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Unique Effects of Spindle Inhibitors on Mammalian Oocyte Meiosis (42164)

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Abstract. The effects of Nocodazole, reported to be a rapidly reversible inhibitor of microtubules in somatic cells (1), and Colcemid, a classic microtubule inhibitor, were studied for their effects on mouse and cow oocyte *in vitro* meiotic resumption. When present throughout the maturation period to Metaphase II/Polar Body I, both compounds predictably inhibited progression at Metaphase I (MI). An unexpected effect was seen on mouse germinal vesicle breakdown (GVB) with both inhibitors, but not in cow oocytes tested with Nocodazole. Recovery from Nocodazole was notably retarded in mouse oocytes even with brief exposure times. Addition of 1 μg/ml of Nocodazole 10 min after commencement of mouse and cow oocyte incubation was sufficient time to allow normal GVB, while inhibition at MI still took place suggesting that the critical events of GVB occur very quickly *in vitro*.

The assembly of the spindle during *in vivo* and *in vitro* resumption of meiosis in mammalian oocytes has been well described (2–5), and synthesis of tubulin during this period has been demonstrated in the mouse oocyte by Schultz *et al.* (6). While no data exist concerning exact homology of cytoskeletal proteins of mammalian meiotic and mitotic spindles, the inhibition of meiotic spindle assembly by classical compounds has been documented in several species, both *in vitro* (7) and *in vivo* (8). In addition, selective arrest of meiotic progression in Chinese hamster oocytes with Colchicine has been shown to produce various degrees of chromosomal nondisjunction at meiosis I, presumably due to inhibition of the formation of "small numbers of microtubules" (9). It was the purpose of the present study to examine in detail the effects on oocyte meiotic resumption of the rapidly reversible microtubule inhibitor, Nocodazole, and to compare it with the classical inhibitor, Colcemid, in an attempt to gain further insights into mammalian oocyte spindle function at the time of meiotic resumption.

Materials and Methods. Mouse and cow oocyte *in vitro* meiotic maturation was studied using previously described techniques (10–12). Equal sized, microscopically normal, cumulus enclosed oocytes were rapidly removed by pricking medium-sized follicles from ovaries of young adult mixed breed dioestrous cows or regularly cycling Swiss mice (CAMM). The cow oocytes were obtained in the autumn at a local slaughterhouse and returned to the laboratory within 20 min. For each species the oocytes were pooled in McCoy's 5A medium (Grand Island Biological Co). Within 5 min of pooling, they were distributed to test petri dishes containing appropriate media. Three replicates were carried out for each experiment. Incubation was carried out at 37°C in a high humidity 5% CO_2/air atmosphere for 16 hr for Metaphase II (MII)/Polar Body I with mouse oocytes or 27 hr for cow oocytes. After appropriate incubation times, oocytes were prepared for cytologic observation by a modification of the method of Tarkowski (13), and stained with 2% toluidine blue in resin. Well-spread, intact nuclei were selected using a 40× bright light objective and endpoints of germinal vesicle (GV), Metaphase I (MI) or II were scored using a Zeiss 100X Planapo objective. The state of germinal vesicle breakdown (GVB) in the second and third series of experiments was subclassified into I, II, and III using a slight modification of the classification of Donahue (14). A further class IV which met criteria of atresia was also designated. Class I of GVB was characterized as a nucleus containing a nucleolus, fine filamentous chromatin, and many chromocenters; Class II referred to a nucleus with a dark staining nucleolus and condensing chro-

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matin filaments; Class III was characterized by visible condensed portions of bivalents and absence of a detectable nucleolus; Class IV germinal vesicles revealed fragmented and disorganized chromatin and an irregular nuclear membrane. The spindle inhibitor Nocodazole (*methyl* [5-2 thienyl/carbonyl)-1H-benzimidazol-2yl] (Sigma, St. Louis, Mo.) carbamate dissolved in dimethylsulfoxide was studied utilizing both species of oocytes. Colcemid (deacety-*N*-methylcolchicine) (Sigma, St. Louis, Mo.) diluted with McCoy's 5A medium was studied with mouse and cow oocytes for selected comparisons to the Nocodazole effects.

In the first series of experiments, Nocodazole and Colcemid concentrations from 50 pg/ml to 1 μg/ml were examined for detecting threshold effects on the stages of *in vitro* mouse oocyte nuclear maturation (Table I) and 500 pg to 1 μg of Nocodazole and 50 pg/ml to 1 μg/ml of Colcemid for effects on cow oocytes (Table I).

The second series studied recovery from exposure to Nocodazole or Colcemid by incubating oocytes with either 500 pg or 1 μg/

TABLE I. EFFECTS OF NOCODAZOLE OR COLCEMID ON GERMINAL VESICLE BREAKDOWN AND MEIOTIC METAPHASE STAGES OF *in Vitro* MATURED MOUSE AND COW OOCYTES

Concentration per ml	Total oocytes (*n*)	Oocytes % (*n*)			
		GV	MI	MII	Total matured
Mouse					
Nocodazole					
0[a]	141	24 (35)	21 (29)	55 (77)	76 (106)
50 pg	46	37 (17)	24 (11)	39 (18)	63 (29)
250 pg	39	46 (18)*	18 (7)	36 (14)*	54 (21)*
500 pg	93	50 (47)*	13 (12)	37 (34)*	50 (46)*
1 ng	81	65 (52)*	12 (10)	23 (19)*	35 (29)*
40 ng	56	64 (36)*	34 (19)*	2 (1)*	36 (20)*
1 μg	78	62 (48)*	33 (26)*	5 (4)*	38 (30)*
DMSO 10 μg	43	19 (8)	23 (10)	58 (25)	81 (35)
Colcemid					
0[a]	84	24 (20)	15 (13)	61 (51)	76 (64)
50 pg	68	34 (23)	15 (10)	51 (35)	66 (45)
500 pg	71	31 (22)	27 (19)*	42 (30)*	69 (49)
1 ng	122	52 (64)*	18 (22)*	30 (36)*	48 (58)*
4 ng	106	64 (68)*	20 (21)*	16 (17)*	36 (38)*
40 ng	136	68 (93)*	32 (43)*	0 (0)*	32 (43)*
1 μg	140	31 (44)	64 (90)*	5 (6)*	69 (96)
Cow					
Nocodazole					
0[b]	101	18 (19)	17 (17)	65 (65)	82 (82)
500 pg	54	19 (10)	37 (20)*	44 (24)*	81 (44)
10 ng	117	16 (19)	59 (69)*	25 (29)*	84 (98)
100 ng	55	20 (11)	71 (39)*	9 (5)*	80 (44)
1 μg	68	15 (10)	78 (53)*	7 (5)*	85 (58)
DMSO 10 μg	34	15 (5)	20 (7)	65 (22)	85 (29)
Colcemid					
0	32	12 (4)	19 (6)	69 (22)	88 (28)
50 pg	60	13 (8)	2 (1)	85 (51)	87 (52)
500 pg	33	18 (6)	12 (4)	70 (23)	82 (27)
1 ng	61	8 (5)	7 (4)	85 (52)	92 (56)
4 ng	18	11 (2)	11 (2)	78 (14)	89 (16)
40 ng	28	21 (6)	25 (7)	54 (15)	79 (22)
1 μg	18	50 (9)*	11 (2)	39 (7)*	50 (9)*

[a] 16 hr culture.
[b] 27 hr culture.
* $P > .05$.

ml of the inhibitor in McCoy's 5A for selected time periods (Table II), washing three times in fresh media, followed by 16-hr further incubation in control media for mouse or 27 hr for cow oocytes. Recovery of mouse oocytes from the effects of 500 pg/ml of Nocodazole was studied because of the unexpected finding of a primary effect at this concentration on GVB. Since 1 μg/ml of Nocodazole affected GVB, as well as Metaphase I, recovery from this concentration was compared to the recovery from 1 μg/ml of Colcemid which affected only Metaphase I. Cow oocyte recovery from the effect of 1 μg/ml of Nocodazole exclusively on Metaphase I was selected for comparison with the more complex mouse oocyte response at this concentration.

In the third series of experiments, 500 pg/ml or 1 μg/ml of the inhibitors was added to the media at different times (Table III) after beginning the oocyte cultures, with subsequent incubation for the appropriate total maturation to attempt correlation of the effects of the inhibitors on some early events of GVB known to occur by specific times of culture (3, 5). In all experiments, data were analyzed by the χ^2 method.

Results. *Series 1: Dose response.* Mouse oocytes (Table I) treated with 250 pg–1 ng/ml of Nocodazole for 16 hr were noted to show inhibition at GVB and a decrease in the number of MII stages achieved, with no discernible effect on the percentage which reached MI. Increasing the concentration to 40 ng/ml inhibited GVB, blocked meiotic progression at MI, and almost completely eliminated progression to MII. A concentration of 1 μg/ml produced identical effects to the 40-ng concentration. Control experiments with 10 μg/ml of DMSO in McCoy's 5A revealed no effect on oocyte maturation of either species. Colcemid, at a concentration of 500 pg/ml increased the number of mouse oocytes held at MI, and hence decreased the maturation to MII, but unlike Nocodazole, did *not* have a significant effect on GVB. A concentration of 1–40 ng/ml inhibited GVB, MI, and progression to MII in a dose–response manner, while a concentration of 1 μg/ml effected a hold at MI with a sharp reduction in MII production, but no significant increase in cells inhibited at GV. Unlike mouse oocytes, cow oocytes (Table I) did not show any effect of 500 pg to 1 μg/ml of Nocodazole on GVB. However, an inhibition at MI appeared with concentrations of 500 pg/ml which increased with the increments to 100 ng and 1 μg wherein almost total inhibition of progress to Metaphase II/Polar Body I resulted. Colcemid at concentrations of 50 pg/ml to 40 ng/ml had no effect on GVB or progression to MI or MII (Table I). A concentration of 1 μg/ml inhibited GVB, and progression to MII.

Series 2: Recovery. Mouse oocytes (Table II) were treated with 500 pg/ml of Nocodazole which had been shown in Series I to primarily inhibit GVB. When they were exposed for 10 min to 2 hr, then placed in fresh media, many oocytes were still inhibited at GV by the end of 16 hr of further incubation, although a few oocytes had proceeded through to MI and MII. Classes I and III of GVB were achieved in these inhibited oocytes in relatively normal numbers despite up to 1 hr of exposure, but increased oocytes at Class II were found. Treatment for 1.5 hr produced an increase of Class III in 31% of the GVB and 2 hr of exposure inhibited GV at the beginning of breakdown at Class I. Two-hour treatment permitted some recovery of the capability to reach MI and MII.

Similar experiments with a concentration of 1 μg/ml (Table II) revealed an increase of Class II GVB after 10 min of treatment. Thirty minutes to 8 hr of exposure to Nocodazole was sufficient to produce an additional inhibition at MI, although 1 hr of treatment did not also affect GVB significantly. All time periods of exposure prevented attainment of MII. Sixteen hours of treatment followed by an equal recovery period resulted in an almost complete inability to proceed beyond GV which showed extensive atresia. The atretic condition of GV (Class IV) was a common feature of almost all of the Nocodazole mouse oocyte recovery studies.

The mouse oocyte experiments testing a concentration of 1 μg/ml of Colcemid revealed a block at GV with exposure of 10 min to 8 hr. Attainment of MII was blocked with treatment for all of these intervals, but MI only at the 10-min and 1-hr periods. Recovery following 16-hr exposure was almost complete, unlike the results with Nocodazole.

A significant population of cow oocytes

TABLE II. RECOVERY OF MOUSE AND COW OOCYTE MATURATION AFTER EXPOSURE TO NOCODAZOLE OR COLCEMID

	Total oocytes (*n*)	Oocytes % (*n*)							
		Total GV	GV classes				MI	MII	Total matured
			I	II	III	IV (atretic)			
Mouse									
Nocodazole (500 pg/ml)									
0[a]	134	24 (32)	9 (12)	8 (11)	7 (9)	0 (0)	24 (32)	52 (70)	76 (102)
10 min[b]	166	65 (108)*	10 (16)	19 (32)*	4 (7)	32 (53)*	9 (15)*	26 (43)*	35 (58)*
30 min	57	88 (50)*	2 (1)	38 (22)*	11 (6)	37 (21)*	4 (2)*	8 (5)*	12 (7)*
1 hr	90	87 (78)*	6 (5)	44 (40)*	9 (8)	28 (25)*	8 (7)*	5 (5)*	13 (12)*
1.5 hr	64	78 (50)*	0 (0)*	11 (7)	31 (20)*	36 (23)*	9 (6)*	13 (8)*	22 (14)*
2 hr	131	61 (80)*	36 (47)*	2 (3)	0 (0)*	23 (30)*	21 (28)	18 (23)*	39 (51)*
Nocodazole (1 μg/ml)									
0	88	21 (18)	9 (8)	5 (4)	5 (4)	2 (2)	27 (24)	52 (46)	79 (70)
10 min	64	70 (45)*	5 (3)	1 (1)*	39 (25)*	25 (16)*	27 (17)	3 (2)*	30 (19)*
30 min	145	48 (70)*	2 (3)*	30 (43)*	10 (15)	6 (9)	47 (68)*	5 (7)*	52 (75)*
1 hr	67	31 (21)	2 (1)*	7 (5)	0 (0)	22 (15)*	66 (44)*	3 (2)*	69 (46)
8 hr	88	60 (53)*	0 (0)*	18 (16)*	3 (3)	39 (34)*	40 (35)*	0 (0)*	40 (35)*
16 hr	51	90 (46)*	0 (0)*	0 (0)*	12 (6)	78 (40)*	4 (2)*	6 (3)*	10 (5)*
Colcemid (1 μg/ml)									
0	72	14 (10)	12 (9)	2 (1)	0 (0)	0 (0)	21 (15)	65 (47)	86 (62)
10 min	88	57 (50)*	15 (13)	2 (2)	0 (0)	40 (35)*	43 (38)*	0 (0)*	43 (38)*
30 min	74	80 (59)*	23 (17)	30 (22)*	9 (7)*	18 (13)*	19 (14)	1 (1)*	20 (15)*
1 hr	46	63 (29)*	15 (7)	20 (9)*	6 (3)*	22 (10)*	37 (17)*	0 (0)*	37 (17)*
8 hr	74	84 (62)*	11 (8)	19 (14)*	9 (7)*	45 (33)*	16 (12)	0 (0)*	16 (12)*
16 hr	26	15 (4)	0 (0)*	4 (1)	0 (0)	11 (3)*	31 (8)	54 (14)	85 (22)
Cow									
Nocodazole (1 μg/ml)									
0	131	23 (30)	15 (19)	2 (3)	2 (3)	4 (5)	16 (21)	61 (80)	77 (101)
10 min	133	37 (49)*	5 (7)*	13 (17)*	0 (0)*	19 (25)*	6 (8)*	57 (76)	63 (84)*
30 min	124	31 (38)	2 (2)*	0 (0)	9 (11)*	20 (25)*	20 (25)	49 (61)*	69 (86)
1 hr	86	58 (50)*	10 (9)	0 (0)	0 (0)	48 (41)*	16 (14)	26 (22)*	42 (36)*
8 hr	99	21 (21)	19 (19)	2 (2)	0 (0)	0 (0)	67 (66)*	12 (12)*	79 (78)

[a] No treatment; 16 hr.
[b] Exposure time.
* $P < 0.05$.

TABLE III. EFFECTS OF ADDING NOCODAZOLE OR COLCEMID TO MOUSE OR COW OOCYTES AT INTERVALS AFTER BEGINNING CULTURE

Time of addition	Total oocytes (*n*)	Oocytes % (*n*)							
			GV classes						
		Total GV	I	II	III	IV (atretic)	MI	MII	Total matured
Mouse									
Nocodazole (500 pg/ml)									
0	114	28 (32)	4 (5)	16 (18)	2 (2)	6 (7)	16 (18)	56 (64)	72 (82)
10 min	82	55 (45)*	15 (12)*	7 (6)*	20 (16)*	13 (11)	15 (12)	30 (25)*	45 (37)*
30 min	120	82 (98)*	6 (7)	30 (36)**	9 (11)	37 (44)*	8 (10)	10 (12)*	18 (22)*
1 hr	125	56 (70)*	17 (21)*	12 (15)*	8 (10)	19 (24)*	16 (20)	28 (35)*	44 (55)*
1.5 hr	99	61 (60)*	6 (6)	23 (22)	7 (7)	25 (25)***	13 (13)	26 (26)*	39 (39)*
Nocodazole (1 μg/ml)									
0	112	19 (21)	9 (10)	4 (4)	6 (7)	0 (0)	23 (26)	58 (65)	81 (91)
10 min	64	19 (12)	1 (1)*	13 (8)*	0 (0)*	5 (3)*	78 (50)*	3 (2)*	81 (52)
30 min	96	25 (24)	14 (13)	8 (8)	0 (0)*	3 (3)	67 (64)*	8 (8)*	75 (72)
1 hr	67	46 (31)*	18 (12)	3 (2)	0 (0)*	25 (17)*	49 (33)*	5 (3)*	54 (36)*
2 hr	44	39 (17)*	16 (7)	7 (3)	12 (5)	4 (2)	57 (25)*	4 (2)*	61 (27)*
Colcemid (1 μg/ml)									
0	106	15 (16)	11 (12)	2 (2)	1 (1)	1 (1)	15 (16)	70 (74)	85 (90)
10 min	87	24 (21)	16 (14)	7 (6)	0 (0)	1 (1)	75 (65)*	1 (1)*	76 (66)
30 min	51	45 (23)*	39 (20)***	4 (2)	0 (0)	2 (1)	49 (25)*	6 (3)*	55 (28)*
1 hr	70	27 (19)*	21 (15)	6 (4)	0 (0)	0 (0)	64 (45)*	9 (6)*	73 (51)*
Cow[b]									
Nocodazole (1 μg/ml)									
0	130	13 (17)	12 (16)	0 (0)	0 (0)	1 (1)	19 (25)	68 (88)	87 (113)
10 min	140	15 (21)	13 (18)	0 (0)	0 (0)	2 (3)	77 (108)*	8 (11)*	85 (119)
30 min	110	30 (33)*	0 (0)*	0 (0)	0 (0)	30 (33)*	68 (75)*	2 (2)*	70 (77)*
1 hr	103	5 (5)*	2 (2)*	1 (1)	0 (0)	2 (2)	91 (94)*	4 (4)*	95 (98)*
8 hr	113	24 (27)*	8 (9)	2 (2)	8 (9)*	6 (7)	72 (81)*	4 (5)*	76 (86)*

[a] 16-hr culture.
[b] 27-hr culture.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

treated with 1 μg of Nocodazole for 10 min (Table II) were at the Class IV stage of GV at the end of the subsequent 27 hr of culture in fresh media, but unlike mouse oocytes, the grossly normal remainder had achieved MII. This capability for reaching MII was diminished by 30-min exposure, and 1 hr produced many atretic germinal vesicles, as well as a decreased number of oocytes achieving MII. Exposure for 8 hr yielded a persistent block at MI after further culture.

Series 3: Latent treatment. Effects of the addition of Nocodazole or Colcemid at intervals after commencement of mouse or cow oocyte incubation are shown in Table III. Utilizing a concentration of 500 pg/ml of Nocodazole, it was noted that addition to mouse oocytes 10 min to 1.5 hr after the culture had begun, inhibited GVB and did not affect MI, but inhibited the capability for attaining MII. The GVB effect was particularly noted in Class II when Nocodazole was added to 30 min. Use of the higher dose of 1 μg/ml at 10 min to 2 hr produced the characteristic block at GV only when added at 1 or 2 hr, but inhibition at MI and MII was seen at all times studied. Independent of the time of addition, 1 μg/ml of Colcemid primarily effected a block of spindle formation at MI, but also inhibited GVB when added at 30 min or 1 hr. Analysis of cow oocytes after addition of 1 μg/ml of Nocodazole at 10 min to 8 hr following culture commencement and a further culture for 27 hr, revealed a loss of spindle assembly at MI, as well as an inhibitory effect on GVB following the addition at 30 min and 8 hr.

Discussion. While the capability of Nocodazole and Colcemid for inhibiting oocyte meiotic progression at MI was predictable from other studies, the marked effect on the breakdown of the GV over a wide dose range in the mouse oocyte was surprising. Such an effect was seen with the bovine oocyte only at the maximal dose tested (1 μg/ml) or when the Nocodazole was added or removed at definite times of maturation possibly indicating a specific metabolic state of the bovine oocyte when removed from the follicle. The separation of the two major cytologic effects on the mouse oocyte was notable at a threshold concentration of 40 ng/ml of Nocodazole which induced the block at GV and an additional block at MI, with a sharp decrease in MII. Such an effect on the process of GVB of mammalian oocytes by compounds held to have principally microtubule inhibitory properties has not been reported heretofore. Effects of such agents other than directly on microtubules have been reported for Nocodazole in neoplastic cells by de Brabander *et al.* (15) who described general cellular disorganization, and Mizel and Wilson (16) who described the inhibition of nucleoside transport in mammalian cells by colchicine. Direct evidence for destructive effects on aspects of GVB such as chromatin condensation, nucleolar breakdown, and the formation of nuclear envelope doublets described by Szollosi *et al.* (5) and Calarco *et al.* (3) cannot be derived from the present data. The only suggestion of a specific target was seen in the mouse oocyte recovery experiments where Colcemid treatment for 10 min or 1 hr was effective but for 30 min was not. Calarco *et al.* (3) have noted that one feature of GVB at 30 min of mouse oocyte *in vitro* maturation was "chromatin condensation along the nuclear envelope," an observation potentially related to the present finding in an unknown way. The suggestion of critically timed events in cow oocytes identified in the Nocodazole latent experiments at 1 hr also remains obscure since no detailed ultrastructural descriptions of early cow GVB are available (4). The presence of a significant number of atretic oocytes of both species in the recovery experiments would indicate that both agents had permanently interfered with essential functions. That this damage was related to drug concentration and time of exposure after commencement of resumption was highlighted by the response of the oocytes to treatment with 1 μg/ml of Nocodazole as seen in Tables II and III. If the Nocodazole exposure of mouse and cow oocytes occurred from time 0 for 10 min (Table II), GVB, but not MI, was inhibited whereas addition 10 min after commencement of culture (Table III) did not significantly alter total GVB, but did block MI. Effects on critical events of GVB during the first 10 min of culture were suggested by such findings.

The lack of recovery of mouse oocytes from the effects of Nocodazole inhibition was unlike the very rapid recovery of mitotic mammalian microtubules which has been described by Zieve *et al.* (1). Particularly

noteworthy was the lack of recovery of the mouse oocytes in the presence of 500 pg or 1 μg/ml of Nocodazole for even short periods. The recovery of the cow oocytes treated for 10 and 30 min was more successful in reaching MII than the mouse oocyte, and a marked decline in this capability was seen only after the longer exposure of 8 hr when significant inhibition at MI was seen. It is known from the detailed ultrastructural studies of Szollosi *et al.* (5) and Calarco *et al.* (3) that some microtubular assembly takes place during the first 30 min of mouse oocyte culture, proceeds to formation of a small aster by 2 hr and formation of the Metaphase I spindle as early as 4.5 hr. That a principal effect on mouse and cow oocytes during these time periods was on the MI spindle assembly was noted above in the studies in which the 1 μg of Nocodazole was added as early as 10 min after culture had begun. The recovery of mouse oocytes from prolonged exposure to Colcemid of 16 hr was concordant with previous *in vivo* studies (7) and contrasted sharply with the damage to the GV induced by such treatment with Nocodazole. The present data would suggest that oocyte meiotic spindles cannot recover from the effects of Nocodazole as rapidly as somatic spindles. This may be due either to intrinsic structural differences as suggested by Murphy (17), different molecular interactions of the meiotic vs the mitotic units with the inhibitor or different metabolic effects (18). It would now seem reasonable in the light of the new findings presented here to utilize these agents further as discriminatory tools for dissecting out the separate phenomena of GVB and meiotic resumption in mammalian oocytes.

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