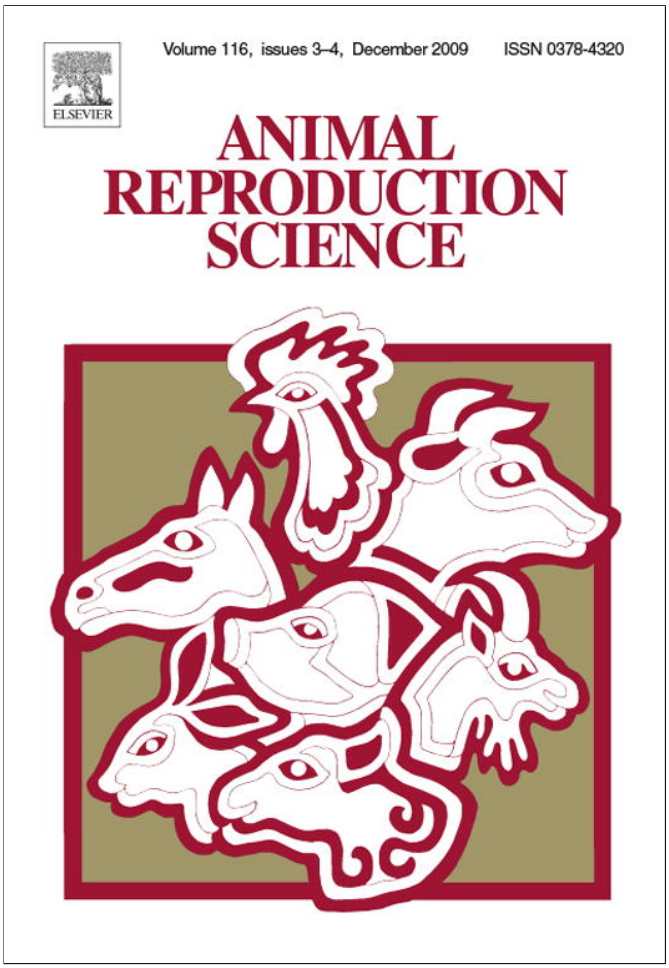

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Short communication

Karyoplast exchange between strontium- and 6-DMAP-parthenogenetically activated zygotes of cattle

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ABSTRACT

Ooplasmic factors drive nuclear organization after fertilization and are also important for re-programming in nuclear transfer procedures, in which artificial activation is essential for reconstructed embryos to progress in development. The present research evaluated the effect of pronuclear transfer (PT) between zygotes parthenogenetically activated with ionomycin followed by strontium (S) or 6-DMAP (D) on early embryonic development. PT was performed in the same zygote to obtain embryos in control groups (S-PT and D-PT) and between zygotes activated with S and D to achieve embryos with differentially activated cytoplasm (C) and nucleus (N) (SCDN and DCSN). PT procedure did not affect cleavage and blastocyst rates, respectively, in PT control groups compared to non-manipulated control (S-PT: 73.6% and 7.3% compared with S-Control: 77.9% and 7.8%; and D-PT: 73.3% and 31.7% compared with D-Control: 83.1% and 41.5%). Cleavage, eight-cell, and blastocyst rates, respectively, were similar between SCDN (76.5%, 36.4%, and 6.8%) and DCSN (69.5%, 25.0%, and 4.9%) embryos. Developmental rates in SCDN were similar to S-PT, but inferior to D-PT.

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Developmental arrest up to eight-cell stage was greater in SCDN and DCSN than in S-PT and D-PT. In conclusion, karyoplast exchange between parthenogenetic zygotes activated with strontium and 6-DMAP can lead to nuclear–cytoplasmic incompatibilities and affect embryonic development to the eight-cell and blastocyst stages.

1. Introduction

After fertilization, ooplasmic factors drive mutual modifications between maternal and paternal genomes, which are essential for embryonic development (Moore and Reik, 1996; Mann and Bartolomei, 2002). The ooplasm ability to re-program the genome also has an important role in the success of somatic cell nuclear transfer (SCNT). Following SCNT, the re-constructed embryo requires an activation stimulus to progress in development. Among several agents used for activation, ionomycin and strontium (Cuthbertson et al., 1981) promote intracellular calcium increase, whereas 6-dimethylaminopurine (6-DMAP; Susko-Parrish et al., 1994) inhibits protein phosphorylation.

Because different activation treatments function through distinct pathways, resulting modifications of the oocyte nucleus and cytoplasm are also variable. From previous observations (Méo et al., 2007), 6-DMAP affects spindle organization and accelerates cell cycle progression when compared to strontium, which is considered a more physiological treatment that promotes periodical intracellular calcium pulses similar to fertilization in mice (Bos-Mikich et al., 1995). Greater developmental rates are achieved when 6-DMAP is used for activation. Thus, there is great interest in searching for more physiological treatments that promote greater rates of activation, especially in cattle given the increased interest in cloning and transgenesis in this species for research and commercial purposes (Niemann and Kues, 2007).

In the present study, zygotes activated with ionomycin followed by strontium or 6-DMAP were submitted to pronuclear transfer (PT). Because interaction between nucleus and cytoplasm begins immediately subsequent to fertilization or activation, PT between zygotes would allow the evaluation of resulting modifications. To our knowledge, this is the first report on karyoplast exchange between activated zygotes of cattle and objectives were to evaluate its effects on early embryonic development and to assess whether 6-DMAP activated cytoplasm and karyoplast were able to improve developmental rates achieved in strontium-activated zygotes.

2. Materials and methods

2.1. Chemicals and media

Chemicals and media were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise stated. Culture conditions during all experiments were in droplets (100 μL) under mineral oil (Dow Corning Co., Midland, MI, USA) with 20–25 oocytes or embryos per drop at 38.5 °C under 5% CO_2 in air and maximum humidity. All media were supplemented with 0.2 mM sodium pyruvate and 83.4 μg/mL amikacin (Instituto Biochimico, Rio de Janeiro, RJ, Brazil).

2.2. Oocyte recovery and in vitro maturation

Follicles (3–7 mm) were aspirated from abattoir-derived bovine ovaries, and oocytes with at least four layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation (IVM). IVM was performed for 24 h in tissue culture medium (TCM-199; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Cripion, Andradina, SP, Brazil), 1.0 μg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Ont., Canada), 50 μg/mL hCG (Profasi™, Serono, São Paulo, SP, Brazil), and 1.0 μg/mL estradiol.

2.3. Artificial activation

After IVM, oocytes were stripped from cumulus cells by gentle pipetting in 0.5% hyaluronidase (Hyalozima™, Aspen, São Paulo, SP, Brazil). Denuded oocytes with an extruded first polar body were activated with 5 μM ionomycin for 5 min in Hepes-buffered synthetic oviductal fluid (HSOF). After that, oocytes were treated with 20 mM strontium ($SrCl_2$; J.T. Baker, Phillipsburg, NJ, USA) and 10 μg/mL cytochalasin D (Cyto D) for 6 h in Ca^{2+}- and Mg^{2+}-free TALP (S); or with 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h in TALP (D). After activation, oocytes were kept in TALP until further utilization.

2.4. Centrifugation

Presumptive zygotes after 16–24 h of activation were centrifuged in HSOF with Cyto D at 15,000 × *g* for 15 min at 25 °C for lipid droplet separation. Pronuclei were visualized using an inverted microscope Olympus IX-70 equipped with Hoffman Modulator Contrast. Activated zygotes submitted to culture after centrifugation and pronuclear evaluation constituted the non-manipulated control groups: S-Control ($n = 77$) and D-Control ($n = 65$).

2.5. Pronuclear transfer

Pronuclear transfer (McGrath and Solter, 1984; Westhusin and De Azambuja, 1996) was performed in HSOF with 7.5 μg/mL cytochalasin B. Four experimental groups were produced: two in the PT control group, where removed karyoplast (pronucleus and adjacent cytoplasm) of one zygote was transferred back to the perivitelline space of the same zygote in S (S-PT; $n = 216$) or D (D-PT; $n = 165$) treatments; and two in the PT differentially activated group, where the karyoplast was removed from a zygote of one activation treatment and was then transferred to a previously enucleated zygote from the other treatment, which allowed the production of embryos with S cytoplasm and D nucleus (SCDN; $n = 255$) and D cytoplasm and S nucleus (DCSN; $n = 279$).

Cytoplasm–karyoplast complexes were fused in a 0.28 M mannitol solution containing 0.1 mM $CaCl_2$ (Merck, Darmstadt, Germany), 0.1 mM $MgSO_4$ (Merck), and 3 mg/mL bovine serum albumin (BSA). Cell fusion was induced by two direct pulses of 1.5 kV/cm for 30 μs each.

2.6. Culture and assessment of early embryonic development

Embryos were co-cultured with granulosa cells in SOF supplemented with 2.5% FCS and 3 mg/mL BSA for 7 days. Cleavage at 46–48 h after activation, eight-cell stage on Day 4, and blastocyst development rates on Day 7 were assessed under a stereoscopic microscope (at 50× magnification).

2.7. Statistical analysis

Experiments were replicated at least three times. Embryonic development rates were analyzed by Chi-square test and, when appropriate, by Fisher's exact probability test using SAS. A level of 5% significance was used.

3. Results

Differences ($p < 0.05$) in pronuclear development rates were observed between activation treatments: 39.1% for S and 43.2% for D. Pronuclear transfer had no effect on cleavage and blastocyst developmental rates, respectively, since the results obtained in S-PT (73.6% and 7.3%) and D-PT (73.3% and 31.7%) were, respectively, similar ($p > 0.05$) to that obtained in non-manipulated control zygotes: S-Control (77.9% and 7.8%) and D-Control (83.1% and 41.5%).

Karyoplast exchange between differentially activated zygotes resulted in embryos with S cytoplasm and D nucleus and D cytoplasm and S nucleus, which were compared to control groups (S-PT and D-PT; Fig. 1). All groups presented similar ($p > 0.05$) cleavage rates (Fig. 1). For eight-cell development rates, S-PT was similar ($p > 0.05$) to D-PT and SCDN, but SCDN was inferior ($p < 0.05$) to D-PT, while DCSN

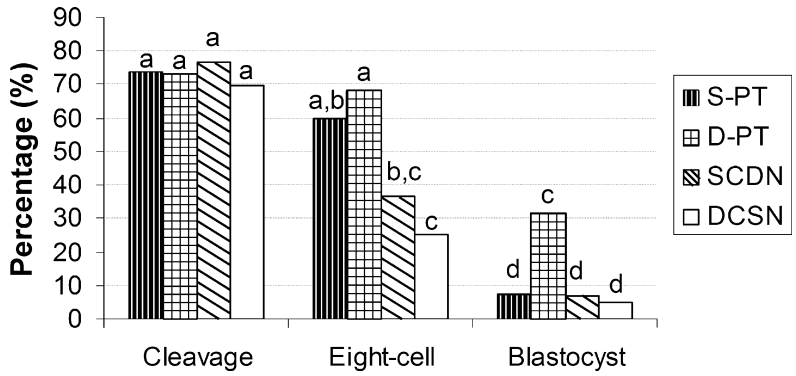

Fig. 1. Cleavage (46–48 h) and development of cattle embryos to eight-cell (Day 4) and blastocyst (Day 7) stages in pronuclear transfer control (S-PT and D-PT) and differentially activated (SCDN and DCSN) embryos. S: oocytes activated with ionomycin + strontium, D: oocytes activated with ionomycin + 6-DMAP, C: cytoplasm, N: nucleus. [abcd]Bars with different superscripts are different ($p < 0.05$).

was inferior ($p < 0.05$) to S-PT and D-PT, but similar ($p > 0.05$) to SCDN (Fig. 1). Regarding blastocyst development, D-PT was superior ($p < 0.05$) to S-PT, SCDN, and DCSN (Fig. 1).

In S-PT and D-PT groups, development rates to the eight-cell stage were similar ($p > 0.05$) to cleavage rates, however, there was a decrease ($p < 0.05$) in blastocyst development rates (Fig. 1). In SCDN and DCSN, there was a decrease ($p < 0.05$) in developmental rates from the two-cell to the eight-cell stage and then also to the blastocyst stage (Fig. 1).

4. Discussion

Strontium and 6-DMAP activation treatments act by distinct pathways. While the first promotes periodical intracellular calcium pulses similar to fertilization in mice (Bos-Mikich et al., 1995), the second inhibits protein phosphorylation. Although activation with strontium would be more physiological, 6-DMAP promotes higher developmental rates (Méo et al., 2007). As expected, in the present study greater pronuclear formation rates were obtained in D compared to S treatment. However, because only zygotes with a visible pronucleus were used for pronuclear transfer procedures, effects of different pronuclear rates between treatments on further embryonic development were minimized.

PT control embryos were produced after removal and transfer of the same karyoplast to the same zygote in each activation treatment (S-PT and D-PT). PT had no detrimental effect on cleavage and blastocyst development rates. This is in agreement with results obtained after pronuclear transfer in embryos of cattle produced by *in vitro* fertilization (Westhusin and De Azambuja, 1996).

After that, PT was performed between strontium and 6-DMAP activated zygotes to obtain SCDN and DCSN embryos. It was expected that embryos re-constructed with the 6-DMAP nucleus (SCDN) would have a similar response to D-PT and improve developmental rates achieved in S-PT, but the opposite was observed: blastocyst development rate in SCDN was similar to S-PT and inferior to D-PT. Because DCSN was also similar to S-PT and inferior to D-PT, it suggests that both the cytoplasm and karyoplast from strontium-activated zygotes negatively affected 6-DMAP-activated zygotes. Also, 6-DMAP-activated karyoplast and cytoplasm were not able to improve developmental rates of S-activated zygotes.

The reason for that interaction between nucleus and cytoplasm of these two activation treatments is not clear. One hypothesis is that cell cycle incompatibilities between these activation treatments may occur because 6-DMAP accelerates pronuclear development and cell cycle progression when compared to strontium (Méo et al., 2007). However, because only zygotes in pronuclear stage were used, in this case the cell cycle would not be an important factor to interfere on further embryonic development.

Based on results from the present study, the detrimental effect of strontium is assumed to be mediated through its cytoplasm (the recipient cytoplasm or the cytoplasm surrounding the transferred pronucleus) and that it was established early in a period between activation and pronuclear formation, because it could not be recovered by karyoplast exchange with 6-DMAP-activated zygotes. This effect

of strontium may be similar to that observed in mouse DDK syndrome (Renard and Babinet, 1986), but the reduced developmental potential was observed as soon as at the eight cell stage. Interestingly, while strontium promotes similar or lesser developmental rates to blastocyst when compared to 6-DMAP for parthenogenetic activation (Yamazaki et al., 2005; Méo et al., 2007), it results in greater rates when used in SCNT procedures (Yamazaki et al., 2005), in which the activation of recipient oocytes is performed after enucleation and transfer of donor nucleus. Therefore, further studies are necessary to investigate the components present on zygotes after each activation treatment that may affect the development of the resulting embryos.

5. Conclusion

Karyoplast exchange between zygotes of cattle activated with strontium and 6-DMAP can generate nucleus–cytoplasmic incompatibility that does not affect cleavage rates, but disturbs development to eight-cell and blastocyst stages.

Acknowledgments

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