

INFLUENCE OF GAMETE CO-INCUBATION TIME, SIRE AND SPECIAL ADDITIVES ON THE *IN VITRO* FERTILIZATION OF CUMULUS-ENCLOSED OR DENUDED BUFFALO OOCYTES

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Abstract: The present study was designed to investigate the influence of cumulus removal before the *in vitro* fertilization step and the impact of co-incubation time, sire, and additives to the fertilization medium on the efficiency of *in vitro* fertilization of buffalo oocytes. *In vitro* matured oocytes were fertilized either as cumulus-oocyte complex (COCs) or after removal of cumulus cells (denuded). Cumulus-enclosed or denuded oocytes were co-incubated with sperm cells for 6, 12 or 18 h (experiment 1), fertilized with sperm cells from one of three sires (experiment 2) or fertilized in medium supplemented with 20µg/ml heparin, 5 mM theophylline or a mixture 20µM penicillamine, 10µM hypotaurine and 1 µM epinephrine (PHE) (experiment 3). In all experiments, a group of oocytes was fixed and stained to evaluate the fertilization pattern (penetration, normal and abnormal fertilization rates) and the rest were cultured up to 8 days to assess the developmental competence (cleavage and blastocyst yield). In all experiments, removal of cumulus cells before fertilization step significantly retarded the fertilization pattern and the developmental competence. Various co-incubation times did not significantly influence the fertilization pattern or the developmental competence of denuded or COCs. However, 6 h tended to decrease the abnormal fertilization (15.74 ± 1.70 vs 28.46 ± 6.06 , $P=0.069$) and to improve the blastocyst/oocyte (11.70 ± 3.41 vs 5.53 ± 1.75) and the blastocyst/cleavage (27.14 ± 6.19 vs 11.98 ± 3.81 , $P=0.082$) when compared with 18 h. Sperm cells from the three sires resulted in similar fertilization pattern and developmental competence in COCs and denuded oocytes. In COCs and denuded oocytes, PHE tended to improve the blastocyst/oocyte (7.44 ± 2.58 vs 14.67 ± 4.29) and blastocyst/cleavage (18.43 ± 6.08 vs 33.13 ± 9.27) in comparison to heparin ($P>0.05$). Thus, none of the investigated factors could counteract the adverse effect of cumulus removal otherwise, the addition of PHE showed promising results but it need further investigations.

Key words: cumulus; co-incubation time; additives; fertilization pattern; developmental competence

Introduction

Limited efficiency of superovulation embryo transfer in buffalo gives the *in vitro* fer-

tilization protocol (IVF) further importance in this species (1). Research in the field of buffalo IVF aims to maximize the normal fertilization pattern and the developmental competence of

presumptive zygotes (2). The fundamental steps of IVF protocol are oocyte maturation, sperm preparation, fertilization and embryo culture. During each step, there are numerous factors influence the protocol efficiency (3). Different laboratories have exerted a lot of efforts to define the optimum conditions of buffalo IVF protocol (4). Otherwise up today most of the applied conditions are adapted from cattle protocol.

Conditions during fertilization step not only influence the fertilization pattern but also the developmental competence of presumptive zygotes. These conditions include gamete co-incubation time (5), sperm cell concentration (6,7), basic fertilization medium (8,9), sperm preparation and capacitation methods (10,11), additives to fertilization medium (12,13) and sires themselves (6,10). Moreover, interactions between the effects of sire and sperm cell concentration (6,14), sire and co-incubation time (15), sire and additives to fertilization medium (16), sperm cell concentration and cumulus existence (7), co-incubation time and basic fertilization medium (17,18), special additives and sperm cell concentration (13) have been reported in different species.

Most of the laboratories co-incubate buffalo gametes for 16-24 h, while fewer laboratories apply 6 h co-incubation time (3). Prolonging the co-incubation time to 16 h has improved the fertilization rate, but further prolongation behind 16h has decreased the blastocyst/oocyte rate in buffalo (5). In cattle, maximum cleavage and blastocyst rates have been achieved after gamete co-incubation for 8 h with no further improvement after longer co-incubation time 12 and 16 h (15). Moreover, extending the co-incubation time from 5 to 10 h significantly improved the cleavage and the blastocyst rates, while further extension to 15 or 20 h significantly improved the cleavage but not the blastocyst rate (19). Basic fertilization medium is usually supplemented with special additives as heparin, caffeine, theophylline or a mixture of penicillamine, hypotaurine, and epinephrine (PHE) (6,13). These additives induce capacitation like changes in sperm cells, enhance sperm cell motility and inhibit the reactive oxygen species (6,13,16,20). Supplementation

of fertilization medium with PHE and theophylline has increased the fertilization and the blastocyst development rates (13). Moreover, incorporation of 10µg heparin/mL into the IVF medium has increased the fertilization rate of some sires, while increasing heparin concentration to 100 µg/ml has improved or retarded the fertilization depending on the sire (6).

Common IVF protocol involves fertilization of cumulus-enclosed oocyte (COCs) followed by complete removal of the cumulus before embryo culture. Fertilization of cumulus-free oocytes (denuded) has negatively affected the fertilization pattern and the developmental competence in buffalo (7) and cow (21-23). However, partial removal of some cumulus layers before fertilization has increased the fertilization and the blastocyst development rates (24). Furthermore, fertilization of denuded cattle oocytes in cumulus cells conditioned medium or in presence of loose cumulus cells slightly improved the cleavage rate but it did not reach the COCs cleavage rate (21,22). Recently, oocyte cryopreservation has been widely practiced and it has been reported that oocyte denudation before vitrification has improved the survival rate but reduced the fertilization and the development rates (25). This point out the urgency of optimizing the fertilization conditions of denuded oocytes. In this direction, previous reports have attempted using cumulus cells conditioned medium, adding free cumulus cells or co-fertilizing denuded oocytes with COCs (21,22,25-27). However, to our knowledge, a few reports have proposed the modification of other fertilization conditions to improve the fertilization and developmental competence of denuded oocytes (7,26,28).

Therefore, the present study was designed to evaluate the effect of cumulus removal before fertilization step. Additionally, we investigated whether modification of co-incubation time, using semen from different sires and supplementation of fertilization medium with heparin, theophylline or PHE could improve the fertilization and the developmental competence of oocytes fertilized as COCs or denuded.

Material and methods

Chemicals and media

All chemicals used for preparation of different media were purchased from Sigma Chemical CO. (St. Louis, MO, USA) with exception of sodium bicarbonate, sodium pyruvate, EDTA, and sodium lactate that were purchased from Oxford (Maharashtra, India).

The basic medium used for oocyte maturation was HEPES-buffered tissue cultures medium 199 (TCM-199, M7528) and the basic medium for sperm preparation and fertilization was Brackett and Oliphant medium (BO) (29). The BO medium was composed of 112.0 mM NaCl (S-5886) 4.02 mM KCl (P-5405), 2.25 mM CaCl₂ (C-7902), 0.83 mM NaH₂PO₄ (S-5011), 0.52 mM MgCl₂ (M-2393), 37.0 mM NaHCO₃ (S-07990), 13.9 mM glucose (S-2837), 1.25 mM sodium pyruvate (S-08302), 30 µg/ml penicillin G sodium (P-3032) and 3 mg/ml bovine serum albumin (A-6003).

The basic culture medium was synthetic oviduct fluid (SOF) contained 107.70 mM NaCl, 7.16 mM KCl, 1.19 mM KH₂PO₄ (P-5655), 1.71 mM CaCl₂, 0.49 mM MgCl₂, 25.07 mM NaHCO₃, 3.30 mM Na lactate (L-7900), 0.33 mM Na pyruvate, 1.50 mM glucose, 5 mg/ml bovine serum albumin, 100 units/mL penicillin G sodium and 50 µg/ml streptomycin (S-1277) (30).

Oocyte collection and maturation

Buffalo oocytes were aspirated from all antral follicles in abattoir-derived ovaries. Oocytes with homogenous cytoplasm and surrounded with ≥ 2 layers of compact cumulus were selected for further experiment. A group of 10-15 COCs was matured in a 100µl droplet of TCM-199 supplement with 10% heat-inactivated fetal calf serum (F-6178), 0.2mM sodium pyruvate and 50µg/ml gentamycin sulfate (G-3632) at 38.5 °C in 5% CO₂ under humidified air for 24h. At the end of maturation and before the *in vitro* fertilization, some oocytes were denuded by vortexing in TCM-199 supplemented with 10IU/ml hyaluronidase (H-4272) enzyme while others were used as COCs.

Sperm preparation and fertilization

For separating motile sperm cells, frozen-thawed sperm cells were allowed to swim up in BO medium supplemented with 0.5mM caffeine (C-0750) for 20 minutes at 38°C. Harvested sperm cells were washed in fertilization medium that constitutes of BO medium supplemented with different additives according to the experiment. After washing in fertilization medium, *in vitro* matured oocytes were fertilized in 100µl droplet of fertilization medium at 38.5°C in 5% CO₂ under humidified air. The duration of the sperm-oocytes co-incubation was varied according to experiment. The final sperm concentration in fertilization droplet was 5x10⁶/ml.

Experimental design

Experiment 1: The effect of different co-incubation time and its interaction with cumulus cells existence.

In this experiment, 266 denuded oocytes and 288 COCs were co-incubated with the sperm cells for 6, 12 or 18 h. The fertilization medium was BO medium supplemented with 20µg/ml heparin (H3149).

Experiment 2: The effect of different sires and its interaction with cumulus cell existence.

In this experiment, sperm cells from different sires were used to fertilize 271 denuded and 293 COCs in BO medium supplemented with 20µg/ml heparin for 18 h.

Experiment 3: The effect different additives to IVF medium and its interaction with cumulus cells existence.

In this experiment, 367 COCs and 280 denuded oocytes were fertilized in BO medium supplemented either with 20µg/ml heparin (28), 5 mM Theophylline (T-1633) (31) or a mixture of 20 µM penicillamine (P-4875), 10 µM hypotaurine (H-1384) and 1 µM epinephrine (E-4250) (32). The sperm oocytes co-incubation time was 18 h.

In all experiments, a group of fertilized oocytes was stained for assessment of the fertilization pattern, and the rest were cultured for 8 days to assess the developmental competence.

Assessment of fertilization pattern

After 18 h from the beginning of fertilization, the cumulus and sperm cells were removed through vortexing in Dulbecco's phosphate buffered saline supplemented with 10 IU/ml hyaluronidase enzyme. Denuded oocytes were fixed in aceto-ethanol (acetic acid: ethanol 3:1 v/v) and then stained with 1% orcein in 45% acetic acid (28). Oocytes with no pronucleus were defined as unfertilized while those with two pronuclei were defined as normally fertilized. Oocytes with one or more than two pronuclei were defined as abnormally fertilized.

In vitro culture and assessment of developmental competence

At the end of gamete co-incubation, the attached sperm cells or cumulus -if present- were removed by vortexing. Presumptive zygotes were washed several times in modified SOF medium then transferred into 200µl of SOF contained a monolayer of cumulus cells. The basic SOF was supplemented with 5% fetal calf serum, 100µg/ml cysteine (C-03147), 50µg/ml myoinostiol (I-7508), 30 µl/mL essential (P-6766) and 10µl/ml non-essential amino acid (M-7145) (13,30).

For preparation of cumulus monolayer, cumulus cells collected during oocyte collection were washed several times in culture medium. The final cell concentration was adjusted to 1×10^5 /ml. The culture droplet was incubated for 48 h before culturing the presumptive zygote. The zygotes were cultivated at 38.5°C under 5% CO₂ in humid air. On day four, half of the droplet (i.e 100µL) was replaced with 100 µL of conditioned SOF supplemented with 10% FCS. Therefore the final concentration of FCS in the culture droplet beginning from day 4 was 7.5%. The cleavage rate was assessed on day 2 and the morula and blastocyst rates were assessed on days 5 and 8, respectively.

Statistical analysis

All experiments were replicated three times. The effects of all variables under investigations were statistically analyzed by ANOVA using the GLM procedures of the IBM SPSS software

(Version 16.0; IBM Corp., Armonk, NY, USA). In all experiments, the model included the fixed effects of either the co-incubation time (6, 12 and 18 h) or the different sires (1, 2 and 3) or the different additives (heparin, theophylline and PHE) in addition to the type of oocytes (COCs vs denuded). The model also investigated the interaction between different *in vitro* fertilization conditions and cumulus cells existence. Multiple mean comparisons were done using Duncan Multiple Range Test. All values are presented as the mean \pm standard error. The differences and interaction were considered significant if $P < 0.05$.

Results

Effect of cumulus removal before IVF step

In all experiments, removal of cumulus cells before the *in vitro* fertilization step adversely affected the fertilization pattern and the developmental competence of buffalo oocytes (Tables 1-3). The penetration rate did not differ significantly between denuded oocytes and COCs in all experiments ($P > 0.05$). In experiment one, in comparison to denuded oocytes fertilization of COCs resulted in significantly higher normal fertilization, cleavage and morula rates ($P \leq 0.05$) and similar abnormal fertilization, blastocyst/oocyte and the blastocyst/cleavage rates. In experiment 2 and 3, removal of cumulus cells prior to fertilization significantly decreased the normal fertilization, the cleavage, the morula, the blastocyst/oocyte and the blastocyst/cleavage rates and significantly increased the abnormal fertilization rate (Tables 1- 3).

Effect of co-incubation time and its interaction with cumulus cells existence

The co-incubation time did not significantly influence the fertilization pattern or the developmental competence of buffalo oocytes (Table 1). However, 6 h of co-incubation tended to none significantly improve the blastocyst/cleavage (27.14 \pm 6.19 vs 16.76 \pm 5.61 and 11.98 \pm 3.81; $P=0.082$). Moreover, prolonging the co-incubation time to 12 and 18 h tended to none significantly increase the mean value of the abnormal fertilization (23.12 \pm 2.31 and 28.46 \pm 6.06 vs 15.74 \pm 1.70; $P=0.069$).

Table 1: The effect of gamete co-incubation time and its interaction with cumulus cells existence on *in vitro* fertilization pattern and *in vitro* development of buffalo-oocytes (mean \pm SE)

Factors		No. of oocytes	Pattern of fertilization			No. of oocyte	In vitro development			
			Penetration	Normal fertilization	Abnormal fertilization		Cleavage	Morula	Blastocyst/oocyte	Blastocyst/cleavage
Co-incubation Time	6 h	81	66.71 \pm 2.59	50.97 \pm 3.98	15.74 \pm 1.70	76	40.67 \pm 4.55	16.98 \pm 3.85	11.70 \pm 3.41	27.14 \pm 6.19
	12 h	101	68.14 \pm 5.21	45.03 \pm 6.33	23.12 \pm 2.31	72	42.50 \pm 5.22	14.76 \pm 2.40	7.93 \pm 2.84	16.76 \pm 5.61
	18 h	121	69.79 \pm 4.90	53.82 \pm 15.5	28.46 \pm 6.06	103	43.72 \pm 5.44	13.21 \pm 1.99	5.53 \pm 1.75	11.98 \pm 3.81
Cumulus cells existence	Denuded	143	63.96 \pm 4.61	37.16 \pm 4.66 ^b	26.80 \pm 4.9	123	34.32 \pm 3.13 ^b	11.39 \pm 1.47 ^b	5.65 \pm 1.60	15.88 \pm 4.48
	COCs	160	72.47 \pm 1.22	62.71 \pm 10.37 ^a	18.08 \pm 1.58	128	50.27 \pm 3.22 ^a	18.57 \pm 2.20 ^a	11.13 \pm 2.42	21.37 \pm 4.43
Interaction*			0.96	0.45	0.64		0.90	0.95	0.78	0.89

Values with different superscript at the same column were significantly different within the same factors ($P < 0.05$).

*P value of interaction between different co-incubation time and cumulus cell existence. Values > 0.05 indicate no interaction between the two factors

Table 2: The effect of different sires and its interaction with cumulus cells existence on *in vitro* fertilization pattern and *in vitro* development of buffalo-oocytes (mean \pm SE)

Factors		No. of oocytes	Pattern of fertilization			No. of oocytes	In vitro development			
			Penetration	Normal fertilization	Abnormal fertilization		Cleavage	Morula	Blastocyst/oocyte	blastocyst/cleavage
Sires	1	125	70.73 \pm 4.15	45.85 \pm 5.96	24.21 \pm 4.73	95	35.98 \pm 4.67	15.81 \pm 3.68	11.65 \pm 3.54	28.96 \pm 8.16
	2	98	65.79 \pm 3.01	46.04 \pm 4.72	19.74 \pm 4.20	70	42.51 \pm 5.04	17.90 \pm 3.09	10.86 \pm 2.93	27.14 \pm 6.19
	3	104	73.92 \pm 2.42	43.74 \pm 6.82	30.17 \pm 6.16	72	37.61 \pm 7.18	13.82 \pm 4.00	9.40 \pm 4.81	23.80 \pm 10.70
Cumulus cells existence	Denuded	154	66.26 \pm 2.60	33.96 \pm 3.11 ^b	32.29 \pm 4.45 ^b	117	26.73 \pm 2.34 ^b	8.60 \pm 1.76 ^b	4.35 \pm 1.45 ^b	16.93 \pm 5.90 ^b
	COCs	173	74.03 \pm 2.76	56.27 \pm 2.66 ^a	17.12 \pm 1.81 ^a	120	50.67 \pm 2.05 ^a	23.09 \pm 1.64 ^a	16.92 \pm 2.71 ^a	36.93 \pm 5.77 ^a
Interaction*			0.856	0.680	0.718		0.300	0.782	0.801	0.795

Values with different superscript at the same column are significantly different within the same factors ($P < 0.05$).

*P value of interaction between sires and cumulus cells existence. Values > 0.05 indicate no interaction between the two factors.

Table 3: The effect of different additives to fertilization medium and its interaction with cumulus cells existence on the *in vitro* fertilization pattern and *in vitro* development of buffalo-oocytes (mean \pm SE)

Factors		No. of oocytes	Pattern of fertilization			No. of oocytes	In vitro development			
			Penetration	Normal fertilization	Abnormal fertilization		Cleavage	Morula	Blastocyst/oocyte	Blastocyst/cleavage
Additive	Heparin	152	68.77 \pm 4.04	40.99 \pm 6.20	27.77 \pm 3.66	102	34.43 \pm 4.43	12.99 \pm 3.09	7.44 \pm 2.58	18.43 \pm 6.08
	Theophylline	109	71.93 \pm 4.16	60.26 \pm 17.73	28.33 \pm 5.24	88	36.66 \pm 4.93	13.19 \pm 3.18	10.23 \pm 3.20	24.13 \pm 5.97
	PHE	107	74.17 \pm 4.47	48.17 \pm 5.93	26.00 \pm 4.17	89	39.73 \pm 4.60	18.78 \pm 4.74	14.67 \pm 4.29	33.13 \pm 9.27
Cumulus cells existence	Denuded	161	69.27 \pm 3.70	35.21 \pm 4.50 ^b	34.06 \pm 2.60 ^b	119	29.37 \pm 3.27 ^b	9.88 \pm 2.23 ^b	5.74 \pm 2.16 ^b	16.14 \pm 5.95 ^b
	COCs	207	73.97 \pm 2.10	64.41 \pm 9.54 ^a	20.68 \pm 2.52 ^a	160	44.52 \pm 2.27 ^a	20.10 \pm 2.83 ^a	15.83 \pm 2.36 ^a	34.33 \pm 4.30 ^a
Interaction*			0.84	0.36	0.84		0.97	0.56	0.84	0.94

Values with different superscript at the same column were significantly different within the same factors ($P < 0.05$).

*P value of interaction between different additives and cumulus cells existence. Values > 0.05 indicate no interaction between the two factors

There was no interaction between cumulus cell existence and co-incubation times for penetration, normal fertilization, abnormal fertilization, cleavage, morula, blastocyst/oocyte, and blastocyst/cleavage; respectively.

Effect of different sires and its interaction with cumulus cells existence

Semen from the three sires under investigation resulted in a similar fertilization pattern and *in vitro* developmental competence

of buffalo oocytes. The differences among sires were very narrow in all judgment criteria (Table 2). There was no interaction between sire and cumulus cell existence.

Effect of different additives and their interaction with cumulus cells existence

Different additives used in the current study did not influence the fertilization pattern or the developmental competence of buffalo oocytes. However, the addition of heparin to fertilization medium significantly decreased the normal fertilization rate in comparison to theophylline and PHE (40.99 ± 6.20 vs 60.26 ± 17.73 and 48.17 ± 5.93). Fertilization of the oocytes in medium supplemented with PHE resulted in none significantly higher blastocyst/oocyte (14.67 ± 4.29 vs 10.23 ± 3.20 and 7.44 ± 2.58 ; $P=0.10$) and blastocyst/cleavage (33.13 ± 9.27 vs 7.44 ± 5.97 and 18.43 ± 6.08 ; $P=0.16$) compared to medium supplemented with heparin and theophylline.

Different additives used in the current study did not influence the fertilization pattern or the developmental competences of buffalo oocytes fertilized as COCs or denude. There was no interaction between the additives and cumulus existence for penetration, normal fertilization, abnormal fertilization, cleavage, morula, blastocyst/oocyte and blastocyst/cleavage; respectively.

Discussion

Undoubtedly research in the field of buffalo IVF aims to define the optimum conditions that maximize normal fertilization and later developmental ability. In the current study, removal of cumulus cells before fertilization step did not influence the penetration rate in all experiments. Some studies have stated a significant reduction in the fertilization rate after fertilization of denuded oocytes (23,26), while others showed no significant difference (33). On the other hand, higher fertilization rate after fertilization of partially denuded oocytes has been reported (24). In the current study, denudation of the oocytes before fertilization increased the abnormal fertilization which is in agreement with most of the previous studies (28,33). Taken the previous results together, it

seems that enclosing the oocytes with cumulus cells during fertilization is important to achieve normal fertilization otherwise defining the necessary threshold volume of cumulus requires further investigation. During fertilization cumulus cells increase sperm motility, influence acrosomal reaction, enhance sperm-zona binding capacity, prevent abnormal spermatozoa from entering cumulus matrix and/or create optimum microenvironment around the oocytes (34).

Additionally, removal of cumulus cells before fertilization step retarded the developmental competence of buffalo oocytes. This result is in agreement with previous reports in cows (22,23) and buffaloes (7). However, partial removal of cumulus cells before fertilization has improved the blastocyst rate (24). Lower cleavage and blastocyst/oocyte rates after fertilization of denuded oocytes may be a sequel of lower penetration rate, while lower blastocyst/cleavage rate may be a subsequent to higher abnormal fertilization rate. Furthermore, cumulus cells enclosed the oocytes may protect the oocyte or the presumptive zygotes from the hazards of oxidative stress (21) or secrete embryotonic substances that improve later developmental competence (22). The positive impact of cumulus cells during fertilization step was restricted to cumulus cells enclosed the oocytes while loose cumulus cells did not have the same effect (26). Otherwise, the addition of loose cumulus cells or using cumulus cells conditioned medium to denuded oocytes slightly improved the cleavage rate but it did not reach the efficiency of COCs (21,22).

In the current study, we observed a tendency for higher abnormal fertilization and lower blastocyst yield after extending the co-incubation time above 6 h in oocytes fertilized as COCs or denuded. Maximum fertilization rate has been achieved after 8 h (26) and maximum cleavage, blastocyst/oocyte or blastocyst/cleavage have been achieved after 6 h (35), 10 h (19) in cow and 16 h in buffalo (5) with no further improvement after longer gamete co-incubation. Moreover, polyspermia has been decreased when sperm cells were removed from oocytes surface at 6-12 h after

fertilization (28). Variation among studies may be due to the interaction between co-incubation time with other factors as sires themselves (15), sperm preparation method (36) and fertilization medium (17). Additionally, the current study didn't detect an interaction between co-incubation time and cumulus cells existence which is in agreement with a previous study stated that timing of sperm penetration was not modified by the cumulus (26). In addition to higher polyspermia, prolonged co-incubation time may adversely affect the developmental competence of presumptive zygotes through increasing the potential damage induced by higher reactive oxygen species (37) or sperm metabolic product (38).

The current study revealed no differences in the ability of the sperm cells from different sires to fertilize or support embryonic development of buffalo oocytes as previously reported (6,10,15,16,19). Differences among sires were attributed to different characteristics (39), different sperm-zona binding ability (40) or interaction with non-sire factors as sperm cell concentration (41), co-incubation time (15) and additive to fertilization medium (16). The three bulls used in the current study showed similar performance in denuded and COCs. This may be due to the fact that the three sires used in the current study were selected sires based on their performance on IVF.

Numerous reports have stated the beneficial effect of heparin (6,16), PHE (13,32) and theophylline (31) on the fertilization pattern and the developmental competence with no previous report has compared the efficiency of the three additives under the same experimental conditions. The current study showed that the blastocyst/cleavage and the blastocyst/oocyte were nearly duplicated after using PHE mixture in comparison to heparin otherwise this difference did not reach a significant level. This promising result was clear in denuded oocytes. Heparin, theophylline and PHE mixture improve the sperm motility, sperm activity, prolong the sperm lifespan and induce capacitation like changes in the sperm cells (6,13,16,20). Additionally, hypotaurine in the PHE may inhibit the reactive oxygen species (42). Since cumulus cells are responsible for

protecting the oocytes from reactive oxygen species (43), the antioxidant activity of PHE mixture may be responsible for improved developmental competence after fertilization of denuded oocytes. Further investigation of this result is indicated.

Conclusion

In conclusion, removal of the cumulus cells before fertilization significantly retarded the fertilization pattern and the developmental competence and none of the investigated factors could counteract this adverse effect. However, supplementation of fertilization medium with a mixture of PHE showed promising improvement in the blastocyst yield after fertilization of denuded oocytes. Moreover, co-incubation time longer than 6 h is not necessary even it may have an adverse effect.

Conflict of interest

None of the authors have any conflict of interest to declare

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