

Vitamin C and ubiquinone have the same ability in reducing the spermatozoa DNA fragmentation index in infertile men at Doctor Soetomo General Hospital, Surabaya, Indonesia

Ayang Halim,^{1,2*} Supardi,^{1,2} Hamdani Lunardhi^{1,2}

ABSTRACT

Background: Infertility is one of the most common health problems in the world. Malefactors contribute to 50% of cases and about 15-30% of infertile men are categorized as unexplained infertility. There have been numerous studies investigating the role of spermatozoa DNA fragmentation in male infertility. Spermatozoa nuclear DNA integrity has been suggested as a better predictor of male infertility and oxidative stress has been known related to it. This study was conducted to determine the oral supplementation of Vitamin C and Ubiquinone in reducing the spermatozoa DNA fragmentation index (DFI) among infertile men.

Methods: This was an experimental clinical trial with a pre-test and post-test group design. Thirty infertile men were randomized double-blindly into 3 groups: Vitamin C and Ubiquinone; Vitamin C and Placebo; Ubiquinone and Placebo groups, each treatment was given for 35 days. The DFI was evaluated by Sperm Chromatin Dispersion (SCD) test, before and after treatment. Data were analyzed using SPSS version 21 for Windows.

Results: The average age of respondents was 32.6 years old, followed by 4.6 years of infertility duration in 5.8 years of marriage. Most of the respondents had a normal Body Mass Index (BMI) (46.7%), no smoking history (75.0%), no history of heat (90.0%) or chemical (73.3%) exposure, and diagnosed with primary male infertility (90.0%). Vitamin C group showed a significant reduction in the spermatozoa DFI (95% CI, $p < 0.05$), while the other two groups showed no differences in the spermatozoa DFIs (95% CI, $p > 0.05$). Comparison analysis showed no differences in the reduction of the spermatozoa DFIs (95% CI, $p > 0.05$) among these three groups.

Conclusion: The recent findings suggest that oral supplementation of vitamin C can reduce the spermatozoa DFI, while ubiquinone and the combination of vitamin C and ubiquinone could not reduce the spermatozoa DFI.

Keywords: Spermatozoa, DNA Fragmentation, Vitamin C, Ubiquinone, Male Infertility

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¹Andrology Department of Doctor Soetomo General Hospital Surabaya, Surabaya, Indonesia

²Biomedicine, Department of Medical Faculty, Universitas Airlangga, Surabaya, Indonesia

*Correspondence to: Ayang Halim; Andrology Department of Doctor Soetomo General Hospital Surabaya, Surabaya, Indonesia; ayang.halim-2015@fk.unair.ac.id

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INTRODUCTION

Infertility is one of the most common health problems in the world. About 15% of reproductive age couples experience infertility problems, which can be caused either by female or malefactors, or both. The malefactor contributes to about 50% of cases.¹ Aetiology of male infertility is still not fully understood. Some aetiologies have been known, but about 30% of the aetiology is idiopathic, where the low concentration, motility, and normal morphology of spermatozoa cannot be explained. About 15-30% of infertile men show normal semen analysis and are categorized as unexplained infertility.^{2,3}

Recently studies on the role of reactive oxygen species (ROS) and spermatozoa DNA fragmentation on male infertility at the molecular level has developed rapidly. Spermatozoa require ROS in the physiological amount to carry out their functions in fertilization.⁴ If the balance between ROS and antioxidant capacity is disrupted, oxidative stress will occur and negatively affects spermatozoa chromatin

by inducing DNA strand breaks.⁵ Spermatozoa DNA integrity is essential for the occurrence of pregnancy and transmission of genetic information. This disorder can be inherited to the offspring. Spermatozoa DNA fragmentation should be suspected in cases of unexplained infertility.⁶

The conventional semen analysis cannot detect spermatozoa DNA damage. Sperm Chromatin Dispersion (SCD) is a simple method of DNA fragmentation examination, entirely accurate, fast, economical, and can be carried out in an Andrology laboratory. The high spermatozoa DNA fragmentation index is inversely proportional to fertility potential. This examination can provide a better diagnosis and prognosis for infertility than semen analysis.³

Antioxidants counteract the adverse effects of oxidants and maintain the balance of ROS in the seminal plasma. Oral antioxidant therapy is of choice because serious side effects are rare. Vitamin

C and Ubiquinone are some of the antioxidants that are frequently used in the treatment of male infertility in daily practice. The effectiveness of Vitamin C in male infertility therapy has been widely reported in various studies. Besides, Vitamin C is widely available at affordable prices, with minimal side effects.

Ubiquinone is known to be able to recycle Vitamin E and has strong antioxidant potential. It is a trans-electron chain component and participates in aerobic cellular respiration through an important oxidative phosphorylation pathway in the formation of ATP. Studies of Ubiquinone and the combination of Vitamin C and Ubiquinone in reducing spermatozoa DNA fragmentation in infertile men are still limited.¹ Based on the mentioned above, this study aims to evaluate the ability of vitamin C and ubiquinone in reducing the spermatozoa DNA fragmentation index in infertile men at Doctor Soetomo General Hospital, Surabaya, Indonesia.

METHODS

An experimental clinical trial study was conducted using a pre-test and post-test group design at Andrology Clinic of Doctor Soetomo General Hospital, Surabaya from August to November 2018. The calculation of the sample size obtained a minimum sample size of 8 subjects. To anticipate the dropout, each study group would consist of 10 subjects. We collected 20 subjects, randomized them in a double-blinded manner into two groups of treatment: combination of Vitamin C and Ubiquinone group; and Vitamin C group. After the completion of treatment, samples of Vitamin C group were washed out for 14 days, and after that would be treated as Ubiquinone group. Each treatment was given for 35 days. The semen of each sample was examined for spermatozoa DFI before and after treatment.

Subjects included in this study were male patients with idiopathic infertility. The inclusion criteria were men age 20-40 years old, sexual abstinence of 48 hours – 7 days, spermatozoa concentration more than 5 million/ml, married and still living with his wife, not taking any antioxidants before, and Erythrocyte Sedimentation Rate 0 - 15 mm/hour. The exclusion criteria included significant genitourinary abnormalities (anorchia, testis volume < 10 ml in at least one testis, varicocele grade III, obstruction of reproductive tract, leucospermia, hematospermia), history of organic disorders, such as kidney function problems, kidney stone, liver function problems, chronic digestive problems, history of physical trauma or surgery of genital region and active chronic inflammatory diseases, and consumption of other drugs such as warfarin.

The independent variable in this study was the use of Vitamin C 1000 mg, Ubiquinone 100 mg, and the combination of Vitamin C 1000 mg and Ubiquinone 100 mg, every day. The dependent variable was spermatozoa DFI, which was counted before and after treatment. The DFI of more than 25% indicates spermatozoa DNA fragmentation. The research materials were Vitamin C 500 mg tablet, Ubiquinone 100 mg soft capsule, and placebo soft capsule, 1 ml and 3 ml syringes, EDTA blood tube, object-glass, glass cover, disposable tip, Eppendorf tube 1.5 ml, tissue, alcohol swab, Spermfunc® DNaf Kit, 70% alcohol, 100% alcohol, aquadest, normal saline (NS). Micropipette, manual differential counter, Olympus™ CX-35 microscope, improved Neubauer, timer, water bath, float cork, 37°C incubator, chemical thermometer, refrigerator, slide tray, and the device of Westergren method for LED examination.

Prospective study samples will get explanations of the purpose and procedure of this study. Then they will undergo the process of history taking, physical examination, semen analysis, and the examination of ESR. Semen samples were obtained from study samples with a period of sexual abstinence of 48 hours to 7 days, by masturbation. Semen analysis carried out based on the 5th edition of the “WHO laboratory manual for examination and processing of human semen” guidelines, 2010. The LED examination was carried out using the Westergren method, recommended by the International Committee for Standardization in Hematology (ICSH). Information for Consent and Informed Consent were obtained before all subjects recruited into this study. The remaining semen samples would be examined for the spermatozoa DFI using the Spermfunc® DNaf Kit. The assessment of spermatozoa DFI was carried out twice for each group (pre-test and post-test). The randomization of the treatment group was recorded and carried double-blindingly by the Pharmacy. Group A was given 1000 mg of Vitamin C tablet in combination with Ubiquinone 100 mg soft capsules, once daily for 35 days. Group B has given Vitamin C tablets 1000 mg once daily for 35 days. After the post-test, samples of Group B would undergo wash-out for 14 days, and then assigned as samples of Group C, given Ubiquinone 100 mg once daily for 35 days. Also, all data was arranged and analyzed with the *Statistical Package for the Social Sciences* (SPSS) version 21.0 program for Windows™.

RESULTS

According to [Table 1](#), the mean age of respondents was 32.6 years old, and the average age of the spouses was 30.7 years old. Most of the respondents also

Table 1 Baseline characteristic of respondents

Variables	Numbers (N=30)
Age (years)(mean)	
Respondents	32.6
Wife	30.7
Occupation (%)	
Government employees	16.7
Private	83.3
Residence (%)	
Surabaya	66.7
Other cities	33.3
Duration (years) (mean)	
Marriage	5.8
Infertility	4.6
History of Infertility Therapy (%)	
Have been treated	40.0
Have never been treated	60.0
Body Mass Index (BMI) (kg/m²)	
Normal (18.5-24.9)	46.7
Overweight (25.0-29.9)	40.0
Obesity (>30.0)	13.3
Smoking History (%)	
Yes	25.0
No	75.0
Diagnosis of Male Infertility (%)	
Primer	90.0
Secondary	10.0
History of Heat Exposure (%)	
Yes	10.0
No	90.0
History of Chemical Exposure (%)	
Yes	26.7
No	73.3

Table 2 Homogeneity Test of Spermatozoa DFI between groups

Data Groups	N	Levene Test	p
Spermatozoa DFI Pre-test	30	2,760	0,081
Spermatozoa DFI Post-test	30	2,523	0,099
Delta of Spermatozoa DFI	30	2,959	0,069

Table 3 Shapiro-Wilk and Paired T-Test among study groups

Study Groups	n	Mean Pre-test	P1	Mean Post-test	P2	Paired T-Test	P3
Vitamin C + Ubiquinone (A)	10	26.592	0.924	20.982	0.705	0.156	0.274
Vitamin C + Placebo (B)	10	34.626	0.349	22.713	0.324	0.047*	0.314
Ubiquinone + Placebo (C)	10	24.035	0.518	20.156	0.196	0.116	0.501

P1: p-value of pre-test; P2: p-value of post-test; P3: delta p-value; *p-value: statistically significant if less than 0.05

work in private sector (83.3%), living in Surabaya (66.7%), never had infertility therapy (60.0%), diagnosis with primary male infertility (90.0%) and normal BMI (46.7%) (Table 1). Besides, the average duration of marriage and infertility among respondents was 5.8 and 4.6 years, respectively. Most respondents also had no history of smoking (75.0%), heat exposure (90.0%), or chemical exposure (73.3%) (Table 1). Besides, the spermatozoa DFI on the three study groups were also tested for homogeneity using the Levene test. Table 2 below showed that the data were all homogenous ($p > 0.05$).

Normality test on the pre-test, post-test, and delta of spermatozoa DFI data of each study groups was conducted using the Shapiro-Wilk test. The normality test results of the pre-test ($p = 0.924; 0.349; 0.518$, respectively), post-test ($p = 0.705; 0.324; 0.196$, respectively) and delta data ($p = 0.274; 0.314; 0.501$, respectively) are all normally distributed ($p > 0.05$) as shown in Table 3.

The normality test results showed that the data on the pre-test and post-test of spermatozoa DFI in the three treatment groups were normally distributed ($p > 0.05$), so the use of the appropriate comparison statistical test was the paired t-test. The mean pre-test of study groups was 26.592 (A), 34.626 (B), and 24.045 (C). Besides, the mean post-test of study groups was 20.982 (A), 22.713 (B), and 20.156 (C) (Table 3). The results in Table 3 showed a significant difference between the pre-test and post-test DFI spermatozoa in group B ($p < 0.05$), but no significant differences in group A and group C ($p > 0.05$).

The microscopic examination pre-test and post-test DFI results of each study group can be seen in Figure 1 as below. In Figure 1, the light microscopy evaluation indicates different types of spermatozoa, such as large halo, medium halo, small halo, no halo, and degraded spermatozoa (Figure 1).

The normality and homogeneity test of the pre-test, post-test, and delta of spermatozoa DFI data in all study groups were normally distributed and homogeneous, so that One Way Anova test were conducted to determine the difference among groups. Table 4 showed there were no significant differences in the pre-test ($p = 0.120$), post-test (0.808) and delta of spermatozoa DFI ($p = 0.319$) among the three study groups (Table 4). Post-Hoc

Table 4 One Way Anova test among study groups

Variables	n	P1	P2	P3
Vitamin C + Ubiquinone (A)	10	0.120	0.808	0.319
Vitamin C + Placebo (B)	10			
Ubiquinone + Placebo (C)	10			

P1: p-value of pre-test; P2: p-value of post-test; P3: delta p-value; *p-value: statistically significant if less than 0.05

Table 5 Post-Hoc Test Multiple Comparison with LSD Method

Variable	Study Groups	P
Post-test	Vitamin C + Ubiquinone (A) Vitamin C + Placebo (B)	0,667
	Ubiquinone + Placebo (C)	0,837
	Vitamin C + Placebo (B) Vitamin C + Ubiquinone (A)	0,667
	Ubiquinone + Placebo (C)	0,526
	Ubiquinone + Placebo (C) Vitamin C + Ubiquinone (A)	0,837
	Vitamin C + Placebo (B)	0,526
Delta	Vitamin C + Ubiquinone (A) Vitamin C + Placebo (B)	0,260
	Ubiquinone + Placebo (C)	0,754
	Vitamin C + Placebo (B) Vitamin C + Ubiquinone (A)	0,260
	Ubiquinone + Placebo (C)	0,154
	Ubiquinone + Placebo (C) Vitamin C + Ubiquinone (A)	0,754
	Vitamin C + Placebo (B)	0,154

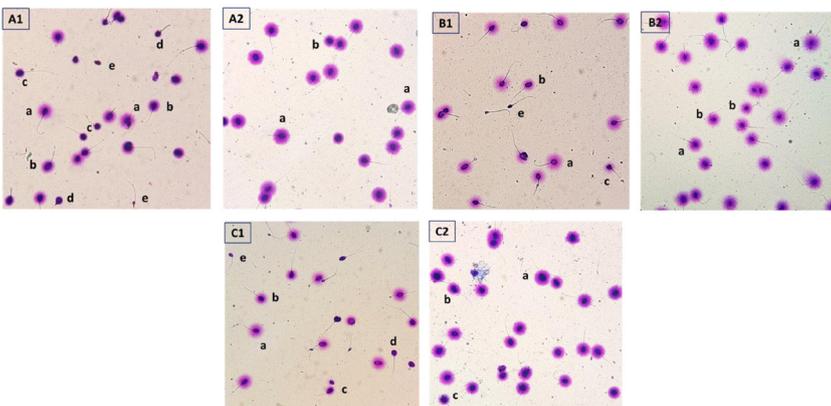


Figure 1 Group A-C Halo. A1,B1,C1 = Pre-test, A2,B2,C2 = Post-test. Notes: (a) Large Halo; (b) Medium Halo; (c) Small Halo; (d) No Halo; (e) Degraded spermatozoa (light microscopy: 10 x 40 magnification)

test multiple comparison with LSD method was also carried out to assess the comparison between intravariation of study groups and also no significant relationship found in this study ($P > 0.05$) (Table 5).

DISCUSSION

ROS is involved in several mechanisms of cellular signalling and can interact with lipids, proteins and DNA. Spermatozoa DNA damage has been reported to be more common in infertile men than fertile men. Currently, assessment of spermatozoa DNA fragmentation has been carried out

in many fertility centres, especially in the setting of IVF. Supplementation of oral antioxidants is a reasonable practice in cases of male infertility. Many studies have shown the effectiveness of antioxidant therapy in reducing spermatozoa DNA damage, both in vivo and in vitro, especially in men with high levels of DNA fragmentation.⁷

The statistical analysis of this study showed that supplementation of Vitamin C for 35 days gave a significant difference in reducing the spermatozoa DFI. Several studies have analyzed the effects of Vitamin C in vivo on the spermatozoa DNA fragmentation, both singly and in combination with other antioxidants, with various doses and durations. Our results agree with those of Vani et al. and Greco et al. which showed that Vitamin C supplementation for 3 months in lead mining workers, and combination of Vitamin C and E for 2 months, reduced the spermatozoa DNA fragmentation significantly.^{8,9} Vitamin C can minimise DNA fragmentation triggered by ROS, recycle inactive Vitamin E, and reduce lipid peroxidation.¹⁰ Studies by Tunc et al. and Menezo et al. on antioxidant combinations, containing Vitamin C, for 3 months, showed improvements in the spermatozoa DNA integrity. Still, in the latter study, there was also an increase in chromatin condensation of spermatozoa which might be mediated by the high redox potential of vitamin C which disrupts disulfide bonds in protamine, especially P2.^{11,12} In vitro studies of Vitamin C have reported significant protection against DNA damage triggered by X-ray radiation.¹³ There is also a significant relationship between decreasing levels of Vitamin C with increasing levels of 8 OHdG, which is a marker of DNA damage. The effects of the combination of Vitamin C with other antioxidants in improving spermatozoa parameters have also been reported in various studies, but some other previously published data are contradictory. The differences between these studies are likely to be related to the type and dose of the antioxidant used, the characteristics of the subjects under treatment, and the duration of the procedure. The improvement in spermatozoa parameters may be due to spermatozoa DNA that is more resistant to ROS than the membrane/mitochondria.^{8,14}

In this study, there was no difference between the pre-test and post-test of Ubiquinone administration for 35 days. To date, studies on the effect of single Ubiquinone administration on spermatozoa DNA fragmentation are still very limited. Previous studies tend to observe improvements only in spermatozoa parameters.

An in vivo study using Ubiquinone 200 mg for 3 months showed no significant difference in the parameters of spermatozoa, but there were

improvements in MDA levels and seminal plasma TAC.¹⁵ Correlation analysis of Ubiquinone 200 mg and Aspartic Acid (D-Asp) 2660 mg combination for 3 months showed that only Ubiquinone was involved in increasing SOD activity, NO reduction, and significant improvement in oxidative DNA damage.¹⁶

Considerable decrease in spermatozoa DNA fragmentation was observed in *in vitro* studies with the combination of Ubiquinone and other antioxidants.¹⁷ High levels of oxidative stress and low antioxidant capacity in varicocele patients have been reported to correlate with changes in Ubiquinone distribution in spermatozoa and seminal plasma. Previous research indicated that Ubiquinone reduced lipid peroxidation when administered in a mouse model with ischemic/reperfusion injury.¹⁸ Studies with Ubiquinone 6 months described increased spermatozoa motility, increased levels of Ubiquinone and Phosphatidylserine in seminal plasma and spermatozoa. The increased motility may be related to the mitochondrial respiratory chain.¹⁹ The positive effects of Ubiquinone on spermatozoa energy metabolism and as antioxidants do not last long after cessation.²⁰ There was a strong correlation between number, sperm motility and seminal Ubiquinol plasma levels. Spermatozoa parameters were reported to increase after 26-week Ubiquinone, and Ubiquinol treatments, but Ubiquinol was more effective in increasing the number and motility of spermatozoa.^{21,22} On the other hand, Ubiquinol is less effective in improving spermatozoa morphology.²²

Some physicochemical and physiological factors are known to inhibit Ubiquinone delivery to mitochondria. The instability of the chemical structure in pharmaceutical preparations triggered by exposure to air, UV, high temperatures, and low oral bioavailability results in barriers to adequate oral delivery. The low oral bioavailability of Ubiquinone is caused by a considerable molecular weight, high lipophilicity, poor solubility in water, regional variations in Ubiquinone permeability in the gastrointestinal tract, and multi transporter involvement.²³

Many studies are currently developing approaches to address these issues, such as the use of solid or dry emulsion formulations, nanoliposomes, nanoparticles with biodegradable surfactant/stabilizer capable of reducing plasma lipid peroxide levels, membrane-penetrating lipophilic cations, and mitochondrial-targeted nano-systems that are effective against oxidative and inflammatory stress.²³

Our data did not show any significant improvement of spermatozoa DNA fragmentation after *in vivo* Vitamin C and Ubiquinone combination

for 35 days. Previous studies that have existed so far used a combination of several types of antioxidants. An improvement of spermatozoa parameters has been described after 3-month treatment with a combination of Vitamin C, Ubiquinone, and other antioxidants, which can be attributed to the partial augmentative effect of each compound. Ubiquinone counteracts ROS and stimulates the production of LH (luteinizing hormone) and FSH (follicle-stimulating hormone), which ultimately results in improved spermatozoa parameters. Vitamin C prevents oxidative stress and enhances the quality of spermatozoa.²⁴ Combination of Vitamin C, Ubiquinone, and other antioxidants for three months also gave a significant increase in DNA integrity, a decrease in the proportion of spermatozoa with degraded DNA, and an improvement in spermatozoa parameters. The mechanism by which they can reduce DNA fragmentation has not been fully known and has only been shown to provide protective effects.²⁵

Comparative tests of these three groups showed no differences in their effectiveness in reducing DFI spermatozoa. This result needs to be confirmed by more extensive and properly designed clinical prospective trials, but it opens up new horizons for monitoring male infertility treatment. Not all patients respond to antioxidants, and it seems that the improvement is more obvious in some cases than the others. This might be due to continuous exposure to ROS sources. This fact indicates that specific markers related to ROS damage are needed to ensure that specific treatment that will be more effective (the type of antioxidants, dosage and duration of treatment) for each particular group of patients according to their particular clinical characteristics.

CONCLUSION

Oral supplementation of Vitamin C 1000 mg for 35 days can reduce the spermatozoa DFI in infertile men. While oral supplementation of Ubiquinone 100 mg; and combination of Vitamin C 1000 mg and Ubiquinone 100 mg for 35 days cannot reduce the spermatozoa DFI in infertile men.

CONFLICT OF INTEREST

None.

ETHICS CONSIDERATION

Ethical clearance No. 0728/KEPK/X/2018 was approved by Health Research Ethic Committee of Doctor Soetomo General Hospital Surabaya.

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AUTHOR CONTRIBUTION

All of authors are equally contributed to the study from the conceptual framework, data gathering, data analysis, until manuscript preparation for publication.

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