EFFECTS OF AN ONCOSTATIC DRUG ETOPOSIDE (VP16–213) ON SPERM MORPHOLOGY AND SPERM COUNT OF MALE SQUIRREL Funambulus pennanti

Sastry M. S.*1 and Chaudhari R.M.2

1. Department of Zoology, R.T.M. Nagpur University, Nagpur (INDIA)
2. P.S.G.V.P. Mandal’s, G.B. Patel Science College, Shahada (INDIA)

Received August 10, 2012

ABSTRACT
Etoposide (VP16–213), an oncostatic drug was administered intramuscularly to six adult male Indian palm squirrel, Funambulus pennanti (Wroughton) during the active breeding period at the dose level of 10 and 30mg/KgBW/day respectively with the object of examining its toxicities on the testis and accessory organs for 30 days. Both the treatments resulted into abnormalities in the sperm morphology, sperm count and motility. The various head defects were extreme elongation, compression, lateral displacement of head, loss of acrosome, amorphous or deformed head, compressed head with across one thrown into bulb, loss of DNA and hence vacuolation in the head, pin-head condition, nuclear swelling, stippling in chromatin, unexpected curvature of the head, pyriform head. The mid-piece defects were swollen mid-piece, loss of mid-piece. The tail defects were unusually long and double tails. The sperm count was also low as compared to vehicle treated control and resulted into oligospermia along with an increased percentage of sloughed off testicular cells in various stages of apoptosis. Sperm motility was of rotational type. Thus the study of etoposide conclusively demonstrated that this drug belongs to the category of antineoplastic, antiandrogenic and anti-spermatogenic leading to infertility when used in the cancer patients.

Key Words : Etoposide, Squirrel, Oligospermia, Oligozoospermia, Anti-spermatogenic

INTRODUCTION
Etoposide (VP16–213) is a semisynthetic derivative of podophyllotoxin which is used as an oncostatic agent against testicular cancer, lymphomas, Hodgkins disease and leukemia. It is a topoisomerase II poison which leads to DNA strand breakage.1–4 A perusal of literature showed that even though an extensive research has been done on toxicity of several drugs on testis but information on sperm analysis is scanty. Therefore it was felt interesting to study the side effects of this anticancer drug.5–7

MATERIAL AND METHODS
Animals and treatment
Adult male squirrels weighing between 100 to 150gms were trapped alive in and around Nagpur city during the breeding period from January to July 2005.8 After a week of acclimatization to laboratory conditions, Etoposide (VP16–213) dissolved in saline was administered intravenously. The control animals received same amount of saline (Table 1). The animals were sacrificed using chloroform 24 hours after the last day of each experiment. Immediately cauda epididymidis was excised.

Sperm analysis
The spermatozoa present in the cauda epididymidis were collected after mincing/slicing the tissue in a cavity block containing 1ml of physiological saline centrifuged at 600rpm for 1 minute and a drop of 5% aqueous eosin.8 Since, Phase contrast microscope was not available an ordinary light microscope was used by lowering the condenser to disperse the light for unstained preparations. All evaluations were done at 100X.

Assessment of sperm motility
Motility of sperms in the experimental and control animals were determined by putting a
Several times observation of caudal epididymidal depths less than 20µm constrains the rotational movement of spermatozoa. The freshly made wet preparation was left to stabilize for approximately one minute. The motility of spermatozoa from treated groups were observed as follows:

a. Rapid progressive motility
b. Slow or sluggish progressive motility
c. Non-progressive motility
d. Immotility
e. Rotational motility

**Table 1: Experimental design for etoposide (VP16-213) treatment**

<table>
<thead>
<tr>
<th>Number of animals and sex</th>
<th>Treatment</th>
<th>Dose (mg/KgBW/day)</th>
<th>Route</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 males (Experimental)</td>
<td>Etoposide</td>
<td>10 mg</td>
<td>I.M.</td>
<td>30 days</td>
</tr>
<tr>
<td>6 males (Experimental)</td>
<td>Etoposide</td>
<td>30 mg</td>
<td>I.M.</td>
<td>30 days</td>
</tr>
<tr>
<td>6 males (Control)</td>
<td>Saline</td>
<td>Equal volume</td>
<td>I.M.</td>
<td>30 days</td>
</tr>
</tbody>
</table>

I.M. = Intra muscular. BW = Body weight

**Sperm count**

Sperm count was done by using Neubauer’s haemocytometer. The sperms were counted in five Thoma ruled chambers after charging the haemocytometer with the above solution and calculated by using the formula 50,000 n x d where ‘n’ is the number of sperms and ‘d’ is dilution which was 1ml.

**Assessment of sperm morphology**

The saline solution of cauda and caput epididymis prepared for studying the sperm concentration was directly observed several times for assessing the sperm morphology.

**Statistical analysis**

To indicate individual variations in sperm count, the mean values and standard deviation (mean ± SD) for measurements from six animals were calculated. The statistical significance of differences for these values were assessed using ‘t-test’\(^{10}\). A significant level of P<0.05 was accepted.

**RESULTS AND DISCUSSION**

**Sperm count**

**Vehicle-treated controls**

As the animal was in breeding period the epididymis of vehicle-treated control showed swarms of spermatozoa and hence a condition of normospermia exited (range 65.00 ± 2.35).

**10mg/KgBW/day treatment**

Reduction in the number of sperms was observed in the aqueous saline solution (range 26.50-29.50 10\(^5\)/ml, Fig.1). Thus a condition of oligospermia was observed. Beside the sperms sloughed off testicular cells (spermatocytes and spermatids) were found to be plenty undergoing apoptosis.

**30mg/KgBW/day treatment**

The range was found to be 15.10 – 18.60 10\(^5\)/ml. A condition of oligozoospermia or diminished sperm count was noted because of extremely low sperm count along with an increased percentage of sloughed off testicular cells in apoptosis (Fig. 1).

**Sperm motility**

**Vehicle-treated controls**

Rapidly fast moving sperms were observed.

**10mg/KgBW/day treatment**

Some sperms were immotile, some showed non-progressive movement remaining others showed either random forward or rotational movement.

**30mg/KgBW/day treatment**

The sperms were extremely low in number, mostly they were found to be immotile or showing rotational movement.

**Sperm morphology**

**Vehicle-treated controls**

The head was oval, acrosomal region was well defined, occupying 40-70 % of the head area. Mid-piece region was slender about one and half times the length of the head and attached axially to the head. The tail region was straight, uniform, and thinner than the mid-piece and uncoiled (Fig. 2 and Fig. 3).

**10mg/KgBW/day treatment**

Several times observation of caudal epididymidal slices in measured quantity of saline showed following types of abnormalities of sperm.

---

<table>
<thead>
<tr>
<th>Number of animals and sex</th>
<th>Treatment</th>
<th>Dose (mg/KgBW/day)</th>
<th>Route</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 males (Experimental)</td>
<td>Etoposide</td>
<td>10 mg</td>
<td>I.M.</td>
<td>30 days</td>
</tr>
<tr>
<td>6 males (Experimental)</td>
<td>Etoposide</td>
<td>30 mg</td>
<td>I.M.</td>
<td>30 days</td>
</tr>
<tr>
<td>6 males (Control)</td>
<td>Saline</td>
<td>Equal volume</td>
<td>I.M.</td>
<td>30 days</td>
</tr>
</tbody>
</table>
Fig. 1: Spermatozoal counts in the cauda epididymidis after 10mg and 30mg/KgBW Etoposide (VP16-213) daily for 4 weeks treatment with vehicle and Etoposide treatment (control, n=6, Etoposide, n=6). There was a significant decrease in the sperm count when compared with the vehicle. *P<0.001

Fig. 2: Few sperms photographed from the vehicle treated control squirrel. Note oval head, slender axially attached midpiece and thin straight tail X 225

Fig. 3: A single sperm photographed from vehicle treated control X 400
Failure of the basal plate to attach to the nucleus at the opposite pole to the acrosome caused the heads and tails to detach on spermiation. The heads were absorbed and only tails were found giving the pin-head defect. Therefore, in the present work, pin-heads were not counted as head defects, however, these were not frequent. We could not keep a count of sperm defects but whatever defects were observed we photographed immediately by staining them in 0.5% aqueous eosin. The morphological defects resulted in head, tail and mid-piece deformities. The head defects included pyriform head, unexceptional curvature of head, nuclear swelling, amorphous head, deformed head, extreme elongation of head, lateral compression of head, vacuolation of head due to loss of DNA, acrosome thrown into bulb, slitting of head, decondensation of swollen head, largeness of head, stippling of chromatin, loss of acrosome, unusual compression of head. The neck defects included swelling of mid-piece, loss, asymmetrical insertion of middle piece and the tail defects were wavy or bent at tip, unusually long tail, bifurcation of tail Fig. 4 and Fig. 5.

Fig. 4: Sperms after slicing the cauda epididymidis in saline solution photographed from low dose treated Etoposide (10mg / KgBW/ day) for 30 days. The sloughing off of testicular cells is remarkable. The various head defects are unusually compressed head (arrow), vacuolated acrosome thrown into bulb (arrow head), Nuclear swelling-a diploid condition (thick arrow), unusually large head, stippling in the chromatin (thin arrow), amorphous head (broken arrow), decondensation of nucleus (long arrow), pin-head condition X 1000

Fig. 5: From the same regimen sperms showing different Head defects – amorphous head (arrow) deformed head (arrow head), extremely elongated and compressed head (thick arrow); lateral displacement of head (thin arrow), unusual compression of head (long arrow), loss of acrosome (short arrow), head compressed and thrown into bulb (broken arrow), loss of DNA and hence vacuolation in the head (arrow), pin-head condition (arrow), bifurcation of tail (arrow) X 1000
30mg/KgBW/day treatment

In high dose treatment sperms showed the defects mostly related with head, neck and tail as described for low dose treatment, however, the defects were more severe Fig. 6 to Fig. 8

Fig. 6: Sperms in the saline solution photographed to show various morphological defects after 30mg / Kg BW / day Etoposide for 30 days. The head defects are pin-head condition (arrow), extremely laterally compressed head (arrow-head), broken head showing vacuolation due to loss of DNA (thick arrow) or decondensed nucleus (thin arrow). Also note huge amount of testicular gonial cells exfoliation (long arrow) X 1000

Fig. 7: Photographs of squirrel epididymal spermatozoa from chronic, daily treatment (30mg KgBW) The fall in count is extreme. Please note vacuolated acrosome thrown into bulb (arrow) and unusually long tail X 1000

Fig. 8: Nucleus is extremely compressed and decondensed (arrow), asymmetrical insertion of middle piece (arrow head), swollen mid-piece (thick arrow) and unusually long tail from the same treatment X 400
The sperm count is the first step in evaluation of fertility in normal and treated groups of animals. Etoposide low dose treatment (10mg/KgBW/day) caused reduction in the number of sperms (oligospermia) whereas high dose treatment (30mg/KgBW/day) caused severe loss of sperms (oligozoospermia) since Etoposide is a potent inducer of apoptosis and the most sensitive cells included pachytene and dividing spermatocytes similarly because of decrease or loss of spermatogonia.

Sperms have two principles namely motility and fertilizing ability. Motility is directly related with fertilizing ability. In the low dose and high dose treatment either immotile with non-progressive movement with random forward movement or rotationally moving sperms were observed. It may be explained that structural abnormalities of the sperm flagella, mid-piece, bifurcation or multifurcation of the tail in the present study has no effect on the progressive motility. According to Huguchi et al., such abnormalities of tail do not affect sperm motion.

The precise sperm morphology governs the fertility of an individual. A perusal of literature revealed that there are no reports on the Etoposide induced morphological changes in sperms therefore the present study have given valuable data on this subject. Even though we could not maintain the percentage of abnormalities but the different types of abnormalities existed were head, middle piece and tail. Thus the treatment manifested antian drogenic and antifertility effects causing reduction in sperm density decrease and in motility with an increase in rotational movement and in structural manifestation of various types of morphological defects.

CONCLUSION
Thus the study of Etoposide conclusively demonstrated that this drug belongs to the category of antigonadotrophic, antiandrogenic and anti-spermatogenic leading to infertility when used in the cancer patients.

REFERENCES
12. Takahashi N., Kadota T., Kawano S., Ohta K., Ishikawa K., Kuroyangi K., Hamajima


