CHAPTER VII

HISTOLOGICAL STUDIES AND THE SPERMATOGENIC ACTIVITY IN RATS AFTER TREATMENT WITH MITOMYCIN C ALONE AND TOGETHER WITH EITHER HCG OR TESTOSTERONE PROPIONATE

The testis is composed of an endocrine (Leydig cells) and the exocrine (Sertoli cells) components. The former is concerned with steroid hormone production, the latter primarily with the formation of male gametes. The quality of the spermatozoa produced depends eventually upon the metabolic exchanges, upon the nuclear and cytoplasmic synthesis and upon the structural transformations occurring during meiotic prophase and spermiogenesis. This aspect of spermatogenesis is still little understood and will be the field of important investigations. Finally, the attainment of spermatozoal populations capable of acquiring a high fertilizing power is in fact one of the essential roles of the normal functions of the testis. The direct study of Christenson and Mason in 1965 (1) suggests that conversion of progesterone-4-C\textsuperscript{14} to 17-OH-progesterone, androstenedione, testosterone and progesterone was 40 to 148 times greater in interstitial tissue than the tubules of the testis. Of the three androgens secreted by testis, testosterone and androstenedione are considerably more potent than dehydroepiandrosterone (2).

The spermatogenic processes in the seminiferous tubules of testis are the sum of the transformations which result in the formation of the spermatozoa (haploid cells). These processes are
in preparation from the time of embryonic life and take place after puberty throughout the life of the animal, owing to a continued renewal of the stem cells.

The primordial germ cells migrate toward the germinal crests and occupy the gonad space sometimes before sexual differentiation. In the fetus and the young male, the gonocytes issuing from these primordial germ cells are contained within the sex cords.

The gonocytes multiply and give rise to spermatogonia. The latter after several mitotic divisions and the differentiations of most of the daughter cells thus obtained (the other remaining in the state of stem cells) form a group of germ cells contained in the parietal layer of the seminiferous tubule. Their last generation gives rise to primary spermatocyte.

The primary spermatocyte (tetraploid nuclei) are the germ cells which undergo meiosis. This comprises an extremely long prophase with pairing of the chromosomes and possible exchange of chromosome material. The reduction in number of chromosomes takes place in the course of two successive divisions (maturation divisions) giving, first, secondary spermatocytes (diploid nuclei), and then spermatids (haploid nuclei).

The spermatids are the post meiotic germ cells of the seminiferous epithelium. They undergo a series of transformations during spermiogenesis, ending in the formation of spermatozoa, the male germ cells so called after their release from the seminiferous epithelium.
The intense proliferation of germ cells and the subsequent release of spermatozoa (end products of their cell population) are constantly renewed from stem spermatogonial cells.

It is generally accepted that both steroidogenic and spermatogenic activity of the testis are under the control of pituitary hormones. Smith (3,4) in a series of classical experiments demonstrated pituitary control of gonadal function in the rat and thus established firmly the importance of gonadotrophin in testicular physiology. The discovery by Zondek (5) of the FSH and of LH or ICSH directed interest toward the possibility of a specific role for each of these gonadotrophine factors and led to the classic concept of FSH control over the spermatogenic process and LH control over the functions of Leydig cells (6,7).

The premeiotic phases of spermatogenesis proceed independently of any known hormonal factors, that reduction division requires gonadotrophins, and that the process of spermiogenesis is either independent of hormones or it may require androgen (8) (DIAGRAM-2, Chapter 1).

Any particular area of any seminiferous tubule of the rat contains a few spermatogonia along the basement membrane, one or several layers of spermatocytes further in, and groups of spermatids next to the lumen of the tubule. The present work is a systematic study of the various modes of association of these cells in what is known as the seminiferous epithelium after treatment with MC alone and together with either HCG or testosterone propionate treatment by the method of Clermont and Leblond (9).
Spermiogenesis may thus be divided into 19 stages. The first 14 stages of spermiogenesis, being well defined and easy to identify.

In immature rat testis, FSH given together with ICSH markedly enhanced androgen production. In individual rats, production of spermatozoa was correlated with high androgen secretion but rate of spermatogenesis is not controlled by the hormone (10). Testosterone is essential for initiation and maintenance of spermatogenesis in the rat. The ability of testosterone propionate (TP) to restore spermatogenesis was investigated in 67 to 70 days hypophysectomized rats. Following TP treatment, for 35 days, the seminiferous tubules contained germinal cells as mature as acrosome phase spermatids (11). After treatment for 90-110 days, the spermatogenesis was completely restored to normal in majority of these animals.

Previously a large number of workers have been reported that MC treatment resulted in a drastic alteration of testicular histology and specifically affected the seminiferous tubular epithelium along with spermatogenic activity (12-17). They carried out the experiments with MC in a high doses (5-7 mg/kg Body wt.) and observed that the drug directly acted over spermatogonia and brought about a chromosomal aberrations along with inhibition of mitosis. In the present investigations a series of experiments have been conducted with MC in low doses (500 μ gm/kg Body wt. treated every alternate day) and under different conditions. However,
in the previous chapters (III and IV) it has been observed that the drug suppressed testicular steroidogenesis. Further experiment in this line (Chapter V) have shown that MC induced reduction in testicular steroidogenesis was improved to a large extent when MC was treated together with HCG. The consideration of the above findings it is reasonable to speculate that as MC reduced testicular steroidogenesis it might also disturbed the spermatogenic activity. Moreover, it is also assumed that the improvement of testicular steroidogenesis in rats treated with MC and HCG togetherly may be the result of overall restoration of the normal testicular physiology. Therefore in the present chapter an experimental design has been undertaken to deliniate the spermatogenic status of the rats after treatment with MC alone and together with either HCG or testosterone propionate.

MATERIALS AND METHODS

Closed colony bred (whister strain) sexually immature (30 days age, weighing 50-60 gms) and mature (90 days age, weighing 140-150 gms) male rats were selected for the present investigations and they were housed at a constant lighting schedule of 12 hrs. light per day and fed on a standard rat diet (Chapter II) with sufficient water. Thirty two such animals, both immature and mature were taken and equally divided into 4 groups, each group consists of 8 rats and treated as follows.
Immature group - 1: MC (500 μg/kg, i.p.) in distilled water (400 μ gm/ml) every alternate day for 30 days started from 30 days of age together with oil vehicle in the same amount as that of group 2.

Immature group - 2: MC as that of immature group 1 together with testosterone propionate (1 mg/animal sc.) in oil (2 mg/ml) every day.

Immature group - 3: MC as that of immature - 1 together with HCG (20 I.U./animal) dissolved in 0.9% NaCl solution injected sc every alternate day.

Immature group - 4: Only 0.9% NaCl solution and oil vehicle in the same amount as that of immature group - 2 and group - 3 and treated as control.

Mature group - 1: MC (500 μg gm/kg i.p.) in distilled water (400 μ gm/ml) every alternate day for 30 days started from 90 days age together with oil vehicle in the same amount as that of group 2.

Mature group - 2: MC as that of mature group - 1 together with testosterone propionate (1 mg/animal, sc) in oil (2 mg/ml) every day.

Mature group - 3: MC as that of mature group 1 together with HCG (20 I.U./animal) dissolved in normal saline and injected sc every alternate day.
Mature group - 4: Only 0.9% NaCl solution and oil vehicle in the same amount as that of mature group 2 and group 3 and treated as control.

After 30 days treatment all the animals were sacrificed and the testes were removed from all the animals of each group for histological preparation. The permanent sections of the testes were prepared following the method mentioned in section D (Chapter II) and spermatogenesis was evaluated by counting the number of germ and sertoli cell nuclei in round cross section of seminiferous tubules at stage VII of the cycle of seminiferous epithelium (18,19) in mature rats.

In mature rat testis the relative number of germ cells was determined by adjusting crude germ cell counts for differences in nuclear diameter by Abercrombie's method (20) and for tubular shrinkage by a sertoli cell correction factor (18,19,21).

The histological structure of the testis in immature rat was examined under microscope and compared with other group of immature rats.

RESULTS

On histological examination of the testes, control sections of mature and immature rats showed normal pattern of cellular association of spermatogenic cells - consisting of spermatogonia, different stages of spermatocytes and spermatids along with mature
sperm and sertoli cells at the peripheral layer of the tubule. Mature spermatozoa were lined around the lumen of tubules and a few residual bodies were observed amongst them (Fig. 1,1a).

After treatment with MC in mature and immature rats, the most of the tubules were appeared to reduce significantly the number of spermatogonia, spermatocyte (resting, primary, secondary) and spermatid together with a good number of formation of "multinucleated giant cells" and pyknotic cells and vacuoles. Some tubules were also appeared to have only sertoli cells and practically devoid of any tubular epithelium specially in immature rats (Fig. 2,2a).

Administration of MC together with testosterone propionate resulted in a profound improvement in the overall testicular histology both in mature and immature rats and microscopical examination showed presence of spermatogonia, different stages of spermatocytes spermatid in a good number of tubules and mature spermatozoa in a fair number of tubules but the Leydig cells remain atrophic. (Fig. 3,3a).

Histological examination of the rats (mature and immature) treated with MC and HCG together, showed more or less same results as that of the rats treated with MC alone with testosterone propionate but here the Leydig cells showed the close appearance with that of control rat (Fig. 4,4a).

The spermatogenic activity of the rats both in mature and immature were examined after administration of MC alone and together
DESCRIPTION OF FIGURES

Fig.1: Testis of mature control rat showing normal process of spermatogenesis. Presence of mature spermatozoa around the lumen (X 240).

Fig.2: Testis of Mitomycin C treated mature rat showing a general tendency of tubular shrinkage, a reduction of spermatocytes and spermatid count as compared to Fig.1 (X 240).

Fig.3: Recovery of spermatogenesis in mature Mitomycin C treated rats with simultaneous testosterone propionate treatment, showing improvement of tubular size and germinal epithelium count as compared with Fig.2 (X 240).

Fig.4: Recovery of spermatogenesis in mature rats treated with Mitomycin C and HCG, showing improvement of tubular size, spermatocytes and spermatid number and appearance of mature spermatozoa, as compared with Fig.2 (X 240).
DESCRIPTION OF FIGURES

Fig.1(a) : Development of spermatogenesis in 60 days old control rat (X 240).

Fig.2(a) : Testis of 60 days old rat treated with Mitomycin C every alternate day for 30 days. A marked reduction in tubular size, pyknotic cells and gross degeneration of testicular epithelium. Compared with Fig.1a (X 240).

Fig.3(a) : Testis of 60 days old rat treated with simultaneous Mitomycin C every alternate day and testosterone propionate every day for 30 days, showing a marked improvement of tubular epithelium. Compared with Fig.2a (X 240).

Fig.4(a) : Testis of 60 days old rat treated with simultaneous Mitomycin C and HCG every alternate day for 30 days, showing HCG restores the inhibitory effect of Mitomycin C on germinal epithelium. Compared with Fig.2a (X 240).
with either HCG or testosterone. Significant fall of the number of spermatid in MC treated rats were restored to a large extent towards normal when MC treatment followed by the administration of either HCG or testosterone (Table 1).

DISCUSSION

The important role of the pituitary gonadotrophins in the regulation of testicular activity via the stimulation of testicular hormone production have been well documented. In the previous chapters (III, IV, V) it has been observed that MC has got a potent inhibitory action over testicular steroidogenesis. In the present chapter MC treatment both in mature and immature rats resulted an involution of testicular histology along with the arrest of spermatogenic activity (Fig. 2,2a). And simultaneously it has also been observed that when MC treatment was followed by the administration of either testosterone or HCG there causes a marked improvement of the overall testicular histology (Fig. 3,3a,4,4a) along with spermatogenic activity (Table 1) towards normal. It has been reported earlier that the testicular degeneration and an arrest of the spermatogenic activity in hypophysectomized rats improved to a large extent after testosterone propionate treatment (11). It has been observed by Kalra and Prasad (22) that in the clomiphene fed immature rats, receiving higher doses (1 mg/day) of TP alone or in combination with FSH advanced spermatogenesis to completion within 30 days of the initiation of treatment. Following treatment of
Table 1 - Relative number of germ cell nuclei per cross-section of seminiferous tubules at stage VII of the cycle of the seminiferous epithelium (each value represents the mean ± S.E.) of testis from six mature rats. The indicated germ cell nuclei were counted in 25 "round" tubular cross-sections at stage VII of the cycle of the seminiferous epithelium. All nuclear counts were corrected for difference in nuclear diameter by Abercrombie's formula and for tubular shrinkage by Sertoli cell correction factor.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dose of the treatment</th>
<th>Type Aspermatogonia</th>
<th>Preleptotene spermatoocytes</th>
<th>Pachytene spermatoocytes</th>
<th>Step 7 spermatids</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>Saline and oil vehicle</td>
<td>0.70 ± 0.010</td>
<td>19.67±0.135</td>
<td>18.66±0.140</td>
<td>69.31±0.809</td>
</tr>
<tr>
<td>II. Mitomycin C (MC) treated</td>
<td>MC 500 µg/kg Body wt. every alternate day</td>
<td>0.42 ± 0.010</td>
<td>13.61±0.362</td>
<td>13.98±0.483</td>
<td>31.78±1.032</td>
</tr>
<tr>
<td>III. Mitomycin C (MC) &amp; testosterone propionate (TP) treated</td>
<td>MC 500 µg/kg Body wt. every alternate day and TP 1 mg/day/rat</td>
<td>0.54 ± 0.005</td>
<td>17.95±0.733</td>
<td>16.93±0.399</td>
<td>65.21±1.975</td>
</tr>
<tr>
<td>IV. Mitomycin C and HCG treated</td>
<td>MC 500 µg/kg Body wt. and HCG 20 I.U. every alternate day</td>
<td>0.66 ± 0.028</td>
<td>18.18±0.775</td>
<td>18.42±0.766</td>
<td>67.29±5.20</td>
</tr>
</tbody>
</table>

The level of significance (P1 values) in different experimental conditions are compared in Table 2.
Table 2 - Comparison of the levels of significance ("P" value) in different experimental conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Type A spermatogonia</th>
<th>Preleptotene spermatocytes</th>
<th>Pachytene spermatocytes</th>
<th>Step 7 spermatids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Group I vs</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group II</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
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<tr>
<td>2. Group I vs</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group III</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.1$ (NS)</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.2$ (NS)</td>
</tr>
<tr>
<td>3. Group I vs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>$P &lt; 0.3$ (NS)</td>
<td>$P &lt; 0.2$ (NS)</td>
<td>$P &lt; 0.8$ (NS)</td>
<td>$P &lt; 0.8$ (NS)</td>
</tr>
<tr>
<td>4. Group II vs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>5. Group II vs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.001$</td>
</tr>
</tbody>
</table>

Groups I, II, III and IV are described in Table 1.

NS - Statistically non-significant.
immature or orchidectomized mammals with exogenous androgen, rapid increase occur in cell renewal and mitotic cycles in accessory glands (23) as well as increase in DNA and RNA levels (24, 25) and synthesis of specific proteins (26). It has been observed that HCG acts directly on testis like ICSH and stimulate androgen production (27). That HCG produced proliferation of Leydig cells in rats as previously reported by Army (28). In vitro studies of Steinberger et al. (29) revealed that HCG stimulate the supporting cells in four daysold rat testis which assume the morphological characteristics of mature sertoli cells. The consideration of the above discussion it is reasonable to speculate that MC (500 µ gm/kg Body wt.) induced testicular degeneration and an arrest of the spermatogenic activity was possibly due to the inhibition of testicular steroidogenesis.

In this connection it is to be considered that the improvement of testicular histology in MC together with either TP or HCG treated rat again confirm the interpretation of the Chapters V and VI, where it has been claimed that MC probably suppressed pituitary gonadotrophin secretion along with it's direct action over testis in the diminution of testicular hormone production.

SUMMARY

In the present investigation, the histological changes of the testis, both in mature and prepubertal animals were observed, after treatment with MC alone and together with either HCG or TP.
After administration of MC most of the tubules were appeared to reduce the number of spermatogonia, spermatocytes and spermatid together with a good number of formation of multinucleated giant cells and pyknotic cells and vacuoles both in case of mature and prepubertal animals. Moreover, some tubules were also appeared to have only sertoli cells and practically devoid of any tubular epithelium in case of prepubertal MC treated testis.

Differential count of germ cells of the seminiferous tubules in the mature rat testis, revealed a significant decrease in the number of spermatids and the other germ cells count remain subnormal and shrinkage of the seminiferous tubules also occurred after administration of MC.

The spermatogenic activity, restored to a large extend towards normal when MC treatment followed by the administration of either HCG or TP both in mature and prepubertal animals but the improvement were more prominent in case of MC and HCG treatment.

These findings, again, support the interpretation of the Chapters V and VI that MC probably suppressed pituitary gonadotrophin secretion along with it's direct action over testis in the diminution of testicular hormone production.
REFERENCES


