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Development of Early Porcine Embryos In Vitro and In Vivo[1]

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ABSTRACT

In vitro development of early porcine embryos under different culture conditions was evaluated and compared to in vivo development. First, one- and two-cell embryos were collected and cultured individually in 20-μl drops under 5% CO_2 in air for 4 days. Embryos from one oviduct were cultured in NCSU-23, and those from the contralateral oviduct were cultured in KSOM/AA. The embryos developed in NCSU-23 had a higher mean number of inner cell mass (ICM) nuclei compared to those developed in KSOM/AA ($p = 0.025$). They also had higher trophectoderm (TE) and total nuclear number ($p = 0.001$), while there was no difference in the average ratio of ICM to TE nuclei ($p = 0.731$). When the effect of different gas atmospheres was tested, the numbers of TE and total nuclei were higher ($p < 0.01$ and $p < 0.025$, respectively) in embryos cultured in an atmosphere with 5% CO_2 in air than in those developed under 5% CO_2:5% O_2:90% N_2. Next the development of embryos cultured in NCSU-23 was compared to that of embryos incubated in vivo. By the end of the 4-day incubation, the cultured embryos had higher nuclear numbers and a higher ratio of ICM to TE nuclei than those developed in vivo ($p < 0.001$).

Finally, the embryos that developed in NCSU-23 or in vivo were transferred into recipients. By Day 40 of pregnancy, 37.1 ± 15.3% of the in vitro- and 53.8 ± 15.3% of the in vivo-incubated embryos formed conceptuses. These results indicate that despite the lower nuclear numbers caused by in vitro conditions, the cultured embryos were developmentally competent.

INTRODUCTION

Maintaining viability and development of early embryos during culture is crucial to a number of applications in the field of animal biotechnology [1]. Efforts to culture different embryos met various successes; for a long time, porcine embryos were regarded to be extremely difficult to culture past the four-cell stage [2, 3]. Later attempts to overcome this ''in vitro developmental block'' included culturing embryos in oviducts maintained in organ culture, coculture with oviductal or granulosa cells, culture in ligated sheep oviducts, supplementation with complex biological fluids such as serum or oviduct fluid, and various modifications of simple culture media (for review see [1, 4]). Although the achievements made early stages of embryonic development feasible in vitro, the adequate culture conditions for the preimplantation porcine embryo have yet to be determined. There are numerous data showing that embryonic development in vitro is retarded and results in fewer cell numbers compared to those in embryos developed in vivo, which is true as well for the pig [5–9]. It was not determined, however, to what (if any) extent this lower cell number compromises the further development of the cultured embryos.

The objective of the present study was to define culture conditions that provide the best in vitro environment for development of early porcine embryos: a) two different culture media, one developed for pigs (NCSU-23 [10]) and one developed for mice (KSOM/AA [11]), were evaluated, and b) two different gas compositions (5% CO_2 in air versus a low O_2 tension atmosphere) were tested. In addition, the early development of porcine embryos under in vitro and in vivo conditions and the developmental competency of these embryos after transfer into recipient animals were compared.

MATERIALS AND METHODS

Embryo Collection

Experiments were conducted according to institutional Animal Care and Use Committee guidelines. Thirty-two naturally cycling Large White gilts were used as embryo donors. They were monitored for estrus once a day by exposure to a mature boar and artificially inseminated at 12 and 24 h after estrus detection. The day of the first insemination was designated as Day 0. Two days after the first insemination (Day 2), the embryos were flushed from the oviducts with 25 ml of the appropriate medium, as described previously [12]. During the experiments in which different culture media were compared, the embryos from one oviduct of each pig were collected using NCSU-23 medium supplemented with 0.1 mg/ml cysteine and 4 mg/ml BSA (both from Sigma Chemical Co., St. Louis, MO), and those from the contralateral oviduct were collected with KSOM/AA (kindly provided by Dr. John Eppig, Jackson Laboratory, Bar Harbor, ME). For the comparison of different gaseous atmospheres, the embryos were collected in NCSU-23 medium from both oviducts; and for evaluation of in vitro and in vivo development, only one oviduct of each animal was flushed (using NCSU-23 medium) on Day 2. In the latter case, embryos from the contralateral oviduct were collected 4 days later, by flushing of the uterus with 50 ml NCSU-23 after cannulating the ovarian end of the uterine horn.

Embryo Culture

After collection on Day 2, the embryos were washed thoroughly and those at the one- or two-cell stage were transferred into 20-μl drops of the same medium that had been used for flushing. They were cultured individually in the microdrops for 4 days, covered with paraffin oil that had previously been equilibrated with the appropriate medium. Generally, the culture was performed at 39°C in a humidified atmosphere containing 5% CO_2 in air (which contains ~20% O_2). When the effect of different atmospheric compositions was tested, the embryos from one oviduct of the animals were cultured in 5% CO_2 in air; those

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from the other oviduct were incubated in an atmosphere containing 5% CO_2:5% O_2:90% N_2. The embryos in the oviducts that were not flushed on Day 2 developed in vivo for the same period of time.

Scores for Morphological Stage of Development

At the end of the culture period (Day 6), in vitro and in vivo embryos were collected and evaluated for stage of development. The end developmental stages were considered linear for the purposes of statistical analysis and scored as follows: 1, morula-stage embryo with no sign of compaction; 2, compact morula; 3, early blastocyst (blastocyst-stage embryo with a barely visible blastocoele); 4, blastocyst (blastocyst-stage embryo that already has a well-defined blastocoele that does not fill the zona pellucida); 5, full blastocyst (a blastocyst without zona pellucida thinning whose blastocoele has reached its final size); 6, expanded blastocyst with zona pellucida thinned; 7, hatching blastocyst.

Differential Staining

Differential nuclear staining of inner cell mass (ICM) and trophectoderm (TE) was undertaken using two chromatin-specific fluorochromes with different fluorescent spectra: propidium iodide, which can enter only cells with damaged cell membranes, and *bis*benzimide (Hoechst 33342), which can pass through both damaged and intact membranes [9, 13, 14]. At the end of the culture period (Day 6) the zonae pellucidae were removed by a brief exposure to 0.5% pronase, and the zona pellucida-free embryos were rinsed in Hepes-buffered Tyrode's medium (HbT; [15]). They were then exposed to a 1:5 dilution of rabbit anti-pig whole serum (Sigma, P-3164; dialyzed in HbT without serum to remove sodium azide) for 1 h. After three 5-min rinses in HbT, the embryos were placed into a 1:10 dilution of guinea pig complement (Sigma, S-1639) for 1 h. Propidium iodide and *bis*benzimide were added to the complement solution to a final concentration of 10 μg/ml. They were then rinsed briefly in HbT, mounted under coverslips, and examined under UV light using a Nikon Diaphot inverted microscope (Nikon Corp., Tokyo, Japan) fitted for epifluorescence. The cell counts were made directly under the microscope; the nuclei of the ICM cells were labeled with *bis*benzimide and appeared blue, while the nuclei of the TE cells were stained with both fluorochromes and thus were fluorescing pink.

Embryo Transfers

In order to examine the effect of different culture conditions on further embryo development, embryos were transferred into recipients to establish pregnancy. At the end of the 4-day incubation the embryos were collected and evaluated for their developmental stages. Some embryos were randomly selected for differential staining to determine cell numbers, while the rest were transferred into 4 recipient gilts that were in estrus 1 day after the donors. One uterine horn of each recipient was tied to prevent migration of the embryos between the uterine horns, and the embryos were transferred to the tip of a uterine horn by means of a tom cat catheter inserted through the uterine wall. In vitro and in vivo embryos from the same donor animal were transferred into one recipient. A second set of corpora lutea was induced in the recipients by injecting them with 1000 IU of eCG on Day 9 of their estrous cycles and with 750 IU of hCG on Day 12. Thirty-five days after the transfers (Day 40 of pregnancy) the recipients were slaughtered, the reproductive tracts were collected, and the number of fetuses present in each uterine horn was determined.

TABLE 1. Characteristics of pig embryos developed in vitro in different culture media.

Items	NCSU-23	KSOM/AA	n
End stage	3.2 ± 0.22	2.8 ± 0.23	75
ICM cell number	5.7 ± 0.59[a]	3.8 ± 0.63[b]	73
TE cell number	25.0 ± 1.5[a]	17.5 ± 1.6[b]	73
Total cell number	30.5 ± 1.8[a]	21.4 ± 1.9[b]	75
Ratio of ICM:TE cell number	0.22 ± 0.064	0.18 ± 0.068	73

[a,b] Values in the same row having different superscripts are significantly different ($p < 0.025$).

Statistical Analysis

Differences between the mean values of different cell numbers and developmental stages were determined by calculating least-significant differences using Multivariate General Linear Hypothesis of Systat [16]. Percentage data were subjected to arc sine transformation before analysis; they were analyzed by ANOVA and Fisher's protected least-significant difference test using the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA) and are presented as mean ± SEM.

RESULTS

A total of 367 embryos were collected from the donor gilts; 80 embryos that had more than two cells on Day 2 were not used in this study. In the first set of experiments, 94 one- and two-cell embryos were cultured in the microdrops of NCSU-23 or KSOM medium; the numbers of one- and two-cell embryos in the two culture media were similar. Seventy-five embryos cleaved and began early embryonic development. Nineteen unfertilized oocytes were omitted from further analyses. The general pattern of development was similar in the two media: 47.7% (21 of 44) of the embryos reached one of the blastocyst stages in NCSU-23 medium by Day 6, while in KSOM/AA this rate was 45.2% (14 of 31). The end developmental stage in both media averaged around the early blastocyst stage (Table 1).

The differential staining revealed, however, that the embryos developed in the NCSU-23 medium had higher numbers of ICM, TE, and total nuclei than those cultured in KSOM/AA ($p < 0.025$). There was no difference ($p = 0.731$) in the ratio of the number of ICM to TE nuclei between the two media. A typical photograph of an embryo stained with the differential staining method is shown in Figure 1.

Since these results suggested that the environment provided by the NCSU-23 medium was more suitable for early porcine embryos, we used it in the second set of experiments to compare development in different gaseous atmospheres. For this purpose, 78 embryos were allocated into the two treatment groups. Two embryos did not cleave and were therefore discarded; the rest developed to the early blastocyst stage in both atmospheres (Table 2). Of the embryos cultured in 5% CO_2, 55.3% (21 of 38) reached one of the blastocyst stages, while 50.0% (19 of 38) of the embryos incubated in 5% O_2:5% CO_2:90% N_2 developed to this stage. The number of ICM nuclei in the embryos developed in 5% CO_2 in air was 3.1 ± 0.40, versus 2.1 ±

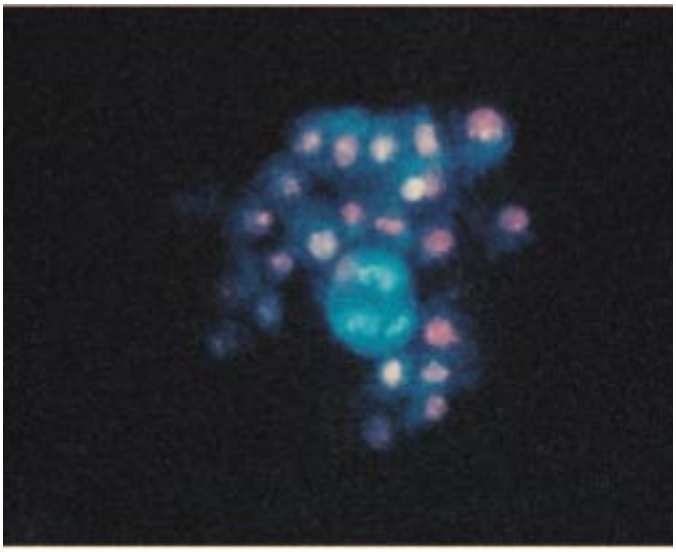

FIG. 1. Porcine embryo developed in vitro for 4 days in KSOM/AA medium and stained with the differential staining method. Nuclei of the ICM stain blue; those of the TE cells are pink.

0.42 in the low-oxygen atmosphere ($p = 0.09$). The number of TE nuclei ($p = 0.01$) and the total number of nuclei ($p < 0.025$) were significantly higher in the embryos cultured in 5% CO_2 in air. There was no difference ($p = 0.198$) in the ratio of the number of ICM to TE nuclei or the developmental stage the embryos reached by the end of the 4-day culture.

Sixty-one embryos were used to compare embryonic development in NCSU-23 medium to that provided by the female genital tract. Results for the embryos that were incubated and successfully stained are shown in Table 3. By Day 6, there was no significant difference in the developmental stage attained by the embryos developed under different conditions. However, in vitro conditions resulted in embryos (n = 30) having significantly ($p < 0.001$) lower numbers of ICM, TE, and total nuclei, and these embryos also had a lower ratio of ICM to TE nuclei by the end of the culture period as compared to those in vivo (n = 31).

The developmental competence of the embryos cultured in NCSU-23 medium for 4 days is shown in Table 4, along with that of the embryos incubated in vivo. Fifty-four embryos obtained from 4 donors were incubated for 4 days either in vitro or in vivo. At the end of culture, 15 of them were used to determine cell numbers, and the rest were transferred into 4 recipient gilts. As in the previous set of experiments, the numbers of ICM, TE, and total nuclei were significantly ($p < 0.01$) lower in the cultured embryos versus the embryos that developed in vivo. But in spite of the low cell numbers, 37.1 ± 15.3% of the cultured embryos were developmentally competent and formed conceptuses by Day 40 of pregnancy, while this rate for the in vivo-developed embryos was 53.8 ± 15.3% ($p = 0.78$).

DISCUSSION

Preimplantation porcine embryos are best cultured in simple media such as those used for mouse embryos [4]. Therefore KSOM/AA, a modified version of a simplex-optimized medium (SOM), was selected to be tested for its ability to support development of early porcine embryos. SOM and KSOM were originally developed for culturing mouse embryos from the one-cell through the two-cell stage [17–20]. When KSOM was supplemented with essential and nonessential amino acids (KSOM/AA), it provided an environment in which preimplantation mouse embryos underwent development similar to that occurring in vivo [11]. It was also used successfully for culturing bovine and rabbit embryos [21, 22]. We compared it to a standard porcine embryo culture system, the NCSU-23 medium [10], that reportedly improved development of early porcine embryos to the blastocyst stage while increasing total cell

TABLE 2. Characteristics of pig embryos cultured in NCSU-23 medium in different gas atmospheres.

Items	5% CO_2:95% air	5% CO_2:5%)$_2$:90% N_2	n
End stage	2.9 ± 0.12	2.7 ± 0.13	76
ICM cell number	3.1 ± 0.40	2.1 ± 0.42	76
TE cell number	21.1 ± 0.90[a]	17.9 ± 0.94[b]	76
Total cell number	24.1 ± 1.1[a]	20.0 ± 1.1[b]	76
Ratio of ICM:TE cell number	0.116 ± 0.016	0.087 ± 0.017	76

[a,b] Values in the same row having different superscripts are significantly different ($p < 0.025$).

TABLE 3. Characteristics of pig embryos developed in vitro or in vivo.

Items	NCSU-23	In vivo	n
End stage	3.2 ± 0.27	3.8 ± 0.24	52
ICM cell number	3.3 ± 2.1[a]	18.1 ± 1.8[b]	52
TE cell number	22.0 ± 2.4[a]	36.7 ± 2.1[b]	52
Total cell number	25.3 ± 3.8[a]	54.9 ± 3.3[b]	52
Ratio of ICM:TE cell number	0.17 ± 0.058[a]	0.5 ± 0.050[b]	52

[a,b] Values in the same row having different superscripts are significantly different ($p < 0.001$).

TABLE 4. Pregnancy results after transfer of embryos developed in vitro or in vivo.

Type of development	ICM cell number	TE cell number	Total cell number	Total embryos transferred (n)	Embryos per recipient	Development to conceptuses (%)
In vitro	8.2 ± 2.7[a]	20.2 ± 2.8[a]	28.4 ± 4.9[a]	23	5.7 ± 0.8	37.1 ± 15.3
In vivo	22.2 ± 2.5[b]	32.9 ± 2.6[b]	59.1 ± 4.5[b]	16	4.0 ± 1.2	53.8 ± 15.3

[a,b] Values in a column having different superscript letters are significantly different ($p < 0.01$).

numbers of the embryos. According to the original description, both media contain BSA fraction V as a protein source; however, it was replaced by a highly purified, essentially fatty acid-free form of BSA (Sigma 0281). It was reported that probably due to the contaminants bound to it, BSA fraction V can inhibit cell proliferation and embryo development [23–25]. In the present experiments, both media supported development of one- and two-cell embryos to the early blastocyst stage. However, the significantly higher cell numbers indicate that NCSU-23 was more appropriate for porcine embryos than KSOM/AA.

It has also been postulated that a low oxygen tension environment has a beneficial effect on early embryo development, although the results are contradictory. Bovine [26], ovine [26, 27], and porcine [28] premorula-stage embryo development was reportedly more superior under a 5% oxygen than in a 20% oxygen atmosphere. Others [29], however, were unable to confirm the beneficial effect of the reduced oxygen tension by culturing bovine embryos in the two gaseous atmospheres. It was suggested that the oxygen effect is due to an alteration in the oxidation-reduction potential in the embryo, which at early developmental stages is most dependent on the NAD+/NADH ratio as well as pyruvate and lactate [30]. The oxygen environment may also influence metabolism. It can alter the rate of aerobic and anaerobic glycolysis and, if coupled with insufficient phosphate supply, the mechanisms of glycolysis and oxidative phosphorylation running simultaneously in the embryo can be disturbed [31]. In the mouse the positive effect of the low-oxygen atmosphere was most pronounced between the one- and two-cell stages [32]. It seems, however, that in NCSU-23 medium, the pig blastocysts that developed in 20% O_2 were of better quality in terms of higher TE and total cell numbers than those cultured in the low oxygen tension atmosphere.

The lower cell number of the embryos developed in vitro compared to those in vivo is not surprising. Incubation in vitro was reported to have a negative effect on the development of early embryos. In culture they show species-specific blocks to development [33, 34]. Mouse embryos were reported to have reduced cleavage and blastocyst rates, and their developmental competence after transfer was also impaired [5–8, 35]. Development of pig preimplantation embryos was also retarded in vitro. When Day 5 and Day 6 porcine embryos were incubated in vitro in Ham's F-12 medium or in immature mouse oviducts for 1–3 days, the morphology of the resulting blastocysts was similar to that of those developed in vivo. Cell counts indicated, however, that these cultured embryos were retarded in terms of total cell number compared to in vivo embryos of the same chronological age [9]. The embryos that developed in the mouse oviduct had a higher number of cells then those developed in Ham's F-12 medium, so it was concluded that the immature mouse oviduct provided a better culture environment. It was also hypothesized after differential staining that the lower cell number observed in cultured embryos was the result of retardation and cell death, since nuclear fragments were seen in several embryos. In the present experiments, in vivo embryos also had significantly higher cell numbers: they were one cleavage division ahead of the in vitro embryos, and they also had a higher ratio of ICM:TE cell numbers. Interestingly, in spite of their apparent retardation the cultured embryos were still developmentally competent. When transferred into recipient gilts following 4 days of culture, similar percentages of the cultured and in vivo embryos were competent for further development and establishment of pregnancy.

The mean number of ICM nuclei detected in the cultured embryos by Day 6 varied between 3.1 ± 0.40 (Table 2) and 8.2 ± 2.7 (Table 4). The reason for this is not known. It is likely that pig variation or slight differences in the culture conditions (replication variation) play a role. The possibility that transferring in vitro-developed embryos with a lower number of ICM nuclei would have resulted in a lower rate of development into conceptuses cannot be excluded.

Because of the nature of the staining technique it was not possible to determine the cell numbers of embryos prior to transfer, so one or two embryos from each group were randomly selected for differential staining and the rest were transferred into the surrogate gilts. Since the number of embryos available for transfer was fairly low in some cases, accessory corpora lutea were induced in recipients. In the pig, the presence of at least four conceptuses is needed to maintain pregnancy; if there are only two, the pregnancy may be terminated. The hormonal treatment used has been reported to cause a second set of corpora lutea to develop, thus extending the luteal phase to maintain a small litter [36].

It is well known that inadequate culture conditions cause cellular trauma in the embryo that can be manifested in different ways, and the overall result is the developmental retardation of the cultured embryo. Cellular functions such as embryo metabolism can be seriously impaired during culture [37–39], which in the mouse was shown to lead to lower viability after transfer [40]. Because of our insufficient understanding of the specific role of certain media components and the complex interactions between them (such as interactions between amino acids or between amino acids and carbohydrates [41]), a number of artifacts may be induced in vitro that do not exist under normal in vivo conditions. The NCSU-23 and KSOM/AA media were both able to support development of one- and two-cell porcine embryos through the so-called four-cell developmental block. This block that can be encountered using certain media is also an artifact caused by inadequate culture conditions. The cell numbers and the ratio of ICM to TE cells of the embryos cultured in these media were significantly lower compared to those of the embryos developed in vivo. The developmental potential of the embryos was not severely impaired, since they could establish pregnancy and develop into fetuses like their in vivo counterparts. Due to the low number of recipients and the low power of the statistical test (.054), we cannot safely compare the developmental potential of the cultured embryos to that of the in vivo embryos; we can only conclude that they were developmentally competent. All these observations suggest that we can culture early porcine embryos to the blastocyst stage without seriously compromising their developmental competence but are still not able to provide an environment that is comparable to in vivo conditions.

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