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M-Phase Promoting Factor (MPF) and Mitogen Activated Protein Kinases (MAPK) Activities of Domestic Cat Oocytes Matured *In Vitro* and *In Vivo*

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ABSTRACT

This work was undertaken in order to examine M-phase promoting factor (MPF) and mitogen-activated protein kinases (MAPK) activities during meiotic progression of cat oocytes cultured in two different media for two different incubation times and preovulatory cat oocytes that reached MII *in vivo*. Oocytes recovered from ovaries of ovariectomized cats were cultured either in TCM 199 or SOF for 24 h and 40 h. *In vivo* matured oocytes were recovered by follicular aspiration from ovaries of domestic cats ovariectomized 24 h to 26 h after hormonal treatment. Results showed that the kinetic of MPF and MAPK activity was similar during meiotic progression of cat oocytes matured in TCM 199 and SOF. After 24 h of incubation, MII oocytes had significantly ($p < 0.001$) higher MPF and MAPK levels than MII oocytes cultured for 40 h in both culture media. MPF and MAPK activity was significantly ($p < 0.01$) lower in the oocytes matured *in vitro* than in those matured *in vivo*. This study provides evidence that the two different maturation media did not determine differences in MPF and MAPK fluctuations and levels during meiotic progression of cat oocytes and that the time of maturation influenced the level of the two kinases. Moreover, it shows that MPF and MPK activity is higher in *in vivo* matured oocytes than in *in vitro* matured oocytes, suggesting a possible incomplete cytoplasmic maturation after culture.

INTRODUCTION

In both domestic and non-domestic cat species, assisted reproduction techniques including *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and embryo development have progressed during the last decade (Fartstad et al., 2000; Pope, 2000). The domestic cat is a valuable model for the establishment of these techniques in non-domestic feline species, most of which are threatened or endangered. *In vitro* oocyte maturation, fertilization, and embryo production has been shown to be relatively effective in the domestic cat and several non-domestic species (Pope, 2000). However, optimal conditions for *in vitro* maturation have not yet fully defined, and IVM efficiency of cat oocytes is generally inferior to those of other species (Johnston et al., 1989, 1993; Goodrowe et al., 1991; Luvoni et al., 1993; Pope et al., 1993; Wood et al., 1995; Schramm and Bavister, 1995; Spindler et al., 1999); only 50–60% of cultured cat oocytes achieve nuclear maturation.

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Moreover, even under optimal culture, *in vitro* matured cat oocytes show poor *in vitro* embryo development compared to those derived from *in vivo* matured oocytes (Swanson et al., 1996; Pope et al., 2000; Gomez et al., 2000). The developmental difficulties of *in vitro* matured oocytes could be related to several factors such as deficiencies in cytoplasmic maturation due to inadequate conditions of *in vitro* maturation systems. The oocyte nuclear and cytoplasmic maturation can be asynchronous as reported for the oocytes of several species (Goudet et al., 1997; Singh et al., 1997; Spindler and Wildt, 1999), and the nuclear assessment of meiotic maturation may not be a good index of viability and developmental potential post-fertilization. Recently, it has been shown that determining cat oocyte energetic-metabolic activity during *in vitro* maturation provides useful information on developmental competence after fertilization *in vitro* (Spindler et al., 2000). Similarly, analysis of the activity of molecules involved in the regulation of the meiotic cycle could be an important indicator of oocyte quality. Among these molecules, the maturation promoting factor (MPF) and the mitogen activated protein kinases (MAPK) are actively involved in the control of the maturation process and play a pivotal role in the mitotic and meiotic process (Dunphy et al., 1988; Nurse et al., 1990; Peter et al., 1992; Verlhac et al., 1993). In fact, the activity of these two kinases is necessary for the onset of germinal vesicle breakdown, the meiotic progression and the arrest of the oocyte at metaphase II (Masui and Merkert, 1971; Naito and Toyoda, 1991; Gavin et al., 1994; Sobajima et al., 1993; Haccard et al., 1993).

The kinetics of MPF and MAPK activity during meiotic progression (Hashimoto and Kishimoto, 1988; Naito and Toyoda, 1991; Goudet et al., 1998; Ledda et al., 2001), and after *in vitro* fertilization and parthenogenetic activation of the oocytes (Bogliolo et al., 1996, 2000; Xu and Greeve, 1988; Kikuchi et al., 1995) have been studied in several species. Moreover, it has been shown that MPF activity is related to oocyte quality and is influenced by the different culture media used for *in vitro* maturation (Naito et al., 1992; Ledda et al., 2001).

Since no information is available on cat oocytes, we assayed the MPF and MAPK activity during meiotic progression of cat oocytes matured *in vitro* in two culture conditions and in preovulatory cat oocytes that reached MII *in vivo*.

MATERIALS AND METHODS

Chemicals

All chemicals in this study were purchased from Sigma Chemical Company (St. Louis, MO) unless stated otherwise.

Recovery and in vitro maturation of oocytes

Ovaries were recovered from domestic cats subjected to ovariectomy or ovariohysterectomy at the veterinary clinic during February–April. Cats were anaesthetized with tiletamine and zolazepam HCl (Zoletil 20, Virbac, Milan, Italy, 10 mg/kg body weight, im), and ovaries were excised aseptically via midventral laparotomy. The ovaries were immediately placed in phosphate-buffered saline (PBS) containing penicillin-G potassium (100 μg/mL) and streptomycin sulphate (100 μg/mL) at 37°C. Within 2–4 h of collection, the ovaries were washed several times in fresh PBS and the oocytes released from the follicle by repeatedly puncturing the ovaries with 200-gauge needles until the tissue was minced. According to previously defined criteria (Johnston et al., 1989), oocytes with a uniform dark, finely granulated ooplasm that filled the zona pellucida and completely surrounded by corona radiata and cumulus cells were selected for *in vitro* maturation.

Selected oocytes were randomly divided in two treatment groups for *in vitro* maturation:

a. TCM 199 + 0.4% BSA + cysteamine (100 mM) + follicle stimulating hormone (FSH, 0.1 IU/mL, Pluset, Serono, Italy) + luteinizing hormone (LH, 0.1 IU/mL, Pluset, Serono, Italy).
b. Synthetic oviductal fluid (SOF, Tervit et al., 1972) + BSA, cysteamine, FSH and LH at the same concentration as in TCM 199.

Oocytes were cultured in 2 mL of medium in groups of 50, in 35-mm Petri dishes at 39°C in a humidified environment of 5% CO_2 in air for 24 h and 40 h. At different times (0, 8, 16, 20, 24, and 40 h) during culture, groups of cumulus–oocyte complexes were stripped of their granulosa cells by pipetting through a narrow-bore pipette and were thereafter briefly evaluated by Hoechst staining (1 μg/mL Hoechst 33342, Sigma) to assess their meiotic stage (germinal vesicle, GV; germinal vesicle breakdown, GVBD; metaphase I,

MI; anaphase-telophase, A/T; metaphase II, MII). Each oocyte was placed in 2 μL of ice cold collection buffer (PBS supplemented with 1 mg/mL polyvinyl alcohol, 5 mM EDTA, 10 mM Na_3VO_4, 10 mM NaF) and then stored at −80°C pending MPF and MAPK assays.

Gonadotrophin treatment and recovery of in vivo matured oocytes

Interoestrual domestic cat females were treated with a total of 12.5 U.I FSH given as a decreasing daily s.c. doses for four days (day 1 = 5; day 2 = 3.5; day 3 = 2.5; day 4 = 1.5). Approximately 85 h after initiation of FSH treatment, donors were given 150 U.I hCG (Corulon, Intervet, Italy).

At 24–26 h after hCG, cats were anaesthetized with tiletamine and zolazepam as described above, and ovaries were excised aseptically via midventral laparotomy. The ovaries were immediately placed in PBS, and oocytes were recovered by aspirating all follicles of 2 mm or larger in diameter with a 20-gauge needle. Cumulus cells were loosened from oocytes by incubation with 0.1% hyaluronidase (type VIII, Sigma), and then those cells that were still attached were mechanically removed by repeated aspiration using a small bore glass pipette. Thereafter, the oocytes were stained with Hoechst 33342 and briefly evaluated as described for *in vitro* matured oocytes. Each oocyte at MII stage was placed in 2 μL of ice cold collection buffer and then stored at −80°C pending MPF and MAPK assays.

Histone H1 kinase assay

MPF activity was determined by measuring the phosphorylation of exogenous histone H1 in cat oocytes at different stages (GV; GVBD; MI; A/T; MII) of their meiotic progression in the two different culture systems and in MII cat oocytes recovered *in vivo*. Histone H1 Kinase was assayed as described by Naito and Toyoda (1991) with some modifications.

After thawing, samples were brought to a final volume of 9 μL with a solution containing 45mM β-glycerophosphate, 12mM p-nitrophenylphosphate, 20mM MOPS-KOH (pH 7.2), 12mM MgCl2, 12mM EGTA, 0.1% mM EDTA, 0.8% mM DTT, 2.3mM NaVO4, 2mM NaF, 0.8 mM PMSF, 15 μg/mL leupeptin, 30 μg/mL aprotinin, 1 mg/mL PVA, 1 mg/mL histone H1 (type III-S from calf thymus, Sigma), 2.2 μM protein kinase inhibitor peptide (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asu-Ala-Ile-His-Asp), and 1.8 MBq/mL γ-[^{3}P]-ATP (166.5 TBq/mmole, ICN Pharmaceuticals, Costa Mesa, CA). The reaction started after addition of γ-

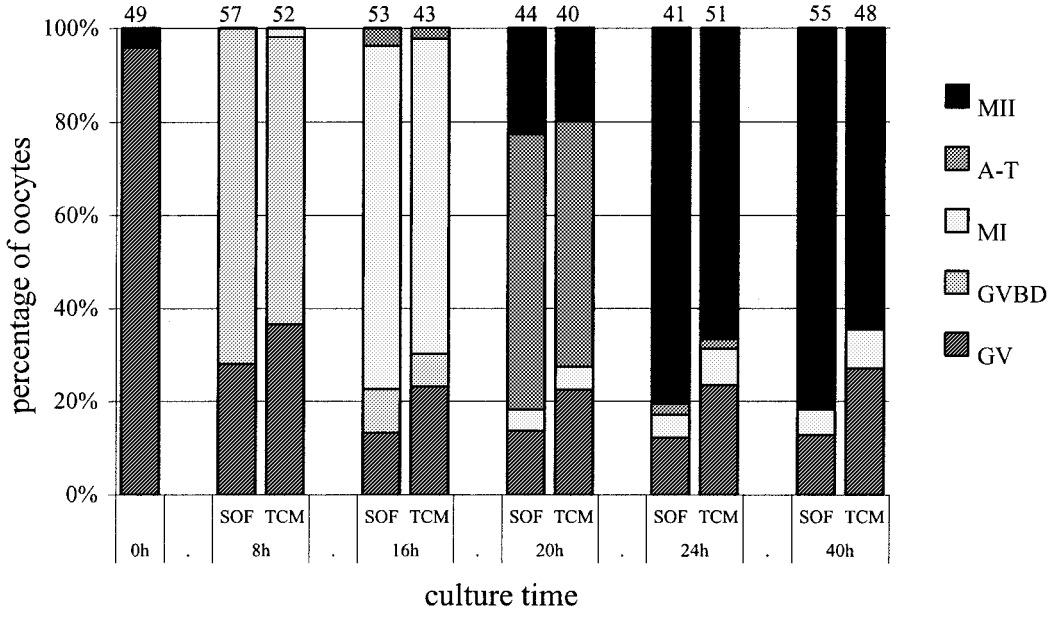

FIG. 1. Stages of meiotic maturation of the cat oocytes at different time intervals during culture in TCM 199 and SOF. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; A-T, anaphase-telophase; MII, metaphase II.

TABLE 1. *IN VITRO* MATURATION OF CAT OOCYTES AFTER 24 H AND 40 H OF CULTURE IN TWO DIFFERENT MEDIA

Culture medium	*Culture time*	*No. oocytes examined*	*No. matured oocytes (%)*
TCM 199	24 h	51	34 (66.7)[a]
	40 h	48	31 (64.6)[a]
SOF	24 h	41	33 (80.5)[b]
	40 h	55	45 (81.8)[b]

[a,b]Values with different superscripts are significantly different ($p < 0.001$; chi-squared test).

[^{32}P]ATP and was performed for 30 min at 37°C. The assay was stopped in 2 × concentrated sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 min. Proteins were separated on 1D-SDS-PAGE electrophoresis as described by Laemmly (1970), and the phosphorylation of substrates was analyzed by autoradiography of the gels.

Myelin basic protein kinase assay

MAPK activity during the meiotic progression of *in vitro* matured cat oocytes in the two different culture systems and in MII cat oocytes recovered *in vivo* was determined by the same protocol as for MPF except for the substitution of histone H1 with myelin basic protein (MBP).

Five replicates were carried out for each time point of meiotic progression in the two different culture media and for MII oocytes recovered *in vitro*.

The activity of both MPF and MAPK of each sample was quantified by measuring the density of the bands in the autoradiographic film with a densitometer. The values were analyzed by analysis of variance (ANOVA). Data were considered different when $p < 0.05$.

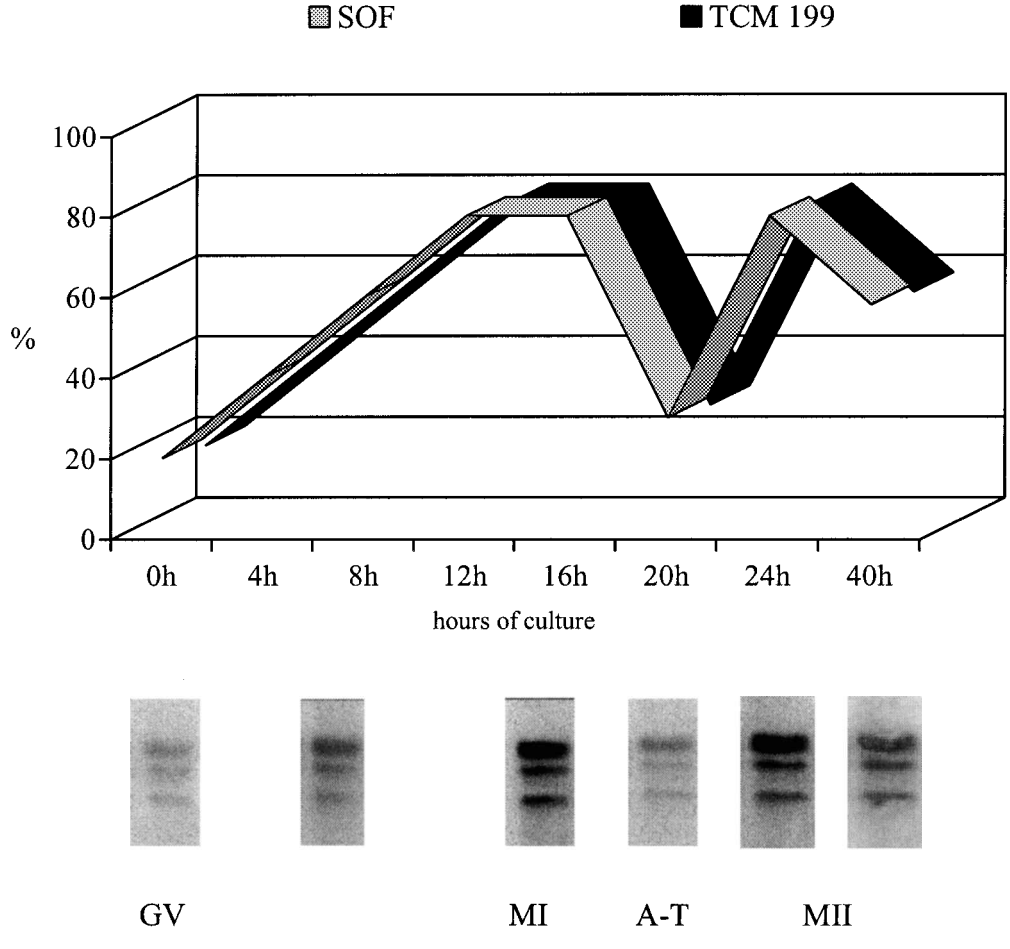

FIG. 2. Changes in MPF activity during meiotic progression of cat oocytes cultured in SOF and TCM 199 for 24 h and 40 h.

RESULTS

Nuclear maturation of cat oocytes

The number of oocytes examined at each time point ranged from 41 to 57 (total $n = 533$).

The stages of meiotic maturation of the oocytes at different time intervals examined are shown in Figure 1.

Results of *in vitro* nuclear maturation of domestic cat oocytes after 24 h and 40 h of culture in TCM 199 and SOF are reported in Table 1. A significantly ($p < 0.001$) higher rate of oocytes reaching MII was recorded following 24 h of incubation with SOF (33/41; 80.4%) than with TCM 199 (34/51; 66.7%). Similar differences ($p < 0.001$) were registered after 40 h of culture (31/48; 64.6% vs. 45/55; 81.8%). A comparison of the maturation rate at 24 h and 40 h reveals that meiosis was completed after 24 h, without any significant differences within each treatment group.

MPF and MAPK activity

MPF activity during the meiotic progression of cat oocytes matured in two different culture media is shown in Figure 2. The biochemical determination of MPF activity during the different stage of meiotic progression of cat oocytes in the two different culture media did not revealed significantly difference in the fluctuations and levels of MPF in the two treatments groups. MPF level was low at GV stage, rose during GVBD, reached two peaks at MI and MII, and exhibited a temporary decrease associated with A-T.

Also MAPK activity was similar during meiotic progression of cat oocytes matured in TCM 199 and SOF (Fig. 3). Its level was very low at GV stage, rose during GVBD and chromosome condensation until MI, and remained high until the MII.

MII cat oocytes matured *in vitro* in the two different media displayed equivalent MPF and MAPK levels.

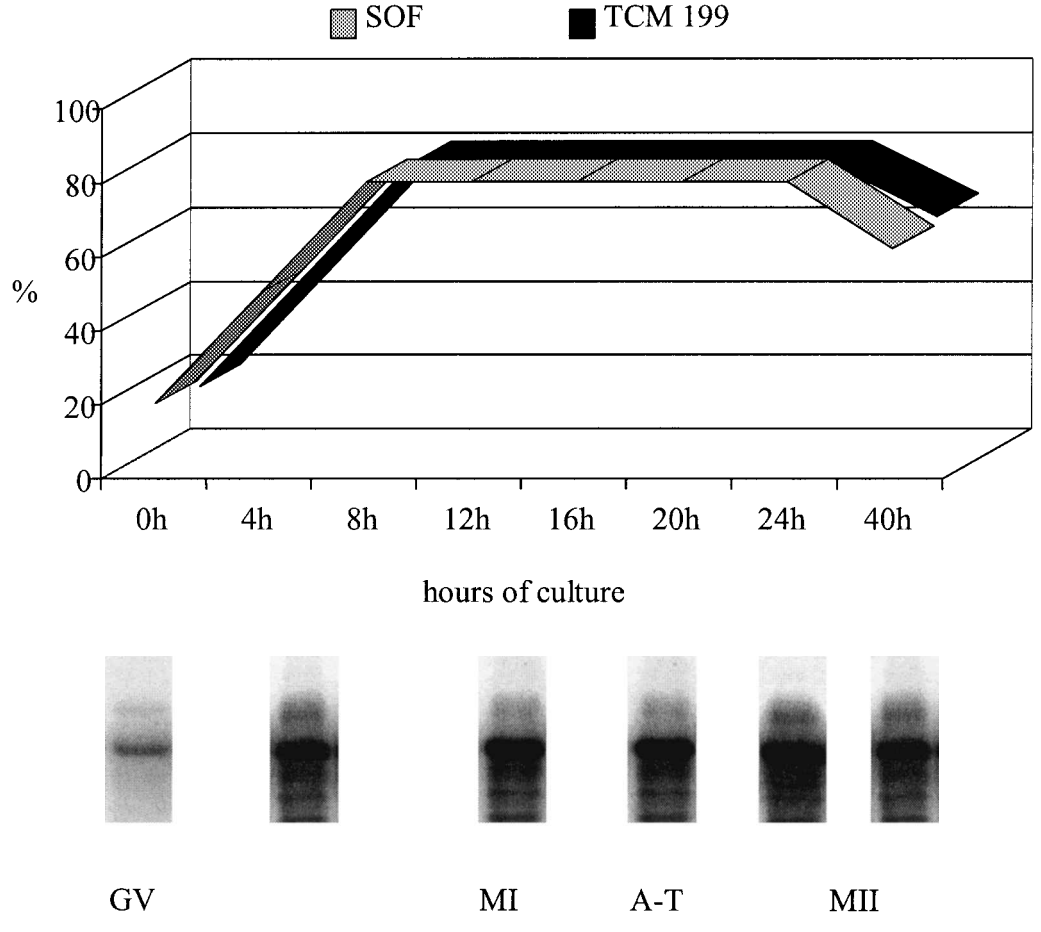

FIG. 3. Changes in MAPK activity during meiotic progression of cat oocytes cultured in SOF and TCM 199 for 24 h and 40 h.

After 24 h of maturation, MII oocytes had significantly higher ($p < 0.001$) levels of MPF and MAPK than MII oocytes incubated for 40 h in both culture media (Figs. 4 and 5). MPF and MAPK activity was significantly lower in *in vitro* matured oocytes than in MII oocytes recovered *in vivo*. Infact, MPF activity was 100 ± 7.48 of *in vivo* matured oocytes, while in MII oocytes matured *in vitro* in SOF and TCM 199 was, respectively, 82.4 ± 9.37 ($p < 0.01$) and 81.2 ± 7.26 ($p < 0.01$) after 24 h of culture and 60 ± 6.63 ($p < 0.001$) and 59 ± 6.63 ($p < 0.001$) after 40 h (Fig. 4).

The activity of MAPK of MII oocytes recovered *in vivo* was 100 ± 6.32, while in MII oocytes matured *in vitro* in SOF and TCM 199 was, respectively, 82.6 ± 10.5 ($p < 0.05$) and 79.2 ± 7.73 ($p < 0.01$) after 24 h of culture and 66.4 ± 6.1 ($p < 0.001$) and 63.2 ± 6.98 ($p < 0.001$) after 40 h of culture (Fig. 5).

DISCUSSION

In the present study, we examined MPF and MAPK activity during meiotic progression of cat oocytes cultured in two different media and in preovulatory cat oocytes that reached MII *in vivo*.

The results provide evidence that the fluctuation patterns and values of the two kinases were almost the same during each stage of meiotic progression in the oocytes matured in TCM 199 and SOF.

As reported in our previous study (Bogliolo et al., 2001), our data showed that a greater proportion of oocytes cultured in SOF achieved complete nuclear maturation compared to those cultured in TCM 199. However, we observed that the activity of the two major cell cycle regulators, as indicator of cytoplasmic maturation, is similar in the complex or simple medium irrespective of

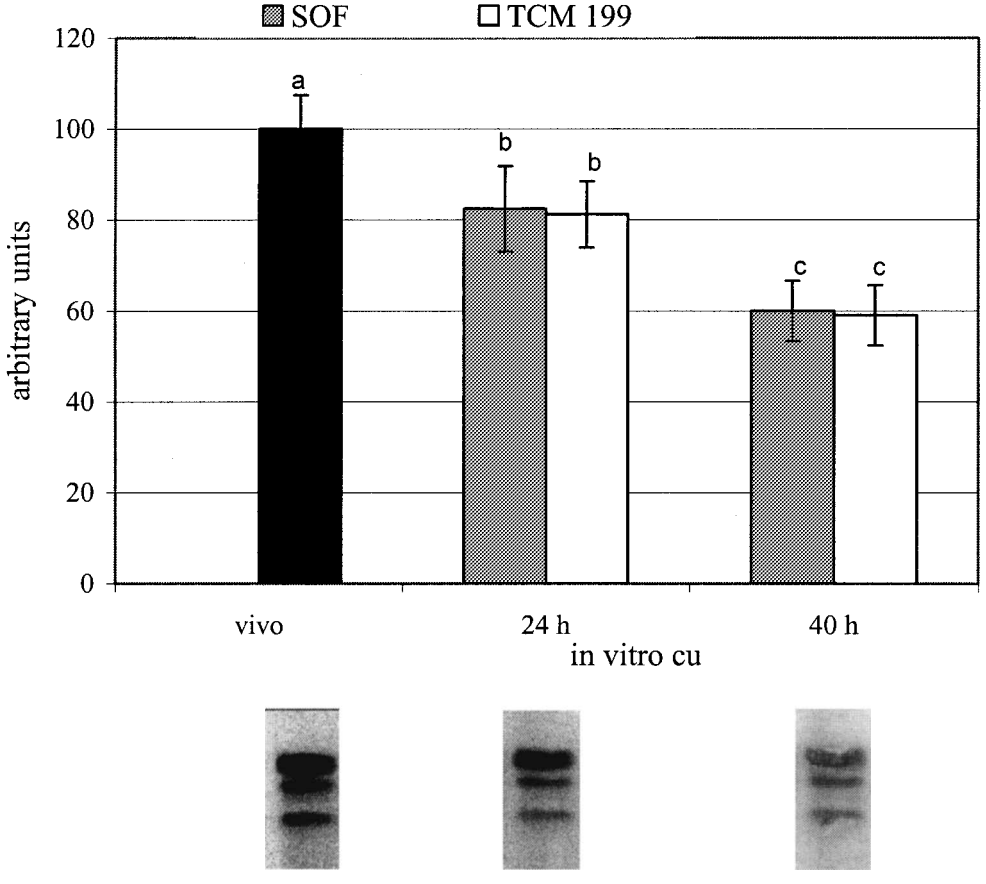

FIG. 4. MPF activity of MII cat oocytes matured *in vitro* in SOF and TCM 199 for 24 and 40 h and matured *in vivo*. Mean (±SD) activity of five replicates is shown. Different superscripts are statistically different (ANOVA: a vs. b, $p < 0.01$; a, b vs. c, $p < 0.001$).

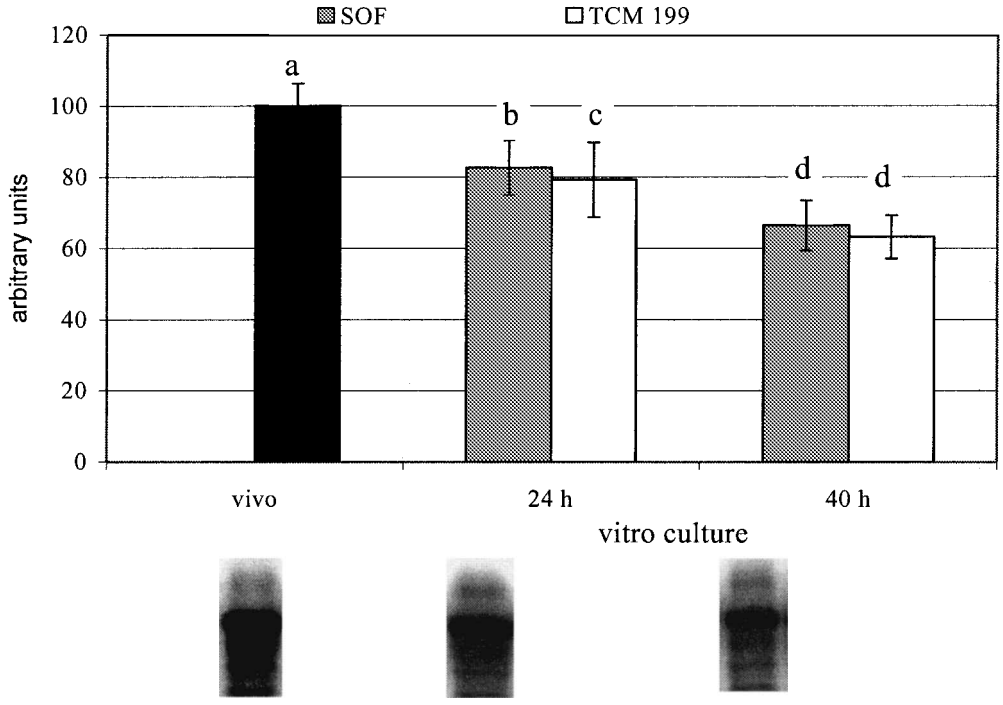

FIG. 5. MPK activity of MII cat oocytes matured *in vitro* in SOF and TCM 199 for 24 and 40 h and matured *in vivo*. Mean (±SD) activity of five replicates is shown. Different superscripts are statistically different (ANOVA: a vs. b, $p < 0.05$; a vs. c, $p < 0.01$p; a, b, c vs. d, $p < 0.001$).

the nuclear maturation percentages. These results were not in agreement with what has been described by other authors, which indicate that different maturation media affect the fluctuation pattern of MPF activity in porcine oocytes (Naito et al., 1992).

Kinetics of MPF and MAPK fluctuation of cat oocytes are entirely consistent with other experiments in mammalian species (Hashimoto and Kishimoto, 1988; Choi et al., 1991; Naito and Toyoda, 1991; Dedieu et al., 1996; Wu et al., 1997), which demonstrated the key regulator role of MPF and MAPK in the meiotic cell cycle. In fact, the increasing level of the two kinases induces the germinal vesicle breakdown and chromosomes condensation before the onset of MI, the reduction of MPF activity but not MAPK triggers the metaphase-anaphase transition and a resurgence of MPF activity and constant high level of MAPK were observed after the release of the first polar body at the MII stage.

Moreover, in the present study, we found that time of maturation (24 h, 40 h) can influence the level of the two kinases. MPF and MAPK activity reached a plateau at 24 h of *in vitro* maturation, corresponding to the time when almost the oocytes were at MII and decreased at 40 h of culture. Our findings are in agreement with other experiments, which indicate a gradual decrease of MPF activity in aged oocytes (Kukuchi et al., 1995a, 2000). Several authors have reported that, when the culture period is prolonged, presumptive cytoplasmic changes affect oocyte quality with reduction of viability and fertilization rates (Kubiak, 1989; Nagai, 1987; Hunter, 1987). Extended period of maturation also increased spontaneous oocyte activation (Kukuchi et al., 1995a, 2000). Consistent with these observations we observed a higher number of oocytes undergoing spontaneous activation after 40 h of culture (unpublished data). This phenomenon could be in part attributed to the decrease of MPF activity of the cat oocytes during a prolonged culture period. The relationship between MPF activity and parthenogenetic activation has been previously documented in other species (Choi et al., 1991; Naito and Toyoda, 1992; Kikuchi et al., 1995, 2000; Bogliolo et al., 2000).

The potential decrease of MPF and MAPK activity after 40 h of culture could explain the results of experiments on *in vitro* developmental competence of cat embryos after somatic cloning

(Skrzyszowska et al., 2002). These studies evidenced that recipient cytoplasm used after 43 h of *in vitro* maturation for somatic cloning decreased the developmental competence of reconstructed embryos to reach blastocyst stage. The authors suggest that the lower development was a consequence of not fully reprogramming the somatic nuclei after their transfer in suboptimal cytoplasmic environment of recipient oocytes.

The comparison between MPF and MAPK activity of *in vitro* and *in vivo* matured oocytes revealed that the levels of these two kinases was higher in *in vivo* MII oocytes than in the oocytes that reached MII *in vitro*. Similar findings have been reported in other species where higher MPF activity was observed in *in vivo* matured oocytes versus *in vitro* matured (Goudet et al., 1998). The relationship between oocyte quality and MPF and MAPK levels has been also described in other species (Christmann et al., 1994; Ledda et al., 2001). In porcine oocytes a lower pronucleus formation ability is associated with a lower histone H1 kinase activity (Naito et al., 1992). The inadequate cytoplasmic maturation after *in vitro* culture could be the main cause of the differences of the developmental competence reported in several studies between *in vivo* and *in vitro* matured cat oocytes (Pope et al., 2000; Gomez et al., 2000, 2002). In fact, it has been observed that IVM oocytes had a lower incidence of cleveage and development to the morula stage after IVF or ICSI compared to *in vivo* matured oocytes (Gomez et al., 2000; Pope et al., 2000).

In conclusion, our results showed that the kinetics and levels of MPF and MAPK are similar in the cat oocytes matured in two culture media and that the activity of the two kinases is higher in MII oocytes matured for a short time compared to those leaved in culture for 40 h. In addition our date provide evidence of a higher MPF and MAPK activity in the oocytes that reached MII *in vivo* versus those matured *in vitro*. These findings suggest that *in vitro* culture conditions for maturation should be improved in order to obtain oocytes of the best quality suitable for *in vitro* cat embryo production and for cloning experiments by nuclear transfer.

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