Effects of Caffeine on Maturation-Promoting Factor (MPF) Activity in Bovine Oocytes and on the Development of Somatic Cell Nuclear Transfer Embryos in White-Hanwoo

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ABSTRACT

The technique of SCNT is now well established but still remains inefficient. The *in vitro* development of SCNT embryos is dependent upon numerous factors including the recipient cytoplast and karyoplast. Above all, the metaphase of the second meiotic division (MII) oocytes have typically become the recipient of choice. Generally high level of MPF present in MII oocytes induces the transferred nucleus to enter mitotic division precociously and causes NEBD and PCC, which may be the critical role for nuclear reprogramming. In the present study we investigated the *in vitro* development and pregnancy of White-Hanwoo SCNT embryos treated with caffeine (a protein kinase phosphatase inhibitor). As results, the treatment of 10 mM caffeine for 6 h significantly increased MPF activity in bovine oocytes but does not affect the developmental competence to the blastocyst stage in bovine SCNT embryos. However, a significant increase in the mean cell number of blastocysts and the frequency of pregnant on 150 days of White-Hanwoo SCNT embryos produced using caffeine treated cytoplasts was observed. These results indicated that the recipient cytoplast treated with caffeine for a short period prior to reconstruction of SCNT embryos is able to increase the frequency of pregnancy in cow.

(Key words : Caffeine, Somatic cell nuclear transfer, Maturation-promoting factor (MPF), Pregnancy, White-Hanwoo)

INTRODUCTION

In somatic cell nuclear transfer (SCNT), the cell cycle stage and quality of the recipient cytoplast play an important role in successful development. In the majority of studies enucleated metaphase II (MII) oocytes have become the cytoplast of choice (Campbell and Alberio, 2003). MII oocytes contain maturation-promoting factor (MPF) activity which can cause dissolution of the nuclear envelope, condensation of the donor chromatin and dispersion of nucleoli in the donor nucleus, events which may be essential for nuclear reprogramming (Collas *et al*., 1992). The role of MPF in nuclear reprogramming is poorly understood, however, studies in cattle have shown that prolonged exposure of the donor nucleus to an MII cytoplasmic environment improves development (Wells *et al*., 1998).

Production of a cytoplast recipient involves enucleation of the oocyte which may have detrimental effects on subsequent development due to removal of oocyte proteins or perturbations in kinase activities. In previous studies (Lee and Campbell, 2006), we have shown that ovine oocytes can be effectively and reproducibly enucleated at anaphase/telophase of the first meiotic division (AI-TI). This procedure removes a minimal volume of oocyte cytoplasm and has no effects on oocyte MPF activity. However, a significant decrease in the ability to induce nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) in the donor nucleus was found in enucleated oocytes. One possible explanation for this observation is that the MPF activity or other factors in the enucleated oocyte is insufficient to induce NEBD. However, even in unenucleated oocytes not all transferred nuclei underwent NEBD suggesting the possibility that the MPF activity in MII ovine oocytes in general is less than is required to induce NEBD in somatic nuclei. This coupled

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with the significant variation in MPF activity between batches of oocytes suggests that in the majority of SCNT studies which follow embryo development, or produce live offspring, the exact fate of the transferred nucleus during the 1st cell cycle in individual embryos is unknown. If NEBD and PCC are important for 'nuclear reprogramming' then the production of cytoplast recipients which consistently induce these effects may be beneficial to development. Although MPF is involved in NEBD other factors may also be important, however, the production of cytoplasts with more uniform MPF activity, or the production of cytoplast recipients with increased kinase activities may influence the occurrence of NEBD and PCC.

MPF is a protein kinase composed of a catalytic subunit ($p34^{cdc2}$) and are gulatory subunit (cyclin B). CyclinB accumulates during interphase and associates with $p34^{cdc2}$ (pre-MPF). During interphase phosphorylation of tyrosine-15 (Y15) and threonine-14 (T14) in the ATP-binding site of $p34^{cdc2}$ by Myt1 and Wee1 kinases prevents MPF activation. At the G2/M transition, dephosphorylation of Y15 and T14 by cdc25 phosphatase brings about MPF activation (active MPF) (Kikuchi *et al*., 2000). In MII oocytes, MPF activity remains high and is stabilised by cytostatic factor, the product of the c-mos proto-oncogene (O'Keefe *et al*., 1991; Sagata *et al*., 1989). However, regulation by phosphorylation and dephosphorylation remains important, an imbalance in the relative rates of these events can change the level of MPF activity. It has previously been demonstrated that treatment of porcine oocytes with caffeine increases the dephosphorylation of pre-MPF and consequently increases MPF kinase activity by inhibiting Myt1/Wee1 activity (Kikuchi *et al*., 2000).

In these studies bovine oocytes were treated with caffeine, the effects on development of parthenogenetically activated oocytes and MPF activity in oocytes were investigated. Finally in order to test the hypothesis that artificially increasing cytoplast MPF activity by the treatment of caffeine may improve development of SCNT embryos and the developmental competence of SCNT White-Hanwoo embryos using caffeine treated enucleated oocytes as recipient cytoplast was examined.

MATERIALS & METHODS

All chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) unless otherwise stated.

In Vitro Oocyte Maturation

Bovine oocytes were aspirated from antral follicles (2~3 mm in diameter) of ovaries collected at a local slaughterhouse. Good quality oocytes with at least thee layers of cumulus cells and homogeneous cytoplasm selected for *in vitro* maturation. Selected oocytes were washed several times in Hepes-buffered TCM199 (Gibco Life Technologies Inc.) containing 10% FBS (Gibco) and then cultured in bicarbonate-buffered TCM 199 (Gibco) supplemented with 10% FBS, 5 μg/ml FSH, 5 μg/ml LH, 1 μg/ml estradiol-17β, 0.3 mM sodium pyruvate, 100 μM cysteamine and 50 μg/ml gentamicin. Groups of 40~45 oocytes were cultured in 500 μl of maturation medium under mineral oil in 4-well dishes (Nunc, Roskilde, Denmark) at 39℃ in a humidified atmosphere of 5% CO_2.

Caffeine Treatment and Parthenogenetic Activation of *In Vitro* Mature MII Oocytes

At 22~24 hours post onset of maturation (hpm), oocytes were stripped of cumulus cells by vortexing in 400 μl of H-SOF medium containing 300 IU/ml of hyaluronidase for 4~5 min in a 20 ml conical polystyrene tube and then followed by three washes in H-SOF. Good quality MII oocytes, those with the first polar body and an evenly pigmented cytoplasm were selected for parthenogenetic activation. Selected oocytes were cultured in maturation medium with different concentrations of caffeine (0 mM, 5 mM, 10 mM or 20 mM) until 28~30 hpm, washed in H-SOF and then immediately activated. Oocytes were activated by 5 min exposure to 5 μg/ml calcium ionophore (A23187), followed by incubation with 10 μg/ml of CHXM and 7.5 μg/ml CB for 5h at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Parthenogenetic embryos were cultured and examined as previously described for reconstructed embryos.

Oocyte Enucleation

Oocytes were enucleated at anaphase/telophase of the 1st meiotic division (AI-TI). Prior to enucleation the cumulus cells were removed at 15 hpm, oocytes were placed in to 400 μl of H-SOF medium containing 300 IU/ml hyaluronidase in a 20 ml conical polystyrene tube. The oocytes were incubated at 39℃ for 2 min and then vortexed for 4~5 min. The denuded oocytes were then incubated in H-SOF containing 5 μg/ml Hoechst 33342 for 15 min. Enucleation was carried out as previously described using a 20~25 μm glass micropipette in H-SOF plus 4 mg/ml BSA and 7.5 μg/ml CB. A portion of cytoplasm was removed containing the extruding AI-TI spindle. Enucleation of oocytes was confirmed by visualization of DNA in the aspirated karyoplast using a short exposure to UV light (0.1 sec). Enucleated oocytes were cultured in maturation medium with or without 10 mM caffeine.

Donor Cell Culture

Primary fibroblasts were isolated from an White-

Hanwoo ear skin as previously described (Wilmut *et al*., 1997) and cultured for two passages in Dulbecco modified Eagle medium (DMEM) supplemented with 1.0% β-mercaptoethanol, 2.0 mM L-glutamine, 1.0% (v/v) penicillin/streptomycin, and 10% FBS. Primary cultures were then stored in liquid N_2 until required. For each experiment, cells were thawed and cultured until approximately 80～90% confluence, quiescence was then induced by reducing the concentration of FBS to 0.1% for a further 2～3 days.

Cell Fusion and Activation

Quiescent primary fibroblasts used as nuclear donors were fused to enucleated cytoplasts with two DC pulses of 1.25 KV/cm for 60 μsec in 0.3 M mannitol in the absence of calcium ions using an Eppendorf Multiporator. Fused couplets were cultured in mSOFaaci containing and 4 mg/ml BSA until activated. Activation was carried out in H-SOF medium containing 5 μg/ml A23187 for 5 min, followed by culture in mSOFaaci medium supplemented with 10 μg/ml of cycloheximide (CHXM) and 7.5 μg/ml CB for 5h at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Culture of Reconstructed Embryos

Following activation, reconstructed embryos were transferred into mSOFaaci medium and cultured in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39℃. On 2 day of culture, embryo cleavage was assessed and 5% FBS added to the culture medium. The blastocysts were incubated in H-SOF containing 5 μg/ml Hoechst 33342 for 15 min. On day 7, development to blastocyst and the total cell number were assessed.

MPF Activity Assay

Groups of 10 oocytes, caffeine treated MII oocytes were prepared for each sample. Oocytes were washed several times in Ca^{2+}-free DPBS containing 0.1% PVA and placed into 5 μl of sample buffer (50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.01% Brij35, 1 mM PMSF, 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate, 1 mM Na-orthovanadate). The samples were snap frozen and stored at −70℃ until analysed. The MESACUP cdc2 kinase assay kit (MBL Medical & Biological Laboratories CO., LTD, Nagoya, Japan) was used for measuring non-radioisotopic cdc2 kinase activity. The kit is based on enzyme linked immunosorbent assay (ELISA) that utilizes a synthetic peptide as a substrate for the cdc2 kinases and a monoclonal antibody recognizing phosphorylation form of the peptide substrate. Cdc2 kinase present in the samples catalyzed phosphorylation of biotinylated MV peptide. The reaction mixture was transferred to anti-phosphoMV peptide monoclonal antibody immobilized microwell. The biotinylated phosphoMV peptides was bound to anti-phosphoMV peptide monoclonal antibody and was subsequently detected with streptavidin conjugated to horseradish peroxidase. The horseradish peroxidase substrate was then added to the microwell and the intensity of the color was measured photometrically at 492 nm.

Statistical Analysis

One-way ANOVA with GenStat was used for evaluation of the results. A probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

MPF Activity at Different Stages of Oocyte Maturation

The MPF activity was assayed at three different stages [germinal vesicle (GV), the first anaphase/telophase (AI-TI) or the second metaphase (MII)] during maturation of ovine oocytes *in vitro*. In non-cultured immature oocytes (0 hour post onset of maturation (hpm); GV), MPF activity was low the basal levels as shown in Fig. 1. During maturation the MPF activity increased significantly reaching maximum activity at MII, 22～24 hpm. (Fig. 1)

Determination of the Optimal Concentration of Caffeine

This experiment was carried out to determine the optimal concentration to caffeine in bovine oocytes. In the first experiment, matured denuded MII oocytes (22～24 hpm) were cultured in maturation medium containing 0 mM, 5 mM, 10 mM or 20 mM caffeine for 6h and then MPF activity determined. The MPF activity in

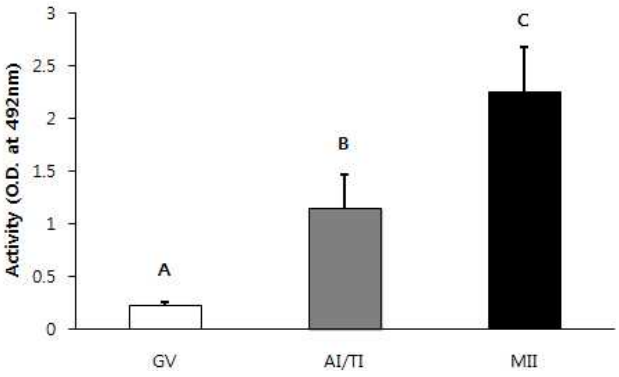

Fig. 1. Changes of maturation-promoting factor (MPF) in bovine oocytes at the different stages of maturation. 10 oocytes were cultured, denuded and then collected in 5 μl sample buffer. GV: germinal vesicle oocyte (0 hpm). AI/TI: anaphase I/telophase I oocytes (15 hpm). MII: metaphase II oocytes (22～24 hpm). Three replicates were performed. Significant increase in activity (p<0.05) compared with that of control.

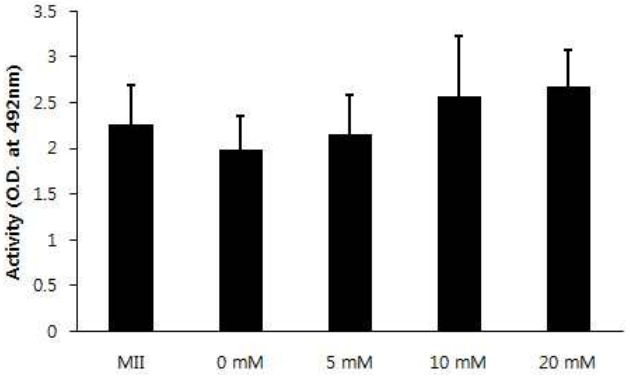

Fig. 2. Effects of caffeine on maturation-promoting factor (MPF) activity in bovine oocytes. Oocytes were denuded at 22~24 hpm, cultured in different concentrations (MII, 0 mM, 5 mM, 10 mM and 20 mM) of caffeine for 6 h and then collected in 5 μl sample buffer before snap freezing/thawing for cdc2 kinase activity. 10 oocytes were analyzed for each concentration of caffeine and three replicates were performed. Bars represent mean±SEM.

bovine oocytes treated with 0 mM and 5 mM caffeine (24~30 hpm) were lower than those in MII oocytes (24 hpm), in contrast treatment with 10 mM and 20 mM caffeine increased the MPF activity as compared to 24 hpm MII oocytes (Fig. 2). In the second experiment, the developmental competence of embryos derived from parthenogenetically activated bovine oocytes was examined. MII (24 hpm) oocytes were treated with 0 mM, 5 mM, 10 mM and 20 mM caffeine for 6 h, activated and development assessed. The results from these studies are presented in Table 1. Treatment with 20 mM caffeine significantly reduced cleavage at all time periods as compared to 0 mM, 5 mM and 10 mM groups. 20 mM caffeine caused a significant reduction in development to the blastocyst stage at all time points, in contrast although 10 mM caffeine increased development to blastocyst, this was not significantly different 5 mM and control groups. 10 mM was chosen for the concentration of caffeine to bovine oocytes.

Table 1. Determination of the optimal concentration of caffeine on the developmental competence of Parthenogenetic bovine embryos

Concentration of caffeine (mM)	No. oocytes	No. cleaved (%) on 2 day	No. blastocysts (%) on 7 day
0	64	62 (96.9)^{b}	27 (42.2)^{b}
5	64	59 (92.2)^{b}	25 (39.1)^{b}
10	64	58 (90.6)^{b}	27 (42.2)^{b}
20	64	50 (78.1)^{a}	15 (23.4)^{a}

MII oocytes were selected 22~24 hpm and cultured with the different concentrations of caffeine for 6 h. Following caffeine treatment, oocytes were activated in H-SOF containing 5 μg/ml calcium ionophore (A23187), cultured in mSOFaaci with 10 μg/ml of CHXM and 7.5 μg/ml CB for 5h and then transferred to mSOFaaci medium supplemented with 4 mg/ml BSA, 5% O_2, 5% CO_2 and 90% N_2 at 39℃. On 2 day cleavage was assessed and cleaved embryos were transferred to fresh culture medium supplemented with 10% FBS. On day 7 development to blastocysts was assessed. ^{a,b} Values in same columns with different superscripts are significantly different (p<0.05).

Determination of the Optimal Period of Exposure to Caffeine

The experiment was examined to determine the optimal period of exposure to caffeine. In the experiment, matured denuded MII oocytes (24 hpm) were cultured in maturation medium containing 10 mM caffeine for 0, 2, 4, 6, 8h and then MPF activity determined. The MPF activity in bovine oocytes treated with 10 mM caffeine for 0, 2 and 4 hours (24~28 hpm) were lower than those in MII oocytes (30 hpm), in contrast treatment with 10 mM for 6 and 8 hours caffeine increased the MPF activity as compared to 24 hpm MII oocytes (Fig. 3). In the second experiment, the developmental competence of embryos derived from parthenogenetically activated bovine oocytes was examined. MII (24 hpm) oocytes were treated with 10 mM caffeine for 0, 2, 4 or 6 hours, activated and development assessed. The results from these studies are presented in Table 2. Treatment with 10 mM caffeine for 8h significantly reduced cleavage at all time periods as compared to 2, 4 or 6 hours groups. 10 mM caffeine for 8h caused a significant reduction in development to the blastocyst stage at all time points (Table 2). 6 hours of exposure to caffeine was chosen for the optimal period in bovine oocytes.

Development of NT Embryos Reconstructed using Caffeine Treated Oocytes as Cytoplast Recipients

The quality of the recipient cytoplast is a major factor to achieve nuclear reprogramming and development

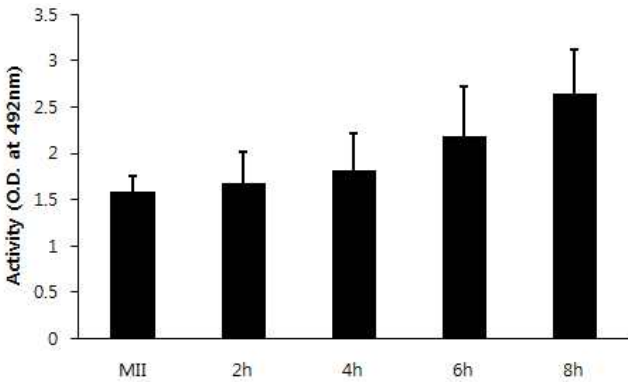

Fig. 3. Effects of treatment of metaphase II (MII) bovine oocytes with caffeine on maturation-promoting factor (MPF) activity. MII oocytes were cultured in different exposures (0, 2, 4, 6 and 8 h) to 10 mM caffeine. 10 oocytes were analyzed for each concentration of caffeine and three replicates were performed. Bars represent mean±SEM.

Table 2. Determination of the optimal period of exposure to caffeine on the developmental competence of Parthenogenetic bovine embryos

Culture period(h)	No. oocytes	No. cleaved (%) on 2 day	No. blastocysts (%) on 7 day
0	63	27 (42.9)[a]	14 (21.9)[a]
2	64	55 (85.9)[bc]	24 (37.5)[bc]
4	64	54 (84.4)[bc]	25 (39.1)[bc]
6	64	58 (90.6)[c]	27 (42.2)[c]
8	64	51 (78.1)[b]	21 (32.8)[b]

MII oocytes were selected 22～24 hpm and cultured with the different periods of exposure (0, 2, 4, 6 and 8h) to 10 mM caffeine for 6h. Following caffeine treatment, oocytes were activated in H-SOF containing 5 μg/ml calcium ionophore (A231-87), cultured in mSOFaaci with 10 μg/ml of CHXM and 7.5 μg /ml CB for 5h and then transferred to mSOFaaci medium supplemented with 4 mg/ml BSA, 5% O_2, 5% CO_2 and 90% N_2 at 39℃. On 2 day cleavage was assessed and cleaved embryos were transferred to fresh culture medium supplemented with 10% FBS. On day 7 development to blastocysts was assessed. [a,b] Values in same columns with different superscripts are significantly different (p<0.05).

in SCNT reconstructed embryos. To examine the effects of caffeine treatment on development of SCNT embryos reconstructed using enucleated bovine oocytes as cytoplasmt recipients, two different treated enucleated oocytes were used as cytoplast recipients. In all groups oocytes were enucleated at 15～18 hpm, Group I: cytoplasts were returned to culture and then transferred into maturation medium (18～24 hpm) and subsequently fused to nuclear donor cells (G0/G1 phase of the cell cycle) at 24 hpm, Group II were returned to culture and then transferred into maturation medium containing 10 mM caffeine (18～24 hpm) and fused at 24 hpm. Fused embryos in all groups were selected and activated 1h following application of the cell fusion pulse. Each of these cytoplast recipients contained different levels of MPF activity. The results of these experiments are summarized in Table 3. The fusion rate of reconstructed embryos with in the different treatments differed significantly between control and caffeine group (88.2% vs. 77.3%) (p<0.05). No significant difference was observed between each treatment for either cleavage rate of the reconstructed embryos (88.1% vs. 81.1%) or their development to the blastocyst stage (20.8% vs. 21.9%). The frequency of development of embryos reconstructed using caffeine treated cytoplast recipients was not greater than that of the control groups. However, the mean cell number of day 7 blastocysts from non-treated SCNT embryos was 76.1±2.7, this was significantly lower than the mean cell number of SCNT embryos treated with 10 mM caffeine (107.8± 6.7) (p<0.05) (Table 3). In addition, when White-Hanwoo SCNT embryos were transferred to the surrogates by the method of non-surgical embryo transfer, the frequency of pregnant on 5 months was significantly increased (p<0.05) (Table 3).

DISCUSSION

In these studies treatment of *in vitro* matured bovine oocytes for 6 hours with 10 mM caffeine did not adversely affect development to the blastocyst stage of parthenogenetically activated oocytes. However, this treatment caused a significant increase in the activity of MPF in oocytes and might result in a significant increase in NEBD and PCC in the donor nucleus when treated oocytes were fused to G0/G1 donor cells. The mechanisms responsible for the increase in NEBD and PCC in caffeine treated oocytes are unknown. The possibility that bovine oocyte MPF levels are simply below that required to induce NEBD is supported by the fact that increasing MPF activity increase the occurrence of NEBD. Whether, the effects of MPF and or MAPK kinase in inducing NEBD are direct on the donor nucleus or whether one or both of these kinases activates other molecules is unknown, however, the effects of increasing kinase activity is to increase occurrence of NEBD

Table 3. Developmental competence of White-Hanwoo SCNT embryos using caffeine treated enucleated oocytes as cytoplasmic recipients

Treatment	No. oocytes	No. fused (%) NT embryos	No. cleaved (%) NT embryos	No. blastocysts (%) on 7 day	No. Cells/blastocyst (mean±SEM)	No. surrogates	No. pregnant on 150 day
Control	229	202 (88.2)[a]	178 (88.1)[a]	42 (20.8)	76.1±2.7	16	2[a]
Caffeine	213	169 (79.3)[b]	137 (81.1)[b]	37 (21.9)	107.8±6.7	15	4[b]

Groups of oocytes were enucleated at 15～18 hpm. Control group: cytoplasts were returned to culture, transferred into maturation medium (18～24 hpm), cultured fused at 24 hpm and activated at 25 hpm. Caffeine group: cytoplasts were returned to culture and transferred into maturation medium containing 10 mM caffeine (18～24 hpm), fused at 24 hpm and activated at 25 hpm. Following activation SCNT embryos were transferred to mSOFaaci medium supplemented with 4 mg/ml BSA, 5% O_2, 5% CO_2 and 90% N_2 at 39℃. On 2 day cleavage was assessed and cleaved embryos were transferred to fresh culture medium supplemented with 10% FBS. On day 7 development to blastocysts was assessed. On 150 day, the pregnant was tested by ultrasonography. [a,b] Values in same columns with different superscripts are significantly different (p<0.05).

and PCC in the transferred nucleus.

In the White-Hanwoo SCNT reconstructed embryos, the developmental competence to the blastocyst stage did not differ between control or caffeine treated cytoplast recipients. However, a significant increase in the frequency of pregnant derived from White-Hanwoo SCNT embryos produced after caffeine treatment was observed. The mechanisms behind this increase in the frequency of pregnant are unknown but may be due to a number of pathways. Injection or electroporation of oocytes with caffeine has been reported to elucidate a calcium release in both porcine and bovine oocytes through the Ryanodine receptor (RyRs) (Petr *et al*., 2002; Viets *et al*., 2001), however, release of calcium through the Ryanodine receptor is thought to be dependent upon a primary calcium release mediated via the IP3 receptor. In these experiments, calcium did not induce activation of the treated oocytes which maintained high levels of MPF, however, it is possible that caffeine may have acted later in development and improved calcium release resulting in increased cell numbers.

A second possible explanation of the increase in pregnancy is related to the occurrence of NEBD and PCC. Previous reports have suggested that NEBD and exposure to the oocyte cytoplasm is more beneficial to development (Collas *et al*., 1992), furthermore prolonged exposure to the oocyte cytoplasm (Wells *et al*., 1998) and the use of young oocytes which contain higher levels of MPF than aged oocytes have both increased the frequency of development to blastocyst of NT bovine embryos. In the present study, no increase was observed in the frequency of development between caffeine treated and untreated oocytes, however, embryos produced using caffeine treated oocytes showed an increased cell number at the blastocyst stage. An increase in pregnancy suggests a change in the rate of cell division during this period of early development. Alterations in cell cycle length have been reported in bovine SCNT and parthenogenetic embryos as compared to *in vitro* produced embryos with major changes during the 4^{th} and 5^{th} cycles, which is coincident with the onset of zygotic transcription (Holm *et al*., 2003). In addition studies of transcription and gene expression in bovine SCNT embryos have shown that MII oocytes result in a greater inhibition of transcription from the transferred nucleus than do activated cytoplasts (Smith *et al*., 1996) and gene expression in blastocysts derived from MII cytoplasts is more normal (Wrenzycki *et al*., 2001). Chromatin condensation has been reported to displace transcription factors from the DNA in the absence of changes in their binding affinity (Martinez-Balbas *et al*., 1995). Therefore inducing PCC in the donor nucleus may remove somatic transcription factors and facilitate binding of embryonic transcription factors which are required for early development.

A third possibility is that NEBD and PCC facilitates earlier initiation or more rapid DNA synthesis during the 1^{st} cell cycle. Although studies have defined the effects of NEBD on the occurrence of DNA synthesis in donor nuclei of different cell cycle phases (Campbell *et al*., 1993), the effects of different activation protocols (Alberio *et al*., 2001b; Motlik *et al*., 2002) and donor cell cycle stage (Kurosaka *et al*., 2002) on the timing of S-phase no single study has investigated the initiation, duration and fidelity of S-phase in relation to cell cycle stages of both donor and recipient cells. However, it is possible that differences occurring during the 1^{st} cell cycle may contribute to the increase in cell numbers observed.

In summary the treatment of *in vitro* matured bovine oocytes with 10 mM caffeine for up to 6 hours affected the development of parthenogentically bovine embryos. The same treatment in AI-TI enucleated oocytes increases MPF activity, produces a cytoplasmic environment which induces NEBD and PCC of the transferred nucleus, in terms of response of the donor nucleus. Furthermore, White-Hanwoo SCNT embryos produced using caffeine treated cytoplasts have increased the total cell number of blastocyst stage embryos and frequency of pregnant on 5 months. The effects on development to term of SCNT embryos produced in this way needs to be established.

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