Surgical Oocyte Retrieval and the Developmental Potential of the Oocytes Derived from Prepubertal Calves

S. P. Yeh, J. C. Ju, J. K. Tseng, P. C. Chou, C. H. Chen, T. Chih and Y. K. Fan*
Department of Animal Science, National Chung Hsing University, Taichung 402, Taiwan, ROC

ABSTRACT : The objectives of this study were to investigate the ovarian responsiveness of juvenile calves to exogenous gonadotropin treatments and to establish the oocyte retrieval technique for prepubertal heifers. Three 78-day-old calves were treated with 4 doses (40, 30, 30 and 30 mg) of FSH (Folltropin V) at 12 h interval up to 229 day-old. Surgical oocyte retrieval was performed 24 h after the last injection of FSH. Calves with good ovarian responses to FSH treatment had an average ovarian size of 5×3 cm compared to 3×2 cm in the less-responsive animals. Large variations were observed in the number of total follicles (51±45), aspirated follicles (39±36), oocytes recovered (23±25) and usable oocytes recovered (11±19) during 78 to 229 day-old. Oocytes derived from prepubertal calves had significantly lower maturation rate than those from cows (34 vs. 100%, $p<0.05$). Mean diameters of calf oocytes (144±1 μm) and ooplasm (110±1 μm) were significantly lower than those of cows (149±1 and 125±1 μm, respectively). The diameter of the ooplasm also increased significantly after *in vitro* maturation (IVM) (108±1 vs. 112±1 μm). However, further studies are required to optimize the IVP system for the oocytes derived from prepubertal heifers. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 2 : 174-178)***

Key Words : Oocyte, Gonadotropin, Calf, Surgical Retrieval

INTRODUCTION

The *in vitro* embryo production system has been widely used in cattle embryos as well as that in other domestic species. Due to its long prepubertal and gestation periods, the efficiencies of genetic improvement and herd expansion have been greatly compromised. The use of juvenile or prepubertal animal's oocytes for IVP provides great potential and could be one of the most effective solutions to this problem.

Calf ovaries contain large numbers of growing and antral follicles (Majerus et al., 1999), which exhibit wave-like patterns of growing (Adams et al., 1994; Evans et al., 1994). As in most mammalian species, folliculogenesis in cattle occurs during fetal development. Antral follicles are observed in the fetal ovaries during late gestation, and as many as 50 antral follicles appear when a heifer reaches 2 months of age (Erickson, 1966). The use of oocytes containing these follicles increases the genetic gain in livestock breeding programs through a reduction of the generation interval because it potentially provides a rich and untapped source of germplasm. Lohuis (1995) showed that a progeny test incorporated with multiple ovulation and embryo transfer (MOET) of cows in combination with IVP of calf (1 to 5 months of age) oocytes increased genetic improvement for milk yield by 22% over a traditional progeny test. It also provides a rapid mean of expanding the animals from a particularly valuable genotype such as transgenic founder animals. The developmental competence of oocytes from calves over other sources has been evaluated by various laboratories and their conclusions are controversial. Nevertheless, calf oocytes have the potential to resume meiosis, reach metaphase II and can be normally fertilized (Armstrong et al., 1992, 1994; Duby et al., 1995; Revel et al., 1995). The use of slaughterhouse ovaries implies using animals whose exact physiological state are variable and their genetic background are unknown. Besides, oocyte viability is justified entirely by morphological characteristics of the oocytes and cumulus-oocyte-complexes (COCs; Martino et al., 1995; Hashimoto et al., 1998). In contrast, collection of oocytes from juvenile animals gives an early access to the large numbers of ovarian oocytes from certainly known donor animals. This technique would benefit programs for rapidly multiplying genetically valuable and endangered species.

The objectives of this study were to establish calf oocyte retrieval technique and to investigate the ovarian responses of juvenile heifers to exogenous gonadotropin treatments. Developmental competence of oocytes from calves in comparison with those from adult cows was also evaluated *in vitro* using maturation rate (MR) and the diameter of oocytes.

MATERIALS AND METHODS

Animals and hormonal treatments

The use of animals was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University. Three healthy Holstein calves, which were selected based on their genetic merits, were purchased from local dairy farmers. Each calf was fed *ad libitum* with Bermuda and alfalfa (0.5 kg/day) hays plus 1 kg/day commercial concentrates (CP 16%) throughout the

* Corresponding Author: Y. K. Fan. Tel: +886-4-2285-3748, Fax: +886-4-2286-0265, E-mail: ykfan@dragon.nchu.edu.tw
Received May 21, 2003; Accepted September 17, 2003

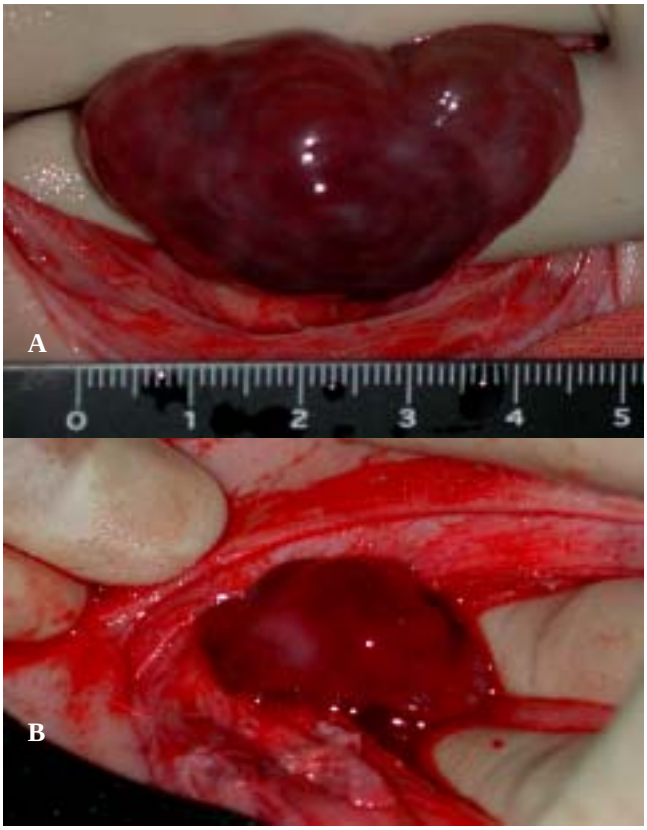

Figure 1. The size of hyperstimulated calf ovaries. Calves were treated with 4 doses of FSH (Folltropin V) at 12 h intervals (40, 30, 30 and 30 mg). Calves with good responses to gonadotropin treatment had an average ovarian size of 5×3 cm (A) compared to 3×2 cm of the less-responsive animals (B).

experiment. Drinking water was freely accessible to all animals. Oocytes were surgically collected from these calves starting at 78 day-old. All calves were treated with gonadotropin and withdrawn of feeds for 24 h and drinking water for 12 h prior to oocyte collection.

The procedures of hormonal treatment were carried out as stated by Taneja et al. (2000). For each round of FSH treatment, the calves were injected with 4 doses of FSH intramuscularly (Folltropin V, Vetrepharm, Ontario, Australia) at a 12 h interval (40, 30, 30 and 30 mg) starting from 78 days of age. Surgical retrieval of oocytes was performed 24 h after the last injection of FSH. The oocyte-retrieved calves were thereafter repeatedly stimulated with exogenous FSH for further retrieval at a 30-day interval till 229 days of age.

Surgical oocyte retrieval

Calf oocytes : Calf oocytes were recovered by midventral laparotomy under general anesthesia with xylazine (0.1 mg/kg BW; Chanazine, Chanelle Pharmaceuticals Manufacturing Ltd.) and ketamine (0.5 mg/kg BW). Antral follicles larger than 4 mm in diameter were carefully aspirated using an 18 gauge needle attached to a 12 mL syringe. The aspiration medium was Dulbecco's PBS (Gibco 11500-030) supplemented with 100 U/mL penicillin (Sigma P-4687), 100 μg/mL streptomycin (Sigma S-6501), 3 mg/mL BSA (Faction V), and 0.05 mg/mL sodium heparin (Sigma H-9399).

The aspirated follicular fluid with oocytes in the syringe were then transferred into Em-Con (Pets Inc., Canton, TX) embryo filters and washed 2 to 3 times with aspiration medium. Filters with washing medium were transported to the laboratory for oocyte searching under a dissection microscope. The collected COCs were washed 3 times in D-PBS prior to IVM.

Cow oocytes : Oocytes in cow ovaries were obtained from a local slaughterhouse. The ovaries were bathing in saline (supplemented with penicillin 600 IU/mL) at 37°C and transported to the laboratory within 3 h after dissection. The ovaries were washed 3 times in washing medium at 38.5°C in the laboratory. COCs were obtained after slicing the ovaries in PBS supplemented with 100 μg/mL streptomycin, 3 mg/mL BSA and 0.05 mg/mL sodium heparin. Oocytes with one or more complete layers of cumulus cells and evenly granulated cytoplasm were selected and washed 3 times with IVM medium before IVM.

Measurement of oocyte diameter

Thirty-two oocytes collected from calves and twenty oocytes from cows were removed of cumulus cells. The diameters of the oocytes were measured using an inverted microscope equipped with a micrometric eyepiece. The measurement of the oocytes was conducted immediately after being moved out from the incubator. Their maximal diameters, including the surrounded zona pellucida, were measured at a magnification of 250× under a microscope.

***In vitro* maturation**

IVM medium for cow oocytes consisted of TCM 199 supplemented with 10% fetal bovine serum (FBS, Hyclon, Logan, UT), 0.5 μg/mL bFSH (NIDDK, bFSH AFP-5332B), 5 μg/mL bLH (NIDDK, bLH AFP-11743B) and 1 μg/mL estradiol-17 β (Ju et al., 1998; Liu et al., 1998; Yang et al., 1994). For calf oocytes, IVM medium consisted of TCM 199 supplemented with 20% FBS, 5 μg/mL bFSH, 5 μg/mL bLH and 1 μg/mL estradiol-17 β as reported by Taneja et al. (2000).

Oocytes were IVM in 50 μL-droplet of the medium for 24 h. Five oocytes were cultured in a droplet covered with mineral oil (Nacalai, Code 26137-85) and then incubated at 5% CO_2 in humidified atmosphere maintaining at 39°C.

Statistical analysis

The variables of interest were analyzed statistically

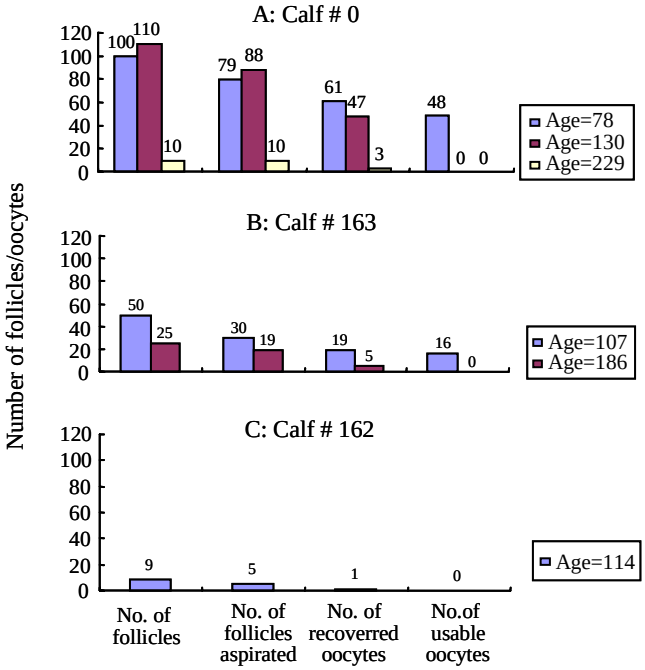

Figure 2. Ovarian responses of juvenile Holstein calves. A total FSH dosage of 130 mg was injected intramuscularly (40, 30, 30 and 30 mg) for each section of oocyte retrieval. (A) Calf # 0 was repeatedly treated with FSH for 3 sessions of oocyte collection at Days 78, 130 and 229 of age. Ovarian responses, including numbers of follicles, oocyte recovered and usable oocytes, to FSH treatment apparently reduced at the third collection. (B) Calf # 163 was repeatedly stimulated with FSH for 2 rounds of oocyte collection at Days 107 and 186 of age. The ovarian responses to FSH treatment also reduced at the second collection. (C) Calf # 162 was only treated with FSH once starting at Day 114 of age due to the poor responseveness of the ovaries.

using the FREQ procedure of SAS (1988). Effects of oocyte sources, collected from calves or cows, were tested using the chi-square test of homogeneity.

RESULTS

Follicle aspiration and responses to gonadotropin stimulations

Follicle aspiration for oocytes retrieval was performed 1 to 3 times for each animal depending on the condition of recovery from surgery. The anesthesia and surgical procedures went smoothly which usually took 20 to 30 min to complete the whole section of the retrieval. When repeated collections were performed, we found that 2 out of 6 calves had organ or tissue adhesion during the second or third surgery, especially at the ovary. Sometimes the ovaries were completely enclosed by other tissues, which often resulted in less follicle development (10 and 19) compared to normal conditions (30 and 84). Calves with good responses to FSH administration had an average ovarian size of 5×3 cm compared to 3×2 cm of the less-responsive animals (Figure 1). Great variations in number of follicles among different rounds of exogenous FSH stimulations within each calf were observed (Figure 2 and Table 1). The ranges and average numbers of follicles, aspirated follicles, recovered oocytes, and usable oocytes were 9 to 110 and 51 ±45, 5 to 88 and 39±36, 1 to 61 and 23±25, and 0 to 48 and 11±19, respectively.

Table 1. Numbers of follicles and oocytes recovered from calf ovaries after FSH[1] treatment (n=3) from 78 to 229 day-old

Parameter	Range	Mean±SEM[2]
Follicles	9-110	51±45
Aspirated follicles	5-88	39±36
Oocytes recovered	1-61	23±25
Usable oocytes	0-48	11±19

[1] A total FSH dosage of 130 mg (40, 30, 30 and 30 mg at a 12 h interval) was injected intramuscularly for each round of oocyte retrieval.
[2] The means were derived from 6 repeated sessions of oocyte retrieval.

Table 2. *In vitro* maturation of cow and juvenile calf oocytes cultured in TCM 199-based media

Group	No. of oocytes	% M II (No.)
Cow[1]	65	100 (65)[a]
Calf [2]	64	34 (22)[b]

[1] Oocytes were cultured in TCM 199 supplemented with 10% FBS, 0.5 μg/mL bFSH, 5 μg/mL bLH and 1 μg/mL estradiol-17 β.
[2] Oocytes were cultured in TCM 199 supplemented with 20% FBS, 5 μg/mL bFSH, 5 μg/mL bLH and 1 μg/mL estradiol-17 β.
[a, b] Percentages with different superscripts in the same column differ significantly (p<0.01).

Table 3. The comparison of oocyte diameters between cows and juvenile calves (mean±SEM)

Oocyte source	No. of oocytes	Diameter of ooplasm, μm	Diameter of oocyte*, μm
Cow	20	125±1[a]	149±1[a]
Calf	32	110±1[b]	144±1[b]

* The diameters include the thickness of the zona pellucida.
[a, b] Values with different superscripts in the same column are significantly different (p<0.01).

IVM development

The MR of the oocytes collected from juvenile calves and adult cows are presented in Table 2. Significantly lower MR was observed in the oocytes collected from prepubertal calves than those from adult cows (34% vs. 100%, p<0.01).

The diameters of oocytes

Average diameters of calf oocytes (144±1 μm) and the ooplasm (110±1 μm) were significantly less than those of cows (149±1 μm and 125±1 μm; p<0.01, Table 3). The diameters of oocytes prior to and after IVM were also compared. Only the diameters of the ooplasms were significantly different between matured and immature oocytes (112±1 vs. 108±1.08 μm, p<0.01; Table 4).

Table 4. The comparison of oocyte diameters after *in vitro* maturation (mean±SEM)

Group	No. of oocytes	Diameter of ooplasm, μm	Diameter of oocyte*, μm
Matured[1]	20	112±1[a]	145±1
Immature[2]	12	108±1[b]	142±1

*The diameters include the thickness of the zona pellucida.
[1] M II oocytes with polar body. [2] Oocytes without polar body.
[a, b] Values with different superscripts within the same column are significantly different ($p<0.01$).

DISCUSSION

In the present study we have investigated the feasibility of oocytes production from elite bovine donors at the age of 78 days, as well as the influence of repeated gonadotropin stimulations on developmental competence of oocytes recovered from the same donor calves of various ages. Many studies (Armstrong et al., 1992; Armstrong, 1993; Damiani et al., 1995; Loony et al., 1995; Revel et al., 1995. Duby et al., 1996; Armstrong et al., 1997; Kelly et al., 1997) including our work showed that these follicles in calf ovaries were stimulated to grow with exogenous gonadotrophins. The calves were subjected to a 2 day gonadotropin stimulation regimen and the responses of the ovaries, however, were characterized by a great variation among these donors. The causes of the variations have been reviewed (Armstrong, 1993) but have not been completely elucidated yet. It is likely that the discrepancies in the rates of cleavage and development of the oocytes in prepubertal calves observed by Armstrong et al. (1994), Looney et al. (1995) and Revel et al. (1995) are at least partially, attributable to the variations of animal ages and/or culture systems used. The variations in the superovulatory response and the morphology of the ovaries in prepuberal heifers (Armstrong, 1993) suggest that follicular waves develop earlier than the onset of puberty. This is further supported by the observation that plasma estradiol-17 β (E_2) levels in gonadotropin-stimulated calves varied and the hormonal levels were correlated to the number of follicles presented at the time of first FSH injection (Testart et al., 1977). Later researches confirmed that follicular waves occurred in heifers beginning at 2 weeks of age (Evans et al., 1994) and waves were also observed at 8 months of age lasting to first ovulation at 12 months (Adams et al., 1994). These studies indicated that the growth of ovarian follicles could be hyperstimulated during prepubertal stage.

Armstrong et al. (1992) have reported live births from *in vivo* matured oocytes collected from hyperstimulated 5 to 6-wk-old heifers. In contrast, Revel et al. (1995) demonstrated that oocytes collected from slaughtered 3-mo-old calves with or without previous gonadotropin stimulations had very limited developmental competence. In their studies, the 3-mo-old calves produced only 9% blastocysts without hormonal stimulation and 11% blastocysts with stimulation. Both the unstimulated and stimulated groups exhibited cleavage rates similar to those of mature cows. Loony et al. (1995) reported that oocytes derived from stimulated heifers under 240 days of age produced no blastocysts compared to 19% blastocysts from older heifers (over 240 days of age) with similarly stimulations.

Our measurements of oocyte diameters showed that oocytes recovered from calf ovaries were smaller than those collected from adult animals, and this result was consistent with previous studies (Duby et al., 1996; Gandolfi et al., 1998). Duby et al. (1996) also reported that the follicle sizes in gonadotropin stimulated prepubertal calves were smaller (10 to 12 mm) than those in adult cows (15 to 20 mm). Although the ooplasm of the majority of oocytes was homogeneous, it was also significantly smaller (119.7 μm) than that obtained from cows (125.6 μm). Oocytes smaller than 120 μm in diameter failed to complete meiosis (Fair et al., 1995; Yang et al., 1998), but prolonged IVM culture increased the number of matured oocytes (Khatir et al., 1998). The relevance of oocyte diameter to the developmental competence in calf oocytes was further strengthened by recent observation (Fair et al., 1995), in which the capability of bovine oocytes reaching metaphase II stage was directly proportional to their diameters. To improve the development of calf oocytes, optimization of the *in vitro* culture system would be necessary.

In conclusion, juvenile or prepubertal calves responded differently to gonadotropin treatment among individuals. When the donor animals were selected critically, calf oocytes might be served as an alternative source of germplasms for *in vitro* embryo production (IVP). In combination with transgenic and/or animal cloning studies, this technology would efficiently accelerate the expansion of genetically superior dairy herds. However, further studies are required to optimize the dosage or combination of hormonal treatments for follicular development and IVP system for juvenile heifers.

ACKNOWLEDGMENTS

Authors wish to thank Drs. Jerry X. Yang and M. Taneja for their technical support in retrieval of calf oocytes. This work was partly supported by a grant from National Science Council, Executive Yuan, Taiwan.

REFERENCES

Adams, G. P., A. C. O. Evans and N. C. Rawlings. 1994. Follicular waves and circulating gonadotrophins in 8-month-old prepubertal heifers. J. Reprod. Fertil. 100:27-33.

Armstrong, D. T., P. J. Kotaras and C. R. Earl. 1997. Advances in production of embryos *in vitro* from juvenile and prepubertal oocytes from the calf and lamb. Reprod. Fertil. Dev. 9:333-339.

Armstrong, D. T., B. J. Irvine, C. R. Earl, D. McLean and R. F. Seamark. 1994. Gonadotropin stimulation regimes for follicular aspiration and *in vitro* embryo production from calf oocytes. Theriogenology 42:1227-1236.

Armstrong, D. T. 1993. Recent advances in superovulation of cattle. Theriogenology 39:7-24.

Armstrong, D. T., P. Holm, B. J. Irvine, B. A. Petersen, R. B. Stubbings, D. McLean, G. F. Stevens and R. F. Seamark. 1992. Pregnancies and live births from *in vitro* fertilization of calf oocytes by laparoscopic follicular aspiration. Theriogenology 38:667-678.

Damiani, P., R. A. Fissore, J. B. Cibelli, J. M. Robl and R. T. Duby. 1995. Evaluation of cytoplasmic maturation of calf oocytes. Theriogenology 43:191 abstr.

Duby, R. T., P. Damiani, C. R. Looney, R. A. Fissore and J. M. Robl. 1996. Prepubertal calves as oocyte donors: promises and problem. Theriogenology 45:121-131.

Duby, R. T., P. Damiani, C. R. Looney, C. R. Long, J. J. Balise and J. M. Robl. 1995. Cytological characterization of maturation and fertilization in prepubertal calf oocytes. Theriogenology 43:202 (abstr).

Erickson, B. H. 1966. Development and senescence of the postnatal bovine ovary. J. Anim. Sci. 25:800-805.

Evans, A. C. O., G. P. Adams and N. C. Rawlings. 1994. Endocrine and ovarian follicular changes leading up to first ovulation in prepubertal heifers. J. Reprod. Fertil. 100:187-194.

Fair, T., P. Hyttel and T. Greve. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol. Reprod. Dev. 42:437-442.

Gandolfi, F., E. Milanesi, P. Pocar, A. M. Luciano, T. A. L. Brevini, F. Acocella, A. Lauria and D. T. Armostrong. 1998. Comparative analysis of calf and cow oocytes during *in vitro* maturation. Mol Reprod Dev. 49:168-175.

Hashimoto, S., K. Saeki, Y. Nagano, N. Minami, M. Uamada and K. Utsumi. 1998. Effects of cumulus cell density during *in vitro* maturation on the developmental competence of bovine oocytes. Theriogenology 49:1451-1463.

Ju, J. C., L. Liu, J. E. Parks and Yang X. 1998. Cytoskeletal reorganization of IVM-derived bovine oocytes after *in vitro* fertilization. Theriogenology 49:286 (abstr).

Kelly, J. C., J. R. Earl and D. T. Armstrong. 1997. Blastocyst production rate from different grades of oocytes collected from 2 to 3 month old calves. Theriogenology 47:291 (abstr).

Khatir, H., P. Lonergan and P. Mermillod. 1998. Kinetics of nuclear maturation and protein profiles of oocytes from prepubertal and adult cattle during *in vitro* maturation. Theriogenology 50:917-929.

Liu, L., J. C. Ju and X. Yang. 1998. Parthengenetic development and protein pattern of newly matured bovine oocytes after chemical activation. Mol. Reprod. Dev. 49:298-307.

Lohuis, M. M. 1995. Potential benefits of bovine embryo-manipulation technologies to genetic improvement programs. Theriogenology 43:51-60.

Looney, C. R., P. Damiani, B. R. Lindsey, C. R. Long, C. L. Gonseth, D. L. Johnson and R. T. Duby. 1995. Use of prepubertal heifers as oocyte donors for IVF: effect of age and gonadotropin treatment. Theriogenology 43:269 abstr.

Majerus, V., R. De Roover, D. Etienne, S. Kaidi, A. Massip, F. Dessy and I. Donnay. 1999. Embryo production by ovum pick up in unstimulated calves before and after puberty. Theriogenology 52:1169-1179.

Martino, A., T. Mogas, M. J. Palomo and M. T. Paramio. 1995. *In vitro* maturation and fertilization of prepubertal goat oocytes. Theriogenology 43:473-485.

Revel, F., P. Mermillod, N. Peynot, J. P. Renard and Y. Heyman. 1995. Low developmental capacity of *in vitro* matured and fertilized oocytes from calves compared with that of cows. J. Reprod. Fertil. 103:115-120.

SAS. 1988. SAS Procedure Guide, Release 6.03 ed. SAS Institute Inc., Cary, NC, USA.

Taneja, M., E. J. Peter, P. E. J. Bols, A. Van De Velde, J. C. Ju, D. Schreiber, M. W. Tripp, H. Levine, Y. Echelard, J. Riesen and X. Yang. 2000. Developmental competence of juvenile calf oocytes *in vitro* and *in vivo*: Influence of donor animal variation and repeated gonadotropin stimulation. Biol. Reprod. 62:206-213.

Testart, J., G. Kann, J. Saumande and M. Thibier. 1977. Oestradiol-17 β, progesterone, FSH and LH in prepubertal calves induced to superovulate. J. Reprod. Fert. 51:329-336.

Yang, X., G. A. Presecce, L. Moraghan, S. Jiang and R. H. Foote. 1994. Synergistic effect of ethanol and cycloheximide on activation of freshly matured bovine oocytes. Theriogenology 41:395-403.

Yang, X., C. Kubota, H. Suzuki, M. Taneja, P. E. J. Bols and G. A. Presicce. 1998. Control of oocyte maturation in cows-biological factors. Theriogenology 49:471-482.