Highlights (for review)

- *In vivo* matured oocytes have higher competence than *in vitro* matured oocytes.

- A single FSH injection prior to OPU can improve oocyte competence.

- *In vitro* pre- maturation enables to improve oocyte competence without FSH priming.

Review Article

Title: Follicle priming by FSH and pre-maturation culture to improve oocyte quality *in vivo* and *in vitro*

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Abstract

Nowadays there is strong demand to produce embryos from premium quality cattle, and we can produce embryos using oocytes collected from living premium animals by ovum-pick up (OPU) followed by *in vitro* fertilization (IVF). However, the developmental competence of IVF oocytes to form blastocysts is variable. The developmental competence of oocytes depends on the size and stages of follicles, and follicle-stimulating hormone priming (FSH-priming) prior to OPU can promote follicular growth and improve the developmental competence of oocytes. Furthermore, following the induction of ovulation using an injection of luteinizing hormone or gonadotropin-releasing hormone after FSH-priming, we can collect *in vivo* matured oocytes from ovulatory follicles, which show higher developmental competence than oocytes matured *in vitro*. However, the conventional protocols for FSH-priming consist of multiple FSH injection for 3 to 4 days, which is stressful for the animal and labor-intensive for the veterinarian. In addition, these techniques cannot be applied to IVF of oocytes collected from bovine ovaries derived from slaughterhouses, which are important sources of oocytes. Here, we review previous research focused on FSH-priming, especially for collecting *in vivo* matured oocytes and a simplified method for superstimulation using a single injection of FSH. We also introduce the previous achievements using *in vitro* pre-maturation culture, which can improve the developmental competence of oocytes derived from non-stimulated animals.

Keywords: FSH; Ovum pick-up; *In vivo* maturation; Single injection; *In vitro* pre-maturation

1. Introduction

In vitro fertilization (IVF) technology is now widely used commercially for producing embryos in cattle [1]. Ultrasound-guided ovum-pick up (OPU) combined with IVF is used to produce embryos in cattle for genetic improvement [1], and the efficiency of embryo production by OPU-IVF is higher than that by *in vivo* embryo production [2, 3]. Although there has been significant progress in the technology of IVF procedures, the rate of development to blastocysts from oocytes recovered from non-superstimulated donors is rarely consistently exceeds 40 to 50% [4, 5]. When donors are superstimulated, a significant increase in the rate of blastocyst is seen but can vary from 50 to 80% [6]. However, *in vivo* maturation will remain the gold standard resulting in the highest rate of blastocyst.

In mono-ovulatory species including cattle, the emergence of follicular growth is induced by a surge-like secretion of follicle-stimulating hormone (FSH). Then, a dominant follicle is selected as the level of FSH decreases due to the inhibitory effect of estradiol-17β and inhibin secreted by follicles themselves. The dominant follicle continues to grow in response to stimulation with luteinizing hormone (LH), and this results in ovulation [7, 8]. In other words, most follicles degenerate at immature stages, and only a small proportion of follicles will develop fully and ovulate in the life time of a cow [7, 8]. During folliculogenesis, the diameter of the oocyte will increase during follicular growth phases. Developmental competence is defined as the ability of an oocyte to acquire nuclear, cytoplasmic and molecular maturation and thus the competence to produce a blastocyst following

fertilization that will result in a healthy offspring after embryo transfer [6]. Although most oocytes in immature antral follicles (≥ 2 mm, diameter of the oocyte: approximately 110 μm) acquire competence for nuclear maturation [9], most oocytes will not have acquired cytoplasmic and/or molecular competence when compared to larger oocytes (120 μm) from follicles grown sufficiently *in vivo* [10, 11].

To enhance follicular growth and the developmental competence of oocytes, administration of FSH before OPU (FSH-priming) has been conducted in many studies [12, 13]. However, conventional FSH treatments require multiple intramuscular (im) administrations, which are stressful for the animal and time-consuming for the veterinarian. In conventional *in vivo* embryo production by superovulation followed by uterine flushing, many researchers have tried to simplify FSH treatment [14]. Application of these methods for OPU should be evaluated.

Although the quality of oocytes before IVM is considered the critical factor for the outcome of *in vitro* embryo production [15-17], there are some cases where exogenous FSH is not applicable to promote the developmental competence of oocytes, such as oocytes derived from slaughterhouses or low-responding animals [18]. Previous studies have suggested that the developmental competence of oocytes can be improved by adding a pre-maturation *in vitro* step (pre-IVM) prior to IVM. Pre-IVM inhibits germinal vesicle breakdown and holds the oocytes at the germinal vesicle (GV) stage to acquire full developmental competence of oocytes *in vitro* during meiotic arrest [19].

In this review, we describe FSH-priming to improve developmental competence before

collection of oocytes. In particular, we focused on collecting *in vivo* matured oocytes and simplifying the priming protocol using a single injection of FSH. In addition, we also examine pre-IVM to improve the developmental competence of oocytes *in vitro*.

2. FSH-priming

2.1. Collecting *in vivo* matured oocytes by FSH-priming following induction of an LH surge

Several studies have suggested that *in vivo* matured oocytes are more developmentally competent than *in vitro* matured oocytes (Table 1) [20-26]. Evidence of an increased developmental competence following *in vivo* maturation include differences in mRNA transcription in oocytes [27-29], size of the meiotic spindle [30], cytoplasmic maturation (distribution of cortical granules [26], ATP content [30]), and developmental kinetics of embryos [26]. Bordignon *et al*. [20] reveal that heifers treated with gonadotropin-releasing hormone (GnRH) after superstimulation exhibited an LH surge within 3 h after treatment (34 h after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection). In the control group subjected to superstimulation and $PGF_{2\alpha}$ injection without a GnRH injection, 40% of heifers also exhibited an LH surge within 47 h (between 41 and 45 h) after $PGF_{2\alpha}$ treatment. Most of the oocytes recovered from control heifers were in metaphase I (MI) stage regardless of the state of expansion of the cumulus, whereas most oocytes (97%) with expanded cumulus cells in the GnRH-treated group were at the metaphase II (MII) stage. As a result, the developmental competence of oocytes with expanded cumulus cells was higher in the GnRH-treated group than the control when oocytes were

directly subjected to IVF without IVM after OPU (control: 40%, GnRH: 60%, Table 1) [20]. Rizos *et al*. [22] showed that *in vivo* matured oocytes had higher developmental competence than oocytes from follicles just before LH surge. These results indicate that a post-LH follicular environment induced by a GnRH injection is essential to improve the developmental competence of bovine oocytes.

The maturation of bovine oocytes is known to initiate 6 h after the LH surge *in vivo* [31]; therefore, some researchers collected oocytes 6 h after an injection of LH or human chorionic gonadotropin [32-34]. Although they suggested promotive effects of exogenous LH injections on developmental competence [33, 34], waiting 6 h after LH was not long enough to permit oocytes to complete maturation *in vivo* as expressed by non-expanded cumulus cells surrounding the oocytes. Matoba *et al*. [24] reported that ovulation occurred at 29 to 32 h after a GnRH injection (average 30.0 h), and *in vivo* matured oocytes could be successfully collected 25 to 26 h after a GnRH injection. Other researchers collected oocytes after 20 to 26 h after GnRH injection, and succeed in collecting *in vivo* matured oocytes with higher developmental competence [20-22, 25, 26], whereas Sprícigo *et al.* [25] reported that they could collect more MII oocytes 24 h after GnRH injection (85%) than at 20 h after GnRH injection (31%). These results indicated that the suitable duration between GnRH injection and OPU for collecting *in vivo* matured oocytes should be 24 to 26 h. Although *in vivo* matured oocytes show higher developmental competence, handling of them will be difficult due to stickiness of expanded cumulus investments. Matoba *et al*. [24] changed conical tubes for collecting oocytes after 5 to 6 follicle aspirations, making it easier to collect individual oocytes instead of clumps.

In vivo matured oocytes with expanded cumulus cells were directly subjected to IVF after OPU in some studies [20, 22, 25], while oocytes in other studies were cultured in IVM medium for 3 to 24 h [20, 21, 23, 24, 26] (Table 1). Bordignon *et al*. [20] reported that when oocytes with expanded cumulus cells were subjected to IVM for 24 h, 29.2% of them underwent spontaneous activation, and caused lower developmental competence than *in vivo* matured oocytes directly subjected to IVF after OPU (Table 1). The total maturation period initiated from LH surge in Bordignon *et al*.'s study was 48 h (*in vivo* maturation for 24 h and *in vitro* maturation for 24 h). Previously, we suggested that IVM for longer than 30 h caused aging of oocytes, and these oocytes showed lower developmental competence than oocytes matured *in vitro* properly for 22 h [35]. Taken together, 22 to 24 h of IVM was too long for *in vivo* matured oocytes. In cattle, OPU for collecting oocytes with FSH treatment was conducted at 25 to 26 h after GnRH injection because ovulation occurred at 29 to 32 h after GnRH injection (average 30.0 h) [24]. Some researchers matured *in vitro* the oocytes for another 3 h after OPU to synchronize the total maturation period *in vivo* and *in vitro* with the time after GnRH injection and ovulation [24, 26]. Further studies are needed to optimize the duration for IVM for *in vivo* matured oocytes.

2.2. Simplification of the regimen for FSH-priming

Superovulation after a single injection of FSH has been developed in conventional *in vivo* embryo production by superovulation followed by uterine flushing [14]. This approach can be done in

one of two ways; (1) using a solvent that enabled FSH to be released slowly, such as polyvinylpyrrolidone (PVP) [36, 37], aluminum hydroxide gel mixes [38], or hyaluronan-based slow release formulation [39, 40], or (2) a single subcutaneous administration of high-dose FSH dissolved in saline [36, 41, 42]. In both treatments, blood FSH is slowly absorbed into the general circulation and induces the growth of multiple ovulatory follicles (PVP [36], aluminum hydroxide gel [38], hyaluronan [43], and FSH dissolved in saline by subcutaneous (sc) injection [42]). The injection methods tested for FSH-priming before OPU to simplify the regimen of FSH injection included PVP [44], aluminum hydroxide gel [26], hyaluronan [43], FSH dissolved in saline by sc injection [33, 45, 46], im injection [33, 47, 48], or epidural injection [49] (Table 2). There is very limited information about the efficiency of a single injection of FSH prior to OPU, but Vieira *et al*. [43] reported that a single im injection of FSH dissolved in 0.5% hyaluronan resulted in similar plasma FSH profiles as twice-daily FSH treatments, and a single injection increased the number of embryos per OPU-IVF. In some studies, researchers conducted a single FSH injection prior to OPU using saline as a solvent of FSH (sc [45], im [47], or a simultaneous injection of im and sc [33, 50]). The efficiency of these treatments on the acquisition of oocyte developmental competence was not reported to be different with that of non-stimulated animals [45, 47] or lower than that of conventional multiple FSH injection [33].

It is known that the interval between the last FSH injection of multiple FSH injections and OPU (“coasting period”) critically affects the developmental competence of oocytes [34, 51]. Nivet *et*

al. [51] suggested that a coasting period between 44 and 68 h showed better developmental competence of blastocysts than a shorter period (20 h), although a longer coasting period (92 h) decreased the developmental competence [51]. During the coasting period, a progressive hypoxia occurs in follicles, which is related to the increase in apoptosis and inflammation of follicles [52]. This follicular environment is similar to several preovulatory changes in the dominant follicle and associated with improvement in the developmental competence of oocytes [19]. Reduction of the FSH level during the coasting period was similar to the growth of a dominant follicle [53], because FSH levels decrease for several days before ovulation in the natural estrous cycle [54]. After the reduction in FSH level, the basal level of LH is supposed to maintain growth and prevent atresia of follicles during the coasting period [13]. Although the coasting period is important to improve developmental competence, there is little information about the suitable interval between a single FSH injection and the OPU. Blondin *et al*. [48] conducted a study where a single bolus FSH injection was administered to beef heifers, and oocytes were collected from animals soon after slaughter at 24, 48, and 72 h after the FSH injection (Table 2). In that case, the developmental competence of oocytes was higher in animals slaughtered 48 h after the FSH injection than those after 24 or 72 h. Furthermore, the developmental competence of oocytes was higher when oocytes were collected 4 to 5 h after slaughter than oocytes collected soon after slaughter (Table 2). This result cannot be applied directly to other single-injection regimens, because the plasma circulation of FSH after a single FSH injection was different between im and sc injections [55], and the type of animal and solvent can affect the transition

of FSH to general circulation from the injection site. For example, some researchers successfully induced superstimulation using a single sc injection of FSH in beef cows [41, 42], but Takedomi *et al*. [36] failed to induce superstimulation using a single sc injection in Holstein heifers. When FSH dissolved in saline was subcutaneously administered into Holstein heifers, the plasma concentration of FSH markedly increased within 3 h and was maintained until 9 h after administration [36]. Plasma concentration of FSH decreased to the basal level after 36 h, and superovulation was not induced [36]. However, a FSH solution dissolved in PVP [36] results in a gradual increase in FSH plasma concentrations that peak 12 h after administration. Then, FSH plasma concentrations decreased gradually but were maintained at a higher level than the basal level for more than 48 h, and superstimulation can be induced in Holstein heifers [36]. For the optimization of OPU followed by a single FSH injection, further studies are needed to find out the appropriate coasting periods based on the plasma dynamics of circulating FSH after the injection.

2.3. Epidural area as an injection site of FSH to induce superstimulation

Although there are some effective methods for superstimulation, the effectiveness of these different treatments varies considerably, probably because of differences in the amount of subcutaneous fat tissue in the animals [36, 41, 42]. To develop a more efficient method to simplify the regimen of FSH injection, we took an idea from a study of human pharmacokinetics, which suggested that alfentanil (an opioid analgesic drug) was slowly absorbed into the general circulation after

epidural administration in humans [56]. Therefore, we firstly compared the outcome of *in vivo* embryo production by superovulation followed by uterine flushing between a conventional multiple FSH injection and a single epidural FSH injection. We collected embryos from five Japanese black cows given twice-daily im FSH administration (totally 20 armour units of Antrin R-10, approximately 200 international units, Kyoritsu Seiyaku, Tokyo, Japan) for 3 d (control) or a single epidural FSH injection (30 armour units, approximately 300 international units). The number of transferable blastocysts after epidural treatment (9.0 ± 6.0) was similar to that in the control group (4.7 ± 3.5, $P = 0.10$). Furthermore, we confirmed the efficiency of a single epidural FSH injection for OPU-IVF of cattle with low productivity by *in vivo* embryo production. We conducted OPU for three Japanese black cows with low embryo productivity given twice daily im FSH administration (total 30 armour units) or a single epidural FSH injection (30 armour units). Although most follicles were less than 6 mm in diameter, and the numbers of follicles and collected oocytes were similar between treatments, the rate of transferable blastocysts in the epidural group was higher than that of the control (Table 3, $P < 0.0001$). Further study is needed to reveal the mechanism of improved developmental competence of oocytes after a single epidural FSH injection, and plasma FSH dynamics after epidural injection to optimize the protocol such as the coasting period.

3. Pre-maturation

3.1. Application of cyclic adenosine monophosphate (cAMP) modulators for pre-maturation

In conventional *in vitro* embryo production including ultrasound-guided OPU-IVF, oocytes derived from antral follicles lager than 2 mm in diameter are used [6, 57], in which the oocytes acquire competence for meiotic resumption [9]. One of the reasons for the lower competence of oocytes is precocious meiotic resumption. If meiotically competent oocytes are isolated from follicles, they can resume meiosis spontaneously without ovulatory stimulation such as an LH surge [58]. However, oocytes collected from living cattle without FSH-priming or from slaughterhouse-derived ovaries originate from follicles of varied developmental stages [6, 9, 59]. This means that all oocytes are not growing enough to acquire developmental competence, resulting in lower developmental competence to the blastocyst stage. Meiotic arrest is caused by an increase in cAMP in oocytes, which is produced by oocytes themselves [60, 61] or supplied from cumulus cells via gap junctions [62, 63]. To improve the acquisition of developmental competence of oocytes, many researchers have cultured oocytes from non-stimulated slaughterhouse-derived ovaries in conditions that prevent meiotic resumption by controlling cAMP concentration before IVM (pre-IVM; Table 3) [64-71]. Reduction of cAMP concentration can be achieved through the addition of phosphodiesterase (PDE), the enzyme that degrades cAMP to 5'-AMP. Thus, inhibition of PDE activity has been applied in pre-IVM culture. 3-isobutyl-1-methylxanthine (IBMX) is a non-specific PDE inhibitor that prevents a reduction in cAMP levels and inhibits meiotic resumption in bovine oocytes [72-75]. Forskolin (FSK) is an activator of adenylate cyclase, which promotes the synthesis of cAMP. Culture conditions with IBMX and forskolin increase cAMP levels in bovine oocytes [76]. A culture system called the "simulated

physiological oocyte maturation" (SPOM) system consists of pre-IVM culture with 500 μM IBMX and 100 μM FSK for 2 h before IVM, which promoted the blastocyst rate [64, 71] and cell numbers in blastocysts [64]. More recently, extending pre-IVM culture from 2 to 6 h was reported to increase the proportion of hatched blastocysts on day 8 and yielded a highest ratio of inner cell mass to total cells on day 8 after IVF by increasing intra-oocyte reduced glutathione, which has important roles as an antioxidant agent in oocyte maturation, fertilization, and embryonic development [66].

3.2. Effect of the diameter of oocytes on the outcome of pre-IVM culture

Otoi *et al.* [11] collected oocytes from follicles 1 to 7 mm in diameter in slaughterhouse-derived ovaries, and morphologically healthy oocytes (three or more dense layers of cumulus cells, evenly granulated cytoplasm) were divided into groups based on their diameters and subjected to *in vitro* embryo production. They showed that the developmental competence of oocytes became higher as the diameter of oocytes became larger [11]. Based on Otoi *et al.*'s studies [11], we speculated that the most suitable duration of pre-IVM culture is dependent on the diameter of oocytes. We collected bovine oocytes from slaughterhouse-derived ovaries and divided them into small-sized (110 to < 115 μm) and large-sized (≥ 115 μm) oocytes and subjected them to pre-IVM culture for 0, 5, or 10 h in medium containing IBMX (500 μM) and a low dose of FSH (2×10^{-6} units/mL, from porcine pituitary) [69]. Before pre-IVM culture, all oocytes were at GV stage in both groups. Although approximately 90% of oocytes were still GV stage after pre-IVM for 5 h in both groups, half of the oocytes reached

metaphase I after pre-IVM for 10 h, indicating the spontaneous meiotic resumption of oocytes during an extending pre-IVM culture in both groups. In large oocytes (≥ 115 μm), the percentage of blastocysts after IVF was not different between the different duration of pre-IVM culture (31%). However, pre-IVM culture for 5 h showed a higher blastocyst rate (16%) than for 0 h (9%) or 10 h (8%) (Table 3). Although the mechanism underlying improved developmental competence in pre-IVM for 5 h is unclear, we previously reported that the mitochondrial activity of *in vitro* grown oocytes (105.9 to 122.7 μm) increased at 10 h of pre-IVM, then decreased after 20 h of pre-IVM [77]. Changes in mitochondrial activity during pre-IVM were accompanied by developmental competence to form blastocysts [77]. Similarly, mitochondrial activity may increase during the first 5 h of pre-IVM, then decrease after 10 h of pre-IVM in *in vivo* grown small-sized oocytes (110 to < 115 μm). Further studies are needed to define the appropriate duration of pre-IVM treatment in more detail.

3.3. Culturing oocyte with natriuretic peptide precursor type C

During follicular development, natriuretic peptide precursor type C (NPPC or CNP) derived from mural granulosa cells, and its receptor derived from cumulus cells (natriuretic peptide receptor 2; NPR2), play important roles for inhibiting meiotic resumption [78]. In mice, NPPC derived from granulosa cells promotes production of cyclic guanosine monophosphate (cGMP) by NPR2 in cumulus cells [78]. Cumulus cell-derived cGMP inhibits the reduction of cAMP concentration by the inhibition of PDE3A, an oocyte-specific phosphodiesterase, which is a trigger for meiotic resumption

[78]. As with mice, bovine cumulus cells express NPR2 mRNA [65, 68, 79] and protein [68], and meiotic resumption of oocytes can be arrested during culture of cumulus-oocyte complexes (COCs) with NPPC [65, 67, 68, 70, 80]. Although NPR2 was not expressed in oocytes in mice [68], some studies reported the expression of NPR2 mRNA [68, 79] and protein [68] in bovine oocyte membranes, and meiotic resumption of denuded oocytes was arrested by NPPC [68]. Some studies suggested that pre-IVM with NPPC (100 nM or 200 nM) for 6 h improved the blastocyst rate [67, 68], the blastocysts cell number [65, 67], and the blastocyst hatching rate [65] (Table 3). The combination of NPPC (100 nM) and IBMX (500 μM) in pre-IVM culture followed by 20 h IVM also showed a higher blastocyst rate [70] (Table 3).

4. Conclusion

In vivo matured oocytes have higher developmental competence than *in vitro* matured oocytes, but there is still a need for further research to optimize IVM conditions that will result in the acquisition of developmental competence for immature bovine oocytes. Although a single injection of FSH prior to OPU can improve the developmental competence of oocytes similar to conventional multiple FSH injection, further study, such as of the coasting period, is needed to maximize the potential of oocytes. For oocytes collected from cattle without FSH-priming, pre-IVM is a candidate method to improve the developmental competence of oocytes. In addition, the diameter of oocytes is an important criterion to affect the optimal duration of pre-IVM culture and the developmental

competence of oocytes.

Competing interests

The authors have no competing interest in publishing findings of this research.

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Table 1. Summary of data published on the developmental competence of *in vivo* matured bovine oocytes.

Authors	Injection manner of FSH or equine chorionic gonadotropin (eCG)	Time between GnRH or LH treatment and OPU	IVM duration for *in vivo* matured oocytes	Developmental competence (%) *
Bordignon *et al.* 1997 [20] **	FSH injection in eight decreasing doses 12 hours apart for 4 d	26 h	0 h or 24 h	Blastocysts on Day 7 FSH-only-IVM 0 h: 40[a] FSH-GnRH-IVM 0 h: 60[b] FSH-only-IVM 24 h: 20 FSH-GnRH-IVM 24 h: 13
van de Leemput *et al.* 1999 [21]	A single injection of eCG followed by an injection of eCG antibody (112 h later)	24 h	Maximally 2 h 54 min	Blastocysts on Day 11 Slaughterhouse: 26.4[a] eCG-GnRH: 49.3[b]
Rizos *et al.* 2002 [22] ***	FSH injection in eight decreasing doses twice daily for 4 d	20 h	0 h	Blastocysts on Day 7 Slaughterhouse (2 to 6 mm): 31.7[a] (> 6 mm): 38.4[ab] FSH-only: 35.3[a] FSH-GnRH: 48.5[b]
Dias *et al.* 2013 [23] ****	FSH injection in eight or 14 consistent doses twice daily for 4 or 7 d (Short FSH or Long FSH)	24 h	6 h	Morulae and blastocysts on Day 9 Short FSH: 24.7[ab] FSH starvation: 18.1[b] Long FSH: 36.6[a]
Matoba *et al.* 2014 [24] *****	FSH injection in eight decreasing doses twice daily for 4 d	25 to 26 h	3 h	Good-quality blastocyst until Day 9 Non-stimulated: 36.1 Dominant follicle ablation: 54.9[a] GnRH: 21.5[b]
Sprícigo *et al.* 2015 [25]	FSH injection in eight decreasing doses twice daily for 4 d	24 h	0 h	Blastocysts on Day 7 Slaughterhouse: 37.9[a] Non-stimulated: 50.6[a] FSH only: 58.8[b] FSH-GnRH: 62.4[b]
Egashira *et al.* 2019 [26]	A single sc FSH injection using aluminum hydroxide gel	25 to 26 h	3 h	Good-quality blastocyst on Day 8 Non-stimulated: 29.5[a] FSH-GnRH: 45.6[b]

*: The definitions of each experimental group are described below (Day 0 = Day of IVF).

FSH-only: Groups of animals subjected to FSH-priming, but without GnRH injection later.

FSH (or eCG)-GnRH: Groups of animals given GnRH after FSH (or eCG)-priming for collecting *in vivo* matured oocytes.

Slaughterhouse: Oocytes were collected from slaughterhouse-derived ovaries to serve as a control.

Non-stimulated: Groups of animals with collected oocytes without FSH-priming and GnRH injection.

**: Collected oocytes were subjected to IVM for 0 or 24 h.

***: Collected oocytes from slaughterhouse-derived ovaries were classified by the diameter of follicles (2 to 6 mm or > 6 mm)

****: Cows were treated with three different FSH treatments as described below.

Short FSH: FSH was administered (im) in eight consistent doses twice daily for 4 d.

FSH starvation: FSH was administered (im) in eight doses twice daily for 4 d, and OPU was conducted 4.5 d after the final FSH injection.

Long FSH: FSH was administered (im) in eight consistent doses twice daily for 4 d.

*****: Developmental competence was compared between two methods (Dominant follicle ablation or GnRH injection) for the follicular wave control 1.5 d prior to FSH-priming.

[a, b]: $P < 0.05$

Table 2. Summary of data published on the developmental competence of bovine oocytes after a single FSH injection.

Authors	Breeds	Injection site for single FSH	Solvent for single FSH	Coasting period	Developmental competence (%) *
Bungartz *et al*. 1994 [47]	Lactating Holstein cow	im	Saline	4 d	Morulas and blastocysts on Day 7 Non-stimulated: 2.9 Single FSH: 3.8
Ooe *et al*. 1996 [44] **	Cyclic lactating Holstein cows	im	30% PVP (10 mL)	48 h	Blastocysts on Day 8 Single FSH on Day 1: 25 Single FSH on Day 7: 28 Single FSH in pregnant cows: 33 Single FSH on Day 8 to 14: 29
Blondin *et al*. 1997 [48] ***	Beef heifers	im	Saline	Experiment 1: 24, 48, or 72 h Experiment 2: 48 h + 1 to 2 h or 4 to 5 h after slaughter	Embryos (≥ 64 cells) on Day 6 Experiment 1: Non-stimulated: 15[a] FSH + 24 h: 5[b] FSH + 48 h: 25[a] FSH + 72 h: 7[b] Experiment 2: Non-stimulated: 18[a] FSH 48 h + 1 to 2 h: 24[ab] FSH 48 h + 4 to 5 h: 41[b]
Goodhand *et al*. 2000 [45]	Cyclic beef × Friesian cows	sc	Saline	Multiple: 1 d Single: 3 d	Transferable embryos on Day 7 Non-stimulated: 43 Single FSH: 33 Multiple FSH: 35
Chaubal *et al*. 2007 [33] ****	Angus cross cows	im and sc	Saline	54 h	Blastocysts on Day 7 Multiple FSH-LH: 21.7 Multiple FSH: 18.7 Single FSH-LH: 18.8 Single FSH: 17.2
Vieira *et al*. 2016 [43]	Non-lactating Holstein cows	im	0.5% hyaluronan	Multiple FSH: 1.5 d Single FSH: 3 d	Blastocysts on Day 6 Non-stimulated: 25.9 Multiple FSH: 30.3 Single FSH: 30.3
Sakaguchi *et al*. 2018 [49]	Japanese black cows	Epidural	Saline (5 mL)	Multiple FSH: 21 to 23 h Single FSH: 75 to 78 h	Blastocysts on Day 7 Multiple FSH: 10.5[a] Single FSH: 26.2[b]
Egashira *et al*. 2019 [26] *****	Japanese black cows	sc	Aluminum hydroxide gel	4.5 d	Good-quality blastocyst on Day 8 Non-stimulated: 29.5[a] Single FSH-GnRH: 45.6[b]
Sakagami *et al*. 2019 [46] ******	Japanese black cows	sc	Saline (50 mL)	72 h	Blastocysts on Day 8 Single FSH: 22.1[a] Single FSH-pre-IVM: 39.1[b]

*: The definition of each experimental group is described below (Day 0 = Day of IVF).

Non-stimulated: Groups of animals with collected oocytes without FSH-priming and GnRH injection.

Single FSH: Groups of animals subjected to a single FSH injection.

Multiple FSH: Groups of animals subjected to multiple FSH injections for few days.

**: There were four experimental groups as described below.

Single FSH on Day 1: FSH was administered (im) on Day 1 (Day 0 = ovulation), and OPU was conducted on Day 3.

Single FSH on Day 7: DFA was conducted on Day 6, FSH was administered (im) on Day 7, and OPU was conducted on Day 9

Single FSH in pregnant cows: FSH was administered (im) on 70, 75, 80, 85, and 90 d of pregnancy and OPU was conducted 48 h later (5 times at 5 d intervals).

Single FSH on d 8 to 14: FSH was administered (im) on Days 8 to 14 and OPU was conducted 48 h later

***: Oocytes were collected from ovaries after slaughter. In experiment 1, animals were slaughtered at 24, 48, or 72 h after a single FSH injection then oocytes were collected soon after (FSH + 24, 48, or 72 h groups). In experiment 2, animals were slaughter at 48 h after a single im FSH injection, and oocytes were collected 1 to 2 h or 4 to 5 h after slaughter (FSH 48 h + 1 to 2 h or 4 to 5 h groups).

****: In the single FSH group, FSH was given simultaneously by two routes (im and sc). In Multiple FSH-LH and Single FSH-LH groups, LH was injected 6 h prior to OPU (48 h after the end of FSH treatment).

*****: In single FSH-GnRH group, GnRH was administered (sc) 25 to 26 h prior to OPU (2 d after a single FSH injection).

******: In single FSH-pre-IVM group, collected oocytes were subjected to pre-IVM for 2 h before IVM.

[a, b]: $P < 0.05$

Table 3. Summary of data published on the developmental competence of bovine oocytes subjected to pre-IVM culture.

Authors	cAMP modulator, NPPC, or FSH in pre-IVM medium	Duration of pre-IVM	Duration of IVM	Developmental competence (%) *
Albuz *et al*. 2010 [64]	FSK (100 µM), IBMX (500 µM)	0 (control) or 2 h	24 h or 30 h	Blastocysts/cleaved on Day 8 Control IVM 24 h: 22[a] Pre-IVM IVM 24 h: 48[b] Control IVM 30 h: 27[a] Pre-IVM IVM 30 h: pre-IVM: 69[c]
Franciosi *et al*. 2014 [65]	Recombinant human FSH (10^{-4} unit/mL) + NPPC (100 nM) or cilostamide (10 µM) or without NPPC and cilostamide (control)	8 h	22 h	Expanded and hatched blastocysts on Day 9 Control: 78[a] Cilostamide**: 94[b] NPPC: 93[b]
Li *et al*. 2016 [66]	FSK (100 µM), IBMX (500 µM)	0 (control), 2, 4, or 6 h	Control: 24 h Pre-IVM 2 h: 24 h Pre-IVM 4 h: 22 h Pre-IVM 4 h: 20 h	Blastocysts/cleaved on Day 8 Control: 26.3[a] Pre-IVM 2 h: 39.2[b] 4 h: 35.2[b] 6 h: 34.2[b]
Zhang *et al*. 2017 [67]	NPPC (200 nM)	0 (control) or 6 h	24, 28, or 32 h	Blastocysts on Day 7 Control IVM 24 h: 32.2[b] IVM 28 h: 15.0[a] IVM 32 h: 0 Pre-IVM IVM 28 h: 51.6[c]
Xi *et al*. 2018 [68]	NPPC (200 nM)	0 (control) or 6 h	24, 26, or 28 h	Blastocysts on Day 7 Control IVM 24 h: 23.5[a] 26 h: 24.1[a] 28 h: 21.7[a] Pre-IVM IVM 24 h: 26.9[a] 26 h: 45.2[b] 28 h: 41.6[b]
Abdel-Ghani *et al*. 2018 [69]	Porcine pituitary FSH (2×10^{-6} units/mL), IBMX (500 µM)	0, 5, or 10 h	22 h	Blastocysts on Day 7 (110 to < 115 µm) *** Control: 9[a] Pre-IVM 5 h: 16[b] 10 h: 8[ab]
Soto-Heras *et al*. 2019 [70]	NPPC (200 nM), IBMX (500 µM)	0 (control) or 6 h	24 (control) or 20 h	Blastocysts on Day 8 Control: 34.5 Pre-IVM: 45.1
Hashimoto *et al.* 2019 [71]	FSK (100 µM), IBMX (500 µM)	0 (control) or 2 h	24 h	Blastocysts on Day 7 Control: 16.7[a] Pre-IVM: 27.5[b] Blastocysts on day 8 Control: 25.0[a] Pre-IVM: 33.3[b]

*: Day 0 = Day of IVF

**: Specific inhibitor of PDE3 (specific PDE of oocytes).

***: Collected oocytes from slaughterhouse-derived ovaries were classified by their diameter (110 to < 115 μm or ≥ 115 μm)

[a, b, c]: $P < 0.05$