

## ORIGINAL ARTICLE

# The Effect of Antioxidant Vitamin C and E Administration on Spermatozoa Quality of White Rat (*Rattus Norvegicus*) Strain Wistar Male Against Allethrin Exposure

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## ABSTRACT

**Introduction:** Allethrin is an active ingredient used in anti-mosquito drugs. Allethrin enters the body through inhalation, allegedly can disrupt the quality of spermatozoa by decreasing its amount, motility, viability and morphology. Many studies suggested that with sufficient intake of vitamin C and E would be able to minimize free radicals caused by allethrin. This study aims to investigate the effect of giving antioxidant vitamin C and E on spermatozoa quality of white rat (*Rattus norvegicus*) strain Wistar male with allethrin exposure.

**Methods:** This research was a post-test design randomized control group design using *Rattus norvegicus* male strains Wistar exposed to allethrin (LPB IPB veterinary stem cell). A total of 16 white rat strains Wistar male divided into 4 groups, one control group (without any treatment) and 3 treatment groups (given antioxidant vitamin C, vitamin E and both). The exposure of mosquito coils was performed 8 hours per day for 45 days and on the 46th day, spermatozoa was observed. The data were analyzed using ANOVA one way and BNT.

**Results:** The results showed that treatment with vitamins affected the amount, motility and viability of rat spermatozoa exposed to allethrin. The mean amount of control group spermatozoa and treatment was 14,93, the mean spermatozoa motility control and treatment groups were 45,13 and the mean spermatozoa viability of control and treatment groups was 58,88. This is shown by the real difference between the control group and the treatment ( $p < 0,05$ ). Similarly, in the control group found morphological abnormalities.

**Conclusion:** Exposure of mosquito coils 8 hours/day for 45 days decreased the spermatozoa quality. Overall, the admission of antioxidant vitamin C and E are able to maintain the quality of spermatozoa instead of one type of vitamin.

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## Introduction

The anti mosquito drug industry in Indonesia is growing rapidly due to the tropical climate in Indonesia causing the fertile breeding of mosquitoes. It causes Indonesia to become one of the potential market in trading mosquito killer product or mosquito repellent. Almost every household use anti mosquito

drug to overcome mosquito disruption. It is often used especially in transition season, because of dengue fever. Based on the Riskesdas data, the highest proportion in the effort to prevent mosquito bites was by using mosquito repellent (48.4%), followed by the use of mosquito nets (25.9%), repellents (16.9%), insecticides (12.2 %), and mosquito netting (8.0%). According to the characteristics, the proportion of the use of mosquito

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repellent drugs in rural areas (50.0%) is higher than in urban areas (46.9%). Likewise, the use of mosquito nets, the proportion in rural areas (39.5%) is higher than in urban areas (12.5%). In contrast, the proportion of households that use repellents, insecticides and wild mosquitoes in urban areas (23.2%, 17.9% and 12.3% respectively) is higher than in rural areas (10.4%; 6 respectively). 4%, and 3.6%).<sup>1</sup>

Anti-mosquito drug has various active ingredients, namely dichlorvos, propoxur, pyrethroid, and diethyltoluamide as well as the ingredients of their combination. Most anti mosquito drugs in Indonesia contain materials such as d-allethrin, transflutrin, bioallethrin, prallethrin, d-phenothrin, cyphenothrin or esbiothrin, which is derivatives of pyrethroid. Pyrethroid is classified into medium-grade insecticide poison, with the effect of irritating the eyes and sensitive skin and causing asthma. Allethrin is one of the active ingredients used in several types / brands of anti-mosquito drugs which have the molecular formula C<sub>19</sub>H<sub>26</sub>O<sub>3</sub> and have 8 stereoisomers. Allethrin that enter the body through inhalation for a long time, in addition to causing disturbances in the lungs such as irritation will also cause the liver will be unable to detox completely. This leads to the emergence of secondary metabolites that can act as free radicals. Furthermore, free radicals will follow the blood circulation to the entire body including testicles.<sup>2</sup>

Testicles are reproductive organs that have 2 functions namely, producing spermatozoa and producing androgenic hormones. Since several decades ago, many researchers have linked male fertility to the quality of spermatozoa. As a reproductive organ that plays a role in spermatogenesis, the testicles are thought to be damaged by exposure to Allethrin active substances present in mosquito coils. Previous study showed that allethrin caused histological changes in the testes, reduced testicular weight and decreased the diameter of the seminiferous tubules. This shows that if the testicles are damaged then spermatogenesis will be disrupted, so that the spermatozoa produced will also decrease.<sup>3</sup>

Antioxidants are substances that the body needs to neutralize free radicals and prevent damage caused by it. Antioxidants stabilize free radicals by supplementing electron deficiencies possessing free radicals, and inhibiting the occurrence of chain reactions from free radical formation which can cause oxidative stress. Oxidative stress is an imbalance between free radicals (prooxidants) and antioxidants triggered by two common conditions of lack of antioxidants, overproduction of free radicals. Vitamin C and E as antioxidants can stop the chain reaction of free radicals. First vitamin E will capture free radicals, but vitamin E then turns into vitamin E radicals that require vitamin C. Vitamin C together with vitamin E can inhibit oxidation reactions by binding to vitamin E radicals that are formed in the process of breaking free radical

reactions by vitamin E into free vitamin E which works again as an antioxidant.<sup>4</sup>

With these different working mechanisms, if both of these vitamins are used it is expected to inhibit free radical activity. Based on the data above, it is necessary to examine the effect of antioxidants in the form of vitamins C and E on the quality of spermatozoa of allethrin-exposed whiterats.

## Methods

This research was an experimental study with post test design randomized control group design. The study was conducted in January - March 2013 at Biology Medicine's Laboratory, Faculty Medicine Universitas Airlangga. The research sample used was 16 white *Rattus norvegicus* weighing between 150-200 grams which were obtained from the Department of Biology Medicine, Faculty Medicine Universitas Airlangga. It was divided into 4 groups, each group consisting of 4 rat, group A (control), group B (vitamin C treatment 0.36 mg), group C (vitamin E treatment 1.44 mg), group D (vitamin C treatment 0.36 mg + vitamin E 1.44 mg).

There are 3 variables used in this study, namely: 1). Independent variables: giving antioxidants in the form of vitamin C dose of 1, 8 mg / day, vitamin E dose 1.44 mg / day, and a combination of vitamin C and vitamin E. 2). Dependent variable: sperm quality which includes number, morphology, motility, spermatozoa viability. 3). Control variables: strain, sex, weight, and treatment cage.

Firstly, rats were acclimated in cages for one week, and then sixteen rats were grouped randomly into 4 groups, 4 each. We measured the initial body weight of mice and mark white mice with picric acid. During the study rats were fed and drank ad libitum. The exposure of mosquito coils was performed 8 hours per day for 45 days. Allethrin exposure in electric insect repellent started at 21.00 - 05.00 and given antioxidants orally for 45 days. On the 46th day, all rats were sacrificed and dissected to get the vas deferens. Data were taken on the amount, motility, viability, and morphology of spermatozoa.

After that, we made a stock solution by putting the vas deferens in a cup containing 0.9% physiological NaCl, sorted and referred to as a stock solution used to determine the quality of spermatozoa as below.<sup>5</sup>

We calculated the number of spermatozoa by taking the stock solution using a hemocytometer until the mark of 0.5 and 0.9% NaCl solution until the mark 101, and the pipette is shaken. Discard a few drops on tissue paper, then drop it on the counting chamber that has been closed with a cover glass and has been prepared in a microscope, then check on the microscope. We used the formula for the number of spermatozoa calculated (s) x 200,000 = million/ml.

Then, we looked at the morphology of the spermatozoa using Giemsa staining and observed under a

microscope with a magnification of 400 times to see abnormalities in the shape of the head, neck, and tail of the spermatozoa.

We calculated the sperm viability by making smear preparations from stock solutions then stained with Giemsa. Observations were made by calculating the average percentage of the 6 fields of view and seen on the head of the spermatozoa, if the color is transparent it means the spermatozoa are still alive and those that die in the colored head region. The spermatozoa viability value is expressed in percent.

Calculating motility by means of a visual field examined and obtain 100 motile sperm found and the percentage calculated. The category used to classify sperm motility is called (a), (b), (c), (d), and is defined as follows: Category (a) if the sperm moves fast and straight to face. Category (b) if the motion is slow or difficult to move straight or move not straight. Category (c) if it does not move forward. Category (d) if sperm do not move (Hermawanto 2000). Motile classification for movement of the spermatozoa category (a) and non-motile classification for category (b), (c), (d) spermatozoa movements to produce a percentage of each motility category.

The data in the form of sperm quality is analyzed statistically with ANOVA one way and if it was succeed, it would be continued with further test of BNT. For data in the form of sperm morphology analyzed descriptively. To be able to find out whether the hypothesis is rejected or accepted, the calculated F value that has been known through one-way ANOVA calculation is compared to the F-table value with the test level of 5%. If the calculation with ANOVA test one way shows the difference significantly, then the calculation continued with the BNT test by comparing the mean (average) of the two treatment groups. If the average value > BNT value is 5% then both treatments are significantly different and if the average value < BNT value is 5% then the two treatments are not significantly different. For analysis we used SPSS for windows, version 20.0 (SPSS, Inc., Chicago, IL, USA).

## Results

### Amount of Spermatozoa

The amount of spermatozoa after vitamin C, E and combination of vitamins C and E with the same dose for each of the different groups was different. Each group showed an increase in the mean value of the treatment group compared with the control group and the highest increase rate was shown by the vitamin C + vitamin E treatment group (table 1).

Based on the results of ANOVA analysis, there were differences between control and antioxidant treatment ( $p < 0,05$ ). This is indicated by the price of F arithmetic (25.93) greater than F table (3.49). Furthermore, to know the different groups, then tested BNT with 5% test level (table 1).

We found that group A was different from group B, C and D. Between group B and group C was not different but with different group D. While between group C with group D was not different. This suggests that the administration of vitamin C and E together results more effectively in preventing free radicals from allethrin than when the vitamin is administered separately.

Table 1. Mean amount of control group spermatozoa and treatment

| Group | Average Amount of Spermatozoa (million/ml ejaculate) | A      | B       | C    | D |
|-------|--|--------|---------|------|---|
| A     | 10.5   | -      |         |      |   |
| B     | 15.25  | 4.75** | -       |      |   |
| C     | 16.5   | 6**    | 1.25 ns | -    |   |
| D     | 17.5   | 7**    | 2.25 ns | 1 ns | - |

BNT = 1.87

A : control.

B : vitamin C treatment 0.36 mg.

C : vitamin E treatment 1.44 mg.

D : vitamin C treatment 0.36 mg + vitamin E 1.44 mg.

ns = non significant, not significantly different at test level 5% .

\*\* = significant, significantly different at test level 5%.

### Spermatozoa Motility

Motile classification for motion spermatozoa category (a) and non motile classification for movement of spermatozoa category (b), (c), (d). The mean spermatozoa motility after treatment increased when compared to the control group without vitamin C or E (table 2).

Table 2. Mean spermatozoa motility control and treatment groups

| Group | Average Spermatozoa Motility (%) | A       | B       | C      | D |
|-------|----------------------------------|---------|---------|--------|---|
| A     | 11.75                            | -       |         |        |   |
| B     | 41.75                            | 30 **   | -       |        |   |
| C     | 60.25                            | 48.5 ** | 18.5 ** | -      |   |
| D     | 66.75                            | 55 **   | 25 **   | 6.5 ns | - |

BNT = 7.12

A : control.

B : vitamin C treatment 0.36 mg.

C : vitamin E treatment 1.44 mg.

D : vitamin C treatment 0.36 mg + vitamin E 1.44 mg.

ns = non significant, not significantly different at test level 5% .

\*\* = significant, significantly different at test level 5%.

Based on the results of ANOVA analysis, it can be seen that the hypothesis is accepted, that there is influence on ( $p < 0,05$ ) for vitamin C and E group from each treatment. This is indicated by the price of F arithmetic (113.5) greater than F table (3.49) and BNT 5% level test.

After a further test with 5% BNT test can be seen that between group A with group B, C and D was different. Group B was different from group C and been able to counteract free radicals indicated by increased sperm motility and if both of these vitamins were given together it indicates better function in warding off free radicals.

**Spermatozoa Viability**

Table 3. Mean spermatozoa viability of control and treatment groups

| Group | Average Spermatozoa Viability (%) | A       | B       | C       | D |
|-------|-----------------------------------|---------|---------|---------|---|
| A     | 30.5                              | -       |         |         |   |
| B     | 47.5                              | 17 **   | -       |         |   |
| C     | 67                                | 36.5 ** | 22.5 ** | -       |   |
| D     | 90.5                              | 60 **   | 43 **   | 23.5 ** | - |

BNT = 8.06

A : control

B : vitamin C treatment 0.36 mg

C :vitamin E treatment 1.44 mg

D :vitamin C treatment 0.36 mg + vitamin E 1.44 mg

\*\* = significant, significantly different at test level 5%

D. This suggests that the administration of either vitamin C 0.36 mg / day and vitamin E 1.44 mg / day has

Mean of spermatozoa viability of treatment group was increased when compared with control group. Based on the results of ANOVA analysis, it can be seen that the hypothesis is accepted, that there is influence on (p <0,05) for vitamin C and E group from each treatment. This is indicated by the price of F arithmetic (97.32) greater than F table (3.49). After further testing with 5% BNT test can be seen that each group has a difference. This shows that the administration of either vitamin C 0.36 mg / day and vitamin E 1.44 mg / day has been able to ward off free radicals with spermatozoa survival viability. When vitamin C and E are together the results are more effective in treating free radicals to maintain viability of spermatozoa of white rat exposed to allethrin.

**Spermatozoa Morphology**

We found some morphological abnormalities of spermatozoa after allethrin exposure. Abnormalities found include:

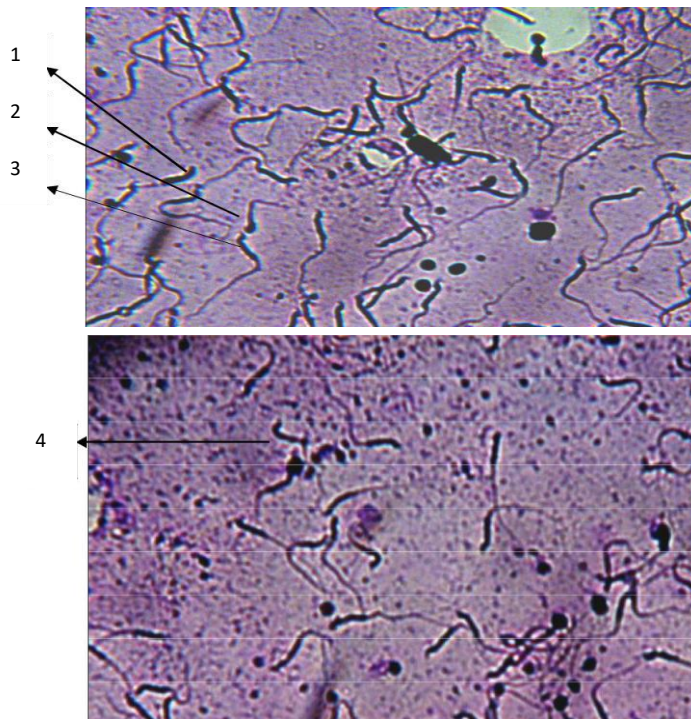


Figure 1. (Control group, 400x, giemsa): 1. short-tailed spermatozoa, 2. small headed spermatozoa, 3. normal spermatozoa consisting of head, neck, and tail, 4. spermatozoa without a tail

The morphological abnormalities of spermatozoa in the control group caused by the presence of interference from free radicals.

Then we compared the morphology with treatment group. We found no abnormalities in the given of vitamin C and E, either separately or simultaneously. Normal morphology is shown by sickle shaped

spermatozoa, neck, as well as tails. This suggests that the antioxidant vitamins C and E are able to counteract the effects of free radicals.

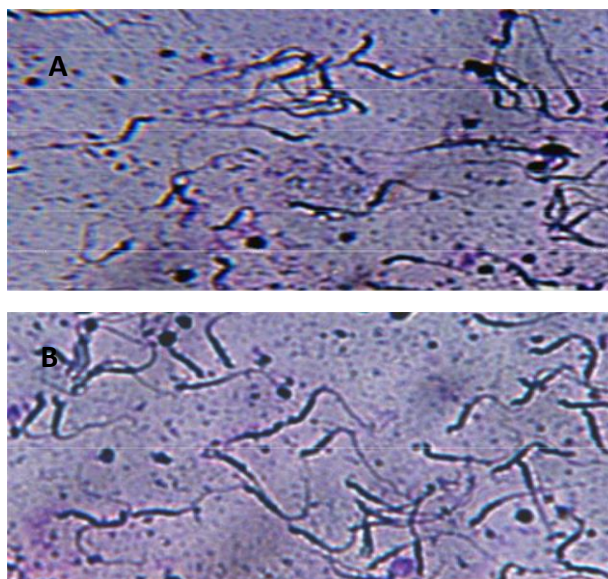


Figure 2. A. Group B, 400x magnification, giemsa, B. Group C, 400x magnification, giemsa shows normal Spermatozoa morphology.

## Discussion

In this study, the control group with 8 hours / day of mosquito coils exposure for 45 days showed the lowest average result for the calculation of the amount, motility and viability of spermatozoa when compared with treatment group given vitamin C, vitamin E and combination of both vitamins C and E. The morphology of spermatozoa in the control group showed the presence of morphological abnormalities whereas in the group who were given vitamins did not find any abnormalities. This suggests that allethrin belonging to the pyrethroid group I, can cause a decrease in the number, motility, and viability of spermatozoa, and cause morphological abnormalities of spermatozoa.<sup>6</sup>

Allethrin in mosquito coils can lead to the formation of free radicals. If free radicals are not stopped, it will damage the mitochondrial cell membrane. In this case the mitochondrial cell is the generating ATP necessary for the conversion of testosterone in leydig cells in the spermatogenesis process. When mitochondria are disturbed or damaged then the process of spermatogenesis will experience interference. This is in accordance with previous study that stated free radicals from pyrethroid can cause damage to the cell membrane of Leydig cell mitochondria, the damage in the form of unpaired mitochondrial cell membranes. Free radicals from pyrethroid interfere with the biosynthesis of testosterone by reducing cholesterol delivery to

mitochondria by Peripheral Benzodiazepine Receptor (PBR) and Steroidogenic Acute Regulatory protein (StAR), as well as lowered cholesterol conversion to pregnenolone catalyzed by cytochrome P450 side-chain cleavage (P450scc), leading to decreased testosterone production. It takes the neutralizing allethrin in the form of antioxidants in order to prevent that problem.<sup>7</sup>

Antioxidants are substances that the body needs to neutralize free radicals and prevent damage caused by free radicals to normal cells, proteins and fat by donating hydrogen electrons to free radicals to become stable free radicals that are not destructive. Antioxidants used in this study were vitamins C and E. The groups given antioxidant vitamins C and E showed an increase in the number, motility and viability of spermatozoa. Likewise with the morphology of spermatozoa that were not found abnormalities in the group treated with antioxidants in the form of vitamins C and E. Vitamin C has the ability to convert reactive vitamin C into stable vitamin C and able to regenerate reactive vitamin E into stable vitamin E again. Giving a combination of vitamin C and E can increase the number, motility, viability of spermatozoa and reduce morphological abnormalities of spermatozoa. This suggests that the synergistic cooperation of vitamins C and E, that is by means of free radicals caused by allethrin in electric mosquito repellent will be captured by vitamin E by donating one electron to a radical which then turns into vitamin E radical and then stabilized by vitamin C. Vitamin C which is radical

due to the loss of electrons will eventually turn into a stable again by antioxidant enzymes in the body.<sup>4</sup>

Average amount of spermatozoa between control and treatment groups were different. Compared with control, antioxidant vitamins C and E were able to counteract free radicals caused by Allethrin in mosquito repellent. The administration vitamin C (group B) or E (group C) only, showed no significant differences. This shows that both vitamin C and E have the same ability in winning free radicals so that the number of spermatozoa can be maintained.<sup>8</sup>

Vitamin C serves to counteract free radicals by neutralizing allethrin in insect repellent in the cytosol because vitamin C has a high solubility in water. Vitamin E has the ability to stop lipid peroxide by donating one of its hydrogen atoms from the OH group to a radical lipid peroxy to a less reactive and non-destructive vitamin E. Between group C and D is different because vitamin E alone is able to counteract the effects of free radicals, so when vitamin E and vitamin C are administered simultaneously shows a less noticeable difference in statistics even though it shows better mean rates in the calculations.<sup>9</sup>

In motility of spermatozoa obtained data showing group A different from group B, C, and D. This proves that the provision of antioxidants in the form of vitamin C and E able to counteract free radicals that can maintain sperm motility by preventing the damage caused by free radicals. Group B shows differences with C and D groups, it shows that when vitamin C works alone it is less able to make a difference, so vitamin C can play an optimal role in counteracting free radicals to maintain sperm motility, vitamin C should work together with vitamin E. This is because vitamin C works synergistically with vitamin E in maintaining the number of spermatozoa by capturing free radicals from Allethrin in mosquito coils, vitamin E subverts one electron to radical which then turns into vitamin E radicals and will further be stabilized by vitamin C. Vitamin C which is radical will then turn into stable again by antioxidant enzyme in the body.<sup>8</sup>

For groups C and D did not show this difference because vitamin E alone was able to counteract the free radical shown by persistence of sperm motility so that when used together with vitamin C can not show any difference even though in the calculation average number shows more numbers. This is because vitamin E has the ability to stop lipid peroxide by donating one hydrogen atom from the OH group to a radical lipid peroxide to a less reactive and less damaging vitamin E. When there are free radicals, lipid peroxide increases because of the reaction between lipids and free radicals. In the early stages of the reaction, hydrogen release from unsaturated fatty acids is homolytic, forming an alkyl radical that occurs due to the initiator (heat, active oxygen, metal or light). In normal circumstances the alkyl radicals react quickly with oxygen to form a peroxy radical in which these peroxy radicals react further with unsaturated fatty acids

forming hydroperoxides with alkyl radicals, then these formed alkyl radicals react with oxygen. Thus the oxidation reaction is a free radical chain reaction. Because the mitochondrial cell membrane is rich in lipids that are sensitive to free radical attack.<sup>8</sup>

On calculation of spermatozoa viability between groups A, B, C and D were different. This suggests that vitamins C and E work better in counteracting free radicals in order to maintain spermatozoa viability when used together. Vitamin C works in neutralizing free radicals from Allethrin in insect repellent in the cytosol because vitamin C has high solubility in water. While vitamin E has the ability to stop lipid peroxide by donating one hydrogen atom from the OH group to a radical lipid peroxy to become less reactive and non-destructive lipid peroxide. Because the mitochondrial cell membrane is rich in lipid that is sensitive to free radical attack caused by Allethrin in mosquito repellent. This shows that vitamin C and vitamin E have been able to ward off free radicals so as to increase the viability of spermatozoa. If the two vitamins used together will work better than the use of one vitamin alone, this is indicated by showing more viability of spermatozoa. This is in accordance with previous study in 2003 which states by different working mechanisms, if both vitamins C and E are used together will be expected to give the optimal effect in the face of free radical activity.<sup>10</sup>

The spermatozoa abnormalities morphology of exposed allethrin was found only in the control group, whereas in the antioxidant group of vitamin C and E and the combination of vitamins C and E there was no abnormalities. This is because the antioxidant vitamins C and E are able to ward off free radicals well, thereby facilitating the stages of spermatogenesis that begins from the process of conversion of testosterone that originated from the transfer of cholesterol into the mitochondrial membranes by PBR and StAR so successfully converted into pregnenolone and catalyzed by P450<sub>scc</sub> on membranes in the mitochondria. The next process in the testes is the Leydig cell where P450<sub>scc</sub> begins the initial enzymatic stage in steroidogenesis. Thereafter pregnenolone leads to a subtle endoplasmic reticulum which is then converted to progesterone by 3 $\beta$ -HSD. Pregnenolone decarboxylation by P450 17 $\alpha$  to form 17-hydroxyprogesterone and androstenedione which is then converted to testosterone by 17 $\beta$ -HSD.<sup>6</sup>

If the above stages are disrupted by the presence of free radicals from phyrethroid then the next step in spermatogenesis and spermiogenesis until the spermatozoa will be disturbed anyway, resulting deformation of spermatozoa.

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## Conclusion

Based on the results of the research and discussion in the previous chapter, it was concluded that giving antioxidant vitamin C and vitamin E can counteract free

radicals from allethrin in electric mosquito repellent against male wistar strains of white rat (*Rattus norvegicus*) spermatozoa. The administration of both vitamins simultaneously shows better result than separately.

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### Conflict of Interest

The author stated there is no conflict of interest.

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### References

1. Riskesdas. Riset Kesehatan Dasar 2013. Jakarta: Badan Penelitian dan Pengembangan Kesehatan Kementerian Kesehatan RI 2013.
2. Intisari. Jangan Asal Semprot, bahaya...! In: Indonesia DKR, (ed.). 2007.
3. Sakr S and Azab A. Effect of Pyrethroid Inhalation on Testis of Albino Rat. *Pakistan Journal of Biological Sciences*. 2001; 4: 498-500.
4. Pavlovic V, Cekic S, Rankovic G and Stoiljkovic N. Antioxidant and Pro-oxidant Effect of Ascorbic Acid. *Acta Medica Medianae*. 2005; 44: 65-9.
5. Soehadi K and Arsyad KM. *Analisis Sperma*. Surabaya: Airlangga University Press, 1983.
6. WHO. Environmental Health Criteria For Allethrins. 1989.
7. Shu-Yun Z, Yuki I, Osamu Y, et al. Permethrin May Disrupt Testosterone Biosynthesis via Mitochondrial Membrane Damage of Leydig Cells in Adult Male Mouse. *The Endocrine Society*. 2007; 148: 3941-9.
8. Hariyatmi. Kemampuan Vitamin E sebagai Antioksidan terhadap Radikal Bebas pada Lanjut Usia. *MIPA*. 2004; 14.
9. Frei. Reactive Oxygen Species and Antioxidant Vitamins: Mechanisms of Action (American Journal Medicine): (1994).
10. Christyaningsih J. Pengaruh Suplementasi Vitamin E dan C terhadap Aktivitas Enzim Super Oxide Dismutase(SOD) dalam Eritrosit Tikus yang Terpapar Asap Rokok Kretek. *JIPTU*. 2003.