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14. Effect of Follic Acid Antagonist Methotrexate (MTX) on Epididymis of Funambulus Pennanti (Wroughton)

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Abstract

Effect of Follic acid antagonist Methotrexate on epididymis studied by intramuscularly injecting 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 the breeding period January. For comparing the effects saline treated vehicle was injected same amount of saline and were maintained for the same duration. The histological alterations in the caput and cauda epididymis were remarkable. A significant decrease in size and irregularity in contour was associated with an increase in the intertubular connective tissue, extreme thickness of lamina propria, severe regression of epithelium, pyknosis of nuclei often resulting into granulation, fragmentation, vacuolation and dencondensation of chromatin material, irregularity in their placement, heavy loss of nuclei sometimes enucleating lot of epithelial area, their accumulation in the lumen, extreme vacuolation of supra and infra region, an increase in the thickness of smooth muscle bands around each tubule, sometimes disruption of basement membrane and therefore infiltration of macrophages and leukocytes in between the secretory epithelium, prevalence of cellular debris-like flocculent fibrous epididymal fluid, sperm debris, appearance of clear cells and halo cells in large numbers in the caput segment of the treated groups but more in high dose group for the clearance of these debris, extrusion of degenerating epithelial cells in the lumen and hence vacuolation in between the secretory epithelium, sometimes complete detachment of epithelium from the basement membrane, extreme regression of basal cells, the stereo-cilia appeared either clumpped into stiff or limp structure, significant reduction of sperms in both the treatment resulted into oligozoospermia, a few apoptotic germinal elements were also observed in the lumen.

Key Words: Methotrexate, antagonist, caput and cauda

Introduction

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.

Objective

Thus present study embodies

1. Histological changes undergone by caput and cauda epididymis

Experimental Protocol

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 organs were excised, and testes, caput, cauda epididymis, prostate, seminal vesicle were 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 Studies

Caput and cauda were heavily invested with fat. The epididymal epithelium rests over a basement membrane surrounded by a thin lamina propria of smooth muscles, 2-3 layers thick and loose connective tissue rich in blood capillaries. The caput epididymis of vehicle treated

control animals presented its cytoarchitecture quite similar to the caput of normal squirrels. It was lined by pseudo-stratified columnar epithelium in which tall columnar or principal cells and basal cells were clearly seen. The apical area of each principal cell bore a tuft of stereo-cilia floating into the lumen. The lumen was full of maturing sperms (figs. 1 and 2). The tubules of cauda epididymis were large and distended with swarms of sperms. The epithelium appeared of low columnar type (figs. 3 and 4). The apical area of each principal cell bore a tuft of stereo-cilia flaying into the lumen.

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

Histopathological study

Caput epididymis

Noticeable damage in the epididymal tubule was evident by their distorted contour, shrinkage in size moderate thickness of fibromuscular coat, tunica propria lining the tubule was also moderately thickened, the epithelium showed regression in height, supra and infranuclear vacuolation, nuclei appeared pseudo-stratified with granular chromatin material, all nuclei appeared of various shapes, vacuolation indicated loss of chromatin material, the clearity between the junctional zone of two cells was not distinct, hence all cells appeared intermingled with each other. The stereo-cilia were either lost or limp.

Some clear cells were seen among the principal cells. Basal cells have also lost their contour. The lumen showed cellular debris, sperm debris, degenerated germinal epithelium or apoptotic bodies, and few sperms (figs.5 and 6).

Cauda epididymis

The tubules appeared shrunk, due to moderate increase in the inter-tubular connective tissue which has been lost, an artifact due to processing of material, the lamina propria covering each tubule also appeared distorted, the secretory epithelium have lost its distinctness, all cells appeared either compacted at some places, or lost at other places within the tubule. When they were compacted at one place the nuclei appeared as granules of chromatin material due to loss of nuclear wall and cytoplasmic wall between two cells. The other region of epithelium was severely affected as evident by total loss of nucleus and cytoplasm, only the skeleton of fibrous material was left. The occurrence of dark granules in the centre of the lumen indicates the accumulation of lost nuclei from the secretory epithelium. The lumen also showed dry flocculent material, may be cytoplasm or epididymal fluid in the centre. The reduction in sperm number indicated an oligozoospermic condition (figs. 7 and 8).

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

Caput epididymis

The epididymal tubules were reduced in size due to shrinkage, even they appeared sparsely distributed, the fibro-muscular tissue separating these tubules have been lost therefore clear spaces appear in between these tubules, these spaces might be due to degeneration of tissue due to high toxic nature of MTX or an artifact during the processing of tissue. Each epididymal tubule was lined by mildly thick lamina propria. The secretory epithelium have been severely affected due to regressive changes such as reduction in the height of principal cells, extensive supra and infranuclear vacuolation in the cytoplasm, extensive pyknosis of the nuclei, dissolution of junctional complexes of the epithelium. An increase in the macrophages in the inter tubular connective tissue compactment have been noted. The lumen of each tubule revealed cellular debris suggestive of oligozoospermic condition (figs. 9 and 10).

Cauda epididymis

The shrunken tubules were separated by thick amount of inter-tubular connective tissue. Each tubule was lined by moderately thick lamina propria. The epithelium lining the tubule have been lost at many places in each tubule, even some of the tubules were broken, the epithelium showed extensive infranuclear vacuolation, simultaneously there was loss of nuclei, each cell was not distinctly demarcated. Few sperms along with cellular debris were observed in the lumen (figs. 9, 10, 11 and 12).

Discussion

The epididymis, one of **the major male accessory organs** is concerned with the **transport of spermatozoa** and their **maturation and storage** (Robaire and Hermo, 1988; Robaire and Viger, 1995). The microenvironment of the epididymal ductal lumen changes constantly from the initial segment to the distal cauda, and it contributes to the physiological maturation of the spermatozoa during their passage along the epididymal duct (Rabaire and Hermo, 1988). Such a change in microenvironment is due to the activity of the different epithelial cell types lining the epididymal lumen. The major cell types in the epithelial compartment of the duct are the principal, clear, narrow, basal and halo cells (Robaire and Hermo, 1988; Trasler et al., 1988 a and b).

Among the various cell types, the principal cell out numbers all the other cell types combined by at least three to one (Reid and Cleland, 1957; Clermont and Flannery, 1970; Glover and Nicander, 1981; Robaire and Viger, 1995). The principal cell is concerned with at least two important roles; the first is secretion of several proteins, glycoproteins and many other substances several of which contribute to the physiological maturation and survival of the

spermatozoa. The second role is endocytotic removal of particulate as well as dissolved substances from the lumen (Robaire and Hermo, 1988; Robaire and Viger, 1995).

Low levels of testosterone after MTX treatment causes involution in the weights of epididymis and its secretory activity, which may be due to the decreased binding of testosterone (Brooks, 1977). MTX being a potent inhibitor of testicular 3α-hydroxy oxido reduction activity, itself binds to the catalytic binding sites of the substrates like DHT (5α-dihydroxysteroid testosterone) thus reducing the ABP production which would have helped in the maintenance of the epididymis and the accessories.

The histological alterations as observed in caput and cauda epididymis induced by MTX low and high dose daily were noteworthy as well as interesting. The decrease in the size of tubules was associated with moderate increase in the intertubular connective tissue, noticeable decrease in the height of secretory (Principal epithelium, either severe pyknosis of nuclei or their total loss, or vacuolization and decondensation of chromatin mass, or pyknosis often resulting into granulation and fragmentation of nuclei, severe cytoplasmic vacuolation, loss of demarcation between cells due to dissolution of junctional complexes ; the accumulation of chromatin material of these lost nuclei in the lumen along with the sperm debris, sometimes complete detachment of epithelium from the smooth muscle bands, increase in the thickness of smooth muscle bands around each tubule, sometimes disruption of basement membrane and therefore infiltration of macrophages and leukocytes in between the secretory epithelium from the intertubular connective tissue because an increase in the number of macrophages was observed simultaneously (Linnetz and Amann, 1967). Prevalence of cellular debris-like flocculent fibrous non-cellular stained material may be the dried epididymal fluid, degenerating sperms, broken heads and tails and remnants of testicular tissue or darkly stained degenerating testicular tissue (few undifferentiated immature cells also). The debris occurred throughout the entire length of the epididymis since Methotrexate has the ability to cross from blood into interstitial space and seminiferous tubule to induce such changes significantly (Saxena et al., 2004). The debris content is of significance because if the content exceeds the absorptive capacity of the epididymis, it creates a back pressure on the testis, which retards the transportation of the sperm from the testis to the caput epididymis. Appearance of clear cells for the clearance of this debris is necessary which has been observed in MTX treated caput segment of the epididymis. Clear cells were abundant in the low dose treated group but were also numerous in the high dose since the high dose causes an increasing percentage of death of sperms as well as damage to the principal cells. The damage being so powerful so that most of

the cell lost their nuclei and side walls. All the sloughed off nuclei were found to be gathered in the lumen along the debris (Mason and Shaver, 1952; Burgos, 1964; Linnetz and Amann, 1967).

Clear cells were also noticed in secretory epithelium of caput epididymis since the ratio of principal cells to clear cells in the caput is much lower (Trasler et al., 1988a and 1988 b) in the vehicle treated group. The appearance of clear cells in the caput epididymis apparently has an important role in absorption of fluid and substances emanating from the testis or in other words sperm debris. After vasectomy, clear cells in the cauda epididymis have been shown to become distended with vacuoles containing large masses of membranous material (Flickinger, 1972) and after treatment of rats with either Cyproterone acetate or Medroxy progesterone acetate and testosterone (Flickinger, 1976; 1977; Sastry and Gupta, 2003; 2006). After MTX treatment a twofold increase in the basal cells in corpus and cauda as compared to the initial segment and caput epididymis were observed. On the contrary in the present **basal cells** appeared reduced in number and extremely regressed.

Methotrexate leads to an alteration in the relative number and distribution of halo cells in the caput epididymis, since this is only the segment where these cells are most numerous. On the basis of histological characteristics several investigators have suggested that these cells represent lymphocytes (Reid and Cleland, 1957; Hoffer et al., 1973 and Dym and Romrell, 1975). Similarly in both the doses of MTX treated caput epididymis appearance of clear cells and halo cells (few in low dose group but more in high dose group) have been observed. Robaire and Hermo, 1988 have suggested that these cells may represent monocytes. Dym and Romrell, 1975 have proposed that halo cells may play a role in segregation of sperm from the general circulation. The relative percentile contribution of **halo cells** is highest in the caput as compared to other epididymal segments (Trasler et al., 1988a, 1988b; Reid and Cleland, 1957). A relative much higher percentage near the lumen suggests that these cells may be responding to a signal, perhaps from the lumen.

We have also observed an **extrusion of degenerating epithelial cells** into the lumen and hence vacuolation in between the secretory epithelium as described by Akbarsha and Averal, 1998 and 1999. Thus the disturbed histological changes described in the present study are almost similar to those described after surgical castration, hypophysectomy and androgen withdrawl on epithelial cells (Hamilton et al., 1975 and Moore and Bedford, 1979). Cyproterone acetate treatment in Rhesus monkey and men (Kaur et al., 1990), efferent duct ligated rats (Paulson et al., 1985) and seasonal regression of epididymis in squirrel (Reddi and Prasad, 1968) or DMPA treated squirrel (Sastry and Gupta, 2003 and 2006).

The stereo-cilia in the high dose treated groups were largely affected than the low dose. The cilia appeared slant, mostly clumpped together but Rajlakshmi et al., 1990 have described little effect and suggested that there appears to exist differential thresholds of androgens for different organelles within the epididymis. They further suggested that while the secretory activity of the epididymal epithelium was selectively inhibited by interference with the action of androgens with the Cyproterone acetate, all the stereo-cilia appeared normal in treated rats. It is likely that stereo-cilia require much lower levels of androgens to maintain their structural integrity than that required for the maintenance of the secretory epithelium. We disagree strongly with Rajlakshmi et al., 1990 and affirm the dependency of stereo-cilia on the testosterone for their maintenance since in the present study we found significantly low levels of testosterone after high dose treatment.

Our observation confirms a sharp **decline in the androgen level,** since the regressive changes undergone by the epididymis reflects hypoandrogenic status of MTX as suggested by Robaire and Hermo, 1988 and Toney and Danzo, 1988). Takeda et al. (1984) has only described the effect of MTX on epididymis weight but not on histopathological changes therefore ours is the pioneer study on epididymis. Beside the histopathological changes the count of spermatozoa was also declined (oligozoospermia) along with a number of head, tail and mid-piece abnormalities. The epididymis is the site where spermatozoa acquire motility and fertilizing potential (Robaire and Hermo, 1988).

From the foregoing it is concluded that the regressive changes in the caput and cauda epididymis were due to the toxic effect of MTX in dose and duration dependent manner and reduction in the level of testosterone since epididymis is the androgen dependent organ. Moreover, it is noteworthy that no earlier workers tried to describe the various histopathological changes undergone by the epididymis.

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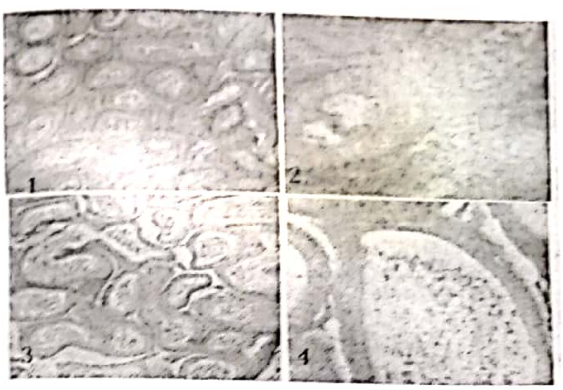

Fig. 1,2,3,4. Vehicle treated control

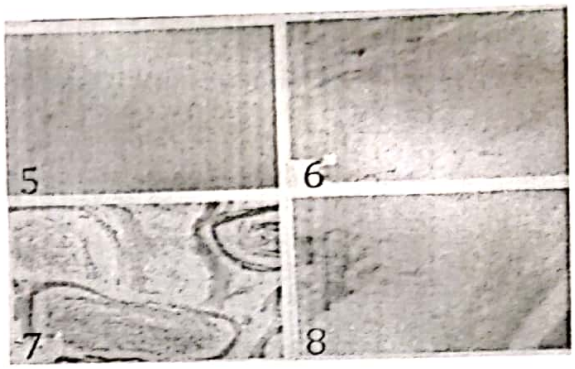

Fig. 5,6,7,8. Low Dose Treatment (6mg/kg BW/day MTX for 15 days)

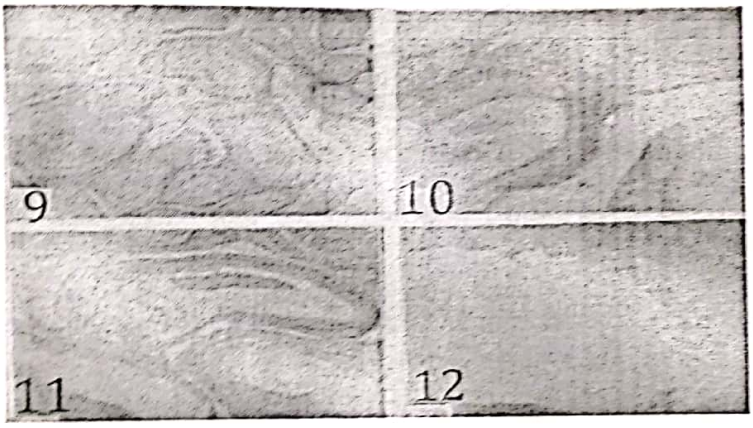

Fig. 9,10,11,12. High Dose Treatment (6mg/kgBW/day MTX for 15 days)