Effect Of Folic Acid Antagonist Methotrexate (MTX) on Testis of *Funambulus Pennanti* (Wroughton)

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Abstract

Methotrexate (MTX) is an effective agent in treatment of cancer, is one of the most versatile antineoplastic agents in spite of severe toxicity problems. The toxic effect of the Methotrexate on testis have been studied by intramuscularly injection of low dose of 3 mg/kg BW/per day and 6 mg/kg BW/day for 15 days to adult male squirrel (Funambulus pennanti) during breeding period. For comparing the effects the saline treated vehicle was injected same amount of saline and was maintained for the same duration. Toxic effect of MTX on the testis was their smallness in size sometimes irregularity in general contour, noticeable thickness of tunica albuginea, irregular appearance of spermatic arteries supplying blood to testis, in both the doses. Since MTX crosses the blood testis barrier, it induces significant reduction in the size of the tubules. From the foregoing it is concluded that Methotrexate has antigonadotrophic, antiandrogenic and antispermatogenic properties which are dose and duration dependent besides being toxic, therefore certainly causing reduction in the fertility rate.

Key words: *Methotrexate, toxicity, antifertility*

Introduction

Methotrexate is structurally related to dihydrofolate (the natural substrate for dihydrofolate reductase) that catalyzes the reduction of dihydrofolate to tetrahydrofolate and is a potent inhibitor of dihydrofolate reductase (DHFR). The inhibition of DHFR leads to an accumulation of dihydrofolate which is unable to act as substrate for any of the reaction converting tetrahydrofolate to its cofactor derivatives and, therefore, its accumulation is associated with depletion of the pool of the reduced folate cofactors. Methotrexate (Rheumatrex) is a medicine that is used to treat Rheumatoid arthritis (RA), psoriatic arthritis, Reiter's syndrome and other conditions. Aside from its antineoplastic activity, Methotrexate has also been used with benefit in the therapy of common skin disease psoriasis (Mcdonald, 1981). Additionally Methotrexate inhibits cell mediated immune reaction and is employed as an immunosuppressive agent, for example, in allogenic bone marrow and organ transplantation and for the treatment of dermatomyositis, rheumatoid arthritis, Wegener granulomatosis and Crohn's disease (Messmann and Allegra, 2001; Feagan *et al.,* 1995). Methotrexate was formerly known as amethopterin, is an antimetabolite drug used in treatment of cancer and autoimmune diseases.

Material and Method

In all three sets of experiments using low and high-doses of Methotrexate (MTX) were performed for the present study for the duration of 15 days (Tables 1& 2).

Animals were sacrificed using chloroform 24 hours after the last day of each experiment. Immediately the testis was excised and used for histological studies.

Table 1: Experimental Design for Low Dose Methotrexate treatment

Number of animals and sex	Treatment	Dose mg/kg BW	Route	Duration
3 males (Experimental)	Methotrexate	3 mg daily	I.M.	15 days
3 males (Control)	Saline	E.V.	I.M.	15 days

Table 2: Experimental Design for High Dose Methotrexate treatment

Number of animals and sex	Treatment	Dose mg/kg BW	Route	Duration
3 males (Experimental)	Methotrexate	6 mg daily	I.M.	15 days
3 males (Control)	Saline	E.V.	I.M.	15 days

Abbreviations: E. V. = Equal volume, I. M. = Intra muscular, B W = Body weight

Observation and Results

Histological and Histopathological Studies :

Testis were fixed in Bouin's fluid for 24hrs and preserved in 70% alcohol. The tissues were dehydrated by passing through graded series of alcohol, cleared in xylol and after embedding in paraffin, blocks were prepared and serial sections were cut at various thicknesses between 5µ to 8µ. For routine histological study the sections were stained with Ehrlich's haematoxylene and counter-stained with eosin. Measurements when necessary were taken with the help of an occular micrometer calibrated to a stage micrometer. The photomicrographs were taken with the help of a Carl Zeiss camera attached to the microscope and enlarged to the required size.

Sexually mature testis reaches a maximum size of 12 x 15 mm. It was covered over by a thin transparent shining tunica albuginea which showed a series of blood vessels running dorsoventrally in a spiral course in the form of distinct well marked strips.

The testis was covered by moderately thick tunica albuginea. The seminiferous tubules were perfectly circular in shape. They were separated from one another by a small amount of interstitial connective tissue (figs. 1 and 2). The fibro-muscular tunica propria, enveloping the seminiferous tubules, was formed by contractile spindle shaped peritubular smooth muscle cells or the myoid cells, collagen fibres and lamina propria was a well defined, non-cellular basal lamina.

The Sertoli cells were elongated, pyramidal, basally resting over the basal lamina with its apical end extending up to the lumen of seminiferous tubules. The nucleus was basal in position. Sertoli cells formed a sheath around the developing germ cells (spermatogenic cells) and the lumen of the seminiferous tubules. Large number of flagelar tails of spermatozoa appeared as tufts, extending from the apical end of the Sertoli cells into the lumen (fig. 2).

The cells of the spermatogenic lineage in seminiferous tubules were stacked in 4-8 layers that occupied the space between the basement membrane and the lumen of the tubules (figs.1 and 2). Germ cells in different stages of development were present including primary spermatocytes, early spermatids with round nuclei, and late spermatids with elongated and condensed nuclei.

A large number of spermatozoa with residual cytoplasm were visible at the luminal side of the tubules. The head of the sperms remain buried in apical cytoplasm of Sertoli cells while the tail hanged freely into the lumen and formed a brush-like whorl in the centre of transversely cut tubule. The space between the seminiferous tubules in control animal was filled with connective tissues, nerve fibers, blood capillaries and lymphatic vessels. The connective tissue was composed of fibroblast cells, collagen fibers, macrophages and the Leydig cells. The fibroblast cells were elongated with tapering ends and a centrally located nucleus. The Leydig cells were rounded or polygonal in shape with a central nucleus (figs. 1and 2).

Low Dose Treatment (3mg/kg BW MTX for 15 days)

The low dose treatment has resulted into partial thickness of tunica albuginea, partial shrinkage of seminiferous tubules, and partial change in the contour of tubules, noticeable increase in the intertubular spaces due to their sparse distribution. Leydig cells were occasionally affected otherwise their distribution and number appeared normal, but mesenchyme lodging the Leydig cells were highly reduced. Distortion of tunica propria appeared hanging as loops all over the tubules therefore the tubules appeared naked, (fig.3). Shrinkage and loss of spermatogonia at many places in the tubule was remarkably noticeable, depletion of germ cells was also remarkable, few spermatocytes, few round and long spermatids were visible along with the cytoplasmic masses in the periphery; lumens in some tubules appeared empty but in some flaying tails of sperms were seen. Degenerating pyknotic nuclei were seen randomly distributed all over the germinal epithelium (fig.4). The Sertoli cells appeared highly regressive and shrunken.

High Dose Treatment (6mg/kg BW/day MTX for 15 days)

The high dose treatment has resulted into severe distortion and disintegration of the seminiferous tubules. The tubules have lost their circular contour, appearing half of their original size. The overall appearance of the testis showed atrophy. Leydig cells also appeared few in number and the mesenchyme lodging them has become reduced to streak-like spaces (figs. 5 and 6). The tubule has been denuded due to loss

of lamina propria. All the germinal elements appeared disrupted and depleted and exfoliated towards the lumen. The spermatogonia were few in number and severely damaged. The Sertoli cells were also severely damaged as they appeared laterally compressed, with little of cytoplasm, nucleus undergoing degeneration, their apical or adluminal compartment lodging few elongated spermatids. The Primary and Secondary Spermatocytes along with few round spermatids appeared pyknotic, the lumen of the tubule showed fibrous elements; some may be sperms with coiled tails. The overall appearance of the seminiferous indicated tubular atrophy (fig. 6).

Discussion

In the present study, following MTX low and high dose treatment, the light microscopic results showed marked changes in the testicular cyto-architecture in the tubular and intertubular compartments and in the Sertoli cells. Regarding the general morphological appearance Shamberger *et al.,* 1981a recorded a **decrease in the size of the testis** with MTX treatment as in the present study, an insignificant with low dose but significant with high dose on the other hand Hensle *et al.,* 1984 described abnormal morphology of testis. The high dose treatment also **caused a remarkable thickening of tunica albuginea and the thickening of blood capillary walls** as well as a decrease in the volume of testis Johnson *et al.,* 1994; but Frick, 1973; observed no thickening of tunica albuginea with norethinodrone.

The seminiferous tubules showed **remarkable shrinkage in their size and also in their contour**. The tubules appeared irregular in shape and because of the remarkable shrinkage in their sizes there was an increase in the intertubular spaces (Saxena *et al.,* 2004) on the contrary Narrod and Narrod, 1977 could register moderate tubular atrophy.

The high and low dose of MTX treatment in the present work also resulted **into disruption and thickness of lamina propria** following deposition of extra-cellular matrix between the cellular compartments as described by Lendon *et al.,* 1978; Hensle *et al.,* 1984; and Saxena *et al.,* 2004. Davidoff *et al.,* 1990 have shown that the various form of hypo spermatogenesis is accompanied by different form of thickening of lamina propria. Our results are similar to Davidoff *et al.,* 1990 as noticeable thickening and disruption of lamina propria has resulted due to marked shrinkage and hypo-spermatogenesis or total arrest of spermatogenesis. The thickening of lamina propria may be due to androgen deficiency also confirmed by our results of decrease in testosterone levels.

In the present work depletion in number and size of **I^{ry} and II^{ry} spermatocytes,** vacuolization and decondensation of chromatin also changed the architecture of the cells was observed as described by Saxena *et al.,* 2004. They noticed significant alteration in the size of primary/secondary spermatocytes, vacuolization and decondensation of chromatin mass.

The **necrosis of germinal elements** has **resulted into formation of large cavities**. These vacuoles were formed either in between the germinal elements or in the basal portion of Sertoli cells. These cavities may be the result of reduction in the population of gonial cells or their extreme shrinkage or their fibrosis due to cytolysis in both the treatment groups. The appearance of these vacuoles is an indication of androgen dependent seminiferous epithelium disruption as evident by a significant fall in the levels of testosterone or antigonadotrophic property of MTX. Since in the present study we did not measure the FSH and LH, a perusal of earlier literature gives an evidence of reduction of gonadotrophins (Shamberger *et al.,* 1981 a, b and Koehler *et al.,* 1986).

Apart from the necrotic changes observed in some experiments the MTX treatment has resulted into partial **arrest of spermatogenesis** at specific stages depending upon the low and high dose (3 and 6mg/kg BW/day for 15 days respectively).

In the low dose treated group the interesting histopathological changes in the seminiferous tubule is **hydropicity or hyalinization or accumulation of watery fluid** in between the germinal epithelial cells. This may be due to accumulation of residual cytoplasm after the disintegration of different stages of

The decreased levels of testosterone was reflected by the atrophy of accessory glands as MTX has a direct effect on Leydig cells thus impairing their normal steroidogenic function (Badri *et al.,* 2000; Gaffan *et al.,* 2003 and Saxena *et al.,* 2004).

Conclusion

From our low and high dose MTX treatment it is concluded that the action of MTX was **antigonadotrophic, antispermatogenic, antispermiogenic** and **antisteroidogenic**.

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