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EMPLOYMENT OF THE HYPO-OSMOTIC SWELLING TEST TO EVALUATE FUNCTIONAL INTEGRITY OF THE WASHED BUFFALO SPERMATOZOAL MEMBRANE

(With 4 Tables and One Figure)

By

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إستخدام إختبار إنخفاض الاسموزيه للتقييم الوظيفى للغشاء الخلوي في الحيوانات المنوية منزوعة البلازما فى الجاموس المصري

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أجريت هذه الدراسة علي عدد ١٢ طلوقة جاموس ناضج جنسياً عند عمر ٣- ٥ سنوات، وزن الجسم الحي ٤٠٠-٥٠٠كجم. وكان الهدف من الدراسة معرفة أفضل الظروف من الضغط الاسموزي عند تخفيف السائل المنوي بمحلول اللاكتوز- سترات الصوديوم عند المستويات المختلفه من الضغط الاسموزي (٥٠، ١٠٠، ١٥٠، ٢٠٠، ٣٠٠ ملي اوسمول/ لتر) سواء فى الحيوانات المنوية منزوعة أو غير منزوعة البلازما. تم تقدير النسبة المئوية لكل من حيوية الحيوانات المنوية، درجة الحركة التقدميه، الذيل الملتف والحيوانات المنوية المنتفخه أثناء التحضين علي درجة حراره ٣٧°م لمدة صفر ، ٥ ، ١٥، ٣٠ و ٦٠ دقيقة. أوضحت النتائج أن نزع بلازما السائل المنوي أدي الي زيادة النسبه المئوية لحيوية الحيوانات المنوية ودرجة الحركه التقدميه والنسبة المئوية للحيوانات المنوية ملتفة الذيل والمنتفخة بدرجه معنوية (على مستوى ٠,٠٥) مقارنة بالحيوانات المنويه الغير منزوعه البلازما. زيادة النسبه المئويه لحيوية الحيوانات المنوية ودرجة الحركه التقدميه بدرجه معنويه (على مستوي٠,٠٥) مع انخفاض النسبة المئوية للحيوانات المنوية ملتفة الذيل والمنتفخه بدرجه معنويه (على مستوي ٠,٠٥) في السائل المنوي سواء منزوع أو غير منزوع البلازما والمخفف بمحلول اللاكتوز- سترات الصوديوم علي مستوي ٢٠٠ أو ٣٠٠ ملي اوسمول / لتر مقارنه بمستوي ٥٠، ١٠٠أو١٥٠ملي اوسمول/ لتر. كان لزيادة فترة تحضين السائل المنوي علي درجة حراره ٣٧°م لمدة ٦٠ دقيقة سواء منزوع أوغير منزوع البلازما والمخفف بمحلول اللاكتوز- سترات الصوديوم عند المستويات المختلفه من الاسموزيه تأثيرا معنويا (على مستوي ٠,٠٥) علي إنخفاض النسبه المئوية

لحيوية الحيوانات المنوية ودرجة الحركه التقدميه بينما زادت النسبه المئويه للحيوانات المنويه ملتفة الذيل والمنتفخه معنوياً (على مستوي ٠,٠٥) مع زيادة فترة التحضين.

SUMMARY

Twelve buffalo bulls of 3-5 years of age and 400 to 500 kg live body weights were used in the present study. The experimental work was carried out to establish the optimum condition of osmotic pressure using lactose-Na-citrate solution at different osmolarities (50, 100, 150, 200, and 300 mOsmol/ L) either washed or non-washed buffalo spermatozoa. The percentage of sperm motility, grade of the progressive sperm motility (score), percentages of spermatozoa with coiled tail, and spermatozoa swelling, were determined during incubation at 37°C for 0, 5, 15, 30 and 60 minutes. The results showed that, washed buffalo bull semen increased significantly ($P<0.05$) the percentage of sperm motility, grade of the progressive sperm motility, percentages of spermatozoa with coiled tails and spermatozoa swelling as compared to the non-washed semen. The percentage of sperm motility and grade of the progressive sperm motility were significantly ($P<0.05$) increased, while the percentages of spermatozoa with coiled tail and spermatozoa swelling were significantly ($P<0.05$) decreased in the extended buffalo bull spermatozoa either washed or non-washed with lactose-Na-citrate solution at levels of 200 or 300 as compared to 50, 100 or 150 mOsmol/L. The advancement of incubation time at 37°C for up to 60 minutes of the extended buffalo bull spermatozoa either washed or non-washed semen with lactose- Na-citrate solution at different levels of osmolarities decreased significantly ($P<0.05$) the percentage of sperm motility and grade of the progressive sperm motility, while increased significantly ($P<0.05$) the percentages of spermatozoa with coiled tail and spermatozoa swelling.

***Key words**: Buffalo bulls, washed semen, sperm membrane, hypo-osmotic, swelling, coiled tails.*

INTRODUCTION

The routine semen analysis relies on assessing a number of parameters for the prediction of male fertility (Correa and Zavos, 1994). It is assumed that these parameters provide information about the status of spermatogenesis and fertilization potential by the sperm and the structural, but not the functional integrity of the sperm plasma membrane (Schradr *et al.*, 1986). However, it has been shown that these

parameters have limitations and cannot be used as reliable predictors of sperm fertilizing ability (Amann, 1989 and Zavos and Centola, 1990).

The study of the sperm membrane integrity is of particular important since an intact and functionally active membrane is required for metabolism, capcitation, acrosome reaction, attachment and penetration into oocyte (Jeyendran *et al.*, 1984). The elucidation of these mechanisms is of fundamental important to resolve cases of infertility and to the choice of optimal conditions for the assisted reproductive techniques such as in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (Langlais and Roberts, 1985). Thus assessment of the sperm membrane functional status appears to be a significant marker for the fertilizing capacity of spermatozoa (Jeyendran *et al.*, 1984 and Zaneveld *et al.*, 1990). The principle of hypo-osmotic swelling test (HOS-test) is based on the observation of the morphological alterations (size increase) in spermatozoa exposed to hypo-osmotic conditions (Drevius, 1972 and Jeyendran *et al.*, 1984). HOS-test assesses the integrity of the sperm membrane (Spittaler and Tyler, 1985 and Zaneveld *et al.*, 1987). HOS-test has been used for evaluation of sperm quality in some species such as man (Jeyendran *et al.*, 1992), dog (Kumi-Diaka and Badtram, 1994), horse (Caiza de laCueva *et al.*, 1997), ram (Moussa, 1999), buffaloes (El-Kishk, 2003) and bull (Zeidan, 2004). In addition, some studies have shown that the hypo-osmotic swelling test is highly predictive of pregnancy in women (Check *et al.*, 1989). On the other hand, the presence of seminal plasma causes an apparent reduction in glucose uptake by the spermatozoa (Flipse, 1954). Decapacitation factors obtained from the seminal plasma of various species inhibit corona penetrating enzyme which is an acrosomal enzyme involved in the passage of fertilizing spermatozoa through the corona radiata surrounding the ovum (Ahmed *et al.*, 1996). However, limited studies, so far, have been conducted on the effects of removal of seminal plasma from buffalo semen prior to extension and the response of spermatozoa to hypo-osmotic solution is still somewhat masked.

The present study was planed to evaluate the response of the washed and non-washed buffalo spermatozoa to the different hypo-osmotic solutions, during incubation at 37°C for 60 minutes.

MATERIALS and METHODS

The experimental work was carried out at Gommaiza Animal Production Research Station, Gommazia Village, Gharbiya Province, located in the northern part of the Nile Delta (31°), belong to Animal

Production Research Institute in cooperation, with Animal Production Department, Faculty of Agriculture, Mansoura University, Egypt. The experimental work was carried out to establish the optimum condition of osmotic pressure using lactose-Na-citrate hypo-osmotic solution at different osmolarities of 50, 100, 150 and 200 mOsmol/L compared to 300 mOsmol/L that cause the maximum number of idéntifiable swollen with washed or non-washed buffalo semen, during incubation at 37°C for up to 60 minutes.

Experimental animals:

Twelve sexually mature buffalo bulls at 3 to 5 years of age and weighed 400 to 500 kg average live body weight were used in the present study. They were housed individually under semi-open shed and partially roofed with asbestos forming 4x4 meters. All bulls were healthy and clinically free from external and internal parasites. Palpation of external genitalia tract showed that they were typically normal. The testicular tone was glandular and the epididymal regions were present and both testicles were almost equal in size and moved freely up and down within the scrotal pouches.

Feeding and management:

During the experimental period, all animals were individually fed co-op concentrate mixture according to NRC (1978) requirements. Dietary allowances were offered twice daily at 07.00 a.m. and 16.00 p.m. Clean and fresh water was offered three times daily.

Semen was collected from bulls twice weekly for 10 weeks throughout the experimental period (winter season) by using artificial vagina between 08.00 and 10.00 a.m. and was immediately evaluated after collection. Two successive ejaculates were obtained from each bull on each day of semen collection. Semen was collected, evaluated, pooled, and divided into two equal portions. The first portion (non-washed) was extended with 1 ml saline solution (0.9% NaCl) and the second portion was washed (treated) at 1000*g* for 15 minutes at room temperature according to Ahmed *et al.* (1996). After centrifugation of semen, seminal plasma was removed and the sperm plugs were re-suspended in lactose-Na-citrate extender to a volume equal to that of the semen before centrifugation.

Aliquots of each sperm rich-fraction (0.1 ml) either washed (treated) or non-washed (control) semen samples were added to 0.9 ml of the mixture lactose-Na-citrate solution. The response of the buffalo spermatozoa to HOS-test was assessed using solution prepared with lactose (1.25%) and Na-citrate (2.90%) in distilled water to give

osmolarity of 300 mOsmol/L using a freezing-point depression osmometer (Osmett A, Model 5002, Fisher Scientific, Pittsury, PA, USA). Semen was divided into five portions and then extended with lactose-Na-citrate solution at different osmalarities (50, 100, 150, 200 and 300 mOsmol/L) and the mixtures were incubated at 37°C for 0, 5, 15, 30 and 60 minutes. After each incubation time, percentages of sperm motility, grade of the progressive sperm motility, spermatozoa with coiled tail and swollen spermatozoa, were estimated. Grade of the progressive sperm motility was graded according to Zavos *et al.* (1994) as follows: Grade 1, oscillating movement but stationary, Grade 2, slow movement with no fixed direction, Grade 3, slow progressive movement and Grade 4, fast progressive movement. After each incubation time (0, 5, 15, 30, and 60 minutes), sperm swelling was assessed by placing 15 µl of well-mixed sample on a warm slide, which was covered with a cover glass before being observed under a phase contrast microscope at x1000. Slides were stained with eosin-nigrosin mixture stain. Two hundred spermatozoa per slide were counted and the percentage of swelling/coiling was determined (number of spermatozoa with swollen/coiled tail divided by the total number of spermatozoa counted multiplied by 100). The proportion of coiled/swollen spermatozoa from a control sample (300 mOsmol/L) was subtracted from the calculations (Vazquez *et al.*, 1997).

Data were statistically analyzed using Least Squares Analysis of Variance according to Snedecor and Cochran (1982). Percentage values were transformed to Arc-Sin values before being statistically analyzed. Duncan's new multiple range test used for the multiple comparisons (Duncan, 1955).

RESULTS

Motility of buffalo spermatozoa (%):

The results obtained in Table 1 showed that the effect of type of the extended semen (washed or non-washed) incubated with the different osmolarities of lactose- Na-citrate solutions on the percentage of motility of buffalo spermatozoa was significant ($P<0.05$). The washed semen was significantly ($P<0.05$) higher in the percentage of sperm motility at the different osmolarities of lactose-Na-citrate solutions than the non-washed semen, during incubation at 37°C for up to 60 minutes. The percentage of motile spermatozoa either washed or non-washed semen was approximately similar at osmolarities ranging between 50 to 150 mOsmol/L, while it was significantly ($P<0.05$) higher at 200 and 300

than 50, 100 and 150 mOsmol/L. The highest (P<0.05) value of the percentage of motile spermatozoa was recorded with osmolarity solution at 300 mOsmol/L and the lowest (P<0.05) value was recorded at 50 mOsmol/L either washed or non-washed semen.

The advancement of incubation time at 37°C decreased significantly (P<0.05) the percentage of sperm motility with the different osmolarities of lactose- Na-citrate solutions either washed or non – washed semen.

The interaction effects between osmolarities level and incubation time on the percentage of sperm motility were significant (P<0.05).

Grade of the progressive motility of buffalo spermatozoa (Score):

Data presented in Table 2 showed that the effect of type of the extended semen (washed or non-washed) incubated with the different osmolarities of lactose-Na-citrate solutions on the grade of the progressive motility of buffalo spermatozoa was significant (P<0.05). The washed semen was significantly (P<0.05) higher in the grade of the progressive sperm motility at the different osmolarities of lactose –Na-citrate solutions than the non-washed semen, during incubation at 37°C for up to 60 minutes. The grade of the progressive motility of spermatozoa either washed of non-washed semen was approximately similar at osmolarities ranging between 50 to 150 mOsmol/L, while it was significantly (P<0.05) higher at 200 and 300 than 50, 100 and 150 mOsmol/L. The highest (P<0.05) value of the grade of the progressive motility of spermatozoa was recorded with osmolarity solution at 300 mOsmol/L and the lowest (P<0.05) value was recorded at 50 mOsmol/L either washed or non-washed semen.

The advancement of incubation time at 37°C decreased significantly (P<0.05) the grade of the progressive sperm motility with the different osmolarities of lactose –Na-citrate solutions either washed or non-washed semen.

The interaction effects between osmolarity level and incubation time on the grade of sperm motility were significant (P<0.05).

Buffalo spermatozoa swelling (%):

Table 3 showed that the effect of type of the extended semen (washed or non-washed) with the different osmolarities of lactose-Na-citrate solutions on the percentages of buffalo spermatozoa swelling was significant (P<0.05). The washed semen was significantly (P<0.05) higher in the percentages of buffalo spermatozoa swelling at the different osmolarities of lactose-Na-citrate solutions than the non-washed semen, during incubation at 37°C for up to 60 minutes. The

percentages of buffalo spermatozoa swelling either washed or non-washed semen was approximately similar at osmolarities ranging between 50 to 150 mOsmol/L, while it was significantly ($P<0.05$) lower at 200 and 300 than 50, 100 and 150 mOsmol/L. The lowest ($P<0.05$) value of the percentage of buffalo spermatozoa swelling was recorded with osmolarity solution at 300 mOsmol/L and the highest ($P<0.05$) value was recorded at 50 mOsmol/L either washed or non-washed semen. A scoring system (HOS-test ranking) based on sperm swelling patterns is shown in Figure 1.

The advancement of incubation time at 37°C increased significantly ($P<0.05$) the percentages of swollen spermatozoa with the different osmolarities of lactose-Na-citrate solutions either washed or non-washed semen.

The interaction effects between osmolarity level and incubation time on the percentage of swollen spermatozoa were significant ($P<0.05$).

Coiled tails of buffalo bull spermatozoa (%):

Table 4 showed that the effect of type of the extended semen (washed or non-washed) with the different osmolarities of lactose-Na-citrate solutions on the percentage of buffalo spermatozoa with coiled tail was significant ($P<0.05$). The washed semen was significantly ($P<0.05$) higher in the percentage of spermatozoa with coiled tail at the different osmolarities of lactose-Na-citrate solutions than the non-washed semen, during incubation at 37°C for up to 60 minutes. The percentages of buffalo spermatozoa with coiled tail either washed or non-washed semen was approximately similar at osmolarities ranging between 50 to 150 mOsmol/L, while it was significantly ($P<0.05$) lower at 200 and 300 than 50, 100 and 150 mOsmol/L. The lowest ($P<0.05$) value of the percentage of spermatozoa with coiled tail was recorded with osmolarity solution at 300 mOsmol/L and the highest ($P<0.05$) value was recorded at 50 mOsmol/L either washed or non-washed semen.

The advancement of incubation time at 37°C increased significantly ($P<0.05$) the percentage of coiled tails of spermatozoa with the different osmolarities of lactose-Na-citrate solutions either washed or non- washed semen.

The interaction effects between osmolarity level and incubation time on the percentage of spermatozoa with coiled tails were significant ($P<0.05$).

Table1: Mean percentage of motility of washed and non-washed buffalo spermatozoa as affected by different hypo-osmotic solutions, during incubation at 37^{0}C for up to 60 minuets.

Incubation time (minutes)	Type of semen											
	Non-washed semen					Means	Washed semen					Means
	Osmolality (mOsmol/L)						Osmolality (mOsmol/L)					
	50	100	150	200	300		50	100	150	200	300	
0	55.43 ± 1.35	55.62 ± 1.26	56.21 ± 1.18	60.25 ± 1.19	65.78 ± 1.22	58.66 ± 1.99 ^{A}	62.19 ± 1.62	62.15 ± 1.43	62.58 ± 1.27	66.28 ± 1.25	70.82 ± 1.15	64.80 ± 1.69 ^{A}
5	43.35 ± 1.16	43.51 ± 1.61	45.11 ± 1.13	56.19 ± 1.54	65.18 ± 1.24	50.67 ± 4.34 ^{B}	56.12 ± 1.23	56.26 ± 1.45	56.49 ± 1.18	63.19 ± 1.16	70.35 ± 1.25	60.48 ± 2.81 ^{B}
15	20.42 ± 1.14	21.13 ± 1.48	21.18 ± 1.18	48.11 ± 1.39	62.45 ± 1.35	34.66 ± 8.72 ^{C}	34.48 ± 1.81	34.73 ± 1.15	35.14 ± 1.33	58.42 ± 1.19	70.14 ± 1.53	46.58 ± 7.46 ^{C}
30	13.15 ± 1.12	13.28 ± 1.65	13.85 ± 1.25	40.18 ± 1.72	60.16 ± 1.19	28.12 ± 9.54 ^{D}	26.29 ± 1.18	26.62 ± 1.13	26.74 ± 1.28	52.25 ± 1.64	69.28 ± 1.37	40.27 ± 8.80 ^{D}
60	6.18 ± 1.42	6.20 ± 1.78	6.76 ± 1.27	30.19 ± 1.65	55.12 ± 1.52	20.89 ± 9.72 ^{E}	15.75 ± 1.24	15.78 ± 1.08	16.13 ± 1.14	43.12 ± 1.12	68.86 ± 1.36	31.93 ± 10.63 ^{E}
Means	27.71^c ± 9.33	27.95^c ± 9.33	28.62^c ± 9.45	46.98^b ± 5.43	61.74^a ± 1.94	38.60^b	38.97^c ± 8.81	39.11^c ± 8.79	39.42^c ± 8.80	56.65^b ± 4.13	69.89^a ± 0.36	48.81^a

a,b,c : Means with different superscripts in the same row, differ significantly (P<0.05) .

A, B, C, D, E: Means with different superscripts in the same column, differ significantly (P<0.05).

Table 2: Mean of grade of the progressive motility (score) of washed and non-washed buffalo spermatozoa as affected by different hypo-osmotic solutions, during incubation at 37^{0}C for up to 60 minutes.

Incubation time (minutes)	Type of semen											
	Non-washed semen					Means	Washed semen					Means
	Osmolality (mOsmol/L)						Osmolality (mOsmol/L)					
	50	100	150	200	300		50	100	150	200	300	
0	3.23 ± 0.12	3.25 ± 0.11	3.31 ± 0.12	3.41 ± 0.19	3.62 ± 0.19	3.36^A ± 0.07	3.45 ± 0.36	3.40 ± 0.41	3.58 ± 0.15	3.84 ± 0.19	3.92 ± 0.22	3.64^A ± 0.10
5	2.58 ± 0.19	2.60 ± 0.12	2.75 ± 0.13	3.20 ± 0.16	3.60 ± 0.18	2.95^B ± 0.20	3.17 ± 0.22	3.42 ± 0.15	3.48 ± 0.23	3.60 ± 0.16	3.90 ± 0.17	3.51^A ± 0.12
15	1.62 ± 0.11	1.76 ± 0.25	1.84 ± 0.14	2.85 ± 0.41	3.52 ± 0.16	2.32^B ± 0.37	2.04 ± 0.36	2.15 ± 0.42	2.18 ± 0.11	3.35 ± 0.22	3.84 ± 0.14	2.71^B ± 0.37
30	1.06 ± 0.13	1.10 ± 0.22	1.12 ± 0.17	2.36 ± 0.10	3.40 ± 0.12	1.81^C ± 0.47	1.35 ± 0.48	1.35 ± 0.17	1.40 ± 0.13	2.84 ± 0.18	3.75 ± 0.16	2.14^B ± 0.49
60	0.32 ± 0.16	0.43 ± 0.21	0.55 ± 0.41	1.86 ± 0.26	3.21 ± 0.37	1.27^C ± 0.56	1.14 ± 0.51	1.20 ± 0.15	1.28 ± 0.21	2.46 ± 0.20	3.64 ± 0.15	1.94^C ± 0.49
Means	1.76^c ± 0.52	1.83^c ± 0.51	1.91^c ± 0.51	2.74^b ± 0.28	3.47^a ± 0.08	2.34^b	2.23^c ± 0.47	2.30^c ± 0.48	2.38^c ± 0.49	3.22^b ± 0.25	3.81^a ± 0.05	2.79^a

a,b,c : Means with different superscripts in the same row, differ significantly (P<0.05) .
A, B, C: Means with different superscripts in the same column, differ significantly (P<0.05).

Table 3: Mean percentage of swollen (hypo-osmotic swelling test response) of washed and non-washed buffalo spermatozoa as affected by different hypo-osmotic solutions, during incubation at 37^{0}C for up to 60 minuets.

Incubation time (minutes)	Type of semen											
	Non-washed semen					Means	Washed semen					Means
	Osmolality (mOsmol/L)						Osmolality (mOsmol/L)					
	50	100	150	200	300		50	100	150	200	300	
0	14.16 ± 0.82	13.26 ± 1.02	13.18 ± 1.10	10.45 ± 0.75	7.54 ± 0.82	11.72^B ± 1.22	17.14 ± 1.06	16.43 ± 1.14	16.25 ± 1.30	12.73 ± 1.15	10.13 ± 0.82	14.54^B ± 1.34
5	16.18 ± 1.12	15.16 ± 1.12	15.4 ± 1.02	10.52 ± 0.85	7.68 ± 0.75	12.94^B ± 1.64	19.87 ± 1.30	19.27 ± 1.43	19.15 ± 1.02	13.18 ± 0.	10.24 ± 0.85	16.34^B ± 1.95
15	18.55 ± 1.16	18.26 ± 1.13	17.10 ± 1.13	12.71 ± 0.86	8.91 ± 0.82	15.11^A ± 1.87	24.75 ± 1.08	24.68 ± 0.82	23.84 ± 1.12	16.12 ± 1.04	11.73 ± 1.02	20.22^A ± 2.67
30	21.14 ± 1.05	20.87 ± 1.02	20.14 ± 0.96	14.22 ± 1.66	9.15 ± 0.88	17.10^A ± 2.36	28.66 ± 1.16	27.43 ± 1.05	27.22 ± 1.23	18.32 ± 0.92	12.58 ± 1.04	22.84^A ± 3.16
60	19.88 ± 1.11	19.65 ± 1.15	19.12 ± 1.14	13.26 ± 0.92	8.47 ± 1.01	16.08^A ± 2.26	25.78 ± 1.15	25.38 ± 1.32	25.20 ± 1.25	17.45 ± 1.20	11.86 ± 1.31	21.13^A ± 2.79
Means	17.98^a ± 1.26	17.44^a ± 1.41	16.94^a ± 1.27	12.23^b ± 0.75	8.35^c ± 0.32	14.59^b	23.24^a ± 2.08	22.64^a ± 2.06	22.33^a ± 2.02	15.56^b ± 1.12	11.31^c ± 0.48	19.01^a

a,b,c : Means with different superscripts in the same row, differ significantly (P<0.05).
A, B: Means with different superscripts in the same column, differ significantly (P<0.05).

Table 4: Mean percentage of coiled tails (occurrence of osmotic shock) of washed and non-washed buffalo spermatozoa as affected by different hypo-osmotic solutions, during incubation at 37^{0}C for up to 60 minuets.

Incubation time (minutes)	Type of semen											
	Non-washed semen					Means	Washed semen					Means
	Osmolality (mOsmol/L)						Osmolality (mOsmol/L)					
	50	100	150	200	300		50	100	150	200	300	
0	30.28 ± 1.40	30.15 ± 1.76	30.14 ± 1.30	12.08 ± 1.20	7.10 ± 1.14	21.95 ^{C} ± 5.11	36.42 ± 1.15	36.40 ± 1.25	36.12 ± 1.73	16.25 ± 1.40	12.62 ± 1.21	27.56 ^{C} ± 5.39
5	33.38 ± 0.85	33.25 ± 1.21	32.19 ± 0.86	13.11 ± 1.12	7.16 ± 1.21	23.82 ^{C} ± 5.67	40.53 ± 0.71	40.38 ± 0.84	39.80 ± 1.15	18.15 ± 1.11	12.91 ± 1.13	30.33 ^{C} ± 6.11
15	36.62 ± 1.42	36.51 ± 0.86	36.21 ± 1.02	15.13 ± 0.78	8.92 ± 0.92	26.68 ^{B} ± 6.06	46.64 ± 1.18	46.43 ± 1.16	45.94 ± 1.12	22.28 ± 0.84	13.08 ± 1.20	34.87 ^{B} ± 7.17
30	41.74 ± 0.82	46.66 ± 0.67	40.13 ± 1.04	17.22 ± 1.10	10.06 ± 0.95	29.96 ^{A} ± 6.76	50.17 ± 0.83	50.11 ± 1.10	50.02 ± 0.88	24.73 ± 1.06	15.12 ± 1.05	38.03 ^{A} ± 7.55
60	39.19 ± 1.03	39.15 ± 1.92	39.12 ± 1.10	16.35 ± 1.84	9.17 ± 1.02	28.60 ^{A} ± 6.56	48.84 ± 1.06	48.65 ± 1.10	48.56 ± 1.13	23.28 ± 1.14	14.13 ± 1.04	36.69 ^{A} ± 7.48
Means	36.24^a ± 2.03	35.94^a ± 1.92	35.56^a ± 1.93	14.78^b ± 0.96	8.48^c ± 0.58	26.20^b	44.52^a ± 2.61	44.39^a ± 2.60	44.09^a ± 2.65	20.94^b ± 1.60	13.57^c ± 0.46	33.50^a

a,b,c : Means with different superscripts in the same row, differ significantly (P<0.05).
A, B, C: Means with different superscripts in the same column, differ significantly (P<0.05).

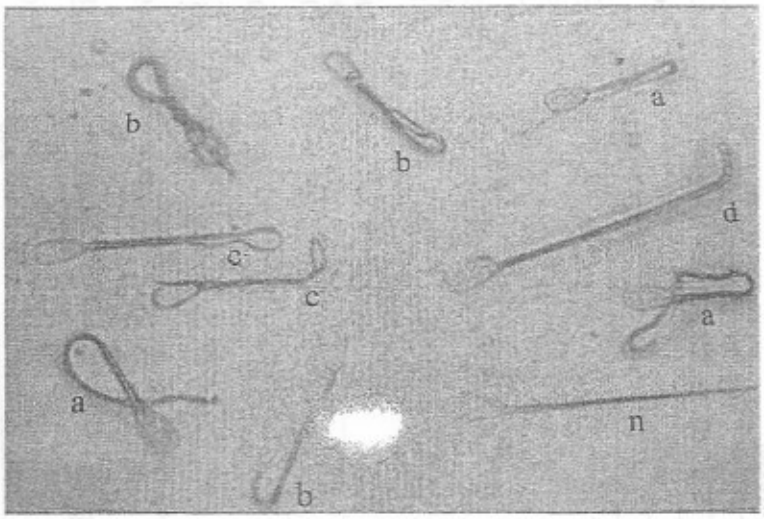

Fig. 1: Micrograph of the spermatozoa swelling patterns as measured by the hypo-osmotic swelling test (HOS-t). Type a: represents maximal sperm swelling. Type b and c: represents intermediate sperm swelling stages. Type d: represents the initial sperm swelling response in the HOS-test. Type n: represents non-swollen spermatozoa that are considered to have a functionally inactive or damaged sperm membrane.

DISCUSSION

The main objectives of the present study were to assess the effects of the different osmolarity levels (50,100,150,200 and 300 mOsmol/L) on the percentage of sperm motility, grade of the progressive sperm motility (score), percentages of spermatozoa swelling and coiled tails of spermatozoa either washed or non- washed buffalo bulls semen. The results obtained indicate that, the percentage of sperm motility and grade of the progressive sperm motility in the washed buffalo semen was higher at the different osmolarities of lactose-Na-citrate solutions than the non-washed semen, during incubation at 37°C for 60 minutes. The results obtained indicate also that, the percentages of sperm motility and grade of the progressive sperm motility either washed or non- washed buffalo were approximately similar at osmolarities of 50, 100 and 150 mOsmol/L, but tended to the higher at osmolarities of 200 and 300 than 50,100 and 150 mOsmol/L. This phenomenon may be due to difference that may exist in the rate of active

transport of the physical and biochemical compounds across the sperm membrane which is considered to have an important biochemical role for maintaining high sperm viability and fertilizing capabilities (Keel and Webster, 1990) or due to an abrupt decrease in osmotic pressure which results in loss of sperm motility (Zavos, 1983). The obtained results are in agreement with those of Jeyendran *et al.* (1984) in man and Correa and Zavos (1994) and Zeidan (2004) in bull spermatozoa. Higher correlations were obtained after exposure of the spermatozoa to the hypotonic solution. These spermatozoa were undergoing induced swelling, which resulted in alteration or progressive loss of motility (Zavos *et al.*, 1996).

Occurrence of osmotic shock as reflected by the percentage of coiled tails of spermatozoa and swollen spermatozoa in the washed buffalo semen was less apparent at the different osmolerities of lactose-Na-citrate solutions than non- washed semen, during incubation at 37°C for 60 minutes. Our results indicate that, the percentages of spermatozoa with coiled tails and spermatozoa swelling either washed or non-washed buffalo semen were approximately similar at osmolarities of 50 ,100 and 150 mOsmol/L, but seemed to be lower at osmolarities of 200 and 300 than 50,100 and 150 mOsmol/L. These results may attributed to that the sperm tail membrane bulges and swells in response to the hypo-osmotic medium because of the influx of fluids into the spermatozoa as has been described in bull (Drevius and Eriksson 1966), and in man (Schrader *et al.*, 1986). Similar results were reported by Correa and Zavos (1994) in bull, Vazquez *et al.* (1997) in boar, El-Kishk (2003) in buffalo and Zeidan and Ahmadi (2004) in camel spermatozoa. The degree of the coiled tails of spermatozoa varied significantly among spermatozoa samples (Moussa, 1999). This variation ranged from cases in which only the distal half of the sperm tail was coiled, to situations in which coiling of spermatozoa to coiling in response to the HOS-test, would imply normal membrane integrity of the HOS-reacted spermatozoa, that is the ability between the fluid compartment of the spermatozoa and external environment (Drevius, 1972 and Jeyendran *et al.*, 1984).

With regard to incubation time, the advancement of incubation time at 37°C for 0, 5, 15, 30 and 60 minutes decreased the percentages of sperm motility and grade of the progressive sperm motility either washed or non- washed buffalo semen at the different osmolarities of lactose-Na-citrate solutions. In contrast, the percentages of spermatozoa with coiled tails and swollen spermatozoa increased by advancing of incubation time. The osmotic shock phenomenon, caused by the

exposure of the extended spermatozoa to hypotonic conditions is characterized by increased coiling of the sperm tail, which results in loss of sperm motility (Zavos, 1983). The degree of spermatozoa swelling is dependent on cellular water uptake per unit of time. Under these conditions, the reliability of the assay was very high as reported by Jeyendran *et al.* (1984) in human spermatozoa. The same authors showed that the capability of human spermatozoa to swell in a hypo-osmotic solution depended on the compounds in the solution. Moreover, the osmotic shock phenomenon, caused by the exposure of extended spermatozoa to hypotonic conditions is characterized by increased coiling of the sperm tail. Similar trend was reported by Correa and Zavos (1995) in bovine, Kumi-Diaka (1993) in canine and Vazquez *et al.* (1997) in boar spermatozoa. It is worth noting that, the maximum reactivity of spermatozoa to HOS-test (spermatozoa with coiled tails and swollen spermatozoa) was reached at 30 minutes of incubation at 37°C. The response of spermatozoa to HOS-test is depending on the cellular water uptake (osmolarity level) per time unit (incubation time). Similarly, Zeidan (2004) found that all reacted bovine spermatozoa showed maximal coiling within 30 minutes of incubation at 37°C.

In conclusion, the removal of seminal plasma of the buffalo semen is a practical and effective technique, which provides a useful alternative to semen stored in liquid form. Our results indicate that buffalo spermatozoa show a clear tail swelling reaction when they were incubated in hypo-osmotic media. The tail swelling was dependent upon the osmolarity level of the media and also upon the time of incubation. It has been suggested that hypo-osmotic swelling test could be used to improve the buffalo semen analysis, identifying male's infertility and in predicting the outcome of *in vitro* fertilization (IVF).

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