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DEVELOPMENTAL TOXICITY OF N-METHYLANILINE FOLLOWING PRENATAL ORAL ADMINISTRATION IN RATS

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Abstract

Objectives: The objective of the study was to assess prenatal toxicity of N-methylaniline (NMA) administered by gavage to pregnant female rats. **Material and Methods:** Pregnant female rats were administered N-methylaniline in corn oil by gavage at daily doses of 0.8 mg/kg of body weight (b.w.), 4 mg/kg b.w., 20 mg/kg b.w. and 100 mg/kg b.w. from implantation (the 5th day post mating) to the day prior to the scheduled caesarean section (the 20th day of pregnancy). General behavior, body weight, food and water consumption, hematological, biochemical analyses and pathomorphological changes of the dams were recorded. **Results:** All the females survived until the end of the study. The test substance was toxic to pregnant females, even at the lowest of the used doses, i.e., 0.8 mg/kg b.w./day. Lower weight gain during pregnancy and significantly higher NMA-dose-dependent absolute weight of the organs were noted in the exposed females. The females from the groups exposed at doses of 20 mg/kg b.w./day and 100 mg/kg b.w./day developed anemia and showed higher concentrations of free thyroxine (FT3) and free triiodothyronine (FT4) thyroid hormones. Total protein concentration exhibited an increase in all the exposed groups of females. In the prenatal toxicity study, administration of N-methylaniline throughout the embryonic and fetal periods produced embryotoxic effects at doses ranging 4–100 mg/kg b.w./day. **Conclusions:** Considering the data obtained in this study, it is reasonable to assume that N-methylaniline administered orally to pregnant rats is toxic for mothers even at a low dose of 0.8 mg/kg b.w./day. However, this dose was not associated with any significant effects to their offspring. This prenatal exposure level may be considered as no-observed-adverse-effect level (NOAEL) for the progeny and a dose of 4 mg/kg b.w./day as the lowest-observed-adverse-effect level (LOAEL) for the progeny.

Key words:
Rat, N-methylaniline, NMA, Developmental toxicity, metHb, Toxicity

INTRODUCTION

Aniline and its derivatives, such as p-nitroaniline, m-nitroaniline, o-nitroaniline, are chemicals with strong methemoglobinogenic potent *in vitro* [1]. N-methylaniline (NMA) is also a methemoglobinogenic chemical. N-methylaniline is used as a solvent for many organic reactions and as an acid acceptor. This substance has been detected in foods, e.g., cheese, vegetables and orange oil. N-methylaniline, when freshly prepared, is a colorless liquid, but when left in the air, it becomes brown [2]. Toxicological data on the test substance are scarce.

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N-methylaniline is well absorbed in the respiratory and gastrointestinal tracts and through the skin. The median lethal dose (LD_{50}) upon administration to rats is 700–800 mg/kg body weight (b.w.) [3]. The lowest lethal dose of NMA to rabbits after administration *per os* is 280 mg/kg b.w. Clinical signs of poisoning (cyanosis, labored breathing, proteinuria and, during the terminal stage, convulsions) resemble those of aniline poisoning [4].

N-methylaniline shows a more potent methemoglobinogenic effect than aniline, as demonstrated by the test results reported below. Methemoglobin (MetHb) concentrations have been evaluated in cats exposed intravenously to NMA or aniline at a dose of 30 mg/kg b.w. After NMA and aniline administration, MetHb concentrations were: 68% and 30%, respectively [5]. Similarly, higher levels of MetHb have been observed in rats after intraperitoneal administration of these substances at a dose of 35 mg/kg b.w. Methemoglobin concentrations after administration of N-methylaniline and aniline were: 46% and 22%, respectively [6].

N-methylaniline is not mutagenic in *Escherichia coli* and does not induce unscheduled synthesis of deoxyribonucleic acid (DNA) in culture of rat hepatocytes, but it induces structural chromosomal aberrations in Chinese hamster lung cells with and without metabolic activation [3]. There is no evidence of carcinogenic effects of NMA in rats and mice [7,8].

It is known that aniline is a chemical compound that can cross the placenta to the fetus [9]. N-methylaniline is an aniline derivative. Because there is no data on the developmental toxicity of N-methylaniline, the aim of our study was to investigate possible disorders in fetuses following exposure to the substance.

MATERIAL AND METHODS

Chemical

The analyzed substance was N-methylaniline – CAS No.: 100-61-8, molecular formula: C_7H_9N (Figure 1), molecular weight: 107.15, synonims: monomethylaniline, N-methylaminobenzene, N-methyl-benzenamine, NMA.

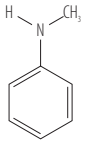

Fig. 1. Chemical structure of N-methylaniline

Animals and dosage

The present study was performed in conformity with the current Polish and European Union (EU) regulations on the protection of animals used for scientific purposes [10,11]. Experimental protocol to be used in our study was approved by the 9. Local Ethical Committee on Animal Research, Łódź (document No. 42/ŁB560/2011 of June 20, 2011).

In our study we used N-methylaniline (CAS No. 100-61-8), lot No. S33936V, 98% pure, obtained from Sigma-Aldrich (Germany).

Adult virgin female and male rats of the Wistar strain were obtained from our own breeding colony. The males and females were acclimated to the laboratory for 7 days prior to the experiment to exclude any intercurrent infections and to acclimate to the new conditions. Rats in good health were selected for study. The females and males were individually identified by ear tag, and were housed in plastic cages containing sterile paddy husk as a bedding material, with a controlled temperature 22±2°C, light/dark cycle 12:12 h (light on at 6:00 a.m.), and relative humidity of 45–55%. The rats received standard rodent chow (Fodder Factory, Motycz, Poland) *ad libitum*.

Virgin female rats, about 12 weeks of age, were mated overnight with 14-week male rats from the same supplier. The day when sperm was detected in the vaginal smear was considered to be the day 0 of pregnancy. Pregnant rats were assigned at random to 5 groups as follows: group 1 – control, which received *per os* corn oil and 4 groups of females that were administered NMA by gavage at daily doses of: 0.8 mg/kg b.w., 4 mg/kg b.w., 20 mg/kg b.w., and 100 mg/kg b.w. (0.1–12.5% LD_{50}).

Each group consisted of 16–17 pregnant females. The test substance was administered daily from implantation (the 5th day post mating) till the day prior to the scheduled caesarean section (the 20th day of pregnancy). The females were housed individually and received the tested substance once daily. The volume of each dose was adjusted to 0.5 ml/100 g b.w., and each of the females received a dose calculated from the actual body weight on a specific day of pregnancy. The test substance used in this study was kept in a sealed container in a cool (max 8°C) and dark place.

Observation and examinations

During the study, general behavior of the dams was observed in terms of the signs of toxicity before and after the daily exposure. Toxicity was evaluated based on the integral toxicity indicators such as: mortality, body weight and body weight gain, daily food and water intake, biochemical and hematological parameters, and the macroscopic and microscopic examination of internal organs at necropsy. All the females were evaluated for body weight, food and water consumption on days 0, 5, 8, 11, 14, 17 and 20 of gestation.

The surviving dams were decapitated on the 20th day of gestation. All the females had a gross-pathological examination performed. Gravid uterus was opened and first of all, the number of live and dead fetuses, and early and late resorptions in uterus were counted. Subsequently, corpora lutea in ovaries were counted.

The fetuses were macroscopically assessed. The live fetuses were sexed, measured for body weight and crown-rump length, and examined for external malformations. Approximately half of the live fetuses from each litter, randomly selected, were fixed in 95% ethanol and stained with alizarin red S for a subsequent skeletal examination [12]. The remaining live fetuses in each litter were fixed in Bouin's solution for a visceral examination using the free-hand razor blade sectioning method [13].

Biochemical, hematological and pathomorphological examination

Blood samples were collected at necropsy on the 20th day of gestation from all the control and exposed dams for biochemical and hematological analyses. The samples were collected in the morning between 7:30–9:30 a.m. to minimize differences arising from the circadian cycle. Before blood sampling, on the day preceding the tests, the animals were left without food between 5:00–6:00 p.m.

Blood was collected by intracardiac puncture into 2 S-Monovette tubes containing ethylenediaminetetraacetic acid (EDTA) or lithium heparin as an anticoagulant. S-Monovette tubes containing EDTA were used for all hematological determinations. Hemoglobin was determined by the use of the cyanmethhemoglobin method with Drabkin reagent.

Lithium heparin tubes were centrifuged (10 min, 5000 rpm/min), plasma was collected, erythrocytes were washed 3 times with 0.9% NaCl and lysed by freeze-thawing. The following parameters were determined in the plasma to assess the overall health: concentration of total protein, blood urea nitrogen (BUN), bilirubin, total cholesterol and activities of alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Determinations of biochemical parameters in serum were performed using kits supplied by Alpha-DIAGNOSTICS (Poland). Precision and repeatability of the method were checked using the reference normal and pathological sera from the same company. Methemoglobin concentration was also determined in the blood collected from the dams during the *post mortem* examination.

Free thyroxine (FT4) and free triiodothyronine (FT3) concentrations in plasma were determined by the electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics, Germany) using kits, calibrators and controls from the same manufacturer. The analysis was performed using Cobas immunoassay analyzer. The limit of detection was 0.6 pmol/l for FT3 and 0.3 pmol/l for FT4.

Internal organs were dissected and weighed (brain, liver, spleen, kidneys, adrenals, thyroid, pituitary gland, uterus, ovaries and placentas). The organs were fixed with 10% formalin in a phosphate buffer. The tissues were prepared using the routine paraffin technique. The 4–6 μm paraffin sections were stained with hematoxylin and eosin in a Varistain Gemini Slide Stainer. Microscopic preparations were assessed qualitatively by means of light microscopy (Nikon Eclipse, Japan).

Statistical analysis

Absolute and relative weight of internal organs as well as hematological and biochemical parameters were compared using the one-way analysis of variance followed by the Dunnett's test. The effect on food and water consumption, body weight and body weight gain was evaluated by the use of analysis of variance, followed by the Dunnett's multiple comparison test if differences were found [14]. The frequency data (litters totally resorbed and sex ratio of the live fetuses with delayed ossification) were analyzed using the Fisher's exact probability test [15]. Statistical analysis of the offspring data was carried out using the litter as a unit. In all statistical analyses, the difference between the compared variables was assumed to be statistically significant at $p < 0.05$. The data were analyzed using STATA 9 package (Stata-Corp-LP, TX, USA).

RESULTS

All the females survived until the end of the study. The female rats that had NMA at a dose of 100 mg/kg b.w. daily administered had tachypnea and an ungroomed hair-coat. In the prenatal toxicity study, administration of N-methylaniline through-out the embryonic and fetal periods produced embryotoxic effects at doses, range 4–100 mg/kg b.w./day.

Weight gain of the female rats exposed to NMA at a daily dose of 100 mg/kg b.w. was significantly lower compared to the control group since the 8th day of pregnancy and compared to the females exposed at doses of 4 mg/kg b.w., and of 20 mg/kg b.w. since the 14th day of gestation. The daily intake of food in the group of females exposed to the highest dose was significantly lower in the 2nd and 3rd week of pregnancy, while in the groups exposed at 4 mg/kg b.w./day and 20 mg/kg b.w./day it was significantly lower in the 2nd week. Water intake in the female groups exposed to NMA at 4–100 mg/kg b.w./day was lower than in the control on the 5th and 11th day of gestation. There was no significant effect of NMA administered at a daily dose of 0.8 mg/kg b.w. on water intake by the female rats (Table 1).

On the 20th day of pregnancy the dams were necropsied. The *post mortem* macroscopic evaluation showed that spleen from the females exposed to NMA at doses of 20 mg/kg b.w./day and 100 mg/kg b.w./day were significantly higher than in the female controls. No macroscopic pathologic changes (e.g., color, structure, dimension) were detected in other internal organs, peritoneal, pleural cavities and on inner integument of the animals.

Table 2 shows terminal body weight of the females as well as the absolute and relative organ weight (ratio of organ weight to body weight). The average body weight and absolute liver weight of the females receiving NMA at a dose of 100 mg/kg b.w/day were significantly lower than in the control group. In contrast, the absolute weight of spleen, kidneys, thyroid in the groups of females receiving NMA at doses of 4 mg/kg b.w./day, 20 mg/kg b.w./day and 100 mg/kg b.w./day were significantly higher than the weight of the corresponding organs of the control females. The relative weight of the spleen, kidneys, pituitary thyroid were higher than in the controls, and increased along with higher doses of NMA, while the relative weight of the liver, brain, adrenal glands and ovaries were significantly higher than in the controls only in the group of females exposed to NMA at a dose of 100 mg/kg b.w./day (Table 2).

Table 3 presents reproductive findings in the rats given N-methylaniline by gavage from the 5th to the 20th day

Table 1. Body weight gain, food and water consumption of the female rats receiving N-methylaniline (NMA) *per os* from the 5th to the 20th day of gestation

Variable	Control group (N = 17)	Groups exposed to NMA			
		0.8 mg/kg b.w./day (N = 16)	4 mg/kg b.w./day (N = 17)	20 mg/kg b.w./day (N = 17)	100 mg/kg b.w./day (N = 16)
Body weight gain during pregnancy [g] (M±SD)					
days 0–5	21.3±8.9	15.4±8.1	17.8±7.0	17.2±4.5	15.7±5.8
days 0–8	26.1±4.7	18.8±6.3	17.1±8.6	16.8±7.7	7.9±7.9**
days 0–11	34.8±5.3	34.4±8.9	26.9±12.0	25.2±10.4	13.3±8.7**
days 0–14	53.1±4.9	44.6±8.5	33.4±13.2**	31.1±10.1**	15.8±10.8**
days 0–17	68.3±6.8	64.7±13.2	50.7±16.5**	49.0±14.0**	16.4±13.0**
days 0–20	101.6±8.2	104.8±19.4	76.5±22.7**	73.2±19.9**	21.6±16.2**
Daily food consumption [g/dam] (M±SD)					
day 0	19.8±2.3	18.7±1.4	19.4±2.0	19.8±1.6	19.5±1.4
day 5	18.6±1.4	20.7±2.0	18.8±3.2	18.8±1.4	19.0±2.7
day 8	19.1±2.1	18.9±2.3	15.1±3.9**	15.1±2.7**	11.7±2.6**
day 11	20.1±1.4	21.1±3.9	15.6±5.4**	16.1±2.1**	13.9±4.2**
day 14	19.8±1.7	18.9±3.8	16.9±1.7**	17.0±1.3**	14.3±2.4**
day 17	19.9±2.1	20.2±2.4	18.4±3.5	19.6±4.1	16.1±3.4**
day 20	18.3±3.0	18.7±2.9	18.2±3.2	17.8±4.1	13.4±2.6**
Daily water consumption [ml/dam] (M±SD)					
day 0	32.5±3.9	31.9±2.1	33.4±2.8	31.7±2.5	32.6±3.3
day 5	37.1±3.2	36.6±2.9	34.8±7.3**	33.6±4.7**	33.3±4.6**
day 8	37.7±4.4	38.9±2.6	36.1±3.8	37.1±2.4	36.6±3.9
day 11	38.1±4.3	37.6±4.0	31.7±6.0**	31.7±7.3**	30.9±7.0**
day 14	37.0±2.6	35.9±7.1	37.1±7.7	34.0±5.3	38.1±9.7
day 17	38.7±3.1	40.4±4.0	38.4±5.4	39.5±7.1	39.0±7.9
day 20	36.7±5.0	41.0±4.8	39.2±5.3	36.2±8.5	39.8±7.6

M – mean; SD – standard deviation; b.w. – body weight; N – number of females examined.
Significantly different from the control: ** $p < 0.01$; * $p < 0.05$.

of gestation. No changes in the percentage of pregnant females in all the inseminated females, number of corpora lutea and implants per litter were observed. No changes in the pregnant/inseminated ratio, number of corpora lutea and implants per litter were observed.

Preimplantation losses were significantly increased in the NMA groups with doses 4–100 mg/kg b.w./day. In the females from the exposed group, embryolethality was observed at daily doses of 4–100 mg/kg b.w., but early resorptions per litter were significantly higher in

Table 2. Absolute and relative organ weight of the female rats receiving N-methylaniline (NMA) *per os* from the 5th to the 20th day of gestation

Examined parameter	Absolute and relative weight[a] (M±SD)				
	control group (N = 17)	groups exposed to NMA			
		0.8 mg/kg b.w./day (N = 16)	4 mg/kg b.w./day (N = 17)	20 mg/kg b.w./day (N = 17)	100 mg/kg b.w./day (N = 16)
Terminal body [g]	304.80±20.40	301.40±32.00	305.70±33.50	296.70±29.10	243.70±21.80**
Liver [g (g%)]	12.38±0.86 (4.08±0.35)	13.31±1.30 (4.42±0.66)	12.48±1.55 (4.08±0.23)	12.34±1.25 (4.14±0.36)	11.07±1.26** (4.56±0.62**)
Spleen [g (g%)]	0.60±0.08 (0.20±0.03)	0.67±0.09* (0.22±0.03)	0.83±0.12** (0.27±0.04**)	1.12±0.15** (0.38±0.05**)	2.21±0.30** (0.91±0.14**)
Kidneys [g (g%)]	1.25±0.17 (0.41±0.04)	1.32±0.16 (0.44±0.06)	1.43±0.14** (0.47±0.05**)	1.42±0.14** (0.48±0.03**)	1.44±0.11** (0.59±0.06**)
Brain [g (g%)]	1.76±0.13 (0.58±0.04)	1.68±0.15 (0.56±0.09)	1.79±0.11 (0.59±0.05)	1.77±0.09 (0.60±0.06)	1.80±0.07 (0.74±0.05**)
Pituitary [mg (mg%)]	9.50±1.41 (3.12±0.42)	10.50±2.22 (3.09±0.52)	10.90±1.34 (3.59±0.40*)	11.50±1.63 (3.85±0.46**)	11.10±1.62 (4.58±0.89**)
Thyroid [mg (mg%)]	10.60±3.52 (3.08±1.48)	11.08±1.15 (3.74±0.72)	13.00±2.75* (4.24±0.86**)	14.80±2.12** (4.96±0.74**)	13.10±2.22** (5.40±0.93**)
Adrenals [mg (mg%)]	61.10±9.60 (20.10±2.89)	64.80±9.74 (21.50±2.35)	67.80±11.67 (22.30±3.74)	64.80±10.02 (21.80±3.83)	66.20±7.15 (27.30±3.64**)
Ovaries [mg (mg%)]	95.90±12.20 (31.50±2.99)	90.10±12.10 (30.20±5.20)	102.80±19.80 (33.60±4.60)	103.30±25.70 (34.40±7.79)	94.70±22.40 (38.90±9.03**)

[a] Relative weight (g% or mg%) is the ratio of organ weight to body weight.
Abbreviations as in Table 1.

Table 3. Developmental toxicity in the rat fetuses prenatally exposed to N-methylaniline (NMA)

Variable	Control group	Groups exposed to NMA			
		0.8 mg/kg b.w./day	4 mg/kg b.w./day	20 mg/kg b.w./day	100 mg/kg b.w./day
Inseminated females [n]	20	20	20	21	21
Pregnant females [n (%)]	17 (85)	16 (80)	17 (85)	17 (81)	16 (76.2)
Corpora lutea/dam (M±SD)	14.30±2.31	14.30±1.49	14.70±1.84	14.30±3.03	15.00±2.48
All litters					
implantations/litter (M±SD)	11.50±2.70	12.50±2.59	8.53±3.92	8.29±3.53	9.13±4.87
preimplantation losses/litter (M±SD)	2.87±2.20	2.10±2.33	6.24±3.65*	5.94±3.90*	5.88±4.59*
postimplantation losses/litter (M±SD)	0.47±0.74	0.90±0.99	1.82±1.21	1.58±2.27	8.69±5.17**
early resorptions/litter (M±SD)	0.44±0.73	0.65±0.99	1.30±1.21*	1.00±1.00	1.40±0.89*
late resorptions/litter (M±SD)	0	0	0.50±1.51	0.12±0.33	7.30±4.97**
totally resorbed litters [n]	0	0	0	1	14

dead female fetuses/litter (M±SD)	0	0	0.06±0.25	0.18±0.53	0
dead male fetuses/litter (M±SD)	0	0	0	0.29±0.69	0
Live litters					
live female fetuses/litter (M±SD)	4.80±2.14	4.90±1.59	2.80±1.68**	3.10±1.90**	0.25±0.77**
live male fetuses/litter (M±SD)	6.30±1.67	6.50±1.43	3.90±2.49**	3.60±2.52**	0.19±0.54**
sex ratio male/female fetuses	100/77	104/80	67/47	62/53	3/4
average male fetal body weight/ litter (M±SD)	3.50±0.58	3.30±0.50	3.30±0.33	3.30±0.34	2.60±0.83
average female fetal body weight/ litter (M±SD)	3.70±0.47	3.50±0.57	3.50±0.29	3.40±0.34	2.90±0.49
average male fetal body length/ litter (M±SD)	3.90±0.15	3.80±0.20	3.90±0.10	3.90±0.11	3.40±0.28
average female fetal body length/ litter (M±SD)	4.00±0.11	4.00±0.22	3.90±0.09	3.90±0.10	3.40±0.04
Gravid uterine weight [g] (M±SD)	61.50±12.85	63.90±16.60	38.30±17.37**	39.90±18.53**	21.40±8.29**
Average placenta weight/litter [g] (M±SD)	0.50±0.06	0.54±0.05	0.61±0.15*	0.57±0.11*	0.55±0.08

Abbreviations as in Table 1.

the 4 mg/kg b.w. and the 100 mg/kg b.w. groups, while late resorptions in the 100 mg/kg b.w. group. Number of postimplantation losses tended to grow with an increasing NMA dose but, due to a high variability of that parameter, the difference was statistically significant only in the 100 mg/kg b.w./day group.

The number of live fetuses of each sex in litters of the females exposed to NMA at daily doses of 4 mg/kg b.w. and higher was significantly lower than in the litters of the control females. Body weight of the fetuses from the 100 mg/kg b.w./day group was lower than that of the control fetuses of the same sex, but the difference was not statistically significant because the number of fetuses in the 100 mg/kg b.w./day group was very small. Weight of gravid uteri in the females from the 4–100 mg/kg b.w./day groups was decreased, while the average weight of placenta in those groups was increased (Table 3).

Results of the reproductive toxicity tests in the females exposed to NMA at the highest dose of 100 mg/kg b.w./day need to be supplemented. Of the 16 litters obtained from fertilized females, 14 were totally resorbed. The two remaining litters comprised only 7 (3 female and 4 male) fetuses. Therefore, the weight of gravid uterus of females in the 100 mg/kg b.w./day group was significantly lower. There was no developmental toxicity in the 0.8 mg/kg b.w./day group (Table 3).

Table 4 presents the summarized results of external, skeletal and visceral examinations of fetuses from the control and NMA-exposed dams. Congenital malformations or significant delays in the development of the skeleton or internal organs were not observed in the fetuses of either the control or the prenatally NMA-exposed females. The frequency of detected minor skeletal variations was similar in the control and in the groups exposed to 0.8–20 mg/kg b.w. daily doses of NMA. Assessment of skeletons of the fetuses exposed to NMA at a dose of 100 mg/kg b.w./day was not feasible because they were damaged during Dawson (1926) [12] staining. Assessment of internal organs showed isolated cases of renal pelvis dilatation in the fetuses exposed prenatally to NMA at doses of 4 mg/kg b.w./day and 20 mg/kg b.w./day; however, the frequency of these changes was not statistically significant.

Table 4. External, skeletal and internal examinations of the fetuses of the rats prenatally exposed to N-methylaniline (NMA)

Variable	Control group (N = 17)	Groups exposed to NMA			
		0.8 mg/kg b.w./day (N = 16)	4 mg/kg b.w./day (N = 17)	20 mg/kg b.w./day (N = 17)	100 mg/kg b.w./day (N = 16)
External examination fetuses/litters	177/17	184/16	114/17	115/16	7/2
Skeletal examination fetuses/litters	87/17	93/16	57/17	58/16	4/2[a]
Fetuses/litters with skeletal malformations	0	0	0	0	–
Fetuses/litters with skeletal variations	3/2	6/3	8/5	7/4	–
sternebrae ossification bipartite	2/1	3/3	3/2	4/4	–
sternebrae ossification absent	1/1	2/1	0	1/1	–
skull incomplete ossification	0/17	3/2	7/5	3/2	–
Internal examination fetuses/litters	90/17	91/16	57/17	57/16	3/2
Fetuses/litters with internal malformations	0	0	0	0	0
Fetuses/litters with internal variations	0	0	1/1	2/1	0
dilatation of renal pelvis	0	0	1/1	2/1	0

[a] All fetuses were damaged during staining process.

Table 5. Biochemical parameters in plasma of the female rats receiving N-methylaniline (NMA) *per os* from the 5th to the 20th day of gestation

Biochemical parameter	Biochemical parameters in plasma (M±SD)				
	control group (N = 17)	groups exposed to NMA			
		0.8 mg/kg b.w./day (N = 16)	4 mg/kg b.w./day (N = 17)	20 mg/kg b.w./day (N = 17)	100 mg/kg b.w./day (N = 16)
Urea [mg/dl]	55.20±7.81	53.30±9.39	49.60±7.23	48.80±6.38	48.80±8.50
BUN [mg/dl]	25.80±3.64	24.90±4.38	20.40±5.18	21.40±4.73	21.90±5.47
Cholesterol [mg/dl]	55.20±12.50	57.90±19.30	60.70±13.20	64.50±16.00	67.00±14.00
Protein (total) [g/dl]	4.42±0.42	4.92±0.66*	5.50±0.64**	5.72±0.71**	6.34±0.74**
Bilirubin [mg/dl]	0.59±0.19	0.58±0.26	0.61±0.19	0.89±0.29*	1.64±0.62**
AST [U/l]	60.10±22.89	68.70±6.75	60.30±12.24	62.80±24.13	84.10±24.45**
ALT [U/l]	46.50±12.09	53.90±12.09	50.30±15.70	46.30±13.82	53.70±14.19

BUN – blood urea nitrogen; AST – aspartate aminotransferase; ALT – alanine aminotransferase.
Other abbreviations as in Table 1.

Table 5 presents the results of biochemical studies of the female rats exposed from the 5th till the 20th day of gestation to NMA administered by gavage. No significant differences in the concentration of urea, BUN, total cholesterol in plasma of the females exposed to the test compound were detected, as compared to the control group. In all the groups of exposed females, the total protein concentration was significantly higher than in the plasma of the control females. A significant increase in bilirubin was observed in the females who were receiving the highest (20 mg/kg b.w./day and 100 mg/kg b.w./day) daily doses of NMA. Activity of alanine amine transferase (ALT) did not change significantly in the plasma of the control and exposed females. In contrast, the activity of aspartate aminotransferase (AST) in plasma of the females exposed to the highest dose of NMA was significantly higher than in the control females (Table 5).

Results of hematological analysis indicate that the concentration of hemoglobin in the blood of the females from the 100 mg/kg b.w./day group was significantly lower than in the control group, and the number of white blood cells was significantly higher than in the control group. The percentage of lymphocytes increased along with the dose of NMA, but a significant difference was observed only in the group of rats that received the highest dose of the substance. In contrast, the percentage of granulocytes was significantly lower in the 100 mg/kg b.w./day group. The number of red blood cells and associated indices (mean corpuscular volume (MCV), red-cell distribution width (RDW), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)) in the groups treated with NMA at doses of 20 mg/kg b.w./day and 100 mg/kg b.w./day were significantly different compared with the control. The number of platelets was significantly lower in the groups administered NMA at daily doses of 4 mg/kg b.w./day and 100 mg/kg b.w./day than in the control group (Table 6).

Table 7 shows concentrations of thyroid hormones in plasma of the females from all groups. It was found that the concentrations of free triiodothyronine (FT3) and free thyroxine (FT4) in the plasma of the females exposed to the 2 highest doses of NMA were significantly higher than in the plasma of the female controls.

Table 6. Hematological parameters in plasma of the female rats receiving N-methylaniline (NMA) *per os* from the 5th to the 20th day of gestation

Hematological parameter	Hematological parameters in plasma (M±SD)				
		groups exposed to NMA			
	control group (N = 17)	0.8 mg/kg b.w./day (N = 16)	4 mg/kg b.w./day (N = 17)	20 mg/kg b.w./day (N = 17)	100 mg/kg b.w./day (N = 16)
Hemoglobin [g/dl]	11.30±0.51	11.20±1.14	11.40±0.80	11.00±0.62	10.60±0.55**
Hematocrit [%]	32.10±1.20	32.30±3.09	31.80±2.40	31.30±1.32	30.60±1.30
Red blood cells [$\times 10^6$/mm^3]	6.28±0.29	6.18±0.63	6.20±0.53	5.56±0.38**	4.18±0.35**
White blood cells [$\times 10^3$/mm^3]	4.60±1.92	4.40±1.02	3.60±1.18	4.00±1.30	6.60±3.84*
Lymphocytes [%]	40.80±5.71	42.90±8.74	43.70±9.60	45.60±6.92	62.10±9.31**
Granulocytes [%]	39.30±6.91	33.80±9.64	38.60±6.42	35.90±7.52	20.50±7.03**
Monocytes [%]	19.90±4.72	22.50±7.13	15.90±3.30	18.50±6.23	17.50±5.33
Mean corpuscular volume (MCV) [ft]	51.30±1.60	50.80±1.50	51.80±1.60	56.90±3.25**	73.70±6.09**
Red-cell distribution width (RDW) [%]	12.90±0.40	12.80±0.76	13.20±0.76	14.20±0.81**	15.60±1.50**
Mean corpuscular hemoglobin (MCH) [pg]	18.00±0.60	18.30±0.63	18.50±0.66	19.90±1.14**	25.20±1.46**
Mean corpuscular hemoglobin concentration (MCHC) [g/dl]	35.20±0.61	35.90±0.89	35.90±1.15	34.90±0.83*	34.30±1.24*
Platelets [$\times 10^3$/mm^3]	817.00±98.50	746.00±91.70	718.00±116.60*	779.00±64.50	655.00±98.10**

Abbreviations as in Table 1.

Table 7. Concentration of plasma thyroid hormone of the female rats receiving N-methylaniline (NMA) *per os* from the 5th to the 20th day of gestation

Thyroid hormone	Thyroid hormones in plasma (M±SD)				
		groups exposed to NMA			
	control group (N = 8)	0.8 mg/kg b.w./day (N = 8)	4 mg/kg b.w./day (N = 8)	20 mg/kg b.w./day (N = 8)	100 mg/kg b.w./day (N = 8)
Free triiodothyronine (FT3) [(pmol/l]	3.42±0.34	3.56±0.60	3.59±0.66	4.23±0.30*	4.76±0.34**
Free thyroxine (FT4) [pmol/l]	15.10±4.60	16.60±1.99	15.40±5.88	19.60±3.87	26.30±8.63*

Abbreviations as in Table 1.

Histopathology of internal organs revealed foci of extramedullary hematopoiesis in the spleen of the females exposed to NMA at doses of 4 mg/kg b.w./day and above. They were large clusters of white blood system cells (myelocytes) and the plate system cells (megakaryocytes). Similar changes also occurred in the females exposed at a dose of 0.8 mg/kg b.w./day; however, the incidence was significantly lower. There were no pathomorphological changes in other internal organs.

DISCUSSION

Methemoglobinemia is the primary toxic effect of aromatic amines (including aniline and its derivatives) [16,17]. The time that elapses from the end of exposure to the time

of blood sampling for an analysis is the factor that affects MetHb concentration in the blood.

Pauluhn [18] has found that the quantitative result requires sampling and processing of blood within the first 5 min since cessation of exposure. Kim and Carlson [19] have shown that MetHb half-life after inhalation exposure to aniline of rats was as short as 75 min (exposure at 400 mg/m^3, for 8–12 h).

Effectiveness of formation of MetHb largely depends also on other factors, such as, e.g., the route of exposure. Pauluhn [20] has conducted a study on beagle dogs exposed to aniline head-only or by gavage in such a way as to achieve a target dose of aniline in the body of animals of ca. 15 mg/kg b.w. Concentration of MetHb in the blood of dogs exposed by gavage was approx. 5-fold higher than in the blood of the animals after 4 h of inhalation exposure.

Accordingly, although in our study, gavage, rather than inhalation, was the favored route of administration, the MetHb concentrations in blood were indistinguishable from those in the control group (data not shown), because the samples were collected the next day after finishing NMA administration. Besides, assessment of blood MetHb concentration is made difficult by the high activity of N-acetylotransferase – the enzyme involved in MetHb metabolism [21].

Clinical analysis of the key parameters confirmed that NMA, as aniline derivative, is hepatotoxic. Comparisons revealed concentration response in all the exposure groups only in total protein, in the case of bilirubin concentrations – a statistically significant increase was noted in the 20 mg/kg b.w./day and 100 mg/kg b.w./day exposure groups. Results of the studies on intragastric exposure of laboratory animals to NMA or the effects of that exposure on the major biochemical and hematological indices have not been published yet. Subacute inhalation study of male rats has shown a significant increase of bilirubin concentration after exposure to 270 mg/m^3 of aniline. Other analyzed parameters differ from control, but dose-effect relationship has not been ascertained [18].

In our study we did not find many statistically significant changes in several biochemical parameters depending on the dose of NMA. However, the trend indicating NMA hepatotoxicity is consistent with that observed after administration of aniline.

In the present study we found anemia in the pregnant females exposed to NMA at the 2 highest doses of 20 mg/kg b.w./day and 100 mg/kg b.w./day. Maternal exposure to high doses of the test compound (20 mg/kg b.w./day and 100 mg/kg b.w./day) resulted in a significant decrease in erythrocyte counts and hemoglobin concentration ($p < 0.01$ compared with control). These changes are probably related to the fact that aromatic amines, including aniline and its derivatives and metabolites, are possible mediators of chemical-induced methemoglobinemia and hemolytic anemia [22,23].

The increased activity of AST in the animals exposed to the highest dose of NMA may be a result of ischemia, caused by NMA induced methemoglobinemia and hemolytic anemia. It is in agreement with the decreased count of erythrocytes, hemoglobin concentration and an increased bilirubin level. Analysis of the effects of exposure to other derivatives of aniline leads to a conclusion that the subchronic toxicity of aniline hydrochloride in rats significantly decreases erythrocyte counts and hemoglobin levels during 30 days of treatment [24]. Similarly as in our study, the mean corpuscular volume and mean corpuscular hemoglobin increased in 60 days and 90 days of exposure [24]. It means that erythrocytes are macrocytic and hyperchromic.

After a subacute inhalation exposure to aniline (9.2 mg/m^3, 32.4 mg/m^3, 96.5 mg/m^3 and 274.9 mg/m^3, 6 h/day, 5 day/week for 2 weeks), after the highest level of exposure, a decrease in hemoglobin level and hematocrit and reticulocyte counts and erythrocyte morphological alterations in blood of male rats have been observed [18].

Similarly to our study, a significantly lower number of red blood cells has been observed in rats after a subchronic

exposure to aniline hydrochloride in comparison with the controls. At the same time indicators such as MCV and MCH have been higher. In addition, in our females from the 20 mg/kg b.w./day and/or 100 mg/kg b.w./day groups, MCHC was significantly lower, while RDW and the number of white blood cells were significantly higher.

Our results of the pathomorphological assessment of internal organs of the females revealed splenomegaly and pathological changes in the spleen. In the red pulp of all the animals exposed to NMA at doses ranging 4–100 mg/kg b.w./day, there were large clusters of myelocytes and a few megakaryocytes indicating an increased splenic extramedullary hematopoiesis. Similar changes, but with a much lower intensity, were observed in the spleen of the females exposed *per os* to NMA at 0.8 mg/kg b.w./day.

These results are in agreement with the observations of rats exposed by inhalation to aniline [18]. Male rats exposed to aniline at 96.5 mg/m^3 and higher for 2 weeks have shown anemia, decreased hemoglobin and hematocrit and toxic effects in the spleen. Hypoxic conditions prevailing in the case of poisoning with methemoglobinogenic agents, intensify the process of extramedullary hematopoiesis. As the amount of bone marrow in rodents is relatively small, some portion of erythrocytes is produced by the spleen [25,26]. It should be noted that the process of hematopoiesis in the spleen continues also in adult rats [27], while in humans it stops right after birth [28].

Assessment of reproductive toxicity in the rats under the influence of NMA, in the presented study, revealed maternal toxicity, as evidenced by a significant decrease in body weight gain, food consumption during pregnancy and an increased absolute and relative organ weight found at doses of 4 mg/kg b.w./day and higher. Our results indicate that the tested chemical when administered by gavage from the 5th to the 20th day of gestation reduces fertility, increasing the number of implantation loss.

The study of prenatal development of the offspring of the females exposed to NMA did not reveal any significant adverse effect of the exposure. Evaluation of morphological development showed similar frequency and type of skeletal variations in the fetuses from all the groups, i.e., from the control and exposed mothers, e.g., extension of fontanels due to incompletely ossified parietals and interparietals bones or bipartiale fifth and/or absent sixth ossification centers of sternabrae. Similar frequency of such changes in control rats has been also observed by other authors [29,30]. In our study, the frequency of delayed ossification was slightly higher in the offspring of the females exposed to NMA compared to the controls. However, the difference was not statistically significant.

The delayed ossification observed in this study could be a consequence of a slight malnutrition of the mothers exposed during pregnancy. Food intake in the female groups receiving higher doses of NMA was in fact significantly lower – by about 12–20% – than in the controls. Similar effects, but with a significant, up to 40%, reduction in maternal food intake during the period of organogenesis have been found in rats by Fleeman et al. [31]. However, it seems reasonable to assume that the observed delay in ossification does not significantly affect postnatal development of the offspring of females exposed to NMA.

The suggestion has been confirmed by the results of Marr et al. [32], who have observed delayed fetal ossification no longer visible in about 2-month-old offspring. Dilated renal pelvis was also noted in individual cases of fetuses from the groups with a higher level of exposure. In accordance with the criteria of evaluation of fetuses, the delayed ossification and the dilatation renal pelvis observed in this study were transient and disappeared during further development of the fetus [29,33,34].

CONCLUSIONS

Considering the data obtained in this study, it is reasonable to assume that N-methylaniline administered orally to pregnant rats is toxic for mothers even at a low

dose of 0.8 mg/kg b.w./day. However, this dose was not associated with any significant effects to their offspring. This prenatal exposure level may be considered as no-observed-adverse-effect level (NOAEL) for the progeny and a dose of 4 mg/kg b.w./day as the lowest-observed-adverse-effect level (LOAEL) for the progeny.

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