

## A Comparison of Toxicity Mechanisms of Cigarette Smoke on Isolated Mitochondria Obtained from Rat Liver and Skin

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### Abstract

Previous studies demonstrated that CSE induces oxidative stress and its consequences on isolated mitochondria obtained from lung, heart and brain which may provide insight into the role of CSE in human health and disease. The present study was carried out to further characterize and compare toxic effect of CSE extract on isolated mitochondria obtained from either a directly contacting tissue (*i.e.* skin) or a vital visceral tissue (*i.e.* liver). We obtained Rat liver and skin mitochondria by differential ultracentrifugation and incubated the isolated mitochondria with different concentrations (1, 10 and 100%) of standardized cigarette smoke extract (CSE). Our results were similar to our previous study which discovered CSE toxicity mechanisms on isolated mitochondria obtained from lung, heart and brain with minor changes. CSE induced a significant rise in ROS formation, lipid peroxidation and mitochondrial membrane potential collapse and mitochondrial swelling on isolated mitochondria obtained from both liver and skin. CSE induced Decrease in ATP concentration on isolated mitochondria obtained from both liver and skin did not include CSE lowest concentration (1%). Our findings showed that CSE-induced toxicity in liver and skin is due to disruptive effect on mitochondrial respiratory chain which can lead to cytochrome c release and apoptosis signaling.

**Keywords:** Cigarette smoke extracts (CSE); Toxicity; Isolated mitochondria; Liver; Skin.

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### Introduction

Cigarette smoking is a complex mixture of 40 different compounds with toxic and/or carcinogenic potential (1). Numerous studies showed the potential hazard of cigarette smoke for infants and children (2). High incidence of respiratory tract diseases and cancer in heavy smokers may reflect cigarette smoking induced impairment in the immune system (3). Controversially; cigarette smoke (CS) negatively

affects on heart diseases, atherosclerosis, fatty liver diseases and premature skin aging (4-6). Other study suggested that CS caused an imbalance in connective tissue matrix components (7) According to some epidemiological studies; Cigarette smoking in low dose in chronic time induces considerable teratogenic and carcinogenic effects by nicotine on new born rat (8).

Mitochondria are dynamic organelles essential for cellular life, death, and differentiation. They are best known for ATP production via oxidative phosphorylation (OXPHOS), and are centers for apoptosis and

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ion homeostasis(9,10). Also mitochondrial respiratory chain is a rich source of reactive oxygen species and the cellular production of hydrogen peroxide and they are also vulnerable to oxidative stress (11). Previous studies also showed that CS increases ROS generation inside and outside of mitochondrial respiratory chain. Reactive oxygen species are promoters of chemical modification and conformational changes in membrane polypeptides and lipids(3). Numerous studies have demonstrated that oxidative stress due to the mitochondrial dysfunction plays a key role in tissue injury and cell apoptosis (12). Therefore, we planned to study and compare the toxicity mechanisms of CS extract on isolated rat mitochondria obtained from a directly contacted peripheral organ (skin) and also a visceral indirectly contacted organ (liver).

## Experimental

### *Chemicals*

All chemicals were purchased from Sigma-Aldrich (Taufkrichen, Germany). All chemicals were of the best commercial grade. Cigarette smoke extract (CSE) was standardized and used at lower concentrations (1, 10, and 100%) by diluting 100% CSE in RPMI 1640 with 10% FBS.

### *Animals*

Male Sprague-Dawley rats (200-300 g) that had access ad libitum to water were used in the experiments in a controlled temperature ( $22 \pm 1$  °C) and humidity of 70-80% under artificial light with 12 h light/dark cycle. All the experiments were carried out according to established ethical standards approved by the Committee of Animal Experimentation in Shahid Beheshti University of Medical Sciences, Tehran, Iran

### *Preparation of mitochondria*

Rats were decapitated and the liver and skin were surgically harvested, minced and homogenized with a glass hand held homogenizer with previous method (13). Protein concentration was determined by the Coomassie blue protein-binding method using BSA as the standard sample (14).

### *In-vitro evaluation of mitochondrial parameters*

The mitochondrial ROS production was assayed by F-2500 fluorescence spectrophotometer (HITACHI) using DCFH-DA in the period of 60 min (15). The activity of mitochondrial complex II (succinate dehydrogenase) was determined by measuring the reduction of MTT (16). The content of the lipid peroxidation marker (MDA) was assessed by measuring the absorbance of the supernatant at 532 nm with an ELISA reader as described in previous study (17). Reduced glutathione (GSH) level was determined in mitochondrial extracts using DTNB reagent using by spectrophotometer. GSH content was expressed as  $\mu\text{g}/\text{mg}$  protein (18). Mitochondrial membrane potential was determined by mitochondrial uptake of rhodamine 123 with fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively (19). Mitochondrial swelling was assayed using a previously reported method by monitoring the absorbance at 540 nm (20). The ATP level and ATP/ADP ratio were measured by luciferase enzyme (21). Finally, concentration of cytochrome c was determined by using the Quantikine<sup>®</sup> Rat/Mouse Cytochrome c Immunoassay kit (Minneapolis, Minn).

### *Statistical analysis*

All experiments were performed with triplicates (N=3). All results are expressed as mean  $\pm$  SD. Probability p-values  $<0.05$  were considered statically significant.

## Results

As shown in Table 1, CSE concentrations (10 and 100%) induced a significant rise at ROS formation on both liver and skin mitochondria. However, lower concentration of CSE (1%) did not significantly increase mitochondrial ROS generation during 60 min of exposure, compared to control skin mitochondria ( $P>0.05$ ). Increased ROS formation at each concentration of CSE is expressed as DCF fluorescence intensity unit (Table 1). As shown in Table 2, 1 h exposure of liver and skin mitochondria to different concentrations of CSE (1, 10 and 100%) results in significant decrease in the mitochondrial

**Table 1.** Aqueous cigarette smoke extract (CSE) induced ROS formation on isolated skin and liver mitochondria.

Groups	ROS				
	5min	15 min	30 min	45 min	60 min
<b>Skin</b>					
Control	0 ± 1	2 ± 1	10 ± 3	20 ± 2	29 ± 2
+CSE (1%)	3 ± 1	18 ± 4	34 ± 5	44 ± 7	59 ± 8
+CSE (10%)	23 ± 5***	125 ± 7***	129 ± 11***	141 ± 9***	154 ± 13***
+ CSE (100%)	29 ± 5***	255 ± 14***	266 ± 18***	284 ± 14***	292 ± 23***
<b>Liver</b>					
Control	0 ± 1	2 ± 1	4 ± 3	9 ± 2	14 ± 2
+CSE (1%)	3 ± 2	18 ± 1**	21 ± 6*	24 ± 5*	29 ± 3*
+CSE (10%)	14 ± 4*	49 ± 6***	51 ± 12***	56 ± 9***	59 ± 9**
+ CSE (100%)	23 ± 7***	75 ± 9***	79 ± 8***	80 ± 11***	88 ± 18***

ROS formation was determined by fluorescence spectrophotometer using DCFH-DA as described in materials and methods and demonstrated as DCF fluorescence intensity unit. Values represented as mean±SD (n=3). \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$  compared with control mitochondria at the same time interval.

reduction of MTT to formazan ( $p<0.05$ ).

On the other hand, addition of concentrations of CSE (10 and 100%) to both liver and skin mitochondria, significantly increased MDA formation compared to their corresponding control mitochondria. However, lower concentration of CSE (1%) did not significantly increase MDA formation on isolated skin mitochondria ( $P>0.05$ ) (Table 3).

Incubation of different CSE concentrations (1, 10 and 100%) significantly decreased GSH

levels on isolated mitochondrial obtained from both skin and liver tissues following 1 h compared to their corresponding control mitochondria ( $P<0.05$ ) (Table 4).

The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the measurement of mitochondrial membrane potential collapse. As shown in Table 5, CSE concentrations (1, 10 and 100%) significantly induced MMP collapse on isolated liver mitochondrial after 30 min of incubation

**Table 2.** Effect of aqueous cigarette smoke extract (CSE) on Succinate dehydrogenase (complex II) activity (%) on both liver and skin mitochondria.

Groups	Succinate dehydrogenase (complex II) activity (%)	
	Liver	Skin
Control	100 ± 1.400 ± 1.4	100 ± 1
+CSE (1%)	85 ± 4.77*	82.08 ± 6.3*
+CSE (10%)	74.9 ± 0.90*	66.06 ± 7.8**
+ CSE (100%)	42.50 ± 0.42***	44.45 ± 1.9***

Succinate dehydrogenase activity was measured using MTT dye as described in Materials and methods. Isolated mitochondria (0.5 mg/mL) were incubated for 1 h with various concentrations of CSE (0, 1, 10 and 100%). Values represented as mean±SD (n=3). \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$  compared with control mitochondria.

**Table 3.** Effect of aqueous cigarette smoke extract (CSE) on lipid peroxidation on both liver and skin mitochondria.

Groups	MDA(µg/mg protein)	
	Liver	skin
Control	4.82 ± 1.82	3.89 ± 1.54
+CSE (1%)	8.05 ± 1.97	7.94 ± 1.28
+CSE (10%)	15.77 ± 0.96**	13.82 ± 0.93**
+ CSE (100%)	27.30 ± 0.86***	17.14 ± 1.07***

Isolated mitochondria (0.5 mg/mL) were incubated for 1h with various concentrations of aqueous CSE(0,1,10 and 100%) .Values represented as mean±SD (n=3). \* $P<0.05$  compared with control mitochondria.

**Table 4.** Effect of aqueous cigarette smoke extract (CSE) on the GSH level on both liver and skin mitochondria.

Groups	GSH( $\mu\text{g}/\text{mg}$ protein)	
	Liver	Skin
Control	54.07 $\pm$ 0.50	13.68 $\pm$ 0.48
+CSE (1%)	37.96 $\pm$ 1.01**	9.07 $\pm$ 1.06***
+CSE (10%)	29.67 $\pm$ 0.78***	7.43 $\pm$ 1.10***
+ CSE (100%)	24.67 $\pm$ 0.49***	4.27 $\pm$ 0.94***

Isolated mitochondria (0.5 mg/mL) were incubated for 1h with various concentrations of aqueous CSE (0,1 ,10 and 100%) .Values represented as mean $\pm$ SD (n=3). \* $P$ <0.05 compared with control mitochondria.

( $p$ <0.05) (Table 5). As shown in Table 5, CSE concentration (1%) did not induce significant MMP collapse after 60 min of incubation.

A decreased light absorbance is consistent with an increase in mitochondrial volume reflected the opening of mitochondrial ion channels and membrane pores. Our result showed that there were a significant decrease in absorbance following incubation of both rat liver and skin mitochondria with different CSE concentrations (1, 10 and 100%) after 45 min of incubation on isolated liver mitochondria and after 1 hour of incubation on isolated mitochondria which is consistent with our MMP collapse and lipid peroxidation results (Table 6).

We also measured the ATP levels on isolated mitochondria obtained from rat liver and skin following the addition of CSE concentrations (1, 10 and 100%). As shown in Table 7, CSE

concentrations (10 and 100%) significantly decreased mitochondrial ATP levels on both skin and liver mitochondria compared to their corresponding control mitochondria. ATP depletion is an indicator of mitochondrial dysfunction (Table 7).

Finally, cytochrome c release, important endpoint of cell death signaling was determined. Our results showed that significant ( $P$ <0.05) cytochrome c release following exposure of isolated liver mitochondria to different concentrations of CSE in a concentration dependent manner (Table 8), while only higher concentrations of CSE (10 and 100%) induced significant ( $P$ <0.05) release of cytochrome c from skin mitochondria. Significantly, the pretreatment of CSE-treated mitochondria with MPT inhibitor of cyclosporine A (Cs A) and butylated hydroxyl toluene (BHT), an

**Table 5.** Effect of aqueous cigarette smoke extract on mitochondrial membrane. Potential MMP collapse ( $\Delta\Psi\%$ ) on both liver and skin mitochondria.

Groups	$\Delta\Psi\%$				
	5 min	15 min	30 min	45 min	60 min
<b>Skin</b>					
Control	0 $\pm$ 2	13 $\pm$ 1	17 $\pm$ 5	21 $\pm$ 4	21 $\pm$ 2
+CSE (1%)	4 $\pm$ 1*	21 $\pm$ 5	26 $\pm$ 4	35 $\pm$ 8	41 $\pm$ 5**
+CSE (10%)	13 $\pm$ 1***	42 $\pm$ 16	48 $\pm$ 7***	55 $\pm$ 11*	58 $\pm$ 4***
+ CSE (100%)	22 $\pm$ 1***	57 $\pm$ 18*	59 $\pm$ 2***	61 $\pm$ 13**	66 $\pm$ 7***
<b>Liver</b>					
Control	0 $\pm$ 1	14 $\pm$ 2	21 $\pm$ 5	31 $\pm$ 1	45 $\pm$ 2
+CSE (1%)	1 $\pm$ 1	17 $\pm$ 4	58 $\pm$ 3**	86 $\pm$ 8***	147 $\pm$ 11***
+CSE (10%)	5 $\pm$ 2	22 $\pm$ 5	63 $\pm$ 9***	94 $\pm$ 9***	149 $\pm$ 15***
+ CSE (100%)	6 $\pm$ 3	22 $\pm$ 3	86 $\pm$ 10***	97 $\pm$ 7***	138 $\pm$ 13***

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 on liver and skin mitochondria were evaluated. The values are expressed as means  $\pm$  SD (n=3). Values represented as mean $\pm$ SD (n=3). \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001 compared with control mitochondria.

**Table 6.** Effect of aqueous cigarette smoke extract (CSE) on the mitochondrial swelling on both liver and skin mitochondria.

Groups	Mitochondrial Swelling percent (%)				
	5 min	15 min	30 min	45 min	60 min
<b>Skin</b>					
Control	0 ± 2	13 ± 1	17 ± 5	21 ± 4	21 ± 2
+CSE (1%)	4 ± 1	21 ± 5	26 ± 4	35 ± 8	41 ± 5**
+CSE (10%)	3 ± 1	42 ± 16	48 ± 7***	55 ± 11*	58 ± 4***
+ CSE (100%)	4 ± 3	57 ± 18*	59 ± 2***	61 ± 13**	66 ± 7***
<b>Liver</b>					
Control	0 ± 1	1 ± 1	2 ± 1	3 ± 1	5 ± 2
+CSE (1%)	6 ± 2	11 ± 2*	13 ± 4	16 ± 1***	17 ± 2***
+CSE (10%)	29 ± 2***	30 ± 4***	40 ± 4**	40 ± 2***	41 ± 2***
+ CSE (100%)	69 ± 9***	69 ± 4***	71 ± 15***	72 ± 4***	73 ± 1***

Mitochondrial swelling was measured by determination of absorbance at 540 nm as described in Materials and methods. Values represented as mean±SD (n=3). \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 compared with control mitochondria.

antioxidant, inhibited cytochrome c release as compared with CSE-treated group (10%), indicating the role of oxidative stress and MPT pore opening in cytochrome c release following cigarette smoke exposure in both liver and skin tissues (Table 8).

### Discussion

According to previous studies, CSE shows liver pathogenesis, including decreased cellular antioxidant levels, increased lipid peroxidation, and increased CYP2E1 induction (22). Besides, fatty liver disease induced by cigarette smoke is associated with cardiovascular disease risk (23). Numerous studies showed CSE caused ROS generation via interaction with mitochondrial respiration which could be associated with pathological conditions such as aging, diabetes and cancers (24,25). We therefore investigated

and compared toxicity mechanisms of CSE on isolated mitochondria obtained from rat skin and liver.

Based on our results, CSE at various concentrations induced increased ROS formation on both skin and liver mitochondria (Table 1). Mitochondria are an important source of ROS formation in mammalian cells (26). Furthermore, our results showed that decreased complex II (succinate dehydrogenase) activity is involved in CSE-induced tissue damage in both rat skin and liver (Table 2). Based on these results the IC<sub>50</sub> values for CSE on skin and liver mitochondria were 14.44%, and 45.76% respectively. This suggests that the skin tissue is much more sensitive than liver tissue against CSE toxicity.

Lipid peroxidation has been proven as a major mechanism of free radicals induced cell damage. It may alter intrinsic membrane

**Table 7.** Effect of aqueous cigarette smoke extract (CSE) on mitochondrial ATP level on both liver and skin mitochondria.

Groups	ATP (μmol/mg protein )	
	Liver	Skin
Control	2.61 ± 0.12	2.78 ± 0.20
+CSE (1%)	2.19 ± 0.04	2.73 ± 0.19
+CSE (10%)	1.72 ± 0.29*	1.28 ± 0.01**
+ CSE (100%)	0.64 ± 0.06***	0.89 ± 0.18***

Isolated mitochondria (0.5 mg/mL) were incubated with CSE concentrations (0, 1, 10 and 100) and ATP levels were determined after 1 h of incubation using *Luciferin/Luciferase* Enzyme System as described in Materials and methods. Values represented as mean±SD (n=3). \*\*P<0.01; \*\*\*P<0.001 compared with control mitochondria.

**Table 8.** Effect of aqueous cigarette smoke extract (CSE) on cytochrome c release on both liver and skin mitochondria.

Groups	Cytochrome C release ( ng/mg protein)	
	Liver	Skin
Control	42 ± 11	42 ± 17
+CSE (1%)	88 ± 17**	50 ± 24
+CSE (10%)	152 ± 30***	101 ± 20*
+ CSE (100%)	254 ± 23***	166 ± 8***
+CSE (10%) +BHT	92 ± 43	84 ± 5
+CSE (10%) +CsA	98 ± 40	80 ± 4

Isolated mitochondria (0.5 mg/mL) were incubated for 1h with various concentrations of aqueous CSE (0,1 ,10 and 100%).The amount of released cytochrome c from mitochondria was determined after 1 h of incubation using Rat/Mouse Cytochrome c ELISA kit as described in Materials. Values represented as mean±SD (n=3). \* $P < 0.05$  compared with control mitochondria.

properties, due to physicochemical changes of oxidized lipids (27). Our results also showed that there was significant MMP collapse on both skin and liver mitochondria after treating with various concentrations of CSE. It seems that oxidation of mitochondrial lipid membranes could result in disruption of mitochondrial membrane potential and MPT pore opening and finally cytochrome c release. Besides, MPT plays a key role in necrotic cell death via oxidative stress including increasing ROS formation, lipid peroxidation and GSH oxidation (28).

Oxidation of thiol groups (GSH) on both mitochondrial outer or inner membranes could cause conformational change in mitochondrial permeability transition pore (MPT) and also MMP collapse, which are generally considered as potential end points in many conditions associated with oxidative stress (17). Moreover, Cs A and BHT pretreatment completely blocked the CSE-induced release of cytochrome c from both liver and skin mitochondria which supports the hypothesis that the apoptosis induction via CSE is due to an oxidative stress and depends on the opening of the mitochondrial transition pore in liver and skin tissues. Our results confirmed the hypothesis that impairment of ETC by cigarette smoke results in reduced ability of mitochondria for ATP synthesis leading to MPT pores opening which is associated with substantial mitochondrial swelling and finally cytochrome c release on mitochondria obtained from rat liver and skin.

Based on the  $IC_{50}$  values (succinate dehydrogenase activity assay) for CSE on skin and liver mitochondria, the skin tissue is much

more sensitive than liver tissue against CSE toxicity. On the other hand, as shown in Table 8, CSE could induce more cytochrome c release and apoptosis signaling in rat liver tissue than skin tissue, perhaps in the latter it favored mostly necrotic mode of cell death.

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