Synchronization of cell division in eight-cell bovine embryos produced *in vitro*: effects of 6-dimethylaminopurine

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The methods used to achieve blastomere cell cycle synchronization in embryos used as nuclear donors during embryo reconstruction have been largely unsuccessful. The aim of this study was to determine the reliability of 6-dimethylaminopurine (6-DMAP), an inhibitor of maturation promoting factor, to halt and to synchronize blastomere division in cleavage stage bovine embryos. A second goal was to assess its reversibility and toxicity *in vitro*. Eight-cell stage embryos obtained at 58 h after insemination were treated with several concentrations of 6-DMAP for 12 h. Treated embryos were assessed for cleavage arrest, chromatin morphology, DNA synthesis, histone H1 and scored for blastocyst formation and for hatching rate. They were subsequently fixed and the number of nuclei counted. Complete arrest of cell division was observed at concentrations of 3 mmol 6-DMAP l⁻¹ and above. At these concentrations, interphase nuclei in arrest were noticeably larger compared with interphase nuclei of eight-cell control embryos. Removal from 6-DMAP led to release from cleavage arrest and was followed by synchronized mitosis, histone H1 kinase deactivation and re-entry into interphase within 4–5 h. Twenty-nine per cent of interphase nuclei were synthesizing DNA at the end of the 12 h treatment as indicated by BrdU analysis. At 2 h after removal from 6-DMAP, an abrupt decrease to 9% BrdU-positive nuclei was observed followed by an increase to 39% by 6 h and a decrease to 28% at 10 h. The ability of treated embryos to reach the blastocyst stage *in vitro* and the number of cells per blastocyst were reduced. These results indicate that 6-DMAP can reversibly arrest and synchronize cleavage to the fifth cell cycle in eight-cell bovine embryos. Although a decrease was observed in the proportion of blastocysts obtained after treatment, it is concluded that 6-DMAP is a useful tool for synchronization studies requiring donor nuclei at metaphase before fusion to recipient oocyte.

Introduction

In mammals, early embryonic processes are characterized by asynchronous cleavage which increases progressively among blastomeres throughout preimplantation development. Asynchronous cleavage appears to play a role in determining the position of blastomeres within the embryo and, in doing so, determines their fate to become inner cell mass (ICM) or trophoblast (Sutherland *et al.*, 1990). Furthermore, blastomere asynchrony is also believed to cause inconsistent nucleocytoplasmic interactions in embryos reconstructed by nuclear transplantation (NT). The negative effects of the absence of cell cycle stage synchrony between nuclear donor and recipient cytoplasm have been shown to reduce the success of NT procedure in mammals (Smith *et al.*, 1988, 1990; Campbell *et al.*, 1994; Otaegui *et al.*, 1994; Stice *et al.*, 1994). The presence of a high concentration of maturation promoting factor (MPF) in host oocytes enucleated at metaphase is

believed to lead to premature chromosome condensation (PCC) of interphase donor nuclei immediately after fusion (Czolowska *et al.*, 1984; Szöllösi *et al.*, 1986; Collas and Robl, 1991; Collas *et al.*, 1992a). Treatments such as ageing, cooling and activation of recipient oocytes before fusion have been used to reduce MPF activity in enucleated oocytes.

Cell cycle synchronization of blastomeres within donor embryos is more difficult to obtain owing to the high asynchrony observed during late cleavage. Since no reliable methods are currently available for synchronizing cell division of bovine embryos, it was deemed important to determine the effectiveness of different cell-cycle-arresting agents which cause minimal effects to normal development. Nocodazole, an inhibitor of tubulin polymerization, has been used to synchronize mouse embryo cleavage (Kato and Tsunoda, 1992; Otaegui *et al.*, 1994; Samaké and Smith, 1996a, b). Although exposure to nocodazole caused limited toxic effects on subsequent development, chromatin dispersion was evident at longer exposure periods in mouse embryos and so could lead to ploidy abnormalities. An inhibitor of maturation promoting factor (MPF), 6-dimethylaminopurine (6-DMAP) has been used

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to prevent germinal vesicle breakdown (GVBD) during invertebrate (Guerrier and Dorée, 1975; Dorée *et al.*, 1983) and vertebrate oocyte maturation (Rime *et al.*, 1989). It was also successfully used to arrest cleavage in four-cell mouse embryos, enabling synchronized entry to the fourth cell cycle (Samaké and Smith, 1996a). Therefore, the aim of this study was to determine the lowest concentration of 6-DMAP necessary to arrest cleavage in bovine eight-cell embryos and to establish the reversibility and viability of treated embryos to develop normally to the blastocyst stage after treatment.

Materials and Methods

Embryo source and culture

Bovine ovaries were collected at a local abattoir, and non-haemorrhagic follicles with a diameter of 1–5 mm were punctured with an 18 gauge needle to collect cumulus–oocyte complexes. Groups of 20 cumulus–oocyte complexes with complete cumulus layers and homogeneous ooplasm were collected and matured for 24 h in 100 μ l TCM-199 bicarbonate-buffered medium (Gibco, Grand Island, NY) supplemented with 10 μ g LH ml^{-1} , 1 μ g FSH ml^{-1} , 2 ng oestradiol ml^{-1} and 10% FCS (Gibco). At the end of maturation, expanded cumulus–oocyte complexes were fertilized by standard protocols (Parrish *et al.*, 1986). Briefly, matured oocytes were transferred to TALP medium containing 10 μ g heparin ml^{-1} and inseminated with frozen–thawed, Percoll gradient-separated spermatozoa (1×10^6 spermatozoa ml^{-1}). At 18 h after insemination, zygotes were transferred to 50 μ l drops of INRA Menezo B2 medium (MB2; Pharmascience, Paris) with bovine oviduct epithelial cells (BOEC) for coculture. Eight-cell stage embryos obtained at 58 h after insemination were selected for this experiment to test the ability of 6-DMAP to arrest cleavage. Furthermore, to assess the reversibility of 6-DMAP treatment, embryos were washed three times in Hepes-buffered TCM-199 and transferred to agent-free MB2 medium, and their ability to resume cleavages to the fifth cell cycle was determined. They were then assayed, during a period of several hours after release from the arrest agent, to determine the percentage of metaphase nuclei as an indication of timing and synchrony of mitotic division, the activity of histone H1 kinase activity, and the pattern of DNA synthesis. The toxicity of treatment was assessed by washing embryos three times in Hepes-buffered TCM-199 medium and then coculturing them for a further 6 days in agent-free MB2 medium and their ability to resume cleavage and develop into normal blastocysts *in vitro* was determined.

Cleavage arrest and chromatin morphology assay

Working solutions of 6-DMAP (Sigma, St Louis, MO) were prepared by appropriate dilution of the stock into TCM-199 medium conditioned with BOEC. Embryos were treated with concentrations ranging from 0 to 5 mmol ml^{-1} of this agent for 12 h to determine the lowest effective dose. Some embryos were harvested at different times during the 12 h treatment to assess the kinetics of changes in chromatin morphology. At the

end of treatment, embryos were evaluated for cleavage and chromatin morphology using a rapid staining procedure for nuclei. Briefly, embryos were fixed in 10% (v/v) formalin and mounted onto a glass slide in a mounting solution (Mowiol; Aldrich, Milwaukee, WI) containing the DNA-specific dye bisbenzimidazole at 1 $\mu\text{g ml}^{-1}$ (Hoechst 33342; Sigma). Nuclear morphology and the number of nuclei per embryo were examined with an inverted microscope equipped with epifluorescence (TMD-Diaphot, Nikon) and an ultraviolet filter block (330–380 nm excitation and 420 nm emission; UV-2A, Nikon).

Histone H1 kinase assay

Protein kinase assays were performed as described by Chesnel and Eppig (1995). Groups of at least ten embryos were collected each hour in 10 μ l of kinase buffer containing 60 mmol l^{-1} β -glycerophosphate, 30 mmol l^{-1} *p*-nitrophenylphosphate, 25 mmol l^{-1} 3-[N-morpholinol]propanesulfonic acid (MOPS, pH 7.2), 15 mmol l^{-1} EGTA, 15 mmol l^{-1} MgCl_2 , 0.1 mmol l^{-1} sodium orthovanadate (Na_3VO_4), 1 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF, and 1 mg BSA ml^{-1} and quickly frozen at -70°C . Experiments were performed twice. After thawing, lysates were centrifuged at 14 000 *g* for 10 min at 4°C before use. Kinase reactions were carried out for 1 h at 30°C in a total volume of 25 μ l in H1 kinase buffer supplemented with 100 $\mu\text{g ml}^{-1}$ histone H1 (type III-S; Sigma), 2.5 mg myelin basic protein ml^{-1} (MBP; Sigma), 1 $\mu\text{g cAMP}$ -dependent protein kinase A inhibitor ml^{-1} (Sigma), and 40 $\mu\text{Ci [}\gamma\text{-}^{32}\text{P] ATP ml}^{-1}$. Reactions were terminated by adding 25 μ l of double-strength Laemmli sample buffer (Laemmli, 1970). Phosphorylation of both substrates was analysed by 15% SDS-PAGE carried out on a horizontal electrophoresis cell (Multiphor II, Pharmacia, Uppsala) attached to a power supply (Multidrive XL; Pharmacia) according to standard methods (Laemmli, 1970). After electrophoresis the gels were fixed for at least 1 h (two washes) in 50% methanol 10% acetic acid, briefly washed in distilled water, dried on Whatman paper sheet at 60°C for 90 min and exposed to X-ray film at 70°C for 72 h. The evaluation of histone H1 phosphorylation was performed by densitometric analysis of the film using a laser densitometer (Ultrascan XL, Pharmacia) with accompanying software (Gelscan XL software Package, Pharmacia). Results are expressed as the ratio of H1 kinase activity of treated embryos to control embryos.

DNA synthesis assay

After release from 6-DMAP, embryos were incubated for 1 h at each 2 h interval in Menezo's B2 medium supplemented with 100 $\mu\text{mol l}^{-1}$ bromodeoxyuridine (BrdU; 5-bromo-2'-deoxyuridine 5'-triphosphate; Sigma) to determine the onset of DNA synthesis over the 10 h after release. At the end of each incubation period, embryos were fixed in 10% (v/v) formalin for 15 min. Plasma membranes were permeabilized in PBS 0.1% (v/v) Triton X-100 (Biopharm, Laval, Canada) for 15 min and embryos were washed in block solution (PBS 3% (v/v) BSA, 0.1% (v/v) Tween-20) for 30 min at room temperature. The

Fig. 1. The ability of 6-dimethylaminopurine (6-DMAP) to induce cleavage arrest *in vitro* at concentrations of 0 to 5 mmol l⁻¹ in eight-cell bovine embryos. ▨, minimal dose at which complete cleavage arrest at eight-cell stage was obtained ($n = 240$ with 20 embryos per concentration in two replicates).

embryos were then incubated for 1 h in a humidified chamber with 10 μ l full-strength anti-BrdU monoclonal antibody (Amersham) containing 1 μ g DNase ml⁻¹. After incubation, embryos were washed twice in block solution for 10 min and incubated for 1 h in 10 μ l fluorescein (DTAF)-conjugated affinity purified goat anti-mouse IgG (Sigma) at 1:100 dilution. The embryos were then washed twice in block solution for 10 min, mounted onto slides in Mowiol containing 1 μ g Hoechst 33342 ml⁻¹ and examined by epifluorescence. Percentages were analysed by the chi-squared test and numbers of cells by ANOVA.

Results

Complete arrest of cleavage was induced with 6-DMAP in these studies. The effects of the agent with respect to dose required for arrest, reversibility and toxicity are described separately below.

Cell cycle arrest

Embryos were considered arrested when containing eight nuclei and considered non-arrested when the number of nuclei was greater than eight at the end of treatment. A total of 240 embryos, 40 embryos per concentration, was exposed for 12 h to concentrations of 6-DMAP ranging from 0 to 5 mmol l⁻¹, after which they were fixed to assess cleavage arrest and chromatin morphology. Whereas a concentration of 1 mmol 6-DMAP l⁻¹ had no effect on cleavage arrest, at 2 mmol l⁻¹ there was cleavage to the fifth cell cycle in 45% (18/40) of the treated embryos. Complete cell cycle arrest was observed at concentrations of 3 mmol l⁻¹ and above ($P < 0.05$) (Fig. 1).

Fig. 2. Cell cycle reversibility of 6-dimethylaminopurine (6-DMAP)-treated bovine embryos. Percentage of nuclei found in metaphase at several periods after removal of arrest agent (●) compared with controls (○) ($n = 180$ in three replicates).

Ninety per cent of the non-treated control embryos had cleaved within 6 h after exposure of treated embryos to 6-DMAP, indicating that treated groups were arrested by the cell-cycle-arrest agent ($P < 0.05$).

Reversibility

The reversibility of 6-DMAP treatment during the period immediately after release from arrest agent was assessed by assaying embryos for the percentage of metaphase nuclei ($n = 180$ embryos), as an indication of the timing and synchrony of mitotic division, histone H1 kinase activity ($n = 206$ embryos) and for DNA synthesis ($n = 240$ embryos). The percentage of nuclei in metaphase increased rapidly after release from the arrest agent, to reach a maximum of 82% by 2 h after agent removal, and then decreased rapidly with complete disappearance of metaphase nuclei within 4 h ($P < 0.05$) (Fig. 2). Although no quantitative assessment was performed on the fixed embryos, an apparent increase in the size of interphase nuclei was observed in all 6-DMAP-treated embryos compared with controls (Fig. 3). Statistical analysis was not possible for the H1 kinase assay; however, there was a noticeable variation in the activity of this kinase. Although absent at the end of treatment, release from 6-DMAP led to an increase in the activity of histone H1 kinase by 3 h, after which it decreased progressively, with complete disappearance by 5 h (Fig. 4a, b). The timing and synchrony of entry into S-phase were assessed by removing embryos from 6-DMAP and assaying for BrdU incorporation at 2 h intervals for the following 10 h (Fig. 5). At the end of treatment 29% of blastomeres were still incorporating BrdU, indicating continued DNA synthesis. This amount of DNA synthesis decreased abruptly to 9% by 2 h, then increased gradually during the next

Fig. 3. Chromatin morphology of eight-cell bovine embryos (a) before exposure to 6-dimethylaminopurine (6-DMAP), (b) control after 12 h (70 h after insemination) and (c) after 12 h exposure to 3 mmol 6-DMAP l⁻¹. Scale bar represents 10 μm.

4 h, reaching a maximum of 39% by 6 h, and then decreased to 28% by 10 h after removal of 6-DMAP ($P < 0.05$). Since BrdU incorporation oscillated during the same period in control embryos, it is unknown whether 6-DMAP treatment had any effect on the synchrony of re-entry into the following S-phase.

Fig. 4. Histone H1 kinase assay showing (a) representative autoradiogram of gel showing phosphorylation of histone H1 by eight-cell bovine embryos 5 h after release from 12 h treatment with 6-dimethylaminopurine (6-DMAP), and (b) densitometric readings of autoradiogram showing histone H1 kinase activity of embryos treated with 6-DMAP. Scale is arbitrary.

Toxicity

The toxicity of 6-DMAP on embryo development *in vitro* was assessed by exposing 180 eight-cell treated embryos for 12 h to 0–5 mmol l⁻¹ of the agent, then washing and culturing them in agent-free MB2 medium for a further 6 days (Fig. 6). All concentrations used in this study, including the minimal effective dose of 3 mmol l⁻¹, significantly reduced the ability of treated embryos to develop to the blastocyst stage ($P < 0.05$). A significant reduction in the number of cells in blastocysts compared with controls was also observed ($P < 0.05$). Furthermore, this toxic effect increased significantly for concentrations above 3 mmol l⁻¹ ($P < 0.05$). Hatching rate per blastocyst at day 6 was not affected in the presence of the minimal effective dose of 3 mmol l⁻¹ relative to controls ($P > 0.05$) (Fig. 6b). However,

Fig. 5. Percentage of blastomeres positively showing BrdU incorporation in 6-DMAP-treated embryos several hours after removal of agent (●) compared with controls (○) ($n = 240$ with 20 embryos h^{-1} in two replicates).

hatching rate was significantly reduced for concentrations above 3 mmol l^{-1} ($P < 0.05$).

Discussion

Our results indicate that bovine eight-cell embryo cleavage can be successfully and reversibly arrested for up to 12 h by 6-DMAP treatment at 3 mmol l^{-1} , but with a decrease of its subsequent development *in vitro* to the blastocyst stage. Furthermore, release from 6-DMAP led to synchronized entry into mitosis, as confirmed by the appearance of histone H1 kinase activity by 1 h and by the increase of metaphase nuclei of blastomeres within 2 h. These blastomeres progressed through mitosis over 2 h and subsequently exited from M-phase to enter into interphase of the fifth cell cycle. This was confirmed by the decrease of metaphase after 1 h and histone H1 kinase activity through 4 h, and the complete disappearance of these two characteristics within 4 h and 5 h, respectively. These observations confirm that the activity of 6-DMAP on cleavage arrest of eight-cell bovine embryos is reversible for up to 12 h.

In this study, a minimum dose of 3 mmol l^{-1} was required for 6-DMAP to successfully and reversibly arrest eight-cell bovine embryos at interphase; a similar dose was required for this agent to arrest four-cell mouse embryos at interphase (Samaké and Smith, 1996a). However, a lower concentration (2 mmol) has been shown to prevent germinal vesicle breakdown (GVBD) of mouse (Rime *et al.*, 1989) and bovine oocytes (Fulka *et al.*, 1991). By preventing the normal phosphorylation of proteins, 6-DMAP interferes with the activity of MPF, and consequently prevents cell division (Néant *et al.*, 1988a, 1989; Rime *et al.*, 1989). MPF cyclic activity has been shown to control the M-phase in somatic and germinal cells (Masui and Markert, 1971; Gerhart *et al.*, 1984; Newport and Kirschner,

Fig. 6. Effects of 6-dimethylaminopurine (6-DMAP) at doses of 0 to 5 mmol l^{-1} on (a) the development *in vitro* of eight-cell bovine embryos to the blastocyst stage, (b) the hatching rate, and (c) the number of nuclei at day 6 after removal of the agent ($n = 180$ with 15 embryos per concentration in three replicates). ▨, effects using the minimal effective dose of 3 mmol l^{-1} . Columns with different letters above are significantly different from one another ($P < 0.05$).

1984). Although the ability of 6-DMAP to arrest cleavage was predictable, it remains unclear why arrest of embryos occurs at higher concentrations (3 mmol l^{-1}) compared with that of oocytes (2 mmol l^{-1}). It is possible that the autonomous nature of cleavage in amphibian (Newport, Kirschner, 1982) as well as in mammalian early embryos (Waksmundzka *et al.*, 1984) leads to a requirement for increased concentrations of 6-DMAP to induce cell cycle arrest.

Since 6-DMAP inhibits the normal phosphorylation of proteins, it is expected that blastomeres of treated embryos would be arrested at late G2 of the cell cycle, just before the rise of MPF activity. However, in this study, 29% of arrested blastomeres were incorporating BrdU and thus synthesizing DNA at the end of 6-DMAP treatment. Although the mechanisms for this phenomenon are unknown, one possible interpretation of these results is that some blastomeres may remain in S-phase instead of terminating replication and arresting in G2-phase. A further observation of the present study was that blastomere nuclei in embryos treated with 6-DMAP appeared larger compared with control embryos. However, quantitative measurements were not performed

owing to possible artifacts caused by blastomere squashing during the whole-mount technique. Large nuclei have also been reported after exposure to 6-DMAP in oocytes and embryos of molluscs (Néant *et al.*, 1988b, 1994), echinoderms (Néant *et al.*, 1989; Dufresne *et al.*, 1991) and mice (Rime *et al.*, 1989; Szöllösi *et al.*, 1991; Samaké and Smith, 1996a). In invertebrate oocytes, nuclear enlargement has been shown to be due to the decondensation of metaphase chromatin, which merges to produce large DNA-synthesizing nuclei (Néant *et al.*, 1988b, 1994; Dubé and Dufresne, 1990). None the less, a recent report using three marine invertebrates has shown that the regulation of DNA replication of the first mitotic division after fertilization and activation remain intact in the presence of 6-DMAP (Néant and Dubé, 1996).

Although we conclude that the mitotic arrest activity of 6-DMAP on eight-cell bovine embryo cleavage with synchronized entry of blastomeres into mitosis is reversible, the ability of treated embryos to develop to the blastocyst stage and the number of cells per blastocyst are reduced by this agent compared with controls. In mice (Samaké and Smith, 1996a) and in bovine embryos in the current study, DNA synthesis was observed during and at the end of 6-DMAP treatment. The toxicity of this agent to both mouse and bovine embryo viability may have been related to ploidy defects caused by continuous DNA synthesis after long periods of exposure to 6-DMAP. Alternatively, the observed lower cell numbers may have resulted from the 12 h arrest present in treated but not in control groups. Experiments in which these 6-DMAP-treated bovine blastocysts will be transferred to synchronized recipients will help to assess the potential for long term toxicity on embryo viability.

The importance of synchrony between the donor and recipient cell cycle stages in embryo reconstitution studies by nuclear transfer has been well established in several species (Smith *et al.*, 1988, 1990; Collas *et al.*, 1992a, b; Barnes *et al.*, 1993; Campbell *et al.*, 1993, 1994; Cheong *et al.*, 1993; Otaegui *et al.*, 1994; Stice *et al.*, 1994). Our results indicate that 6-DMAP can be used to synchronize nuclear donor cells at late G2-phase before fusion. The fully reversible activity of 6-DMAP makes it a useful choice for studies on cell cycle reversibility. Furthermore, in contrast to nocodazole, synchronization of donor cells at metaphase can be achieved with 6-DMAP as early as 2 h after release from the arrest agent. This synchronization is useful when nuclei are required at this specific cell cycle stage prior to fusion to recipient oocytes. The opportunity to obtain such nuclear donor cells will enable further studies on the effects of nucleocytoplasmic asynchrony and its relevance to embryo cloning procedures.

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