CHROMOSOMAL MOSAICISM IN MOUSE TWO-CELL EMBRYOS AFTER PATERNAL EXPOSURE TO ACRYLAMIDE

Francesco Marchetti*†, Jack Bishop‡, Xiu Lowe*†¶ and Andrew J. Wyrobek*†

*Biosciences Directorate, Lawrence Livermore National Laboratory, Livermore, CA 94550

†Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

‡National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

¶Department of Psychiatry, Kaiser Permanente Medical Group, Inc, Hayward, CA 94545

Send correspondence to:
Dr. Francesco Marchetti
Life Sciences Division, MS74R0157
Lawrence Berkeley National Laboratory
1 Cyclotron Rd
Berkeley CA 94720
Telephone: (510) 486-7352
Telefax: (510) 486-6691
e-mail: fmarchetti@lbl.gov

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ABSTRACT

Chromosomal mosaicism in human preimplantation embryos is a common cause of spontaneous abortions, however, our knowledge of its etiology is limited. We used multicolor fluorescence in situ hybridization (FISH) painting to investigate whether paternally-transmitted chromosomal aberrations result in mosaicism in mouse 2-cell embryos. Paternal exposure to acrylamide, an important industrial chemical also found in tobacco smoke and generated during the cooking process of starchy foods, produced significant increases in chromosomally defective 2-cell embryos, however, the effects were transient primarily affecting the postmeiotic stages of spermatogenesis. Comparisons with our previous study of zygotes demonstrated similar frequencies of chromosomally abnormal zygotes and 2-cell embryos suggesting that there was no apparent selection against numerical or structural chromosomal aberrations. However, the majority of affected 2-cell embryos were mosaics showing different chromosomal abnormalities in the two blastomeric metaphases. Analyses of chromosomal aberrations in zygotes and 2-cell embryos showed a tendency for loss of acentric fragments during the first mitotic division of embryogenesis, while both dicentrics and translocations apparently underwent proper segregation. These results suggest that embryonic development can proceed up to the end of the second cell cycle of development in the presence of abnormal paternal chromosomes and that even dicentrics can persist through cell division. The high incidence of chromosomally mosaic 2-cell embryos suggests that the first mitotic division of embryogenesis is prone to missegregation errors and that paternally-transmitted chromosomal abnromalities increase the risk of missegregation leading to embryonic mosaicism.

Key words: acrylamide, in vivo, fluorescence in situ hybridization, spermatogenesis

INTRODUCTION

Constitutional chromosomal mosaicism indicates the presence of two or more chromosomal complements within an individual (Kalousek, 2000). The most common case of mosaicism is the presence of trisomic cells within normal diploid cells, although other chromosomal abnormalities such as sex chromosome monosomy or structural aberrations are also observed (Kalousek, 2000). Mosaic aneuploidy is found in 1-2% of prenatal diagnoses of chorionic villus samples (Kalousek et al., 1991), 0.3% in those performed by amniocentesis (Hsu, 1992) and 0.02% among livebirths (Hassold et al., 1996). Fluorescence in situ hybridization (FISH) analyses suggest that as many as 50% of early human embryos are mosaic (Bielanska et al., 2002a; Bielanska et al., 2002b; Delhanty et al., 1997; Gonzalez-Merino et al., 2003; Munné et al., 1998; Munné et al., 1994; Vorsanova et al., 2005). Mosaicism for chromosomal structural abnormalities has also been reported, although it is relatively rare (Hsu, 1992).

A critical question in the development of mosaicism is the time at which the second cell line appears. Mosaicism occurring at the first cleavage division results in an embryo in which all cells are abnormal; mosaicism occurring at the second cleavage results in an embryo in which half of the cells are abnormal and so on. When the second cell line arises at or shortly after the first postzygotic division, is likely that mosaicism will be present in both the fetus and the placenta (Kalousek and Vekemans, 1996). However, if mosaicism arises late during the preimplantation development, mosaic cells will constitute only a small portion of the blastocyst. In these cases, because the fetus is derived from only few of the cells that make up the blastocyst (Markert and Petters, 1978), mosaicism is generally confined to the extra embryonic tissues (Lestou et al., 2000).

Despite the high incidence of chromosomal mosaicism in human conceptuses, our knowledge of its etiology is limited. Mitotic nondisjunction and anaphase lag are thought to be the most common error producing mosaicism in early embryos (Kalousek, 2000). Mitotic errors account for the generation of chromosome 21 mosaicism in the majority of mosaic embryos (Katz-Jaffe et al., 2004; Katz-Jaffe et al., 2005), and in ~4% of all Down syndromes (Antonarakis et al., 1993). Anaphase lag is considered the most common error producing 45,X/46,XX mosaicism (Robinson et al., 1995). There is also evidence that chromosomal aberrations can predispose to the formation of mosaicism during early development. High rates of mosacisms have been observed in the embryos from Robertsonian (Emiliani et al., 2003) or reciprocal translocation carriers (Iwarsson et al., 2000).

Acrylamide (AA) is an important industrial chemical classified as a probable human carcinogen (IARC, 1994). Tobacco smoking is a significant source of AA exposure in the human population (Smith et al., 2000; Vesper et al., 2007). In addition, AA is formed in starchy foods as a by-product of high-temperature cooking processes (Mottram et al., 2002; Rosen and Hellenas, 2002; Stadler et al., 2002; Tareke et al., 2002). Daily human exposures to AA are estimated in 1.4-18.6 μg/kg for daily workplace inhalation exposure and 0.75 μg/kg/d via food consumption (Manson et al., 2005). A recent assessment of the reproductive risks of AA exposure concluded that there is some concern for adverse reproductive and developmental effects, including heritable effects, for exposure in occupational settings (Manson et al., 2005). AA has been extensively studied both in vivo and in vitro, although at doses much higher than those encountered by humans (Dearfield et al., 1995; Manson et al., 2005). In mice, AA induces chromosomal damage at first-cleavage (Marchetti et al., 1997; Marchetti et al., 1996; Pacchierotti et al., 1994), abnormalities in preimplantation development (Titenko-Holland et al.,

1998), dominant lethal (DL) mutations (Shelby et al., 1986), heritable translocations (HT) (Adler, 1990; Adler et al., 1994; Shelby et al., 1987) and specific locus mutations (Ehling and Neuhauser-Klaus, 1992; Russell et al., 1991), particularly after treatment of late spermatids and early spermatozoa. The high sensitivity of these cells is thought to result from two factors. Protamines, basic proteins that replace histones during spermiogenesis (Meistrich et al., 1978), are considered the primary site of AA-alkylation (Sega et al., 1989). Secondly, sperm and late spermatids have reduced DNA repair capacity as compared with early spermatids and the other spermatogenic cells (Baarends et al., 2001; Olsen et al., 2005; Sega, 1974; Sega, 1979; Sega et al., 1989; Sotomayor and Sega, 2000). Therefore, DNA or chromatin lesions induced during late spermatogenesis can accumulate and be transmitted to the zygote where they can be converted into chromosomal aberrations (Marchetti and Wyrobek, 2008).

We developed a method for detecting both stable and unstable chromosomal rearrangements in mouse zygotes using a combination of multicolor chromosome painting probes (PAINT) and 4,6-diamidino-2-phenylindole (DAPI) staining (Marchetti et al., 1996) and demonstrated that unstable and stable chromosomal aberrations in mouse zygotes are predictive of embryonic death after implantation and of translocation carriers at birth (Marchetti et al., 2004; Marchetti et al., 2001; Marchetti et al., 2007; Marchetti et al., 1997). Here we report the application of our PAINT/DAPI method to investigate the genesis of chromosomal mosaicism in 2-cell embryos after treatment of male mice with AA, and specifically to determine whether: (a) the presence of chromosomal aberrations in zygotes predisposes to chromosomal missegregation during the first mitotic division; and (b) unstable chromosomal aberrations persisted during the early embryonic stages of mammalian development.

MATERIALS AND METHODS

Animals, chemical treatment of males, and hormone administration of females.

Male and female B6C3F1 mice (Harlan Sprague-Dawley Inc., Indianapolis, IN, USA) between 8 and 12 weeks of age at the beginning of the experiments were used. They were maintained under a 12 hr light/12 hr dark photoperiod (light from 6.00 am to 6.00 pm) at room temperature of 21-23° C and relative humidity of 50 ± 5%. Pelleted food and sterilized tap water were provided *at libitum*. The use of animals in these experiments was approved by the Lawrence Livermore National Laboratory Institutional Animal Care and Use Committee.

Human exposure to AA occurs mostly through oral and inhalation routes (Manson et al., 2005). However, because of the inherent animal-to-animal variation in exposure associated with these routes of exposures, and because previous animal studies have shown that different routes of exposures (ip, dermal, drinking water etc) produced qualitatively similar results (Dearfield et al., 1995), AA was administered intraperitoneally (i.p.). Male mice received 5 consecutive daily doses of 50 mg/kg AA (CAS No. 79-06-1, Fisher Scientific, Fair Lawn, NJ) dissolved in sterile distilled water. AA solution was prepared fresh every day and was administered i.p. at the final volume of 0.01 ml/g b.w. every morning. Groups of 12 similarly treated males were mated with untreated females at 2.5, 6.5, 9.5, 12.5, 20.5, 27.5, 41.5 and 48.5 days after the last AA injection. Rather than mimicking the doses humans are exposed to, the AA dose and timepoints utilized in this study were chosen to allow comparisons with previous studies in mouse zygotes (Marchetti et al., 1997; Pacchierotti et al., 1994) and in developing embryos (Shelby et al., 1987; Shelby et al., 1986). For each time point, at least three repetitions (each with a different group of treated males) were performed and the data combined. Control males received 5 daily injections of sterile distilled water (0.01 ml/g b.w.), and were mated with untreated females at 2.5, 6.5, 9.5 and

12.5 days after the last injection. No differences in the frequencies of embryos with chromosomal aberrations were found among the 4 groups of control matings and the results were pooled.

B6C3F1 females were superovulated using an i.p. injection of 7.5 I.U. of pregnant mare's serum (PMS, Sigma Chemical Co., St. Louis. MO, USA), followed 48 hr later by an i.p. injection of 5.0 I.U. of human chorionic gonadotrophin (HCG, Sigma Co.). Immediately after HCG injection (1.00 pm), the females were caged with treated or untreated males (1:1). The following morning females were checked for the presence of vaginal plugs. Mated females received an i.p. injection of 0.08 mg of colchicine (CAS No. 64-86-8, Sigma Co.) dissolved in 0.2 ml of distilled water, 44 hr after HCG to arrest embryonic development at the metaphase stage of the second cleavage division. Females were euthanized by CO_2 inhalation 6 hr after colchicine injection and 2-cell embryos were harvested and processed according to the mass harvest procedure (Mailhes and Yuan, 1987).

Fluorescence in situ hybridization

Prepared slides were examined with a 10x objective under phase contrast to determine the number of eggs that were fertilized, the number of fertilized eggs that progressed past the first mitotic division, and the number of 2-cell embryos that reached the second-cleavage metaphase stage. Slides were then stored in nitrogen atmosphere at -20˚C until used for FISH.

Five composite DNA painting probes were used: four biotin-labeled probes specific for chromosomes 1, 2, 3 or X plus a digoxigenin-labeled probe specific for chromosome Y (Applied Genetics Laboratories, Inc., Melbourne, FL). Hybridization and washing conditions were as previously described (Marchetti et al., 1996). Amplification of the fluorescent signals was

achieved using the Biotin-Digoxigenin Dual Color Detection Kit (Oncor, Gaithersburg, MD) following the manufacturer specifications. DAPI at 0.25 μg/ml diluted in antifade mounting medium (Oncor) was used for counterstaining. The 5-probe cocktail produced two sets of fluorescent patterns in normal male and female 2-cell embryos. Male embryos showed one red signal for the Y chromosome and 7 green signals corresponding to the three couples of autosomes and one copy of the X chromosomes in each metaphase (Figure 1A), while female embryos showed 8 green signals per metaphase (the three couples of autosomes plus two copies of the X chromosome).

Metaphase analysis and scoring

A Zeiss Axioplan fluorescent photomicroscope was equipped with a double-bandpass excitor (81P102, Chroma Technology, Brattleboro, VT) for visualizing the red (rhodamine) and green (FITC) signals; a triple-bandpass filter set (61002, Chroma Technology) for the simultaneous detection of the red, green, and blue (DAPI) signals; and another DAPI filter set (487901, Zeiss) for visualizing DAPI fluorescence only. Metaphase 2-Cl embryos were analyzed both by DAPI staining to detect aberrations not involving painted chromosomes and by PAINT analysis to detect aberrations involving painted chromosomes. Scoring of the painted aberrations was done according to the PAINT nomenclature system (Marchetti et al., 1996; Tucker et al., 1995). A metaphase was considered abnormal regardless of whether the chromosome aberration was detected by PAINT or DAPI analysis. Also, an embryo was considered abnormal even when the chromosomal aberration was present in only one of the two metaphases.

Statistical analysis

The ANOVA test was used for the analysis of the results. When the numbers of events was less than 5, the Fisher's exact test was used.

RESULTS

Baseline levels of chromosomal abnormalities in mouse 2-cell embryos

The baseline frequencies of chromosomal abnormalities in 2-cell embryos after mating of untreated male and female mice are reported in Table 1. Six out of 358 2-cell embryos obtained from untreated mice showed chromosomal structural aberrations; five cases were a chromosomal acentric fragment and one was an interstitial chromatid deletion. In all cases, the chromosomal aberration was present in only one metaphase, while the other appeared normal. Affected embryos were therefore mosaics for chromosomal structural aberrations.

Among 2-cell embryos analyzed for numerical abnormalities, 0.6% were aneuploid (each metaphase contained 41 chromosomes), and 3.4% were triploid (each metaphase contained ~60 chromosomes). Three 2-cell embryos (0.9%) were numerical mosaic embryos (41 chromosomes in one metaphase and 39 in the other), presumably originated from a nondisjunctional event during the first mitotic division after fertilization.

To assess the effects of spontaneous chromosomal abnormalities on embryonic development, we compared the frequencies of chromosomally abnormal 2-cell embryos recovered in control matings from the AA exposure study with those in zygotes collected from control matings from other experiments that were undergoing in our laboratory at the same time of the 2-cell embryo experiment. The comparison (Table1) showed that showed that numerical abnormalities were present at similar frequencies between the two embryonic stages and that there was a marginally significant (P=0.08) three-fold increase in the frequencies of 2-cell embryos with chromosomal structural aberrations with respect to zygotes. These results indicate that in unexposed matings neither structural nor numerical abnormalities, including the presence

of an additional haploid genome, affected embryonic development up to the 2-cell metaphase stage.

Pre- and post-fertilization toxicity induced by paternal exposure to acrylamide

We investigated the effects of AA exposure of male germ cells on pre- and post fertilization toxicity. The former is manifested as a reduction in the number of eggs that are fertilized; the latter is manifested as a reduction in the number of fertilized eggs that proceed past the first mitotic division and reach the metaphase stage of the second cell division. AA treatment of male mice prior to mating induced pre-fertilization toxicity only after matings that sampled late spermatids (9.5 dpt). At this time point the frequencies of fertilized embryos were reduced to 71% versus 83% in control matings ($P<0.01$; Table 2). There was little evidence for an effect on the number of fertilized eggs that were unable to complete the first cell cycle of development and proceed to the 2-cell stage. As shown in Table 2, although the frequencies of one-cell embryos were higher than controls at all mating points, no statistically significant differences were found due to large variation among control matings in the frequencies of one-cell embryos (range 3.4-20.1). This large variation is most likely an artifact of the preparation procedure. In fact, the great majority of embryos classified as one-cell embryos had one set of 40 mitotic chromosomes and most likely represented 2-cell embryos in which the metaphase from the second blastomere was lost during the fixation process rather than zygotes that had arrested at the metaphase stage of the first cleavage division.

AA treatment of male mice prior to mating induced a significant reduction in the numbers of 2-cell embryos that reached the metaphase stage in matings within the repair deficient window of spermatogenesis (2.5-12.5 dpt) with respect to the control value ($P<0.05$, last column of Table

2). As shown in Table 2, this was due to a significant increase in the percentages of 2-cell embryos that were still in interphase at the time of collection suggesting that paternal exposure to AA induced cell cycle delay, an effect also found in bone marrow cells (Gassner and Adler, 1996). Finally, the percentages of asynchronous 2-cell embryos (i.e. those embryos in which one cell was at the metaphase stage and the other in interphase at the time of collection, Figure 1B) were not affected by paternal exposure to AA.

Chromosomal structural aberrations detected by PAINT/DAPI analysis

As shown in Table 3, paternal treatment with AA induced high frequencies of chromosomally abnormal 2-cell embryos (>50%) after treatment of epididymal sperm (2.5 dpt), testicular sperm (6.5 dpt) and late spermatids (9.5 dpt). Testicular sperm were the most sensitive cell type for the induction of chromosome structural aberrations (statistically different from 2.5 and 9.5 dpt) with 82% of the 2-cell embryos presenting with chromosome structural aberrations. The frequencies of embryos with structural aberrations decreased to 19% after treatment of elongating spermatids (12.5 dpt) and 5.0% after treatment of round spermatids (20.5 dpt). Chromosomal aberrations were not significantly increased with respect to the control value after treatment of pachytene spermatocytes (27.5 dpt), differentiating spermatogonia (41.5 dpt) or stem cells (48.5 dpt).

Testicular spermatozoa were also the germ cell type with the highest amount of chromosomal damage (Table 4). For this analysis, each aberrant chromosome was considered independently, that is, the two translocated chromosomes produced by an exchange event were treated separately. Similarly, acentric fragments produced by an exchange event and associated with dicentrics were included in the acentric fragment class. The data for PAINT analysis are

given in cell-equivalents (Marchetti et al., 1996; Tucker et al., 1995). As shown in Table 4, each type of aberration was most prevalent at 6.5 dpt mating time when the total number of chromosomal aberrations per embryo was above 3.3 by both PAINT and DAPI analyses, while it was less than 2.4 per embryo at 2.5 dpt and decreased to around 1.5 at 9.5 dpt. In addition, there was good agreement between the data obtained by PAINT and DAPI analyses suggesting that the painted chromosomes were not preferentially involved in aberration formation.

Chromosomal mosaicism in 2-cell embryos

PAINT/DAPI analysis of mouse 2-cell embryos showed that paternal treatment with AA induced mosaicism for structural chromosomal aberrations (Table 3). The majority of abnormal embryos, irrespective of mating time point, were mosaics, i.e., the two metaphases had a different karyotype (Figure 1C-D). In the treated groups, the majority of 2-cell embryos had chromosomal structural aberrations in both metaphases, but the aberrations were of different types (mosaic abnormal/abnormal in Table 3). Conversely, all abnormal embryos found in the control group had one metaphase with a chromosomal aberration while the other appeared to be normal (mosaic abnormal/normal in Table 3). A third group of embryos were structurally abnormal with the apparently same chromosomal aberration in both metaphases (non-mosaic in Table 3).

Numerical abnormalities in 2-cell embryos

Paternal exposure to AA also induced numerically abnormal 2-cell embryos (Figure 1E). As shown in Table 5, the majority of the embryos were hypodiploid in one or both metaphases. At 6.5 dpt, the most sensitive time for the induction of chromosomal structural aberrations (Table

3), over 72% of the embryos were hypodiploid. Hyperdiploid non-mosaic embryos (41 chromosomes in both metaphases) and triploid non-mosaic embryos (60 chromosomes in both metaphases) were also found, but their frequencies were not different with respect to controls. Numerically abnormal mosaic 2-cell embryos (those having 41 chromosomes in one metaphase and 39 in the other; Figure 1E) were increased at least three-fold at 2.5, 6.5 and 9.5 dpt, however, none of these values were statistically different from controls. When these mating points were pooled, the increase was statistically significant ($P<0.01$, Fisher's exact). This indicates that high levels of chromosomal aberrations induced by paternal exposure to AA during the repair deficient window of spermatogenesis may result in the production of mosaic aneuploidy in 2-cell embryos.

Persistence of chromosomal aberrations

We analyzed the persistence of the various types of chromosomal structural aberrations in 2-cell embryos (present study) versus zygotes (Marchetti et al., 1997) to determine whether the number per metaphase of stable aberrations would remain the same in zygotes and embryos, while the number of unstable aberrations would decrease. For these comparisons, we divided by two the data shown in Table 4 to obtain the number per metaphase of each class of abnormality to account for the fact that each 2-cell embryo has two metaphases, while zygotes have only one metaphase. We then compared these numbers with those obtained in the prior zygote experiment. As shown in Figure 2A, the sum of the total number of aberrations per metaphase was slightly lower in 2-cell embryos than in zygotes (4.14 vs 4.71). Interestingly, this was due mostly to a loss of acentric fragments while dicentric frequencies were similar between zygotes and 2-cell embryos. Figures 2B and 2C compare the number of dicentrics and acentric fragments found in

zygotes and 2-cell embryos at the four mating times with the highest incidence of chromosomal aberrations. The frequencies of acentric fragments were consistently lower in 2-cell embryos vs zygotes but the time course was similar. As expected, the frequencies of translocations were similar between zygotes and 2-cell embryos (data not shown). These results suggest that there is a tendency for loss of acentric fragments during the first mitotic division, while both dicentrics and translocations apparently undergo proper segregation without loss.

Comparisons with dominant lethality and heritable translocation data

In previous studies (Marchetti et al., 2004; Marchetti et al., 2001; Marchetti et al., 2007; Marchetti et al., 1997), we reported a very good correlation between the percentages of zygotes with unstable chromosomal aberrations and embryonic lethality and between stable chromosomal aberrations and the percentages of offspring with reciprocal translocations. We therefore determined whether PAINT/DAPI analysis of 2-cell embryos also provided good estimates of embryonic lethality and of translocation carriers at birth. The study on the induction of dominant lethality after paternal exposure to 5 x 50 mg/kg AA (Shelby et al., 1986) reported frequencies of dead implants in each of four 3-day post-treatment mating intervals as 67% (0.5-3.5 dpt), 68% (4.5-7.5 dpt), 37% (8.5-11.5 dpt) and 12% (12.5-13.5 dpt). Each one of our first 4 mating times is contained within one of these intervals. Using PAINT/DAPI analysis, the frequencies of 2-cell embryos with unstable aberrations, which are expected to die in utero because of loss of genetic material, were: 66% (98 out 149), 80% (115 out 143), 48% (102 out 212), and 19% (57 out 307) at 2.5, 6.5, 9.5 and 12.5 dpt mating times, respectively. As shown in Figure 3, the cytogenetic data for 2-cell embryos agreed very well with both the DL results (Shelby et al., 1986) and our previous study in zygotes (Marchetti et al., 1997). It is interesting to

note that the concordance between 2-cell embryos with unstable aberrations and DL results is already good without considering embryos with one normal and one abnormal metaphase, suggesting that such mosaic 2-cell embryos, which have 50% of normal cells, survive and produce viable offspring.

Two studies investigated the induction of translocation carriers in the offspring of mice treated with AA (Adler, 1990; Shelby et al., 1987). In both studies, male mice treated with 5 x 50 mg/kg AA were bred with untreated females between 7 and 10 days after the last injection. (Shelby et al., 1987) analyzed only the male offspring and found that 39% had heritable translocations. (Adler, 1990) analyzed both male and female offspring and reported a combined frequency of 22% (Figure 4). Using our 9.5 dpt data point, which is within their 7-10 day window, we analyzed 200 2-cell embryos which corresponded to 74 cell-equivalents (Table 4): 41 2-cell embryos showed unstable aberrations, 8 had reciprocal translocations and 25 were without aberrations. Assuming that the 41 2-cell embryos with unstable aberrations will result in embryonic lethality, a total of 33 embryos are expected to produce viable offspring. As shown in Figure 4, the proportion of 2-cell embryos with stable aberrations (24 %, 8 out 33 2-cell embryos) is in agreement with the frequencies of offspring with heritable translocations reported using the standard HT method and paralleled the findings obtained in zygotes (Marchetti et al., 1997).

Overall, the findings shown in Figures 3 and 4 indicate that the frequencies of chromosomal aberrations in zygotes and 2-cell embryos are consistent with each other and are a good predictor of embryonic fate for death after implantation and birth with reciprocal translocations.

DISCUSSION

We applied PAINT/DAPI analysis to mouse 2-cell embryos to investigate the induction of chromosomal mosaicism after paternal treatment with AA and the effect of chromosomal abnormalities on embryonic development from first cleavage to second cleavage. Chromosomal aberrations of paternal origin were significantly increased in embryos produced from matings that sampled treated postmeiotic cells. We also found that paternal exposure to AA resulted in extensive chromosomal mosaicism in 2-cell embryos. Comparisons with results obtained in zygotes indicated that there was no apparent selection against numerical or structural chromosomal aberrations between the first and second metaphase after fertilization and that dicentrics persisted through the first cell division at unchanged frequencies.

Paternal exposure to AA before mating significantly increased the frequencies of 2-cell embryos with chromosomal aberrations of paternal origin but the effects were transient primarily affecting the postmeiotic stages of spermatogenesis. The time course of AA-induced chromosomal aberrations in mouse 2-cell embryos after treatment of male germ cells observed in our study compared very well with the prior experiment in mouse zygotes (Marchetti et al., 1997) (Figure 2) and is in agreement with the general pattern of sensitivity of male germ cells to alkylating agents (Marchetti and Wyrobek, 2005). At variance with our study in zygotes, we could not repeat the finding of a significant increase in paternally-transmitted chromosomal aberrations after AA treatment of pachytene spermatocytes (28.5 dpt). However, the frequencies of zygotes and 2-cell embryos with chromosomal aberrations observed in matings that sampled pachytene spermatocytes were not different (3.4% vs 4.8%, p=0.48) and the major difference between the two studies was the 3-fold increase in the frequencies of 2-cell embryos with chromosomal aberrations found in controls (Table 1). In all six chromosomally abnormal 2-cell

embryos observed in controls, the chromosomal aberration was present in only one metaphase suggesting that the aberration had been formed during the second cell cycle of embryonic development or because of an asymmetrical distribution at first cleavage. The higher baseline level of chromosomal abnormalities in 2-cell embryos with respect to zygotes, a finding that supports results obtained with a different strain of mice (Tusell et al., 2004), suggests that the analysis of mouse zygotes may be a more sensitive approach for detecting small effects.

A major finding of our study was the presence of extensive chromosomal structural mosaicism in 2-cell embryos after paternal exposure to AA. Of the 425 2-cell embryos with chromosomal aberrations over 80% were mosaics, and two thirds of these had chromosomal aberrations in both blastomeric metaphases. The differential sensitivity of the various stages of spermatogenesis to the clastogenic effects of AA highlighted a direct correlation between the amount of chromosomal damage and the fraction of mosaic abnormal/abnormal embryos. 2-cell embryos mosaic abnormal/abnormal were highest after matings that sampled testicular sperm (6.5 dpt), followed by epididymal sperm (2.5 dpt) and late spermatids (9.5 dpt). These three timepoints were also those with the highest percentages of chromosomally abnormal 2-cell embryos (Table 3) and the highest amount of chromosomal damage (Table 4). The positive correlation between amount of chromosomal aberrations and rate of mosaicism is undoubtedly a consequence of the fact that the higher the number of chromosomal aberrations in a zygote the lower is the chance that all the aberrations will be distributed equally between the two daughter cells. There is also evidence suggesting that the first mitotic division in the embryo may be prone to chromosome missegregation regardless of the presence of chromosome structural abnormalities (Bean et al., 2001). Our results show that paternally-transmitted chromosomal aberrations can be an etiological cause of chromosomal mosaicism during the first cleavage

division of mammalian embryogenesis and may be partly responsible for the high levels of mosaicism observed in human preimplantation embryos (Bielanska et al., 2002a; Bielanska et al., 2002b; Delhanty et al., 1997; Gonzalez-Merino et al., 2003; Munné et al., 1998; Munné et al., 1994; Vorsanova et al., 2005).

Comparisons of the present results with those obtained in zygotes (Marchetti et al., 1997) allowed us to study the persistence of specific types of chromosomal aberrations between the first and second cleavage division. We found similar frequencies of different types of chromosomal aberrations in zygotes and 2-cell embryos (Figure 2). Only the frequencies of acentric fragments were consistently lower in 2-cell embryos suggesting that some acentric fragments were lost during the first cleavage division. The frequencies of dicentrics were similar between zygotes and 2-cell embryos, which is seemingly at variance with a great body of evidence from somatic cells that shows that dicentric frequencies tend to decline with every mitotic division (Bauchinger et al., 2001; Carrano, 1973a; Carrano, 1973b; Tucker et al., 2005). These findings can be explained by the fact that the decline in dicentric frequencies in somatic tissues is more a consequence of the elimination of cells with unstable aberrations by apoptosis (Carrano, 1973a; Carrano, 1973b) than the actual loss of dicentrics. Apoptosis, however, is not observed during the early embryonic stages (Brison and Schultz, 1997; Hardy et al., 2001). A dicentric in a zygote (or a somatic cell for that matter) has two segregation possibilities: the first involves a parallel orientation, in which both centromeres of a chromatid orient to the same spindle pole and each blastomere inherits an intact dicentric. The second possibility involves a non-parallel orientation in which the two centromeres of a chromatid orient to opposite poles and the original dicentric undergos the well-characterized breakage-fusion-bridge cycle (Gisselsson et al., 2000; Koshland et al., 1987; Lukaszewski, 1995; McClintock, 1939), with the result that

each blastomere would still inherit a dicentric. Therefore, regardless of centromeric orientation, the persistence of 2-cell embryos with unstable aberrations assures that the frequencies of dicentrics per cell would remain constant between zygotes and 2-cell embryos. Indeed, data in oocytes suggest that dicentrics can survive the two meiotic divisions and persist into the first few embryonic divisions (Koehler et al., 2002).

Our results of similar frequencies of zygotes and 2-cell embryos with chromosomal abnormalities seems at variance with a previous study that found that chromosomal aberrations were significantly higher in zygotes than in 2-cell embryos after exposure of male mice to ionizing radiation (Tusell et al., 2004). The authors suggested that this was due to the unstable nature of most of the detected aberrations and/or death of zygotes carrying chromosomal aberrations. We found little evidence for the death of zygotes with chromosomal aberrations. In fact, the frequencies of 2-cell embryos with chromosomal aberrations were very similar to those of chromosomally abnormal zygotes at all mating times (Table 3 and Figure 5). Additionally, as in previous studies with zygotes (Marchetti et al., 2004; Marchetti et al., 2001; Marchetti et al., 2007; Marchetti et al., 1997; Marchetti and Wyrobek, 2008), the estimates of embryonic lethality based on the frequencies of 2-cell embryos with chromosomal aberrations matched the reported frequencies of dominant lethality (Figure 3). This provides compelling evidence that chromosomal aberrations do not affect embryonic development during the preimplantation period. Indeed, a recent study demonstrated that mosaic embryos can develop and implant into the uterine tissue but die soon thereafter (Lightfoot et al., 2006). There are several differences between our study and that of (Tusell et al., 2004), including the agents utilized which induce different spectra of DNA lesions, and the fact that we allowed embryonic development to

proceed in vivo, while (Tusell et al., 2004) collected 2-cell embryos by culturing zygotes for 24 hr. It is possible that these differences may be responsible for the differing outcomes.

Our study shows that the PAINT/DAPI analysis of mouse 2-cell embryos has both strengths and weaknesses with respect to the analysis of mouse zygotes. As the PAINT/DAPI analysis of mouse zygotes (Marchetti et al., 2004; Marchetti et al., 2001; Marchetti et al., 2007; Marchetti et al., 1997), the analysis of 2-cell embryos is a powerful approach for investigating paternally-transmitted chromosomal abnormalities and their consequences for embryonic development. Unlike mouse zygotes, 2-cell embryos allow to study the persistence of chromosomal aberrations and the induction of mosaicism and other complex abnormalities that are thought to arise after fertilization. However, it may be at a disadvantage with respect to zygotes for detecting small effects of paternal exposure because of the higher background level of chromosomal aberrations seen in untreated matings that reduces the sensitivity of the assay.

The analysis of mouse 2-cell embryos seems less efficient in detecting mutagen-induced effects on fertilization than the analysis of mouse zygotes. In the present study, pre-fertilization toxicity was confined to matings that sampled late spermatid (9.5 dpt) only, while in the zygote experiment we observed effects up to 41.5 dpt (Marchetti et al., 1997). In addition, the fertilization rate at each mating time in the 2-cell experiment was consistently higher than that in the zygote experiment (Figure 6). This was probably due to a preferential loss of unfertilized eggs during the fixation procedure in the 2-cell embryo experiment. Unfertilized eggs begin to degenerate about 10 hr after ovulation (Marston and Chang, 1964). In our experiment, cells were collected about 40 hr after ovulation and it is possible that once the zona pellucida was removed during the fixation process, degenerating unfertilized eggs were prone to lysis and this resulted in an artificial increase in the fertilization rate.

In conclusion, we have shown that after AA exposure high levels of paternally-inherited chromosomal aberrations in zygotes are not selected against and lead to extensive chromosomal mosaicism during the first mitotic division with the majority of the resulting 2-cell embryos having different chromosomal aberrations in the two blastomeric metaphases. The high incidence of chromosomal mosaicism observed in 2-cell embryos suggests that paternally-transmitted chromosomal structural aberrations increase the chance of missegregation during the first mitotic division and may be partly responsible for the high levels of mosaicism observed in human preimplantation embryos.

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Table 1 - Baseline frequencies of various chromosomal abnormalities in mouse zygotes and 2-cell embryos

	Zygotes[a]			2-cell embryos		
	Total	No.	% ± S.E.M.[b]	Total	No.	% ± S.E.M.[b]
Aneuploidy	883	9	1.0 ± 0.3	327	2[c]	0.6 ± 0.5
Numerical mosaic				327	3[d]	0.9 ± 0.4
Triploidy	883	27	3.1 ± 0.7	327	11[e]	3.4 ± 0.5
Structural aberrations	922	5	0.5 ± 0.3	358	6[f]	1.7 ± 0.2[g]

[a]Data from Marchetti et al. 1996; 1997; 2001.

[b]Standard error of the mean.

[c]2-cell embryos with 41 chromosomes in both metaphases.

[d]2-cell embryos with 41 chromosomes in one metaphase, and 39 in the other.

[e]2-cell embryos with 60 chromosomes in both metaphases.

[f]All six 2-cell embryos were structural mosaic, i.e., one metaphase showed the aberrations while the other metaphase appeared normal.

[g]P=0.08 vs zygotes (Fisher's exact test).

Table 2 - Effects of paternal treatment with acrylamide (5 x 50 mg/kg) on fertilization rate and embryonic progression through the first two cell cycles of development mice.

						2-Cell embryos							
Days	Total	Fertilized[b]		1-cell embryos		Total[c]		Interphase[e]		Asynchronous[e]		Metaphase[e]	
p.t.[a]	embryos	No.	% ± S.E.M.[d]	No	% ± S.E.M.[d]	No.	% ± S.E.M.[d]	No.	% ± S.E.M.[d]	No.	% ± S.E.M.[d]	No.	% ± S.E.M
control	614	512	83.4 ± 2.3	61	11.9 ± 3.4	451	88.1 ± 3.4	12	2.7 ± 1.2	10	2.2 ± 0.6	429	95.1 ± 1.
2.5	717	552	77.0 ± 2.1	118	21.4 ± 3.1	434	78.6 ± 3.1	112	25.8 ± 2.7[f]	34	7.8 ± 4.4	288	66.4 ± 6.2
6.5	936	713	76.2 ± 1.7	153	21.5 ± 2.2	560	78.5 ± 2.2	179	32.0 ± 3.8[f]	15	2.7 ± 0.8	366	65.4 ± 3.
9.5	1178	838	71.1 ± 2.8[f]	155	18.5 ± 2.5	683	81.5 ± 2.5	161	23.6 ± 3.2[f]	37	5.4 ± 1.0	485	71.0 ± 3.
12.5	872	684	78.4 ± 1.4	86	12.6 ± 1.2	598	87.4 ± 1.2	83	13.9 ± 2.0[f]	17	2.8 ± 1.1	498	83.3 ± 3.(
20.5	925	758	81.9 ± 3.4	136	17.9 ± 2.5	622	82.1 ± 2.5	78	12.5 ± 5.1[g]	20	3.2 ± 0.2	524	84.2 ± 5.
27.5	981	820	83.6 ± 0.8	177	21.6 ± 3.0	643	78.4 ± 3.0	57	8.9 ± 3.0	16	2.5 ± 1.0	560	87.1 ± 4.
41.5	669	589	88.0 ± 1.6	72	12.2 ± 1.3	517	87.8 ± 1.3	32	6.2 ± 1.7	12	2.3 ± 1.0	476	91.5 ± 2.
48.5	440	362	82.3 ± 0.1	56	15.5 ± 3.1	306	84.5 ± 3.1	6	2.0 ± 1.1	6	2.0 ± 1.4	294	96.1 ± 0.

[a]Post-treatment; days between the last AA injection and mating.

[b](Unfertilized eggs/Total number of eggs and embryos); Indicator of prefertilization toxicity

[c](2-cell embryos/Fertilized eggs); indicator of postfertilization toxicity

[d]Standard error of the mean.

[e]Percentages on total 2-cell embryos

[f]P<0.01 vs. controls (ANOVA)

[g]P<0.05 vs. controls (ANOVA)

Table 3 – Mosaicism for structural chromosomal aberrations detected by PAINT/DAPI analysis in 2-cell embryo metaphases after paternal treatment with acrylamide (5 x 50 mg/kg) in mice

Days p.t.[a]	No. of embryos	Mosaic		Non-Mosaic	Total	% ± S.E.M.[b]
		Abnormal/ Abnormal[c]	Abnormal/ Normal[d]	Abnormal/ Abnormal[e]		
control	358	0	6	0	6	1.7 ± 0.2
2.5	149	58	17	27	102	68.5 ± 5.7[f]
6.5	143	89	17	11	117	81.8 ± 1.6[f,g]
9.5	212	57	33	29	110	51.9 ± 4.4[f]
12.5	307	25	20	13	58	18.9 ± 4.1[f]
20.5	303	1	13	1	15	5.0 ± 1.9[h]
27.5	298	0	6	4	10	3.4 ± 1.6
41.5	344	0	5	0	5	1.5 ± 0.3
48.5	233	0	2	0	2	0.9 ± 0.6

[a]Post-treatment.

[b]Standard error of the mean.

[c]Mosaic embryos with chromosomal aberrations in both metaphases but of different type.

[d]Mosaic embryos with chromosomal aberrations in only one metaphase.

[e]Embryos with apparently the same type of chromosomal aberrations in both metaphases.

[f]$P<0.001$ vs controls (Chi-square).

[g]$P<0.05$ vs 2.5 and 6.5 dpt (Chi-square).

[h]$P<0.05$ vs controls (Chi-square).

Table 4 – Comparison of numbers and types of chromosomal structural aberrations in mouse 2-cell embryo metaphases after paternal treatment with acrylamide (5 x 50 mg/kg) detected by PAINT/DAPI analysis

Days p.t.[a]	2-Cell embryos	DAPI analysis				PAINT analysis						
		Total	Dicentrics	Fragments	Other	Cell-eq[b]	Total	Dicentrics	Fragments	Transl[c]	Insertions	Other
control	358	0.02	0	0.01	0.01	103	0	0	0	0	0	0
2.5	149	2.37	0.85	1.27	0.30	55	2.38	0.60	0.58	1.15	0.04	0.02
6.5	143	3.80	1.15	1.98	0.66	39	3.36	0.61	0.67	1.87	0.15	0.05
9.5	212	1.58	0.52	0.72	0.34	74	1.48	0.27	0.20	0.89	0	0.12
12.5	307	0.42	0.17	0.14	0.11	109	0.46	0.11	0.05	0.25	0.01	0.04
20.5	303	0.05	0.02	0.03	0.01	112	0.04	0	0	0.04	0	0
27.5	298	0.03	0	0.02	0.02	110	0.05	0	0	0.05	0	0
41.5	344	0.02	0.01	0.01	0.01	126	0.01	0	0	0	0	0.08
48.5	223	0.01	0	0.01	0	83	0	0	0	0	0	0

[a]Post-treatment.

[b]Cell-equivalents.

[c]Translocations.

Table 5 - Numerical aberrations in mouse 2-cell embryo metaphases after paternal treatment with acrylamide (5 x 50 mg/kg) detected by PAINT/DAPI analysis

Days p.t.[a]	No. of embryos	Embryos with numerical abnormalities (%)					
		Diploid[b]	Hypodiploid[c]		Hyperdiploid[d]	Triploid[e]	Mosaic[f]
control	327	87.3	4.4	6.6	0.6	3.4	0.9
2.5	107	49.5	29.0	16.8	0.9	0.9	3.7[g]
6.5	101	20.8	61.4	14.9	0	4.7	3.0
9.5	153	60.8	22.9	13.1	0	2.5	3.3
12.5	255	82.4	7.5	8.6	0	1.5	1.6
20.5	267	66.3	14.2	18.4	0.4	1.8	0.7
27.5	246	77.6	5.7	14.6	0.8	2.3	1.2
41.5	301	82.4	10.3	82.4	1.7	2.3	1.3
48.5	197	91.9	1.5	4.1	1.0	1.5	1.5

[a]Post-treatment.

[b]Embryos with 40 chromosomes in both metaphases.

[c]Embryos with <40 chromosomes in one (first percentage) or both metaphases (second percentage).

[d]Embryos with 41 chromosomes in both metaphases.

[e]Embryos with ~60 chromosomes in both metaphases.

[f]Embryos with 41 chromosomes in one metaphase and 39 in the other.

[g]P=0.067 (Fisher Exact).

FIGURE LEGENDS

Figure 1. Photomicrographs of 2-cell embryo metaphases analyzed by the PAINT/DAPI method. Cells were hybridized with chromosome-specific composite DNA probes for chromosomes 1, 2, 3, and X (labeled with biotin and signaled with the green dye FITC) and chromosome Y (labeled with digoxigenin and labeled with the red dye rhodamine). **A:** PAINT image of a normal 2-Cl embryo. In each metaphase the Y chromosome (in red) and 7 painted chromosomes (in green), three couples of autosomes and one copy of the X chromosomes, can be observed. **B:** Asynchronous 2-cell embryo with one blastomere already at the metaphase stage (right) and the other still in interphase (left). **C-D**: The two metaphases of a mosaic 2-Cl embryo showing different chromosomal abnormalities. Arrowheads indicate acentric fragments, while arrows indicate dicentrics. **E:** Numerically mosaic embryo originated by the nondisjunction of chromosome Y during the first mitotic division. Note that one metaphase has two chromosomes Y and the other none. Bars represent 10 μm.

Figure 2. Comparisons of the number and types of chromosomal aberrations detected in zygotes (white columns) and 2-cell embryos (gray columns) by PAINT/DAPI analysis after paternal exposure to 5 x 50 mg/kg acrylamide. **A:** Sums of total aberrations, dicentrics, acentric fragments and translocations. **B:** Number of dicentrics per metaphase in zygotes and -2-cell embryons at the four time points with the highest levels of abnormal embryos. **C:** Number of acentric fragments per metaphase in zygotes and -2-cell embryons at the four time points with the highest levels of abnormal embryos. Bars represent the standard error. See text for explanation.

Figure 3. Comparison of the percentages of 2-cell embryos with unstable chromosome aberrations versus the percentages of zygotes with unstable aberrations (gray column) and of dead implants (black columns) by PAINT/DAPI analysis after paternal exposure to 5 x 50 mg/kg acrylamide. For 2-cell embryos, the fraction of mosaic embryos with unstable aberrations in one, or both metaphases, and non-mosaic embryos with the apparently same type of abnormality is given. Bars represent the standard error.

Figure 4. Comparison of the frequencies of 2-cell embryos with stable aberrations by PAINT/DAPI analysis after paternal exposure to 5 x 50 mg/kg acrylamide with those obtained in zygotes (Marchetti et al 1997) and in standard tests heritable translocation test (Adler, 1990; Shelby et al 1987). Bar represent the 95% confidence interval.

Figure 5. Comparison of the time course of induction of chromosomal aberrations in zygotes (white circles) and 2-cell embryos (black squares) after paternal exposure to 5 x 50 mg/kg acrylamide. Bars represent the standard errors. Timepoints without bars represent data from a single mating. The last 4 timepoints are presented in expanded version in the insert.

Figure 6. Comparison of the time course of induction of prefertilization toxicity between the studies in zygotes (white circles) and 2-cell embryos (black squares) after paternal exposure to 5 x 50 mg/kg acrylamide. The percentages of unfertilized eggs are reported. Bars represent the standard errors. Timepoints without bars represent data from a single mating.

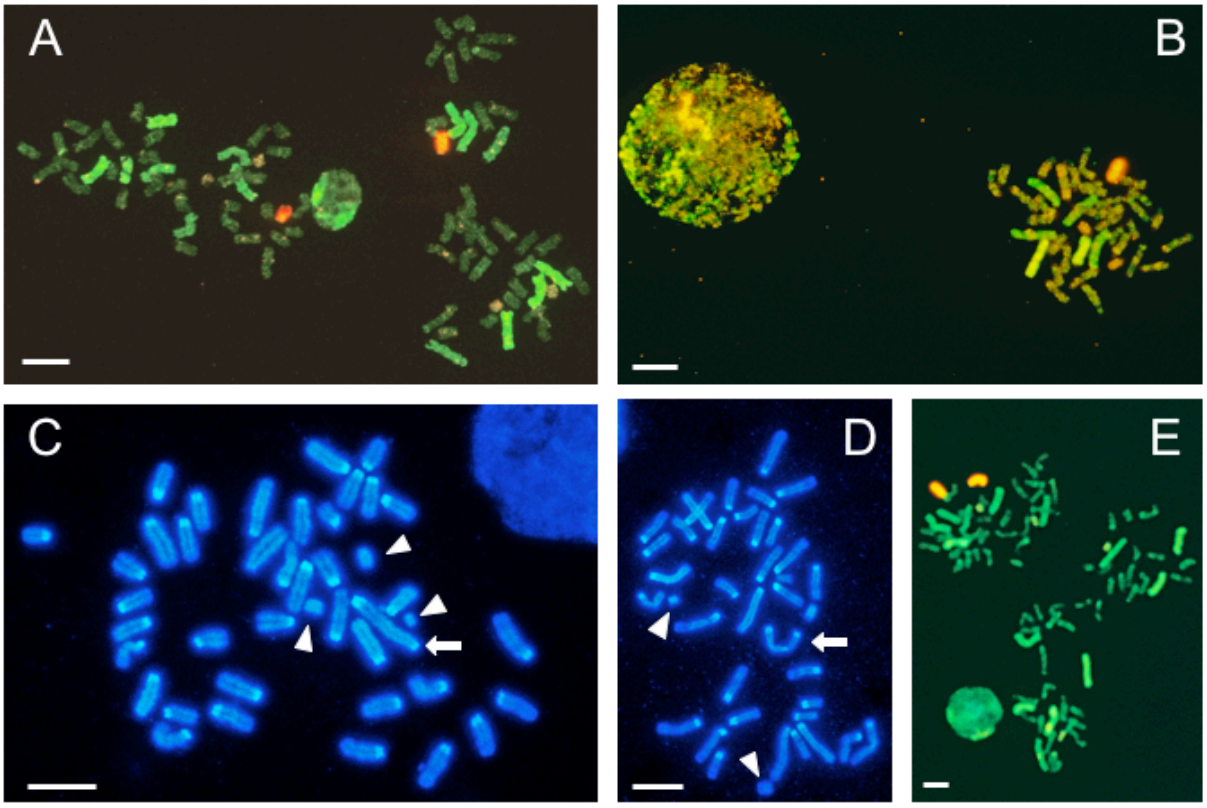
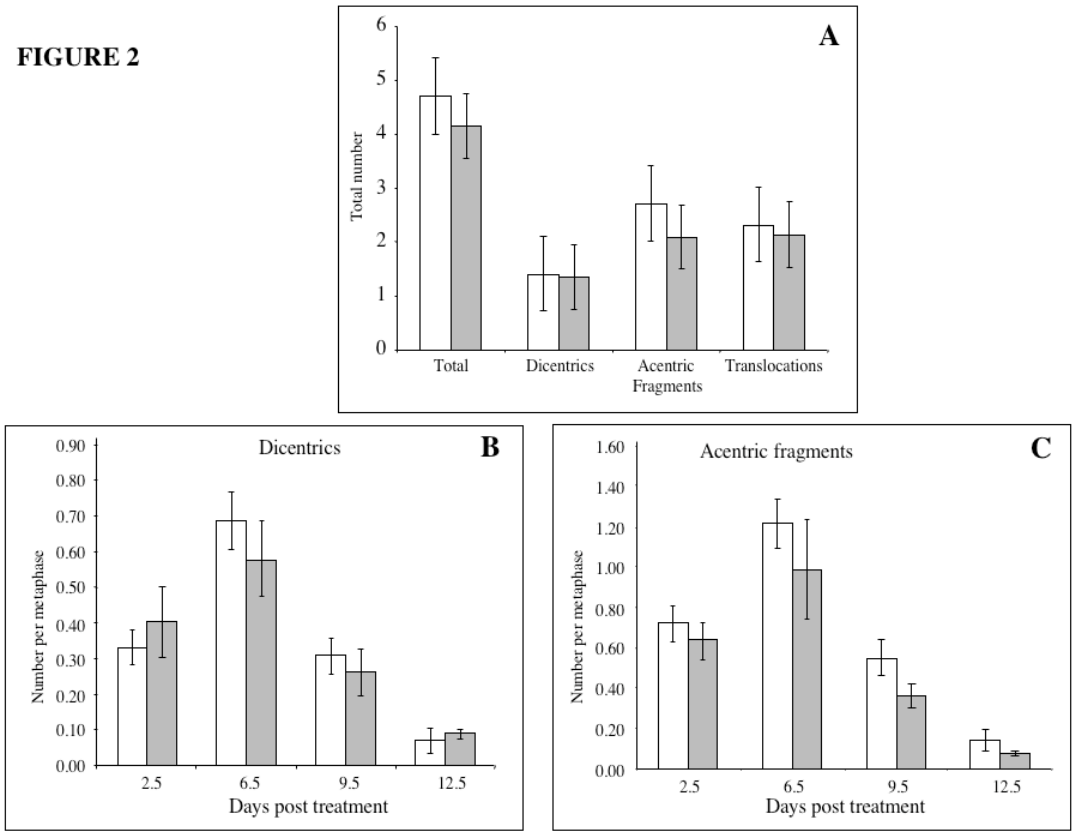


Figure 1

FIGURE 2


A
Total number
0
1
2
3
4
5
6
Total
Dicentrics
Acentric Fragments
Translocations
B
Dicentrics
Number per metaphase
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
2.5
6.5
9.5
12.5
Days post treatment
C
Acentric fragments
Number per metaphase
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
2.5
6.5
9.5
12.5
Days post treatment

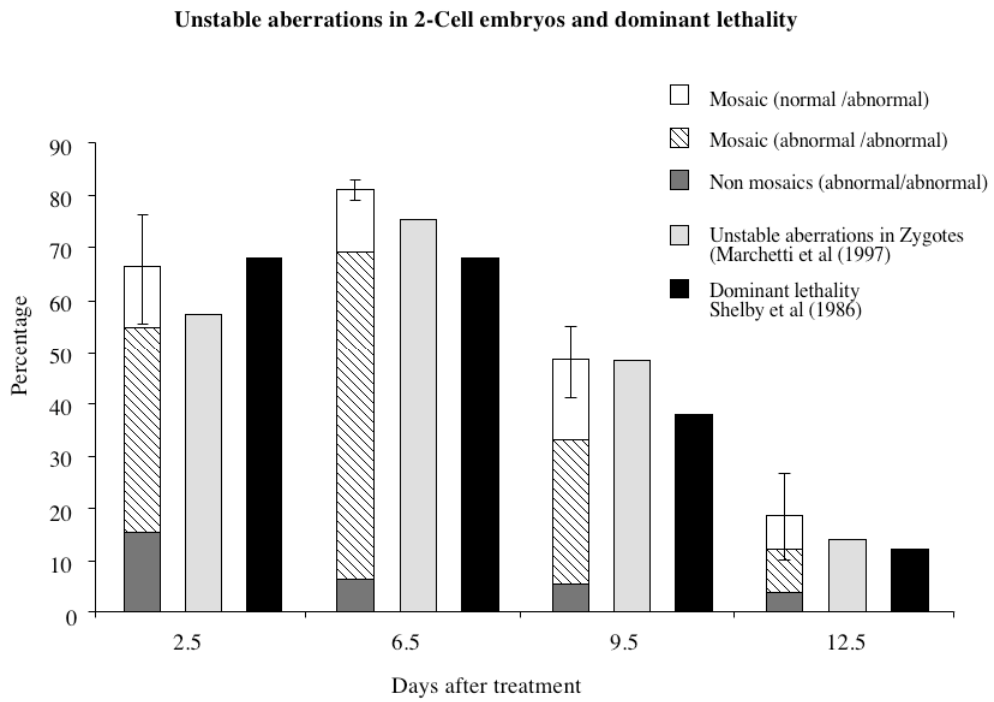

Unstable aberrations in 2-Cell embryos and dominant lethality
Mosaic (normal /abnormal)
Mosaic (abnormal /abnormal)
Non mosaics (abnormal/abnormal)
Unstable aberrations in Zygotes (Marchetti et al (1997)
Dominant lethality Shelby et al (1986)
Percentage
0
10
20
30
40
50
60
70
80
90
2.5
6.5
9.5
12.5
Days after treatment

Figure 3

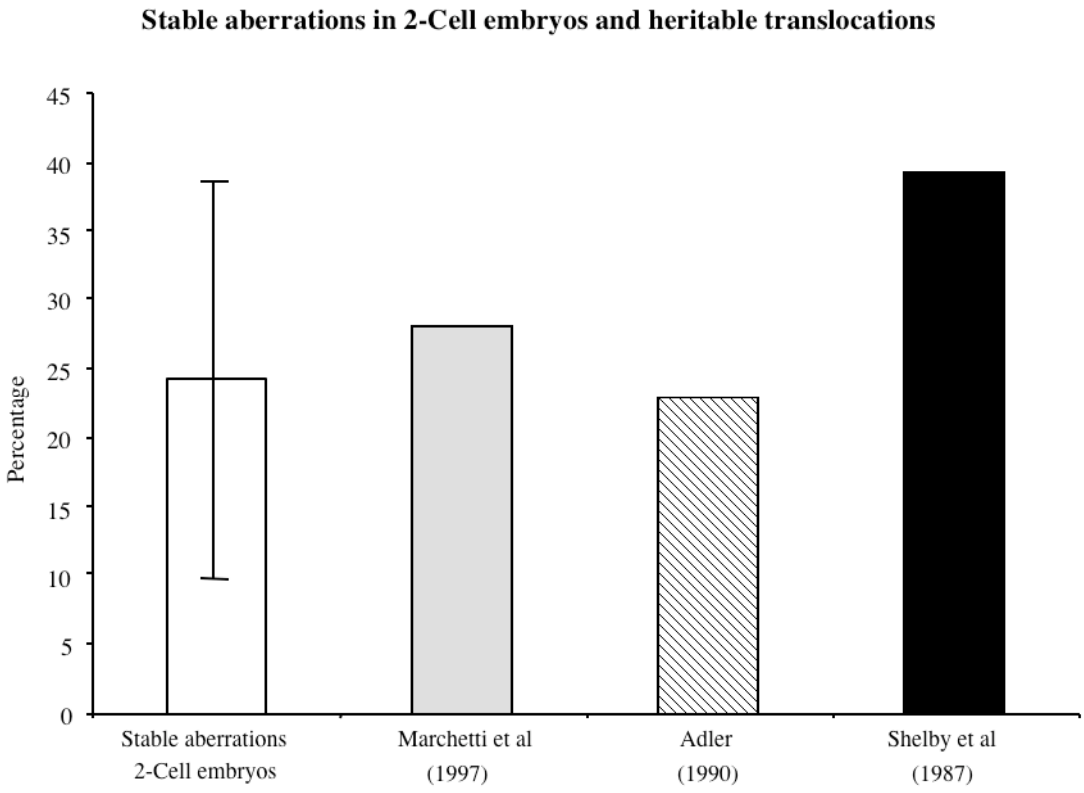

Stable aberrations in 2-Cell embryos and heritable translocations
Percentage
45
40
35
30
25
20
15
10
5
0
Stable aberrations
2-Cell embryos
Marchetti et al
(1997)
Adler
(1990)
Shelby et al
(1987)

Figure 4

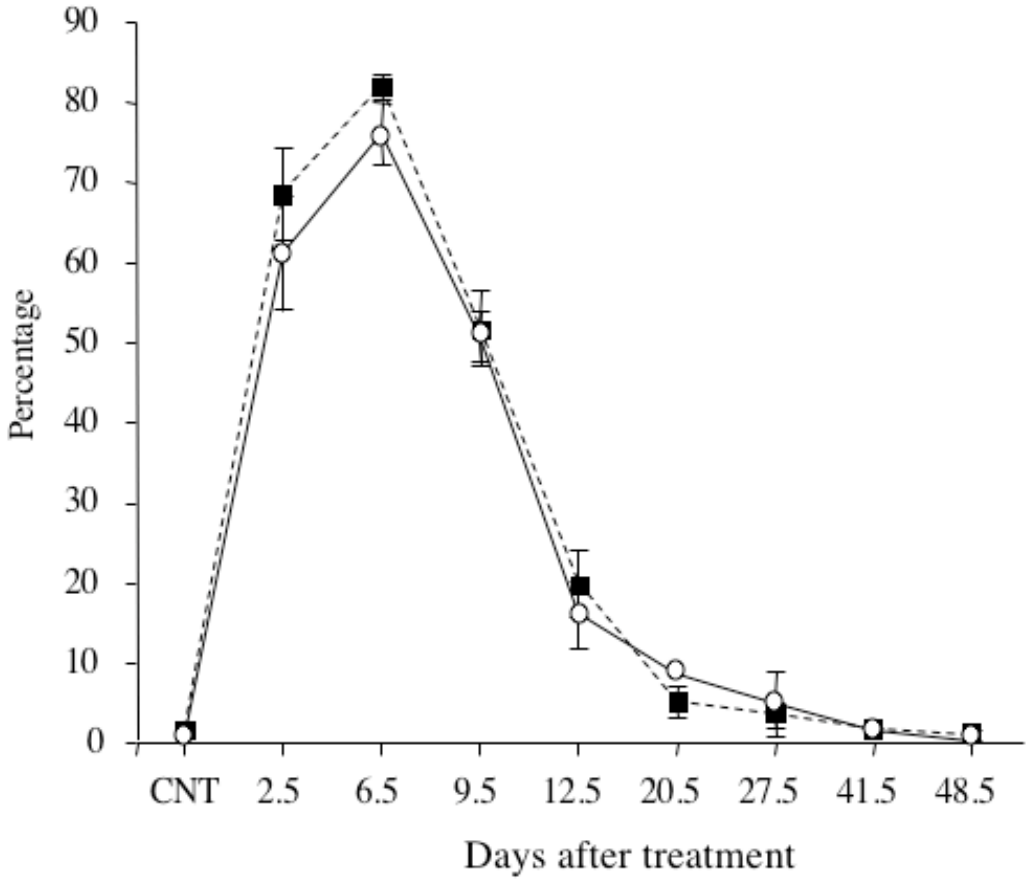

90
80
70
60
50
40
30
20
10
0
Percentage
CNT
2.5
6.5
9.5
12.5
20.5
27.5
41.5
48.5
Days after treatment

Figure 5

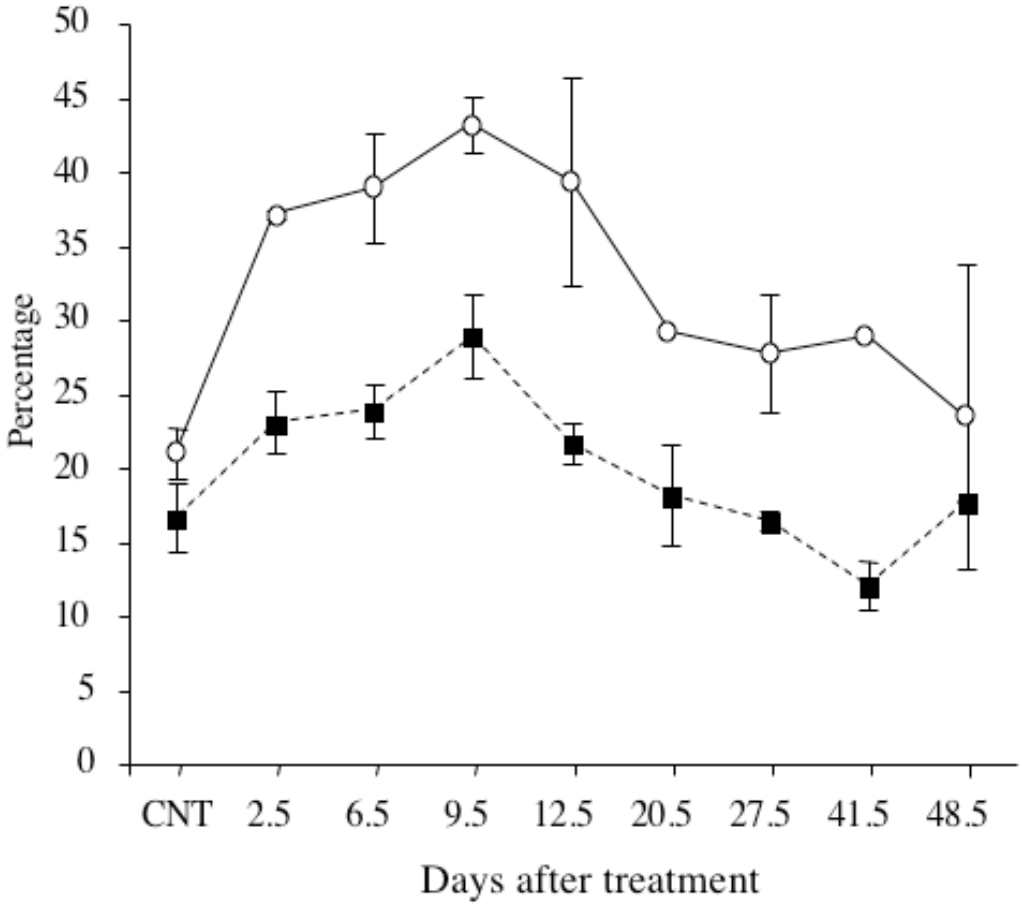

50
45
40
35
30
25
20
15
10
5
0
Percentage
CNT
2.5
6.5
9.5
12.5
20.5
27.5
41.5
48.5
Days after treatment

Figure 6