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The Blastocyst Production Rate and Incidence of Chromosomal Abnormalities by Developmental Stage in *In Vitro* Produced Porcine Embryos

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Abstract. The present study was conducted to determine the relationship between embryonic development speed at different stages (the cleaved stage at 52 h and the blastocyst stage at 6 days post insemination) and incidences of chromosome abnormalities in *in vitro* produced porcine embryos. Porcine oocytes were collected from 3–6-mm ovarian follicles obtained at a slaughterhouse and matured in modified NCSU-37 medium for 44–46 h. Following *in vitro* fertilization with a final concentration of 1×10^5 sperm/ml for 3 h, all oocytes were cultured *in vitro* for 52 h. Day-2 (52 h after insemination) embryos were classified according to their cleaved stages into 2-cell, 3- to 4-cell, 5- to 8-cell, and >8-cell stages; these were cultured separately for additional 4 days (Day 6). The resultant Day-6 blastocysts were classified according to the morphological diameter into 3 grades: Grade A, expanded blastocysts; Grade B, expanding blastocysts; and Grade C, early blastocysts. They were then analyzed chromosomally. The 3- to 4-cell and 5- to 8-cell embryos had significantly high blastocyst development rates (46.1 and 36.9%, respectively), and these blastocysts contained significantly more cells (40.2 and 42.4 cells, respectively) than those derived from 2-cell embryos and >8-cell embryos (28.6 and 26.5 cells, respectively). The incidence of chromosomal abnormalities was significantly higher in the blastocysts derived from 2-cell and >8-cell stage embryos than in the blastocysts derived from the other stage embryos. Furthermore, the grade A blastocysts had the lowest incidence of chromosomal abnormalities (35.3%) and contained the most cells (48.7 cells). Porcine *in vitro* production (IVP) yielded a high blastocyst rate and an excellent embryo quality when 3- to 4-cell and 5- to 8-cell stage embryos were selected on Day 2 after insemination. The same criteria yielded a higher quality of expanded blastocysts based on the stage of embryo development and morphology.

Key words: Blastocyst, Chromosomal abnormality, Embryo, *In vitro* production (IVP), Pig
(J. Reprod. Dev. 54: 22–29, 2008)

In mammals, the technology of *in vitro* production (IVP) of embryos, which involves *in vitro* maturation and fertilization of oocytes and *in vitro* culture of the resultant embryos, has become important for increasing the number of offspring from selected high-quality animals and for reducing the generation intervals for breed improvement in livestock [1, 2]. Moreover, IVP technology has been used in the production of cloned and transgenic animals, which can be used in biomedical research [3–5]. In swine, although embryos have been successfully produced using the IVP system, the development rates to the blastocyst stage and to full term after embryo transfer are still low [6–8]. The viability of porcine embryos produced by IVP is low compared with that of *in vivo*-derived porcine embryos. For example, porcine embryos produced by IVP are of poor quality in terms of the number of cells compared with those produced *in vivo* [9].

Although there are undoubtedly multiple causes for the developmental failure that occurs in embryos produced by IVP, chromosomal abnormalities appear to be a major cause of early

embryonic mortality [10, 11]. Embryos produced by IVP have a high incidence of chromosomal abnormalities, including mixoploidy and polyploidy; these have been linked to a high frequency of polyspermic fertilization [12–14]. Cytogenetic analysis of porcine blastocysts produced *in vitro* has revealed that about 40% of the blastocysts are abnormal [15]; this is higher than the 7.3% reported for *in vivo*-derived embryos [16]. In cattle, the incidence of mixoploidy is about three times higher in embryos produced by IVP than in those produced *in vivo* [17]. Embryos with chromosomal abnormalities may result in reproductive failure that is manifested by embryonic developmental arrest or early embryonic death. In humans, cytogenetic studies have shown that the high incidence of chromosomal abnormalities in embryos produced by IVP is responsible for high rates of embryonic death [18] and abortion [10, 11].

To select the best quality embryos for transfer, embryo competence to develop to parturition must be carefully evaluated from the early stages. Quality assessment of preimplantation embryos based on morphological criteria has been used in some species, such as cattle [19, 20] and humans [21, 22]. It appears that the timing of cleavage after insemination is linked to blastocyst formation. Bovine embryos produced by IVP that cleave faster to the 2-cell

stage after insemination display significantly higher blastocyst rates than embryos that cleaved more slowly [23–25]. Furthermore, it has been reported that the number of blastomeres of embryos at any given time linked to embryo viability and chromosome abnormalities. In the hamster [26], the development potential to the blastocyst stage of embryos that reach the 8-cell stage within a normal time frame is significantly greater than that of <4-cell, 4-cell and 5- to 7-cell stage embryos. Moreover, the morulae/blastocysts derived from the 8-cell embryos have better competency to develop to term after embryo transfer. In comparison of human Day-3 IVF embryos with moderately cleaving ones, both slow- and fast-cleaving embryos have low blastocyst formation rates [27] and high incidences of chromosome abnormalities [28].

Despite the relative abundance of cytogenetic data with respect to embryos produced by IVP in other species, few cytogenetic studies of porcine embryos produced by IVP are available. Furthermore, no work has been done to determine the chromosomal quality of porcine embryos based on their early cleaved stages and embryo development stages. In polytocous animals such as swine, although the strategy for efficient embryo transfer is more complicated than that in monotocous animals such as cattle and humans, it is important to eliminate porcine embryos produced by IVP that carry chromosomal abnormalities and to select excellent quality embryos for embryo transfer. Furthermore, determination of the relationship between embryonic development and the incidences of chromosome abnormalities in porcine embryos produced by IVP is required. The present study was conducted to evaluate early cleaved porcine embryos for their developmental competence to the blastocyst stage and for their incidence of chromosome abnormalities.

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were obtained from prepubertal gilts at the Utsunomiya Slaughterhouse (Tochigi Prefecture, Japan) and then transported in a sterile saline solution of 0.85% (w/v) NaCl at 35–37 °C to our laboratory within 1 or 2 h. Cumulus-oocyte complexes (COCs) were collected from 3–6-mm-diameter follicles in phosphate buffer solution (PBS) supplemented with 3 mg/ml of bovine serum albumin, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Only COCs that had a uniform cytoplasm and were surrounded by compact cumulus cells were selected for *in vitro* maturation. The selected COCs were cultured for 20–22 h in modified North Carolina State University (NCSU)-37 medium as describe by Kikuchi *et al.* [8]; the medium contained 10% (v/v) porcine follicular fluid (pFF), 0.6 mM cysteine, 1 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich Chemicals, St. Luis, MO, USA), 10 IU/ml equine chorionic gonadotropin (eCG), 10 IU/ml human chorionic gonadotropin (hCG), 0.1 mM 2-mercaptoethanol (2-ME) and antibiotics. They were subsequently cultured in the same modified NCSU-37 medium without dbcAMP and hormonal supplements for 24 h. The maturation culture was carried out under 5% O₂, 5% CO₂ and 90% N₂ at 39 °C. The pFF was collected in advance by aspirating the fluid of 3–6 mm follicles with a syringe, and it was then centrifuged at 3,000 rpm for 1 h; the supernatant was stored at –20 °C

until use.

In vitro fertilization (IVF) of porcine oocytes and in vitro culture (IVC) of the zygotes

IVF and IVC were conducted according to the previous report of Kikuchi *et al.* [8]. Briefly, the medium used for IVF was a modified Pig-FM [29] consisting of 90 mM NaCl, 12 mM KCl, 25 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 10 mM sodium lactate, 10 mM HEPES, 8 mM CaCl₂, 2 mM sodium pyruvate, 5 mg/ml caffeine, 5 mg/ml bovine serum albumin and antibiotics. Cryopreserved epididymal spermatozoa from Landrace swine [30] were thawed and preincubated for 15 min at 37 °C in medium 199 with Earle's salts and HEPES; the pH was adjusted to 7.8. A small portion (10 µl) of the preincubated spermatozoa was introduced into 90 µl IVF droplets containing about 20 COCs surrounded by expanded cumulus cells; they were then co-incubated at 39 °C under 5% O₂, 5% CO₂ and 90% N₂ for 3 h. The final sperm concentration was adjusted to 1 × 10⁵ sperm/ml. The day of insemination was defined as Day 0. After removal of the cumulus cells and the spermatozoa attached to the surface of the zona pellucida by passing the oocytes several times through a narrow glass pipette, all oocytes were transferred into IVC medium. From Days 0 to 2, IVC was performed in IVC-PyrLac medium and, from Days 2 to 6, IVC was performed in IVC-Glu medium [8]. The IVC-PyrLac medium consisted of NCSU-37 [31] without glucose that was supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 50 µM 2-ME. On Day 2 (52 h after insemination), the embryos that were not degenerated and that consisted of approximately equal blastomeres having neither very light nor very dark cytoplasm were determined to be normal. The Day-2 normal embryos were classified under a stereomicroscope based on their cleaved stages into: 2-cell, slow cleaved embryos; 3- to 4-cell, moderately slow cleaved embryos; 5- to 8-cell, moderately fast cleaved embryos; and >8-cell, fast cleaved embryos. They were then cultured separately to the blastocyst stage in IVC-Glu medium consisting of NCSU-37 supplemented with 4 mg/ml BSA and 50 µM 2-ME. IVC was carried out at 39 °C under 5% O₂, 5% CO₂ and 90% N₂ for 6 days.

Chromosomal analysis

Day-6 blastocysts in each cell stage group were classified into the following three grades according to stages of embryonic development and morphological diameter: Grade A embryos at the expanded stage, Grade B embryos at the expanding stage and Grade C embryos at the early blastocyst stage. After grading, we measured the diameters of the individual embryos in each group. The diameters of the embryos were > approximately 180 µm (191.3 ± 9.5) in the Grade A embryos, 150–180 µm (166.3 ± 5.1) in the Grade B embryos, and < about 150 µm (140.8 ± 3.1) in the Grade C embryos. The blastocysts were cultured for 17 h in IVC-Glu medium containing 30 ng/ml vinblastine sulfate as a mitotic inhibitor. Chromosome preparation was performed in a high humidity room according to the method of Yoshizawa *et al.* [32]. Briefly, the blastocysts were placed in a 0.4 ml hypotonic solution of 1% (w/v) sodium citrate for 13–15 min and then fixed mildly by pouring about 0.02 ml acetic alcohol (1 part acetic acid: 1 part methanol) into the hypotonic solution. After few minutes, each

blastocyst was individually placed onto each clean glass slide, and the blastomeres were separated using a very small droplet of acetic acid. Finally, their chromosomes were spread using several drops of acetic alcohol fixative (1 part acetic acid: 3 parts methanol). The chromosome samples were sufficiently air-dried and then stained with 2% (w/v) Giemsa solution for 10 min.

Cytogenetic criteria

The total number of cells with nuclei, analyzable metaphases (intact and non-overlapping), and chromosomes were examined under a microscope (Olympus, Tokyo, Japan) with a magnification of 600 (40×15) or 900 (60×15). The mitotic index was determined as the ratio of the number of metaphase plates of the total number of cells. Embryos that had two sets of chromosomes ($2n=38$) in all countable metaphases were defined as normal diploids, and those in which all analyzable metaphases (nuclei) contained one set of chromosomes ($n=19$) were defined as haploids. Embryos containing more than two sets of chromosomes in all countable nuclei were defined as polyploids ($3n$, $4n$, $5n$, etc.), and those with a mixture of diploid cells and haploid ($n/2n$), triploid ($2n/3n$), tetraploid ($2n/4n$) or other type of polyploid cells were defined as mixoploids.

Statistical analysis

The chi-square test was used to analyze the differences among the blastocyst rates of the various cell stage embryos and to assess the differences in chromosomal abnormalities among the blastocyst groups. Data for the average number of cells per blastocyst were assessed using the Kruskal-Wallis test and Dunn's multiple comparisons test. A value of $P<0.05$ was considered statistically significant.

Results

Blastocyst production and grading

Of a total of 1,601 oocytes used for IVF, 1,202 (75.1%) cleaved on Day 2 after insemination, and 1,006 (83.7%) of the cleaved embryos were found to be normal (Table 1). The Day-6 total blastocyst rate, which was calculated based on the total number of inseminated oocytes, was 23.1% (370/1,601), while the rate for the cleaved normal embryos was 36.8% (370/1,006).

On Day 2 (52 h after insemination), there were significantly more 3- to 4-cell and 5- to 8-cell embryos than 2-cell and >8-cell embryos (Table 2). The 3- to 4-cell and 5- to 8-cell embryos had

significantly ($P<0.05$) higher blastocyst rates (46.1 and 36.9%, respectively) than the 2-cell and >8-cell embryos (17.5 and 16.8%, respectively). Many of the blastocysts from the 3- to 4-cell and the 5- to 8-cell stage embryos developed into expanded blastocysts (Grade A); their rates of development into expanded blastocysts (52.9 and 51.2%, respectively) were significantly ($P<0.05$) higher than those of the 2-cell (15.0%) and >8-cell embryos (17.6%). On the other hand, many of the blastocysts derived from the 2- and >8-cell stage embryos developed into Grade C blastocysts.

The number of cells and incidences of chromosomal abnormalities in blastocysts derived from various Day-2 cell stage embryos

A total of 370 Day-6 porcine blastocysts produced by IVP were prepared for chromosomal analysis, including 20, 206, 127 and 17 blastocysts derived from 2-cell, 3- to 4-cell, 5- to 8-cell and >8-cell embryos, respectively (Table 3). Overall, 62.4% (231/370) of the blastocysts prepared as chromosome samples were analyzed, including 75.0, 67.5, 54.3 and 47.1% of the 2-cell, 3- to 4-cell, 5- to 8-cell and >8-cell groups, respectively. Overall, the mean number of cells per blastocyst was 39.8 ± 15.7 . The numbers of cells per blastocyst were similar in the 3- to 4-cell and 5- to 8-cell embryos (40.2 ± 15.4 and 42.4 ± 16.8 , respectively) and were significantly ($P<0.05$) higher than those of the blastocysts derived from the 2-cell and >8-cell embryos (28.6 ± 6.9 and 26.5 ± 5.8 , respectively). There were no significant differences in the number of metaphases per blastocyst or mitotic index between the blastocysts derived from any of the Day-2 embryo groups.

The overall incidence of chromosomal abnormalities was 45.9% (Table 4). Notably, chromosomal abnormalities were significantly ($P<0.05$) more common in blastocysts derived from 2-cell embryos (80.0%) and >8-cell embryos (87.5%) than in blastocysts derived from 3- to 4-cell embryos (41.0%) and 5- to 8-cell embryos

Table 1. Results of *in vitro* production of porcine embryos

No. of oocytes used in IVF	1,601
No. of embryos cleaved at day 2 (%)	1,202 (75.1)
No. of normal embryos (%)	1,006 (83.7)
No. of degenerated embryos (%)	196 (16.3)
No. of blastocysts (%*)	370 (23.1)
Blastocysts/cleaved embryos	(370/1,006: 36.8)

*The percentage was calculated based on the total number of inseminated oocytes.

Table 2. Results of development of embryos at various cell stages to the blastocyst stage and grading of the blastocysts

	Embryo cell stages				Total
	2 cells	3 to 4 cells	5 to 8 cells	>8 cells	
No. of embryos on Day 2 (%)	114 (11.3) ^a	447 (44.4) ^b	344 (34.2) ^b	101 (10.1) ^a	1,006
No. of blastocysts on Day 6 (%)	20 (17.5) ^a	206 (46.1) ^b	127 (36.9) ^b	17 (16.8) ^a	370 (36.8)
Grade A (%*)	3 (15.0) ^a	109 (52.9) ^b	65 (51.2) ^b	3 (17.6) ^a	180 (48.6)
Grade B (%*)	8 (40.0)	63 (30.6)	35 (27.6)	4 (23.6)	110 (29.8)
Grade C (%*)	9 (45.0) ^a	34 (16.5) ^b	27 (21.2) ^b	10 (58.8) ^a	80 (21.6)

The values with different superscripts (a–b) in the same row are significantly ($P<0.05$) different. *The percentages for Grades A, B and C were calculated from the number of blastocysts on Day 6 for each cell stage group of embryos.

Table 3. Cell numbers and mitotic indexes of blastocysts derived from embryos at various cell stages

	Embryo cell stages				Total
	2 cells	3 to 4 cells	5 to 8 cells	> 8 cells	
No. of prepared blastocysts	20	206	127	17	370
No. of analyzed blastocysts (%)	15 (75.0)	139 (67.5)	69 (54.3)	8 (47.1)	231 (62.4)
No. of cells per blastocyst*	28.6 ± 6.9 ^a	40.2 ± 15.4 ^b	42.4 ± 16.8 ^b	26.5 ± 5.8 ^a	39.8 ± 15.7
No. of metaphases per blastocyst*	1.6 ± 0.9	2.6 ± 1.9	2.5 ± 1.7	1.5 ± 0.5	2.6 ± 2.4
Mitotic index***	5.6 ± 2.6	6.3 ± 4.6	5.9 ± 3.6	5.6 ± 1.7	6.3 ± 4.1

*The data are presented as means ± SD. The values with different superscripts (a–b) in the same row are significantly ($P < 0.05$) different.

**The mitotic index was calculated as the ratio of the number of metaphases for the total number of cells.

Table 4. Incidence of chromosomal abnormalities in blastocysts derived from embryos at various cell stages

	Embryo cell stages				Total
	2 cells	3 to 4 cells	5 to 8 cells	> 8 cells	
No. of analyzed blastocysts	15	139	69	8	231
No. of normal blastocysts (%)	3 (20.0) ^a	82 (59.0) ^b	39 (56.5) ^b	1 (12.5) ^a	125 (54.1)
No. of abnormal blastocysts (%)	12 (80.0) ^a	57 (41.0) ^b	30 (43.5) ^b	7 (87.5) ^a	106 (45.9)
Haploid (%*)	2 (13.3)	2 (0.4)	1 (1.5)	0 (0.0)	5 (2.2)
Polyploid (%*)	6 (40.0) ^a	27 (19.4) ^b	11 (15.9) ^b	5 (62.5) ^a	49 (21.2)
Mixoploid (%*)	4 (26.7)	28 (20.1)	18 (26.1)	2 (25.0)	52 (22.5)

The values with different superscripts (a–b) in the same row are significantly ($P < 0.05$) different. *The percentages of chromosome abnormalities; the percentages for haploid, polyploid and mixoploid were calculated from the number of analyzed blastocysts.

Table 5. Cell numbers and mitotic indexes of the blastocysts according to morphological grade

	Blastocyst grades			Total
	Grade A	Grade B	Grade C	
No. of prepared blastocysts	180	110	80	370
No. of analyzed blastocysts (%)	136 (75.6) ^a	60 (54.5) ^{ab}	35 (43.8) ^b	231 (62.4)
No. of cells per blastocyst*	48.7 ± 13.5 ^a	28.0 ± 4.4 ^b	19.9 ± 4.1 ^c	39.8 ± 15.7
No. of metaphases per blastocyst*	3.5 ± 2.3	1.9 ± 1.7	1.2 ± 0.6	2.6 ± 2.4
Mitotic index***	7.3 ± 5.9	6.6 ± 3.1	5.5 ± 3.3	6.3 ± 4.1

*The data are presented as means ± SD. The values with different superscripts (a–c) in the same row are significantly ($P < 0.05$) different. **The mitotic index was calculated as the ratio of the number of metaphase plates of the total number of cells.

(43.5%). On the other hand, the frequency of normal diploidy was significantly ($P < 0.05$) higher in the blastocysts derived from 3- to 4-cell embryos (59.0%) and 5- to 8-cell embryos (56.5%) than in the blastocysts derived from 2-cell embryos (20.0%) and >8-cell embryos (12.5%).

Haploidy, polyploidy and mixoploidy were found in all groups, with the exception that no haploidy was observed in blastocysts derived from >8-cell embryos. Of the blastocysts with chromosomal abnormalities, those derived from 2-cell embryos and >8-cell embryos had significantly ($P < 0.05$) higher incidences of polyploidy than the blastocysts derived from 3- to 4-cell and 5- to 8-cell embryos. The blastocysts derived from 2-cell embryos had a high haploidy rate but there was no significant difference compared with the blastocysts derived from the other groups of embryos.

The number of cells and incidences of chromosomal abnormalities in blastocysts based on morphology

A total of 370 blastocysts were statistically analyzed based on grade (Table 5). Almost all the Grade A blastocysts (75.6%) could be analyzed chromosomally; significantly more Grade A blastocysts could be analyzed chromosomally than Grade C blastocysts (43.8%, $P < 0.05$). The Grade A blastocysts had significantly ($P < 0.05$) more cells (48.7 ± 13.5) than the Grade B (28.0 ± 4.4) and Grade C (19.9 ± 4.1) blastocysts. Although the Grade A blastocysts had more metaphases per blastocyst and a high mitotic index, no significant differences were found among the three grades of blastocysts. The Grade A group had a significantly ($P < 0.05$) lower incidence of chromosomal abnormalities (35.3%) than the B (51.7%) and C (77.1%) groups (Table 6). Haploidy, polyploidy (triploidy, 3n; Fig. 1; tetraploidy, 4n; and others) and mixoploidy (n/2n, 2n/3n, 2n/4n; Fig. 2; and others) were found in all grades of blastocysts. There were no significant differences in the incidences

Table 6. Incidence of chromosomal abnormalities in the blastocysts according to morphological grade

	Blastocyst grades			Total
	Grade A	Grade B	Grade C	
No. of analyzed blastocysts	136	60	35	231
No. of normal blastocysts (%)	88 (64.7) ^a	29 (48.3) ^b	8 (22.9) ^c	125 (54.1)
No. of abnormal blastocysts (%)	48 (35.3) ^a	31 (51.7) ^b	27 (77.1) ^c	106 (45.9)
Haploid (%*)	1 (0.8)	1 (1.7)	3 (8.6)	5 (2.2)
Polyploid (%*)	18 (13.2) ^a	16 (26.7) ^b	15 (42.8) ^b	49 (21.2)
Mixoploid (%*)	29 (21.3)	14 (23.3)	9 (25.7)	52 (22.5)

The values with different superscripts (a–c) in the same row are significantly ($P<0.05$) different. *The percentages of chromosome abnormalities; the percentages for haploid, polyploid and mixoploid were calculated from the analyzed blastocysts.

Fig. 1. A microphotograph of a triploid (3n) metaphase seen in an *in vitro* produced porcine blastocyst. There are 57 chromosomes in this metaphase. **Fig. 2.** A microphotograph of mixoploidy (2n/4n) seen in an *in vitro* produced porcine blastocyst. Diploid (2n: right) and tetraploid (4n: left) metaphases can be seen in this image.

of haploidy and mixoploidy among the three grades. However, the incidence of polyploidy was significantly ($P<0.05$) lower in the Grade A, 13.2%, than in the Grades B and C, 26.7% and 42.8%, respectively.

With respect to the relationship between chromosomal abnormality and the numbers of cells in the blastocysts, it was found that diploid blastocysts had significantly ($P<0.05$) more cells than polyploid and haploid blastocysts (41.4 ± 13.9 , 28.2 ± 10.2 and 25.8 ± 7.7 , respectively; Fig. 3).

Discussion

In the present study, the overall blastocyst rate (23.1%) for all the inseminated oocytes was similar to the rate previously reported by Kikuchi *et al.* [8] (25.3%); however, it was slightly lower than the rates reported by Somfai *et al.* [33] (32%) and Karja *et al.* [34] (31.3%). When the embryos were classified by their cleaved stages on Day 2, the blastocyst rate of the moderate stage embryos (3- to 4-cell and 5- to 8-cell embryos) was significantly higher than the rates of the delayed or slow (2-cell embryos) and fast stage (>8 -cell embryos) embryos. Alikani *et al.* [27] noted similar results with *in vitro*-derived human Day-3 embryos; they reported that slow (<7

Fig. 3. The cell numbers (means $\pm$ SD) of *in vitro* produced Day-6 porcine blastocysts based on their chromosome complements. Different letters (a, b, c) denote statistically different values ($P<0.05$).

cells) and fast (>8 cells) cleavage of embryos had a significantly negative effect on normal blastocyst formation. On the other hand, it has been reported that cattle embryos that divided faster into 2

cells before 30–31 h after insemination had a significantly higher proportion of morula-blastocysts by Day 8 than embryos that divided later [23]. McKiernan and Bavister [26] have also reported that hamster embryos (<4 cells) with slower cleavage within a time frame were less likely to reach the blastocyst stage than embryos that reached the 8-cell stage within a normal time frame when they were cultured *in vitro*. Mateusen *et al.* [35] have shown that porcine embryos with fragmentation exceeding 15% of the embryo volume have a significantly negative impact on embryonic development. In the present study, the small number of cells present in the Day-2 embryos with >8 cells may reflect a high frequency of fragmentation; this is considered to be a cause of a low blastocyst rate. Furthermore, Day-2 embryos with >8 cells may be products of abnormal cleavage (3 or more cells) caused by polyspermy at the first cleavage division [36]. Abnormal cell division was also observed in porcine embryos produced by IVF at the first cleavage division (unpublished our data).

The average number of cells per blastocyst found in the present study was similar to that previously reported by Kikuchi *et al.* [8], Somfai *et al.* [33] and Karja *et al.* [34]. Blastocysts derived from 3- to 4-cell embryos and 5- to 8-cell embryos had significantly more cells than those from the other cell stage embryos. This result is not in agreement with our hypothesis that, on Day 2, embryos with >8 cells might have more cells at the blastocyst stage than 3- to 4-cell embryos and 5- to 8-cell embryos; this indicates that the development of >8-cell embryos slows down after Day 2. The results of the present study, which found that expanded blastocysts had more cells than expanding and early blastocysts, are in agreement with the report of Jakobsen *et al.* [37], which showed that hatched bovine blastocysts (fast development) had more cells than non-expanded blastocysts (slow development). In the present study, the expanded blastocysts were evaluated as being of better quality than the other blastocysts based on the general criterion that “the larger the number of cells in embryos the better the quality,” as discussed by Jakobsen *et al.* [37] and Farin *et al.* [38]. Furthermore, the blastocysts that developed from 3- to 4-cell embryos and 5- to 8-cell embryos were also evaluated as being of high quality because they contained a large number of cells at the blastocyst stage.

The mitotic index, which is the proportion of mitotic metaphases among all nuclei, reflects embryo viability. In the present study, the low mitotic index (6.3) was similar to that reported in Day-6 porcine blastocysts produced by IVP (7.7) by McCauley *et al.* [15]; this indicates that relatively few cells had undergone mitosis. A mitotic index of 20% has been reported for bovine blastocysts produced by IVP and treated with vinblastine for 17 h [33], while a higher mitotic index (92%) has been reported for mouse morulae derived from natural mating after vinblastine treatment for 10 h [39]. The low mitotic indices of porcine blastocysts produced by IVP compared with bovine and mouse embryos suggest that cell division occurs more slowly in porcine embryos than in bovine and mouse embryos. Moreover, porcine embryos produced by IVP may be less viable than bovine embryos produced by IVP.

In the present study, the total incidence of chromosomal abnormalities in the Day-6 porcine blastocysts was 45.9%; this was higher than the incidence of 39.1% reported by McCauley *et al.* [15]. The chromosomal abnormalities observed in the present

study were mainly polyploidy and mixoploidy. During *in vitro* fertilization in mice [12, 13], cattle [14] and swine [15], the high incidences of polyploidy and mixoploidy are the result of polyspermy. It is likely that the polyploidy and mixoploidy noted in the present study were also the result of polyspermy.

In the present study, classification at the 2-day embryo stage showed that the incidence of chromosomal abnormalities differed among the various blastocyst groups; the blastocysts derived from 3- to 4-cell embryos and 5- to 8-cell embryos had fewer chromosomal abnormalities. In human cleaved embryos on day 3, however, Magli *et al.* [28] reported that the incidence of chromosomal abnormalities was significantly lower in 7–8-cell stage embryos than in slow-cleaving embryos (4-cell), moderately slow-cleaving embryos (5–6-cell) and very fast-cleaving embryos (≥ 9 -cell). In regard to incidence of chromosomal abnormalities, it may be derived from the difference between porcine and human embryo development *in vitro* that the 3- to 4-cell and 5- to 8-cell stages produced good results for Day-2 porcine embryos produced by IVP, while the 7–8-cell stage produced good results for human IVF on day 3. Therefore, it could be that the speed of cleavage during early embryonic development is related to the incidence of chromosomal abnormalities. Furthermore, it may be concluded that the stage of embryo development and the embryonic morphology are related to the incidence of chromosomal abnormalities, since there was a significant difference in the incidence of chromosomal abnormalities among the expanded, expanding and early blastocysts. This relationship between the timing of development and the incidence of chromosomal abnormalities was not observed by Jakobsen *et al.* [37]; they found no significant differences among the chromosomal abnormalities of hatched, expanded and non-expanded bovine blastocysts produced by IVP. Furthermore, in contrast to the results of the present study, they reported a high incidence of mixoploidy in all embryo groups, but they did not observe polyploidy or haploidy.

In the present study, the poor embryonic development of the blastocysts derived from the >8-cell embryos, which was manifested as a small number of cells and a high incidence of chromosomal abnormalities, may have been caused by the abnormal cleavage attributed to polyspermy as reported by Han *et al.* [36]. Furthermore, the small number of cells of blastocysts was related to the presence of polyploidy. This is in agreement with the report concerning pig embryos produced by IVP, which showed that the incidence of polyploidy decreased in embryos with many cells [15]. Mixoploidy may not be detrimental to blastocyst development *in vitro* because the mixoploid and normal diploid blastocysts contained many cells in the present study. Viuff *et al.* [40] reported that the frequency of mixoploidy in *in vivo*-derived embryos increases as the embryos develop from Day 2 to Day 5 after ovulation. Viuff *et al.* [41] reported that mixoploidy did not affect the developmental competence of embryos, since mixoploid embryos were generally observed on Day 5 in their fast-developing group of bovine embryos produced by IVP. In addition, in the present study, blastocysts derived from slow (2-cell embryos) and fast (>8-cell embryos) cleaved Day-2 embryos displayed a significantly higher incidence of polyploidy than those derived from moderate cleaved embryos (3- to 4-cell embryos and 5- to 8-cell

embryos); furthermore, normal embryonic development of the slow and fast cleaved embryos was adversely affected. Kawarsky *et al.* [42] found that bovine haploid and polyploid embryos produced by IVP develop slower than diploid and mixoploid embryos. In the present study, although it was not statistically significant due to an insufficient number of samples, the slow cleaved embryos had a higher incidence of haploidy than the moderate and fast cleaved embryos; this result might support the finding that mouse haploid parthenogenotes develop more slowly in culture during the preimplantation period than fertilization-derived diploid embryos [43].

In conclusion, the present study indicates that Day-6 porcine blastocysts produced by IVP and derived from moderate cleaved stage Day-2 embryos (3- to 4-cell and 5- to 8-cell embryos) are superior in terms of quality, based on the ability of the embryo to develop, number of cells and incidence of chromosome abnormalities of the resultant blastocysts compared with slow (2-cell embryos) and fast (>8-cell embryos) cleaved stage embryos on Day 2. Furthermore, among the blastocysts graded by stages of embryo development and morphology, expanded blastocysts had the highest quality based on the fact that they had the largest number of cells and the lowest incidence of chromosomal abnormalities.

The results of the present study can be used for selection of high quality embryos at the Day-2 cleaved embryo and Day-6 blastocyst stages of development.

Acknowledgements

We thank the management and staff of Utsunomiya Slaughterhouse in Tochigi Prefecture for their help in procuring porcine ovaries.

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