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FACTORS AFFECTING IN-VITRO FERTILIZATION OF EGYPTIAN BUFFALOES WITH SPECIAL REFFERENCE TO SPERM-ZONA PELLUCIDA BINDING ASSAY

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Abstract

Assays based on spem-zona pellucida binding have been developing as diagnostic tests to predict the fertilization potential of bull spermatozoa. In this study a similar assay for buffalo sperm was used.

The results showed a significant variation (P<0.01) in accessory sperm number between the fresh semen of three bulls, in spite of the same physical characters. Also three bulls, in their physical characters aand number of accessory sperm. The overall mean of accessory sperm was significantly increased (P<0.01) by fresh semen than by frozen semen (181.0 ± 3.0 Vs 30.7 ±7 ± 2.2). The effect of (2mM) caffeie and/or (10 µg/ml) heparin on the number of accessory sperm was significantly obvious (P<0.01) when compared with the control, while, there was no significant variations between them. Addition of both caffeine and heparin in the incubation media improved the number of accessory sperm than in fertilization media, but without significant variations. The number of the accessory sperm was insigificantly increased when the oocytes co-cultured in sperm droplets than when the sperm co-cultured with oocytes in fertilization medium.

INTRODUCTION

The early events of mammalian fertilization involve binding of spermatozoa to receptors on the zona pellucida, the acrosome reaction, and penetration of the spermatozoa through the zona pellucida. Therefore, sperm zona binding and/or sperm-zona penetration are pre-requisted steps in the evaluation of fertilization rates than routine examination of semen (First and Parrish, 1987). Recently, sperm binding to the zona pellucida has been used as a diagostic test of male fertility in, human (Muller, 1992), bull (Fazeli *et al.* 1993) boar (Ivanova and Mollova, 1993) and stallions (Meyers *et al.* 1996). Spermatozoa trapped in the zona pellucida after penetration of the first sperm are termed "accessory sperms". The number of these

sperms is positively associated with fertility in swine (Pursel, 1982), sheep (Hawk and Cooper, 1984) and cattle (Hawk and Tanable, 1986).

The aim of the present investigation was applied to indicate the factors affecting in vitro fertilization in Egyptian buffalo-bulls such as individual variation between bulls, difference between fresh and frozen semen, type of capacitator factor and method of their addition and finally, the techique of fertilization.

MATERIALS AND METHODS

Fresh ejaculates from three fertile buffalo-bulls (Code no: 34, 100 & 117) at Animal Reproduction Research Institutes, and frozen semen of three bulls (Code no: 221, 223 & 227) from Abbasia freezing Center of buffalo semen, were used in the present study. Samples were evaluated for motility (before and after washing and washing and 2 hours post-incubation), viability and morphological characters. Individual or pooled semen was prepared by layering under 1 ml of washing media that contained Ham's F10 (Sigma, N. 1387) + 10 µl/ml sodium pyruvate (Sigma, P.2256); 3 mg/ml bovine serum albumin fraction V "BSAV" (BDH chemicals Ltd pooled England); 50 µl/ml gentamycin (Sigma, G. 1264) and, capacitator factors. After one hour incubation in a water bath at 39°C, the top portion (0.85ml) of the medium was removed from the tube and centrifuged at 2000 rpm/10 minutes. The lowere portion containing the spermatozoa was washed twice in the media by centrifugation at 2000 rpm for 10 minutes. The resulting sperm pellets were resuspended with the same media to be in a final concentration of 2×10^{6} spermatozoa/ml of media.

Buffalo-ovaries were obtained from the Cairo slaughter house one day before the experiments and were kept in phosphate buffer saline (pH 7.4) at 4°C. Follicular fluids were aspirated from antral follicles with a diameter of 2-7 mm. Cumulus oocyte complexes were recovered from the follicular fluid, and vortex in PBS for 4 minutes to remove cumulus cells, then washed with PBS.

Sperm zona pellucida binding assay was carried out according to the procedure of Fazeli *et al*, (1993). Forty-eight microlitre droplets of fertilization medium were placed under paraffin oil, and equilibrated for 2 hours at 39°C in 5% CO2 in air with high humidity (95%). The fertilization media consisted of Ham's F10+6 mg/ml BSA fatty acid free (Sigma, A. 6003), 10 µl/ml sodium pyruvate and 50 µl/ml gentamycin. Two microliters of semen suspension was added to denuded oocytes in the fertilization media and incubated for 2 hours. The oocyte-sperm complexes were

rinsed five times with PBS to remove the loosely attached spermatozoa. They were fixed with 2.5% glutaraldehyde in PBS for 10 minutes, and washed again in PBS. Then, stained with "Hoechst 33342" dye (bis Benzimide, Sigma, B. 2261), 1 mg/ml in normal saline, for 10 minutes and washed in PBS. The perm-oocyte comples were placed on slides, slightly compressed with coverslip and sealed. The number of spermatozoa bound to the zona pellucida of the oocytes (accessory sperm) was counted under the inverted microscope.

The experiment was designed to express the individual variations between bulls and the difference between fresh and frozen semen. Also, it showed the best capacitator factor for buffalo semen, when added to the sperm or in the fertilized medium. The capacitator factors used were caffeine (Sigma, C.0705) in final concentration 2mM/ml or heparin (Sigma, H 5640) in final concentration 10µl/ml or both. This experiment was conducted to achieve a high fertilization rate either by by co-culturing of sperm in fertilization droplet or by co-culturing the oocyte in sperm droplets.

The data were statistically analysed for analysis of variance with the aid of the general linear mode procedure of the statistical analaysis system (SAS, 1987).

RESULTS

Table 1 showed a non-significant variation in physical characters of fresh semen collected from three bulls, while, their accesory sperm numbers were significantly different in between ($P<0.01$). The significant variations ($P<0.01$) in physical characters of frozen semen derived from other three bulls were parallel with the significant variation ($P<0.01$) in their numbers of accessory sperm. The overal number of accessory sperm in fresh semen (181.0 ± 3.0) was significantly higher ($P<0.001$) than that in frozen semen (30.7 ± 2.2).

Inclusion of caffeine, heparin or both in the fertilization or incubation media had significantly improved sperm zona pellucida binding assay when compared with that observed following the use of basic media without additives (Table 2). There was no significant variation in accessory sperm number between the different types of capacitator factors. It is obvious that addition of both caffeine and heparin in the incubation media was better than in other treatment.

Table 3 revealed non-significant changes by using using either fertilization drop (addition of sperm to oocytes) or sperm drop (addition of ova to sperm) on sperm zona pelluucida bionding assay.

DISCUSSION

The assessment of sperm motility and morphological observation are commonly used to evaluate semen samples of many species, and widely used as fudamental indicator of male fertility. However, the result don't provide a prognostic a prognostic information of fertility in vivo (Dunphy *et al.*, 1989), as the sperm needs ability to penetrate the cumulus and the zona pellucida (ZP) of the oocyte. Sperm zona pellucida test can be applied to prediet the fertilizing ability of sperm, embryo quality and subsequent development (Dejarnette *et al.*, 1992, Saacke *et al.*, 1994).

In the present study, immature stored buffalo oocytes were used to count the accessory sperms that bound to the ZP. It has found that the sperm binding was not influenced by oocyte quality or maturity assessed morphologically (Liu *et al.*, 1989, Niwa *et al.*, 1991, Ocampo, *et al.*, 1993). The mechanism of attachement of spermatozoa to ZP is based upon the intraction between glycoprotein constituent of ZP and their complementary sperm proteins. Following fusion of sperm and egg, the ZP becomes refractory to both binding of free-swimming sperm and peetration by sperm that had partially penetrated the extracellular coat prior of fertilization (Wassarman, 1994 and Berger, 1996). Betteridge (1995) suggested that ZP filtered out structurally abnormal sperm so that the only morphologically normal sperm wtih acrosomal reactions can bind ZP. The sperm/oocyte interaction was normal sperm with acrosomal reactions can bind ZP. The sperm/oocyte interaction was incubated for 2 hours which is enough for the binding of denuded oocytes (Park et al., 1989).

Our results (Table 1) showed that fresh buffalo semen with high quality (motility, viability and morphology) illustrated a significant variation ($P<0.01$) in the mean number of accessory sperms between different bulls. In parallel with our results, Fazeli *et al.* (1993) reported that there were differences between established fertile bulls in relative numbers of spem cells bound to the ZP of a bath of oocytes. It was suggested that there was a relationship between the sperm ZP binding capacity and the fertility of bulls. Moreover, Malchow and Arns (1994) recorded that using ZP biding test can be more informative in predicting fertilization potential than those determining sperm motility, morphology and concentration. Berger (1996) explained the variation in sperm interact with the ZP to the number of sperm receptors in the ZP, or may be due to variation in number of affiity of molecules interacting with the ZP. Interaction with receptors on the oolemma appears to contribute to variation in sperm fertilizing potential observed within relatively fertile population.

Frozen buffalo semen samples with significant individual variations (P<0.01) in physical characters (Table 1) were positively correlated with the number of accessory sperms. Our results agree with Ohgoda *et al.*, (1988), who found that fertilization rates and subsequent development to blastocysts rate in-vitro fertilization of bovine sire was highly individual variable. It may be due to differences in metabolic characterstic of sperm cell (Brackett and Oliphant, 1975), male age (Sirard and Lamber, 1985) and difference in seminal plasma content and the ratio of seminal plasma volume to sprm number (Fukui *et al.*, 1988). The low number of accessory sperm in the present study was related to the lower rate of motility, viability and morphology (Liu *et al.*, 1989, Liu and Baker, 1992), or it may be due to the effect of freezability on buffalo semen (Muer *et al.*, 1988). In addition, the motility of frozen sperm was decreased after washing and precincubated with capacitator factor, in contrast with results obtained in the fresh semen. The overall mean number of accessory sperm was significantly decreased (P<0.001) in frozen semen than in fresh semen (30.7 ± 2.2 vs. 181.0 ± 3.0) as a conclusion in Table 1. Long *et al.* (1994) suggested that source of semen is one of the factors affecting invitro frtilization rate and subsequent evelopment to blastocysts. In buffalo. In buffalo, Totey *et al.* (1992) found that fertilization rate was significantly improved when fresh ejaculated spermatozoa was used, than with frozen-thawed spermatozoa. Madan *et al.* (1994) reported that fragility of frozen buffalo semen resulted in low cleavage rate and embryo production.

Several studies have been conducted to improve in-vitro fertilization of buffalo oocytes by addition of diffrent capacitator factors either in the pre-incubated media of sperms or in the fertilization media (Totey *et al.*, 1992). Our results (Table 2) showed a significant increase (P<0.01) of accessory sperm when caffeine and/or heparin was used, while, addition of both in the incubation media of sprm improved the number of accessory sperm (150 ± 7.2) than in the fertilization media (138 ± 6.3) but without significant varition. It has been demonstarted that significantly higher proportion of oocytes was penetrated by pre-incubated sperm with both caffeine and heparin (Park *et al.*, 1989) in bovine and Kadoom (1995) in Egyptian buffaloes. Niwa and Ohgoda (1988) indicated that caffeine and heparin acted synergistically to induce capacitation and/or acrosome reaction of spermatozoa and stimulated in-vitro fertilization of cattle oocytes. Caffeine stimulates sperm motility through inhibition of phosphodiestrase activity and the subsequent accumulation of cyclic nucleotides, especially cyclic adenosine monophosphate "cAMP" with sperm (Hicks *et al.*, 1972), or by maintaining the level of the calcium

Table 1. Influence of physical characters of fresh and frozen semen on sperm zona pellucida binding assay.

Source of semen	Code No of bulls	No of sam - ples	Physical characters					Spem-ZP binding	
			Motility (%)			Viability (%)	Morphology (%)	No of oocytes	No of oocytes
			Initial	After wash	After incubation				
Fresh	34	5	80±3.5	75±3.5	85±3.5	99.6±0.4	96±1.6	20	190±3.6a
	100	5	70±3.5	70±3.5	80±3.5	99.6±0.6	96±1.5	20	168±6.2b
	117	5	80±3.5	76±3.5	85±3.5	96.0±1.3	96±1.1	20	185±4.3a
Frozen	227	5	65±3.5a	60±3.5a	45±3.5a	75±1.7a	80±1.7a	20	50±0.7a
	221	5	55±3.5a	50±3.5a	40±3.5a	76±1.3a	75±27a	20	32±1.6b
	223	5	30±3.5b	25±3.5b	20±3.5b	55±1.8b	50±1.7b	20	10±0.9c

Table 2. Effect of capacitator factors and their method of inclusion on sperm zona pellucida binding assay.

Capacitator factors		Sperm-zona pellucida binding	
		No of oocytes	No of accessory sperm
Basic media (without capacitator factor)		20	91 ± 1.7b
Caffeine	Fertilization media	20	128 ± 6.9a
	Incubation media	20	132 ± 8.8a
Heparin	Fertilization media	20	133 ± 6.9a
	Incubation media	20	145 ± 7.8a
Caffeine + Heparin	Fertilization media	20	138 ± 6.3a
	Incubation media	20	150 ± 7.2a

Means with different superscripts were significant at ($P<0.01$)

ions within the sperm cell. Heparin as a complex glycoproteins (Lee and Ax, 1984) binds directly to spermatozoa, where it is considered to facilitate, but not directly induce acrosome reaction. However, many authors declared that either caffeine (Crister *et al.*, 1984) in bovine or heparin (Williams *et al.*, 1991) in rams or both together (Wang *et al.*, 1991) in swine stimulated acrosome reaction or fertilization after sperm/oocyte incubation. In addition, in swine, Kano *et al.* (1994) detected that the percentage of penetrated oocytes was zero when caffeine was added to the incubation media of sperm.

The dose of caffeine used (2mM) was in accordance with optimum concentration for activity of water buffalo spermatozoa (EL-Menoufy *et al.*, 1988), while, Niwa *et al.* (1988) found that the highest penetration of bovine oocytes with negligible polyspermy was obtained at 5mM caffeine. The concentration of heparin used (10 µg/ml) in the present study was the optimum dose needed for the highest rate of bovine oocyte penetration recorded by Saki *et al.*, (1995).

The co-culturing of sperm into fertilization drop conained oocytes was considered as a routine system in in-vitro embryo production field. Recently, fertilization of occytes by introducing them in sperm droplet was applied by several ivestigatiors (Goodrow and Hay, 1993 and Fazeli *et al.* 1993). Co-culturing of oocytes in sperm droplets gave a better results but without sigificant variation (Table 3). Further studies are needed in this respect.

From the aforementioned results, we can conclude that, pooled fresh semen has to be used to avoid the individual variation effect on the IVF. Addition of 2mM caffeine and 10 µg/ml heparin in incubation media of sperm improved their fertilizing ability. Co-culturing of buffalo oocytes in sperm droplets gave better results in IVF.

Table 3. Effect of technique of fertilization on sperm zona pellucida binding assay.

Technique of fertilization	Sperm-zona pellucida binding	
	No of oocytes	No of accessory sperm
Fertilization droplets	20	132 ± 8.7
Sperm droplets	20	139 ± 3.5

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العوامل المؤثرة علي الإخصاب خارج الرحم في الجاموس المصري باستخدام تجربة ارتباط الحيوان المنوي بالقشرة الشفافة للبويضة

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تعد تجربة ارتباط الحيوان المنوي بمنطقة القشرة الشفافة للبويضة خطوة هامه للاخصاب الناجح خارج الرحم. في هذه التجربة قمنا بالعد المجهري للحيوانات المنوية المرتبطة بالقشرة الشفافة للبويضات (سميت بالحيونات المنوية الثانوية أو المساعدة) وذلك بعد غسل البويضات لعدة مرات واستخدام الماصة الدقيقة لأزالة الحيوانات المنوية الغير ثابتة ... وبهذه التجربة تم دراسة تأثير العوامل الفردية ومصدر السائل المنوي (غير مجمد أو مجمد) وذلك باستخدام سائل منوي من طلائق مختلفة كل علي حدة. كما تم دراسة تأثير نوع العوامل المكثفة وطرق اضافتها وأيضاً طرق الأخصاب. أوضحت النتائج أن هناك فروقا معنوية في الحيوانات المنوية الثانوية من السائل المنوي الطازج (الغير مجمد) لثلاث طلائق مختلفة بالرغم من تشابة صفاتهم الفيزيقية (الحركة الفردية ، الحيوية والشكل) وأيضا وجود فروق معنوية في عدد الحيوانات المنوية من السائل المنوي المجمد المستخدم من ثلاث طلائق مختلفة تمشياً مع الأختلاف المعنوي في صفاتهم الفيزيقية. ولقد لوحظ أيضاً أن السائل المنوي الطازج يزيد من عدد الحيوانات المنوية الثانوية زيادة ملحوظة عن السائل المنوي المجمد.

وباستخدام ٢ مللي عياري كافيين أو ١٠ ميكروجرام / ملليلتر هيبارين أو الأثنين معاً زاد من عدد الحيوانات المنوية الثانوية بالمقارنة بالمجموعة الضابطة. ولقد وجد أن أضافة هذه المكثفات في بيئة التحضين للحيوانات المنوية يزيد من عدد الحيوانات المنوية الثانوية عن أضافتها في بيئة الأخصاب للبويضات ولكن بفرق غير معنوي. كما أن اضافة كل من الكافيين مع الهيبارين كان أفضل من إستخدام كل منهما علي حدة.

كما أوضحت النتائج أن طريقة الأخصاب: زرع البويضات في قطرات من السائل المنوي يزيد من عدد الحيوانات المنوية الثانوية زيادة غير معنوية بالمقارنة إضافة السائل المنوي إلي قطرات من بيئة الإخصاب التي تحتوي علي بويضات.