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Research Article

Adverse Effect of Dactinomycin on Reactive Oxygen Species and Important Steriodogenic Enzymes in Male Mice

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Abstract

Dactinomycin, known as actinomycin D, is a chemotherapy medication used in the treatment of Wilms tumor, rhabdomyosarcoma, Ewing's sarcoma, trophoblastic neoplasm, testicular cancer and certain types of ovarian cancer. Therefore, current study was carried out to investigate induction of the reactive oxygen species (ROS), apoptosis and alteration of some steroidogenic enzymes following Dactinomycin treatment. In this study, Swiss albino male mice weighing 25g were divided into four groups I, II, III and IV (n = 5). Group I served as control and received vehicle, group II (1 mg/kg/bwt Dactinomycin), group III (3 mg/kg/bwt Dactinomycin) and Group IV (5 mg/kg/bwt Dactinomycin) via intraperitoneal for 21 days daily. Effects LH, FSH and Testosterone hormone levels were evaluated by ELISA. The role of apoptosis was assessed by evaluating CASP-3 and CASP-9 activities. The expressions of steroidogenic acute regulatory (StAR), Cholesterol side-chain cleavage enzyme (CYP450scc), 17β-hydroxylase (P450c17), 17β-Hydroxysteroid dehydrogenase1 (17βHSD1) and 3β-hydroxysteroid dehydrogenase (3βHSD) and their mRNA levels were detected by Western blot and real-time polymerase chain reaction (real-time PCR) respectively. The results showed that treatment with Dactinomycin results in the generation of reactive oxygen species and induction of apoptosis leading to the alteration of Steroidogenic enzymes resulting in male reproductive dysfunction of mice. Also, Significant decrease of proteins expression and simultaneous decrease in their mRNA expressions as compared with the control group suggests that Dactinomycin may have an induction effect on protein expressions and enzyme activities, such effect might be related to the posttranscriptional regulation of its protein expression. This study confirmed generation of reactive oxygen species leading to the downregulation of vital enzymes in steroidogenesis, other drug along with dactinomycin should be considered, this will enhance its fast and easy metabolism and elimination from the system.

Keywords: *Dactinomycin; Reactive Oxygen Species; Apoptosis; Hormones; Steroidogenesis; Testes*

Introduction

Chemotherapy involves one or several antineoplastic drugs which inhibit cancer cells. Dactinomycin is an example of an anticancer drug that interferes with cellular reproduction and is used in the treatment of certain inflammatory disease. Dactinomycin is the first antibiotic used for treating cancer; these cancers include Wilms tumor, rhaddomyosarcoma, Ewing's sarcoma, testicular cancer and certain types of ovarian cancer [1-3]. Dactinomycin has been reported to intercalate into DNA, preventing the progression of RNA polymerases [1,4]. Dactinomycin inhibits RNA synthesis by binding to guanine residues and inhibiting DNA-dependent RNA polymerase [5-7]. Dactino-

mycin has been reported to be a known DNA-interacting transcription blocker with an anti-cancer activity [5,8-9], working as a cytotoxic inducer of apoptosis against tumor cells [5,7]. Dactinomycin inhibits transcription by binding DNA at the transcription initiation complex and preventing elongation of RNA chain by RNA polymerase [10]. Dactinomycin also interferes with DNA replication and thus protein synthesis, although other chemicals such as hydroxyurea are better suited for use in the laboratory as inhibitors of DNA synthesis. The affinity of these stains/compounds for GC-rich regions of DNA strands makes them excellent markers for DNA. 7-AAD binds to single stranded DNA; therefore it is a useful tool in determining apoptosis and distinguishing between dead cells and lives ones [11]. Because of the importance of this drug, this study was therefore designed to investigate the adverse effect of Dactinomycin on hormones and ROS, its role in apoptosis and also to check if there is any alteration in the levels of Steroidogenic regulatory enzymes using mouse model.

Materials and Methods

Animals grouping and treatments

Specific pathogen-free, healthy young adult male mice of 10 to 12 weeks were used in this study. Male mice weighing about 20g were obtained from animal house of College of Medicine, University of Ibadan. The animals were maintained under standard conditions of humidity (50 +/- 5%), temperature (25 +/- 2°C) and dark and light cycles (12h each) with free access to food and water.

Male mice were divided into four groups (n = 5) and treated daily with single dose of Dactinomycin for 21 days via intraperitoneal route as follows; Group I: Control; Group II: 1 mg/kg; Group III: 3 mg/kg and Group IV: 5 mg/kg. Animals were sacrificed after the treatment.

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 10,000g for 20 minutes. The supernatant was used to estimate T and LH levels using ELISA, and were expressed in mg/ml.

Measurement of reactive oxygen species (ROS) level

The ROS assay was performed by the method of Hayashi., *et al.* (2017). In brief, 50 µl of testicular tissue homogenate and 1400 µl sodium 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 (Molecular Devices.) ROS levels from the tissue were calculated from a calibration of H_2O_2 and expressed as U/mg of protein (1 unit = 1.0 mg H_2O_2/L).

CASP- 3 and CASP- 9 activities

Caspase 3 and Caspase 9 activities were estimated by using manufacturer protocol. Briefly, the assay was based on monitoring of DEVD-p- nitroaniline (pNA) through time. The temperature was maintained at 37ºC and the kinetics of the reaction were followed for 3 hr by measuring absorbance at 405 nm every minute on each well using the plate reader.

RNA extraction and real-time PCR

Total RNA was extracted from testis samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA were done using standardization of cDNA amplification condition and optimization of annealing temperature for primer use. Primers for PCR amplification were as follows: for STARD1up: 5'-GGACCTTGTAGAACCTCTTTGC-3'; STARD1dn: 3'-TCATCTGAGTCGTCACCCTTT-5'; CYP11A1up: 5'-GGATGCGTCGATACTCTTCTCA-3'up; CYP11A1dn: 3'-GGACGATTCGGTCTTTCTTCCA-5'; CYPc17up: 5'-AGTCAAAGACACCTAATGCCAAG-3dn; CYPc17dn: 5'-ACGTCTGGGGAGAAACGG-3'; 17βHSD1up: 5'-ACATCTCTGATAACACCCCTCA-3'; 17βHSD1dn: 5'TGGTAAACACGCTATTTAGCACT3'; βHSDup: 5'GGATGCGTCGATACTCTTCTCA-3'; 3βHSDdn: 5'-GGACGATTCGGTCTTTCTTCCA-3'. As a control, β-actin was amplified with primers: β-actinup: 5'-TGCGTGACATCAAGGAGAAG-3'; β-actindn: 5'-TGCCAGGGTACATTGTGGTA-3'. In brief, 10 ng of

total RNA were used to perform fluorescent-based real-time PCR quantification. The conditions for the PCR reactions were: pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 31 seconds for 45 cycles. The SYBR Green fluorescence was collected at the end of each cycle.

Expression of StAR, CYP450scc, CYP450c17, 17βHSD1 and 3βHSD proteins by western blotting

Total proteins were isolated from testes of mice using urea lysis buffer. 50 mg of total protein from each sample was separated on a 15% SDS-PAGE and transferred on to nitrocellulose membrane for immunodetection by a semi-dry electro-blotting apparatus (GE Health, UK). The membrane was blocked in 5% non-fat dry milk in phosphate buffered saline tween-20 (PBST) for 1 h at room temperature and incubated with primary antibodies of StAR, CYP450scc, CYP450c17, 17βHSD1 and 3βHSD at a dilution of 1:1000 in 2.5% non-fat dry milk in PBST overnight, followed by another incubation of horseradish peroxidase- conjugated goat anti rabbit secondary antibody at a dilution of 1:10,000. Immunoblots were reprobed with β-actin monoclonal antibody to confirm equal amount of protein loading. The expression levels of the proteins detected by immunoblotting were quantitated using the program IMAGE (National Institutes of Health) for the integrated density of each band. Quantitative Western blot data were calculated from densitometric analysis of bands with the NIH image J software. The values were normalized to β-actin, which served as a loading control.

Statistical analysis

All statistical comparisons between the groups were made using analysis of variance (ANOVA) by Prism statistics software. Results were presented as mean +/- SEM (Standard Error Mean). Values of $p < 0.05$ were considered as statistically significant.

Results

Effect on T, FSH and LH levels

The study showed that the level of Testosterone, FSH and LH was significantly decreased ($p < 0.05$) in testes of mice treated with Dactinomycin in dose dependent manner in all the three parameters.

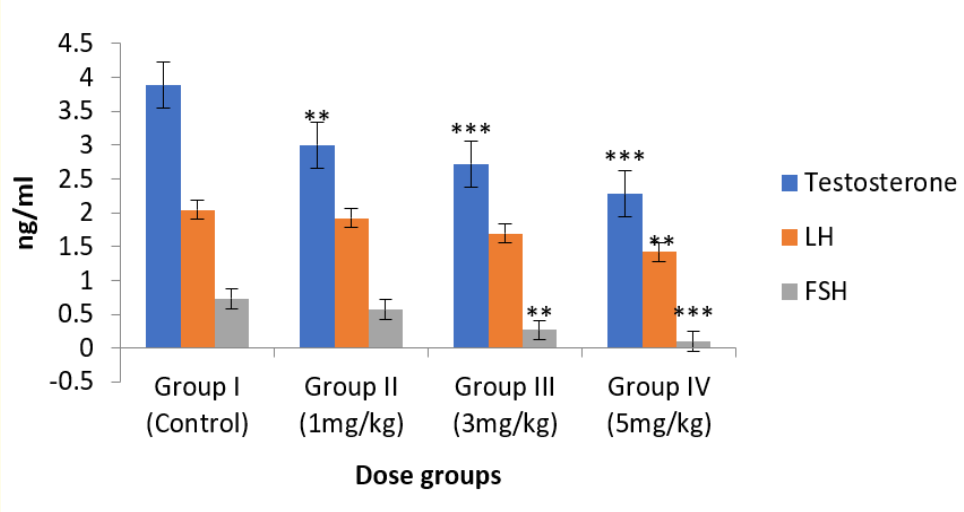


Figure 1: *Effect of dactinomycin on the levels of testosterone, lutenizing hormone and follicle stimulating hormone. Note: *, and ** Indicate significantly different as compared to controls at (p < 0.01), (p < 0.05) respectively.*

Effect of dactinomycin on reactive oxygen species

Significant increase level ($p < 0.5$) of reactive oxygen species in dose dependent pattern was seen following treatment with dactinomycin when compared to the control. The result suggests induction of oxidative stress following dactinomycin exposure.

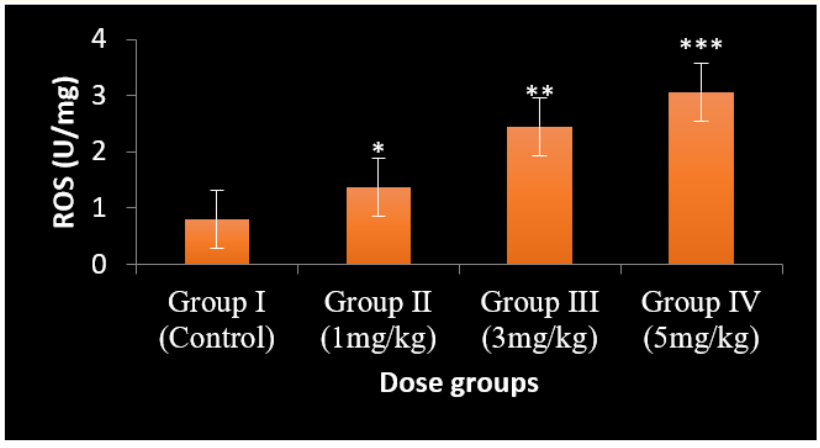


Figure 2: *Bar chart showing the increasing level of Reactive Oxygen species in dose dependent pattern. Note: *, ** and ** Indicate significantly different as compared to controls at ($p < 0.001$), ($p < 0.01$), ($p < 0.05$) respectively.*

Effect on CASP- 3 and CASP-9 activities

There is a significant increase in CASP-3 and CASP-9 activities across the groups when compared to the control.

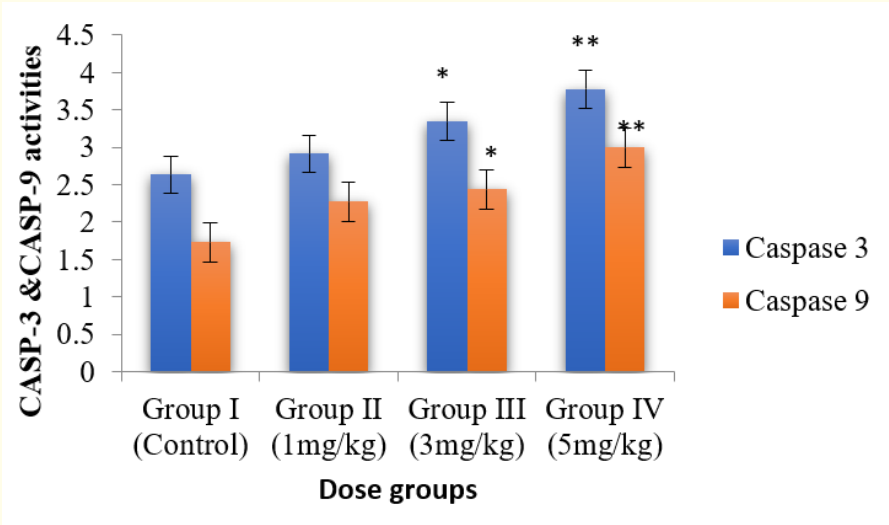
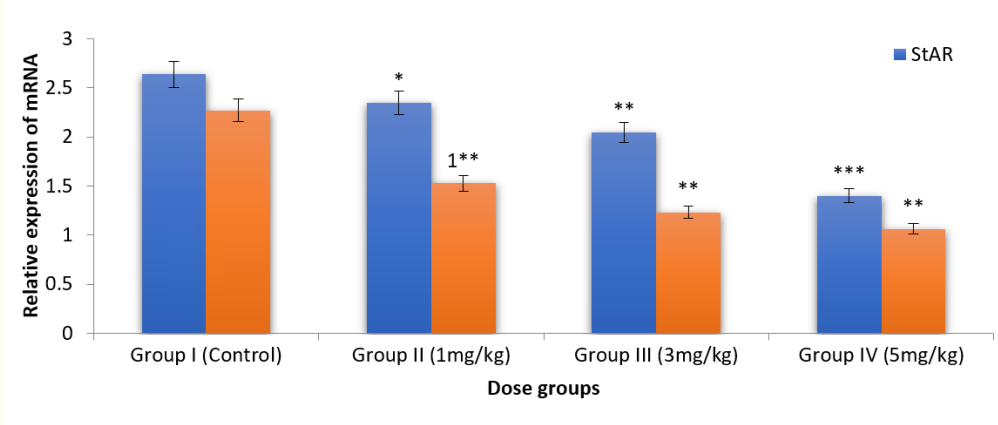


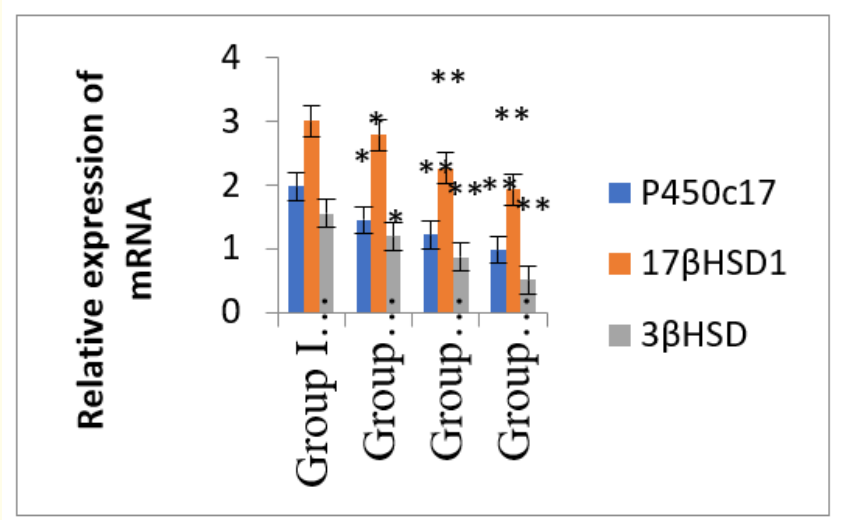
Figure 3: *Bar chart showing adverse effect of Dactinomycin on CASP-3 and CASP- 9. The data showed significant decrease levels across the groups when compared to the mice in control group in dose-dependent manner. Note: *, ** and ** Indicate significantly different as compared to controls at ($p < 0.001$), ($p < 0.01$), ($p < 0.05$) respectively.*

Effects of dactinomycin on StAR, CYP450scc, P450c17, 17βHSD1 and 3βHSD mRNA expression

P450scc level was significantly decreased ($p < 0.01$) in all treated groups when compared with control group. StAR was also seen significantly decreased when compared with the control Group I. The reduced level of these mitochondrial enzymes as shown in this study indicates impairment of the cell organelles which is suggestive of severe adverse effect of dactinomycin in the testicular cells of mice.



a



b

Figure 4a and 4b: *Effects of Dactinomycin on StAR, CYP450scc, P450c17, 17βHSD1 and 3βHSD mRNA expression. The data was expressed as means ± SEM of triplicate measurements of five samples. Note: *, ** and ** Indicate significantly different as compared to controls at (p < 0.001), (p < 0.01), (p < 0.05) respectively.*

Effect of dactinomycin on StAR, CYP450scc, P450c17, 17βHSD1 and 3βHSD protein expression

The study showed that the levels of 3βHSD, 17βHSD1, P450c17 proteins were significantly decreased ($p < 0.05$) following treatment with Dactinomycin, when compared to the control.

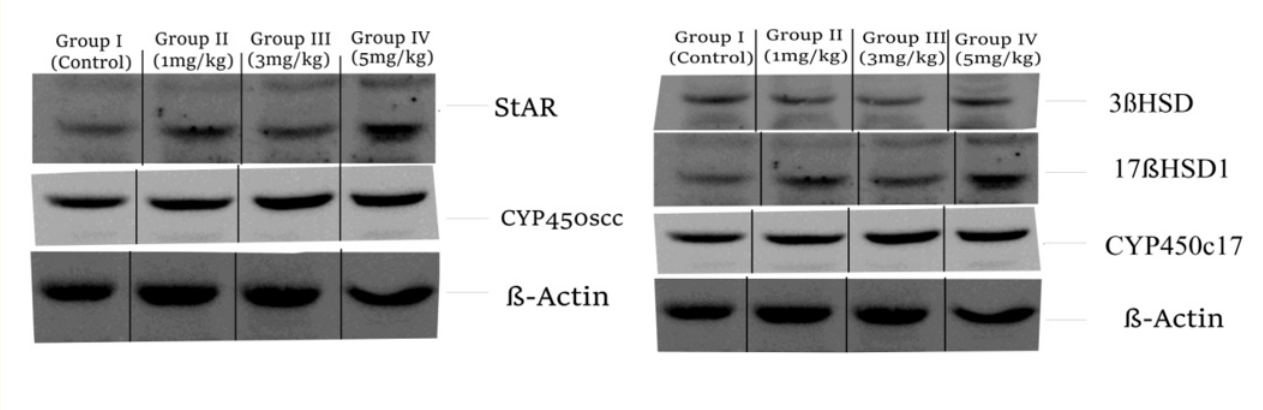


Figure 5a: *Western blot of testes microsomes of testis of mice treated with Dactinomycin anti-human/rat/mouse StAR, CYP450scc, CYP450c17 and anti-human 3βHSD, 17βHSD1 polyclonal antibodies and β-actin monoclonal antibodies.*

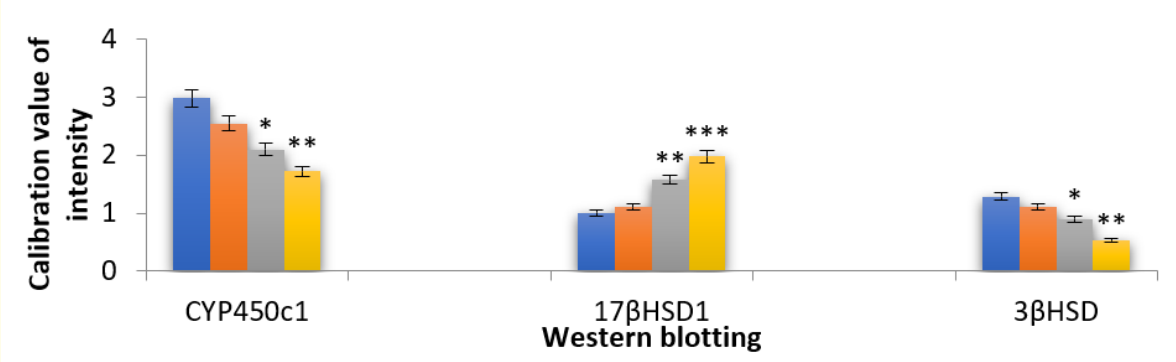


Figure 5b: *Effect of Dactinomycin on CYP450c17, 17βHSD1 and 3βHSD proteins expression. Each bar chart shows the intensity ratio calibrated by β-actin. Values are the mean ± SEM (n = 5). Note: *, ** and ** Indicate significantly different as compared to controls at (p < 0.001), (p < 0.01), (p < 0.05) respectively.*

Discussion

Dactinomycin, a chemotherapy medication used to treat a number of types of cancer. The clinical use of Dactinomycin for countless numbers of human malignancies has been impeded due to its toxic effects. Therefore, this studied the effect of dactinomycin on reproductive hormones and also investigated its effect on activities of Steroidogenic enzymes. The role of apoptosis following administration of dactinomycin was also evaluated. Our results showed that Dactinomycin caused a decrease in the Testosterone, LH, and FSH. Since testosterone is essential in the regulation of sexual behavior, accessory sex organ functions, epididymal sperm maturation, and spermatogenesis, drugs implicated in cytotoxicity of the testis either inhibit biosynthesis of testosterone or its secretion thus having intense effects on the processes required for the timely deposition of viable spermatozoa into the reproductive tract of the female [13]. Leydig cells in

adjacent position to the seminiferous tubules are responsible for the production of testosterone in the presence of luteinizing hormone (LH). As LH level is significantly low in the testis of the dactinomycin -treated mice compared to the vehicle control, this might be the reason for the decreased level of testosterone in the testis of the mice administered with dactinomycin in a dose dependent manner. The level of FSH in the mice is reduced due to its relationship with the luteinizing hormone (LH). Exogenous factors as been reported to lead to the generation of ROS in different parts of the male reproductive tract. Mahsa., *et al.* [14] highlighted the negative impact of oxidative stress on the regulation and cross-talk between the reproductive hormones. Disruptive effects of ROS on male reproductive hormones by dactinomycin as confirmed by this study would necessitate further study on the prevention of ROS-mediated hormonal imbalances.

Results showed that Dactinomycin caused an increased level of Caspase 3 and Caspase 9 when compared to the control. The increased levels of CASP- 3 and CASP- 9 can induce excessive program cell death, Similar studies have examined the induction of apoptosis in germ cells following chemical insult or androgen withdrawal, Animals treated with mono-(2-ethylhexyl) phthalate (MEHP), ciprofloxacin and diethyl maleate (DEM) were found to have increased germ cell apoptosis, whereas germ cells undergoing apoptosis following androgen withdrawal exhibited increased levels of caspase 3 and caspase 9 [14-18]. Therefore, different cellular insults to the testis may trigger more than one possible apoptotic pathway. Caspase-9 function is required for apoptosis, leading to the normal development of the central nervous system [19]. Without correct function, abnormal tissue development can occur leading to abnormal function, diseases and premature death. The action of caspases is regulated on several levels, including blockade of activation of caspases at the DISC as well as inhibition of enzymatic caspase activity. c-FLIP proteins are well-known inhibitors of death receptor-induced apoptosis.

It has been shown that caspases have effect on the mitochondria and on upstream events of intrinsic apoptosis. Caspase 9 has been shown to uncouple the mitochondria and increase ROS production, while cells deficient in caspase 3 show a delay in the mitochondria events of intrinsic apoptosis [20]. Apoptosis is important for normal spermatogenesis in mammals, maintaining cellular homeostasis, and safeguarding the organism by getting rid of damaged or infected cells that may interfere with normal function with the supportive ability of the Sertoli cells. It is also characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Reports have been made available on the effect of apoptosis on the male reproductive system [21].

Steroidogenesis which entails processes by which cholesterol is converted to biologically active steroid hormones examples of the steroid hormone is the testosterone which can be affected if there is defect in the synthesis of conversion of cholesterol to testosterone, and they are catalyzed by some enzymes involved in the Steroidogenesis pathway examples are: StAR, p450c17, 17βHSD1, 3βHSD. In addition to this study done, result showed that there was decrease value in StAR, p450c17, 17βHSD1, 3βHSD enzymes involved in Steroidogenesis when compare to the control. StAR is a transport protein that regulates cholesterol transfer within the mitochondria, which is the rate-limiting step in the production of steroid hormones [22] present in steroid-producing cells, including theca cells and luteal cells in the ovary, Leydig cells in the testis and cell types in the adrenal cortex [23]. With decreased level of StAR, a protein that facilitates the transport of cholesterol from the outer membrane to the inner membrane, less cholesterol would be available to initiate the synthesis of the steroids, ultimately testosterone needed for quality sperm production. Also, Cholesterol P450scc enzyme (CYP11A1), a mitochondrial enzyme that catalyzes conversion of cholesterol to pregnenolone the first enzymatic step in all steroid synthesis. Significant decrease in the value of p450scc enzymes when compared to the control value indicate that the synthesis of pregnenolone which is also an intermediate used in the synthesis of other steroid hormones would be affected. Decreased level of P450c17 signifies that the production of androgenic and oestrogenic sex steroids which is done by converting 17α-hydroxy pregnenolone to dehydroepiandrosterone (DHEA) will be inhibited as P450c17 catalyses the reaction involved in this process. 17βHSD1 and 3βHSD decreased level signifies an inhibition in the Steroidogenesis pathway. In view of these observations, it is relevant to presume that dactinomycin, being chemotherapies cause an induction of apoptosis by evaluating Caspase 3 and Caspase 9 activities and alteration of Steroidogenic acute regulatory enzyme (StAR), Cyt P450scc and other important enzymes in Steroidogenesis pathway.

Conclusion

This present study show that dactinomycin, an anticancer agent interferes with germ cells and it has adverse effect on FSH, LH, testosterone hormones by decreasing the activities in dose dependent pattern. Also, this study revealed that dactinomycin treatment leads to induction of apoptosis and alteration of steroidogenic regulatory enzyme and other enzymes involved in synthesis of testosterone. Further study is required to fully understand mechanic by which dactinomycin causes damage to germ cells.

Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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