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Research Article
Approaching Insights to *in vitro* Production of Bovine Embryos and the Growth Rate to Blastocyst

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Abstract

Background: Cattle oocytes were collected from live animals by Ovum Pick Up (OPU) or from Slaughter House Ovaries (SHO) by aspiration using needle. The *in vitro* fertilization (IVF) technique consists of *in vitro* maturation (IVM) of oocytes, fertilization and *in vitro* cultures (IVC) of the embryos. Mature oocytes are cultured with sperm for fertilization resulting in zygotes formation. Those zygotes are cultured for cleavage till reach the blastocyst stages. Those embryos are transferred to recipients directly or cryopreserved. **Materials and Methods:** This study presents the characteristics of OPU-embryo growth from alive Japanese black cattle (n = 635) and those originated from SHO (n = 10,147). The ova were processed for selection, cleavage, maturation and fertilization parallel to handling of good spermatozoa used for fertilization. Our methods were commonly used with slight modifications. Differences between OPU and SHO-originated embryos at each growth rate were tested by statistical significance by chi-square. **Results:** Cleavage rates by 48 h after the IVF of the oocytes were 76.1 and 81.7% for the OPU and SHO, respectively. However, the embryo growth to the blastocyst by 6-9 days later was 38.6 and 28.5%, respectively. The percentage of blastocysts (day 9) is lower than expected because the development of some embryos is arrested at earlier stages in particular those originated from the SHO. **Conclusion:** The growth results of an OPU origin ovum and those of a SHO origin ovum are almost the same. The growth rate to the blastocyst of an OPU origin ovum is strongly subject to the influence of a sire and donor cow and its variation is large. This study presents a simple lab technique for cattle IVF and recommends the slaughter house ovaries (SHO)-originated oocytes, not risky and easily applied, than those originated by ovum-picking up (OPU) from alive animals. Future studies are required to improve the oocyte maturation percent into blastocyst.

Key words: Oocytes, sperm, embryos, *in vitro*, OPU

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The *in vitro* production (IVP) of embryos as one of the Assisted Reproduction Technologies (ART) (Gardner *et al*., 2011) is now a days a biotechnique widely used commercially in animal breeding as well as a treatment of the infertility problems (Duszewska *et al*., 2010; Freitas and Melo, 2010; Dang-Nguyen *et al*., 2011; Zhao *et al*., 2011). Offspring of some farm animal species that have been obtained after transfer of embryos obtained *in vitro* to recipients include sheep (Paramio, 2010) and cattle (Lonergan and Fair, 2008). There are many potential benefits of using *in vitro* fertilization (IVF) to produce embryos. The most obvious is that by transporting embryos from distant locations into a local region a complete set of new genes can be introduced. These embryo transfers provide greater genetic gains over that obtained with artificial insemination, whereby only the paternal half of the genome can be introduced. Embryo transportation is also considerably easier than live animal transportation. Importantly, the chances for disease transmission are greatly reduced when compared to whole animal or semen introductions. There are two origins of the bovine oocytes; the slaughter house ovaries (SHO) and the ovum-picking up ovum (OPU) from a live animal. Obtaining farm animal embryos *in vitro* is a multi-step procedure comprising; *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). The most important factors responsible for success in obtaining embryos *in vitro* include: (1) The source of oocytes and sperm (including the age and physiological state of female and male), (2) Technical factors and (3) Highly skilled laboratory and veterinary staff.

The key technical factors are consisted of the media used for maturation, fertilization and embryo culture (containing water, amino acids, vitamins, hormones, growth factors and additives obtained from albumin, serum, etc.), conditions of maturation, fertilization and culture (temperature, CO_2 level and humidity), the timing of oocyte maturation, fertilization and embryo development (Duran, 2000; Camargo *et al*., 2006). This study aimed to investigate if there is a difference in the growth maturation of bovine embryos originated from the SHO and those from the live animals with regard to the same laboratory technique of the oocyte handling and maturation.

MATERIALS AND METHODS

Media: Seven types of liquid media used for all procedures from collection of the oocytes up to *in vitro* fertilization and freezing of embryos, including: (1) H199 medium (HEPES-M199 medium for oocytes collection), (2) Maturation medium (M199), (3) Fertilization medium (IVF-CDM), (4) Percoll preparation, (5) Culture media; CDM-1 and CDM-2 and (6) Freeze liquid medium: 1.5 M ethyleneglycol+0.1 M sucrose+mPBS. All the media for IVM/IVF/IVC used in this investigation were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

Collection of oocytes, maturation, fertilization and culture: The oocytes are collected by two methods; the first is applied from the slaughter house origin as following: (1) The ovaries are collected from the slaughtered cows, (2) They are placed in a thermos flask containing sterile sodium chloride solution (0.9%) supplied with 100 i.µ., penicillin and 100 µg streptomycin per milliliter (Samardzija *et al*., 2006), (3) The ovaries-containing thermos flask is transferred within 4 h at 27-35°C, (4) On arrival at laboratory, the saline in the thermos flask is changed and the ovaries in the flask are washed at least twice with a sterile saline at 37°C to be ready for aspiration of the oocytes, (5) Each ovary is dried with a tissue paper and blow out with 70% alcohol and (6) The oocytes are aspirated from 2-8 mm follicles using a sterile 18 G needles connected to a sterile 10 mL syringe.

The second method of obtaining oocytes is the ovum-picking up (OPU) from a live cow. It can be performed by using one cow (donor) every 4-10 days with or without ovarian stimulation. The donor cow can give 5-50 oocytes which are immature and located within small sized follicles. Ovarian stimulation does not increase the number of oocytes but only enhance the growing up of the present follicular sizes. For ovarian stimulation, FSH (5-10 i.µ.) is injected intramuscularly a day before OPU. The PMSG is also used for ovarian stimulation but not preferred as it contains LH which may lead to ovulation or follicular atresia. The technique is performed by using transvaginal ultrasound probe supported with a long needle and receiving the oocytes in a 50 mL tube containing PBS.

Oocyte washing: The H199 medium is used for washing the oocytes, the slaughter house origin and those by OPU, either. The medium containing oocytes are collected in a sterile 15 mL test tube or a 50 mL universal container. The content of the tube is allowed to settle down and then 3/4 of the supernatant fluid is removed. The remaining content is transferred into a sterile 60 mm petri dish with rinsing the test tube or the universal container with 2 mL of H199 medium at least twice. Add sufficient H199 medium to facilitate searching of the oocytes. Search for oocytes in the petri dish under stereo microscope with 10-15X magnification and the oocytes are transferred into a standard petri dish containing 3 mL

H199 medium. Check the dish at least three times to ensure that all oocytes have been removed.

Oocytes selection: Following collection, oocytes are evaluated and only good oocytes are used for *in vitro* maturation (IVM). The following criteria must be met by a good oocyte; surrounded by at least 4-5 layers of cumulus cells, bright, ungranulated, even cytoplasm and those cumulus cells are unexpanded, compact and even. Finally, the oocytes are washed twice with pre-equilibrated H199 medium and then once more with pre-equilibrated M199 medium.

Selection and *in vitro* maturation: The selected oocytes are transferred into a 4-well plate containing 1 mL of M199 medium as 40-50 oocytes/well and then incubated at 39°C, 5% CO_2 with maximum humidity for 22-24 h.

In vitro fertilization and culture

Preparation of fertilization drops: Prepare 40 μL droplets with IVF-CDM medium in a 60 mm petri dish covered and with sterile mineral oil. Equilibration; after preparation of droplets and then incubate at 39°C, 5% CO_2 and maximum humidity for 2 h prior to use.

Semen preparation: Semen was obtained and spermatozoa processed for separation by the method previously described by Parrish *et al*. (1986, 1995), but with modification using percoll (Avery and Greve, 1995; Mendes *et al*., 2003). Into a sterile 15 mL conical tube, dispense 2 mL of 90% percoll then layer 2 mL of 45% percoll on the top and incubate at 39°C, 5% CO_2 with maximum humidity for at least 2 h prior to use. This is enough for 1-2 straw of frozen semen. One straw of sexed frozen semen (at a sperm number of 4×10^6/straw and 100 oocytes to be inseminated) is thawed by direct immersion into warm water at 37°C for 30 sec. Wipe the outer wall of the straw with tissue paper dampened with 70% alcohol and then checked for the sperm motility. Centrifugation: Spin semen at 2500 rpm for 20 min and directly after centrifugation, carefully remove the top layers of the tube and leave loose pellet of live sperm (around 100 μL in volume) at the bottom of the tube. Thereafter, 1 mL of IVF-CDM medium is added and spanned one more time at 1500 rpm for 5-10 min. Finally, the top layers of the tube are removed leaving loose pellet of live sperm (around 50 μL in volume) at the bottom of the tube. Sperm quality parameters were evaluated after thawing and after sperm preparation for IVF. Sperm concentration was determined with a thoma chamber according to Herak (1991). Progressive motility of semen was subjectively assessed by visual estimation under a microscope according to Hammerstedt *et al*. (1988). Compensate sperm pellet by adding IVF-CDM medium to obtain a sperm concentration of 10×10^6 mL^{-1}. This semen is ready for insemination.

Oocyte preparation: The cumulus cells are partly removed from the oocyte by repeatedly dispensing in H-CDM until 4-5 layers of cumulus cells are left around the oocyte. Then, the oocytes are washed twice in H-CDM medium and once more in IVF-CDM medium and finally transferred into the fertilization drops (10-15 oocytes per drop).

Fertilization: According to Edwards *et al*. (1997) with modifications, 5 μL of the prepared semen is added to the oocytes into the fertilization drop if the final concentration is 1 million per milliliter. then, the oocytes and spermatozoa are incubated at 39°C, 5% CO_2 with maximum humidity for at least 6-7 h. About 6-7 h after insemination, the cumulus cells are completely removed from the presumptive zygotes by repeatedly pipetting in the fertilization drops and washing the zygotes three times in drops containing pre-equilibrated CDM-1 medium under mineral oil. The fertilized oocytes are removed from the washing drops to 4-well plate containing CDM-1 medium with 40-50 zygotes per well and culture at 39°C, 5% CO_2, 90% N_2 with maximum humidity for 89-90 h.

Check of cleavage and embryo growth: After culture of zygotes in CDM-1 for 89-90 h check the cleavage and remove the uncleaved oocytes from the well and replace with 0.6 mL pre-equilibrated CDM-2 medium. Early embryos are continuously cultured under the same condition (39°C, 5% CO_2, 90% N_2 with maximum humidity) for 72-96 h. Bovine embryos were evaluated according to IETS standards: On day 2 of culture we registered the number of cleaved embryos (fertilization rate); on day 6 up to 9 the number of morulas and blastocysts (Stringfellow, 1998).

Statistical analysis: The percentage of embryos developing into cleaved morulae (day 2) and blastocysts (day 6-9) was expressed as the proportion of all oocytes cultured for maturation. Differences were tested for statistical significance by chi-square test.

RESULTS AND DISCUSSION

The number of morulae and blastocysts appeared after cleavage of the *in vitro* fertilized oocytes collected from alive cows by OPU (N = 635) and from SHO (N = 10147) and that the

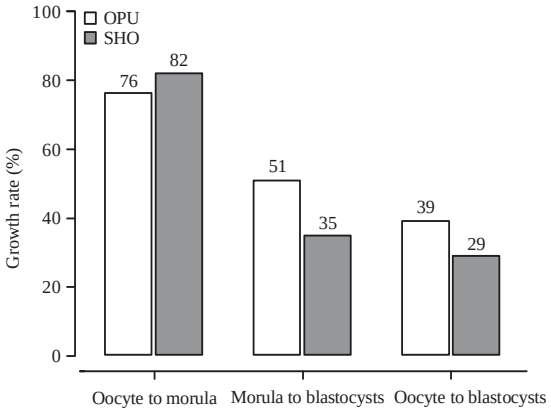

Fig. 1: Growth rate (%) of the embryos fertilized *in vitro* from the early stage of oocyte up to the blastocyst stage. The oocytes collected from alive cattle by OPU (N = 635 oocytes) and those collected from the SHO (N = 10147 oocytes)

Table 1: Number and percentage of morulae and blastocysts appeared after cleavage of the fertilized oocytes *in vitro*

Collection method	Oocytes (n)	Cleavage rate 48 h later of IVF (%)	Blastocysts rate (day 6-9) (%)
OPU	635*	76.1	38.6**
SHO	10147#	81.7	28.5##

*Denaturing oocytes, only zonae pellucidae is included, **The best results, 46 of blastocyst (80.7%) from 57 immature ova collected from 3 donors, #Denaturing oocytes is included. ##A harmful experiment is included to an ovum. The oocytes were collected from two origins; from alive cows by ovum picking up (OPU; N = 635 oocytes) and from slaughter house ovaries (SHO; N = 10147 oocytes). The cleavage rates by 48 h and after IVF were 76.1 and 81.7%, respectively. The blastocysts rates by 6-9 days after IVF were 38.6 and 28.5%, respectively

cleavage and growth rates are shown in Table 1 and Fig. 1. The cleavage rates of the oocytes after IVF into morulae by 48 h were 76.1 and 81.7% for the OPU and SHO, respectively. The growth rates into blastocysts by 6-9 days after IVF were 38.6 and 28.5%, respectively. Collectively, the growth rate to a blastocyst is about 20-45%, suggesting the regulatory role played by growth factors in the ovarian function. Thus, future studies are required to improve the oocyte maturation into blastocysts especially those originated from the slaughter house ovaries.

Cleavage and growth rates of the oocytes into morulae, the morulae into blastocysts and the oocytes into blastocysts had shown non-significant differences between the OPU and SHO, either (Chi square 3.4, df 2) (Fig. 1). Representative microscopic images of oocytes after fertilization *in vitro* are shown in Fig. 2. The methods of oocyte aspiration from the SHO are shown in Fig. 2a and b. The collected oocytes in H199 medium before and after selection are shown in Fig. 2c and d, respectively. The cleavage of oocytes into morulae and growth of the blastocysts throughout 9 days after IVF are shown in Fig. 2e and f.

Several studies were established for producing bovine embryos *in vitro*. *In vitro* fertilization (IVF) is the process by which the sperm was allowed to fertilize the oocyte in a petri dish outside the uterus. Oocyte retrieval after the donor's death can be performed by aspiration from the follicle using a needle and syringe. On the other hand, retrieval of oocytes from living donors is performed by laparoscopy to visualize the ovaries, in the small animals, or ultrasound imaging with a vaginal probe and needle, in the large animals like cattle (Fortune *et al.,* 1991).

The Ovum Picking Up (OPU) by the aid of ultrasound in live animals could be performed twice a week without a harmful effect on the animal's fertility (Galli *et al.,* 2001; Chastant-Maillard *et al*., 2003). After maturation, oocytes and sperm are co-incubated 6-8 h using frozen semen. After co-culture, sperm are washed away and the embryos are cultured for an additional 9 days, allowing blastocyst formation to occur. The development of embryos produced in different culture systems was evaluated by their ability to develop *in vitro* into blastocysts and hatched blastocysts at day 9 after fertilization. It was found that about 7 days after fertilization of the oocytes, up to 40%, developed into embryos (Brackett and Zuelke, 1993). However, the percentage of blastocysts developed was lower than expected (Krefft, 1993) which is agreed to this study whatever the source of the oocytes. For the both methods either, the earlier arresting of the embryonic growth could be attributed to presence of growth factors that may play a regulatory role in the functioning of the ovary (Echternkamp *et al*., 1994).

In the case of expensive, imported semen, many oocytes could be fertilized using a single straw. Rather than insemination of one animal, many IVF embryos could be transferred to recipients, making economical use of the straw. In addition, IVF and embryo transfer can be performed far away from the herd of interest. The drawbacks of IVF compared to other breeding methods are substantial, even when compared to artificial insemination. The primary problem is the cost. The IVF requires skilled labor, a controlled laboratory environment and expensive equipment. Fertilization and pregnancy rates are not as high as with natural mating or artificial insemination, leaving some recipients open.

Embryos with more manipulation, such as those that have been frozen have even lowered survivability. The matured

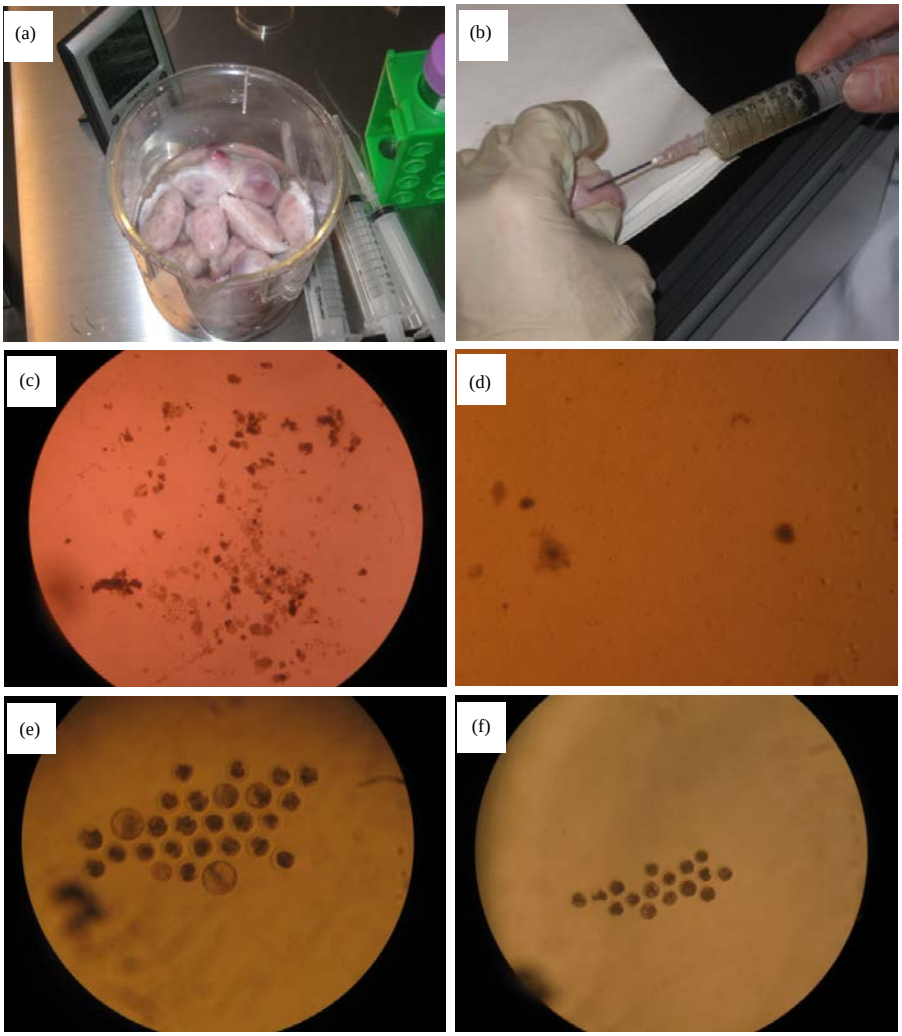

Fig. 2(a-f): Representative microscopic images of oocytes and morulae after fertilization *in vitro* (a, b) The methods of oocyte aspiration from the SHO, (c, d) The collected oocytes in H199 medium before and after selection in images, respectively and (e, f) The cleavage of oocytes into morulae and growth of the blastocysts throughout 9 days after IVF

oocyte is characterized by an evenly dispersed corona radiata and cytoplasm without any signs of degeneration, fragmentation or vacuolization. The stage of maturation is determined indirectly on the basis of dispersion of the granulosa cells of the corona radiata (Paramio, 2010; Dang-Nguyen *et al.,* 2011). There are also differences in the timing of oocyte maturation *in vitro* among species (Banwell and Thompson, 2008).

In cows, sheep, goats and oocytes reach maturity after 24 h of the culture. The next step in obtaining embryos *in vitro* is the fertilization of matured oocytes. Usually, thawed sperm is used for fertilization, however, since in sheep and horses problems with freezing sperm occur, fresh sperm is used in these species (Morrell and Rodriguez-Martinez, 2010). Spermatozoa used in *in vitro* fertilization have to undergo capacitation, which starts with removal of the glycoprotein coat. This subsequently leads to structural modifications and changes in the intramembrane protein topography of the head and tail of the spermatozoon.

The changes in the sperm head enable the acrosomal reaction to occur, whereas changes in the tail lead to hyperactivation (augmented movement of the sperm). *In vitro* capacitation is a reversible process, in contrast to the acrosomal reaction, which is an irreversible process allowing the sperm to penetrate the zona pellucida (Tulsiani *et al*., 2007; Bailey, 2010; Gadella and Evans, 2011). The acrosomal reaction consists of the release of enzymes that allow the sperm to penetrate the zona pellucida. The entry of sperm leads to oocyte activation, completion of the II meiotic division resulting in the extrusion of the II polar body. Subsequently,

two pronuclei are formed, DNA replication taking place in each of them and the first mitotic division is initiated when the chromosomes of the oocyte and sperm form a common metaphase plate. Differences among species can pertain to asynchrony in pronuclei formation and the moment of replication initiation (Gadella, 2010; Ikawa *et al*., 2010; Tulsiani and Abou-Haila, 2011).

CONCLUSION

The present study aims to find a significant difference in the growth rate of the bovine embryos originated from the slaughter house ovaries and those originated from the known alive cows by ovum-picking up (OPU). No significant difference was recorded but the OPU-originated embryos tended to grow better than the former origin. Furthermore, in the materials and methods part we stated our modified method for the laboratory *in vitro* fertilization methodology.

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