Original Article

Histomorphometric effects of gemcitabine on Swiss albino mice spermatogenesis

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Abstract

Background: Spermatogenesis is a highly conserved and regulated process and it is sensitive to fluctuations in the physical and chemical environment. Gemcitabine is a novel antimetabolic anticancer drug used frequently in the treatment of many cancers. This study aimed to investigate the histomorphometric effects of gemcitabine on spermatogenesis in Swiss albino mice.

Materials and Methods: Gemcitabine in high and low doses (80 and 40 mg/kg) injected intraperitoneally to inbred Swiss albino mice. Gross testicular features and seminiferous tubular histomorphometry was studies at the end of 7th, 14th day and at 2 months sperm shape abnormalities were studied.

Results: Seminiferous tubular morphology was altered significantly, showing a reduction in height, perimeter and area in a dose dependent manner. Sertoli cell number decreased. Basement membrane thickness was reduced and it appeared to be permanent, with statistically insignificant changes even after 2 months. There was a reduction of intertubular spaces. Sperms have shown banana heading, decapitation and loss of normal hook of head. The effects were partially reversible at the end of 2 months.

Conclusion: It was concluded that gemcitabine affects the process of spermatogenesis adversely in a dose and time dependent manner and the effects are partially reversible.

Key Words: Gemcitabine, seminiferous tubules, Sertoli cells, spermatogenesis, Swiss albino mice

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Received: 08.06.2013, **Accepted:** 15.02.2014

INTRODUCTION

Progressive division and differentiation of spermatogonia in seminiferous tubules and production of spermatozoa constitutes spermatogenesis. Seminiferous tubules are composed of supporting somatic cells (Sertoli cells) and germ cells (spermatogonia, spermatocytes and spermatids). Spermatogenesis has in three phases. The process of mitotic division of primary spermatogonia referred as spermatocytogenesis. Second phase is of reductional (meiotic) division. This is followed by a phase of differentiation of spermatozoa called spermiogenesis.[1] This process is highly sensitive to fluctuations in the physical and chemical environment.[1] List of chemicals affecting the sperm production is increasing day by day. As the gametogenesis involves rapid division of cells, drugs especially acting on the cell cycle have adverse effects on it. Chemotherapeutic agents have been shown to have lethal effects on the actively dividing cells involved in spermatogenesis. During "S" phase of the

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.150423

How to cite this article: Viveka S, Udyavar A, Shetty B, Kuriakose S, Sudha MJ. Histomorphometric effects of gemcitabine on Swiss albino mice spermatogenesis. Adv Biomed Res 2015;4:29.

cell cycle, replicating deoxyribonucleic acid (DNA) is susceptible to damage. Purine and puramidine analogs get incorporated in DNA and prevent normal synthesis of genetic material and thereby altering normal cell division.[2-4] The anticancer drug, gemcitabine being a fluorinated nucleoside analog can arrest DNA replication and hence used in the treatment of various carcinomas, including small cell carcinoma lung, breast cancer and pancreatic cancer. As the drug affects not only rapid dividing cells of the tumor, but also normal dividing cells, it can be expected to result in myelosuppresion.[5,6]

MATERIALS AND METHODS

Total 40 inbred adult Swiss albino male mice were subjected into the study, dividing them into three experimental G1, G2 and G-late, of 10 each and a control group having 10. Mice were fed on standard feed and hosted in a 12 h night day cycle environment and allowed water *ad libitum*. Optimum temperatures maintained throughout the experiment. The procedures and the handling of the mice were reviewed and approved by the Institutional Animal Care and Use committee. All mice received humane care in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Intraperitoneal gemcitabine of 40 mg/kg and 80 mg/kg administered to the experimental G1 and G2, respectively. It has been shown that in mice, gemcitabine lethal dose is 333 mg/kg. A dose $1/8^{th}$ of it is selected as a baseline for group G1 mice and half of it is selected for group G2 mice in order to get maximum effects of the drug.[7] Control group mice were treated with intraperitoneal saline. After 7 days, the half the mice from each group were sacrificed by sodium pentobarbital injection and testis with epididymis were dissected out. The G-late group got 40 mg/kg of gemcitabine and half the mice were sacrificed at the end of 30 days and the rest at the end of 60 days. Their testes were studied in order to observe the reversibility of the effects.

Gross features of testis were noted. The specimens were fixed with Bouins' solution. Semen was milked out of the epididymis and vas deferens – smears were prepared.

Drug

Gemcitabine hydrochloride (Gemita lyophilized) is procured from Fresenius Kabi Oncology Ltd. It was reconstituted with distilled water as recommended by the manufacturer (10 ml/vial). The whole reconstituted drug was used for the study at once, within 24 h, in order to maintain the potency of the reconstituted drug. Any unused reconstituted drug is discarded. The reconstituted drug was taken in insulin syringe and administered intraperitoneally.

Histology

The mice testis with epididymis was fixed using Bouin's solution (prepared using 75 ml of picric acid and 25 ml of 10% formalin) for 24 h. Fixed specimens are sectioned at 3-5 μm thickness using a microtome and standard hematoxylin and eosin staining done. The slides thus prepared were observed under the morphometric microscope and analyzed using Jenoptik Optical Systems Digital morphometry microscope, Business Unit Digital Imaging, Goeschwitser Str. 25, 07745 Jena, Germany. Software used: ProgRes® Capture Pro 2.7 release 003.

Three non-serial testicular sections were used for the morphometric analysis and in each section 10 tubules were quantified, adding to 30 tubules per animal. The following parameters were analyzed and tabulated. The seminiferous tubular dimensions and epithelial cell height were studied.

Statistical analysis

All data observed were expressed as mean ± standard deviation and statistical significance of the histomorphometric data observed was performed by one-way ANOVA and Student's *t*-test ($P < 0.05$ was accepted as statistically significant).

RESULTS

Gross morphology

There was significant weight loss of testis in G1 and G2 group both at 7 and 14 days in comparison to control group. G-late group showed a significant increase of weight at the end of 2 months in comparison mice groups studied at 7 and 14 days. All parameters noted are tabulated in Table 1. There was statistically no difference between the control group (119.3 ± 11.4) and G-late group (117.7 ± 10.5) in terms of weight of testis. There was decreased visible vascularity of the tunica albuginea over testis.

The seminiferous tubular histomorphometry

Normal histoarchitecture appreciated in control group [Figure 1]. G1 and G2 showed a significant decrease in diameter perimeter and area of the seminiferous tubules both at 7 and 14 days ($P < 0.01$). The seminiferous epithelial height in the control group was 74.41 ± 10.9 μ. In the group treated with drug, the epithelium was reduced to a single layer in few of the tubules [Figure 2]. The epithelial height in G1 and G2 at the end of 7 days were 33.74 ± 3.02 μ and 29.72 ± 5.53 μ, respectively. The reduction was

Table 1: Tabulation of gross testicular features, histomorphometric parameters of seminiferous tubules and sperm shape abnormalities in control, G1 and G2 groups at the end of 14 days (for consideration of acute effects of drug) and in G-late group at the end of 2 months (for considering reversibility of effects of drug)

Parameter	Control	G1	G2	G-late
Gross testicular features				
Mean weight (in g)	22.9±11.2	18±0.5*	17.2±1.6*	18.4±1.7*
Testis+epididymis weight (in mg)	119.3±11.4	108.2±8.3*	100.9±8**	117.7±10.5†
Length of testis	6.6±0.8	5.6±0.9*	5.2±1.1*	6.3±0.9†
Breadth of testis	5.5±0.5	4.9±0.8*	4.6±0.9**	5.3±0.6†
Histomorphometry of seminiferous tubules				
Diameter (in μm)	227.42±72.5	143.94±68.3*	162.8±53.7*	182±25.57†
Perimeter (in μm)	688.27±56.5	422.2±70.59*	410.45±60.0	544.62±77.67
Area (in sq. μm)	33345.2±12988.8	12513.7±2409.82*	8849.67±1670.57**	17977.5±541.31*
Epithelial height (in μm)	74.41±10.9	33.74±3.02*	29.72±5.53*	40.6±10.2*
Sertoli cell nuclear area (in sq. μm)	36.19±13.59	16.59±1.96*	10.78±2.96**	27.09±8.65†
Basement membrane thickness (in μm)	8.13±0.61	2.55±0.33*	2.43+0.65*	2.29±0.61*
Sperm shape abnormalities (number of abnormal sperms/500 sperms counted)				
Hookless	0.4	1.4*	2.6*	0.6
Banana	0.5	1.6*	2.5*	0.4
Amorphous	0.5	0.8*	1.9*	0.5
Double head/tail	0	0	0	0
Total	1.4	3.8*	7**	1.5†

*P<0.05; **P<0.01; †P>0.1

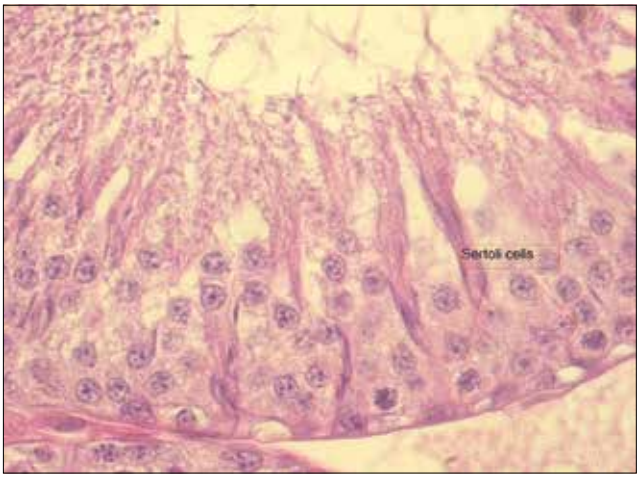

Figure 1: Seminiferous epithelium, control mice, H and E, high magnification

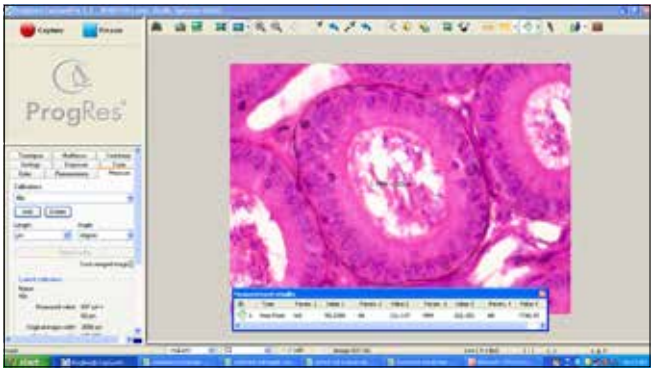

Figure 2: Screen shot of the measurement of seminiferous tubular dimensions using morphometric microscope and ProgRes Software, ×40

highly significant (P < 0.01). The spermatogenic cells were detached from the basement membrane. Spermatogenic activity was halted. Intraepithelial bleb noted [Figure 3]. The sperms in some of the tubules (especially in mice exposed to acute effects of higher dose of gemcitabine, as in G2 group after 7 days) had clumped to a corner [Figure 4]. In later stages when the effects of the drug wear off (in G1 group after 14 days) spermatogenic cells filled the entire lumen of the tubule. In both group at the end of 14 days – spermatogenic cells showed intense nuclear hyperchromatism. Differentiation of primary and secondary spermatogonia was not possible.

There was thinning out of basement membrane in mice treated with drug during initial days (8.13 μ in control vs. 2.55 μ in G1 group, P < 0.01). Sertoli cell nuclear dimensions were also reduced in G1 and G2 mice at 7 and 14 days [Table 1]. G-late group showed a significant increase in seminiferous epithelial height, diameter, perimeter and areas of cut section of seminiferous tubules in comparison to mice administered with drug at 7 and 14 days. The area of seminiferous tubules in control and G-late groups were 33345.2 and 17977.5 μ^2 respectively (P > 0.1). The seminiferous tubular cytoarchitectural observations of G-late group were almost similar to control group. The sequential changes in the seminiferous epithelial height are shown Figure 5. Basement thickness and Sertoli cell nuclear dimensions were similar to control

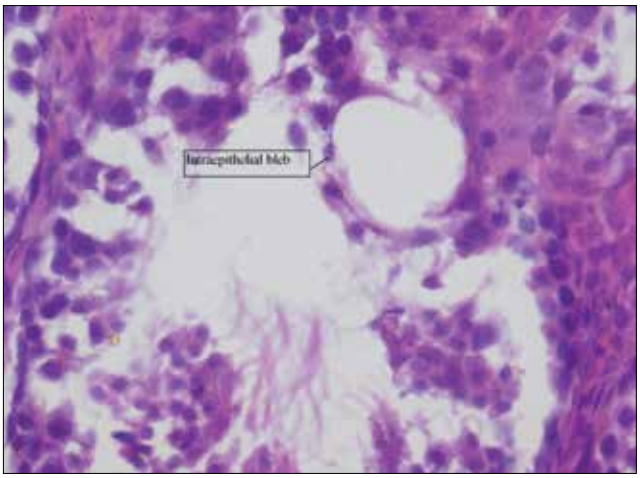

Figure 3: Seminiferous tubule from G2 at 7 days, showing intraepithelial bleb, H and E, ×40

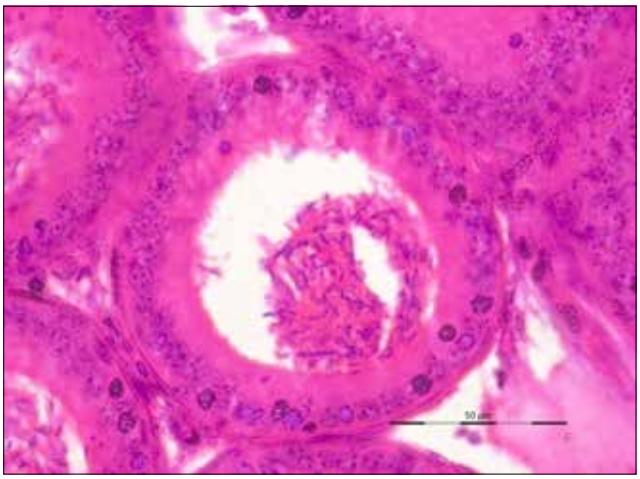

Figure 4: Seminiferous tubule from G1 at 7 days, showing marked decrease in the tubular dimensions, with epithelium virtually reduced to single layer and sperms clumped to a corner

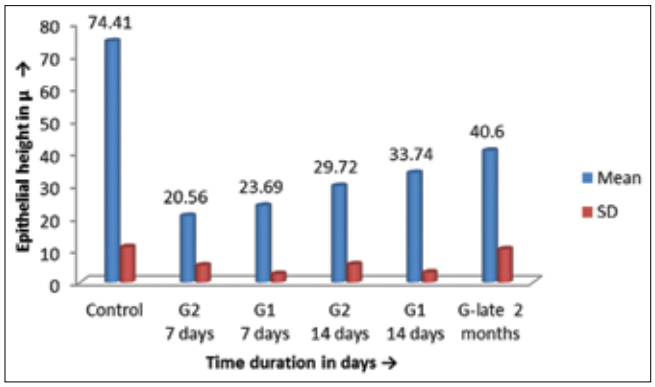

Figure 5: Representation of mean and standard deviation of seminiferous tubular height in the groups studied, shows sequential increase in the seminiferous epithelial height with decrease in dose and increasing time

group mice. Gemcitabine has resulted in a dose and time dependent statistically significant decrease in the mean height of seminiferous epithelium and the effects are reversible, though not completely.

Sperm shape abnormalities

The sperms from the G1 and G2 group showed several abnormalities: Lack of usual hook, banana such as head, amorphous head, folded sperms, bent head, bent neck, decapitated sperms and vacuole in the sperm head.

DISCUSSION

The testis has very active prosurvival and proapoptotic systems that work together to regulate the extent of germ cell apoptosis. The germ cell proliferation is tightly regulated system of physiological apoptosis optimizing the output to a level sustainable by the seminiferous epithelium. Apoptosis will be markedly increased after exposure to toxic insult, indicating that the seminiferous epithelium is dysfunctional with suboptimal support.[8] The seminiferous tubular epithelium may recover if the toxicant is not too severe or exposure is for short duration.

The drug has resulted in decrease in weight of mice testis. The magnitude of the loss of weight in the group treated with therapeutic dosage of gemcitabine is up to 15% and 27%, respectively at the end of 7 and 14 days. There is statistically significant reversible decrease in weight of the animal immediately after the administration of the drug, accounted by its acute toxic effect on the mucosa of digestive tract; recovery of which leads to subsequent sequential increment in weight of the animal. Similar reduction in the combined weight of testis and epididymis is noted with decrease in its visible vascularity; the acute toxic effect of the drug on the endothelium of the vessels possibly decrease the blood supply to the testis and resulted in gross decrease in its weight. Similar alterations in the weight are also reported by Meistrich *et al*., in their experiment where they have observed the effect of 14 different toxics on mice testis and spermatogenesis.[9] Chemotherapeutics in particular, being very active against actively dividing cells, have more effect on spermatogenesis and in turn weight of testis.[10] Similar and comparable decrease in the weight of mice at the end of 5th week is reported using vincristine sulfate.[11]

It has been conclusively shown in this study that gemcitabine arrest the normal spermatogenesis at early stage (primary spermatocytic cycle) in majority of the seminiferous tubule as evidenced by the significant decrease in the seminiferous tubular dimensions, decrease in the seminiferous epithelial height and

by morphological changes in the spermatogenic cells. The effects are dose and time dependent. As the mice exposed to the supranormal dose shown statistically significant more disruption of seminiferous tubular morphology than the mice exposed to therapeutic dose. Similar dose dependent decrease in the spermatocytic activity is reported using 14 various mutagens on mice.[12] The changes in the spermatogenic cells have been observed by various authors using array of toxic chemicals, ranging from heavy metals to the modern day most sophisticated chemotherapeutic agents, from plant extracts to physical constraints like prolonged hypoxia on testis. Most of the studies do correlate with human beings in that comparable effects can be seen in humans naturally exposed to these chemical and physical agents. Gemcitabine being a deoxycytidine analog requires an intracytoplasmic phosphorylation to convert to active gemcitabine-nucleotides, gemcitabine 2',2'-difluoro 2'-deoxycytidine diphosphate and gemcitabine 2',2'-difluoro 2'-deoxycytidine triphosphate, which in turn inhibit DNA synthesis. This possibly explains the arrest of the spermatogonial cells in the early stages of meiosis where DNA synthesis has to occur; and to a lesser extent to the formation of relatively unstained crescents surrounding the chromatin, perceived as perinuclear halo in the histology.

In the present study, the spermatogenic cells have been reduced to single layer, showing complete halt of spermatogenesis. In most of the tubules studied from the G2 and G1 mice testis the spermatogenetic halt was evident by reduced epithelial cell height and lack of sperms in the lumen. Injection of imatinib mesylate to mice gives similar results, in less than 2 weeks.[13] Decrease in epithelial height reported after injection of doxorubicin to mice.[14] As the meiosis in most of the germ cells have halted in early stage, in this study it was not possible to differentiate the primary and secondary spermatocytes in most of the tubules during acute phase of the drug administration.

The detachment of spermatogenic cells from the basement membrane, if preparation artifacts are effectively removed, will indicate an altered interaction with basement membrane. Similar kinds of alterations are also observed after use of many anticancer drugs especially reported with mitomycin C.[15,16] Appearance of intraepithelial vacuolations may be due to intraepithelial edema and altered intercellular connections, due to acute cytological toxicity of the drug used. Similar intraepithelial vacuolations are reported in mice treated with Neem extract,[17] Brahmi leaves[18] and diazacholesteroldihydrochloride.[19] Occasional such intraepithelial vacuolations are observed in the current study [Figure 3].

Clumping of the sperms inside the seminiferous tubules are the indication of halt of normal spermatogenesis, loss of junctional complexes between the adjacent Sertoli cells, mitochondrial membrane damage, plasma membrane damage with profound disturbances in the membrane functions of spermatozoa in the lumen as a result of hypoxic and hyponutritive environments prevailing in the seminiferous tubules under the influence of the drug. Aggregates of sperms are well-documented in many mice spermatogenesis studies.[12,20,21]

All the abnormalities observed have undergone statistically significant reversal after 30 and 60 days interval. Though the seminiferous tubular morphology has regained near normal morphology, there exists statistically significant difference with the control group. The wearing effect of the drug and recovery of spermatocytic cells from acute toxic effects of the drug, may account for such a recovery. The permanent loss of spermatogonial stem cells may explain lack of complete recovery.

Though it has shown that spermatogenesis is inhibited in most initial stages, the cells which are already recruited in the process showed some structural alterations in sperms such as hookless, banana shaped, amorphous, folded sperms, bent heads. Significant number of sperms ware decapitated. Misshapen sperm count is dose and time dependent. The highest mean values of total abnormal sperm and most other kinds of abnormalities were in G2 at 7th day. The proportion of misshapen sperm may be influenced by genetic background.[22] According to human data, gemcitabine resulted in moderate to severe hypospermatogenesis, decreased number fertility reversible.[23] Animal data also found that the drug resulted in decreasing spermatogenesis and fertility in male albino mice.[7] It is plausible that gemcitabine causes structural chromosomal aberrations in spermatozoa of treated animals these chromosomal abnormalities reflect on sperm head morphologies.[24,25]

The increases in the fractions of abnormal sperm shapes, specifically to hookless and banana headed types is probably due to the disruptive effect of gemcitabine on the DNA synthesis. This leads to changes in differentiation during gene expression, including later steps of transcription and translation of the genetic message.

CONCLUSION

With this study it is concluded that the spermatogenesis is highly regulated and conserved process. Gemcitabine even in therapeutic doses adversely affects the process

of sperm production and caution is advised during administration of drug for reproductively active males. There is significant reversal of adverse effects caused by the drug in 2 months of duration after a single therapeutic dose administration in mice.

ACKNOWLEDGMENT

Dr. Ramakrishna Avadhani, Prof and Head, Department of Anatomy, Yenepoya Medical College, Mangalore, Dr. Seethalakshmi, Professor, Department of Pharmacology, A J Institute of Medical Sciences, Mangalore, Dr. Mohandas Rai, Prof and Head, Department of Pharmacology, A J Institute of Medical Sciences, Mangalore.

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Source of Support: Nil, **Conflict of Interest:** None declared.