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

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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

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...
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.

| 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 | 4-cell embryos to morulae | Blastocysts | No. embryos transferred | No. of piglets born |
| 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).

#### 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 M 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 (Pubrogen 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 CO2 and N2 adjusted to 5% and 90%, respectively (5% O2).

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 from the gilts on day 5 or 6 after the initial artificial insemination.

Embryo transfer

Recipient 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 IVF 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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