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# Study on the protective effect of melatonin against testicular dysfunction and DNA fragmentation in Etoposide-treated mice

**Ojo Olajumoke Omolara, Odesanmi Elijah Olalekan, Sanni Jamiu Folorunsho**
Department of Biochemistry, Ekiti- State University Ado-Ekiti, Nigeria.
*Corresponding author email: *olajumoke.ojo@eksu.edu.ng*

## Abstract

Etoposide (ETP)) compound is an antitumor and antimicrobial agent, however, different reports have shown its adverse effect on male reproductive system. Therefore, this work was designed to investigate protective effect of melatonin against etoposide-induced testicular tissue damage using mouse model. In this study seventy-five Swiss albino mice were randomly selected into 3 groups (n=10). Group 1 served as control while II, III and IV groups received 5mg/kg,10mg/kg and 20mg/kg of etoposide while Group V received (20mg/kg/bwt ETP+ 5mg of Melatonin). Three animals from each group were sacrificed at intervals of 6hr,12hr and 24hr after the treatment. Effect on testosterone (T) and luteinizing hormone (LH) and role of apoptosis was estimated were estimated by enzyme immunosorbent assay (ELISA). Biochemical assays and sperm parameters were also measured. It was observed that etoposide leads to depletion of the glutathione level and other antioxidants measured in a dose-dependent manner with simultaneous increase in malonialdehyde level. Etoposide leads to significant decrease in the testicular testosterone level. This study gives insight on the adverse effects of etoposide compound on male reproductive system and possible protective effect of melatonin. This study recommend that safe dose of etoposide should be put into consideration before its administration and also gives insight on the use of melatonin supplement during the treatment regime.

**Keywords:** Etoposide; Oxidative Stress; DNA Damage; Melatonin; Apoptosis

## Introduction

Chemotherapy is an aggressive form of chemical drug therapy meant to destroy rapidly growing cells in the body. It is usually used in the treatment of cancer cells which grow and divide faster than other cells. Etoposide, is one of the chemotherapy medication used in the treatments of different types of cancer including testicular cancer, lung cancer, lymphoma and ovarian cancer. Etoposide forms a ternary complex with DNA and the topoisomerase II enzyme, which is an enzyme that aids in relaxing negative or positive supercoils in DNA [1]. Topoisomerase II

normally will form a double-stranded break in one DNA double-strand, allow another to pass through, and re-ligate the broken strands. Etoposide's binding prevents topoisomerase II from re-ligating the broken DNA strands, which causes the DNA breaks made by topoisomerase II to stay broken, and also prevents the topoisomerase II molecule from leaving the site and relieving tension elsewhere. This results in a double-strand break in the DNA that can have various deleterious effects on the cell, and depletion of topoisomerase II available to relieve further tension [1]. Cancer cells rely on this enzyme more than healthy cells, since they divide more rapidly. Therefore, this causes errors in DNA synthesis and promotes apoptosis of the cancer cell [2]. Also reports have shown, decrease in the number of spermatozoa and in their motility, with simultaneous increase in morphologically abnormal spermatozoa following chemotherapy treatment [3]. Several chemotherapeutic drugs have been found to be cytotoxic and also responsible for the major male reproductive organs dysfunction following the treatment. Therefore this study focused on evaluating the induction of oxidative stress and apoptosis following exposure of etoposide compound in testicular tissue and also investigate possible protective effect of melatonin.

## Materials and Methods

### Ethical Approval and experimental procedure

The use of animals in this studies were conducted in accordance with the standards and permission established by The Ethics Committee of Animal, Ekiti State University Ado-Ekiti, Nigeria. Male Swiss-albino mice were housed in room at 22 ± 2°C with 40% relative humidity and with a 12-hrs light ± dark cycle. They were fed with a standard rat chow and tap water ad libitum.

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### Dose grouping

Male mice were divided into five groups of fifteen animals each and treated intraperitoneally as following:

Group1 (Control),
Group1I (5mg/kg/bwt ETP)
Group1II (10mg/kg/bwt ETP),
Group IV (20mg/kg/bwt ETP) and
Group V (20mg/kg/bwt ETP+ 5mg of MT).
The animals were autopsied at 6hrs, 12hrs and 24hrs.

### Tissues samples for biochemical studies

At autopsy, one part of testis was kept in tube and immediately stored in -20$^0$C. Other parts of the tissues were fixed in 10% formal saline for microscopy analysis.

### Testicular Testosterone (T) and Luteinizing hormone (LH) concentrations

The testicular testosterone and luteinizing hormone levels in three mice from each group were measured. Briefly, testicular proteins were extracted with phosphate buffer (50 mM, pH 7.4) and centrifuged at 10,000 g for 20 min. The supernatant was used to estimate T and LH levels using ELISA, and were expressed in ng/ml [4].

### Biochemical Analysis

Testis homogenate (10%, w/v) was prepared in chilled 100 Mm Tris-HCL buffer (pH 7.4) and the homogenate was used to measure GSH, CAT, and SOD activities by using spectrophotometry method. Protein content in the samples was estimated, according to Lowry et al. All the parameters were expressed per mg protein [6,7,8,9]

## Measurement of reactive oxygen species (ROS) level

The ROS assay was performed. In brief, 50 µl of testicular tissue homogenate and 1400 µl sodiu m acetate buffer were transferred to a cuvette. After then, 1000 ul of reagent mixture (N,N-diethyl paraphenylenediamine 6 mg/ml with 4.37 µM of ferrous sulfate dissolved in 0.1M sodium acetate buffer pH- 4.8) was added at 37°C for 5 minutes. The absorbance was measured at 505 nm using spectrophotometer (10).

## Measurement of MDA

Testes tissues were homogenized in 10 ml TCA (trichloroacetic acid) which is at the rate of 10%, and then centrifuged at +4°C for 15 minutes. 750 µl of the supernatant which was obtained was mixed with 0.67% TBA (thiobarbituric acid) in a ratio of 1:1. Afterwards, the solution was left in the water bath for 15 minutes. Finally, the absorbance was measured spectrophotometrically at 535 nm.

## Sperm Parameters

Caudal epididymidis was removed from each mouse and cleaned off from the epididymal fat pad, and minced in a pre-warmed Petri dish containing 500µl phosphate buffer saline solutions (PBS, pH 7.4) at 37$^{o}$C. Sperm motility was estimated and expressed as percentage incidence [11]. For sperm count, an aliquot of this suspension was charged into the Neubauer's counting chamber and the spermatozoa were counted under light microscope. Total sperm count was calculated as the average of the spermatozoa count (N) in each chamber X multiplication factor (106) X dilution factor and was expressed in millions/ml. The sperm morphology was also evaluated [6]. Briefly, a smear of sperm was made on a clean slide and stained with haematoxylin and eosin and were examined under a light microscope with an oil immersion lens. The morphology of spermatozoa was also scored [12,13]

## Effect on CASP-3 and CASP-9 Activities

Spermatogenic cells were used for the detection of CASP- 3 and 9 activities by using colorimetric assay kits according to the manufacturer's protocol. Briefly, spermatogenic cells were washed with 1X PBS buffer 2 times at 500 x g for 5 minutes the pellet was dissolved in the extraction buffered and processed for activity assay [8].

## Estimation of Bcl-2 and Bax by ELISAs

Bcl-2 and Bax mitochondrial level was measured by ELISAs. Briefly, 1g of mitochondrial protein in PBS was sealed overnight at 4°C. Bcl-2 and Bax peptide standards were included with a concentration range from 1 g/ml to 4 ng/ml. The plates were washed with buffer containing PBS with 0.02% sodium azide and 0.05% Tween-20. The wells were blocked with 300 l of 1% BSA in PBS with 0.02% sodium azide and incubated at room temperature for 60 min. After washing of samples four more times, primary antibody at a concentration of 5 g/ml diluted in 1% BSA in PBS/azide was added to each well, and the plate was incubated for 60 min at room temperature. Each well was washed four times before the addition of the secondary antibody. Secondary diluted 1:2000 in 1% BSA in PBS/azide was then added to each well, and the plate was incubated again for 60 min at room temperature. The washing procedure was then repeated. Substrate containing paranitrophenyl phosphate at a concentration of 1 mg/ml in substrate buffer [carbonate-bicarbonate (pH 9.6)] was added. The plate was then incubated at room temperature for 60 min, after which, absorbance at 405 nm was read [14,15].

## Statistical analysis

All statistical comparisons between the groups were made using analysis of variance (ANOVA) by Prism statistics software. Results were presented as mean ± S.E.M (Standard Error Mean). Values of $p<0.05$, $p<0.01$, $p<0.001$ were considered as statistically significant.

## Results

### Effect of Testosterone levels on mice induced with etoposides and potential attenuative effect of Melatonin.

The study shows the Testosterone levels in testes of etoposides-induced mice. The mean percentage of testosterone concentration in etoposide -treated groups (Grp II to Grp IV) significantly ($p<0.05$) decreased which is proportional to the weight and dosage of the etoposide compared with the control. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the level of Testosterone improves compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin as seen in table 1 below:

**Table 1**: Testosterone level in Etoposide-induced mice and ameliorative effect of Melatonin.

| Dose Groups | 6hrs | 12hrs | 24hrs |
|---|---|---|---|
| Group I(Control) | 8.563±0.24 | 8.453±0.09 | 7.975±0.12 |
| Group II(5mg/kg.bwt ETP) | 7.231±0.07*[a] | 6.234±0.02*[a] | 5.897±0.05*[a] |
| Group III(10mg/kg.bwt ETP) | 5.545±0.03*[a] | 4.657±0.06*[a] | 3.768±0.03**[a] |
| Group IV(20mg/kg.bwt ETP) | 4.124±0.01**[a] | 3.456±0.05**[a] | 2.466±0.07**[a] |
| GroupV(20mg/kg.bwt ETP+5mg/kg.bwt MT) | 6.752±0.02*[ab] | 5.789±0.08*[ab] | 5.003±0.03*[ab] |

**Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively.'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

### Effect on Luteinizing Hormones level

The study shows the Luteinizing Hormone level in testes of etoposides induced mice. According to Table 1, the mean percentage of luteinizing hormone concentration in etoposide- groups (Grp II to Grp IV) significantly ($p<0.05$) increased which is proportional to the weight and dosage of the etoposide compared with the control. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the level of Luteinizing Hormone decreased compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin as shown in the table below:

**Table 2: Luteinizing hormone level in Etoposide-induced mice and ameliorative effect of Melatonin.**

| Dose Groups | 6hrs | 12hrs | 24hrs |
|---|---|---|---|
| Group I(Control) | 4.091±0.01 | 4.132±0.09 | 4.456±0.12 |
| Group II(5mg/kg.bwt ETP) | 6.052±0.03*[a] | 7.566±0.07*[a] | 6.464±0.02*[a] |
| Group III(10mg/kg.bwt ETP) | 9.045±0.08*[a] | 10.867±0.02*[a] | 11.676±0.13*[a] |
| Group IV(20mg/kg.bwt ETP) | 12.897±0.12**[a] | 14.076±0.12**[a] | 14.985±0.21**[a] |
| Group V (20mg/kg.bwt ETP+5mg/kg.bwt MET) | 6.987±0.22*[ab] | 0.777±0.14**[ab] | 0.653±0.04**[ab] |

**Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone

## Relative Oxygen Species (ROS) Activity

The relative oxygen species activity was increased across the concentration and time when treated with etoposide in comparison with the vehicle treated control.

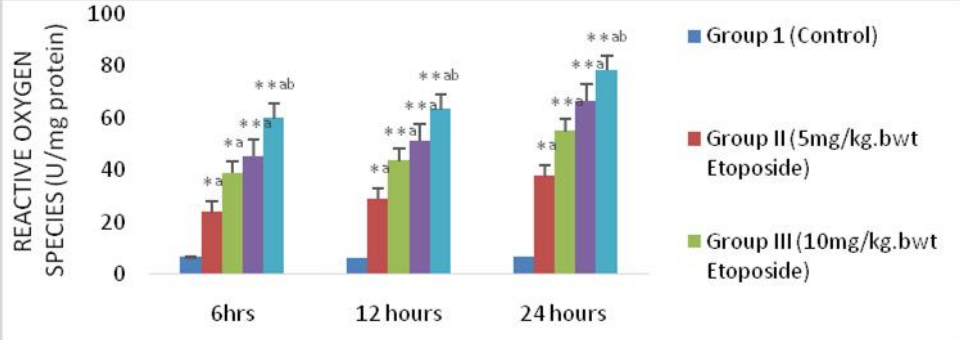


**Figure 1: Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

## Malondialdehyde Activity

The Malondialdehyde activity was increased when treated with etoposide across the concentration and time in comparison with the vehicle treated control.

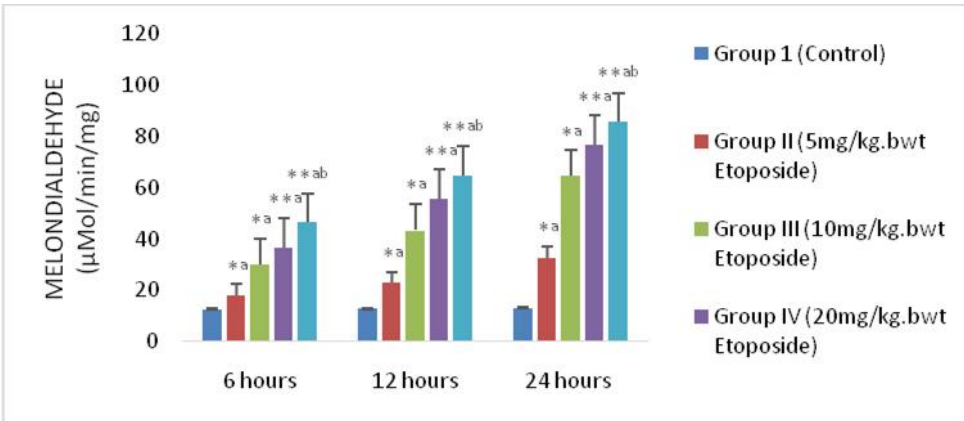


**Figure 2:** The effect of on Malondialdehyde (MDA) level. **Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone

## Antioxidant status

### Superoxide Dismutase Activity

The level of superoxide dismutase decreased drastically when treated with etoposide across the concentration and time in comparison with the control group. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of superoxide improve compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin Fig 3.

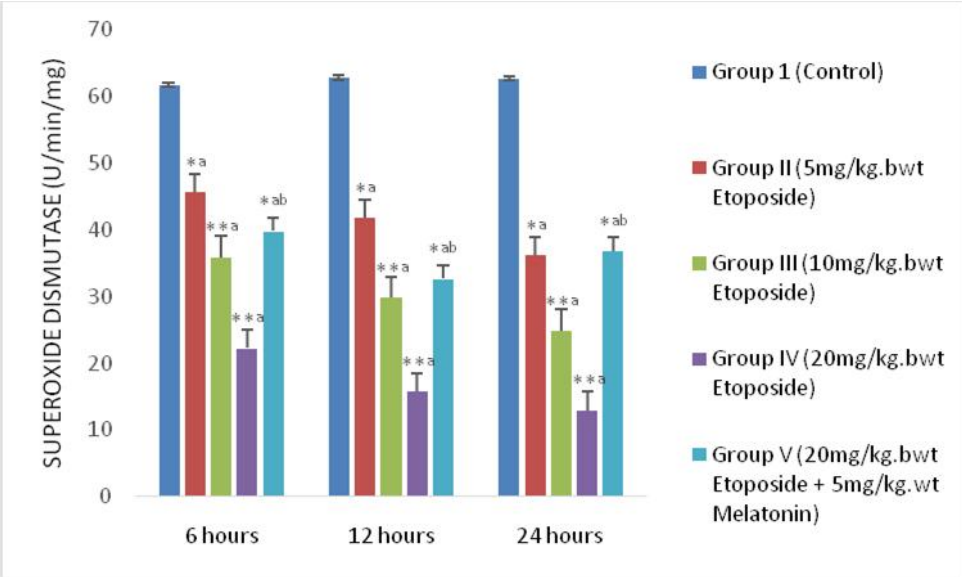


**Figure 3**: The effect on Superoxide Dismutase (SOD) level. **Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone

### Catalase Activity

The level of catalase decreased significantly when treated with etoposide across the concentration and time in comparison with the control group. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of catalase improve compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin which shows recovery in catalase activity in the testes of the mice.

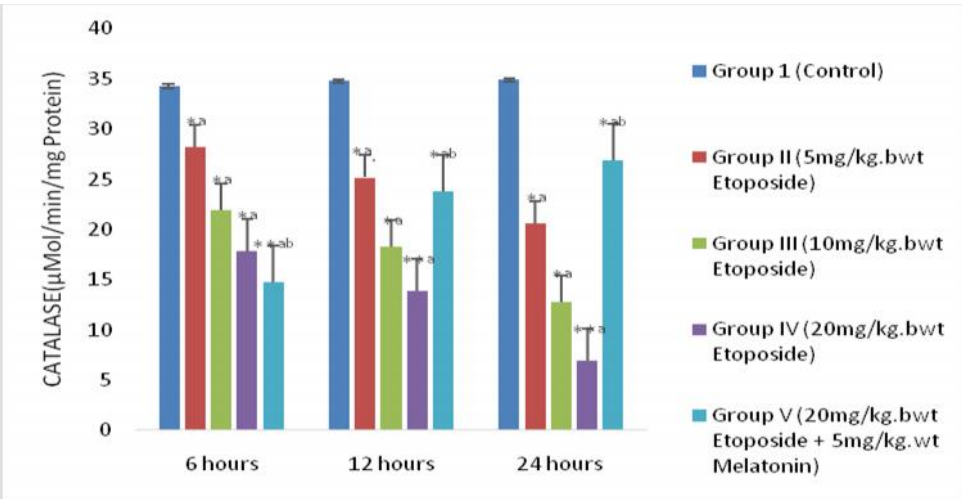


**Fig 4:** The effect on Catalase (CAT) level. **Note:**All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

## Glutathione Activity

The level of Glutathione decreased drastically when treated with etoposide across the concentration and time in comparison with the control group. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of Glutathione improve compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin as shown in Fig 5.

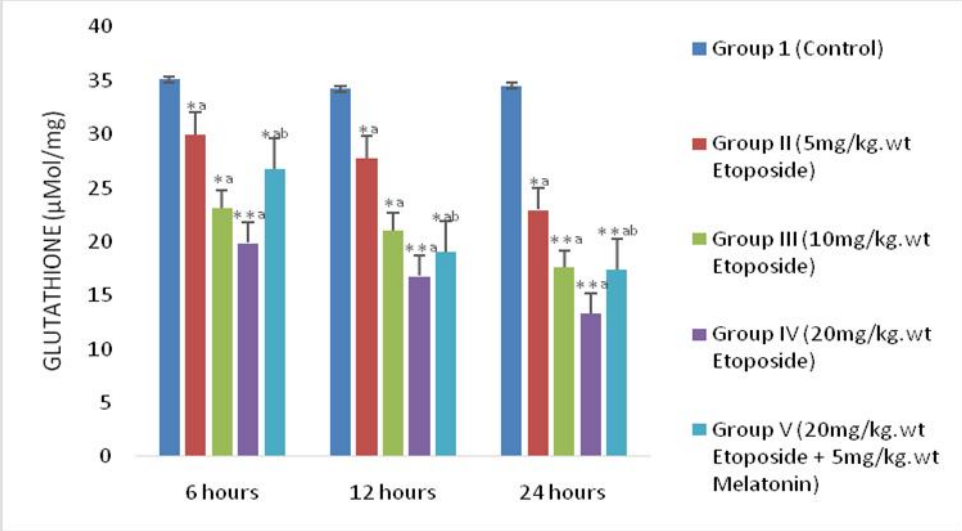


**Fig 5**: The effect on Glutathione (GSH) level. **Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone

## Cell Viability Activity

The spermatozoa viability numbers decreased drastically when treated with etoposide in comparison with the control group. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of cell viability activity improve compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin as shown in Fig 6.

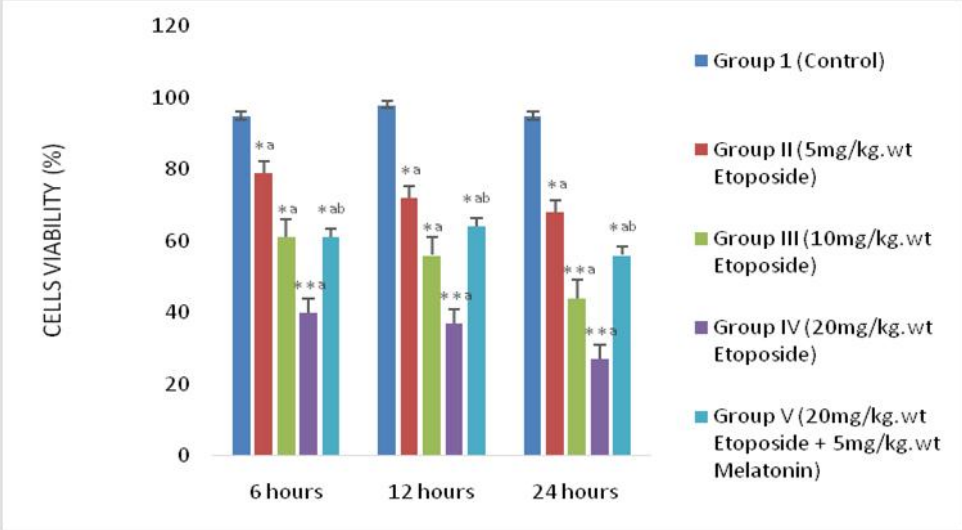


**Figure 6**: The effect on Cell viability. **Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

## BAX Activity

The BCL-2 Protein activity was increased when treated with etoposide in comparison with the vehicle treated control. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of BAX reduces compared to Group IV (20mg/kg.bwtEtoposide) due to the addition of 5mg/kg.bwt melatonin as shown in figure 7.

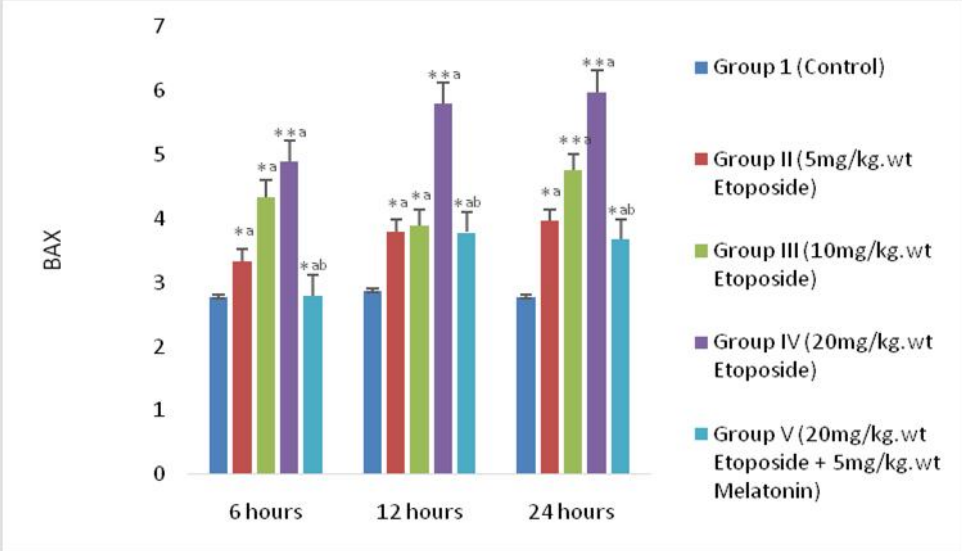


**Figure 7:** The effect on the BCL-2 Protein activity. **Note:** All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

## BCL- 2 Activity

The level of BCL- 2decreased drastically when treated with etoposide in comparison with the control group. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of BCL-2 improve compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin as shown in Fig. 8.

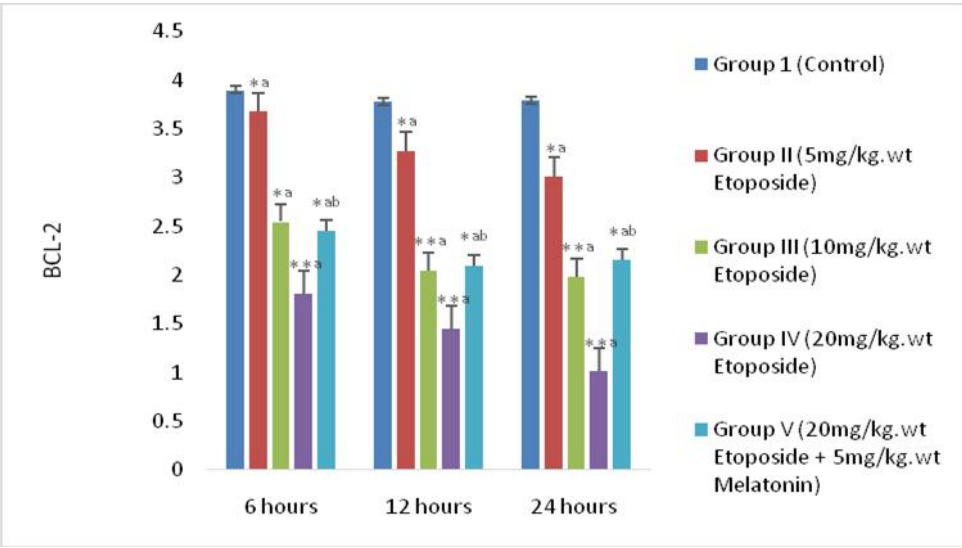


**Figure 8:** The effect of on BCL- 2 activity**. Note:**All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

### Sperm DNA Damage Comet Assay Activity

The Sperm DNA Damage Comet Assay activity was increased when treated with etoposide in comparison with the vehicle treated control. However, in Group V (20mg/kg.bwt Etoposide + 5mg/kg.bwt melatonin), the activity of Sperm DNA Damage Comet Assay improve compared to Group IV (20mg/kg.bwt Etoposide) due to the addition of 5mg/kg.bwt melatonin as shown in figure 9 below.

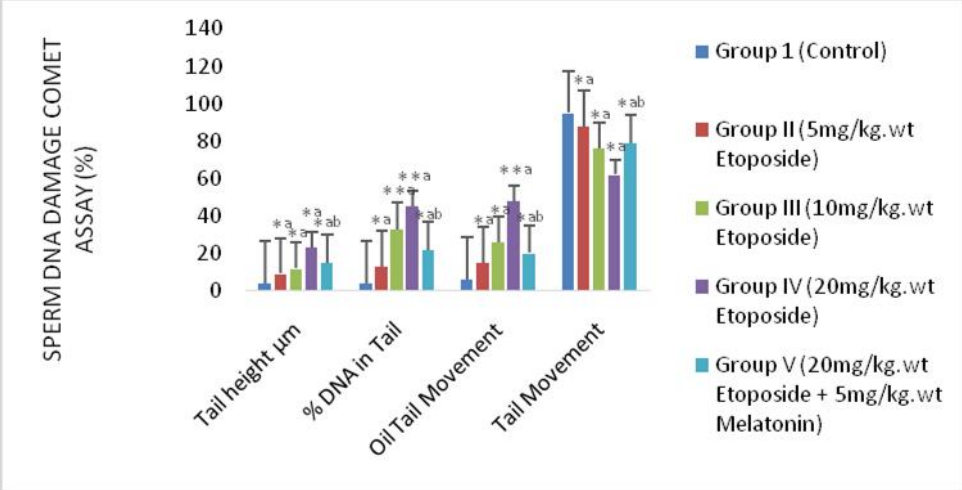


**Figure 9:** The effect on Sperm DNA Damage Comet Assay level. **Note:**All data are expressed as mean ± standard error mean (SEM), (n=5). * and ** indicates significant differences as compared with controls at ($p<0.01$) and ($p<0.05$) respectively. 'a'- ETP –treated groups vs. control and 'b' ETP + MT vs. 20mg/kg of ETP alone.

## Discussion

The clinical use of Etoposide as an anticancer drug for a countless number of human malignancies, has been a source of concern due to its toxic effects, one of which is male reproductive toxicity. Present study investigates the induction of oxidative stress and apoptosis following etoposide treatment and possible ameliorative effect of melatonin. Analysis of etymological and biochemical profile of organs are widely used as indicators to access the functional status of the animal health and the internal environment of the organism [16]. Enzymes such as superoxide dismutase, glutathione, catalase etc and bio-molecule such as Malondialdehyde served as good bio-indicators. In this induction of oxidative stress and apoptosis was confirmed by the depletion of the antioxidants enzymes measured. The group co-treated with melatonin shows signs of recovery.

Also, there was significant reduction in the concentration of testosterone in the testis of the etoposide treated mice. Since testosterone is essential in the regulation of accessory sex organ functions, epididymal sperm maturation, sexual activities and spermatogenesis [17] and drugs implicated in causing toxicity to the testes and epididymis either by inhibiting biosynthesis of testosterone or its secretion thus having a profound effect on the processes required for the timely deposition of viable spermatozoa into the reproductive tract of the female [18, 19]. Luteinizing hormone level is significantly increased in the testis of the Etoposide-treated mice compared to the vehicle control, Etoposide initiate primary hypogonadism (hypergonadotropichypogonadism) whereby pituitary produces high level of LH and FSH to try and stimulate the testes to produce more testosterone. However, as the testes are impaired, they're unable to respond to the increased levels

of gonadotropin and little or no testosterone are produced leading to decreased level of testosterone in the testis of the mice administered with Etoposide in a dose dependent manner.

Generation of Reactive oxygen species (ROS) in the testes which is the major evidence of increased oxidative stress, itself impairs sperm function by causings damage to the male genital tract leading to the infertility. Relative oxygen species can be from the mitochondria and a variety of enzymes including the NADPH-oxidases, and the cytochrome P450s. These enzymes specialize in the professional generation of ROS or produce these toxic metabolites as an inadvertent consequence of their biochemical activity. In order to address this risk, the testes have developed a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents. Concerning the enzymatic constituents of this defense system, the induction of oxidative stress in the testes precipitates a response characterized by the NFκB mediated induction of mRNA species for superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities.

This current study showed signs of oxidative stress as shown by increased MDA activity in the epididymis of the etoposide -treated mice, the drug-treated mice also showed a defective antioxidant response as evident from diminished activity of antioxidant enzymes such as CAT, SOD, and GSH. Similar reports have been likewise made available [20]. Oxidative stress is a common pathology that has been implicated in male infertility. Induction of oxidative stress can be as result of increasing the free radical generation in testis and epididymis resulting in degeneration of spermatogenesis and ultimately infertility [21].

In addition to this, decrease in sperm count was observed in the caudaepididymidis of etoposide treated mice indicating cytotoxicity of etoposidein the epididymis of the mice .The effect of etoposideon the early germ cells in the spermatogenic cycle led to its abnormality, thereby increasing the normal of abnormal spermatozoa. Etoposide treatment resulted in an adverse effect on spermatozoa function, significant decrease of sperm motility was observed. Etoposide treatment resulted in the distortion of the normal sperm morphology, significant amount of abnormal spermatozoa was observed in etoposide -treated mice. Oxidative stress in the epididymis might be the reason for this effect on the sperm parameters of the etoposide treated mice. As stated above, melatonin is a potent anti-oxidant; it was observed that there was no significant effect on sperm parameters in the melatonin treated mice when compared to the vehicle-control. Oxidative stress has been suspected to be the major reason responsible for the decrease in sperm count, impaired sperm motility and abnormal spermatozoa. The induction of oxidative stress and its effect was found to be prevented by melatonin treatment.

In the present study, caspase-9, caspase-3 and a downstream effector of the Bcl-2-mediated mitochondrial apoptosis pathway were measured, indicating an increase in apoptotic activity in testicular tissue. Bcl-2 is a multidomain, pro-survival protein that regulates apoptosis by preventing the release of proapoptogenic factors from the mitochondria (e.g., cytochrome c) and subsequent caspase activation. It was observed that there was a significant decrease in the activities of caspase 3 and caspase 9 and significantly reduced level of the anti-apoptic protein Bcl-2. Apoptosis is mediated by the activities of caspase 3 and 9. Apoptosis also known as programmed cell death (PCD), is important for normal spermatogenesis in mammals, maintaining cellular homeostasis, and ensuring an adequate number of germ cells are eliminated in order to maintain a precise germ cell population in compliance with the supportive ability of the sertoli cells. It could be assumed that the induction of oxidative stress led to the reduced activities of caspase 3. It could likewise be assumed that this decreased activities could be responsible for infertility since they affect the overall process of spermatogenesis. Similarly,

reports have been made available on the effect of apoptosis on the male reproductive system [22].

Studies have shown that the mechanisms induced in germ cells in response to oxidative damage varies with age, that DNA repair efficiency declines, and both sperm DNA damage and spontaneous mutations increase. However, it is not known whether the altered response with age is a cause, or consequence, of an age-associated change in cell susceptibility to genetic damage. Conclusively the present study demonstrates the cytotoxicity effect of Etoposide on Swiss Albino mice. Oxidative stress was induced by Etoposide treatment as confirmed by the simultaneous significant decrease in SOD, GSH and CAT levels and increase in MDA and ROS level and Sperm DNA damage comet Assay level. Apoptosis is mediated by the activities of caspase 3 and 9. Decrease in their activities can induce apoptosis. The observed reduced Spermatozoal count might be due to the administration of this compound. A strong anti-oxidant should be used alongside during administration. Hence, this study gives insight on the use of melatonin to alleviate the harzardious effect of etoposode and also recommend further study to fully understand the mechanism involved.

## Acknowledgments

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