

## Research Article

### Demecolcine Can Overcome Rat Oocyte Spontaneous Activation

Jie Zhu<sup>1,4\*</sup>, Dasari Amarnath<sup>1,2</sup>, Inchul Choi<sup>1,3</sup>, Yukio Tsunoda<sup>2</sup>, Keith Campbell<sup>1,4\*</sup>

<sup>1</sup>Division of Animal physiology, School of Biosciences, The University of Nottingham, Sutton Bonington campus, College road, Sutton Bonington, Loughborough, Leicestershire, LE12 5RD, the UK

<sup>2</sup>Laboratory of Animal Reproduction, College of Agriculture, Kinki University, 3327-204, Nakamachi, Nara, Japan

<sup>3</sup>Department of Animal Biosystem Sciences, College of Agriculture and Life Sciences, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon 305-764, Korea

\*Corresponding author: Keith Campbell. BSc, DPhil. Professor of Animal Development, The University of Nottingham

Dr. Jie Zhu, Department of Biological Resources, Mary Lyon Centre, MRC Harwell, Harwell Science and Innovation Campus Didcot, Oxfordshire, OX11 0RD England, the UK, Email: j.zhu@har.mrc.ac.uk

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## Abstract

To inhibit oocyte spontaneous activation is an important key for the success of rat somatic cell nuclear transfer. In order to achieve this we focused on optimizing rat oocyte activation with strontium chloride (SrCl<sub>2</sub>), timing of oocyte activation and extension of *in vitro* culture of rat oocytes without compromising their developmental competence. First, we proved that 10mM SrCl<sub>2</sub> combined with either 5µg/mL CytochalasinB (CB) or 2mM 6-dimethylaminopurine (DMAP) could equally, effectively activate rat oocytes and resulted in blastocyst rate of around 50%, also no difference in the mean cell number/ blastocyst was observed between the two groups. Second, we found that rat oocytes collected from 14-22 h after hCG injection had equally developmental potential after being activated. Third, we applied MG132, a protease inhibitor to maintain rat oocytes at the metaphase of the second meiotic division (MII stage) for 2 hours, and then activated them with an optimized rat oocyte activation protocol. 60% of the treated oocytes developed to the blastocyst stage after activation. Finally, we demonstrated that rat oocytes could be maintained in 0.05µg/ml demecolcine containing KSOM medium up to 5 hours after oocyte collection without compromising the developmental competence. Therefore, we conclude that demecolcine treatment could effectively inhibit rat oocyte's spontaneous activation *in vitro* for up to 5 hours and this method may potentially benefit rat oocyte manipulation *in vitro* such as somatic cell nuclear transfer, rat oocyte IVF or ICSI and oocyte cryopreservation.

## Introduction

Since the first success of rat somatic cell nuclear transfer has been reported in 2003 [1] this success has unfortunately not been repeated to date. Although many problems such as animal strains [2,3], quality of oocytes [2-5], timing of oocyte collection [6], the oocyte activation method [2,6,7], embryo culture system [1,8], the synchronization of cloned embryos and recipient animals [9-12] and so on, would be factors responsible for success in rat somatic cell nuclear transfer. However, rat oocyte spontaneous activation could

be responsible for the failure in somatic cell nuclear transfer, which is significant species specific in this species. Little has been known the reasons of why rat oocyte activation spontaneously occurs shortly after oocyte collection. However the phenomenon has been well known, for instance, rat oocytes in culture enter a so called meiotic III stage, namely at which the metaphase spindle in the oocyte disappears in 1.5 hours of culture and chromosomes spread or scattered in the cytoplasm accompanied with the decrease of maturation promoting factor (MPF) [13-15], such oocyte would very likely lose the capability of its developmental competence.

The observation and description of rat oocyte spontaneous activation have been reported in many studies [13,14,16]. To date, despite successes in rat oocyte activation have world widely been reported by many laboratories [10,11,17,18], some of which showed that rat oocytes developed to the blastocyst stage at over 50% with different activation protocols and a variety of stimuli, such as electrical stimulation [10,19], ethanol [7,20], ionomycin [21,22], strontium chloride [6,12,23,24], DMAP [7,24] as well as roscovitine [11]. However, the main problem, oocyte spontaneous activation in rats, has still not completely been resolved as in these successes most oocytes were activated immediately once they were collected from *in vivo*. Moreover some data [11,12,25] also show that the percentage of blastocyst formation after activation dramatically declined with extension of *in vitro* culture. Obviously, to overcome rat oocyte spontaneous activation is requisite for manipulating oocytes such as nuclear transfer, rat oocyte IVF and cryopreservation. Several attempts [6,7,12,26] have been made to inhibit rat oocyte spontaneous activation by adding MG132 or cytochalasin B (CB) into culture KSOM medium after oocyte collection from *in vivo* so that oocytes could be arrested at MII stage in culture. More importantly, the developmental competence of these rat oocytes could still be maintained even after several hours of *in vitro* culture. The first one [26] led the birth of rats successfully cloned with somatic cells by holding oocytes in MG132 an inhibitor of proteasomes and calpains, which could maintain the most of oocytes at the metaphase of the second meiotic division for 3 h. The second [7] used CB to inhibit oocyte spontaneous activation for 6 h and after subsequently parthenogenetic activation the blastocyst rate could reach to over 50%, indicating developmental competence in these oocytes was well maintained. Controversially [6], supplemented 10  $\mu\text{g}/\text{ml}$  CB in culture medium and failed in inhibiting rat oocyte spontaneous activation after 4 and 6 h of culture of rat oocytes, the rate of blastocyst formation decreased from 38% in the controls (10 min after oocyte collection) to 4% in 4h culture and 1% in 6h culture, respectively. The conflict results may reflect a negative effect of cytochalasin B on rat oocyte developmental potential as in the Galat *et al* experiment [7], after rat oocytes were exposed to 3  $\mu\text{g}/\text{ml}$  cytochalasin B in the culture medium for 6 h, the oocytes were also rinsed and cultured in the medium without adding CB for another 4 h before activation, whereas in the latter, rat oocytes after 4 and 6 h CB treatment were immediately activated. Perhaps this difference in handling oocytes could explain why the results from the similar experiments were so different, which suggests that in some cases rat oocytes may need time to recover fully from stress after a long time of drug treatment. Also, this recovery time seems to be very essential for resuming developmental potential of the treated rat oocytes in the case. Therefore, CB could be used to effectively inhibit rat oocyte spontaneous activation for several hours but oocytes need 4 hours extra culture in the CB free medium to recover before use.

Here, we aimed at establishment of effective and reliable protocols for rat oocyte activation, rat embryo culture as well as prolonging *in vitro* culture of rat oocytes. In order to achieve these we carried out a series of experiments. First,

we focused on optimizing conditions related to rat oocyte activation in order to obtain an effective and reliable protocol for rat oocyte activation with  $\text{SrCl}_2$  as  $\text{SrCl}_2$  can trigger  $\text{Ca}^{2+}$  oscillations in a mature oocyte as those seen in a fertilized oocyte [27,28]. Second, based on the protocol we expected to be able to examine and to compare developmental competence of oocytes after treated *in vitro* with MG132 and demecolcine for several hours. Also, we were more interested in demecolcine, a stable factor of spindle due to several reasons: (1) It has been commonly used to synchronize somatic cells or oocytes at the metaphase stage [29]. (2) To assist enucleation of oocytes as recipient cells in somatic cell nuclear transfer [30,34], the more important is that this treatment has led the birth of many healthy cloned animals, indicating that demecolcine is less toxic, (3) demecolcine treatment could increase maturation promoting factor (MPF) in goat [28] and bovine oocytes [35], MPF is responsible for nuclear envelope breakdown. Therefore, demecolcine may benefit rat somatic cell nuclear transfer. Taken all these considerations together we hypothesized that demecolcine may logically be a more suitable chemical for inhibiting rat oocyte spontaneous activation.

Here we reported that addition of demecolcine at a concentration of (0.05  $\mu\text{g}/\text{mL}$ ) in culture medium can not only effectively inhibit rat oocyte spontaneous activation, but also maintain the developmental competence of the oocytes after 5 h treatment of demecolcine which has been proven by parthenogenetic activation. The chromosome status of rat oocytes cultured in the medium containing demecolcine show that all observed oocytes arrested at the MII stage after 5 hours of the treatment, whereas in MG 132 treated group some considerable number of treated oocytes already activated after the same period of culture, indicating that demecolcine may be more reliable than MG132 for this purpose. In conclusions, this approach may be useful to widen "a time window" for manipulating rat oocyte, such as nuclear transfer, ICSI or oocyte cryopreservation.

## Materials and Methods

### Chemicals

In these experiments all chemicals were purchased from Sigma unless otherwise stated.

### Animals

Sprague-Dawley (SD) female rats (21-28 days old) were purchased from commercial companies either Charles River (Japan) or Harlan (UK). All animals were kept at a temperature of  $21 \pm 2^\circ\text{C}$  in a 12 h light / dark cycle with a humidity of  $65 \pm 5\%$ . And free access a commercial rat food and filtered water. All experimental protocols were performed in accordance with the guidelines of the Home Office in the UK and were approved by the ethics committee of Department of Animal Sciences, the University of Nottingham, or by the ethics committee of College of Agriculture, Kinki University in Japan.

### Superovulation protocol and oocyte collection

SD female rats were intraperitoneally (i.p) injected with 10 IU / animal PMSG (Intervet, UK) at 21:00, they were then given 15IU/animal hCG. i.p injection 48 h later. Time of oocyte collection depended on individual experiments; however we generally collected rat oocytes at 15 h after hCG injection. SD rats were sacrificed by cervical dislocation and oocyte-cumulus complexes were immediately released from the oviducts within 5 min and transferred into a 30mm Petri dish containing 0.1% hyaluronidase in M2 medium and kept on a heating stage at 37 °C for 2 min. These complexes were then gently, repeatedly pipetted by a small glass hand pipette to strip cumulus cells off from oocytes. After cumulus cells were removed, denuded oocytes were rinsed in M2 or transferred into KSOM medium (Specialty Media, the UK) and kept in an incubator at 37°C, 5%CO<sub>2</sub> in air before use.

### Oocyte activation and embryo culture

Oocytes were rinsed several times in KSOM medium and subsequently cultured in activation medium: 10mM SrCl<sub>2</sub> + 2mM DMAP or 10mM SrCl<sub>2</sub> + 5µg/ml CB in KSOM for 4 or 6 h in an incubator at 37°C, 5% CO<sub>2</sub> in air. After 4 or 6 hours in activation medium oocytes were rinsed several times in KSOM medium to remove all SrCl<sub>2</sub> and DMAP or CB and cultured in the medium for 20-24 hours in the incubator at 37°C, 5%CO<sub>2</sub> in air. Twenty to twenty-four hours after cultured in KSOM medium, oocytes were rinsed several times in the modified rat 1 cell embryo culture medium (m R1ECM)(Miyoshi and Niwa, 1997) supplemented with 0.3% BSA and cultured in droplets of the medium, in groups of 10 oocytes/50µl covered with mineral oil in the incubator at 37°C, 5% CO<sub>2</sub> in air for further 4 days [1]. In some experiments we also supplemented with 5% fetal calf serum (FCS) into 50µl droplets containing embryos at the morula stage to achieve better development.

### Collection of D5 *in vivo* embryos

An adult Sprague Dawley (SD) female rat was mated with a fertile male in a mating cage at 17:00 pm and in the next morning the plugged female was separated from the male and kept in an individual cage, the time was regarded as 0.5 day of gestation. 5 days later the female was killed in a CO<sub>2</sub> gas chamber at 14:00 pm and embryos were flushed with M2 medium and collected blastocysts were immediately fixed in 4% Paraformaldehyde (PFA) at room temperature for 20 min.

### Maintenance of oocytes *in vitro*.

Rat oocytes were collected and rinsed several times in KSOM and in groups of 10 oocytes /50µl cultured in the medium supplemented with 5M MG132 or 0.05 µg/ml demecolcine (WAKO, Japan) covered with mineral oil in an incubator at 37°C, 5%CO<sub>2</sub> in air. According to experiments different durations of demecolcine treatment could be applied.

### Experimental design

**Experiment 1.** Establishment of a method for rat oocyte activation

#### (1) Effect of SrCl<sub>2</sub> concentration on development of activated rat oocytes

The objective of this experiment was to determine effect of SrCl<sub>2</sub> concentration on the developmental competence of activated rat oocytes. Oocytes were collected from superovulated SD female rats at the age of 21 – 28 days 16-18 h after hCG injection and immediately treated with 0.1% hyaluronidase in M2 medium for 3-5 min and then denuded oocytes were rinsed several times in KSOM medium, subsequently transferred into KSOM supplemented with 0, 2, 5 and 10 mM SrCl<sub>2</sub> + 5µg/ml CB and cultured in the medium for 5-6 h in an incubator at 37°C, 5%CO<sub>2</sub> in air. After activation all oocytes were rinsed several times in KSOM medium and cultured in the medium for 20-24 h in the same conditions. Subsequently all oocytes were rinsed in mR1ECM medium and cultured in the same conditions for extra 4 days.

#### (2) Effects of CB and DMAP on developmental potential of activated rat oocytes

We intended to compare CB and DMAP in this experiment to optimize our protocol. Oocyte were collected from superovulated SD female rats 16-18 h after hCG injection and treated in 0.1% hyaluronidase in M2 and oocytes from individual animals were divided evenly into two groups in order to minimum variations among animals and activated either in 10 mM SrCl<sub>2</sub> + 5 µg/ml CB for 5-6 h or in 10mM SrCl<sub>2</sub> + 2 mM DMAP for 4 h. Meanwhile SrCl<sub>2</sub> alone for 4 h was as a control group. The subsequent procedures were conducted the exactly same as described in the previous experiment. In order to compare mean cell numbers of *in vitro* and *in vivo* produced rat embryos we also collected *in vivo* fertilized embryos on day 5 after naturally mated animals plugged as another control group.

**Experiment 2.** Timing of *in vivo* rat oocyte activation

In order to find the best timing of *in vivo* ovulated rat oocyte activation we collected rat oocytes from superovulated female animals from 14- 22 h after hCG injection with an interval of every 2 h. Oocytes were then treated in 0.1 % hyaluronidase in M2 and immediately activated in 10 mM SrCl<sub>2</sub> + 2mM DMAP for 4 h. then cultured in KSOM for 18-20 h and in mR1ECM for extra 4 days.

**Experiment 3.** MG 132 and demecolcine inhibit spontaneous activation and maintains development potential of rat oocytes

In the third series of experiments, we focused on extending *in vitro* culture of rat oocytes with MG 132 or demecolcine treatment and expected to maintain the developmental competence after the treatment.

### (1) Effect of MG 132 concentration on the developmental competence of activated rat oocytes after 2 hours *in vitro* culture.

We collected rat oocytes at 19- 20 h after hCG administration and denuded oocytes were cultured in KSOM supplemented with 5  $\mu$ M MG132 for 2 h and the oocytes were rinsed several times to remove MG 132 completely. Oocytes were activated in KSOM supplemented with 2, 5 and 10 mM SrCl<sub>2</sub> + 5  $\mu$ g/ml CB for 5-6 h respectively, after activation they were continually cultured according to the same procedures as described above. On day 4 morulae were observed and 2.5  $\mu$ l of pre-warmed FCS was injected directly into 50  $\mu$ l droplets. Subsequently the dish containing embryos were placed back into the incubator at 37°C, 5% CO<sub>2</sub> in air and continually cultured in the same conditions.

### (2) Effect of demecolcine concentration on developmental potential of activated rat oocytes.

Oocytes were collected 14 h after hCG injection as described above and oocytes were cultured for 2 h in 0, 0.05, 0.1 and 0.5  $\mu$ g/ml demecolcine at 37°C, 5% CO<sub>2</sub> in air, respectively and they were then activated in 10mM SrCl<sub>2</sub> + 2 mM DMAP for 4 h, meanwhile oocytes were activated at 0 h of oocyte collection as controls. Subsequently embryos culture was performed as the exactly same in the previous experiments.

### (3) Effects of different durations of demecolcine treatment on development of activated rat oocytes

Oocytes were collected 19-20 h after hCG injection described above and maintained in KSOM supplemented with 0.05  $\mu$ g/ml demecolcine in the incubator at 37°C, 5%CO<sub>2</sub> in air for 1, 2, 3, 4 and 5 h, respectively. Subsequently they were activated in 10 mM SrCl<sub>2</sub> +5  $\mu$ g/ml CB for 5-6 h at 1, 2, 3, 4 and 5h after the demecolcine treatment, respectively. Again, when morulae were observed on day 4, 2.5  $\mu$ l of pre-warmed FCS injected directly into 50  $\mu$ l droplets containing embryos and embryos were continually cultured in the same conditions for another day.

### Cell number counting

Fixed *in vivo* blastocysts and *in vitro* produced blastocysts collected on day 5 were rinsed in PBS and gently mounted on clean glass slides and stained with DAPI or 5  $\mu$ g/ml Hoechst33342, observed directly under a microscope attached with an ultraviolet light unit and cell number were counted and recorded or the slides were kept in a fridge until use for cell counting. No more than 10 blastocysts were mounted on each slide to avoid repeatedly counting.

### Data collection and analysis

Numbers of oocytes used and cultured were recorded in each experiment and all experiments were repeated at least three times. Pronuclear formation, number of day 1 cleaved

oocytes, number of Day 4 compact morulae and number of Day 5 blastocysts were observed in each treatment and recorded. Comparisons of treatments were statistically analysed with a  $\chi^2$  - test, whereas a comparison of cell numbers between treatments was analysed with Student T- test. The value of P less than 5 % or 1% was regarded as a significant or highly significant difference.

## Results

**Experiment 1.** Establishment of a method for rat oocyte activation

### (1) Effect of SrCl<sub>2</sub> concentrations on rat oocyte activation

The results are shown in Table 1. blastocyst rates were only 5 % (3 blastocysts/59 oocytes used) and 3% (2/63) in 0 and 2mM SrCl<sub>2</sub> + 5  $\mu$ g/ml CB treatments, respectively, whereas the rate reached to 59 (61/108) and 67% (78/119) when oocytes were activated with 5mM and 10 mM SrCl<sub>2</sub> + 5  $\mu$ g/ml CB, respectively, which were significantly higher than those in the former two ( $\chi^2$ -test; P< 0.01). However, the mean cell number / blastocyst was 33  $\pm$  6, 29  $\pm$  3, 32  $\pm$  7 and 41  $\pm$  10 in 0, 2, 5 and 10 mM SrCl<sub>2</sub> + 5  $\mu$ g/ml CB, respectively and no statistical differences in mean cell number/blastocyst between groups ( $\chi^2$ -test; P> 0.05) were observed although that in 10 mM SrCl<sub>2</sub> treatment appeared to be higher than others. In this experiment 5 mM or 10 mM SrCl<sub>2</sub> could equally, effectively activate rat oocytes and led over 50 % of oocytes developing to the blastocyst stage. Therefore, the concentration of 10 mM SrCl<sub>2</sub> was used for the late experiments.

SrCl <sub>2</sub> (mM)	No.Oocytes (N)	PN (N)	D1Cleaved (N)	D4 M (N)	D5Blast. (N)	D5Blast. %	Mean Cell Number Mean $\pm$ SD
2mM	27	13	12	8	6	22.2*	46 $\pm$ 11
5mM	72	69	67	47	45	62.5	48 $\pm$ 11
10mM	81	79	79	57	49	60.5	49 $\pm$ 10

**Table 1.** Effect of SrCl<sub>2</sub> Concentrations on Rat Oocyte Activation

PN: the pronuclear formation,

D1 Cleaved: the number of oocytes cleaved on day 1 after activation.

D4 M: the number of oocytes developed to the morula stage on day 4 after activation,

D5 Blast: the number of oocytes developed to the blastocyst stage on day 5 after activation

D5 Blast. %, the percentage of blastocyst formation on day 5 was calculated based on the total number of oocytes used.

SD: Standard Deviation

### (2) Effects of CB and DMAP on developmental potential of activated rat oocytes

SrCl<sub>2</sub> has been commonly combined with DMAP to activate rat oocytes and thought to be more effective than SrCl<sub>2</sub> combined with CB (Jiang et al., 2002). However, our results showed that SrCl<sub>2</sub> combined with either DMAP or CB could equally; effectively activate rat oocytes (seen in Table 2). Pronuclear formation, day 1 cleavage rate, the number of day 4

compact morulae or earlier blastocysts and number of day 5 blastocysts as well as the mean cell number/ blastocyst in SrCl<sub>2</sub> combined with CB or DMAP were 139/140 vs 139/140, 130/140 vs.129/140, 84/140 vs.106/140 and 76/140 vs. 78/140 as well as 39.5±13.8 (N=31) vs. 39.0±14.0 (N=32), respectively. No differences in all observed parameters were found between these two groups ( $\chi^2$ -Test, P>0.05 and Student T-test; P>0.05). Interestingly, although pronuclear formation (51/53) and day1 cleavage rate (52/53) in SrCl<sub>2</sub> treatment alone were very similar to the other two groups unfortunately unlike mouse oocytes, no rat oocyte could develop beyond the 2-cell stage, suggesting that diploidisation of rat oocytes during oocyte activation may be essential for *in vitro* blastocyst formation.

Treatment	No.Oocytes (N)	PN (N)	D1 Cleaved (N)	D4. M (N)	D5.Blast. (N)	D5 Blast. (%)	Mean cell number Mean±SD
CB	140	139	130	84	76	54.23	39.5±13.8 (N=31)
DMAP	140	139	129	106	78	55.71	39.0±14.0 (N=32)
Controls	53	51	52	0	0	0	-
<i>In vivo</i> fertilized	37	-	-	-	-	-	57.92 ±17.5 *

**Table 2.** Effect of Cytochalsin B (CB) and DMAP Treatment on Developmental Potential of Activated Rat Oocytes

\* shows significant differences between the values in the same columns ( $\chi^2$  - test, P< 0.01)  
 PN: the pronuclear formation,  
 D1 cleaved: the number of oocytes cleaved on day 1 after activation.  
 D4 M: the number of oocytes developed to the morula stage on day 4 after activation,  
 D5 Blast: the number of oocytes developed to the blastocyst stage on day 5 after activation  
 D5 Blast. %, the percentage of blastocyst formation on day 5 was calculated based on the total number of oocytes used.  
 SD: Standard Deviation

**Experiment 2: Timing of *in vivo* rat oocyte activation**

Timing of *in vivo* rat oocyte activation is important for rat somatic cell nuclear transfer as oocyte enucleation and subsequent reconstitution of a somatic cell and an enucleated oocyte should be performed at the right time when the oocyte as a recipient could be activated more effectively. Our results were shown that the blastocyst rates in all groups were 43.50% (47 blastocysts/108 oocytes), 56.50% (61/108), 53.20% (59/111), 54.22% (90/166) and 52.50% (21/40) with oocytes collected and activated at 14, 16, 18, 20 and 22 h after hCG injection, respectively (table 3). Furthermore, there were no statistical differences in all observed parameters cross these time points of oocyte collection from 14 to 22 h after hCG injection ( $\chi^2$ -test; P > 0.05) although the blastocyst rate of only 43.50 % in oocytes collected at 14 h seemed to be lower than the others. These results indicate that oocytes collected from 14 h to 22 h after hCG could be used for rat somatic cell nuclear transfer (table 4).

Concentration	No. oocytes (N)	PN (N)	D1Cleaved (N)	D4 M (N)	D5 Blast. (N)	D5 Blast. (%)	Mean cell number Mean±SD
0 mM	59	2	5	4	3	5	33±6
2 mM	63	4	8	5	2	3	29±3
5 mM	108	103	94	65	61	59**	32±7
10 mM	119	114	111	83	78	67**	41±10

**Table 3.** Timing of Rat Oocyte Collected at Different Time Points by Activation

There are no differences among treatments, ( $\chi^2$  - test, P > 0.05)  
 PN: the pronuclear formation,  
 D1 cleaved: the number of oocytes cleaved on day 1 after activation.  
 D4 M: the number of oocytes developed to the morula stage on day 4 after activation,  
 D5 Blast.:the number of oocytes developed to the blastocyst stage on day 5 after activation  
 D5 Blast: %, the percentage of blastocyst formation on day 5 was calculated based on the total number of oocytes used.  
 SD: Standard Deviation

Hours after hCG	No Oocytes (N)	PN (N)	D1. Cleaved (N)	D4 M (N)	D5 Blast. (N)	D5 Blast. %
14h	108	108	108	82	47	43.5
16h	108	103	106	85	61	56.5
18h	111	108	108	71	59	53.2
20h	166	162	146	83	90	54.2
22h	40	39	37	21	21	52.5

**Table 4.** Developmental Potential of Rat Oocytes Exposed to MG 132 for 2 Hours before activation

\* There are significant differences among the values in the column  
 The oocytes used were collected at 19-20 h after hCG injection.  
 PN: Pronuclear formation  
 D1 Cleaved: Number of oocytes cleaved on day 1 after activation,  
 D4 M: Number of morulae observed on Day 4 after activation,  
 D5 Blast: Number of blastocysts observed on Day 5 after activation.  
 D5 Blast %: Percentage of blastocysts observed on Day 5 based on the number of oocytes used  
 \* there are significant differences among the values in the column ( $\chi^2$  - test; P< 0.01)

**Experiment 3.** MG132 and demecolcine inhibits spontaneous activation and maintains development potential of rat oocytes

In this series of experiments, we found MG 132 treated oocytes could be well maintained at the MII stage for 2 h without decrease of the developmental competence.

After activation with 5 or 10 mM SrCl<sub>2</sub> + 5 µg/ml CB, over 60% of MG132 - 2 h treated oocytes could develop to the blastocyst stage, whereas only 22% of 2 mM SrCl<sub>2</sub> + 5 µg/ml CB treated oocytes reached to the blastocyst stage, which was highly significantly lower than those in the other groups ( $\chi^2$  - test, P< 0,01). The result is similar to that shown in Table 1, which also reveals that 5 and 10 mM SrCl<sub>2</sub> both can effectively activate rat oocytes. However, with extending culture duration up to 5 h spontaneous activation occurred in a considerable number of MG132 treated oocytes, observed by Hoechst 33342 staining (data not shown), which was consistent with the previous report. In addition, we cultured rat oocytes in the presence of demecolcine at different concentrations of 0, 0.05, 0.1 and 0.5 g/ml for 4h, respectively. The blastocyst rate was 43.24% (32 blastocysts / 74 oocytes) in the control, 2% (1/50) in 0 µg/ml, 28.95% (22/76) in 0.05µg/ml, 24.69% (20/81) in 0.1µg/ml and 27.03% (20/74) in 0.5µg/ml (seen in Table 5), respectively. There were no significant differences in the blastocyst rate between these groups including the control except for the 0 µg/ml demecolcine group ( $\chi^2$ -test, P >0.05) although that

in 0 hour controls appeared to be higher. However the proportion of blastocyst formation in 0  $\mu\text{g/ml}$  demecolcine was highly significantly lower than the others ( $\chi^2$ -test,  $P < 0.01$ ), which indicates that demecolcine could effectively inhibit spontaneous activation in rat oocytes as we expected. Based on the result the concentration of 0.05 $\mu\text{g/ml}$  demecolcine was used in the last experiment.

Demecolcine ( $\mu\text{g/ml}$ )	No.Oocytes (N)	PN (N)	D1Cleared (N)	D4 M/B (N)	D5 Blast. (N)	D5 Blast. (%)
0H Control	74	70	73	43	32	43.2
0 $\mu\text{g/ml}$	50	44	41	7	1	2**
0.05 $\mu\text{g/ml}$	76	72	71	32	22	29.0
0.1 $\mu\text{g/ml}$	81	73	67	29	20	24.7
0.5 $\mu\text{g/ml}$	74	64	61	34	20	27.0

**Table 5.** Effect of Demecolcine Concentration on Developmental Potential of Activated Rat Oocytes

0h: oocytes were immediately cultured in 10mM  $\text{SrCl}_2$  +2mM DMAP for 4h

Oocytes in the rest groups were cultured in KSOM medium containing DEMO for 2 h before activation, whereas oocytes cultured in KSOM alone for 2h were regarded as controls.

\*\*There were no significant differences among groups ( $\chi^2$ -test;  $P > 0.05$ ) except for 0 $\mu\text{g/ml}$  ( $\chi^2$ - test;  $P < 0.01$ )

## (2) Effects of different durations of demecolcine treatment on development of activated rat oocytes

In the last experiment, the result shows that the blastocyst rate was 64.29% (45 blastocysts/70 oocytes), 42.42% (28/66), 54.10% (33/61), 42.25% (30/71) and 43.06% (31/72) ) in oocytes cultured in 0.05 $\mu\text{g/ml}$  demecolcine for 1, 2, 3, 4 and 5 h before activation with 10 mM  $\text{SrCl}_2$  + 5  $\mu\text{g/ml}$  CB for 5-6 h, respectively (seen in Table 6). Additionally, no significant differences in the blastocyst rate was observed between all the groups ( $\chi^2$  - test,  $P > 0.05$ ), which indicate that 0.05 $\mu\text{g/ml}$  demecolcine could inhibit rat oocyte spontaneous activation and could maintain the developmental competence of rat oocytes cultured *in vitro* for at least 5 h. Moreover we also could not find any significant differences in the mean cell numbers / blastocyst between the groups ( $\chi^2$  - test,  $P > 0.05$ ). In order to confirm the status of cell cycle in the demecolcine treated oocytes we stained randomly collected oocytes and the results show that all the oocytes (100%) arrested at the MII stage after 5 h culture in demecolcine.

DEMO treatment	No. Oocytes (N)	PN (N)	D1 Cleaved (N)	D4 M (N)	D5 Blast. (N)	D5. Blast. (%)	Mean cell number Mean $\pm$ SD
1H	70	69	63	48	45	64	54 $\pm$ 11
2H	66	63	58	34	28	43	49 $\pm$ 4
3H	61	60	59	39	33	54	50 $\pm$ 2
4H	71	65	68	33	30	42	41 $\pm$ 7
5H	72	71	70	31	31	43	47 $\pm$ 9

**Table 6.** Effects of Different Durations of Demecolcine Treatment on Development of Activated Rat Oocytes

There are no significant differences among the treatments ( $\chi^2$ - test;  $P > 0.05$ )

PN: the pronuclear formation,

D1 cleaved: the number of oocytes cleaved on day 1 after activation.  
D4 M: the number of oocytes developed to the morula stage on day 4 after activation,

D5 Blast: the number of oocytes developed to the blastocyst stage on day 5 after activation

D5 Blast. %: the percentage of blastocyst formation on day 5 was

calculated based on the total number of oocytes used.

SD: Standard Deviation

DEMO Demecolcine. The concentration of 0.05 $\mu\text{g/ml}$  Demecolcine was used in this experiment

## Discussion

$\text{Ca}^{2+}$  is essential for rat oocyte survival during activation with  $\text{Sr}^{2+}$

Several reports have shown that rat oocytes can be activated effectively by butyrolacton I [26], electrical stimuli, calcium ionophore, ionomycin, DMAP and  $\text{SrCl}_2$  [19]. The first success of rat somatic cell nuclear transfer was achieved by butyrolacton I activating reconstituted embryos. Unfortunately Btyrolactone I is expensive and also did not produce consistent results in our laboratory. Instead, we decided to use  $\text{SrCl}_2$  as it can trigger multiple  $\text{Ca}^{2+}$  oscillations in mouse oocytes which are similar to those seen in a fertilised oocyte, whereas the others such as electrical stimuli or ionomycin and so on, trigger only one big spike of  $\text{Ca}^{2+}$  in oocyte cytoplasm [27]. Additionally, mouse oocytes can be activated effectively with  $\text{SrCl}_2$ +CB in  $\text{Ca}^{2+}$  free -CZB medium and can develop to the blastocyst stage at over 90%. This protocol has been routinely used for mouse somatic cell nuclear transfer and resulted in many births of healthy cloned mice. However this seemed to be not the case in rats as we initially applied the exact same protocol into rat oocytes, oocytes hardly survived and all 34 oocytes lysed within 60 min of 10 mM  $\text{SrCl}_2$ +5 $\mu\text{g/ml}$  CB treatment in  $\text{Ca}^{2+}$  free medium. Subsequently we activated rat oocytes using the same protocol but with CZB medium supplemented with  $\text{Ca}^{2+}$ , the results were dramatically improved, not only all oocytes survived but also over 50 % of rat oocytes activated could develop to the blastocyst stage, which indicate that  $\text{Ca}^{2+}$  containing medium is essential for efficient activation of rat oocytes by 10mM  $\text{SrCl}_2$ +5  $\mu\text{g/ml}$  CB. However, interestingly, Galat, *et al* [16] reported that 12.6% of rat oocytes activated in  $\text{Ca}^{2+}$  free and  $\text{Mg}^{2+}$  free CZB medium with 2.5mM  $\text{Sr}^{2+}$ , 3 $\mu\text{g/ml}$  CB for 1.5h at 37°C in a  $\text{CO}_2$  incubator could develop to the blastocyst stage and they found DMAP more efficient than  $\text{Sr}^{2+}$  for oocyte activation. Moreover, Popova, *et al* [12] incubated rat oocytes in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  -free M16 medium containing 2mM  $\text{Sr}^{2+}$  for 30 min at 37°C in a  $\text{CO}_2$  incubator and achieved a blastocyst rate of around 36%, from these experiments, we could conclude that rat oocytes could tolerate  $\text{Ca}^{2+}$  medium for a short time and could survive and develop to the blastocyst stage after a short incubation of a low concentration of  $\text{Sr}^{2+}$ +CB; however the developmental competence may be comprised when a high concentration of  $\text{SrCl}_2$  combined with a long duration exposes to rat oocytes in a culture medium in the absence of  $\text{Ca}^{2+}$ . Obviously,  $\text{Ca}^{2+}$  in the activation medium seems to play an important role in rat oocyte survival during activation with  $\text{SrCl}_2$ , which we have not yet seen other report in other species. The reason for this remains unknown. Recently, Yoo, *et al* [22] observed that rat oocyte spontaneous activation was significantly decreased in calcium-free condition although oocytes failed to survive. They also concluded that rat oocytes have very sensitive response to extracellular cal-

cium. Therefore,  $\text{Ca}^{2+}$  in the medium may balance the extra- and intracellular calcium concentrations in oocytes so that oocyte damage could be avoided by adding  $\text{Ca}^{2+}$  into culture medium during activation with  $\text{SrCl}_2$ . Clearly, further study needs to be done to understand the role of  $\text{Ca}^{2+}$  in rat oocyte activation by  $\text{SrCl}_2$ .

### **Effect of $\text{SrCl}_2$ concentration on developmental competence of activated rat oocytes.**

Several laboratories have shown that rat oocytes could be successfully activated with different concentrations of  $\text{SrCl}_2$  such as 2mM (Popova, 2008), 2.5mM, 10mM [36]. We optimized  $\text{SrCl}_2$  concentration in our culture conditions and found that 5 and 10mM  $\text{SrCl}_2$ +5 $\mu\text{g}/\text{ml}$  CB could yield a blastocyst rate of 59% and 67%, respectively, which was significantly higher (Table.1) than those *et al*; observed in the other concentrations ( $X^2$ -test,  $P<0.01$ ). Although no difference in the blastocyst rate was observed between 5 and 10mM  $\text{SrCl}_2$  + 5 $\mu\text{g}/\text{ml}$  CB treatments there was a significant difference in the mean cell number/blastocyst, the mean cell number/blastocyst in 10mM  $\text{SrCl}_2$  + 5 $\mu\text{g}/\text{ml}$  CB treatment was  $41\pm 10$ , where only  $32\pm 7$  in 5mM  $\text{SrCl}_2$  + 5 $\mu\text{g}/\text{mL}$  CB treatment (T-test;  $P<0.05$ ), indicating that  $\text{SrCl}_2$  concentration may influence not only activation rate and blastocyst rate but also the quality of blastocysts. Unfortunately in the experiment we could not test any  $\text{SrCl}_2$  concentration higher than 10 mM, our results however showed that in the experiment 10mM  $\text{SrCl}_2$  could effectively activate rat oocytes and resulted in a blastocyst rate of over 50%.

Additionally, we investigated the effect of CB and DMAP on developmental competence on oocytes activated with  $\text{SrCl}_2$  combined with CB or DMAP. Unlike mouse oocyte activation no rat oocyte activated with  $\text{SrCl}_2$  alone would be able to develop into the blastocyst stage although almost oocytes could form pronuclei and cleaved on day 1 after activated with 10 mM  $\text{SrCl}_2$  alone, however, these activated oocytes with perhaps haploid chromosomes stopped growing at 2-cell stage, whereas either 10mM  $\text{SrCl}_2$  combined with CB or DMAP could achieve a blastocyst rate of over 50 %. This result shows that haploid rat oocytes activated with  $\text{SrCl}_2$  may not be able to develop to the blastocyst stage *in vitro*. However, Popova, *et al* (2009)[12] activated rat oocyte in  $\text{SrCl}_2$  for 30 min without depolarization and claimed that a subsequent blastocyst rate of about 35 % oocytes was achieved. It is relevant that in the experiment most activated oocytes should perhaps be haploid rather than diploid, suggesting that haploid rat oocytes were able to develop the blastocyst stage. Obviously, only diploid embryos have an advantage in the developmental competence, which has been proven in several species [37-39] . Therefore, further investigation on this issue needs to be done including karyotyping oocytes after activation with the same protocol to confirm that the activated oocytes would be haploid or diploid. So far in most experiments of rat oocyte activation [19], DMAP, a protein kinase inhibitor, has commonly been used to activate oocytes or to block the second polar body extrusion and achieved a high developmental rate in rats compared with

CB. Also some healthy cloned animals were born after using DMAP. However, DMAP could also cause chromosomal abnormalities which have been well known [40]. These have been thought to be one of factors influencing death of cloned embryos during early gestation [41]. Previous experiments showed that  $\text{SrCl}_2$  combined with DMAP instead of CB would yield more blastocysts in rat oocyte activation [19]. However, in our experiment the data showed that there was no significant difference between DMAP and CB treatments in either developmental competence or the mean cell number/blastocyst, which suggests that CB would be used to replace DMAP to reduce the chromosomal abnormality, also this would benefit rat somatic cell nuclear transfer.

### **Timing of rat oocyte activation**

It has been noticed in several studies that rat oocyte activation would improve with oocyte age. For example, blastocyst rate increased from 16 to 23 hours of hCG injection, whereas the rate was significantly lower when oocytes were activated at 14h after hCG injection [7,19]. However, we did not observe any significant differences in the blastocyst rates using oocytes collected from 14 to 22 hours after hCG injection ( $X^2$ -test,  $P>0.05$ ) although blastocyst rate of oocytes activated at 14 h after hCG injection was slightly lower than other groups, This difference may be due to different activation methods or different culture systems applied. Obviously, regardless of  $X^2$ -statistical analysis the developmental competence of the oocytes collected at 14 h after hCG injections is relatively low compared to others of oocytes collected at 16 h to 22 h after hCG, which suggests that oocyte cytoplasm maturation rather than nuclear maturation *in vivo* at that time may not be fully capable of being activated. Our result on timing of rat oocyte activation clearly shows that oocytes collected from 14 -22 h after hCG injection can be equally effectively activated by  $\text{SrCl}_2$  + CB or DMAP, which indicates that rat oocytes as recipients for somatic cell nuclear transfer could be used theoretically from 14h to 22 h after hCG injection. However, in practice, spontaneous activation of rat oocytes occurs rapidly after oocyte collection, oocyte chromosomes scatter and maturation promoting factor (MPF) which we believe very important for somatic cell reprogramming also decreases very quickly, due to these reasons, the time of reconstituting nuclear transfer embryos is very limited. Therefore we focused next on overcoming this problem in order to delay or to inhibit the spontaneous activation in rat oocytes.

### **Inhibition of rat oocyte spontaneous activation by demecolcine**

In the first success of rat somatic cell nuclear transfer [1] it was believed that the use of MG132, a protease inhibitor was the key step of overcoming the spontaneous activation so that oocytes could be cultured in the drug for 3 h without compromising their developmental competence, which gave enough time to manipulate oocytes. However they found that this drug also reversibly stabilized most oocyte MII metaphases (77%) for up to 3 hours. Furthermore, Ito,

*et al*; (2005) [42] reported that the decreased level of p34 cdc2 kinase activity in aged or enucleated rat oocytes is responsible for their inability to support the premature chromosome condensation (PCC) of microinjected cumulus cell nuclei and that inhibition of p34 cdc2. Kinase inactivation by chemicals such as MG132 is in part effective for rat oocyte to promote PCC and further development. However on the other hand MG 132 dose not inhibit all rat oocyte at MII stage as the mentioned above and also has adverse effects on chromosome in mouse oocytes [43], these reports seemed to show some limitations of use of MG 132 in rat somatic cell nuclear transfer. More recently, Galat, *et al*; [7] reported that rat oocytes were treated with cytochalasin B for 6h since they were collected and that the rate of *in vitro* development of cytochalasin B treated rat oocytes after activation with DMAP was comparable to parthenogenetic controls, while nocodazole and demecolcine, a microfilament inhibitor, produced oocytes that developed at low frequencies, which indicates that CB is an alternative to inhibit rat oocyte spontaneous activation.

### Potential benefits of demecolcine for rat somatic cell nuclear transfer

In our experiment with demecolcine we first tested the effect of demecolcine on developmental potential of activated rat oocytes and found that 0.05µg/ml demecolcine seemed to be best although there were no differences between 0.05, 0.1 and 0.5µg/ml. In the later experiment, we cultured rat oocytes with 0.05µg/ml demecolcine for 1 to 5 h and activated with our standard activation protocol, after 5 days of culture we could not observe any significant differences between the treatments in the blastocyst rate or in the mean cell numbers/blastocyst (Table 6). This observation difference from Galat' s [7] may be due to different activation protocol and culture system being applied. Demecolcine is a microfilament inhibitor and often used to synchronise somatic cells at the M phase or oocytes at the MII stage. In fact, demecolcine not only can synchronise oocytes at the MII stage but also would increase maturation promoting factor (MPF) in cultured, goat and bovine oocytes in recent reports [44,45] which indicates that oocytes cultured in demecolcine containing medium may be better potentially for rat somatic cell nuclear transfer, especially using G1/G0 donor cells.

Therefore, we could conclude that demecolcine as expected could be used in rat oocytes to effectively inhibit oocyte spontaneous activation for at least 5h without compromising the developmental potential after activation with SrCl<sub>2</sub> + CB or DMAP. Obviously, use of demecolcine could widen the "window" of micromanipulation of rat oocytes in rat somatic cell nuclear transfer, rat oocyte ICSI.

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