Induction of Apoptosis by Bleomycin Compound in testis of Swiss albino mice

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Abstract: Bleomycin, a glycopeptides derived antibiotics used in the treatment of squamous cell cancers, testicular cancer, Hodgkin's and non-Hodgkin's lymphoma has been reported to show male reproductive toxicity. However the clinical importance of this antibiotic necessitates thorough evaluation of its safety; hence, the study investigated the possible mechanisms involved in its toxicity. 5, 10 and 20mg/kg of bleomycin were administered via intaperitoneal injection for 14 consecutive days; animals in control group received the vehicle while animals in recovery groups were sacrificed 14 days later. At autopsy, animals were sacrificed and the testes were excised. One of the testes was fixed in 10% formal saline for histological observation while the other half was stored in 20°C biochemical analyses. Role of bleomycin in apoptosis in-vivo were investigated by using different techniques. Increased level of LPO, CAT and decreased GSH content was estimated (p < 0.05) in a dose-dependent manner. Decrease level of sperm count and motility with increased sperm morphology was seen. Bleomycin treatment results in down regulation of CASP 3 and CASP 9 protein expressions. This study reveals the adverse effect of bleomycin on the testicular germ cells of the mice. The administration of Bleomycin leads to generation of oxidative stress and induction of apoptosis in testis tissue of mice, resulting to impairment of function of testis.

Keywords: Bleomycin, Apoptosis, oxidative stress, Hormones,

I. INTRODUCTION

Bleomycins are family of glycopeptides derived antibiotics isolated from the bacterium Streptomyces verticillus. Bleomycin has a common name called Bleoxane; Bleomycin is classified as an antitumor antibiotic. It is used in the treatment of squamous cell cancers, melanoma, sarcoma, testicular cancer, Hodgkin’s and non-Hodgkin’s lymphoma. It may also be administered inside the chest to help prevent the recurrence of a fluid around the lung due to cancer (Perry, 2001). As mammalian sperm is an excellent model to monitor the potential damage induced by chemical agents, more so, reproductive system is an essential target for toxicity of drugs. Thwaini (2015) reported that bleomycin induced male rat reproductive toxicity, however mechanism involved in bleomycin induced male reproductive toxicity is not clear, therefore this study focused on investigating possible mechanism by which bleomycin induced male reproductive toxicity and to achieve this, effect on oxidative and anti-oxidative marker enzymes was investigated (Ojo et al., 2013, Qiao-FengWei, et al., 2016). Sperm parameters- sperm count, motility and morphology were estimated. Effect on the levels of CASP 3 and CASP 9 activities Histoarchitectural change of testes by transmission electron microscopy was also observed.

II. METHODOLOGY

Animals, grouping and treatments
Male swiss albino mice weighing about 20 g were obtained from the Laboratory Animal Division of the Institute. The animals were maintained under standard conditions of humidity (50 ± 5%), temperature (25 ± 2°C) and dark and light cycles (12 h each) with free access to food and water. 5, 10 and 20mg/kg of bleomycin were administered to mice for 14 days, animals in control group I received the vehicle. Groups V, VI, VII and VII were sacrificed after 14 days of the recovery.

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 (50mM, 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.
Biochemical estimations in tissue samples
Testsis from each mouse (n=5) were stored at -80°C for different biochemical assays such as Lipid peroxidation (LPO), GSH, superoxide dismutase (SOD) and catalase (CAT). Protein quantity was estimated according to Lowry’s method. 10% tissue homogenates (w/v) were prepared in 100 mM Tris-HCL buffer (pH 7.4) using Cole Parmer tissue homogenizer. The values were expressed per mg of protein.

Lipid peroxidation (LPO)
The lipid peroxidation was estimated by a spectrophotometric method in terms of thiobarbituric acid reactive substances. Briefly, one volume of homogenate was mixed with two volumes of stock solution (15% w/v trichloroacetic acid in 0.25 N HCL and 0.375% w/v thiobarbituric acid in 0.25 N HCL) in a centrifuge tube, vortexed and heated for 15 min at 95°C in water bath. The mixture was cooled and centrifuged at 5000 rpm for 5 min and the absorbance of the supernatant was read at 532 nm.

Superoxide dismutase (SOD) activity
Superoxide dismutase (SOD) activity was estimated by a spectrophotometric method. Assay mixture containing sodium pyrophosphate buffer (pH 8.3, 0.052M), phenazine methosulfate (186 μM), nitroblue tetrazolium (300 μM) and NADH (780 μM) were diluted with appropriate enzyme in total volume of 3 ml. The mixture was incubated at 37°C for 90 sec and reaction was stopped by addition of glacial acetic acid. The reaction mixture was mixed vigorously by adding n-butanol and allowed to stand for 10 min before the collection of butanol layer. The intensity of chromogen in butanol was measured at 520 nm.

Catalase (CAT) activity
Catalase activity was quantified by measuring the decomposition of hydrogen peroxide (H₂O₂). Assay mixture consisting of 0.01M phosphate buffer (pH 7), 0.2 M hydrogen peroxide and tissue homogenate was incubated at 37°C for 1 min. The reaction was stopped by addition of potassium dichromate (5% w/v) and acetic acid. The remaining hydrogen peroxide was determined by measuring chromium acetate after heating the assay mixtures in a boiling water bath for 15 min. The absorbance was read at 570 nm (Aebi, 1983; Alberts, 2002).

Glutathione (GSH) content
Glutathione (GSH) content was estimated by centrifuging an aliquot of 10% homogenates of the tissues in 100 mM Tris-HCL buffer (pH 7.4) containing 0.16 M KCL at 1000 g for 5 min. The supernatant was used to measure the rate of reduction of 5’ 5’-dithiobis-(2 nitrobenzoate) to 2-nitro-5 thiobenzoate. The absorbance was read at 412 nm. Glutathione content was expressed in μM/mg protein. For determination of GSSG content 0.1M NaOH was used instead of Tris-HCL buffer.

Testicular cells preparation
Testicular cells were prepared for apoptosis analysis following a protocol adapted from Malkov et al. (1998). Briefly, testes were removed and decapsulated by making a small incision in the testis. The contents of the testes were collected through the incision into a 15 ml tube containing 5 ml ice-cold 1X PBS buffer (pH -7.4) and the contents were incubated for 40 min at 37°C with vigorous shaking. Then, the tubes were placed on ice and incubated to allow the seminiferous tubules to settle. The supernatants were discarded and the seminiferous tubules were washed twice in 10 ml of PBS twice.

Preparation of cDNA
cDNA was prepared according to manufacturer’s instructions (Applies Biosystem). Total reaction volume of 20 μl RT enzyme mix, 0.5 μl RNA (up to 5 μg) and 7.5 μl RNase free water were mixed gently and incubated at 25 °C for 10 min, 50°C for 30 min and terminated the reaction at 85°C for 5 min. Reaction mixture was chilled on ice. Reverse Transcriptase PCR reactions were performed in PCR tubes (Axygen, California) using Peltier-based thermal cycler (PTC 100, MJ Research). Gradient PCR reactions were done for standardization of cDNA amplification condition and optimization of annealing temperature for primer use.

Determination of mRNA levels using quantitative RT-PCR
Real time PCR analysis was performed according to the supplier’s instruction (Roche Diagnostics Germany). The components of the reaction were SYBR Green PCR Master Mix, cDNA template, forward primer and reverse primers and nuclease-free water. PCR reactions were performed in Light Cycler® 480 Real-Time PCR instrument and analyzed according to accompanying software instructions Light Cycler® 480 Soft ware release 1.5.0 (Roche Diagnostics Ltd Germany). Respective samples were pooled and experiments were carried out in triplicates after that Cp values were calculated. β -actin was used as an internal control to normalized ratios between the samples. For each primer pair, a melting curve analysis was performed according to the instrument. The program in brief was an initial incubation of 50°C for 2 min and 95°C for 10 min was followed by 40
cycles at 95°C for 15 sec 60°C for 60 sec. Relatives changes in mRNA level between control and treated groups were calculated by using $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

**Western blotting assay**

Total proteins were isolated from tissues by using modified protocol of Ghribi et al. (2001). Tissues were homogenized in urea lysis buffer (7 M Urea, 2 M Thiourea, 65 mM DTT, 4 % CHAPS). Cellular debris were spun down at 20,000 x g for 30 mins at 4°C and supernatants were used as whole protein extract. Isolated proteins were quantified using Bradford reagent. 50 µg proteins from each sample was separated on 15 % SDS-PAGE and transferred on to a nitrocellulose membrane using a semi-dry electro-blotting apparatus (GE Health, UK). Transfer was examined by Ponceus S stain and washed in distilled water until the stain disappeared. Membrane was incubated in 5 % Non–Fat dried milk at 4°C overnight. Blocked membrane was washed with 0.1 % PBST and probed with primary antibodies. After primary antibody incubation further washing was done in 0.05 % PBST. Membrane was incubated in HRP conjugated secondary antibody and washed three times again. Enhanced chemi-luminescent detection reagent was used to develop the blots which were further used for densitometric analysis (UVP CA) and normalization. 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.

**III. RESULTS**

The levels of reproductive hormones in Swiss mice.

The levels of T and FSH were obviously decreased by bleomycin administration in Bleomycin treated swiss mice. The level of LH was significantly increased by bleomycin treatment in a dose-dependent manner (Fig 1).

*: significantly different from the control group (* $p <0.05$; ** $p <0.01$; *** $p <0.001$) in comparison to controls.

![Fig 1.](image)

**Biochemical assay**

Bleomycin treatment resulted in significant increase ($p <0.01$) in the concentration of thiobarbituric acid reactive substances in testis tissue homogenates of mice. Bleomycin induced depletion in the protein level in testis in a
dose dependent manner. Oxidative stress was induced by bleomycin treatment as confirmed by the significant decrease ($p < 0.01$) in GSH, CAT, LDH and SOD levels in testicular tissues (Fig 2).

![Graphs showing the effect of bleomycin treatment on oxidative and anti-oxidative enzymes](image)

**Fig 2.** Effect on oxidative and anti-oxidative enzymes following 14 days Bleomycin treatment and after 14 days withdrawals in male mice. The asterisks indicate significant differences ($^* p < 0.05; ^{**} p < 0.01; ^{***} p < 0.001$) in comparison to controls.

**Sperm motility, count and morphology**

Bleomycin treatment caused significant decrease ($p < 0.001$) in epididymal sperm count and motility (Fig. 3a,& b) across the bleomycin treatment groups. A marked increase ($p < 0.01$) in abnormal morphology was observed in epididymal spermatozoa (Fig 3c ) in all bleomycin treated mice.
Fig 3. Effect of Bleomycin on sperm count, motility and morphology

**Effect of Bleomycin of apoptosis in Testis**
Bleomycin treatment results in increased percentage of Annexin V-positive in group II, III, IV that received 5, 10 and 20 mg/kg respectively in dose dependent pattern (Fig 4). The ability of Bleomycin to induce apoptosis in the both cells after withdrawal of the treatment was also noticeable. Increased necrosis of spermatocytes cells was also seen after the addition of Propidium Iodide. Annexin staining, indicative of exposure of phosphoserine residues, is an additional hallmark of early apoptotic cell death thus, an appropriate method to quantify apoptosis.

Fig 4. Flow cytometric analysis of annexin-V/propidium iodide (PI) staining on primary spermatogenic cells following Bleomycin administration for 14 days and after 14 days withdrawal of the treatment.

**Effects on mRNA gene expression**
Bleomycin treatment at doses 10 and 20 mg/kg results in significant increase in the expression of Bax and Casp-9 gene. The expressions of these genes were also detected after 14 days withdrawal of the treatment. However, markedly reduce expression of the anti apoptotic gene Bcl-2 was seen in all Bleomycin treated groups as compared with the control groups. Statistical analysis indicates that increased levels of both the apoptotic genes; Bax and Casp-9 were significantly different from mice in control groups (Fig 5).

Fig 5. Quantitative RT-PCR analysis of Caspase-9, Bax and Bcl-2 in genes of testes with different doses of Bleomycin (Control, 5, 10 & 20 mg/kg) for 14 days and after 14 days of withdrawal of the treatment.

**Effect on the expression levels of BAX, BCL-2 and CASP-9 proteins**
Bleomycin increased BAX and CASP-9 protein expression in a dose-dependent manner within total cell lysates. Down regulations expression of Bcl-2 was seen (Fig 6 a & b). Similar effect was observed in the renal cell lysate following Bleomycin exposure.
IV. DISCUSSION AND CONCLUSION

Investigation of cytotoxic drugs using mouse model have been described to mimicked human response hence this study explored the role of apoptosis by Bleomycin in testis of Swiss albino mice. Results revealed decrease level of testosterone and follicle stimulating hormone with raised luteinizing hormone, this result is similar to Thwaini (2015) observation in rat. Based on these data, it can be stated that bleomycin induces germ cells injury by increasing lipid peroxidation. There was depletion of primary antioxidant enzymes that protect cells from oxidative stress such as SOD, glutathione-s-transferase, catalase, and glutathione peroxidise. Glutathione reductase is important antioxidant enzyme that protect from oxidative stress via elimination of reactive oxygen species. The deleterious effect of Bleomycin is therefore seen in the depletion of GR activity especially in the group that was treated with higher dose of 20mg/kg. However, increase in these antioxidants enzymes is necessary so as to protect the organs from the toxic agent (Meister & Anderson, 1983; Jurczuk et al., 2004). The toxicity of Bleomycin has been studied extensively (Al-Snaifi, 2013 and Thwaini, 2015). However, the mechanism by which the compound induced germ cells injuries are largely unknown. In this study, it is found that bleomycin treatment leads to generation of oxidative stress and induction of apoptotic cell death in spermatocyte cells through a direct pathway from caspase-independent release of cytochrome c after intracellular redistribution of cell death activator Bax. Bleomycin treatment results in the inhibition of ATP hydrolysis in mitochondria with subsequent decreased cell energy production and death (Aleman et al., 2007). Bleomycin induces a decrease in mitochondrial membrane potential, cytochrome c release into cytosol, and activation of caspase-9. Bax translocation to the mitochondria has been shown to reduce mitochondrial membrane potential, enhance cytochrome c release from the mitochondria, and activate caspases (Zhang, 2000, Kreuser et al., 1990). Administration of Bleomycin leads to the decrease in both caspase -3 and 9 with in dose dependent manner. Generation of oxidative stress was also seen after bleomycin treatment. The data indicate that bleomycin administration leads to impairment of testes tissue due to the formation of oxidative stress and induction of caspase dependent apoptosis.

REFERENCES


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