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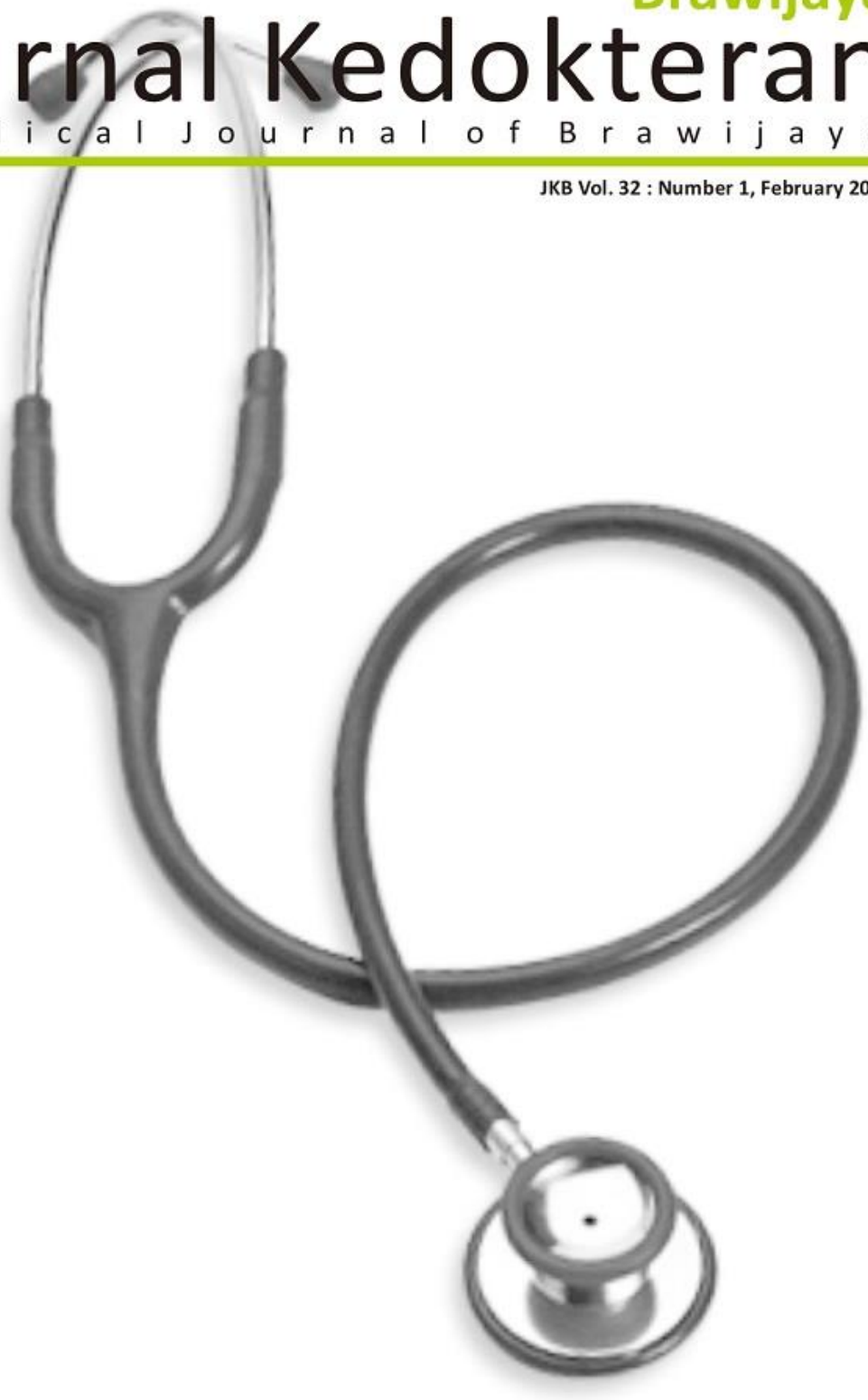

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Research Article

Combination of Vitamin C and E Improves Spermatogenesis of White Male Rat Model of Paradoxical Sleep Deprivation Stress

Kombinasi Vitamin C dan E Memperbaiki Spermatogenesis pada Tikus Putih Jantan Model Stres Paradoxical Sleep Deprivation

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ABSTRACT

Paradoxical Sleep Deprivation (PSD) elevates glucocorticoid and Reactive Oxygen Species (ROS) levels that cause oxidative stress, trigger spermatogenic cells damage, and reduce the number of sertoli cells. Vitamin C and E are antioxidants that could prevent spermatogenesis damage by preventing free radical formation. The study aimed to determine the effect of single and combined doses of vitamin C and E in improving spermatogenesis of white male rats (Rattus norvegicus) model of PSD. This is experimental research with post-test only and control group design on 28 white male rats distributed into four groups, i.e. group I (control, PSD), group II (PSD+vitamin C), group III (PSD+vitamin E), and group IV (PSD+a combination of vitamin C and E). Testicular preparations were stained using Hematoxylin-Eosin staining, quantitative scores of spermatogenic cells were measured using the Johnsen method, and the number of sertoli cells was counted in 10 seminiferous tubules in each of the three testicular sections. Group I has the lowest mean of Johnsen score (5.27±0.28), and group IV has the highest mean score (8.95±0.62), while the Mann-Whitney test showed a significant difference (p<0.05) between group II, III and IV compared to group I (control group). Mean sertoli cells number of group I is the lowest (10.66±1.04), and group IV has the highest sertoli cells number. The post-hoc LSD test showed a significant difference (p<0.05) between group IV and the other groups. Thus, the combination of vitamins C and E improves Johnsen score and sertoli cells number of male rats (Rattus norvegicus) experiencing PSD.

Keywords: *Paradoxical sleep deprivation, spermatogenesis, vitamin C, vitamin E*

ABSTRAK

Paradoxical Sleep Deprivation (PSD) meningkatkan kadar hormon glukokortikoid dan *Reactive Oxygen Species* sehingga menyebabkan stres oksidatif dan memicu kerusakan sel spermatogenik dan menurunkan jumlah sel Sertoli. Vitamin C dan E merupakan antioksidan untuk mencegah kerusakan spermatogenesis dengan mencegah reaksi akibat radikal bebas. Tujuan penelitian adalah mengetahui pengaruh pemberian vitamin C dan E dengan dosis tunggal dan kombinasi dalam perbaikan spermatogenesis pada model stres *Paradoxical sleep deprivation* (PSD) pada tikus putih (*Rattus norvegicus*) jantan. Metode penelitian adalah *experimental post test only with control group design* menggunakan tikus putih jantan berjumlah 28 ekor dibagi menjadi 4 kelompok perlakuan. Kelompok kontrol dengan PSD (KI), kelompok PSD dan vitamin C (KII), kelompok PSD dan vitamin E (KIII), dan kelompok PSD dengan vitamin C dan E (KIV) selama selama 7 hari. Preparat testis diwarnai dengan pewarnaan Hematoksilin-Eosin, skor kuantitatif sel spermatogenik diukur dengan metode Johnsen dan jumlah sel Sertoli dihitung pada 10 tubulus seminiferus pada tiap tiga potongan testis. KI memiliki rerata skor Johnsen terendah (5,27±0,28) dan tertinggi pada KIV (8,95±0,62). Uji Kruskal-Wallis menunjukkan perbedaan signifikan (p<0,05) dan dilanjutkan Uji Mann-Whitney menunjukkan perbedaan signifikan (p<0,05) antara kelompok II,III, dan IV dengan kelompok KI. Pada jumlah sel Sertoli, rerata jumlah terendah pada KI (10,66±1,04) dan tertinggi pada KIV(14,26±1,70), uji Post-Hoc LSD menunjukkan perbedaan yang signifikan (p<0,05) antara KIV dan kelompok lain. Dapat disimpulkan bahwa kombinasi vitamin C dan E memberikan perbaikan skor Johnsen dan sel Sertoli tikus putih (*Rattus norvegicus*) jantan yang mengalami PSD.

Kata Kunci: *Paradoxical sleep deprivation,* spermatogenesis, vitamin C, vitamin E

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INTRODUCTION

Paradoxical sleep deprivation (PSD) is a stress model on experimental animals similar to stress due to sleep deprivation among humans resulted from work demands and busy activity in this globalization era. Paradoxical sleep deprivation experiment on experimental animals for four days can activate Hypothalamus-hypophysis-adrenal (HHA) axis, which induces corticosterone hormone elevation (1). Induction of PSD (paradoxical sleep deprivation) on white male rats Wistar strain in 96 hours can reduce serum testosterone level (436.0±68.0 ng/dl) compared with normal rats (58.0±8.2 ng/dl), elevate corticosterone hormone level, and change prostate histology structure (2). PSD stress model in experimental animals in 48-72 hours increases thiobarbituric acid reactive substance (TBARS) production, increases lipid peroxidation, reduces glutathione (GSH) antioxidant, and triggers oxidative stress (3). Oxidative stress on the testes impairs Deoxyribose Nucleic Acid (DNA) and disturbs cells membrane integrity, which in turn causes programmed cell death (apoptotic) of germ cells and disturbances of sperms' morphology and motility. Endocrine impairment caused by stress contributes 20% of spermatogenesis disturbance (4), and ROS adds the remaining 25-40% (3). Decreases in hormone production and oxidative stress can damage Sertoli cells and disrupt spermatogenesis, thus lowering the Johnsen score.

Vitamin C and E are non-enzymatic antioxidants that have huge role in spermatogenesis to fight free radicals, improve the Johnsen score, and prevent DNA damage due to free radicals (5). Recent animals research found that vitamin C has a radioprotective effect on spermatogonia, increase the number of spermatozoa, decrease the percentage of spermatozoa with abnormal morphology, neutralize hydroxyl radicals, such as superoxide and hydrogen peroxide, and prevent sperm agglutination, while vitamin E has the main role in preventing cell damage from lipid peroxidation and increasing the activity of total anti-oxidation competence (T-AOC), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) (1). The combination of vitamin C and E provides effective effect to prevent free radicals by elevating the testosterone level and improve the histology appearance of seminiferous tubules (6), reduce malonic dialdehyde (MDA) level, increase the number of spermatogonia and spermatid (7), increase SOD, GSH, catalase (CAT) antioxidants, reduce spermatid and spermatozoa degeneration (8), increase animals' body weight, testis volume, seminiferous tubules diameter, and Johnsen score (9). Vitamin C and E are exogenous antioxidants proven to inhibit free radicals in the testes, including in the Sertoli cells, and improve Johnsen score and spermatogenesis. However, research on the effect of vitamin C and E combination on the PSD stress model has not been done yet.

METHODS

This research was conducted with a post-test experimental with control group design using 28 white male Wistar strain rats (*Rattus norvegicus*), because is most widely used in biomedical research such as aging, teratogenic toxicology testing, efficacy testing and reproductive testing with the inclusion criteria were healthy, aged 3-4 months old and weighed 200-300 grams, and was obtained from Pharmacology Laboratory of Gadjah Mada University. This research has received ethical approval from the Medical Research Ethical Commission of Medical Faculty, Jenderal Soedirman University, through Ethics Approval number 061/KEPK/VIII/2019 on 18 August 2019. Research material included AD II feed from Comfeed®, vitamin C 50 mg from IPI®, vitamin E (α-tocopherol) tablet 400 mg from Santa-E®, and sesame oil as vitamin E solvent.

Animals were randomly divided into four groups, seven rats each. All groups were treated with the PSD stress model using the modified multiple platform method (MMPD). The rats were placed in a tank sized 123 x 44 x 44 cm containing water as high as 1 cm above the surface and provided with 14 platforms with a distance of 10 cm between platforms equipped with a muscle atonia tool which was turned on every 10 minutes for 18 hours during 04.00-22.00 (GMT+7) so that the rats were unable to sleep. Seven days before the treatment, rats were acclimatized in a water tank for 30 minutes/day and freely moved in the tank by jumping from one platform to the others and interacted with the other rats. After the treatment, rats were returned to the cage and left slept for 6 hours from 22.00 to 04.00 (GMT+7). The PSD treatment was carried out simultaneously by administering antioxidants for seven days which were given 2 hours before administering the PSD stress model (10).

Antioxidants given to the experimental animals were vitamin C and E based on the dose of vitamins in humans weighing 70 kg that were 1000 mg/day for vitamin C and 600 mg/day for vitamin E. The dose used was the preventive dose in humans converted to mice weighing 200 grams with a conversion factor of 0.018. The calculated vitamin C dose in rats (200 gram) was human dose x conversion factor = 1000 mg/day x 0.018 = 18 mg/200 grBW/day, thus the vitamin C dose in rats (per gram) was 0.09 mg/grBW/day. The maximum oral vitamin C solution in rats was 5 ml/grBW, and because it is lipophilic, every 9 mg of vitamin C was dissolved in 1 ml of distilled water. The dose of vitamin E in rats (200 gr) was human dose x conversion factor = 600 mg/day x 0.018 = 10.8 mg/200 grBW/day, so the vitamin E dose in rats (per gram) was 0.054 mg/grBW/day. The maximum oral vitamin E solution was 5 ml/100 grBW, thus every 54 mg solution was dissolved with 1 ml of distilled water. Animals were divided into control group (group 1) given PSD exposure and placebo, treatment group I (group 2) given PSD and vitamin C antioxidant dose of 0.09 mg/grBW/day, treatment group II (group 3) given PSD and vitamin E antioxidant dose of 0.054 mg/grBW/day, and treatment group III (group 4) given PSD and a combination of vitamin C 0.09 mg/grBW/day and vitamin E 0.054 mg/grBW/day (11).

Left testes were taken after the rats were inhaled with Chloroform. The testes were rinsed with NaCl, weighed, put in tubes and fixed with PBF 10% solution, cut to 5 µm thickness, and stained with Hematoxylin Eosin. The spermatogenic quantitative score was measured using Johnsen methods; score 10 (complete spermatogenesis and mature spermatozoa), score 9 (lots of spermatozoa with seminiferous tubules damage with irregular epithelial and spermatogenic exfoliation from basal membrane), score 8 (visible spermatozoa (<10)), score 7 (no visible spermatozoa but many spermatids), score 6 (no visible spermatozoa and few spermatids (5-10)), score 5 (no

visible spermatozoa and spermatid, but many spermatocyte), score 4 (no visible spermatid and spermatozoa, and spermatocyte <5), score 3 (visible spermatogonia), score 2 (visible sertoli cell), and score 1 (no visible cell in seminiferous tubules). The number of Sertoli cells was counted in 10 seminiferous tubules randomly selected for each of three testicular sections, and the scores for each rat were added and averaged to obtain the group scores. Observations were done using Nikon Eclipse E100® light microscope with 400x magnification equipped with Optilab® and Image Raster v 2.1 software. Data normality test was done using Shapiro Wilk, and homogeneity of variance test was done using Levene's test. Since the Johnsen score data did not show a normal distribution despite the transformation even though the variance was the same, the Kruskal-Wallis test was performed and continued with the Mann-Whitney test to determine the differences between groups. Analysis of differences in the number of Sertoli cells between treatments was done using the One-Way Analysis of Variance (ANOVA) test and continued with the Post-Hoc LSD test.

RESULTS

Table 1 shows that group I has the lowest mean Johnsen score (5.27±0.28) and group IV has the highest mean Johnsen score (8.95±0.62), and the homogeneity of variances test showed that the data variance was the same (p = 0.503). The Mann-Whitney test showed that the spermatogenesis scores of all treatment groups receiving vitamin C and E were higher and statistically significant than the control group. Group IV, with a combination dose of vitamins C and E, also had the highest mean number of Sertoli cells (14.26 ± 1.70), while the lowest number was found in group I (control group) (10.66 ± 1.04). The results of the Post-Hoc LSD test showed that a significant difference in the mean number of Sertoli cells was only found between the control group and the treatment group with the vitamin combination (group IV, p = 0.014), while the treatment with a single dose of vitamin C or E showed no difference compared to normal (p = 0.686 and p = 0.855), and II, III, control; and between the two groups (p = 0.831). The administration of a combined dose also showed a higher Johnsen score and Sertoli cell counts than a single dose of vitamin C or E.

Table 1. Mean Johnsen score and Sertoli cells in all four experiment groups

No	Groups	∑ Sample	Johnsen score	Sertoli cell
1	Group 1	6	5.27 ± 0.28*	10.06 ± 1,04^{+}
2	Group 2	6	7.12 ± 0.84*	11.15 ± 1,56^{x}
3	Group 3	6	7.72 ± 0.55*	10.89 ± 2.58$^{\#}$
4	Group 4	6	8.95 ± 0.62*	14.26 ± 1.70$^{+x\#}$

Note: Group I (PSD + placebo), Group II (PSD + vitamin C), Group III (PSD + vitamin E), Group IV (PSD + vitamin C and E)

*significant difference between group I and II-IV
$^{+}$ significant difference between group I and IV
^{X} significant difference between group II and IV
$^{\#}$ significant difference between group III and IV

Observations on the Johnsen score showed differences in the histological features of the spermatogenic cells in each

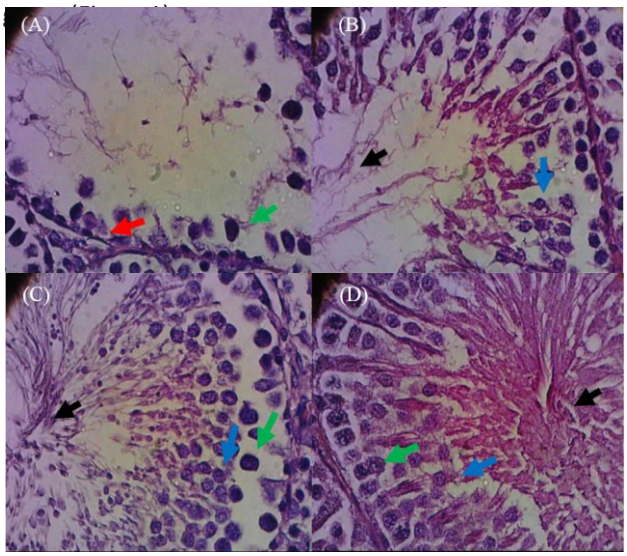

Figure 1. Johnsen score of histological feature of rat left testicle (Hematoxylin-Eosin stain, 400 X magnification)

Note: (a) Group I (control + PSD + placebo) scored 4; (b) Group II (PSD + vitamin C) scored 8; (c) Group III (PSD + vitamin E) scored 9; (d) Group IV (PSD + vitamin C dan E) scored 10; red arrow = spermatogonia; green arrow = primary spermatocyte; blue arrow = spermatid; black arrow = spermatozoa. Group 1, scored 4, showed spermatogonia and many primary spermatocytes without any spermatid and spermatozoa. In group II, scored 8, there were two spermatozoa (less than 10). Group III, scored 9, showed complete spermatogenic cells, but seminiferous tubules epithelia were irregular, characterized by spermatogenic cells exfoliation from the basal membrane. Group IV had a complete and regular Johnsen score, and mature spermatozoa were found in the seminiferous tubular lumen so that it has a score of 10.

DISCUSSION

The results of this study proved that treatment on the PSD stress model showed the lowest Johnsen score and the number of sertoli cells. The administration of a combination of vitamin C and E as antioxidants can improve the Johnsen score and the number of sertoli cells so it can be recommended as adjuvant therapy for the treatment of male infertility because it can improve one of reproduction paramater but also increases LH and FSH levels and inhibit excessive secretion of glucocorticoid

Spermatogenic Cell Count

The low Johnsen score in the control group (PSD stress model) was due to the effect of PSD stress on spermatogenesis through hormonal disturbances in the form of increased activation of HHA and free radicals. HHA axis activation escalates glucocorticoids, release GnIH that inhibits the release of LH, FSH, and testosterone, and impacts impaired spermatogenesis (12). Glucocorticoids that bind to spermatogenic cells trigger mitochondrial damage in the form of decreased membrane potential and loss of electron transport chains which increase the release of free electrons and trigger the formation of free radicals and decrease the main antioxidants in the intratesticular, i.e. glutathione, superoxide dismutase, catalase and glutathione s-transferase (3). The decrease in intratesticular antioxidants results in oxidative stress that triggers damage to lipids, proteins, nuclear DNA, and mitochondrial DNA, which stops the mitosis process accompanied by an increase in apoptosis of spermatogenic cells (5). The increase in superoxide anion radicals trigger

spermatocytes to release cytochrome c from the mitochondria to the cytosol and stimulates the Sertoli cells to express Fas ligand (FasL), activating the intrinsic pathway of germ cell apoptosis, whereas FasL activates the extrinsic pathway of germ cell apoptosis. The Sertoli cell FasL binds to the Fas receptor on spermatocytes and activates the Fas-associated death domain, which activates pro-caspase 8, caspase-8, caspase-3 sequentially, resulting in apoptosis of spermatocytes. Glucocorticoid activates Fas system and proapoptotic molecules (Bax, Bak, Bad), and also inhibits antiapoptotic molecules (Bcl-2, Bcl-xL, Bcl-W), which induce germ cell apoptosis and reduces Johnsen score (13). The mean Johnsen score in group II (PSD + vitamin C) was higher than that of group I (control group), proving that vitamin C enters seminiferous tubules and acts on germ cells by binding to receptors in sertoli cells, i.e. sodium-dependent vitamin C transporter (SVCT) 1 and 2, react with superoxide anions, hydrogen peroxide, and tocopherol radicals, which reduce the concentration of free radicals in the testes and prevent germ cell apoptosis (14).

The administration of a single dose of vitamin E showed a higher mean of Johnsen score compared to the control group. Vitamin E is a lipophilic antioxidant that is highly contained in spermatocytes and works on cell membranes by breaking the main chain of superoxide anion free radicals, hydroxyl radicals (4), and suppressing MDA formation as a lipid peroxide product, and increasing total antioxidant competence (T-AOC), superoxide dismutase, and glutathione peroxidase (5). The highest mean of the Johnsen score of a combined dose compared to a single administration of vitamin C or E proves that the combination of vitamin C and E can increase seminiferous tubular diameter, spermatogenic index, and daily sperm production, decrease lipid peroxidation, increase enzymatic antioxidants (8), decrease MDA level, increase the number of spermatogonia and spermatid, and improve the weight, testis volume, seminiferous tubules, and Johnsen score (7). Vitamin E as a lipophilic component will work initially on the mitochondrial membrane to inhibit oxidized polyunsaturated fatty acid (PUFA) reactions and increase the enzymatic antioxidants catalase and glutathion peroxide. The combination of vitamins C and E works more effectively in reducing free radicals; vitamin E turns into tocopherol radicals, which are free radical compounds that are less reactive, then vitamin C reacts with tocopherol radicals and converts them back into tocopherols which can function as antioxidants.

Sertoli Cell Count

The lowest mean number of sertoli cells was found in the control group, a rat model of PSD stress; since the negative effect of PSD could activate the hypothalamic-pituitary-adrenal (HHA) axis, it triggers oxidative stress (15). HHA axis activation causes an increase in systemic glucocorticoid levels, which bind to glucocorticoid receptors in the hypothalamus, which inhibit the hypothalamus-pituitary-gonad (HHG) axis, resulting in inhibition of FSH and LH secretion (10). The administration of PSD stress for seven days reduces LH hormone level due to increasing glucocorticoids which had a negative effect in the form of decreasing the number of Sertoli cells in white rat experimental animals (13). Glucocorticoids also increase mitochondrial Ca^{2+}, which triggers free radical formation and disrupts mitochondrial membrane potential. Mitochondrial dysfunction disrupts the electron transport chain (ETC), resulting in an increased release of free electrons that react with molecular oxygen to form reactive oxygen species (ROS). In stressful situations, excess glucocorticoids inhibit antioxidant defenses against increased free radicals, causing oxidative stress that damages DNA/RNA, lipids, and proteins (15). PSD treatment for 72 hours increases ROS and lipid peroxidation, decreases antioxidant glutathione (GSH), and induces damage to the mitochondrial membrane of sertoli cells (16); thus it results in the entry of proapoptotic protein Bax into the mitochondria and release of cytochrome C from the mitochondria to the cytosol causing the cell death process, one of which is through the apoptotic caspase cascade process. Cytochrome C released into the cytosol binds to apoptosis protease activating factor-1 (APAF-1) and binds to pro-caspase 9 to activate caspase 9. Caspase 9 as a caspase initiator works to activate caspase executor/effector, namely caspase 3, 6 and 7, which trigger apoptosis of Sertoli cells by cutting intracellular proteins as poly (ADP-ribose) polymerase, actin, and gleosin (17).

The administration of a single dose of Vitamin C or E in the rat model of PSD stress did not show a difference in the mean number of sertoli cells compared to the positive control. This indicates that the administration of vitamins C and E alone does not provide a protective effect against oxidative damage that occurs in experimental animals and the improvement came from the combination with other supplements but in this study, the results could be influenced by the determination of the dose of vitamins and the variables of reproductive function (9). Administration of vitamin C and vitamin E combination in the group of rats receiving PSD showed the highest mean number of Sertoli cells than if only one vitamin were given (9). Vitamin C is the most important hydrophilic antioxidant in extracellular fluid and works to neutralize free radicals before lipid peroxidation by donating two electrons from the double bond between the second and third carbon to bind to free radicals and turn into ascorbyl radicals. The ascorbyl radical changes to dehydroascorbic acid after losing a second electron and is reduced by the GSH-dependent dehydroascorbate reductase enzyme to be returned to the active form of ascorbic acid. Ascorbic acid in plasma enters the sertoli cells via the sodium vitamin C transporter (SVCT), which is then stored for use under certain conditions or transferred across the sertoli cell membrane to the spermatogenic cells located in the adluminal compartment of the seminiferous tubules. Vitamin E (α-tocopherol) is a lipophilic antioxidant that works as an antioxidant that breaks the oxidative chain reactions in cell membrane phospholipids (6), thus preventing damage to cellular structures due to oxygen free radicals and reactive products from lipid peroxidation, as well as functioning as a cell membrane stabilizer (18).

The administration of vitamin C and E combined can provide a more optimal effect in overcoming oxidative stress because it has a mutually synergistic working mechanism. Vitamin C and E can work synergistically to reduce peroxidative damage through a combination of the hydrophilic properties of vitamin C and lipophilic properties of vitamin E. Vitamin E will work on cell membranes, while vitamin C acts on the cytosol and extracellularly (9). Vitamins C and E that accumulate in the adrenal glands play the same role in inhibiting excess glucocorticoid synthesis from adrenocortical cells, thereby preventing inhibition of the HHG axis and

oxidative stress. The combination of vitamins C and E was able to improve spermatogenesis in PSD stress model mice by increasing the number of spermatogenic cells and sertoli cells.

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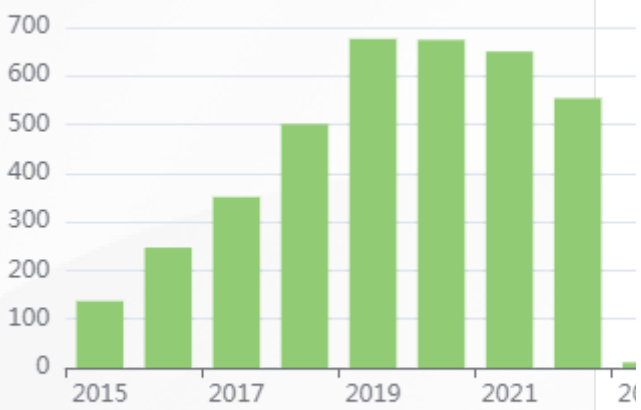

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