# The combined treatment of calcium ionophore with strontium improves the quality of ovine SCNT embryo development

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## Summary

Poor embryo quality is a major problem that contributes to the failure of pregnancy in somatic cell nuclear transfer (SCNT). The aims of this study were to improve the quality of ovine SCNT embryos by modifying the conventional activation protocol with the addition of $SrCl_2$. In order to achieve this objective we conducted a series of experiments with *in vitro*-matured oocytes to optimize conditions for oocyte activation with strontium, and subsequently applied the protocol to SCNT embryos. The results showed that *in vitro*-matured oocytes could be activated effectively by 10 mM $SrCl_2$ + 5 mg/ml cytochalasin B (CB) for 5 h in the absence of $Ca^{2+}$ and that the blastocyst rate on day 7 (33.2%) was similar to that in the control group (31.0%) (5 M calcium ionophore [IP] A23187 for 5 min and cultured in CB/cycloheximide [CHX] for 5 h; $P > 0.05$). In SCNT experiments, the total cell number/blastocyst (104.12 ± 6.86) in the IP + $SrCl_2$/CB-treatment group was, however, significantly higher than that in the control group (81.07 ± 3.39; $P < 0.05$). Apoptotic index (12.29 ± 1.22%) was significantly lower than the control (17.60 ± 1.39%; $P < 0.05$) when a combination of IP and $SrCl_2$/CB was applied to SCNT embryos. In addition, karyotyping of the SCNT embryos showed that the percentage of diploid blastocysts in the IP + $SrCl_2$/CB-treatment group was slightly higher than that in the control ($P > 0.05$). We conclude that the modified activation protocol with IP + $SrCl_2$/CB can improve significantly the quality of ovine SCNT embryos in terms of total cell number, apoptosis and ploidy.

Keywords:   Apoptosis, Embryo development, Oocyte activation, Somatic cell nuclear transfer, Strontium

## Introduction

Although successful in a range of species, the low efficiency of development to term of embryos reconstructed by somatic cell nuclear transfer (SCNT; <5%) has been a major problem that has restricted its application in both basic and applied research. SCNT is a very complex technique that involves multiple procedures, such as enucleation, transfer of the donor nucleus by fusion or injection, parthenogenetic activation, embryo culture or subsequent transfer to a suitable synchronised recipient; the failure of any of these steps may have serious consequences on success. Since the birth of the first mammal cloned from somatic cells, Dolly the sheep (Wilmut *et al.*, 1997), and despite significant research the total efficiency is still low, however the rate of blastocyst formation in mice (Ribas *et al.*, 2006; Kishigami *et al.*, 2007; Mohammed *et al.*, 2008; Dai *et al.*, 2009), pigs (Prather *et al.*, 2004; Ma *et al.*, 2009) and cattle (Akagi *et al.*, 2003, 2008; Yang *et al.*, 2006; Iager *et al.*, 2008; Alexopoulos & French, 2009; Ma *et al.*, 2009) has improved steadily. Therefore, it is probable that poor quality of SCNT embryos is a major factor responsible for pregnancy failure. Activation of the reconstructed embryos is perhaps the most variable of all steps in current SCNT procedures. For example, different activation protocols have been reported to affect the frequency of *in vitro* development of both parthenogenetic and SCNT embryos (Bos-Mikich *et al.*, 1995; Levron *et al.*,

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1995; Escriba & Garcia-Ximenez, 2000; Liu *et al.*, 2005; Garcia-Mengual *et al.*, 2008; Alexopoulos & French, 2009; Gupta *et al.*, 2009; Whitworth *et al.*, 2009), total cell number of blastocysts (Liu *et al.*, 2005; Gupta *et al.*, 2009), karyotype of both parthenotes and SCNT embryos (Gupta *et al.*, 2009), apoptosis of blastocyst (Liu *et al.*, 2005; Gupta *et al.*, 2009;) and gene expression in embryos (Wrenzycki *et al.*, 2001; Cervera *et al.*, 2009). In domestic animals most of the currently used activation protocols are regarded as non-physiological. These protocols include electrical stimulation (Ware *et al.*, 1989; Stice *et al.*, 1994; Nussbaum & Prather, 1995), treatment with ethanol (Minamihashi *et al.*, 1993; Atabay *et al.*, 2003), $Ca^{2+}$ ionophore (Ware *et al.*, 1989; Rhoton-Vlasak *et al.*, 1996; Tao *et al.*, 2000; Atabay *et al.*, 2003) or ionomycin (Campbell *et al.*, 2007; Heytens *et al.*, 2008), all of which trigger only a single spike of $Ca^{2+}$ in the oocyte. In contrast, treatment with strontium has been reported to induce a series of intracellular calcium rises in mouse oocytes, through $IP_3$ (inositol-1,4,5,-trisphosphate) receptors, resembling the calcium oscillations observed following normal fertilization (Bos-Mikich *et al.*, 1995; Zhang *et al.*, 2005). In mouse, strontium has been used routinely following SCNT and has led to the birth of healthy pups (Ogura *et al.*, 2000; Rideout *et al.*, 2000; Ono *et al.*, 2001; Yamazaki *et al.*, 2005). Furthermore, stimulation of repetitive $Ca^{2+}$ oscillations has been shown to affect a range of parameters in subsequent embryonic development including cell allocation, such as inner cell mass/trophectoderm ratio as well as lead to modest improvements in implantation rate and post-implantation development (Bos-Mikich *et al.*, 1997). It is believed that the improved developmental competency is due to the calcium oscillatory pattern; hypo-activation or hyper-activation by influx of calcium regulates both early and later cellular events such as cortical granule exocytosis, cell cycle resumption and recruitment of maternal mRNA (Ducibella *et al.*, 2002; Ozil *et al.*, 2005). Together these observations suggest that the improvement of development of preimplantation and post-implantation in the mouse may be due to an improvement in the quality of embryos induced by strontium activation. Several studies have reported that strontium can induce oocyte activation and support development to the blastocyst stage in bovine and porcine SCNT embryos. Furthermore, strontium-activated bovine SCNT embryos can develop to term (Meo *et al.*, 2005, 2007; Yamazaki *et al.*, 2005; Che *et al.*, 2007). It has also been reported that, in cattle, oocyte activation using a combination of strontium and ionomycin can increase the *in vitro* developmental capacity of reconstructed embryos, and that such treatments were influenced less by oocyte age (Meo *et al.*, 2007). However, no differences in the ratio or number

of inner cell mass (ICM) and trophectoderm (TE) cell numbers or total cell number were observed in blastocyst stage embryos (Meo *et al.*, 2005). From these studies it is clear that further investigation is needed to clear the confusion. To date, to our knowledge, no similar information on strontium activation in ovine SCNT is available. Therefore, these studies examined the ability to activate ovine oocytes using strontium and the effects of strontium on embryo development and quality in parthenotes following SCNT.

To test the hypothesis, preliminary studies were carried out using *in vitro*-matured ovine oocytes in order to determine and optimize the activation conditions needed for strontium, both alone and in combination with other agents. The optimized activation conditions were then applied to fused ovine SCNT couplets and the development and quality of the resultant embryos were determined.

## Materials and methods

All chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

### Ovary collection and *in vitro* oocyte maturation

Ovine ovaries were collected from a local slaughterhouse and transported to the laboratory in pre-warmed phosphate-buffered saline (PBS) solution at 25°C. Before aspiration, ovaries were rinsed several times with warmed PBS. Cumulus–oocyte complexes (COCs) were aspirated from follicles (2–3 mm in diameter) using a 10 ml syringe fitted with a 21-gauge needle. Aspirated follicular fluid was placed into 50 ml centrifuge tubes and maintained at 39°C to allow settling; after approximately 15 min the upper two-thirds of the fluid was discarded and the remainder transferred into a 90 mm tissue culture dish. COCs with at least three layers of cumulus cells and a homogenous cytoplasm were selected under a dissection microscope (Leica MZ125; Leica, Wetzlar, Germany). Selected oocytes were washed in HEPES-buffered tissue culture medium 199 (TCM199; Gibco, Paisley, Renfrewshire, UK) supplemented with 10% fetal bovine serum (FBS, Gibco) and then in maturation medium, bicarbonate-buffered TCM199 (TCM199 with Earle's salts; Gibco) supplemented with 10% fetal bovine serum (FBS), 5 g/ml follicle stimulating hormone (FSH; Ovagen, ICP, Auckland, New Zealand), 5 g/ml luteinizing hormone (LH), 1 g/ml estradiol-17β, 0.3 mM sodium pyruvate, 100 M cysteamine and 50 g/ml gentamycin. The COCs were cultured in groups of 40 in 500 l of maturation medium under mineral oil in four-well dishes (Nunc, Roskilde,

Denmark) in a humidified atmosphere of 5% $CO_2$ in air at 39°C.

## Somatic cell nuclear transfer

Fifteen hours after onset of oocyte maturation (hpm), cumulus cells were removed from oocytes by repeated pipetting in HEPES-buffered synthetic oviduct fluid (H-SOF) that contained 300 IU/ml hyaluronidase. Denuded oocytes at anaphase–telophase of the first meiotic division (AI–TI) were selected for enucleation and then incubated in H-SOF that contained 5 g/ml bisbenzimide (Hoechst 33342) and 7.5 g/ml cytochalasin B (CB) for 15 min. Enucleation was performed by laser-assisted aspiration using a XYclone laser (Hamilton Thorne, Beverly, MA, USA) in H-SOF that contained 4 mg/ml fatty acid-free bovine serum albumin (FAF-BSA) (H-SOF/BSA) and 7.5 g/ml CB. Removal of the extruding AI–TI spindle was confirmed by exposure of the aspirated karyoplast to ultraviolet (UV) light. After enucleation, cytoplasts were washed twice in H-SOF/BSA and incubated in maturation medium until reconstruction. At 22 hpm, a single fetal fibroblast cell (three to five passages) was inserted into the perivitelline space of an enucleated recipient oocyte in H-SOF/BSA. Couplets were washed in H-SOF/BSA and then transferred into 200 l of 0.3 M mannitol solution supplemented with 0.5 mM HEPES, 0.05% FAF-BSA and 0.1 mM $MgSO_4$ between the electrodes of an electrofusion chamber with a 200 M electrode gap. Fusion was induced by applying two DC pulses of 1.25 kV/cm for 30 s in the Eppendorf Multiporator (Eppendorf, Hamburg, Germany). Reconstructed oocytes were cultured in modified SOF supplemented with essential and non-essential amino acids, myo-inositol, sodium citrate (mSOFaaci) and 4 mg/ml FAF-BSA (C-SOF) for 1–2 h at 39°C under a humidified atmosphere of 5% $CO_2$ in air until activation.

## Parthenogenetic activation

After *in vitro* maturation (24 hpm), cumulus cells were removed by vortexing the COCs in H-SOF medium supplemented with 300 IU/ml hyaluronidase for 5 min. Oocytes with a visible first polar body and a homogenous cytoplasm were selected and allotted randomly to each treatment group. Selected oocytes were activated using 5 M calcium ionophore A23187 (IP) in H-SOF/BSA for 5 min, followed by incubation in $Ca^{2+}$-free Chatot, Ziomet and Bavister (CZB) medium supplemented with 5 mM, 10 mM or 15 mM $SrCl_2$ + 5.0 g/ml CB for 4 h at 39°C in a humidified atmosphere of 5% $CO_2$ in air. Oocytes were treated with 10 mM $SrCl_2$ to examine the effect of strontium alone, of strontium in combination with cycloheximide (CHX) or the effects of medium that

contained $Ca^{2+}$ on the activation. This concentration was based on the results obtained following activation of *in vitro*-matured metaphase II (MII) oocytes in $Ca^{2+}$-free or regular CZB medium with or without CHX. Reconstructed oocytes were activated chemically at 26 hpm, using 5 M calcium ionophore A23187 in combination with 10 mM $SrCl_2$ (IP + $SrCl_2$/CB), or using 10 mM $SrCl_2$ alone without an initial treatment ($SrCl_2$/CB) for 4 h at 39°C in a humidified atmosphere of 5% $CO_2$ in air.

For the control groups, MII and SCNT-reconstructed oocytes were treated with calcium ionophore A23187 (5 M for 5 min) dissolved in H-SOF/BSA, rinsed several times in the same medium and then cultured in C-SOF medium supplemented with 10 l/ml CHX and 5.0 g/ml CB (IP + CHX/CB) for 5 h at 39°C in a humidified atmosphere of 5% $CO_2$ in air.

## Assessment of activation in parthenotes

Oocytes treated with strontium either in $Ca^{2+}$ or in Ca-free CZB medium were evaluated for activation efficiency by determination of pronucleus formation at 14 h post activation (hpa). Oocytes were fixed in 4% paraformaldehyde (PFA) for 30 min and stained with 5 g/ml bisbenzimide (Hoechst 33342) in PBS supplemented with 4 mg/ml polyvinylpyrrolidone (PBS/PVP) for 10 min. Pronucleus formation was determined by visualization of DNA using an epifluorescence microscope (Leica, Germany). Oocytes with two pronuclei (2PN) were regarded as activated diploid oocytes whilst oocytes with one pronucleus (1PN) were considered to be haploid. The remaining oocytes without pronuclei were considered to be non-activated.

## Embryo culture

After activation, both parthenotes and SCNT embryos were rinsed in C-SOF medium; 10–15 embryos were cultured in 30 l microdrops of the same medium covered with mineral oil at 39°C in a humidified atmosphere of 5% $O_2$, 5% $CO_2$ and 90% $N_2$. The embryos were observed and recorded for cleavage at 20, 24, or 48 hpa and for development to blastocyst on day 7.

## Apoptosis assay

Apoptosis in parthenogenetic and SCNT blastocysts was detected by the terminal transferase dUTP nick end labelling (TUNEL) assay using an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany). Embryos were rinsed twice in PBS/PVP, then fixed with 4% (v/v) neutral buffered PFA in PBS that contained 10 g/ml Hoechst 33342 for 30 min at room temperature, followed by two washes in

**Table 1.** Parthenogenetic development of ovine oocytes after various activation treatments

| Activation protocol | No. of oocytes | 24 hpa (%) | 48 hpa (%) | No. of blastocysts on D-7 (%) | Total cell no. of blastocyst (mean $\pm$ SD) |
|---|---|---|---|---|---|
| IP + SrCl$_2$/CB in Ca$^{2+}$-free CZB | 269 | 175 (65.1)[a] | 211 (78.4)[a,b] | 93 (34.6) | 100.1 $\pm$ 29 |
| SrCl$_2$/CB in Ca$^{2+}$-free CZB | 265 | 169 (63.8)[a] | 223 (84.2)[a] | 88 (33.2) | 105.6 $\pm$ 32 |
| SrCl$_2$/CB/CHX in Ca$^{2+}$-free CZB | 189 | 97 (51.3)[b] | 135 (71.4)[b] | 54 (25.6) | 99.6 $\pm$ 32 |
| IP + CHX/CB in Ca$^{2+}$-free CZB | 145 | 92 (63.4)[a] | 122 (84.1)[a] | 45 (31.0) | 100.1 $\pm$ 30 |

[a,b]Values with different superscripts in the same column are significantly different (chi-squared test, $P < 0.05$). IP (5 M calcium ionophore A23187) for 5 min in HEPES-buffered synthetic oviduct fluid (H-SOF); SrCl$_2$ (10 mM strontium chloride)/CB (5 g/ml cytochalasin B)/CHX (cycloheximide) for 5 h in calcium-free Chatot, Ziomet and Bavister (CZB) medium. SD, standard deviation.

PBS/PVP. The fixed embryos were permeabilized with 0.01% Triton X-100 in PBS/PVP for 15 min at room temperature. The blastocysts were rinsed three times in PBS/PVP and once in TUNEL reaction buffer, the blastocysts were then placed in 50 l of the TUNEL reaction mixture at 39°C for 45 min in a humid chamber. Next, the blastocysts were rinsed in PBS/PVP and mounted on diagnostic microscope slides (Erie Scientific Co. Portsmouth, UK) under a coverslip in VectaShield mounting medium that contained 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc. Burlingame, CA, USA) and examined using an epifluorescence microscope (Leica, Germany).

### Chromosomal analysis of parthenotes and SCNT embryos

Day 7 blastocyst stage embryos derived from two different treatments (IP + CHX/CB and IP + SrCl$_2$/CB) and *in vitro* production groups (parthenotes and SCNT embryos) were prepared. Their karyotype was then examined using a modification of the procedure described by Dyban (1983). Briefly, embryos were incubated in C-SOF that contained 0.1 g/ml demecolcine (Wako, Japan) for 4 h to arrest and maintain chromosome metaphase plates. The zona pellucida was then digested partially with 0.5% pronase until it visibly thinned. The embryos were then transferred into a hypotonic solution (0.9% sodium citrate) in a pre-cooled watch glass at 4°C for 1 h, followed by 75.0 mM KCl treatment for 3 min, after which they were fixed with 3:1 mixture of methanol/glacial acetic acid at –20°C for 3 min. Individual embryos were then placed on clean slides with a drop of the fixative and gently dispersed by addition of several drops of softening solution, a mixture of 75% pre-cooled methanol and acetic acid (1:1), followed by a drop of the fixative to achieve final spreading and adherence to the slide. The slides were then air dried, stained with 2% Giemsa (Gibco) at pH 6.8 for 20 min, rinsed with distilled water,

dried in air at room temperature and then examined microscopically at ×1000 magnification (Leica).

### Statistical analysis

Oocyte activation, cleavage, blastocyst development, and chromosome complement of embryos were compared by chi-squared test analysis. Total cell numbers and numbers of apoptotic nuclei in blastocysts were compared using one-way analysis of variance (ANOVA). Statistical significance was $P < 0.05$ unless specified in the text. The entire statistical analysis was carried out using Sigma-Stat software (Jandel Scientific, USA).

## Results

### Preimplantation development and pronucleus formation in parthenotes activated by strontium

We examined if strontium could be used as an agent for parthenogenetic activation of *in vitro*-matured ovine oocytes. As summarised in Table 1, ovine oocytes activated by strontium, or by any combination of strontium with IP or CHX in Ca$^{2+}$-free CZB medium, developed to blastocyst. The blastocyst development rates and total cell numbers on day 7 were not different from that of the control group (IP + CHX/CB) ($P > 0.05$). However, the SrCl$_2$/CB/CHX-treated group showed a relatively slower first cleavage at 24 hpa and had lower cleavage rates ($P < 0.05$).

We also examined the effects of strontium, composition of activation medium, and action of other activating agents such as CB and CHX on efficiencies of ovine oocyte activation. Formation of 2PN was used as an indicator for completion of activation of oocytes treated with strontium (Table 2). In the Ca$^{2+}$-free CZB medium, all oocytes were successfully activated by strontium alone or by strontium combined with other chemicals such as ionophore or CHX. However, only 1PN was observed in oocytes that had been incubated

**Table 2.** Effect of strontium-induced different activation protocols on pronucleus formation of ovine at 14 hpa

| Activation protocol | No. of oocytes | MII | AII | TII | 1PN (%) | 2PN (%) |
|---|---|---|---|---|---|---|
| IP + SrCl$_2$/CB in Ca$^{2+}$-free CZB | 74 | 8 | 0 | 3 | 0 (0.0) | 63 (85.1) |
| SrCl$_2$/CB in Ca$^{2+}$-free CZB | 50 | 1 | 0 | 1 | 3 (6.0) | 45 (90.0) |
| SrCl$_2$ in Ca$^{2+}$-free CZB | 24 | 4 | 0 | 0 | 20 (83.3)[a] | 0 (0.0)[a] |
| SrCl$_2$/CB/CHX in Ca$^{2+}$-free CZB | 22 | 1 | 0 | 0 | 0 (0.0) | 21 (95.5) |
| SrCl$_2$/CB in CZB | 44 | 42 | 1 | 0 | 0 (0.0) | 1 (2.2)[a] |

MII (metaphase II), AII (anaphase II), TII (telophase II), PN (pronucleus formation), IP (5 M calcium ionophore for 5 min in HEPES-buffered synthetic oviduct fluid (H-SOF)); SrCl$_2$ (10 mM strontium chloride)/CB (5 g/ml cytochalasin B)/CHX (cycloheximide) for 5 h in calcium-free or containing Chatot, Ziomet and Bavister (CZB) medium. [a]Significantly different in the pronucleus formation (chi-squared test, $P < 0.01$). hpa, hours post activation.

**Table 3.** Cleavage and blastocyst development of *in vitro*-matured ovine oocytes activated with a combination of 5 M calcium ionophore A23187 and various concentrations of SrCl$_2$

| | IP + CHX/CB | IP + 5 mM Sr/CB | IP + 10 mM Sr/CB | IP + 15 mM Sr/CB |
|---|---|---|---|---|
| Total no. oocytes | 197 | 109 | 122 | 89 |
| No. cleaved 20 hpa (%) | 60 (30.5)[a,c] | 41 (37.6)[c,d] | 58 (47.5)[b,d] | 24 (27.0)[c] |
| No. cleavage at 24 hpa (%) | 122 (61.9)[a] | 61 (56.0)[a] | 78 (64.0)[a] | 35 (39.3)[b] |
| No. cleaved 48 hpa (%) | 169 (85.8) | 83 (76.1) | 105 (86.1) | 69 (77.5) |
| No. blastocysts day 7 (%) | 66 (33.5)[a] | 23 (21.1)[b] | 39 (32.0)[a,b] | 19 (21.3)[a,b] |

[a–d]Values with different superscripts in the same column are significantly different (chi-squared test, $P < 0.05$). CB (cytochalasin B), CHX (cycloheximide), IP (calcium ionophore A23187), Sr (strontium chloride). hpa, hours post activation.

without CB during strontium-induced activation. Interestingly, unlike the findings for mouse oocytes, most ovine oocytes could survive and remain arrested at the MII stage when the oocytes were cultured with strontium in Ca$^{2+}$-containing CZB medium. However, when we rinsed these MII-arrested ovine oocytes 1–2 h later and transferred them to the activation medium in the absence of Ca$^{2+}$, the rate of blastocyst formation on day 7 was similar to that observed after other strontium treatments (data not shown).

**Effect of concentration of strontium chloride on activation and preimplantation development of ovine parthenotes**

To optimize the activation protocol with strontium, matured ovine oocytes were exposed to 5 mM, 10 mM and 15 mM of SrCl$_2$ in Ca$^{2+}$-free CZB medium with CB for 5 h after calcium ionophore treatment in H-SOF for 5 min. Subsequently, cleavage and blastocyst development rates were assessed. The results are summarised in Table 3. Cleavage rates at 48 hpa were similar in all groups and ranged from 77.1 to 86.1% ($P > 0.05$), compared with a control group (85.78%) treated with IP + CHX/CB for 5 h. However, parthenotes activated by calcium ionophore treatment, followed by treatment with 10 mM SrCl$_2$

and 7.5 mg/ml CB in Ca$^{2+}$-free CZB showed a significantly higher frequency of cleavage at 20 hpa (47.5%, $P < 0.05$) in comparison with the control and the 15 mM SrCl$_2$/CB-treated groups (30.5 and 27.0%, respectively). There were no significant differences in blastocyst development between the control and treatment groups with various concentration of SrCl$_2$, except for the 5 mM SrCl$_2$-treatment group ($P < 0.05$). The results demonstrated that matured ovine oocytes could be activated effectively by strontium treatment in combination with Ca$^{2+}$ ionophore to induce the initial rise in intracellular Ca$^{2+}$ concentration.

**Effect of strontium chloride on activation and preimplantation development of ovine nuclear transfer embryos**

To determine whether strontium can effectively activate reconstructed SCNT embryos, reconstructed oocytes were treated at 26 hpm with 5 M calcium ionophore in H-SOF for 5 min and 10 mM SrCl$_2$ in Ca$^{2+}$-free CZB medium supplemented with 7.5 g/ml CB for 4 h or SrCl$_2$/CB without calcium ionophore treatment, based on cleavage timing and blastocyst development results found in parthenotes (Tables 1, 2 and 3). A greater proportion of reconstructed oocytes treated with IP + SrCl$_2$/CB had cleaved at

**Table 4.** Cleavage and blastocyst development of ovine SCNT embryo activated with combination of 5 M calcium ionophore A23187 and 10 mM SrCl2.

| | IP + CHX/CB | IP + SrCl$_2$/CB | SrCl$_2$/CB |
|---|---|---|---|
| Total no. fused oocytes | 66 | 93 | 95 |
| Cleavage at 20 hpa (%) | 18 (27.3)[a] | 45 (48.4)[b] | 32 (33.7)[a,b] |
| Cleavage at 24 hpa (%) | 30 (45.4) | 56 (60.2) | 51 (53.7) |
| Cleavage at 48 hpa (%) | 60 (90.9) | 75 (80.6) | 80 (84.2) |
| Blastocysts at day 7 (%) | 21 (31.8)[a,b] | 31 (33.3)[a] | 19 (20.0)[b] |

[a–c]Values with different superscripts in the same row are significantly different (chi-squared test, $P < 0.05$). CB (cytochalasin B), CHX (cycloheximide), IP (calcium ionophore A23187), Sr (strontium chloride). hpa, hours post activation.

**Table 5.** Total number of cell and apoptotic nuclei in blastocyst embryos treated with combination of calcium ionophore A23187 (5 M for 5 min) and SrCl$_2$ (10 mM for 4 h)

| | SCNT | | Parth | |
|---|---|---|---|---|
| | IP + CHX/CB | IP + SrCl$_2$/CB | IP + CHX/CB | IP + SrCl$_2$/CB |
| No. of blastocysts | 27 | 24 | 32 | 20 |
| Total cell no. (mean ± SEM) | 81.07 ± 3.39[a] | 104.12 ± 6.86[b] | 104.50 ± 6.09[b] | 107.90 ± 7.4[b] |
| Total no. apoptotic nuclei (mean ± SEM) | 13.44 ± 0.73[a,c] | 11.33 ± 0.65[c,d] | 9.15 ± 0.94[b,d] | 6.40 ± 0.54[b] |
| Apoptotic index (%) (mean ± SEM) | 17.60 ± 1.39[a] | 12.29 ± 1.22[b] | 9.59 ± 1.05[b,c] | 6.74 ± 0.93[c] |

[a–c]Values with different superscripts in the same row are significantly different (analysis of variance (ANOVA) test, $P < 0.05$). CB (cytochalasin B), CHX (cycloheximide), IP (calcium ionophore A23187), Parth (parthenotes), SCNT (somatic nuclear transfer embryo), SrCl$_2$ (strontium chloride). SEM, standard error of the mean.

20 hpa, compared with the control group treated with IP + CHX/CB (48.4 versus 27.3%, respectively; $P < 0.01$), but at 48 hpa there was no significant difference among the groups (Table 4). The developmental rates to blastocyst stage in SCNT groups did not differ when IP + CHX/CB and/or IP + SrCl$_2$/CB were used as activating agents (31.8 versus 33.3%, respectively). However, IP + SrCl$_2$/CB treatment resulted in improved blastocyst development compared with SrCl$_2$/CB treatment (33.3 versus 20.0 %, respectively; $P < 0.05$).

Total cell number and number of apoptotic nuclei were assessed in blastocysts to determine if exposure to strontium after treatment of calcium ionophore improved the quality of embryos, as it had been observed that embryos produced by the two sequential activating agents seemed to be morphologically more compact and developed earlier to the blastocyst stage. As shown in Table 5, the total cell number of blastocysts was significantly higher in SCNT embryos that had been activated by IP + SrCl$_2$/CB than the number in the SCNT embryos activated by IP + CHX/CB. Although the total number of apoptotic nuclei did not differ between the two SCNT groups, the apoptotic index (%; total number of apoptotic nuclei/total cell no. of blastocyst) in the IP + SrCl$_2$/CB-treated SCNT group was significantly lower than that in the control SCNT group. However, no differences were observed in the total cell numbers and apoptotic nuclei, and apoptotic index in the parthenogenetic blastocysts between IP + CHX/CB and IP + SrCl$_2$/CB treatment. These results proved the hypothesis that strontium could improve the quality of ovine SCNT embryos, not only by the increase in total cell numbers/blastocyst, but also by reduction in the value of the apoptotic index, which is an important parameter for pregnancy after embryo transfer.

**Chromosomal complement of blastocysts**

To examine the effect of strontium treatment on chromosomal complement, 81 blastocysts derived from IP + CHX/CB, or IP + SrCl$_2$/CB activation were karyotyped. As shown in Table 6, 155 metaphase chromosomal spreads of blastocysts after IP + CHX/CB treatment, and 132 after IP + SrCl$_2$/CB treatment were counted. In parthenotes, the percentages of diploid nuclei of blastomeres were 81.5% from the IP + CHX/CB group and 80.8% from the IP + SrCl$_2$/CB group; after SCNT the percentages were 84.1 and 86.4%, respectively. More cells with normal diploid karyotypes were observed in SCNT embryos regardless of which activation treatment was used, however there was no statistically significant difference between parthenotes and SCNT embryos. In addition, no significant differences were observed in the rates of chromosomally normal diploid nuclei between the IP + CHX/CB (83.1%)-treatment and IP + SrCl$_2$/CB

**Table 6.** Chromosome complement and ploidy in blastocyst stage SCNT and parthenogenetic embryos activated with calcium ionophore A23187, followed by CHX or SrCl₂ treatment

| | Activation treatment | 1n (%) | 2n (%) | 3n (%) | 4n (%) | No. of metaphase plates | Haploid (%) | Diploid (%) | Polyploid (%) | Mixoploid (%) | No. of blastocysts |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Parthenotes | IP + CHX/CB | 3 (3.3) | 75 (81.5) | 4 (4.3) | 10 (10.9) | 92 | 2 (9.1) | 7 (31.8) | 1 (4.5) | 12 (54.6) | 22 |
| | IP + SrCl₂/CB | 8 (11.0) | 59 (80.8) | 2 (2.7) | 4 (5.5) | 73 | 1 (4.3) | 9 (39.1) | 0 (0) | 13 (56.5) | 23 |
| SCNT | IP + CHX/CB | 3 (4.8) | 53 (84.1) | 2 (3.2) | 5 (8.0) | 63 | 1 (5.6) | 8 (44.4) | 2 (11.1) | 7 (38.9) | 23 |
| | IP + SrCl₂/CB | 2 (3.4) | 51 (86.4) | 0 (0) | 6 (10.2) | 59 | 1 (7.7) | 6 (46.2) | 1 (7.7) | 5 (38.5) | 13 |

CB (cytochalasin B), CHX (cycloheximide), IP (calcium ionophore A23187), SCNT (somatic nuclear transfer embryo), SrCl₂ (strontium chloride).

(84.4%)-treatment groups ($P > 0.05$), a finding that indicated that most nuclei in both parthenotes and SCNT blastocysts were of normal ploidy. However, most blastocysts were chromosomally abnormal due to mixoploidy. The rates of completely normal diploid embryos were 31.82% (7/22) in parthenotes and 44.1% (8/18) in SCNT embryos after IP + CHX/CB treatment. Whereas 39.13% (9/23) parthenotes and 46.15% (6/13) SCNT blastocysts possessed diploid nuclei without either haploid or polyploidy chromosomes after IP + SrCl₂/CB treatment, respectively. There was no significant difference between the two activation treatments or between parthenote and SCNT groups ($P > 0.05$).

## Discussion

Meo *et al.* (2005) showed that strontium could be used for bovine oocyte activation in $Ca^{2+}$- and $Mg^{2+-}$free TALP-IVF (*in vitro* fertilization) medium, however the frequencies of pronuclear formation, cleavage and blastocyst development were reduced (Meo *et al.*, 2005). In contrast, Yamazaki *et al.* (2005) demonstrated that the combination of calcium ionophore with strontium was more effective for bovine oocyte activation in parthenotes and SCNT, compared with strontium alone, but that there were no differences in total cell number at blastocyst stage (Yamazaki *et al.*, 2005). However, as seen in Table 1, ovine oocytes treated with strontium alone, or combined with IP or CHX, were activated effectively in $Ca^{2+}$-free CZB medium and developed to blastocysts, a finding that suggested that strontium could activate oocytes successfully and support preimplantation development in sheep. We suggest that the discrepancies in the effectiveness of the use of strontium for activation between bovine and ovine may be attributed to the activation medium used rather than species differences, because $Ca^{2+}$-free CZB medium that contained 0.11 mM EDTA may chelate residual $Ca^{2+}$ added by BSA. Although Meo *et al.* (2005) claimed that bovine oocytes were activated by strontium in $Ca^{2+}$- and $Mg^{2+}$-free TALP-IVF medium, in fact the '$Ca^{2+}$- and $Mg^{2+}$-free medium' probably contained $Ca^{2+}$ as 2.5% serum was added – and thereby could be related to the poor development (Meo *et al.*, 2005). To confirm if absence of $Ca^{2+}$ in the activation medium is essential for strontium-induced oocytes activation, we examined pronuclear formation of oocytes treated with strontium in $Ca^{2+-}$free CZB or $Ca^{2+}$-containing CZB medium. As seen in Table 2, regardless of combinations with other activating agents such as IP and CHX, strontium treatment successfully activated oocytes in $Ca^{2+}$-free medium; however, only a single oocyte was activated when

regular CZB medium was used, indicating that a $Ca^{2+}$-free environment is a prerequisite for oocyte activation using strontium. These findings are consistent with previous studies in both cattle and mice (Ma *et al.*, 2005; Yamazaki *et al.*, 2005). It has been reported that addition of a membrane-impermeable chelator of $Ca^{2+}$, ethylene glycol-bis-N,N,N′,N′-tetraacetic acid (EGTA), to standard culture media can support activation and development in the mouse by chelating $Ca^{2+}$ (Kishigami & Wakayama, 2007a,b). We also observed that ovine oocytes were activated and developed to blastocysts when 5 mM EGTA was added to regular C-SOF medium supplemented with 10 mM $SrCl_2$, or when oocytes were rescued by returning them to $Ca^{2+}$-free medium, following 1–2 h of strontium treatment in the presence of $Ca^{2+}$ (data not shown). This result suggested that the presence of $Ca^{2+}$ in activation medium supplemented with strontium may interfere with strontium-mediated oocyte activation, whereby $Sr^{2+}$ can release internal stored $Ca^{2+}$ (Kline & Kline, 1992).

The optimal activation conditions for the use of strontium (such as concentration, duration of incubation and combinations with other activating agents) varies between species. For example, regimes of 10 mM for 2.5 h in mice, 2 mM for 15 min or 2 h in rats, ionomycin combined with 20 mM $SrCl_2$ for 6 h in cattle, and ionomycin, followed by 10 mM $SrCl_2$ for 4 h in pigs (Krivokharchenko *et al.*, 2003; Ma *et al.*, 2005; Meo *et al.*, 2005; Yamazaki *et al.*, 2005; Che *et al.*, 2007) have been tried. Interestingly, bovine oocytes showed improved activation efficiencies and blastocyst development with an increase in total cell number when exposed to a single rise in $Ca^{2+}$, followed by strontium treatment, compared with strontium treatment alone (Yamazaki *et al.*, 2005). Thus, we needed to examine the effects of varying the concentration of $SrCl_2$ when a single $Ca^{2+}$ influx in oocyte is induced by calcium ionophore for oocyte activation, because the single rise in $Ca^{2+}$ is enough to activate ovine oocytes (Loi *et al.*, 1998). Consequently, a lower concentration of $SrCl_2$ could be used for the activation, or the higher/lower concentration may have detrimental effects on early embryo developmental kinetics. We found that the 5 mM and 10 mM $SrCl_2$-treated groups tended to have a slightly delayed first cleavage (15 mM $SrCl_2$ at 20 and 24 hpa; $P < 0.05$, compared with 10 mM $SrCl_2$) and lower blastocyst rates (5 mM $SrCl_2$; $P < 0.05$, compared with control group) (Table 3). In contrast, the 10 mM $SrCl_2$-treated group showed a relatively rapid first cleavage and similar blastocyst rates compared with control, a finding that suggested that use of the optimum concentration of strontium as an activating agent is important for early embryo kinetics, and that the optimum is different across species. Taken together, our studies demonstrated that, in ovine, the options

of a lower strontium concentration (10 mM) and a shorter incubation period (5 h) were sufficient for oocyte activation, compared with bovine oocytes.

As seen in Tables 1, 2 and 3, oocytes were activated successfully using strontium without treatment with CHX, an compound that has been known to delay PN formation in the mouse when compared with strontium treatment. In addition, combination of strontium with CHX, or a single rise in $Ca^{2+}$ by ethanol did not accelerate PN formation. This observation suggested that repetitive $Ca^{2+}$ oscillations have a marked influence on the timing of PN formation when an inhibitor of protein synthesis is not used (Rogers *et al.*, 2006). This result is consistent with our findings that oocytes treated with strontium in the absence of CHX started to cleave earlier, whilst any CHX - activated cells did not (Tables 1 and 3).

Interestingly, treatment with IP + 10 mM $SrCl_2$/CB induced earlier first cleavage than other treatments that contained CHX at 20 hpa (Tables 3 and 4), although cleavage rates at 48 hpa and development to blastocyst were not different significantly in both parthenotes and SCNT embryos. These results confirm that the delayed first cleavage is attributable to treatment with CHX, an unspecific inhibitor of protein synthesis, during the first cell cycle (Yang *et al.*, 1998; Holm *et al.*, 2003). More significantly, several studies have reported that embryos that cleaved earlier to the 2-cell stage showed increased development to the blastocyst stage in bovine and significantly improved pregnancy rates in human. This observation suggested that earlier cleavage is associated with embryo competence in terms of preimplantation and post-implantation (Lonergan *et al.*, 1999; Bos-Mikich *et al.*, 2001; Windt *et al.*, 2004). Therefore, in subsequent experiments, CHX was excluded from activation medium that contained $SrCl_2$. Although there is agreement that inhibition of protein kinases by treatments such as CHX or 6-dimethylaminopurine (6-DMAP), and use of a single $Ca^{2+}$ influx, improved activation and development to blastocysts (Bos-Mikich *et al.*, 1995; Alexander *et al.*, 2006).

It was observed (Tables 1, 2 and 3) that the use of a single treatment (10 mM $SrCl_2$/CB) or sequential treatments (IP + $SrCl_2$/CB) gave a better outcome than using other various concentrations of strontium or a combination with CHX, in regard to developmental competence as assessed by the timing of first cleavage, cleavage and blastocyst rates, and total cell number of blastocysts. Therefore, the same assessment was performed in SCNT embryos under two different strontium-induced activation protocols, and compared with the control group (IP + CHX/CB). Similar results were observed in SCNT embryos treated with IP + $SrCl_2$/CB (10 mM) as in parthenotes that had been give the same activating agents (Tables 3 and

4). However, in a single-treatment group ($SrCl_2/CB$), blastocyst development was lower and the timing of first cleavage was delayed ($P < 0.05$). These results suggested that strontium-induced activation is an applicable protocol in SCNT, but that strontium activates recipient oocytes in a slightly different or more complicated way compared with its effect in parthenotes (Ma *et al.*, 2009). In SCNT, nuclei in donor cells exhibited totally different characteristics from those in oocytes. Oocytes should, therefore, be replaced with donor nuclei (in terms of cell cycle, epigenetic status, nuclear membrane) and/or comparatively aged recipient oocytes, which have been selected at 16 hpm and activated for 26 hpm. In parthenotes, oocytes are selected and activated for 24 hpm. This finding might be seen as an explanation for the discrepancies in results between SCNT and parthenotes even though the same activation method was employed.

In the mouse, a higher incidence of apoptosis occurred when oocytes were activated by CHX compared with those cells activated by strontium. Subsequently an increase in total cell number was observed in the strontium-activated group. The poor development could be rescued when the CHX-treated oocytes were exposed to a $Ca^{2+}$ spike or oscillations, implying that increase in $Ca^{2+}$ during activation had a great influence on embryo development (Ma *et al.*, 2009). In bovine SCNT and parthenotes, blastocyst rates were higher in the ionophore plus strontium-activated groups compared with the strontium-alone groups, but total cell numbers did not increase. However, increased blastocyst development was reported in the SCNT group compared with the strontium-alone, and strontium/6-DMAP groups (Yamazaki *et al.*, 2005). It is not clear if a series of $Ca^{2+}$ oscillations by strontium confers benefit on embryo development (particularly in aspects such as total cell number, apoptosis, and cell allocations) or if a single large $Ca^{2+}$ increase by calcium ionophore is enough. Our data suggest that a serial calcium increase such as a single spike $Ca^{2+}$ and repetitive $Ca^{2+}$ oscillations could produce more developmentally competent embryos after SCNT because a prolonged rise in intracellular $Ca^{2+}$ seems to ensure initial activation. Subsequently $Ca^{2+}$ oscillations guarantee or promote the latter events of activation (such as pronucleus formation without treatment of non-specific protein synthesis inhibitor, CHX) that induce delay in initiation of DNA synthesis in the first cell cycle in bovine SCNT (Alberio *et al.*, 2001). In this study, we found that recipient oocytes activated by IP + $SrCl_2/CB$ cleaved earlier and that embryos developed to blastocysts with an increase in total cell numbers and less apoptotic proposition in SCNT ($P < 0.05$). Moreover, the accessed parameters such as timing of first cleavage, total cell number, and apoptosis were more informative for the judgement

of embryo competence in accordance with previous studies (McKiernan & Bavister, 1994; van Soom *et al.*, 1997; Brison & Schultz, 1998; Byrne *et al.*, 1999; Lonergan *et al.*, 1999). Thus, our data suggested that a $Ca^{2+}$ increase induced by IP + $SrCl_2/CB$ treatment during activation played a crucial role in the first cell cycle and led to competent embryo development.

It is widely accepted that a higher incidence of inaccurate ploidy is observed in embryos derived from any chemically activated oocytes, resulting in the higher developmental failure in both parthenotes and SCNT embryos compared with IVF embryos (Alexander *et al.*, 2006; Bhak *et al.*, 2006; Wang *et al.*, 2008). Thus, it is necessary to investigate whether the addition of strontium may have detrimental effects on chromosome complement, as reported in the CHX or 6-DMAP treatment that is routinely used as a secondary agent to maintain the basal level of maturation promoting factor (MPF) activities after exposure of the oocyte to a calcium ionophore or ionomycin in oocyte activation (Presicce & Yang, 1994; Susko-Parrish *et al.*, 1994). In addition, several studies have revealed that CHX treatment is superior when used in SCNT in terms of blastocyst development and in regard to normal diploidy, when compared with 6-DMAP addition, which resulted in increased incidence of ploidy such as polyploidy and mixoploidy (Cha *et al.*, 1997; Winger *et al.*, 1997; De La Fuente & King, 1998; Loi *et al.*, 1998) – although increased blastocyst development rates were observed in parthenotes (Alexander *et al.*, 2006; Booth *et al.*, 2001).

As shown in Table 6, there were no differences in the percentages of total diploid nuclei of blastomeres and in the percentages of blastocysts that contained complete diploid blastomeres between two different embryos groups (parthenotes and SCNT), as well as between the two different activation groups (IP + CHX/CB and IP + $SrCl_2/CB$). One possible explanation of these results could be that non-physiological activation may not ensure cytokinesis, because theoretically the addition of CHX or strontium without CB treatment leads to completion of the second meiotic division, resulting in haploid cells. That is, diploidization depends on CB, rather than CHX or strontium (Liu *et al.*, 2002; Alexander *et al.*, 2006). This result was also supported by our experiments in which two pronuclei were formed in cell groups following any CB-treated activation protocol (Table 2). Another explanation could be that CHX may induce unknown broad-spectrum effects on apoptosis, because a higher incidence of apoptosis after IP + CHX/CB treatment may reduce aneuploid blastomeres. Subsequently the proportion of diploid blastomeres may increase as seen in Table 6.

In conclusion, strontium can be used as an efficient agent for activation of reconstructed oocytes as well

as *in vitro*-matured ovine oocytes, with regard to early development kinetics and blastocyst development. In particular, sequential treatment of recipient oocytes with calcium ionophore and strontium improved the developmental potential of SCNT embryos in terms of earlier first cleavage and an increase in total cell number of blastocyst, with less apoptosis and without any adverse effects on early embryo development, such chromosome complement. However, more studies are needed to understand the mechanism of strontium on activation of ovine oocytes and subsequent embryonic development and to elucidate zygotic gene expression with regards to somatic cell nuclear reprogramming.

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