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# Can Vitamin E and Selenium Prevent Cigarette Smoke-Derived Oxidative mtDNA Damage?

## [E Vitamini ve Selenyum Sigara Dumanı Kaynaklı Oksidatif mtDNA Hasarını Önleyebilir mi?]

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**ABSTRACT**

**Objectives:** The reactive oxygen species and their active intermediate metabolites produced by cigarette smoke may create oxidative damage on mitochondrial DNA. Damages of mtDNA may trigger mitochondrial dysfunction. Mitochondria contribute to cardiac dysfunction and myocyte injury via a loss of metabolic capacity and by the production and release of toxic products. Reactive oxygen species induce myocyte hypertrophy, apoptosis, and interstitial fibrosis. These cellular events play an important role in the development and progression of maladaptive cardiac remodeling and failure. Oxidative mtDNA damage that have been created by cigarette smoke and possible protective effects of Vitamin E and selenium which are powerful antioxidants were measured in the heart tissue of *Mus musculus* in this study.

**Methods:** Quantitative polymerase chain reaction method was used to measure oxidative damage and mtDNA copy number in heart tissue. It is highly sensitive for measuring DNA damage and repair. 6 groups were designed for the Cigarette Smoke, Vitamin E and Selenium applications.

**Results and Conclusion:** According to our results cigarette smoke induced the oxidative mtDNA damage in the heart tissue of mice. Also these results have been obtained to suggest that the antioxidant supplementation may prevent this damage. This is important for the children who had smoker parents and all second-hand smokers especially.

**Key words:** cigarette smoke, mitochondrial DNA, oxidative damage, vitamin E, selenium, heart

**ÖZET**

**Amaç:** Sigara dumanı tarafından üretilen reaktif oksijen türevleri ve aktif ara metabolitler, mitokondrial DNA da oksidatif hasar yaratabilirler. mtDNA hasarları mitokondri fonksiyonlarında bozulmaları tetikleyebilir. Bu durum toksik ürünlerin üretimi ve metabolik kapasitenin azalması ile kardiyak fonksiyon bozukluğu ve miyositlerde hasar meydana getirir. Reaktif oksijen türevleri ayrıca miyosit hipertrofisi, apoptozis ve interstitial fibrozisi indükler. Bu hücresel olaylar kalp kasında işlev kaybı ve kalp krizi gelişimi ve ilerlemesinde önemli bir rol oynar. Bu çalışmada sigara dumanının *Mus musculus*'un kalp dokusunda ortaya çıkarttığı oksidatif mtDNA hasarı ve güçlü antioksidanlar olan E vitamini ve selenyumun olası koruyucu etkileri araştırılmıştır.

**Yöntem:** Kalp kasında oksidatif hasar ve mtDNA kopya sayısının ölçülmesinde kantitatif PCR metodu kullanılmıştır. Kantitatif PCR metodu DNA hasar ve tamirini ölçmek için çok duyarlı bir metoddur. Sigara dumanı, E vitamini ve selenyum uygulamaları için 6 grup oluşturulmuştur.

**Bulgular ve Sonuç:** Bizim yaptığımız çalışmanın sonuçlarına göre sigara dumanı farelerin kalp dokusunda oksidatif mtDNA hasarı meydana getirmektedir. Yine elde ettiğimiz sonuçlara göre antioksidan desteği bu hasarı önleyebilir. Bu sonuç özellikle ebeveynleri sigara içen çocuklar açısından ve tüm ikincil içiciler açısından önemlidir.

**Anahtar Kelimeler:** Sigara dumanı, mitokondrial DNA, oksidatif hasar, E vitamini, selenyum, kalp

## Introduction

DNA damage that is originated by cigarette smoke in various organs is declared by some research [1, 2]. Tobacco smoking contains many thousands of chemicals including a plethora of mutagens. Many carcinogens undergo metabolic activation in mammalian tissues to reactive intermediates that interact with and modify informational macromolecules, such as DNA with potentially mutagenic consequences [3]. PAHs (Polycyclic aromatic hydrocarbons) cause irreversible DNA damage via covalent binding or oxidation [4]. However genetic damage reflecting individual exposure and susceptibility to PAH may play a role in disease development [5].

Tobacco smoke contains major classes of carcinogens that include PAHs, aromatic amines and tobacco-specific nitrosamines. In addition, toxic compounds such as formaldehyde, acetaldehyde, acrolein, short-lived radicals and reactive oxygen intermediates generated by redox cycling from catechol and hydroquinone and nitric oxide (NO) may also contribute to the toxic and carcinogenic effects of tobacco smoke. Direct DNA-damaging compounds that are present in cigarette smoke (CS) have previously been reported to include reactive oxygen intermediates, peroxynitrite, ethylating agents and unidentified compounds [6].

Many carcinogens in the cigarette smoke like PAHs, nitrosamine and cisplatin bind mitochondrial DNA (mtDNA) preferentially [7]. Mammalian cells have hundreds of mitochondria and thousands of mtDNA. Although there is no nucleotide excision repair in mammalian mtDNA, there is effective base excision repair [8].

mtDNA damages may trigger mitochondrial dysfunction. Mitochondria contribute to cardiac dysfunction and myocyte injury via a loss of metabolic capacity and by the production and release of toxic products [9]. Recent experimental and clinical studies have suggested that oxidative stress is enhanced in heart failure. Chronic increases in oxygen radical production in the mitochondria can lead to a catastrophic cycle of mtDNA damage as well as functional decline, further oxygen radical generation, and cellular injury. Reactive oxygen species induce myocyte hypertrophy, apoptosis, and interstitial fibrosis by activating matrix metalloproteinases. These cellular events play an important role in the development and progression of maladaptive cardiac remodeling and failure [10].

A substantial number of studies suggest that altered levels of oxidative and nitrosoxidative stress within the cardiovascular environment are essential in the development of cardiovascular disease; however, the impact of such changes on the subcellular or organellar components and their functions that are relevant to cardiovascular disease inception are less understood. In this regard, studies are beginning to show that mitochondria not only appear susceptible to damage mediated by increased oxidative and nitrosoxidative stress, but also play significant roles in the regulation of cardiovascular cell function. In addition, accumulating evidence suggests that a common theme among cardiovascular disease development and cardiovascular disease risk factors is increased mitochondrial damage and dysfunction [11].

The antioxidants are used frequently as food supplements may be effective to preventing cigarette smoke damage on mtDNA. Damages that are created by CS may be prevented by vitamin E (Vit E) and selenium (Se) which are powerful antioxidants. CS originated mtDNA damages in cardiac tissue and effects of Vit E and Se were investigated in this study. Quantitative polymerase chain reaction (QPCR) method was used to measure oxidative damage. The lesion present in the DNA, block the progression of any thermostable polymerase on the template. So the DNA amplification decreases in damaged template. QPCR method is highly sensitive for measuring DNA damage and repair [12].

## Materials And Methods

### *Animal Care and Experimental Applications:*

Two months old male mice (*Mus musculus*, Balb/C) were used in these experiments. The Ethic Council Report to allow this search was given by the Akdeniz University. The animals were housed in plastic cages on sawdust bedding. Mice were fed with "Low selenium diet rat modified" from MP Biomedicals and tap water *ad libitum*. The animal room temperature was 23 ± 2 ˚C with relative humidity of %45 and 12h day-night light cycle.

After 5 days acclimatization the mice were randomly divided into six groups. Mice were exposed to cigarette smoke (CS) generated by one cigarette during 20 minutes, namely 3 cigarettes and totally 60 minutes per day for up to 20 weeks. Some groups were treated daily with selenium

(L-selenomethionine, 3.5 µg / kg body weight) [13] and/ or vitamin E (dl α tocoferol acetate, 2.5 mg/ kg body weight) [14] which has been started exactly the same day as their exposure to CS.

Whole body exposure mainstream cigarette smoke was obtained by using filter-tipped commercial cigarette (Maltepe, Turkish tabac). Mice undergoing the smoke treatment were placed in a 110x43x20 cm closed fiberglass chamber. Smoke homogenisation in the chamber was achieved by a fan (0.4 watt, 6 cm radius).

Experimental groups were: "CS", "CS+Vit E", "CS+Se", "CS+VitE+Se", "Vit E+Se" (without smoke) and "control" (neither smoke nor Vit E and Se). Each of these groups was designed for 5 months application time. There were 7 mice in each experimental group.

After their respective treatments, mice from each group

were anesthetized. The heart excised and immediately perfused with cold serum physiologic on ice. The samples were kept at –80 ºC until the experiment day.

Quantitative PCR:

SIGMA G1N-350 GenElute Mammalian Genomic DNA kits were used for total DNA isolation according to the technical bulletin.

Molecular Probes Pico Green dsDNA quantitation kit were used for both template DNA quantitation and the analysis of PCR products as fluorometrically 485 nm excitation, 530 nm emission. A crucial step of quantitative PCR is the concentration of the DNA sample. In fact, the accuracy of the assay relies on initial template quantity because all of the samples must have exactly the same amount of DNA. The Pico Green kit has not only proved efficient in regarding to template quantitation but also to PCR product analysis [12].

SIGMA Jump Start ready mix Taq were used for PCR. In this mix Taq polymerase combines the performance enhancements of Taq antibody for hot start. When the temperature is raised above 70ºC in the first denaturation step of the cycling process, the complex dissociates and the polymerase becomes fully active. DMSO as 4% of total volume and 20 ng of template total DNA were added into the each PCR tube.

Mouse 117 bp mtDNA fragment (small fragment) primers were:

13597 5'- CCC AGC TAC TAC CAT CAT TCA AGT- 3'

13688 5'- GAT GGT TTG GGA GAT TGG TTG ATG- 3'

Mouse 10 kb mtDNA fragment (long fragment) primers were:

3278 5'- GCC AGC CTG ACC CAT AGC CAT AAT AT- 3'

13337 5'- GAG AGA TTT TAT GGG TGT AAT GCG G- 3'

[12].

For long fragment PCR amplification, DNA was denatured initially at 75ºC for 2 min and 95ºC for 1 min, and then the reaction underwent 21 PCR cycles of 94ºC for 15 sec, 59ºC for 30 sec, and 65 ºC for 11 min. Final extension was allowed to proceed at 72ºC for 10 min.

For small fragment PCR amplification, DNA was denatured initially at 75 ºC for 2 min and 95 ºC for 15 sec, and then the reaction underwent 19 PCR cycles of 94ºC for 30 sec, 50ºC for 45 sec, and 72 ºC for 45 sec. Final extension was allowed to proceed at 72ºC for 10 min.

We were always run a 50% template control and a nontemplate control in PCR. This control should given a 50% reduction of the amplification signal. The nontemplate control would detect contamination with spurious DNA or PCR products [12].

QPCR method was used to measure oxidative damage. The lesion present in the DNA, block the progression of any thermostable polymerase on the template. So the DNA amplification decrease in damaged template. QPCR method is highly sensitive for measuring DNA damage and repair. mtDNA damage was quantified by comparing the relative efficiency of amplification of long fragments of DNA and normalizing this to gene copy numbers by the amplification of smaller fragments, which have a statistically negligible likelihood of containing damaged bases. To calculate relative amplification, the long QPCR values were divided by the corresponding short QPCR results to account for potential copy number differences between samples (mtDNA/total DNA value may be different in 20 ng template total DNA of each PCR tube) [12,15]. The copy number results not indicate the damage.

### *Statistical Analysis:*

Minitab Release 13.0 statistical software was used for analysis. The results were estimated with Kruskal Wallis test.

## Results and Discussion

Quantitative PCR method is highly sensitive for measuring oxidative DNA damage. The lesion present in the DNA, blocks the progression of any thermostable polymerases on the template; decreasing DNA amplification in the damaged template. To calculate relative amplification, the long QPCR values were divided by the corresponding short QPCR results (copy number results) to account for potential copy number differences between samples [12,15]. Although the QPCR is a relatively new method for measuring of DNA damage, the method is used in many studies in literature (12, 15, 16, 17, 18).

Decreased relative amplification is an indicator of the damaged DNA in the QPCR method. Relative amplification results were shown in Table 1 and Figure 1.

According to these relative amplification results showing mtDNA damage, "CS" group was significantly different from all other groups ($p<0.05$). mtDNA damage was significantly higher in the "CS" group than the other groups. However "CS+Vit E+Se" group had lowest mean damage. Selenium and vitamin E might be showed an additive protective effect. Vitamin E regulates mitochondrial peroxide formation by serving as an antioxidant in mitochondria [19] and selenium prevents malignant transformation of the cells as an antimutagenic agent [14] . According to our results, Vit E and Se were individually effective to prevent oxidative damage in the mtDNA and they were significantly more effective together to prevent damage ($p<0.05$, Table 1).

mtDNA damage may be important at heart tissue. Recent experimental and clinical studies have suggested that oxidative stress is enhanced in heart failure. The production of oxygen radicals is increased in failing heart, whereas antioxidant enzyme activities are preserved as normal. Chronic increases in oxygen radical production in the mitochondria can lead to a catastrophic cycle of

**Table 1.** Relative amplification results (Decreased relative amplification is an indicator of the damaged DNA)

| Groups | Mean ± SE | Median | Min. | Max. |
|---|---|---|---|---|
| Control | 1,56 ± 0,08 | 1,48 | 1,26 | 1,86 |
| CS | 0,23 ± 0,04[1] | 0,21 | 0,17 | 0,30 |
| CS+Vit E | 1,48 ± 0,16 | 1,41 | 1,00 | 2,40 |
| CS+Se | 1,29 ± 0,07 | 1,31 | 1,05 | 1,50 |
| CS+Vit E+Se | 1,94 ± 0,15[2] | 1,93 | 1,38 | 2,50 |
| Vit E+Se | 1,50 ± 0,20 | 1,26 | 0,93 | 2,90 |

SE standart error, Min. minimum, Max. maximum
[1] Value is statistically different from control and the other groups ($p<0.05$)
[2] Value is statistically different from CS, CS+Se and CS+Vit E groups ($p<0.05$)

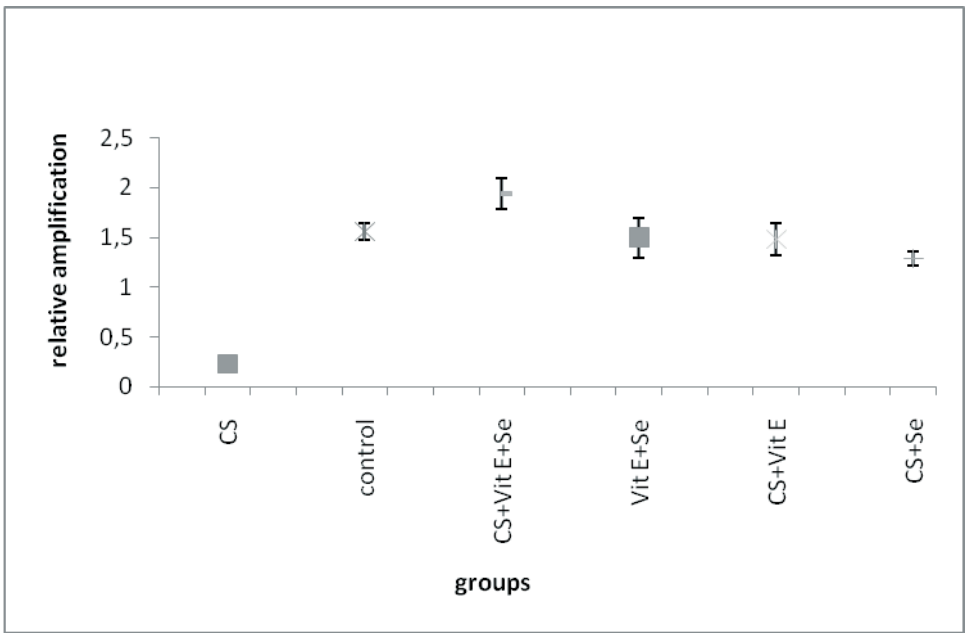


**Figure 1**. Relative amplification results

mtDNA damage as well as functional decline, further oxygen radical generation, and cellular injury.

Reactive oxygen species induce myocyte hypertrophy, apoptosis, and interstitial fibrosis by activating matrix metalloproteinases. These cellular events play an important role in the development and progression of maladaptive cardiac remodeling and failure [10].

There are many articles about relationship between cigarette smoke and nuclear DNA damage. The differences of application time, daily application doses or investigation of different specific damages may cause different results. For example cigarette's effect on light and heavy smokers is different [20]. However our previous studies carried out under the same conditions (smoking time, vit E and Se applications etc.) showed that the cigarette smoke increased the malondialdehyde and 8-hydroxydeoxyguanosine amount in serum and effected some of the antioxidant enzyme activities of brain, liver and kidney [21- 23].

Copy numbers were achieved by the amplification of small fragment, which had a statistically negligible likelihood of containing damaged bases. The copy number results did not indicate the mtDNA damage. mtDNA copy number results were shown in Table 2 and Figure 2.

In cigarette smoke application groups with the exception of CS+VitE, mtDNA copy number was capable of to decrease. This reduction of mtDNA number may be the result of oxidative damage. Vit E alone is effective to prevent the decrease of the copy number.

Suematsu et al. reported that tumor necrosis factor-α application caused ROS production in cardiac myocytes and decreased mtDNA copy number. Cells were viable at the times of mtDNA level decrease so the mtDNA

**Table 2.** Small fragment amplification (mtDNA copy number) results

| Groups | Mean ± SE | Median | Min. | Max. |
|---|---|---|---|---|
| Control | 22,00 ± 2,85 | 20 | 9 | 40 |
| CS | 15,17 ± 1,58[1] | 15 | 10 | 20 |
| CS+Vit E | 21,73 ± 2,24 | 20 | 13 | 40 |
| CS+Se | 17,67 ± 2,01 | 16 | 13 | 25 |
| CS+Vit E+Se | 14,4 ± 2,14[2] | 12,5 | 8 | 29 |
| Vit E+Se | 25,33 ± 2,56 | 27 | 12 | 36 |

SE standart error, Min. minimum, Max.maximum

[1] Value is statistically different from VitE+Se and CS+VitE groups ($p<0.05$)

[2] Value is statistically different from VitE+Se and CS+VitE groups ($p<0.05$)

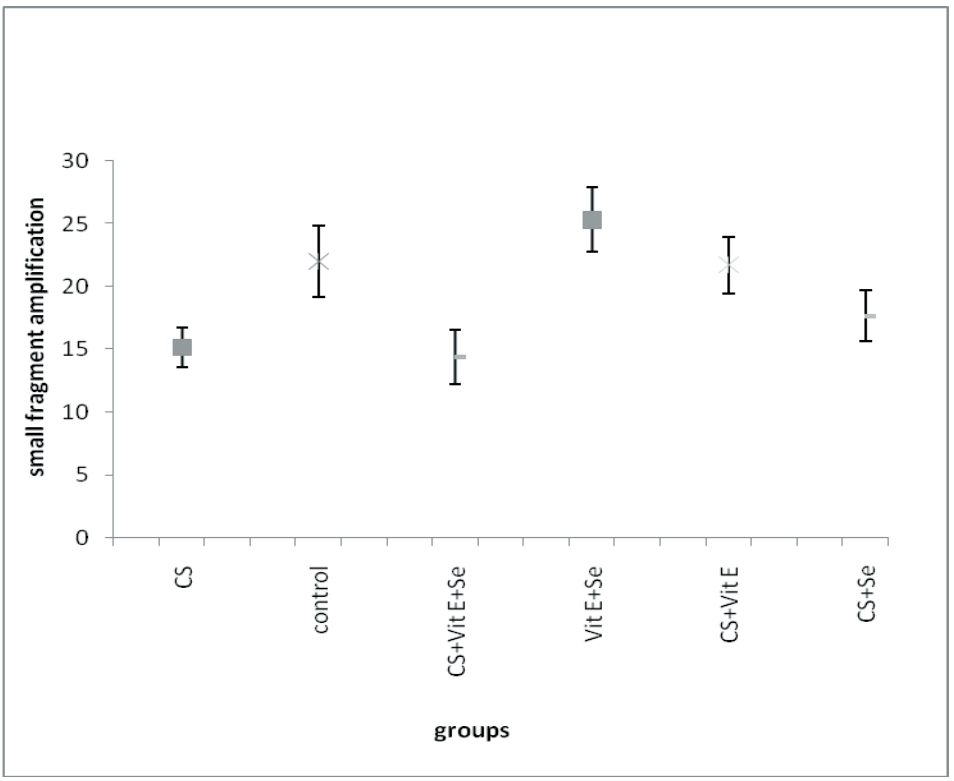


**Figure 2**. Small fragment amplification (mtDNA copy number) results

copy number was not caused by cell death [24]. Ling et al. indicated that the removal of DNA damage could be explained such as selective amplification of undamaged mtDNA and/or selective elimination [25].

Over expression of mitochondrial transcription factor A (TFAM) could ameliorate the decline in mtDNA copy number and preserve it at a normal level in failing hearts. Consistent with alterations in mtDNA, the decrease in oxidative capacities was also prevented. Therefore, the activation of TFAM expression could ameliorate the pathophysiological processes seen in myocardial failure. Inhibition of mitochondrial oxidative stress and mtDNA damage could be novel and potentially very effective treatment strategies for heart failure [10, 26].

Copy number alterations of mtDNA also correlate with carcinogenesis. The copy number of mtDNA has been significantly decreased in hepatocellular carcinoma (HCC). Yin and co-workers demonstrated that reduced mtDNA copy number, impaired mitochondrial biogenesis and somatic mutations in mtDNA occurred concomitantly in HCC [27].

De Grey has discussed in his review that although mtDNA mutations could theoretically accumulate, mitochondria housing mutant mtDNA would be preferentially eliminated by turnover, resulting in a low and nonincreasing level of mutant mtDNA. Another possibilty according to de Grey was wholesale destruction of damaged mtDNA molecules (coupled with replication of undamaged ones) as a repair mechanism [28].

Murdock et al. has shown that mitochondrial T414 G mutations accumulated in muscle but not in brain. Either mtDNA base substitution rate was unusually slow or base substitutions were eliminated in brain, this

elimination could be via apoptosis or selective autophagy of mutant mitochondria according to authors [29]. Also mitochondria may have an autonomous system to achieve self destruction. Skulachev indicated that the mitochondrial populations in the cell can purified from the ROS-overproducing or damaged organelles [30].

In summary, CS created the mtDNA damage and mtDNA copy number reduction in the heart tissue of mice. Our results also suggest that the Vitamin E and selenium supplementation may prevent this damage. To our knowledge, this is one of the first studies that evaluate protective effect of Se and vitamin E together on mtDNA oxidation in the heart. This is important for the children who had smoker parents especially.

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