



Toxicity mechanisms of Cigarette Smoke on Eye and Kidney using Isolated Mitochondria

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Abstract

Cigarette smoking is one of the main risk factors for premature human death associated to a variety of respiratory and vascular diseases, and cancer due to containing Hundreds of toxicants. Rat mitochondria were obtained by differential ultracentrifugation and incubated with different concentrations (1, 10 and 100%) of standardized cigarette smoke extract (CSE). Our results showed that cigarette smoke extract (CSE) induced a rise in mitochondrial ROS formation and mitochondrial membrane potential decrease before mitochondrial swelling ensued in isolated eye and kidney mitochondria. Disturbance in oxidative phosphorylation was also confirmed by decrease in ATP concentration in the CSE treated mitochondria. In addition, collapse of mitochondrial membrane potential (MMP) and mitochondrial swelling caused release of cytochrome c via outer membrane rupture or MPTpore opening. Our results suggested that cigarette smoke induced toxicity in both external directly exposed and visceral tissues is the result of disruptive effect on mitochondrial respiratory chain that leads to ROS formation, lipid peroxidation, mitochondrial membrane potential decline and cytochrome c expulsion which starts apoptosis signaling and cell loss.

Keywords: Cigarette smoking extracts (CSE), Cytochrome c release, isolated mitochondria; mitochondrial dysfunction.

1. Introduction

Cigarette smoking is one of the important risk factors for premature human death, several diseases, and cancer [1,2]. According to some epidemiological studies, cigarette smoke studies suggested that premature death is associated to a variety of respiratory and vascular role in the development of cancers and it is a major health hazard [3]. It is proven that cigarette smoke is a

complex and dynamic aerosol consisting of thousands of chemicals; the most recent estimate is 5600 individual smoke components of which approximately 158 have toxicological properties [4]. Besides, Hydrogen peroxide (H₂O₂), another constituent of cigarette smoke has been shown to participate in the mechanism of oxidative stress which makes cigarette smoke highly oxidative and is responsible for most of the damages at the origin of chronic obstructive pulmonary disease and emphysema [2].

The acute irritation of cigarette smoke on eyes and nose, and its annoying smell by means of a questionnaire, and the related effects, recorded various complaints in healthy and allergic people are all related to the quantity of smoked cigarette [3,4]. Andersson and Dalhamn

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[5] Noticed some irritating effects and headache when people were exposed to cigarette smoke. In these experiments, the concentration of particulate matter sometimes exceeded values of 10 mg/m, but no quantitative relations between the complaints and the concentration of Cigarette smoke were examined [6,7]. Distributed between the particulate and gaseous fractions and sometimes presented in both are chemicals known to be associated with various smoking related diseases. Aldehydes in the gaseous phase (formaldehyde, acrolein, acetaldehyde) are associated with chronic obstructive pulmonary disease (COPD) and lung toxicity, Polycyclic aromatic hydrocarbons (PAHs), tobacco specific nitrosamines (TSNAs), arsenic, cadmium and chromium in the particulate phase can be linked with various cancers [4-8]. There is an experimental and epidemiologic evidence that smoking-associated alteration of the antioxidant status can be attenuated by dietary antioxidant supplementation or therapy [9]. It is estimated that exposure to CSE may increase the risk of coronary heart disease due to platelet activation, increase in arterial stiffness, enhanced oxidative stress, decrease antioxidant defense, induction of endothelial dysfunction and precipitation of inflammation [10]. Besides, numerous epidemiological studies reported relationship between smoking and neurodegenerative diseases including Alzheimer and Parkinson. However, there is an evidence concerning a protective effect of smoking in Parkinson, but not a clear effect in Alzheimer [11].

It seems that the protective effect of smoking in Parkinson is related to nicotine that interacts with the mitochondrial complexes respiratory chain and decreases ROS generation. The increasing evidences suggested that in rat eye and kidney, nicotine induced oxidative stress *in vivo*, increasing ROS formation, lipid peroxidation, and inducing glutathione-S-transferase (GST) activity [11]. Increasing

interest of public in the tobacco products caused the tobacco industry to invest on better understanding of the mechanisms of toxicity caused by cigarette smoke in different vital tissue such as eye and kidney. According to the previous literature, these tissues are the main storage site and most important targets organs in CSE toxicity [11, 12]. Another study suggested that F2-isoprostanes, a marker of lipid peroxidation products increases in smoker's plasma, indicating that CSE can cause increases in lipid peroxidation *in vivo* [12]. On the other hand, Oxidative stress resulted from the imbalance between ROS production and detoxification enzymes could target cells leading to several tissues oxidative damage from the interaction of reactive oxygen with critical cellular macromolecules [3]. As ROS is mainly generated by mitochondria, we sailed that mitochondria is involved in CSE-induced ROS generation. So, we planned to study the toxic mechanisms of CSE in isolated rat mitochondria precisely by measuring different mitochondrial toxic parameters in eye and kidney tissues .

2. Materials and Methods

2.1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany) in the best commercial grade. Aqueous cigarette smoking extract was known as 100% CSE and used at lower concentrations (1, 10, and 100%) by diluting 100% CSE in deionized water.

2.2. Cigarette Smoke Extract Preparation

Cigarette smoke extract (CSE) was standardized by bubbling the smoke from one 1R5F research grade cigarette (1.67 mg tar, 0.16 mg nicotine, and 2.08 mg total particulate matter per cigarette; The University of Kentucky, Lexington, KY) into 10 mL of RPMI 1640 with 10% FBS over 3 min using a cigarette smoking apparatus. The CSE was pH corrected (7.4), then filter sterilized and the absorbance value read at

320 nm using a Tecan GENios plate reader (Basel, Switzerland), and only CSE preparations with an absorbance value of 0.42 ± 0.03 were used. The resulting CSE was known as 100% CSE and used at lower concentrations (1, 10, and 100%) by diluting 100% CSE in RPMI 1640 with 10% FBS. Vehicle control medium was made by bubbling air through RPMI 1640 with 10% FBS for 3 min followed by filter sterilizing.

2.3. Animals

Male wistar rats (250-300 g) were fed with a normal standard chow diet and tap water ad libitum. All experiments were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran. After the animals were decapitated, desire tissues (eye and kidney) were quickly dissected out; the tissues were pooled and rapidly rinsed using isotonic saline buffer. These samples were used for the isolation of mitochondria as described below.

2.4. Preparation of Mitochondria

Mitochondria were prepared from the Wistar rats eye and kidney using differential centrifugation [13]. The desired tissues was removed and minced with a small scissor in a cold mannitol solution containing 0.225 M D-mannitol, 75 mM sucrose and 0.2 mM EDTA. The minced desired tissues were gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at $1000 \times g$ for 10 min at 4 °C to remove the nuclei, unbroken cells, and other non-subcellular debris. The supernatants were centrifuged at $10,000 \times g$ for 10 min. The dark packed lower layer (mitochondrial fraction) was re-suspended in the mannitol solution and re-centrifuged twice at $10,000 \times g$ for 10 min. Mitochondrial sediments were suspended in Tris solution containing 0.05 M Tris-HCl buffer (pH = 7.4), 0.25 M sucrose, 20 mM KCl, 2.0 mM MgCl₂, and 1.0 mM Na₂HPO₄ at 4 °C before the assay. Aliquots of the suspension were used to determine the multi-parameters of oxidative stress. All tests were

performed three times. The concentrations of CSE (%) (1, 10 and 100) were chosen based on our pilot study.

2.5. Protein Concentration

Mitochondrial protein concentration was determined by the Coomassie blue protein-binding method using BSA as the standard [14].

2.6. Quantification of Mitochondrial ROS Level

Isolated mitochondria were incubated with CSE concentration (%) (1, 10 and 100) in respiration buffer containing 0.32 mM sucrose, 10 mM Tris, 20 mM MOPS, 50 μM EGTA, 0.5 mM MgCl₂, 0.1 mM KH₂PO₄ and 5 mM sodium succinate [15]. Following the addition of CSE concentration (%) (1, 10 and 100), a sample was taken and DCFH-DA was added (final concentration, 10 μM) to the mitochondria and was then incubated for 15 min.

2.7. Estimation of complex II activity

The activity of mitochondrial complex II (succinate dehydrogenase) was assayed through the measurement of MTT reduction and the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) [17].

2.8. Mitochondrial Membrane Potential Assay

The mitochondrial uptake of the cationic fluorescent dye, rhodamine123, has been used for the determination of mitochondrial membrane potential. The mitochondrial suspensions (500 Rg protein /mL) were incubated with various concentrations of CSE and then 10 RM of rhodamine123 was added to the mitochondrial solution in the respiration buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH₂PO₄, 2 mM MgCl₂, 50 μM EGTA, 5 mM sodium succinate, 10 mM

HEPES, 2 μ M Rotenone). The fluorescence was measured using Shimadzu RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively [20]. The capacity of mitochondria to uptake the rhodamine123 was calculated as the difference (between control and treated mitochondria) in rhodamine123 fluorescence. Our data was shown as the percentage of mitochondrial membrane potential collapse (% $\Delta\Psi_m$) in all treated (test) mitochondria groups.

2.9. Determination of Mitochondrial Swelling

Isolated mitochondria were suspended in swelling buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate and 1 RM of rotenone) and incubated at 30 °C with different concentration of CSE concentration (%) (1, 10 and 100) [17]. The absorbance was measured at 540 nm at 10 min time intervals with an ELISA reader (Tecan, Rainbow Thermo, Austria).

2.10. Statistical Analysis

Results are presented as means \pm SD. All statistical analyses were performed using the SPSS software, version 17. Assays were performed in triplicate and the mean was used for the statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test. Statistical significance was set at $P < 0.05$.

3. Results and Discussion

3.1. Effects of CSE Concentration (1, 10 and 100%) on Mitochondrial ROS Production

As shown in Table 1, CSE concentration (1, 10 and 100%) induced significant ROS formation rise on rat eye and kidney mitochondria that CSE-induced mitochondrial ROS production was concentration dependent in eye and kidney.

Table 1. aqueous cigarette smoking extract (CSE) induced ROS formation in isolated Mitochondria.

Group	DCF fluorescence intensity (%) after Groups 15 min incubation	
	Eye	Kidney
Control	10.2 \pm 3.0	52.48 \pm 5
+CSE (1%)	24.1 \pm 2*	78.9 \pm 2*
+CSE (10%)	48.8 \pm 2*	90.14 \pm 2*
+CSE (100%)	60.9 \pm 3.2*	97.5 \pm 2.1*

ROS formation was determined by flow cytometry using DCFH-DA as described in materials and methods and demonstrated as fluorescence intensity of DCF. Values represented as mean \pm SD (n=3). Control (no mitochondria) contains respiration buffer plus DCFH-DA (10 μ M). * $P < 0.05$ compared with control mitochondria at the same time interval.

3.2. Effect of CSE on Mitochondrial Complex II Activity

Succinate dehydrogenase (complex II) activity, the measure for mitochondrial functionality, was also assessed using the MTT test after 1h incubation of mitochondria obtained from eye and kidney with different CSE concentrations (1, 10 and 100%). Fig.1 shows a significant decrease in the mitochondrial metabolic conversion of MTT to formazon ($p < 0.05$) following addition of different

can decrease the activity of mitochondrial succinate dehydrogenase down to 50%.

According to our results the order of sensitivity of these tissues against cigarette smoke toxicity is kidney > eye.

3.3. Effect of CSE on mitochondrial membrane potential

The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the measurement of mitochondrial membrane

Table 2. Effect of aqueous cigarette smoking extract on mitochondrial membrane potential MMP collapse ($\Delta\Psi\%$) in isolated mitochondria.

Groups		$\Delta\Psi\%$				
Eye	5min	15min	30min	45min	60min	
Control	0±1	4±1	24±3	28±2	34±5	
+CSE (1%)	40±3.1*	52±3*	72±4*	47± 2 **	66±21***	
+CSE (10%)	70±6.2*	102±4*	129±6**	156±17**	161±16***	
+CSE (100%)	103±3*	108±5*	148±7***	192±15**	204±21***	
Kidney						
Control	2±1	8±2	10±4	14±2	17±3	
+CSE (1%)	76±20*	88±2**	93±7**	192±21***	95±4***	
+CSE (10%)	107±19*	184±24***	18±17***	282±1 ***	185±20***	
+CSE (100%)	204±7*	297±10***	304±24***	325±25*	341±11*	

Mitochondrial membrane potential collapse ($\Delta\Psi\%$) was measured by Rhodamine 123 as described in Materials and Methods. The effect of aqueous CSE concentration% (0, 1, 10 and 100) on the mitochondrial membrane potential decrease in Eye and kidney mitochondria was evaluated. The values are expressed as means \pm SD (n=3). Values represented as mean \pm SD (n=3). * $P < 0.05$. compared with control mitochondria.

concentrations of CSE (1, 10 and 100%) in two mentioned tissues. As shown in Fig. 1A, CSE concentration (1%) did not cause a significant decrease in the enzyme activity only in the eye tissue. Based on our results the IC₅₀ values for CSE on eye and kidney tissues are as follows: eye: 3.4%, kidney:11.4%. The IC₅₀ value is defined as a concentration of xenobiotics which

potential collapse. As shown in Table 2, CSE concentrations (1, 10 and 100%) significantly decreased the MMP in all the mitochondrial test groups obtained from eye and kidney in a concentration and time dependent manner ($p < 0.05$). CaCl₂ (50 RM), a known inducer of mitochondrial permeability transition (MPT) and

MMP collapse was used as a positive control (Table 3).

3.4. Effect of CSE on mitochondrial swelling

Any change of absorbance at 540 nm (A540) was assayed as mitochondrial swelling, which is an indicator of mitochondrial membrane permeability transition (MPT). Addition of CSE concentrations (1, 10 and 100%) to isolated mitochondria obtained from all the two tissues leads to mitochondrial swelling (Table 3).

Cigarette smoking is one of the main risk factors for premature human death which associated to a variety of cancer, respiratory and cardiovascular diseases, oxidative stress, and the age-related neurodegenerative disorders [2]. Many epidemiological reports suggested that cigarette smokers are at a greater risk of other cancers such as oropharynx, stomach, pancreas, liver, kidney, urinary bladder, colon, breast and prostate [3]. Cigarette smoke extract (CSE) contains many known potent carcinogens, that increases the oxidative burden of the cell, which when persisted may lead to many pathological conditions [3]. Although the underlying mechanisms involved in the pathogenesis associated with CSE are not still clear. It seems that free radical-induced oxidative damage plays a major role in the pathogenesis of numerous smoking-related disorders. In the present study, we ,therefore, investigated oxidative stress related toxic mechanisms of CSE on isolated mitochondria obtained from rat Eye and kidney to clarify the role of CSE on these vital organs toxicity.

Exposure to CSE compounds other than nicotine caused inhibition of viability, proliferation and differentiation of cell culture [21].The main irritants in cigarette smoke are-besides the particulates-the aldehydes, especially acroleine, mammonia, some hydrocarbons, and to some extent the nitrogen oxides[22, 23]. In

this investigation we observed several important findings that warrant additional discussion. First of all, Based on our results the IC50 values for CSE on eye and kidney mitochondria were 3.4%, 11.4% respectively. This suggests that the order of sensitivity of these tissues against cigarette smoke toxicity is: eye<kidney. Secondly, a rapid increase in ROS formation was observed in all investigated tissues following addition of CSE on isolated mitochondria. This confirmed the probable involvement of mitochondrial ROS in CSE induced toxicity mechanisms (Table 1). Besides, acute exposure to CSE adversely affected vascular function by promoting oxidative stress and endothelial dysfunction via increased superoxide production in vascular wall [24, 25].The reactive radicals can act as initiators and promoters of carcinogenesis, cause DNA damage, activate pro carcinogens, and alter the cellular antioxidant defense system. The effective detoxification mechanism comprises SOD and catalase, which work in a sequential manner in the disposal of superoxide radical and conversion of hydrogen peroxide to water [28,29]. Changes in GSH homeostasis have also been implicated in the etiology and progression of a number of pathological diseases. Murai and Oya studied that Kidney cancer accounts for 1.9% of all malignancies, with approximately 189 000 new cases diagnosed globally each year and the incidence varying more than 10-fold around the world, with the highest rates generally observed in central European countries and among blacks in the United States. Smoking is an established risk factor, although the increase in risk is moderate. [21, 30].

Shapiro and Bergstrom had studied that other possible risk factors are hypertension and obesity, with potentially differing risks for men and women [30]. On the other hand ,application of herbal medicine such as lemon grass and chamomile and essential oils of ambrette seeds and citronella could ameliorate toxicities in rat

eye and kidney against cigarette smoke extract via high free radical scavenging potential leading to decreased lactate dehydrogenase (LDH) activity, and increased glutathione reductase, xanthine oxidase and catalase activities [25]. Secondly, the effect of CSE on succinate dehydrogenase (complex II) activity showed a significant reduction in function of complex II activity following CSE incubation in isolated desired tissues. Reduction in Complex II activity ameliorates mitochondrial respiration rates as indicator of the mitochondrial oxidative capacity [26]. It seems that oxidation of mitochondrial lipid membranes could result in disruption of mitochondrial electron transfer chain and consequently collapse of MMP and cytochrome c release.

Our results in this investigation confirmed that collapse of mitochondrial membranes potential and mitochondrial swelling which both events lead to increased ROS formation and disturbance in mitochondrial electron transfer. Other study showed that the morphological and inflammatory changes induced by chronic CSE exposure are accompanied by increase in TNF- α (an inflammatory marker), thickening of the airway epithelium, goblet cell hyperplasia, and reduce in anti-oxidative stress markers (total GSH levels). This report demonstrated that mitochondria were damaged after exposure of isolated mitochondria to CSE due to down regulation of an ATP synthase subunit. This leads to mitochondrial dysfunction.

4. Conclusion

The precise mechanism of cigarette extract toxicity is still unknown but the present study suggested that oxidative stress in tissues of Eye and kidney is directly involved in CSE toxicity mechanism. Our results showed that addition of CSE to isolated mitochondria causes electron transfer chain impairment at complexes I, II

and/or III leading to increased ROS production, failure of oxidative phosphorylation, mitochondrial swelling and finally release of cytochrome c which can then promote cell death signaling. Besides, our study showed nearly similar results in the isolated mitochondria obtain from three different tissues (i.g. liver and skin) This can help to better understanding of CSE toxicity mechanism. To our knowledge this is the first study to address and compare CSE - induced oxidative stress and its consequences on isolated rat Eye and kidney mitochondria which may provide insight into the role of CSE in human health and disease. Funding: The authors received no funding from national or international sources. The research was funded by the authors. The investigation was carried out in the Professor J. Pourahmad's laboratory at Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Declaration of Interests: The authors have no competing interests with anybody in the world.

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