Global Veterinaria 3 (2): 144-150, 2009
ISSN 1992-6197

Evaluation of Sephadex Filtration for Freezability and *in vitro* Fertilizing Ability of Buffalo Semen

[1]T.H. Scholkamy, [2]Karima Gh. M. Mahmoud, [1]F.A. El Zohery and [1]Maha S. Ziada

[1]Department of Artificial Insemination and Embryo Transfer,
Animal Reproduction Research Institute, Al-Ahram, Giza, Egypt
[2]Department of Animal Reproduction & A.I, National Research Center, Dokki, Giza, Egypt

Abstract: The objective of this study was to assess the effect of sephadex filtration on the quality, freezability and *in vitro* fertilizing ability of buffalo bull semen. Various grades of sephadex G-25, G-50, G-75, G-100, G-200 and G 50-200 were used. Semen samples were collected from four buffalo bulls, diluted 1:20 with Tris buffer, loaded in different grades of sephadex columns and kept for 4-5 minutes at 37° C. After examination of the effect of six types of sephadex on semen quality, the semen showed best grades of sephadex was selected for evaluation of their freezability and *in vitro* fertilizing ability. All types of sephadex filtration had a significant effect on sperm motility and percentage of live spermatozoa. Sperm motility and acrosomal defects in diluted and post-thawed semen samples were significantly improved after filtration with sephadex 75. There were no differences in fertilization percentages between filtered and non filtered semen separated by sephadex 75. In conclusion, sephadex filtration can effectively enhance the quality of buffalo semen before and after freezing but not for the *in vitro* fertilizing capacity.

Key words: Sephadex filtration · Buffalo bull · Semen quality · *in-vitro* fertilization

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 [1]. The recovery of morphologically normal, intact, motile sperm from semen is required for use in most assisted reproductive techniques [2].

The negative influence of dead and abnormal spermatozoa on the remaining sperm population [3], as well as on fertility has long been known [4]. Separation of dead and morphologically abnormal spermatozoa is performed in the female genital tract [5]. Various centrifugation gradients [6,7], filtration columns [8,9], or by methods based on active sperm movements that is swim up [10], have been used to separate motile from immotile cells and to enhance the quality of ejaculates. Graham and Graham [8] were the first to report a significant improvement of fertility (as non-return rates) for low fertility bulls after removal of dead and abnormal spermatozoa from extended ejaculates using sephadex filtration. The mechanism by which sephadex retain dead, damaged or capacitated spermatozoa [11] is still not well understood. 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 [12]. It was speculated that there was a physicochemical reaction between sperm plasma membrane bound proteins and sephadex particles[13].

Generally, semen ejaculates with low initial motility are discarded as they usually show reduced post thaw motility. In buffalo, it was recorded that 31 % of the total semen ejaculates produced per month had to be discarded due to poor initial quality [14]. 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 [15-17].

In previous studies, semen quality assessment after filtration was usually based on subjective estimation of sperm motility, viability and appearance of acrosome and

Corresponding Author: Dr. Karima Gh. M. Mahmoud, Department of Animal Reproduction & A.I, National Research Center, Dokki, Giza, Egypt

in some cases morphology [18]. 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 [2,19,20]. The abnormal shaped spermatozoa cannot participate in fertilization and these effects were exerted by the zona pellucida [21].

The objective of the present investigation was to make a comparative evaluation of various grades of sephadex filtration for improving the quality of buffalo semen. Also, to test if selecting a sperm population before freezing reduces the deleterious effects of cryopreservation and consequently improve the fertilizing ability.

MATERIALS AND METHODS

Semen Sample Preparation

Semen Collection: Semen samples were collected by means of artificial vagina from four healthy buffalo bulls, kept at Animal Reproduction Research Institute farm, AL -Harm, Giza Province. Two ejaculates were collected 10 min apart twice weekly, assessed for subjective motility analysis. Semen subjected to spermiograms within acceptable limit and had an initial motility of 70% and more than 80% morphologically normal spermatozoa was used.

Preparation for Sephadex Filtration: Slurries of sephadex G-25 (12% w/v), G-50 (6.0%w/v), G-75 (4.2%w/v), G-100 (3.3%w/v), G-200 (1.8%w/v) and G 50-200 (5% w/v) were prepared by allowing them to swollen in 3% sodium citrate buffer for 4 hrs at 5°C [22]. The filtration column was prepared according to Januskauskas *et al*. [18] 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. Different types of sephadex was gently layered over the glass wool and allowed to settle for 3-4 min. The syringes were placed in a test tube rack for allowing the free drainage of fluid into collecting vessel [23] and the rack was kept in an incubator at 37°C prior for filtration. The complete filtration process took about 4.5 min in all columns.

Semen Analysis: Control and filtered semen samples of different grades of sephadex were evaluated using standard techniques for individual motility (%), sperm concentration (X 10^9/ml). Percentages of alive and abnormal spermatozoa in stained smears using eosin - negrosine stain according to Blom [24]. Percentages of spermatozoa with abnormal acrosomes were counted in stained smears by fast green stain according to Wells and Awa[25].

Freezing of Semen: Good quality filtered semen samples (filtered through sephadex columns of grades 75 and 50-200) were centrifuged at 800g for 10 min. The supernatant was discarded. Control and 2 ml of sediment parts of filtered and centrifuged samples were diluted in Tris based diluent (Optidyl ™ medium which is commercial, Biovet France. It is composed of Tris, ionized egg yolk and antibiotics; penicillin, streptomycin, spectinomycine and lincomycin). Diluted samples were cooled to 5°C through 45 min., equilibrated at 5°C for 2 hrs. and packaged in 0.25 ml. French straws at 5°C. The straws were then frozen horizontally in liquid nitrogen vapor in foam box according to Mohammed *et al*. [26]. The straws were then rapidly plunged in liquid nitrogen for storage. Frozen straws were thawed at 35°C for 30 sec. Samples were evaluated for post-thaw sperm motility, percentages of spermatozoa with acrosomal defects. Viability indices for thawed samples were recorded.

Evaluation of Fertilizing Ability of Buffalo Bulls

Oocyte Recovery and Selection: Ovaries were collected from buffaloes within 2 hrs of slaughter. Ovaries were transported in physiological saline (0.9%, w/v, NaCl) with antibiotic (100 µg/ml streptomycin and 100 IU/ml penicillin) maintained at 30° C to the laboratory. Ovaries were washed three times in phosphate buffered saline (PBS). Oocytes were aspirated from 2-5 mm follicles with a 20-gauge needle attached to a 5-ml syringe containing PBS with 3% bovine serum albumin, fraction V and antibiotics (100 µg/ml streptomycin and 100 IU/ml penicillin). Oocytes were collected using a low power (20X) stereomicroscope. Only oocytes with intact layers of cumulus cells and homogenous cytoplasm were selected [27].

Oocyte Maturation: Selected oocytes were washed 3 times in TCM-199 with Earl's salts and 25mM HEPES supplemented with 10% fetal calf serum (heat treated at 56° C for 30 min.) and 50 µg/ml gentamycin sulfate. Oocytes were cultured in 4-well plastic Petri-dishes containing 100 µl of culture medium (the same as washing medium), prepared 24 hrs before culturing of oocytes.

Each drop of media contains about 10-15 oocytes/100 µl of medium covered with a layer of mineral oil. These culture dishes were incubated for 24-26 hrs at 38.5°C in 5% CO_2 in air and 95% humidity.

***In vitro* Fertilization and Culture:** The procedure was performed as described by Niwa and Ohgoda [28]. Straws of frozen buffalo semen (Control and filtered by sephadex 75) were thawed in a water bath at 35 - 37 ° C for 1 min. Sperms were washed twice by centrifugation (800 g for 10 minutes) in BO medium [29] without BSA containing 10 µg /ml heparin and 2.5 mM caffeine. The sperm pellets were diluted with BO medium containing 20 mg/ml bovine serum albumin to adjust the concentration of spermatozoa to 12.5 x 10^6 sperm/ml. After removing the cumulus cells, matured oocytes were washed three times in BO medium containing 10 mg/ml BSA and were introduced into 100 µl droplets of sperm suspension (about 5-10 oocytes/ droplet) under paraffin oil. The sperm and oocytes were co-cultured for 5 hrs under the same culture conditions, 5% CO_2, 38.5°C, 95% humidity. Groups of 10-20 oocytes were again cultured with previously prepared co-culture 100 µl droplet consisting of TCM-199 + 10 % calf serum.

Fertilization Assay: After 20 hrs of culture, oocytes were placed in the refrigerator at 4° C until fixation [30]. For fixation, 5-10 oocytes were placed on glass slides according to the procedure described by Tarkowski [31]. Oocytes were fixed in a solution of 3 methanol: 1 glacial acetic acid then stained with 1 % acetio- orcin stain. Oocytes were considered fertilized if the sequence of total sperm penetration into ooplasm described by Xu and Greve [32] were presented, i.e. sperm tail, sperm head decondensation, completion of second meiotic division and male and female pronuclear development. Fertilization percent was calculated by dividing total numbers of ova penetrated with sperm over total numbers of ova inseminated multiplied by 100.

Statistical Analysis: The results were tabulated in a way to indicate the mean values of the various parameters studied and their standard errors. Data were subjected to ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by Duncan's Multiple Range Test. Differences were considered to be significant at $P < 0.05$. Bulls were compared for the significant between filtered and non filtered semen by paired T-test.

RESULTS

Semen Picture after Filtration: Different types of sephadex G-25, G -50, G-100, G-200 and G50-200 were used. Mean initial sperm motility values after semen collection were 62.50 ± 1.00% (Table 1). All types of sephadex filtration had a significant effect on sperm motility. Samples filtered through both Serphadex G-75 and G 50-200 revealed higher semen quality than sephadex 25, 50, 100 and 200. The mean percentage of live spermatozoa after filtration by different types of sephadex was significantly improved than control samples. More improvement was observed with sephadex 75 and sephadex 50-200 than other types of sephadex (Table 2).

Semen Picture During and after Freezing: Table 7 illustrates means of sperm motility of diluted semen samples which have been filtered through sephadex columns of choice (G-75 and G 50-200). Diluted sperm motility was improved ($P<0.05$) after filtration with sephadex 75 than sephadex 50-200 (Table 7). Also, percentage values of post-thawed motile spermatozoa were improved ($P<0.05$) by filtration in sephadex 75 than sephadex 50-200 (Table 4). Acrosomal defects in both diluted and post-thawed semen samples decreased ($P<0.05$) in sephadex 75 than sephadex 50-200 (Table 5 and 6, respectively). Also viability indices in post-thawed semen samples improved ($P<0.05$) in sephadex 75 than sephadex 50-200 (Table 3).

Table 1: Effect of filtration in different columns of sephadex on percentages of different bull sperm motility (Means±SE)

Sephadex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
Control	61.66±1.66	63.33±3.33	63.33±1.66	61.66±1.67	62.50±1.00 ^{c}
Sephadex 25	71.66±1.67	66.66±3.32	71.66±4.41	66.66±1.66	69.16±1.48 ^{b}
Sephadex 50	71.66±1.66	70.00±2.89	71.66±1.67	73.33±1.67	71.66±0.94 ^{b}
Sephadex 75	78.33±1.67	86.66±1.66	85.00±2.89	85.00±2.87	83.75±1.39 ^{a}
Sephadex 100	76.66±1.66	83.33±1.67	83.33±1.67	83.33±3.33	81.66±1.28 ^{b}
Sephadex 200	68.33±1.67	76.66±1.67	70.00±2.89	75.00±2.94	72.50±1.44 ^{b}
Sephadex 50- 200	81.66±1.67	83.33±1.66	86.66±1.68	85.00±2.89	84.16±1.03 ^{a}
Over all mean	72.85±1.49 ^{A}	75.71±2.05 ^{A}	75.95±2.03 ^{A}	75.71±2.08 ^{B}	75.05±0.96

Values with different superscript within the same column and raw differ significantly ($p<0.05$)

Table 2: Percentages of live spermatozoa of semen samples of different bulls after filtration in different sephadex columns (Means±SE)

Sephadex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
Control	65.33±1.45	65.66±3.18	66.66±2.40	64.33±1.85	65.5±1.02 [d]
Sephadex 25	75.00±1.25	69.66±2.85	73.66±3.84	69.01±2.08	71.83±1.39 [c]
Sephadex 50	74.33±1.85	73.00±1.74	74.67±2.03	77.00±2.08	74.75±0.93 [c]
Sephadex 75	83.66±1.86	90.01±1.17	89.33±2.33	88.66±3.18	87.91±1.22 [ab]
Sephadex 100	83.66±1.85	86.00±0.58	85.67±2.03	85.73±2.85	85.16±0.89 [b]
Sephadex 200	70.33±1.20	81.02±2.51	72.01±2.32	75.66±3.18	74.75±1.63 [c]
Sephadex 50- 200	87.00±1.53	89.66±1.20	91.33±0.88	88.67±1.76	89.16±0.76 [a]
Over all mean	77.04±1.73	79.28±2.15	79.04±2.12	78.38±2.14	78.44±1.01

Values with different superscript within the same column differ significantly (P<0.05)

Table 3: Diluted sperm motility after filtration in sephadex columns (Means±SE)

Sephdex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
control	65.0±2.04	71.25±2.39	68.75±1.25	66.25±3.75	67.81±1.29 [b]
Sephadex 75	85.0±2.04	87.50±1.44	88.75±1.25	82.50±3.23	85.93±1.14 [a]
Sephadex 50 -200	68.75±1.25	70.00±2.04	70.00±2.04	66.25±3.75	68.75±1.16 [b]
Over all mean	72.91±2.78	76.25±2.62	75.83±2.88	71.66±2.97	74.16±1.39

Values with different superscript within the same column differ significantly (P<0.05)

Table 4: Post-thawed sperm motility frozen after filtration in sephadex columns (Means±SE)

Sephdex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
control	41.25±4.73	48.75±1.25	56.25±3.75	35.00±6.46	45.31±2.87 [b]
Sephadex 75	58.75±5.91	63.75±1.25	71.25±2.39	47.50±4.79	60.31±2.87 [a]
Sephadex 50 -200	43.75±4.27	48.75±1.25	56.25±3.75	32.50±7.50	45.31±3.08 [b]
Over all mean	47.91±3.50 [B]	53.75±2.23 [B]	61.25±2.76 [A]	38.33±3.86 [C]	50.31±1.95

Values with different superscript within the same column and raw differ significantly (P<0.05)

Table 5: Diluted sperm acrosomal defects of semen samples filtered in Sephadex columns (Means±SE)

Sephdex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
control	6.50±0.96	6.25±0.75	5.75±0.75	6.00±0.41	6.12±0.34 [a]
Sephadex 75	2.25±0.48	1.75±0.25	2.50 ±0.50	2.00±0.58	2.13±0.22 [b]
Sephadex 50 -200	6.75±0.85	7.00±1.00	5.25±0.95	6.00±0.41	6.25±0.41 [a]
Over all mean	5.16±3.46	5.00±3.71	4.50±2.71	4.66±2.87	4.83±0.34

Values with different superscript within the same column differ significantly (p<0.05)

Table 6: Post-thawed sperm acrosomal defects of semen samples frozen after filtration in sephadex columns (Means±SE)

Sephdex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
control	14.25±1.18	15.75±1.44	15.25±1.44	16.00±1.42	15.31±0.64 [a]
Sephadex 75	7.75±0.75	7.50±0.50	8.25±0.48	8.75±0.95	8.06±0.34 [b]
Sephadex 50 -200	13.5±1.26	14.75±1.25	13.25±1.31	15.75±1.44	14.31±0.64 [a]
Over all mean	11.83 ±1.04	12.66±1.26	12.25±1.07	13.50±1.22	12.56±0.56

Values with different superscript within the same column differ significantly (p<0.0001)

Table 7: Viability index of post-thawed semen samples frozen after filtration in sephadex columns (Means±SE)

Sephdex	Bull 1	Bull 2	Bull 3	Bull 4	Over all mean
control	95.00±3.54	93.75±6.50	121.87±6.64	52.50±18.93	90.78±7.99 [a]
Sephadex 75	134.37±8.68	151.625±4.02	165.62±1.88	99.37±25.69	137.75±8.87 [b]
Sephadex 50 -200	95.0±3.54	96.87±1.88	115.00±6.54	52.50±18.92	89.84±7.48 [a]
Over all mean	108.125±6.36 [B]	114.08±8.35 [B]	134.16±7.34 [A]	68.125±13.02 [C]	106.125±5.63

Values with different superscript within the same column and raw differ significantly (p<0.05)

Table 8: Fertilization percentages of frozen semen treated with or without sephadex 75 filtration (Mean±S.E)

	Non filtered semen			Filtered semen		
Bull number	No of examined oocytes	No of fertilized oocytes	% of fertilized oocytes	No of examined oocytes	No of fertilized oocytes	% of fertilized oocytes
1	73	29	39.72±1.21[b]	97	42	42.77±1.98[b]
2	114	52	45.56±1.24[c]	99	47	47.81±1.18[c]
3	100	18	18.17±0.72[a]	100	20	20.28±1.69[a]
4	110	18	16.22±0.72[a]	112	20	17.57±0.83[a]
Total	397	117	29.92±3.92	408	129	32.11±4.07

Data was 3 replicates for each bull. Values with different superscript within the same column differ significantly (P<0.0001). No = number

***In vitro* Fertilizing Ability:** Fertilizing ability of filtered semen by sephadex 75 from different bulls was recorded in Table 8. Filtration by sephadex 75 did not have a significant effect on sperm fertilizing ability of different bulls. There was a significant affect of bull (P<0.0001) for sperm fertilizing ability variable in both filtered and non filtered semen.

DISCUSSION

The quality of semen after filtration with six types of sephadex in respect of sperm motility and live sperm percent of buffalo bulls was improved significantly in all types of sephadex as compared to the unfiltered controls. Similar result was obtained in semen of buffalo [15,17]. G-75 and G50-200 sephadex columns were significantly higher than other types. This also showed that these higher grades had better power of separation of immotile and dead spermatozoa as compared to lower grades. It could probably be due to the firm nature of packed beads of smaller diameter of higher grades of sephadex as compared to lower grades G-50 and G-25.

The influence of sephadex gel filtration on sperm quality prior to and following cryopreservation provide further proof of the value of the technique to recover post-thaw sperm of high quality. The result of the present study demonstrated that sephadex filtration significantly improved sperm quality in terms of post-dilution motility and post-thaw sperm motility. The improvement in semen motility is dependent on semen quality before filtration. In accord with the present study, Anzar and Graham [9] and Ahmad *et al*. [16] reported significant improvement in sephadex filtered sperm motility after dilution of bovine and buffalo semen, respectively. Another study by Januskauskas *et al.* [18] did not observe any significant effect of filtration on motility values.

In the present study, post-thaw viability indices improved significantly for sephadex filtered semen samples. Similar to our results, [17,18] found significant enhanced viable sperm for filtered semen samples compared to non-filtered samples. In this work, acrosomal defected spermatozoa had significantly lower percentages after dilution and post-thawing of filtered samples. Previous studies documented significant improvements in the percentage of morphologically normal acrosomes in filtered semen [8,16,17].

In this study, there was no significant increase in fertilization rates of buffalo bulls after filtration with sephadex. In this respect, [33] found that selecting sperm by glass wool/Sephadex filtration or Percoll separation prior to insemination did not affect pregnancy outcome (P=0.422) in mare. The non significant differences in the percentages of oocytes fertilized with separated or control sperm, indicating the non toxic effect of sephadex. In this respect, [34] indicated that sephadex filtration and washing procedures were somewhat ineffective in protecting spermatozoa from damage caused by the freezing process. Regardless the separation methods, [35,36] found that there was no effect of the separation procedure (swim-up or Percoll) on *in vitro* fertilizing capacity of separated spermatozoa. Moreover, [37] found that sperm Prep sephadex column washes were toxic to human sperm and mouse in vitro fertilization.

In the present work, sephadex filtration can select motile and living spermatozoa but can not enhance the *in vitro* fertilizing ability of these spermatozoa with originally low *in vitro* fertility. It seems that fertilization success does not simply depend on the absolute number of vital, motile, morphologically normal spermatozoa but more importantly on their functional competence. Irrespective to filtration treatments, bull were found to be significantly differed in their post-thaw sperm motility, viability and *in vitro* fertilizing ability. Freezing and thawing procedures are mostly harmful to sperm membranes [38,39], since temperature and osmotically caused changes occur in the organization, fluidity, permeability and lipid composition of these membranes [18]. So it seems that sephadex filtration can not remove the effect of bull factor on *in vitro* fertilization.

In conclusion, G-75 sephadex grade gave more balanced picture of semen quality as compared to the other grades. Selecting a sperm population before freezing didn't reduce the bull effect and the deleterious effects of cryoprservation.

REFERENCES

1. Foote, R.H., 2003. Fertility estimation: a review of past experience and future prospects. Animal. Reproduction. Sci., 75: 119-139.
2. Abdel Dayem, A.M.H., K. Gh. M. Mahmoud, M.F. Nawito, M.M. Ayoub and T.H. Scholkamy, 2008. Fertility evaluation in Egyptian buffalo bulls using zona pellucida binding and *in vitro* fertilization assays. Livestock. Science. (in press).
3. Lindemann, C.B., M. Fisher and M. Lipton, 1982. A comparative study of the effects of freezing and frozen storage on intact and demembranated polyspermatozoa. Cryobiology, 19: 20-28.
4. Hancock, J.L., 1959. The morphologic characteristics of spermatozoa and fertility. International Journal of Fertility, 4: 347.
5. Saacke, R.G., 1984. Semen quality: Importance of and influencing factors. In: Proceeding of the Tenth NAAB Technical Conference on Artificial Insemination in Reproduction; Milwaukee, WI; 30-36.
6. Lessley, B.A. and D.L. Garner, 1983. Isolation of motile spermatozoa by density gradient centrifugation in Percoll. Gamete Res., 7: 49-61.
7. White, L.M., W.E. Beal, J.H. Bame, R.G. Saacke and C.E. Marshall, 1984. Characterization of bovine spermatozoa after migration through serum albumen gradient. J. Animal Sci., 59: 454-459.
8. Graham, E.F. and J.K. Graham, 1990. The effect of whole ejaculate filtration on the morphology and the fertility of bovine semen. J. Dairy Sci., 73: 91-97.
9. Anzar, M. and E.F. Graham, 1993. Filtration of bovine semen. I. Development of a Sephadex ion-exchange filter. Animal Reproduction Sci., 31: 187-195.
10. Parrish, J.J. and R.H. Foote, 1987. Quantification of bovine sperm separation by a swim-up method. Relationship to sperm motility, integrity of acrosomes, sperm migration in polyacrylamide gel and fertility. J. Andrology, 8: 259-266.
11. Samper, J.C. and B.G. Crabo, 1993. Assay of capacitaed, freeze-damaged and extended stallion spermatozoa by filtration. Theriogenology, 39: 1209-1220.
12. Landa, C.A., J.O. Almquist and R.P. Amann, 1980. Factors influencing sephadex separation of bovine and ovine spermatozoa. J. Dairy Sci., 63: 277-282.
13. Samper, J.C., D.W. Hamilton, J.L. Bryor, K.J. Loseth, M.H. Trodsson and B.G. Crabo, 1995. Mechanism of Sephadex trapping of capacitated stallion spermatozoa. Biology of Reproduction., 1: 270-285.
14. Zafar, A.H., N. Ahmad and K.H. Shah, 1988. Effect of seasonal variation on semen production of Nili-Ravi buffalo bulls. Buffalo Journal, 1: 61-68.
15. Chauhan, S.S., G. Mohan, S. Kumar and K.L. Sahni, 1993. Comparative evaluation of various grades of sephadex for improving the quality of buffalo semen. Indian J. Animal. Sci., 63: 246-250.
16. Ahmad, Z., M. Anazar, M. Shahab, N. Ahmad and S.M.H. Andrabi, 2003. Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low-grade buffalo semen ejaculates. Theriogenology, 59: 1189-1202.
17. Ziada, M.S., M. Alaa El-Deen, A.A. H. Zaky and K.D. Hassanin, 2006. Assessment of the efficacy of sephadex filtration of buffalo spermatozoa for cryopresevation. Vet. Med. J., 54: 295-307.
18. Januskauskas, A., K. Lukoseviciute, S. Nagy, A. Johannisson and H. Rodriguez-Martinez, 2005. Assessment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for cryopreservation. Theriogenology, 63: 160-178.
19. Mahmoud, K.G.M., M. Ziada and T.H. Scholkamy, 2004. Freezability of buffalo semen in relation to its *in-vitro* fertilizing capacity and chromosomal picture. J. Egyptian Vet. Med. Assoc., 64: 239-251.
20. Abdel Dayem, A.M., 2008. The use of *in vitro* fertilization technique to evaluate the fertility of buffalo bulls with special emphasis on genotype analysis. M.V.Sc. Thesis (Theriogenology), Fac. Vet. Med. Cairo Uni., Egypt.
21. Penfold, L.M., J. Jost, D.P. Evenson and D.E. Wildt, 2003. Normospermic versus teratospermic domestic cat sperm chromatin integrity evaluated by flow cytometery and intracytoplasmic sperm injection. Biology of Reproduction, 69: 1730-1735.
22. Graham, E.F., I.A. Vasquez, M.K. Schmchl and B.K. Evensen, 1976. An assay of semen quality by use of Sephadex filtration. In: Proceedings of Eighth Intl. Congress of Animal Reproduction, 4: 896-899.
23. Faycmi, L.O., B.G. Crabo and E.F. Graham, 1979. Assay of frozen boar semen with Sephadex filtration. Theriogenology, 12: 13-17.

24. Blom, E., 1983. The spermiogram of the bull. Nordisk Veterinary Medicine, 35: 105.
25. Wells, M.E. and O.A. Awa, 1970. New technique for assessing acrosomal characteristics of spermatozoa. J. Dairy Sci., 53: 327-332.
26. Mohammed, K.M.E., M.S. Ziada and G.M. Darwish, 1998. Practical trials for freezing semen of buffalo and Friesian bulls. Effect of various regimens of freezing, different milk extenders and types of straws packages on post thawing semen characters. Assiut Vet. Med. J., 39: 70.
27. Warriach, H.M. and K.R. Chohan, 2004. Thickness of cumulus cell layer is a significant factor in meiotic competence of buffalo oocytes. J. Vet. Sci., 5: 247-251.
28. Niwa, K. and O. Ohgoda, 1988. Synergistic effect of caffeine and heparin on in vitro fertilization of cattle oocytes matured in culture. Theriogenology, 30: 733-741.
29. Brackett, B.G. and G. Oliphant, 1975. Capacitation of rabbit spermatozoa *in vitro*. Biology of. Reproduction, 12: 260-274.
30. Thundathil, J.A., T. Palasz, A.D. Barth and R.J. Mapletoft, 2001. The use of *in vit*ro fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. Animal Reproduction Sci., 65: 181-192.
31. Tarkowski, A.K., 1966. An air-drying method for chromosome preparations from mouse eggs. Cytogenetic, 5: 394-400.
32. Xu, K.P. and T. Greve, 1988. A detailed analysis of early events during in-vitro fertilization of bovine follicular oocytes. J. Reproduction and Fertililty, 82: 127-134.
33. Nie, G.J., K.E. Johnson and G.W. Wenzel, 2003. Pregnancy outcome in mares following insemination deep in the uterine horn with low numbers of sperm selected by glass wool/Sephadex filtration, Percoll separation or absolute number. Animal. Reproduction Sci., 79: 103-109.
34. Goyal, R.L., R.K. Tuli, G.C. Georgie and D. Chand, 1996. Comparison of quality and freezability of water buffalo semen after washing or Sephadex filtration. Theriogenology, 46: 679-686.
35. Parrish, J.J., A. Krogenaes and J.L. Susko-Parrish, 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology, 44: 859-869.
36. Suzuki, K., M. Geshi, N. Yamauchi and T. Nagai, 2003. Functional changes and motility characteristics of Japanese black bull spermatozoa separated by Percoll. Animal Reproduction Sci., 77: 157-172.
37. Scott, L. and S. Smith, 1997. Mouse in vitro fertilization, embryo development and viability and human sperm motility in substances used for human sperm preparation for assisted reproduction. Fertility Sterility, 67: 372-381.
38. El-Sisy, G.A., W.S. El-Nattat and R.I. El-Sheshtawy, 2006. Effect of different extenders on viability of frozen buffalo spermatozoa. Proceeding of the.3rd International Confernce Of the Veterinary Research Division, National Research centre, Cairo, Egypt. pp: 17-22.
39. El-Sisy, G.A., W.S. El-Nattat and R.I. El-Sheshtawy, 2007. Buffalo semen Quality, antioxidants and peroxidation during chilling and cryopreservation. Online J. Vet. Res., 11: 55-61.

(Received: 18/01/2009; Accepted: 1/2/2009)