

Comparison of Bovine Embryonic Development of Parthenogenetically Activated Oocytes by Different Combinations of Chemical Treatments

Siti Khadijah, I., Rahman, M. M., Abdullah*, R. B. and Wan Khadijah, W. E.

Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

*Corresponding author: ramli@um.edu.my

Abstract

Oocyte activation is the efficient method or step for successful animal cloning. This study was carried out to evaluate the embryonic development for both parthenotes and fertilised embryos according to the oocyte grades (Grades A, B and C). Recovered oocytes according to the oocyte grades were divided into various combination treatments: (i) A23187+6-DMAP, (ii) Iono+6-DMAP, (iii) EtOH+6-DMAP, (iv) Iono+CHX, and (v) IVF (control). Treatment with combination of 10 μ M Iono+2 mM 6-DMAP indicated the highest embryonic development rates for 8-cell, morula and blastocyst stages compared to the other combination treatments. There were significant differences between combination of Iono+6-DMAP and IVF control at morula (24.3 vs. 3.4%) and blastocyst stages (7.1 vs. 0.0%), respectively. There was no consistent relationship between oocyte grades and embryo development rates. The data also showed that no differences were observed in blastocyst rates for each oocyte grade for all activation treatments including IVF control. In conclusion, to activate bovine oocytes for later embryonic development stages (8-cell, morula and blastocyst), the best combination treatment obtained in this study was the combination of 10 μ M Iono (5 min)+2 mM 6-DMAP (4 h), regardless of the grades of oocytes, including the importance of the optimal combination of chemical activation to produce bovine embryos *in vitro*.

Key words: bovine oocyte, chemical treatment, parthenogenesis, oocyte grade

Introduction

The mechanism of parthenogenetic activation in oocytes is based on oscillation of intracellular calcium concentration $[(Ca^{2+})_i]$, which leads to meiosis resumption and extrusion of the second polar body. Consequently, pronuclei are formed, DNA synthesis is begun and embryonic cleavage is initiated (Grabeic *et al.*, 2007). For adequate activation of the oocyte, additional stimuli are often required during *in vitro* production processes. Parthenogenetic activation often used as model in order to study the biochemical changes and morphological characteristics that occurred during early embryonic development

(Collas *et al.*, 1993). Parthenogenetic activation has been conducted in order to mimic the action carried out by the sperm cells during fertilisation to activate oocytes which are arrested at metaphase II (MII) of meiosis division (Meo *et al.*, 2007).

Wide ranges of activation agents have been applied to activate mammalian oocytes during the cloning of mice (Szollosi *et al.*, 1993), sheep (Alexander *et al.*, 2006), goats (Shen *et al.*, 2006), pigs (Leal and Liu, 1998) and cattle (Shen *et al.*, 2008). However, activation protocols must be optimised for use in each species (Krivokharchenko *et al.*, 2003) as different species have different specific response to the different kinds of chemicals. There were also various results

that were discovered by several researchers, when combination treatments were applied to induce oocyte activation in bovine. For example, the combination of calcium ionophore (A23187) and 6-dimethylaminopurine (6-DMAP) was reported to be better than the combination of ionomycin and 6-DMAP (Shen *et al.*, 2006), while another researcher reported that combination of ionomycin and 6-DMAP gave higher blastocyst rate compared to those treated with the combination of ethanol (EtOH), cycloheximide (CHX) and cytochalasin B (Wang *et al.*, 2008). Therefore, it is necessary to determine the optimal combination treatments to activate bovine oocytes as well as to compare the embryonic development between parthenotes and IVF embryos. This study was conducted to evaluate the embryonic development for both parthenotes and fertilised embryos according to the oocyte grades (Grades A, B and C).

Materials and Methods

Recovery of Oocytes

All activation and culture media used were based on modified potassium simplex optimisation medium (KSOM) as stated by Lawitts and Biggers (1993). The bovine ovaries were obtained from the local abattoir complex. The collected ovaries from slaughterhouse were transported to the laboratory within 1 to 2 h in warm saline medium which were maintained between 30 to 38°C. The ovary was sliced individually by checker-board incision technique on the whole ovarian surface (Kwong, 2012). According to oocyte grades, the cumulus oocyte complexes (COCs) were searched and washed in TL-Hepes and later cultured in *in vitro* maturation (IVM) medium inside CO₂ incubator (5%) at 38.5°C.

Cumulus Oocyte Complexes (COCs) Denuding and Grading

After the respective IVM duration, the COCs were transferred into hyaluronidase droplet (0.1%) and the COCs were sucked through a narrow-bore pipette to remove their cumulus cells. Only the matured oocytes with first extruding polar body were chosen for the subsequent experiments.

Activation of Oocytes

The retrieved oocytes grouped into Grades A (COC with complete 5 layers of cumulus cells with evenly granulated cytoplasm), B (COC with partially 5 layers or complete 3-4 layers of cumulus cells with evenly granulated cytoplasm) and C (COC with partially 3-4 layers or complete 1-2 layers of cumulus cells with evenly granulated cytoplasm) based on the number layer of cumulus cells (Wurth and Kruij, 1992). Recovered oocytes according to the oocyte grades (Grades A, B and C) were divided into various combination treatments: (i) A23187+6-DMAP, (ii) Iono+6-DMAP, (iii) EtOH+6-DMAP, (iv) Iono+CHX, and (v) IVF (control). For A23187+6-DMAP treatment, oocytes were treated in A23187 for 5 min in CO₂ incubator and rinsed in TL-Hepes three times followed by equilibrated KSOM three times before transferred into 6-DMAP for 4 h. The culture dish was cultured inside CO₂ incubator. The procedures of activation were similar for the other combination treatments (Iono+6-DMAP, EtOH+6-DMAP and Iono+CHX) whereby the oocytes were treated in the first activation chemicals (Iono or EtOH) for 5 min followed by the second activation chemicals (6-DMAP or CHX) for 4 h. The activated oocytes were subsequently cultured *in vitro* in CO₂ incubator.

In Vitro Fertilisation (IVF) of Bovine Oocytes

For control, cumulus cells of COCs were partially removed by gentle repeated pipetting to leave few layers of corona cells surrounding the oocytes in order to make oocytes ready for IVF procedure. Oocytes were transferred into IVF-TALP culture dish and cultured inside CO₂ incubator to allow oocytes ready for insemination later. Meanwhile, straws (straw size: 0.5 mL; approximate 1.0×10^6 total spermatozoa/mL) of frozen semen were pre-thawed at room temperature (25°C; 1 min) followed by thawing in a water bath (37°C; 3 min). The semen was released into the centrifuge tube and centrifuged for 5 minutes. The supernatant of the semen was sucked out to leave the sediment and a small amount of the supernatant. The sediment was mixed well with the supernatant residue and sucked out and dispensed at the bottom of the tube containing equilibrated Sp-TALP and centrifuged again. The supernatant was discarded and the bottom most part of supernatant was sucked out and dispensed at the bottom part of a microcentrifuge tube containing 1 mL of equilibrated Sp-TALP. The tube was cultured inside CO₂ incubator for 45 min to 1 h to allow the sperm swim-up (Habsa, 1998).

After 1 h of sperm capacitation (Siti Khadijah, 2013) and when oocytes were ready for insemination process, 10 µL of sperm suspension (incubated earlier) from Sp-TALP microcentrifuge tube was taken out and dispensed on the glass slide and observed under inverted microscope to examine the sperm motility and availability. When the sperm confirmed present (alive; approximate concentration 1.0×10^6 sperm/ml), 20 µL of sperm suspension was dispensed into each IVF-TALP microdroplet (20 µL/5-10 oocytes) and allowed to inseminate in CO₂ incubator for 18 to 24 h.

In Vitro Culture (IVC) of Activated and Fertilised Embryos

For respective treatment, all the activated and fertilised embryos (shown in Tables 1 and 2) were washed three times in TL-Hepes followed by equilibrated KSOM after the few hours. Activated oocytes and fertilised embryos were then transferred into KSOM microdroplet (10-15 oocytes/microdroplet) and subsequently cultured in CO₂ incubator (5%; 38.5°C) for later embryonic developmental rates such as maturation rate and cleavage rate (2-cell, 4-cell, 8-cell, morula and blastocyst).

Statistical Analysis

All data obtained in this study were expressed as mean± standard error. The results were analysed using one-way analysis of variance (ANOVA) using SPSS (ver. 17.0, SPSS Inc., Chicago, IL, USA). Means comparison was accomplished using Duncan's Multiple Range at P value of <0.05 considered statistically significant.

Results and Discussion

When bovine oocytes were treated with different combinations of chemicals (Table 1), treatment with combination of 10 µM Iono+2 mM 6-DMAP showed the highest embryonic cleavage rates for 8-cell, morula and blastocyst stages. Treatment with Iono+CHX showed significantly lower (P<0.05) embryonic cleavage rate than other combination treatments at 8-cell stage. There were significant differences (P<0.05) between combination of Iono+6-DMAP and IVF control at morula (24.3 vs. 3.4%) and blastocyst stages (7.1 vs. 0.0%), respectively.

When the development of bovine parthenotes were compared according to the oocyte grades for every activation protocols (Table 2), generally there was no consistent

relationship between oocyte grades and embryo development rates. In addition, no differences ($P>0.05$) were observed in blastocyst rates for each oocyte grade for every activation protocols and IVF control.

When bovine oocytes were treated in the combination of 5 μM Iono (5 min)+1.9 mM 6-DMAP (3 h), the cleavage rate (20 vs. 18%) was higher than those treated with the combination of 5 μM Iono (5 min)+10 $\mu\text{g/mL}$ CHX (5 h), whereas only few cleaved oocytes developed to blastocyst (2 vs. 1%), respectively (Abdalla *et al.*, 2009). In the present study, the cleavage rates for 2-cell (73.6 vs. 63.5%), 4-cell (61.0 vs. 46.3%), 8-cell (42.4 vs. 19.7%) and blastocyst (7.1 vs. 2.2%) obtained when bovine oocytes were treated with the combination of 10 μM Iono (5 min)+2 mM 6-DMAP (4 h) and 10 μM Iono (5 min)+10 $\mu\text{g/mL}$ CHX (4 h), respectively were comparatively higher than those reported by Abdalla *et al.* (2009), although they only observed on cleaved and development on blastocyst, and no specific study was conducted on 2-, 4-, 8-cells and morula stages. Results in the present study showed that the combination treatment of Iono+6-DMAP gave higher cleavage and blastocyst rates compared to those treated with the combination of Iono+CHX, which supported the present results as obtained by Abdalla *et al.* (2009).

Bhak *et al.* (2006) showed that the cleavage (52 vs. 38%) and blastocyst (12 vs. 5%) rates of bovine oocytes activated by combination of 5 μM Iono (5 min)+1.9 mM 6-DMAP (3 h) was significantly higher than those treated by the combination of 5 μM Iono (5 min)+10 $\mu\text{g/mL}$ CHX (3 h). This finding is in agreement with the current research where bovine oocytes treated with combination of Iono+6-DMAP, which showed the better embryonic development. In a separate study, when bovine oocytes were treated with the combination of 5 μM Iono (5 min)+2 mM 6-DMAP (3 h), the

pronuclear formation (67.1%), cleavage (69.2%) and blastocyst (28.0%) rates were significantly ($P<0.05$) higher compared to other treatments of a previous study (Wang *et al.*, 2008). In the current study, the cleavage rate is lower (73.6% for 2-cell, 61.0% for 4-cell and 42.4% for 8-cell) when bovine oocytes treated with Iono+6-DMAP than those reported (67.1% for 2 to 8-cells) by Wang *et al.* (2008), including lower in blastocyst rate (7.1 vs. 28.0%) and these results indicate the necessity of protocol improvement with efficiency.

Previous study also reported that the treatment of bovine oocytes post-ICSI with combination of Iono+6-DMAP effectively improved blastocyst yield (Ock *et al.*, 2003; Oikawa *et al.*, 2005). Also, Galli *et al.* (2003) suggested that using CHX instead of 6-DMAP improved the cleavage rate but not improved the blastocyst yield. Moreover, both activation treatments (Iono+CHX or Iono+6-DMAP) resulted in obviously low blastocyst rate (2-7%). Therefore, the significantly lower blastocyst yields might be due the adverse effects of the inhibitors (CHX and 6-DMAP) on the activity of other kinases or protein synthesis involved in cell functions (Soloy *et al.*, 1997). Perecin *et al.* (2007) suggested that the side effects of CHX or 6-DMAP can be avoided by using more specific kinase inhibitors for cell cycle regulation.

Oikawa *et al.* (2005) and Shen *et al.* (2008) reported that ionomycin in combination with 6-DMAP could optimise the activation of the reconstructed oocytes compared with other activation treatments. In addition, when 6-DMAP was replaced with CHX and CB, the blastocyst rates of the reconstructed oocytes were significantly lowered. Using combination of ionomycin and 6-DMAP has been shown particularly effective in inducing parthenogenesis in bovine oocytes (Wells *et al.*, 1999). This might be because ionomycin was a stronger

stimuli in inducing $[(Ca^{2+})_i]$ than other intracellular calcium elevator. Low concentrations of ionophore and ionomycin do not allow adequate rise in the $[(Ca^{2+})_i]$ to permit the cells to enter anaphase II (Uranga *et al.*, 1996). The oocytes actually start meiosis again, emit the second polar body and enter a new metaphase (metaphase III) where they are finally halted (Vincent *et al.*, 1992).

Protein phosphorylation is inhibited by 6-DMAP after oocyte activation by ionomycin, however, 6-DMAP itself does not induce the inactivation of histone H1 kinase in metaphase II-arrested oocytes (Oikawa *et al.*, 2005). In addition, post-fertilisation events such as the formation of the interphase network of microtubules, the remodeling of sperm chromatin and pronucleus formation were controlled by 6-DMAP-sensitive kinases (Szollosi *et al.*, 1993). It was reported that 6-DMAP could enhance the speed of pronuclear formation compared with CHX in bovine and ovine oocytes (De La Fuente *et al.*, 1998; Alexander *et al.*, 2006). On the other hand, CHX, a protein synthesis inhibitor, restricted the synthesis or re-accumulation of cyclin B and subsequently blocked the re-synthesis of maturation promoting factor (MPF) activity, as well as prohibited the synthesis of proteins such as cytosolic factor (CSF) and MPF, resulting in low CSF concentration and decreased MPF activity (or inactivation) which later led to the resumption of second meiotic division and the activation of oocytes (Presicce & Yang, 1994).

Winger *et al.* (1997) reported that ethanol, ionomycin or calcium ionophore with or without combination of CHX were not enough to stimulate parthenogenetic development in bovine oocytes. Thus, induction of diploidisation by combining the calcium oscillation-inducing activators with

cytochalasin or 6-DMAP is necessary to improve the parthenogenetic development of bovine oocytes (Meo *et al.*, 2004). In the present study, the rate of blastocyst development in oocytes activated with Iono+DMAP was highest (7.1%). These findings are in agreement with previous studies, in that 6-DMAP treatment of artificially activated oocytes increased the developmental potential of the parthenotes in bovine, murine and porcine (Leal & Liu, 1998). Similarly, a previous study has shown that combining Iono+6-DMAP was particularly effective in inducing bovine oocyte activation and has been used in nuclear transfer studies (Bhak *et al.*, 2006).

Wang *et al.* (2008) indicated that bovine oocyte activation is more achievable using combination of Iono+6-DMAP or EtOH+CHX than other combinations or single treatment. Presicce & Yang (1994) also reported that oocyte activation was high for both young and aging bovine oocytes using combination of EtOH+CHX treatment. The activation with ionomycin alone and in combination of CHX caused an increase in haploid parthenotes, whereas the 6-DMAP treatment resulted in diploid parthenotes is the reason why oocytes treated in 6-DMAP is more competent than CHX-treated oocytes.

In the current study, it seemed that grades of oocytes did not give significant effect on cleavage of IVF embryos. This phenomenon would suggest that developmental potential of the individual oocyte might not possibly be predicted based on the visual appearance of the oocytes (De Wit & Kruip, 2001). Other aspects had been reported to affect competency of oocytes such as diameter of the oocytes as well as morphology and size of both follicle and ovary (Hendriksen *et al.*, 2000).

Table 1: Comparison of *in vitro* embryonic development of bovine parthenotes after activation by various combination treatments

Treatment	No. of oocytes retrieved	Maturation rate (%)	Embryo cleavage rate (%)				
			2-cell (Mean \pm SE)	4-cell (Mean \pm SE)	8-cell (Mean \pm SE)	Morula (Mean \pm SE)	Blastocyst (Mean \pm SE)
A23187+DMAP	209	52.2 (n=109)	74.9 \pm 5.6 ^a (n=83)	57.0 \pm 3.7 ^a (n=62)	41.8 \pm 4.3 ^b (n=44)	17.9 \pm 3.6 ^{bc} (n=20)	4.2 \pm 2.5 ^a (n=5)
Iono+DMAP	223	65.9 (n=147)	73.6 \pm 4.7 ^a (n=109)	61.0 \pm 4.3 ^a (n=89)	42.4 \pm 4.9 ^b (n=63)	24.3 \pm 4.0 ^c (n=35)	7.1 \pm 1.4 ^a (n=13)
EtOH+DMAP	169	53.9 (n=91)	74.2 \pm 2.8 ^a (n=64)	56.0 \pm 3.5 ^a (n=51)	37.5 \pm 3.7 ^b (n=36)	18.6 \pm 4.5 ^{bc} (n=18)	0.0
Iono+CHX	188	72.3 (n=136)	63.5 \pm 9.1 ^a (n=87)	46.3 \pm 7.1 ^a (n=63)	19.7 \pm 4.4 ^a (n=27)	8.8 \pm 2.9 ^{ab} (n=12)	2.2 \pm 1.6 ^a (n=3)
IVF (control)	129	61.2 (n=79)	73.0 \pm 7.2 ^a (n=53)	62.9 \pm 9.3 ^a (n=45)	37.6 \pm 8.7 ^b (n=29)	3.4 \pm 2.0 ^a (n=4)	0.0

n, number of oocytes/embryos.

Values sharing same letters in a column are not significantly different ($P>0.05$).

Table 2: Comparison of *in vitro* embryonic development of bovine parthenotes according to oocyte grades from every activation protocols

Treatment	Oocyte grade	Total oocytes retrieved	Maturation rate (%) (Mean±SE)	Embryos cleavage rate (%)				
				2-cell (Mean ±SE)	4-cell (Mean ±SE)	8-cell (Mean ±SE)	Morula (Mean ±SE)	Blastocyst (Mean ±SE)
A23187 +DMAP (4 hr)	A	88	60.7±4.1 ^{abc} (n=53)	80.1±8.0 ^{ab} (n=45)	64.0±5.8 ^a (n=36)	49.5±8.9 ^{bc} (n=27)	20.8±4.3 ^{abcde} (n=12)	8.0±5.0 ^a (n=4)
	B	31	50.3±6.5 ^a (n=16)	86.1±9.0 ^{ab} (n=14)	58.3±9.4 ^a (n=8)	52.8±10.0 ^c (n=7)	25.0±9.4 ^{bcde} (n=4)	0.0
	C	34	61.0±7.0 ^{abc} (n=20)	84.5±7.8 ^{ab} (n=16)	57.1±7.1 ^a (n=11)	29.8±8.5 ^{abc} (n=6)	16.7±8.9 ^{abcde} (n=3)	2.4 ^a (n=1)
Iono +DMAP (4 hr)	A	62	74.7±8.2 ^{bcd} (n=49)	85.9±8.5 ^{ab} (n=40)	69.2±9.9 ^a (n=31)	50.2±6.6 ^{bc} (n=22)	28.7±1.6 ^{cde} (n=14)	8.9±2.4 ^a (n=5)
	B	69	64.7±3.0 ^{abcd} (n=45)	72.8±4.9 ^{ab} (n=35)	63.4±4.2 ^a (n=29)	39.4±7.0 ^{abc} (n=20)	25.8±8.1 ^{bcde} (n=13)	8.3±3.6 ^a (n=6)
	C	57	72.8±9.0 ^{abcd} (n=39)	75.6±7.7 ^{ab} (n=26)	66.7±10.3 ^a (n=22)	50.7±16.2 ^{bc} (n=16)	34.7±16.4 ^c (n=8)	3.6±2.3 ^a (n=2)
EtOH +DMAP (4 hr)	A	78	62.4±7.0 ^{abc} (n=52)	77.2±7.0 ^{ab} (n=40)	65.3±7.9 ^a (n=32)	47.8±5.9 ^{bc} (n=24)	33.1±7.5 ^{de} (n=15)	0.0
	B	24	61.1±8.4 ^{abc} (n=13)	81.95±8.7 ^{ab} (n=10)	63.9±11.7 ^a (n=7)	51.4±16.7 ^{bc} (n=5)	-	0.0
	C	22	56.3±4.0 ^{abc} (n=13)	92.85±7.2 ^b (n=11)	73.2±15.5 ^a (n=7)	13.4±7.8 ^a (n=3)	9.8±6.1 ^{abcd} (n=2)	0.0
Iono +CHX (4 hr)	A	55	85.2±6.5 ^d (n=44)	68.4±13.2 ^{ab} (n=27)	49.5±10.2 ^a (n=20)	19.1±4.1 ^{ab} (n=9)	8.0±3.5 ^{abc} (n=4)	1.4 ^a (n=1)
	B	43	77.3±1.8 ^{cd} (n=33)	60.4±11.7 ^a (n=21)	41.5±9.0 ^a (n=14)	20.9±6.9 ^{abc} (n=7)	12.2±5.6 ^{abcde} (n=4)	2.9 ^a (n=1)
	C	58	78.9±2.8 ^{cd} (n=46)	69.4±7.9 ^{ab} (n=34)	51.9±5.0 ^a (n=25)	20.8±5.3 ^{abc} (n=10)	8.0±3.8 ^{abc} (n=4)	2.0 ^a (n=1)
IVF control	A	44	54.6±8.2 ^{ab} (n=27)	79.1±13.0 ^{ab} (n=17)	74.6±15.3 ^a (n=15)	42.7±4.6 ^{abc} (n=10)	4.4±2.6 ^{abc} (n=2)	0.0
	B	41	58.6±9.3 ^{abc} (n=25)	75.0±10.2 ^{ab} (n=17)	62.5±16.1 ^a (n=16)	39.4±17.6 ^{abc} (n=10)	3.1 ^{ab} (n=1)	0.0
	C	41	64.3±6.9 ^{abcd} (n=27)	71.9±3.7 ^{ab} (n=19)	55.6±7.9 ^a (n=14)	33.8±6.9 ^{abc} (n=9)	3.1 ^{ab} (n=1)	0.0

n, no. of oocytes/embryos.

Values sharing same letters in a column are not significantly different (P>0.05).

Conclusion

A protocol that produced bovine embryos through parthenogenetic approach in Malaysia was developed in this study, in which generally lower blastocyst rate was obtained than those which had been previously reported (Shen *et al.*, 2008; Wang *et al.*, 2008). The optimal combination treatment to activate bovine oocytes for 8-cell, morula and blastocyst stages was by using 10 μ M Iono (5 min)+2 mM 6-DMAP (4 h). All oocyte grades (Grades A, B and C) were competent throughout the embryonic development. These findings are useful to be considered in future experiments involving nuclear transfer protocols and other advanced reproductive technologies in mammalian species. However, further experiment is necessary to improve the present protocol for optimum embryonic development rate.

Acknowledgement

This study was supported by IPPP research grant (PS293/2010A) funded by University of Malaya.

References

- Abdalla, H., Shimoda, M., Hirabayashi, M. and Hochi, S. 2009. A combined treatment of ionomycin with ethanol improves blastocyst development of bovine oocytes harvested from stored ovaries and microinjected with spermatozoa. *Theriogenology* 72: 453-460.
- Alexander, B., Coppola, G., Di Berardino, D., Rho, G.J., St John, E., Betts, D.H. and King, W.A. 2006. The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. *Mol. Reprod. Dev.* 73: 20-30.
- Bhak, J.S., Lee, S.L., Ock, S.A., Kumar, B.M., Choe, S.Y. and Rho, G.J. 2006. Developmental rate and ploidy of embryos produced by nuclear transfer with different activation treatments in cattle. *Anim. Reprod. Sci.* 92: 37-49.
- Collas, P., Sullivan, E.J. and Barnes, F.L. 1993. Histone H1 kinase activity in bovine oocytes following calcium stimulation. *Mol. Reprod. Dev.* 34: 224-231.
- De La Fuente, R. and King, W.A. 1998. Developmental consequences of karyokinesis without cytokinesis during the first mitotic cell cycle of bovine parthenotes. *Biol. Reprod.* 58: 952-962.
- De Wit, A.A.C. and Kruip, T.A.M. 2001. Bovine cumulus-oocyte-complex-quality is reflected in sensitivity of α -amanitin, oocyt-diameter and developmental capacity. *Anim. Reprod. Sci.* 65: 51-65.
- Galli, C., Vassiliev, I., Lagutina, I., Galli, A. and Lazzari, G. 2003. Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol. *Theriogenology* 60: 1467-1480.

- Grabiec, A., Max, A. and Tischner, M. 2007. Parthenogenetic activation of domestic cat oocytes using ethanol, calcium ionophore, cyclohexamide and magnetic field. *Theriogenology* 67: 795-800.
- Habsah, B.B. 1998. IVF performance of different qualities of oocyte using co-culture and chemically-defined medium in Malaysian cattle. A PhD thesis, University of Malaya, Kuala Lumpur, Malaysia.
- Hendriksen, P.J.M., Vos, P.L.A.M., Steenweg, W.N.M., Bevers, M.M. and Dieleman, S.J. 2000. Bovine follicular development and its effect on the *in vitro* competence of oocytes. *Theriogenology* 53: 11-20.
- Krivokharchenko, A., Popova, E., Zaitseva, I., Vil'ianovich, L., Ganten, D. and Bader, M. 2003. Development of parthenogenetic rat embryos. *Biol. Reprod.* 68: 829-836.
- Kwong, P.J. 2012. Development of intra- and interspecies somatic cell nuclear transfer protocols using ear fibroblast cells as donor karyoplasts for production of cloned caprine embryos. A PhD thesis, University of Malaya, Kuala Lumpur, Malaysia.
- Lawitts, J.A. and Biggers, J.D. 1993. Culture of preimplantation embryos. *Meth. Enzymol.* 255: 153-164.
- Leal, C.L. and Liu, L. 1998. Differential effects of kinase inhibitor and electrical stimulus on activation and histone H1 kinase activity in pig oocytes. *Anim. Reprod. Sci.* 30: 51-61.
- Meo, S.C., Leal, C.L.V. and Garcia, J.M. 2004. Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. *Anim. Reprod. Sci.* 81: 35-46.
- Meo, S.C., Yamazaki, W., Ferreira, C.R., Perecin, F., Saraiva, N.Z., Leal, C.L.V. and Garcia, J.M. 2007. Parthenogenetic activation of bovine oocytes using single and combined strontium, ionomycin and 6-dimethylaminopurine treatments. *Zygote* 15: 296-306.
- Ock, S.A., Bhak, J.S., Balasubramanian, S., Lee, H.J., Choe, S.Y. and Rho, G.J. 2003. Different activation treatments for successful development of bovine oocytes following intracytoplasmic sperm injection. *Zygote* 11: 69-76.
- Oikawa, T., Takada, N., Kikuchi, T., Numabe, T., Takenaka, M. and Horiuchi, T. 2005. Evaluation of activation treatments for blastocyst production and birth of viable calves following bovine intracytoplasmic sperm injection. *Anim. Reprod. Sci.* 86: 187-94.
- Perecin, F., Meo, S.C., Leal, C.L.V. and Garcia, J.M. 2007. Oocyte activation and preimplantation development of bovine embryos obtained by specific inhibition of cyclin-dependent kinases. *Brazilian J. Vet. Anim. Sci.* 59: 280-287.
- Presicce, G.A. and Yang, X. 1994. Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. *Mol. Reprod. Dev.* 37: 61-68.
- Shen, P.C., Lee, S.N., Liu, B.T., Chu, F.H., Wang, C.H., Wu, J.S., Lin, H.H. and Cheng, W.T.K. 2008. The effect of activation treatments on the development of reconstructed bovine oocytes. *Anim. Reprod. Sci.* 106: 1-12.

- Shen, P.C., Lee, S.N., Wu, J.S., Huang, J.C., Chu, F.H., Chang, C.C., Kung, J.C., Lin, H.H., Chen, L.R., Shiau, J.W., Yen, N.T. and Cheng, W.T.K. 2006. The effect of electrical field strength on activation and development of cloned caprine embryos. *Anim. Reprod. Sci.* 92: 310-320.
- Siti Khadijah, I. 2013. Production of parthenogenetic embryos by using chemical activation of oocytes in murine, bovine and caprine species. An MSc thesis, University of Malaya, Kuala Lumpur, Malaysia.
- Soloy, E., Kanka, J., Viuff, D., Smith, S.D., Callesen, H. and Greve, T. 1997. Time course of pronuclear deoxyribonucleic acid synthesis in parthenogenetically activated bovine oocytes. *Biol. Reprod.* 57: 27-35.
- Szollosi, M., Kubiak, J.Z., Debey, P., De Pennart, H., Szollosi, D. and Maro, M. 1993. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. *J. Cell Sci.* 104: 861-872.
- Uranga, J.A., Pedersen, R.A. and Arechaga, J. 1996. Parthenogenetic activation of mouse oocytes using calcium ionophores and protein kinase C stimulators. *Int. J. Dev. Biol.* 40: 515-519.
- Vincent, C., Cheek, T.R. and Johnson, M.H. 1992. Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. *J. Cell Sci.* 103: 389-396.
- Wang, Z.G., Wang, W., Yu, S.D. and Xua, Z.R. 2008. Effects of different activation protocols on preimplantation development, apoptosis and ploidy of bovine parthenogenetic embryos. *Anim. Reprod. Sci.* 105: 292-301.
- Wells, D.N., Misica, P.M. and Tervit, H.R. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* 60: 996-1005.
- Winger, Q.A., De La Fuente, R., King, W.A., Armstrong, D.T. and Watson, A.J. 1997. Bovine parthenogenesis is characterized by abnormal chromosomal complements: implications for maternal and paternal co-dependence during early bovine development. *Dev. Genet.* 21: 160-166.
- Wurth, Y.A. and Kruip, T.A.M. 1992. Bovine embryo production in vitro after selection of the follicles and oocytes. In: *Proceedings of the 12th International Congress of Animal Reproduction, 1992; The Hague, The Netherlands. The Hague: ICAR. v.1, pp. 387-389.*