The effect of sperm-motility enhancing agents on the *in vitro* fertilization of *Bubalus bubalis* oocytes and their subsequent development to blastocyst

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Abstract: Effects of sperm-motility enhancing agents on the *in vitro* fertilization of buffalo oocytes and their subsequent development *in vitro* were examined in this study. Buffalo oocytes from ovaries taken at slaughter were matured *in vitro* for 24—26 h at 38.5 ℃ under a humidified 5% CO_2 in air, and then fertilized *in vitro* using buffalo spermatozoa washed with 50 μg/mL trypsin in experiment 1, treated with different concentration of caffeine (0, 2.5, 5.0, and 10.0 mmol/L) in experiment 2, calcium-ionophore A23187 (0, 0.1, 1.0 and 2.5 μmol/L) in experiment 3 or PHE only, PHE+2.5 mmol/L caffeine+2.5 μmol/L A23187, and PHE+2.5 μmol/L A23187 in experiment 4. At 24 to 26 h post insemination, the oocytes were co-cultured with granulosa cell monolayer in droplets containing culture medium to evaluate their embryonic development. There was a significant decrease (12.5% vs 2.6%, $P<0.05$) in the proportion of cleaved embryos that developed to blastocyst after sperm treatment with trypsin. The treatment of spermatozoa with caffeine and calcium-ionophore A23187 had no significant effect on cleavage and blastocyst yield. There was a minor improvement in blastocyst yield when PHE combined with calcium-ionophore was employed during IVF. However, high concentrations of caffeine seem to be detrimental to cleavage rate and blastocyst development.

Key words: *Bubalus bubalis*; *in vitro* fertilization; sperm-motility enhancing agent; spermatozoa; embryo
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不同促精子活动剂对水牛卵母细胞体外受精及随后胚胎发育的影响

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摘要: 主要探讨精子活动促进剂对水牛 *Bubalus bubalis* 卵母细胞体外受精及随后胚胎发育的影响. 来自屠宰场水牛卵巢的卵母细胞和卵丘细胞复合体, 在体积分数为 5% CO_2 的培养箱中培养 24~26 h, 然后通过体外受精测定其受精和胚胎发育能力. 试验 1: 解冻的精子用 50 g/mL 的胰蛋白酶处理 30 min, 然后进行受精; 试验 2: 受精在含不同浓度咖啡因 (0, 2.5, 5.0 和 10.0 mmol/L) 的受精液中进行; 试验 3: 在含不同浓度的钙离子载体 A23187 (0, 0.1, 1.0 和 2.5 μmol/L) 的受精液中进行体外受精; 试验 4: 在 PHE、咖啡因和 A23187 不同组合(空白, PHE, PHE+2.5 mmol/L 咖啡因+2.5 μmol/L A23187 和 PHE+2.5 μmol/L A23187) 的受精液中进行体外受精. 试验结果表明, 精子用胰蛋白酶处理后的受精卵囊胚发育率显著下降 (12.5% 和 2.6%, $P<0.05$), 而用咖啡因和钙离子载体 A23187 处理, 对卵母细胞的卵裂率和囊胚发育率没有明显影响. 在体外受精中 PHE 和钙离子载体的组合使用能提高受精卵的胚胎发育率, 而高浓度的咖啡因则降低卵母细胞的卵裂率和胚胎发育率.

关键词: 水牛; 体外受精; 精子活力促进剂; 精子; 胚胎

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In buffalo, the overall fertilization efficiency is lower than in cattle. A major limiting factor is the quality of the frozen semen[1]. A high fertilization rate was observed when fresh ejaculated semen was used, whereas fertilization rate was reduced significantly when diluted semen or frozen semen was used. These results confirmed the observation that preservation and freezing of buffalo semen significantly reduced fertilization capacity. It has been demonstrated that freezing results in acrosomal damage, leakage of enzymes, alterations in ionic strength and pH, complete withdrawal of the hydration shell of protein in the solution, and loss of motility[2]. It has also shown, with electron microscopy studies, that the plasma membrane of buffalo spermatozoa is more fragile, particularly at the acrosomal region. Even if higher fertilization rates are obtained when IVF is performed with fresh semen[3], its use is limited because of impracticality.

For penetration of mammalian egg investments (cumulus oophorus and zona pellucida) spermatozoa must be vigorously motile and have undergone additional changes, termed capacitation, outside the male tract[4]. The time-course of sperm capacitation has been correlated with changes in substrate metabolism (HARRISON R A P. The metabolism of mammalian spermatozoa[A]. GREEP R O, KOBLINSKY M A. Frontiers in Reproduction and Fertility Control[C]. Part 2. Cambridge: MIT, 379—401.), altered calcium fluxes, removal of sperm surface components[4], and/or alterations of lipid domains on the sperm head[5], increases in cyclic nucleotides (GARRBERS D L, KOPF G S. The regulation of spermatozoa by calcium and cyclic nucleotides[A]. GREENGARD P, ROBINSON G A. Advances in Cyclic Nucleotide Research: Vol. 13 [C]. New York: Raven Press, 251—306.), and alterations of sperm flagellar beat and swimming trajectory[6].

In cattle, several methods have been developed to induce capacitation of the sperm, whereas in buffalo, the only successful procedure involves the use of heparin. Different motility-inducing agents are used during IVF. One group of stimulators consisting of penicillamine, hypotaurine and epinephrine (PHE) was initially developed for *in vitro* sperm studies in hamsters[7,8] and has been employed in cattle[9]. In addition to PHE, caffeine[10] and glutathione have also been employed to enhance bull sperm motility. Moreover, treatment with caffeine[11], calcium ionophore A23187[12], calcium ionophore A23187 in combination with caffeine[13] or lysophospholipids and trypsin[14] have also been reported to capacitate bovine spermatozoa and calves have been obtained or pregnancies established using caffeine[11] or A23187 plus caffeine[13].

Many researchers have reported on the effect of sperm motility enhancing agents in terms of rate of sperm motility, *in vitro* induction of acrosome reaction, oocyte penetration and fertilization rate. Although these criteria could be useful, the ultimate goal in IVF procedure is to obtain transferable embryos. Therefore, the present study was undertaken to ascertain the effect of some of these agents on buffalo spermatozoa and oocytes IVF and their subsequent cleavage and balstocyst development.

1 Materials and methods

1.1 Oocyte maturation, fertilization and embryo culture

Buffalo ovaries were obtained from a local slaughterhouse excised after slaughter and stored in thermos flasks containing normal saline solution at 25—30 ℃. The ovaries were transported to the laboratory within 3 to 4 h after slaughter. Oocytes were aspirated from follicles of 2 to 6 mm diameter using a 5 or 10 mL disposable syringe with an 18-guage needle. Oocytes possessing compact cumulus cells were selected for maturation. The oocyte *in vitro* maturation, *in vitro* fertilization and embryo *in vitro* culture were performed according to the methods reported previously[15].

1.2 Experimental design

1.2.1 Effects of trypsin on the *in vitro* fertilization of buffalo oocytes This experiment was designed to determine the effect of treating buffalo sperm with trypsin on the IVF of buffalo oocytes. The trypsin solution was prepared by using Ca^{2+}, Mg^{2+}, and BSA free TALP supplemented with 50 μg/mL trypsin. Frozen thawed buffalo semen was swim-up separated after which the separated sperm was incubated with trypsin solution for 30 min at 38.5 ℃, 5% CO_2 in humidified air. Matured oocytes in each batch were randomly allocated into two groups contained in the same fertilization medium. One group was inseminated with trypsin treated sperm and the other group with untreated sperm.

1.2.2 Effects of caffeine concentration on the *in vitro* fertilization of buffalo oocytes This experiment was designed to determine the effect of caffeine concentration in

the IVF medium on cleavage rate and subsequent development to blastocysts. Both sperm preparation medium and basic fertilization medium contained caffeine (Sigma) at different concentrations of 0, 2.5, 5.0, or 10.0 mmol/L.

1.2.3 Effects of calcium ionophore A23187 concentration on the *in vitro* fertilization of buffalo oocytes This experiment was designed to determine the effect of calcium ionophore A23187 concentration in the IVF medium on cleavage rate and subsequent development to blastocysts. Four concentrations (0, 0.1, 1.0 and 2.5 μmol/L) of A23187 were employed in both the sperm preparation medium and basic fertilization medium.

1.2.4 Effect of caffeine, calcium ionophore A23187 and PHE on the *in vitro* fertilization of buffalo oocytes This experiment was designed to determine the combined effect of caffeine, calcium ionophore A23187, and PHE in the fertilization medium on cleavage rate and subsequent development to blastocysts. Both the sperm preparation medium and the fertilization medium contained 10 μmol/L penicillamine, 1 μmol/L hypotaurine, 20 μmol/L epinephrine, 2.5 μmmol/L caffeine, and 2.5 μmol/L calcium ionophore A23187.

1.3 Statistical analysis

The present study evaluated the IVF of buffalo oocytes by the proportion of inseminated oocytes that cleaved and subsequently developed to blastocysts. The difference between treatments within each experiment was analyzed by Chi Square.

2 Results

2.1 Effects of trypsin on the *in vitro* fertilization of buffalo oocytes

As shown in Table 1, the treatment of buffalo sperm with 50 μg/mL of trypsin resulted in a significant decrease ($P<0.05$) in cleavage rate and blastocyst yield as compared to the control (12.5% vs 2.6%).

Tab. 1　Effect of sperm treatment with trypsin on IVF of buffalo oocytes[1)]

treatment	oocytes insem.	No. of cleavage	cleavage rate/%	No. of blastocysts developed	blastocysts developed rate/%
control	64	17	26.5	8	12.5a
50 μg/mL	115	18	15.6	3	2.6b

1) Within columns, values with different small letters are significantly different ($P<0.05$, Chi Square test)

2.2 Effects of caffeine concentration on the *in vitro* fertilization of buffalo oocytes

As shown in Table 2, there was a no significant difference in the effect of caffeine on the development to blastocyst compared to the control. However, there was a minor decrease in proportion of cleaved eggs and blastocyst yield with increased caffeine concentration.

2.3 Effects of calcium ionophore A23187 concentration on the *in vitro* fertilization of buffalo oocytes

The effect of different treatments of calcium ionophore A23187 on the IVF of buffalo oocytes is shown in Table 3. There was no significant difference in the effect of calcium ionophore treatment on the proportion cleaved eggs and blastocyst yield.

Tab. 2　Effect of caffeine concentration on IVF of buffalo oocytes[1)]

caffeine/(mmol·L^{-1})	oocytes insem.	No. of cleavage	cleavage rate/%	No. of blastocysts developed	blastocysts developed rate/%
0	78	23	29.5a	7	9.0a
2.5	105	20	19.0a	8	7.6a
5.0	176	29	16.5a	9	5.1a
10.0	141	20	14.2a	7	5.0a

1) Within columns, values with same small letters are not significantly different ($P>0.05$, Chi Square test)

Tab. 3　Effect of calcium ionophore A23187 on the IVF of buffalo oocytes[1)]

A23187/(μmol·L^{-1})	oocytes insem.	No. of cleavage	cleavage rate/%	No. of blastocysts developed	blastocysts developed rate/%
0	47	13	27.7a	5	10.6a
0.1	152	30	19.7a	17	11.2a
1.0	241	64	26.6a	30	12.4a
2.5	118	35	29.7a	15	12.7a

1) Within columns, values with same small letters are not significantly different ($P>0.05$, Chi Square test)

2.4 Effect of caffeine, calcium ionophore A23187, and PHE on the *in vitro* fertilization of buffalo oocytes

As shown in Table 4, there was no significant difference in the proportion of inseminated oocytes that cleaved. However, blastocyst yield was significantly higher when PHE and calcium-ionophore A23187 (2.5 μmol/L) were combined as compared to the control (17.2% vs 10.0%), respectively. When caffeine (2.5 mmol/L) was added to this medium, blastocyst yield dropped (17.2% vs 9.1%). PHE alone did not have any significant effect on blastocyst yield.

Tab. 4 Effect of PHE, caffeine and A23187 on the IVF of buffalo oocytes[1)]

treatments			oocytes insem.	No. of cleavage	cleavage rate/%	No. of blastocysts developed	blastocysts developed rate/%
PHE	caffeine /(mmol·L^{-1})	A23187 /(μmol·L^{-1})					
−	−	−	50	16	32.0a	6	10.0a
+	−	−	120	43	35.8a	13	11.6a
+	2.5	2.5	88	28	31.8a	8	9.1a
+	−	2.5	145	60	41.3a	25	17.2a

1) Within columns, values with same small letters are not significantly different ($P > 0.05$, Chi Square test)

3 Discussion

After *in vitro* maturation, fertilization represents a very critical step of the entire procedure of *in vitro* embryo production. In addition to capacitation, high sperm motility is required to accomplish fertilization. Many researchers have reported on the effect sperm motility enhancing agents in terms of rate of sperm motility, *in vitro* induction of acrosome reaction, oocyte penetration and fertilization rate. Although these criteria could be useful, however, the ultimate goal in IVF procedure is to obtain transferable embryos. Therefore, the present study evaluated the effect of some capacitation and sperm-motility enhancing factors on the proportion of inseminated *in vitro* matured oocytes using frozen-thawed buffalo spermatozoa, which cleaved and subsequently developed to blastocysts. Researchers[16, 2] reported that the use of frozen buffalo semen for IVF is not an effective strategy because of damage during freezing as revealed by acrosomal enzyme leakage. The culture conditions for heparin-treated IVF (base medium) of buffalo oocytes in this study resulted in 28% cleavage rate and 10.8% blastocyst yield, which was comparable with previous reports[17]. A very low fertilization rate (19.6%) was observed when frozen-thawed spermatozoa treated with 10 μg/mL heparin in Hepes-Talp medium were used[2]. Also a similar result was observed in buffalo IVF, although maturation rate was more than 75%[18]. Heparin is a glycosaminoglycan that is able to induce hypermotility, capacitation and thus binding of the acrosome of buffalo spermatozoa; the use of heparin in IVF increases fertilization, cleavage, and blastocyst development[19].

Treatment of buffalo spermatozoa with trypsin significantly decreased ($P < 0.05$) both the proportion of cleaved eggs and blastocyst yield. It was reported that trypsin treatment facilitated the loss of acrosome integrity of bovine spermatozoa, resulting in a rapid but small increase in the number of spermatozoa exhibiting non-intact acrosomes[14]. This was also supported by similar findings with hamster sperm[7]. The plasma membrane of buffalo spermatozoa was already made fragile due to freezing and washing with trypsin may have damaged the acrosome region of the spermatozoa leading to low fertilization and cleavage rates. One possible explanation put forward is that exogenous proteases may act alone or synergistically with endogenous proteases in altering the membrane glycoproteins and dispersing the acrosomal matrix during the acrosome reaction[7].

In this study, treatment of buffalo spermatozoa with caffeine during fertilization had no significant effect on the proportion of cleaved eggs or blastocyst yield. However, there was a slight decrease in proportion of cleaved eggs and blastocyst yield with increased caffeine concentration. According to one report, caffeine was found to increase zona binding, oolema penetration, and fertilization, but decreased cleavage with no difference in the development of the remaining embryos[17]. This suggests that caffeine can increase capacitation and fertilization, but at high concentration caffeine is detrimental to embryonic development.

Induction of acrosome reaction after calcium

ionophore treatment has been reported for ram[20], guinea pig[10], stallion[21, 22], and boar[23]. In this study, the proportion of cleaved eggs and blastocyst yield obtained when buffalo spermatozoa was treated with calcium ionophore A23187 was not significantly different when compared to the control. There was also no significant difference in cleavage rate and blastocyst yield when frozen-thawed buffalo spermatozoa were treated with PHE alone. However, there was an improvement in the proportion of cleaved eggs and blastocyst yield when preparation of spermatozoa was carried out in base medium treated with PHE and 2.5 μmol/L calcium ionophore A23187 (17.2% vs 9.1%) but not with caffeine.

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