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Supplementary Material

Improving functional oocyte enucleation by actinomycin D for bovine somatic cell nuclear transfer

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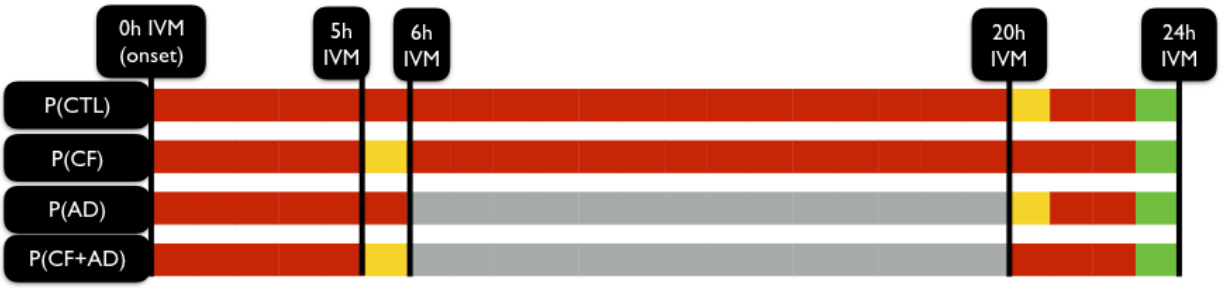

Fig. S1. Experimental design to address the effect of cumulus cells on actinomycin D (AD) toxicity during bovine oocyte *in vitro* maturation (IVM) and further inhibition of parthenogenetic embryonic development. P(CTL): cumulus-enclosed oocytes under standard IVM conditions for 24 h (parthenogenetic control); P(CF): Cumulus-free oocytes from 5 h of IVM onward and further subjected to standard IVM until completion of 24 h of IVM; P(AD): cumulus-enclosed oocytes treated with 1 µg mL^{-1} AD for 14 h (from 6 to 20 h of IVM) and further subjected to standard IVM until completion of 24 h of IVM; P(CF+AD): cumulus-free oocytes from 5 h of IVM onward, treated with 1 µg mL^{-1} AD for 14 h (from 6 to 20 h of IVM) and further subject to standard IVM maturation until completion of 24 h of IVM. Red: standard IVM medium alone. Yellow: denuding to obtain cumulus-free oocytes. Gray: AD treatment. Green: maturation rates were determined by the numbers of eggs with a polar body at the end of IVM (24 h). All eggs were subject to chemical activation at 24 h of IVM to determine parthenogenetic development.

Fig. S2. Experimental design to investigate the effect of gene transcriptionon actinomycin D (AD) toxicity during bovine oocyte *in vitro* maturation (IVM) and subsequent inhibition of parthenogenetic embryonic development. P(CTL): cumulus-enclosed oocytes under standard IVM conditions for 24 h (parthenogenetic control); P(α):cumulus-enclosed oocytes treated with 50 μg mL^{-1} α-amanitin from 3 h to 20 h of IVM and further subjected to standard IVM until completion of 24 h of IVM; P(AD): cumulus-enclosed oocytes treated with 1 μg mL^{-1} AD for 14 h (from 6 to 20 h IVM) and further subjected to standard IVM until completion of 24 h of IVM; P(α+AD): cumulus-enclosed oocytes treated with 50 μg mL^{-1} α-amanitin alone (from 3 h to 6 h of IVM), subsequently treated with 50 μg mL^{-1} α-amanitin and 1 μg mL^{-1} AD (from 6 h – 20 h IVM), and further subjected to standard IVM until completion of 24 h of IVM. Black: hours after the onset of IVM. Blue: α-amanitin treatment. Gray: AD treatment. Yellow: denuding to obtain cumulus-free oocytes. Green: maturation rates were determined by the numbers of eggs with a polar body at the end of IVM (24 h). All eggs were subject to chemical activation at 24 h of IVM to investigate parthenogenetic development.

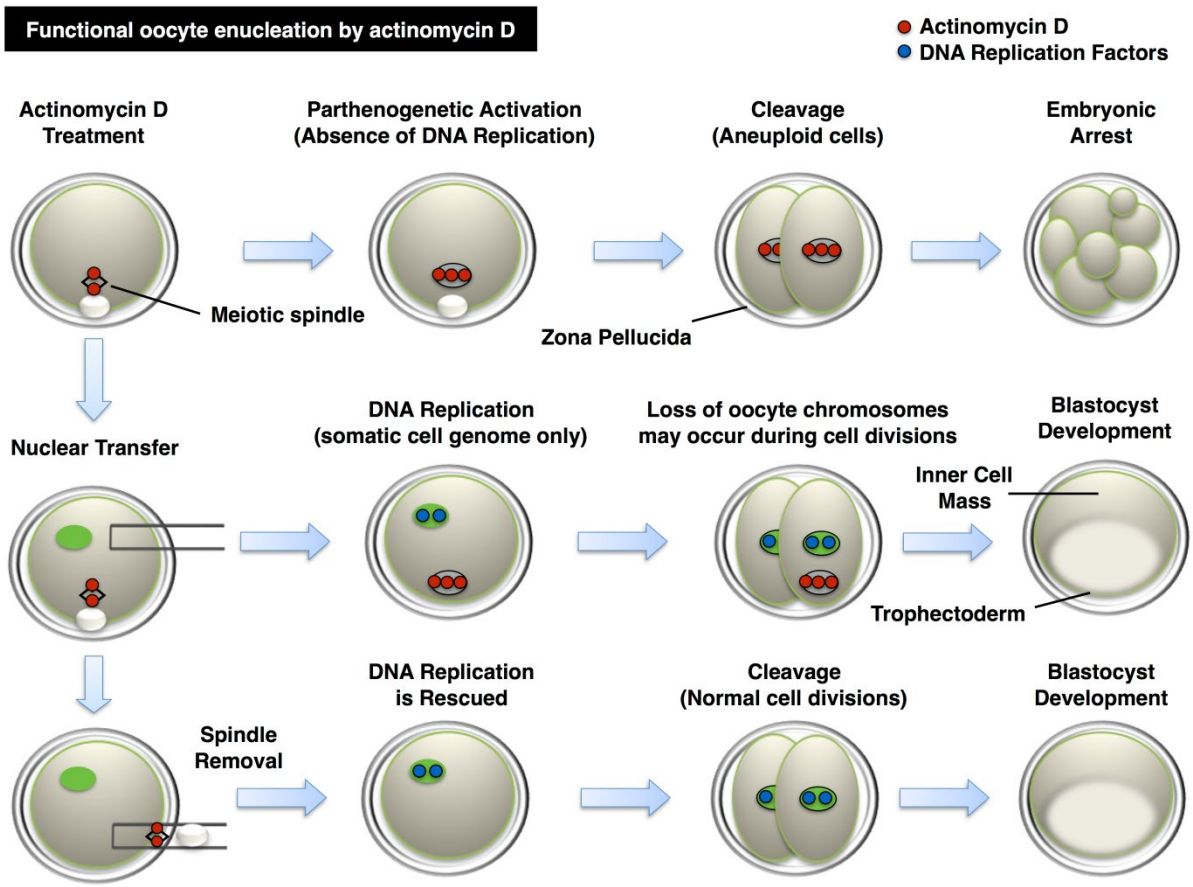

Fig. S3. Working hypothesis for functional oocyte enucleation by actinomycin D for bovine somatic cell nuclear transfer.

Table S1. Maturation rates and inhibition of bovine parthenogenetic development using different actinomycin D (AD) concentrations under a two-hour incubation period

Five replicates. Maturation: eggs with a polar body at 24 h of *in vitro* maturation. Cleavage: embryos with two or more cells at 48h post-activation. D7/D8: Blastocysts after 7 and 8 days post-activation, respectively. *AD concentration in μg mL^{-1}. CTL: cumulus-enclosed oocytes under standard IVM conditions for 24 h (parthenogenetic control). Different superscripts (^{a,b,c}) on same column differ significantly ($P < 0.05$)

AD* (h)	Maturation (%)	Cleavage (%)	Blastocyst (%)	
			D7	D8
0.0 (CTL)	248/284 (87.33)^{a}	225/284 (79.23)^{a}	103/284 (36.27)^{a}	126/284 (44.37)^{a}
1.0 (14 h)	243/296 (82.10)^{b}	173/296 (58.45)^{b}	7/296 (02.37)^{b}	9/296 (03.04)^{b}
5.0 (2)	256/287 (89.20)^{a}	136/287 (47.39)^{c}	0/287 (00.00)^{c}	0/287 (00.00)^{c}
10.0 (2)	252/290 (86.90)^{ab}	131/290 (45.18)^{c}	0/290 (00.00)^{c}	0/290 (00.00)^{c}
15.0 (2)	258/287 (89.90)^{a}	125/287 (43.56)^{c}	0/287 (00.00)^{c}	0/287 (00.00)^{c}
20.0 (2)	247/285 (86.67)^{ab}	147/285 (51.58)^{bc}	0/285 (00.00)^{c}	0/285 (00.00)^{c}

Table S2. Maturation rates and inhibition of bovine parthenogenetic development using 5 µg mL^{-1} actinomycin D (AD) under varying incubation periods

Five replicates. Maturation: eggs with a polar body at 24 h of *in vitro* maturation. Cleavage: embryos containing two or more cells at 48 h post-activation. D7/D8: Blastocysts after 7 and 8 days post-activation, respectively. Different superscripts (^{a,b}) on same column differ significantly ($P < 0.05$)

Incubation (h)	Maturation (%)	Cleavage (%)	Blastocyst (%)	
			D7	D8
0.0	220/268 (82.09)^{a}	207/268 (77.24)^{a}	102/268 (38.05)^{a}	116/268 (43.29)^{a}
1.0	233/287 (81.19)^{a}	121/287(42.16)^{b}	0/287 (00.00)^{b}	0/287 (00.00)^{b}
1.5	239/280 (85.36)^{a}	113/280 (40.36)^{b}	0/280 (00.00)^{b}	0/280(00.00)^{b}
2.0	222/274 (81.03)^{a}	125/274 (45.62)^{b}	0/274(00.00)^{b}	0/274(00.00)^{b}

Table S3. Maturation rates and inhibition of bovine parthenogenetic development using 5 µg mL^{-1} actinomycin D (AD) under incubation periods shorter than 1 h

Three replicates. Maturation: eggs with a polar body at 24 h of *in vitro* maturation. Cleavage: embryos containing two or more blastomeres at 48 h post-activation. D7/D8: Blastocysts after 7 and 8 days post-activation, respectively. Different superscripts (^{a,b,c}) on same column differ significantly ($P < 0.05$)

Incubation (h)	Maturation (%)	Cleavage (%)	Blastocyst (%)	
			D7	D8
0.0	162/193(83.94)^{a}	135/192(70.32)^{a}	6/192(33.85)^{a}	81/192(42.19)^{a}
0.25	168/195(86.16)^{a}	97/195(49.75)^{bc}	0/195(00.00)^{b}	0/195(00.00)^{b}
0.50	159/196(81.13)^{a}	101/196(51.53)^{b}	0/196(00.00)^{b}	0/196(00.00)^{b}
1.0	177/202(87.63)^{a}	81/198(40.91)^{c}	0/198(00.00)^{b}	0/198(00.00)^{b}

Table S4. Maturation rates and inhibition of bovine parthenogenetic development using different actinomycin D (AD) concentrations under a 15-min incubation period

Three replicates. Maturation: eggs with a polar body at 24 h of *in vitro* maturation. Cleavage: embryos containing two or more cells at 48 h post-activation. D7/D8: Blastocysts after 7 and 8 days post-activation, respectively. Different superscripts (^{a,b,c,d,e}) on same column differ significantly ($P < 0.05$)

Act. D conc. ($\mu g\ mL^{-1}$)	Maturation (%)	Cleavage (%)	Blastocyst (%)	
			D7	D8
0.0	141/164 (85.98)^{a}	140/164 (85.37)^{ab}	55/164 (33.54)^{a}	71/164 (43.30)^{a}
0.5	150/166 (90.36)^{a}	146/166 (87.95)^{a}	66/166 (39.75)^{a}	72/166 (43.37)^{a}
1.0	148/169 (87.57)^{a}	143/169 (84.61)^{ab}	45/169 (26.62)^{a}	50/169 (29.58)^{b}
1.5	142/158(89.87)^{a}	126/158 (79.74)^{b}	16/158 (10.12)^{b}	27/158 (17.08)^{c}
2.0	148/167 (88.62)^{a}	134/167 (80.23)^{ab}	10/167 (05.98)^{b}	14/167 (08.38)^{d}
5.0	136/163 (83.43)^{a}	114/163 (69.93)^{c}	0/163 (00.00)^{c}	1/163 (00.61)^{e}

Table S5. Effect of somatic cell nuclear transfer using actinomycin D (AD) functional oocyte enucleation on cleavage-stage embryonic development

Three replicates. P(CTL): cumulus-enclosed oocytes subject to 24 h IVM; P(AD): eggs treated with 5 μg mL^{-1} AD for 15 min at 17 h IVM and moved to standard IVM medium until completion of 24 h IVM;NT(CTL): NT control using egg enucleation and reconstruction by micro-manipulation; NT(AD): eggs treated with 5 μg mL^{-1}AD for 15 min and reconstructed with a somatic cell by micro-manipulation; NT(AD+SR): eggs treated with 5 μg mL^{-1} AD for 15 min, followed by spindle removal (1–5 h after AD treatment) and reconstruction with somatic cell by micro-manipulation. Cleavage: embryos containing two or more cells at 48 h post-activation. Different superscripts (^{a,b,c,d,e}) on same column differ significantly ($P < 0.05$)

Group	Non-cleaved (%)	Cleavage (%)	Fragmentation (%)	Cleavage with fragmentation (%)
P(CTL)	13/194(06.70)^{a}	147/194(75.77)^{a}	16/194(08.24)^{a}	18/194(09.27)^{a}
P(AD)	39/166(23.49)^{b}	67/166(40.36)^{c}	39/166(23.49)^{b}	21/166(12.65)^{ab}
NT(AD)	26/178(14.60)^{c}	102/178(57.30)^{b}	20/178(11.23)^{a}	30/178(16.85)^{b}
NT(AD+SR)	16/194(08.24)^{ac}	140/194(72.16)^{c}	15/194(07.73)^{a}	23/194(11.85)^{ab}