Pharmacology Biochemistry & Behavior, Vol. 15, pp. 185–189, 1981. Printed in the U.S.A.

Alcohol Withdrawal Reactions After Chronic Intake of Chlordiazepoxide and Ethanol[1]

ARTHUR W. K. CHAN[2], FLORENCE W. LEONG, DONNA L. SCHANLEY AND SUSAN M. HOWE

Research Institute on Alcoholism, New York State Division of Alcoholism and Alcohol Abuse
1021 Main Street, Buffalo, NY 14203

Received 25 March 1981

CHAN, A. W. K., F. W. LEONG, D. L. SCHANLEY AND S. M. HOWE. *Alcohol withdrawal reactions after chronic intake of chlordiazepoxide and ethanol.* PHARMAC. BIOCHEM. BEHAV. 15(2) 185–189, 1981.—Withdrawal reactions were compared in C57BL/6J mice, which had been fed an ethanolic liquid diet containing chloridazepoxide (CDP, 3.2 or 6.4 mg/100 ml, group B or C, respectively) with those which had been administered an ethanol diet alone (group A) for 15 days. Group A showed a significantly more pronounced decrease in rectal temperature (4 to 10 hr) and a higher withdrawal score (4 to 14 hr) than mice in groups B and C. The differences in withdrawal signs still persisted even after mice were fed an ethanol diet without CDP for one extra day before withdrawal. The presence of metabolites of CDP in the blood during withdrawal could only account for a minor contribution to the protective effect. Our data are more suggestive of an increased rate of ethanol metabolism leading to lower blood alcohol levels during diet intake period as being the major factor. However, we cannot rule out the alternative possibility that CDP or its metabolites might interfere with the development of tolerance to and physical dependence on alcohol.

Chlordiazepoxide Ethanol diet Alcohol withdrawal reactions

THE consumption of both the benzodiazepines and alcohol together is not uncommon [8]. However, not much is known about the clinical consequences of their combined use [1]. Recently an atypical withdrawal syndrome was reported for a small number of alcoholics who had a mixed alcohol-benzodiazepine addiction [1]. This was characterized by a delay in appearance of a withdrawal syndrome which closely resembled that of withdrawal from benzodiazepines.

We have recently investigated [2] the influence of chloridazepoxide (CDP) on the consumption of an ethanolic liquid diet in mice. In light of the supra-additive effect of CDP on ethanol sleep time [3], it can be speculated that mice receiving the diet containing CDP might be more affected by the combination of drugs, and this in turn might influence the development of tolerance to and physical dependence on alcohol. On the other hand, since CDP and other benzodiazepines are commonly used as drugs to treat alcohol withdrawal [10], the chronic administration of both CDP and alcohol might retard or abolish the development of tolerance to and physical dependence on alcohol. This in turn might affect the withdrawal manifestations which follow cessation of diet administration. Therefore, we have compared the withdrawal reactions exhibited by mice that had been fed chronically an ethanolic diet with or without the incorporation of CDP. Two common measures of withdrawal signs were chosen, namely, rectal temperature [12], and withdrawal scores based on convulsions on handling [7].

METHOD

Male C57BL/6J mice (8–9 weeks old) were purchased from the Jackson Laboratories, Bar Harbor, ME. They were housed singly in plastic cages in a controlled environment room (21–22°) on a 12/12 hr light-dark cycle and received Teklad mouse diet (Teklad Mills, Winfield, IA) and tap water ad lib for 7 days before the beginning of an experiment.

Mice (N=33) were fed ad lib a chocolate-flavored Nutrament liquid diet (distributed by the Drackett Products Co., Cincinnati, OH), fortified with a vitamin mixture (ICN Pharmaceuticals, Inc., Cleveland, OH) and containing 3.5% (v/v) ethanol (from 95% ethanol). Fresh diet was prepared each day according to published procedure [6]. Daily diet intake was recorded. Another group of mice (N=33) was fed the same liquid diet except that an isocaloric sucrose solution was substituted for ethanol. The amount of diet administered for each of the 3 days was equal to the daily mean

[1]Supported in part by Grant 1732 from the New York State Health Research Council.
[2]Also faculty member of Department of Pharmacology and Experimental Therapeutics, State University of New York at Buffalo, Buffalo, NY 14214.

intake of those mice receiving the ethanol diet (first day intake determined from pilot experiments). After 3 days of acclimatization with the liquid diet, mice that had received the ethanol diet were arranged in 3 groups (A, B, C, N=11 each) such that one member of each group matched closely with the corresponding members of the other two groups in terms of their mean diet intake. Mice that had been fed the sucrose diet were randomly divided into 3 groups (D, E, F; N=11 each). Group A mice continued to receive the 3.5% ethanol diet ad lib for another 3 days. The concentration of ethanol in the diet was then increased by 1.5% every 3 days up to 8% (total of 12 more days after acclimatization). Mice in groups B and C were fed ad lib the same ethanol diets except that CDP (3.2 mg and 6.4 mg/100 ml, respectively) were incorporated each day. The hydrochloride of CDP (Hoffman-LaRoche, Inc., Nutley, NJ) was dissolved in the volume of water used to solubilize the vitamin mixture [6]. The diet tubes were wrapped in foil to protect CDP from light. We have tested (unpublished results) by high-pressure liquid chromatography, that CDP was stable for at least 24 hr in aqueous ethanol solutions (from 3.5 to 15%). Mice in group D were pair-fed with group A the corresponding isocaloric sucrose diets, while those in groups E and F were pair-fed with groups B and C, respectively, the isocaloric sucrose diet containing the respective CDP concentrations. After the 8% diet had been administered for 3 days, the mice were withdrawn from the ethanol diet at 8 a.m. on the next day and every group received an isocaloric (for 8% ethanol) sucrose diet containing no CDP. Rectal temperature [12] and withdrawal scores based on convulsions on handling [7] were recorded every 2 hr for 14 hr and at 24 hr after withdrawal.

The above experiment was repeated with the exception that 3 times the number of animals were used for groups C and F, other groups remaining the same. One third of the mice in groups C and F only were sacrificed at 10:30 a.m. on the day that ethanol diet was withdrawn. Blood and brain samples were collected and assayed for CDP and its N-demethyl metabolite (NDCDP). The extraction procedure and analytical methods were the same as those previously reported [9]. Groups B and E were not used for these analyses because our results showed that withdrawal signs were similar between groups B and C, and between groups E and F. The other mice in groups A, B and C were administered diet A (8% ethanol) for one extra day before withdrawal. This was to minimize the presence of blood and brain levels of CDP or its metabolites on the next day. Likewise, control mice were pair-fed an isocaloric sucrose diet containing no CDP. At 8:00 a.m. the next morning, the mice were withdrawn from the ethanol diet. Half of the mice in (the remaining) groups C and F were sacrificed and blood and brain samples were collected and analyzed as above; the remaining animals (including groups A, B and E and the rest of C and F) were monitored for withdrawal signs as described above.

In another experiment similar to the first one, but involving only groups A and C, tail blood samples (20 μl) were collected on the day of withdrawal at $^1/_2$ and $2^1/_2$ hr after removal of ethanol diet. These were analyzed for ethanol according to published procedure [11].

Student's *t*-test and analysis of variance were used for the statistical evaluation of the data on rectal temperature. The Mann-Whitney U test was utilized for ordinal measurements such as withdrawal scores.

RESULTS

We have demonstrated previously [2] that mice in groups B and C showed a sharp increase in diet intake (20–40%, compared with group A, $p<0.001$; unpublished results) on the first day that CDP was incorporated in the 3.5% diet (diets B and C). The magnitude of increase was the same for mice consuming diets B and C. However, there was no significant difference in the volume of diet consumed among mice in groups A, B and C on the second and third days that CDP was present in the 3.5% diets. The same was true for the six days when CDP was included in the ethanol diet which contained 5 or 6.5% ethanol (v/v, 3 days each). On the next 3 days when the diet contained 8% ethanol, mice in groups B and C showed an increase (10–20%, compared with group A) in the intake of the diets containing CDP. Except for the increase in diet intake mentioned above, the average daily ethanol intake ranged from 12 to 18 g/kg/day, irrespective of the concentration of ethanol in the diet. This is because the volume of diet consumed varied inversely to the ethanol concentration. Thus the daily CDP intake actually decreased with time as exemplified by a mean intake of about 20 mg/kg/day on the first day (day 4 of diet administration) for group B mice and of about 10 mg/kg/day on the last day (day 15 of diet administration). The corresponding CDP intakes for group C mice were about 40 and 20 mg/kg/day, respectively. The mean body weight for each group of mice was not significantly different from the others before and after diet treatment, e.g., group A had a mean body weight of 24.23 g ($\pm$ 0.30, SEM). There was an increase in weight (about 1 to 1.5 g) during the first week of diet administration for all groups, but a gradual decline to pre-experimental value occurred in the next week.

Figure 1A depicts changes in rectal temperature at various times after withdrawal from ethanol diet. There were very little and insignificant changes in temperature for mice in control groups D, E and F; therefore, only the result in group D is shown. It is seen that mice fed the ethanol diet alone (group A) had significantly lower temperature (compared to groups B, C and D) at 4 to 10 hr. Mice that had been fed the ethanol diet containing CDP (groups B and C) showed a significant decrease (compared to group D) in temperature only at 4 hr and the magnitude of decrease was significantly smaller than that found for group A at the same time (Fig. 1A). Furthermore, there was no significant difference in temperature between groups B and C. An analysis of variance indicates that at 4 to 10 hr significant interaction effects (ethanol $\times$ CDP) were observed, e.g., at 4 hr, $F(1,40)=22.49$, $p<0.001$.

Withdrawal scores at various times after diet withdrawal are shown in Fig. 2A. The data depicted for group D, namely zero score for the entire period, were similar to those (not shown) exhibited by groups E and F. Mice in groups B and C had significantly ($p<0.01$) lower withdrawal scores than group A mice from 4 to 14 hr after withdrawal. The number of mice exhibiting signs of withdrawal such as body tremor and convulsions on handling was also significantly larger in group A than groups B or C; for example, at 4 hr, 9 mice (N=11) in group A showed the above withdrawal signs compared to 4 mice (N=11) in group B or C.

Analysis of blood alcohol concentration (BAC) at $^1/_2$ and $2^1/_2$ hr after withdrawal revealed that group A mice had BAC (mg% $\pm$ SEM) of 176.5 $\pm$ 29.4 and 55.7 $\pm$ 19.4, (N=8), respectively, while the corresponding values for mice in group C were 111.1 $\pm$ 15.7 and 13.5 $\pm$ 3.8 (N=7). The differences between the two groups were not statistically significant, because of large variations in each group. At $2^1/_2$ hr after withdrawal, blood and brain samples taken from mice that

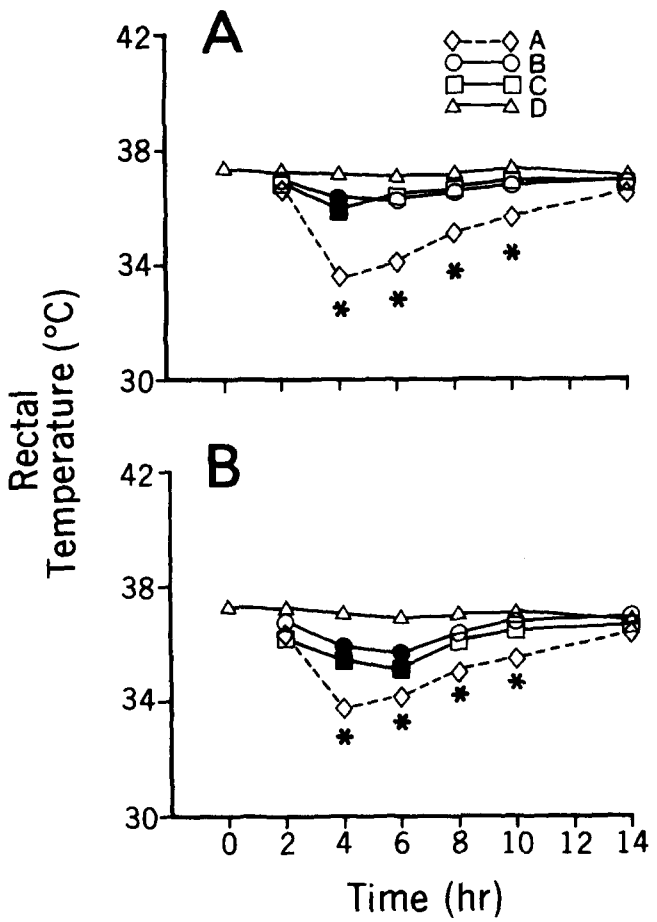

FIG. 1. Effect of combined administration of chlordiazepoxide and ethanol on rectal temperature during withdrawal. Zero hour was the moment that the ethanol diet was withdrawn. Mean values (N=11 for each group) was depicted. Closed symbols represent values significantly different ($p<0.01$) from corresponding values in group D. Group A: ethanol diet alone. Group B: ethanol diet + CDP (3.2 mg/100 ml). Group C: ethanol diet + CDP (6.4 mg/100 ml). Group D: isocaloric sucrose diet. *Significantly different from groups A, B and C, $p<0.05$. (B) was different from (A) in that there was an extra day of diet administration before withdrawal, in which groups A, B and C received the 8% ethanol diet containing no CDP and groups D, E and F (the latter two groups not shown; see text) received an isocaloric sucrose diet.

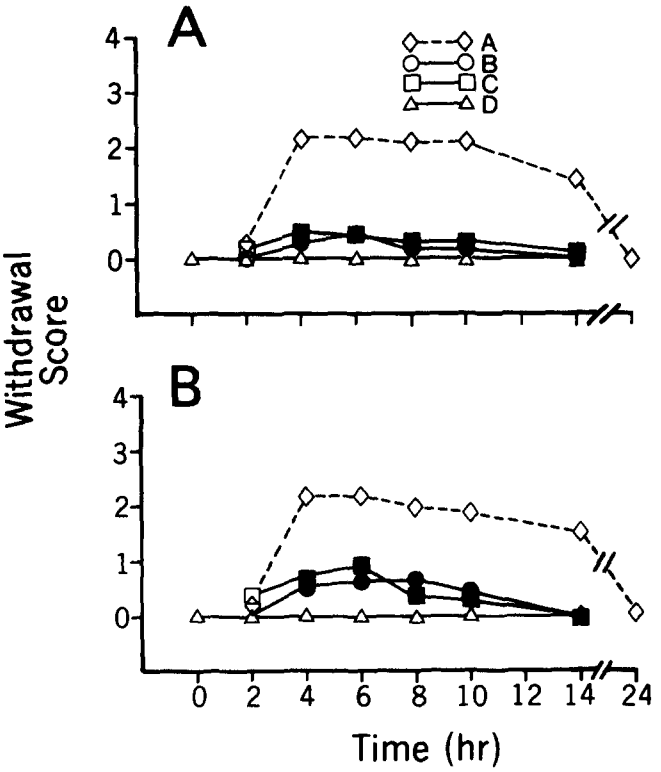

FIG. 2. Effect of combined administration of chlordiazepoxide and ethanol on withdrawal score during withdrawal. Zero hour was the moment that ethanol diet was withdrawn. Mean values (N=11 for each group) are depicted. Closed symbols represent values significantly different ($p<0.02$) from corresponding values in group A. Treatments for the various groups (A to D) were identical to those shown in legend for Fig. 1. The difference between (A) and (B) was as indicated in legend of Fig. 1.

had consumed the CDP/ethanol diet (group C) and the control diet containing CDP (group F) showed no measurable level of CDP for either group. There was also no detectable level of NDCDP in group F; however, group C showed a mean blood NDCDP concentration of 2.61 ± 0.47 μg/ml (N=8).

In the experiment where mice which had received the CDP-containing diet for 12 days (excluding 3 days of acclimatization) were fed a diet containing no CDP for one extra day before withdrawal, blood and brain samples taken at 1/2 hr after withdrawal did not show any detectable levels of CDP and NDCDP. Nevertheless, results of measurement of rectal temperature (Fig. 1B) and withdrawal scores (Fig. 2B) were qualitatively similar to those described previously for mice not receiving an extra day of liquid diet (Figs. 1A and 2A). There were slight increases in withdrawal scores in groups B and C (Fig. 2B, compared with Fig. 2A), and a more pronounced decrease in rectal temperature for groups B and C (Fig. 1B compared with Fig. 1A), especially at 4 and 6 hr. Nevertheless, the intensity of these withdrawal manifestations remained significantly less than those exhibited by mice in group A.

DISCUSSION

One or more factors may be responsible for the attenuation of withdrawal signs seen in mice which had been administered an ethanolic liquid diet containing CDP. Firstly, the chronic co-administration of CDP and ethanol may lead to an increase in the rate of metabolism of ethanol such that lower blood alcohol levels (BAL) were maintained (compared to those ingesting ethanol diet alone), especially during the latter part of the period of diet administration. It has been demonstrated [7] that the intensity of withdrawal reactions was directly correlated with BAL during chronic ethanol intake. We have observed at two time intervals after withdrawal, higher BAL in mice fed the ethanol diet alone (group A) compared to those in mice fed the CDP/ethanol diet (group C), although the difference was not statistically significant. These data are suggestive of an enhanced rate of ethanol metabolism in group C mice, especially in light of our

observation that there was a significant increase (10–20%) in the intake of the 8% diet by these animals. However, since we did not monitor the pattern of diet intake each day, we cannot rule out the possibility that the feeding pattern could be different in the two groups of animals, thereby leading to the apparent differences in BALs. Another less likely possibility is that chronic CDP treatment might alter the absorption of ethanol. However, we have previously reported [3] that mice injected intraperitoneally with an acute dose of ethanol plus CDP did not have different BALs compared to those injected with ethanol alone.

Another plausible factor contributing to the attenuation of withdrawal signs may be the presence of long-lasting metabolite(s) of CDP during withdrawal. No blood or brain CDP level was detectable during the withdrawal period. However, blood levels of NDCDP were appreciable at 2½ hr after withdrawal. It has been reported [5] that in mice the antipentylenetetrazol activity of CDP appeared to parallel the brain levels of NDCDP rather than those of the parent compound or its lactam metabolite (LCDP). Therefore, the presence of blood and brain NDCDP or its metabolites during withdrawal might lessen the severity of withdrawal symptoms. However, our results do not provide a firm support for this hypothesis. The cessation of CDP administration one day before withdrawal led to no detectable blood and brain levels of CDP and NDCDP, yet we still observed considerable attenuation of withdrawal reactions. In this instance there was, however, a trend for a slightly more severe manifestation of withdrawal reactions (compare Figs. 1B and 2B with Figs. 1A and 2A). These results suggest a minor contribution of NDCDP in attenuating the withdrawal reactions seen in Figs. 1A and 2A. The fact that groups B and C, having different CDP intake (one was about doubled), had very similar withdrawal signs also suggests a minor contribution of NDCDP. Although we cannot rule out the possibility that some other long-lasting metabolite (e.g., LCDP) might still be present, it is not likely. It has been reported [5] that after a single, oral administration of CDP (20 mg/kg) in mice, blood CDP could be detected up to 4 hr, but brain CDP was measurable up to 2 hr only; NDCDP was present in the blood and brain up to 18 hr, and polar conjugates could not be detected at this time; LCDP was not present in the brain at 18 hr, but could still be measured in the blood at 24 hr, having a mean value of 0.85 μg/g. Since the intake of diet in the present investigation was spread out throughout the whole day (total daily intake of CDP for group C mice amounted to no more than 20 mg/kg) lower blood and brain levels of CDP and its metabolites (compared to one single oral dose) would be maintained. For the period that attenuation of withdrawal signs was observed in the second experiment (Figs. 1B and 2B) mice would have been off the CDP diet for at least 28–38 hr. Therefore, it is not likely that appreciable amounts of metabolites of CDP would have been present in the blood or brain of these animals.

A third factor that can also account for the diminished intensity of withdrawal signs may be related to a probable ability of CDP to interfere with the mechanisms by which mice develop tolerance to and physical dependence on alcohol. There is no prior report suggesting such an action of CDP, but our preliminary results (unpublished) indicate that mice which had consumed the CDP/ethanol diet had a shorter ethanol sleep time (when challenged with an IP injection of ethanol, 3.5 g/kg; 35.91 ± 3.75 min, N=7) compared to mice receiving the isocaloric diet (96.74 ± 3.27 min, N=7) or the ethanol diet alone (55.57 ± 7.37 min, N=7). However, further experimentation is needed to identify the basis (metabolic or functional tolerance) for the observed difference.

Our results differ from those reported for human studies [1] in that there was no delay in the appearance of withdrawal signs in mice which were administered CDP and ethanol; in fact the time course for appearance of withdrawal signs was identical in mice receiving ethanol diet alone or ethanol diet plus CDP, although the intensities and duration differed significantly. Because of the complexity of differences between animal and human investigations, an extrapolation of the present data to the human situation is premature and unwarranted. The withdrawal syndrome exhibited by patients having a mixed alcohol-benzodiazepine addiction started 2–10 days after abrupt discontinuation of drugs, and the nature of the specific symptoms resembled withdrawal from benzodiazepines [1]. Patients addicted only to alcohol had withdrawal reactions within 24 hr after the last drink. It is probable that the attenuation of alcohol withdrawal reactions observed in mice could also be operative in the human situation. However, because of the longer elimination half-lives of benzodiazepines in humans, the attenuated alcohol withdrawal syndrome would have been suppressed by the presence of these drugs and their active metabolites. The end result was the appearance of a benzodiazepine withdrawal syndrome. In our investigation, we have monitored the mice each day for 1 week after the initial withdrawal and we did not detect any delayed appearance of withdrawal signs.

In summary, we have found that in mice chronic intake of CDP/ethanol diet resulted in a marked suppression of withdrawal signs upon termination of diet administration. The presence of blood NDCDP during withdrawal could account for a minor contribution to the protective effect. Our results are more suggestive of an increased rate of ethanol metabolism during chronic diet intake as being the major factor for the observed attenuation of withdrawal signs, although we cannot rule out the alternative possibility that CDP or its metabolites might interfere with the development of tolerance to and physical dependence on alcohol. Further investigations are needed to pinpoint the exact mechanisms.

ACKNOWLEDGEMENTS

We thank Dr. Ben Morgan Jones for reviewing this manuscript, Dr. W. E. Scott of Hoffman-LaRoche for providing CDP and its metabolites, Mr. Joseph Kaminski for ethanol analysis, and Miss Iris McLaughlin for analysis of CDP and its metabolites in blood and brain samples.

REFERENCES

1. Benzer, D. and P. Cushman. Alcohol and benzodiazepines: Withdrawal syndromes. *Alcoholism: Clin. exp. Res.* **4:** 243–247, 1980.

2. Chan, A. W. K. Influence of chlordiazepoxide on alcohol drinking. *Pharmacologist* **22:** 280, 1980.

3. Chan, A. W. K., H. B. Greizerstein and W. Strauss. Interaction of chlordiazepoxide with ethanol. *Pharmacologist* **20:** 159, 1978.
4. Chan, A. W. K. and P. Heubusch. The combined effect of chlordiazepoxide and ethanol on brain cyclic nucleotides. *Alcoholism: Clin exp. Res.* **4:** 211, 1980.
5. Coutinho, C. B., J. A. Cheripko and J. J. Carbone. Relationship between the duration of anticonvulsant activity of chlordiazepoxide and systemic levels of the parent compound and its major metabolites in mice. *Biochem. Pharmac.* **18:** 303, 1969.
6. Freund, G. Alcohol withdrawal syndrome in mice. *Archs Neurol.* **21:** 315–320, 1969.
7. Goldstein, D. B. Relationship of alcohol dose to intensity of withdrawal signs in mice. *J. Pharmac. exp. Ther.* **180:** 203–215, 1972.
8. Greenblatt, D. J. and R. I. Shader. Benzodiazepines (first of two parts). *New Engl. J. Med.* **291:** 1011–1015, 1974.
9. Greizerstein, H. B. and I. G. McLaughlin. The high-pressure liquid chromatographic determination of chlordiazepoxide and its n-demethyl metabolite in mouse brain. *J. liquid Chromat.* **3:** 1023–1030, 1980.
10. Keeler, M. H. and W. C. Miller. Selection among benzodiazepines for alcohol withdrawal. *Sth. Med. J.* **70:** 970–973, 1977.
11. LeBlanc, A. E. Micro-determination of alcohol in blood by gas liquid chromatography. *Can. J. Physiol. Pharmac.* **46:** 665–667, 1968.
12. Ritzmann, R. F. and B. Tabakoff. Body temperature in mice. A quantitative measure of alcohol tolerance and physical dependence. *J. Pharmac. exp. Ther.* **199:** 158–170, 1976.