APPLIED TECHNOLOGY OF OPU-IVF FOR LIVESTOCK INDUSTRY AND EARLY PREGNANCY DIAGNOSIS BY ULTRASONOGRAPHY IN CATTLE

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ABSTRACT

In recent decades, the development of a practical system for calf production using ovum pick-up and in vitro fertilization has contributed to a worldwide increase in the activity of embryo transfer and genetic improvement in cattle industry. According to the latest statistics of the International Embryo Transfer Society, in the year 2013 the half of the total embryos produced (also transferred) for transfer in the world were derived from OPU-IVF. The success of embryo production by OPU-IVF greatly depends on the efficacy of each technology involved, such as the number of healthy oocytes aspirated from the follicles during OPU and the culture conditions during in vitro maturation, IVF and subsequent embryo culture. After the transfer of produced embryos to the recipient cows, an early pregnancy diagnosis is important to save breeding costs for farmers by improving reproductive efficiency. For this purpose, the use of an ultrasonography is beneficial not only for the detection of early pregnancy but also for the determination of fetus sex and twin pregnancy. Compared with rectal palpation, an ultrasonography enables a more accurate evaluation of normality of embryonic/fetal development with less damage to the ovary and uterus. This paper describes an applied protocol for OPU-IVF technology and early pregnancy diagnosis developed to enhance the genetic improvement and reproductive management in cattle.

Keywords: Cattle, Ovum Pick-up, In Vitro fertilization, Early Pregnancy, Fetus, Ultrasound

INTRODUCTION

The combination of transvaginal oocyte recovery (ovum pick-up; OPU) with in vitro embryo production (IVP) has proven to be a valuable tools to enhance genetic improvement in the cattle-breeding and embryo transfer industry of both beef and dairy cattle (Galli et al. 2001; Merton et al. 2003). The large scale production of calves from cattle of high genetic value facilitates the improvement in the selection intensity and shortening of the generation intervals (Merton et al. 2013; Ponsart et al. 2013). The successful of OPU-in vitro fertilization (IVF) systems play a key role to increase the number of produced embryos and calves derived from one superior donor for the selection intensity (Ponsart et al. 2013; Schaeffer 2006; van Wagendonk-de Leeuw 2006). Furthermore, application of embryo production is needed for valuable donors that have poor in vivo embryo production due to reproductive disorders. An ultrasound scanner with an adequate endovaginal probe and a guided needle is required to perform this procedure. A scanner with good resolution and with a probe of at least 6 MHz is used generally to envisage follicles more than 2-3
mm in diameter and also to view the needle during follicle aspiration (Galli et al. 2003). Using OPU, oocytes are collected transvaginally from live donor animals with minimal invasiveness under an ultrasound-guidance and subjected to IVF (Callesen et al. 1987; Pieterse et al. 1988).

Reproductive performance is a major factor affecting production and economic efficiency in dairy and meat production in farms. Pregnancy diagnosis is considered as one of the most important technologies in the reproductive management not only on post embryo transfer but also on postinsemination. The ultrasonic visualization is a useful tool to determine a non-invasive conception at an earlier stage of conception using a transrectal transducer than by rectum palpation (Curran et al. 1986a; Curran et al. 1986b). In addition, the use of transrectal ultrasonography causes no damage to the embryo/fetus (Curran et al. 1986b; Kähn 1992; Pieterse et al. 1990).

The purpose of this paper is to review the applied procedures of the OPU-IVF for producing high numbers of embryos and the ultrasonography determination of early pregnancy and fetal gender in cattle.

APPLIED TECHNOLOGY OF OPU-IVF FOR LIVESTOCK INDUSTRY IN CATTLE

Potential for the applied technology of OPU-IVF

In dairy cattle, reproductive performance has been declining for the past 50 years due to an antagonistic relationship between milk production and fertility (Rodriguez-Martinez et al. 2008). To accelerate an efficient calf production, multiple ovulation and embryo transfer (MOET) is practical use. Ovarian response of donor cows to superstimulation in MOET programs still remains a problem, with little improvement in the yield of transferable embryos per superstimulation cycle over the past 30 years (Hasler 2003; Merton et al. 2003; Perry 2013). Acquiring oocytes from live donors means that the genetic merit and health status of the donor animal are precisely known. OPU-IVF is an alternative and is potentially much more efficient as a means of producing embryos (Imai et al. 2006; Merton et al. 2003) compared with MOET and requires far fewer sperm than required for artificial insemination (AI). After calving, OPU can be performed soon around 2-3 weeks postpartum in dairy lactating cows (Hasler et al. 1995; Matoba et al. 2012). One of the advantages of OPU is that it can be repeated relatively frequently to maximize the number of oocyte recovered (Pieterse et al. 1991; Pieterse et al. 1992; Simon et al. 1993). Moreover, OPU-IVF has been applied as a method to produce embryos from cattle with reproductive disorders (Imai et al. 2006; Looney et al. 1994), pregnant donors as 90-150 days of gestation (Eikelmann et al. 2000; Guyader Joly et al. 1997; Imai et al. 2006; Kruij et al. 1994; Merton et al. 2003; Reinders and van Wagendonk-de Leeuw 1996), fattening cattle (Ohya et al. 2005), and calves (Brogliatti and Adams 1996; Duby et al. 1996; Fry et al. 1998; Majerus et al. 1999; Tagawa et al. 2008; Taneja et al. 2000).

Timing and frequency of follicle aspiration

In cattle, follicular growth in the ovary involves either two or three waves in the estrous cycle (Ginther et al. 1989b; Savio et al. 1988; Sirois and Fortune 1988). One selected follicle develops to be dominant according to the emergence in each follicle wave and the other remainders become subordinates. Then, a dominant follicle becomes non-ovulatory (in the first wave during two-follicular-wave-cycle or the first two waves during three-follicular-wave-cycle) or ovulatory (in the second wave during two-follicular-wave-cycle or the third wave during three-follicular-wave-cycle) and subordinate follicles undergo an atresia after a shot growing phase (Ginther et al. 1989a; Ginther et al. 1989b). The presence of a dominant follicle negatively affects the development of the other follicles through the secretion of inhibin and estradiol (Wolfsdorf et al. 1997). Therefore, the stage of follicle wave in the estrous cycle affects intrafollicular oocyte developmental competence (Hagemann et al. 1998). To achieve an optimal protocol for OPU, different timing of follicle puncture has been submitted on days 3-4, 9-10 and 15-16 (day 0 is defined as the day of estrus) (Pieterse et al. 1991). Although the largest number of follicles were recovered on days 3-4, similar oocyte recovery was observed in all the timing. Development to the blastocyst stage was higher in oocytes aspirated from healthy follicle periods in the follicular growth phase (on days 2 and 10) than those from dominance and atresia periods (on days 7 and 15 after estrus) (Hagemann et al. 1999). Other protocols have been also reported with different intervals between OPU sessions described as once a week (Goodhand et al. 1999), or twice a week (Gibbons et al. 1994). The twice-a-week protocol results in the maximum number of competent oocytes in a given period of time (Garcia and Salaheddine 1998; Goodhand et al. 1999).
**Improvement of the quality of embryos produced by OPU-IVF; efficient production of sexed embryos**

The ability to produce calves of the desired sex is an attractive reproductive technology for dairy farmers wishing to breed replacement heifers. Recently, sexed frozen semen is commercially available for AI and the accuracy of the sexing procedure has been reported to be around 90% (Schenk et al. 2009; Seidel 1999). However, the potential use of this technology, conception rates after AI with sex-sorted semen are still lower than those achieved with conventional non-sorted semen (Hutchinson et al. 2013). Moreover, when cows were superstimulated, received AI and their uteri were flushed the number of produced embryos was 0.58-1.1/head/ MOET session in dairy cattle (de Feu et al. 2008; Nakagawa 2013). According to these recent results, it is difficult to produce one calf post-transfer per a MOET session. On the other hand, alternative technologies of OPU combined with IVF have been known to be an efficient method of embryo production compared with AI. IVP procedures in cattle have been efficiently improved for several decades since the first successful birth of calves derived from in vitro maturation (IVM) and IVF of cattle was reported from Hanada et al. (1986). IVP embryos are normally produced from oocytes collected by aspiration of antral follicles (2-6 mm in diameter) in the ovaries. However, less than half of the recovered oocytes are capable of developing to the blastocyst stage following the process of IVM-IVF-IVC (Lonergan et al. 2003a; Lonergan et al. 2003b). In contrast, blastocyst development from in vivo matured oocytes is greater than that from those from in vitro matured oocytes (Dieleman et al. 2002; Greve et al. 1987; Marquant-Le Guienne et al. 1989; Matoba et al. 2012; Rizos et al. 2002). Moreover, taken together, IVF of in vivo matured oocytes with sex-sorted sperm is considered an efficient approach to maximize the number of sex-determined blastocysts produced through applied reproductive technologies in dairy cattle. To achieve this, we have confirmed that OPU must be performed 25 h to 26 h after GnRH administration for induction of ovulation to harvest of in vivo matured oocytes from superstimulated Holstein cows and 30 h after GnRH administration was determined as the optimum time for the IVF (Matoba et al. 2012) (Fig. 1).

![Fig. 1. Scheme of production of in vitro produced embryos using sex-sorted sperm and in vivo matured oocytes in Holstein cows (based on Matoba et al. 2014). CIDR = controlled internal drug release (intravaginal progesterone-releasing device; Pfizer, Tokyo, Japan); DFA = all follicles ≥ 8 mm in diameter were aspirated, dominant follicle ablation; FSH = 30 Armor Uunits (AU) of follicle stimulation hormone (6, 6, 4, 4, 3, 3, 2 and 2 AU, respectively) was administered twice daily for four days in decreasing doses (Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan); PGF2α = d-cloprostenol (Dalmazin; Kyoritsu Seiyaku Co.); GnRH = 100 μg of a GnRH analog (fertirelin acetate, Supolnen; Kyoritsu Seiyaku Co.); OPU = ovum pick up; IVF = in vitro fertilization; morning = 0900 h; evening = 1650 h; OPU = 1000 to 1100 h and IVF = 1500 h (and 1300 to 1400 h).](image-url)

Recently, we have established an efficient system for the production of female embryos in dairy cows by OPU-IVF using in vivo matured oocytes and X-sorted sperm after superstimulated coupled with induction of ovulation by GnRH administration (Matoba et al. 2014) (Table 1). This system allows the production of in average 7.1 transferable (58.0% of transferable blastocyst formation) and 4.1 freezeable good quality blastocysts (54.9% of...
freezable good quality blastocyst formation) per head per session. Moreover, in this system, extra immature oocytes (25.6% of recovered oocytes in total are submitted immature oocytes) can be subjected to IVM for 22-23 h (Fig 1) which can also be used for IVF to increase the number of sexed embryos produced from a single cow.

Table 1. Efficient embryo production by IVF using in vivo matured oocytes and X-sorted sperm in Holstein cows.

| Oocytes or blastocysts | Superstimulated OPU, 
<table>
<thead>
<tr>
<th>Dominant follicle removal</th>
<th>Non-stimulatedb</th>
<th>OPU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DFAc (n=9)</td>
<td>GnRHc (n=9)</td>
</tr>
<tr>
<td>Oocytes [no. (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live oocytes</td>
<td>12.4 ± 2.3</td>
<td>14.7 ± 3.6</td>
</tr>
<tr>
<td>Cleaved</td>
<td>10.9 ± 2.5 (83.8 ± 3.6)</td>
<td>12.1 ± 3.0 (84.0 ± 4.4)</td>
</tr>
<tr>
<td>Blastocysts [no. (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7-9 blastocyst</td>
<td>7.6 ± 2.5 (58.0 ± 10.9)</td>
<td>6.9 ± 2.3 (52.8 ± 8.6)</td>
</tr>
<tr>
<td>Transferable blastocyst</td>
<td>7.1± 2.1 (85.1 ± 6.0)</td>
<td>5.5 ± 1.9 (62.3 ± 11.9)</td>
</tr>
<tr>
<td>Good quality blastocyst</td>
<td>4.1± 1.0** (54.9 ± 7.5*)</td>
<td>0.9 ± 0.2** (21.5 ± 11.8*)</td>
</tr>
</tbody>
</table>

Taken from Matoba et al. 2014. aData are mean ± SEM per head per ovum pick up (OPU) session in each group. bImmature oocytes were inseminated after 22 to 24 h of maturation in vitro. cDFA: ≥ 8-mm dominant follicle ablation. dAdministration of GnRH i.m. eNon-degenerated oocytes with expanded cumulus cells used for in vitro fertilization (IVF) of 9 of 12 animals in the DFA and the GnRH groups. *P < 0.05; **P < 0.01 (significant differences within a row).

**EARLY PREGNANCY DIAGNOSIS BY ULTRASONOGRAPHY IN CATTLE**

Early pregnancy diagnosis
The earliest positive pregnancy has been visually recognized by a fetal heartbeat using a high resolution scanner coupled with a probe on day 20.3 ± 0.3 of gestation (Curran et al. 1986b). When scanning performed on days 26-33 of postinsemination, the sensitivity and specificity of the pregnancy diagnosis (97.7% and 87.8%, respectively) were higher than those (44.8% and 82.3%, respectively) conducted on days 21-25 postinsemination (Pieterse et al. 1990). Depending on the sequential diagnosis, fetal growth can be detected as time-dependent increases in specific characteristics of the fetus and related reproductive tracts (Kitahara 2015; Tatami 2015) (Table 2).

Table 2. Relationship among the gestation days, the size of the amniotic cavity, the crown rump length and uterine diameter by an ultrasonographic diagnosis.

<table>
<thead>
<tr>
<th>Days after insemination</th>
<th>Diameter of amniotic cavity (mm)</th>
<th>Crown rump length (mm)</th>
<th>Uterine diameter with/without fetus (mm)</th>
<th>Minutely heat beat</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>10</td>
<td>5-7</td>
<td>32 / 31</td>
<td>140-150</td>
</tr>
<tr>
<td>30</td>
<td>18-20</td>
<td>8-12</td>
<td>32 / 31</td>
<td>160-180</td>
</tr>
<tr>
<td>35</td>
<td>20-25</td>
<td>13-17</td>
<td>38 / 35</td>
<td>170-190</td>
</tr>
<tr>
<td>40</td>
<td>30-35</td>
<td>17-24</td>
<td>38 / 35</td>
<td>170-190</td>
</tr>
<tr>
<td>45</td>
<td>23-26</td>
<td>47 / 38</td>
<td>180-200</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>35-45</td>
<td></td>
<td>180-200</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>45-60</td>
<td>53 / 37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>60-70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>140-150</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Adapted from Kitahara 2015; Tatami 2015.

The accuracy of detecting early pregnancy has been increased when the presence of a visualized fetus with heartbeats can be confirmed more easily and rapidly (Beal et al. 1992). Therefore, the most practical timing for scanning cows is after 26 days of gestation and the peak of accurate pregnancy is reached after 45 days (representative image in Fig 2A). The establishment of the early pregnancy diagnosis by ultrasonography has also
allowed to characterize the timing and rate of embryonic losses (Diskin and Morris 2008; Santos et al. 2004). In a previous study, 12.8% (ranging from 3.2 to 42.7%) of dairy cows that were diagnosed pregnant on around the 30 days of gestation showed embryo loss at the second diagnosis on the 41-58 days of gestation due to milk yield and heat stress (Santos et al. 2004). These data indicate an average rate of pregnancy loss of 0.85% (ranging between 0.23-2.67%) per day in dairy cows and these rates were higher than those in heifers or beef cattle (Santos et al. 2004). Therefore, it is important to assess embryonic mortality at least twice (until 41 and after 58 days of gestation, respectively). Furthermore, ultrasonographic scanning provides the additional information for the evaluation for the ovarian morphology, twin fetuses and fetal sex (see next section).

**Determination of fetal sex**

Examinations of fetal gender are generally required a transrectal ultrasound scanner equipped with a liner-array or convex-array, and a transducer with more than 5 MHz frequency. Diagnosis can be given to view a fetus in its entirety in sequential frontal, cross-sectional or sagittal axis by operating in three-dimensions (Curran 1992). The areas of an umbilicus, hind limbs and tail can be examined to recognize the position of a genital tubercle (Curran 1992). In an earlier study, fetal sex has been detected with more than 90% accuracy between 73-120 days of gestation by the images of the scrotum and mammary glands of male and female fetuses (Müller and Wittkowski 1986). Several decades before, the accuracy of sex diagnosis at 48-60 days after estrus has been examined using identified position of genital tubercle (Curran et al. 1989). The accuracy of this way of sex determination for either female or male fetuses gradually increased from Day 48 (no sex determined) to 52, reached the maximum on Day 53 and kept to until Day 60. The fetus was determined as male when the genital tubercle was located immediately caudal to the abdominal attachment of the umbilical cord and female when the tubercle was located in the area of the tail (Curran 1992). The presence of genital tubercle has been shown at the front of the tail in female or just cranial the hind limbs in male fetus in a frontal view, respectively (Fig 2BC).

![Fig. 2. Representative images of bovine fetuses on an early pregnancy diagnosis by ultrasonography. (A) Day-40 (1.78 cm of crown rump length), (B) Day-68 female and (C) Day-65 male fetuses. Circles with dotted line indicate genital tubercles. Day 0 = in vitro fertilization or estrus day.](image)

**Table 3. Relationship between accuracy of sex diagnosis and certainty scores.**

<table>
<thead>
<tr>
<th>Certainty Score</th>
<th>Criterion</th>
<th>Number of correct fetus diagnosis</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sex diagnosis not determinable</td>
<td>0/7 0/9 0/16</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Minimal</td>
<td>3/5 7/8 10/13</td>
<td>76.9</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate</td>
<td>12/12 9/9 21/21</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Maximal</td>
<td>24/24 34/34 58/58</td>
<td>100</td>
</tr>
</tbody>
</table>

Based on Curran et al. 1989.
CONCLUSION

Bovine OPU-IVF technologies have been improved for the production of embryos and calves after transfer to the recipient cows in these decades in livestock industry. Usefulness of this technology offers a repeatable treatment and possibility to use oocytes of animals with reproductive disorder, early pregnancy and those of fattening cattle. Furthermore, it allows the production of embryos of the desired sex without damages to them and embryo production from calves. In recent a decade, half of the total embryos produced for transfer (and also those which were transferred) in the world were derived from OPU-IVF. The success of embryo production by OPU-IVF greatly depends on the efficacy of each technology involved, such as the number of healthy oocytes aspirated from the follicles during OPU and the culture conditions during IVM, IVF and subsequent embryo culture. More applied introduction and production of sexed embryos are needed for the breeding industry. Recent results have demonstrated that high numbers of female embryos and high rates of good-quality blastocysts can be obtained by in vitro fertilization with X-sorted sperm using in vivo matured oocytes collected by OPU from superstimulated dairy cows. After embryo transfer, management of early pregnancy diagnosis for saving opened periods is important. Moreover, determination of fetus gender is also important to plan the production for next female generation, and sire and/or genomic evaluation. Further improvement of OPU technology, and to avoid false pregnancy diagnosis an adequate training for operators for rectal palpation and the practical experience are required.

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