

The Effects of Hg²⁺ on Endothelial Function in Electronic Cigarette Smokers with Coronary Artery Disease

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The present available data on the harmful effects of electronic cigarettes (EC) is inconsistent and contradicting. Even though EC is considered a healthier alternative to the traditional cigarettes, several studies reported a large range of toxic compounds. The objective of this study is to determine whether the mercury found in the solutions used for electronic cigarettes is involved in the initiation and aggravation of endothelial dysfunction found in smoking persons with coronary artery disease (CAD). Endothelial function was evaluated by performing organ bath studies of vascular rings from patients with CAD, and was determined as a response to cumulative doses of endothelial-dependent/independent vasodilators, in the presence of Hg²⁺. Hg²⁺ extraction was realised using ion exchanging resin PUROLITE S920 from the liquid solution used in EC. The results of this study suggests that Hg²⁺ may be responsible for the aggravation of the endothelial dysfunction found in smoker of EC patients with CAD.

Keywords: mercury, endothelial dysfunction, electronic cigarette, smoking

Coronary artery disease (CAD) is considered the leading cause of morbidity and mortality worldwide, accounting for seven million deaths annually [1]. Numerous studies demonstrated that the risk of dying from CAD is two times higher in smokers as compared to non-smokers. At the moment, the most concerning fact is that, according to World Health Organisation, at least 6 million people die each year due to diseases caused by smoking [2].

Even if in the present the mechanisms by which smoking is involved in CAD are not fully elucidated and understood, many processes are incriminated, such as: promotion of endothelial dysfunction, acceleration of the formation of atherosclerotic plaques, impaired haemostatic process and increase of oxidative stress and lipid peroxidation [3]. Among these offending mechanisms, probably the most important change induced by smoking is endothelial dysfunction.

The vascular endothelium is considered to be a barrier delimiting the complex surface of all blood vessels and represents an auto- / paracrine organ with multiple and various roles, among which the most important is the modulation of vessel response [4]. Hence, endothelial dysfunction represents the changing of the phenotype of normal endothelium and is defined as the decrease of the bioavailability of vasodilatory substances, like nitric oxide (NO) and the increase of the bioavailability of vasoconstrictor substances, like endothelin (ET-1). Thus, endothelial dysfunction is involved in the development of CAD [5].

In the last decade, due to continuously rising awareness of the harmful effects of cigarette smoking on general health, the attention has shifted towards the use of electronic cigarettes (EC), as a long-awaited healthier alternative to the conventional cigarettes. However, due to the relative short time that had passed since their invention, the long-term health effects have not been studied completely. Moreover, due to the continuously increasing demand of solutions to charge the EC (e-liquid), on the

market we can find e-liquid produced by uncertified manufacturers that can contain even more toxic substances.

It is stated that EC are safe products that do not produce the harmful effects observed in traditional smoking [6,7], whereas the e-liquid contains various combinations of glycerine, propylene glycol, nicotine, tobacco extracts and different flavourings. Even though toxic chemicals like heavy metals, phenols and polycyclic aromatic hydrocarbons that are carcinogenic were not detected, traces of mercury (0.17 ng per EC), formaldehyde and acetaldehyde were found [8]. Regarding the physico-chemical stability and characterisation of tobacco and related products, several instrumental methods were employed, including thermal stability and kinetic degradation [9,10], by well-known methodologies, according to literature published for diverse compounds, including bioactive or potentially bioactive compounds [11-21]. Also, studies including the analysis of the smoke and its composition [22] were carried out.

The aim of this study was to determine whether the mercury found in the solutions used for electronic cigarettes is toxic and whether it can contribute to the endothelial dysfunction found in smoking persons with coronary artery disease (CAD).

Experimental part

Materials and methods

This study was realised over a period of 7 months and included 28 patients (40% females and 60% males, with ages between 57 to 71) that were previously diagnosed with coronary artery disease by coronarography. All the experiments were realised after the patients signed a written consent and were in accordance with the Declaration of Helsinki that states the ethical principles for medical research that involves human subjects [23].

Patients were divided into two groups:

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- Control : non-smokers with CAD that underwent by-pass surgery (n=15)

- Smokers: smokers of electronic cigarettes with CAD that underwent by-pass surgery (n=13)

The solution (e-liquid) used in electronic cigarettes (V=100 ml) is commercially available and was purchased from Ruyan, RY4 (nicotine content 36 mg/unit, China).

Separation of Hg^{2+} form e-liquid

The extraction of Hg^{2+} was realised after a method previously described by Andoni et al. [24], in an efficient way, namely by using ion exchange resin PUROLITE S920. The glycerine solution was diluted 1:10 in water to obtain an aqueous solution from which Hg^{2+} was extracted. PUROLITE S920 (1.6 g/L) was added, followed by system shaking for 15 minutes. The concentration of Hg^{2+} was measured by atomic absorption spectrophotometry (spectrophotometer Varian AA 110).

Organ bath studies

The function of the vascular endothelium was analysed by measuring the vasomotor effect of vascular rings, at increasing doses of vasomotor agents, after a method previously described by Saviou et al [25]. Fragments of internal mammary artery were obtained during the coronary by-pass procedure. Further the fragments were cleaned of adherent residual tissue and sectioned into circular rings (l= 2.5-3 mm). The rings were introduced in an organ bath (BIOPAC MP 100, System Inc, USA) filled with Krebs - Henseleit tampon solution (V=10 mL/well, NaCl 118 mmol/L, glucose 11.1 mmol/L, KCl 4.7 mmol/L, $NaHCO_3$ 25 mmol/L, CaCl₂ 1.6 mmol/L, $MgSO_4$ 1.2 mmol/L and KH_2PO_4 1.2 mmol/L; pH=7.4) maintained at 37°C and continuously aerated with carbogen. All the reagents used in this study were purchased from Sigma Aldrich and Invitrogen. Isometric tension was measured continuously by means of an isometric force transducer FORT 10 (World Precision Instruments, Inc.).

Experimental procedure

The vascular rings were pre-tensioned at 1.5 grams/force and equilibrated one hour. Indomethacin (10^{-5} M) was added in the organ bath to eliminate the influence of prostaglandin synthesis. The viability was tested by KCl

additions (70 mmol/L) that allowed the measurement of the maximal contractile response. Phenylephrine (Phe) was added at increasing concentrations until we obtained a contractile response that was at least 80% of the one obtained after KCl addition. The endothelial-dependent vasodilator response was tested at cumulative doses of 5'ADP (10^{-9} M- 10^{-4} M) and the endothelial-independent vasodilator response was tested at cumulative doses of sodium nitroprusside (NPS), against a pre-contraction induced by 10^{-5} Phe. We compared the data obtained from before and after the incubation of vascular rings were with Hg^{2+} (0.25 ng/mL).

Statistical analysis

The data obtained were expressed as means \pm standard deviation and analysed using one-way ANOVA and t-Student test (GraphPad Prism5, USA). The accepted significance level was $p < 0.05$.

Results and discussions

In table 1 is presented the vascular reactivity of mammary artery rings, before and after preincubation with Hg^{2+} , in order to assess the direct effect of this substance. When we compared the initial parameters with the parameters obtained after the addition of Hg^{2+} , we observed a significant decrease when the endothelial-dependent relaxation was assessed. No significant differences were obtained when we assessed precontraction induced by Phe, neither endothelial-independent relaxation induced by NPS.

Even though EC facilitates smoking cessation as is considered a healthier alternative to normal cigarettes, some studies and users are concerned about products safety and toxicity [26]. Indeed, EC delivers fewer total chemicals and carcinogens as compared to the traditional tobacco-burning cigarettes, but the e-liquid cartridge and the vapour emissions varies among products and they are not yet very well characterized. Changes in blood pressure were reported in 3.5% of EC users, whereas increased heart rate was observed when levels of plasma nicotine increased, after EC use [27,28]. At pulmonary level, although several beneficial effects were observed after EC usage compared to the traditional cigarettes, studies

Parameters	Initial	Hg^{2+}	p
Precontraction induced by Phe			
Emax (g force)	3.35 \pm 0.29	3.42 \pm 0.45	0.83
EC50 (Log[M])	-5.52 \pm 0.15	-5.6 \pm 0.21	0.61
Endothelial-dependent relaxation induced by 5'ADP (%)			
Emax (%)	72.65 \pm 1.9	40.18 \pm 3.23	<0.001
EC50 (Log[M])	-5.87 \pm 0.3	-4.31 \pm 0.55	<0.05
Endothelial-independent relaxation induced by NPS (%)			
Emax (%)	94.36 \pm 5.02	95.23 \pm 3.95	0.83
EC50 (Log[M])	-6.45 \pm 0.58	-6.5 \pm 0.13	0.89

Table 1
PARAMETERS DETERMINED BY MEANS OF THE HILL EQUATION REGARDING THE EFFECTS OF Hg^{2+} ON INITIAL VASCULAR REACTIVITY IN CONTROL GROUP. Phe: PHENYLEPHRINE, ADP: ADENOSINE DYPHOSPHATE AND NPS: SODIUM NITROPRUSSIDE.

reported negative effects such as coughing or choking, *tightening of the lungs, difficulty breathing* that suggest an increased pulmonary resistance and a decrease in exhaled nitric oxide levels, similar to effects seen when traditional burning-cigarettes are used [27]. Other negative effects included chest pain, allergies, vomiting, nausea, palpitations, dizziness, headache, black tongue, confusion, and fatigue [27,29]. There are a large number of complaints that are blaming EC for hospitalization for illnesses like congestive heart failure, pneumonia, hypotension, possible aspiration pneumonia, chest pain and arrhythmias, disorientation and seizure [30].

Various errors regarding e-liquid have been reported, such as errors in labelling nicotine concentrations, use of diethylene glycol, which is a known toxicant [27]. Harmful additives, such as coumarin, were found in e-liquid [31]. Another study found a large quantity of silicate beads in the aerosol [32]. Moreover, a study reported that the emissions resulted from EC smoking contained acetaldehyde and mercury [6] while another study found metals in fluid in low concentration, here including mercury [33].

The results of the assessment of vascular reactivity in human mammary artery rings form Control vs. Smokers group are presented in figure 1 and 2.

The results revealed a significant change in the endothelial-dependent vasodilator response to 5'ADP after Hg^{2+} was added to both Control and Smokers group, respectively ($p < 0.05$) (fig. 1). Moreover, we found a significant decrease of the endothelial-dependent vasodilator response to 5'ADP when we compared the Smokers group, before and after Hg^{2+} addition with the Control group, before Hg^{2+} (Initial) (fig. 1).

When the endothelium-independent relaxation response to NPS was analysed, no significant differences were obtained between the studied groups.

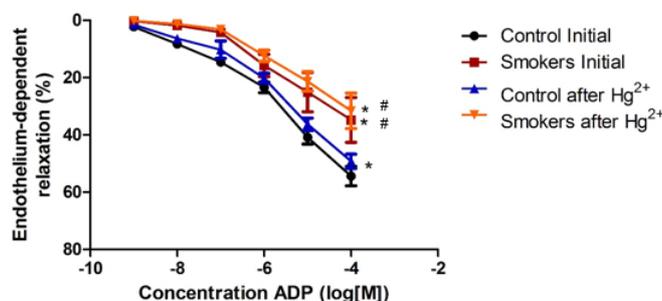


Fig. 1. Dose-response curve of human mammary artery rings before and after preincubation with Hg^{2+} , to cumulative doses of 5'ADP, Control vs. Smokers group. * $p < 0.05$ vs Control Initial; # $p < 0.05$ vs. Control after Hg^{2+}

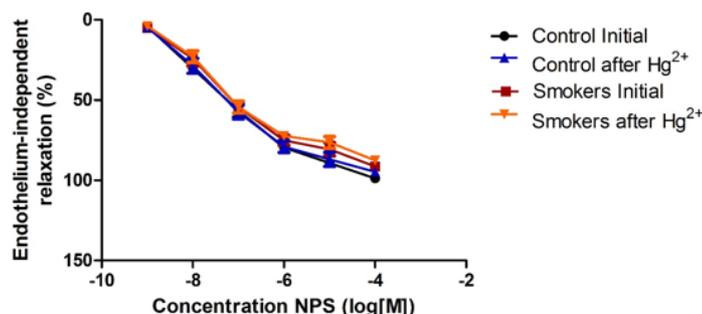


Fig. 2. Dose-response curve of human mammary artery rings before and after preincubation with Hg^{2+} , to cumulative doses of NPS, Control vs. Smokers group. When the endothelium-independent relaxation response to NPS was analysed, no significant differences were obtained between the studied groups

Mercury is a liquid metal that can be found as elemental mercury (Hg^0 , is very little soluble in water), mercuric ion (Hg^{2+}), mercurous ion (Hg_2^{2+} , is much more soluble in the water and has an increased affinity for inorganic and organic ligands) and as alkylmercury that accumulates in living organisms very toxic for central nervous system [34]. In human metabolism, mercury has no known role and there is no mechanism that allows the metabolism or excretion of mercury, hence during life, mercury will accumulate [35].

A large number of studies have well identified and characterised the negative effects of mercury on organism health. It was discovered that mercury can increase oxidative stress, reduced oxidative defence, thrombosis, mitochondrial dysfunction, hyperlipidaemia, inflammation and vascular/endothelial dysfunction [36,37], all of which can contribute to cardiovascular disease development and progression by initiating the development of atherosclerosis. It was demonstrated that endothelial function can be improved by the treatment with atorvastatin, in the case of patients with hyperlipidaemia, while the measurement of intima media thickness (IMT) together with the determination of high sensitive C reactive protein (hsCRP) can have a predicting value for future CAD events [38,39].

In our study we obtained a significant difference between the Control group (Initial) and Smokers group (Initial), which suggests the presence of endothelial dysfunction in EC smokers. Furthermore, we obtained a significant decrease of the endothelial-dependent vasodilator response after Hg^{2+} was added to both Control and Smokers, together with no difference in the endothelium-independent relaxation response, results that suggest that mercury may be responsible for the endothelial dysfunction observed. Indeed, the mechanisms that produces a reduced vasodilator response may include a reduced NO generation and an increased oxidative stress, in endothelial dysfunction [40].

Moreover, at vascular level, mercury was shown to increase free radical production, oxidation of LDL (oxLDL), platelet aggregation and to inactivate antioxidant defence, changes that can produce consecutively atherosclerosis. These effects will lead to DNA damage, depletion of vitamin C and E, an immune reaction and thrombosis. Moreover, endothelial cell formation and migration from endothelial progenitor cells is reduced, process that will in turn reduce vascular endothelial repair and decrease NO bioavailability and finally produce endothelial dysfunction [37,41].

The consequences of these pathophysiologic mechanisms can explain why mercury is involved in cardiovascular diseases [35,42,43].

Conclusions

Mercury addition to normal human mammary artery rings impairs endothelial function. The addition of mercury potentiates the already installed endothelial dysfunction observed in smoker of EC. Mercury was not responsible for modifications in the endothelial-independent response to NPS. This study may suggest that mercury in the doses observed in e-liquid are responsible for the endothelial dysfunction observed in EC smokers.

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Manuscript received: 9.08.2016