Down-regulation of BMP8A, SMADs 1/5/8 and BAX Proteins Following Methotrexate-treatment in Testicular Tissue of Swiss Albino Mice

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Authors' contributions

This work was carried out in collaboration among all authors. Author OOO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OOA and BTO managed the analyses of the study. Author OOA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Several studies on the adverse effects of methotrexate have been reported, especially its implication in the degeneration of spermatogenesis, reduced sperm count and ultimate male infertility. As an antagonist, methotrexate (MTX) uses folic acid to obstruct the production of some biomolecules involved in synthesis of DNA, RNA and protein. It is used in the treatment of cancer and other diseases such as psoriasis, and rheumatism. Reports have also revealed that the expression of Bone Morphogenetic Protein (BMP8a) promotes spermatogenesis and fertility through the induction and activation of signaling sets of transcription factors, SMAD1/5/8. Hence, the expressions of these proteins and role of apoptosis are crucial to understand the mechanism involved in Methotrexate-induced infertility. In view of this, albino mice (Swiss strain) were randomly sorted to four groups. Group I served as control while groups II, III & IV (n=5) were treated with 5,

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10 and 20mg/kg/day of Methotrexate (IP) respectively. Expressions of *BMP8A* and *SMADs 1/5/8* were done by PCR and Western blotting techniques. Germ cell apoptosis was measured by flow cytometry and immunohistochemistry techniques. Ultrastructural changes were assessed in leydig cells as well as sertoli cells. The results of this study reveal a down-regulation of *BMP8A* and *SMAD1/5/8* proteins in a dose-dependent pattern. Induction of apoptosis was also confirmed by the expression of primary apoptotic Bax antibody. The sertoli cells which play their major roles of nourishing and protecting the development of sperm cells were severely impaired too. These findings suggest that the function of *BMP8A* and *SMAD1/5/8* proteins in promoting proliferation and differentiation of spermatogonia was severely disrupted following methotrexate exposure. Caution should therefore be taken when administering this drug.

Keywords: Bone morphogenetic proteins (BMPs); methotrexate; apoptosis; TEM.

1. INTRODUCTION

Methotrexate (MTX) is one of the many anticancer drugs used in chemotherapy, although it is also known for its use in the treatment of autoimmune diseases like rheumatoid arthritis and psoriasis [1]. MTX functions as a competitive inhibitor of enzymes which utilize folate as coenzyme. With this ability, it is able to disrupt the process of DNA replication in cancer cells [2]. Interestingly, MTX is reportedly used majorly by males of reproductive age for cancer treatment [3]. Male infertility, paternal teratogenicity, hypotension and allergic reactions are some of the complications resulting from MTX-treatment as suggested by previous studies [4]. It was reported in a recent study that MTX treatment in male mice led to reduction in the levels of testosterone and luteinizing hormone which are crucial hormones related to male fertility [5]. Another study also suggested that MTX causes oxidative damage to testicular tissue in male rats [6,7]. Despite these reports, there is still insufficient clarification of the mechanism responsible for MTX-induced male infertility.

Bone Morphogenetic Proteins (BMPs) and SMADs are important molecules in signal transduction pathways which are pertinent to fertility in males. BMPs are proteins classified under a large family of growth-factor proteins [8]. In the male reproductive system, BMPs play an important role in spermatogenesis and germ cell maintenance and development of epididymis. More specifically, *BMP8A* has been reported to be a central player in the preservation of structure and functions of the epididymis [9,10]. SMADs are known as the major signal transducers responsible for the regulation of cell growth and development in many organs of the body. The activity of SMADs is activated by BMPs [11]. Thus, BMPs and SMADs function

together in activating pathways which stimulate spermatogenesis [12].

Furthermore, programmed cell death (apoptosis) has been linked with incidence of male infertility. Apoptosis is an indispensable biological process required for spermatogenesis in mammals. Besides degenerated spermatogenesis, apoptosis is also relevant to the pathogenesis of male infertility whether independently or in synergy with genetic disorders [13,14]. Spermatogenic processes have their intricacies alongside maturity of germ cells. The sertoli and leydig cells are at the helm of affairs in the development, nourishment, protection and preservation of germ cells as they are known to increase in number [15]. Sertoli cells are activated by follicle-stimulating hormone (FSH) to secrete androgen-binding proteins. This process makes for the sustenance of spermatogenesis. On the other hand, leydig cells are important in that they are responsible for the production of testosterone and are stimulated by luteinizing hormone (LH) [16]. Apoptosis aids the selective regulation of number of germ cells according to the ability of the Sertoli cells [17]. The pro-apoptotic protein, BAX, is a signaling molecule encoded by the *BAX* gene. It is a common indicator of apoptosis in cells [18]. Similarly, Annexin V is also known to be another indicator of apoptotic cells due to its affinity for phosphatidylserine which is an apoptotic marker on the extra-cytosolic side [19].

Evidences are yet to be considerably established with regard to the effect of MTX usage male infertility and the mechanism supporting these observations. Hence, the purpose of this study was to investigate possible mechanisms through which MTX could induce male infertility vis-à-vis gene expressions of *BMP8a*, *SMADs 1/5/8* and *BAX*, coupled with apoptosis in testicular tissue of male mice.

2. METHODOLOGY

2.1 Care of Experimental Animals

The Institutional Animal Ethics Committee of College of Medicine, Ekiti State University, Ekiti-State Nigeria, approved the protocols for animal handling for this study. Strict compliance with all the animal care and handling guidelines as recommended by the committee was ensured.

2.2 Animal Dosing

Twenty (20) swiss albino mice were randomly sorted to four experimental groups (n=5). Group I served as control while groups II, III & IV were treated with 5, 10 and 20mg/kg/day of Methotrexate respectively via intraperitoneal injection. The treatment lasted for twenty-one (21) days.

2.3 Tissue Sample Preparation

At autopsy, animals were killed by using over dose of anesthesia to avoid any suffering to the animals. Testis of one side were excised and cleaned with cold saline. Excess blood and moisture was also discarded. Tissues were frozen using liquid nitrogen and stored at -80°C (Ultra-cold Freezer, Thermo Fisher Scientific, UK) until required for further analysis. Other side testes were fixed in appropriate fixative for ultrastructural and immunohistochemistry examinations.

2.4 Isolation Testicular Germ Cells (TGCs)

Testicular germ cells (TGCs) were collected from testes of experimental animals by incision using a surgical blade. The cells were subjected to centrifugation (5 min at 800 xg). The cells were thoroughly washed with DMEM/F12 medium. TGCs were cultured and counted in a hemocytometer. Trypan blue was also used as staining agent for determination of cell viability. Spermatocytes (>90% pachytene) and round spermatids were also purified [20,21].

2.5 Apoptosis Detection by Flow Cytometry

FITC Annexin V Apoptosis Detection Kit protocol was used for detecting apoptosis via flow cytometry. The analysis investigated induction of apoptosis in testicular germ cells. Annexin V-

fluorescein isothiocyanate/Propidium Iodide (PI) detects primary phases of the apoptotic process by binding to phosphoserine while permitting direct quantification. Testis tissue was homogenized in PBS (1500xg/5mins). Cells were washed, and re-suspended in phosphate-buffer saline (PBS, pH-7.0). Fluorescein isothiocyanate conjugated Annexin V and PI.

2.6 Immunohistochemical (IHC) Staining

After deparaffinising and rehydrating the tissue sections, they were kept in a dark space (30 mins) with 3% hydrogen peroxide to deactivate peroxidase present. Thereafter, the sections were rinsed and placed in 0.01% Tween 20 with 0.01M PBS for 5 minutes (pH 7.4). The tissue sections were further incubated for 30 minutes and then overnight both at 4°C with Bax primary antibodies (1:1000). After sufficient washing in 0.01% Tween 20, sections were incubated with anti-immunoglobulin G for 1 hour (37°C). Furthermore, the tissue sections were washed and 3, 3'-diaminobenzidine was used for antigen detection.

2.7 Testicular Ultrastructure

The ultrastructure of testis was studied [22]. Tiny testicular fractions were excised and fixed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.3) for 12 hours (4°C). After washing with a buffer solution, tissues were fixed for 1 hour (1% osmium tetroxide and 0.1M phosphate) at 4°C . Sample dehydration was carried out using increasing levels of acetone. Infiltration of the samples was followed by embedding using araldite (CY 212 /TAAB, UK). 1 μm slices were cut and mounted on clean glass slides. This was immediately followed by staining using blue toluidine stain. The slices were viewed under a light microscope for gross scrutiny of the area and quality of the fixation process. Thin slices of grey-silver colour interference (70-80 nm) were mounted onto 300 mesh-copper grids for electron microscopy. Uranyl acetate (alcoholic) and lead citrate (alkaline) were used as staining agents. The slices were further washed and observed under a Morgagni 268D transmission electron microscope (FEI Company, Netherlands).

2.8 Total RNA Extraction

Total RNA extraction from testicular germ cells was carried out using Trizol reagent.

Spectrophotometry was used to quantify isolated total RNA as well as its level of purity by measuring the optical density (OD) (260 and 280nm). Optical density ratios (260nm/280nm) for all the samples ranged from 1.8 to 2.0. The amplification and quantification of target genes were performed in a reaction of 20 mL for 40 cycles (SYBR Green Kit Germany) [23].

2.9 Expression of BMP8A protein by Western Blotting

Total protein was extracted from testicular tissue with urea lysis buffer according to modified protocol of [24]. Protein separation and transfer was probed using BMP8B protein and β - actin antibodies. Bound antibody was detected using HRP conjugated secondary antibody. Blot development was done via enhanced chemiluminescent detection reagent which was further used for densitometric analysis and normalization. Data from western blotting (quantitative) will be calculated from densitometric analysis of bands with the NIH image J software. The values were normalized with β -actin which served as loading control.

2.10 Statistical Analysis

Analysis of variance (ANOVA) was used to analyze statistical comparisons between the experimental groups (GraphPad Prism software). Results were expressed as mean $\pm$ Standard Error Mean (SEM). Statistically significant values were considered at $p < 0.05$.

3. RESULTS

3.1 MTX and Viability of Spermatogenic Cells

The viability determined by trypan blue staining showed significant decrease of germ cells in all the MTX- treated samples when compared with the control group in a dose-dependent manner (Fig. 1).

3.2 Methotrexate Effect on Spermatogenic Cells

Increased concentrations of Methotrexate resulted in an increased percentage of Annexin V-positive spermatogenic cells in dose dependent pattern (Fig. 2). Annexin V positivity is an attribute of apoptotic cells; hence, an appropriate method to quantify apoptosis.

3.3 Immunohistochemical Analysis of BAX in Testes of Mice

Positive staining of BAX antibody was observed in the testes of mice treated with 5, 10 and 20mg/kg/day (Fig. 3a). BAX positive staining in 5mg/kg was significantly intense in the spermatocytes and spermatogonia cells, indicating increased rate of apoptosis among the Methotrexate treated groups. Positive staining of BAX antibody was also seen in the nucleus of elongated spermatids (Est), spermatogonia (Sg), round spermatids (RSt) and pachytene spermatocytes (PSc) (Fig. 3b).

3.4 Effect of MTX on Expression of BMP8A and Bax Genes in Testis

A significant difference was observed in RT-PCR results ($p < 0.05$) in gene expression levels of *BMP8a* and *Bax* when compared with the control group (Fig. 4).

3.5 Effect of MTX on Testicular Ultrastructure

Leydig cell ultrastructure in MTX-treated mice indicated elevation in hypertrophy with shrunken nuclei. There was also a corresponding rise in the number of lipid droplets in comparison to the control group. Condensed mitochondria were detected in mice administered 10 and 20 mg/kg MTX (Fig. 5a, b, 'c and d').

Series of abnormal and irregular morphological characteristics were seen in Sertoli cell ultrastructure following MTX-exposure. These include increased vacuole number in the cytoplasm and expanded endoplasmic reticulum. Shrunken mitochondria and intact cell structures were also visible (VI 'b, c, d'). Control group sertoli cells showed intact mitochondria with their cristae in the cytoplasm. The cell nucleoli were also intact as well as the chromatin structures (Fig. 6b).

3.6 Effect of Methotrexate on BMP8a and SMAD1/5/8 Proteins

Methotrexate differentially altered the levels of BMP8a and SMAD1/5/8 protein expressions in a concentration-dependent manner in total spermatogenic cells. Significant downregulation of the protein was seen across the groups (Fig. 6 a and b).

Fig. 1. Graph showing Effect MTX on viability of spermatogenic cells
All the values are expressed as mean $\pm$ SEM, (n=5), ***P<0.001, **P<0.01 and *P<0.05

Fig. 2. Bar chart showing rate of Annexin V positivity in spermatogenic cells of MTX-treated male mice

Fig. 3a. Bar chart representing detection of BAX antibody in MTX-treated testicular tissue

Fig. 3b. Quantitative analysis of BAX immunohistochemistry in MTX-treated male mice

Fig. 4. Bar chart represents the fold change of BMP8a and BAX genes in testes of mice treated with MTX for 21 days

The data are expressed as mean $\pm$ SD (n=5). *, **, and *** indicate significant difference as compared to controls at ($p < 0.05$), ($p < 0.01$), and ($p < 0.001$) respectively

Fig. 5a. Electron micrographs of testes sections focusing on Leydig cells, a: controls and MTX-treated mice (b: 5 mg/kg; c: 10mg/kg and d: 20mg/kg)

Fig. 5b. Electron micrographs of testes sections focusing on Sertoli cells of MTX-treated mice. Note: controls: a, b: 5 mg/kg, c: 10mg/kg and d: 20mg/kg, (200X)

Fig. 6. Effect of MEP on BMP8a, SMAD1, SMAD5 and SMAD 8 protein expression. (a) Western blot (b) Bar chart shows representative of the densitometric quantitation of the Western blot. The data are expressed as mean $\pm$ SD (n=5)

4. DISCUSSION

This study investigated the effect of a 21-day treatment of male mice with Methotrexate (MTX) on expression of BMP8A and SMADs 1/5/8

which are key molecules relevant to male infertility in mice. MTX is majorly used in the treatment of cancer as well as autoimmune diseases. However, the side effects that accompany the use of MTX have raised serious

concerns. One of such side effects is the induction of infertility in male mice. From previous studies, it has been proposed that treatment with MTX causes male infertility through increased oxidative stress and reduction of DNA integrity in sperm [5,25]. Our findings from this study revealed a significant ($p < 0.05$) downregulation of BMP8A gene in mice testes, with the lowest expression of BMP8A observed at 20mg/kg/day dose. This is quite profound because BMP8A is important in the sustenance and maintenance of spermatogenesis via the activation of SMADs 1/5/8 as well as SMADs 2/3 in spermatogonia [12]. Hence, BMP8A activates promotes spermatogenesis via proliferation and differentiation of spermatogonia [12]. Thus, any impairment in the expression of BMP8A will negatively regulate SMAD 1/5/8 signaling, which in turn causes reduced spermatogonial proliferation and differentiation culminating in infertility. Hereafter, downregulated expression of BMP8A observed from our findings show a relationship between MTX treatment in mice testis and infertility. To further confirm this, SMADs 1/5/8 expression result from this study also showed a significantly dose-dependent downregulation ($p < 0.05$). SMADs 1/5/8 activation by BMP8A is responsible for spermatogonial diversification [26]. The observed downregulation of SMADs 1/5/8 can be linked with the downregulation of BMP8A observed in mice spermatogonia. Based on these observations, we propose that downregulated expressions of BMP8A and SMADs 1/5/8 are likely signaling mechanisms that are associated with MTX-induced male infertility in mice.

To obtain a closer perspective of the involvement of apoptosis in MTX-induced infertility, this study also assessed the expression of BAX gene, a pro-apoptotic molecule, in the testes of mice. BAX is a regulator of apoptosis involved in a series of signaling reactions which culminate in the release of cytochrome C, a potent death signal, as well as other pro-apoptotic factors [27,28]. Result from expression of BAX gene in MTX-treated mice showed a dose-dependent increase in BAX expression. This indicates that MTX treatment is capable of inducing production of BAX antibodies, thus increasing the rate of apoptosis in mice spermatogonia. This is attributed mainly to increased level of caspases released from the mitochondria causing proliferation of apoptotic cells and ultimately programmed cell death. In addition to this, immunohistochemical analysis showed presence of BAX in mice testes of all the groups treated

with MTX (5, 10 and 20mg/kg/day). These observations are further supported by the clear positive staining of BAX antibody observed in nuclei of spermatogonia, spermatocytes and spermatids in MTX-treated mice. Annexin V, a signaling protein which detects the presence of apoptotic cells by signaling expression of surface phosphatidylserine, was also determined in mice spermatogenic cells. Increasing Annexin V positivity from increased doses of MTX in this study confirms the presence of apoptotic cells in MTX-treated mice. These observations further confirm that MTX treatment may also induce male infertility by inducing apoptosis in spermatogonia.

In addition to the aforementioned parameters, we also assessed the ultrastructures of sertoli and leydig cells in testes of MTX-treated rats to check for structural irregularities. Results showed that the structure of the sertoli cells was compromised in MTX-treated mice. The compromise of sertoli cell structure by MTX will result in failure to secrete androgen-binding proteins necessary for spermatogenesis. Also, it was revealed from this study that the number of lipid droplets increased as MTX dose increases when compared to the control rats. This abnormality can be attributed to the cytotoxic effect of MTX on the structure of the leydig cells, especially in mice treated with 10 and 20mg/kg. The observed increase in lipid droplets in the MTX-treated rats indicates impairment in lipophagy. Lipophagy is an autophagic process of breaking down lipid droplets; a process used by most eukaryotic cells to produce energy. Lipophagy modulates breaking down of cholesteryl esters to free cholesterol which is the precursor for the production of testosterone [29,26]. Previous research also reported the importance of lipophagy in stimulation of spermatogenesis [30,31]. It is possible that lipophagy was compromised in MTX-treated rats due to decreased production of Luteinizing hormone (LH) which positively regulates lipophagy. In a study by [6], it was reported that MTX-treated mice showed significantly decreased LH and testosterone levels. Thus, impaired lipophagy induced by MTX could be linked with reduced testosterone production from spermatogenesis, ultimately causing male infertility.

5. CONCLUSION

This study suggests that apoptosis via downregulation of BMP8A and BAX genes

occurs in spermatogonia following methotrexate-treatment in testicular tissue of swiss albino mice. This might be the likely mechanism through which MTX induces male infertility.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

The Institutional Animal Ethics Committee of College of Medicine, Ekiti State University, Ekiti-State Nigeria, approved the protocols for animal handling for this study. Strict compliance with all the animal care and handling guidelines as recommended by the committee was ensured.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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