Anais da Academia Brasileira de Ciências (2017) 89(1 Suppl.): 635-647
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201720160483
www.scielo.br/aabc

ACADEMIA BRASILEIRA DE CIÊNCIAS 1916 2016

Differences between NMRI and DBA/2J mice in the development of somites and susceptibility to methylnitrosourea-induced skeleton anomalies

IBRAHIM CHAHOUD[1] **and FRANCISCO J.R. PAUMGARTTEM**[2]

[1] Institut für Klinische Pharmakologie und Toxikologie, Charité - Universitätsmedizin Berlin, Campus Charité Mitte, Therapieforschung, Charitéplatz 1, 10117 Berlin, Deutschland
[2] Laboratório de Toxicologia Ambiental, Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz, Av. Brasil, 4036, salas 101-104 e 913, 21040-361 Rio de Janeiro, RJ, Brazil

Manuscript received on July 25, 2016; accepted for publication on January 5, 2017

ABSTRACT

The development of DBA/2J mouse strain embryos is nearly 12 h - or 6 somite pairs - delayed as compared to the outbred NMRI mouse embryos of the same age on gestation days (GD) 8-12. To evaluate inter-strain differences in susceptibility to teratogens, dams were treated with methylnitrosourea (MNU, 5 mg/kg body weight i.p.) on defined gestation days (NMRI: GD 9, $9^{1/2}$ or 10; DBA/2J: GD 10 or $10^{1/2}$). Skeletal anomalies produced by MNU on both mouse strains varied with the GD of treatment. The pattern of anomalies produced by MNU on a given GD markedly differed between the two mouse strains, yet they were similar –with a few exceptions- when exposures at equivalent embryonic stages are compared. Findings from this study indicated that strain-dependent differences in the developmental stage of mouse embryos of the same gestational age occur, a possibility that has been often neglected when inter-strain differences in susceptibility to developmental toxicants are interpreted.

Key words: alkylating agents, embryo development, phase specificity, mouse strains, skeleton anomalies, teratogenicity.

INTRODUCTION

The phase specificity of teratogens, or the idea that the susceptibility to teratogenic agents varies with the developmental stage at the time of exposure, is one of the principles of teratology enunciated by James Wilson over 50 years ago (Wilson 1959). It was demonstrated by Stockard's pioneering studies on the "critical periods" of vulnerability of fish (*Fundulus* sp.) embryos to teratogens (Stockard 1921), and further substantiated by several mammalian studies in which mothers were exposed to toxicants at a sequence of defined stages of embryo development (Frohberg 1969, Shenefelt 1972, Horton et al. 1985, Cherrington and Chernoff 2002). Along this line, the classical work by Clarke Fraser on the susceptibility to cleft palate in A/Jax and C57BL mice was one of the earliest studies to illustrate phase specificity principle in mammalian

Correspondence to: Francisco José Roma Paumgartten
E-mail: paum@ensp.fiocruz.br

* Contribution to the centenary of the Brazilian Academy of Sciences.

embryos (Clarke Fraser 1977). Clarke Fraser noted that the shelves moved to a horizontal position later in A/Jax than in C57BL embryos thereby making the former strain embryos relatively more susceptible than the latter to any environmental factor that delays shelf movement. The phase specificity of developmental toxicants was also illustrated by Nowack's characterization of human pregnancy periods of susceptibility to thalidomide-induced embryopathy (Nowack 1965).

Owing to phase specificity, the developmental stages at which embryos are exposed are taken into account when teratogenic effects are extrapolated between species. Moreover, it is known that, in polytocous mammals, the stage of development of individual embryos varies within the litter and between litters at a same gestational age. Nonetheless, a strain-dependent difference in the development of embryos of the same age has seldom been considered as a possible cause for inter-strain differences in susceptibility to teratogens (Linval et al. 1982, Moreno and Epstein 1987, Biddle 1988, Juriloff et al. 1991, Biddle et al. 1993, Hall et al. 1997, Datta et al. 2008, Downing et al. 2010). Along this line, it should be mentioned that a comparative study of the embryo development in four mouse strains (NMRI, BALB/cJ, DBA/2J and C57BL/6J) revealed remarkable differences among them in somite counts on defined gestation times (GD) between GD9 and GD12 (Thiel et al. 1993). Additionally, it was also noted that, for a given mouse strain on a same GD, there was a pronounced variability of somite counts within and between litters (Thiel et al. 1993). Inter-strain differences on the average number of somite pairs of embryos of the same gestational age were clearly shown by the aforementioned comparative study (Thiel et al. 1993).

In this study we investigated the hypothesis that the development of outbred NMRI and inbred DBA/2J mouse embryos does not proceed synchronously and that, owing to this fact, distinct patterns of skeleton anomalies would occur after pregnancy-timed exposures to methylnitrosourea (MNU) during organogenesis (GD8-12). Since one pair of somites develops in nearly 2 h in rats and mice (Vickers 1983, Goedbloed and Smits-van Prooije 1986), we used a DNA-alkylating teratogen (MNU) with a very short half-life ($t_{1/2}$ <15 min) (Swann 1968) so that the length of exposure of individual embryos was much shorter than the time needed for the development of a somite pair.

MATERIALS AND METHODS

ANIMALS

The inbred DBA/2J and outbred NMRI mice used in this study were supplied by the *Zentralinstitute für Versuchstierzucht*, Hannover, Germany. The animals were kept under specific pathogen free (*spf*) conditions with free access to a standard rodent pellet diet (Altromin® 1324, Fa Altromin Lage, Germany) and tap water. Animals` quarters photoperiod (lights on from 09:00 a.m. to 9:00 p.m.), temperature (21±1ºC), and relative humidity (55±5%) were controlled. All mice were adapted to the conditions of our animal facilities for 3 weeks before being used in experiments. Animals received humane care and protocols complied with institutional (Charité - Universitätsmedizin Berlin) and legal guidelines.

MATING PROCEDURE

Five untreated virgin females were placed into the cage of one male for 2 h each day (07:00-09:00 a.m.) and subsequently examined for the presence of a vaginal plug. The first 24 h period following the detection of a vaginal plug was considered as gestation day (GD) 0. Females which were treated or scored for somite numbers on days $9^{1/2}$ or $10^{1/2}$ were mated between 07:00 p.m. and 09:00 p.m.. At the time of mating, body weights of DBA/2J and NMRI mice ranged from 20 to 25 g, and from 30 to 35 g, respectively.

TREATMENT

N-Methyl-*N*-nitrosourea (MNU, *CAS* Nr 684-93-5) was purchased from Sigma Chemical Co. Since MNU decomposes substantially faster with increasing pH values, it was dissolved in saline phosphate buffer at pH 5 immediately before being administered to mice. To exclude any possibility of a circadian variation in the teratogenic response, all treated mice were injected with MNU at 9:00 a.m. Based on previous data on the dose response relationships of embryotoxic effects of MNU in mice (Platzek et al. 1988), we chose a dose as high as 5 mg/ kg body weight, given by the intraperitoneal route, for this study. The injection volume was 10 µL per g of body weight.

DETERMINATION OF THE NUMBER OF SOMITES

For counting somite pairs, pregnant females were killed by cervical dislocation at 9:00 a.m., the NMRI dams on gestation days (GD) 8, 9, $9^{1/2}$, 10, $10^{1/2}$, 11 and 12, and the DBA/2J dams on GD9, 10, $10^{1/2}$, 11 and 12. Immediately after death, uteri were opened, implantation sites were recorded and all living embryos were removed and examined under a stereomicroscope with 40-fold magnification. The yolk sac was carefully removed and embryos were illuminated with a white light for determining the number of somite pairs. Only undamaged embryos with completely developed somites were evaluated.

EVALUATION OF FETAL SKELETON ANOMALIES

For evaluating the skeleton anomalies caused by pregnancy-timed exposures to MNU, untreated controls and MNU-treated dams were killed by cervical dislocation on GD18 at 9:00 a.m.. The gravid uterus was weighed, opened and examined to determine the number of implantations, and living, dead and resorbed fetuses. Fetuses were removed from the uterus, blotted, weighed, examined for externally-visible abnormalities and fixed in a 5% buffered formaldehyde solution. After fixation, fetuses were cleared and stained with alizarin red S for skeleton evaluation.

STATISTICAL ANALYSIS

Statistical evaluation was performed using a SAS statistical software package (SAS Institute, SAS User`s Guide, SAS Institute, Cary, NC, USA). Parametric variables were analyzed by ANOVA and Duncan`s post hoc test. A non-parametric test (Mann-Whitney U test) was used to compare somite counts between NMRI and DBA/2 mice. Proportions were compared by the Fisher exact test or by chi-square test. In any case, differences were considered as statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

DEVELOPMENT OF SOMITES OF NMRI AND DBA/2J MOUSE EMBRYOS ON GD8-12.

As shown in Table I, the number of somite pairs steadily increased during the embryo development between GD8 and GD12 in both mouse strains. The mean of the number of somite pairs of DBA/2J embryos was lower (Mann-Whitney U test, $P<0.05$) than that of NMRI embryos at GD9, 10, $10^{1/2}$, 11 and 12 (Table I). Nonetheless, DBA/2J embryos somite pair means on GD10 and $10^{1/2}$ were comparable to those of NMRI embryos half a day earlier, i.e., on GD$9^{1/2}$ and 10, respectively (Table I). The foregoing data indicated that the development of DBA/2J embryos was – on average - approximately 12 hours delayed as compared to the development of NMRI embryos of the same age. The length of the development delay of DBA/2J embryos compared to NMRI embryos was 5 somite pairs on GD9, 6.8 on GD10, 7.6 on GD$10^{1/2}$, 4.6 on GD11 and 4.5 on GD12, or an average 5.7 somite pairs difference during the GD9-12 period. Therefore, the 12 h development difference between DBA/2J and MNRI embryos corresponds to an average

TABLE I
The number of somite pairs of NMRI and DBA/2 mouse embryos on gestation days (GD) 8-12.

Mouse Strain:	NMRI					DBA/2J				
Gestation Day (GD)	Embryos (litters), N	Not evaluated Embryos+, N	Somite pairs (N)			Embryos (litters), N	Not evaluated Embryos+, N	Somite pairs (N)		
			Range (Min-Max)	Mean ± SD	Median (Q1-Q3)			Range (Min-Max)	Mean ± SD	Median (Q1-Q3)
8	132 (13)	7	2-7	5.1±0.8	5 (5-6)	-	-	-	-	-
9	384 (35)	45	8-21	15.3±2.4	15 (14-17)	112 (28)	26	5-18	10.3±2.4	10(9-12) *
$9^{1/2}$	117 (12)	18	12-24	19.0±2.2	19 (17-21)	-	-	-	-	-
10	384 (40)	47	17-35	27.7±3.2	28 (26-30)	217 (48)	34	9-34	20.9±3.8	21 (19-23) *
$10^{1/2}$	42 (4)	2	30-35	32.6±1.4	33 (31.7-34)	56 (12)	7	19-30	25.0±2.2	25 (24-27) *
11	212 (20)	21	31-47	40.4±4.9	42 (40-43)	108 (29)	10	27-40	35.8±2.9	36 (34-38) *
12	212 (20)	18	43-57	51.4±2.3	52 (51-53)	112 (27)	9	39-52	46.9±3.4	47 (45-50) *

+Only undamaged embryos with completely developed somites were evaluated. An asterisk (*) indicates that the median of number of somite pair differ (Mann-Whitney U test, $P<0.05$) from that of NMRI mice. Q1-Q3: interquartile range.

somite count of nearly 6. The foregoing estimate of the length of the developmental delay of DBA/2J embryos as compared to NMRI embryos of the same gestation age is consistent with previous estimates that, in rats and mice, one pair of somites develops in approximately 2 h (Vickers 1983, Goedbloed and Smits-van Prooije 1986). Since organogenesis proceeds very fast on GD8-12, such an inter-strain difference of nearly 6 somite pairs in embryo development at a defined gestation time (GD) is expected to result in distinct responses to short exposures to a developmental toxicant.

It is of note that, in the outbred (NMRI) mouse breeding stock as well as in the inbred (DBA/2J) mouse strain, somite pair counts at a defined GD exhibited a marked variation so that, for a same strain, ranges of individual somite counts overlap to some extent between two 12 h-apart gestation times (GDs) (Table I). The foregoing finding is consistent with previous observations that there is a considerable within and between litter variation of somite counts at defined GDs, irrespective of whether mouse strain is outbred or inbred (Thiel et al. 1993, van Maele-Fabry et al. 1992). The underlying causes for such a wide within litter variation of somite counts are not entirely clear. Since within-litter variation of somite counts occurs in inbred strains as well, it cannot be explained by genetic differences among littermates. Random differences among embryos in fertilization and/or implantation time, and/or differences related to the position of the embryo within the uterus can not be ruled out as contributing factors for the within-litter variability of somite pair numbers.

Differences of copulation time that occur even if mating period is limited to a few hours, and other maternal factors possibly contribute to the inter-litter variation of somite counts, while genetic and epigenetic factors can not be excluded in the case of inter-strain differences. Data provided by Ciriani and Diewert (1986) and also by Collins et al. (2006) suggested that inter-strain differences in somite development may arise from imprinting of maternal genes and or from an influence of maternal uterine environment. Collins et al. (2006) found that crosses between C57BL/6N females and SWV males yielded embryos that were more developmentally advanced than those produced by crosses between SWV females and C57BL/6N males. A similar directional dominance toward the maternal strain had also been noted by Ciriani and Diewert (1986) who made reciprocal intercrosses of C57BL/6 and A/WySn mouse strains. Genetic imprinting involves methylation and histone modifications that lead to monoallelic gene expression (Isles and Holland 2005). The idea that somite development depends on maternal genes that are preferentially expressed in the conceptus is plausible because it is known that many imprinted genes in mammals are involved in pre and postnatal growth as well as in metabolism (Isles and Holland 2005). Moreover, as commented by Bourc'his and Proudhon (2008), experiments involving construction - by nuclear transfer - of uniparental conceptuses indicated that conceptuses with two maternal genomes (parthenogenetic) or with two paternal genomes (androgenetic) did not progress beyond mid gestation. Furthermore, uniparental conceptuses also exhibited an imbalance in embryo:placenta growth which depended on the parental origin of the nucleus. These studies led to the long-standing generalization that maternal genome is required for the development of the embryo while the paternal genome promotes the development of the extraembryonic structures (placenta) (Bourc'his and Proudhon 2008).

A considerable variability in the developmental stage among embryos at a given gestational age has also been observed in humans and non-human primates (Neubert et al. 1988, Shiota 2009). Possible causes of such a variation in human embryonic development were discussed by Shiota (2009) who analyzed Nishimura's unique collection of human embryos with over 44,000 specimens

from therapeutic abortuses performed in Japan. Although recognizing that inaccurate estimates of fertilization time is a contributing factor for the developmental variability, Shiota (2009) concluded that variability also arises from the fact that prenatal development does not proceed at the same speed in every embryo

Results presented here and findings from previous studies (van Maele-Fabry et al. 1992, Thiel et al. 1993) showed that gestation age is an imprecise indicator of the developmental stage of rodent embryos as well. Although the developmental stage of early post-implantation embryos is generally defined by number of somite pairs, variations in morphological stages of a given structure among embryos with the same number of somite pairs have been found in NMRI mice (Vickers 1983). Taken together these observations illustrate that the development of mouse embryos, even that of those with an almost identical genotype, proceeds differently during the organogenesis period.

SKELETON ANOMALIES INDUCED BY TIMED EXPOSURES TO MNU BETWEEN GD9 AND $10^{1/2}$ IN NMRI AND DBA/2J MICE

It had been reported that, at the dose level selected for this study (5 mg/kg body wt i.p.), MNU induced a variety of skeleton defects, while producing no or very low embryolethality, and no overt maternal toxicity in mice (Platzek et al. 1988). Caesarean section data of untreated controls and MNU-treated dams on GD18 confirmed that this DNA-alkylating agent, at the tested dose, was not overtly embryolethal. As shown in Table II, except for a slight increase in the number of resorptions per litter (0.22±0.43 vs 0.77±1.31, P=0.04) among DBA/2J mice treated on GD10, MNU did not produce any other discernible increase in the resorption rate, nor did it decrease the litter size (number of live fetuses per litter) regardless the mouse strain or the GD of treatment (Table II). A significant increase in the number of implantations per litter was found in NMRI mice treated with MNU on GD10 and in DBA/2J mice treated with this alkylating agent on $GD10^{1/2}$ (Table II). A larger litter size was also detected in the group of DBA/2J mice treated with MNU on $GD10^{1/2}$ (Table II). A larger litter is consistent with a greater number of implantations per litter but the toxicological relevance of these findings is unclear. A reduction of fetal body weight was observed in the offspring of NMRI mice treated on GD10 and in the offspring of DBA/2J treated on $GD10^{1/2}$, but not among NMRI and DBA/2J fetuses exposed at earlier GDs (Table II).

The skeleton anomalies produced by this sequence of timed exposures to MNU between GD9 and $10^{1/2}$ in NMRI and DBA/2J mice are shown in Table III. Despite being rapidly eliminated from the maternal organism (Swann 1968), a single dose of MNU, administered between GD9 and $10^{1/2}$, caused a variety of skeleton anomalies in the offspring of NMRI and DBA/2J dams. As expected from phase specificity, incidences of most skeleton anomalies in both NMRI and DBA/2J mice depended on the gestation day (GD) at which the embryos were exposed to MNU (Table III).

Since at a same gestation age (GD) development of DBA/2J embryos is nearly 12 h delayed as compared to that of NMRI embryos, maximum incidences of these anomalies in the two strains were in most instances produced by exposures at distinct GDs yet similar developmental stages (Tables II and III).

The induction of cleft palate by MNU illustrates the aforementioned time-dependent difference in susceptibility to a particular type of anomaly. The maximum incidences of cleft palate were noted in NMRI mice treated on GD10 (89.5%), and in DBA/2J mice treated on $GD10^{1/2}$ (62.7%), i.e., in embryos which were exposed when their average somite counts were 27.7±3.2 and 25.0±2.2, respectively (Table III). Strain differences in the susceptibility to cleft palate have been reported in the literature. Kusanagi (1985), for instance,

TABLE II
Caesarean section data of dams from NMRI (outbred) and DBA/2J (inbred) mouse strains treated with a single injection of methylnitrosourea (MNU, 5 mg/kg body weight ip) on a defined time of pregnancy (gestation days 9, $9^{1/2}$, 10 or $10^{1/2}$).

Mouse strain:	NMRI				DBA/2J		
Gestation day (GD) at treatment (MNU):	Untreated control	GD9	GD$9^{1/2}$	GD10	Untreated control	GD10	GD$10^{1/2}$
Litters (N)	11	14	22	10	18	30	9
Implantations (N)	130	176	237	144	73	128	56
Implantations/Litter	11.9±2.4	12.5±1.9	10.8±2.9	14.4±2.5*	4.0±2.1	4.2±1.9	6.2±1.9*
Resorptions (N)	5	6	17	10	5	19	5
Resorptions/Litter	0.55±0.82	1.21±3.2	0.82±1.56	1.05±1.16	0.22±0.43	0.77±1.31*	0.56±0.88
Fetuses (N)	125	170	220	134	68	109	51
Fetuses/Litter	11.4±2.5	11.3±3.3	10.0±3.3	13.4±2.5	3.8±2.0	3.4±1.7	5.7±1.5*
Fetal body weight (g)	1.03±0.14	1.04±0.09	0.96±0.15	0.82±0.08*	0.95±0.12	0.71±0.11	0.63±0.08*

Gestation day 0 = first 24 h period after mating. Data are shown as means ± SD and were analyzed – separately for each stock/strain - by ANOVA and Duncan's post hoc test. An asterisk (*) indicates that the mean differs ($P<0.05$) from that of the untreated control from the same strain.

TABLE III
Occurrence of skeleton anomalies in the offspring of NMRI and DBA/2J mice treated with a single injection of methylnitrosourea (MNU, 5 mg/kg body weight ip) on a defined time of pregnancy (i.e., on gestation days 9, $9^{1/2}$, 10 or $10^{1/2}$). On GD18, all fetuses were removed by C-section and examined for skeleton anomalies after clearing and staining with alizarin red S.

Mouse strain:	NMRI				DBA/2J		
Gestation day (GD) at treatment (MNU):	Untreated control	GD9	GD$9^{1/2}$	GD10	Untreated control	GD10	GD$10^{1/2}$
Somite pairs at treatment, (N, mean±SD):	**-**	**15.3±2.4**	**19.0±2.2**	**27.7±3.2**	**-**	**20.9±3.8**	**25.0 ±2.2**
Examined fetuses (N):	125	170	220	134	68	109	51
Anomalies (%) in:							
Skull							
Cleft palate	0.8	8.2*	10.0*	89.5*	0	22.9*	62.7*
Fontanelle (enlarged)	4.0	4.7	21.8*	93.2*	1.5	27.5*	74.5*
Os zygomaticum (absent)	0	0	0.4	31.3*	0	11.0*	23.5*
(smaller)	0	0	2.3	44.7*	0	9.2*	3.9
Os tympanicum (fused)	0	0	0	14.9*	0	0	3.9
(shorter)	0	0	0.4	0	0	9.2*	11.7*
Proc.j.Os squamosum (absent)	0	0	0.4	25.3*	0	3.7	15.6*
Os supraoccipitale (absent)	0	0	2.7	14.9*	0	0	0
(smaller)	0	1.8	7.7*	53.7*	0	1.8	0
(misshapen)	9.6	48.2*	39.0*	0	1.5	27.5*	9.8
Os frontale (additional o.c.)	0	3.5	5.4*	0	16.1	26.6*	41.1*
(fused with O.parietale)	0	0	0	0	0	0.9	5.8

TABLE III (continuation)

Mouse strain:	**NMRI**				**DBA/2J**		
Gestation day (GD) at treatment (MNU):	**Untreated control**	**GD9**	**GD9$^{1/2}$**	**GD10**	**Untreated control**	**GD10**	**GD10$^{1/2}$**
Somite pairs at treatment, (N, mean±SD):	**-**	**15.3±2.4**	**19.0±2.2**	**27.7±3.2**	**-**	**20.9±3.8**	**25.0 ±2.2**
Vertebral column							
Atlas (wider)	0	0	11.8*	0	0	41.2*	43.1*
(misshapen)	2.4	40.5*	20.0*	0.7	0	7.3*	3.9
Thor.ventr. (oc dumbbell)	0	13.5*	12.2*	4.5	0	16.5*	3.9
Lumb.ventr. (o.c.dumbbell)	0	2.9	7.3*	14.1*	0	31.1*	11.7*
Ribs							
(misshapen)	0	2.3	5.9*	11.9*	0	11.9*	0
Cervical (rudimentary, extra)	10.4	0*	28.1*	0*	22.0	59.6*	41.1*
Lumbar (supernumerary)	46.4	55.2	52.2	60.4*	4.4	2.8	0
Sternum							
Sternebrae (fused)	0	0	0.4	14.1*	0	6.4*	7.8*
(misaligned)	1.6	6.5*	1.8	0	4.4	1.8	0
(misshapen)	13.6	14.1	38.1*	51.4*	5.9	49.5*	54.9*
(absent)	0.8	1.8	3.6	58.9*	0	24.7	21.5*
½ (fused)	0	1.8	1.4	4.5	0	9.2*	2.0
2/3 (fused)	0	3.5	1.4	0.7	0	0	0
Forelimbs							
(misplaced)	0	0	0.9	44.7*	0	8.2*	21.5*
Clavicle (bent)	0	7.6*	4.1*	15.6*	0	25.6*	27.4*
(misshapen)	0	0.6	0	0	0	1.8	3.9
Finger 1 (polydactyly)	0	0.6	0.4	9.0*	0	2.8	0
Finger 2 (polydactyly)	0	0	0.9	14.1*	0	4.6	2.0
(thicker)	0	0	0.4	6.0*	0	4.6	2.0
Fingers ½ (syndactyly)	0	0	0.4	10.4*	0	0.9	0
Forepaws (misplaced)	0	0	0.9	6.7*	0	11.9*	0
Humerus (misshapen)	0		0.4	0.7	0	4.6	0
(absent)	0	0	0	0	0	1.8	0
(shorter)	0	0	0.9	68.6*	0	8.2*	7.8
Metacarpus 2 (absent)	0	0	0.4	3.7	0	2.8	0
Proc. deltoid (absent)	0	5.3*	10.0*	0.7	0	3.7	5.9
(smaller)	4.0	10.5*	20.9*	0	0	0.9	0
Radius/humerus (fused)	0	0	0	26.8*	0	1.8	15.6*
Radius (bent)	0	3.5	3.6	30.5*	0	21.1*	27.4*
(absent)	0	0	0.4	3.0	0	0	0
(shorter)	0	0	0.4	76.1*	0	6.4*	15.6*

TABLE III (continuation)

Mouse strain:	**NMRI**				**DBA/2J**		
Gestation day (GD) at treatment (MNU):	**Untreated control**	**GD9**	**GD9$^{1/2}$**	**GD10**	**Untreated control**	**GD10**	**GD10$^{1/2}$**
Somite pairs at treatment, (N, mean±SD):	-	**15.3±2.4**	**19.0±2.2**	**27.7±3.2**	-	**20.9±3.8**	**25.0 ±2.2**
Scapula (misshapen)	0	0	0.9	18.6*	0	36.6*	11.7*
(absent)	0	0	0	0	0	0.9	0
Spina scapulae (absent)	0	0	0	41.7*	0	25.6*	54.9*
Ulna/humerus (fused)	0	0	0	22.3*	0	9.2*	17.6*
Ulna/radius/humerus (fused)	0	0	0	32.0*	0	5.5*	13.7*
Ulna/radius (fused)	0	0	0	6.0*	0	6.4*	3.9
Ulna (bent)	0	0.6	0	11.9*	0	1.8	31.3*
(absent)	0	0	0	18.6*	0	0.9	0
(shorter)	0	0	0.4	63.4*	0	3.7	23.5*
Hindlimbs							
(misplaced)	0	0	1.8	58.2*	0	24.7*	33.3*
(absent)	0	0	0	6.7*	0	4.6	0
Femur (absent)	0	0	0	80.5*	0	42.2*	60.7*
(shorter)	0	2.4	5.0*	9.0*	0	10.0*	11.7*
Fibula (absent)	0	0	0	79.1*	0	16.5*	31.3*
Hindpaws (misplaced)	0	10.0*	2.7	11.9*	0	44.9*	23.5*
Ilium (absent)	0	0	0	58.2*	0	9.2*	39.2*
Ischium (fused with os pubis)	0	0	0	39.5*	0	3.7	19.6*
Ischium (absent)	0	0	0	10.4*	0	1.8	2.0
(shorter)	0	0	16.3*	80.5*	2.9	23.8*	39.2*
Metatarsus 1 (absent)	0.8	2.4	10.0*	23.1*	0	7.3*	2.0
Os pubis (absent)	0	0	0.4	21.6*	0	3.7	2.0
(shorter)	0	0	16.8*	83.5*	2.9	23.8*	39.2*
Tibia (bent)	0	1.2	2.7	63.4*	0	32.1*	47.0*
(absent)	0	2.9	2.7	17.9*	0	3.7	0
(shorter)	0	3.5	3.6	75.3*	0	16.5*	25.4*
Toe 1 (adactyly)	0	1.8	1.4	18.6*	0	2.8	0
(polydactyly)	0	0	0	20.1*	0	4.6	7.8*
Toe 2 (polydactyly)	0	1.2	3.6	21.6*	0	4.6	3.9
Toes ½ (syndactyly)	0	0	5.0	38.8*	0	12.8*	5.9

Proportions (%) different ($P<0.05$, Fisher exact test) from those of untreated controls of the same strain are indicated by an asterisk (*).

showed that horizontalization and fusion of the palatal shelves occurred earlier in C57BL/6 than in SWV embryos, but fusion of the primary palate with the secondary palate occurred later. Owing to these differences in the length of susceptibility period, C57BL/6 and SWV differed regarding their vulnerability to palatal slit and cleft palate induction by triamcinolone (Kusanagi 1985). In this study, however, frequencies of cleft palate were similar in NMRI and DBA/2J mice exposed to MNU at equivalent embryonic stages.

A similar dependency of the effect on the embryonic stage at treatment was observed for a number of other anomalies induced by MNU in both strains such as *fontanelle* enlarged, *os zygomaticum* absent, *Proc. j. os squamosum* absent, *sternebrae* anomalies (fused, misshapen, absent), forelimbs misplaced, *clavicle* bent, *radius-humerus* fused, *radius* bent, *radius* shorter, *scapula spina* absent, *ulna-humerus* fused, *ulna-radius-humerus* fused, *ulna* (bent, shorter), hindlimbs misplaced, *femur* (absent, shorter), *fibula* absent, *ilium* absent, *ischium-os pubis* fused, *ischium* shorter, *os pubis* shorter, and *tibia* (bent, shorter) (Table III, Figure 1).

The foregoing examples are consistent with the idea that, provided that NMRI and DBA/2J embryos are exposed to MNU at the same developmental stage, effects on the occurrence of skeleton anomalies are similar between strains.

Some exceptions, however, were noted. Incidences of a few anomalies were enhanced in NMRI fetuses exposed to MNU on GD10 (27.7±3.2 somite pairs), but not in DBA/2J fetuses treated at a similar developmental stage (GD10$^{1/2}$, 25.0±2.2 somite pairs). In NMRI mice, for instance, the occurrence of *os zygomaticum* smaller increased from 2.3% on GD9$^{1/2}$ to 44.7% on GD10, while in the DBA/2J strain the incidence of this anomaly decreased from 9.2% on GD10 to 3.9% on GD10$^{1/2}$ (Table III). Along the same vein, *os supraoccipitale* absent (14.9%) and smaller (53.7%) were observed in NMRI mice treated on GD10, but not in DBA/2J treated either on GD10 or on GD10$^{1/2}$ (Table III). The incidence of *humerus* shorter also increased from 0.9% (GD9$^{1/2}$) to 68.9% (GD10) in NMRI stock, while it remained almost unaltered in DBA/2J mice treated at comparable embryonic developmental stages (8.2% on GD10 and 7.8% on GD10$^{1/2}$) (Table III). Moreover, marked increases in the frequencies of hindpaw digit anomalies (adactyly, polydactyly and syndactyly) were noted among MNRI mice treated on GD10 as compared to those treated earlier, while incidences of toe anomalies were lower and exhibited only minor changes between DBA/2J treated on GD10 and those exposed on GD10$^{1/2}$ (Table III).

A few anomalies, on the other hand, were enhanced in DBA/2J mice treated on GD10$^{1/2}$ but not in NMRI mice exposed to MNU at a similar embryonic stage (GD10). The frequency of *Atlas* wider, for instance, was substantially increased in DBA/2J treated either on GD10 (41.2%) or on GD10$^{1/2}$ (43.1%) as compared to untreated control incidence (0%), while in the NMRI stock the occurrence of this anomaly was 11.8% and 0% after treatment on GD9$^{1/2}$ and 10, respectively (Table III). In DBA/2J mice, the incidence of hindpaws misplaced fell from 44.9% to 23.5% as treatment time advanced from GD10 to GD10$^{1/2}$, while in the NMRI stock incidences of this anomaly produced by treatments at similar embryonic stages were 2.7% and 11.9%, respectively (Table III). In the DBA/2J strain, frequencies of *lumbar vertebrae o.c.* dumbbell-shaped also fell from 31.1% to 11.7% as treatment time advanced from GD10 to GD10$^{1/2}$, while in NMRI mice the incidence of this anomaly increased from 7.3% (GD9$^{1/2}$) to 14.1% (GD10) with treatments at equivalent embryonic developmental stages (Table III).

The aforementioned differences in the occurrence of some skeleton anomalies, even when NMRI and DBA/2J embryos were exposed to MNU at similar developmental stages, suggest

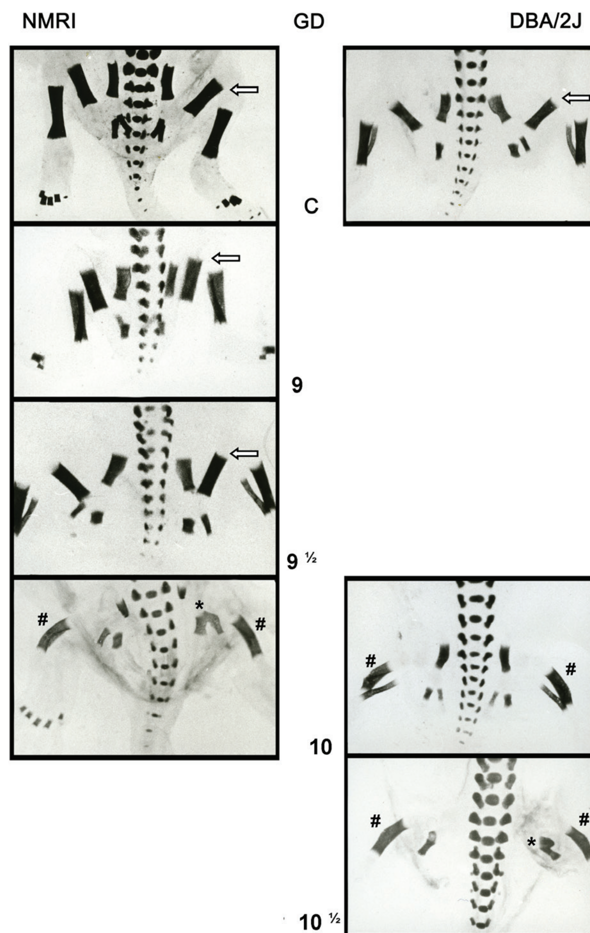

Figure 1 - Skeleton anomalies in the hindlimbs induced by a single administration of methylnitrosourea (MNU; 5 mg/kg body weight, ip) to NMRI and DBA/2J mice on gestation day (GD) 9, $9^{1/2}$, 10 or $10^{1/2}$. Fetuses (GD18) were cleared and stained with alizarin red S for skeleton evaluation. Femurs on the right side of each photo are indicated by an arrow. Right and left femurs are missing in fetuses exposed to MNU on GD10 and $10^{1/2}$ but not in those treated earlier. *Os pubis* fused with *os ischium* (*) is noted in NMRI fetuses treated on GD10 and in DBA/2J fetuses treated on GD$10^{1/2}$, while tibia bones are bent (#) in fetuses treated on GD10 and $10^{1/2}$. C: untreated controls. GD0= 24 h period following copulation.

that periods of susceptibility to these anomalies possibly also differ between the strains.

Collins and coworkers (Collins et al. 2006, Lee et al. 2010) reported inter-strain differences in the response to all-trans-retinoic acid that cannot be attributed simply to differences in either embryo developmental stage at the time of exposure, or kinetics of the teratogenic agent. The authors noted that, although exhibiting an almost identical number of somites at treatment on GD 9.5, C57BL/6N mice were more susceptible than SWV mice to induction of forelimb ectrodactyly (missing digits), whereas the opposite was noted for the induction of thymus agenesis, gastroschisis and cleft palate (Collins et al. 2006). A locus on mouse chromosome 11 (*Rafar*) was found to be associated with the difference between C57BL/6N and SWV strains susceptibility to all-trans-retinoic acid induced forelimb ectrodactyly (Lee et al. 2005). Furthermore, it was also found that the C57BL/6N mouse strain is susceptible, whereas SWV strain is resistant to induction of forelimb ectrodactyly by $CdCl_2$ (4 mg/kg ip) on GD 9 [30]. By using a proteomic approach Chen et al. (2008) noted that, 24 h after $CdCl_2$ administration (GD 10), 38 proteins had identifiable differences in abundance levels in cadmium-treated limb buds between the two strains. Since of those 38 proteins, 14 could be associated with the unfolded protein response process and 7 are associated with actin polymerization, Chen et al. (2008) advanced a hypothesis that the differential murine strain response to cadmium-induced forelimb defects results from differences in their pathways for the unfolded protein response and / or actin polymerization.

A study by Downing et al. (2010) also found differences in the teratogenic response of C57BL/6J and DBA/2J mice to a single dose of valproic acid given on GD 9, i.e., while C57BL/6J fetuses were more susceptible than DBA/2J fetuses to induction of digit (fused or missing digits) and vertebral anomalies (fused, missing and asymmetrical arches and centra), the latter strain was more susceptible than the former mouse strain for the induction of rib anomalies (fused, missing, bifurcated, and wavy/bulbous ribs). Since the authors did not determine the number of somite pairs, inter-strain differences

of developmental stages at the time of treatment cannot be ruled out as contributing factors for these differences in susceptibility to VPA-induced teratogenicity. Actually, Thiel et al. (1993) reported that C57BL/6J embryos are at a more advanced stage of development than DBA/2J embryos of the same gestation age on GDs 9-12.

Treatments with MNU between GD9 and GD10$^{1/2}$ had only a minor impact on the incidence of cervical rudimentary ribs as well as lumbar supernumerary ribs, skeleton variations that occur spontaneously at relatively high rates in these mouse strains (Table III).

CONCLUSIONS

Data from this study showed that the effect of MNU on the occurrence of skeleton anomalies varied with the gestation time (GD) at which NMRI and DBA/2J mice were treated. Furthermore, a comparison between the two mouse strains demonstrated that, at all gestation times analyzed in this study (GD8-12), the development of DBA/2J embryos was nearly 12 h -or 6 somite pairs- delayed as compared to the developmental stage of NMRI embryos. Since on GD8-12 embryo development -as indicated by somite counts- proceeds differently in the two mouse strains, the teratogenic effects of MNU on NMRI and DBA/2J fetuses were not comparable when treatments took place at the same gestation time. Except for a few cases, occurrences of fetal skeleton anomalies were similar when NMRI and DBA/2J mice had been exposed to MNU at equivalent embryonic developmental stages. The aforementioned findings highlighted that inter-strain differences in susceptibility to teratogenic agents may eventually result from distinct developmental stages of the strains at a same gestation time, a possibility that has been often neglected when inter-strain differences in susceptibility to developmental toxicants are interpreted.

ACKNOWLEDGMENTS

The authors are indebted to the late Ms Briggite Woeffle, formerly a technician of the Institute of Clinical Pharmacology and Toxicology, Charité Medical University, for excellent technical assistance and help in preparing the photos of skeleton anomalies. FJRP was the recipient of a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

BIDDLE FG. 1988. Genetic differences in the frequency of acetazolamide-induced ectrodactyly in the mouse exhibit directional dominance of relative embryonic resistance. Teratology 37: 261-265.

BIDDLE FG, MULHOLLAND LR AND EALES BA. 1993. Penetrance and expressivity of acetazolamide-ectrodactyly provide a method to define a right-left teratogenic gradient that differs between C57BL/6 and WB/ReJ mouse strains. Teratology 47: 603-618.

BOURC'HIS D AND PROUDHON C. 2008. Sexual dimorphism in parental imprint ontogeny and contribution to embryonic development. Mol Cell Endocrinol 282: 87-94.

CHEN H, BOONTHEUNG P, LOO RR, XIE Y, LOO JA, RAO JY AND COLLINS MD. 2008. Proteomic analysis to characterize differential mouse strain sensitivity to cadmium-induced forelimb teratogenesis. Birth Defects Res A Clin Mol Teratol 82: 187-199.

CHERRINGTON JW AND CHERNOFF N. 2002. Periods of vertebral column sensitivity to boric acid treatment in CD-1 mice in utero. Reprod Toxicol 16: 237-243.

CIRIANI D AND DIEWERT VM. 1986. A comparative study of development during primary palate formation in A/WySn,C57BL/6, and their F1 crosses. Craniofac Genet Dev Biol 6: 369-377.

CLARKE FRASER F. 1977. Relation of animal studies to the problem in man. In: Wilson JG and Clarke Fraser F (Eds), Handbook of Teratology, Volume 1 General Principles and Etiology. New York and London: Plenum Pres, New York, USA, p. 75-96.

COLLINS MD, ECKHOFF C, WEISS R, RESNICK E, NAU H AND SCOTT JR WJ 2006. Differential teratogenesis of all-trans-retinoic acid administered on gestational day 9.5 to SWV and C57BL/6N mice: emphasis on limb dysmorphology. Birth Defects Res A Clin Mol Teratol 76: 96-106.

DATTA S, TURNER D, SINGH R, RUEST LB, PIERCE JR WM AND KNUDSEN TB. 2008. Fetal alcohol syndrome

(FAS) in C57BL/6 mice detected through proteomics screening of the amniotic fluid. Birth Defects Res A Clin Mol Teratol 82: 177-186.

DOWNING C, BIERS J, LARSON C, KIMBALL A, WRIGHT H, ISHII T, GILLIAM D AND JOHNSON T. 2010. Genetic and maternal effects on valproic acid teratogenesis in C57BL/6J and DBA/2J mice. Toxicol Sci 116: 632-639.

FROHBERG H. 1969. Teratogenversuche zur Feststellung der sensiblen Phase der Tächtigkeit an Mäusen. Naunyn Schmiedebers Arch Exp Pathol 263: 210-211.

GOEDBLOED JF AND SMITS-VAN PROOIJE AE. 1986. Quantitative analysis of the temporal pattern of somite formation in the mouse and rat. A simple and accurate method for age determination. Acta Anat 125: 76-82.

HALL JL, HARRIS MJ AND JURILOFF DM. 1997. Effect of multifactorial genetic liability to exencephaly on the teratogenic effect of valproic acid in mice Teratology 55: 306-313.

HORTON VL, SLEET RB, JOHN-GREENE JA AND WELSCH J. 1985. Developmental phase-specific and dose-related teratogenic effects of ethylene glycol monomethyl ether in CD-1 mice. Toxicol Appl Pharmacol 80: 108-118.

ISLES AR AND HOLLAND AJ. 2005. Imprinted genes and mother-offspring interactions. Early Hum Dev 81: 73-77.

JURILOFF DM, HARRIS MJ, TOM C AND MACDONALD KB. 1991. Normal mouse strains differ in the site of initiation of closure of the cranial neural tube. Teratology 44: 225-233.

KUSANAGI T. 1985. Strain differences between C57BL/6 and SWV mice in time of palate closure and induction of palatal slit and cleft palate. Teratology 31: 279-283.

LEE GS, CANTOR RM, ABNOOSIAN A, PARK E, YAMAMOTO ML, HOVLAND JR DN AND COLLINS MD. 2005. A gene(s) for all-trans-retinoic acid-induced forelimb defects mapped and confirmed to murine chromosome 11. Genetics 170: 345-353.

LEE GS, LIAO X, SHIMIZU H AND COLLINS MD. 2010. Genetic and pathologic aspects of retinoic acid-induced limb malformations in the mouse. Birth Defects Res A Clin Mol Teratol 88: 863-882.

LINVAL LR, DEPASS LR AND WEAVER EV. 1982. Comparison of teratogenic effects of aspirin and hydroxyurea in the Fischer 344 and Wistar strains. J Toxicol Environ Health 10: 297-305.

MORENO PA AND EPSTEIN CJ. 1987. Enhanced susceptibility of mouse embryos heterozygous for oligosyndactyly (Os/+) to mitomycin C-induced skeletal abnormalities. Teratology 35: 261-265.

NEUBERT D, HEGER W, MERKER HJ, SAMES K AND MEISTER R. 1988. Embryotoxic effects of thalidomide derivatives in the non-human primate *Callithrix jacchus*. II. Elucidation of the susceptible period and of the variability of embryonic stages. Arch Toxicol 61: 180-191.

NOWACK E. 1965. Die sensible Phase bei der Thalidomide-Embryopathie. Humangenetik 1: 516-536.

PLATZEK T, BOCHERT G, PAULI B, MEISTER R AND NEUBERT D. 1988. Embryotoxicity induced by alkylating agents: 5. Dose-response relationships of teratogenic effects of methylnitrosourea in mice. Arch Toxicol 62: 411-423.

SHENEFELT RE. 1972. Morphogenesis of malformations in hamsters caused by retinoic acid: Relation to dose and stage at treatment. Teratology 5: 103-118.

SHIOTA K. 2009. Variability in human embryonic development and its implications for the susceptibility to environmental teratogenesis. Birth Defects Res A Clin Mol Teratol 85: 661-666.

STOCKARD CR. 1921. Developmental rate and structural expression: An experimental study of twins, “double-monsters” and single deformities and the interaction among embryonic organs during their origin and development. Am J Anat 28: 115-227.

SWANN PF. 1968. The rate of breakdown of methyl methanesulfonate, dimethyl sulphate and N-methyl-N-nitrosourea in the rat. Biochem J 110: 49-52.

THIEL R, CHAHOUD I, JÜRGENS M AND NEUBERT D. 1993. Time-dependent differences in the development of somites of four different mouse strains. Teratogen Carcinogen Mutagen 13: 247-257.

VAN MAELE-FABRY G, DELHAISE F AND PICARD JJ. 1992. Evolution of the developmental scores of sixteen morphological features in mouse embryos displaying 0 to 30 somites. Int J Dev Biol 36: 161-167.

VICKERS TH. 1983. The chronology of somites in rat embryos. Teratology 28: 457-460.

WILSON JG. 1959. Experimental studies on congenital malformations. J Chronic Dis 10: 111-130.