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EFFECTS OF IMIPRAMINE, NITRITE, AND DIMETHYLNITROSAMINE ON REPRODUCTION IN MICE

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Abstract

Administration of the tricyclic dibenzazepine drug imipramine, a tertiary amine, in the food (100 mg/kg) or sodium nitrite (1 g/liter) or dimethylnitrosamine (0.1 ppm) in the drinking water of Swiss CD-1 mice before and during pregnancy, resulted in increased perinatal death of the offspring compared to controls. Administration of imipramine and nitrite together had no effect on perinatal survival, but instead resulted in infertility or delayed impregnation in some females. A biological synergism or *in vivo* chemical interaction of the two chemicals is suggested.

Introduction

The tricyclic dibenzazepine drug, imipramine, 5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-dibenz[b,f]azepine hydrochloride, is widely prescribed for adult endogenous depression and juvenile enuresis. It is a tertiary amine which might react with dietary or salivary nitrite to yield the toxicant and carcinogen, dimethylnitrosamine (DMN). We have treated mice chronically with imipramine, nitrite, imipramine plus nitrite, or a low dose of DMN, in order to determine effects on

reproductive parameters, including rates of fertility and perinatal survival, litter sizes, and weanling weights.

Methods and Results

Association of Feeding and Drinking

In these experiments two chemicals with the potential for interaction were given to mice simultaneously but in different vehicles, imipramine in the food and nitrite in the drinking water. This mode of administration eliminated the possibility of preingestion interaction of the chemicals, but required that the mice eat and drink in close temporal proximity. Several workers have noted that food and water consumption by rats are closely associated in time (Siegel and Stuckey, 1947; Spengler, 1960), a fact that was demonstrated quantitatively by the elegant experiments of Kissileff (1969). Kissileff found that for normal rats, 22% of the total water intake occurs less than 1/2 min after eating, 84% within 20 min before or after eating, and only 6% at times greater than 1 hr before or after eating.

We have confirmed a similar association between eating and drinking in mice. Adult female mice were housed in clear plastic cages and their eating and drinking observed under dim light for three 1-hr periods at the beginning of the daily dark cycle. Of 21 nibbles or meals taken by 4 control mice, 15 (71%) were associated with water drinking. Four females given imipramine-containing chow and nitrite-containing water ate little during these observation periods. It was assumed that because of the bitter taste of the drug, these mice ate fewer, larger meals, only when hungry. They were, therefore, deprived of food overnight, but given free access to nitrite-containing water.

When food was supplied in the morning, all of the mice soon commenced meals of 4-23 minutes duration, and all of these meals were punctuated with frequent drinking. These observations show that feeding was usually accompanied by drinking in both control and imipramine-plus-nitrite-treated mice, insuring adequate opportunity for *in vivo* interaction of the chemicals.

Experiment 1 (Preliminary Study)

Fifty CD-1 female mice (5-6 weeks old, Charles River Breeding Farms) were housed in groups of 10 and treated as follows. Group 1 received imipramine (Bio-Craft Laboratories) thoroughly mixed (100 mg/kg) with powdered Purina Laboratory Chow. Group 2 received 1 g/l $NaNO_2$ in their drinking (tap) water. Group 3 received both imipramine and nitrite. Group 4 received in their water 0.1 ppm DMN (Schuchardt Co., Munich) diluted twice weekly from a stock solution kept cold and dark and prepared fresh monthly. All mice were housed in plastic cages with hardwood shavings and filter bonnets, in a room at 24 ± 2°C, 40% relative humidity, and a 14/10 hr light/dark cycle. After 10 weeks, CD-1 males were added. The females were separated to individual cages when a vaginal plug was found and treatment was continued. Near term they were inspected for births once daily, except weekends. Females showing no signs of pregnancy were returned to the males for a second fertilization.

The reproductive success of each group was measured by the number of females with surviving litters and the total number of offspring at weaning (Table 1). Only in the control group did each of the 10 females have surviving offspring. Fewer offspring were weaned in the DMN-,

Table 1

Experiment 1: Effects of imipramine, nitrite, and dimethylnitrosamine on progeny yield[1]

	Treatment Group				
	Imipramine	Nitrite	Imipramine + Nitrite	DMN	Control
# ♀s with litters[2] at weaning / # ♀s starting	9/10	7/10	9/10	9/10	10/10
# Offspring	61*	52*	86	74	100
♀	29	25	39	34	51
♂	32	27	47	40	49

* Significantly different from control group ($p<.01$, Chi square test).
[1] Adult CD-1 ♀ mice received the treatment indicated for 10 weeks and were then bred. Litters were counted at weaning.
[2] Remainder of females did not become pregnant after 2 vaginal plugs or had no surviving offspring.

imipramine-, and nitrite-treatment groups, compared with the controls. This effect was of statistical significance for the imipramine group and the nitrite group. However, imipramine and nitrite administered together did not have an additive adverse effect of reproduction; indeed the progeny yield in this group was not significantly different from that in the control group, even though 1 female had no litter.

Experiment 2

After Exp. 1 indicated potentially interesting effects of the chemicals on reproductive success, Exp. 2 was carried out to study these effects in more detail. Variables recorded in this experiment were: preconception water consumption and weight gain, conception time, litter size, rates of stillbirth and neonatal death, histopathology of the major organs of dead pups, and weaning weights and sex ratios of the offspring.

One hundred weanling female mice, divided into 5 groups of 20, were housed in subgroups of 10. After 1 week on Purina Mouse Chow, preconception treatment was begun and the experiment proceeded as in Exp. 1, except that the imipramine was mixed into Mouse Chow before pelleting by the Purina Co. Waters were changed and measured twice weekly. The mice were weighed at 2-week intervals. After 75 days' preconception treatment, a male was added to each cage. The females were examined daily and were removed to individual cages when found with a vaginal plug or when obviously pregnant. Females with vaginal plugs proving not pregnant after 22 days were returned to the male. Those who did not become pregnant after a total of 60 days' exposure to the male were judged infertile. Vaginal lavage, palpation, and weighing of the mothers and newborns were omitted to avoid possible disruption of conception, gestation, or maternal care. Near term, each female was transferred to a sterilized cage and observed twice daily for birth (once a day on weekends). Newborns were counted and carefully inspected. All stillborn fetuses and dead neonates were necropsied and viscera and brains fixed in Bouin's solution.

Preconception weight gain was similar in all groups. Data related to conception and birth is presented in Table 2, and that collected at weaning in Table 3.

Imipramine Group. These females consumed more water than controls (7.7 ± 0.9 vs. 6.2 ± 0.7 ml/mouse/day ± S.D.), probably because of the taste. They required on the average 3 more days to become pregnant, and more females delivered litters at intervals greater than 30 days after addition of the males. The primary adverse effect of the

Table 2

Experiment 2: Effects on impregnation and perinatal survival[1]

	Treatment Group				
	Imipramine	Nitrite	Imipramine + Nitrite	DMN	Control
# Not pregnant[2]	0	1	5*	0	0
# Days, ♂ to birth					
Range	20-38	19-50	21-42	20-42	21-37
Average	28(17)[3]	30(13)	26 (14)	28(14)	25(14)
# Births after 30 days	6	6	4	3	3
Total # born	176	159	141[4]	185	182
Avg. #/litter[5]	8.8	8.4	9.4	9.5	9.1
Total dead (% of total born)	36**(20.4%)	23(14.5%)	14(9.9%)	38**(20.0%)	18(9.9%)
# Stillborn	24	18	7	19	5
# Neonatal deaths[6]	12	5	7	19	13
# Litters with 100% dead	2(10%)	2(10.5%)	1 (7%)	1(5%)	0
# Litters with some dead	11(55%)	9(47%)	7 (50%)	11(55%)	8(40%)

* Differs significantly from all other groups combined ($p<.01$, Chi square test).
** Differs significantly from control group ($p<.05$, Chi square test).
[1] Four-week old CD-1 ♀ mice were treated in groups of 20 as indicated for 75 days before 1 ♂ was added to each cage of 10 ♀s. Females were removed to individual cages when observed with a vaginal plug or obviously pregnant.
[2] # of ♀s of 20 not pregnant after a total of 60 days with a ♂ of known fertility.
[3] # of ♀s included (pregnant at time of first separation from ♂s).
[4] One ♀ dying during delivery was omitted from all progeny data.
[5] Live + dead young/# pregnant ♀s.
[6] Offspring dead <48 hr after birth.

Table 3

Experiment 2: Data at weaning

	Treatment Group				
	1 Imipramine	2 Nitrite	3 Imipramine + Nitrite	4 DMN	5 Control
Total # weaned	140	134	119	152	164
♀	71	56	56	51	84
♂	69	78	63	101*	80
Data for litters of 8-12[1]					
# Litters	9	10	10	10	12
Average # weaned	9.9	9.7	10.2	10.3	9.8
♀	4.8	4.1	4.8	2.9	4.9
♂	5.1	5.6	5.4	7.4	4.9
Average weanling weight (g) ± S.D.					
♀	13.8±1.9**	11.1±2.5	12.7±1.9**	12.2±2.5	11.6±1.9
♂	14.8±2.6**	11.5±3.0**	13.3±2.2	13.6±3.2	13.2±3.0

* Differs significantly from expected 1:1 ratio ($p<.001$, χ^2 test).
** Differs significantly from weights of control weanlings ($p<.005$, Student's t test).
[1] Smaller and larger litters were omitted to minimize effects of litter size.

imipramine was a significant increase in the percentage of pups dying perinatally. The majority of these were stillborn, whereas in most cases death of control offspring occurred in the immediate postnatal period. The greater incidence of dead offspring counted in the litters of imipramine-treated mothers was not due to fewer dead babies eaten by the drugged mothers since the total average litter size (live + dead young) was similar to controls. At weaning the imipramine-treated animals in litters consisting of 8-12 offspring were slightly but significantly heavier than controls.

Nitrite Group. Compared to control values, water consumption was somewhat lower (4.7 ± 0.7 ml/mouse/day), average conception time was 5 days longer, and 3 more females littered more than 30 days after the males were added. Litters were on the average slightly smaller than in other groups. Perinatal death was more common than among the controls; most of the dead young were stillborn. At weaning the offspring were somewhat smaller than controls, a difference of statistical significance for the males.

Imipramine-plus-Nitrite Group. Initially, water consumption was as great as in the imipramine group, but declined to control levels before mating. Conception time and litter size for this group were similar to control values. Imipramine and nitrite together did not act additively to result in a large number of perinatal deaths. In fact, the percent of total perinatal deaths was the same in the control and imipramine-plus-nitrite groups, although the percent stillborn was higher in the latter. At weaning the females were slightly but significantly heavier than controls, though not as heavy as those in the imipramine treatment group. Imipramine-plus-nitrite was not without adverse effect, however, since one female died during parturition, and 5 of the 20 females failed to become pregnant, even after 60 days' exposure to males of known fertility. This number of infertile females was of statistical significance ($p<.01$) when compared with all other groups combined, and its significance is further supported by the low infertility rate of CD-1 mice (about 2% in our colony).

DMN Group. Water consumption and litter size of the DMN-treated females were comparable to controls, but conception time was on the

average 3 days longer. DMN treatment was associated with a doubling of perinatal mortality, with stillbirths and neonatal deaths contributing equally. Weanlings were comparable in size to controls, but included twice as many males as females, even in litters with low perinatal mortality.

Distribution and Pathology of Dead Offspring. In each group the dead pups were distributed among 40-55% of the litters and the effects of the chemicals resulted primarily in an increase in the number and percent dead per litter containing dead pups, with a smaller increase in the number of mothers with dead offspring. Stillborn fetuses and neonatally dead pups in all groups were of normal size and appearance and exhibited no obvious lesions or abnormalities. Histological sections (7μ, hematoxylin and eosin staining) were made of liver, kidney, gastrointestinal tract, spleen, heart, lungs, and brain from at least 5 pups in each group. Many lungs were atelectatic, indicating death before commencement of air breathing, but there were no other lesions or abnormalities.

Experiment 3

Since in Exp. 2 25% of the females in the imipramine-plus-nitrite group did not become pregnant, this effect was studied further in Exp. 3, under conditions that provided maximum opportunity for rapid conception: each female was given her own male and was left with him until obviously pregnant. Forty weanling female mice were housed under the same conditions as in Exp. 2, with 30 receiving imipramine and nitrite and 10 serving as controls. Forty males of the same age received Purina Mouse Chow and water. After 77 days, the females and

males were paired until, near term, each female was housed alone in a steam-sanitized cage and observed twice daily for birth. One female in the imipramine-plus-nitrite group was killed by her mate and is excluded from the data.

Under these conditions, all of the females became pregnant. The 10 control females each delivered their young 21-28 days (avg. 23.3 days) after addition of the males (Table 4).

Table 4

Experiment 3: Effects of imipramine-plus-nitrite on fertility[1]

	Treatment Group	
	Imipramine + Nitrite	Control
# ♀ Bred	29	10
# ♀ Impregnated 21-28 days after mating	23	10
Avg. # days, ♂ to birth	23.6	23.3
# ♀ Impregnated >30 days after mating	6	0
Avg. # days, ♂ to birth	37.8	
Total born	246	101
Total dead before weaning	54 (22.0%)	19 (18.8%)
# Stillborn	20	10
# Neonatal deaths	23	4
# Deaths at 3-7 days	11	5
Average weight of weanlings (g) ± S.D.		
♀	13.6 ± 2.7	13.8 ± 2.5
♂	13.7 ± 2.9	13.7 ± 2.7

[1] Female CD-1 mice were maintained on the treatment indicated for 7 weeks and each housed with a male as treatment continued. Females were housed alone just before term. Offspring were weaned at 21 days.

Of the 29 imipramine-plus-nitrite females, 23 delivered 22-27 days (avg. 23.6 days) and 6 gave birth 30-58 days (avg. 37.8 days) after housing with males. One female died during parturition. The incidence of perinatal death was similar in imipramine-plus-nitrite and control mice.

Discussion

The results of Exps. 1 and 2 showed an adverse effect of imipramine, nitrite, and dimethylnitrosamine on reproduction of mice. Imipramine caused a doubling of perinatal mortality, due largely to an increase in stillbirths. Fetotoxic effects of imipramine have also been demonstrated in rabbits and rats (Harper *et al.*, 1965; Stenger *et al.*, 1965; Jelinek *et al.*, 1967; Singer and Coyle, 1973). Harper *et al.* (1965) saw no effects on fetal mice, but treatment was stopped and the mothers sacrificed several days before birth. The lack of preconception or postnatal toxicity of imipramine in our experiments suggested a special sensitivity of the fetus near term. Chlorpromazine, a phenothiazine derivative similar in structure to imipramine, caused reduced litter size and increased perinatal mortality when administered to mice during gestation until birth (Ordy *et al.*, 1966). Three human newborns from mothers who had taken imipramine throughout pregnancy showed severe physiological distress symptoms that could be attributed to imipramine toxicity (Eggermont *et al.*, 1972).

Benzodiazepine tranquilizers also affect reproduction in mice. Guerriero and Fox (1976) demonstrated reduced birth weight of pups exposed to such agents during gestation. The number of live-born young surviving to weaning and the weights at weaning were significantly

reduced by the benzodiazepine drugs (Guerriero and Fox, 1977), in contrast to our findings with imipramine. The latter resulted in no deaths after the neonatal period and was associated with a small but significant increase in weanling weights, perhaps reflecting greater average birth weights in the imipramine litters included in the comparison or more rapid postnatal weight gain.

Sodium nitrite, at a dose of 1000 ppm, significantly reduced overall progeny yield in Exp. 1 and in Exp. 2 had small decremental effects on litter size, perinatal survival, and weanling weight. Detrimental effects of nitrite on reproduction have been reported for the guinea pig and rat (Sleight and Atallah, 1968; Sinha and Sleight, 1971; Shuval and Gruener, 1972; Gruener *et al.*, 1973). The formation of methemoglobin and resulting anemia in the mother and/or offspring has been implicated.

Administration of 0.1 ppm DMN in the drinking water caused a doubling of perinatal deaths in Exp. 2. Fetal and young rats are known to be sensitive to toxicity of diethylnitrosamine at high concentrations (Thomas and Bollman, 1968); effects of chronic low-dose exposure to nitrosamines apparently have not been tested. It is becoming evident that DMN has detectable biological effects at lower doses than heretofore supposed. For example, in a recent study with carcinogen-susceptible Syrian hamsters, 1 ppm DMN or 1 ppm diethylnitrosamine in the drinking water caused gastric carcinoma in all treated animals (Homburger *et al.*, 1976). It may be noted that cigarette smoke, which contains nitrosamines, has been associated with human stillbirth (e.g., Butler *et al.*, 1972; Meyer *et al.*, 1975).

There were no consistent gross or histopathological changes in the stillborn or neonatally dead young mice exposed to DMN. Although DMN is hepatotoxic in adult animals, lack of lesions in the fetal liver was consistent with the results of Bhattacharyya (1965), who observed only occasional and unpredictable lesions in the livers of fetal rats exposed to high doses of DMN.

DMN treatment in Exp. 2 was also associated with an alteration of the sex ratio in favor of male offspring. Since the sex ratio was significantly different from one, even with no elevation in perinatal mortality, the effect did not seem to be due to selective sensitivity of the female fetuses at term. In Exp. 1 there was only a slight excess of males in the DMN group. The cause and reproducibility of this phenomenon require further study.

Imipramine and nitrite administered together in our experiments had a qualitatively different effect than each given separately. The reduction in litter size associated with nitrite, and the increase in perinatal death observed with both imipramine and nitrite, did not occur when the two were given simultaneously. Instead, there was in Exps. 2 and 3 a diminution of fertility. In addition, 2 females treated with imipramine-plus-nitrite, 1 in each experiment, died during parturition, an occurrence unique to this treatment group. Since such deaths are unusual in young CD-1 mice, they probably were related to treatment.

The results obtained with imipramine-plus-nitrite administration may have reflected biological synergism of the two chemicals. Rats injected i.p. with imipramine failed to deliver viable offspring because

of resorption of the fetuses (Singer and Coyle, 1973). Benzodiazepine tranquilizers have been shown to suppress the estrus cycle in C57BL/6 mice (Guerriero and Fox, 1975) and to depress mating performance in Swiss-Webster mice (Guerriero and Fox, 1976). Perhaps in our experiments disruption of reproductive physiology and/or fetal resorption occurred as a result of additive or potentiative action of imipramine and nitrite.

Another possibility was that imipramine and nitrite interacted chemically within the gastrointestinal tract or elsewhere. Increased perinatal death seen with imipramine or nitrite given singly did not occur when the chemicals were administered together. This suggested involvement of the two in an extensive chemical interaction. Dimethylnitrosamine, a possible product of such an interaction via dealkylating nitrosation, did not mimic the effects of imipramine-plus-nitrite. The possibility that DMN was formed but more rapidly metabolized in this group was rendered unlikely by the finding that the rate of blood clearance of ^{14}C-DMN was the same in mothers from all groups (data not shown). Although formation of DMN from imipramine and nitrite cannot be ruled out, some other form of chemical interaction seemed more likely, perhaps nitrosation of the heterocyclic ring or noncovalent interaction of the nitrite with cationic sites on the imipramine molecule. The formation of such products or complexes and their involvement in infertility and maternal toxicity should be investigated, especially since imipramine and other psychotropic drugs are sometimes administered to infertile (Rogers, 1966; Rogers *et al.*, 1970), as well as pregnant (e.g., Sim, 1972) women.

Although the roles of nitrite, nitrosamines, and drug-nitrite interactions in carcinogenesis are often discussed, their participation in other public health problems such as infertility and fetal wastage has been largely overlooked. Stillborn human fetuses, like those born to our imipramine-, DMN-, or nitrite-treated mice, sometimes have no gross or histopathological abnormalities (Franklin, 1974; Bhargava *et al.*, 1976). The need for investigation of chronically acting toxicants in public health problems has recently been emphasized (Train, 1977). The effects of such agents on human reproductive success might be a fruitful area for study.

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