STUDIES ON QUALITY AND SEX OF *IN VITRO* PRODUCED BOVINE EMBRYOS

(牛体外受精胚の品質と性に関する研究)

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2006

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
(Ph.D.)

UNITED SCHOOL OF VETERINARY SCIENCE
LABORATORY OF ANIMAL REPRODUCTION

UNIVERSITY OF YAMAGUCHI

2006

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ACKNOWLEDGEMENTS

I extend my sincere gratitude and appreciation to many people who made this doctoral thesis possible. Special thanks are due to the Government of Japan (MONBUKAGAKUSHO), for awarding me a scholarship for my Ph.D.

I would like to express my deepest gratitude to my main supervisor Prof. Takeshige Otoi whose help, stimulating suggestions and encouragement helped me in all the time of research and for writing of this thesis. I owe him lots of gratitude for having me shown his way of research. He could not even realize how much I have learned from him. Also I would to thank to Prof. Tatsuzuki Suzuki for accepting me to pursue a doctoral degree in his laboratory. His positive approach to the life, kindness and cordiality are the best. I would like also to thank my second supervisor Prof. Toshihiko Nakao and Prof. Shunichi Kamimura who have been a source of advice and encouragement during my study. Sincerely thanks to Dr. Takashi Nagai from Department of Research Planning and Coordination, National Institute of Livestock and Grassland Science, Tsukuba for revising of my manuscripts, suggestions, encouragement and inspiration.

I want to thank to all the staffs of the Meat Inspection Office of Kitakyushu-shi for their kind hospitality and supply of the ovaries for my research. Many thanks go to all laboratory members for their support as both co-workers and friends.

I also want to thanks my former colleagues from the Department of Animal Reproduction for supporting me in my research work. Finally, I would like to recognize the work done by my lovely wife Agustina Dwi Wijayanti and my son Rizky Ikhsan Imrani, also my parents and whole family for their patient and unconditional love and

generosity in Indonesia. Thank you for all the patience, help and support. I couldn't have gotten this far without you.

ABSTRACT

My studies consist of two chapters; The first chapter was conducted to examine the relationship between the oxygen consumption rate and sex ratio of bovine *in vitro* fertilized embryos on each day of blastocyst formation. The quality of blastocysts collected on day 7, 8, and 9 after *in vitro* fertilization (IVF) were categorized as ranks A and B (excellent and good, respectively) based on the microscopic observation for the morphology. The oxygen consumption rate and sex of individual blastocysts were evaluated using two novel techniques: scanning electrochemical microscopy (SECM) and loop-mediated isothermal amplification (LAMP), respectively. The oxygen consumption rates of embryos of rank A were significantly higher ($p<0.05$) than those of rank B, irrespective of the day of blastocyst appearance after IVF. Neither did the proportion of male embryos of ranks A and B differ significantly from each other at any of the days examined, nor from the average proportion (53%).The oxygen consumption rate of embryos of rank B collected on day 8 was significantly higher ($p<0.05$) in female embryos than that in male embryos collected on the same day. However, there was no apparent difference in oxygen consumption rates at each day of blastocyst appearance between male and female embryos in rank A. These results indicate that the oxygen consumption rate of individual embryos reflects their quality, but does not correlate with the sex ratio of embryos in excellent rank quality.

The second chapter was conducted to investigate whether the sex ratio of *in vitro* fertilized embryos is influenced by the maturation culture period of the oocytes. It has been suggested that the maturational stage of oocytes at time of insemination influences the sex ratio of resulting embryos. However, there are very few reports concerning the relationship between the maturation culture period of oocytes and the sex ratio of resulting embryos. The objective of this study was to investigate the effects of *in vitro*

maturation culture period for bovine oocytes on the sex ratio of *in vitro* produced blastocysts using a novel technique of LAMP. Cumulus-oocyte complexes were collected from the ovaries of slaughtered cows, and then matured *in vitro* for various periods (16, 22, 28, and 34 h). After maturation culture for each period, the oocytes were inseminated with frozen-thawed spermatozoa, and then cultured *in vitro*. Blastocysts were harvested on Day 7 after insemination, and the sex of the embryos was examined using the LAMP method. The rates of oocytes matured to the metaphase II stage were significantly lower ($P<0.05$) in the 16-h maturation group than that in the other groups. The proportion of blastocyst formation after insemination was significantly higher ($P<0.05$) in the 22-h maturation group than in the other groups. The proportion of male blastocysts increased with the increase in maturation culture period. The proportion of male blastocysts derived from oocytes matured for 34 h was significantly higher ($P<0.05$) than that derived from oocytes matured for 16 and 22 h. These results indicate that the sex ratio of *in vitro* fertilized embryos is apparently influenced by the maturation culture period of the oocytes.

GENERAL INTRODUCTION

Techniques for *in vitro* production of embryos have developed on both basic research and practical applications in farm animals (Gordon, 1994). *In vitro* produced bovine embryos have many potential in animal agriculture and research, but their utilization still has many problems. Recent advances in embryo transfer technology have enabled to produce bovine embryos by the fertilization and development of *in vitro* matured oocytes derived from slaughtered cows. Since the first IVF calf had been born (Bracket et al. 1982), most research has been conducted to improve *in vitro* production of bovine blastocysts. *In vitro* manipulation of gametes for the production of embryos was first successful in mice in 1958 and in rabbits in 1959. These advances allowed for the production of viable embryos from oocytes isolated from ovaries obtained at the slaughterhouse. In cattle, the improvement of *in vitro* techniques for production of bovine embryos has been also shown successfully result in offspring. Blastocyst transfer has been reported to improve success rates by the evaluation of quality of transferred embryos. However, conception rates of *in vitro* produced (IVP) embryos are still lower than those of AI or *in vivo* embryos. Moreover, pregnancy rates with IVP embryos have remained very variable. It is assumed that this variation relates to the quality of the embryos which can be affected by some factors such as the culture procedure, culture media and selection of embryos before freezing and /or transfer. Moreover, the loss of embryos after transfer has been suggested to occur frequently during the first 30 d of pregnancy and throughout gestation. In this case, embryo quality has an important factor for the success of embryo transfer. To date, several parameters have been investigated as potential indicators of embryo viability. In the assessment of the viability of embryos, morphological evaluation has been most widely used as a non-invasive assessment, but the correlation between the morphological criteria and the embryo's ability to result in

offspring is not high (Butler and Biggers 1989). To date, several methods for embryo evaluation have been described, but most of these methods are invasive and less useful for evaluation of individual embryos. Therefore, morphological evaluation of embryos has been useful for predicting the pregnancy rates following transfer.

On the other hand, oxygen consumption is a useful parameter for evaluating embryo quality, since it provides a valuable indication of overall metabolic activity (Trimarchi et al. 2000). Shiku et al. (2001) reported that oxygen consumption of an individual bovine IVF embryo was strongly related to its morphological quality. It has been suggested that the oxygen consumption reflects the cell number or the number of mitochondria in the embryo. Moreover, oxygen consumption by individual bovine embryos has been non-invasively quantified by scanning electrochemical microscopy (SECM). If there is some correlation between the oxygen consumption and the sex of bovine IVF embryos, the non-invasive assessment of oxygen consumption of each embryo may potentially be used as a predictive indicator of embryo sexing.

Sexing at time of pre-selection of embryos increases the benefit and efficiency of embryo transfer. The sexed embryos is beneficial to the livestock production because embryo transfer can be planned to produce a calf with specific sex. In addition, genetic progress by the sexing contributes to other advantages on the management and efficiency of livestock industry. The sexing of embryo has a big economic and social implication in beef and dairy farm. Male calves are needed for the beef farm, and the dairy farm needs the female ones. Therefore, the field application of sexing to bovine embryo transfer is beneficial for the both farms.

Fertilization of a mammalian oocyte establishes the sex of embryo and initiates a cascade of events that leads to sexual differentiation. It has been reported that bovine male embryos reach the blastocyst stage faster than female embryos and have more cells

(Bredbacka and Bredbacka, et al. 1996). If the timing of insemination *in vitro* can be used to control the gender of offspring in cattle, it would have an economic benefit and could be applied to livestock production. It has been suggested that the maturational stage of oocytes at time of insemination influences the sex ratio of resulting embryos (Dominko and First, 1997). Moreover, the maturational stage of oocytes at the time of insemination has a great impact on the sex ratio of embryos (Kochar et al. 2003). However, there are few reports concerning the relationship between the maturation culture period of oocytes and the sex ratio of resulting embryos.

Sex differentiation of embryos is currently performed by PCR and DNA analysis of embryonic cells recovered by bisection of biopsy. Recently, the new technique called LAMP, which is both rapid and sensitive, has been developed (Notomi et al. 2000). The LAMP method is simpler and more sensitive than the PCR method for sex determination of bovine embryos (Hirayama et al. 2003). This method reaction is an auto-cycling, strand displacement DNA synthesis carried out using a DNA polymerase with high strand displacement activity and a set of specific primers that recognize a total of six distinct sequences on the target DNA. Therefore, it is expected that it will amplify the target sequence with high selectivity (Notomi et al. 2000)

This thesis consists of two studies. In the first study, we conducted to investigate the relationship between oxygen consumption rate and sex ratio of *in vitro* fertilized bovine embryos on each day of blastocyst formation. In the second study; the effects of the maturation culture period of oocytes on the sex ratio of *in vitro* produced bovine blastocysts were investigated.

REVIEW OF LITERATURE

Embryo quality

A high quality of bovine embryo is very important to increase the rates of pregnancy rate after embryo transfer. An *in vitro* culture system has been established to produce bovine embryos following *in vitro* maturation and fertilization (IVF) of oocytes collected from ovaries (Kajihara et al. 1987; Xu et al. 1987; Goto et al. 1988; Fukuda et al. 1990). However, the quality of *in vitro* embryo is still lower than that of *in vivo* embryos. A variety of parameters such as cell numbers of the embryo (Hardy et al. 1989; Iwasaki et al. 1990; Van Soom et al. 1997, Enright et al. 2000; Mori et al. 2002), incidence of apoptosis (Knijn et al. 2003), relative abundance of gene transcripts (Gardner et al. 1994; Wrenzycki et al. 1996, 2003; Eckert and Niemann 1998; Niemann and Wrenzycki 2000; Lonergan et al. 2003; Gutierrez-Adan et al. 2004; Tesfaye et al. 2004) and chromosomal configuration (Viuff et al. 1999, 2001; Gutierrez-Adan et al. 2004) have been analyzed. Among these parameters, embryo morphology assessment is most popular method for embryo selection prior to transfer of embryos in both cattle and human. The primary criterion for embryo selection after IVF is a morphological appearance based on a combination of cell number and fragmentation (Van Royen et al, 2001). In the past few years, the possibilities of viable embryo selection at the early cleavage stages have been improved substantially by the introduction of non-invasive scoring criteria applicable as early as the pronuclear stage and by refining the scoring criteria for cleaving embryos. However, recent research has been focused on the correlation between bovine embryo morphology and ultra structure, gene expression and cryotolerance (Van Soom et al, 2003). Morphological features such as color of the

blastomeres, the extent of compaction, kinetics of development, timing of blastocyst formation and expansion, and diameter of the embryo at hatching could be linked with embryo quality (Hoelker et al.2006). The number of blastocyst cells is one of good indicators for evaluation of embryonic quality and health. The appropriate proportion of inner Cell Mass (ICM) is important for normal embryonic development after transfer and is closely related to the viability of embryos. Furthermore, *in vitro* produced bovine embryos differ from those produced *in vivo* in many important aspects (Massip et al 1995; Wright and Ellington, 1995) including cytoplasm with dark color, lower density (Pollard and Leibo, 1994), swollen blastomeres (Van Soom et al. 1992), more fragile zonapellucida (Duby et al. 1997), slower growth rate and higher thermal sensitivity (Leibo and Loskutoff, 1993). However, it is unclear whether parts of the process during embryo production are important on the blastocyst yields and blastocyst quality. The viability of bovine IVF embryos has been found to be affected by the age of the embryo, the stage of embryonic development and embryo quality (Han et al. 1994; Takagi et al. 1994; Carvalho et al. 1996). Wrenzycki et al. (1999) demonstrated that the culture system of embryos is the major determinant factor on the blastocyst quality, irrespective of the origin of oocytes. The culture environment can also have a significant effect on the embryo metabolism, which may be related to embryo quality (Rizos et al. 2002) It has been shown that embryos generated in a completely defined medium show lower rates of glycolysis than those in serum (Krisher et al. 1999). Khurana and Niemann (2000) examined the energy metabolism of *in vivo*- and *in vitro*-derived bovine embryos. They demonstrated that the pattern was similar, but IVP embryos exhibited a 2-fold higher rate of anaerobic glycolysis and produced more lactate. Culture for 72 h of *in vivo* produced blastocysts resulted in lactate production which is similar to that of *in vitro* produced blastocysts. On the other hand, Thompson et al. (1996) investigated the consumption of

oxygen, uptake of pyruvate and glucose, and the lactate production of bovine embryos produced *in vitro* from the one-cell stage (day 0 - 6 of culture) was determined. They reported that the oxygen consumption was relatively constant from days 0 - 4 of culture, then increased with the initiation of compaction and continued to increase until the formation and expansion of the blastocoels. Furthermore, when plotted against oxygen consumption, the uptake of both pyruvate and glucose increased significantly. ATP production showed a similar pattern to that of oxygen consumption at the time of compaction and blastulation of embryos. It has been shown that ATP production is derived from oxidative phosphorylation, and could be accounted for the uptake of pyruvate (Enright et al. 2000). Therefore, it seems that bovine embryos produced *in vitro* utilize little endogenous substrates when appropriate exogenous substrates are present in the culture medium.

The other method for evaluation of embryo quality is a scoring of embryonic morphology. The current practice in most IVF laboratories uses the scores of embryonic morphology, such as cytoplasmic appearance and number of blastomeres per embryo. Embryo quality has also been suggested to correlate with oocyte and zygote morphology before/after IVF, i.e. appearance of the cytoplasm, pronuclei and polar bodies (Tesarik and Greco, 1999). Moreover, early cleavage stage of embryos is positively correlated with their quality, implantation and pregnancy rates in the human (Lundin et al. 2001; Wharf et al. 2004) and cattle (Van Soom et al. 1997; Lonergan et al. 1999). In more detailed studies, the timing of blastocyst formation has been shown to be correlated with total cell number (Van Soom et al. 1997), relative abundance of gene transcripts (Wrenzycki et al. 2003) and pregnancy rates (Niemann et al. 1982). However, most of the techniques described above are invasive and impossible for use of any parameter. Consequently, non-invasive selection of embryos by morphology assessment has been

still a popular method for embryo selection before transfer both in human and bovine assisted reproduction.

Embryo sexing

Sexual control of offspring has been investigated in both sperm and embryonic levels. Sexuality of embryos was confirmed currently by PCR and DNA analysis of embryonic cells recovered by bisection of biopsy (Lee et al. 2004). Sex embryo is an additional advantage for the management and efficiency for livestock production. Benefit of sexual embryo in cattle and pig was economically allowing for the production of male and female crossbred lines.

Fertilization of a mammalian oocyte establishes the sex of embryo and initiates a cascade of events that leads to sexual differentiation. A cytogenetic evaluation of IVP embryos has revealed that the proportion of males and females vary under the laboratory conditions (Yadav et al. 1993 ; Lonergan et al. 1999). Various factors including the length of gamete interaction and embryo culture conditions are known to influence the rate of development and sex ratio of mammalian embryos produced *in vitro*. The duration of gamete interaction deemed optimum would vary, depending upon the species. Mechanisms of *in vitro* fertilization (IVF) explain the unequal distribution of males and females among the IVP bovine embryos, indicating there may be some factors favoring Y-chromosome bearing spermatozoa or X-chromosome bearing spermatozoa at the time of fertilization with oocytes, in which the factors has been shown to be correlated with post-fertilization development of embryos with X- or Y-chromosome under *in vitro* conditions (Ward et al. 2002). The length of time for oocyte maturation and gamete interaction is thought to be one of factors that influences the rate of fertilization and the

quality of the embryos produced (Dominko and First, 1997). In the bovine embryo transfer, the sexing of preimplantation embryos is a beneficial management tool for animal production. Sexing based on detection of Y chromosome-specific sequences has been used to predict the sex of offspring. Recently, sexing based on detection of Y polymerase chain reaction (PCR) and primer-extension preamplification-PCR is routinely used (Chrenek et al. 2001).

Recently, the new technique called LAMP, which is both rapid and sensitive, has been developed (Notomi et al. 2000). The LAMP reaction is an auto-cycling, strand displacement DNA synthesis carried out using a DNA polymerase with high strand displacement activity and a set of specific primers that recognize a total of six distinct sequences on the target DNA. Therefore, it is expected that it will amplify the target sequence with high selectivity (Notomi et al. 2000). Hirayama et al. (2003) applied a LAMP technique for the sexing of bovine embryos and showed that sex evaluation using this technique was more sensitive than polymerase chain reaction (PCR). Polymerase chain reaction (PCR) (Chen, et al. 1999; Shea, 1999) is routinely used in the field for sexing; this technique enables amplification of a target sequence from a small number of blastomeres. However, these methods require technical skill and are time consuming. Furthermore, PCR has the risk of false positives because of DNA contamination during the handling of the PCR products in duplicate PCR procedures and/or electrophoresis. The feature of LAMP is specific DNA amplification under isothermal conditions. DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis within the range of 60 – 65°C. LAMP employs a set of four specific primers (termed inner and outer primer sets) that recognize a total of six distinct sequences on the target DNA. Furthermore, an additional primer set (termed loop primers) is used to accelerate LAMP reaction (Takahashi et el. 1996). An inner primer

initiates primary DNA synthesis, and the following strand displacement DNA synthesis by an outer primer releases a singe-stranded DNA derived from the inner primer. The initial steps produce a stem-loop DNA structure, which is a characteristic DNA structure in LAMP, and then an extremely large amount of DNA is amplified from a stem-loop DNA by the auto-cycling reaction. Accordingly, a white precipitate of magnesium pyrophosphate (a by-product of DNA synthesis) is produced (Takahashi, et al. 1998). Therefore, amplification of a target sequence can be judged by measurement of turbidity in a reaction solution. It is noteworthy that LAMP does not need special reagents or electrophoresis to detect the amplified DNA. Embryo sexing based on LAMP is thus more suitable for field application than PCR. Therefore, wide use of LAMP for embryo sexing in the bovine embryo transfer seems to be a simple, rapid, and precise sexing method.

It has been reported that male IVP embryos develop faster and a higher percentage of them reach the blastocyst stage than female IVP embryos (Avery et al. 1991, 1992; Van Soom et al. 1997 Lonergan et al. 1999; Gutierrez-Adan et al. 2001). Gender distribution of calves produced by artificial insemination has been reported to be approximate 1:1 with 52.9% males to 47.1% females (Foote, 1975). This trend has been demonstrated in embryos and calves produced by artificial insemination following superovulation (King et al. 1991; King et al. 1995). However, the sex ratio of calves derived from IVP embryos differs from an expected result (Van Soom and de Kruif, 1992; Massip et al. 1995; Hasler et al.1995). Some studies demonstrated an association between gender and developmental rates of bovine IVP embryos. Male bovine IVP embryos can grow faster in culture compared to female embryos during the first 7 to 8 d after *in vitro* fertilization, suggesting that sex-related gene expression affects development of embryos after activation of the embryonic genome. Furthermore, male bovine embryos develop to

more advanced stages in culture than female embryos (Guiterrez –Adan et al. 1999; Dominko and First, 1997). This may explain the disproportionate number of males among calves from IVP embryos. A cytogenetic evaluation of IVP embryos has revealed that the proportion of male and female vary under the laboratory conditions (Yadav et al. 1993; Lonergan et al. 1999). However, other investigators found no significant overall deviation of sex ratio under a variety of *in vitro* conditions (Lonergan et al. 1999). This factor such as culture condition might not alter the sex ratio of embryos. Therefore, it remains unclear whether the culture condition could affect the sex ratio of IVP embryos.

The length of time allowed for oocyte maturation and gamete interaction are thought to be factors influencing the rate of fertilization and the quality of the embryos produced (Gianaroli et al. 1996; Dominko and First 1997). It has also been shown that these factors may affect the sex ratio of the IVP embryos (Gutierrez-Adan et al. 1999). Gamete interaction process allows for the penetration of oocyte by sperm, pronuclear formation and the subsequent cleavage, and the efficacy of this process depends on the vitality and capacity of the sperm as well as on the maturational status of the oocyte at the time they encounter sperm. Penetration of *in vitro* matured (IVM) bovine oocytes by *in vitro* capacitated spermatozoa occurs within 6 h of insemination, while the pronuclear formation, synkaryosis and cleavage take place much later (Xu and Greve, 1988). Therefore, several factors include the duration of the first cell cycle (Yadav et al. 1993; Plante and King, 1994; Van Soom et al. 1997), chromosome complement of the zygote (Yadav et al. 1993; King et al. 1995; Kawarsky et al. 1996) and the culture conditions (Gutierrez-Adan et al. 1999).

More embryos with normal chromosome that had completed their first cell cycle within 30 h post-insemination (hpi) have the developmental competence to the morula stage by day 5 post-insemination and to the blastocyst stage by day 7 post-insemination

(Van Soom et al. 1997). Investigators have also reported that a majority of the embryos that had completed the first cell cycle within 30 hpi were males (Yadav et al. 1993 and Dominko and First, 1997). Dominko and First (1997) demonstrated that the maturational status of the oocyte at the time of insemination has a great impact on the sex distribution of the embryos and that more males than females are recovered when gamete interaction is delayed. However, it is not known whether the more male embryos in these studies (Yadav et al. 1993; Dominko and First, 1997) is due to earlier fertilization with Y-chromosome bearing spermatozoa, a more rapid growth in male embryos compared with that in female embryos. Regardless of the mechanisms leading to the sex-dependent bifurcation of developmental process, male blastocysts have been reported to have more cells than female blastocysts, at day 8 of *in vitro* development (Xu et al. 1992). Skjervold and James (1979) demonstrated that a primary sex ratio with >60% in dairy cows might be related with the early embryonic mortality and number of abortions occurring in male cows.

CHAPTER 1

Relationship between Oxygen Consumption and Sex of Bovine *In Vitro* Fertilized Embryos

Abstract

The present study was conducted to examine the relationship between the oxygen consumption rate and sex ratio of bovine *in vitro* fertilized embryos on each day of blastocyst formation. The quality of blastocysts collected on day 7, 8, and 9 after *in vitro* fertilization (IVF) were categorized as ranks A and B (excellent and good, respectively) based on microscopic observation of the morphology. The oxygen consumption rate and sex of individual blastocysts were evaluated using two novel techniques: scanning electrochemical microscopy (SECM) and loop-mediated isothermal amplification (LAMP), respectively. The oxygen consumption rates of embryos of rank A were significantly higher ($p<0.05$) than those of rank B, irrespective of the day of blastocyst appearance after IVF. Neither did the proportion of male embryos of ranks A and B differ significantly from each other at any of the days examined, nor from the average proportion (53%). The oxygen consumption rate of embryos of rank B collected on day 8 was significantly higher ($p<0.05$) in female embryos than in male embryos collected on the same day. However, there were no apparent differences of oxygen consumption rates at each day of blastocyst appearance between male and female embryos of rank A. These results indicate that the oxygen consumption rate of individual embryos reflects their quality but does not correlate with the sex ratio of embryos of excellent quality

Introduction

An *in vitro* culture system has been established to produce bovine embryos following *in vitro* maturation and IVF of oocytes collected from ovaries (Kajihara et al. 1987; Xu et al. 1987; Goto et al. 1988; Fukuda et al. 1990). Of the inseminated oocytes, 30-40% routinely develops to the blastocyst stage (Enright et al. 2000). However, the viability of bovine IVF embryos has been found to be affected by the age of the embryo, stage of embryonic development and embryo quality (Han et al. 1994; Takagi et al. 1994; Carvalho et al. 1996). To date, several parameters have been investigated as potentially predictive indicators of embryo viability *in vitro*. In assessing the viability of embryos, morphological evaluation has been most widely used as a non-invasive assessment, but the correlation between the morphological criteria and an embryo's ability to result in an offspring is not high (Butler and Biggers 1989). With the exception of morphological evaluation, most viability assays that have been developed target a metabolic parameter that may be predictive with respect to embryo survival *in vivo*. Unfortunately, these assays are generally invasive and variably perturb the viability of embryos because embryos are exposed to cell-permeating fluorescent dyes, UV illumination or radioactive probes (Overstrom 1996). More recently, oxygen consumption by individual bovine embryos has been shown to be non-invasively quantified by scanning electrochemical microscopy (SECM) (Shiku et al. 2001). In this method, a microelectrode probe is used to scan the surface of a single embryo in the culture media and to measure the oxygen reduction during *in vitro* culture. Shiku et al. (2001) reported that oxygen consumption of an individual bovine IVF embryo was strongly related to its morphological quality. It has been suggested that the oxygen consumption reflects the cell number or the number of mitochondria in the embryo (Trimarchi et al. 2000). On the contrary, it has been demonstrated that the later developing blastocysts are of poor quality as judged by the cell

number (Jiang et al. 1992), and that the cell number of IVF embryos decreases with increasing embryo age (Mori et al. 2002). Yadav et al. (1993) showed that the proportion of male embryos is significantly higher among bovine embryos that cleave to the two-cell stage within the first 30 h post-insemination, the time of the first cleavage being related to the cell number of embryos. Moreover, bovine male embryos seem to reach the blastocyst stage faster than female embryos (Avery et al. 1992) and to have more cells than females (Xu et al. 1992). Therefore, if there is correlation between the oxygen consumption and the sex of bovine IVF embryos, the non-invasive assessment of oxygen consumption of each embryo may potentially be used as a predictive indicator of embryo sex. At present, polymerase chain reaction (PCR) for sex determination of bovine embryos is used in many laboratories (Avery et al. 1992; Grisart et al. 1995; Kochhar et al. 2003). However, this method needs time-consuming post-amplification operations and special equipments. Recently, LAMP has been developed which is rapid and sensitive (Notomi et al. 2000). Hirayama et al. (2003) applied the LAMP technique for the sexing of bovine embryos and showed that sex diagnosis by this technique was more sensitive than that of PCR. In the present study, we quantified the oxygen consumption of single bovine embryos by SECM, and used LAMP technology for their sex diagnosis. The relationship between the oxygen consumption rate and sex of IVF blastocysts collected on day 7, 8, and 9 after *in vitro* fertilization was evaluated.

Material and Methods

Embryo production

The procedures of oocyte collection, *in vitro* maturation, IVF of oocytes, and *in vitro* embryo culture were described by Abe et al. (1999). The serum-free media used for maturation of oocytes and embryo culture were the IVMD101 and IVD101 media

(Research Institute for the Functional Peptides, Yamagata, Japan), respectively, the formulations of which are detailed by Yamashita et al. (1999) and Abe et al. (2002). Cumulus oocyte complexes (COCs) were obtained by aspiration from small ovarian follicles and then cultured in the maturation medium (IVMD101) for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Cryopreserved semen from a single Japanese Black bull was thawed at 37°C and then washed twice with IVF100 medium (Research Institute for the Functional Peptides) by centrifugation at 550 *g* for 7 min. The pellet of sperm was resuspended in IVF100 medium to give a concentration of 5 x 10^6 spermatozoa/ml. The COCs were transferred into the sperm microdroplets for insemination and incubated for 6 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After IVF, inseminated oocytes were cultured in IVD101 for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2/5% O_2/90% N_2. These embryos were then denuded by pipetting with capillary pipettes to completely remove the cumulus cells, then transferred to a fresh IVD101 medium, and incubated in the same conditions. Embryos at the blastocyst stage were collected on day 7, 8, and 9 after insemination and categorized as ranks A, B and C (excellent, good, and fair, respectively) based on microscopic observation of the morphology (Lindner and Wright 1983). Blastocysts categorized as ranks A and B showed a clear differentiation of the trophoblastic cells and inner cell mass, and a distinguishable blastocoele. Only embryos of ranks A and B were selected and used for this study. Moreover, the blastocysts were divided into two groups, according to their morphological development: blastocyst (a blastocoele was visible, the inner cell mass was becoming distinct, but the overall diameter of the embryo, including zona pellucida, was unchanged), and expanded blastocyst (the diameter of the embryo was increased and the thickness of the zona pellucida could be reduced to approximately half of the original thickness).

Estimation of oxygen consumption

Oxygen consumption by individual bovine embryos was non-invasively quantified by the recently developed SECM measuring system (Abe et al. 2004). A single blastocyst was transferred into a plate filled with 5 ml of embryo respiration assay medium-1 (ERAM-1; Research Institute for the Functional Peptides) and the embryos dropped individually to the bottom of the microwell. The medium temperature were maintained at 37° C by a warming plate (MATS502NLR; Tokai Hit, Shizuoka, Japan) on the microscope stage. The measurement instruments were covered with a plastic sheet and water saturated 5% CO_2 and 95% air was allowed to flow. The measurement of oxygen consumption was carried out according to the procedure previously described by Shiku et al. (2001). Briefly, Pt-microdisc electrodes, sealed in a tapered soft-glass capillary (PG10165-4: World Precision Instruments, Sarasota, FL, USA), were fabricated according to the literature (Matsue et al. 1993). A tip potential was held at −0.6 V vs Ag/AgCl with a potentiostat (HA1010mH1B; Hokuto Denko Co., Tokyo, Japan) to monitor the local oxygen concentration in the solution. The tip scanning rate was 19.1 μm/s. A microelectrode with a Pt-disc radius <1.4 μm was selected so that the oxygen reduction current of the electrode was <1.0 nA. The accurate tip radius was determined by cyclic voltammetry in a 5.0-mM $K_4Fe(CN)_6$, 0.1 M KCl solution before the experiments. The XYZ-stage and the potentiostat were controlled by a notebook computer (FMV-BIBLO NE7/800; Fujitsu, Tokyo, Japan). Voltammetry of the Pt-microdisc electrode in ERAM-1 solution showed a steady-state oxygen reduction wave. No response from other electrochemically active species was observed near the embryo surface. The oxygen consumption rate of embryos was calculated by software, in which the oxygen concentration difference between the bulk solution and sample surface (ΔC), and the oxygen consumption rate (F) of a single sample was estimated according to the

spherical diffusion theories (Shiku et al. 2001). We repeatedly scanned the electrode back and forth, more than three times, to estimate the mean ± standard deviation ($n \geq 6$) of the ΔC for each sample. After measurement of oxygen consumption, each embryo was frozen in a tube containing phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) supplemented with 0.3% polyvinylpyrrolidone (PVP) and stored at 80°C until sex determination.

Sex determination

The sex determination of embryos was conducted using a bovine embryo-sexing kit (Eiken Chemical Co., Ltd., Tochigi, Japan). The sexing kit consisted of an extraction solution, a Reaction Mix I that contains dNTPs, buffer and male specific primers, a Reaction Mix II that contains dNTPs, buffer and male female common primers and *Bst* DNA polymerase. Accessory spermatozoa surrounding the frozen thawed embryos were removed by removing the zona pellucida by brief exposure to 0.2% pronase (Actinase E; Kaken Kagaku, Tokyo, Japan) in PBS and by washing three times in PBS with 0.3% PVP. After washing, each embryo, suspended in the PBS with 0.3% PVP (about 6 μl), was put into a sterilized tube containing 6 μl of extraction solution. The mixture was incubated for at least 5 min at room temperature (sample solution). The sample solution (5 μl) was added to two reaction tubes, one tube containing 20 μl of Reaction Mix I and 1 μl of *Bst* DNA polymerase for male reaction and the other tube containing 20 μl of Reaction Mix II and 1 μl of *Bst* DNA polymerase for male-female common reaction. As a positive control, 5 μl of control DNA was added to the two reaction tubes instead of sample solution. As a negative control, a 1 : 1 mixture of extraction solution and embryo washing solution (PBS with 0.3% PVP) was added to the two reaction tubes. After the addition of the sample solution, positive solution and negative solution to each reaction tube, the tube was placed

into the reaction block of Loopamp End Point Turbidimeter (Teramecs, Kyoto, Japan). Amplification reaction was carried out at 63°C for 35 min and then at 80°C for 2 min to terminate the reaction. After amplification reaction, the tube was transferred from reaction block to detection position in the turbidimeter for sex determination. The sex of the embryo was judged to be male when both reactions were positive, while the sex of the embryo was judged to be female when male female common reaction alone was positive.

Statistical analysis

The oxygen consumption rates of embryos were analyzed by one-way analysis of variance. The significance of difference between means was compared by a *post hoc*, Fisher's protected least significant difference test (PLSD test). Sex ratios were compared by chi-square analysis with a Yates' correction. When some expected values were ≤ 5, Fisher's exact probability test was used. Differences at a probability $p \leq 0.05$ were considered significant. Data are expressed as mean ± SEM.

Results

A total of 93 bovine embryos were examined to evaluate the oxygen consumption and sex ratio, of which 76% (16/21), 42% (5/12), and 82% (9/11) of the embryos of rank A collected on day 7, 8, and 9 after IVF, respectively, were expanded blastocysts, and all of the embryos categorized as rank B were blastocysts. Initially, the data for each morphological stage (blastocyst and expanded blastocyst) of embryos of rank A were analysed to investigate any effects of the morphological stage on the oxygen consumption rates. There were no significant differences in the overall average rates of oxygen consumption between the blastocysts and expanded blastocysts (1.59 ± 0.16 vs 1.78 ± 0.08; $p > 0.05$). The consumption rate of expanded blastocysts collected on day 7

was significantly higher ($p<0.05$) than that of blastocysts on the same day (1.75 vs 0.11 vs 1.26 vs 0.07), while the rates of expanded blastocysts on both days 8 and 9 did not differ from those of the blastocysts (day 8, 2.18 vs 0.13 vs 1.88 vs 0.25, $p>0.05$; day 9, 1.59 vs 0.12 vs 1.44 vs 0.56, $p>0.05$).

As shown in Table 1, the oxygen consumption rates of embryos of rank A were significantly higher ($p<0.05$) than those of rank B, irrespective of the day of blastocyst appearance after IVF. The average proportion of male embryos (53%) did not differ significantly from the expected 50%, and neither did the proportion for male embryos of rank A or B differ significantly from each other at any of days examined nor from the average proportion. As shown in Fig.1, there were no significant differences in oxygen consumption rates between male and female embryos of rank A on each day of blastocyst appearance. However, the oxygen consumption rate of embryos of rank B collected on day 8 was significantly higher ($p<0.05$) in female embryos than in male embryos of the same day (1.38 ± 0.18 vs 0.84 ± 0.29).

Discussion

In the present study, we applied two novel techniques, one for assessing oxygen consumption from a single embryo by SECM, and one for sex determination by the LAMP method. In assessing the viability of preimplantation embryos, the predictive values of metabolic parameters have been reported to be superior to morphological evaluation (Gardner and Leese 1987; Rondeau et al. 1995). Oxygen consumption as a parameter for assessing the viability of embryos has been studied using spectrophotometric, fluorescence, and electrochemical techniques (Magnusson et al. 1986; Overstrom et al. 1992; Houghton et al. 1996), which are inappropriate for rapid quantitative measurement and for a single embryo. The SECM procedure used in the

present study can directly measure the concentration profiles within a few minutes to estimate the oxygen consumption of a single blastocyst and allow the measurement without any addition of labeling reagent, in which the measurement conditions can be close to the embryo culture conditions. Therefore, the benefit of this method is that the oxygen consumption can be non-invasively obtained from a single embryo without any damage to the embryos. Indeed, after measurement of the concentration by SECM and subsequent culture, bovine morulae that consumed more oxygen developed to the blastocyst stage at higher rates than did those that consumed less oxygen (Shiku et al. 2001; Abe et al. 2004).

The LAMP method used in the present study is simpler and more sensitive than the PCR method for sex determination of bovine embryos (Hirayama et al. 2003). For example, there is no necessity of heat denaturation of the template DNAs, which is needed for the PCR. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature of 63 °C. The judgment eliminates the need for any laborious and time-consuming post-amplification operations such as hybridization and electrophoresis, as well as the need for special equipment. Magnusson et al. (1986) suggested that the oxygen consumption of human embryos correlates with their morphological quality. Shiku et al. (2001) also confirmed the result using bovine morula. Consistent with these findings, we demonstrated that the oxygen consumption rates in the embryos of inferior quality (rank B) were lower than those of excellent quality (rank A), irrespective of the day of blastocyst appearance. Moreover, the majority of all oxygen consumed by embryos at the blastocyst stage is utilized by mitochondria to generate ATP for the plasma membrane Na/K-ATPase (Benos and Balaban 1980; Trimarchi et al. 2000). Shiku et al. (2001) demonstrated that bovine IVF morulae with higher oxygen consumption developed into more advanced blastocysts

after culture. Therefore, these findings indicate that the oxygen consumption rate reflects the embryo quality. It has been demonstrated that the cell number of bovine IVF embryos is affected by the embryonic stage and age (Jiang et al. 1992; Mori et al. 2002). The cell numbers of bovine expanded blastocysts have been shown to be higher than those of blastocysts, irrespective of the day of blastocyst appearance after IVF (Mori et al. 2002). Trimarchi et al. (2000) reported that the oxygen consumption reflects the cell number or the amounts of mitochondria in the embryo. So it was speculated that the oxygen consumption of expanded blastocysts was higher than that of blastocysts. Contrary to expected results, we found that the oxygen consumption rates of expanded blastocysts collected on both day 8 and 9 did not differ significantly from those of blastocysts. The reason for these unexpected results is not so clear. It was reported that when the morphological stage of bovine IVF blastocysts were derived according to the developmental stage, the cell number of embryos had a large variation even in the same morphological stage on the same day of blastocyst formation (Mori et al. 2002). However, only a limited number of embryos of rank A were compared for evaluation of oxygen consumption between blastocysts and expanded blastocysts, and the cell number of individual embryos was not investigated in the present study. Thus further studies will be needed to make this point clear. Iwasaki et al. (1990) suggested that a bovine embryo developing quickly to the blastocyst stage was of a higher quality than one that developed more slowly. Bovine male embryos are thought to develop faster than female embryos, at least to the blastocyst stage (Avery et al. 1992; Xu et al. 1992; Gutierrez-Adan et al. 1996), indicating sex ratio biases by the day of blastocyst appearance (Avery et al. 1992). Moreover, the *in vitro* culture system influences embryonic growth rates (Pegoraro et al. 1998), and the culture medium affects the sex ratio (Bredbacka and Bredbacka 1996). Bredbacka and Bredbacka (1996) reported that faster development occurs for bovine male

embryos in a cell free culture system with the presence of a high concentration (5.56 mM) of glucose. In the present study, the embryos were cultured in serum-free medium without somatic cells, in which the medium contained 2.22 mM of glucose. We found no significant differences in the total sex ratio and the sex ratio for each day of blastocyst appearance, irrespective of morphological quality. Gutierrez-Adan et al. (2001) demonstrated that the presence of serum in the culture medium increased male embryos, but the absence did not alter the sex ratio. Other reports with different culture systems for sex ratio studies have shown no sex ratio deviation for *in vitro* development of the embryos (King et al. 1992; Grisart et al. 1995). Therefore, some differences in the procedure used may be the basis of sex ratio alteration of *in vitro*-produced bovine blastocysts. The sex of embryos has been generally determined using their blastomeres for detecting Y chromosome-specific sequences, in which the microsurgical procedure for collection of the blastomeres variably perturbed the viability of embryos. Therefore, a simple and non-invasive selection procedure for predicting male and female embryos has been required for efficient embryo transfer. If there is a correlation between the oxygen consumption rate and sex ratio, the oxygen consumption rate may potentially be used for non-invasive sex selection of embryos. In the present study, however, there were no apparent differences of oxygen consumption rates at each day of blastocyst appearance between male and female embryos of rank A, which are usually used for embryo transfer. This finding indicates that the oxygen consumption rate of embryos does not correlate with the sex ratio. In the present study, the oxygen consumption and sex of individual embryos were evaluated using two novel procedures: the SECM and LAMP techniques, respectively. The results demonstrate that the oxygen consumption rate of individual embryos reflects their quality but does not correlate with the sex ratio of embryos. However, considering fact that only a limited number of embryos were used for

evaluation of oxygen consumption and sex determination in the present study, further investigations are needed to clarify whether the oxygen consumption of embryos correlates with embryonic development stage and sex.

CHAPTER 2

Effect of Maturation Culture Period of Oocytes on the Sex Ratio of *In Vitro* Fertilized Bovine Embryos.

Abstract.

It has been suggested that the maturational stage of oocytes at time of insemination influences the sex ratio of resulting embryos. However, there are very few reports concerning the relationship between the maturation culture period of oocytes and the sex ratio of resulting embryos. The objective of this study was to investigate the effects of *in vitro* maturation culture period for bovine oocytes on the sex ratio of *in vitro* produced blastocysts using a novel technique of loop-mediated isothermal amplification (LAMP). Cumulus-oocyte complexes were collected from the ovaries of slaughtered cows, and then matured *in vitro* for various periods (16, 22, 28, and 34 h). After maturation culture for each period, the oocytes were inseminated with frozen-thawed spermatozoa, and then cultured *in vitro*. Blastocysts were harvested on Day 7 after insemination, and the sex of metaphase II stage were significantly lower ($P<0.05$) in the 16-h maturation group than in the other groups. The proportion of blastocyst formation after insemination was significantly higher ($P<0.05$) in the 22-h maturation group than in the other groups. The proportion of male blastocysts increased with the increase in maturation culture period. The proportion of male blastocysts derived from oocytes matured for 34 h was significantly higher ($P<0.05$) than from oocytes matured for 16 and 22 h. These results indicate that the sex ratio of *in vitro* fertilized embryos is apparently influenced by the maturation culture period of the oocytes

Introduction

Fertilization of a mammalian oocyte establishes the sex of the embryo and initiates a cascade of events that leads to sexual differentiation. If the timing of insemination *in vitro* can be used to control the gender of offspring in cattle, it would have an immense economic benefit and would be applied to livestock production. To date, various factors, including embryo culture conditions (Bredbacka and Bredbacka, 1996; Peipo et al.2001; and Kimura et al. 2005) and the duration of sperm-oocyte coincubation (Kochhar et al.2003), have been shown to influence the sex ratio of bovine embryos produced *in vitro*. It appears that a high concentration of glucose in the culture medium accelerates development of male embryos (Bredbacka and Bredbacka, 1996). Kochhar et al. (2003) suggest that a short duration of sperm-oocyte coincubation leads to a skewed sex ratio. Moreover, the maturational stage of oocytes at the time of insemination has been shown to have a great impact on the sex ratio of embryos (Dominko and First., 1997). Although the relationship between the maturation culture period of oocytes and the sex ratio of bovine embryos at the early cleavage stage (from the 2-cell to 8-cell stages) has been evaluated in previous studies (Dominko and First., 1997 and Guiterrez-Adan et al.1999), the influence of the maturation culture period on the sex ratio of blastocysts developed after *in vitro* fertilization (IVF) and culture has not been fully discussed.

A new technique called LAMP, which is both rapid and sensitive, has been developed (Notomi et al.2000) It is expected that it will amplify the target sequence with high selectivity (Notomi et al.2000 and Nagamine et al.2002). Moreover, Hirayama et al. (2004) applied a LAMP technique to the sexing of bovine embryos and showed that sex diagnosis using this technique was more sensitive than polymerase chain reaction (PCR).

This study was conducted to investigate the effects of the maturation culture period of

oocytes on the sex ratio of *in vitro* produced bovine blastocysts using LAMP technology for sex diagnosis.

Materials and Methods

In vitro maturation

The methods used for *in vitro* maturation, fertilization, and subsequent culture in this study were modified from the procedure reported by Otoi et al.(1993). Ovaries were collected from cows at a local abattoir and brought to the laboratory in physiological saline (0.85% [w/v] NaCl) at 30° C within 4 h after slaughter of the cows. The oocytes in the follicular fluid (5 - 10 per ovary) were aspirated from surface-visible follicles (1-7 mm in diameter) with a 5-ml syringe fitted with an 18-gauge needle. After being washed twice with modified phosphate-buffered saline (Embryoteck; Nippon Zenyaku, Fukushima, Japan), only healthy-looking cumulus-oocyte complexes (COCs) with 3 or more dense layers of cumulus cells were suspended in maturation medium (TCM199 medium [Earle's salts]) with 25 mM HEPES buffer (Invitrogen Corp.. Carlsbad, CA, USA), 5% fetal bovine serum (FBS; Invitrogen Corp.), 0.02 mg/ml FSH (Denka, Kawasaki, Japan), and 50 μg/ml gentamicin (Sigma, St. Louis, MO, USA). About 50-60 COCs were incubated for 16, 22, 28, and 34 h in the maturation medium (2.5 ml) under a layer of mineral oil (Sigma) in a polystyrene culture dish (35 x 10 mm; Falcon, Lincoln Park, NJ, USA) at 38.5 C in an atmosphere of 5% $C0_2$ and 95% air with high humidity.

At the end of maturation culture, some oocytes were mechanically denuded from cumulus cells in Dulbecco's phosphate-buffered saline (PBS; Invitrogen Corp.) supplemented with 1 mg/mL hyaluronidase (Sigma). Denuded oocytes were then mounted onto a glass slide and fixed with acetic acid: ethanol (1: 3 v/v) for 48-72 h. The

fixed oocytes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. The precise maturation stage of each oocyte was determined based on the changes in the chromosome configuration and nuclear membrane.

In vitro fertilization and culture

After maturation culture for each period, the COCs were fertilized *in vitro* with frozen-thawed semen in BO medium (Brackett and Oliphant, 1975). Frozen semen was thawed in a water bath at 36 C, and the spermatozoa were processed to induce capacitation *in vitro*. An aliquot of semen was transferred into BO medium without bovine serum albumin (BSA), but supplemented with 5 mM caffeine (Sigma). The sperm was then washed twice by centrifugation at 500 x g for 5 min. The pellet of sperm was resuspended in BO medium that contained BSA (3 mg/ml; Sigma), heparin (10 μg/ml; Novo Industry A/S, Osaka, Japan), and 2.5 mM caffeine to give a concentration of 5 x 10^6 cells/ml. The COCs were transferred into the sperm microdroplets for insemination. After incubation for 5 h, the COCs were transferred into culture medium (TCM199 supplemented with 5%. FBS, 50 μg/ml gentamicin and 5 μg/ml insulin [Sigma]) in a 4-well culture dish (Nunc A/S, Roskilde, Denmark) and incubated at 38.5 C in 5% $C0_2$ and air with high humidity. The culture medium was replaced with fresh medium 72 h after insemination, and the cumulus cells surrounding the embryos were removed by pipetting. The cumulus layer attached to the bottom of the 4-well culture dish was not removed. The developmental stage of the embryos was evaluated under a microscope (x 200; Olympus IMT-2) every 24 h, and culture continued for 7 days after insemination. At the end of culture, morphologically normal blastocysts were harvested to evaluate their sex, and the numbers of cleaved embryos and blastocysts were recorded.

Sex determination

Evaluation of sex embryo has done by LAMP method. Briefly, sex determination of the embryos was conducted using a bovine embryo sexing kit (Eiken Chemical Co., Ltd., Tochigi, Japan). The sexing kit consisted of the following: an extraction solution; Reaction Mix I, which contains dNTPs, buffer, and male specific primers; Reaction Mix II, which contains dNTPs, buffer, and male-female common primers; and Bst DNA polymerase. Accessory spermatozoa surrounding the embryos harvested on Day 7 (Day 0 is the day of insemination) were removed by removing the zona pellucida by brief exposure to 0.2%. pronase (Actinase E. Kaken Kagaku, Tokyo, Japan) in PBS and by washing three times in PBS with 0.3% polyvinylpyrrolidone (PVP). After washing, each embryo, suspended in PBS with 0.3% PVP (about 6 μ/1), was put into a sterilized tube containing 6 μl of extraction solution. The mixture was incubated for at least 5 min at room temperature (sample solution). The sample solution (5 μl) was added to two reaction tubes, one containing 20 μ1 of Reaction Mix I including I μl of Bst DNA polymerase for male reaction, and the other containing 20 μl of Reaction Mix 11 including 1 μl of Bst DNA polymerase for male-female common reaction. As a positive control, 5 μl of control DNA was added to two reaction tubes instead of the sample solution. As a negative control, a 1:1 mixture of extraction solution and embryo washing solution (PBS with 0.3%. PVP) was added to two reaction tubes. After addition of the sample solution, positive solution, and negative solution to each reaction tube, the tubes were placed into the reaction block of a Loopamp End Point Turbidimeter (Teramecs, Kyoto, Japan). An amplification reaction was carried out at 63 C for 35 min and then at 80 C for 2 min to terminate the reaction. After the amplification reaction, the tubes were

transferred from the reaction block to the detection position in the turbidimeter for sex determination. The sex of the embryo was judged to be male when both reactions were positive, while the sex of the embryo was judged to be female when the male-female common reaction alone was positive.

Statistical analysis

The proportions of oocytes that reached metaphase II (MII) and the proportions of embryos that cleaved and developed to the blastocyst stage were subjected to arc sine transformation before being subjected to a one-way analysis of variance. The significance of differences between means was compared by a post-hoc Fisher's protected least significant difference test (PLSD test). The sex ratios of the blastocysts were analyzed by Chi Square analysis with a Yates' correction. Differences at a probability value (P) of 0.05 or less were considered significant. Data were expressed as means ± SEM.

Results

When the meiotic stage of the oocytes cultured for the various periods was examined, the proportion of oocytes reaching MII was significantly lower ($P<0.05$) in the 16-h maturation group than in the other maturation groups (Table 2).

As shown in Table 3, the proportions of cleavage and development to the blastocyst stage in the 22-h maturation group were significantly higher ($P<0.05$) than in the other groups. When the sex of the blastocysts harvested on Day 7 after insemination was examined by the LAMP method, the sex of all embryos was correctly determined. The proportions of male blastocysts increased with the increase in maturation culture period.

The proportion of male blastocysts derived from oocytes matured for 34 h was significantly higher ($P<0.05$) than oocytes matured for 16 and 22 h.

Discussion

To date, various factors have been shown to influence the sex ratio of bovine embryos produced *in vitro*. In particular, male embryos are thought to develop faster than female embryos, at least to the blastocyst stage (Avery et al. 1992; Xu et al. 1992; Gutierez et al. 1996) indicating that the sex ratio of embryos is influenced by the day of blastocyst appearance (Avery et al.1992,) In the present study, therefore, *in vitro* produced blastocysts were harvested on only Day 7 for evaluation of their sex ratio. Our results using the LAMP method showed that the sex ratio of blastocysts is affected by the maturation culture period. These results are in agreement with the experiment of Dominko et al. (1997) who reported that inseminating oocytes immediately after extrusion of the first polar body increased the proportion of female embryos, whereas delaying insemination for 8 h (after polar body extrusion) increased the proportion of male embryos. Similarly, Gutierrez-Adan et al. (1999) demonstrated that delaying insemination enhanced the proportion of male embryos. These findings from previous studies indicate that the capacity of oocytes for selective processing of X- or Y-chromosome bearing spermatozoa may be dependent on their maturational status, and that delaying insemination allows MII-arrested oocytes to process Y-chromosome bearing spermatozoa more effectively than X-chromosome bearing spermatozoa.

Delay of insemination allows a greater proportion of oocytes to reach MII before insemination, but the prolonged maturation culture induces aging of the oocytes. In the present study, we observed that the proportion of oocytes matured to MII reached the

maximum level after being cultured for 22 h, and the proportions did not differ among the oocytes cultured for 22, 28, and 34 h. However, the proportions of blastocyst formed from oocytes cultured for more than 28 h decreased compared with oocytes cultured for 22 h. It has been demonstrated that aged oocytes exhibit abnormal morphological characteristics before fertilization, such as the disappearance of the microfilament-rich area over the mitotic spindle, disruption of the spindle location, and chromatin disorganization (Webb et al. 1986). Aged oocytes have been shown to have enhanced ability to be activated, resulting in a higher rate of fragmentation, and to decrease activity of maturation promoting factor (Kikuchi et al.1995, 2000) Moreover, cytoplasmic changes affecting oocyte quality (for example, decreased ability to achieve fertilization and decreased capacity for development) occur when the meiotic arrest period is prolonged(Chian et al.1992). Therefore, the significantly lower developmental competence of oocytes cultured for more than 28 h observed in the present study may indicate morphological and cytoplasmic changes in the oocytes. On the other hand, the results showed that the proportion of oocytes reaching MII was lower in the 16-h maturation group than in the other maturation groups. Moreover, the blastocysts derived from the 16-h maturation group skewed the sex ratio in favor of female embryos, whereas the prolonged maturation culture (more than 28 h of culture) increased the proportion of male embryos. Although the sex ratio of bovine embryos produced *in vitro* has been shown to be influenced by culture conditions (Bredbacka and Bredbacka, 1996), there was no apparent deviation from a 1:1 sex ratio in the 22-h maturation group. Therefore, the skewing of the sex ratio observed in the 16-h, 28-h, and 34-h maturation groups may be influenced by both the maturational state of the oocytes and the time kinetics of spermatozoa and oocyte interaction (Dominko et al. 1997; Guitierez-Adan et al. 1999), but not by culture conditions.

In conclusion, these results using the LAMP method indicate that the maturation culture period has an effect on the sex distribution of blastocysts appearing on Day 7, and that more males than females are recovered when the oocytes are cultured for more than 28 h and subsequently fertilized with spermatozoa.

OVERALL DISCUSSION AND CONCLUSIONS

Artificial reproductive technologies offer tremendous advantages for improvement of the quality of transferable embryos in order to increase the pregnancy rate and the number of offspring. In human, recent reports have suggested that an examination of the morphology of zygotes can predict the outcome of IVF (Scott and Smith, 1998; Scott et al., 2000; Tesarik et al., 2000). For example, a pregnancy rate of 49.5% could be achieved after the transfer of high quality zygotes (Scott et al. 2000). Sjoblom et al. (2006) reported that morphological characteristics of oocytes are related with blastocyst formation. Furthermore, a specific pattern of nucleolar alignment has been suggested to be related with high quality of embryos (Tesarik and Greco, 1999). When genetically excellent embryos can be transferred to recipients, the increased numbers of genetically superior animals would replace the herd. However, these technologies still have some problems concerning *in vitro* production and embryonic development after transfer when compared with *in vivo* embryos. The viability of IVP embryos is lower than that of embryos produced *in vivo* (Farin and Farin 1995). Differences in gene expression exist between *in vitro* and *in vivo* embryos, which may indicate the differences in their quality. Wrenzycki et al. (1996) demonstrated that expression of the connexin 43 gene at the blastocyst stage differs between bovine embryos produced *in vitro* and *in vivo*. This gene is involved in the formation of a protein that gives rise to gap junctions between embryonic cells. Poor gap junction formation is associated with poor cell compaction and commonly occurs in IVP embryos. Therefore, the expression of connexin 43 gene may be related with the embryo quality, which is an important factor for the success on the development of embryos after transfer. To date, several parameters have been investigated as potentially predictive indicators of embryo viability *in vitro*. In assessing the viability

of embryos, morphological evaluation has been most widely used as a non-invasive assessment, but the correlation between the morphological criteria and an embryo's ability to result in an offspring is not high (Butler and Biggers 1989).

In the first study, I applied two novel techniques, one for assessing oxygen consumption from a single embryo by SECM, and the other for sex determination by the LAMP method. In assessing the viability of preimplantation embryos, the predictive values of metabolic parameters have been reported to be superior to morphological evaluation (Gardner and Leese 1987; Rondeau et al. 1995). The SECM procedure used in the present study can directly measure the concentration profiles within a few minutes to estimate the oxygen consumption of a single blastocyst and allow the measurement without any addition of labeling reagent, in which the measurement conditions can be closed to the embryo culture conditions. Therefore, the benefit of this method is that the oxygen consumption can be non-invasively obtained from a single embryo without any damage to the embryos. However, almost all of the techniques are invasive and it is thereby not possible to correlate any parameter with development to term. Unfortunately, these assays are variably perturbing the viability of embryos (Overstrom 1996). Furthermore, the viability of embryo has effects on the rate of implantation, pregnancy and viability of offspring. Consequently, non-invasive embryo selection for quality by morphology assessment is still a method for embryo selection before embryo transfer in both human and bovine assisted reproduction. The results demonstrated that the oxygen consumption rates in the embryos of inferior quality (rank B) were lower than those of excellent quality (rank A), irrespective of the day of blastocyst appearance. Magnusson et al. (1986) suggested that the oxygen consumption of human embryos correlates with their morphological quality. Shiku et al. (2001) also confirmed the result using bovine morula. Indeed, after measurement of the concentration by SECM and subsequent culture, bovine

morulae consumed more oxygen developed to the blastocyst stage at higher rates than those that consumed less oxygen (Shiku et al. 2001; Abe et al. 2004). Shiku et al. (2001) demonstrated that bovine IVF morula with higher oxygen consumption developed into more advanced blastocysts after culture. Therefore, these findings indicate that the oxygen consumption rate reflects the embryo quality. Thompson et al. (1996) reported that the oxygen consumption was relatively constant from days 0 - 4 of culture, then increased with the initiation of compaction and continued to increase with the formation and expansion of the blastocoels. The uptake of both pyruvate and glucose increased significantly, and ATP production followed a similar pattern of oxygen consumption. Moreover, the majority of oxygen consumed by embryos at the blastocyst stage is utilized by mitochondria to generate ATP for the plasma membrane Na/K-ATPase (Benos and Balaban 1980; Trimarchi et al. 2000). Furthermore, the cell numbers of bovine expanded blastocysts have been shown to be higher than those of blastocysts, irrespective of the day of blastocyst appearance after IVF (Mori et al. 2002). Trimarchi et al. (2000) reported that the oxygen consumption reflects the cell number or the amounts of mitochondria in the embryo. Therefore, it was speculated that the oxygen consumption of expanded blastocysts was higher than that of blastocysts.

The sexing technology allows controlling the gender of offspring by the use of genetic markers to identify embryos expressing desirable sex for production traits. Predetermination of the sex of offspring is very important for genetic improvement in livestock farm. Some studies demonstrated an association between gender and developmental rates of bovine embryos produced *in vitro* (Lonergan et al. 1999; Gutierrez-Adan et al. 2001). The development of male embryos was faster than that of female embryos during the first 7 to 8 d after *in vitro* fertilization, suggesting that sex-related gene expression affects development of embryos by activation of the

embryonic genome (Dominko and First 1997). In the present study, we conducted to clarify whether the oxygen consumption of embryos correlates with the sex ratio of embryos. Our results showed no significant differences in the total sex ratio and the sex ratio for each day of blastocyst appearance, irrespective of morphological quality. Moreover, there were no apparent differences of oxygen consumption rates at each day of blastocyst appearance between male and female embryos in rank A, which are usually used for embryo transfer.

In bovine embryo transfer, sexing of preimplantation embryos is an important management tool. Recently, sexing based on detection of Y chromosome-specific sequences has been used to predict the sex of offspring (Hirayama et al.2004). Polymerase chain reaction (PCR) enables amplification of a target sequence from a small number of blastomeres (Shea, 1999), which is routinely used in the field for sexing. However, the method requires technical skill and is time consuming. Furthermore, PCR has the risk of false positives because of DNA contamination during the handling of the PCR products in duplicate PCR procedures and/or electrophoresis. The LAMP method used in the present study is simpler and more sensitive than the PCR method for sex determination of bovine embryos (Hirayama et al. 2003). For example, there is no necessity of heat denaturation of the template DNAs, which is needed for the PCR. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature of 36 °C. The judgment eliminates the need for any laborious and time-consuming post-amplification operations such as hybridization and electrophoresis, as well as the need for special equipment. Furthermore, rapid determination by the LAMP method may improve the viability of transferable embryos when compared with PCR method.

In the second study, I have investigated whether the maturational stage of

oocytes at time of insemination influences the sex ratio of resulting embryos. If the timing of insemination *in vitro* can be used to control the gender of offspring in cattle, it would have an immense economic benefit and would be applied to livestock production. To date, various factors, including embryo culture conditions (Guiterez et al.1996) and the duration of sperm-oocyte co-incubation (Kochhar, 2003) have been shown to influence the sex ratio of produced bovine embryos *in vitro*. In the present study, therefore, *in vitro* produced blastocysts were harvested on only Day 7 for evaluation of their sex ratio. These results using the LAMP method showed that the sex ratio of blastocysts is affected by the maturation culture period. These results are in agreement with the experiment of Dominko et al. (1997) who reported that inseminating oocytes immediately after extrusion of the first polar body increased the proportion of female embryos, whereas delaying insemination for 8 h (after polar body extrusion) increased the proportion of male embryos. Similarly, Gutierrez-Adan et al. (1999) demonstrated that delaying insemination enhanced the proportion of male embryos. These findings from previous studies indicate that the capacity of oocytes for selective processing of X- or Y-chromosome bearing spermatozoa may be dependent on their maturational status, and that delaying insemination allows MII-arrested oocytes to process Y-chromosome bearing spermatozoa more effectively than X-chromosome bearing spermatozoa. Kochhar et al. (2003) suggested that a short duration of sperm-oocyte coincubation leads to a skewed sex ratio. Moreover, the maturational stage of oocytes at the time of insemination has been shown to have a great impact on the sex ratio of embryos (Dominko and First, 1997). On the other hand, delay of insemination allows a greater proportion of oocytes to reach MII before insemination, but the prolonged maturation culture induces aging of the oocytes. Moreover, Krisher (2004) reported that successful completion of maturation is independent on nuclear maturation and is collectively

referred to cytoplasmic maturation. An oocyte that had not completed cytoplasmic maturation before insemination is determined as a poor quality, because of resulting in abnormal development. In the present study, we observed that the proportion of oocytes matured to MII reached the maximum level after being cultured for 22 h, and the proportions did not differ among the oocytes cultured for 22, 28, and 34 h. However, the proportions of blastocyst formed from oocytes cultured for more than 28 h decreased, as compared with oocytes cultured for 22 h. Therefore, the optimal culture period is important for the production of IVP embryos.

In conclusions, the rate of oxygen consumption reflects the embryo quality and the non-invasive method can be alternative to determine the quality for bovine IVP embryos. In the present study, however, there was no apparent difference of oxygen consumption rates at each day of blastocyst appearance between male and female embryos. This finding indicates that the rate of oxygen consumption of embryos does not correlate with the sex ratio. However, further investigations are needed to clarify whether the oxygen consumption of embryos correlates with embryonic development stage and sex. In the second study, these results showed that the maturation culture period has an effect on the sex distribution of blastocysts harvested on Day 7, and that more males than females were recovered when the oocytes are cultured for more than 28 h and subsequently fertilized with spermatozoa. These findings indicate that the IVP technique may have a possibility for the production of cattle with desired sex using the change of maturation culture period.

This study will contribute to the increase of our knowledge on factors that would be related with embryo quality and production of sexed bovine embryos. These technologies could be used to select the embryos with high quality and desired sex in the cattle production.

TABLES AND FIGURES

Table 1. Relation between embryo quality, oxygen consumption, and sex ratio of bovine embryos collected on day 7, 8, and 9 after IVF

Day after IVF	Quality of embryos	No. of embryos examined	Oxygen consumption rate ($F\times10^{14}mols^{-1}$)*	Male (%)
7	A	21	$1.64 \pm 0.09^{a,d}$	61.9
	B	10	$1.24 \pm 0.13^{b,c}$	40.0
8	A	12	2.01 ± 0.15^{a}	50.0
	B	18	1.08 ± 0.19^{b}	55.6
9	A	11	$1.56 \pm 0.12^{c,d}$	45.5
	B	21	1.08 ± 0.08^{b}	52.4
Total		93	1.40 ± 0.06	52.7

*Values for oxygen consumption rate are presented as mean ± SEM.

[a-c] Values with different superscripts in the same column are significantly different ($P<0.05$).

Table 2. Effects of maturation culture period on the maturation of bovine oocytes*

Maturation culture period (h)	No. of oocytes examined	No. of oocytes reaching MII (%; Mean ± SEM)
16	103	67 (63.6 ± 3.6)[a]
22	92	78 (85.3 ± 4.6)[b]
28	101	82 (81.3 ±1.9)[b]
34	146	116 (80.3 ± 3.5)[b]

* Seven replicated trials were carried out.

[a,b] Values with different superscripts in the same column are significantly different ($P<0.05$).

Table 3. Effects of maturation culture period on the development of bovine oocytes after IVF and sex ratio*

Maturation culture period (h)	No. of oocytes examined	No. of cleaved embryos (%; Mean ± SEM)	No. of blastocysts (%; Mean ± SEM)	No. sexed	No. of males	No. of females	% of males
16	278	189 (67.4 ± 3.0)^{a,c}	41 (13.5 ± 1.8)^{a}	22	7	15	31.8^{a}
22	219	177 (80.2 ± 1.8)^{b}	52 (22.7 ± 2.3)^{b}	22	9	13	40.9^{a,b}
28	274	193 (69.9 ± 2.0)^{a}	44 (15.2 ± 1.4)^{a}	22	15	7	68.2^{b,c}
34	435	268 (61.7 ± 1.7)^{c}	55 (11.7 ± 1.2)^{a}	22	16	6	72.7^{c}

* Seven replicated trials of IVF were carried out, and the blastocysts were harvested on Day 7.

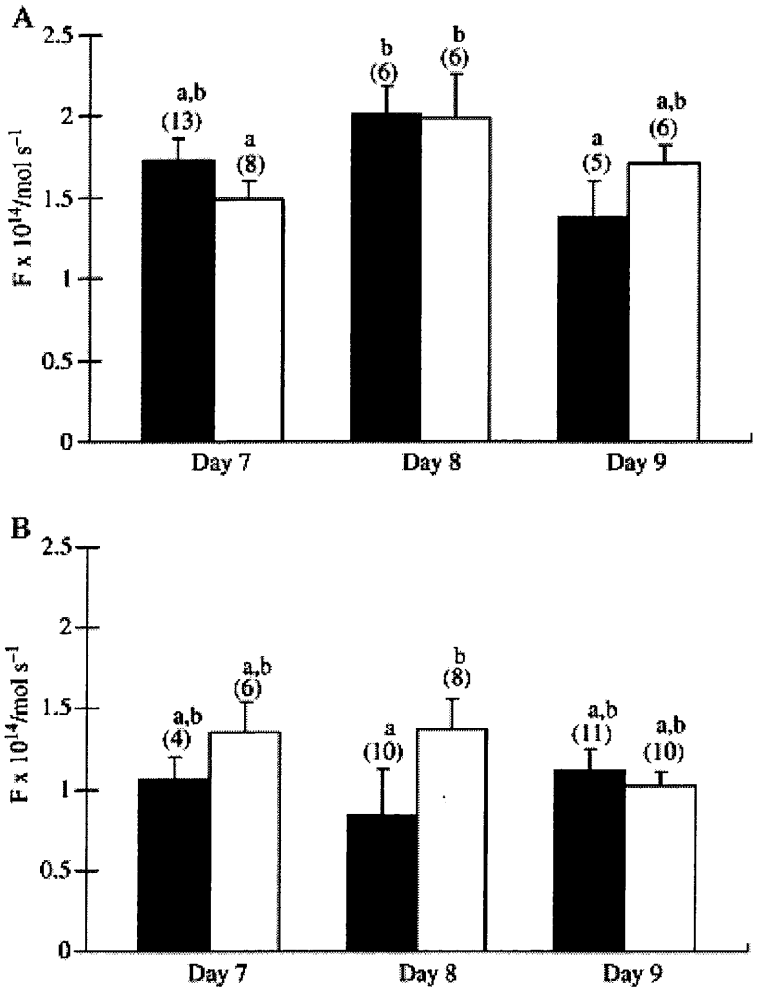

Fig.1. Relationship between the oxygen consumption and sex of bovine blastocysts collected on day 7, 8, and 9 after IVF. Oxygen consumption of blastocysts of rank A (A) and rank B (B) was measured by scanning electrochemical microscopy and then the sex of male (solid bar) and female (open bar) of each blastocyst was determined by the method of loop-mediated isothermal amplification. The values are expressed as means ± SEM. Bars with different letters differ significantly ($p<0.05$). Numbers in parenthesis indicate the total number of blastocysts examined

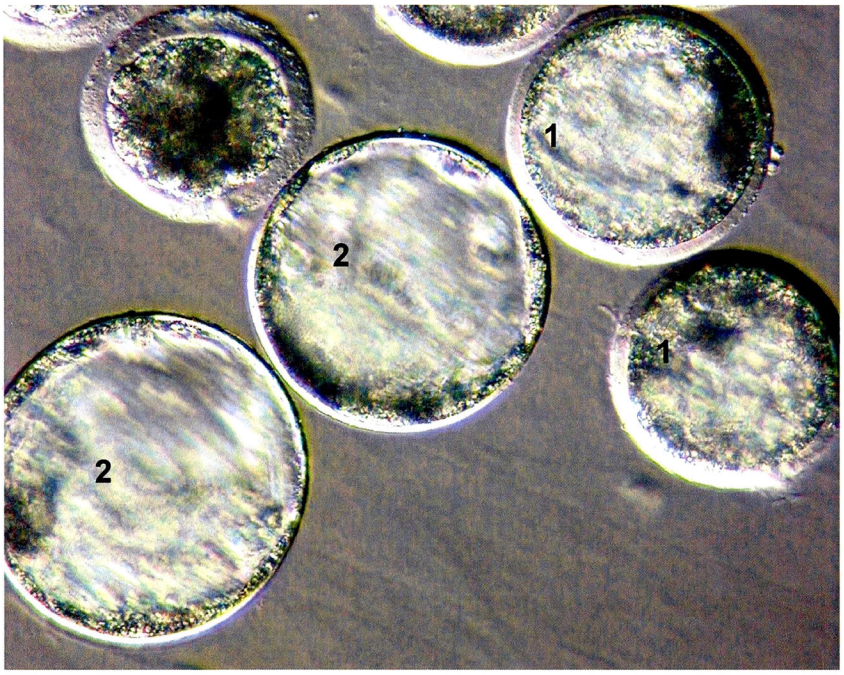

Fig. 2. *In vitro* produced bovine blastocysts in A rank. Blastocysts (1) and expanded blastocysts (2) were showing a clear differentiation of the trophoblastic cells and inner cell mass, and a distinguishable blastocoel. The photographs were taken with phase-contrast microscope (x 200).

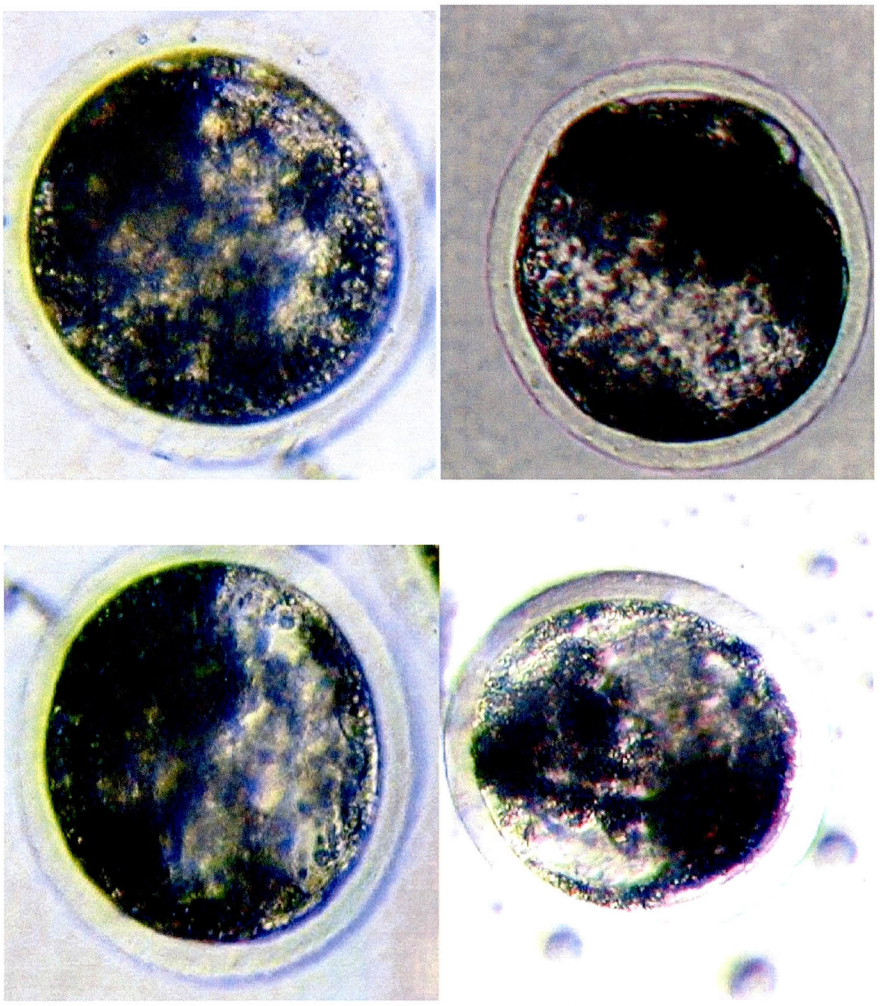

Fig.3. *In vitro* produced bovine blastocysts in B rank. These blastocysts showed unclear differentiation of the trophoblastic cells and inner cell mass. All of the photographs were taken with phase-contrast microscope (x 200).

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