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EFFECT OF FILTRATION OF POST-DILUTED SEMEN BY SEPHADEX ON FREEZING ABILITY AND FERTILIZING CAPACITY OF BUFFALO SEMEN

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ABSTRACT

This study aimed to evaluate the effect of Sephadex column filtration technique of post-diluted semen on freezing ability and fertilizing capacity of buffalo semen. Four Egyptian buffalo bulls were used for semen collection. Semen was extended (37ºC) at a rate of 1:20 and filtered using Sephadex column with G-75 (T2) or G-100 (T3) as compared to un-filtrated semen (T1). Semen in all treatments was evaluated for progressive motility, livability, abnormality and intact acrosome spermatozoa in post-diluted, post-equilibrated and post-thawing semen. Pregnancy diagnosis was performed at 50 d post-insemination. Results showed that T2 and T3 improved ($P<0.05$) percentage of motility, livability, abnormality and intact acrosome spermatozoa in post-diluted, post-equilibrated and post-thawed semen as compared to T1, being better with T2 (G-75) than T3 (G-100). For buffalo cows inseminated with post-thawed semen, conception rate was 73.3, 63.3 and 56.7% in T2, T3 and T1, respectively ($P<0.05$).

The present results indicate that using Sephadex (G-75) column filter technique has beneficial effects on improving semen quality and fertilizing capacity of buffalo spermatozoa by removal the abnormal and dead spermatozoa in buffalo semen, which can have an adverse effect on their fertilizing potential..

Keywords: Buffalo semen, filtration, Sephadex, sperm characteristics, fertility.

INTRODUCTION

Male fertility is an important factor influencing the reproductive efficacy of the herd. Most progress in improving reproductive efficiency can be made by accurate estimation of the fertility of males and their selective use (Foote, 2003). Mammalian spermatozoa are characterized by marked morphological heterogeneity in an ejaculate. Dead and abnormal spermatozoa have toxic (Shannon and Curson, 1972) and lytic (Lindemann *et al.*, 1982) effect on companion cells in semen, and consequently reduce fertility (Saacke and White, 1972). In natural mating, cervical mucus differentially selects motile spermatozoa and acts as a barrier to immotile ones (Saacke, 1984). This cervical selection is by-passed in artificial insemination (AI). Methods of separation of motile, normal, or live from immotile, abnormal, or dead ones received little attention in the earlier reports.

Various centrifugation gradients (Lessley and Garner, 1983; White *et al.,* 1984) filtration columns (Graham and Graham, 1990; Anzar and. Graham, 1993) or by methods based on active sperm movements that is swim up (Parrish and Foote, 1987), have been used to separate motile from immotile cells and to enhance the quality of ejaculates. After filtration through Sephadex column filter technique, a significant increase in the percentage of

motility and livability of spermatozoa had occurred in bull semen (Maki-Laurila and Graham, 1968; Graham *et al.,* 1976), in goat semen (El-Saidy, 2000) and in buffalo and Friesian semen (Abdel-Khalek *et al.,* 2008).

The separation of spermatozoa was probably on the basis of complex and interacting properties of sperm plasma membrane, the medium suspending the sperm and the Sephadex particles. It was speculated that there was a physicochemical reaction between sperm plasma membrane bound proteins and Sephadex particles (Samper *et al.,* 1995). A good deal of heterogenesity in spermatozoal morphology is encountered in mammalian semen and significant reduction in the percentages of dead and abnormal spermatozoa has been reported following Sephadex filtration of buffalo semen (Ziada *et al.,* 2006). In previous studies, semen quality assessment after filtration was usually based on subjective estimation of sperm motility, viability and appearance of acrosome and in some cases morphology (Januskauskas *et al.*, 2005).

There were shortages in literature about the evaluation of fertilizing capacity of frozen semen after filtration through Sephadex. From the previous work on *in vitro* fertilization of buffalo bulls, it was reported that bulls have different ability to fertilize oocytes (Mahmoud *et al.,* 2004; Abdel Dayem, 2008). The abnormal shaped spermatozoa cannot participate in fertilization and these effects were exerted by the zona pellucida (Penfold *et al.*, 2003).

The objective of the present study was to make a comparative evaluation of filtration of post-diluted semen by two grades (G-75 and G-100) of Sephadex on freezing and fertilizing ability of buffalo semen. Also, to test if selecting sperm population before freezing reduces the deleterious effects of cryopreservation and consequently improves the fertilizing ability of buffalo spermatozoa.

MATERIALS AND METHODS

This study was conducted at the International Livestock Management Training Center (ILMTC), Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

Semen collection:

Semen was collected twice weekly from 4 fertile Egyptian buffalo bulls (3-5 years) morning before feeding at 8 a.m. for 5 weeks using the conventional artificial vaginal method. A bull was used as teaser animal for sexual preparation. On collection day, only ejaculates having mass motility of ≥70% were pooled (4 ejaculates for each run) were taken immediately to the laboratory and pooled.. The pooled semen was diluted and divided into three parts, the 1st was kept as control (non-filtered), while the 2nd and 3rd were passed for filtration thought G-75 and G-100 Sephadex column filter, respectively. Semen was evaluated pre-filtration (T1) and post-filtration with Sephadex G-75 (T2) and G-100 (T3) post-dilution, post-equilibration and post-thawing processes.

Sephadex column-filtration test:

Semen dilution:

Semen was diluted in heated (37^{o}C) Tris extender at a rate of 1:20. The Tris extender was prepared with 3.025 g Tris (hydroxymethyl amino methane, 1.675 g citric acid, 0.75 g glucose, 15% egg yolk, 7%, glycerol, 0.005 g streptomycin, 0.25% lincomycin and distilled water (3 times) to 78 ml.

***Preparation of slurries* and Sephadex filters**:

Both extender and ejaculates were kept and mixed at 37^{o}C. Slurries of Sephadex G-75 (4.2% w/v) and G-100 (3.3% w/v) were prepared by allowing them to swollen in 3% sodium citrate buffer for 4 h at 5°C (Graham *et al.,* 1976). The filtration column was prepared according to Januskauskas *et al*. (2005) in a 10 ml disposable plastic syringe. A hole (1.6 mm) was drilled at an 8 ml level in the syringe barrel to allow air bubbles in the barrel to escape when the plunger was lowered. A small amount of glass wool was compressed with the plunger to the bottom of the barrel to prevent loss of Sephadex. Sephadex was gently layered over the glass wool and allowed to settle for 3 min. The syringes were placed in a test tube rack for allowing the free drainage of fluid into collecting vessel (Faycmi *et al.,* 1979) and the rack was kept in an incubator at 37°C prior filtration. The complete filtration process took about 4.5 min in all columns.

Filtration of extended semen:

Extended semen (20 ml) was gently placed on the column using pipette through 3-4 doses at 37^{o}C. It was allowed to drain from the column to complete filtration for about 10-15 minutes for each test. The filtrate was collected in 50 ml volumetric flasks.

Semen evaluation:

Semen of T1, T2 and T3 was evaluated for the percentages of progressive motility (Amman and Hammerstede.1980) Livability (Hancock, 1951), abnormality (Bloom.1983) and intact acrosome (Watson, 1975) of spermatozoa in post-diluted, post-equilibrated and post-thawed semen. Percentage of spermatozoa with intact acrosome was also examined using Giemsa stain (Sigma Chemical Co. St. Louis, Mo.).

All sperm characteristics were determined using research microscope supplied with a hot stage adjusted to 37^{o}C. One drop from the diluted semen was placed on a slide and was covered by a warmed cover slip and immediately examined under the high power magnification (x400). Recovery rate (RR) was calculated in post-thawed semen as the following:

RR (%) = (% in post-thawed/ % in post-diluted semen) x 100.

Equilibration period of semen was 4 h and post thawing kept in water bath at 37^{o}C for performing evaluation tests. Semen was frozen at -196 ^{o}C for at least one month.

Fertility trail:

Total of 90 buffalo cows in heat were divided into 3 groups (30 in each). Buffalo cows in each group were inseminated with frozen/thawed semen from T1, T2 and T3, respectively. Frozen semen of each treatment was thawed at 37^{o}C for 30 seconds and immediately post-thawing, gun of insemination was used to artificial insemination. Pregnancy diagnosis was performed after 50 day post insemination using rectal palpation.

Statistical analysis:

Results were statistically analyzed according to Snedecor and Cochran (1982) using SAS system (1985). The differences among means were tested using Duncan's new multiple range test (Duncan, 1955). The percentage values of sperm progressive motility, livability, abnormality and intact acrosome were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed

RESULTS

Sperm characteristics in post-diluted semen:

Results presented in Table (1) showed that percentage of motility, livability, abnormality and intact acrosome of spermatozoa significantly ($P<0.05$) improved in filtrated (T2 and T3) than in non-filtrated semen (T1). Moreover, filtration of post-diluted semen with Sephadex G-75 (T2) had significantly ($P<0.05$) superior those filtrated with Sephadex G-100 (T3).

Table (1): Effect of filtration with different types of Sephadex on sperm characteristics in diluted semen.

Item	Non-filtration	Filtration	
	Control (T1)	Seph. G-75 (T2)	Seph. G-100 (T3)
Sperm motility(%)	70.5±1.30^{c}	85.0±1.09^{a}	77.3±1.14^{b}
Sperm livability (%)	71.2±1.34^{c}	86.6±1.00^{a}	78.7±1.04^{b}
Intact acrosome (%)	73.7±1.19^{c}	89.0±0.86^{a}	81.2±0.82^{b}
Sperm abnormality (%)	13.9±0.38^{a}	3.2±0.28^{c}	8.2±0.21^{b}

a, b and c: Means within the same row with different superscripts are significantly different at P<0.05.

Sperm characteristics in post-equilibrated semen:

Results in Table (2) showed the same improvement post-filtration in post-equilibrated semen with both grades of Sephadex (T2 and T3) as compared to non-filtrated semen, but percentages of all sperm characteristics in post-equilibrated semen was lower than in post-diluted semen.

Table (2): Effect of filtration with different types of Sephadex on sperm characteristics in post-equilibrated semen.

Item	Non-filtration	Filtration	
	Control (T1)	Seph. G-75 (T2)	Seph. G-100 (T3)
Sperm motility (%)	62.5±1.68^{c}	78.25±1.32^{a}	69.25±1.67^{b}
Sperm livability (%)	63.6±1.75^{c}	79.9±1.37^{a}	71.1±1.7^{b}
Intact acrosome (%)	67.0±1.91^{c}	81.7±1.13^{a}	74.6±1.36^{b}
Sperm abnormality (%)	15.5±0.29^{a}	5.2±0.32^{c}	9.8±0.30^{b}

a, b and c: Means within the same row with different superscripts are significantly different at P<0.05.

Sperm characteristics in post-thawed semen:

Also, the same results of all sperm characteristics after filtration by Sephadex in post-diluted (Table 1) and post-equilibrated (Table 2) were obtained in post-thawed semen (Table 3).

Table (3): Effect of filtration with different types of Sephadex on sperm characteristics in post-thawed semen.

Item	Non-filtration	Filtration	
	Control (T1)	Seph. G-75 (T2)	Seph. G-100 (T3)
Sperm motility(%)	35.5±1.45^{c}	54.5±1.58^{a}	43.8±1.45^{b}
Sperm livability (%)	39.5±1.96^{c}	58.6±2.06^{a}	48.7±2.46^{b}
Intact acrosome (%)	50.3±1.3^{c}	64.3±0.79^{a}	57.05±0.72^{b}
Sperm abnormality (%)	17.1±0.43^{a}	6.5±0.55^{c}	11.5±0.52^{b}

a, b and c: Means within the same row with different superscripts are significantly different at P<0.05.

It is of interest to note that improving all sperm characteristics by Sephadex filtration reflected significantly (P<0.05) the highest recovery rates of sperm characteristics in post-thawed semen (Table 4).

Fertility trail:

Results presented in Table (5) revealed that conception rate (CR) of buffalo cows inseminated with filtrated frozen semen with Sephadex G-75 (T2) was significantly (P<0.05) higher than those inseminated with non-filtrated (T1) semen (73.3 vs. 56.7%).

Table (4): Effect of filtration with different types of Sephadex on recovery rate (%) of sperm characteristics in post-thawed semen

Item	Non-filtration	Filtration	
	Control (T1)	Seph. G-75 (T2)	Seph. G-100 (T3)
Sperm motility(%)	50.06±1.3^{c}	64.03±1.56^{a}	61.45±1.3^{b}
Sperm livability (%)	55.16±2.18^{b}	67.56±2.14^{a}	61.65±2.72^{ab}
Intact acrosome (%)	68.3 ±1.53^{b}	72.30±0.80^{a}	70.39±1.12^{ab}

a, b and c: Means within the same row with different superscripts are significantly different at P<0.05.

Also, CR of buffalo cows inseminated with filtrated frozen semen with Sephadex G-100 (T3) was higher than those inseminated with non-filtrated (T1) semen (63.3 vs. 56.7%), but did not differ significantly from those in T2 and T1 (Table 5).

Table (5): Effect of filtration with different types of Sephadex on conception rate of buffalo cows.

Item	Non-filtration	Filtration	
	Control (T1)	Seph. G-75 (T2)	Seph. G-100 (T3)
Inseminated animals	30	30	30
Non-conceived animals	13	8	11
Conceived animals	17	22	19
Conception rate (%)	56.7^{b}	73.3^{a}	63.3^{ab}

a and b: Means within the same row with different superscripts are significantly different at P<0.05.

It is worthy noting that the differences in conception rate were not significant between animals inseminated with semen filtrated with Sephadex G-100 and those with Sephadex G-75 or control post-thawed semen.

DISCUSSION

Based on the obtained results in this study, semen quality of buffalo bulls in terms of motility, livability, abnormality and intact acrosome of spermatozoa, significantly (P<0.05) improved after filtration with two types of Sephadex in post-dilution, post-equilibrated and post-thawed semen as compared to the unfiltered control semen. In accordance with the present results, Scholkamy et al. (2009) reported that the quality of semen after filtration with different Sephadex grades (G-25, 50, 75, 100 and 200) in respect of sperm motility and livability percent of buffalo spermatozoa was improved significantly in all grades of Sephadex as compared to the unfiltered controls. Similar results were obtained in buffalo semen (Chauhan, *et al.,* 1993; Ziada *et al.,* 2006), in bovine (Graham and Graham, 1990; Januskauskas *et al.,* 2005) and in Friesian and buffalo semen (Abdel-Khalek *et al*., 2008).

The mechanism of separation of immotile, dead and abnormal spermatozoa during filtration was suggested by Graham *et al.* (1976), who mentioned that filtration of spermatozoa on Sephadex column appears to be physico-chemical reaction with Sephadex particles providing a barrier, allowing these types of spermatozoa to agglomerate. Increasing percentage of sperm livability in post- as compared to pre-filtration may be due to that after the death of spermatozoa, positively charged components appear on the sperm membrane, which interact with negatively changes Sephadex particles and are trapped (Abdel-Khalek *et al*., 2008), because the live spermatozoa usually have a net negative surface charge bound to their plasma membrane and pass (Hammerstedt *et al.*, 1979; Holt, 1980).

On the other hand, membrane damage of immotile and abnormal spermatozoa may lead to the exposure of different macromolecules, which might bind to the Sephadex particles (Lodhi and Crabo, 1984). Also, the retention of spermatozoa in the Sephadex column may be due to interaction between buffers and Sephadex beads and between buffers and abnormal spermatozoa and cells with damaged acrosomal membrane. Moreover, previous studies documented significant improvements in the percentage of morphologically normal acrosomes in filtered semen (Graham and Graham, 1990; Ahmad et al., 2003; Ziada *et al.,* 2006).

According to the obtained results, filtration by Sephadex G-75 had better power of separation of immotile, dead, abnormal and damaged acrosome spermatozoa as compared to Sephadex G-100. In this respect, Scholkamy et al. (2009) found that Sephadex G-75 showed significantly the highest impact than other grades (G≤50 or G>75). Also, the effect of Sephadex grade was strong enough to overlap the positive effect of these treatments separately (Ahmad *et al*., 2003). This finding could probably be due to the firm nature of packed beads of appropriate diameter of Sephadex G-75 as compared to G-100 for buffalo semen filtration. In this line, Abdel-Khalek et al. (2008) found marked differences in post-filtrated semen quality between Friesian and buffalo bulls.

The influence of Sephadex gel filtration on sperm quality prior to cryopreservation may provide further proof of the value of the technique to recover post-thaw sperm of high quality (Scholkamy et al., 2009). The present results in this study supported this finding in term of improving freezing ability and fertilizing capacity of buffalo spermatozoa post-filteration. As indicated for all sperm characteristics, the present fertility results are in agreement with many researchers, who have reported promising relationship between fertility and percentage of progressive sperm motility (Linford *et al*., 1970) and separation of weak and immotile spermatozoa through filtration is responsible for increasing progressive sperm motility (Roberts, 1972). Also, the intactness of acrosome has a significant relationship with fertility (Saacke, 1970). The present study indicated strong relationship between sperm characteristics and fertility rate, in particular with progressive sperm motility percentage. The present finding also support theories of separation of normal active motile spermatozoa using Sephadex column. In this study, there was decrease in the sperm abnormalities as compared to non-filtered semen. This decrease

was compensated by proportionate increase in the percentage of live, motile and morphologically active sperm which in turn could lead to improved conception rate through AI with filtered frozen semen.

Sephadex filtration can select motile, living and abnormal spermatozoa and can enhance fertilizing capacity of cryopreserved spermatozoa. It is worthy noting that fertilization success depends more importantly on the functional competence of spermatozoa rather than their counts.

The present results indicate that using Sephadex column filter technique with grades G-75 and G-100 was more useful in improving sperm livability in buffalo semen, being the best with Sephadex G-75.

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تـأثير ترشـيح السـائل المنـوي المخفـف بواسـطة عمـود السـيفادكس علـي القـدرة التجميدية والأخصابية للسائل المنوي للجاموس

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تم جمع السائل المنوي من أربعة طلائق جاموس بالغه جنسيا وتخفيفه بمعدل 1:20 علي 37 درجة مئوية ثم الترشيح بأستخدام عمود السيفادكس (G75 and G100) مقارنة بالكنترول (السائل المنوي المخفف غير المرشح). تم تقييم النسبة المئوية للحركة التقدمية والحيوانات المنوية الحية والحيوانات المنوية الشاذة والحيوانات المنوية ذات الأكروسوم السليم في السائل المنوي المرشح وغير المرشح بعد التخفيف وبعد فترة الموازنة وبعد التجميد .

أظهرت النتائج ما يلي:

1- أظهر السائل المنوي المرشح بالسيفادكس، المعاملة الثانية (G75 والثالثة G100، تحسنا معنويا في النسبة المئوية للحيوية والحيوانات المنوية الحية والحيوانات المنوية ذات الأكروسوم السليم مقارنة بالكنترول (المعاملة الأولي) بعد التخفيف وبعد فترة الموازنة وبعد التجميد.

2- تحسن معدل الأخصاب معنويا في المعاملة الثانية والثالثة مقارنة بالكنترول (73.3-63.3 و56.7%،علي التوالي) بعد الأسالة والتلقيح بالسائل المنوي المجمد.

أشارت النتائج الي أن استخدام الترشيح بواسطة عمود السيفادكس (G75) كان لة تأثير مفيد في تحسين جودة السائل المنوي للجاموس وزيادة القدرة التجميدية للحيوانات المنوية من حيث زيادة الحركة وعدد الحيوانات المنوية الحية وتقليل نسبة الحيوانات المنوية الشاذة.

قام بتحكيم البحث

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