6. COMPARATIVE STUDY ON THE EFFECTS OF DIAZEPAM, CHLORDIAZEPOXIDE AND NITRAZEPAM ON MITOTIC, MEIOTIC AND POST-MEIOTIC GERM CELLS

In the foregoing chapters the effects of three benzodiazepine tranquilizers on the mitotic and spermatogenic cells of mice were studied with different protocols. The results on the qualitative and quantitative aspects as well as the probable mechanisms of action of each drug have already been presented. Therefore, at present we would not take them into consideration, rather we would attempt to generalise the results as a whole as far as practicable. Since the present investigation deals with three tranquilizers of the same chemical class a protocol-wise comparison of their data will naturally throw some light on the better understanding of their relative effectiveness. Thus in the present chapter we would like to bring out the common points only from the data obtained in connection with the treatment of each drug for proper comparison. However, in this attempt we would not be facing much difficulty because this was our original idea while planning the investigation and for that dose and time factors were kept constant for all the three drugs. To reveal a comparative picture the quantitative data obtained for the treatment of three drugs were presented through graphs and histograms protocol-wise (Figs. 21-29). For convenience control data have also been presented in the figures.

For comparison of the data on the metaphase chromosome analysis of bone marrow cells only the breakage frequencies (items 3-7) and total aberration frequencies (items 1-7, vide Tables 1, 2, 3, 12, 13, 14 and 23) of the structural aberrations of chromosomes were considered. As the metaphase chromosome analysis of the bone
marrow cells did not reveal striking numerical changes, they were not taken under consideration. Further, single treatment series was not done with nitrazepam. Time-response curves for breakage frequencies for diazepam and chlordiazepoxide (CDZ) after single treatment (Fig. 21) formed mirror-image for each other. The curves for CDZ peaked at 32 h and the peak was at higher level than that obtained with diazepam at 4 h, which clearly indicated higher effectiveness of chlordiazepoxide. However, when we compared the time-response curves of total aberration frequencies for two drugs the curve for diazepam was found to remain at higher level almost at all the sampling hours. This was due to higher incidences of gap obtained in diazepam treated series.

Dose-response curve also exhibited the same picture (Fig. 22). Breakage frequency curve for CDZ remained at higher level at three dose levels and the peak value was also more than that obtained with diazepam. But with regard to total aberration frequency curve for diazepam showed distinct higher effectiveness. Thus with regard to breakage frequencies both dose-response and time-response analyses after single treatment revealed higher effectiveness of CDZ; but for total aberration frequency diazepam was found to be more effective. Bender et al. (1974) categorised chemicals producing chromosomal aberrations into four heads on the basis of types of aberrations produced in relation to the stage of the cell cycle. Higher incidence of gaps after single treatment of diazepam and its non-delayed effect seem to indicate that diazepam comes under category 1 of Bender et al. (1974) which includes compounds producing gaps and deletions in late S and G2 cells. Under this category come FUDR, AdR, cytosine arabinoside and hydroxyurea and all are
Fig. 21: Time-response curves for bone marrow metaphase chromosome aberrations after single treatment (0.5 mg) of diazepam (DZ) and chlordiazepoxide (CDZ) (data taken from Tables 1 and 12)
Fig. 22: Dose response curves for bone marrow metaphase chromosome aberrations after single treatment of diazepam (DZ) and chlordiazepoxide (CDZ). (data taken from Tables 2 and 13).
inhibitors of biosynthesis of DNA and DNA precursors. Inhibitory effect of diazepam on DNA synthesis has already been demonstrated by Ober (1974). In contrast, CDZ probably comes under category 3 of Bender et al. (1974) which includes compounds like alkylating agents, nitroso-compounds and some antibiotics. These compounds are characterised for production of chromatid aberrations of all types of cells treated in G₁ and early S. Comparatively higher frequency of breakages and lower frequency of gaps in CDZ series as well as its delayed effect support our assumption on its inclusion under category 3. However, its action with DNA or DNA precursors is yet to be known. Thus diazepam and CDZ though chemically closely related seem to exhibit differential sensitivity with respect to different stages. Chemically related compounds causing chromosome aberrations by different mechanisms are not unknown.

As an example, cases of FUdR and BUdR can be cited (Kihlman, 1966).

In contrast to single treatment series, 15 days repeated treatment failed to show any striking effect in any post-treatment week (Figs. 23a, b). Breakage frequency data for CDZ and nitrazepam (NZ) at wk 1 only were significantly higher than the corresponding control values. All other values for all the three drugs remained at the control range. As the values were almost at the control level it would be unreasonable to assess which one was most effective. Still then among the three drugs the breakage frequency curve for CDZ (Fig. 23b) was found to be slightly at higher level. Similarly data on the total aberration frequency for CDZ at wk 1 only was at significantly higher level, all other data were within the control range.
Graphical presentations of the data on total aberrations (a) and breaks (b) induced by repeated treatment of three tranquilizers in bone marrow chromosomes at different post-treatment weeks (data taken from Tables 3, 14, and 23.)
The data on the incidences of micronucleated erythrocytes of the dose-response analyses for three drugs are presented in Fig. 24. The three doses tested for each drug were 0.25, 0.5 and 1.0 mg. NZ failed to show any appreciable effect at any dose level in any cell type. With diazepam the intermediate dose produced the maximum response in two types of erythrocytes taken separately or combinely. However, picture is different for CDZ. Maximum effect was produced with the highest dose tested in poly- and normochromatic erythrocytes taken separately or combinely. Thus, dose-response patterns exhibited by poly- and normochromatic erythrocytes were same for any given benzodiazepine. After single treatment with the intermediate dose (0.5 mg) diazepam was more effective in producing MN in comparison to CDZ (Table 29). Mitotic inhibition is supposed to be the probable reason for the low incidence of MN with the highest dose for diazepam. So, it is not reasonable to compare the data obtained from the highest dose. With the lowest dose the incidences of MN induced by diazepam, CDZ and NZ in poly- and normochromatic erythrocytes remained almost at the same level and it was very close to the control level. If we consider intermediate dose diazepam and CDZ are almost equally effective. With still higher dose, irrespective of mitotic inhibition, CDZ seemed to be the most effective. Thus it is clear that among the three benzodiazepines nitrazepam is the least effective in producing MN. As the incidence of MN in the nucleated cells is less reliable, here in the comparative analysis that portion is omitted. Thus MNT proved to be a sensitive and reliable cytogenetic assay system for the study of induced mutagenesis.
Fig-2.4 Histogram analysis of dose-response data on the incidences of micronucleated erythrocytes induced by diazepam (DZ), chlordiazepoxide (CDZ) and nitrazepam (NZ). (data taken from Tables 7, 18 and 25).
Table 29. Results of micronucleus test after single treatment of diazepam (DZ) and chlordiazepoxide (CDZ) (0.5 mg) at 32 h. (Figures in parentheses are MN/cells scored).

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of Animals</th>
<th>P+ec. with MN Mean %± S.E.</th>
<th>N+ec. with MN Mean %± S.E.</th>
<th>P+N+ec. with MN Mean %± S.E.</th>
<th>Nucleated Cells Erythrocytes/100 nucleated cells Mean ± S.E.</th>
<th>P/N ratio Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZ</td>
<td>6</td>
<td>0.907±0.149a</td>
<td>0.683±0.149a</td>
<td>0.794±0.139a</td>
<td>12.111±0.318</td>
<td>0.826±0.029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(45/4675)</td>
<td>(32/4699)</td>
<td>(77/9374)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDZ</td>
<td>0.299±0.055</td>
<td>0.426±0.088b</td>
<td>0.363±0.071c</td>
<td>13.054±0.912</td>
<td>0.809±0.037</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(21/6860)</td>
<td>(30/6865)</td>
<td>(51/13755)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
<td>0.297±0.029</td>
<td>0.220±0.022</td>
<td>0.262±0.018</td>
<td>11.383±0.989</td>
<td>0.968±0.099</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>(73/23850)</td>
<td>(55/23865)</td>
<td>(128/47715)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ t \text{ test: } a = p < 0.001, \quad b = p < 0.01, \quad c = p < 0.05 \]
In the present investigation structural changes of the meiotic chromosomes induced by diazepam, CDZ and NZ were not so much marked (though positive in certain weeks for diazepam and CDZ). For that reason we are not going to compare here the data on the structural changes induced by them. The data on the incidence of polyploidy and aneuploidy induced by the said benzodiazepines are presented in Figs. 25 and 26 respectively. When the data on polyploidy of the treated series were compared with those of control both diazepam and CDZ were found to induce it significantly in almost all the test weeks, while NZ failed to do it except at wk 1. Fig. 25 clearly demonstrates higher effectiveness of diazepam to induce polyploidy in most of the test weeks. However, at wk 8 the values for diazepam and CDZ were same and at wk 6 the value for CDZ was slightly at higher level. With regard to aneuploidy diazepam and CDZ exhibited completely reverse picture (Fig. 26). CDZ in general seemed to be most effective to induce aneuploidy. The highest frequency obtained at wk 3 was 16.03%. In contrast, diazepam is least effective. But nitrazepam though at early weeks its effect was not so pronounced, at later weeks (wks 6 and 8) showed the highest effectiveness. The reason for the reverse picture for polyploidy and aneuploidy for diazepam and CDZ is not clear. However, it may presumably be due to the fact that diazepam is more effective than CDZ in causing spindle disruption. For that, the extent of mitotic inhibition by diazepam is also more, which results into higher incidence of polyploidy. As the effect of CDZ on spindle apparatus is assumed to be comparatively mild the affected cells may not exhibit complete inhibition rather result aneuploidy. Our
Fig. 25 Incidences of polyploidy in diakinesis-metaphase-1 spermatocytes after diazepam (DZ), chlordiazepoxide (CDZ) and nitrazepam (NZ) treatment at different weeks. (data taken from Tables 8, 19 and 26).
Fig. 26. Incidences of aneuploidy in diakinesis-metaphase-I spermatocytes after diazepam (DZ), chlordiazepoxide (CDZ) and nitrazepam (NZ) treatment at different weeks (data taken from Tables 8, 19 and 26).
assumption on the relative effectiveness of diazepam and CDZ on the mitotic apparatus is supported by our findings obtained from the MNT.

Attempt was also made to know the relative effectiveness to cause dominant lethality. However, nitrazepam could not be tested for want of time. So comparative analysis included data of only diazepam and CDZ. For the sake of convenience and simplicity, only data on mutagenic index which was calculated as percentage of dead implants were plotted graphically (Fig. 27). Both diazepam and CDZ were found to be effective in causing dominant lethality in mice. But the interesting point was that two benzodiazepines acted on two different types of spermatogenic cells to cause dominant lethality. Diazepam acted on the post-meiotic germ cells, while CDZ acted on the pre-meiotic germ cells. In case of CDZ the curve formed plateau at wks 4-6 and that plateau revealed maximum response for it. On the other hand, in diazepam dominant lethality was peaked at wk 1 and the value was a little less than twice the maximum value obtained with CDZ. Thus higher effectiveness of diazepam was noted in causing dominant lethality in mice.

Fig. 28 summarises the data on the epididymal sperm count of control and three drug treated series. In case of diazepam in all the test weeks the counts remained below the control level; the same picture was found for nitrazepam also except last week (wk 8). But in case of chlordiazepoxide the count was in certain weeks just above the control level and in certain other cases just below it. If data of three drug treated series only were compared, diazepam yielded the lowest count almost in all the test weeks. On the other hand, CDZ revealed the highest count in all the weeks. Thus among the three benzodiazepines diazepam seems to be the most
Fig.- 27 Incidences of mutagenic indices in diazepam and chlordiazepoxide treated and control mice. (data taken from Tables 9 and 20)
Fig. 28 Histogram analysis of the effects of three tranquilizers on the epididymal sperm count at different post-treatment weeks (data taken from Tables 10, 21 and 27).
effective in causing spermatozoal depletion and chlordiazepoxide is the least effective. Whether that depletion has any adverse effect or not has been discussed earlier in the respective sections.

Histogram analysis of the data on the sperm head abnormalities induced by three drugs (Fig. 29) clearly revealed that all the three drugs produced maximum effect at wk 6 and minimum effect at wk 1 (wk 1 data for diazepam was slightly higher than that of wk 3 and 4). If we consider the data of wk 6 only, diazepam seems to be the most effective. However week-wise data for the three drugs did not show the same picture. For CDZ, from wk 1-6 the increase was gradual; for diazepam the increase at wk 6 was abrupt, the values in other weeks remaining at very low level; and for N2 the data for five test weeks did not exhibit much differences and they remained at high level. Thus no particular drug is found to be highly effective at all the test weeks. Except wk 6 in all other weeks nitrazepam showed the higher effectiveness. So far qualitative aspect was concerned types of abnormalities encountered after nitrazepam treatment were also more. In general three benzodiazepines exhibited almost similar trend in the induction of sperm head abnormality. Wyrobek and Bruce (1978) provided a list of 60 chemicals tested for their sperm head abnormality inducing capacity in mice. They were tested at 1, 4 and 10 post-treatment weeks and maximum response in most of the cases was found at wk 4. Though wk 10 data are wanted in the present investigation from the maximum effect at intermediate week (here at wk 6 not wk 4) the present results seem to be at par with those of other chemicals tested earlier. The reason for this type of effect with diverse
Fig. 29. Histogram analysis of data on sperm head abnormalities induced by diazepam, chlordiazepoxide and nitrazepam at different post-treatment weeks (data taken from Tables II, 22 and 28).
types of chemicals is not clear.

One very important point has emerged from the analysis of sperm head abnormality. Both diazepam and chlordiazepoxide revealed positive response from cytogenetic study (MNT and meiotic chromosome analysis) as well as sperm head analysis, while nitrazepam demonstrated negative results from cytogenetic analysis but positive from sperm head analysis. This fact indicates that sperm head abnormality has least or no relation with chromosome aberration and thus supports the view of Wyrobek and Bruce (1975, 1978). Some genetic factor(s) may be supposed to be responsible, as mentioned earlier, for the abnormal sperm head. However, such type of observation (negative from cytogenetic study and positive from sperm head analysis) was also reported earlier with other chemicals eg. dichlorvos, EMS, etc. (Wyrobek and Bruce, 1975).

The formation of normal sperm head involves a series of intricate morphological and biochemical steps. The reason for production of sperm of abnormal head shape is still obscure. Though earlier attempt was made to correlate sperm abnormality with the chromosome abnormality (Bruce et al., 1974), subsequent studies (Wyrobek et al., 1975; Wyrobek and Bruce., 1975, 1978; Bennett, 1975; Krzanowska, 1976; Wolfe et al., 1977; Hugenholtz and Bruce, 1977) refute that general relationship. Alternative to chromosomally oriented hypothesis it is now suggested that late changes may be due to changes in the genes responsible for spermatogenesis (Wyrobek and Bruce, 1975). Differential spontaneous incidences of sperm head abnormalities in different strains of mice (Krzanowska, 1972; Bruce et al., 1974) support the view. Besides, several genetic factors are now known to control sperm abnormalities in mouse. The Y-chromosome linked factors have been shown (Krzanowska, 1976b) to control overall percentage of abnormal sperms, and autosomal factors
for the types of abnormalities seen. Again many mutations in the mouse such as T-locus (Bennett, 1975), hop-sterile (Johnson and Hunt, 1971), p-sterile (Wolfe et al., 1977), quaking (Bennett et al., 1971) and perhaps certain X-linked factors (Hugenholtz and Bruce, 1977) are associated with abnormalities in sperm morphology. All these facts tell about the genetic control of sperm head shape. Therefore, higher incidence of sperm head abnormalities induced by a given agent may be considered as a measure of genetic damage caused and it has been discussed in detail by Wyrobek and Bruce (1978).

The fate of the sperms with head shape abnormalities is not yet clearly understood. Some murine mutants with gross morphological sperm abnormality are infertile (Wyrobek et al., 1976). On the other hand, Hugenholtz and Bruce (1977) identified one mutant fertile mouse which produced only abnormal sperms. Perhaps, as suggested by Clavert et al. (1975), sperms having abnormality in flagellum cannot reach the egg and thereby cannot participate in fertilization, while having abnormalities in head shape only can still fertilize. Anyway, it needs verification.