ANIMAL EXPERIMENTATION

Effect of Intracellular Ca^{2+} Chelation with the Acetoxymethyl Ester-Derived Form of Bis(*o*-Aminophenoxy)Ethane-*N,N,N,N'*,*N'*-Tetraacetic Acid on Meiotic Division and Chromosomal Segregation in Mouse Oocytes

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Purpose: Our purpose was to ascertain the effect of intracellular Ca^{2+} chelation on the chromosomal distribution and segregation of mouse oocytes during maturation in vitro.

Methods: Germinal vesicle oocytes were loaded with the acetoxymethyl ester-derived form of bis(*o*-aminophenoxy)ethane-*N,N,N,N'*,*N'*-tetraacetic acid (BAPTA-AM). Chromosomal distribution and segregation of control and BAPTA-AM-treated metaphase II (MII) oocytes were evaluated at 16 hr, and intracellular ATP content at 0, 1, and 16 hr after BAPTA-AM loading.

Results: BAPTA-AM treatment decreased ($P \leq 0.05$) the potential for in vitro maturation, increased ($P \leq 0.0001$) the percentage of oocytes displaying an abnormal distribution of metaphase II chromosomes in the meiosis II spindle and aneuploidy, and decreased ($P \leq 0.005$) the ATP content at 0, 1, and 16 hr of culture compared to the control groups.

Conclusions: These findings raise some concern about any other condition/drug that may directly or indirectly decrease the intracellular Ca^{2+} concentration in human oocytes.

KEY WORDS: aneuploidy; ATP; BAPTA-AM; intracellular calcium chelation; intracellular pH.

INTRODUCTION

The presence of an intact cytoskeleton in the mammalian oocyte is essential for maintenance of the chromosomal organization. During the first meiotic maturation of the oocyte, the separation of homologue chromosomes depends on the presence of an intact spindle and microfilament system. In fact, microtubules play an important role not only in a large variety of cellular events such as cell shape change, intracellular transport, secretion, and changes in membrane fluidity, but also in chromosome movement. On the other hand, microfilaments are involved in anaphase I progression, spindle migration and attachment to the cell periphery, and formation of a cleavage furrow during cytokinesis (1).

Microtubules and microfilaments are labile structures that rapidly assemble and disassemble within a dynamic equilibrium. For this reason, disturbances in tubulin/actin polymerization/depolymerization may induce mistakes in chromosomal distribution and/or segregation during the first and second meiotic division and, so, increase the risk of having a chromosomally aberrant conception. Among many other factors such as ATP levels and a reduced glutathione (GSH)/glutathione disulfide (GSSG) ratio (2), it appears that polymerization/depolymerization of tubulin and actin is closely associated with and controlled by changes in the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). In fact, it is well-known that an increase in $[\text{Ca}^{2+}]_i$ pre-

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vents the polymerization of dimeric tubulin and causes rapid depolymerization of preformed microtubules in *in vitro* (3–11) and *in vivo* (3,12–14) systems. Likewise, it appears that increased $[Ca^{2+}]_i$ is involved in alterations of actin microfilaments and actin-binding proteins associated with the formation of multiple protrusions (blebs) on the cell surface (4,15–17). Recently, Tarín (2) proposed a relationship between the $[Ca^{2+}]_i$ of oocytes and/or surrounding ovarian cells and the maternal-age dependent increase in aneuploidy. According to this view, mitochondria of oocytes/ovarian cells from middle-aged females would exhibit a high production of reactive oxygen species which would decrease the intracellular GSH/GSSG ratio and this, in turn, would cause impairment of Ca^{2+} transport and subsequent perturbation of intracellular Ca^{2+} homeostasis, resulting in a sustained increase in the cytosolic Ca^{2+} concentration.

Although literature on the effects of decreased levels of intracellular Ca^{2+} on cytoskeletal fibers is scarce or fragmentary, it has been shown in the mouse model that formation of the first polar body and resumption of the second meiotic division and associated events, such as cortical granule release, are slightly and strongly inhibited, respectively, by intracellular Ca^{2+} chelation with the acetoxymethyl ester-derived form of bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) (18,19). It has also been reported that nocodazole-treated inseminated oocytes, on removal from the drug to a medium containing BAPTA-AM, show multiple groups of condensed chromosomes but fail to form complete spindles as in the case of oocytes washed out of nocodazole into a normal culture medium. Furthermore, the addition of cytochalasin D (to prevent microfilament-dependent dispersal of the chromosomes) to a nocodazole-containing fertilization medium followed by transfer to a nocodazole-free medium containing BAPTA-AM form a full-sized spindle around the single group of condensed chromosomes, but the spindle poles are pointed rather than having the usual barrel shape seen in control oocytes (19). These data suggest, therefore, that reduced intracellular levels of Ca^{2+} may affect the dynamic equilibrium of polymerization/depolymerization of tubulin and actin, which, in turn, may induce mistakes in chromosomal distribution and segregation. In the present study, we examined the effects of intracellular Ca^{2+} chelation on the chromosomal normality of the mouse oocyte during maturation *in vitro*. Fully grown immature germinal vesicle (GV) mouse oocytes were cultured after BAPTA-AM loading and the normality of the chromosomal distribution in the meta-

phase II (MII) spindle and segregation of chromosomes during the first meiotic division were determined.

MATERIALS AND METHODS

Oocyte Collection and Culture

Fully-grown GV-stage oocytes were obtained from unstimulated 6 to 8-week-old ICR or (C57BL × CBA)F₁ hybrid female mice by tearing the ovaries apart in medium M2 (20) supplemented with 4 mg bovine serum albumin (BSA; fraction V; Sigma Chemical Co. St. Louis, MO)/ml and 0.1 mM dibutyryl cyclicAMP (dbcAMP; Sigma Chemical Co.). GV oocytes were denuded of granulosa cells (if present) by repeated passage through a micropipette. Oocytes were cultured for 1 hr at 37°C in a humidified atmosphere of 5% CO₂ in air in the presence or absence of 10 μ M BAPTA-AM (Molecular Probes, Inc., Eugene, OR). This particular concentration of BAPTA-AM was chosen based on a previous study (21) demonstrating that it can chelate elevations of intracellular Ca^{2+} in mouse oocytes. As a 1000× stock solution of BAPTA-AM was made in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany), a second control group of oocytes was incubated in 0.1% DMSO without BAPTA-AM. The culture media used were medium KSOM (22) for oocytes from the ICR strain and medium M16 (23) for oocytes from F₁ hybrid female mice. Culture medium was supplemented with 4 mg BSA/ml and 0.1 mM dbcAMP. Oocytes were then incubated for 15 min in dbcAMP-free medium for deesterification of intracellular BAPTA-AM before washing out the AMP analogue and permitting *in vitro* maturation for 16 hr. In all the experiments, 200- μ l volumes of the respective culture medium were used for *in vitro* maturation of oocytes. No mineral oil was used to cover the surface of the culture medium owing to its ability to alter the composition of the medium by absorbing and/or transferring certain types of compounds (24).

Chromosomal Analysis

The chromosomal distribution of *in vitro* matured MII oocytes from ICR female mice was determined by fluorescence microscopy after exposure of oocytes for 5 min to a 150 μ M concentration of the fluorescent DNA probe 4',6-diamino-2-phenylindole (DAPI; Sigma Chemical Co.) in medium M2. Oocytes were fixed before staining with 3.7% formaldehyde in phos-

phate-buffered saline (PBS; pH 7.2) for 5 min and washed overnight at 4°C in medium M2. Anomalies in chromosomal distribution were classified into six groups: (i) chromosome clustering, (ii) chromosome scattering, (iii) chromosomes/DNA linked to a polar body DNA mass, (iv) no chromosomes within the oocyte, (v) no chromosomes within the polar body, and (vi) two cells with metaphase chromosomes.

For karyotyping analysis, *in vitro* matured MII oocytes from F₁ hybrid mice were exposed to a hypotonic 7.5 mM potassium chloride solution at 4°C for 20 min to 3 hr and fixed in methanol:glacial acetic acid (3:1) at -20°C (25). Chromosomes were C-banded (26) to ensure the unequivocal identification of whole chromosomes and single chromatids. Criteria for removing a cell from the cytogenetic study were insufficient C-banding to discriminate between whole chromosomes and single chromatids, overlapping or clumped chromosomes that precluded an accurate count, and excessive chromosome scatter. The frequency of aneuploidy was calculated as twice the frequency of hyperhaploidy because an unknown proportion of hypohaploid cells may arise from technical artifacts during slide preparation.

ATP Content Measurement

The ATP content of mouse oocytes was measured at 0, 1, and 16 hr after BAPTA-AM loading at the GV stage. At these times, oocytes were frozen and stored at -80°C in 200 µl of ultrapure water. The ATP content of oocytes was determined in a Berthold LB9501 luminometer by measurement of the luminescence pro-

duced in an ATP-dependent luciferin-luciferase bioluminescence assay. Oocytes were thawed immediately prior to the automated addition of luciferin-luciferase mixture (stock solution, 160 µg/ml luciferin/60 µg/ml luciferase; ATP Bioluminescence Somatic Cell Assay System; Sigma Chemical Co.). This mixture was diluted 25-fold with ATP assay buffer (70 mM Tris-acetate, pH 7.75, 0.1 mM EDTA, 5 mM magnesium acetate, 0.5% BSA). In each series of analyses, a standard curve containing 14 ATP concentrations from 10 to 5 pmol was generated. Control and BAPTA-AM-treated oocytes from several experiments were analyzed simultaneously to reduce potential variability between samples.

Statistical Analysis

One- and three-way analyses of variance (ANOVA) were applied for comparisons of means. In order to stabilize variances, proportions were transformed to arcsine before carrying out mean comparisons. When one-way ANOVA showed statistical differences, the Student-Newman-Keuls (SNK) test was used to discriminate between groups. Chi-square test was applied for comparisons of frequencies. Significance was defined as $P \leq 0.05$. The entire statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS).

RESULTS

Table I shows the chromosomal distribution of MII mouse oocytes matured *in vitro* after BAPTA-AM

Table I. Chromosomal Distribution of MII Mouse Oocytes Matured *In Vitro* After BAPTA-AM Loading at the GV Stage

	Treatment		
	Control	DMSO (0.1%)	BAPTA-AM (10 µM)
No. of experiments	5	5	5
Percentage of MI oocytes	22.6 ± 2.5 ^a	12.0 ± 4.0	38.4 ± 7.3*
Percentage of MII oocytes	77.4 ± 2.5	81.2 ± 3.6	59.7 ± 6.5*
No. of MII oocytes analyzed	54	68	222
Chromosome distribution			
Normal	54 (100.0) ^{b,**}	66 (97.1)	161 (72.5)
Chromosome clustering	—	—	18 (8.1)
Chromosome scattering	—	2 (2.9)	13 (5.9)
Chromosomes/DNA linked to a polar body DNA mass	—	—	13 (5.9)
No chromosomes within the oocyte	—	—	9 (4.1)
No chromosomes within the polar body	—	—	3 (1.4)
Two cells with metaphase chromosomes	—	—	5 (2.3)

^a Values are means ± SE.

^b Percentages given in parentheses.

* Value significantly different from the control and DMSO groups ($P \leq 0.05$).

** Distribution significantly different among groups ($P \leq 0.0001$).

Fig. 1. Chromosomal distribution of MII oocytes matured in vitro after BAPTA-AM loading at the GV stage. Oocytes were fixed, stained with DAPI, and examined by fluorescence microscopy. (A) Normal distribution of chromosomes on the MII spindle. (B) Chromosome clustering. (C) Chromosome scattering. (D) Oocyte chromosomes/DNA linked to a polar body DNA mass. (E) Oocyte with all the DNA fluorescence within one polar body. (F) Oocyte with all the DNA fluorescence within two polar bodies. (G) No DNA staining within the polar body. (H) Two cells with metaphase chromosomes.

loading at the GV stage. BAPTA-AM-treated oocytes showed a decreased potential for in vitro maturation as evidenced by a higher proportion of oocytes that did not extrude the first polar body (metaphase I) compared to the control and DMSO groups ($P \leq 0.05$). Furthermore, BAPTA-AM treatment significantly decreased ($P \leq 0.0001$) the percentage of oocytes displaying a normal distribution of chromosomes in the meiosis II spindle (Fig. 1A) compared to the control groups. This effect of BAPTA-AM was due basically to the presence of oocytes showing chromosome clustering (Fig. 1B), chromosome scattering (Fig. 1C), chromosomes/DNA linked to a polar body DNA mass (Fig. 1D), all the DNA fluorescence within one ($n = 3$) (Fig. 1E) or two ($n = 6$) (Fig. 1F) polar bodies, no DNA staining within the polar body (Fig. 1G), and two cells with metaphase chromosomes (Fig. 1H).

Table II shows the chromosomal constitution of MII oocytes matured in vitro after BAPTA-AM loading at the GV stage. As shown in Table I, the percentage of oocytes arrested at metaphase I was significantly ($P \leq 0.05$) higher in BAPTA-AM-treated oocytes compared to the DMSO and control groups. The percentage of aneuploidy ($2\times$ hyperhaploidy) was also significantly ($P \leq 0.0001$) higher in oocytes exposed to BAPTA-AM at the GV stage compared to the other groups. All the hyperhaploid oocytes showed a complement of 21 chromosomes (Fig. 2).

Table III shows the ATP content of mouse oocytes at different times after BAPTA-AM loading at the GV stage. Three-way ANOVA showed no effect of trial or time after BAPTA-AM loading. BAPTA-AM-treated oocytes, however, exhibited a consistent decrease in ATP content ($P \leq 0.005$) compared to the DMSO and control groups.

DISCUSSION

The experiments reported here demonstrate that in vitro maturation of mouse oocytes after loading with BAPTA-AM at the GV stage is associated with a decrease in ATP content and an increase in the proportion of oocytes showing a meiotic block during the first meiotic division, an abnormal distribution of chromosomes in the MII spindle, and mistakes in chromosomal segregation during the first meiotic division. These results therefore support the notion that an abnormally low intracellular level of free Ca^{2+} may disturb the dynamic equilibrium of polymerization/depolymerization of tubulin and actin of oocytes and so induce mistakes in distribution and segregation of chromosomes. The high percentage of hyperhaploid oocytes found after BAPTA-AM treatment (16%; 7 of 44 oocytes analyzed) makes this model especially suitable for aneuploidy studies because of the charac-

Table II. Chromosome Constitution of Mouse Oocytes Matured In Vitro Until the MII Stage After BAPTA-AM Loading at the GV Stage

Treatment	No. of experiments	% of MI oocytes	% of MII oocytes	No. of fixed oocytes	No. of analyzed oocytes	No. of chromosomes				Aneuploidy
						18	19	20	21	
Control	20	6.1 ± 2.0 ^a	78.9 ± 4.5	119	41 (34.5) ^b	1	1	39	—	0 (0.0)
DMSO (0.1%)	20	3.2 ± 1.6	86.0 ± 3.5	131	42 (32.1)	2	5	34	1	2 (4.8)
BAPTA-AM (10 μM)	20	14.9 ± 4.0*	65.7 ± 6.6**	106	44 (41.5)	3	4	30	7	14 (31.8)***

^a Values are means ± SE.^b Percentages given in parentheses.* Value significantly different from the control and DMSO groups ($P \leq 0.05$).** Value significantly different from the DMSO group ($P \leq 0.05$).*** Distribution significantly different among groups ($P \leq 0.0001$).

teristic low rates of aneuploidy observed in mouse oocytes.

Although the mechanism by which BAPTA-AM induces these anomalies remains to be elucidated, several lines of evidence support a multifactorial origin. First, as mentioned above, cells need intracellular free calcium to correct spindle organization and undergo normal chromosomal segregation during meiotic division. Kiehart (13) proposed the presence of local gradients of free Ca^{2+} which may orchestrate growth and shortening of microtubules in the spindle to coordinate chromosome movement during mitosis. Subsequently, Alderton *et al.* (27) suggested that regulatory pulses of Ca^{2+} may be highly localized at the kinetochore, where microtubule polymerization/depolymerization is presumed to occur. In this context, it has been suggested by Hepler (28) that BAPTA-AM may act as a shuttle that binds Ca^{2+} on the high side of the gradient and releases the ion into the low side. This effect of BAPTA-AM may disturb the local gradient of Ca^{2+} and, consequently, the cell division process.

Second, there is an interrelationship between the $[\text{Ca}^{2+}]_i$ and the intracellular pH (pH_i), such that changes in $[\text{Ca}^{2+}]_i$ are always accompanied by changes in pH_i (29–31). It is known, for instance, that avian neural crest cells (29), avian heart fibroblasts (30), and human epidermoid A-431 cells (32) treated with BAPTA-AM exhibit a significant and consistent decrease in pH_i . Disturbances in pH_i may play an important role in chromosomal segregation and must, therefore, be related to spindle structure and function (33–35). In fact, pH levels lower and higher than normal affect tubulin polymerization (36,37) and cause chromosomal displacement in human lymphocytes in vitro (33,35).

Finally, a third mechanism by which BAPTA-AM may induce anomalies in chromosomal distribution and segregation in in vitro matured mouse oocytes may be related to the consistent decrease in ATP content exhibited by oocytes (see Table III). Although, it appears that the cytoplasmic microtubule network is not affected by intracellular deprivation of ATP caused

Table III ATP Content of GV and MII Mouse Oocytes Matured In Vitro After BAPTA-AM Loading at the GV Stage

Treatment	No. of experiments	0 hr		1 hr		16 hr	
		<i>n</i>	GV	<i>n</i>	GV	<i>n</i>	MI
Control	3	19	1.02 ± 0.05 ^a	18	1.07 ± 0.07	19	1.10 ± 0.05
DMSO(0.1%)	3	19	1.15 ± 0.06	16	0.99 ± 0.05	20	1.04 ± 0.05
BAPTA-AM (10μM)	3	19	1.01 ± 0.05	18	0.87 ± 0.07	20	0.90 ± 0.04

Three-way ANOVA

Trial

ns

Treatment

 $P \leq 0.005$

Time

ns

Trial × treatment

ns

Trial × time

ns

Treatment × time

ns

3-way interactions

ns

^a Values are means ± SE picomoles per oocyte.

Fig. 2. C-Banded MII chromosomes from an in vitro matured oocyte after BAPTA-AM loading at the GV stage. The oocyte shows a hyperhaploid complement of 21 chromosomes.

by prolonged incubation of cells with metabolic inhibitors (e.g., sodium azide, oligomycin, uncouplers) (38), it is known that cellular ATP depletion caused by inhibitors of energy metabolism induces a gradual breakdown of actin-containing microfilament bundles (39,40). Such an effect is accompanied by a reduction of monomeric (G) actin and a concomitant increase in polymeric or filamentous (F) actin from a detergent-soluble or labile pool (41).

In conclusion, we have shown here that the divalent cation chelating agent BAPTA-AM disturbs the normality of chromosomal distribution in the MII spindle and segregation of chromosomes during the first meiotic division. Although any extrapolation to any other condition/drug that may directly or indirectly decrease the $[Ca^{2+}]_i$ in oocytes should be made with caution, the present findings raise some concern about the treatment of certain disorders such as hypertension, dysmenorrhea, bladder instability, etc., by calcium channel blockers in women wishing to get pregnant. It should be stressed, for instance, that although calcium channel blockers do not appear to have any teratogenic or adverse effect on the fetus (42), it has been shown that they reduce the potential for maturation of mouse and rabbit oocytes (43). Another matter of concern relates to the use of Ca^{2+}/Mg^{2+} -free media to disrupt the intercellular junctions among blastomeres and so facilitate conceptus biopsy. Although this procedure does not appear to affect the survival potential in vitro of mouse conceptuses (44), potential detrimental effects on the chromosomal distribution and segregation of

conceptus cells should not be ignored. In fact, Tombes *et al.* (18) have reported that external Ca^{2+} deprivation induces abnormalities in microtubules and chromatin configuration, at least during meiosis I.

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