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Folic acid facilitates *in vitro* maturation of mouse and *Xenopus laevis* oocytes

Xiaoli Huang[1,2], Shu Gao[2], Wei Xia[3], Shaoying Hou[2] and Kun Wu[2]*

[1]*Department of Nutrition and Food Hygiene, School of Public Health, Shandong University, Jinan, People's Republic of China*

[2]*Department of Nutrition and Food Hygiene, Harbin Medical University, Heilongjiang, 157 Baojian Road, Harbin 150081, People's Republic of China*

[3]*Department of Children Health and Hygiene, Harbin Medical University, Heilongjiang, People's Republic of China*

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Abstract

The water-soluble B vitamins, folate and folic acid, play an important role in reproductive health, but little is known about the effects of folic acid on infertility. The present study tested the hypothesis that folic acid affects oocyte maturation, a possible cause of female infertility. We have studied the *in vitro* maturation of mouse and *Xenopus* oocytes. Hypoxanthine (Hx) was used as an inhibitor of mouse oocyte maturation to mimic *in vivo* conditions by maintaining high levels of cyclic-AMP. The frequency of first polar body (PB1) formation and germinal vesicle breakdown (GVBD) in mouse oocytes was decreased by Hx. This effect was counteracted by folic acid added to the medium. PB1 extrusion and GVBD percentages rose to 27·7 and 40·0 % from 12·8 and 19·9 %, respectively, by exposure to 500 μM-folic acid. Folic acid also restored the spindle configuration, which had been elongated by Hx, as well as normalising the distribution of cortical granules (CG). In folic acid-treated *Xenopus* eggs, extracellular signal-regulated kinase 1 was phosphorylated, cyclin B2 and Mos were up-regulated and the frequency of GVBD was accelerated. Taken together, the findings suggest that folic acid facilitates oocyte maturation by altering the expression and phosphorylation of proteins involved in M-phase-promoting factor and mitogen-activated protein kinase pathways, as well as causing changes in spindle configuration and CG migration.

Key words: **Folic acid: Oocytes: *In vitro* maturation: Mise: *Xenopus laevis***

Infertility is a prevalent clinical issue with significant impacts on families, society and healthcare services. Poor-quality oocytes may be one of the causes of female infertility. The competence of oocytes is determined by a number of processes throughout oogenesis, with the final steps of oocyte maturation being especially important[1,2]. Oocyte maturation consists of two interlinked and mutually dependent processes: cytoplasmic maturation, characterised by an asymmetric distribution of cortical granules (CG) in the cortex with no CG about the metaphase II (MII) spindle[3,4], and nuclear maturation, characterised by chromatin changes leading to the extrusion of the polar body. A variety of environmental factors, such as heavy metals, endogenous steroids and chemicals, have been shown to induce meiotic abnormalities that render oocytes incapable of fertilisation[5,6], suggesting that agents that improve oocyte maturation may help to prevent infertility.

Folate (naturally occurring in the body and in foods) and folic acid (synthetic form applied in supplements and fortified foods) are members of the B vitamin family, which serve as important biosynthetic cofactors. Folate derivatives are the source of methyl groups that re-methylate homocysteine into methionine and also play an important role in the synthesis of pyrimidines and purines. Folic acid supplements prevent megaloblastic anaemia during pregnancy and protect against neural tube defects[7]. However, data on the effects of folic acid on infertility are limited. There is some evidence to suggest that folate supplementation is beneficial in males, increasing the sperm count in both sub-fertile and fertile men[8]. In contrast, little is known about the impact of folic acid on oocyte development, although the existence of an endogenous uptake system for folate in *Xenopus laevis* oocytes suggests that it plays an important role[9]. Preconception folic acid treatment has been found to affect the microenvironment of maturing oocytes in human subjects[10]. Moreover, it has been reported that the percentage of oocytes in first and second degree of maturity was higher in a group of women with folic acid supplementation and lower

Abbreviations: CG, cortical granules; ERK1, extracellular signal-regulated kinase 1; GVBD, germinal vesicle breakdown; Hx, hypoxanthine; MII, metaphase II; MAPK, mitogen-activated protein kinase; MPF, M-phase-promoting factor; PB1, first polar body.

* **Corresponding author:** K. Wu, fax +86 451 8750 2885, email wukun_15000@126.com

homocysteine concentration in follicular fluid, which suggests that there is a correlation between follicular fluid homocysteine concentration and oocyte maturity[11]. Whether folic acid or homocysteine directly affects oocyte maturity remains elusive.

We hypothesised that folic acid facilitates *in vitro* maturation of mouse and *Xenopus* oocytes. We assessed the nuclear status, spindle configuration and CG distribution of mouse oocytes at the end of culture and attempted to elucidate the possible mechanisms of action of folic acid in *Xenopus* oocytes. *In vivo*, keeping concentrations of cyclic-AMP high in the oocyte inhibits resumption of meiosis of oocytes. Hypoxanthine (Hx) has been shown to suppress the spontaneous meiotic maturation of mammalian oocytes *in vitro* to mimic *in vivo* conditions by maintaining high levels of cyclic-AMP[12,13]. In the present study, Hx was used as an inhibitory substance of mouse oocyte maturation.

Materials and methods

Animals

Female Kunming mice were purchased from the Experimental Animal Center of the Second Hospital of Harbin Medical University. The animals were maintained in a 14 h light–10 h dark photoperiod under constant temperature and relative humidity. Female *X. laevis* were purchased from Maoshen Biotech. All experiments were conducted in accordance with the policies on the care and use of animals of the Ethics Committee of Harbin Medical University.

Chemicals

Bovine serum albumin was purchased from Promega. Pregnant mare serum gonadotropin was obtained from Ningbo Second Hormone Factory. Rabbit anti-mouse tubulin antibody was obtained from Lab Vision Corporation and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Zhongshan Goldenbridge Biotechnology Company Limited. Antibodies against *Xenopus* cyclin B2 and those against Mos, extracellular signal-regulated kinase 1 (ERK1) and phospho-ERK1 were purchased from Abcam and Santa Cruz Biotechnology, respectively. Triton X-100 was obtained from Ameresco. Folic acid, Hx, collagenase type I, progesterone, fluorescein isothiocyanate-conjugated *Lens culinaris* agglutinin, M2 and M16 media and all other chemicals were purchased from Sigma. The M2 and M16 media did not contain folic acid.

Mouse oocyte collection and culture

Fully grown mouse oocytes were obtained from the ovaries of 4- to 6-week-old mice, killed by cervical dislocation 46–48 h after they were primed with 1 mg of pregnant mare serum gonadotropin. The follicles were punctured under a stereomicroscope using a 29-G needle fixed to a 1 ml disposable syringe and aspirated into M2 medium supplemented with 60 μg/ml of penicillin and 100 μg/ml of streptomycin. Denuded mouse oocytes containing their nucleus or germinal vesicle were subsequently selected, washed three times and cultured in 50 μl drops of M16 medium under mineral oil. Mouse oocytes were matured for 24 h in an atmosphere of 5 % CO_2 at 37°C under the following conditions: (1) Hx-free medium; (2) 4 mmol/l Hx medium and (3) Hx medium with folic acid (20, 100 and 500 μmol/l).

Xenopus laevis oocyte collection and culture

Adult *X. laevis* females were primed with 5 mg of pregnant mare serum gonadotropin, 1 week before the ovaries were surgically removed. Ovary clumps were fully defolliculated in 2 g/l of collagenase type I for 1–2 h at room temperature. Fully grown *Xenopus* oocytes (stage VI) were manually separated using watchmaker forceps, rinsed extensively with Ca-free ND-96 buffer, left to equilibrate overnight at room temperature and then treated with 0, 125, 250, 500 or 1000 μmol/l of folic acid for 6 h. Progesterone at the final concentration of 5 μg/ml served as positive control.

Nuclear maturation of oocytes

After 24 h of culture, the status of oocyte nuclear maturation was recorded using an inverted microscope (Olympus). The oocytes exhibited an intact nucleus (germinal vesicle) and subsequently either initiated resumption of maturation (germinal vesicle breakdown, GVBD) or emitted the first polar body (PB1). The percentage of GVBD (including PB1) per total number of oocytes and that of PB1 formed relative to the total number of oocytes were calculated.

Meiotic spindle and chromosome immunofluorescent staining

After 24 h of culture, mouse oocytes matured under different experimental conditions were labelled as previously described[14]. Briefly, oocytes were fixed for 30 min at room temperature with 4 % paraformaldehyde and permeabilised for 15 min at 37°C in incubation buffer containing 0·5 % Triton X-100 in 20 mM-HEPES, 3 mM-$MgCl_2$, 50 mM-NaCl and 300 mM-sucrose. The mouse oocytes were then blocked in 1 % bovine serum albumin for 1 h and incubated overnight at 4°C in the presence of rabbit anti-α-tubulin antibody, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as a secondary antibody for 1 h at 37°C. After three 5-min washes, the chromosomes were stained with 5 μg/ml of propidium iodide. Finally, the samples were placed in droplets of 1,4-diazobicyclo(2,2,2)octane and mounted on slides.

Assessment of cortical granule distribution

After 24 h of culture, matured mouse oocytes were fixed, permeabilised, blocked, incubated and mounted as for spindle immunofluorescent staining, except that 100 μg/ml of fluorescein isothiocyanate-conjugated *L. culinaris* agglutinin was used to stain oocytes to assess the distribution of CG.

Analysis of spindle size

After fluorescence staining, the spindle, chromosomes and CG were visualised using a Nikon TE-2000 confocal laser scan microscope at constant settings, including the numerical aperture, gain and neutral density filter. Images were recorded electronically. Spindle size was measured as previously described[15].

Cell lysis and Western blot analysis

After 6 h of culture, *Xenopus* oocytes (*n* 60 per group) at metaphase I were washed and homogenised in 5 μl of buffer per oocyte (50 mM-Tris, 150 mM-NaCl, 0·1 % NP-40, 0·5 % deoxycholic acid (pH 7·4), 1 mM-phenylmethylsulfonyl fluoride and 10 μg/ml of protease inhibitor cocktail). Protein homogenates from oocytes collected at indicated times were separated on 10 % SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed using ERK1, phospho-ERK1, cyclin B2 and Mos antibodies. The membrane was then incubated with the secondary alkaline phosphatase-conjugated IgG and detected with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

Statistical analysis

All experiments were performed at least three times. Data were expressed as the mean with their standard errors, and the values were analysed by ANOVA. Percentages were analysed using Pearson's χ^2 analysis and generalised estimating equations. $P<0·05$ was considered statistically significant.

Results

Oocyte nuclear maturation

Mouse oocytes were cultured for 24 h in medium containing 4 mM-Hx and increasing concentrations of folic acid (from 20 to 500 μM) and then assessed for GVBD and PB1 extrusion as a marker of nuclear maturation. As shown in Fig. 1(a), more than 90 % of the untreated oocytes (475/492) underwent GVBD spontaneously and this rate was reduced substantially when the oocytes were treated with Hx (19·9 %, 320/1611). There was a significant increase in the rate of GVBD formation in the Hx-treated oocytes exposed to folic acid ($P<0·001$). The rate of PB1 emission (Fig. 1(b)) was similar to that of GVBD, with almost 80 % (388/492) of untreated oocytes extruding PB1 compared to only 12·8 % (207/1611) of those treated with Hx. The rates were significantly higher ($P<0·001$) at 27·7 % (211/762) in oocytes treated with Hx plus 500 μM-folic acid.

We also examined the effect of folic acid on GVBD in *Xenopus* oocytes. As shown in Table 1, the percentages of GVBD at both 4- and 6-h time points were significantly higher in the folic acid-treated groups compared with the control group. This compares favourably with the response to progesterone, a known *in vitro* promoter of *X. laevis* oocyte maturation, which was used as positive control[16].

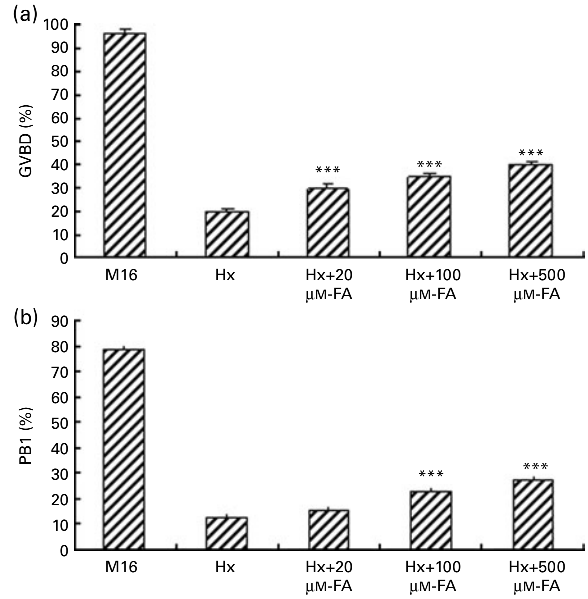

Fig. 1. Status of nuclear maturation. (a, b) Mouse oocytes were cultured for 24 h in M16 medium and in medium containing 4 mM-hypoxanthine (Hx) plus increasing concentrations of folic acid (FA). Bars indicate the percentage of oocytes at germinal vesicle breakdown (GVBD) and the first polar body (PB1) stage of meiotic maturation. Values are means of three experiments, with their standard errors represented by vertical bars. ***Mean values were significantly different from those of control (Hx medium) ($P<0·001$).

Chromosomal arrangement and quantitative analysis of spindles

As shown in Fig. 2, when mouse oocytes were cultured in M16 medium for 24 h, the oocytes at MII exhibited a barrel-shaped spindle with well-aligned chromosomes in the metaphase plate. Mouse MII oocytes exposed to Hx with or without folic acid all possessed well-organised spindles with well-aligned chromosomes located at the spindle equator. Quantitative analysis of spindle formation (Table 2) showed that Hx increased the spindle length, width and area of oocytes at MII. MII spindles might not be damaged severely enough by Hx, as the mouse oocytes possess normal chromosomes. When oocytes were cultured for 24 h in Hx medium in the presence of 500 μM folic acid, there was a significant decrease ($P<0·001$) in spindle area (210·04 (SEM 7·59) μm^2) compared with the Hx medium group (319·47 (SEM 15·85) μm^2).

Table 1. Effect of folic acid (FA) on germinal vesicle breakdown rate (%) in *Xenopus* oocytes

	Time (h)			
Group	0	2	4	6
Control	0	1·7 (1/60)	6·7 (4/60)	13·3 (8/60)
125 μM-FA	0	1·7 (1/60)	21·7 (13/60)*	50·0 (30/60)*
250 μM-FA	0	3·6 (2/60)	28·3 (17/60)*	61·7 (37/60)*
500 μM-FA	0	8·3 (5/60)	51·7 (31/60)*	88·3 (53/60)*
1000 μM-FA	0	11·7 (7/60)*	56·7 (34/60)*	93·3 (56/60)*
5 μg/ml Progesterone	0	33·3 (20/60)*	81·7 (49/60)*	100·0 (60/60)*

* Values were significantly different compared with the control group ($P<0·05$).

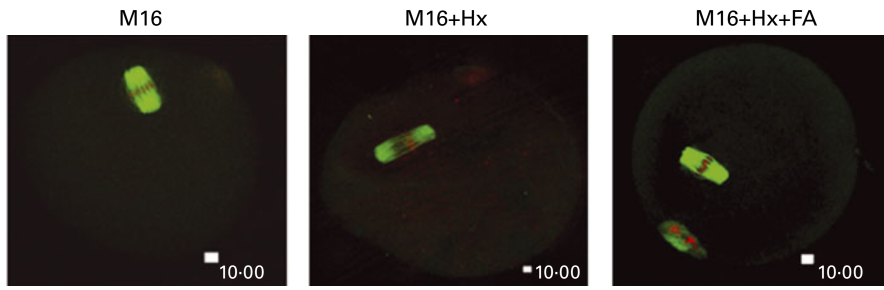

Fig. 2. Spindle configuration and chromosomal arrangement. Mouse oocytes were cultured for 24 h in hypoxanthine (Hx) medium supplemented with or without 500 μM-folic acid (FA). Oocytes at MII were then stained immunocytochemically with anti-α-tubulin monoclonal antibody and fluorescein isothiocyanate to observe the spindle (green) and counterstained with propidium iodide to detect the chromosomes (red). Scale bar represents 10 μm. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Moreover, the alterations in the spindle length and width of MII oocytes exposed to folic acid were consistent with those in the spindle area. The spindle length and width of Hx medium with 500 μM-folic acid group were found to be 24·38 (SEM 0·58) μm and 9·41 (SEM 0·20) μm, respectively, which were found to be lower when compared with Hx medium group (27·88 (SEM 0·70) μm and 13·60 (SEM 0·38) μm, respectively, $P<0·001$). Moreover, there was no difference in the spindle width of the Hx + folic acid groups and Hx-free medium group.

Distribution of cortical granules

The distribution of CG was assessed by immunofluorescent staining to evaluate cytoplasmic maturation[17]. We found that following 24-h culture, CG had migrated to the cortex and formed a continuous layer under the oolemma in all groups. When oocytes were treated with 4 mM-Hx, CG were distributed throughout the cytoplasm (Fig. 3(b) and (c)). However, there were fewer cytoplasmic CG in the Hx + folic acid groups compared with the Hx medium group (Fig. 3(c)). Large CG-free domains form as the germinal vesicle breaks down[18] and these were detected in the control and in the Hx + 500 μM-folic acid-treated groups, but not in the Hx medium group (Fig. 3(d)–(f)).

Activation of M-phase-promoting factor and mitogen-activated protein kinase signalling pathways

Xenopus oocytes were studied by immunoblotting to examine the role of M-phase-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) pathways. As shown in Fig. 4, following 6-h culture in medium containing folic acid, there was a dose-dependent induction of ERK1 phosphorylation and a corresponding increase in the expression of Mos and cyclin B2.

Discussion

In nearly all vertebrates, oocytes within the ovary are arrested at the diplotene stage of the first meiotic prophase until ovulation. In response to the pre-ovulatory hormonal surge or removal from their antral follicles, meiotically competent oocytes resume meiosis up to MII and are subsequently held in meiotic arrest again until fertilisation[19,20]. This process of oocyte maturation is accompanied by nuclear envelope breakdown, meiotic spindle assembly and PB1 extrusion[21]. The present study shows that folic acid added to the medium increases the percentage of PB1 formation, increases GVBD, restores the spindle configuration and maintains the normal distribution of CG in mouse oocytes inhibited by Hx. The percentages of GVBD of *Xenopus* oocytes were also significantly higher when the medium contained supplementary folic acid. The results suggest that this is associated with the activation of MPF and MAPK signalling pathways. These data suggest that folic acid has the potential to enhance maturation of oocytes *in vitro*.

Spindle analysis has been widely used to assess oocyte quality and the effects of chemicals on oocytes[22–24]. Spindles of meiosis II oocytes are characteristically barrel shaped[15,25]; however, exposure to low temperature or treatment with chemicals, such as thioglycolic acid, leads to the elongation of metaphase spindles and the depolymerisation

Table 2. Effect of folic acid (FA) on the spindle configuration of mouse oocytes treated for 24 h
(Mean values with their standard errors)

		Spindle length (μm)		Spindle width (μm)		Spindle area (μm^2)	
Group	Oocytes (*n*)	Mean	SEM	Mean	SEM	Mean	SEM
M16	58	22·21	0·58	9·87	0·23	183·95	6·89
M16 + Hx	40	27·88*	0·70	13·60*	0·38	319·47*	15·85
M16 + Hx + FA	66	24·38*†††	0·58	9·41†††	0·20	210·04*†††	7·59

Hx, hypoxanthine.
* Mean values were significantly different compared with the M16 group ($P<0·05$).
††† Mean values were significantly different compared with the M16 + Hx group ($P<0·001$).

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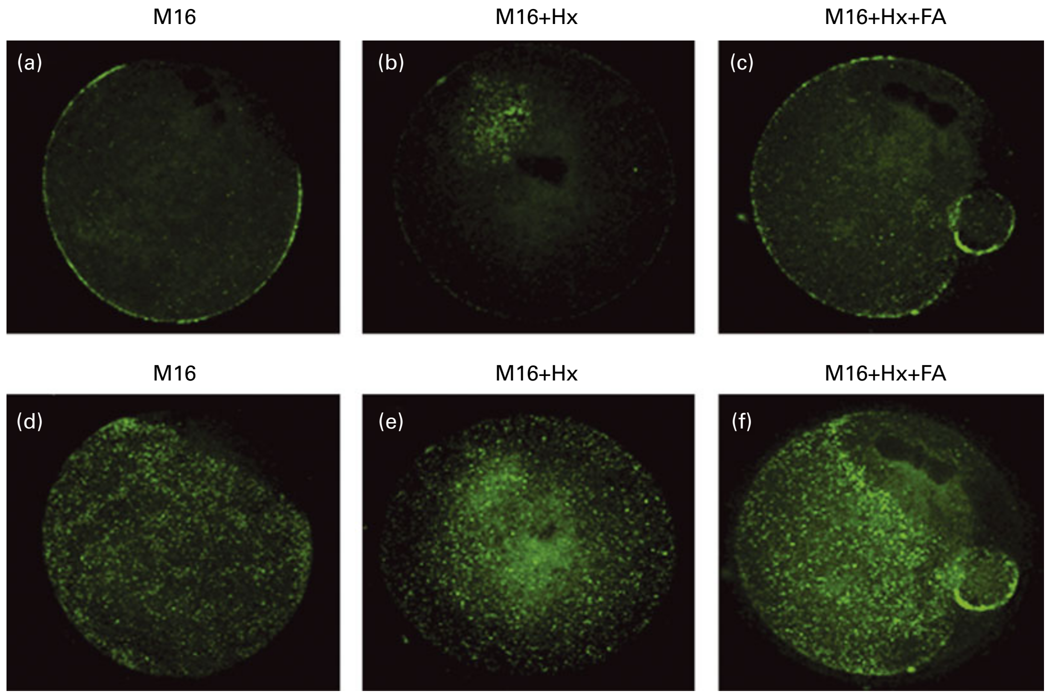

Fig. 3. Laser scanning confocal microscopic images of cortical granule (CG). Mouse oocytes were cultured in maturation medium with hypoxanthine (Hx) alone or in Hx medium with folic acid (FA) for 24 h, and MII oocytes from this were stained with fluorescein isothiocyanate-conjugated *Lens culinaris* agglutinin and then observed. Green fluorescence indicates the distribution of CG. (a–c) CG formation along the membrane of matured oocytes from each group. (d, f) Matured oocytes with a CG-free domain (CGFD). (e) Matured oocytes without a CGFD. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

of microtubules[26–29]. This was also the case for MII mouse oocytes from the Hx medium group, which also had an elongated barrel-shaped spindle, although the chromosomes were still well-aligned in the metaphase plate. Whilst spindle abnormalities have been reported to induce chromosomal errors[30], the oocytes treated with Hx appeared to possess normal chromosomes, suggesting that the changes to the MII spindles were not severe enough to affect the chromosomes. The elongated spindle configuration was recovered by folic acid, suggesting that folic acid may be a protective factor; however, because of the limited availability of material, we were only able to study the effect of folic acid at relatively high (500 μM) concentrations. Whether folic acid supplements are able to affect oocyte maturation *in vivo* remains to be established.

During oocyte maturation, CG migrate to the cortex and form a continuous layer under the oolemma. Changes in their distribution have been used to evaluate cytoplasmic maturation[17,31]. Treatment with Hx postponed CG migration and prevented CG-free domain formation of mouse oocytes, as reported previously[32]. However, the folic acid-treated oocytes showed normal migration of CG to the cortex and CG-free domain formation appeared to be normal. These data suggest that folic acid might facilitate the cytoplasmic maturation of mouse oocytes under *in vitro* conditions.

Oocyte maturation is mainly regulated by the activation of MPF and MAPK pathways, which play crucial roles in chromosome condensation, GVBD, microtubule assembly, spindle formation and CG migration during meiosis[33,34]. MPF, a heterodimer of cyclin B and the cell division control protein 2 (Cdc2) protein kinase, is maintained in an inactive state by Cdc2 phosphorylation on threonine 14 and threonine 15[35]. Moreover, Mos, a protein kinase that can phosphorylate and activate MAPK kinase MARK/ERK Kinase 1, promotes

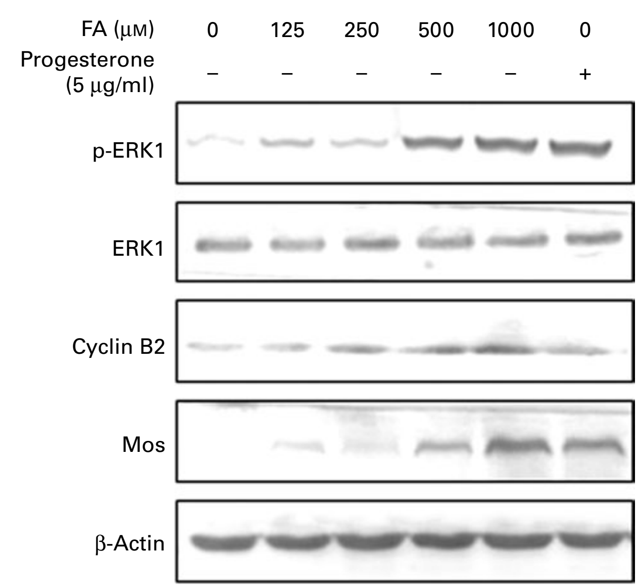

Fig. 4. Activation of M-phase-promoting factor and mitogen-activated protein kinase signalling pathways. *Xenopus* oocytes were treated with 125, 250, 500 and 1000 μmol/l of FA and 5 μg/ml of progesterone for 6 h. Cell lysates were subjected to Western blot analysis using anti-cyclin B2, anti-Mos, anti-phospho-extracellular signal-regulated kinase 1 (p-ERK1) and anti-ERK1 IgG. β-Actin and progesterone were used as loading control and positive control, respectively.

Cdc2 activation[36]. The present study shows that when *Xenopus* oocytes are cultured in the presence of folic acid, there is an increase in the phosphorylation of phospho-ERK1 and an induction of Mos and cyclin B2 expression. This finding suggests that folic acid might be acting through activation of the MPF and MAPK pathways.

In conclusion, the present study has shown that folic acid facilitates the maturation of mouse and *Xenopus* oocytes *in vitro*. In addition to increasing the rates of PB1 and GVBD formation, folic acid also eliminated the elongated spindle configuration and the abnormal distribution of CG by the activation of MPF and MAPK pathways. However, whether folic acid facilitates oocyte maturation *in vivo* remains unclear and thus warrants further investigation.

Acknowledgements

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