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ACRYLAMIDE CAUSES PREIMPLANTATION ABNORMALITIES IN EMBRYOS AND INDUCES CHROMATIN-ADDUCTS IN MALE GERM CELLS OF MICE

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Abstract — Acrylamide, a known male postmeiotic germ cell mutagen, caused a dose-dependent increase in the frequency of morphologic abnormalities in preimplantation embryos. Single-cell eggs, growth retardation, and blastomere lysis were detected after paternal treatment with acrylamide (10 to 50 mg/kg, 5 d). The major effects were seen at weeks 1 to 3 after male treatment, with the highest level of abnormalities at the first week (>90% vs. 5% in control). The frequency of abnormal four-day embryos was similar to preimplantation loss assessed at 15 to 16 d p.c. A >100-fold elevation of chromatin adducts in sperm was observed during 1st and 2nd week after treatment, after which adduct levels decreased to baseline level. However, morphologic defects in embryos are not fully explained by the spermatid adduct curve. These findings demonstrate the effects of paternal exposure to acrylamide on preimplantation development and indicate a potential risk to the offspring of men exposed to acrylamide. © 1999 Elsevier Science Inc.

Key Words: acrylamide; genotoxicity; chromatin-adducts; sperm; preimplantation abnormalities.

INTRODUCTION

Male-mediated genotoxicity is an area of growing interest (1,2). Since the first publications on dominant lethality (DL) (3), considerable information has accumulated showing that paternal treatment can result in adverse outcomes in the survival and health of offspring (4–6). Epidemiologic evidence of associations between male exposure to exogenous agents and abnormal reproductive outcomes (e.g. fetal loss, birth defects, childhood cancer) is also growing (2). However, little is known about the underlying mechanisms linking male germ cell exposure and abnormal developmental reproductive outcomes. Whereas it is very difficult to study these effects in humans, the mouse is a good model for genotoxic and developmental defects.

There are several methods to determine the effect of paternal exposure to environmental mutagens on early stages of human or mammalian embryogenesis. The DL assay provides only a rough estimation of viability and does not allow the study of morphologic or cytogenetic abnormalities in preimplantation embryos. Direct analysis of preimplantation embryos has many advantages for mechanistic studies. Several studies have investigated the induction of preimplantation abnormalities after paternal exposure by alkylating agents (7–10). Morphologic abnormalities were also detected after maternal treatment with chemical mutagens, freezing of the eggs and superovulation (11–14). It would be useful to know whether DL and preimplantation loss can be alternative outcomes of the same toxic process during spermatogenesis, or whether they show different patterns and thus represent discrete mechanisms of toxicity.

In the assessment of male exposure, estimation of the dose of a reactive mutagen reaching the critical molecular target and the ability of that dose to result in a biologic effect is important. Accelerator mass spectrometry (AMS) allows for the ultrasensitive (1 adduct per 10^{11} bases) measurement of radiolabeled compounds and thus is well suited for measuring mutagen-DNA-adducts following an extremely low-dose exposure (15). AMS has been utilized for such assessment with the food carcinogen MeIQx (16) and benzene metabolites (17). The major advantage of AMS is that the cumulative dose at the target (sperm) is measured in relation to biologic effect in progeny (embryo) at doses that are much lower than are feasible with any other analytical method. We attempted to evaluate the dose of acrylamide in the germ cells of treated males using AMS and determine whether

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there was a temporal association with the risk for early preimplantation defects.

Acrylamide is a widely used chemical to which more than 100,000 people are exposed occupationally in the US per year (18,19). Substantial data on molecular changes and genotoxic effects induced by acrylamide in experimental systems have been recently reviewed (20). Acrylamide is not only genotoxic but also considered to be a potential carcinogen. It is metabolized in mice, rats, and humans to the epoxide glycidamide. Solomon et al. (21) found that acrylamide reacts very slowly with DNA in vitro, and the principal site of reaction in calf thymus DNA was the N-1 of adenine. Recent studies have demonstrated that N-7(2-carbamoyl-2-hydroxyethyl)-guanine formed by the acrylamide metabolite glycidamide is the predominant adduct formed in the DNA of rats and mice exposed to ^{14}C-radiolabeled acrylamide (22). However, when ^{14}C acrylamide was injected intraperitoneally (i.p.), radiolabel in male germ cells was primarily associated with protamines of sperm (23). Acrylamide exposure in hamsters caused testicular atrophy with damage to spermatids and mature spermatozoa (24). Reduced sperm motility and impaired fertility have also been reported in treated mice and rats (25). Acrylamide caused chromosomal aberrations in mammalian cells in vitro and in vivo (26,27). Micronuclei were found in mouse bone-marrow cells and splenocytes following exposure to acrylamide (27). In germ cells, the effects of acrylamide are predominantly postmeiotic as indicated by heritable translocations and dominant lethality in mice (28–32). Relatively low pregnancy rates were recorded, suggesting that conceptuses died prior to implantation. The chromosome aberrations detected in first cleavage zygotes are induced predominantly during postmeiotic stages of spermatogenesis (13,33). The cytogenetic effects on meiotic and premeiotic cells are of less magnitude (34). However, Allen et al. showed effects of acrylamide on the synaptonemal complex in spermatocytes (35).

Here, we describe time- and dose-dependent effects of acrylamide treatment of paternal postmeiotic and meiotic germ cells on preimplantation morphologic abnormalities in mouse embryos. The dominant lethal assay was used to assess preimplantation genotoxicity of acrylamide in the same group of animals. Chromatin-adducts in sperm were measured using the newly developed technique of AMS.

MATERIALS AND METHODS

Strains of mice, dosing, and mating

Male C57Bl/6J and female C3H/J mice (7 to 12 weeks of age) were purchased from Simonson Laboratories (Gilroy, CA). Males were housed singly in plastic cages; females were housed 4 or 5 per cage. The animal room was maintained on a 12 h light/dark cycle, the temperature range was 20 to 24°C, and the humidity range was 60 to 70%. Mice were fed standard breeding granulated diet, and water was supplied ad libitum. Animal husbandry was in accordance with the Guide for Care and Use of Laboratory Animals (36).

Our choice of the treatment doses and regimen was based on the data of Shelby et al. (28) who showed that male mice can tolerate a maximum single dose of acrylamide of 125 mg/kg and that a multiple 5-d treatment schedule (total dose of 250 mg/kg) was more effective than a single 125 mg/kg injection at inducing dominant lethality. Young healthy C57Bl/6J male mice (10 to 12 weeks) were randomly assigned to control and

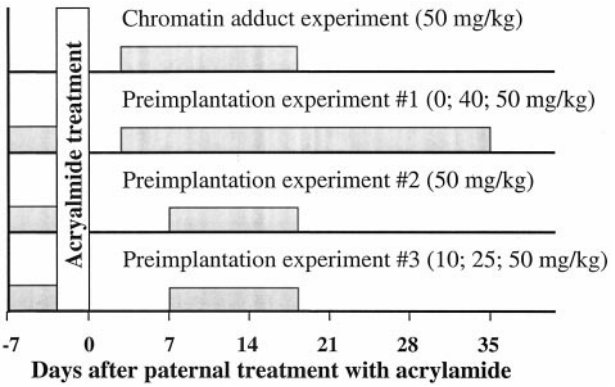

Fig. 1. Scheme of experiments. Shaded areas represent periods when: a) sperm cells were isolated for chromatin adduct analysis; or b) males were mated to obtain preimplantation embryos.

treatment groups (8 to 10 males per each group). Three sets of experiments were performed, as illustrated in the following scheme:

First, two groups of male mice received 40 or 50 mg/kg body weight i.p. injections ($\leq$0.7 mL) of acrylamide (BioRad, Hercules, CA) in sterile PBS (phosphate-buffered saline (PBS)) for five consecutive days and the control group received concurrent injections of PBS. Untreated C3H/J female mice (10 to 12 weeks old) were mated with treated C57Bl/6J mice for up to five weeks after the end of the treatment affecting postmeiotic and meiotic stages of spermatogenesis. The germ cell stages affected, as approximated by the interval between treatment and fertilization, were spermatozoa (week 1), spermatids (weeks 2 and 3), spermatocytes (weeks 4 and 5) (37). Animals were mated 4 or 5 times each week.

In a second set of experiments, three doses of acrylamide (10, 25, and 50 mg/kg, for 5 consecutive

Table 1. Effect of paternal acrylamide treatment on mating performance and embryo recovery

Dose of acrylamide mg/kg, 5 d	Number of males	Number of matings	Number of pregnant females	Success, %[a]	Female body weight, g mean ± SD	Number of CL per female[b] mean ± SD	Number of embryos per female[c] mean ± SD	Efficiency of recovery, %[d]
0 (control)	15	215	42	19.5	24.3 ± 4.9	8.7 ± 1.4	8.1 ± 1.2	93.1
10 (Experiment 3)	10	90	15	12.1	21.5 ± 1.6	8.1 ± 1.5	7.1 ± 1.4	87.6
25 (Experiment 3)	10	90	22	22.2	22.4 ± 1.9	8.7 ± 1.3	7.5 ± 1.1	85.9
40 (Experiment 1)	8	200	35	17.5	22.6 ± 4.6	8.9 ± 1.5	7.4 ± 1.3	82.7
50								
(Experiment 1)	8	200	28	14.0	21.4 ± 3.2	8.4 ± 1.2	6.4 ± 1.5	76.9
(Experiment 2)	38	334	89	26.6	23.7 ± 3.8	8.6 ± 1.1	8.2 ± 0.8	94.8
(Experiment 3)	9	81	20	19.7	24.1 ± 2.9	7.8 ± 1.6	6.4 ± 0.9	82.1
Totals, Experiments 1–3:	55	615	137	22.3	23.1 ± 1.7	8.4 ± 1.4	7.6 ± 1.1	90.5
	98	1210	251	20.7	23.0 ± 1.5	8.5 ± 1.3	7.5 ± 1.1	88.2

C57B1/6J male mice were treated with acrylamide (10 to 50 mg/kg) for 5 consecutive days and mated with untreated C3H/J female mice for 5 weeks (4 or 5 matings per week). Females were sacrificed 4 d p.c. [a]Success of mating was estimated as the portion of females found with vaginal plugs of the total number of females placed with fertile males. [b]Potential fertility or number of ovulated eggs per female was estimated by the number of corpora lutea on the ovaries. [c]Number of embryos per female was counted under a dissecting stereomicroscope before counting CL to avoid bias. [d]Efficiency of recovery was calculated as the portion of eggs/embryos recovered from the uterus compared with the number of CL. No significant difference was observed between females mated with control and treated males in either CL, body weight, embryos per female, or efficiency of recovery. Males did not differ among groups in mating success.

days) were administered, and mating continued through the second and third weeks after cessation of treatment. The third experiment involved treatment of 42 male mice with 50 mg/kg of acrylamide for 5 d and subsequent mating 2 and 3 weeks later.

There was ~10% loss of treated males at the highest dose (50 mg/kg) of acrylamide within 24 h of the last injection but no detectable effects on mating activity of the survivors (Table 1).

Embryo isolation and characterization

One male and 3 or 4 females were grouped overnight, and the onset of the pregnancy was determined by checking for vaginal plugs in the morning. Females were sacrificed by cervical dislocation ~86 to 88 h after mating, which was estimated to have taken place at midnight. Embryos were collected by flushing the uterine horns with warm tissue culture media (RPMI 1640, Gibco). The number of ovulated eggs was estimated by counting corpora lutea (CL) on the ovaries. Approximately 6 to 10 embryos were recovered per female, and the number of embryos was ~85% of the number of CL, a result that appeared to be unrelated to acrylamide exposure. The 15% loss may be explained by delay in transit through the oviducts, premature lysis of abnormal embryos lacking a zona pellucida, or technical errors (38).

Normally, mouse embryos at 4 d of embryogenesis are blastocysts or morulas (38). Morulas have between 16 and 32 compact cells. Normal blastocysts consist of more than 32 cells, which are differentiated into an inner cell mass and trophoblast cells surrounding a blastocele. Until hatching of the embryo at around 4.5 to 5 days postcoitum (p.c.), the embryo is covered by the zona pellucida.

The gross morphology of the preimplantation embryos was evaluated under a dissecting stereomicroscope (Nikon, 1.6 × 10 × 2–4) to determine the frequency and types of morphologic abnormalities. The number of normal blastocysts and morulas (Figure 2a), as well as the number of abnormal embryos, were recorded for each female. Three types of abnormal embryos were differentiated: 1) embryos with retarded cleavage and a small (fewer than 10) number of blastomeres (Figure 2b), 2) embryos with lysis or abnormal structure of cells (Figure 2c), and 3) unfertilized eggs or zygotes that failed to undergo cleavage (Figure 2d).

Measurement of chromatin-adducts with accelerator mass spectrometry

tk;2AMS was used to analyze sperm chromatin-adducts induced by acrylamide. This technique has been described elsewhere (39). Briefly, ten males were treated with ^{14}C-acrylamide (3 mCi/mol, 50 mg/kg) and two of them were sacrificed every three days by cervical dislocation following CO_2 anesthesia. Cauda epidymides were removed and used to isolate sperm by mincing into fragments using fine scissors and a syringe needle to create a sperm suspension (40). The suspensions were incubated in a 37°C water bath for 1 h, aspirated 10 times, and filtered through mesh screen. Sperm suspensions were centrifuged at 6300 *g* for 12 min and processed to isolate sperm nuclei. The suspension was sonicated for approximately 20 s, followed by centrifugation. This process was repeated 3 times so that the final pellet contained nuclei. Five hundred (500) μL of the suspension were put into a quartz 6 × 50 mm tube and centrifuged at 21,200 *g* three times for 10 min, dried

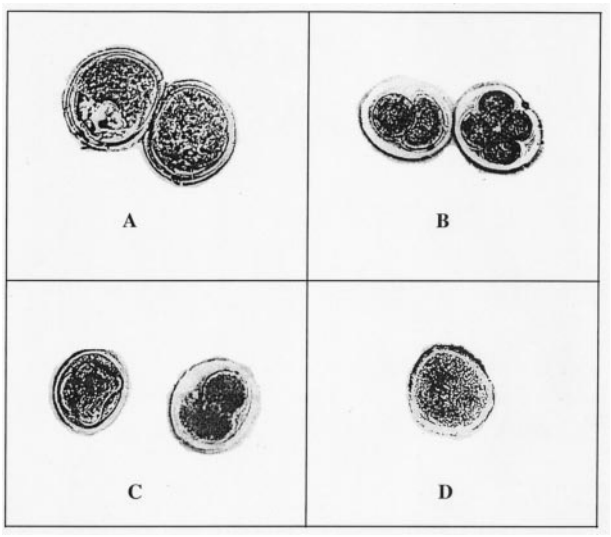

Fig. 2. Preimplantation mouse embryos after paternal treatment with acrylamide during postmeiotic stages of spermatogenesis. Embryos were isolated four days after mating of untreated C3H/J female mice with acrylamide treated C57Bl/6J mice (50 mg/kg, 5 d). Phase contrast, Nikon, x400: A. Morphologically normal embryos: blastocyst (with blastocele) and late morula. Both embryos surrounded by zona pellucida. After DAPI staining these embryos had 45 and 30 cells, respectively. B. Abnormal embryos with arrested cleavage after the 1st division (2 cells) and 2nd division (4 cells). C. Abnormal embryos with lysis or abnormal cellular structure. Zona pellucida remains intact. After DAPI staining these embryos had only 1 to 3 cells. Nuclear fragmentation, cytoplasmic fragments and anucleate cells are typical for embryos with lysis. D. An abnormal egg or one-cell zygote at 4-d p.c. No sign of fertilization or cleavage was visible, zona pellucida still intact. DAPI staining shows one cell with different degree of cytoplasm fragmentation.

under vacuum, and converted to graphite for ^{14}C analysis by AMS.

Tissue samples were converted to graphite to ensure uniform sample composition and high ionization efficiency for AMS measurements (39). Samples for this study were converted to graphite by combustion of the samples to CO_2, followed by reduction of the CO_2 to filamentous graphite on cobalt, in quartz 6 $\times$ 50 mm tubes at 900°C (41). Because the graphitization reaction is most efficient with 1 mg of carbon, fractions were supplemented with carrier carbon having low, well known ^{14}C content before conversion to graphite (41). AMS measures the ratio of radiocarbon relative to ^{13}C (42). We normalized the $^{14}C/^{12}C$ ratio of 1950 carbon using the Australian National University sugar standard as reference (39,43). Adduct level was determined as previously described (43) as acrylamide equivalents (μg) in a gram of the tissue. Sensitivity depends on the specific activity of the test agent and is ~1 adduct per 10^{11} bases of DNA by this technique (17).

Dominant lethality assay and preimplantation loss

The dominant lethality assay was performed according to a standard protocol (5,28) using the same group of treated C57Bl/6J male mice as with preimplantation abnormality experiments. Females with vaginal plugs were randomly assigned to either experiment. For dominant lethality assay, females were sacrificed 15 to 16 d p.c. The uterine contents were inspected for the number of total implants, early death (moles), early resorptions (implants that died at 6 to 12 d of pregnancy), and late death (completely developed embryos that died after 13 d of pregnancy). The CL were counted under a dissecting Nikon stereomicroscope. Preimplantation loss was calculated as the difference between number of CL and number of implants, divided by the number of CL. Postimplantation loss was estimated as a percent of embryos that died at the different stages of postimplantation development (or total dead implants), divided by the total number of implants.

Statistical methods

Fisher's exact test was used to compare frequencies of abnormalities in exposed vs. control embryos. A logistic regression modeling approach was applied to analyze the effects of dose and time of acrylamide treatment on preimplantation abnormalities. The statistical analyses were performed using generalized linear model routine of S language (44). Effects of potential confounding factors were also assessed by fitting more complex logistic regression models that considered experiments, female body weight, and number of CL in addition to dose and time. An ANOVA deviance analysis was conducted to evaluate the significance levels of those factors as well as their interactions. A value of $P \leq 0.05$ was used as the criterion for statistical significance. Effect of sire was not significant by multifactorial regression analysis and results are expressed based on a pooling of embryos with dose groups.

RESULTS

Formation of acrylamide-adducts in mouse male germ cells

Chromatin adducts, which measure bioactivated component of acrylamide reaching sperm nuclei, were determined using radiolabeled acrylamide and AMS. Figure 3 shows the time-dependent formation of acrylamide adducts in sperm nuclei after treatment of male mice with 50 mg/kg acrylamide. While the baseline level of chromatin adducts was 0.12 μg/g sperm, three days after the acrylamide treatment it was increased to 16.5 $\pm$ 2.7 μg/g and reached a peak of 19.3 $\pm$ 2.6 in the beginning of the second week after treatment. This sharp

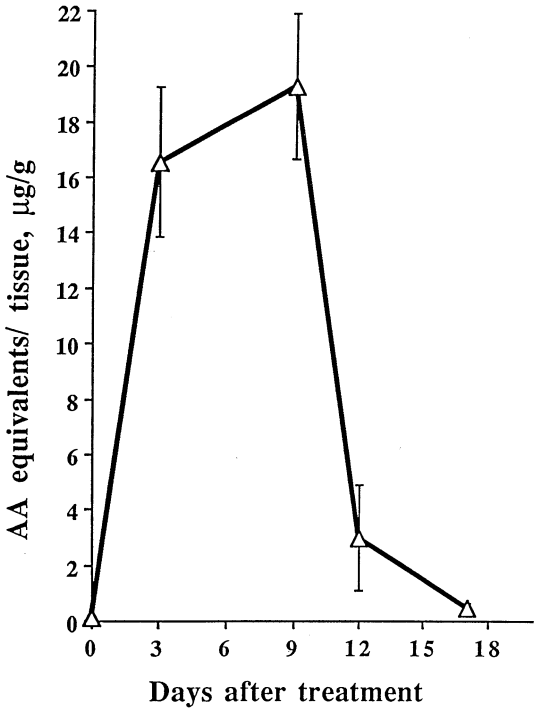

Fig. 3. Chromatin adducts in mouse sperm induced by acrylamide (50 mg/kg). Male mice were treated with ^{14}C-acrylamide (3 μC/mol) and two of them were sacrificed every three days. Sperm was isolated from epidymides and analyzed by accelerator mass spectrometry. Adduct level was determined as acrylamide equivalents in a gram of the tissue. Each time-point represents an average of two independent measurements. Significant increase above baseline level of chromatin adducts (0.12 μg/g) was observed between 3rd and 9th days after treatment (Student's *t*-test, $p < 0.001$) with sharp decline at 12th day ($P < 0.05$) and return to the baseline by 17th day ($P > 0.05$).

increase in the nuclear concentration of acrylamide adducts in the first and second weeks after treatment was followed by a significant decrease at 12 d (3.0 ± 1.9 μg/g) and return to baseline by the third week (0.46 ± 0.20 μg/g). This result indicated that sperm (epididymal and testicular) and late-step spermatids had the highest level of adduct formation.

Characteristics of acrylamide effect on matings of mice and recovery of embryos

Three experiments were performed to characterize the time-course and dose-dependence of preimplantation abnormalities related to paternal exposure to acrylamide. Table 1 presents data for treated and control groups related to the mating activity of male C57Bl/6J mice. Usually, males were placed with 3 or 4 females 5 times each week. Acrylamide treatment did not noticeably affect the mating performance of males ($P > 0.05$).

The average rate of ovulations, estimated by the number of CL in the ovaries of pregnant females 4 d p.c., was between 7.8 and 8.9 per female (Table 1). There was no difference in the number of CL between treated or control groups or between experiments. The average number of CL was 8.5 ± 1.3. At 4 d p.c., we expected the majority of embryos to be found in the uterine horns (38). We were able to recover between 76.9% and 94.8% of the embryos expected based on the number of CL. Moreover, considering that we counted CL before counting embryos, the estimated efficiency of recovery (average for all mice was 88.2%) is likely to reflect a relatively small technical loss and possibly a physiologic loss due to delay in the Fallopian tubes or premature disintegration of the eggs/zygotes. As shown in Table 1, there was no apparent dose effect of acrylamide on the efficiency of embryo recovery.

Preimplantation abnormalities caused by acrylamide treatment at different stages of spermatogenesis

A 6.8% background frequency of preimplantation abnormalities among 144 embryos was determined before the C57Bl/6J male mice were separated into control and treated groups. After acrylamide treatment, both treated and control groups of embryos were analyzed simultaneously for each of the 5 following weeks. In the concurrent control group, the average frequency of abnormalities during 5 weeks of mating was 3.4% (range 0–5%). There was no significant change in the background frequency of preimplantation abnormalities before or throughout the five weeks of the experiment (Figure 4).

Table 2 summarizes data on the three different types of abnormalities in 4-d preimplantation embryos sired by C57Bl/6J males treated with acrylamide. The effect of acrylamide on preimplantation embryo morphology was dose-dependent with the higher dose (50 mg/kg b.w., 5 d) inducing 86.2% abnormalities in embryos in the first week after treatment of males, and the lower dose (40 mg/kg b.w., 5 days) inducing 61.1% ($P < 0.01$) (Table 2). The frequency of total abnormalities remained elevated through the second week after treatment. A gradual decline in the frequency of abnormalities indued by 50 mg/kg acrylamide occurred at weeks 3, 4, and 5. At the 5th week, frequencies remained elevated only for the 40 mg/kg group (Table 2). There was an excess of abnormal embryos in the 40 mg/kg treatment group at 4 weeks compared to the 50 mg/kg group ($P = 0.033$ by Fisher's exact test) (Figure 4).

Different types of abnormalities were analyzed separately. For weeks 1 through 3 of mating, corresponding to exposure of spermatozoa and spermatids, unfertilized eggs or single-cell zygotes were the most frequently recovered type of embryos (39.2%), equaling 62.3% of all abnormals (Table 2). They appeared to be less common at 4 and 5 weeks, when they accounted for only

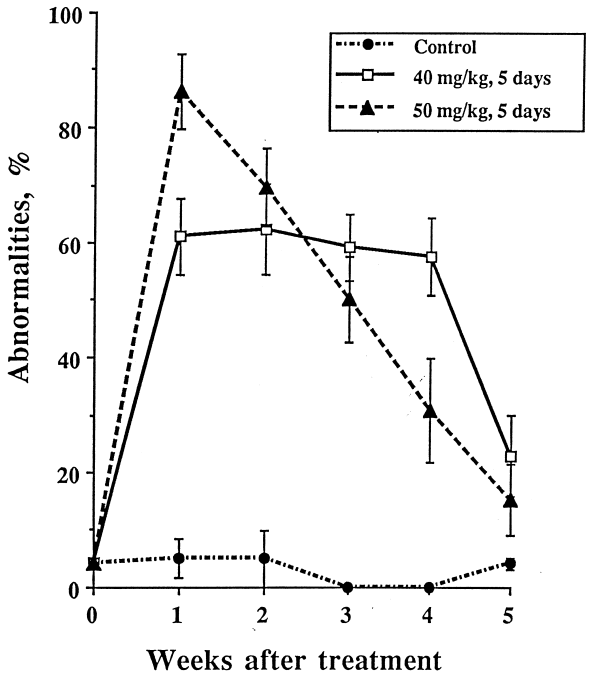

Fig. 4. Morphologic abnormalities in preimplantation mouse embryos after paternal treatment with acrylamide at the different stages of spermatogenesis. The number of embryos analyzed in control and treated groups were 116, 251 (40 mg/kg) and 180 (50 mg/kg), respectively. There was no change in the control level of morphologic abnormalities at any of the sampling times, while in the treated groups the frequency of abnormalities was significantly increased ($P < 0.05$). The highest increase was observed at the 1st week (treatment of spermatozoa) ($P < 0.01$). The plateau at ~60% abnormalities over 4 weeks was observed for the 40 mg/kg treatment group.

8.1% of all recovered embryos or 23.1% of all abnormals ($P < 0.001$). When the other two types of morphologic abnormalities were compared for the same time-periods (1 through 3 weeks and 4 through 5 weeks), their relative frequencies did not change significantly and were 9.9% and 10.1% for embryos with retarded cleavage and 18.0% and 14.2% for embryos with lysis, respectively.

The experiment employing a dose of 50 mg/kg of acrylamide was replicated with a larger number of males ($N = 38$) in order to obtain sufficient numbers of embryos to measure the frequencies of abnormal embryos daily during the second and third weeks (Table 3). Five matings were performed each week and the total number of embryos recovered was 402 and 325, respectively. The average frequency of abnormalities in the second week was 93.5%, compared to 81.8% in the third week ($P = 0.003$). The level of abnormalities in this experiment appears to be higher than in the first (Table 2), while the relative decrease in the frequency of abnormalities from the second to third week is the same for both experiments. We performed a time-dependency

Table 2. Preimplantation abnormalities in 4-d mouse embryos fathered by C57Bl/6 males treated with acrylamide

Weeks after treatment	Characteristics of embryos	Control	40 mg/kg 5 d	50 mg/kg 5 d
1	Females/embryos	5/40	9/54	6/29
	Abnormal embryos, %	5.0	61.1[a]	86.2[a,b]
	Lysis	–	25.9	17.2
	Arrested cleavage	2.5	–	–
	Zygotes/eggs	2.5	35.2	69.0
2	Females/embryos	3/20	6/37	6/46
	Abnormal embryos, %	5.0	62.1[a]	69.5[a]
	Lysis	–	18.9	13.0
	Arrested cleavage	–	8.1	4.3
	Zygotes/eggs	5.0	35.1	52.2
3	Females/embryos	5/32	9/71	9/46
	Abnormal embryos, %	0	59.1[a]	50.0[a]
	Lysis	–	14.1	19.6
	Arrested cleavage	–	12.7	4.3
	Zygotes/eggs	–	32.4	26.1
4	Females/embryos	0/0[c]	6/54	3/26
	Abnormal embryos, %	0	57.4[a,d]	30.8[a]
	Lysis	–	20.4	15.4
	Arrested cleavage	–	22.2	–
	Zygotes/eggs	–	14.8	15.4
5	Females/embryos	3/24	5/35	4/33
	Abnormal embryos, %	4.2	22.8[a]	15.1
	Lysis	–	2.8	15.1
	Arrested cleavage	–	2.8	–
	Zygotes/eggs	4.2	11.4	–

No statistically significant difference was observed in control group at 1 to 5 weeks.
[a]Significantly different from pooled control ($P < 0.01$). Frequencies of abnormal embryos (with arrested cleavage, lysis, or zygotes/eggs) in treated groups were above control at all time-points at the 5th week except the 50 mg/kg group ($P = 0.149$ by Fisher's exact test).
[b]Significant difference compared with 40 mg/kg-treated group ($P = 0.015$) by Fisher's exact test.
[c]No females mated with control males were found with vaginal plugs during 4th week.
[d]Significantly higher than an average level of control ($P < 0.001$) and 50 mg/kg-treated group ($P = 0.033$) by Fisher's exact test.

analysis using logistic regression modeling that also confirmed a significant trend ($P < 0.001$) for a lower frequency of abnormal embryos as time progresses after cessation of the treatment with acrylamide.

Dose-response of acrylamide-induced preimplantation abnormalities

While both the 40 and 50 mg/kg doses produced a strong postmeiotic effect, certain differences were observed between the two doses (Table 2). To further analyze the dose-response relationship, the range of doses of acrylamide was expanded to 10, 25, and 50 mg/kg (5 d). The analysis focused on the spermatid stage of spermatogenesis and accordingly embryos were collected during the second and third weeks after treatment (Table 4). Acrylamide at a dose of 10 mg/kg did not change the baseline frequency of preimplantation morphologic abnormalities at either 2nd or 3rd weeks (0 and 6.6%, $P < 0.01$). A strong dose-effect was observed with

Table 3. Preimplantation abnormalities in 4-d mouse embryos fathered by C57Bl/6 males treated with 50 mg/kg of acrylamide, 5 consecutive days at 7 through 18 d after cessation of treatment (postmeiotic stages of spermatogenesis)

Days after last of 5 injections	Total embryos	Total abnormalities, %	Eggs/zygotes, %	Arrested cleavage, %	Lysis, %
Second week					
7	70	85.5	31.9	43.5	10.1
8	95	96.8	31.6	50.5	14.7
9	50	92.0	40.0	42.0	10.0
10	97	96.9	20.6	57.7	18.6
11	90	93.3	36.7	51.0	5.6
Total:	402	93.5	31.2	50.1	12.2
Third week					
14	144	89.6	27.8	54.2	7.6
15	35	34.3	14.3	14.3	5.7
16	35	96.4	36.4	36.4	23.6
17	40	77.5	35.0	37.5	5.0
18	51	80.4	7.8	60.8	11.8
Total:	325	81.8[a]	25.2[b]	45.8[c]	10.5[c]

[a]Lower than the frequency of abnormalities at the 2nd week ($P = 0.003$ by Fisher's exact test). Time-dependency was characterized by a significant negative trend ($t = -4.27$, $P < 0.001$).
[b]Lower than the frequency of eggs/zygotes at the 2nd week ($P = 0.048$ by Fisher's exact test).
[c]Embryos with arrested cleavage and lysis occurred at similar rates in the second and third weeks ($P = 0.16$ and $P = 0.27$, respectively).

25 and 50 mg/kg acrylamide. At the highest dose (50 mg/kg), the frequency of abnormalities was 72.5% and 45.8% in the second and third weeks, respectively. This frequency is relatively close to the levels observed in the first and second experiments (Tables 2 and 3).

Data from all three experiments were pooled together to analyze the dose- and time-dependency of the frequency of preimplantation embryos after acrylamide treatment. This approach allowed for analysis of the effects of variation between experiments, as well as other confounding factors including the number of ovulations (CL) and female body weight. Logistic regression models with up to the second order interactions were used to study the data. The following model was selected to describe the data after all nonsignificant factors and interactions were deleted:

$$\text{logit}(\text{preimp.abn.}) = \text{Dose} + \text{Female body weight} + \text{Experiment} + \text{CL} + \text{Dose:Week} + \text{Week:Expt.}$$

Coefficients for the dose and dose:week interactions were identical for all three experiments, while week effects were different. This result suggests that dose-responses share the same pattern in all three acrylamide experiments. An ANOVA deviance analysis is presented in Table 5. These data show that dose and time, as well as experiment variability, were the most significant factors in defining the frequency of preimplantation embryos after acrylamide treatment. Two of these factors (female body weight and number of CL), though signif-

Table 4. Preimplantation abnormalities in 4-d mouse embryos fathered by C57Bl/6 male mice treated with 10 to 50 mg/kg of acrylamide

Dose of acrylamide mg/kg, 5 d	Total embryos	Total abnormalities, %	Eggs/zygotes, %	Arrested cleavage, %	Lysis, %
Second week					
10	76	6.6[a]	4.0	1.3	1.3
25	132	28.8	18.9	9.8	0
50	80	72.5[b]	38.8[c]	31.2[c]	2.5
Third week					
10	30	0	0	0	0
25	32	6.3	3.1	3.1	0
50	48	45.8[d]	27.1	14.6	4.2

[a]Lower than the frequency of abnormalities in the 50 mg/kg group and higher than in the 10 mg/kg group at the 2nd week and the 25 mg/kg group at the 3rd week ($P < 0.01$ by Fisher's exact test).
[b]Higher than the frequency of abnormalities at the 3rd week ($P < 0.01$ by Fisher's exact test).
[c]Higher than the frequencies in the 10 and 25 mg/kg groups ($P < 0.05$) at the 2nd and 3rd weeks.
[d]Higher than the frequency of abnormalities in the 25 mg/kg group at the 3rd week ($P < 0.01$ by Fisher's exact test).

Table 5. ANOVA table of significant variables and their interactions

Parameters of the model	Deviance[a]	Degree of freedom	Significance (*P*-value)
Corpus luteum	6.72	1	0.01
Female body weight, g	39.41	1	<0.001
Dose:week	10.34	1	0.001
Week: experiment	13.66	2	<0.001
Experiment + week: experiment	123.02	4	<0.001
Week + week: experiment + week : dose	55.74	4	<0.001
Dose + dose:week	203.41	2	<0.001

Data from three experiments, including 1680 embryos were analyzed. Logistic regression model was used to assess effects of time and dose of paternal acrylamide treatment, as well as maternal body weight and potential fertility, on the frequency of morphologically abnormal preimplantation embryos. [a]Deviance is the sum of variability introduced to the outcome of the model by the single factor or interaction between two or three significant factors.

icant ($P < 0.01$), had less effect on the frequency of preimplantation abnormalities than interaction between week after treatment, dose of acrylamide, and difference between experiments. Figure 5 presents results of modeling of the dose- and time-dependency of the frequency of total preimplantation abnormalities, based on evaluation of 1680 embryos in 3 separate experiments. The dose-curve for acrylamide demonstrates the strongest effect of acrylamide treatment on spermatozoa (1 week). Each following week (earlier stages of spermatogenesis), less damage to male germ cells was observed, with the resulting frequency of preimplantation abnormalities at the 5th week (treatment of meiotic germ cells) only slightly above that of control. These results of statistical modeling based on the total number of abnormalities were further analyzed separately for each of the three different types of abnormalities. Logistic regression model also revealed a significant dose-response for eggs/zygotes ($P < 0.001$) and embryos with retarded cleavage ($P < 0.001$), while for embryos with blastomere lysis this connection was not significant ($P > 0.05$).

Thus, a strong dose-response was demonstrated for the induction of preimplantation abnormalities by paternal treatment with acrylamide, with 10 mg/kg being a "no-effect" dose and 50 mg/kg producing ~ 90% abnormal embryos. A significant time-dependence was also observed, with the postmeiotic effect being the most prominent.

Preimplantation and postimplantation loss at the 15 to 16th day of pregnancy

The same treated C57Bl/6J male mice (50 mg/kg acrylamide, 5 d) used in the first experiment to analyze preimplantation morphologic abnormalities in embryos were simultaneously mated with the same group of C3H/J female mice, with the difference that impregnated females were kept until 15 to 16 days p.c. For each group, ~6 females were analyzed according to the dominant lethal protocol (28). Figure 6 shows the increase in the level of preimplantation and postimplantation loss estimated by the end of the pregnancy compared with the increase in the frequency of morphologically abnormal embryos at 4 d p.c. The control level (12.3% and 7.3% for preimplantation and postimplantation loss, respectively) was subtracted from induced levels to assess acrylamide-related increases. The highest increase in preimplantation loss (72.1%) at 15 to 16 d was observed at 1 to 2 weeks after paternal acrylamide treatment. The increase was significantly lower at 3 to 4 weeks (41.3%) and the 5th week (18.5%) ($P < 0.05$). An increase in acrylamide-induced morphologically abnormal embryos closely coincided with this assessment for all three time intervals (72.9%, 40.1%, and 10.1%, respectively). The increase in postimplantation loss was also the largest at 1 to 2 weeks (12.7%), though it was also slightly above control at 3 to 4 and the 5th weeks (8.2% and 10.1%).

DISCUSSION

The most common test for analysis of paternal genotoxicity is the DL assay (45,46). DL caused by acrylamide in rats and mice effectively shows that it is active predominantly in postmeiotic germ cells (28,30, 31). Preimplantation loss can be estimated by the DL assay only indirectly as the difference between the number of ovulated eggs (equivalent to the number of corpora lutea) and the number of implantations, divided by the number of ovulations. To evaluate DL in our treatment group, we used the same treated males involved in the preimplantation abnormalities experiments. Preimplantation loss for the control group in our experiment was 12.3%, and total postimplantation loss was 7.3%, mostly as a result of early deaths or moles. This result agrees well with published data for C3H/J and C57Bl/6J mice (47). We observed a high level of DL (73.3%) in the first week after treatment with 50 mg/kg of acrylamide but did not record a statistically significant effect during the following weeks of breeding. This finding is very similar to the DL response (83%) to multiple (5 d) injections of the same dose of acrylamide described in experiments with (C3Hx101)F1 mice by Shelby et al. (28). In their study, Shelby et al. (28) also recorded a fast decrease in DL at the second week after treatment compared to the first. Significantly, when they used a single injection of 125 mg/kg of acrylamide, some low DL (1 to 12%) was observed through the fifth week after treatment. This result corroborates our finding of

increased prevalence of morphologic abnormalities up to the fifth week.

The other parameter that suggested that acrylamide has an adverse effect on preimplantation development was the rate of mated females (with vaginal plugs) that were found pregnant at 15 to 16 days p.c. While in our control group all females with vaginal plugs became pregnant, in the group of females mated with treated males, only 90% and 54% became pregnant after 5 d treatment with 40 and 50 mg/kg of acrylamide, respectively. Thus, our findings confirm data on DL of acrylamide in mice reported earlier (28), and allow this substantial data set to be used as a reference point for our preimplantation abnormalities experiments.

The main advantage of the preimplantation abnormalities assay is that it allows a direct morphologic assessment of embryos to be performed immediately preceding implantation. The most striking effect of acrylamide was observed at the first week after treatment with 50 mg/kg of acrylamide (86.2%). The majority of abnormalities at the first week, as well as at 2 to 3 weeks, were unfertilized eggs (or one cell zygotes). In our experiments we also recorded a significant number of embryos with retarded cleavage or lysis. All these abnormalities presumably contribute to preimplantation loss because such embryos have a much higher chance to be eliminated during implantation (48,49). In fact, we observed a very close similarity between the preimplantation loss estimated at the end of pregnancy and the total frequency of morphologic abnormalities shortly before implantation (Figure 5). The striking effect of acrylamide at the first week after treatment indicates direct damage to spermatozoa and late spermatids that likely results in fertilization failure or the failure to form a zygote capable of further development. Our findings are supported by information on reduced sperm motility (25) and high frequency of morphologically abnormal spermatozoa (40) caused by acrylamide. It is noteworthy that

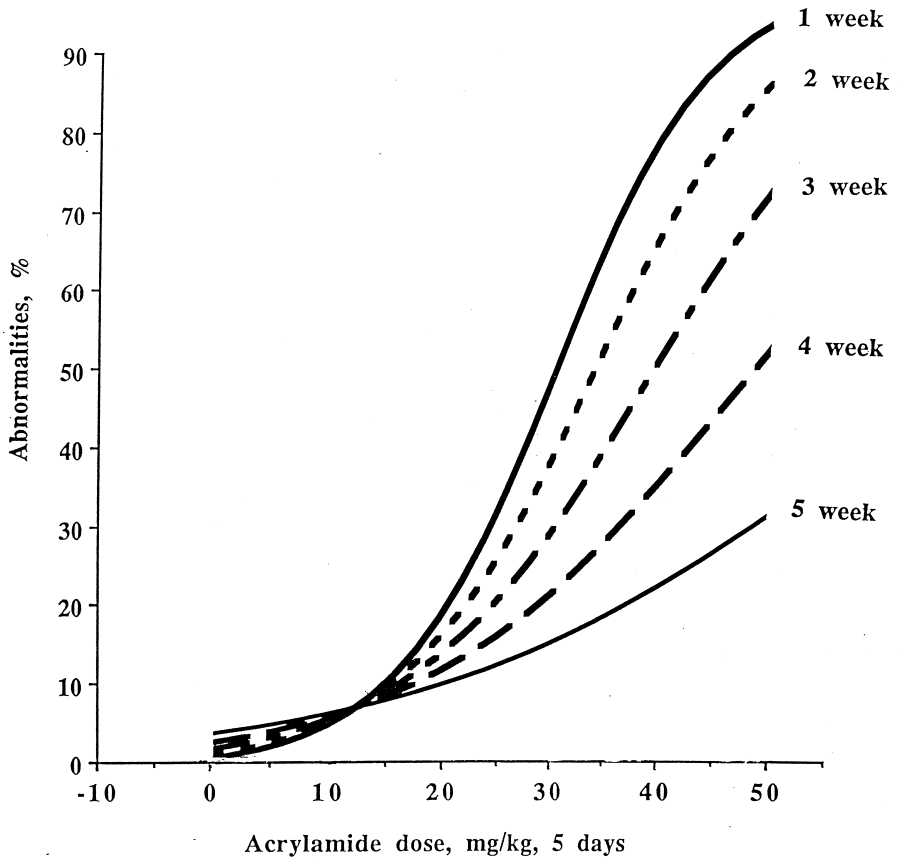

Fig. 5. Time- and dose-dependency of preimplantation abnormalities in mice induced by paternal treatment with acrylamide. Generalized linear model was used (StatSci) to create dose-curves for each of the five weeks after treatment using all data-points from three treatment experiments (total 1816 embryos). Model was corrected for the variability between experiments and for the effect of CL which were found significant ($P < 0.05$). The effect of acrylamide dose was highly significant ($P < 0.001$), as was an interaction between the dose and the week of the experiment ($P < 0.01$), reflecting the role of the stage of spermatogenesis when male germ cells were treated. The highest response was observed in spermatozoa with gradual decrease of the effect for spermatids (weeks 2 and 3) and spermatocytes (weeks 4 and 5).

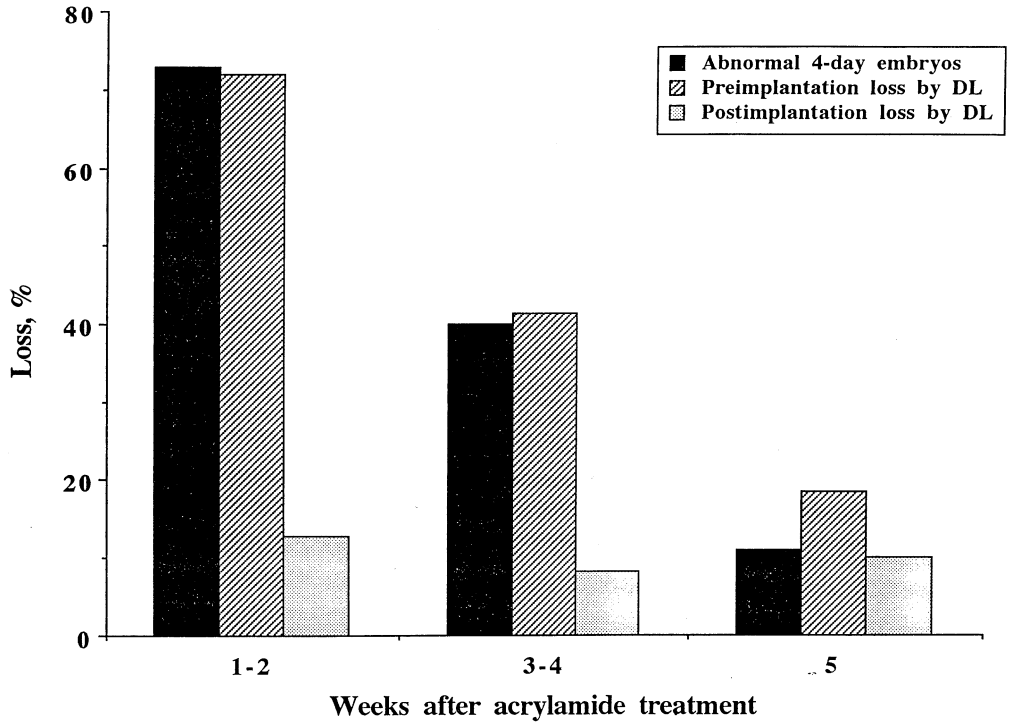

Fig. 6. Comparison of acrylamide-induced preimplantation and postimplantation loss estimated at 4th and 15 to 16th days of pregnancy. All morphologic abnormalities found by evaluation under dissecting microscope at 4 d p.c. were considered a potential preimplantation loss. The dominant lethality assay was performed at 15 to 16 days p.c. according to standard protocol using the same group of treated C57Bl/6J male mice as with preimplantation abnormalities experiments. Preimplantation loss was calculated as the difference between number of CL and number of implants, divided by the number of CL. Postimplantation loss was estimated as a percent of embryos that died at different stages of postimplantation development (early resorptions and dead implants), divided by the total number of implants. To show an increase in loss attributable to acrylamide treatment, loss found in the control group was subtracted from loss found in the treated group. The highest increase (more than 70%) in preimplantation loss was observed at 1 to 2 week after paternal treatment (late postmeiotic period). Correlation between assessment of preimplantation loss at 4 weeks and 15 to 16 days was 0.85 ($P < 0.01$).

mating behavior of the males was not affected while the quality of sperm was compromised.

The important aspect of the dose-response analysis was finding the no-effect dose. At the second week after treatment no-effect dose was 10 mg/kg of acrylamide when no increase in abnormalities was observed. At the third week, the frequencies of morphologic abnormalities did not differ from control for both 10 and 25 mg/kg of acrylamide. Thus, we observed a very broad variation in the frequency of abnormalities, from more than 90% to no effect. To explore different factors contributing to this variability, a statistical model was used. Acrylamide treatment, with both dose and time, was found to be the most significant factor. However, other factors, including weight of females and their potential fertility, estimated by the number of corpora lutea, were also statistically significant.

There are several publications in which authors have reported data on the findings of delayed or abnormal embryos before implantation as a result of exposure to mutagens. To date, exposure to alkylating agents has mainly been assessed. For example, when male mice were treated with thioTEPA at the late spermatid stage, 39% of preimplantation embryos died or delayed their development at the 2 to 22 blastomeres stage (7). It was also shown that the paternal genotype may affect the frequency of preimplantation morphologic abnormalities. Baseline frequencies of abnormalities from homo- and heterogeneous crosses between mice of three inbred strains C57Bl/6, CBA and BALB varied from 5.5% to 37.5%, depending on the combination of the paternal and maternal genotypes (50). All these data imply that preimplantation abnormalities are a common biologic feature that occur spontaneously and can be greatly increased by mutagenic exposure of the father. A noticeable similarity between the frequency of preimplantation loss assessed by the dominant lethal assay, on the one hand, and the frequency of morphologic abnormalities directly assessed before implantation, on the other, shows that these endpoints are likely descriptors of the

same process of early embryonic loss related to the postmeiotic acrylamide treatment of male germ cells. It seems logical to suggest that there are multiple mechanisms causing preimplantation loss. Some mechanisms are physiologic and could be related to direct morphologic damage of sperm by mutagens, as discussed earlier. Some other embryo abnormalities could be caused by cytogenetic defects or molecular changes in sperm transmitted to the progeny.

To study the molecular damage induced by acrylamide in sperm cells, accelerator mass spectrometry was used. This method measures with high precision the level of radioactive acrylamide in sperm nuclei but does not allow recognition of the molecular target. There are data available that show that labeled acrylamide mainly interacts with protamines (23), while other data suggest direct interaction with DNA (22). Further studies will be needed to confirm the target of acrylamide in the germ cells of male mice. A strong response in the level of chromatin adducts was observed in spermatozoa and late spermatids (first-second week after treatment). High levels of adducts also could be a result of pharmokinetics of distribution of acrylamide in the testicular tissue. This corroborates fairly well our finding that the greatest impact of paternal acrylamide on preimplantation abnormalities also occurs at the late postmeiotic period. The time-course was similar to a DL response observed in the study of Shelby et al. (28) and in our group of males. We suggest that chromatin adducts are likely to be converted into cytogenetic abnormalities when they are exposed to DNA repair. Since there is almost no repair occurring in mature sperm due to its very compact structure, it is possible that in a zygote with an active DNA repair machinery, introduced by an egg, potential molecular damage in male chromosomes will be converted to chromosome breaks and other rearrangements. The study of Marchetti et al. (10), using cytogenetic analysis of first cleavage zygotes, showed that almost all zygotes had at least one structural aberration after paternal treatment with acrylamide (using the same treatment protocol as this study). Moreover, all aberrations were found in the male pronucleus. Further, direct comparison of abnormalities in our 4-day mouse embryos with the number of unfertilized eggs and abnormal zygotes observed by Dr. Marchetti et al. (10) in the first cleavage zygotes reveals a close correlation for sperm treatment (84% versus 93%, respectively).

We speculate that some morphologic abnormalities in preimplantation embryos, as well as DL, were caused by direct interaction of acrylamide with DNA and other macromolecules, which may have resulted in chromosome abnormalities. To test this hypothesis, we used slides accumulated in the course of the current study. Normal and abnormal embryos (total = 443) were processed and analyzed separately using a number of cytogenetic and cytologic criteria (51). We found that the frequency of micronucleated cells, reflecting the level of cytogenetic damage, was increased 20-fold in abnormal and tenfold in morphologically normal preimplantation embryos after postmeiotic treatment with 50 mg/kg acrylamide. Proliferation delay, assessed by cell number per embryo, and cell death, assessed by the presence of fragmented nuclei, were also significantly affected by acrylamide. There was a highly significant correlation between morphologic and cytologic characterization of preimplantation abnormalities. All of these findings suggest that the preimplantation embryo is a sensitive indicator of paternally transmitted effects on early development and can be effectively used to study male-mediated genotoxicity. These data add to the information about genotoxicity and carcinogenicity of acrylamide and possible implications to humans.

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REFERENCES

1. Wyrobek AJ. Methods and concepts in detecting abnormal reproductive outcomes of paternal origin. Reproductive Toxicology. 1993;7:3–16.
2. Preston RJ. Future of germ cell cytogenetics. Environ Mol Mutagen. 1994;23:Suppl.24:54–8.
3. Russell WL. X-ray-induced mutations in mice. Cold Spring Harbor Symp Quant Biol. 1951;16:327–336.
4. Russell LB, Shelby MD. Test for heritable genetic damage and for the evidence of gonadal exposure in mammals. Mutat Res. 1985; 154:69–84.
5. Green S, Auletta A, Fabricant J, Kapp R, Manandhar M, Sheu C-Ju, Springer J, Whitfeld B. Current status of bioassys in genetic toxicology - the dominant lethal assay. A report of the U.S Environmental Protection Agency Gene-Tox Program. Mutat Res. 1985;154:49–67.
6. Shelby MD. Human germ cell mutagenesis. Environ Mol Mutagen. 1994;23:Suppl.24:30–4.
7. Malashenko AM, Semenov KK, Selezneva GP, Surkova NI. Studies of mutagenic effect of chemical compounds in laboratory mice. Genetica (Rus). 1978;14(1):52–61.
8. Binkert F, Schmid W. Preimplantation embryos of Chinese hamster. II. Incidence and type of karyotype anomalies after treatment of the paternal post-meiotic germ cells with an alkylating mutagen. Mutat Res. 1977;46:77–86.
9. Albanese R. Mammalian male germ cell cytogenetics. Mutagenesis. 1987;2:79–85.
10. Marchetti F, Lowe X, Moore II D, Bishop J, Wyrobek A. Paternally transmitted chromosome structural aberrations detected in mouse first-cleavage zygote metaphases by multicolor FISH painting. Chromosome Research. 1996;8:604–13.
11. Kitaev EM, Nikitin AI, Lipovski SM, Lovchikov VA. Effects of benzene on the parameters of early embryogenesis in rats. Akusherstvo i Ginekologiia (Rus). 1979;4:51–3.

12. Kola I, Vogel R, Spielman H. Co-administration of ascorbic acid with cyclophosphamide (CPA) to pregnant mice inhibits the clastogenic activity of CPA in preimplantation murine blastocysts. Mutagenesis. 1989;4(4):297–301.
13. Pampfer S, De Hertogh R, Michiels B, Vercheval M. Decreased inner cell mass proportion in blastocysts from diabetic rats. Diabetes. 1990;39(4):471–476.
14. Van der Elst J, Nerinckx S, Van Steirteghem AC. Association of ultrarapid freezing of mouse oocytes with increased polyploidy at the pronucleate stage, reduced cell numbers in blastocysts and impaired fetal development. J Reproduction and Fertility. 1993;99: 25–32.
15. Felton JS, Turteltaub KW. Accelerator mass spectrometry for measuring low dose carcinogen finding to DNA. Environ Health Perspectives.: 1994;102:450–452.
16. Turteltaub KW, Felton JS, Gledhill BL, Vogel JS, Southon JR, Caffee, MW, Finkel RC, Nelson DE, Proctor ID, Davis JC. Accelerator mass spectrometry in biomedical dosimetry:relationship between low-level exposure and covalent binding of heterocyclic amine-carcinogens to DNA. PNAS USA. 1990;87:5288–5292.
17. Creek MR, Vogel JS, Turteltaub KW. Extremely low dose benzene pharmacokinetics and macromolecular binding in B6C3F1 male mice. The Toxicologist- Abstracts of the Annual meeting of the Society of Toxicology, Dallas, TX, March 13–15, 1994 #1704.
18. US Environmental Protection Agency. Assessment of health risks from exposure to acrylamide (unnumbered). EPA Office of Toxic Substances, Washington DC. 1990.
19. IARC working group on the evaluation of carcinogenic risks to humans: some industrial chemicals. Lyon, 15–22 February 1994. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. 1994;60:1–560.
20. Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ. Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk. Mutat Res. 1995;330:71–99.
21. Solomon JJ, Fedyk J, Mukai F, Segal A. Direct alkylation of 2′-deoxynucleosides and DNA following in vitro reaction with acrylamide. Cancer Res. 1985;45:3465–3470.
22. Segerback D, Calleman CJ, Schroeder JL, Costa LG, Faustman EM. Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and rat following i.p. administration of (^{14}C)acrylamide. Carcinogenesis. 1995;16(5):1161–5.
23. Sega GA, Valdivia Alcota RP, Tancongco CP, Brimer PA. Acrylamide binding to the DNA and protamine of spermiogenic stages in the mouse and its relationship to genetic damage. Mutat Res. 1989;216:221–230.
24. Sakamoto J, Kurosaka Y, Hashimoto K. Histological changes of acrylamide-induced testicular lesions in mice. Exp Mol Pathology. 1988;48:324–334.
25. Zenick H, Hope E, Smith MK. Reproductive toxicity associated with acrylamide treatment in male and female rats. J Toxicol Environ Health. 1986;17:457–472.
26. Backer LC, Dearfield KL, Erexson GL, Campbell JA, Westbrook-Collins B, Allen JW. The effects of acrylamide on mouse germline and somatic cell chromosomes. Environ Mol Mutagen. 1989; 13:218–226.
27. Collins BW, Howard DR, Allen JW. Kinetochore-staining of spermatid micronuclei: studies of mice treated with X-radiation or acrylamide. Mutat Res. 1992;281:287–294.
28. Shelby MD, Cain KT, Hughes LA, Braden PW, Generoso WM. Dominant lethal effects of acrylamide in male mice. Mutat Res. 1986;173:35–40.
29. Shelby MD, Cain KT, Cornett CV, Generoso WM. Acrylamide: induction of heritable translocations in male mice. Mutat Res. 1987;173:35–40.
30. Working PK, Bentley KS, Mohr KL. Comparison of the dominant lethal effects of acrylonitrile and acrylamide in male Fischer 344 rats. Mutagenesis. 1987;2:215–220.
31. Sublet VH, Zenick H, Smith MK. Factors associated with reduced fertility and implantation rates in females mated to acrylamide-treated rats. Toxicology. 1989;55:53–67.
32. Adler ID. Clastogenic effects of acrylamide in different germ-cell stages of male mice. In: Biology of Mammalian Germ Cell Mutagenesis (Banbury Report 34). Cold Spring Harbor: CSH Press. 1990:114–31.
33. Pacchierotti F, Tiveron C, D'Archovio M, Bassani B, Cordelli E, Leter G, Spano M. Acrylamide-induced chromosomal damage in male mouse germ cells detected by cytogenetic analysis of one-cell zygotes. Mutat Res. 1994;309:273–284.
34. Nutley EV, Lowe XR, Van Beek MEA, Ma M, Tcheong A, Bishop J, Wyrobek AJ. Use of multi-color fluorescence in situ hybridization to detect sperm aneuploidy and spermatid micronuclei in mice treated with chloral hydrate. Environ Mol Mutagen. 1994;23(23): 50.
35. Allen JW, Poorman-Allen P, Backer LC, Westbrook-Collins B, Moses MJ. Synaptonemal complex analysis of mutagen effects on meiotic chromosome structure and behavior. In: Allen J, Bridges B, Lyon M, Moses M, Russell L, eds. Banbury Report 34: Biology of Germ Cell Mutagenesis. Cold Spring Harbor, NY: Cold Spring Harbor Lab:1990:155–69.
36. NIH Publication no. 78–023, revised, 1985.
37. Bateman AJ, Epstein SS. Dominant lethal mutations in mammals. In: Hollaender A, ed. Chemical Mutagens: Principles and methods for their detection. New York: Plenum Press; 1971;2:541–568.
38. McLaren A. Early mammalian development. Prog Clin Biol Res. 1985;163A:29–41.
39. Turteltaub KW, Frantz CE, Creek MR, Vogel JS, Fultz E. DNA adducts in model systems and humans. J Cell Biochem. 1993; Suppl:17F, 138–148.
40. Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. Proc Nat Acad Sci USA. 1975;72:4425–4429.
41. Vogel JS, Turteltaub KW. Biomolecular tracing through accelerator mass spectrometry. Trends Anal Chem. 1992;11:142–149.
42. Vogel EW, Szakmary A. Basic principles and evaluation of results of assays measuring genotoxic damage of somatic cells of Drosophila, in mutation and the environment. Part B, Wiley-Liss, New York. 1990:149–158.
43. Turteltaub KW, Vogel JS, Frantz CE, Shen NH. Fate and distribution of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyrimidine (PhIP) in mice at a human dietary equivalent dose. Cancer Res. 1992;52:4682–4687.
44. Chambers JM, Hastie TJ.: Statistical models in S. Pacific Grove, California: Wadsworth & Brooks/Cole Advanced Books & Software. 1992:1992.
45. Ehling UH, Machemer L, Buselmaier W, Dycka J, Frohberg H, Kratochvilova J, Lang R, Lorke D, Mueller D, Peh J, Roehrborn G, Roll R, Schulze-Schencking M, Weimann H. Standard protocol for the dominant lethal test on male mice set up by the work group "Dominant lethal mutations of the ad hoc committee chemogenetics." Arch Toxicol. 1978;39:173–185.
46. Russell LB, Russell WL, Rinchik EM, Hunsicker PR. Factors affecting the nature of induced mutations. In: Allen J, Bridges M, Lyon M, Moses M and Russell LB, eds. Banbury Report 34: Biology of Germ Cell Mutagenesis. Cold Spring Harbor, NY: Cold Spring Harbor Lab:1990:217–289.
47. Fabricant JD, Dunn G, Schneider EL. Maternal age related pre- and post-implantation fetal mortality: a strain survey. Mech of Aging & Development. 1978;8:227–231.
48. McLaren A, Orsini MW. The fate of unfertilized eggs in mice. J Reprod Fert. 1968;15:181–3.
49. Austin CR. Recent progress in the study of eggs and spermatazoa. Insemination and ovulation to implantation. Physiology. 1974;8: 95–131.
50. Titenko NV, Evsikov VI. Micronuclei and cytogenetic damage in embryos of mice before implantation. Cytology and Genetics (Rus). 1977;11:1:17–22.
51. Titenko-Holland N, Ahlborn T, Lowe X, Shang X, Smith MT, Wyrobek AJ. Micronuclei and developmental abnormalities in 4-day mouse embryos after paternal treatment with acrylamide. Environ Mol Mutagen. 1998;31:206–217.