STUDIES ON THE TESTICULAR STEROIDOGENESIS AFTER TREATMENT WITH MITOMYCIN C ALONE AND TOGETHER WITH HUMAN CHORIONIC GONADOTROPHIN: AN IN VIVO EXPERIMENT

The biosynthetic capacity and secretory function of the testis has been well documented and the subject has been most ably reviewed (1,2,3). The collective data of the previous chapters (Chapter III and IV) clearly indicate that MC has an potent inhibitory action over testicular steroidogenesis. But the mechanism of action of MC concerning the inhibition of testicular steroidogenesis whether directly on the testicular tissues (Leydig cells) or through the suppression of pituitary gonadotrophin secretion is not yet clear. In this connection it is to be remembered that the mechanism controlling the secretory function of the testis is not very well understood, although three major types of control have been fairly well established, stimuli from the hypothalamus in the form of releasing factors upon the anterior pituitary to liberate gonadotrophins which in turn stimulate the testis to secrete steroid hormones. The steroid hormone in turn exert control on the hypothalamus and pituitary via negative feedback. A third form of control concerns the direct action of steroid hormones on the various enzyme systems involved in steroidogenesis. From above it can be concluded that release of pituitary gonadotrophins and its stimulation over testicular tissue (Leydig cell) plays a vital role in the regulation of the testicular steroidogenesis. From the observation of fall in weight of pituitary and also decreased LCNA
after treatment with MC as observed both in case of mature and immature rats (Chapter III and IV), it is reasonable to speculate that MC may exert some influence over the normal secretory function of the gland and thereby affect testicular steroidogenesis. Therefore it seems reasonable to investigate the secretory status of the pituitary gonadotrophin after treatment with MC.

Human chorionic gonadotrophin (HCG) is of placental origin and is present in plasma and also in the urine of pregnant women. Its biological actions resembles LH with little FSH like activity in females. It acts like ICSH in male. When HCG is administered to hypogonadotrophic eunuchoid males the interstitial mesenchymal cells develop into mature androgen producing Leydig cells. The seminiferous tubules enlarge the sertoli cells differentiate and spermatogenesis begins (43). On consideration of the above facts an attempt have been made in the present chapter to delineate the involvement of pituitary in the reduction of testicular steroidogenesis after treatment with MC alone and together with HCG.

MATERIALS AND METHODS

36 mature (90 to 92 days age, weighing 140 - 150 gms body weight) and 36 immature (30 - 32 days age, weighing 50-60 gms body weight) male rats of wister strain were selected for the present investigation. They were housed at a constant lighting schedule of 12 hrs. light/day and fed on a standard rat diet with a free access of water. Both mature and immature rats were divided equally into
three groups, each comprising of 12 rats. MC was dissolved in distilled water (400 μg/ml) and the amount of single dose of the drug was 500 μg/kg body weight. Human chorionic gonadotrophin dissolved in 0.9% NaCl solution (50 IU/ml) and the amount of single dose of the HCG was 20 IU. The dose of the drug and the HCG were same both in case of mature and immature rats. The nature of treatment of the different groups of animals are mentioned below.

Mature Group 1. Treatment with MC every alternate day for 30 days
Mature Group 2. Treatment with MC together with HCG every alternate day for 30 days.
Mature Group 3. Treatment with 0.9% NaCl solution and noted as control.

Immature Group 1. Treated with MC every alternate day for 30 days.
Immature Group 2. Treatment with MC together with HCG every alternate day for 30 days.
Immature Group 3. Treated with 0.9% NaCl solution and noted as control.

HCG was injected 1 hour after the administration of MC in case of all the rats both mature and immature.

Parameters studied

1. **Organs weight**: Immediate after sacrificing the testis, sex accessories and the pituitary were dissected out, made free of fat bodies and the weights were recorded.
2. Measurement of Leydig cell nuclear area: Fresh testis was fixed in Bouin's fluid, sectioned at 6 μm and stained with haematoxylin and eosin for the measurement of Leydig cells nuclear area. The measurements of LCNA were performed on the slides according to the method of Deb et al. (4).

3. Histochemical localization of enzymes activity: Fresh frozen sections of testis were cut at 20μm in a cryostat at -20°C and placed on cover slips. These unfixed frozen sections were incubated in appropriate substrate media at 37°C for demonstrating the activities of Δ^5-3β-OHD (5) and G-6-PDH enzymes (6,7). Parallel sections were incubated in substrate free media and served as controls. Following incubations the sections were fixed in 10% neutral formalin and mounted in glycerine jelly.

4. Biochemical estimation of total cholesterol, ascorbic acid and DNA content: The biochemical determination of ascorbic acid was performed according to Roe and Kuether (8). The estimation of cholesterol in the testis was determined by adopting the modified method of Sperry and Webb (9). The half portion of right testis were used for ascorbic acid and the other half for cholesterol. Left testis were taken for the measurement of DNA by the modified method of Croft and Lubrum (10,10a).

RESULTS

Histochenmical preparation showed that Δ^5-3β-OHD activity was localized mainly in the interstitial cells of the testis.
On administration of MC every alternate day for 30 days the enzyme activity appeared to fall significantly in comparison to control rats (Figs. 1, 2 and 1a, 2a). Treatment with MC together with HCG showed a marked improvement of the \( \Delta^5-3\beta-OHD \) activity in comparison to the rats treated with MC alone (Figs. 2, 3 and 2a, 3a).

Histochemical demonstration of G-6-PDH appeared to be localized in the interstitial and tubular epithelial cells of the testis in control rats (Figs. 4, 4a). After administration of MC every alternate day for 30 days the enzyme activity reduced to a large extent in comparison to control rats (Figs. 4, 5 and 4a, 5a). On the other hand when MC was treated together with HCG, enzyme activity appeared to improve to a recognizable extent in comparison to rats treated with MC alone (Figs. 5, 6 and 5a, 6a).

No detectable enzymes (\( \Delta^5-3\beta-OHD \) and G-6-PDH) activities were evident in the control sections incubated in corresponding substrate free media. The intensity of enzyme activities in the testis were similar among the animals of the same group.

The weights of the testis, prostate, seminal vesicle and pituitary were reduced remarkably in rats treated with MC alone (Tables 1, 4). The above organ weights except pituitary regained towards normal in rats treated MC together with HCG (Tables 1, 4). LCNA also showed the similar type of results (Tables 3, 6) as that of organ weights and enzymes activity either after treatment with MC alone or together with HCG.
DESCRIPTION OF FIGURES

Fig. 1: \( \Delta^5-3\beta-\text{OHD} \) activity in the testis of 0.9% NaCl treated control mature rat (X 120).

Fig. 2: \( \Delta^5-3\beta-\text{OHD} \) activity in the testis of mature rat after treatment with Mitomycin C every alternate day for 30 days. A significant fall of enzyme activity in comparison to control rat. Fig. 1 (X 120).

Fig. 3: \( \Delta^5-3\beta-\text{OHD} \) activity in the testis of mature rat after treatment with Mitomycin C together with HCG every alternate day for 30 days. A marked improvement of enzyme activity in comparison to Mitomycin C treated rat. Fig. 2 (X 120).

Fig. 4: G-6-PDH activity in the testis of 0.9% NaCl treated control mature rat (X 120).

Fig. 5: G-6-PDH activity in the testis of mature rat after treatment with Mitomycin C every alternate day for 30 days. A significant fall of enzyme activity in comparison to control rat. Fig. 4 (X 120).

Fig. 6: G-6-PDH activity in the testis of mature rat after treatment with Mitomycin C together with HCG every alternate day for 30 days. A marked improvement of enzyme activity in comparison to Mitomycin C treated rat. Fig. 5 (X 120).
DESCRIPTION OF FIGURES

Fig. 1(a) : $\Delta^5$-3p-OHD activity in the testis of 0.9% NaCl treated 60 days old control rat (X 120).

Fig. 2(a) : $\Delta^5$-3p-OHD activity in the testis of 60 days old rat after treatment with Mitomycin C every alternate day for 30 days. A significant fall of enzyme activity in comparison to control rat. Fig. 1a (X 120).

Fig. 3(a) : $\Delta^5$-3p-OHD activity in the testis of 60 days old rat after treatment with Mitomycin C together with HCG every alternate day for 30 days. A marked improvement of enzyme activity in comparison to Mitomycin C treated rat. Fig. 2a (X 120).

Fig. 4(a) : G-6-PDH activity in the testis of 0.9% NaCl treated 60 days old control rat. (X 120).

Fig. 5(a) : G-6-PDH activity in the testis of 60 days old rat after treatment with Mitomycin C every alternate day for 30 days. A significant fall of enzyme activity in comparison to control rat. Fig. 4a (X 120).

Fig. 6(a) : G-6-PDH activity in the testis of 60 days old rat after treatment with Mitomycin C together with HCG every alternate day for 30 days. A marked improvement of enzyme activity in comparison to Mitomycin C treated rat. Fig. 5a (X 120).
Biochemical measurement of testicular DNA content (mg/100 gm of tissue) also showed a significant reduction in the rats treated with MC alone in comparison to control rats and again the marked increase have been noted in the rats which received MC together with HCG (Tables 3 and 6).

Biochemical measurement of both total cholesterol and ascorbic acid showed a great accumulation in the testis in MC treated rats when it was compared to control rats. When MC and HCG were treated simultaneously, the cholesterol and ascorbic acid showed nearly normal level (Tables 2, 5).

Similar results were noted both in mature and immature rats.

DISCUSSION

On the basis of the results obtained in the previous chapters (III and IV), it is fairly conclusive that MC treatment resulted in a remarkable reduction in the production of testicular hormones both in mature and immature rats. But the mode of action of the drug either directly or through pituitary is still obscure. To clarify the problem and to find out whether the pituitary is involved in the reduction of testicular steroidogenesis in such condition, HCG was administered simultaneously with MC both in mature and immature rats. Histochemical demonstration of Δ⁵-3β-OHD and G-6-PDH showed that the significant fall of the above enzymes in MC
Table 1 - Relative organ weights (mg/100 gm body wt.) in mature rats after treatment with Mitomycin C alone and together with Human chorionic gonadotrophin.

Values are means + S.E., from 6 rats in each group.

<table>
<thead>
<tr>
<th>Treatment and group</th>
<th>Testis (both sides) (mg)</th>
<th>Seminal vesicle (mg)</th>
<th>Prostate (mg)</th>
<th>Pituitary (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution control</td>
<td>2462.48 ± 6.43</td>
<td>180.01 ± 3.48</td>
<td>207.94 ± 1.65</td>
<td>6.69 ± 0.045</td>
</tr>
<tr>
<td>Mitomycin C every alternate day for 30 days</td>
<td>1410.11 ± 6.34</td>
<td>51.19 ± 2.90</td>
<td>54.50 ± 2.11</td>
<td>4.66 ± 0.235</td>
</tr>
<tr>
<td>Mitomycin C and HCG every alternate day for 30 days</td>
<td>1910.52 ± 8.77</td>
<td>149.06 ± 4.18</td>
<td>164.26 ± 6.70</td>
<td>4.83 ± 0.141</td>
</tr>
</tbody>
</table>

\[ P \text{ Value} \]
- Control vs MC: <0.001
- Control vs MC + HCG: <0.001
- MC vs MC + HCG: <0.001

*NS = Statistically non-significant.
Table 2 - Total cholesterol and ascorbic acid concentration in the testis of mature rats after treatment with Mitomycin C alone and together with Human chorionic Gonadotrophin.

Values are means ± S.E., from 6 rats in each group

<table>
<thead>
<tr>
<th>Treatment and groups</th>
<th>Cholesterol (mg/100 gm tissue)</th>
<th>Ascorbic acid (μg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution control</td>
<td>238.29 ± 3.14</td>
<td>5.43 ± 0.304</td>
</tr>
<tr>
<td>Mitomycin C every alternate day for 30 days</td>
<td>305.45 ± 6.38</td>
<td>6.94 ± 0.098</td>
</tr>
<tr>
<td>Mitomycin C and HCG every alternate day for 30 days</td>
<td>281.53 ± 3.08</td>
<td>5.88 ± 0.104</td>
</tr>
</tbody>
</table>

P value

Control vs MC  \(<0.001\)  \(<0.001\)

Control Vs MC + HCG  \(<0.001\)  NS*

MC vs MC + HCG  \(<0.001\)  \(<0.001\)

* NS = Statistically non-significant
Table 3 - Total DNA content in the testis and Leydig cells nuclear area in mature rats after treatment with Mitomycin C alone and together with Human chorionic gonadotrophin.

Values are means ± S.E. from 6 rats in each group.

<table>
<thead>
<tr>
<th>Treatment and groups</th>
<th>DNA (mg/100 gm tissue)</th>
<th>Leydig cell nuclear area (mm²) (Camera Lucida X800)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution control</td>
<td>120.40 ± 1.42</td>
<td>68.91 ± 1.19</td>
</tr>
<tr>
<td>Mitomycin C every alternate day for 30 days</td>
<td>67.85 ± 3.39</td>
<td>37.72 ± 2.45</td>
</tr>
<tr>
<td>Mitomycin C and HCG every alternate day for 30 days</td>
<td>102.31 ± 2.20</td>
<td>51.36 ± 1.98</td>
</tr>
</tbody>
</table>

P Value

- Control vs MC <0.001
- Control vs MC + HCG <0.001
- MC vs MC + HCG <0.001
Table 4 - Relative organ weights (mg/100 gm body wt.) in immature rats after treatment with Mitomycin C alone and together with Human chorionic gonadotrophin. Values are means ± S.E., from rats in each group.

Treatment started on 30 days age and sacrificed at 60 days age

<table>
<thead>
<tr>
<th>Treatment and groups</th>
<th>Testis (both sides) (mg)</th>
<th>Seminal vesicle (mg)</th>
<th>Prostate (mg)</th>
<th>Pituitary (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution control</td>
<td>1419.25 ± 5.24</td>
<td>126.45 ± 2.24</td>
<td>145.50 ± 2.46</td>
<td>5.84 ± 0.143</td>
</tr>
<tr>
<td>Mitomycin C every alternate day for 30 days</td>
<td>427.82 ± 7.02</td>
<td>38.39 ± 2.32</td>
<td>49.01 ± 2.83</td>
<td>3.77 ± 0.181</td>
</tr>
<tr>
<td>Mitomycin C and HCG every alternate day for 30 days</td>
<td>889.75 ± 4.54</td>
<td>76.57 ± 3.17</td>
<td>81.13 ± 4.04</td>
<td>3.90 ± 0.066</td>
</tr>
</tbody>
</table>

P Value

| Control vs MC | <0.001 | <0.001 | <0.001 | <0.001 |
| Control vs MC + HCG | <0.001 | <0.001 | <0.001 | <0.001 |
| MC vs MC + HCG | <0.001 | <0.001 | <0.001 | NS*  |

* NS = Statistically non-significant.
Table 5 = Total cholesterol and ascorbic acid concentration in the testis of immature rats after treatment with Mitomycin C alone and together with Human chorionic gonadotrophin. Values are means ± S.E., from 6 rats in each group. Treatment started on 30 days age and sacrificed at 60 days age.

<table>
<thead>
<tr>
<th>Treatment and group</th>
<th>Cholesterol (mg/100 gm tissue)</th>
<th>Ascorbic acid (µg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution control</td>
<td>228.28 ± 3.87</td>
<td>5.87 ± 0.086</td>
</tr>
<tr>
<td>Mitomycin C every alternate day for 30 days</td>
<td>289.47 ± 3.66</td>
<td>7.20 ± 0.209</td>
</tr>
<tr>
<td>Mitomycin C and HCG every alternates day for 30 days</td>
<td>240.93 ± 4.18</td>
<td>6.02 ± 0.085</td>
</tr>
</tbody>
</table>

P Value

- Control vs MC: <0.001
- Control vs MC + HCG: <0.05
- MC vs MC + HCG: <0.001

* NS = Statistically non-significant.
Table 6 - Total DNA content in the testis and Leydig cells nuclear area in immature rats after treatment with Mitomycin C alone and together with Human chorionic gonadotrophin.
Values are means ± S.E., from 6 rats in each group. Treatment started on 30 days age and sacrificed at 60 days age.

<table>
<thead>
<tr>
<th>Treatment and groups</th>
<th>DNA (mg/100 gm tissue)</th>
<th>Leydig cell nuclear area (mm²) (Camera Lucida X800)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution control</td>
<td>568.17 ± 5.15</td>
<td>69.16 ± 1.97</td>
</tr>
<tr>
<td>Mitomycin C every alternate day for 30 days</td>
<td>339.78 ± 13.50</td>
<td>36.27 ± 1.98</td>
</tr>
<tr>
<td>Mitomycin C and HCG every alternate day for 30 days</td>
<td>521.86 ± 7.82</td>
<td>50.25 ± 2.44</td>
</tr>
</tbody>
</table>

P Value

Control vs MC <0.001 <0.001
Control vs MC + HCG <0.001 <0.001
MC vs MC + HCG <0.001 <0.01
treated rats improved to a large extent when MC treatment was accompanied with administration of HCG (Fig. 3 and 6). Under the same condition similar improvement have been noted in the weights of testis and sex accessories (Table 1) both in mature and immature rats. Role of G-6-PDH of the pentose phosphate pathway in the production of NADPH for steroid hydroxilation reaction and the direct involvement of $\Delta^5$-3\beta-OH in the biosynthesis of steroid hormones have been discussed previously and confirmed by a large number of workers (11-26). The sex accessory organ weights have been also generally accepted as a reliable index to access the status of testicular steroid hormone production (27,28). Therefore the improvement of the enzyme activity along with sex accessory organ weights in the rats treated with MC and HCG simultaneously, suggest an increase in the production of testicular hormones. Additional support in favour of the improvement of testicular steroidogenesis is also provided from the results of LCNA (Tables 3,6). It has been reported earlier by different workers that LCNA varies directly according to the rate of production of steroid hormones (29,30). The importance of cholesterol and ascorbic acid in the production of steroid hormone have been discussed earlier. Previously a number of workers have been noted that hypofunctioning condition of the gonads generally associated with the accumulation of cholesterol and ascorbic acid (31-33).

On the other hand several authors are of same opinion that enhanced steroidogenesis always accompanied with a reduction in ascorbic acid content of the steroidogenic gland (18,19,34).
Therefore, the fall of cholesterol and ascorbic acid in the testis (Tables 2, 5) in rats treated with MC and HCG simultaneously in comparison to the rats treated with MC alone again suggest an improvement of the testicular biosynthesis of steroid hormones.

The increase in the weight of the testis (Tables 1, 4) along with its DNA content in rats treated with MC and HCG simultaneously have been noted (Tables 3, 6). It has already been mentioned that MC has got a direct action over the proliferating cells of the seminiferous tubule (35-38) and it has also been claimed that the MC is a potent inhibitor of mitosis (39, 40). Therefore significant reduction of testicular weight and DNA content in rats treated with MC alone may be interpreted as a direct action on the tubular epithelial cells to shrinkage the seminiferous tubule and the involution of tubular epithelium. The reduction of spermatogenic activity along with involution of tubular epithelium have also been noted in case of the testis where the testicular steroidogenesis have been diminished (41). Kalra and Prasad (42) reported that in hypophysectomized condition tubular epithelium regresses to a large extent in immature rats. In the same rats the improvement of the tubular epithelium towards normal have been noted after treatment with testosterone alone and together with FSH. The histochemical results of the present chapter along with previous chapters (III and IV) clearly delineate that MC treatment resulted an inhibition of testicular steroidogenesis. Therefore the action of MC on the tubular epithelium may be explained on the basis of it's direct action over tubular epithelium and indirectly through the suppression
of the testicular steroid biogenesis. In the present chapter on the basis of histochemical results in case of rats treated with MC and HCG an increased steroidogenic activity towards normal level has been noted (Fig. 1-6 and 1a-6a). Therefore, it is logical to assume that increased testicular steroid synthesis in the rats treated with MC and HCG may be responsible for improvement in the tubular epithelium along with increase in the weight of testis and its DNA content which has been noted in the present chapter (Table 1, 3 and 4, 6).

In the present chapter the improvement of MC induced reduction of testicular steroidogenesis after administration of HCG is an interesting finding (Table 1, 1a), because fall of pituitary weight both in mature and immature rats after treatment with MC in the previous chapters and in the present chapter lead to suggest the involvement of pituitary i.e, diminution of gonadotrophin secretion in the reduction of testicular steroidogenesis in this condition. The improvement of testicular steroidogenesis in this condition clearly indicate that MC induced reduction of testicular steroidogenesis is some way related to suppression of pituitary gonadotrophin secretion. However, the direct action of MC on the Leydig cells also cannot be ruled out. Therefore an in vitro studies have been undertaken in the following experiment.

**SUMMARY**

On the basis of the results obtained in the previous chapters (III and IV) it has been concluded that MC treatment
resulted in an inhibition of testicular steroidogenesis. But the manner through which the drug exerted its effect, either directly on the Leydig cells or via the alteration of pituitary gonadotrophin secretion is not clear. To find out the role of pituitary in the MC induced reduction of testicular steroidogenesis the present work has been undertaken. In the present chapter MC was administered along with HCG and the testicular steroidogenic activity was assessed on the basis of different parameters which are directly and indirectly related to the rate of testicular steroid biogenesis. Histochromically increased activity of testicular steroidogenic enzymes ($\Delta^5$-3$\alpha$-OHD and G-6-PDH) along with the improvement of the weights of sex accessories and testes towards normal suggest an overall stimulation of testicular steroidogenesis. Other parameters such as testicular content of cholesterol, ascorbic acid, DNA and LCNA also provide additional evidence in support of the increased steroidogenic activity in this condition. The above results were noted both in case of mature and immature rats. The fall of pituitary weight in the present chapter was consistent with the previous chapters even when MC treatment was followed by the administration of HCG. Therefore it is reasonable to speculate that pituitary may alter its normal secretory pattern in MC treated rat and thereby affects testicular steroidogenesis. Later on the improvement of testicular steroidogenesis in the rats treated with MC and HCG togetherly lead to suggest that the suppression of pituitary gonadotrophin secretion may be one of the responsible factor in connection with MC induced reduction in the testicular steroidogenesis. However, the direct action of the drug on Leydig cell through it's inhibitory action over DNA synthesis cannot be ruled out.
REFERENCES


