α-Tocopherol and L-ascorbic acid increase the in vitro development of IVM/IVF swamp buffalo (Bubalus bubalis) embryos

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This study was conducted to investigate the effects of capacitating agents added at in vitro fertilization (IVF) and antioxidants supplemented during in vitro culture (IVC) on the development of buffalo embryos. In experiment I, in vitro embryo development of buffalo embryos was compared when the IVF medium was supplemented with heparin, caffeine and calcium ionophore A23187 either alone or in combination. There was no significant difference (P > 0.05) in the cleavage rates of oocytes among the treatment groups but the development rate to the blastocyst stage and the cell numbers of blastocyst in the heparin-treated group were significantly higher (P < 0.05) than that of other treatments. In experiment II, in vitro embryo development of buffalo embryos was compared when IVC medium was supplemented with either α-tocopherol (250 and 500 μM) or L-ascorbic acid (250 and 500 μM). The rate of development to the blastocyst stage of embryos cultured in medium supplemented with 250 μM α-tocopherol (33%, 41/123) and 250 μM L-ascorbic acid (31%, 38/123) was significantly higher (P < 0.05) than that of those cultured in medium alone (19%, 20/108) but not significantly different (P > 0.05) from medium supplemented with either 500 μM α-tocopherol (24%, 30/123) or 500 μM L-ascorbic acid (25%, 33/133). These results suggest that buffalo spermatozoa treated with heparin were suitable for IVF and that α-tocopherol and L-ascorbic acid added during IVC increased the rate of buffalo embryo development.

Keywords: buffalo, embryo, development, α-tocopherol, L-ascorbic acid

Introduction

Since the birth of the first buffalo calf from an in vitro fertilized (IVF) oocyte (Madan et al., 1991), a number of articles on in vitro embryo production (IVP) have been published. However, success, in terms of production of transferable stage embryos and birth of calves following embryo transfer, has been limited (Gasparrini, 2002; Nandi et al., 2002). Moreover, the efficiency of IVP in buffalo is much lower than that in cattle (Nandi et al., 2002; Gasparrini, 2002). IVP in cattle can be considered to be a mature technology and available for commercialization (van Wagendonk-de Leeuw, 2006); on the other hand, IVP systems in buffalo are sub-optimal and require substantial improvements (Nandi et al., 2002; Gasparrini, 2002). Several factors might affect the IVF in this species, such as inadequate oocyte maturation, an inappropriate timing of insemination and a non-optimal time of gamete co-incubation. Moreover, the important factors that might affect embryo development are the chemical agents used for capacitation of spermatozoa during IVF and the oxidative damage of embryos during in vitro culture (IVC). Several agents have been used to induce capacitation of buffalo spermatozoa. Heparin is structurally similar to glycosaminoglycans, which were shown to induce capacitation and acrosome reaction (AR) in bovine spermatozoa (Parish et al., 1988) and several mammalian species including buffalo (Chauhan et al., 1997; Kitiyanant et al., 2002). Caffeine stimulates capacitation (Funahashi and Nagai, 2001) and has been included in IVF protocols, especially with frozen-thawed boar spermatozoa (Wang et al., 1991; Funahashi and Day, 1993). Heparin combined with caffeine has been used to capacitate spermatozoa in the production of IVF buffalo embryos (Abdoon et al., 2001; Ravindranatha et al., 2003). Moreover, calcium ionophore...
A23187 has been successfully used to induce AR in buffalo spermatozoa (Sidhu et al., 1984; Kitiyanant et al., 2002). However, there is no report comparing the effects of using these capacitating inducers either alone or in combination on IVF and subsequent development of buffalo oocytes.

Reactive oxygen species (ROS) are physiologically generated during cell metabolism, even under basal conditions. ROS may originate directly from the embryos, and exogenous factors, such as oxygen consumption, metallic cations, visible light, amine oxidase and spermatozoa, can increase the amount of ROS produced by embryos (Goto et al., 1993; Nasr-Esfahani et al., 1990; Alvarez et al., 1996). Oxidative stress seems to damage embryos by causing peroxidation of membrane phospholipids and altering most types of cellular molecules (Nasr-Esfahani et al., 1990). Early mammalian embryos are susceptible to damage from ROS (for review see Johnson and Nasr-Esfahani, 1994), and they increase the production of oxygen free radicals when cultured in vitro (Goto et al., 1993). Vitamins C (L-ascorbic acid) and E (α-tocopherol and derivatives) are well-known antioxidants and are believed to be the primary free radical scavengers in mammalian cell membranes (Niki et al., 1989). Vitamin E added in culture media increased survival rates of explanted rat conceptuses in vitro (Steele et al., 1974) and increased the viability of mouse embryos exposed to heat shock (Arechiga et al., 1994). Moreover, vitamin E, which can suppress oxidation and cell membrane injury caused by ROS, increased the rate of development to the blastocyst stage of bovine (Olson and Seidel, 2000) and porcine (Kitagawa et al., 2004) IVF embryos. However, there have been no reports on the effects of vitamins C and E on the in vitro development of buffalo embryos. The aims of the present study were to (1) investigate the effects of heparin, caffeine and calcium ionophore A23187 either alone or in combination on the in vitro development of buffalo embryos and (2) to examine the effects of the addition of α-tocopherol or L-ascorbic acid to the IVC medium on the subsequent development of cleaved embryos to the blastocyst stage.

Material and methods

Oocyte collection and in vitro maturation

Buffalo oocytes were collected and matured in vitro by the method previously described (Pavasuthipaisit et al., 1992). Cumulus oocyte complexes (COCs) from abattoir ovaries were collected by aspirating the antral follicles (2 to 6 mm) using an 18-gauge needle containing TALP-HEPES. After being washed three times, COCs were morphologically assessed under a stereomicroscope (×200) and only oocytes with compact and homogeneous cytoplasm were selected for in vitro maturation. All selected COCs were cultured in 50 μl drops of maturation medium (TCM 199) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT, USA), 0.2 mm pyruvate and 5 μM/ml follicle-stimulating hormone (Sigma, St Louis, MO, USA) in a humidified atmosphere of 5% CO2 at 39°C. After culturing for 22 h, matured oocytes were subjected to IVF.

In vitro fertilization and in vitro culture

Experiment I. Effects of different capacitating agents on IVF and subsequent development of buffalo embryos.

IVF of buffalo oocytes was based on procedures reported in our previous study (Pavasuthipaisit et al., 1992) with minor modifications. Frozen ejaculated semen was thawed at 37°C and motile sperm were prepared using a swim-up technique. A 10 μl aliquot (5 × 106 cells/drop) of sperm after the swim-up was placed in a culture dish with 50 μl of glucose-free TALP containing different capacitating inducers: (1) 10 μg/ml heparin (Pavasuthipaisit et al., 1992); (2) 10 μM calcium ionophore (Kitiyanant et al., 2002); (3) 5 mM caffeine (Totey et al., 1992); (4) 10 μg/ml heparin + 10 μM calcium ionophore; (5) 10 μg/ml heparin + 10 μM caffeine; and (6) 10 μM calcium ionophore + 10 μM caffeine. Spermatozoa were incubated for 1 h under a humidified atmosphere of 5% CO2 at 39°C, and aliquots of each treatment recovered for the AR were assessed using the staining methods described previously (Rathi et al., 2001). Briefly, spermatozoa were stained with 10 μg/ml Fluorescein isothiocyanate-conjugated Arachis hypogea agglutinin (FITC-PNA) for 30 min and sperm suspension was placed onto a slide, covered with a cover slip and observed under fluorescence microscopy. A green fluorescent sperm head denotes an acrosome-reacted/reacting sperm; a non-fluorescent sperm head indicates an acrosome-intact sperm. Ten in vitro-matured COCs were added to each fertilization drop containing capacitated sperm. After 18 to 20 h of insemination, presumptive zygotes were removed from fertilization drops and transferred to IVC. They were co-cultured with buffalo rat liver (BRL) cells in 50 μl of TCM 199 supplemented with 10% FCS (IVC medium) under a humidified atmosphere of 5% CO2 at 39°C for 7 days. Cleavage and embryonic development to the blastocyst stage were recorded on days 2 and 7 of culture, respectively. Blastocysts derived from each treatment were stained with 10 μg/ml Hoechst 33342 for 10 min and the total number of their nuclei was counted under fluorescence microscopy.

Experiment II. Effects of α-tocopherol and L-ascorbic acid supplemented in IVC medium on the development of IVF buffalo embryos to the blastocyst stage.

Based on the results of experiment I, a higher blastocyst rate was obtained when oocytes were fertilized with spermatozoa capacitated with 10 μg/ml heparin as compared to other treatments. Therefore, heparin was used for the capacitation of spermatoza during IVF. Only cleaved oocytes produced by the IVF method as described above were used in this experiment. These two-cell stage embryos were divided into five groups and were co-cultured with BRL cells in IVC medium supplemented with antioxidants as follows: (1) medium alone (control); (2) medium + 250 μM α-tocopherol; (3) medium + 500 μM α-tocopherol; (4) medium + 250 μM L-ascorbic acid; and (5) medium + 500 μM L-ascorbic acid. Embryonic development to the 8 to 16 cells, morula and blastocyst stages were examined on days 3, 5 and 7 of culture, respectively. Blastocysts derived from
each treatment were stained with 10 μg/ml Hoechst 33342 for 10 min and the total numbers of their nuclei were counted under fluorescent microscopy.

Statistical analyses
The data of cleavage and in vitro development of embryos were collected from 14 replicates. The rate of embryonic development produced by different treatments was analysed using the $\chi^2$ test. The data of total cell number of blastocysts were analyzed using one-way ANOVA. Differences between treatments were considered statistically significant at $P < 0.05$.

Results

Experiment I
The differences in the percentage of AR spermatozoa after treatment with heparin, caffeine or A23187 alone or in combination were not statistically significant (range from 70% to 80%, data not shown). Data of embryonic development using heparin, caffeine and calcium ionophore either alone or in combination are shown in Table 1. There was no significant difference in the cleavage rate of oocytes among the different treatments. The rate of embryonic development to the blastocyst stage and average cell numbers of blastocyst in heparin group were significantly higher ($P < 0.05$) than those of calcium ionophore and their combination groups, but not statistically different from the caffeine group. Combination of heparin, caffeine or calcium ionophore did not increase the percentage of embryos developed to the blastocyst stage.

Experiment II
Table 2 shows the effects of $\alpha$-tocopherol and L-ascorbic acid on in vitro development to the blastocyst stage of IVF-derived embryos. The cleavage rate of buffalo IVF oocytes was 52% (610/1173). The rate of development to the blastocyst stage of two-cell stage embryos cultured in medium supplemented with either 250 μM $\alpha$-tocopherol (33%, 41/123) or 250 μM L-ascorbic acid (31%, 38/123) was significantly higher ($P < 0.05$) than that of those cultured in medium alone (control; 19%, 20/108). No significant differences were observed in blastocyst development when two-cell stage embryos were cultured in medium supplemented with either 500 μM $\alpha$-tocopherol (24%, 30/123) or 500 μM L-ascorbic acid (25%, 33/133) as compared to the control. The mean number of cells on day 7 blastocyst developed from two-cell stage embryos cultured in 500 μM $\alpha$-tocopherol was significantly lower ($P < 0.05$) than the control and other treatments.

Discussion
The results of the present study highlight two major findings: (1) in vitro development to the blastocyst stage of IVF embryos produced by spermatozoa treated with heparin alone was higher than those treated with caffeine, calcium ionophore or their combination treatments and (2) IVC medium supplemented with either $\alpha$-tocopherol or L-ascorbic acid at a concentration of 250 μM enhanced the rate of embryonic development to the blastocyst stage when compared to medium alone.

It has been reported that the rate of cleavage and development of IVF buffalo embryos were higher when IVF was carried out with spermatozoa treated with a combination of caffeine and heparin than those treated in heparin alone (Totey et al., 1992). In contrast, the results of the present study demonstrated that spermatozoa treated with a combination of heparin + caffeine, heparin + calcium ionophore or caffeine + calcium ionophore failed to

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**Table 1** Effects of heparin, caffeine and calcium ionophore supplemented in in vitro fertilization medium on development of buffalo embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of oocytes fertilized</th>
<th>Cleavage (%)</th>
<th>Blastocyst (%)</th>
<th>Cell number (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>235</td>
<td>127 (54)</td>
<td>35 (15)$^a$</td>
<td>100.6 ± 11.3$^d$</td>
</tr>
<tr>
<td>Caffeine</td>
<td>220</td>
<td>112 (51)</td>
<td>26 (12)$^{a,b}$</td>
<td>78.5 ± 9.4$^b$</td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>230</td>
<td>120 (52)</td>
<td>21 (9)$^b$</td>
<td>69.1 ± 10.4$^b$</td>
</tr>
<tr>
<td>Heparin + Caffeine</td>
<td>215</td>
<td>112 (52)</td>
<td>17 (8)$^b$</td>
<td>71.4 ± 7.6$^b$</td>
</tr>
<tr>
<td>Heparin + Calcium ionophore</td>
<td>225</td>
<td>131 (58)</td>
<td>23 (10)$^b$</td>
<td>70.2 ± 7.9$^b$</td>
</tr>
<tr>
<td>Caffeine + Calcium ionophore</td>
<td>240</td>
<td>129 (54)</td>
<td>15 (6)$^b$</td>
<td>56.3 ± 6.4$^b$</td>
</tr>
</tbody>
</table>

Different superscripts within columns are significantly different ($P < 0.05$).

**Table 2** Effects of $\alpha$-tocopherol and L-ascorbic acid supplemented in in vitro culture medium on development of buffalo embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of embryos cultured</th>
<th>8 to 16 cells (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
<th>Cell number (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>108</td>
<td>63 (58)</td>
<td>35 (32)</td>
<td>20 (19)$^a$</td>
<td>85.8 ± 5.2$^a$</td>
</tr>
<tr>
<td>Medium + 250 μM L-ascorbic acid</td>
<td>123</td>
<td>84 (68)</td>
<td>53 (43)</td>
<td>38 (31)$^b$</td>
<td>82.0 ± 6.4$^a$</td>
</tr>
<tr>
<td>Medium + 500 μM L-ascorbic acid</td>
<td>133</td>
<td>78 (59)</td>
<td>48 (36)</td>
<td>33 (25)$^{a,b}$</td>
<td>69.0 ± 8.5$^{a,b}$</td>
</tr>
<tr>
<td>Medium + 250 μM $\alpha$-tocopherol</td>
<td>123</td>
<td>84 (68)</td>
<td>54 (44)</td>
<td>41 (33)$^b$</td>
<td>90.5 ± 9.1$^{a,b}$</td>
</tr>
<tr>
<td>Medium + 500 μM $\alpha$-tocopherol</td>
<td>123</td>
<td>85 (69)</td>
<td>53 (43)</td>
<td>30 (24)$^{a,b}$</td>
<td>58.9 ± 7.8$^b$</td>
</tr>
</tbody>
</table>

Different superscripts within columns are significantly different ($P < 0.05$).
increase the percentage of cleavage and development to the blastocyst stage of IVF buffalo embryos as compared to heparin treatment alone. Another study reported that treatment of spermatozoa with caffeine and heparin resulted in a higher fertilization rate than did treatment with heparin alone but development to the blastocyst stage was inhibited (Tatham et al., 2003). The substitution of heparin with caffeine resulted in a decreased fertilization rate as well as a delay in pronuclei formation (73.0% and 25.4%, respectively) (Pavloket al., 2001). Developmental failure of embryos produced by caffeine-containing medium may be due to the high concentration of caffeine (Tatham et al., 2003) or polyspermy (Mao et al., 2005). Tatham et al., (2003) suggested that caffeine can increase capacitation and fertilization, but at a high concentration caffeine is detrimental to embryonic development. It was found that caffeine may have caused hyper-motility of spermatozoa and resulted in polyspermy. A high incidence of polyspermic penetration in caffeine-containing IVF spermatozoa and resulted in polyspermy. A high incidence of polyspermic penetration in caffeine-containing IVF medium has been reported (Mao et al., 2005).

Calcium ionophore has been successfully used to induce capacitation and AR in buffalo spermatozoa (Kitiyayanget al., 2002; Kaul et al., 2001): however, the effect of this agent on IVF and on the subsequent development of buffalo embryos has not been reported. The present study shows that calcium ionophore used alone or combined with either heparin or caffeine failed to improve cleavage rate and blastocyst development in IVF buffalo embryos compared to other treatments. We also noticed that calcium ionophore decreased sperm motility within 30 min of incubation and this may have impaired the development of embryos produced in calcium ionophore containing medium. Treatment of stallion sperm with concentrations of calcium ionophore over 3 μM has been reported to greatly reduce stallion sperm motility (Li et al., 1995).

Although the embryo culture medium (TCM 199) used in this study has been supplemented with natural antioxidants, an addition of antioxidants such as vitamins C and E (Olson and Seidel, 2000), selenium (Uhm et al., 2007) as well as melatonin (Rodriguez-Osorio et al., 2007) have been shown to increase the in vitro development of embryos. Our results demonstrate that culture medium supplemented with either α-tocopherol or L-ascorbic acid at a concentration of 250 μM increased the quality of IVF-derived buffalo embryos, blastocyst rate and blastocyst cell number. Higher concentration of either α-tocopherol or L-ascorbic acid (500 μM) failed to improve in vitro embryo development. Vitamin C (ascorbic acid) is an important water-soluble antioxidant that reduces sulphhydrils, scavenges free radicals and protects against endogenous oxidative DNA damage (Fraga et al., 1991). Vitamin C may become a pro-oxidant when free transition metals are present (Guerin et al., 2001; Yamamoto and Niki, 1988). Supplementation of ascorbic acid to an embryo culture significantly improved the blastocyst development rate (Wang et al., 2002). Vitamin E (tocopherol and derivatives) is a lipid-soluble antioxidant that suppresses peroxidation of membrane lipids (Tappel, 1980; Chow, 1991). It protects cells from oxygen radicals in vivo (Miller and Brzezinska-Slebodzinska, 1993) and in vitro, and is believed to be the primary free radical scavenger in mammalian cell membranes (Chow, 1991). Vitamin E (α-tocopherol) has been successfully used to improve the in vitro development of bovine embryos (Olson and Seidel, 2000). In other studies, vitamin E partially protected early murine embryos from the effects of heat shock (Arechiga et al., 1994), a cytotoxic event that likely is mediated by free radicals (Loven, 1988). Recently, vitamin E (Trolox®, water-soluble analog) added to culture medium was shown to decrease the H2O2 content and increase the developmental ability to the blastocyst stage and the cell number in porcine IVF embryos (Kitagawa et al., 2004). Moreover, Feugang et al. (2004) reported that vitamin E could protect bovine morulae/blastocysts exposed to oxidative stress generated through an increase in ROS production or a decrease in antioxidant protection. However, increasing concentrations of vitamin E are toxic to bovine embryos as also observed in mouse embryos (Wang et al., 2002). Indeed, concentrations above 400 μM induced a dose-dependent decrease in blastocyst development and blastocyst cell number (Feugang et al., 2004). In conclusion, buffalo spermatozoa treated with a combination of heparin, caffeine and calcium ionophore failed to increase embryo development compared to heparin alone. In vitro development of buffalo embryos was enhanced by adding α-tocopherol and L-ascorbic acid to IVC.

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