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Tamoxifen-induced alterations in meiotic maturation and cytogenetic abnormalities in mouse oocytes and 1-cell zygotes

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Summary

Alterations in the rate of oocyte meiotic maturation (OM) and the timing of the metaphase–anaphase transition may predispose oocytes to premature centromere separation (PCS) and aneuploidy. Tamoxifen has the potential for perturbing the rate of OM since it can function as a calcium antagonist by binding to calmodulin and inhibiting the formation of a calcium–calmodulin complex which is needed for activating calmodulin-dependent cAMP phosphodiesterase and initiating OM. The objective of this study was to test the hypothesis that tamoxifen alters the rate of OM and predisposes oocytes to PCS and aneuploidy. Different does of tamoxifen were administered by oral gavage to female mice preovulation. Metaphase II oocyte and 1-cell zygote chromosomes were C-banded and cytogenetically analysed. Tamoxifen treatment resulted in a modest, but significant ($p < 0.05$), increase in oocytes with PCS. Similar frequencies of hyperploidy and oocytes with unpaired, single chromatids (SC) were found. Metaphase I, diploid and premature anaphase (PA) oocytes were not detected. Hyperploidy, polyploidy, PCS, PA and SC were not detected in zygotes. These data indicate that the levels of tamoxifen-induced PCS found in mouse oocytes did not predispose zygotes to aneuploidy. Tamoxifen did, however, reduce the proportion of females exhibiting oestrus.

Key words: Aneuploidy, Oocytes, Premature centromere separation, Tamoxifen, Zygotes

Introduction

Accurate segregation of chromosomes is an essential cellular process; errors result in aneuploidy. The paramount features of human germ cell aneuploidy are its association with maternal age and its more frequent occurrence during maternal meiosis I (Hassold & Jacobs, 1984; Hook, 1985). Although numerous hypotheses have been proposed about the aetiology of aneuploidy (Hook, 1985; Hassold & Sherman, 1993), very little is known about its mechanisms. During the last decade, several investigators have supported the premise that certain endogenous and exogenous compounds can alter the normal temporal sequence of oocyte maturation (OM) and predispose oocytes to aneuploidy (Hansmann & Pabst, 1992; Eichenlaub-Ritter, 1993; Mailhes & Marchetti, 1994; Plachot, 1997; Yin *et al.*, 1998). When preovulatory mammalian oocytes are exposed to chemicals that alter microtubular structure and function, increased frequencies of metaphase I (MI) and diploid metaphase II (MII) oocytes are usually found in the ovulated oocytes. These MI and diploid oocytes have undergone a delay in their rate of OM and are usually accompanied by aneuploid MII oocytes (Mailhes, 1995). Compounds other than those that damage microtubules can also alter the initiation and rate of OM (Eppig & Downs, 1984; Racowsky, 1986). Tamoxifen may be one such compound.

Tamoxifen is a non-steroidal, triphenylethylene derivative widely used for inducing ovulation in certain anovulatory patients and as an adjuvant in breast cancer patients. In addition to its clinical use, tamoxifen has been shown to function as a calcium and calmodulin antagonist by occupying calcium-binding sites on calmodulin (Lam, 1984; Lipton & Morris, 1986; Sato, 1990; Edwards *et al.*, 1992). This antagonism interferes with the formation of a calcium–calmodulin complex (Lipton & Morris, 1986; Edwards *et al.*, 1992;

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Maruska *et al*., 1984; Rasmussen & Means, 1989) which is needed for coordinating cell-growth regulators (Home *et al*., 1993; Carroll *et al*., 1994; De Koninck & Schulman, 1998) that are essential for the resumption and completion of meiosis in mice (Jagiello *et al*., 1982; Maruska *et al*., 1984; Sato, 1990). When tamoxifen (instead of calcium) is bound to calmodulin, calmodulin-dependent cAMP-phosphodiesterase activity is inhibited (Bornslaeger *et al*., 1984; Edwards *et al*., 1992). This results in an inhibition of OM because this enzyme is needed for decreasing cAMP levels prior to initiating OM in mice (Chafouleas *et al*., 1981; Schultz *et al*., 1983). Besides its role in the initiation and rate of OM, calmodulin may also participate in the events comprising chromosome segregation and the metaphase–anaphase transition (MAT). Calmodulin is found in the pericentriolar region of the mitotic spindle (Welsh *et al*., 1979; Geiser *et al*., 1993), it depends on kinetochore microtubules for proper localisation (Sweet & Welsh, 1988) and it is needed for formation of the spindle apparatus (Geiser *et al*., 1993).

Besides altering intracellular calcium levels, tamoxifen has also been shown to activate specific kinases and phosphatases (Issandou *et al*., 1990). This activity seems relevant to aneuploidy because numerous kinase and phosphatase reactions play fundamental roles during the initiation of OM (Dorée *et al*., 1995), the timing of anaphase (Chesnel *et al*., 1994; Eppig *et al*., 1994) and chromosome segregation (Dorée *et al*., 1995; Dekel, 1996). Thus, the ability of tamoxifen to interfere with formation of a calcium–calmodulin complex, to alter cell cycle kinetics in somatic cells (Osborne *et al*., 1983; Francavilla *et al*., 1989), to alter kinase and phosphatase reactions (Issandou *et al*., 1990) and to delay the rate of OM in mouse oocytes (Sato, 1990; De Felici *et al*., 1991; Tombes *et al*., 1992) suggest that tamoxifen may predispose oocytes to abnormal chromosome segregation.

In addition to its potential for altering the temporal sequence of events during OM, tamoxifen has demonstrated genotoxicity both *in vivo* (Vijayalaxmi & Rai, 1996) and *in vitro* (White *et al*., 1992; Crofton-Sleigh *et al*., 1993; Pathak & Bodell, 1994; Styles *et al*., 1994). Tamoxifen has also been reported to induce aneuploidy in mammalian cells *in vitro* (Tsutsui *et al*., 1997) and *in vivo* (Sargent *et al*., 1994, 1996; Styles *et al*., 1997). Nevertheless, information about the ability of tamoxifen for inducing cytogenetic damage in germ cells has not been reported. Such data are needed for identifying potential human aneugens and for gaining insight into some of the numerous potential mechanisms of aneuploidy. The objective of this study was to test the hypothesis that tamoxifen alters the rate of OM and predisposes mouse oocytes to aneuploidy *in vivo*.

Materials and methods

Animals

In all experiments, ICR (Harlan Sprague-Dawley, Indianapolis, IN) male and female mice between 8 and 12 weeks of age (25–34 g) were used. They were housed under a 12-h light/12-h dark photoperiod, ambient temperature of 21–23 °C and relative humidity of 50 ± 5%. Feed and water were provided *at libitum*. This research was approved by the Louisiana State University Medical Center Animal Resources Advisory Committee.

Oocytes and zygotes

Follicular maturation was augmented by administering 7.5 IU of pregnant male serum (PMS; Folligon, Intervet, Cambridge, UK) and 5.0 IU of human chorionic gonadotrophin (hCG; Ayerst, Philadelphia, PA) was given 48 h later to induce ovulation. Tamoxifen citrate (CAS# 54965–24–1) was purchased from Sigma Chemical Co. (no. T9262, St Louis, MO). Solutions were prepared in olive oil (Sigma Chemical Co., no. 0–1500) and mixed on a magnetic stirrer for 45 min prior to administration by oral gavage.

In the MII oocyte experiments, doses of 5, 50, 250 and 500 mg/kg tamoxifen were given to females at each of three different times (–2.0, 0 and +2 h hCG). Thus, females in the 5 mg/kg group actually received this dose at three different times for a total dosage of 15 mg/kg. Controls received olive oil (0.1 ml/10 g body weight) at –2.0, 0 and +2 h hCG. The tamoxifen doses were based on data obtained from a preliminary toxicity study. When 1000 mg/kg tamoxifen was administered at –2.0, 0 and +2 h hCG, the number of ovulated oocytes was reduced relative to the four tamoxifen doses used in the study groups. An average of only 16 ovulated oocytes was obtained from mice that received 1000 mg/kg tamoxifen, whereas the means of ovulated oocytes from the four tamoxifen study groups and the controls were 36 and 31, respectively. Also, in the females that received 1000 mg/kg tamoxifen, one animal died after the first dose and two others died after the third dose.

In the 1-cell zygote experiments, the protocol was similar to that of MII oocytes except that 3.5 h after hCG females were caged (1:1) with males for 16.5 h. Females exhibiting a vaginal plug (evidence of mating) were given 2×10^{-3} M colchicine (Sigma no. C9754) by intraperitoneal injection 26 h post-hCG. Zygotes were collected 18 h after colchicine. One-cell zygote data were not obtained from females given 500 mg/kg tamoxifen because only 3 of 40 (8%) females exhibited a vaginal plug. As discussed later, relatively high doses of tamoxifen can inhibit oestrous behaviour in rodents.

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Oocyte harvest and slide preparation

For the MII oocyte and 1-cell zygote experiments, females were killed by CO_2 inhalation 18 h and 42 h, respectively after the last tamoxifen treatment. Oocytes and zygotes were harvested on multiple days for each treatment group and were prepared for cytogenetic analysis during a 3 h period according to a procedure (Mailhes & Yuan, 1987) that involves processing the oocytes *en masse* from 10–15 females.

Similar to cytogenetic techniques for other cell types, the number of oocytes actually placed onto slides was less than the number collected because some cells were lost during the hypotonic and fixation steps. Also, the number of oocytes analysed was less than the number placed onto a slide because some cells could not be objectively analysed.

Cytogenetic analysis and statistical analysis of data

To distinguish between complete dyads (MII chromosomes) and disjoined dyads (comprising two single chromatids), chromosomes were C-banded (Salamanca & Armendares, 1974). In each cell analysed, the number of chromosomes and/or chromatids was counted at ×1250 magnification to provide data for calculating aneuploidy. The numbers of hypoploid (n = 10–19½), euploid (n = 20), hyperploid (n = 20½–29½) and diploid (n = 30–40) oocytes were recorded. An oocyte classified as 20½ contains 20 dyads and one chromatid or one-half dyad. The frequencies of each ploidy class were divided by the total number of MII oocytes analysed excluding diploidy. The frequencies of MII oocytes displaying premature centromere separation (PCS), single chromatids (SC) and premature anaphase (PA) were also calculated relative to the total number of MII oocytes analysed. Since MI and diploid oocytes are distinct categories and are disregarded when computing aneuploidy, their frequencies were calculated relative to the total number of oocytes analysed. When analysing 1-cell zygotes, the frequencies of hypoploid (2N = 30–39½), euploid (2N = 40), hyperploid (2N = 40½–49½) and polyploid (PP; 2N = 50–80) were calculated; polyploid cells were not included when calculating aneuploidy. The number and type of structural chromosome aberrations were also recorded when present.

The criteria for eliminating a cell from analysis included: inadequate C-banding for discriminating between intact dyads and those separated at the centromere, overlapped or clumped chromosomes, or excessive chromosome scatter that precluded an objective analysis of numerical or structural aberrations. The frequencies of hyperploid cells were used as a measure of aneuploidy because an unknown proportion of the hypoploid cells is influenced by technical artefact resulting from excessive chromosome scatter during slide preparation. Chi-square analysis and the Fisher's Exact Test were used for data analyses.

Results

The frequencies of PCS (Fig. 1*a*) in oocytes from each of the tamoxifen groups were significantly ($p < 0.05$) higher than in controls (Table 1). We point out, however, that the degree of PCS observed in the tamoxifen groups is similar to frequencies previously reported for controls (Mailhes *et al*., 1997, 1998). Due to the numerous mechanisms whereby PCS may occur (Mailhes *et al*., 1998) and the variation in dose–responses curves for cytogenetic parameters used to estimate alterations in the rate of OM (Mailhes, 1995; Marchetti *et al*., 1996), the lack of a tamoxifen dose-response in PCS frequencies is not unexpected. Apparently, 5 mg/kg tamoxifen was a threshold dose for PCS induction. Additionally, the frequencies of hyperploidy and SC (Fig. 1*b*) were not significantly ($p > 0.05$) different between the tamoxifen groups and the controls. MI oocytes, diploid oocytes and those exhibiting PA were not found.

The frequencies of hyperploidy (Fig. 1*c*) and PP (Fig. 1*d*) in 1-cell zygotes were similar ($p > 0.05$) among all groups (Table 2). Zygotes exhibiting PCS, SC and PA were not found. The only noticeable effect of tamoxifen in the 1-cell zygote data was the reduced oestrous behaviour of females following higher doses of tamoxifen. Thus, due to the relatively (although statistically significant) low frequencies of PCS found in oocytes, an adequate test of the contention that PCS predisposes MII oocytes to aneuploid zygotes cannot be made. However, the oocyte and zygote data do show that tamoxifen did not increase the levels of hyperploidy and that it reduced the proportion of females exhibiting oestrous behaviour.

Discussion

The finding that higher doses of tamoxifen reduced oestrous behaviour (sexual receptivity) in mice was not unexpected (Table 2). When overiactomised Syrian hamsters were given 500 µg tamoxifen or 500 µg tamoxifen plus 5 µg oestradiol benzoate (EB) subcutaneously daily for 17 days, the duration of oestrous was significantly ($p < 0.05$) less than in females that only received EB (Wade & Powers, 1993). Another study involving ovariectomised adult rats reported that oestrous behaviour was significantly ($p < 0.05$) reduced in females receiving a single dose of either 0.7 or 7.0 mg/kg tamoxifen when compared with those receiving the same doses of tamoxifen plus 100 µg/kg EB (Bowman *et al*.,

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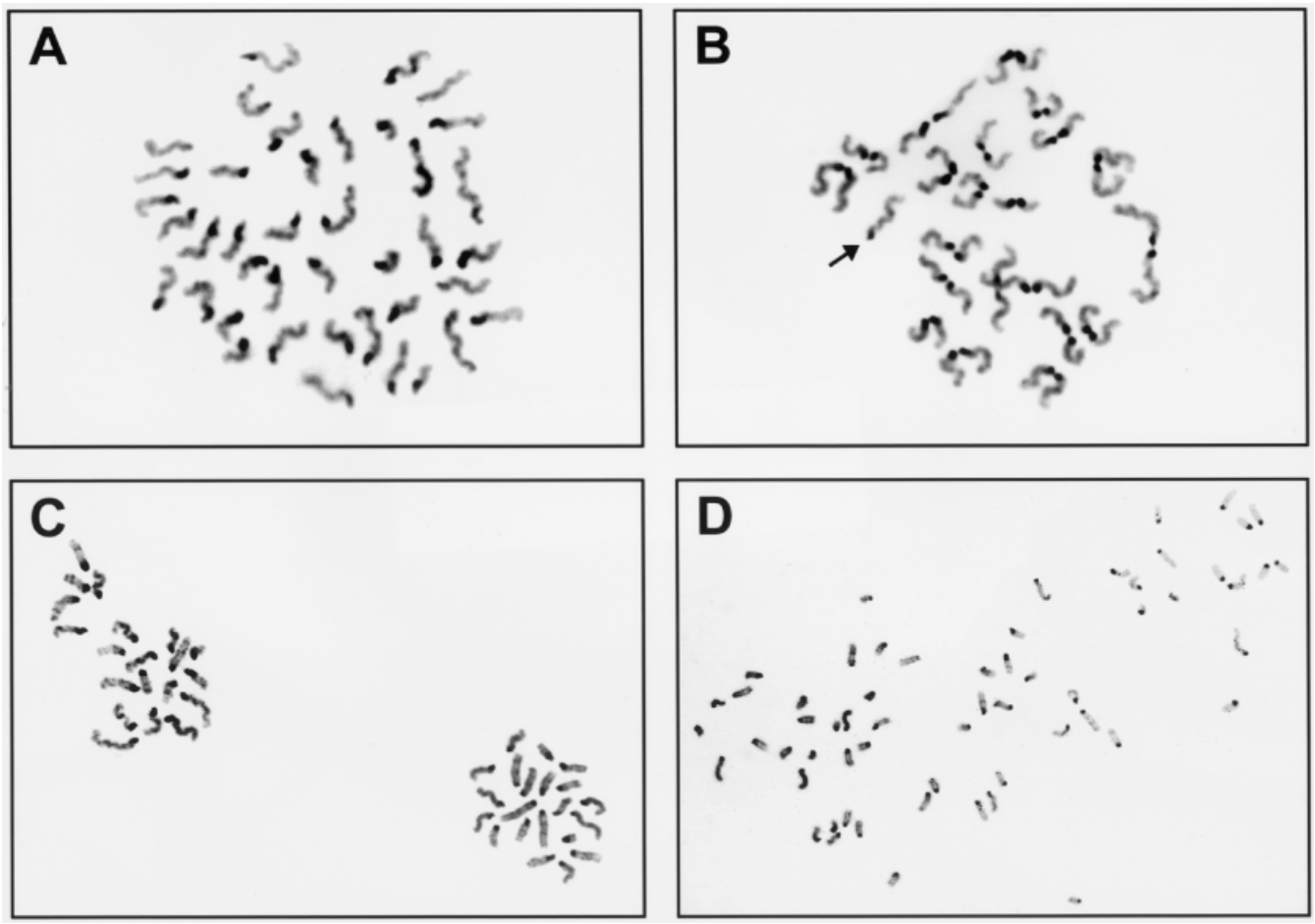

Figure 1 Cytogenetic analysis of mouse oocytes and zygotes following tamoxifen treatment. Cytogenetic analysis was performed as described above; chromosome configurations are shown. (*A*) MII oocyte, premature centromere separation, $n = 20$ (40 chromatids). (*B*) MII oocyte, hyperploid, single chromatid, $n = 21½$ (21 dyads and 1 chromatid); arrow indicates the single, unpaired chromatid. (*C*) One-cell zygote, hyperploid, 2N = 41. (*D*) One-cell zygote, polyploid, 2N = 60.

1983). These studies indicate that tamoxifen, like other anti-oestrogens, antagonises the effects of oestradiol on sexual receptivity in both rats and hamsters (Wade & Baulstein, 1978; Meisel *et al*., 1987).

Cell type and species appear to influence tamoxifen-induced aneuploidy data. The effect of these variables is readily apparent when the *in vivo* oocyte aneuploidy data in Table 1 are compared with aneuploidy data obtained from rats treated orally with tamoxifen and their hepatocytes subsequently cultured *in vitro*. Although *in vivo* tamoxifen treatment did not elevate aneuploidy levels in mouse oocytes (Table 1), 0.3 and 35.0 mg/kg tamoxifen induced 71% and 85% aneuploidy in rat hepatocytes, respectively (Sargent *et al*., 1994, 1996). Part of the difference between the oocyte and hepatocyte data apparently stems from scoring criteria, because aneuploidy in hepatocytes was considered as a deviation in chromosome number from either diploidy or tetraploidy. Since 70% of the hepatocytes were reported as tetraploid, and we did not include oocytes with multiples of the haploid number in our aneuploid analysis, this different in scoring criteria influenced the data. These aneuploidy data may also have been influenced by differences in the bioavailability of tamoxifen among species and in target organ specificity of tamoxifen activity. Pharmacological studies showed that the same dose of tamoxifen resulted in tamoxifen serum levels 4-fold lower in mice than in rats (Robinson *et al*., 1991). Also, mice given 45 mg/kg tamoxifen for 4 days had 30–40% fewer DNA adducts in the liver than those detected in rats. The absence of detectable adducts in extrahepatic organs suggested a selective activation in the liver, possibly mediated by cytochrome P450 (White *et al*., 1992).

PCS was described in postovulatory aged mouse oocytes more than 25 years ago (Rodman, 1971). Although the PCS levels noted in oocytes from tamoxifen-treated mice were significantly ($p > 0.05$) greater

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Table 1 Tamoxifen-induced cytogenetic abnormalities in mouse oocytes *in vivo*

Tamoxifen (mg/kg)	No. of MII oocytes analysed (%)					
	Total[a]	Hypoploid[b]	Haploid[c]	Hyperploid[d]	PCS[e]	SC[f]
Control[g]	322	9 (2.8)	311 (96.6)	2 (0.6)	1 (0.3)	2 (0.6)
5 (×3)	271	19 (7.0)	247 (91.1)	3 (1.1)	8 (3.0)*	2 (0.7)
50 (×3)	218	15 (6.9)	202 (92.7)	1 (0.5)	9 (4.1)*	0
250 (×3)	263	7 (2.7)	255 (97.0)	1 (0.4)	7 (2.7)*	1 (0.4)
500 (×3)	264	22 (8.3)	241 (91.3)	1 (0.4)	6 (2.3)*	0

[a]The total numbers are less than the sum of the five categories because some cells had more than one abnormality.
[b]MII oocytes (n = 10–19.5).
[c]MII oocytes (n = 20).
[d]MII oocytes (n = 20.5–29.5).
[e]PCS, premature centromere separation.
[f]SC, single, unpaired centromere.
[g]Olive oil solvent.
MI, diploid MII (n = 30–40), and premature anaphase oocytes were not found.
*$p < 0.05$ versus control.

Table 2 Tamoxifen-induced cytogenetic abnormalities in mouse 1-cell zygotes *in vivo*

Tamoxifen (mg/kg)	No. one-cell zygotes analysed (%)					
	Total	Hypoploid[a]	Euploid[b]	Hyperploid[c]	PP[d]	No. mated/ no. mice[e]
Control[f]	196	16 (8.2)	177 (90.3)	1 (0.5)	2 (1.0)	49/89 (55.1)
5 (×3)	209	10 (4.8)	198 (94.7)	0	1 (0.5)	30/50 (60.0)
50 (×3)	219	14 (6.5)	203 (92.7)	0	2 (0.9)	28/70 (40.0)
250 (×3)	191	10 (5.3)	178 (93.2)	2 (1.1)	1 (0.5)	20/89 (22.5)
500 (×3)	–	–	–	–	–	3/40 (7.5)

[a]One-cell zygotes (2N = 30–39.5).
[b]One-cell zygotes (2N = 40).
[c]One-cell zygotes (2N = 40.5–49.5).
[d]PP (polyploid), 2N = 50–80.
[e]16.5 hour mating period.
[f]Olive oil solvent.
MII oocytes with premature centromere separation (PCS), premature anaphase (PA) and single chromatids (SC) were not found.

than in controls, the levels were similar to those previously reported for controls (Mailhes *et al*., 1997, 1998). Nonetheless, the biological significance of PCS and PA is that they appear to predispose oocytes to aneuploidy (Rao *et al*., 1996; Mailhes *et al*., 1997, 1998; Kajii *et al*., 1998) and they may have value in cancer risk assessment (Rao *et al*., 1996; Major *et al*., 1999). If sister chromatids precociously separate during prometaphase and prior to stable microtubular attachment during metaphase II, the chromatids have an increased probability of random segregation during anaphase II. Consequently, if both sister chromatids segregate to the secondary oocyte, trisomy will result in the 1-cell zygote following fertilisation; if neither chromatid segregates to the oocyte, the zygote will be monosomic. Also, premature separation of chromatids during meiosis I may result in MII oocytes with single and unpaired chromatids (Angell, 1991; Angell *et al*., 1991).

The potential mechanisms whereby alterations in the biochemical pathways associated with OM and the MAT might predispose cells to PCS, PA and aneuploidy have been largely unexplored. Abnormalities in the spindle checkpoint (Elledge, 1996; Allshire, 1997), kinase and phosphatase reactions (Dekel, 1996), anaphase promoting complex (APC) activity (Cohen-Fix & Koshland, 1997; Nasymth, 1999) and sister chro-

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matid cohesion proteins (Dorée *et al*., 1995; Gorbsky *et al*., 1999; Biggins & Murray, 1999) all seem to have the potential for predisposing cells to chromosome mis-segregation. The complexity of these processes can be illustrated by considering the MAT. Although it appears straightforward from a cytogenetic viewpoint, the MAT actually consists of a series of events involving the coordination of independent processes that depend on spindle checkpoint release prior to activation of the APC (Skibbens & Hieter, 1998; Craig *et al*., 1999). These events include: the mechanistically independent events of chromatid arm separation and centromere separation (Sluder & Rieder, 1993; Rieder & Salmon, 1998); the ability of sister chromatids to separate in the absence of a spindle (Rieder & Palazzo, 1992); the occurrence of anaphase spindle dynamics in the absence of chromosomes (Zhang & Nicklas, 1996); and the ability of chromatids to separate when MPF activity is high (Sluder & Rieder, 1993).

This study appears to be the first involving the effects of tamoxifen on OM and aneuploidy in mammalian oocytes. Caution should be exercised in extrapolating these results due to the reported variation among species and cell types to tamoxifen. We conclude that tamoxifen did not appreciably alter the rate of OM and predispose mouse oocytes to aneuploidy. However, tamoxifen did decrease the proportion of females exhibiting oestrous.

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