Journal of Reproduction and Development, Vol. 67, No 3, 2021

—SRD Innovative Technology Award 2018—

Production, dissemination activities, and supplying-system development for *in vitro* fertilized bovine embryo

Seizo HAMANO[1)]

[1)]*Maebashi Institute of Animal Science, Livestock Improvement Association of Japan, Inc., Gunma 371-0121, Japan*

Abstract. This review discusses the production and sale of fertile oocytes for *in vitro* fertilization technology, calf production through transplantation and delivery, and the current circulation of calves produced by *in vitro* production (IVP) embryos.
Key words: Bovine, *In vitro* production, Supplying system

(**J. Reprod. Dev. 67:** 167–175, 2021)

In vitro fertilization technology was developed in the 1970s, primarily using experimental animals [1–4]. However, its application in livestock breeding took some time. During this period, with the birth of the first *in vitro* fertilized human baby in 1978 [5], this technology began to attract public attention.

The first successful *in vitro* fertilization in cows involved the insemination of cattle oocytes in 1977 by Iritani and Niwa [6]. Since then, several research organizations have been studying artificial insemination. However, in spite of the current penetration rate for artificial insemination being over 95%, *in vitro* fertilization technology is thought to lack practical value for use in calf production.

Nine years later, Hanada *et al*. [7] collected immature oocytes from bovine ovary, which were matured *in vitro*, and successfully produced embryos after *in vitro* fertilization. The embryos were then transplanted, and the birth of a calf was reported for the first time in history. It was argued in this report, that bovine ovaries cannot be used to produce calves. The production of blastocysts completely *in vitro* was not yet possible when this technology was developed. Therefore, transplantation of eight cell embryos into the oviduct of a rabbit, and subsequent culturing *in vivo* for five days, was adopted as the preferred technique.

The author conducted embryo transplantation of Japanese Black cattle by *in vitro* production (IVP) of embryos over a period of 2 years beginning in 1987, with the objective of producing 100 calves. An exhibition was held to showcase the calves, for advertising the feasibility of this technology (Fig. 1).

Finally, a culturing method to generate blastocysts by co-culturing with cumulus cells *in vitro* was established by Goto *et al*. [8].

This success was the turning point, and the bovine ovary, which was until then disposed of as non-edible meat in Japan, was recognized as a genetic resource with the potential to produce individual organisms. Since then, *in vitro* fertilization using bovine oocytes has become a preferred technology for calf production, and through research and development, has evolved into its current form. However, the resulting technology was not readily adopted for cattle production.

It took about 30 more years since then to improve the method of production of IVP embryos, to make it suitable for operational use by producers, and today, more than 30,000 IVP embryos are transplanted every year to produce feeder calves.

This paper will discuss the production and sale of fertile oocytes for *in vitro* fertilization, calf production through transplantation and delivery, and the current circulation of calves produced by IVP embryos.

Background of Development of IVP Technology

In recent years, there has been a decrease in the population of Japanese Black cattle, a genetic resource indigenous to Japan.

A number of underlying social problems are responsible for this decrease, such as difficulty in securing workers due to aging of breeders and their departure from farms, or the deterioration of the environment surrounding the cattle raising areas. However, Japanese Black cattle are essential for the production of beef that best suits the Japanese pallet, and the supply does not meet the demand. Therefore, an increase in the breeding of Japanese Black cattle was planned, and the calf breeding system for producing Japanese Black cattle oocytes by *in vitro* fertilization and embryo transfer was commercialized in 1991, as a part of national measures to increase production for the purpose of improving the sufficiency of domestically produced beef.

In addition, the starting point for large scale adoption this technology was the liberalization of the import of beef and oranges in the 1990 GAT/WTO Uruguay Round. With the liberalization of beef import, it was thought that the management of domestic farmers would be constrained. Therefore, it was proposed that the embryo transfer technique, which had become popular by then, be used to produce high-value-added Japanese Black cattle.

IVP of Bovine Embryos

Although many studies concerned with *in vitro* fertilization using cattle oocytes have been reported recently, this paper will describe the production method of IVP implemented for commercial purposes by the author [9].

Received: November 16, 2020
Accepted: February 8, 2021
Advanced Epub: March 2, 2021

Correspondence: S Hamano (e-mail: hamano@liaj.or.jp)

Fig. 1. The objective of producing 100 calves obtained from IVP embryos in 1987 in Gunma prefecture.

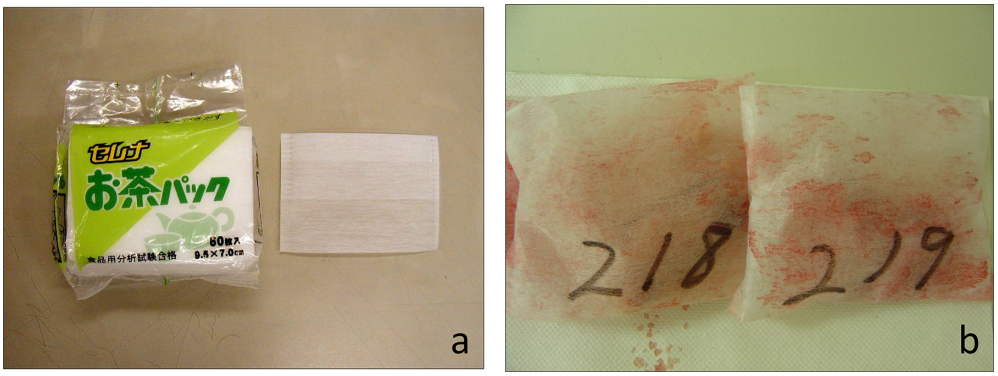

Fig. 2. The ovaries were obtained from individuals and transported to the laboratory in a tea bag (a). The collection number is recorded on the tea bag (b).

Collection of ovaries

Live cattle were shipped from various parts of Japan to a slaughterhouse at TOKYO Meat Market Co., Ltd. (subsequently, Kobe Branch was added in 1992 at Western KOBE Meat Market, and Fukuoka Branch was added in 2016 at Fukuoka Wholesale Meat Market).

Donors were evaluated before slaughtering, distinguishing Japanese Black from other breeds by individual identification (ID) number. Live cattle that passed the veterinary inspection were slaughtered and dressed. Ovaries from Japanese Black and other breeds were obtained from individuals and transported to the laboratory within 1 h in a tea bag (Fig. 2) at room temperature.

An ID number was assigned to individual animals in order to establish their history, as was required after the outbreak of bovine spongiform encephalopathy (BSE) in Japan. Using the information from the ID number, we were able to sort out the Japanese Black cattle from other breeds.

The maximum time from ovary collection to oocyte collection was approximately 2 h. The ovary was collected without being placed in saline solution. The average number of ovaries collected per day was 70. Ovary collection was done from Monday to Thursday every week.

Oocyte collection

All visible follicles (2 to 5 mm in diameter) at the ovarian surface were aspirated from oocytes in follicular fluid, using a 5 ml syringe and an 18-gauge needle. Cumulus-oocyte complexes (COCs) were washed several times in the maturation medium under mineral oil (HUMCO, Austin, TX, USA) in a culture dish (35 × 10 mm, Nunc 153066; Thermo Fisher Scientific, Waltham, MA, USA).

Oocytes collected were classified according to the characteristics of their cumulus cells as follows: Rank A oocytes were surrounded by more than six layers of cumulus cells adhering to the zona pellucida, while Rank B and Rank C oocytes were surrounded by three to five and one to two layers of cumulus cells, respectively. Naked oocytes were assigned to Rank D.

We used individual syringes for each animal ovarian sample. The average number of oocytes obtained by aspiration per donor in 20 COCs.

Oocyte maturation in vitro

Rank A and B COCs were washed several times and cultured in a CO_2 incubator (2% CO_2 in air with high humidity at 38.5°C) for 21 to 22 h [10], in droplets of maturation medium (one oocyte / 10 µl medium) under mineral oil in a multidish (24 well, Nunc 144530). Generally, 5% CO_2 in air is used for *in vitro* culture. However, when we used this concentration, the pH of the medium changed due to the acidity of the co-culture system. Therefore, I developed a strategy using 2% CO_2 to maintain the pH at a neutral level.

The maturation medium was TCM-199 (Earle's salts with 25 mM HEPES, Life Technologies Inc., Grand Island, NY, USA) supplemented with 5% heat-treated fetal bovine serum (FBS, Thermo Trace Ltd., Melbourne, Australia), and antibiotics.

Cultures of Japanese Black cattle immature oocytes were matured *in vitro*, and the rates reaching the second metaphase (Met II) are indicated in Table 1. Compared to the 75% rate of Met II at 18 h after culturing, the rate increased with the increase in cultivation time, reaching 91.5% and 97.5% at 21 and 22 h, respectively [10].

Sperm capacitation

Sperm capacitation was induced using the method described by Parrish *et al*. [11].

After thawing frozen semen at 38°C, the semen was suspended in glucose-free BO medium [12] containing 10 mM theophylline (Sigma-Aldrich Co., St. Louis, MO, USA), and was washed twice by centrifugation for 5 min at 800 × *g*. After washing, the concentration of spermatozoa was adjusted to 2×10^7/ml. The sperm suspension was then diluted two-fold with BO medium containing 10 mg/ml bovine serum albumin (Fraction V, Fujifilm Wako Pure Chemical Co., Osaka, Japan) and 10 µg/ml heparin (NOVO-Heparin Injection 1000, Aventis Pharma Ltd., Tokyo, Japan).

In vitro fertilization

At the end of culture maturation, the COCs were washed by pipetting 2 or 3 times in the medium, and were then introduced into the drops (0.5 ml) of sperm suspension. Oocytes and spermatozoa were cultured together for 5 to 6 h, while the maturation medium from which the oocytes were removed, although it still contained cumulus cells, was kept in a CO_2 incubator. All of the inseminated oocytes, still surrounded by cumulus cells along with the attached motile spermatozoa, were then introduced into the maturation medium that had been kept with the cumulus cells in the CO_2 incubator.

In vitro culture

At 48 h after insemination, the oocytes were removed from the cumulus cell mass by pipetting, and were observed for their stage of cleavage. Four to eight cell embryos were further cultured with a cumulus cell monolayer. I found that the occurrence of the blastocyst stage improved significantly on adding β-merchaptoethanol to the culture medium for 72 h after insemination [13]. Embryos were assessed for development of the blastocyst stage between days 7 to 8 of post-insemination (Day 0 = day of insemination).

Table 1. Time required for *in vitro* maturation of bovine follicular oocytes

Culture periods [1)]	No. of oocytes examined	No. (%) of oocytes at stage of MII [1)]
18	80	60 (75.0 [a)])
19	80	62 (77.5 [a)])
20	80	69 (86.3 [ab)])
21	82	75 (91.5 [bc)])
22	80	78 (97.5 [bc)])

[1)] MII: metaphase II. Values with different superscripts differ significantly ($P < 0.05$).

Freezing and thawing of IVP embryo

The development of embryos to the blastocyst stage was examined under a microscope. CodeIblastocysts [14] were used for cryopreservation. Embryos were removed from the culture medium and placed directly into the equilibration medium. The equilibration medium was modified-199 (TCM-199 (Earle's salts without sodium bicarbonate, Powder, Gibco, Thermo Fisher Scientific) containing 20 mM HEPES and 0.35 g/l sodium bicarbonate (adjusted to pH 7.35)) [9], supplemented with 20% calf serum (CS, HyClone, GE Healthcare UK Ltd., Buckinghamshire, UK), 10% glycerol, and antibiotics. Equilibration was performed for 15 min at 25°C. After equilibration, embryos were transferred to freezing medium (modified-199 containing 20% CS, 10% glycerol, and 0.25 M sucrose) and subsequently loaded with cryoprotectant into 0.25 ml plastic straw (Plastic Cassou AI mini straw, IMV Technologies, L'Aigle, France) and ultrasonically sealed.

The straws were placed directly into a programmable freezer (alcohol bath) at 6°C. After 1 min, the straws were seeded, maintained at –6°C for another 9 min, cooled to –25°C at –0.33°C /min, and then plunged into liquid nitrogen.

The straws were thawed by allowing them to stand in air for 10 sec, followed by immersion in 30°C water for 10 sec.

Maintenance of an IVP system

Badge check of fetal bovine serum is an important factor in maintaining the developmental rate of the blastocyst stage obtained by IVP [15]. The productivity is also influenced by the culture dish and mineral oil used in IVP.

Embryo transport

Developed blastocysts were transported to farms across Japan in two conditions:

1) Non-frozen via air or ground shipping using a cell transport container. The CodeIgrade blastocysts were transferred to a transport medium (modified-199 containing 20% CS, 100 µM β- merchaptoethanol and antibiotics [16]), and subsequently loaded into 0.25 ml plastic straws and ultrasonically sealed. Then, we put the straw in a portable incubator containing a small battery (Fig. 3: Cell transporter, Fujihira Industry Co., Ltd., Tokyo, Japan) and transported it thereafter. The IVP embryos were kept warm at 38ºC within the portable incubator during transit. With home delivery, the embryos were shipped from our center in the evening, and were expected to reach the farmhouse early in the morning the following day. We

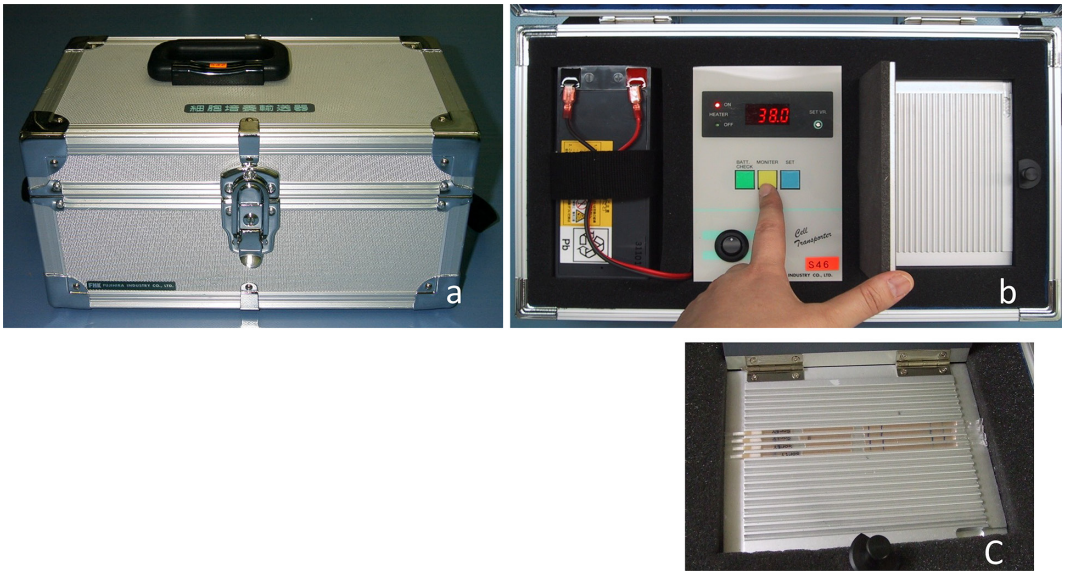

Fig. 3. Cell transporter: Portable incubator (a) containing a small battery, was kept warm at 38°C (b). The container for transporting the straw tube containing the fresh IVP embryo. Survey and the inside of a portable incubator. Twenty 0.25 ml straws can be placed on the metallic plate (c).

used modified-199 medium, which provides a modified atmosphere to prevent pH change during the transport of embryos.

2) Cryopreserved in glycerol for direct transfer.

Types of IVP embryos

The following four types of IVP embryos were used to produce feeder calves.

(1) IVP embryos in Japanese Black cattle, wherein only the bull was identifiable. The obtained oocytes were collected randomly, with 100 oocytes per culture dish, so that the individual females could not be identified. This was done to improve the efficiency of production of the oocytes fertilized *in vitro*, and to prevent attention from being focused on a particular family line. Next, blastocysts produced *in vitro* were morphologically observed, and only those blastocysts that were determined to have excellent implantation ability were used to increase the production of feeder calves, whose breed was known to be Japanese Black cattle. Only the bulls used in the *in vitro* fertilization were identifiable, although the family line or carcass grade of the female was unknown.

(2) Sex determination of blastocysts: The blastocysts were determined to be male based on sex determination performed on a part of the blastocyst produced in (1). Sex determination was performed on the blastocysts determined to be excellent in quality by morphological observation under a microscope, and a portion of trophoblast cells were removed by biopsy conducted according to the LAMP method (Eiken Chemical Co., Ltd., Shimotsuga-gun, Tochigi, Japan). Although the primary objective was to increase the production of feeder calves as in (1), another objective was to achieve greater sales of the calves in the livestock markets. Subsequently, it was developed into IVP using sex-sorted semen.

(3) Holstein Friesian IVP embryo: The ovaries of alternating Holstein Friesian cows were collected and IVP embryo was produced using sex-sorted semen.

(4) Crossbreed IVP embryo: Holstein Friesian matured oocytes were inseminated with frozen-thawed semen from a Japanese Black bull.

In vitro production of bovine embryos

The results of *in vitro* production are shown in Table 2. Semen from six bulls was used; however, the rate of production was determined depending on the marketability of the calves. The results of *in vitro* production were consistent, with ratios showing no significant difference between the bulls in terms of the rate of division into more than 2 cells at 48 h after insemination, or in terms of incidence of blastocysts.

The results of the sex determination test of bovine blastocysts obtained from *in vitro* fertilization conducted in 2005 and 2006 are shown in Table 3. The sex determination of extracted embryos indicated that there were more male fertilized eggs, with a ratio of 6:4.

Utilization of IVP Embryos

This method is expected to reduce the costs of beef production, since the purchase price of IVP embryos is relatively low compared to IVP embryos through cooperation between dairy farmers and fattening farmers.

The purpose of the transfer of Japanese Black cattle blastocysts was to produce Japanese Black calves using Holstein cattle that were determined to not produce female calves as recipients. Therefore, approximately 95% of the recipients were Holstein. There were also cases of transfer to other recipients, such as crossbred cattle (Holstein × Japanese Black) or Japanese short horn cattle.

The only disadvantage of the cattle blastocysts produced by the authors is that the calves produced by transplantation are not eligible for calf registration. The registration of Japanese Black cattle in

Table 2. Production results of cattle oocytes fertilized *in vitro*

Bull	No. of oocytes cultured	No. (%) of embryos cleaved [1)]	Total No. (%) of Blastocysts on day 8 [2)]
A	122438	81321 (66.4)	31825 (26.0)
B	62721	44917 (71.6)	19910 (31.7)
C	51182	37534 (73.3)	15023 (29.4)
D	13401	9820 (73.3)	4641 (34.6)
E	9306	6083 (65.4)	2649 (28.5)
F	2898	2021 (69.7)	1039 (35.9)

[1)] Oocytes were examined 48 h after insemination. [2)] Day 0 = day of insemination.

Table 3. Results of sex determination of cattle IVP embryos

Year	No. of blastocysts [1)] used for sexing	No. (%) of male embryos
2005	3600	2340 (63.2)
2006	5793	3354 (57.9)

[1)] Expand blastocysts at day 7 (Day 0 = day of insemination).

Japan is conducted according to the registration regulations of the Wagyu Registry Association Corp (calves produced by registered cattle may be registered, but those produced by only a registered female may not be).

The cattle from which the ovaries are collected for the production of cattle IVP embryos are almost all fattened cattle meant for shipment, that have only calf registration. Therefore, the calves produced by the transfer of IVP embryos produced by the authors are not permitted for calf registration.

As a result, the calves produced from IVP embryos were often confused with crossbreds when sold in livestock markets, and were thus frequently sold at a low price. As a result, recognition of the value of IVP embryos was low.

Considering these circumstances, 5 years after the start of sale of calves produced from IVP embryos, the following three systems were established with the objective of confirming the sale of the produced calves before transplantation (Fig. 4):

(1) Selling calves through contract with fattening farmers

(2) Selling calves in livestock markets

(3) Developing a consecutive process from the production to the fattening of calves

Much effort was also made to establish a distribution method for the final product. However, afterwhen the fattening of the calves produced by transfer of IVP embryos was complete, and as it became clear that beef produced was high quality, the calves began to be actively traded livestock markets across the country. Ultimately, the calves were freely and willingly adopted by farmers, and in recent years, these calves have been traded at a stable price in livestock markets.

Sale of IVP Embryos

The number of IVP embryos sold since 1989 is indicated in a bar graph (Fig. 5).

In 1991, we sold only 1,311 IVP embryos. In the following years,

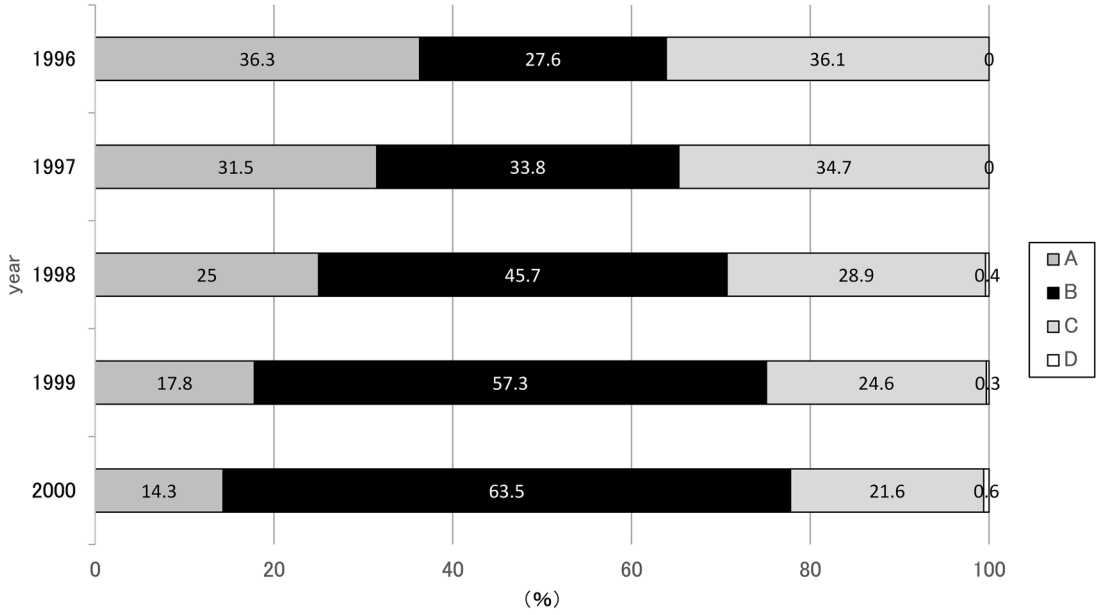

Fig. 4. Change in the sales system of calves obtained from IVP embryos. A: Selling calves through contract with fattening farmers. B: Selling calves at livestock markets. C: Developing a contract process from the production to the fattening of calves. D: Other.

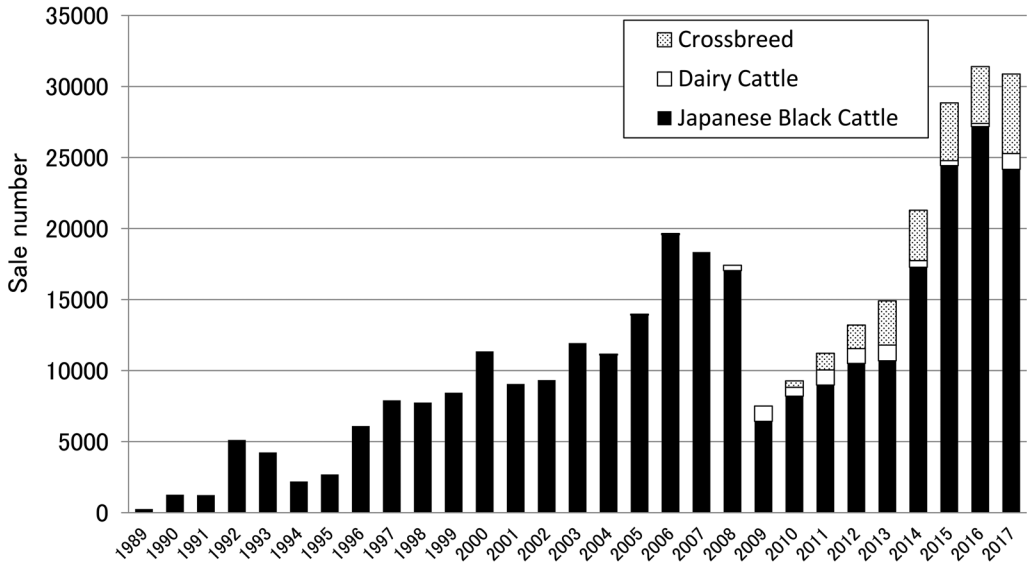

Fig. 5. Change in the number of sales of the IVP embryos.

sales volumes did not increase as per our expectations. There were two major reasons for this. Firstly, the technique of *in vitro* fertilization was not pervasive, and secondly, the conception rate after transplantation was low. The rate of conception is an important factor determining the usefulness of embryo transfer technology in the production of calves.

A workshop of the fusion method was held in the area where IVP embryos were used for calf production. I prepared the manual describing how to use IVP embryos, and made an effort to improve the conception rate.

Initially, only frozen embryos were sold, and fresh embryos were introduced in 1997.

Around 1997, carcass results of IPV embryo customers began to become clear. As a result of this, and due to the gradual improvement in conception rate and the evaluation of meat quality, the number of units sold has started to increase since 1998.

From these experiences, I felt the need to shift the focus from research and development to the dissemination of technology. Since then, the number of units sold by IVF has repeatedly increased and decreased due to circumstances stemming from the state of the economy, and therefore beyond our control. The selling price of a calf in a livestock market rose, and the number of embryos used gradually increased as well.

The BSE outbreak that occurred in Japan in 2001 was the first such experience for the country. Consumers were therefore discouraged from buying beef, and so the number of IVPs sold also stagnated. However, the government's response kept food safety unaffected and recovery was seen by 2006, when 19,500 IVPs were sold.

Later, the foot-and-mouth disease outbreak that occurred in 2010, and the financial crisis of 2007-2008, caused the sales numbers, that had been steadily increasing, to decrease once again. After these crises, it took four years for the sales numbers to recover. Meanwhile, we started producing and supplying the embryos of dairy and crossbred cattle, in order to diversify the use of IVP.

Marketing of Calves Produced from IVP Embryos

As a part of meat quality assessment, the establishment of a route to sell IVP embryo-derived calves was also necessary. Although a number of options can be considered for the utilization of embryos produced by *in vitro* fertilization in farming, the cash sales of the calves produced is the most important aspect of business, and marketing in livestock markets enables this exchange in a short period. Japanese Black cattle are reared for approximately 10 months before being listed as breeding cattle, but the calves from IVP embryos are predominantly sold at a young age of approximately 2–3 months.

Actual sales in Japan were found to be predominantly in the Kumamoto prefecture. The sale price of IVP derived calves is also 20,000–30,000 yen higher than that of cross breeds (Holstein × Japanese Black) at the beginning of the sale. However, in recent years, the sales prices of male cattle have stayed at 100,000–200,000 yen or higher, and for females they have stayed at approximately 90,000 yen or higher. The cattle have been recognized by farmers as products of high value.

The calves reflected in the sales results originated from the IVP embryos, but their maternal family lines cannot be identified and they have no calf registration.

Grade of Beef of Calves Produced from IVP Embryos

As long as the breed of IVP embryos that we produce is Japanese Black cattle, the price of the final beef product will have a large impact on the sales of calves in livestock markets and the sales of IVP embryos (Fig. 6). Although as much data as possible was collected on the grade of the beef produced, since the data covers an extended period of time and includes data reflecting the economic conditions in each period, a simple comparison of average prices was not considered useful. Therefore, a demonstration of the economics

Fig. 6. 1st Annual ET Wagyu Beef contest obtained by IVP embryo in Tokyo Meat Market Co. (18th June, 1999).

of Japanese Black cattle produced from IPV was held at the Tokyo Meat Market to evaluate the varieties.

As indicated in Table 4, the weight of the carcass was higher than that reported in the data from the national Japanese Black cattle castration, and the quality was also higher. The calves derived from IVP embryos were found to have superior productivity by meat market officials. As a result, IVP embryos were recognized as having great value for various purposes throughout the distribution line, from the farmers using transplantation for calf production to the meat markets handling the carcasses.

Beginning with the establishment of a system to produce large quantities of Japanese Black cattle embryos at a low price using *in vitro* fertilization, we have arrived at a point where technology now exists for the production of the more profitable gender of cattle.

Conditions for Calf Production (Large Offspring Syndrome: LOS)

As indicated in Fig. 2, the transfer of IVP embryos to increase the production of feeder calves in Japanese Black cattle has been expanding every year. On the other hand, there are reports of miscarriages and risk of LOS resulting from conception by transfer of IVP embryos [17]. Therefore, an investigation was conducted on the conditions of delivery of embryos derived from *in vivo* and *in vitro* fertilization, transferred in the same area and in the same time period.

Table 5 shows the gestation period after transfer of embryos produced through *in vivo* and *in vitro* fertilization. The gestation periods for male and female births by transfer of *in vivo* embryos were 281.4 and 280.2 days, respectively. In comparison, the gestation period in cases of male and female births by implantation of IVP embryos were 283.6 and 281.4 days, respectively, indicating longer gestation period for both male and female calves originating from IVP embryos.

As indicated in Table 6, the birth weights of male and female calves originating from *in vivo* embryos were 32.5 and 29.8 kg respectively, and the birth weights of male and female calves originating from IVP embryos were 33.8 and 31.2 kg respectively, indicating that

Table 4. Comparison of carcass weight and marbling score in Japanese Black Cattle [1] obtained by transfer of *in vivo* and *in vitro* produced bovine embryos

Embryo origin	*In vivo*		*In vitro*	
Sex	Steer	Female	Steer	Female
n	95	40	88	15
Final days of age	29.4	29.7	28.9	28.6
Carcass weight (kg)	550.0	511.7	547.6	503.9
'Rib-eye' muscle area (cm^2)	73.2	72.2	72.1	67.2
Beef marbling standard score	8.6	8.1	8.3	7.4

[1] Annual ET Wagyu Beef contest in Tokyo Meat Market Co. at 2015, 2016, 2017 and 2018.

Table 5. Gestation length (days) of the calves [1] obtained by transfer of *in vivo* development and *in vitro* produced bovine embryos

Embryo origin	Sex	Gestation length (days)
In vivo	Male	281.4
	Female	280.2
In vitro	Male	283.6
	Female	281.4

[1] No. of calves: *in vivo* 107, *in vitro* 788.

Table 6. Birth weight (kg) of the calves obtained by transfer of *in vivo* development and *in vitro* produced bovine embryos

Embryo origin	Sex	Birth weight (kg)
In vivo	Male	32.5
	Female	29.8
In vitro	Male	33.8
	Female	31.2

the calves originating from IVP embryos were heavier than those originating from *in vivo* embryos.

For the calves originating from IVP embryos, the gestation periods for *in vitro* fertilization are indicated by bull in Table 7. For the calves originating from the five bulls, the gestation periods for the male and female calves from bull A were 285.4 and 283.5 days respectively, which were the longest among the gestation periods of calves originating from all five bulls. Similarly, the birth weights of calves from *in vitro* fertilization are indicated by bull in Table 8. For the calves originating from the five bulls, the birth weights for male and female calves from bull A were 36.0 and 33.2 kg, respectively, which were the heaviest among the calves originating from all the bulls.

From these findings, it can be speculated that there are more incidences of prolonged gestation for calves derived from IVP embryos compared to those from *in vivo* embryos, and the type of bull is also a factor. However, the underlying cause has not yet been identified. As a measure to improve this situation, in cases where there is no sign of calving after the due date, induction of labor may be performed after consultation with a veterinarian.

During the transplantation of IVP embryos, the occurrence of LOS is a significant problem. The occurrence rate approached zero after the use of bull, which became a problem only and up to now was canceled, but it isn't a zero.

Table 7. Gestation length (days) of the calves sired by five bulls and developed from embryos produced *in vitro*

Bull	Calf sex	Gestation length (day)
A	Male	285.4
	Female	283.5
B	Male	283.6
	Female	283.4
C	Male	281.4
	Female	280.4
D	Male	281.8
	Female	275.1
E	Male	280.9
	Female	277.3

Table 8. Birth weight (kg) of the calves sired by five bulls and developed from embryos produced *in vitro*

Bull	Calf sex	Birth weight (kg)
A	Male	36.0
	Female	33.2
B	Male	28.9
	Female	28.8
C	Male	31.2
	Female	28.8
D	Male	29.5
	Female	27.2
E	Male	31.8
	Female	28.7

Future Prospects

We have been exploring the novel use of IVP embryos in which a portion of beef cattle production is relegated to dairy farmers and employs unused Japanese Black cattle oocytes, a genetic resource indigenous to Japan, in order to satisfy the needs of customers.

The practice of transfer of IVP embryos, which began with the proposed production of Japanese Black feeder calves with high added value, has been ongoing for 15 years since the beginning of national distribution. It has been proven that these cattle are not different from Japanese Black calves produced from artificial insemination, or those originating from *in vivo* embryos, and yet have a higher production value.

As a result of improvements in the conditions of *in vitro* fertilization, semen from various breeds can now be used, and by increasing the variations of IVP embryos, the calves are now beginning to attract higher prices in livestock markets. In addition, producers are now beginning to search for ways to use this technology to improve their own business.

Due to these developments, there have been a number of requests from farmers who, until now, were not interested in using *in vitro* fertilization, and from farmers who tried and abandoned it in the past after unsuccessful attempts. This indicates an increased expectation among farmers with regard to *in vitro* fertilization. Further development of this technology is expected to deliver better results to farmers who wish to employ it.

Acknowledgements

I would like to thank the conference for receiving the prestigious award. In addition, I would like to extend my gratitude to the Animal Bio-Technology Center staff and related persons, who have continued to support this work. Finally, I would like to specially thank the Japanese dairy farmers who believed and used IVP technology.

References

1. **Yanagimachi R, Chang MC.** Fertilization of hamster eggs *in vitro*. *Nature* 1963; **200**: 281–282. [Medline] [CrossRef]
2. **Pickworth S, Change MC.** Fertilization of Chinese hamster eggs *in vitro*. *J Reprod Fertil* 1969; **19**: 371–374. [Medline] [CrossRef]
3. **Toyoda Y, Yokoyama M, Hoshi T.** Studies on the fertilization of mouse eggs *in vitro*. I. *In vitro* fertilization of eggs by fresh epididymal sperm. *Jpn J Anim Reprod* 1971; **16**: 147–151. [CrossRef]
4. **Yanagimachi R.** Fertilization of guinea pig eggs *in vitro*. *Anat Rec* 1972; **174**: 9–19. [Medline] [CrossRef]
5. **Steptoe PC, Edwards RG.** Birth after the reimplantation of a human embryo. *Lancet* 1978; **2**: 366. [Medline] [CrossRef]
6. **Iritani A, Niwa K.** Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. *J Reprod Fertil* 1977; **50**: 119–121. [Medline] [CrossRef]
7. **Hanada A, Suzuki T, Shioya Y.** Birth of calves from nonsurgical transfer of blastocysts originated from in vitro fertilized oocytes matured *in vitro*. Proc 78th Ann Meet Jpn Spci Zootech Sci 1986; 18 (Abst).

8. **Goto K, Kajihara Y, Kosaka S, Koba M, Nakanishi Y, Ogawa K.** Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in-vitro matured follicular oocytes. *J Reprod Fertil* 1988; **83**: 753–758. [Medline] [CrossRef]
9. **Hamano S, Kuwayama M.** *In vitro* fertilization and development of bovine oocytes recovered from the ovaries of individual donors: A comparison between the cutting and aspiration method. *Theriogenology* 1993; **39**: 703–712. [Medline] [CrossRef]
10. **Hamano S, Tuchiya H, Yoshikawa M.** *In vitro* aging of bovine follicular oocytes. *Jpn J Embryo Transfer* 2015; **37**: 107–112.
11. **Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL.** Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* 1986; **25**: 591–600. [Medline] [CrossRef]
12. **Brackett BG, Oliphant G.** Capacitation of rabbit spermatozoa *in vitro*. *Biol Reprod* 1975; **12**: 260–274. [Medline] [CrossRef]
13. **Hamano S, Kuwayama M, Takahashi M, Okamura N, Okano A, Nagai T.** Effect of β-merchaptoethanol on the preimplantation development of bovine embryos fertilized *in vitro*. *J Reprod Dev* 1994; **40**: 355–359. [CrossRef]
14. **Wright JM.** Photographic illustrations of embryo developmental stage and quality codes. In : Stingferrow DA, Givens MD (eds), Manual of the International Embryo Transfer Society, 4th Edition. Savoy Il: IETS; 2010: 141-144.
15. **Hamano S, Kuwayama M.** Effect of featl bovine serum added to the medium on the development of bovine oocytes matured and fertilized *in vitro*. *Jpn J Anim Reprod Tech* 1992; **14**: 6–16.
16. **Takahashi H, Kuwayama M, Hamano S, Takahashi M, Okano A, Kadokawa H, Kariya T, Nagai T.** Effect of β-mercaptoethanol on the viability of IVM/IVF/IVC bovine embryos during long-distance transportation in plastic straws. *Theriogenology* 1996; **46**: 1009–1015. [Medline] [CrossRef]
17. **Kurip TAM, den Daas JHG.** *In vitro* produce and cloned embryos: Effects of pregnancy, parturition and offspring. *Theriogenology* 1997; **47**: 43–52. [CrossRef]