ACUTE EFFECTS OF GEMCITABINE ON SEMINIFEROUS TUBULAR HISTOMORPHOMETRY IN MICE MODEL

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ABSTRACT
Chemotherapeutic agents have adverse effects on spermatogenesis. The gonadotoxic effects of these agents are of significance in young cancer patients undergoing chemptherapy. Gemcitabine is a novel antimetabolic anticancer drug used frequently in treatment of many cancers. This study was designed to evaluate the acute effects of gemcitabine on spermatogenesis in mice model. Gemcitabine in high and low doses (80 and 40 mg kg⁻¹) injected intraperitoneally to inbred Swiss albino mice. Gross testicular features and seminiferous tubular histomorphometry was studies at the end of 7th, 14th day. Sperm shape abnormalities studied. We noted that seminiferous tubular morphology was altered significantly, showing reduction in height, perimeter and area in a dose dependent manner. Sertoli cell number decreased. Basement membrane had reduced thickness. Intertubular spaces has reduced. Sperms had abnormal shapes with banana heading, decapitation. It was concluded that gemcitabine affects the process of spermatogenesis adversely in a dose dependable manner.

Keywords: Swiss albino mice, spermatogenesis, Gemcitabine, seminiferous tubules, Sertoli cells
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. This process is highly sensitive to fluctuations in the physical and chemical environment. 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 cell cycle, replicating DNA is susceptible to damage. Purine and pyrimidine analogues get incorporated in DNA and prevent normal synthesis of genetic material and thereby altering normal cell division. The anticancer drug, gemcitabine being a fluorinated nucleoside analogue can arrest DNA replication and hence used in treatment of various carcinomas, including small cell carcinoma lung, breast cancer and pancreatic cancer. As the drug affects not only rapid dividing cells of tumor but also normal dividing cells, it can be expected to result in myelosuppresion. There is paucity of literature regarding its effect on gametogenesis. Its effects on Sertoli cells haven’t been reported in literature to the best of our knowledge. The aim of the current study is to investigate the gross and histological changes in Swiss albino male mice treated with intraperitoneal Gemcitabine in clinical and supraclinical doses. It was also attempted to record the reversibility of the acute effects due to administration of the drug. During study, a new method to quantify the Sertoli cell damage by considering its nuclear area was evaluated.

MATERIALS AND METHODS
Total forty adult Swiss albino male mice were subjected into the study, dividing them into 2 experimental G1 and G2 of 10 each and a control group having 10. Mice were fed on standard feed and hosted in a 12 hour 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 CPCSEA.
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.[6]

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. Gross features of testis were noted. The specimens were fixed with Bouins’ solution. Semen was milked out of 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 per vial). The whole reconstituted drug was used for the study at once, within 24 hours, 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 hours. Fixed specimens are sectioned at 3-5µm thickness by using a microtome and standard haematoxylin and eosin staining done. The slides thus prepared were observed under the morphometric microscope and analysed 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 ten tubules were quantified, adding to 30 tubules per animal. The seminiferous tubular dimensions and epithelial cell height were studied.

**Statistical analysis**

All data observed were expressed as Mean ± SD (standard deviation) and statistical significance of the histomorphometric data observed was performed by one-way ANOVA and Student ‘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 & 14 days in comparison to control group. All parameters noted are tabulated in table 1. There was decreased visible vascularity of the tunica albugenia over testis.

Table 1: Tabulation of gross testicular features, histomorphometric parameters of seminiferous tubules and sperm shape abnormalities in control, G1 & G2 groups at the end of 14 days (for consideration of acute effects of drug)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>G1</th>
<th>G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean weight (in g)</td>
<td>22.9 ±11.2</td>
<td>18 ± 0.5*</td>
<td>17.2 ± 1.6*</td>
</tr>
<tr>
<td>Testis</td>
<td>119.3 ± 11.4</td>
<td>108.2 ± 8.3*</td>
<td>100.9 ± 8**</td>
</tr>
<tr>
<td>Length of testis</td>
<td>6.6±0.8</td>
<td>5.6±0.9*</td>
<td>5.2±1.1*</td>
</tr>
<tr>
<td>Breadth of testis</td>
<td>5.5±0.5</td>
<td>4.9±0.8*</td>
<td>4.6 ±0.9**</td>
</tr>
<tr>
<td>Diameter (in µm)</td>
<td>227.4±72.5</td>
<td>143.94±68.3*</td>
<td>162.8±53.7*</td>
</tr>
<tr>
<td>Perimeter (in µm)</td>
<td>6.88 ±5.6.5</td>
<td>422.2 ±70.59*</td>
<td>410.45±60.*</td>
</tr>
<tr>
<td>Area (in sq. µm)</td>
<td>33345.2±12988.8</td>
<td>12513.7±2409.82</td>
<td>8849.67±1670.57**</td>
</tr>
<tr>
<td>Epithelial height (in µm)</td>
<td>74.41 ±10.9</td>
<td>33.74 ±3.02*</td>
<td>29.72 ±5.53*</td>
</tr>
<tr>
<td>Sertoli cell nuclear area (in sq. µm)</td>
<td>36.19±13.89</td>
<td>16.59±1.96*</td>
<td>10.78 ±2.96**</td>
</tr>
<tr>
<td>Basement membrane thickness (in µm)</td>
<td>8.13±0.61</td>
<td>2.55±0.33*</td>
<td>2.43+0.65*</td>
</tr>
<tr>
<td>Sperm shapes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hookless</td>
<td>0.4</td>
<td>1.4*</td>
<td>2.6*</td>
</tr>
<tr>
<td>Banana</td>
<td>0.5</td>
<td>1.6*</td>
<td>2.5*</td>
</tr>
<tr>
<td>Amorphous</td>
<td>0.5</td>
<td>0.8*</td>
<td>1.9*</td>
</tr>
<tr>
<td>Double head/tail</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1.4</td>
<td>3.8*</td>
<td>7 **</td>
</tr>
</tbody>
</table>

Note: * = p<0.1, ** = p<0.01

The seminiferous tubular histomorphometry
Normal histoarchitecture appreciated in control group (figure 1). G1 and G2 showed significant decrease in diameter perimeter and area of the seminiferous tubules both at 7 & 14 days (p<0.01). The seminiferous epithelial height in control group was 74.41±10.9µ. In the group treated with drug, the epithelium was reduced to single layer in few of the tubules.
The epithelial height in G1 and G2 at end of 7 days and 14 days were 33.74±3.02µ and 29.72±5.53µ respectively. The reduction was statistically significant (p<0.01). The spermatogenic cells were detached from the basement membrane. Spermatogenic activity was halted. Intraepithelial bleb noted. 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 3). In later stages when the effects of drug wear off (in G1 group after 14 days) spermatogenic cells filled the entire lumen of the tubule. In both the group at end of 14 days – spermatogenic cells showed intense nuclear hyperchromatism. Differentiation of primary and secondary spermatogonia was not possible. Changes in tubular epithelial height shown in figure 4.

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. 40x. The picture depicts seminiferous tubular epithelium reduced to single layer.
There was thinning out of basement membrane in mice treated with drug during initial days (8.13µ in control vs 2.55µ 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). The sequential changes in the seminiferous epithelial height are shown figure 4. Basement thickness and Sertoli cell nuclear dimensions were similar to control group mice. Gemcitabine has resulted in a dose dependent statistically significant decrease in the mean height of seminiferous epithelium.

**Sperm shape abnormalities**

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

Figure 3: Seminiferous tubule from G2 at 7 days, showing marked decrease in the tubular dimensions, with epithelium reduced to single layer and sperms clumped to a corner.

![Image](image_url)

**Figure 4:** Representation of mean and standard deviation (SD) of seminiferous tubular height in the groups studied, showing sequential increase in the seminiferous epithelial height with decrease in dose and increasing time.
DISCUSSION
Seminiferous tubular cell growth and differentiation is highly regulated process. Tubular germ cell and supporting cells undergo apoptosis if they are exposed to high doses of toxic insults.[7] In the present study the insulting drug has halted the process of spermatogenesis as evidenced by histoarchitectural changes. 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 were dose dependant. Similar dose dependent decrease in the spermatocytic activity is reported using 14 various mutagens on mice.[8] These studies if correlated to humans, implicates that these toxic substances will alter the fertility of patients undergoing treatment.

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.[9] Decrease in epithelial height reported after administration of doxorubicin to mice.[10] 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. Clumping of the sperms inside the seminiferous tubules are the indication 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. Similar changes in tubular cells are reported in literature. Aggregates of sperms are well documented in many mice spermatogenesis studies.[8,11,12]

Though it has shown that spermatogenesis was inhibited in most initial stages, the cells which were recruited in the process showed some structural alterations in sperms like hookless, banana shaped, amorphous, folded sperms, bent heads. Significant number of sperms were decapitated. Abnormalities of sperm count ware dose dependant.

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.

REFERENCES


