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Suppression of meiosis by inhibitors of m-phase proteins in horse oocytes with low meiotic competence

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Summary

Germinal vesicle (GV)-stage horse oocytes with diffuse chromatin are meiotically incompetent and degenerate in culture, whereas horse oocytes having condensed chromatin within the GV are meiotically competent. Degeneration of incompetent oocytes in culture may be related to premature GV breakdown, which could possibly be prevented by inhibition of m-phase protein activity. We examined the effects of 6-dimethylaminopurine (6-DMAP), butyrolactone and roscovitine on GV-stage horse oocytes. Culture in the presence of 2 mM 6-DMAP for 24 h suppressed meiosis (2% MI or MII compared with 38% for untreated oocytes). The proportion of GV-stage oocytes having condensed chromatin was not different between 6-DMAP culture and directly fixed controls; however, the proportion of oocytes with diffuse chromatin was significantly lower, and more oocytes with diffuse chromatin had atypical chromatin than did controls ($p < 0.01$). Culture with butyrolactone at 100 μM suppressed meiosis (5% MI + II). Again, this treatment maintained GV-stage oocytes having condensed chromatin, but the proportion of oocytes with diffuse chromatin was significantly reduced compared with directly fixed controls ($p < 0.05$). Culture with roscovitine at 25 μM was also effective in maintaining GV-stage oocytes having condensed chromatin; however, culture with 100 μM roscovitine did not suppress meiosis or maintain oocytes in the GV stage. These results indicate that meiosis in GV-stage horse oocytes having condensed chromatin may be suppressed by inhibitors of m-phase protein activity; however, oocytes originally having diffuse chromatin appear to degenerate in culture even in the presence of these inhibitors.

Keywords: Butyrolactone, 6-DMAP, Maturation, MPF, Roscovitine

Introduction

Horse oocytes having compact granulosa originate largely from histologically viable follicles, but have low rates of maturation *in vitro* (Hinrichs & Williams, 1997; Hinrichs & Schmidt, 2000). This low meiotic competence is associated with the presence of diffuse chromatin within the germinal vesicle (GV), whereas oocytes with high meiotic competence have condensed chromatin within the GV. Chromatin condensation within the GV, and nuclear maturation rates, increase with increasing follicle size, and a major increase occurs when the follicle diameter reaches >20 mm (Hinrichs & Schmidt, 2000). Thus, oocytes with diffuse chromatin appear to be meiotically incompetent because they are juvenile.

Over 90% of visible horse follicles are ≤20 mm diameter (Hinrichs & Schmidt, 2000), so it would be desirable to develop an *in vitro* method to increase the proportion of meiotically competent oocytes, i.e. to bring those juvenile oocytes, having diffuse chromatin, to a state of meiotic competence. In other species, a suggested method for increasing meiotic and develop-

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mental competence has been to maintain oocytes in the GV stage for a period of culture, during which the cell may gain cytoplasmic maturity (Sirard & Coenen, 1995; Lonergan *et al*., 1997; Saeki *et al*., 1997). In the horse, however, it may be difficult to maintain oocytes having the diffuse chromatin configuration, as the chromatin of these oocytes appears to degenerate in culture (Alm & Hinrichs, 1996; Hinrichs & Williams, 1997; Hinrichs & Schmidt, 2000). Degeneration of incompetent oocytes in culture may be due to initiation of precocious GV breakdown, at a time when the chromatin is inadequately prepared for the resumption of meiosis.

Only one previous report is available on pharmacological treatment for meiotic suppression of horse oocytes. In that report, culture with cycloheximide resulted in maintenance of oocytes having a condensed chromatin configuration within the GV, but GV oocytes with a diffuse chromatin configuration degenerated during treatment (Alm & Hinrichs, 1996). Cycloheximide acts to suppress meiosis by blocking the synthesis of stage-specific proteins, including kinases needed for activation of m-phase proteins (Kastrop *et al*., 1990; Tatemoto & Horiuchi, 1995). The major proteins involved in resumption of meiosis include $p34^{cdc2}$/cyclin B (maturation-promoting factor, MPF) and mitogen-activated protein kinase (MAPK). It has been shown in some species, such as mouse and goat, that incompetent oocytes lack the $p34^{cdc2}$ component of MPF, although they appear to have adequate amounts of cyclin B and MAPK (De Vantery *et al*., 1996; Harouk & Clarke Hugh, 1995; Dedieu *et al*., 1998); competent oocytes have adequate amounts of $p34^{cdc2}$. The situation is not as clear in other species, such as the cow and pig (Levesque & Sirard, 1996; Kubelka *et al*., 1995; Naito *et al*., 1995). In the horse, MPF and MAPK were detected in GV-stage oocytes (Goudet *et al*., 1998), but no work has been done on differences between competent and incompetent GV-stage oocytes in this species.

Specific meiotic inhibitors have been identified in recent years. These include 6-dimethylaminopurine (6-DMAP), butyrolactone I and roscovitine. 6-DMAP, an alkylpurine, is an inhibitor of serine/threonine protein kinases. Culture of oocytes in the presence of 6-DMAP inhibits the burst of phosphorylation that precedes GVBD (Rime *et al*., 1989). Whereas cycloheximide treatment does not suppress GVBD in murine oocytes, 6-DMAP is effective in doing so (Rime *et al*., 1989). In porcine oocytes, cycloheximide treatment suppresses GVBD but fails to inhibit chromatin condensation, whereas 6-DMAP suppresses both events (Kubelka *et al*., 1995). Both cycloheximide and 6-DMAP block meiosis in the majority of bovine oocytes. 6-DMAP is effective at a concentration of 1–2 mM (Levesque & Sirard, 1995; Fulka *et al*., 1991).

Butyrolactone I is a competitive inhibitor of ATP that specifically inhibits the activity of cyclin-dependent kinases, including MPF. However, treatment of bovine oocytes with butyrolactone I results in inhibition of both MPF and MAP kinase activity, indicating that MAP kinase activation may be linked to MPF activation (Kubelka *et al*., 2000). Butyrolactone I inhibits meiotic maturation in bovine oocytes in a dose-dependent manner, with maximum suppression in bovine serum albumin (BSA)-containing medium occurring at about 100 μM (Motlik *et al*., 1998; Kubelka *et al*., 2000).

Roscovitine has been only recently described. It is structurally related to, but more potent than, olomoucine and inhibits binding of ATP to the cyclin B component of MPF. Roscovitine inhibited fertilisation-induced calcium oscillations in mouse oocytes at 50 μM (Deng & Shen, 2000), and maintained cattle oocytes at the germinal vesicle stage at 25 μM, an effect that was fully reversible (Mermillod *et al*., 2000).

The aim of this study was to determine the effects of 6-DMAP, butyrolactone and roscovitine on suppression of meiosis and maintenance of the GV in horse oocytes. We desired especially to identify a treatment that would support incompetent oocytes (those oocytes having diffuse chromatin within the GV) and prevent them from degenerating in culture.

Materials and methods

Horse ovaries were obtained from two horse slaughterhouses and were transported 3–4 h in phosphate-buffered saline (PBS) at room temperature (22–25°C). All visible follicles were opened with a scalpel blade. The granulosa layer of each follicle was scraped using a 0.5 cm bone curette, and the cells were washed from the curette into individual Petri dishes using holding medium consisting of M199 with Hanks salts and 12.5 mM Hepes (Gibco Life Technologies, Grand Island, NY), with added ticarcillin (0.1 mg/ml; SmithKline Beecham Pharmaceuticals, Philadelphia, PA).

The contents of the Petri dishes were examined using a dissection microscope at ×10–20. Cumulus–oocyte complexes (COCs) were classified as being compact (Cp) or expanded (Ex), on the basis of both cumulus and mural granulosa cell morphology. If any aspect of the recovered granulosa cells showed signs of expansion (ranging from cells protruding from the surface layer to full expansion with copious intercellular matrix), the COC was classified as Ex even the cumulus appeared compact. Only those COC having entirely compact granulosa cells in addition to a tightly compact cumulus were classified as Cp. Only Cp oocytes were used for this study.

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Oocyte culture

Basic culture medium was M199 with Earles salts (Gibco) and 25 μg/ml gentamicin (Gibco), with added treatments as described under each experiment below. Oocytes intended for culture were incubated in groups in droplets of medium at a ratio of 10 μl medium per oocyte, at 38.2 °C in a humidified atmosphere of 5% CO_2 in air. After a 24 h incubation period, the cumulus was removed and the oocytes were fixed, stained and evaluated as described below.

Experiment 1: 6-DMAP

6-DMAP stock solution was prepared by dissolving 6-DMAP (Sigma, St Louis, MO) in water at 25 mg/ml. The stock solution was frozen in aliquots until used.

In part 1, oocytes were divided into three groups as they were recovered: direct control oocytes were denuded and fixed immediately; matured control oocytes were incubated in basic culture medium with 10% fetal bovine serum (FBS); and 6-DMAP oocytes were incubated in basic culture medium with 10% FBS and 2 mM 6-DMAP.

In part 2, oocytes were divided into two groups as they were recovered: hold/direct oocytes were placed in holding medium until all oocytes were collected (0–3 h) then were denuded and fixed; and hold/culture oocytes were placed in holding medium containing 2 mM 6-DMAP until all oocytes were collected, and then were incubated in culture medium with 6-DMAP as for part 1.

Experiment 2: Butyrolactone I

Butyrolactone (Biomol Research Laboratories, Plymouth Meeting, PA) was prepared as a 10 mM stock solution in dimethylsulfoxide (DMSO). Aliquots were frozen until use. Oocytes were divided into four groups as they were recovered: direct control oocytes were placed in holding medium until all oocytes were collected, and then were denuded and fixed; control (0 B) oocytes were placed in holding medium until all oocytes were collected then were incubated in basic culture medium with 1.5 mg/ml BSA and 10 μl/ml DMSO; 50 B oocytes were placed in holding medium containing 100 μM butyrolactone I until all oocytes were collected, then incubated in basic culture medium with 1.5 mg/ml BSA and 50 μM butyrolactone I; and 100 B oocytes were placed in holding medium containing 100 μM butyrolactone I until all oocytes were collected, then incubated in basic culture medium with 1.5 mg/ml BSA and 100 μM butyrolactone I.

Experiment 3: Roscovitine

Roscovitine (Biomol) was prepared as a 5 mM stock solution in DMSO. Aliquots were frozen until use. Oocytes were divided into four groups as they were recovered: direct-control oocytes were placed in holding medium until all oocytes were collected, and then were denuded and fixed; 0 R oocytes were placed in holding medium until all oocytes were collected then were incubated in basic culture medium with 1.5 mg/ml BSA and 20 μl/ml DMSO; 25 R oocytes were placed in holding medium containing 25 μM roscovitine until all oocytes were collected, then incubated in basic culture medium with 1.5 mg/ml BSA and 25 μM roscovitine; and 100 R oocytes were placed in holding medium containing 100 μM roscovitine until all oocytes were collected, then incubated in basic culture medium with 1.5 mg/ml BSA and 100 μM roscovitine.

Experiment 4: DMSO

Because the control media in the above studies differed (M199/FBS vs M199/BSA with added DMSO) according to the carrier used for the compound tested, a trial was conducted to determine whether culture in serum-free (BSA-containing) medium or addition of DMSO affected rates of maturation and degeneration in horse oocytes in comparison with M199/FBS. Oocytes were incubated in one of three media: basic culture medium containing 10% FBS, basic culture medium containing 1.5 mg/ml BSA, or basic culture medium containing 1.5 mg/ml BSA and 20 μl/ml (2%) DMSO. Oocytes were cultured for 24 h and assessed for stage of development.

Oocyte assessment

For chromatin evaluation, oocytes were denuded of cumulus by pipetting in a solution of 0.25% trypsin and 1 mM EDTA in Hank's salts without $CaCl_2$, $MgCl_2$ or $MgSO_4$ (Gibco). The oocytes were fixed in buffered formol saline at room temperature. Fixed oocytes were labelled for chromatin evaluation by placing them on a glass slide with 6.5 μl of mounting medium (3:1 glycerol : PBS containing 2.5 μg/ml Hoechst 33258). The oocytes were evaluated using a fluorescence microscope with a 365 nm excitation filter. Chromatin configuration within the GV was classified as either condensed (Fig. 1*a*) or diffuse (Hinrichs *et al*., 1993*a*, *b*; Hinrichs & Williams, 1997). Diffuse chromatin within the GV was further subdivided into: *fibrillar*, oocytes having individual strands of dense chromatin visible throughout the GV (Fig. 1*b*, previously classified as 'diakinesis'; Hinrichs & Schmidt, 2000); *FN*, having a

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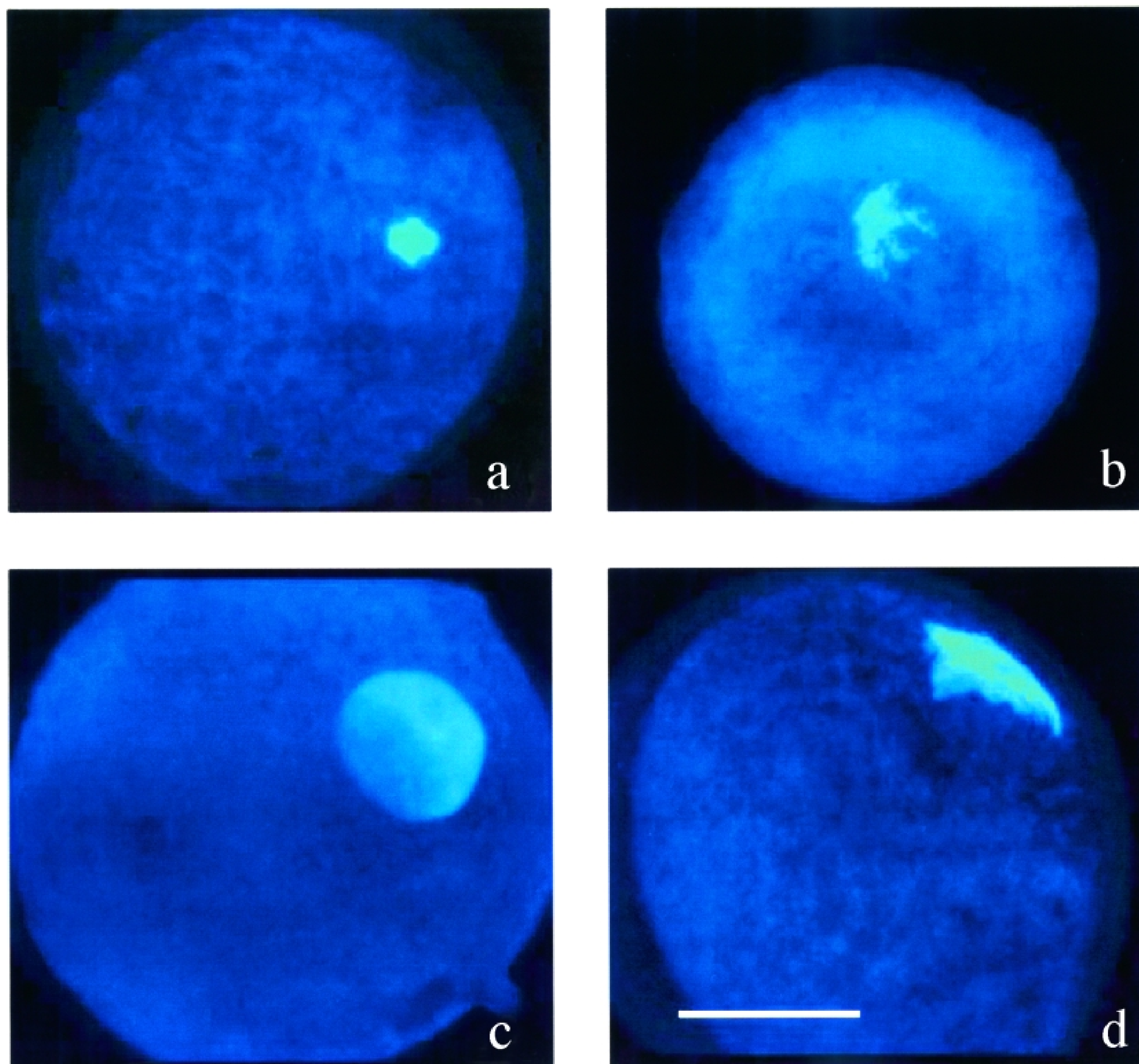

Figure 1 Chromatin configurations of germinal vesicle (GV)-stage horse oocytes: (*a*) condensed chromatin (CC); (*b*) fibrillar; (*c*) homogeneously fluorescent nucleus (FN); (*d*) fibrous-GV. Scale bar represents 50 μm.

homogeneously fluorescent nucleus with or without aggregates of chromatin (Fig. 1*c*); or *fibrous-GV*, collapsed, irregular or atypical GV in which the chromatin had a net-like appearance (Fig. 1*d*). Other classifications were metaphase I, metaphase II or degenerating (abnormal chromatin configurations or no chromatin visible).

Differences in proportions of GV stage configurations were compared among groups using chi-square analysis, with Fisher's exact test used when a value of less than 5 was expected for any parameter.

Results

Experiment 1: 6-DMAP

For part 1, 1379 follicles were scraped. A total of 352 Ex, 300 Cp and 21 degenerating oocytes were recovered. One hundred and twenty Cp oocytes were used for a separate study. Eleven replicates were performed. The proportion of oocytes having the different chromatin configurations in the direct control, matured control and 6-DMAP treatments is presented in Table 1.

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Culture with 6-DMAP effectively suppressed maturation in treated oocytes, as only 1 of 55 oocytes (2%) reached metaphase I or II after 24 h culture, versus 22 of 58 (38%) for oocytes in the maturation control ($p < 0.001$). The proportion of oocytes with condensed chromatin was not significantly different between the direct control and the 6-DMAP groups (17/60, 28% and 17/55, 31% respectively, $p > 0.1$). The proportion of oocytes considered to have diffuse chromatin (FN, fibrous-GV and fibrillar combined) was significantly lower in the 6-DMAP group than in direct controls ($p < 0.05$). However, 6-DMAP appeared to maintain a portion of GV oocytes having diffuse chromatin, as there were significantly more oocytes in this configuration in the 6-DMAP treatment than in maturation controls ($p < 0.05$). Within the diffuse chromatin classification, the 6-DMAP group had significantly fewer oocytes in the FN and fibrillar groups, and significantly more oocytes in the fibrous-GV group than did the direct controls ($p < 0.01$).

For part 2, 1012 follicles were scraped, and 321 Ex, 157 Cp and 32 degenerating oocytes were recovered. Five replicates were performed. There was no significant difference between parts 1 and 2 in the proportions of oocytes in the different chromatin configurations after 6-DMAP culture ($p > 0.1$).

Experiment 2: Butyrolactone

For this study, 1786 follicles were scraped, and 561 Ex, 280 Cp and 56 degenerating oocytes were recovered. Nine replicates were performed. The proportion of oocytes having the different chromatin configurations in the direct control, and 0, 50 and 100 μM butyrolactone groups are presented in Table 2.

Culture with butyrolactone at 50 μM was not effective in suppressing maturation (oocytes reaching MI or II were 9/65, 14% for 50B vs 13/60, 22% for 0B; $p > 0.1$); however, a significantly higher proportion of the maturing oocytes were in MI in 50 B than in 0 B (6/9

Table 1 Chromatin configurations of oocytes fixed directly upon removal from the follicle (Dir), or cultured for 24 h in the presence of 0 or 2 mM 6-DMAP

		Germinal vesicle stages					Maturing oocytes			Degenerating (%)
		Diffuse chromatin				CC (%)	MI (%)	MII (%)	Total maturing	
	n	FN (%)	Fibrillar (%)	Fibrous-GV (%)	Total diffuse					
Experiment 1										
DIR	60	10 (17)	16 (27)	2 (3)[a]	28 (47)[a]	17 (28)[a]	0	0	0[a]	15 (25)[a]
0	58	2 (3)	4 (7)	1 (2)[a]	7 (12)[b]	3 (5)[b]	7 (12)	15 (26)	22[b]	26 (45)[b]
2 mM	55	1 (2)	3 (5)	12 (22)[b]	16 (29)[c]	17 (31)[a]	0	1 (2)	1[a]	21 (38)[a,b]
Experiment 2										
DIR	77	20 (26)	4 (5)	6 (8)	30 (39)	25 (32)	0	0	0	22 (28)
2 mM	77	6 (8)	0	14 (18)	20 (26)	22 (29)	1 (1)	0	1 (1)	34 (44)

FN, homogeneously fluorescent nucleus; CC, condensed chromatin.
Within columns, values with different superscipts are significantly different ($p < 0.05$).

Table 2 Chromatin configurations of oocytes fixed directly upon removal from the follicle (Dir), or cultured for 24 h in the presence of 0, 50 or 100 μM butyrolactone I

		Germinal vesicle stages					Maturing oocytes			Degenerating (%)
		Diffuse chromatin				CC (%)	MI (%)	MII (%)	Total maturing	
	n	FN (%)	Fibrillar (%)	Fibrous-GV (%)	Total diffuse					
DIR	58	19 (33)	1 (2)	3 (5)	23 (40)[a]	12 (21)[a]	0	0	0[a]	23 (40)[a]
0	60	5 (8)	1 (2)	1 (2)	7 (17)[b]	2 (3)[b]	2 (3)	11 (18)	13 (22)[b]	38 (63)[b]
50	65	3 (5)	1 (2)	7 (11)	11 (17)[b]	8 (12)[a,b]	6 (9)	3 (5)	9 (14)[b,c]	37 (57)[a,b]
100	64	2 (3)	4 (6)	0	6 (9)[b]	11 (17)[a]	0	3 (5)	3 (5)[a,c]	44 (69)[b]

FN, homogeneously fluorescent nucleus; CC, condensed chromatin.
Within columns, values with different superscipts are significantly different ($p < 0.05$).

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(67%) vs 2/13 (15%; $p < 0.05$). Culture in 100 B did suppress meiosis; the proportion of oocytes reaching MI or MII in 100 B (3/64, 5%) was significantly lower than that for 0 B (13/60, 22%, $p < 0.01$). There was no decrease in the prevalence of GV oocytes having condensed chromatin in 100 B, compared with directly fixed controls, indicating that meiosis was suppressed in these oocytes. However, the proportion of GV oocytes in the diffuse configuration was significantly lower in the 100 B group compared with directly fixed controls (6/64, 9% and 23/58, 40% respectively; $p < 0.05$), and the proportion of degenerating oocytes was significantly higher (44/64, 69% and 23/58, 40% respectively, $p < 0.01$).

Experiment 3: Roscovitine

For this study, 1569 follicles were scraped, and 530 Ex, 307 Cp and 41 degenerating oocytes were recovered. Nine replicates were performed. The proportion of oocytes having the different chromatin configurations in the direct control, 0, 25 and 100 μM roscovitine treatments are presented in Table 3.

Culture with roscovitine at either 25 or 100 μM was not effective in suppressing maturation (oocytes reaching MI or II; 8/78, 10% and 15/77, 20% respectively, vs 11/77, 14% for 0 R); however, in 100 R a significantly higher proportion of maturing oocytes were in metaphase I (15/15 for 100 R vs 6/11 for 0 R; $p < 0.01$) Both roscovitine treatments had a significantly lower proportion of oocytes in the diffuse GV stages than did the direct controls (12/78 (16%) and 10/78 (13%) for 25 and 100 R, respectively, vs 42/78 (54%) for direct controls; $p < 0.001$). The proportion of GV oocytes having condensed chromatin was also significantly reduced in the 100 R treatment when compared with directly fixed controls ($p < 0.05$), but there was no significant difference in this parameter between 25 R and directly fixed controls.

Experiment 4: DMSO

For this study, 1160 follicles were scraped and 362 Ex, 202 Cp and 61 degenerating oocytes were recovered. The proportions of oocytes in the different chromatin configurations after culture in basic culture medium containing 10% FBS, basic culture medium containing 1.5 mg/ml BSA, or basic culture medium containing 1.5 mg/ml BSA and 20 μl/ml (2%) DMSO, is given in Table 4. There were no significant differences in proportions of metaphase or degenerating oocytes among the three media. Interestingly, the maturation and degeneration rates in the two control media used for the above experiments (FBS or BSA + DMSO) were similar, whereas the maturation rate for oocytes in BSA alone tended ($p = 0.1$) to be lower than for the other two media.

Discussion

Of the compounds tested, both 6-DMAP and 100 μM butyrolactone appeared to be effective in suppressing meiosis in those GV-stage oocytes originally having condensed chromatin. With both these treatments there was no significant reduction in this chromatin configuration in cultured oocytes compared with directly fixed oocytes, and significantly fewer oocytes had resumed meiosis than in maturation controls. However, with all three compounds tested, and in maturation controls, the proportion of GV oocytes having diffuse chromatin decreased significantly after culture in comparison with directly fixed controls, and the proportion of degenerating oocytes showed a proportional increase, indicating that these oocytes degenerated during culture. In a previous study, it was found that oocytes with diffuse chromatin degenerated when cultured with cycloheximide (Alm & Hinrichs, 1996).

Culture with 6-DMAP appeared to maintain the GV in a proportion of the oocytes having diffuse chromatin; however, the chromatin configurations differed

Table 3 Chromatin configurations of oocytes fixed directly upon removal from the follicle (Dir), or cultured for 24 h in the presence of 0, 25 or 100 μM roscovitine

		Germinal vesicle stages					Maturing oocytes			Degenerating (%)
		Diffuse chromatin				CC (%)	MI (%)	MII (%)	Total maturing	
	n	FN (%)	Fibrillar (%)	Fibrous-GV (%)	Total diffuse					
DIR	78	37 (47)	3 (4)	2 (3)	42 (54)[a]	17 (22)[a]	0	0	0[a]	19 (24)[a]
0	77	0	5 (6)	1 (1)	6 (8)[b]	3 (4)[b]	6 (8)	5 (6)	11 (14)[b]	57 (74)[b]
25	78	5 (6)	5 (6)	2 (3)	12 (15)[b]	15 (19)[a]	2 (3)	6 (8)	8 (10)[b]	43 (55)[c]
100	77	4 (5)	4 (5)	2 (3)	10 (13)[b]	2 (3)[b]	15 (19)	0	15 (20)[b]	50 (65)[b,c]

FN, homogeneously fluorescent nucleus; CC, condensed chromatin.
Within columns, values with different superscipts are significantly different ($p < 0.05$).

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Table 4 Chromatin configurations of oocytes cultured for 24 h in medium with FBS, medium with BSA, or medium with BSA and 2% DMSO

	n	Germinal vesicle stages					Maturing oocytes			Degenerating (%)
		Diffuse chromatin				CC (%)	MI (%)	MII (%)	Total maturing	
		FN (%)	Fibrillar (%)	Fibrous-GV (%)	Total diffuse					
FBS	67	0	3 (5)	4 (6)	7 (10)	5 (8)	4 (6)	12 (18)	16 (24)	38 (57)
BSA	67	0	8 (12)	2 (3)	10 (15)	3 (5)	2 (3)	6 (9)	8 (12)	46 (69)
BSA/DMSO	66	1 (2)	6 (9)	2 (3)	9 (14)	1 (2)	11 (17)	6 (9)	17 (26)	40 (60)

FN, homogeneously fluorescent nucleus; CC, condensed chromatin.
There were no significant differences in chromatin configuration among the three media tested.

significantly between 6-DMAP-cultured and directly fixed oocytes. Cultured oocytes had a lower prevalence of the FN and fibrillar configurations and a higher prevalence of the fibrous-GV configuration, suggesting that the morphology of oocytes with diffuse chromatin changed during culture.

We hypothesise that degeneration of diffuse-chromatin oocytes in culture is related to precocious GV breakdown. If this is so, the greater effectiveness of 6-DMAP in relation to that of butyrolactone or roscovitine, used at doses effective in other species, suggests that GV breakdown in incompetent oocytes in culture cannot be prevented by specific inhibition of MPF or MAPK, but may be suppressed by inhibiting the phosphorylation of a separate protein. However, the fibrous-GV configuration, which appeared after 6-DMAP culture, is rare in directly fixed oocytes and its meaning – whether it is a variation of normal chromatin, or whether it represents a step toward degeneration – is unknown. There appears to be a continuum between the network-like appearance of the fibrous GV and an abnormal chromatin configuration in which net-like chromatin is spread throughout the oocyte. Washing and maturation of oocytes after 6-DMAP treatment is needed to help define the status of the fibrous-GV configuration; however, since FN oocytes degenerate in culture, yet are associated with follicle viability (Hinrichs & Williams, 1997), nuclear maturation in the currently available media may not be a definitive test for viability of horse oocytes.

The data on roscovitine action are somewhat confusing. Treatment with 25 μM roscovitine appeared to maintain the condensed chromatin configuration, similar to what was seen with 6-DMAP and 100 μM butyrolactone, and previously with cycloheximide (Alm & Hinrichs, 1996). The 25 R treatment had fewer oocytes maturing (10%) than did the maturation control. These data suggest that 25 R was partially effective in suppressing maturation. However, increasing the concentration of roscovitine appeared to reverse this trend, as 100 R had essentially no effect on resumption of meiosis, with few oocytes remaining in either diffuse or condensed GV stages, and more maturing oocytes than in the maturation control. Nevertheless, oocytes cultured in 100 R were apparently delayed, as maturing oocytes were still in MI at 24 h culture.

Arrest of oocytes at MI may be related to the failure of MPF activity to rise again after the first metaphase, as this is necessary to initiate the MI to MII transition. After MI, MPF activity falls, and the metaphase plate is maintained in association with high levels of MAPK (Moos *et al*., 1995). The function of an MPF inhibitor may be to cause a situation similar to that seen in juvenile but partially competent oocytes in other species. These oocytes resume meiosis but arrest at MI; in these oocytes the amount of $p34^{cdc2}$ is generally lower than for completely competent oocytes (Dedieu *et al*., 1998; De Vantery *et al*., 1997). Thus, in both instances the failure of oocytes to proceed through MI may be related to insufficient MPF activity.

The maturation rate seen in the maturation control group in the 6-DMAP experiment was higher than that seen in the maturation controls for the butyrolactone or roscovitine studies (38% vs 21% and 14%, respectively). This could possibly have been related to the maturation medium used: the maturation control in the 6-DMAP study was media with added FBS, whereas the maturation controls in the subsequent studies were media with BSA. BSA was used in the latter two experiments because serum decreases the effectiveness of butyrolactone (Kubelka *et al*., 2000), and this is likely to be the case with roscovitine as well. However, when maturation was compared among control media (experiment 4), there was no difference in maturation (MI + MII) between medium containing FBS and that containing BSA with DMSO. Oocytes at either MI or MII at the end of 24 h culture were considered to be maturing, as maturation to MII in Cp oocytes takes 24–32 h (Hinrichs *et al*., 1993*b*).

One factor which may have affected maturation rates was that from the time of experiment 1 (6-DMAP) to the subsequent experiments, the proportions of

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oocytes designated as Cp decreased. In experiment 1, 300 of 652 (46%) non-degenerating oocytes were designated Cp, whereas in the subsequent experiments the proportion of Cp oocytes was 280 of 841 (33%), 307 of 837 (37%), and 202 of 564 (36%). The Cp:Ex ratio should have been similar among the seasons in which the experiments were performed (Hinrichs & Schmidt, 2000). The decrease in proportion of Cp oocytes may reflect the adoption of more stringent classification criteria. In our current classification scheme, oocytes having any signs of expansion of either cumulus or mural granulosa are designated Ex (high meiotic competence), as the highest maturation rate is seen in horse oocytes from follicles in the earliest stage of atresia (Hinrichs & Williams, 1997). Since in early atresia approximately half the oocytes have an expanded cumulus and half a compact cumulus (Hinrichs & Williams, 1997), the categorisation of these oocytes can greatly affect the observed maturation rate. More stringent criteria for designation of oocytes as Cp would tend to eliminate these 'marginal' oocytes from the Cp group and thus lower the observed maturation rate for this group. Laboratories working with equine oocytes differ in their criteria for cumulus classification; therefore the oocyte recovery rate and proportions of Ex and Cp oocytes recovered were given for each experiment so that comparisons with data from other laboratories may be possible.

In part 1 of the 6-DMAP experiment, oocytes were denuded or placed into culture immediately after collection; however, we felt that this may have been affecting the incubation environment negatively and we therefore examined the effect of holding oocytes in suppressive medium at room temperature until all oocytes had been collected (part 2). The timing of placement into culture did not affect the suppressive action of 6-DMAP. We have found that holding of oocytes at room temperature before culture does not affect maturation rate (Love *et al*., 2002); however, holding before fixation does affect the appearance of the initial chromatin configuration, with diffuse chromatin apparently shifting from the fibrillar to the FN configuration. This is reflected in the different proportions of FN oocytes in the direct controls of the 6-DMAP experiment part 2 compared with those of part 1. We are currently studying the effect of holding time of ovaries and oocytes on oocyte chromatin configuration and meiotic competence.

In conclusion, 6-DMAP and butyrolactone, and to a lesser extent roscovitine, were effective in suppressing meiosis in horse oocytes originally having condensed chromatin within the GV. GV oocytes having diffuse chromatin degenerated in culture with these compounds. 6-DMAP appeared to be partially effective in maintaining GV oocytes with diffuse chromatin, but the chromatin configuration within the GV changed during culture. This is the first study evaluating the effects of these inhibitors on meiosis in horse oocytes. Work is needed to determine the amounts and activities of the different m-phase proteins in GV-stage horse oocytes, and to determine the differences in these proteins between oocytes with condensed and diffuse chromatin. Development of a method to prevent degeneration, and increase meiotic competence, of oocytes with diffuse GV chromatin will greatly increase the efficiency of research in assisted reproduction in the horse.

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