Three-to-5-day weaning-to-estrus intervals do not affect neither efficiency of collection nor in vitro developmental ability of in vivo-derived pig zygotes

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Revised

Three-to-5-day weaning-to-estrus intervals do not affect neither efficiency of collection nor *in vitro* developmental ability of in vivo-derived pig zygotes

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Abstract

An efficient system to collect large numbers of vital zygotes is a pre-requisite for application of zygote genome-editing technology, including development of efficient models for xenotransplantation using pigs. Owing to the sub-optimal *in vitro* production of zygotes in pigs, efficient collection of *in vivo* developed zygotes is required. Timing of ovulation is a key factor to sustain efficiency since the interval between pronuclear formation and the first division is very short in pigs. The weaning-to-estrus interval can, due to its inverse relation with length of estrus and time of ovulation, interfere with ovulation and make it asynchronous, which reduces the probability of obtaining zygotes. This retrospective study compared the effects of three weaning-to-estrus intervals of 3, 4 or 5 days on zygote collection efficiency in a total of 17 trials over a 3-year period including 223 sows. Donor sows in groups of 10-15 animals were superovulated with eCG 24 h after weaning and those in estrus at 48–72 h post-eCG were immediately treated with hCG, followed by insemination 6 and 24 h thereafter. Collected structures during laparotomy on Day 2 (Day 0: onset of estrus) were morphologically evaluated and only those with a single cell and two visible polar bodies were considered as zygotes. Zygotes were injected with CRISPR-Cas9 editor mixture and cultured for 6 days to evaluate their developmental ability against non-injected control zygotes. Of all recovered structures (N=5,468), 67.4%, 30.8% and 1.8% were zygotes, 2-cell embryos and oocytes-degenerated embryos, respectively. The different weaning-to-estrus intervals did not affect either the percentages of collected zygotes (range: 64.1% to 70.0%) or the percentages of sows with zygotes at collection time (range: 69.0% to 73.3%). The weaning-to-estrus intervals did not affect the *in vitro* developmental ability of zygotes. After 24 h of culture, $78.1 \pm 2.0\%$ and $95.1 \pm 0.6\%$ ($P < 0.05$) of injected (N=2,345) and non-injected (N=335) zygotes, respectively, developed to 2-to-4-cell embryo stage. The total efficiency of the system was $64.1 \pm 2.2\%$ and $85.8 \pm 1.5\%$ ($P < 0.05$) for injected and non-injected zygotes, respectively. In conclusion, the results indicate that neither the efficiency of collecting *in vivo* derived porcine zygotes from superovulated sows nor the zygote ability to develop to blastocyst after cytoplasmic genome-editing injection were affected by a weaning-to-estrus interval between 3-to-5 days.

Keywords: zygote, pig, embryo collection, CRISPR-Cas9, cytoplasmic injection, weaned to estrus interval

1. Introduction

Zygote genome-editing engineering with programmable nucleases has initiated a revolution in agriculture and biomedical research, where pig is one of the most popular large animal models due to its similitude to humans in anatomy, organ size, physiology, and genomic and cell cycle characteristics [1]. Zygote genome-editing technology requires efficient methods to obtain large numbers of good-quality zygotes. Porcine zygotes can be produced by *in vitro* fertilization (IVF) of oocytes matured *in vitro*. However, and despite progress achieved in the last decades, current porcine IVF procedures remain inefficient and are associated with high levels of polyspermy and low embryo quality and blastocyst formation rates compared with *in vivo*-derived embryos [2-4]. For these reasons, *in vivo*-derivation is currently the main source of porcine zygotes for zygote genome-editing [5]. Surprisingly, information available on the efficiency of the procedures for collection of *in vivo*-derived porcine zygotes is scarce.

Post-weaned primiparous and multiparous sows have great potential as a zygote source for zygote genome-editing porcine programs, since weaning is an efficient physiological method of synchronizing estrus and ovulation in a group of sows. Most weaned sows exhibit a fertile estrus between 3 and 5 days post-weaning, with more than 90% exhibiting estrus within 7 days post-weaning [6,7]. In addition, superovulation with equine chorionic gonadotropin (eCG) 24 h after weaning followed by human chorionic gonadotropin (hCG) at the onset of estrus increases the number of viable embryos without affecting the number of unfertilized oocytes or the quality of the collected embryos [8].

Timing of ovulation is a key factor for the efficient recovery of *in vivo* zygotes. Since male and female pronuclei are formed 3 to 5 h post-fertilization, and the first division occurs 14–16 h later [9], zygote collection has to be accomplished over a very narrow window of time. In addition, there is a high variability in the ovulation time between individual sows [10], even after hCG treatment [11], which reduces the chance of obtaining embryos at the zygote stage during collection. In this context, the weaning-to-estrus interval plays an important role because it has a strong inverse relationship with length of estrus and time of ovulation [6,10,12]. A weaning-to-estrus interval longer than 6 days or shorter than 2 days decreases reproductive performance of sows [13,14] probably as consequence of alterations in the duration of estrus and time of ovulation. Moreover, high variability (10 h to 60 h) in the interval between the onset of

estrus and period of ovulations has been also reported in sows with a weaning-to-estrus interval of 3 to 6 days [15]. For practical reasons, zygote collection programs normally use donor sows with a specific weaning-to-estrus interval (3 to 5 days) and these sows are usually grouped and not examined independently, which strongly limits our information on relevant embryo parameters.

Because these weaning-to-estrus intervals could still influence the duration of estrus and the timing of ovulations, this study compared the effects of three weaning-to-estrus intervals (3, 4 or 5 days) on the efficiency of zygote collection in donor sows superovulated after weaning and on the *in vitro* developmental ability of genome-editing injected zygotes to achieve the blastocyst stage.

2. Materials and Methods

2.1. Animals

This work was conducted in a pig farm located in Southeastern Spain (Agropor S.L., Region of Murcia, Spain). Weaned sows (Landrace × Large-White) from 2 to 7 parities were selected as donors of zygotes. The sows were housed in individual gestation stalls in a mechanically ventilated facility. Mature boars (1-3 years of age) from a commercial artificial insemination station (AIM Iberica, Murcia, Spain) were used as semen donors. The animals were fed commercial diets according to their nutritional necessities and had *ad libitum* access to water. All the experimental procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments and were reviewed and approved by the Ethical Committee for Experimentation with Animals (code: 69/2014), the Research Ethics Committee (code: 1086/2015) of the University of Murcia, Spain; the Murcia Regional Ministry of Agriculture and Water (code: 273.705), and the Murcia Regional Ministry of Health (code: 061015), Spain.

2.2. Superovulation, detection of estrus and insemination of donors

Weaning was used to synchronize the estrus in donor sows. Only sows with a weaning-to-estrus interval of 3 to 5 days were selected as donors. Superovulation was performed using eCG (1000 IU, im) (Foligon, Intervet, Boxmeer, The Netherlands) 24 h post-weaning. Estrus was checked twice per day (07:00 h and 16:00 h) by snout-to-snout contact with a mature boar while applying manual backpressure. Sows with well-defined signs of estrus at 48–72 h post-eCG were treated with hCG (750 IU, im)

(Veterin Corion, Divasa, Farmavic S.A., Barcelona, Spain) at the onset of estrus. Donors were post-cervically inseminated at 6 and 24 h after the onset of estrus with 1.5×10^9 fresh spermatozoa extended in 45 mL of Beltsville thawing solution extender [16].

2.3. Embryo collection and evaluation

Zygote collections were carried out in an operating room inside the farm at 48-52 h after onset of estrus. Sows were sedated with azaperone (2 mg/kg body weight, im) and anesthetized with sodium thiopental (7 mg/kg body weight, iv). Anesthesia was maintained with isoflurane (3-5% in air) and buprenorphine (0.3 mg/sow, im) was used as post-surgical analgesic drug. The genital tract was exposed through mid-ventral laparotomy, and the number of corpora lutea on each ovary was counted. Zygotes were retrieved by flushing each oviduct with 30 mL of modified Tyrode's lactate-HEPES-polyvinyl alcohol medium (TL-HEPES-PVA) [11,17]. Collected structures were washed, placed in tubes containing 1.5 mL of the same medium and transported at 39 °C to our laboratory at the University of Murcia within 1 h after collection. Embryos were then carefully assessed under a stereomicroscope at a magnification of 60 x and only embryos with a single cell and two visible polar bodies were considered as zygotes.

2.4. Intracytoplasmic injection of zygotes

Zygotes were injected by using the laser-assisted cytoplasmic microinjection protocol previously described by [18]. Briefly, the zygote was immobilized by suction with a holding pipette and positioned with the polar bodies at either 6 or 12 o'clock. Then a hole was created in the zona pellucida at 3 o'clock position by using a laser and an injection pipette was inserted through the hole and advanced into the zygote until the plasma membrane was broken by aspiration of ooplasm into the injection pipette. Then, the aspirated ooplasm and the editor mixture (100 ng/ μ L Cas9 mRNA and 20 ng/ μ L sgRNA) were injected into the cytoplasm.

2.5. In vitro embryo culture and assessment of in vitro embryo development

After injections, zygotes were placed into a 4-well multi-dish (40 zygotes per well) containing 500 μ L of NCSU-23 medium [19] supplemented with 0.3 mM pyruvate and 4.5 mM lactate for 24 h and then transferred to fresh NCSU-23 medium supplemented with 5.5 mM glucose for 5 additional days. Embryos were cultured at 39 °C, 5% CO₂ in

air and 95–97% relative humidity. At Day 5, culture medium was supplemented with 10% fetal calf serum. Embryo development was assessed under a stereomicroscope at 24 h and 144 h of culture to determine cleavage and blastocyst formation rates, respectively.

2.6. Experimental design

In this retrospective study, data related to the zygote collection was recorded over a 3-year period in 17 trials using a total of 223 sows with a lactation length of 21.8 ± 0.1 days and good body condition and reproductive history. In each trial, groups of 10-15 sows with a weaning-to-estrus interval of 3, 4 or 5 days were selected as donors. Donors from the three groups in each trial were inseminated with sperm doses from the same boar. To evaluate the effect of the weaning-to-estrus intervals on the efficiency of zygote collection, the numbers of viable zygotes, 2-cell embryos and oocytes/degenerated embryos recovered in each donor were counted. Recovery rate was defined as the ratio of the number of embryos and oocytes/degenerated embryos recovered to the number of corpora lutea present. Fertilization rate was defined as the ratio of the number of viable embryos to the total number of embryos and oocytes/degenerated embryos collected. Within each group, zygotes collected from each sow were genome-editing injected and cultured *in vitro* to evaluate their ability for blastocyst development compared to control non-injected zygotes. An embryo that had cleaved to the 2-to-4-cell stage was defined as cleaved, and an embryo with a well-defined blastocoel and an inner cell mass and trophoblast totally discernible was defined as a blastocyst. The cleavage rate was the percentage of zygotes that had divided to the 2-to-4-cell stage. Blastocyst formation was the percentage of 2-to-4-cell embryos that developed to the blastocyst stage. The total efficiency was described as the percentage of the total number of cultured zygotes that reached the blastocyst stage.

2.7. Statistical analysis

The data were analysed with the IBM SPSS 24.0 Statistics package (IBM, Chicago, IL, USA). Percentage data were compared using Fisher's exact test. The continuous variables were evaluated using the Kolmogorov–Smirnov test for normality and groups were compared by ANOVA or Student's t-test, as appropriate. The ANOVA model included the main effect of weaning-to-estrus interval and the random effect of the replicate. When the ANOVA indicated a significant effect, the means were compared

by Bonferroni's test. Differences were considered significant at $P < 0.05$. The results are presented as mean $\pm$ SEM.

3. Results

A total of 217 out of 223 donors (97.3%) had embryos at collection. Neither mean ovulation rate (27.3 ± 0.5 corpora lutea) nor the mean number of structures (25.2 ± 0.6) collected in these sows differed between weaning-to-estrus interval groups ($92.3 \pm 1.0\%$ total recovery rate) (Fig. 1). Of all recovered structures ($N = 5,468$), $67.4 \pm 2.9\%$, $30.8 \pm 2.8\%$ and $1.8 \pm 0.4\%$ were zygotes, 2-cell embryos and oocytes-degenerated embryos, respectively (98.2% fertilization rate). The different weaning-to-estrus intervals did neither affect the percentages of collected zygotes (range: 64.1% to 70.0%) (Fig. 2) nor the percentages of sows with zygotes at the collection (range: 69.0% to 73.3%) (Fig. 3). Table 1 shows the total efficiency of the procedure.

The developmental competence of injected zygotes to the blastocyst stage is shown in Table 2. The weaning-to-estrus intervals did not affect the *in vitro* developmental ability of injected zygotes. After 24 h of culture, $78.1 \pm 2.0\%$ and 95.1 ± 0.6 ($P < 0.05$) of injected and non-injected zygotes, respectively, developed to 2-to-4-cell embryo stage and $82.0 \pm 1.6\%$ and $90.2 \pm 0.7\%$ of them, respectively, achieved the blastocyst stage by day 6 of culture. The total efficiency of the system was $64.1 \pm 2.2\%$ for injected and $85.8 \pm 1.5\%$ for non-injected control zygotes ($P < 0.05$) (Fig. 4).

4. Discussion

The present study demonstrates that weaning-to-estrus intervals between 3 and 5 days do not affect neither the efficiency of collecting *in vivo* derived porcine zygotes nor the zygote ability to develop to blastocyst after cytoplasmic injection and further *in vitro* culture for 6 days. In addition, herein we describe an efficient protocol for obtaining high-quality porcine zygotes from post-weaning multiparous sows.

To develop a practical and effective protocol for zygote collection, we decided to use *in vivo* derived zygotes and post-weaning multiparous sows as embryo donors by two main reasons: firstly, although the production of porcine zygotes *in vitro* may be a good alternative with lower costs and shorter time compared with *in vivo* collection, the current IVF systems remain sub-optimal; and secondly, although zygotes can be obtained *in vivo* from prepubertal and pubertal gilts, we chose multiparous sows firstly because superovulation treatments in prepubertal gilts and synchronized mature gilts

produced high proportions (25-50%) of poor quality embryos, unfertilized oocytes and degenerated embryos [20-23] as well as increased embryonic losses post-implantation [24,25]. It was suggested that a percentage of ovulated oocytes in synchronized-superovulated females remains in the germinal vesicle stage (immature) and would not be fertilized [26,27]. To confirm this speculation, we collected over 40% of immature oocytes on day 6 of the cycle from pubertal gilts superovulated with 1000 IU eCG at the end of the synchronization treatment, in previous experiments (unpublished). Together, these factors make inadvisable the use of these female categories as embryo donors. Secondly, superovulation with eCG 24 h after weaning followed by a dose of hCG at the onset of estrus markedly increased the number of viable embryos without negative effects on the number of unfertilized oocytes or the quality of the collected embryos [8]. Moreover, high reproductive performance of recipients was observed after non-surgical transfers of superovulated embryos collected from post-weaning sows [8,28], indicating that these embryos retained their potential to develop to term.

In the present study, we only used weaned 2 to 7 parity sows as donors due to their high accessibility on-farm and because they usually have farrowing rates and litter sizes higher than those of primiparous sows. Moreover, primiparous sows have longer weaning-to-estrus intervals than multiparous sows [29-31], which is associated with differences in duration of estrus and timing of ovulation [13]. Differences in these parameters between primiparous and multiparous sows could be large enough to have important implications for the programming of protocols for zygote collection.

We only selected donor sows with weaning-to-estrus intervals between 3-to-5 days because more than 80% of the superovulated donor sows start estrus within that period after weaning [8]. Therefore, the laparotomies for collecting embryos (which were performed in all cases at day 2 after onset of estrus) could be concentrated in only three consecutive days in each trial. Although weaning-to-estrus interval play an important role in the duration of estrus and hence in the time of ovulations [10], the results of the present study indicate that weaning-to-estrus intervals between 3-to-5 days did not affect the percentage of sows with zygotes at surgical collection, which suggest that such weaning-to-estrus intervals did not influence the period of ovulations. This apparent discrepancy can be justified by the extension of the weaning-to-estrus interval. Thus, while short (< 2 days) or long (> 6 days) weaning-to-estrus intervals decrease reproductive performance of sows [13,14] by increasing the probability of too early or

post-ovulatory inseminations, weaning-to-estrus intervals between 3 and 5 days were associated to high fertility and prolificacy [32].

Embryos at the zygote stage could be collected from approximately 60% of the sows and 30% and 10% of the remaining sows had cleaved embryos and a mixed of zygotes and cleaved embryos at collection, respectively. These results indicate that despite of the weaning and superovulation treatment used, ovulations occurred before than expected in some sows. Although variability in the ovulation period among sows is reported [10], even among sows with weaning-to-estrus intervals from 3 to 5 days [15], other factors could be implicated. In our study, the estrus detection was performed twice per day and that it is likely that some sows diagnosed in estrus in the morning could really had started estrus even 12 h earlier, which would affect ovulation time and, consequently, the developmental stage of the embryos at collection.

Our results also indicate that weaning-to-estrus intervals did not affect the developmental ability of the zygotes to achieve the blastocyst stage. In addition, with the protocol described here, we obtained a high global efficiency of blastocyst formation (number of blastocysts formed to the number of zygotes used) (~64%). That efficiency was much higher than those previously reported using parthenogenetic embryos [33,34] or IVF presumed zygotes [35] where the efficiency of blastocyst formation rates ranged between 10% and 15%. These results indicate that the number of blastocysts formed from *in vivo* derived zygotes may be approximately six times greater compared to those formed from *in vitro* produced embryos.

In the present study, the developmental ability of injected zygotes (~64%) was lower than that of non-injected counterparts (~85%), suggesting that zygote cytoplasmic injection had an adverse effect on further embryonic development. This can be due to differences in the sensitivity to the culture conditions between injected and non-injected zygotes, as well as the culture environment more easily affects nuclear transfer than IVF produced embryos [36]. Our finding disagree with those obtained using parthenogenetic and IVF embryos where no differences in embryo development were reported between genome-editing injected and control non-injected 1-cell embryos [33-35]. Differences in the sensitivity to manipulation during genome-editing injection or to culture conditions between *in vitro* produced and *in vivo* developed zygotes could be responsible for that discrepancy.

4.1. Conclusions

In conclusion, this study clearly demonstrates that weaning-to-estrus interval (3 to 5 days) did not affect neither the percentages of sows with embryos at the zygote stage at the collection nor the number and quality of the collected zygotes. After genome-editing injection, 80% of the zygotes cleaved by 24 h of culture and 80% of them developed to the blastocyst stage by day 6 of culture. The protocol used in this study was very reliable through the different trials (n=17) and could be used in multiple applications where high-quality zygotes are needed. Post-weaning sows could be super-ovulated and inseminated on (commercial) farms and the laparotomies for zygote collection could be performed on the same farm or after transportation of the animals to an experimental unit. Because the donors can be healthy weaned sows with five or more parities, just before they are culled for farm strategies, the cost of the animals is low and the available number of animals is high.

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Author contributions

JC Izpisua and EA Martinez conceived and designed the study. EA Martinez, H Rodriguez-Martinez, C Cuello and MA Gil directed the experiments. CA Martinez, JM Cambra, I Parrilla, X Lucas, EA Martinez, C Cuello and MA Gil performed the experiments. CA Martinez, JM Cambra, I Parrilla, EA Martinez, H Rodriguez-Martinez, C Cuello and MA Gil analysed and interpreted the data. CA Martinez wrote the manuscript. I Parrilla, EA Martinez, H Rodriguez-Martinez, JC Izpisua, C Cuello and MA Gil revised and discussed the manuscript. All authors read and approved the manuscript for publication. JC Izpisua, MA Gil and EA Martinez secured funding.

Declaration of interest

None of the authors have any conflicts of interest to declare.

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Figure legends

Figure 1. Influence of weaning-to-estrus-interval (WEI) on the reproductive parameters in fertilized donor sows.

Figure 2. Influence of weaning-to-estrus-interval (WEI) on the frequency distribution of zygotes, 2-cell embryos and oocytes retrieved from donor sows.

Figure 3. Influence of weaning-to-estrus-interval (WEI) on the frequency distribution of sows with embryos at different stages at the collection.

Figure 4. *In vitro* developmental efficiency of *in vivo* derived zygotes microinjected with Cas9 mRNA/sgRNA

Table 1. Global efficiency of the protocol used for zygote collection from superovulated weaned sows (n= 217)

Parameter	
Corpora lutea (mean±SEM)	27.3±0.5
Oocytes and embryos collected (mean±SEM)	25.2±0.6
Percentage of zygotes collected (%)	67.4
Percentage of 2-cell embryos collected (%)	30.8
Percentage of oocytes and degenerated collected (%)	1.8
Sows with only zygotes (%)	59.4
Sows with zygotes and 2-cell embryos (%)	12.4
Sows with only 2-cell embryos (%)	28.1

Table 2. Influence of weaning to estrus interval on the *in vitro* developmental ability of *in vivo* derived zygotes microinjected with Cas9 mRNA/sgRNA

Weaned to estrus interval (days)	Injected zygotes (N)	Cleavage rate N (mean±SEM) [#]	Blastocyst formation rate [*] N (mean±SEM) [#]	Efficiency rate ^{**} N (mean±SEM) [#]
3	799	620 (78.6±3.4)	512 (84.0±3.0)	512 (63.6±3.5)
4	1312	1009 (77.5±2.9)	840 (82.4±1.9)	840 (64.4±3.1)
5	234	195 (81.3±4.8)	149 (79.9±3.6)	149 (63.0±4.8)

^{*}Number of blastocysts/total of cleaved embryos. ^{**}Number of blastocyst/total of zygotes injected. [#]Mean±SEM from 17 trials.