

THE EFFECT OF VITAMIN C AND E COMBINATION ON SPERM QUALITY AND CEMENT 8-OHdG LEVEL OF SMOKE EXPOSED RATS

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Abstract—Introduction: Cigarette smoke causes oxidative stress which results in reduced sperm concentration, motility and morphology, also increased levels of 8-OHdG as a marker of DNA damage. Vitamin C and E have potential role in repairing spermatozoa damages. The aim of this study was to determine the effect of vitamin C and E combination on sperm quality and cement 8-OHdG level of smoke exposed rats.

Methods: This study used a post test only control group design among 18 male Wistar rats subject, aged 8 week, 150-200 grams body weight (BW). The subject was randomly divided into 3 groups, K1: control, K2: cigarettes smoke exposed, K3: cigarettes smoke exposed and given a combination of 0.045 mg/gBW vitamin C and 0.036 IU/gBW vitamin E per oral. Analysis was done on day 21 using one-way ANOVA and post-hoc LSD for sperm concentration, motility and morphology; using Kruskal-Wallis and Mann-Whitney tests for cement 8- OHdG levels.

Results: The lowest sperm concentration was found in K2 (K2 32.59 million/mL, K1 47.91 million/mL, K 339.43 million/mL); the lowest normal sperm motility was found in K2 (K 238.97%, K 164.57%, K3 51.43%); the lowest normal sperm morphology was found in K2 (K2 27.56%, K 138.36%, K 331.18%); and the highest cement 8- OHdG level was found in K2 (K2 20.18ng/mL, K1 3.43ng/mL, K3 5.28ng/mL).

Conclusion: Combination of vitamin C and E can improve sperm concentration, motility and morphology and decrease cement 8-OHdG levels of smoke exposed rats.

Keywords: Sperm quality, 8-OHdG, vitamin C, vitamin E.

INTRODUCTION

Smoking is associated with increased levels of reactive oxygen species (ROS) which also causes increased oxidative stress level.¹ Under normal

conditions, there is a balance between ROS and antioxidants. If the balance is disturbed, it will cause oxidative stress. Oxidative stress may exceed the antioxidant repair capacity in seminal plasma and can be toxic to sperm, which causes structural damage to spermatozoa.^{1,2} Hydrogen peroxide is a major product of ROS which, in high concentrations, causes lipid peroxidation which causes cell death.³ In addition to damaging cell structure, ROS can also damages DNA molecule through single and double chain DNA termination, deletion and modification of nucleoside.⁴ Antioxidants act as free radical scavengers which protect spermatozoa from the ROS effects. Non-enzymatic antioxidants, such as vitamin C, vitamin E, pyruvate, carnitine, and glutathione, have the ability to compensate the damage to sperm cytoplasmic enzyme; an enzyme which functions in the endogenous defense and repair mechanisms.³ Combination of vitamin C and vitamin E aims to prevent the formation of free radicals. Vitamin E is a tocopheroxyl radical which can be reduced by vitamin C back into tocopherol.⁵ Researches on the role of vitamin C in cigarette smoke exposed rats showed positive effect for the quality of spermatozoa and the mean score of spermatogenesis.⁶⁻⁷ Studies of vitamin E showed positive effect on spermatogenesis and sperm function in rats who suffered from spinal cord injuries.⁸⁻⁹ Until now, there has not been any experiment which suggest that combination between vitamin C and vitamin E can provide protective effects on the fertility of smokers with sperm quality and levels of cement 8-hydroxy-2'-deoxyguanosine (8-OHdG) as indicators.

Globally, there were approximately 1.8 billion smokers and 80% of them live in low-income countries.¹⁰ In Indonesia, 34.8% or 57.6 million adult population are smokers. Data obtained from National Socioeconomic Survey, Baseline Health Research and the Adult Global Tobacco Survey showed an increase in the number of smokers in Indonesia from 53.9% in 1995 to 67.0% in 2011. In 2008, Indonesia became a country with the third largest cigarette

consumption in the world. According to the data, prevalence of male smokers account for 67% of overall men population, which means the number reached around 57.6 million men.¹¹ Data from WHO noted that the biggest prevalence of smokers are young adult males in reproductive ages (46% of smokers age 20-39 years old). Moreover, in 30-50% of couples with infertility, the most common cause is abnormal seminal components. Several studies from multiple centers around the world had been researching the effects of smoking on cement quality, especially in heavy smokers and long-term smokers. The results showed that smoking resulted in decrease of cement parameters, such as viability, concentration, motility and morphology.^{1,12,13} Other studies showed the occurrence of sperm DNA damage, such as a marked increase in 8-OHdG level; a parameter of DNA damage caused by oxidative stress, which can be caused by cigarettes.^{2,3,14}

Research by Claudia et al,⁶ concluded that vitamin C can improve sperm quality (motility, concentration and morphology) of male rats exposed by cigarette smoke. Research by Intania⁷ also showed that vitamin C increased the average score of spermatogenesis in male rats exposed to cigarette smoke. Therefore it is concluded that administration of vitamin C may affect the sperm of smoke-exposed rats. Vitamin C has been known as an antioxidant supplement, but its effect on 8-OHdG is still controversial. Fraga et al,¹⁵ reported that intake of high doses of vitamin C has protective effects against the formation of 8-OHdG in human cement DNA. On the other hand, Loft et al,¹⁶ stated that vitamin C intake did not correlate with urinary excretion of 8-OHdG. In the study conducted by Inoue et al,¹⁴ increased levels of 8-OHdG among smokers abstinent participant that were given extra vitamin C for 14 days did not show statistically significant differences with ones who received placebo. Research conducted by Subekti et al,⁸ concluded that administration of vitamin E can improve spermatogenesis in cigarette smoke exposed rats, but did not show a significant increased in sperm count.¹⁷ The study by Wang et al⁹ in rats with spinal cord injury, vitamin E demonstrated a benefit in improving sperm function. Bruno et al,¹⁸ concluded that smoking caused an increased need of vitamin E due to the reduction of vitamin E plasma level. Research by Umami Kalsum et al,¹⁹ using a combination of vitamins C and E on monosodium

glutamate exposed rats showed an improvement in weight and testical volume, seminiferous tubule diameter and number of spermatogenic testes.

The process of smoking itself is divided into two phases: gas and particulate phase. Smoke cigarettes produce 4,000 kinds of substance through the process of hydrogenation, pyrolysis, oxidation, decarboxylation and dehydration. Elements which have the greatest impact on health are nicotine, tar generated in the particle phase, and carbon monoxide produced in the gas phase.²⁰ Carbon monoxide (CO) will be broken down by hemeoxygenase enzyme (HO) into a pro-oxidant environment which initiates the formation of ROS.²¹ ROS is a collection of various radical form. The most numerous form of ROS in spermatozoa are O₂⁻ (superoxide), H₂O₂ (hydrogen peroxide) and OH⁻ (hydroxyl ions).² Pro-oxidant that exceeds the healing ability of antioxidants in cells will cause oxidative stress. This situation can cause cell damage as a result from the decomposition of important molecules such as DNA, proteins, and lipids. DNA is the main target of damage due to oxidation reaction by endogenous oxidant. DNA damage occurrence can be measured by levels of 8-OHdG.¹⁴

Therefore, this study was done to determine whether the combination of oral vitamin C and E can improve sperm quality and sperm 8-OHdG levels in smoke exposed rats.

METHODS

This study used a post test only control group design. The study was conducted in the Laboratory of Food and Nutrition Studies Center, Gadjah Mada University. A total of 18 rats (*Rattus norvegicus*) Wistar strain male 8 week old, weight 150-200 grams, divided into 3 groups randomly, K1: control, K2: cigarettes smoke exposed, K3: cigarettes smoke exposed and given a combination of 0.045 mg/g BW vitamin C and 0.036 IU/g BW vitamin E per oral for 14 days. On day 21, all mice were terminated. Sample of epididymises were retrieved by cutting the epididymises from the caudal until their ampullae. Epididymises taken were placed inside the petri dishes containing 0.5 mL physiologic saline fluid. Sperm samples were retrieved by the means of epididymal massage.

Analysis was done using one-way ANOVA and post-hoc LSD for sperm concentration, motility

and morphology; using Kruskal – Wallis and Mann – Whitney tests for cement 8- OHdG levels.

RESULTS

The result of this study is shown in Table 1. According to Table 1, the result of this study showed

K2 (27.56 ± 1.41). Given the data were normally distributed (Shapiro-Wilk, $p > 0.05$) and homogenous (Levene test, $p > 0.05$), with one way ANOVA test for statistical analysis ($p = 0.000$).

The lowest levels of 8-OHdG was K1 (3.43 ± 0.33), followed by K3 (5.28 ± 0.42), and lastly, K2

Table 1. Mean Value of Body Weight, Sperm Concentration, Motility, Morphology, and 8-OHdG level.
Group

Variable	K ₁	K ₂	K ₃	P
	(n=6)	(n=6)	(n=6)	
	(x ± SE)	(x ± SE)	(x ± SE)	
BW (g) before	194.83±4.79	190.00±3.51	186.5±1.75	0.284
after	213.67±4.05	208.17±3.10	207.17±1.42	0.300
Concentration (million/ml)	47.91±0.51	32.59±0.47	39.43±0.22	
<i>Shapiro-wilk</i>	0.240	0.537	0.535	
<i>Levene test</i>				0.117
Statistic analysis	<i>One way ANOVA</i>			0.000
Motility (%)	64.57±0.40	38.97±0.42	51.95±0.30	
<i>Shapiro-wilk</i>	0.837	0.210	0.814	
<i>Levene test</i>				0.705
Statistic analysis	<i>One way ANOVA</i>			0.000
Morphology (%)	38.36±0.79	27.56±0.58	31.18±0.33	
<i>Shapiro-wilk</i>	0.121	0.725	0.771	
<i>Levene test</i>				0.200
Statistic analysis	<i>One way ANOVA</i>			0.000
8-OHdG (ng/ml)	3.43±0.13	20.18±0.60	5.28±0.17	
<i>Shapiro-wilk</i>	0.435	0.692	0.855	
<i>Levene test</i>				0.001
Statistic analysis	<i>Kruskal-Wallis</i>			0.001

Note: K₁ : control; K₂ : cigarettes smoke exposed; K₃ : cigarettes smoke exposed and given a combination of 0.045mg/g BW vitamin C and 0.036IU/g BW vitamin E for 14 days.

that the highest sperm concentration was K1 (47.91 ± 0.51), followed by K3 (39.43 ± 0.22) and K2 (32.59 ± 0.47). Group with the highest sperm motility percentage was K1 (64.57 ± 0.97), followed by K3 (51.95 ± 0.73) and K2 (38.97 ± 1.03). Group with the highest sperm morphology percentage was K1 (38.36 ± 1.94), followed by K3 (31.18 ± 0.80) and

(20.18 ± 1.46). Given the data were normally distributed (Shapiro-Wilk, $p > 0.05$) and was not homogeneous (Levene test, $p = 0.001$), and the statistical analysis used was Kruskal – Wallis test ($p = 0.001$).

To analyze which groups have significant differences in terms of concentration, sperm motility

and morphology, post-hoc LSD test is performed, which is shown in Table 2. The results of post-hoc

peroxidation, resulting in DNA damage and cell apoptosis on spermatozoa. Spermatozoal plasma

Table 2. Post-hoc LSD test on sperm concentration, motility and morphology

Group	Variable					
	Sperm Concentration		Motility		Morphology	
	Mean Diff	P	Mean Diff	P	Mean Diff	P
	(χ)		(χ)		(χ)	
K ₁ > K ₂	15,313	0,000	25,595	0,000	10,795	0,000
K ₁ > K ₃	8,476	0,000	12,613	0,000	7,186	0,000
K ₂ > K ₃	-6,846	0,000	-12,981	0,000	-3,618	0,001

LSD test on sperm concentration, motility and morphology shows the results differ significantly ($P < 0.05$) in all groups: K1 compared to K2 and K3; K2 compared to K3.

As for levels of 8-OHdG, Mann-Whitney test was performed which results are shown in Table 3. The results of Mann-Whitney test on levels of 8-OHdG indicated that the results differ significantly ($P < 0.05$) in all groups.

Table 3. Mann-Whitney test on 8-OHdG levels

Group	Mean Diff (χ)	P
K ₁ > K ₂	-16,747	0,004
K ₁ > K ₃	-1.847	0,004
K ₂ > K ₃	14,900	0,004

DISCUSSION

This study results showed that K1 (control group) have better sperm indicator levels than K2 group which get 4 cigarettes worth of smoke exposure a day for 14 days. There were impairments in concentration, motility and morphology of spermatozoa and an increased level of 8-OHdG cement in K2 compared to K1. This result proved that cigarette smoke caused increased oxidative stress, which then caused excessive lipid

membrane largely consists of PUFA, which is susceptible to ROS impact. Lipid peroxidation in spermatozoal plasma membrane would result in a formation of leakage in membranous structure (cellular and organelles) and function (transport process, ions and metabolites regulation, and also signal transduction by the receptor). These conditions cause intracellular ATP to disappear rapidly, causing damage to axon, and decreasing viability of spermatozoa, therefore resulted in decrease of sperm motility.

Another theory states that H₂O₂ can diffuse through plasma membrane into cell and inhibit enzymatic activity of G6DP (glucose 6-phosphate dehydrogenase), which leads to decrease of NADPH ability simultaneously with the glutathione oxidase. These leads to decreasing defensive ability of antioxidants and peroxidation of membranes.^{2,22} Another target of damage caused by oxidative stress in response to cigarette smoke exposure is the DNA. ROS induces a modified form of the DNA bases guanine primarily through lipid peroxides and alkaloxile radicals. Oxidative stress also causes increase of single and double chain DNA termination. ROS can also induce -SH groups in proteins and DNA, which then would alter the structure and function of spermatozoa, thereby increasing susceptibility to macrophages. The results of DNA base guanine oxidation can be observed in the form of 8-OHdG.^{12,14,22} This result is similar to a research done by Batubara et al,²³ which provided cigarette smoke exposure as much as 1, 2, 3, and 4

cigarettes a day for 30 days. The study showed impairment in concentration, motility and morphology of spermatozoa compared to those not receiving cigarette smoke exposure, which concluded that more amount of cigarette smoke exposure caused greater impairment of concentration, motility and morphology of spermatozoa. Likewise, in the study conducted by Somwanshi et al,¹² which compared 50 cement samples of non-smokers to smokers. The study result showed significant decrease in number and motility of spermatozoa of smokers.

Levels of 8-OHdG in the K2 group showed significant increase compared to group K1. This is consistent with a similar study conducted by Inoue et al,¹⁴ which compared the levels of 8-OHdG between smokers and non smokers. The results showed levels of 8-OHdG were significantly higher in smokers compared to nonsmokers.

Group K3; namely groups that received exposure to cigarette smoke 4 cigarettes a day and given vitamin C 0.045 mg/gBW and vitamin E 0.045 mg/gBW per oral, showed better concentration, motility and morphology of sperm, and cement 8-OHdG level compared to group K2. This result proved that administration of vitamin C and E as antioxidants can prevent cell damage caused by smoking. Furthermore, external provision of vitamin C and E prevented decrease of internal vitamin C and E antioxidant level in seminal plasma due to cigarette smoke exposure.²⁴ This suggests that simultaneous administration of vitamin C and E have protective effect against cigarette smoke exposure. The result was similar to other studies that used combination of vitamin C and E in rats (*Mus musculus*) that were given monosodium glutamate (MSG). The study showed positive effect of the combination of vitamin C and E which recovered subject's seminiferous tubules volume and the number of spermatogenic testes.¹⁹ In another study, administration of selenium and vitamin E to prevent testicular tissue damage due to cigarette smoke exposure can further reduce degeneration of testicular tissue.²⁵ Research by by Moslemi et al,²⁶ which administered a combination of selenium and vitamin E in 690 men with infertility, demonstrated beneficial and protective effect of vitamin E especially in sperm motility. Other research, which administer only vitamin C to male rats (*Mus musculus L*) after cigarette smoke exposure, showed that vitamin C can improve sperm

quality.⁶ In vitro study proved that vitamin E can protect sperm from damage caused by oxidative stress, loss of motility, and morphological changes.²²

In conclusion, combination of vitamin C and E can improve sperm concentration, motility, and morphology, and also decrease cement 8-OHdG levels of smoke exposed rats.

Conflicts of Interest

The authors of this paper declare there is no conflict of interest.

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