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Production of Middle White Piglets after Transfer of Embryos Produced *In Vitro*

Koji MISUMI[1)], Yuri HIRAYAMA[1)], Misae SUZUKI[1)#], Michiko NAKAI[2)], Hiroyuki KANEKO[2)], Junko NOGUCHI[2)] and Kazuhiro KIKUCHI[2)]

[1)]*Department of Technology, National Livestock Breeding Center, Fukushima 961-8511, Japan*
[2)]*Division of Animal Sciences, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan*
[#]*Present: National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan.*

Abstract. The present study was conducted to examine the feasibility of *in vitro* embryo production and transfer technologies for producing Middle White piglets. After collection from three retired Middle White sows, a total of 222 oocytes were matured, fertilized and cultured *in vitro*, and a total of 50 embryos from the 4-cell to blastocyst stage were produced by the 4th or 5th day. These embryos were transferred individually into three recipients along with 5 *in vivo*-derived Duroc blastocysts. All of the recipients became pregnant, and they farrowed a total of 9 Middle White and 9 Duroc piglets. These results suggest that *in vitro* embryo production using ovaries from retired sows is useful for reproduction of pigs of pure breeds including the Middle White for breeding activities and conservation/utilization of genetic resources.
Key words: Embryo transfer, *In vitro* embryo production, Middle White, Piglet

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Recently, *in vitro* embryo production (IVP) of porcine species has become possible, resulting in piglet production in laboratories worldwide [1−9]. This technology is expected to contribute to various areas of porcine reproductive technology and is also expected to contribute to production of piglets of pure breeds for breeding activities. For efficient embryo or piglet production, a specialized IVP method for each pig breed has been expected to be established. To our knowledge, however, even IVP using ovaries resulting in production of piglets has been limited in the Large White [1], whereas other successes have been in crossbreeds or unidentified breeds [2−9]. In addition, the ability to utilize ovaries or oocytes from retired sows has been desired. This would offer the possibility of an additional chance for oocyte collection, thus contributing to production of the next generation. However, this has been reported only in our laboratory using the Duroc breed [10].

One of the rare and endangered breeds is the Middle White (or Middle Yorkshire). This breed of pig was established in 1884 in England and was first exported to Japan in 1906. In the world, they exist in only Great Britain and Japan. It has good meat qualities, shows early maturation and is easier to manage than other pig breeds because of its smaller and more compact body size (http://www.britishpigs.org.uk/breed_mw.htm). These characteristics make it advantageous for small farmers, and it became the most dominant breed (over 80%) in Japan around the mid 1960s. Thereafter, however, other commercial breeds such as the Large White and Landrace were introduced into Japan because of their advantages for large-scale pig farming, and by the 1970s, these breeds had largely replaced the Middle White in Japan; in 2010, the reported remaining sow population was 270 (calculated from the report of the Japan Pork Producers Association, http://www.jppa.biz/). The Middle White has been highly valued in Japan because of "its outstanding meat qualities", as the meat is considered very special in terms of its fat content. However, the Middle White has been kept only in small numbers in limited areas and purchased as a "brand pork" with added value (e.g., *koza-ton* in Kanagawa Prefecture, Japan). In Great Britain, a similar situation has been reported by the British Pig Association, with the total number of this breed being just over 100 in 2012 (http://www.britishpigs.org.uk/).

The present report describes the potential utility of conventional IVP and embryo transfer technologies using ovaries from retired sows for the Middle White, a pure breed, the genetic resources of which are very limited worldwide.

As shown in Table 1 and Fig. 1, when a total of 50 Middle White IVP embryos and 15 Duroc *in vivo*-derived embryos were transferred into 3 recipients, all the recipients became pregnant and farrowed to term, resulting in 9 Middle White and 9 Duroc piglets. However, one of the recipients farrowed only Duroc piglets. Two of the 9 Middle White piglets died purely through accidents in handling and in fact showed no abnormality. The other 7 piglets grew normally and produced a subsequent generation without any problems.

As described above, most successful attempts at piglet production using IVP have been limited to crossbreeds or unidentified breeds [2−9]. On the other hand, successful embryo production in pure Large White pigs has been reported [1], and recently, we have also achieved this for the Duroc breed in our laboratory, but piglet production has not reported yet [10]. The present study clearly demonstrates that the conventional IVP procedure can improve the chance for reproduction in pure breeds and offers the opportunity

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Correspondence: K Kikuchi, (e-mail: kiku@affrc.go.jp)

Table 1 *In vitro* production and transfer of MW embryos

MW			*In vitro* development of MW embryos					Embryo transfer			
No.	Age (mo)	No. oocytes subjected to IVM	No. (%) oocytes completed IVF	No. (%) cleaved embryos on day 2	No. (%) 4-cell embryos ≤ on day 4	No. (%) on day 5		No. embryos transferred		No. of piglets born Total (male + female)	
						4-cell embryos to morulae	Blasto-cysts	MW	Duroc	MW	Duroc
1	25	82	46 (56.1)	27 (58.7)	17 (37.0)	11 (23.9)	6 (13.0)	17**	5$^{\$\$}$	4 (0 + 4)	2 (2 + 0)
2	19	75	48 (64.0)	28 (58.3)	18 (37.5)	–	–	18*	5$^{\$}$	5 (2 + 3)	3 (0 + 3)
3	21	65	45 (69.2)	24 (53.3)	15 (33.3)	12 (26.7)	3 (6.7)	15**	5$^{\$\$}$	0	4 (1 + 3)
Total		222	139 (62.6)	79 (56.8)	50 (36.0)	23 (25.3)$^{\#}$	9 (9.9)$^{\#}$	50	15	9 (2 + 7)	9 (3 + 6)

MW, Middle White; IVM, *in vitro* maturation; IVF, *in vitro* fertilization. Day 0 was defined as the day of IVF. The MW embryos on days 4* and 5** and Duroc embryos on days 5$^{\$}$ and 6$^{\$\$}$ after artificial insemination were transferred. $^{\#}$The percentages were calculated using the numbers of oocytes that completed IVF in MW sows Nos. 1 and 3 (=91).

to utilize oocytes from slaughtered pigs, thus extending the scope of regular reproduction or breeding activities. In IVP from cycling gilts or sows, the most appropriate timing for oocyte collection is reported to be around the 16th day after onset of estrous (estimated as the late luteal to follicular stage in ovaries) [10]. In the present study, we collected oocytes at around this time, and the blastocyst production rate was 9.9% on day 5, being lower than the value on day 6 (19.2%) reported previously for the Duroc breed. Although the timing of evaluation after embryo culture differed between the two studies, the discrepancy between the two values may be attributable to the difference in breed. Further research on this issue to confirm the specialized IVP method for each pig breed will be necessary.

The efficacy of piglet production is related to the number of viable embryos before implantation [11]. It is generally accepted that pigs have a unique reproductive characteristic, i.e., sows require a critical pregnancy signal from several embryos in order to establish and maintain pregnancy [12]. Usually, successful piglet production by IVP using embryos generated from crossbred pigs is feasible after transfer of 18−25 embryos [13]. In the present study, we obtained a slightly lower number (15−18) of Middle White IVP embryos. To ensure a solid pregnancy and farrowing, in consideration of the fact that the embryos were generated in a pure breed, we cotransferred Duroc *in vivo*-derived embryos because it has already been reported that cotransfer of parthenogenetic oocytes is effective for assisting pregnancy involving a small number of *in vivo*-derived [14] and ICSI [15] embryos.

In conclusion, IVP procedures will be useful for reproduction of pigs of pure breeds including the Middle White for breeding activities and conservation/utilization of genetic resources. In the future, cryopreservation of oocytes and embryos will enhance the value of IVP technology.

Methods

Animals

All animals used in the present study were maintained and fed in accordance with the manuals issued by the Animal Care and Use Committee of the National Livestock Breeding Center (NLBC). Middle White sows at least 19 months old and Duroc gilts around 10 months old were used as oocyte and embryo donors, respectively.

Fig. 1. Piglets generated from *in vitro*-produced Middle White blastocysts (4 head) and *in vivo*-derived Duroc blastocysts (2 head). These piglets were born from a single recipient (Recipient No. 1 in Table 1). The photo was taken 1 day after birth.

Landrace × Large White crossbred gilts around 8 months old were used as recipients for the embryos.

IVP of Middle White embryos

Three Middle White sows were checked for signs of estrus (standing to be mounted, congested and swollen vulva and clear mucus streaming from the vulva) and slaughtered on the 16th day after onset of the natural estrous cycle in an abattoir located at the NLBC. Their ovaries were preserved for 1 h at 38 C in Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical, Tokyo, Japan). Oocyte collection, *in vitro* maturation and fertilization were carried out as described previously [9, 11]. The ovaries were washed several times, and the hila were removed with scissors. All follicles were opened with a surgical blade in Medium 199 (with Hanks' salts; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan) and antibiotics (100 IU/ml penicillin G potassium (Sigma-Aldrich) and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich)). Cumulus-oocyte

complexes (COCs), in which the cumulus cells comprised several layers, were collected. The basic maturation medium was bovine serum albumin (BSA)-free NCSU-37 solution [16] containing 10% (v/v) fetal bovine serum, 0.6 mM cysteine, 50 μM β-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan), 10 IU/ml hCG (Puberogen 500 U; Novartis Animal Health, Tokyo, Japan) and antibiotics. Approximately 20 COCs were cultured in 100 μl of maturation medium in 35-mm plastic dishes (TC dish 35 × 10, Nalge Nunc International, Roskilde, Denmark) for 20−22 h. They were then cultured in each medium without dbcAMP and hormones for 24 h. Maturation culture was carried out at 39 C under CO_2 and N_2 adjusted to 5% and 90%, respectively (5% O_2).

In vitro fertilization (IVF) was carried out using the COCs after *in vitro* maturation. The fertilization medium used for porcine oocytes was Pig-FM [17], with addition of 2 mM caffeine and 5 mg/ml BSA (Fraction V, Sigma-Aldrich). The COCs were then transferred to fertilization medium.

The ejaculated spermatozoa used for IVF were collected from a 2 year-old Middle Yorkshire boar and then frozen [18]. These spermatozoa were thawed and preincubated for 15 min at 37 C in Medium 199 (with Earl's salts, Gibco) adjusted to pH 7.8 [19]. A portion (10 μl) of the preincubated spermatozoa was introduced into 90 μl of fertilization medium containing about 20 COCs. The final sperm concentration was adjusted to 1×10^5/ml. Coincubation was carried out for 3 h at 39 C under 5% O_2. The cumulus cells and/or spermatozoa attached to the zona pellucida were removed mechanically with a fine-bore pipette. *In vitro* culture (IVC) was carried out as described previously [2]. Two types of medium were prepared: (1) IVC-PyrLac (which was NCSU-37 containing 0.4% (w/v) BSA, 50 μM β-mercaptoethanol, 0.17 mM sodium pyruvate and 2.73 mM sodium lactate) was used from day 0 (the day of IVF was defined as day 0) to 2; and (2) IVC-Glu (which was NCSU-37 containing 0.4% (w/v) BSA, 50 μM β-mercaptoethanol and 5.55 mM glucose) was used from days 2 to 5. IVC was carried out at 38.5 C under 5% O_2. The rates of development of embryos, ≥4-cell embryos and 4-cell embryos to morulae or blastocysts on days 2, 4 and 5, respectively, were evaluated.

Collection of Duroc embryos

In the present study, to ensure pregnancy, the embryos obtained *in vivo* from Duroc gilts were cotransferred. Donor gilts were prepared according to the method used in previous studies [20, 21]. In brief, they were checked for signs of estrus and inseminated artificially with semen from a 1-year-old Duroc boar. Between 14 and 30 days after artificial insemination, they were injected with 0.526 mg prostaglandin F2α analogue (cloprostenol-Na, Planate, MSD Animal Health, Tokyo, Japan) to induce miscarriage. Thereafter, the same amount of cloprostenol-Na plus 1000 IU eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) was injected at 24 h, followed by 500 IU hCG (Puberogen 1500 U; Novartis Animal Health, Tokyo, Japan) 72 h later. Artificial insemination was carried out three times, at 24, 31 and 48 h after hCG injection. Blastocysts were collected surgically from the gilts on day 5 or 6 after the initial artificial insemination.

Embryo transfer

Recipients were prepared using the same treatments as those described above. Pregnant Landrace × Large White gilts at days 20−25 of gestation were treated with cloprostenol-Na, eCG and hCG. Ovulation was expected to occur around the time of IVF, 41−42 h after hCG injection. The Middle White IVP embryos on day 4 or 5 after IVF were introduced surgically into the uterus of a synchronized recipient on day 5 after hCG injection with IVC-Glu supplemented with 20 mM HEPES, the osmolality of which had been adjusted to 285 mOsm/kg, as a transfer medium. Five *in vivo*-derived Duroc embryos on day 5 or 6 were cotransferred simultaneously to the uterus. Pregnancies in all recipients were allowed to progress to term.

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