Effect of Duwet fruit (*Syzgium cumini*) extract on MDA level and Caspase 3 expression in Rat (*Rattus sp*) Testes exposed to cigarette smoke

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Abstract. This research evaluates the MDA levels, number and apoptosis of spermatogenic cells in Rat provided with duwet fruit extract and exposed to cigarette smoke towards. The study involved 4 experimental animal groups and 6 replications, where K0 was the negative control group, K1 was exposed to the cigarette smoke, K2 was given orally duwet fruit, and K3 was given orally duwet fruit after being exposed to cigarette smoke. Therefore, data obtained were analyzed using One way Anova test. The results showed significantly lower number of spermatocytes and MDA levels in the testes in all groups compared to K1 at p <0.05. Spermatogenic cell apoptosis occurred in all groups, and there was also a high tendency for reduction in K2 compared to K1, although not significant at p > 0.05. In addition, apoptosis was expressed by Caspase 3 in the testes. The highest MDA levels found in K1. This phenomenon was assumed to have resulted in lower numbers of spermatocytes. These decreased number of spermatocytes were related with the decline in number of spermatogenic cells prone to apoptosis. In conclusion, duwet fruit had the ability to reduce free radicals with the tendency to inhibit spermatogenesis. This case features an increase in spermatocytes and a potential decline in the incidence of spermatogenic cell apoptosis.

Keywords: Cigarette smoke, duwet fruit, apoptosis, spermatogenesis, Caspase 3

1. Introduction

The effects of cigarette smoking include an increase in free radicals [1], as well as a disruption of the quantitative balance in the body’s physiology. In addition, studies have shown reduced antioxidant defense mechanism, oxidative stress production [2] and lipid peroxidation development [3].

This exposure affects and damages the organ functions [4] including the reproductive system [5]. Particularly, uncontrolled free radicals induce DNA damage and increase spermatogenic cell apoptosis in the testes, consequently developing disorders related to spermatogenesis [6].

Mice exposed to cigarette smoke for 35 days indicated a significantly low number of pachytene spermatocytes and spermatids [7]. This further induced testicular cell death in the adult variant, with increased apoptosis and testicular DNA damage in new-borns [8]. Furthermore, cell death is also influenced by various environmental factors or physiological stress, including UV radiation [9], free radicals [10], exposure to toxicants, and reducing in hormone levels.

The Spermatogenesis process is complex and organized, characterized by germ cell proliferation and differentiation to produce spermatozoa in the seminiferous tubules [11]. In addition, time required to complete each growth step is different, as various combinations of germ cells and seminiferous tubules occur in each part of the tubule. These associations are formed to develop the epithelial stage. Furthermore, Oakberg & Rugh divided the epithelium into 12 levels, ranging from I - XII [11]. This development in rats extended through a 35.5 day period after completing 4 seminiferous epithelial cycles, at 207 ± 6 hours each [12].
During normal spermatogenesis, most cells demonstrate apoptosis [13] in response to a physiological or pathological stimuli. Also, the correct number of germ cells is related to the function of Sertoli, which serve as support for physiological growth and development in this stage [14]. This process functions to eliminate the germ units susceptible to damage and abnormalities, maintain genetic integrity by cleaning mutated varieties, and prevent the transmission of mutations to offspring. [15]. Furthermore, the number of apoptotic spermatogenic form exceeds a portion of the differentiated variants in this process. Meanwhile, a limited amount was detected in histochemical preparations resulting phagocytosis within a short time by Sertoli cells [16]. The apoptotic mechanism in germ cells during spermatogenesis occurs through extrinsic and intrinsic pathways. Also, Fas and FasL respectively detected in germ and Sertoli units, have proven to be essential for this process [17]. The participation of Testicular Fas is triggered by hormone depletion, UV radiation, toxicity, and oxidative stress. Moreover, an increase in protein expression facilitates the activity of caspase 3 after androgen /progesterone administration, and ultimately initiates a decline in spermatozoa concentration. The proteins are usually expressed in spermatogonia, spermatocytes, and spermatids. In addition, the linear regression and a significant positive correlation between these parameters indicate a possible decline in spermatozoa concentration [18,19]. The effects of Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), include lipid peroxidation in membranes, free radical formation, and the induction of apoptosis through the intrinsic and extrinsic pathways. Furthermore, analysis with western blot demonstrates the expression of caspase 9, cytochrome c, and caspase 8 proteins [19].

The mechanism for resistance and balance of free radicals within the body requires antioxidant activity, particularly the secondary metabolites obtained from various parts of plants. These materials act by preventing a chain and uncontrolled oxidative stress formation. According to previous research, duwet fruit contains bioinorganic complexes, between Fe metal and aromatic cyanidin compounds. These complex possess the ability to reduce DPPH free radicals intensity [20,21]. The product application in cigarette filters potentially modifies the smoke characteristics to a significantly safer level for living systems. This is evidenced by the crystal formed during XRD analysis, changes in the absorption of functional group waves in FTIR study, and the magnetic character of free radicals evaluated using ESR [22]. The purpose of this study, therefore, was to determine the effect of duwet fruit extract as an antioxidant ingredient in spermatogenesis disorders towards protein caspase 3 expression in the testes of white rats (Rattus norvegicus) exposed to cigarette smoke.

2. Methods
2.1. Duwet Fruit Extraction
The duwet fruit powder was freeze-dried, filtered using a 250 µM sieve and extracted by maceration with distilled water. Subsequently, the filtrate obtained was filtered, and re-dried[23]

2.2. Experimental Animal
A total of 24 male rats aged ± 12 weeks, with a body weight of 200-250 grams were randomly divided into 4 groups. Each consisted of 6 animals as replications, where K0 denoted as the negative control. The rats were given water orally. K1 were positive control with the rats exposed to cigarette smoke. K2 were the rats given duwet fruit extract orally (300 mg/kg body weight) and K3 were rats given the fruit extract after being exposed to cigarette smoke. The rats were placed in a ventilated glass room and exposed to cigarette smoke every day, at a rate of 1 stick per animal for 48 days. This smoke treatment was obtained from combustion using a smoking pump with suction speed of 0.23 m / s, streamed through the glass room. After 6 hours of exposure on the same day, as much as 0.5 cc treatments in the form of duwet fruit extract at 300 mg / kg BW was administered orally / day for 48 days

2.3. MDA Observations
The MDA analysis for testes was performed using the Espinosa Mansila’s method. This measurement is based on the amount of malondialdehyde estimated to have reacted with the trichloroacetic acid reagent using a spectrophotometer, based on the color absorption formed from the reaction between TBA and MDA.

2.4. Spermatogenesis
After treatment, animal surgery was performed to formulate testicular microanatomy preparations using the paraffin method. The fixative used is Normal Buffer Formalin, and staining was conducted with Hematoxylin Ehrlich - Eosin. The treatment processes include dehydration, clearing, infiltration, embedding, cutting, and staining. Therefore, the preparations were observed under a light microscope at 200 times magnification on the general structure of normal and changed networks. Also, histopathological data were analyzed descriptively. The observations were carried out towards the number of spermatogonia A, pachyten spermatocytes and spermatids, using a light microscope.

2.5. Caspase 3 Expression
The apoptosis of spermatogenic cells through protein caspase 3 expression was examined after preparations were formulated using immunohistochemical methods. These samples were then attached to a poly-L-lysine coated frosted slide after deparaffinization and hydration. Furthermore, retrieval antigen was applied in treatment, using boiling buffer citrate, endogenous blocking with 3% H2O2, background blocking with sniper, primary antibody using rabbit anticaspase 3, secondary antibody from Trekkie Universal Link, and TrekAvidin-HRP. Therefore, the tissue was visualized using DAB (Diaminobenzidin) chromogen dye, and Hematoxylin was applied as a counterstain. The examinations involved counting the number of spermatogenic cells estimated to have expressed caspase 3, including cells assumed to impart a positive reaction to DAB dye by showing a brown color. Subsequently, observations were made using a light microscope with 40 x magnification.

2.6 Statistical analysis
The experiment involved a completely randomized design (CRD), and the data obtained were analyzed by ANOVA. Therefore, the test was continued on instances where the treatment has a significant effect, using the Duncan difference test at the 5% level to determine the variations between treatments.

3. Results
Cigarette smoke is a free radical source characterized by the ability to form oxidative stress in the body. This was accompanied with increased MDA levels as a free radical marker in the testes, and interferes with spermatogenesis, therefore causing related disorders. Furthermore, the syndromes were analyzed based on differences in the number of spermatogenic cells, testes microanatomy and apoptosis expressed through the caspase 3 protein.

The statistical analysis showed significantly higher testicular MDA levels at p < 0.05 in the K1 and K3 groups compared to the K0 and K2. In addition, further test with Duncan affirmed no significant differences between K1 and K3, and between K0 and K2 (table 1). This indicated the ability for duwel fruit extract to significantly reduced endogenous free radicals in the testes, although this caused in the body by cigarette smoke was not significant compared to the control.

Table 1. Mean average number and apoptosis of Spermatogenic cells, MDA testes level of Rat norvegicus exposed to cigarette Smoke after administration of java plum fruit extracts

| Variable   | Treatment | F count | P value |
|------------|-----------|---------|---------|
|            | K0        | K1      | K2      | K3      |
| Spermatogonia | 74±12,1       | 71±8,3  | 70± 19,4 | 71±13,6 | 2,11 | 0,130 |
| Spermatocytes | 87±8,33a     | 80± 9,5b | 85±11,8a | 81± 8,2b | 3,50 | 0,033* |
| Spermatids    | 138±12a      | 134±16,7b | 139±11a | 137±22b | 1,40 | 0,026* |
| Apoptosis     | 25±0,5       | 27±0,3  | 24±0,3  | 27±0,41 | 2,83 | 0,065 |


Spermatogenic Cells

| MDA testes (μmol/g) | 1.17±1.7<sup>a</sup> | 4.14±0.6<sup>b</sup> | 1.73±0.37<sup>a</sup> | 4.18±1.60<sup>b</sup> | 71.70 | 0.005* |

Means with different superscripts letter (a, b) in each column are significantly different (p<0.05).

MDA was one of the free radical markers formed in the body as a result of lipid peroxidation. This process occurred in Poly-unsaturated fatty acid (PUFA) and cholesterol from cell membranes, and the intrinsic multiple bonds were susceptible to free radicals. The potential reaction is chained through the initiation stage, while propagation formed lipid peroxyl as well as lipid hydroperoxyl radicals [24]. In addition, high MDA levels in K1 were instigated by smoking as a source of exogenous free radicals in the body, implicated in the poor ability for the bodily antioxidants to maintain balance, therefore it initiated the formation of oxidative stress. However, these challenges are possibly alleviated using an external antioxidant source.

The low MDA levels in K2, compared to K1, resulted from the ability of duwet fruit extract to reduce the intensity of endogenous free radicals. Previous studies have shown the tendency for varieties containing bioinorganic compounds to act as free radical scavengers. Based on the ESR analysis results, duwet fruit extract prompted a reduction in DPPH free radical intensity[20]. The complex compounds present alongside transition metals serve as the central ion, while the ligand antioxidant compounds are the electron source for the free radicals. Furthermore, the transition metal as the atom center acts as an electron transfer regulator. Also, the radicals tend to release or gain electrons from the central ion of bioinorganic compounds where radical complex formation does not occur. Meanwhile, metal ions, including Fe<sup>2+</sup> and Fe<sup>3+</sup> only modify the magnetic properties of complex compounds from paramagnetic to diamagnetic after a lost or gain in electrons.

The duwet fruit extract significantly increased the quantity of spermatocytes in K2 and K0 groups. However, there was no substantial effect on rats exposed to cigarette smoke (K1 and K3), as the total number recorded had no significant increase. The groups exposed to both duwet fruit extract and cigarette smoke showed insignificant differences at p > 0.05 in relation to the number of spermatogonia and spermatids, as well as spermatogenic cell apoptosis.

Normal testicular microanatomy showed the layered arrangement of spermatogenic cells from the basement membrane to the lumen according to the development level. These were in the order spermatogonia A and B, Spermatocytes, round spermatids, elongated spermatids and full spermatozoa filling the lumen, as shown in K0. Furthermore, Sertoli cells extend into the lumen, and Leydig cells between the seminiferous tubules. However, the spermatogenic cell arrangement in the K1 group became looser than the other (Figure 1). This phenomenon occurred due to the reduction of spermatogenic cells in the inner membrane, especially from the spermatocyte layer. In addition, these components were assumed to be damaged, continued with degeneration and phagocytosis by the Sertoli cells. Moreover, the tubule lumen at stage VII contained spermatozoa lower than the quantity recorded in K0, due to reduced spermatocytes and spermiogenesis disorders. Figure 1 shows the cellular arrangement in K0 and K2, which appear denser than K1.
Figure 1. Seminiferous testicular tubules at K0, K1, K2, and K3 in level VII, with spermatogenic cell arranged from the basement membrane to the lumen. 1. Spermatogonia. 2 spermatocytes. 3 round spermatids. 4. Elongated spermatid. 5 spermatozoa in the lumen 6. Sertoli cells.

The lowest apoptosis percentage of spermatogenic cells through caspase 3 expression in the seminiferous testes was observed in the group treated with duvet fruit extract, while those exposed to cigarette smoke had the highest value, as shown in Table 1. Specifically, the total number in the K1 group reportedly increased, while the elevation in value for K2 group was small, and the difference was not significant at p > 0.05 (Table 1).

In addition, the K0 group demonstrated a positive reaction towards DAB dye, as shown by a brown color expression of caspase 3, while a negative reaction (not expressing caspase 3) was observed on spermatogonia, spermatocytes, spermatids, and spermatozoa. Moreover, the inverse was reported with K1 group for both parameters. Figure 2 showed both positive and negative reactions of K2 and K3 to spermatogonia, spermatocytes, spermatids, and spermatozoa.

Based on this findings, free radicals and oxidative stress induced by cigarette smoke instigates an increase in apoptosis as observed with spermatogenic cells, particularly the spermatocytes, spermatids, and spermatozoa. However, spermatogonia are more resistant to free radicals, resulting from the relatively higher dominance of ZnSOD compared to others [25]. This is congruent with the outcome of a study by Everitt, which acknowledged the greater resistance to external disturbances. In contrast, spermatocytes tend to be very susceptible to free radicals, consequently leading to a significant decline in the total number, as well as apoptosis in spermatogenesis.
Figure 2. Testes with Caspase Expression 3. Positive reactions (brown) indicate apoptosis of spermatogenic cells. A negative reaction (blue) indicates normal cells. K0: Animals without treatment. K1: Animals treated with cigarette smoke. K2: Animal administered *duvet* fruit extract. K3: Animals treated with cigarette smoke and *duvet* fruit extract.

The testes is known to contain a PUFA membrane on carbon atom No. 20 with 4 double bonds (20: 4) and No. 22 with 6 double bonds (20: 6) [26]. This component is susceptible to free radicals, hence the formation of H₂O₂ and others through lipid peroxidation [27], subsequently leading to the development of chain radicals. Furthermore, the products have a tendency to interfere with the spermatogenesis process involving the function of Leydig and Sertoli cells, FSH and LH hormones, as well as gene transcription [26]. Moreover, spermatocytes are also known to be very susceptible towards external disorders with the capacity to instigate apoptosis [28]. The FSH plays an important regulatory role towards Sertoli cell functionality and the initiation of spermatogenesis, especially in spermatogonia division and differentiation into spermatocytes. Therefore, the free radicals present in Leydig cells have the ability to stimulate testosterone synthesis, but exposure to oxidative stress increase DNA oxidative modification [29,30] and decrease the hormone testosterone synthesis [31]. Research conducted by [32] showed a decline in the activity of enzymes catalase, SOD, and glutathione, and an increase in MDA levels, after rat exposure to cigarette smoke every day for 42 days.

This treatment is a source of free radicals with the potential to form oxidative stress, instigate lipid peroxidation processes, and consequently damage the cell membranes. Furthermore, the destruction of integrity leads to DNA damage, apoptosis, and motility disorders of spermatozoa (Zanocka and Kurpisz 2004). Also, ROS has been implicated in the potential mutilation of inner and outer mitochondrial membrane. This prompts the release of cytochrome c from the mitochondria, and induction of DNA damage [3]. Moreover, there are two types of DNA, including the nDNA and mtDNA identified in the nucleus and mitochondria, respectively. The possible destruction
correspondingly interferes with the division process in spermatogenic cells and respiratory chain associated with energy source for spermatozoa.

Generally, apoptosis is characterized by a decline in cell size, blebbing of the membrane, condensation of chromatin and fragmentation of nucleus. In addition, a total of 2 apoptotic pathways have been established, including the extrinsic and extrinsic paths through cell surface receptors and mitochondria, respectively [33]. Specifically, the energy possessed by cells in the intrinsic pathway is used to accumulate Ca**++ in the mitochondria. The presence of these ions instigates the formation of reactive oxygen, which simultaneously opens the mitochondrial membrane pores after a combination with Bax protein. Subsequently, the mitochondria demonstrate blebbing activities, and further releases one of the cytochrome-c intermembrane proteins into the cytosol. This process is controlled by Bel 2, while the released cytochrome C binds to Apaf-1 (Apoptotic protease activation factor-1) and CARD (Caspase recruitment domain). Furthermore, the oligomer from Apaf-1 attaches to procaspase-9 in the cytosol to form apoptosomes (Caspase-9 activation complex). Therefore, Caspase-9 triggers the catalytic maturation of procaspase -3, which consequently terminates the cascade, and facilitates apoptosis [33].

The spermatogenesis balance in the body is hindered by the disruption in antioxidant defence mechanisms. This phenomenon is possibly managed by administering exogenous antioxidants, commonly sourced from duwet fruit. This product contains various forms of antioxidants from the flavonoid class, comprising quercetin, tannins, catechins, and anthocyanins. In addition, LCMS analysis ascertained the presence of cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. These anthocyanins are one of the aromatic ring compounds with easy structural characteristics required to chelate transition metals, especially from the catechol and gallol groups, known to form complex or bioinorganic compounds.

Also, duwet fruit extracts are predicted to contain one of the complex compounds, comprising Fe**2+ and 3 Cyanidin 3,5, -O diglucoside. Jabeen et al [34] reported on the relatively stronger antioxidant activity of metal complexes compared to single compounds. In addition, anthocyanin is considered to confer antioxidant activities due to the presence of hydroxyl group on one of the intrinsic rings. The electrons present serve as an source for free radicals, through the mechanisms of atomic donors, electron transfer, and metal chelates [35]. Furthermore, anthocyanin is able to form more stable flavonoid as well as phenoxy radicals, which specifically delocalizes unpaired electrons from the double bond C2-C3 ring B of aromatic compounds to form non-reactive species. This phenomenon occurs through a radical termination reaction.

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