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A Novel Assay System of Chemicals Using In-vitro Maturation of Mouse Oocytes: Effects of Carbendazim and Griseofulvin

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Abstract: *In order to develop an in-vitro assay system for detection of cytogenetic toxicity of chemicals, we cultured mouse oocytes in vitro with two kinds of spindle poisons, carbendazim (MBC) and griseofulvin (GF). When cultured for 15 h with MBC (6 μg/ml), the majority of the oocytes arrested maturation at the metaphase in the first meiosis. This effect of MBC could be achieved with the latter half of the exposure during 15 h for the entire culture. In contrast, a significant proportion of the oocytes cultured with GF (10 μg/ml) could not continue meiosis from the germinal vesicle stage. Therefore, we characterized the difference in the effects of MBC and GF on meiotic progression of mouse oocytes by using this in-vitro assay system, demonstrating that the system would be useful for detection of cytogenetic toxicity of chemicals.*
Key words: *Meiosis, Oocyte, Mouse, Carbendazim, Griseofulvin*

Introduction

The cytogenetic toxicity of chemicals has been studied and evaluated in various *in-vivo* and *in-vitro* systems [1]. Strategies for the detection of cytogenetic toxicity generally involve a variety of approaches which involve exposing test chemicals to various stages of somatic cell division. Nevertheless, some chemicals may cause damage to germ cells but not to somatic cells, due to the differences between meiosis and somatic cell division. Such differences may arise from the biological characteristics in the process of cell division.

The first meiotic division of oogenesis occurs during the embryonic stage, where the cells are arrested at diakinesis [2]. They remain at this stage, but the liberation of oocytes from antral follicles into a chemically defined medium is followed by synchronized progress of meiosis and completion of the first meiotic division [3]. As pointed out by Donahue [4], the spontaneous resumption of meiosis together with the ease in recognizing each stage makes the *in-vitro* culture system suitable for screening chemicals for cytogenetic toxicity [5].

The object of the present study was to develop the *in-vitro* assay system for detection of the cytogenetic toxicity of chemicals. To achieve this purpose, we cultured mouse oocyte *in vitro* with two kinds of chemicals, carbendazim (MBC) and griseofulvin (GF), both of which are known as a spindle poison for various somatic cells. MBC is a broad-spectrum benzimidazole fungicide with low toxicity [6]. At high doses, inhibition of fungal mitotic microtubule formation was reported [7]. GF, an oral antibiotic fungicide used for treating dermatophytosis [8], is known to have a cytogenetic effect in various mammalian cells [9]. It can bind to microtubule-associated proteins (Maps) and affects

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their incorporation into microtubules, thereby influencing the stability of microtubules [10].

Materials and Methods

Animals and collection of oocytes

Female mice (Slc:ICR) were obtained from Japan Slc Inc. (Hamamatsu, Japan) at 3 weeks of age. They were reared under a 12L:12D lighting schedule with lights on at 0700, and provided water and commercial diet *ad libitum*.

Oocytes were isolated from 3- to 4-week-old female mice. Follicular development was stimulated by i.p. injection of 5 I.U. pregnant mare's serum gonadotropin (PMSG; Peamex, Sankyo-ale, Tokyo). The animals were sacrificed by cervical dislocation 48 h after the PMSG treatment. The ovaries were removed immediately, and each pair of ovaries was placed in a tissue culture dish (Falcon 3001, Becton Dickinson Labware, Franklin Lakes, NJ) containing 0.5 ml of modified Whitten's medium [11] supplemented with 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical, St. Louis, MO) to arrest the oocytes at diakinesis of the meiosis until the beginning of culture. Cumulus-oocyte complexes (COCs) were collected by follicular puncture with a 27-gauge needle. In this way, 20–40 COCs were obtained from each mouse.

In-vitro maturation of oocytes

After washing twice with modified Whitten's medium without IBMX, COCs were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 15 h with or without various concentration of MBC (1H-benzimidazol-2-ylcarbamic acid methyl ester; Aldrich Chemical Co. Inc., Milwaukee, WI) or GF (7-chloro-2', 4, 6-trimethoxy-6'-methylspiro[benzofuran-2(3H),1'-[2]cyclohexene]-3,4'-dione; purity 95%, Sigma Chemical, St. Louis, MO). MBC and GF were dissolved in dimethyl sulfoxide (DMSO) and freshly prepared solutions in modified Whitten's medium were used to treat COCs. DMSO never exceeded 0.1%, and no adverse effects of DMSO on oocyte maturation were observed at this concentration.

All media were sterilized by passing through a 0.22 μm Millipore filter and were equilibrated to culture conditions for at least 3 h before use.

Analysis of oocytes

At the end of culture, cumulus cells were removed from COCs by gentle pipetting. Oocytes were examined microscopically for the presence of intact germinal vesicle (GV) and the first polar body. When the GV and the first polar body was not observed, the oocyte was classified as at the metaphase in the first meiosis (Met I), and when the first polar body was observed, the oocyte was classified as at the metaphase in the second meiosis (Met II) [12].

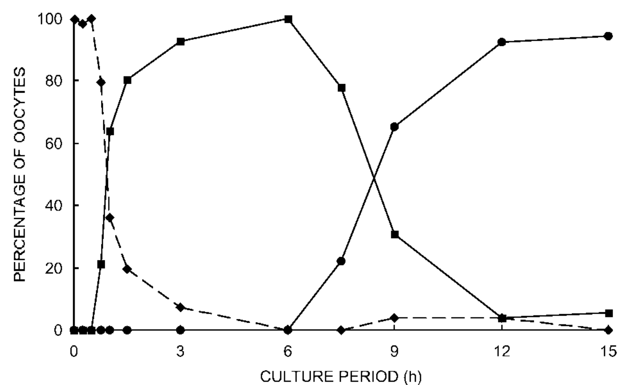

Fig. 1. Time course of oocyte maturation in modified Whitten's medium. The oocytes obtained from ICR mice were cultured in modified Whitten's medium for 0 (50), 0.25 (55), 0.5 (46), 0.75 (54), 1 (47), 1.5 (53), 3 (27), 6 (29), 7.5 (9), 9 (26), 12 (26) or 15 (36) h (number of oocytes in parenthesis). Each oocyte was observed to be classified as follows: germinal vesicle stage (◆); metaphase in the first meiosis (■); metaphase in the second meiosis (●).

Statistical analyses

All of the data obtained were statistically analyzed by Fisher's exact test [13]. All statements of significance were based on a probability level of <0.05.

Results

When oocytes were isolated from ICR mice after PMSG treatment and cultured in modified Whitten's medium, most of them spontaneously resumed meiosis (Fig. 1). Almost all oocytes exhibited intact GV until 30 min of culture and then the percentage of GV oocytes decreased acutely until 1.5 h and then gradually decreased until 6 h (Fig. 1). The proportion of Met I oocytes increased after 30 min of culture and reached a peak at 6 h following decrease until the end of culture (Fig. 1). The Met II oocytes were first observed at 7.5 h of culture and almost all oocytes were arrested at Met II after 15 h of culture (Fig. 1).

In the next experiment, oocytes were cultured with various concentrations of MBC (0.6, 2 or 6 μg/ml) or GF (1, 3 or 10 μg/ml) for 15 h to examine the effects of

Table 1. Effects of MBC and GF on oocyte maturation *in vitro*

Concentration of MBC or GF (μg/ml)	No. of oocytes analyzed	GV (%)	Met I (%)	Met II (%)
MBC 0	63	4 (6.3)	3 (4.8)	56 (88.9)
MBC 0.6	57	2 (3.5)	2 (3.5)	53 (93.0)
MBC 2.0	89	13 (14.6)	27 (30.3)**	49 (55.1)**
MBC 6.0	67	9 (13.4)	45 (67.2)**	13 (19.4)**
GF 0	50	8 (16.0)	0 (0)	42 (84.0)
GF 1	65	9 (13.8)	3 (4.6)	53 (81.5)
GF 3	62	15 (24.2)	9 (14.5)**	38 (61.3)**
GF 10	71	26 (43.3)**	45 (56.7)**	0 (0)**

The oocytes obtained from ICR mice were cultured in modified Whitten's medium containing various concentration of MBC or GF at 37°C. After 15 h of culture, each oocyte was observed to be classified as follows: GV; germinal vesicle stage, Met I; metaphase in the first meiosis, Met II; metaphase in the second meiosis. An asterisk denotes a significant difference from the value when cultured without chemicals of each corresponding stage. **; P<0.01.

Table 2. Stage specific effects of MBC and GF on mouse oocyte maturation *in vitro*

Time of exposure (h)	No. of oocytes analyzed	GV (%)	Met I (%)	Met II (%)
MBC 0 (0–15)	56	5 (8.9)	3 (5.4)	48 (85.7)
MBC 15 (0–15)	51	2 (3.9)	15 (29.4)**	34 (66.7)*
MBC 7 (0–7)	52	6 (11.5)	1 (1.9)	45 (86.5)
MBC 8 (7–15)	48	3 (6.3)	20 (41.7)**	25 (52.1)**
GF 0 (0–15)	34	3 (8.8)	4 (11.8)	27 (79.4)
GF 15 (0–15)	42	24 (57.1)**	18 (42.9)**	0 (0)**
GF 7 (0–7)	34	2 (5.9)	9 (26.5)	23 (67.6)
GF 8 (7–15)	34	9 (26.5)	17 (50.0)**	8 (23.5)**

The oocytes obtained from ICR mice were exposed in 2.0 μg/ml of MBC or 10 μg/ml of GF for 15 h during the entire *in-vitro* maturation, 7 h between GV and Met I, 8 h between Met I and Met II, respectively. After the culture, all oocytes were classified into each meiotic stage as follows: GV; germinal vesicle, Met I; metaphase in the first meiosis, Met II; metaphase in the second meiosis. An asterisk denotes a significant difference from the value when cultured without chemicals for each corresponding stage. *; P<0.05, **; P<0.01.

these chemicals on the meiotic progress. As shown in Table 1, the oocytes treated with MBC showed a dose-dependent inhibition of the meiotic progress. More than 60% of the oocytes cultured with 6 μg/ml of MBC arrested the meiosis at Met I. In corresponding to the increase in Met I oocytes, the percentage of Met II oocytes was significantly decreased in the presence of MBC.

The meiosis in the oocytes exposed to more than 3 μg/ml of GF was inhibited dose-dependently, and more than 40% of the oocytes cultured with 10 μg/ml of GF were arrested at GV stage (Table 1). Corresponding to the increase in GV stage oocytes, the proportion of Met II oocytes was significantly decreased. No oocyte at the Met II stage was observed when cultured with 10 μg/ml of GF.

In the next series of experiments, we cultured mouse oocytes with 2 μg/ml of MBC or 10 μg/ml of GF for 15 h, for the initial 7 h, or for the latter 8 h of culture, to evaluate the stage-specific effects of exposure to these chemicals during meiotic progression. When the oocytes were exposed to MBC for the initial 7 h and continued to culture without chemical until 15 h, the progression of meiosis was similar to the control (Table 2). On the other hand, a similar effect of MBC to the entire 15 h of exposure was observed in the latter 8 h of exposure (Table 2).

Whereas exposure of the oocytes to GF for 15 h caused significant arrest of meiosis at the GV stage, a similar proportion of Met II oocytes as in the control culture were detected in the oocytes exposed to GF for the first 7 h of the culture (Table 2). The inhibition of

meiotic progress of the oocytes was shown in the exposure to GF during the latter half of the culture period (Table 2).

Discussion

In the present study, we examined the effects of MBC and GF, known as spindle poison, on meiotic progression in maturing mouse oocytes *in vitro*.

MBC exposure interfered with meiotic cell cycle progression by delaying advancement to Met II in a dose-related manner. It was reported that MBC exposure for 7 h induced a delay in cell cycle progression of mouse oocytes during *in-vitro* maturation [14]. In the observation of stage-specific effects in the present experiment, oocytes exposed to MBC for 7 h and cultured in control medium for an additional 8 h recovered the delay of cell cycle progression to Met II. Similar reversibility was reported in diethylstilbestrol-induced meiotic delay in mouse oocytes during *in-vitro* maturation [15].

The effects of GF on meiotic progression in maturing mouse oocytes *in vitro* were shown for first time. There were some reports in which GF treatment induced meiotic delay during *in-vivo* oocyte maturation [16–18]. In their reports, GF treatment at Met I resulted in the induction of meiotic delay as demonstrated by the detection of ovulated Met I oocytes. In this study, oocytes arrested at the GV stage were prominent in the treatment with 10 μg/ml of GF for 15 h of culture. Although we have not observed the meiotic stage after 7 h of culture with GF, it could be speculated that most of the oocytes not able to progress beyond the GV stage can resume meiosis during the additional 8 h of culture without GF.

Our findings indicate that there may be some differences of the effect of spindle poisons on the first and second meiotic divisions. In the first meiotic division, homologous chromosome pairing and crossing-over occur before segregation of homologous chromosomes at anaphase I. The process of the second meiotic division, similar to the mitotic cell division, ended the chromatid segregation at anaphase II. It is known that MBC disrupts mitosis by binding to tubulin [19] and preventing polymerization [20], and GF can reduce the incorporation of Maps into microtubules and interact directly with tubulin [21]. Nevertheless, in view of the scarcity of information on the effects of spindle poisons in meiosis, it is necessary to conduct a validated study of aneuploid induction during meiosis.

There is evidence for a very narrow time window for activity for some chemicals in cell division [22, 23]. It is necessary to conduct a precise time-course study, with treatment times narrowly spaced, to maximize the chance of detecting a toxicity of chemicals. Therefore, *in-vitro* maturating mouse oocytes are a useful model for the identification of the cytogenetic toxicity of chemicals and for the detection of stage-specific effects of chemicals during meiosis.

References

1) Sofuni, T. (1993): Japanese guidelines for mutagenecity testing. Ministry of Agriculture, Forestry and Fisheries; Ministry of Health and Welfare; Ministry of Labor. Environ. Mol. Mutagen., 21, 2–7.
2) Borum, K. (1961): Oogenesis in the mouse. A study of the meiotic prophase. Exp. Cell Res., 24, 495–507.
3) Eppig, J.J., O'brien, M. and Wigglesworth, K. (1996): Mammalian oocyte growth and development in vitro. Mol. Reprod. Dev., 44, 260–273.
4) Donahue, R.P. (1968): Maturation of the mouse oocyte in vitro. I. Sequence and timing of nuclear progression. J. Exp. Zool., 169, 237–250.
5) Eichenlaub-Ritter, U. and Boll, I. (1989): Nocodazole sensitivity, age-related aneuploidy, and alterations in the cell cycle maturation of mouse oocytes. Cytogenet. Cell. Genet., 52, 170–176.
6) Seiler, J.P. (1975): Toxicology and genetic effects of benzimidazole compounds. Mutat. Res., 32, 151–152.
7) Davidse, L.C. (1973): Antimitotic activity of methyl benzimidazol-2-yl carbamate in *Aspergillus nidulans*. Pest. Biochem. Physiol., 3, 317–320.
8) Grove, J.F. (1963): Griseofulvin. Q. Rev. Chem. Soc., 17, 1–19.
9) Carli, L.D. and Larizza, L. (1988): Griseofulvin. Mutat. Res., 195, 91–126.
10) Roobol, A., Gull, K. and Pogson, C.J. (1977): Evidence that griseofulvin binds to a microtubule associated protein. FEBS Lett., 75, 149–153.
11) Katsuki, M. (1984): Mouse embryo culture *in vitro* and micromanupilation. Seitai no Kagaku, 35, 90–95 (in Japanese).
12) Eichenlaub-Ritter, U. and Betzendahl, I. (1995): Chloral hydrate induced spindle aberrations, metaphase I arrest and aneuploidy in mouse oocytes. Mutagenesis, 10, 477–486.
13) Hollander, M. and Wolfe, D.A. (1999): Nonparametric statistical methods. 2nd ed. (Hoboken, N.J., ed.) John Wiley & Sons.
14) Can, A. and Albertini, D.F. (1997): Stage specific effects of carbendazim (MBC) on meiotic cell cycle progression in mouse oocytes. Mol. Reprod. Dev., 46, 351–362.
15) Can, A. and Semiz, O. (2000): Diethylstilbesterol (DES)-induced cell cycle delay and meiotic spindle disruption in mouse oocytes during in-vitro maturation. Mol. Hum. Reprod., 16, 154–162.
16) Tiveron, C., Marchetti, F., Bussani, B. and Pacchierotti, F.

(1992): Griseofulvin-induced aneuploidy and meiotic delay in female mouse germ cells. I Cytogenetic analysis of metaphase II oocytes. Mutat. Res., 266, 143–150.

17) Mailhes, J.B., Marchetti, F. and Aardema, M.J. (1993): Griseofulvin-induced aneuploidy and meiotic delay in mouse oocytes: effect of dose and harvest time. Mutat. Res., 300, 155–163.

18) Marchetti, F. and Mailhes, J.B. (1994): Variation of mouse oocyte sensitivity to griseofulvin-induced aneuploidy and meiotic delay during the first meiotic division. Environ. Mol. Mutagen., 23, 179–185.

19) Davidse, L.C. and Flach, W. (1977): Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. J. Cell Biol., 72, 174–193.

20) Jacobs, C.W., Adams, A.E.M., Szaniszlo, P.J. and Pringle, J.R. (1988): Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol., 107, 1409–1426.

21) Mailhes, J.B. and Marchetti, F. (1994): Chemically-induced aneuploidy in mammalian oocytes. Mutat. Res., 320, 87–111.

22) Mailhes, J.B. and Yuan. Z.P. (1987): Differential sensitivity of mouse oocytes to colchicines-induced aneuploidy. Environ. Mol. Mutagen., 10, 183–188.

23) Mailhes, J.B. and Marchetti, F. (1994): The influence of postovulatory aging on the retardation of mouse oocyte maturation and chromosome segregation induced by vinblastine. Mutagenesis, 9, 541–545.