMC. The frequency of T-lymphocyte subsets was evaluated using the following monoclonal antibodies: allophycocyanin (APC)-conjugated mouse anti-rat CD3 and phycoerythrin (PE)-conjugated mouse anti-rat anti-CD8a (from BD Biosciences, Franklin Lakes, NJ, USA), (FITC)-conjugated mouse anti-rat CD4 (from eBioscence, San Diego, CA, USA).

Cells were incubated with appropriate fluorochrome-conjugated isotype controls to gate nonspecific fluorescence signals. Flow cytometry analysis was carried out by the FACSAria I (BD Biosciences) equipped with three lasers (488 nm, 633 nm and 407 nm), and data were analysed using the FlowJo software, version 7.2.5 (Tree Star, Inc., Ashland, OR, USA) and the BD FACSDiva Software version 6.1.3 (BD Biosciences).

Statistical analysis Experiments were performed at least 3 times and the results are expressed as mean ± standard deviation (S.D). Statistical analysis was done by one-way ANOVA and post-hoc T3 Dunnett test was used to evaluate differences between groups. All statistical analyses were performed using SPSS software package for Windows, version 21.0 and the probability level was set at 5%.

RESULTS

Smoke exposure device validation. By using the house-made apparatus described in method section and based on incorporation of smoke though vacuum pump aspiration, smoke from 1, 2 and 3 MS cigarettes were bubbled through 5 ml of FBS in 4, 8 and 12 minutes, respectively. As oxidation markers, nitrite and TBARS were determined and their levels proportionally increased with the cigarette number (data not shown). The toxic effect of substances generated by smoke was assessed measuring cell viability of PBMC cultured for 24 h in medium containing smoke exposed FBS. No difference in BrdU absorbance was observed in cells cultured in presence of FBS exposed to 1 or 2 MS cigarettes (0.18 ± 0.01 each) while FBS exposed to 3 MS caused cell death as confirmed by Tripan blue microscopy staining. Thus, further experiments to compare TCS and ECS were done with FBS exposed to 2 MS cigarettes and with 300 µl of e-liquid both corresponding to 1.2 mg of nicotine.

Nitrite and TBARS in smoke- exposed FBS. *Table 1* shows nitrite and TBARS levels present in FBS after that 2 MS cigarettes and 300 µl of e-liquid both containing 1.2 mg nicotine were aspirated in 5 ml FBS by means of vacuum pump apparatus. Compared to control, TCS and ECS generated an increase of both substances, but those generated by TCS were higher than those generated by ECS. The same parameters were measured in the complete medium used to plate cells and containing 10% of FBS. While nitrite levels decreased about 8/10-fold as expected for dilution, the decrease of TBARS was just helved.

Oxidative markers in cells cultured in TCS and ECS exposed medium. With the aim to establish the behavior of immune cells cultured in medium containing TCS or ECS, PBMC, spleen and lymph node cells isolated from rats were grown in these medium for 1 day, after that nitrites and TBARS were measured in supernatants. The results reported in *Figure 1* show that there are deep differences between TCS and ECS and among cell species. Indeed, nitrite levels in supernatants of all the 3 kinds of cells grown in the medium with ECS were the same and were higher than that present in the medium before cell culture. When cultured in TCS exposed medium, PBMC were able to reduce nitrites in the medium. On the contrary both spleen and lymph node cells doubled it. Further, all the 3 kinds of cells grown in medium with ECS reduced TBARS present in the medium at the same level and similarly to PBMC grown in TCS exposed medium. However spleen and lymph node cells in TCS medium were less efficient than PBMC to reduce TBARS. Values of superoxide released by cells after supernatant removal are reported in *Figure 2* and confirm differences among cells. Although in untreated medium the release of O_2^- from PBMC was not detectable, when cultured in TCS and ECS exposed serum large O_2^- anion release were observed, especially after TCS exposure. On the contrary, basal superoxide released from spleen and lymph node cells was detectable but it did not change when cultured in TCS exposed FBS, but just after ECS exposure.

Nicotine effects on nitrite and TBARS. To investigate whether nicotine could influence nitrite and TBARS levels, smoke from 300 µl of e-liquid nicotine free (nic-) was collected, by means of the device, in 5 ml of FBS. Compared to values of untreated FBS reported in *Table 1*, no differences were observed in nitrite and TBARS levels after exposure to ECS nic- (1.71 ± 0.07 and 4.29 ± 0.29 µmol/l respectively). Similarly, nitrites and TBARS in supernatants of cells grown in medium containing untreated and ECS nic- FBS were not different (*Figure 3*). However a different behavior among cells was observed as PBMC had values always lower

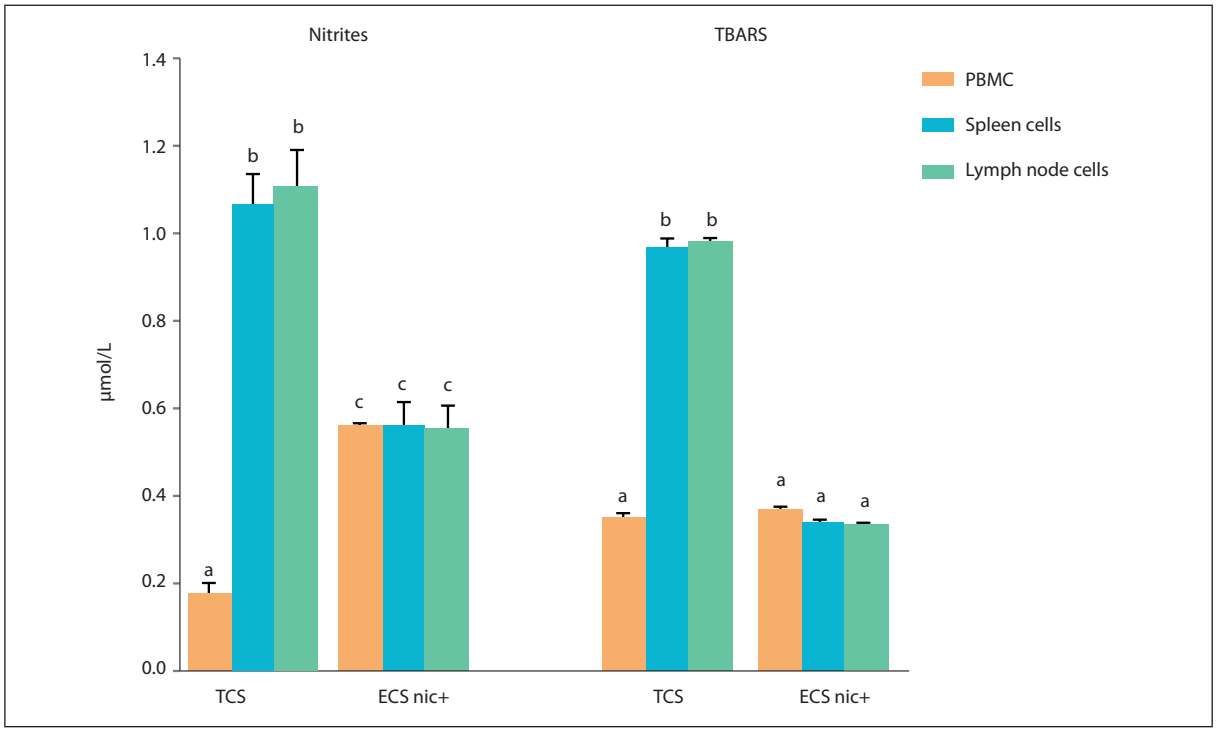

Figure 1
Nitrite and thiobarbituric acid-reactive substances (TBARS) levels in smoke exposed medium after 24 h of cell culture.
Peripheral blood mononuclear cells (PBMC), spleen and lymph node cells were grown for 1 day in the medium containing fetal bovine serum (FBS) exposed to tobacco cigarette smoke (TCS) and e-cigarette smoke (ECS) after that nitrites and TBARS were measured. Values are expressed as mean ±S and those with different letters are significantly different ($p < 0.05$).

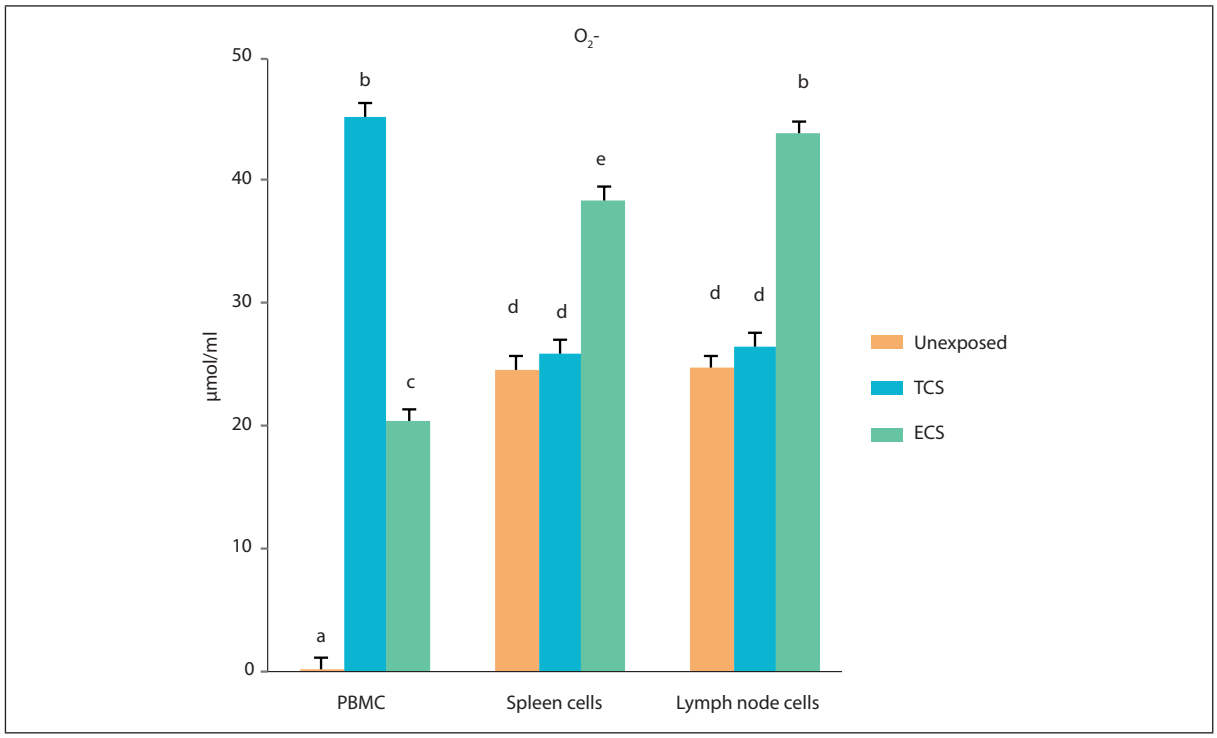

Figure 2
Superoxide anion released after 24 h of cell culture.
Peripheral blood mononuclear cells (PBMC), spleen and lymph node cells were grown for 1 day in untreated medium and in medium containing fetal bovine serum (FBS) exposed to tobacco cigarette smoke (TCS) and e-cigarette smoke (ECS) (nic+) and after supernatant removal superoxide anion was measured. Values are expressed as mean ± SD and those with different letters are significantly different ($p < 0.05$).

Table 1
Nitrite and thiobarbituric acid-reactive substances (TBARS) levels generated by tobacco cigarette smoke (TCS) e and e-cigarette smoke (ECS) in fetal bovine serum (FBS) and present in complete medium. Values are expressed as mean ± SD and those with different letters are significantly different ($p < 0.05$)

	Unexposed	TCS exposed	ECS exposed
FBS			
Nitrites (µmol/L)	1.67 ± 0.05^a	4.74 ± 0.16^b	3.63 ± 0.09^c
TBARS (µmol/L)	4.51 ± 0.39^a	18.57 ± 0.25^b	16.23 ± 0.27^c
Medium			
Nitrites (µmol/L)	0.19 ± 0.00^a	0.59 ± 0.02^b	0.35 ± 0.00^c
TBARS (µmol/L)	0.84 ± 0.08^a	9.51 ± 0.12^b	6.5 2± 0.06^c

compared to spleen and lymph node cells which were equal to each other.

T cell subtypes. Since the above reported results showed that TCS and ECS affected oxidative parameters which could contribute in mediating the inflammatory state and influence the T cell subtype frequency, PBMC and spleen cells were analyzed by flow cytometry. The results reported in *Table 2* show that these cells grown with unexposed FBS had a similar percent distribution of $CD3^+CD4^+$ and $CD3^+CD8^+$ although $CD3^+$are more frequent in PMBC respect to spleen. No significant differences were observed when cells were grown in medium containing TCS or ECS although $CD3^+$ percentage in PBMC and spleen cells were increased after ECS exposure either nic^+ and nic-.

DISCUSSION

It is thought that tobacco cigarette smoke is more dangerous for health than e-cigarette smoke since it is the process of combustion to generate the toxic compounds [16]. Less it is known about ROS generation, although is well established that oxidative toxicity and inflammation are associated with increased risk of lung diseases caused by conventional tobacco products.

The purpose of this study was to investigate the impact of ECS and TCS exposure on production of ROS (specifically nitrites and superoxide) and their effect on cells involved in immunity and isolated from rats. Although it is not possible to compare the *in vivo* effects of e-cigarettes and tobacco cigarettes because the exposures are completely different, data obtained by *in vitro* studies are very useful to evaluate whether the use of e-cig could be considered a "safe" alternative to tobacco smoke or if the health risk of e-cig is underestimated.

Firstly, an house-made apparatus to simulate a smoking machine in efficient and reproducible way was set up. Then, ECS and TCS were collected in FBS which was used in a complete medium where some cells deputed to immunity were grown.

For that concerns nitrite and TBARS production, combustion does not seem to be so influent as both

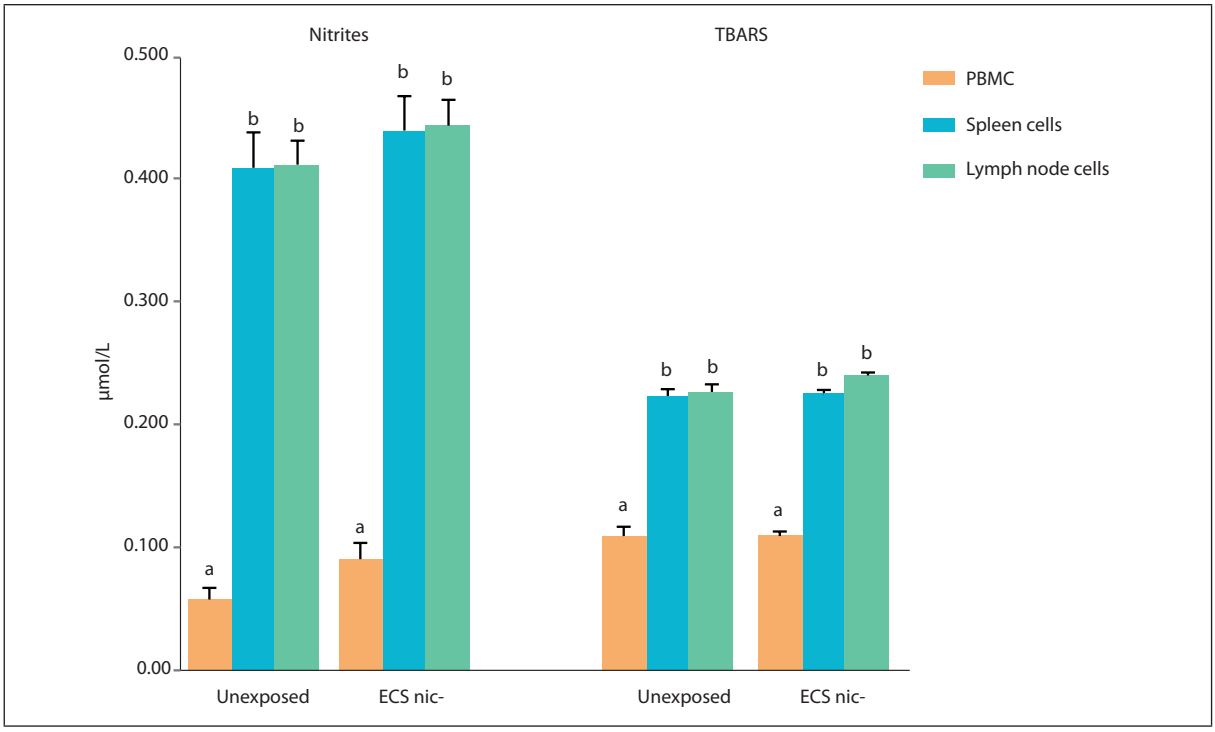

Figure 3
Nitrite and thiobarbituric acid-reactive substances (TBARS) levels in medium after 24 h of cell culture.
Peripheral blood mononuclear cells (PBMC), spleen and lymph node cells were grown for 1 day in untreated medium and in medium containing fetal bovine serum (FBS) exposed to e-cigarette smoke (ECS) nicotine free after that nitrites and TBARS were measured. Values are expressed as mean ±SD and those with different letters are significantly different ($p < 0.05$).

Table 2
$CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ prevalence in splenocytes and peripheral blood mononuclear cells (PBMC) cultured in medium containing fetal bovine serum exposed to tobacco cigarette smoke (TCS) and e-cigarette smoke (ECS). Values are expressed as mean ± SD

	Unexposed	TCS exposed	ECS exposed	ECS nicotine free exposed
Spleen				
$CD3^+$	3.11 ± 1.66	1.65 ± 1.94	4.71 ± 2.61	4.02 ± 2.69
$CD3^+CD4^+$	81.59 ± 1.06	83.56 ± 4.21	77.32 ± 6.5	82.44 ± 8.86
$CD3^+CD8^+$	18.41 ± 1.06	18.79 ± 1.5	22.68 ± 6.51	28.91 ± 12.6
$CD4^+/CD8^+$Ratio	4.44 ± 0.32	5.4 ± 1.8	3.72 ± 1.62	2.93 ± 1.65
PBMC				
$CD3^+$	24.29 ± 14.90	25.76 ± 20.99	34.07 ± 17.79	32.23 ± 22.05
$CD3^+CD4^+$	81.33 ± 5.01	81.56 ± 5.54	81.01±4.45	83.48 ± 3.92
$CD3^+CD8^+$	18.67 ± 4.75	18.45 ± 5.54	18.99 ± 4.45	16.52 ± 3.92
$CD4^+/CD8^+$Ratio	4.65 ± 1.68	4.72 ± 1.47	4.48 ± 1.40	5.28 ± 1.45

TCS and ECS-exposed serum caused a remarkable increase of nitrites and TBARS compared to unexposed serum. However, these parameters were significantly higher after TCS exposure compared to ECS (*Table 1*). In the complete medium containing 10% of FBS, nitrite levels decreased about 8/10-fold as expected for dilution factor while TBARS were just helved. As thiobarbituric acid-reactive substances are essentially due to the presence of malondialdehyde, final product of oxidation, it is possible that the oxidative reactions triggered by smoke continued.

TCS and ECS medium were used to culture three kind of cells involved in immunity: PBMC, spleen and lymph node cells isolated from rats. Nitrites formed in TCS exposed medium were efficiently reduced by PBMC while in presence of the other cells they even increased. When cultured in ECS exposed medium, an increase of nitrites in presence of all the three kinds of cells was observed, however it was lower than that caused by TCS in presence of spleen and lymph node cells. Similarly, TBARS present in TCS exposed medium decreased more efficiently in presence of PBMC compared to spleen and lymph node cells, whilst those present in ECS exposed medium were metabolized in the same measure by the three kinds of cells. Stated that TBARS are unspecific marker of oxidative products, it could be suggested that smoke from e-cigarette and tobacco cigarette cause production of different oxidative molecules and those due to TCS are hardly metabolized by spleen and lymph nodes. The different response of PBMC, spleen and lymph node cells is confirmed by the determination of superoxide anion released by cells after TCS and ECS exposure. Although without smoke exposure superoxide released by PBMC is extremely low, in presence of TCS it considerably increases, while no differences were observed in spleen and lymph node cells. In presence of ECS, all the 3 kind of cells increased O_2^- release, but PBMC more than the other cells. These differences could be due to a different susceptibility to oxidative damage among cells from different source as result of different membrane fatty acid composition, different oxygen concentration, different antioxidant enzyme equipment and so on.

Nicotine is the main toxic component of cigarette smoking with genotoxic and immunotoxic effects, other being an active compound on the nervous system. Its role on oxidative stress has been evaluated in *in vivo* and *in vitro* experiments. Yildiz *et al.*, [17] reported that nicotine has been recognized to result in oxidative stress by inducing the generation of reactive oxidative species by various mechanisms. Thus, being available nicotine-free liquid for e-cigarettes, we set up some experiments using FBS exposed to ECS nicotine-free (nic-). Our results show that values of nitrite and TBARS in ECS nic- FBS exposed were not different from those of untreated FBS (1.71 ± 0.07 and 4.29 ± 0.29 µmol/L respectively) validating the suggested role of nicotine in reactive oxidative species formation. However they are in contrast with those by Lemer *et al.*, [13] who detected the presence of ROS, measured by fluorescent 2'-7'-Dichlorodihydrofluorescein diacetate probe, higher in e-cig vapor without nicotine than in e-cig vapor with nicotine. He suggested that one of the sources of OX/ROS appears to be the heating element status (new *versus* used) and depends on flower additive as flavors containing sweet or fruit flavors were stronger oxidizers than tobacco flavors. Our experiments were performed with e-liquids with the same components and without flowers but differing for nicotine presence only. The role of nicotine in ROS production was further confirmed by results in *Figure 2*, showing no difference in nitrite and TBARS levels after cell incubation in ECS nic- exposed medium compared to untreated medium.

These results are in agreement with data obtained by *in vivo* experiments. Muthukumaran *et al.* [18], found that elevation of the levels of TBARS in tissues of nicotine-treated rats, while Ahmeda *et al.* [19] found increased level of NO in spleen tissues of nicotine treated rats. NO plays an important role in a diverse range of

physiological processes. It reacts with the superoxide anion to generate peroxynitrite, which is a selective oxidant, and nitrating agent that interact with numerous biological molecules, thereby damaging them.

Based on the increased $CD8^+$ T cell numbers found in the airways of smokers in the early phase of COPD, it has been suggested that T cells have a role in the pathogenesis of COPD [20-22]. Furthermore, literature data indicate that cigarette smoke exposure per se would trigger the activation of $CD8^+$ T cells and a possible mechanism could be induction of oxidative stress by components in the tobacco smoke [23]. Since cells from lymph node and spleen cells gave the same results in ROS production, we used only splenocytes and PBMC to characterize the effects of traditional and electronic cigarette smoke on cell subtype population. The subtypes were marked by cell type-specific markers for T lymphocytes ($CD3^+$), helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$), and the double positive cells were measured by flow cytometry.

Our results show that in spite of oxidative product increase after smoke exposure, no statistically significant differences in T cell subtypes were observed. However, $CD8^+$ T percentage in splenocytes exposed to ECS either nic^+ and nic- was higher than that of unexposed and TCS- exposed FBS. This suggests that additional studies are required to characterize the $CD8^+$ subpopulations which could reveal the implication of e-cig in cytotoxic T cell activation. However, other mechanisms such as viral infections or bacterial colonization [24-26] have been suggested to provoke the cytotoxic T cell response observed in COPD patients.

In conclusion, although our data do not confirm the correlation of ROS and increased number of $CD8^+$ cells, they highlight that ECS similarly to TCS generate some oxidative substances and this may pose a health risk that is underappreciated.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Received on 1 February 2018.
Accepted on 3 August 2018.

REFERENCES

1. Cummings KM, Dresler CM, Field JK, Fox J, Gritz ER, Hanna NH, Ikeda N, Jassem J, Mulshine JL, Peters MJ, Yamaguchi NH, Warren G, Zhou C. E-cigarettes and cancer patients. J Thorac Oncol. 2014;9(4):438-41. DOI: 10.1097/JTO.0000000000000129
2. Farsalinos KE, Polosa R. Safety evaluation and risk assessment of electronic cigarettes as tobacco cigarette substitutes: a systematic review. Ther Adv Drug Saf. 2014;5(2):67-86. DOI: 10.1177/2042098614524430
3. Goniewicz ML, Knysak J, Gawron M, Kosmider L, Sobczak A, Kurek J, Prokopowicz A, Jablonska-Czapla M, Rosik-Dulewska C, Havel C, Jacob P, Benowitz N. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. Tob Control. 2014;23(2):133-9. DOI: 10.1136/tobaccocontrol-2012-050859
4. Farsalinos KE, Romagna G, Tsiapras D, Kyrzopoulos S, Voudris V. Characteristics, perceived side effects and benefits of electronic cigarette use: A worldwide survey of more than 19,000 consumers. Int J Environ Res Public Health. 2014;11(4):4356-73. DOI: 10.3390/ijerph110404356
5. Farsalinos KE, Romagna G, Tsiapras D, Kyrzopoulos S, Voudris V. Evaluation of electronic cigarette use (vaping) topography and estimation of liquid consumption: implications for research protocol standards definition and for public health authorities' regulation. Int J Environ Res Publ Health. 2013;10(6):2500-14. DOI: 10.3390/ijerph10062500
6. Palazzolo DL. Electronic cigarettes and vaping: A new challenge in clinical medicine and public health. A literature review. Front Publ Health. 2013;1(art 56). DOI:10.3389/ fpubh.2013.00056
7. Williams M, Villarreal A, Bozhilov K, Lin S, Talbot P. Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. PLoS One. 2013;8(3):e57987. DOI:10.1371/journal.pone.0057987
8. Uchiyama S, Ohta K, Inaba Y, Kunugita N. Determination of carbonyl compounds generate from the e-cigarette using couples silica cartridges impregnated with hydroquinone and 2,4-dinitrophenylhydrazine, followed by high-performance liquid chromatography. Anal Sci. 2013:29(12):1219-22. DOI.org/10.2116/analsci.29.1219
9. Comer DM, Elborn JS, Ennis M. Inflammatory and cytotoxic effects of acrolein, nicotine, acetylaldehyde and cigarette smoke extract on human nasal epithelial cells BMC. Pulm Med. 2014;14:32. DOI: 10.1186/1471-2466-14-3
10. Jaspers I. Cigarette smoke effects on innate immune mechanisms in the nasal mucosa potential effects on the microbiome. Ann Am Thorac Soc. 2014;11(Suppl. 1):S38-S42. DOI: 10.1513/AnnalsATS.201306-154MG
11. Vassallo R, Kroening PR, Parambild J, Kitac H. Nicotine and oxidative cigarette smoke constituents induce immune-modulatory and pro-inflammatory dendritic cell responses. Mol Immunol. 2008;45(12):3321-9. DOI: 10.1016/j.molimm.2008.04.014
12. Cervellati F, Muresan XM, Sticozzi C, Gambari R, Montagner G, Forman HJ, Torricelli C, Maioli E, Valacchi G. Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells. Toxicol in Vitro. 2014;28(5):999-1005. DOI: 10.1016/j.tiv.2014.04.012
13. Lerner CA, Sundar IK, Yao H, Gerloff J, Ossip DJ, McIntosh S, Robinson R, Rahman I. Vapors produced by electronic cigarettes and E-Juices with flavorings induce toxicity, oxidative stress, and inflammatory response in lung epithelial cells and in mouse lung. PLoS One. 2015;10(2):e0116732. DOI:10.1371/journal.pone.0116732
14. Rowell TR, Tarran R. Chronic e-cigarette use cause lung disease? Am J Physiol Lung Cell Mol Physiol. 2015; 309(12):L1398-L1409. DOI: 10.1152/ajplung.00272.2015. Epub 2015 Sep 25. Review.
15. Fremont L, Gozzèlino M T, Franchi M P, Linard A. Dietary flavonoids reduce lipid peroxidation in rats fed polyunsaturated or monounsaturated fat diets. J Nutr.

1998;128(9):1495-502.
16. Pisinger C, Døssing M. A systematic review of health effects of electronic cigarettes. Prev Med. 2014;69: 248-60. DOI: 10.1016/j.ypmed.2014.10.009. Epub 2014 Oct 16. Review.
17. Yildiz D, Ercal N, Armstrong DW. Nicotine enantiomers and oxidative stress. Toxicology. 1998;130(2-3):155-65.
18. Muthukumaran GSH, Sudheer AR, Menon VP, Nalini N. Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. Toxicology. 2008:243(1-2)207-15.
19. Ahmed MA, Hassan KH, Hassanein KMA, Walyb H. Role of vitamin C and selenium in attenuation of nicotine induced oxidative stress, P53 and Bcl2 expression in adult rat spleen. Pathophysiology. 2014;21(3):211-7. DOI: 10.1016/j.pathophys.2014.07.003
20. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, Fabbri LM. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1998;157(3Pt 1):822-6. DOI: 10.1164/ajrccm.157.3.9709027
21. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Paré PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med. 2004;350(26):2645-53. DOI: 10.1056/NEJMoa032158
22. Nurwidya F, Damayanti T, Yunus F. The role of innate and adaptive immune cells in the immunopathogenesis of chronic obstructive pulmonary disease. Tuberc Respir Dis. (Seoul) 2016;7(1):5-13. DOI: 10.4046/trd.2016.79.1.5
23. Chen G, Zhou M, Chen L, Meng ZJ, Xiong XZ, Liu HJ, Xin JB, Zhang JC. Cigarette smoke disturbs the survival of CD8+ Tc/Tregs partially through muscarinic receptors-dependent mechanisms in chronic obstructive pulmonary disease. PLoS One. 2016;11(1):e0147232. DOI: 10.1371/journal.pone.0147232. eCollection 2016.
24. de Jong JW, van der Belt-Gritter B, Koeter GH, Postma DS. Peripheral blood lymphocyte cell subsets in subjects with chronic obstructive pulmonary disease: association with smoking, IgE and lung function. Respir Med. 1997;91(2):67-76. DOI.org/10.1016/S0954-6111(97)90070-6
25. Allie SR, Randall TD. Pulmonary immunity to viruses. Clin Sci (Lond). 2017;131(14),1737-62. DOI 10.1042/CS20160259
26. Kolsum U, Donaldson GC, Singh R, Barker BL, Gupta V, George L, Webb AJ, Thurston S, Brookes AJ, McHugh TD, Wedzicha JA, Brightling CE, Singh D. Blood and sputum eosinophils in COPD; relationship with bacterial load. Respir Res. 2017;18(1):88. DOI: 10.1186/s12931-017-0570-5