Improvement of Developmental Ability to the Blastocyst Stage by Addition of Hyaluronic Acid to Chemically Defined Medium in Diploid Porcine Eggs Matured In-Vitro and Subsequently Electro-Activated

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Abstract: *This study was designed to examine whether hyaluronic acid improved development of the electro-activated porcine diploid eggs. In-vitro matured oocytes were subjected to a single square pulse of direct current for 100 μsec at 1,500 V/cm for activation. The activated eggs were treated with 5 μg/ml cytochalasin B for 4 h. Eggs with only the first polar body that were judged as diploids were further cultured either in the modified KRB solution (mKRB) or Whitten's medium, each with or without addition of 0.5 mg/ml hyaluronic acid. The frequency of eggs developed to blastocysts was significantly higher in Whitten's medium (12% at 144 h and 14% at 168 h after electro-activation) than in mKRB (6% both at 144 h and 168 h) (P<0.05). The frequency of development to blastocysts increased to 13% in mKRB and to 28% in Whitten's medium by addition of hyaluronic acid when measured at 168 h. At this time, frequencies of abnormal eggs were lower in both media in the presence of hyaluronic acid. Expanded blastocysts were observed at 168 h but only when the medium included hyaluronic acid. There were no significant differences in the mean number of cells per blastocyst among the four media, and the number of cells per blastocyst ranged from 21 to 61 at 168 h.*

Key words: *Porcine follicular oocytes, Parthenogenesis, Hyaluronic acid.*

Parthenogenetic activation of porcine *in-vitro* matured oocytes can be induced by electrostimulation [1–4]. The activation of oocytes is an important technique for various studies following nuclear transplantation and for cloning of embryos [5–7]. The developmental capacity of activated eggs themselves, however, is still unclear in pigs [4, 8].

This study was designed to determine the ability, to develop to blastocysts, of the electro-activated diploid porcine eggs that had been matured *in-vitro*. Diploid eggs were chosen because the developmental capacity of activated diploid eggs was known to be superior to that of haploid eggs in mice [9–11] and in cattle [12]. We previously reported that electro-activated porcine eggs ceased its development before compaction when the eggs were cultured in complex TCM-199 medium [4], but that *in-vivo* fertilized porcine eggs successfully developed to the blastocyst stage in Whitten's medium containing hyaluronic acid [13]. In the present study, activated diploid porcine eggs were cultured in simple media, modified KRB solution and Whitten's medium, and whether the addition of hyaluronic acid to these media improved the development of activated eggs to the blastocyst stage or not was examined.

Materials and Methods

Collection and maturation in-vitro of follicular oocytes: Ovaries were collected from gilts at local slaughterhouses and brought to the laboratory at 20°C within 1 h. The method for collection of follicular oocytes was based on Kano *et al.* [14]. Briefly, oocyte-cumulus-granulosa cell complexes (OCGCs) were picked with two pairs of forceps from the inner surface of follicles of 4–6 mm in diameter without detachment of granulosa cells and/or

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an oocyte from the follicle wall. Fewer than 50 OCGCs were transferred to a single plastic dish (35×10 mm; Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) which contained 2 ml of a maturation medium, TCM-199 (Earl's salt; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% (v/v) heat-treated fetal calf serum (FCS; Biocell Inc., Carson, CA, U.S.A.), and containing 0.1 mg/ml sodium pyruvate (Nacalai Tesque Inc., Kyoto, Japan), 0.08 mg/ml kanamycin sulphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.1 IU/ml human menopausal gonadotropin (Pergonal; Serono, Rome, Italy). OCGCs were cultured with two thecal shells from healthy follicles of 4–6 mm in diameter that had been freed of follicular fluid and cells of membrana granulosa in a CO_2 incubator with gentle agitation under an atmosphere of 5% CO_2 in air at 38.5°C with high humidity for 48 h.

Electro-activation: After maturation culture, as described above, expanded cumulus cells were dispersed by the treatment with 0.01% (w/v) hyaluronidase (Sigma) and removed by pipetting. Denuded oocytes were washed three times with a modified TCM-199 (mTCM), TCM-199 supplemented with 10% (v/v) FCS, and containing 0.1 mg/ml sodium pyruvate and 0.08 mg/ml kanamycin sulphate. Oocytes with the first polar body were selected for experiments under an inverted microscope. The oocytes were washed three times with 0.3 M solution of mannitol containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ [15], then 10 to 20 oocytes were transferred to the same solution between parallel electrodes, separated by 2 mm, in a chamber (FTC-03; Shimadzu Co., Ltd., Kyoto, Japan). Oocytes were subjected to a single square pulse of direct current for 100 μsec at 1,500 V/cm from an electric cell-fuser (EFC-2001; Riko Chemical Co., Kyoto, Japan).

Treatment of activated eggs with cytochalasin B: Electro-activated eggs were transferred to mTCM within 1 min and washed three times with mTCM and then cultured in mTCM containing 5 μg/ml cytochalasin B (CB; Sigma) for 4 h to generate diploid eggs. Some activated eggs were cultured in mTCM without the CB-treatment. After treatment with CB, the eggs were washed five times with mTCM and further cultured in mTCM.

Six h after electro-activation, some eggs that had been treated with CB and all eggs treated without CB were whole-mounted on glass slides and fixed with Carnoy's fixative for 48 h and stained with 1% aceto-orcein. The eggs were then examined under a Nomarski differential interference microscope, and the number of polar bodies and nuclei was determined.

Culture of activated eggs and assessment of their development: Eggs that had been treated with CB were examined for the number of polar bodies under an inverted microscope 4 h after electro-activation. Eggs with single polar bodies were judged as diploids, and were used for experiments to determine the developmental capacity. The diploid eggs were cultured in a modified KRB solution (mKRB) [16] or Whitten's medium [17], each supplemented with 0.4% (w/v) bovine serum albumin (BSA; Intergen, New York, U.S.A.) and with or without 0.5 mg/ml hyaluronic acid sodium salt (from human umbilical cord; Nacalai) added. Culture was performed in droplets (1 egg/μl of each culture medium) under paraffin oil (Nacalai) in a plastic dish (35×10 mm; Becton Dickinson Labware) under an atmosphere of 5% CO_2 in air at 38.5°C with high humidity. The stage of embryonic development was evaluated under an inverted microscope at 24-h intervals for 168 h. Eggs that were fragmented or had deteriorating cytoplasm were recorded as degenerated eggs. Blastocysts were stained with 2 μg/ml Hoechst 33342 (Polysciences Inc., Warrington, PA, U.S.A.), and their cells were counted under an epifluorescence microscope [4]. Any cell in the process of mitosis was counted as two cells.

Statistical analysis: The results were analyzed by one-way ANOVA. A probability of less than 0.05 was considered significant.

Results

Table 1 shows the effect of CB on the types of activation. The frequencies of activation were 96% and 95% in the eggs without and with CB-treatment, respectively. In the activated eggs without CB-treatment, 95% had two polar bodies and a single nucleus. In the eggs treated with CB, 91% had a single polar body and two nuclei. The CB-treatment employed was not deleterious, as no eggs had more than two nuclei or had scattered abnormal chromosomes.

Table 2 shows the results of culture of activated eggs. Eggs that had gone through compaction are classified as being at the morula stage (Fig. 1-A), irrespective the number of cells. Since it appeared in this experiment that normal activated eggs had the potential to cleave approximately every 24 h before the morula stage, eggs delayed by two or more developmental stages are taken as delayed eggs. Also, all eggs that were at stages before compaction 144 h after activation and all eggs that had not developed to blastocysts at 168 h are

Table 1. Effects of cytochalasin B (CB) on the blocking of extrusion of the second polar body in electro-activated porcine eggs

Treatment with 5 μg/ml CB	Number of eggs		Number (%)* of eggs with		
	Examined	Activated (%)	2PB1N	1PB2N	1PB1N
−	104	100 (96)	95 (95)	3 (3)	2 (2)
+	73	69 (95)	3 (4)	63 (91)	3 (4)

All eggs were fixed 6 h after electro-activation. *: Proportion to the number of activated eggs. 2PB1N, Two polar bodies and a nucleus; 1PB2N, one polar body and two nuclei; 1PB1N, one polar body and one nucleus.

Table 2. Effects of hyaluronic acid on development of electro-activated porcine eggs in simple media

Medium used*	No. of eggs used	Period (h) of culture	Number of eggs at each stage (%)						% of abnormal eggs Dg+D1=T**
			1-cell	2-cell	3- to 4-cell	5- to 16-cell	Morula	Blastocyst	
mKRB	131	24	22 (17)^{ac}	98 (75)^{a}	1 (1)				8+ 0= 8
WM	78		39 (50)^{b}	22 (28)^{b}	6 (8)				14+ 0=14
mKRBHA	152		27 (18)^{a}	106 (70)^{a}	7 (5)				8+ 0= 8
WMHA	98		27 (28)^{c}	50 (51)^{c}	8 (8)				13+ 0=13
mKRB		48		15 (11)^{a}	90 (69)^{a}	0 (0)^{a}			15+ 5=20^{a}
WM				9 (12)^{a}	26 (33)^{b}	5 (6)^{b}			28+21=49^{b}
mKRBHA				27 (18)^{ab}	96 (63)^{ac}	2 (1)^{a}			14+ 4=18^{a}
WMHA				22 (22)^{b}	50 (51)^{c}	4 (4)^{ab}			17+ 5=22^{a}
mKRB		72			74 (56)^{ab}	12 (9)	11 (8)		18+ 8=26^{a}
WM					28 (36)^{b}	8 (10)	0 (0)		32+22=54^{b}
mKRBHA					96 (63)^{a}	11 (7)	1 (1)		17+12=29^{a}
WMHA					37 (38)^{ab}	17 (17)	5 (5)		20+19=40^{c}
mKRB		96				18 (14)	43 (33)		33+21=53^{a}
WM						18 (23)	15 (19)		38+19=58^{a}
mKRBHA						39 (26)	45 (30)		26+19=45^{ab}
WMHA						33 (34)	24 (24)		27+15=42^{b}
mKRB		120					26 (20)^{a}	3 (2)	40+37=78
WM							16 (21)^{ab}	0 (0)	40+40=79
mKRBHA							34 (22)^{ab}	5 (3)	31+43=74
WMHA							28 (29)^{b}	1 (1)	27+44=70
mKRB		144					16 (12)	8 (6)^{a}	48+34=82^{ab}
WM							4 (5)	9 (12)^{b}	41+42=83^{a}
mKRBHA							15 (10)	16 (11)^{ab}	37+43=80^{ab}
WMHA							11 (11)	16 (16)^{b}	32+41=72^{b}
mKRB		168						8 (6)^{a}	62+32=94^{a}
WM								11 (14)^{b}	44+42=86^{b}
mKRBHA								20 (13)^{b}	47+39=87^{b}
WMHA								27 (28)^{c}	36+37=72^{c}

*: mKRB, Modified KRB solution supplemented with 0.4% (w/v) BSA; WM, Whitten's medium supplemented with 0.4% (w/v) BSA; mKRBHA, mKRB containing 0.5 mg/ml hyaluronic acid; WMHA, WM containing 0.5 mg/ml hyaluronic acid. **: Total % of abnormal eggs = % of degenerated eggs + % of delayed eggs. $^{a-c}$: Numbers with different superscripts in the same column at the same period are significantly different ($P<0.05$).

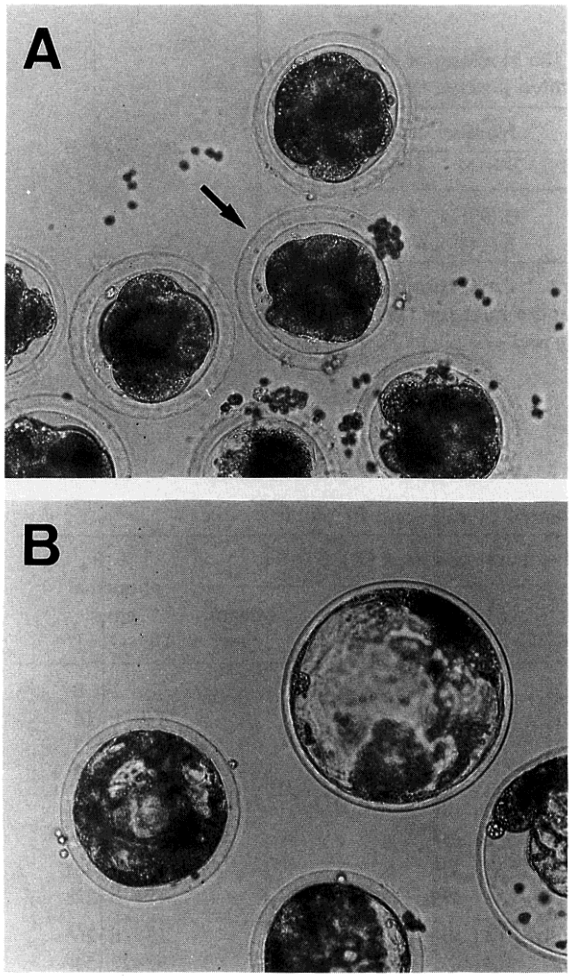

Fig. 1. Photomicrographs of electro-activated porcine diploid eggs cultured in Whitten's medium containing 0.5 mg/ml hyaluronic acid. × 140. A. A morula (arrow) 96 h after electro-activation. B. An expanded blastocyst and a blastocyst 168 h after electro-activation.

counted as delayed eggs.

At 24 h, the first cleavage had already finished in 75% of the activated eggs in mKRB, while only 28% of the eggs were at the 2-cell stage in Whitten's medium. The frequency of eggs at the 2-cell stage was significantly higher in mKRB than in Whitten's medium ($P<0.01$). At 48 h, the frequency of eggs at the 3- to 4-cell stage was also significantly higher in mKRB (69%) than in Whitten's medium (34%, $P<0.01$). In eggs cultured in mKRB, morulae appeared at 72 h (8%), and 33% of the eggs had developed to morulae at 96 h. In Whitten's medium, no morulae were observed at 72 h, and 20% of the eggs had developed to morulae at 96 h. These frequencies of eggs at the morula stage between mKRB and Whitten's medium, however, were not significantly different both at 72 h and 96 h. In mKRB, eggs that had developed to blastocysts were observed at 144 h (6%) and 168 h (6%). In Whitten's medium, 12% and 14% of the eggs at the blastocyst stage were observed at 144 h and 168 h, respectively. The frequencies of eggs at the blastocyst stage were significantly higher in Whitten's medium than in mKRB both at 144 h and 168 h ($P<0.05$). The frequencies of abnormal eggs, a total of degenerated and delayed eggs, were significantly higher in Whitten's medium (49% at 48 h and 54% at 72 h) than in mKRB (20% at 48 h and 26% at 72 h) both at 48 h and 72 h ($P<0.05$). On the other hand, the frequency of abnormal eggs was lower in Whitten's medium (86%) than in mKRB at 168 h (94%, $P<0.05$).

In mKRB with or without addition of hyaluronic acid, there were no significant differences in frequencies of eggs at each developmental stage throughout the period of culture except for those at 168 h. At 168 h, the frequency of eggs at the blastocyst stage was significantly higher in mKRB with hyaluronic acid (13%) than in mKRB alone (6%). In Whitten's medium with hyaluronic acid added, 51% of the eggs were at the 2-cell stage and 51% of eggs were at the 3- to 4-cell stage at 24 h and 48 h, respectively. These frequencies were significantly higher than those in Whitten's medium alone ($P<0.05$). Addition of hyaluronic acid to Whitten's medium resulted in the highest frequency (28%) of development to the blastocyst stage at 168 h ($P<0.05$). Addition of hyaluronic acid to Whitten's medium decreased the frequencies of abnormal eggs at most of the period of culture. Expanded blastocysts were observed but only by the addition of hyaluronic acid in both mKRB (5/20) and Whitten's medium (9/27, Fig. 1-B) at 168 h.

Table 3 shows the numbers of cells in blastocysts. There were no significant differences in the mean number of cells per blastocyst obtained after culture in the four media. The number of cells per blastocyst ranged from 21 to 61.

Discussion

The frequency of activation was high (95–96%), and 91% of the activated eggs treated with CB had a single polar body and two nuclei (Table 1). From these results, the estimated frequency of diploid activated eggs

Table 3. Numbers of cells in blastocysts derived from activated and cultured porcine eggs

Medium used*	No. of blastocysts examined	Number of cells	
		Mean	Range
mKRB	8	37.6	29–55
WM	11	40.1	21–60
mKRBHA	20	38.9	29–57
WMHA	27	40.2	25–61

*: mKRB, Modified KRB solution supplemented with 0.4% (w/v) BSA; WM, Whitten's medium supplemented with 0.4% (w/v) BSA; mKRBHA, mKRB containing 0.5 mg/ml hyaluronic acid; WMHA, WM containing 0.5 mg/ml hyaluronic acid.

was 95% to 96% out of the eggs that were used for culture in the present experiments.

In the absence of hyaluronic acid, frequencies of eggs at the 2-cell stage 24 h after activation and the 4-cell and 5- to 16- cell stages at 48 h were significantly higher in mKRB than in Whitten's medium. However, frequency of eggs that had developed to the blastocyst stage at 168 h was significantly higher in Whitten's medium (14%) than in mKRB (6%). Eleven out of 16 morulae at 120 h in Whitten's medium developed to the blastocyst stage at 168 h, while 8 out of 43 morulae at 96 h in mKRB developed to blastocysts in mKRB at 168 h (Table 2). These results suggest that Whitten's medium supports the development of activated eggs from morulae to blastocysts better than mKRB and/or that eggs with low developmental capacity were selected out at earlier stages in Whitten's medium than in mKRB. A greater number of fertilized porcine eggs developed to the morula or blastocyst stage in Whitten's medium than in mKRB [18]. The developmental ability of porcine embryos was affected more by NaCl concentration than osmolarity, and the higher concentration of NaCl in mKRB than in Whitten's medium thus appeared to impair development of the embryos [18].

The addition of hyaluronic acid improved developmental capacity of activated eggs both in Whitten's medium and mKRB. In Whitten's medium, frequency of developing eggs increased before the 5- to 16-cell stages when hyaluronic acid was added. The effect was also clear in later stages; 27 out of 28 morulae at 120 h developed to blastocysts at 168 h in the presence of hyaluronic acid, while 11 of 16 did so in the absence of hyaluronic acid (Table 2). As frequency of abnormal eggs also decreased throughout culture in the presence of hyaluronic acid, these improvements clearly resulted from the decrease of abnormal eggs. Expanded blastocysts were observed at 168 h in both Whitten's medium and mKRB but only in the presence of hyaluronic acid. Sato *et al.* [19] reported that hyaluronic acid-like glycosaminoglycan derived from porcine follicular fluid promoted the viability of porcine oocytes *in-vitro*. Whitten's medium containing hyaluronic acid supports *in-vitro* development of 1- and 2-cell porcine eggs *in-vivo* fertilized to the blastocyst stage and hyaluronic acid decreases the frequency of degenerated embryos [13]. Miyano *et al.* [13] observed that 70% of fertilized eggs developed to the blastocyst stage in Whitten's medium containing hyaluronic acid, while 28% of activated eggs developed to blastocysts in the same medium in the present study. This may be due to the difference between the eggs fertilized and activated by a single pulse. It has been shown that the pattern of the intracellular Ca^{2+} oscillation induced by electrostimulation was different from that induced by sperm penetration [20]. Rabbit eggs activated by repeated electrostimulation which was similar pattern of the oscillation to that seen in normal fertilization show high developmental ability to the blastocyst stage [21].

In spite of the presence of hyaluronic acid, there were no differences in the mean number of cells per blastocyst obtained in the four media (Table 3). The number of cells per blastocyst ranged from 21 to 61. Since it was reported that the number of cells in porcine blastocysts collected from uterus ranged from 9 to 64 [22], it may be concluded that the blastocysts derived from activated eggs in the present study have the same normal number of cells.

The present results demonstrate that the addition of hyaluronic acid to media improves developmental ability to the blastocyst stage of electro-activated diploid porcine eggs. The improvement is prominently enhanced when hyaluronic acid is added to Whitten's medium rather than to mKRB.

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体外成熟由来ブタ２倍体活性化卵子のヒアルロン酸添加による胚盤胞への発生率の改善

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電気刺激により得られたブタ２倍体活性化卵子の発生能力が，培養液へのヒアルロン酸添加により改善されるか，否かについて調べた。体外成熟卵母細胞に1,500 V/cm DC，パルス幅100 μsecの単矩形波を負荷して，活性化卵子を作出した。活性化卵子を５μg/mlのサイトカラシンＢに４時間浸漬した後，第１極体のみを有する卵子を２倍体と判定し，これらを発生培養に供した。修正KRB液とWhittenの培養液，および各々の培養液に0.5 mg/mlのヒアルロン酸を添加した４種類の培養液を用いた。胚盤胞への発生率は修正KRB液（電気刺激144および168時間後，6%）よりWhittenの培養液（144時間後，12%；168時間後，14%）の方が高かった（$P<0.05$）。ヒアルロン酸を添加したとき，168時間後における胚盤胞への発生率は，修正KRB液で13%に，Whittenの培養液では28%に上昇し，また異常卵子の割合は減少した。電気刺激168時間後には，ヒアルロン酸を添加した培養液にのみ拡張胚盤胞が認められた。得られた胚盤胞の平均細胞数に培養液による有意な差は認められず，その数は21から61個であった。

キーワード：ブタ卵胞卵母細胞，単為発生，ヒアルロン酸．