Advanced In Vitro Production of Pig Blastocysts Obtained through Determining the Time for Glucose Supplementation

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Abstract. The objective was to determine the effect of glucose supplementation on development (to the blastocyst stage) of in vitro matured (IVM) porcine oocytes that were either in vitro fertilized (IVF) or electrically activated (EA). Embryos were incubated for 46 or 58 h post insemination (hpi) in an NCSU37-based medium containing 0.17 mM sodium pyruvate and 2.73 mM sodium lactate (IVC-PyrLac), and then transferred to an NCSU37-based medium containing 5.55 mM glucose (IVC-Glu) and cultured until Days 6 (Day 0 = day of EA or IVF). The proportions of oocytes that had formed full blastocysts by Day 6 following transfer to IVC-glu at 46 hpi was 23.5 and 41.2% in the IVF and EA groups respectively; these were lower (P<0.001) than the proportions of oocytes that formed full blastocysts after transfer at 58 hpi (60.3 and 78.7%). However, there was no significant difference in total cell number (at Day 6) between embryos transferred at 46 vs 58 hpi. We inferred that in vitro-derived pig embryos can efficiently use glucose as an energy source starting at approximately 58 hpi; exposure to glucose at that time enhanced development to the blastocyst stage as well as blastocyst quality.

Key words: Pig embryo, Blastocyst, Glucose usage, In vitro fertilization, Activation

Swine are increasingly used in transgenic technologies, including gene manipulation and nuclear transfer; both of these technologies require in vitro production of large quantities of matured oocytes and embryos. Although substantial progress has been made in in vitro culture, maturation and fertilization (IVC, IVM and IVF, respectively) of porcine oocytes, the overall efficiency of in vitro production of porcine embryos remains relatively low. Several critical aspects should be considered, i.e. variation in oocyte sources and quality of IVM [1–6], and increased polyspermy [7, 8] and morphological abnormalities [4, 9] in porcine embryos produced by IVF. Although substantial efforts have been made to optimize culture conditions for preimplantation porcine embryos, the metabolic needs of the embryo are still not well understood. In many mammals, the utilization of glucose increases as preimplantation development progresses; glycolysis becomes the dominant pathway during preimplantation development [10–13]. In the pig (as in many other mammals), glucose utilization in highest in morulae and blastocysts [13, 14], consistent with the energy required for the formation and expansion of blastocysts. Furthermore, glucose supplementation enhanced in vitro development of pig embryos [4, 16, 17], with
the exception of the earliest preimplantation stages, where glucose appeared to have a deleterious effect on embryo development [4]. It is noteworthy that glucose was not compatible with in vitro development of preimplantation hamster embryos [18,19] and that development of mouse embryos to the blastocyst stage was supported by pyruvate but not glucose [20]. Despite substantial evidence that the earliest preimplantation stages of porcine embryos can be supported by pyruvate and lactate [4,8,21] and several other studies on the metabolism of pig embryos cultured in vitro [13,14,16], the exact time during the early cleavage stages when glucose is the preferred energy source has not yet been determined. We previously reported that glucose supplementation on Day 2 after fertilization enhanced in vitro development of in vitro-derived pig embryos [4]. The objective of the present study was to more precisely determine when glucose is a preferred energy source for in vitro produced porcine embryos.

Materials and Methods

Culture media

The culture media used in this study were essentially the same as those described previously [4]. Briefly, the medium used for oocyte maturation (IVM medium) was BSA-free NCSU-37 medium supplemented with 10% (v:v) porcine follicular fluid (collected from 3 to 6 mm follicles), 0.6 mM cysteine, 1 mM dibutyryl cAMP (Sigma Chemical Co., St. Louis, MO, USA), 10 IU/mL eCG (PMS 1000 Toni NZ; Nihon Zenyaku Kogyo, Koriyama, Japan), and 10 IU/mL hCG (Puberogen; Sankyo, Tokyo, Japan). The fertilization medium (Pig-FM) [22] consisted of 90 mM NaCl, 12 mM KCl, 25 mM NaHCO3, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 8 mM CaCl2, 10 mM sodium lactate (Kanto Chemical Co., Inc., Tokyo, Japan), 2 mM sodium pyruvate (Sigma), 5 mM caffeine, 10 mM Hepes, 5 mg/mL BSA (Fraction V; Sigma), and no antibiotics. The basic culture medium for embryo development (IVC) was NCSU-37 medium supplemented with 4 mg/mL BSA and 50 μM β-mercaptoethanol. This medium was supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate (IVC-PyrLac) or 5.55 mM D-glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (IVC-Glu).

Oocyte collection and IVM

Ovaries were collected from prepubertal gilts (Landrace × Large White) at a local abattoir and transported to the laboratory at 35 C. Immature oocytes were recovered by puncturing 3 to 6 mm follicles with a scalpel. Follicular content was collected in Medium 199 with Hanks salt (Gibco, Life Technologies Inc., Grand Island, NY, USA), supplemented with 10% (v:v) fetal bovine serum (Gibco), 20 mM Hepes (Dojindo Laboratories, Kumamoto, Japan), 100 U/mL penicillin G potassium (Sigma), and 0.1 mg/mL streptomycin sulfate (Sigma). Oocytes with a multilayered, compact cumulus and evenly granulated cytoplasm were selected and washed three times with IVM medium. Then, groups of 30 to 40 oocytes were placed into 0.5 mL of this medium in each well of 4-well dishes (Nuclon Multidishes; Nalge Nunc International, Denmark) and cultured under 5% O2, 5% CO2 and 90% N2 at 39 C. After 22 to 24 h, oocytes were washed twice and cultured in 0.5 mL of the same IVM medium (without dbcAMP, eCG and hCG) for 24 h under the same conditions.

In vitro fertilization, activation and embryo culture

Ejaculated spermatozoa were collected from a boar of the Landrace breed and frozen [23]. Groups of 15 to 20 matured oocytes surrounded by expanded cumulus cells were fertilized (insemination = Day 0) in vitro in 90-μL drops of Pig-FM, using frozen-thawed ejaculated spermatozoa that had been at a final concentration of 1 × 10^4 cells/mL. Four hours postinsemination (hpi), oocytes were freed from the cumulus cells and attached spermatozoa, and transferred into individual wells of a 4-well dish with 0.5 mL IVC-PyrLac medium. Some oocytes were fixed 10 to 11 hpi in acetic acid ethanol (1:3) to examine fertilization status. At 22 to 28 h of culture in IVC-PyrLac, cleaved embryos were selected and continuously cultured in the medium until 46 or 58 hpi, then transferred to IVC-Glu medium and cultured until Days 6 to 7.

For parthenogenetic activation, matured oocytes freed from cumulus cells were inspected for the presence of the first polar body, washed three times in a solution composed of 0.3 M mannitol solution (supplemented with 0.05 mM CaCl2 and 0.1 mM MgSO4) and then placed in an electroactivation chamber (FTC-23W; Shimadzu Co., Ltd., Osaka,
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Japan) filled with the same solution. Activation was achieved by application of a single electric pulse (1,500 V/cm, 100 µsec) created by a Somatic Hybridizer SSH-2 (Shimadzu). After being washed three times in IVC-PyrLac medium, the activated oocytes were incubated for 3 h in the presence of cytochalasin B (5.0 mg/mL; Sigma) to produce parthenogenetic diploids. Following culture, cleaved embryos were processed in the same way as fertilized embryos.

Embryo evaluation

On Day 6, embryo development was evaluated by counting the number of blastocysts (full blastocysts), malformed (part-blastocyst, single blastomere cavitation), and dead embryos. The total cell number in a blastocyst was determined after nuclear staining in 20 µg/mL Hoechst 33342 in IVC-PyrLac-PVA for 15 min. Blastocysts were then put on a microscopic slide, covered with a coverslip, and observed under a fluorescent microscope (Axioplan 2, Carl Zeiss Jena GmbH, Germany). Images were recorded digitally and analyzed using Adobe Systems Photoshop software.

Statistical analysis

The percentages of embryos classified as full blastocyst, malformed or dead were arc-sin transformed and the effect of time of transfer to IVC-Glu (46 vs 58 hpi) for IVF and for EA embryos was determined by Student’s t-test. Blastocyst cell number (at Day 6) was compared among the four groups by ANOVA. All statistical analyses were conducted with Smith’s statistical software (http://www.economics.pomona.edu/StatSite/framepg.html/).

Results

The experiment was replicated six times and the results are summarized in Table 1. The proportions of oocytes that had formed full blastocysts by Day 6 following transfer to IVC-glu at 46 hpi was lower (P<0.001) than the proportions of oocytes that formed full blastocysts after transfer at 58 hpi. Furthermore, the proportions of malformed and dead embryos were higher (P<0.001) in embryos transferred at 46 vs 58 hpi. However, there was no significant difference among the four groups in the total number of cells in the Day-6 blastocysts. Representative blastocysts are shown in Fig. 1. Most blastocysts in the EA group transferred at 58 hpi were large (140 to 180 µm) and of excellent quality (Fig. 1A) with an average cell number of 42.5 (Table 1, Fig. 1C).

Discussion

Unlike other mammalian embryos, porcine embryos can use glucose as their primary energy substrate throughout preimplantation development. Metabolic assays initiated at the earliest cleavage stages indicated that glucose metabolism and usage increased as preimplantation development progressed [13, 14, 15].

Table 1. Effect of time of first exposure to glucose (46 or 58 h post-insemination) on preimplantation development of pig embryos produced from in vitro matured oocytes by in vitro fertilization or electric activation

<table>
<thead>
<tr>
<th>Duration of culture</th>
<th>No. of cleaved oocytes</th>
<th>No. (%) of embryos classified as</th>
<th>Total no. of cells in Day 6 blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Full blastocyst</td>
<td>Malformed</td>
</tr>
<tr>
<td>In vitro fertilization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 h</td>
<td>532</td>
<td>125 (23.5%)</td>
<td>231 (43.4%)</td>
</tr>
<tr>
<td>58 h</td>
<td>667</td>
<td>402 (60.3%)</td>
<td>157 (23.5%)</td>
</tr>
<tr>
<td>Electric activation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 h</td>
<td>488</td>
<td>201 (41.2%)</td>
<td>170 (34.9%)</td>
</tr>
<tr>
<td>58 h</td>
<td>345</td>
<td>429 (78.7%)</td>
<td>66 (12.1%)</td>
</tr>
</tbody>
</table>

a Cell numbers are expressed as the mean ± SEM.
b,c For in vitro fertilization and for electric activation, within a column, percentages with a different superscript are different (P<0.001).
consistent with increased energy demands during maternal-to-zygotic transition, compaction, and blastocoele development. Data from metabolic measurement experiments were confirmed by results of the present study and a previous study [4] where glucose supported development of in vitro produced pig embryos until the blastocyst stage. Although in vivo derived pig embryos have developed to blastocysts in the presence of glucose [16, 17, 24], it was recently reported that pig embryos produced under current in vitro conditions have altered metabolic activity [13] that may compromise their viability.

Preincubation of IVM-IVF embryos in the presence of pyruvate and lactate and subsequent culture in a glucose-supplemented medium enhanced preimplantation development, with the production of high-quality blastocysts and the birth of piglets [4]. In the present study, the developmental potency of IVM-IVF and IVM-EA porcine embryos and the quality of blastocysts obtained varied significantly, depending on when exposure to glucose began. Exposure to glucose starting 58 hpi facilitated the development of both EA- and IVF-produced embryos; the majority (78.7 and 60.3%, respectively) developed into full blastocysts (with average cell numbers of 42.9 and 45). However, earlier (46 hpi) exposure of both EA- and IVF-produced embryos resulted in roughly half the rate of full blastocyst formation, and concurrently a substantial increase in the incidence of malformed and dead embryos.

In metabolic measurement studies, glycolysis was detected in in vitro produced porcine embryos.
at the 2-cell stage (20 to 22 h after fertilization) [13].
Perhaps the deleterious effects of early glucose
supplementation are due to the cytotoxic effect of
methylglyoxal, a metabolic by-product of
glycolysis that inactivates the intracellular
 glutathione peroxidase responsible for scavenging
 oxygen free radicals [25]. Therefore, glucose
 supplementation during very early stages of
development may cause oxidative stress on
genome activation or difficulty in overcoming the
four-cell block (at about Day 2 of IVC of porcine
embryos). Methylglyoxal is detoxified by
glyoxalase I [26]; perhaps this enzyme is minimally
active in early porcine embryos, with a substantial
increase in activation at approximately 58 hpi,
corresponding to the late four-cell stage (third cell
cycle after fertilization), when activation of the
porcine embryonic genome begins [27]. Similarly,
the presence of glucose in the culture media before
embryonic genome activation had a detrimental
effect on in vitro development of bovine
preimplantation embryos [28]. That porcine
embryos can efficiently utilize glucose as an energy
starting around 58 hpi is consistent with metabolic
measurement experiments that showed porcine
embryos can use glucose via glycolysis from the
earliest stages of the preimplantation development,
with an increase in glycolytic activity after the
eight-cell stage (78 to 80 h of culture) for
in vitro-derived embryos, and at the blastocyst stage for in vivo-derived embryos [13].
Although there was an apparent beneficial effect
of preincubating embryos in the presence of
pyruvate and lactate prior to exposure to glucose in
the present study, the importance of pyruvate and
lactate throughout preimplantation development of
porcine embryos has not been established. In
that regard, these compounds did not unilaterally
support the high developmental capacity of IVM-
IVF pig embryos to the blastocyst stage previously
observed [4, 16, 29, 30]. Furthermore, metabolic
measurement experiments showed minimal use of
pyruvate by both in vitro- and in vivo-derived
embryos until the blastocyst stage [13]. However,
pyruvate may offer the embryo protection against
the stress of the environment in vitro as an
antioxidant [31]. Lactate and pyruvate are the
preferred energy substrates at the early cleavage
stages in other species such as mice [20], cows [12,
28], sheep [10] and humans [32, 33]. In the pig,
these chemicals may be beneficial at the beginning
of preimplantation development, supporting
minimal energy needs until the proper usage of
glucose as the energy source begins.
In conclusion, including glucose in the culture
medium starting at 58 hpi resulted in the majority
of IVM-IVF and IVM-EA porcine embryos
developing to the full blastocyst stage, although
including glucose at 46 hpi substantially reduced
the proportion of embryos that reached the full
blastocyst stage, with a corresponding increase in
the number of malformed and dead embryos.
These findings provide insight into the regulation
of metabolic processes in early porcine embryos
and may improve the efficiency of in vitro
production of large quantities of these embryos for
biomedical research.

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