The Effects of Sequential Media System in the Production of Water Buffalo Embryo in Vitro

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Abstract

Effects of sequential media system in improving development of preimplantation stage water buffalo embryos were examined. Oocytes were collected from slaughterhouse-derived ovaries and cultured and fertilized in vitro (IVF). After IVF, presumptive zygotes were divided into two groups; the treatment group (SMS group) was in vitro cultured (IVC) by sequential media system while the control group was by the traditional culture system. Sequential media system involved IVC of fertilized oocytes in 1% PL medium (1% Fetal Bovine Serum (FBS) in TCM 199 supplemented with 0.4 mM pyruvate (P) and 5 mM lactate (L)) for the first 72 hr of IVC; then in 15% PL medium (15% FBS) from ~72 to 120 hr of IVC; and in 15% PLG medium (15% FBS and 1 mg/ml glucose) from ~120 hr onwards. Culture medium in the control group was 10% FBS in TCM 199 throughout the culture period. Cleavage and blastocysts developments were assessed. Cleavage rate was not different between treatments; 68.6% vs. 66.3%. However, morula (38.5% vs. 22.7%) and blastocysts (30.1% vs. 19.31%) developments where significantly higher ($P<0.01$) in SMS group than in Control group. Surprisingly, blastocysts development assessed from the number of morula showed significantly ($P<0.01$) decreased rate in SMS groups than Control, 78.1% vs. 84.8%, suggesting a down-regulation effect of glucose on embryo development. These results suggest beneficial effect of sequential media system but addition of glucose on Day 5 of IVC is detrimental. Further examination is recommended to determine the perfect timing of glucose supplementation for improved blastocysts production in water buffaloes.

Keywords: Buffalo oocyte, Culture system , In vitro embryo production, Serum..

1. Introduction

Recently, reported births of water buffalo calves (2n=50) out of vitrified embryos produced in a co-culture system with cumulus cell monolayers in a riverine buffalo (2n=50) and swamp buffalo (2n=48) recipients (Hufana et al., 2007). The in vitro culture medium used in the production of these embryos was Tissue Culture Medium (TCM) 199 with 10% FBS and 100 IU/mL penicillin and 100 µg/mL streptomycin. The production of live, normal calves makes in vitro production (IVP) of embryos an attractive tool in the propagation of riverine buffaloes for milk and meat purposes in a swamp buffalo dominated countries like the Philippines. However, the success rate of in vitro embryo production in water buffaloes following the above system is still low and this was also observed by others when compared to cattle. Refinement of the in vitro culture system is still needed before the technology could be used regularly in buffalo breeding.

Nandi et al., (2002) reviewed and reported the current efficiency in water buffalo embryo IVP as high rate of maturation (70–90%), fertilization (60–70%) and cleavage (40–50%) with a moderate to low rate of blastocyst formation (15–30%) and calf production (10.5%). Cellblock is one of the major constraints which accounts to about 60% of the zygotes failed to develop to blastocysts, a problem also found in cattle. The energy requirement of water buffalo embryos has not yet fully defined. In cattle, determination of the energy requirements was difficult due to the 8-16 cellblocks. Several researchers

cultured bovine embryos past the "block" stages by co-culture with oviductal cells, trophoblastic vesicles, or cumulus cells or by addition of growth factors (Larson et al., 1990).

The use of sequential media system to cater the changing requirements of the water buffalo embryos in culture has not been studied. Early studies on the development of pre-implantation mouse embryos in culture showed that the development of the zygote to the 2-cell stage had an absolute requirement for pyruvate while development from the 2-cell stage is supported by both pyruvate and lactate and glucose could support embryo development from the late 4-cell/8-cell stage (Takahashi and First, 1992). In support of these early studies, subsequent analysis of nutrient uptake showed that initially the early mouse embryo takes up pyruvate preferentially. After the 8-cell stage, pyruvate uptake declines, and by the blastocyst stage, glucose is the preferred nutrient. In bovine, Rosenkrans et al.(1993) reported that lactate:pyruvate ratio is less important than the total lactate or pyruvate concentration, a case that appears as well in hamster embryos. Considering the different developmental stages that a fertilized oocyte undergoes during development to preimplantation embryo stages, a sequential media system might be needed to cater the changing requirement of the developing zygotes. Hence, this study was conducted with the aim of determining the effect of sequential media system using increasing concentration of serum (1% for the first 72 hrs of IVC then 15% from 72 hr onwards) and glucose supplementation on 120 hr (Day 5) with lactate and pyruvate present throughout the IVC period in water buffalo in vitro embryo production system.

2. Materials and Methods

In Vitro Maturation and Fertilization of Oocytes

Cumulus-oocyte complexes (COCs) were aspirated from slaughterhouse-derived ovaries. COCs surrounded by >3 layers of compact cumulus cells and with granulated cytoplasm were selected and cultured for in vitro maturation in TCM 199 (Earle's salts with 25 mM HEPES, Gibco-BRL, Life Technologies Inc., Grand Island, NY, USA) containing 10% FBS (Gibco) and antibiotics (100 unit penicillin/mL and 100 µg streptomycin/mL) covered with mineral oil. Oocytes were matured through in vitro in a water-jacketed incubator for 22–24 hrs at 39 °C in an atmosphere of 5% CO_2.

For in vitro fertilization (IVF), frozen semen was thawed at 37°C for 15 min. Sperm cells were washed with a pre-incubated modified Brackett and Oliphant medium (BO medium, Brackett and Oliphant, 1975) without Bovine Serum Albumin (BSA) (sperm washing medium) by centrifugation at 800 x g for 5 min. The supernatant was discarded leaving the sperm pellet. Sperm concentration was determined and adjusted by dilution with sperm washing medium. To make the IVF droplets, BO medium containing 10 mM Caffeine, 4 units heparin/mL and 10 mg BSA (Fraction V, Wako Pure Chemical Ind., Osaka, Japan) (sperm dilution solution) were added with the equal volume of the adjusted sperm suspension making a final sperm concentration of 1 x 10^6 sperm cells/mL, 5 mM Caffeine, 2 units heparin/mL and 5 mg BSA/mL. Droplets for IVF were prepared in 100 µL droplets in a culture dish and covered with equilibrated mineral oil. In vitro matured oocytes were co-cultured with the sperm cells for in vitro fertilization at 39° C in an atmosphere of 5% CO_2 for 6-8 hrs.

In Vitro Culture for Embryo Development

The methods of in vitro culture of embryos by co-culture with cumulus cells described in bovine by Hamano and Kuwayama (1993) and adopted in water buffalo by Hufana et al. (2004) was used as control. The in vitro culture media containing pyruvate and lactate and different concentration of serum and presence of glucose as described by Hufana (1996) on the in vitro culture of bovine embryos were adopted for SMS group with few modifications as described below;

To prepare the IVC dishes with cumulus cell co-culture, in vitro matured oocytes were slightly denuded from their cumulus cells and the cumulus cells were separated by vorexing in PBS. Separated cumulus cells were washed three times in pre-incubated IVC medium and concentration determined by hemacytometer. Approximately 50,000 cumulus cells were added in IVC droplets of 50 µL volume for 10 oocytes. In SMS group, after 6-8 hrs of sperm-oocyte co-culture, oocytes were washed to remove excess sperm cells and incubated from 0 to 72 hr in 1% PL medium (TCM 199 with 1% FBS, 0.4 mM sodium pyruvate (P) and 5 mM sodium lactate (L)). At 72 hr of IVC, the culture medium was replaced with 15% PL medium (TCM 199 containing 15% FBS and same concentration of sodium lactate and sodium pyruvate) and incubated for until 130 hr. At ~120 hr of IVC, the culture medium was replaced with 15% PLG medium (15% PL containing 1mg/mL glucose (G)). For the Control group, the same process was made but IVC medium used was TCM 199 (Earle's salts with 25 mM HEPES, Gibco) containing 10% FBS (Gibco) and antibiotics (100 units penicillin/mL and 100 µg streptomycin/mL, both from Sigma Chemical Co., St. Louis, MO) as described by Hufana et al. (2004). All media used for medium replacements were pre-incubated at least 3 hrs prior to use.

Embryos that reached morula at Day 5 and blastocysts on Day 7 of IVC were recorded.

Statistical analysis

Results on cleavage, morula and blastocysts development rates were expressed in percentages and analyzed using Chi-square and Fisher's exact test for any significant difference.

3. Results and Discussion

A total of 806 water buffalo oocytes matured and fertilized in vitro were used. After the sperm-oocyte co-culture for in vitro fertilization, 402 oocytes were cultured in SMS while 404 oocytes were used for Control (Table 1).

There was no significant difference in the cleavage rate between the two groups, 68.6% vs. 66.3%. However, development to morula (38.5% vs. 22.7%) on Day 5 and blastocysts (30.1% vs. 19.31%) on Day 7 of in vitro culture were significantly ($P<0.01$) higher in SMS group than in Control group. When blastocysts development was assessed from the total number of cleaved zygotes (Table 2), same results were achieved showing significantly ($P<0.01$) higher blastocysts development in SMS group than the Control group, 43.8% vs. 29.10%, respectively. Surprisingly, when blastocyst development was assessed from the total number of embryos that reached the morula stage on Day 5 of in vitro culture, a significantly ($P<0.01$) lower blastocysts development rate was observed in SMS group (78.1%) than in Control (84.8%) (Table 3) suggesting a down-regulation effect of glucose supplementation on Day 5 of in vitro culture.

The results demonstrate that sequential media system using increasing concentration of serum (1 to 15%) has beneficial and positive effects on water buffalo in vitro embryo production. The presence of low concentration serum (1%) during the initial stage of in vitro culture contributed on better development of the putative zygote stage. This could be supported by several reports indicating that serum has a biphasic effect as observed in bovine embryo development during culture (Thompson and Peterson, 2000). It was indicated that the first two days after IVF are the prerequisite stage for embryonic genome activation where presence of serum during early cleavage inhibits development but development is accelerated when serum is present from the initiation of compaction which is at the morula stage (Leese, 2003). In the SMS group, 1% serum was provided in the culture medium during the first 72 hours of in vitro culture to enhance the attachment of the cumulus cells at the bottom of the culture dish and cater the nutrient requirement of the zygotes. This was followed by provision of 15% serum at 72 hours onwards where embryos are at around 8 cells and late cleavage stages. It could be deduced from these results that an adequate energy source has enhanced the embryonic potential for genomic activation resulting to higher development of the zygotes to pre-implantation stage embryos.

Additionally, the presence of lactate and pyruvate instead of high concentration of serum in the sequential media system during the early stage of in vitro culture may synergistically improve the development potential of the zygotes. It has been accepted for many years that the pre-implantation embryo has an initial preference for pyruvate at the cleavage stages. In fact, there were reports indicating that zygotes failed to cleave when lactate is the sole energy substrate despite the observation that the zygote can oxidize lactate from the medium. Division to the 2-cell stage is supported only by pyruvate or phosphoenolpyruvate, while from the 2-cell stage; development can be supported by both pyruvate and lactate (Rosenkrans et al., 1993).

Surprisingly, blastocyst development assessed from the total number of embryos that reached the morula stage was lower in SMS group than in control group. These results suggest that addition of glucose on Day 5 of IVC may exhibits a down-regulation effect that caused a decrease in the development of morula stage embryos to the blastocysts. It has to be noted that at the 5th day of in vitro culture, developed embryos are mostly at the 16 cells to morula and compact morula stages. In mouse, glucose uptake has been detected in the earlier stages and embryos underwent a switch in their metabolism around the 8 cell/morula stage of development which enabled them to use glucose predominantly. In cattle, glucose metabolism is low during the first cleavages and increases sharply after the major resumption of the genome which is at 8-16 cells stage. Whether the down regulation effect that was observed in this study was due to the timing of addition of glucose in the culture medium or due to high concentration of glucose in the medium is not clear and requires further study. It is worth mentioning that TCM 199 used as basic medium in this study is a complex medium that already contains glucose at 1mg/mL concentration. Addition of 1 mg/mL glucose on the 5th day of IVC has increased the final glucose concentration to 2 mg/mL. Since the in vitro culture system involves co-culture with cumulus cell, the cumulus cells should take part in the utilization of glucose, hence, decreases the glucose concentration in the culture medium. Addition of glucose on the 5th day of IVC where water buffalo embryos are mostly at morula stage was thought to support the glucose requirement for cavitation as observed in other species (Houghton et al., 1996), thus increase the utilization of glucose in the medium. However, a negative effect translated in terms of lower number of morula stage embryos that developed to blastocysts stage was observed. In porcine, Karja et al. (2004) observed that the developmental potency of IVP embryos and the quality of blastocysts obtained is depended on when exposure to glucose began. On the other hand, Leese (2003) indicated that although glucose consumption rate does increase in late cleavage, oxidative phosphorylation and not glycolysis is the primary source of energy production in blastocysts across the species due to the corresponding elevation in oxygen consumption.Fleming et al.(2004) pointed out that elevated glucose metabolism, particularly via glycolysis, can be viewed as a stress response in embryos and mouse blastocysts showing high glycolytic rates have a lower capacity for implantation. In cattle, it has been shown that the absence of glucose in the later preimplantation stage embryos caused a decrease in the blastocyst total cell number. With these findings, it could be deduced from the results of the present study that the addition of glucose is beneficial in blastocyst production but the timing of supplementation i.e. 5th day of in vitro culture may not be appropriate because the embryos are at different developmental stages. Since significantly ($P<0.01$) higher blastocysts development was observed in SMS group than in control group, addition of glucose in the culture medium seemed beneficial in blastocyst production but an embryo stage-dependent supplementation may be necessary.

With these results, it appeared that species differences exist when it comes to glucose uptake and the stage of the embryos, concentration of glucose, and timing of addition of it in the culture medium need to be considered in order to exhibit a positive effect.

4. Conclusion and Recommendation

These results suggest beneficial effect of sequential media system on water buffalo in vitro embryo production. The increasing concentration of serum and presence of pyruvate and lactate in the in vitro culture medium for embryo development exhibited beneficial effects that lead to improved developmental competence of the in vitro matured and fertilized oocytes to the morula and blastocysts stage. Addition of glucose, however, needs further examination to clear up the down-regulation effect and determine the perfect timing of glucose supplementation for improved blastocyst production.

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