REACTION OF HOLSTEIN BULL SPERMATOZOA TO VARIOUS HYPO-AND HYPER-OSMOLARITY LEVELS UNDER DIFFERENT INCUBATION TIMES

BY

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ABSTRACT

The current study aimed to evaluate the response of Holstein spermatozoa to osmotic shock under hypo- and hyper-osmolarity levels. Semen was collected from five healthy mature Holstein bulls for 10 weeks (50 ejaculates). Osmotic test was designed to establish various levels of osmolarity and incubation times that would give the maximum percentage of curled spermatozoa at different hypo-osmotic levels, (0, 50,, 100, 150 and 200 mOsm) or to establish percentage of shrunk spermatozoa at hyper-osmotic levels(400 and 600 mOsm) as compared to 300 mOsm at 0, 15, 30, 45 and 60 minutes incubation times. Percentage of total curled spermatozoa significantly ($P<0.05$) increased by decreasing the osmotic level from 600 down ward to 0 mOsm, showing the highest reactivity at 0 mOsm solution (74.9%) and the lowest one at 600 mOsm one (13.1%). The significant ($P<0.05$) increase in total curling was associated with increase ($P<0.05$) in the frequency distribution of B and C curling types and significant ($P<0.05$) decrease in the frequency distribution of type A curling. Percentage of total curled spermatozoa also increased by increasing incubation time. The maximum percentage ($P<0.05$) was observed after 30 min incubation (51.1%), thereafter insignificant increase was detected up to 60 min incubation (54.4%). A significant ($P<0.05$) decrease in type A curling and increase in type C were observed after 30 minutes. Significant ($P<0.05$) increase in type B curling was observed after 60 minutes. Percentage of shrunk spermatozoa and total response were significantly ($P<0. 01$) higher at 600 than 400 mOsm (50.6 and 63.7 vs. 41.4 and 59.4%, respectively). Percentage of shrunk spermatozoa significantly ($P<0.05$) increased by increasing incubation time from 0 up tp 45 min (54.7 and 70.2%, respectively) with highest rate of increase between 15 and 30 min.

The present results indicated that osmotic test provide a precise technique for measuring alterations in sperm viability and membrane integrity of spermatozoa, in particular at 0 mOsm. Thus water test could be used as an important additional indicator of male fertility.
Keyword: Bull, spermatozoa, lactose, osmolarity, incubation time.

INTRODUCTION

Membrane functionality of spermatozoon is essential to maintaining sperm motility and carrying out all the events related to its fertilizability (Jeyendran *et al.*, 1984). Spermatozoa behave as osmometers (Drevius, 1972) and undergo curling in different levels of hypo-osmotic (Watson *et al.*, 1992) or shrinkage in hyper-osmotic (Liu and Foote, 1998) solutions. Assessment of this functional aspect is a useful complement to semen analysis.

The osmotic responses of spermatozoa and the relationship of the responses to success in cryo-preservation have been studied by several authors (Choudhry *et al.*, 1995; and Peris *et al.*, 2000). A simple but effective test can be performed to evaluate the physical and functional integrity of the sperm membrane through exposure to hypo-osmotic conditions (i.e. the hypo-osmotic-swelling test. HOS-test). The HOS-test evaluates the ability of cells to swell or curl in a hypo-osmotic solution, thus indicating whether an intact membrane is also biochemically active or, in other words, determining functional integrity of sperm membranes (Correa and Zavos, 1994 and Engel and Petzoldt, 1994).

Some authors reported different responses of sperm reactivity, ranging between occurrence of swelling (Bakst, 1980) and lysis (Watson *et al.*, 1992) in water alone (0 mOsm). This response was almost depending on time of incubation.

The present work aims to study the response of Friesian spermatozoa to different hypo- (0, 50, 100, 150 and 200 mOsm) and hyper- (400 and 600 mOsm) osmotic levels as compared to 300-mOsm solution, associated with different incubation times (0, 15, 30, 45 and 60 min).

MATERIALS AND METHODS

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

Animal:

A total of five Holstein bulls (4-10 years) raised at the ILMTC was used in this study. All bulls were sexually mature and free of any diseases with healthy appearance. Bulls were individually fed daily ration composed of 8 kg concentrate fed mixture (CFM), clover hay (4 kg) and rice straw (4 kg). Bulls were housed individually under semi-open sheds.

Semen collection:

Semen was collected twice a week from each of the experimental bulls using the conventional artificial vaginal method. Only one ejaculate from each bull was weekly taken immediately to the laboratory and pooled for osmotic test, while the other ejaculate was cryo-preserved as a common practice in ILMTC. Total of 50 ejaculates were collected (5 bulls for 10 weeks) before feeding at 8.00 a.m. An infertile bull was used as teaser animal for sexual preparation and mounting during semen collection. The collection of semen for this study was undertaken during the period from October to December 2003. Only semen with mass motility of 70% or more was used in this study.

Osmotic solutions:

The response of spermatozoa to osmotic test was assessed using solution prepared with lactose (2.5%) and Na-citrate (5.8%) in triple distilled water to give osmolarity of 600 mOsm using a freezing-point depression osmometer (Osmett A, Model 5002, Fisher Scientific, Pittsbury, PA, USA).

The final osmolarity of the tested solutions was prepared from 600 down ward to 50 mOsm via serial dilutions by distilled water to obtain different six osmotic solutions (50, 100, 150, 200, 300 and 400 mOsm). While, tripled distilled water was used as 0 mOsm solution.

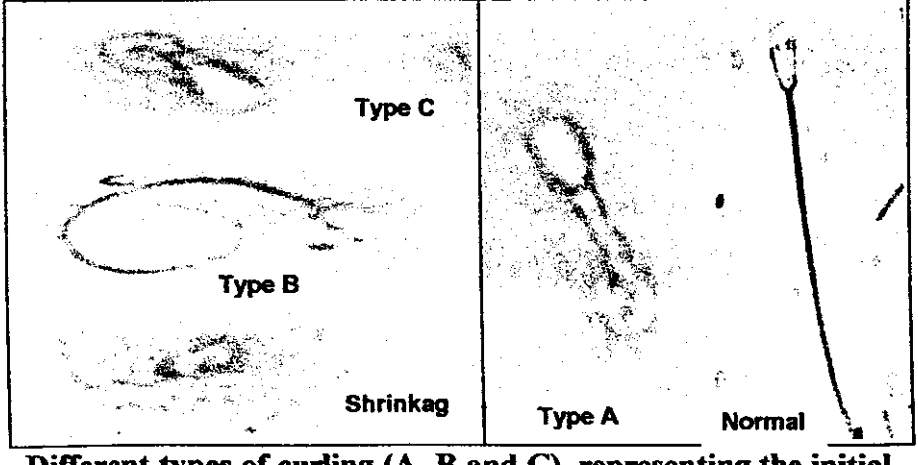
Incubation time:

Total of 5 aliquots were used from the weekly-pooled semen of five Holstein bulls for 10 collection weeks. After

collection and semen pooling, specimens from each aliquot (50 µl) were added to one ml of each of the predetermined osmotic solutions into clean and warm glass tube and the mixtures were immediately examined for zero time (0 time), then they were examined after semen incubation for 5, 15, 30, 45 and 60 min at 37 °C, respectively.

At each incubation time, numbers of spermatozoa with curled tail at all osmolarity levels or shrunk spermatozoa at hyper-osmotic levels were assessed by placing about 15 µl of well-mixed samples on a warm slide (37°C). A semen smear from the mixture was prepared and dried at the same temperature. The slides were stained with eosin-nigrosin mixture stain. All prepared slides were examined using research microscope at a high power magnification (x 400). Two hundred spermatozoa per slide were counted and the number of spermatozoa having each of different types of curling (A, B and C) or shrunk spermatozoa was counted. Thereafter, percentage of total spermatozoa having curled tails or shrunk spermatozoa was computed and frequency distribution of each type of curling (number of spermatozoa with each type of curling divided by the total number of curled spermatozoa counted, multiplied by 100) was calculated in each microscopic field.

A scoring system (Test ranking) of the osmotic test based on sperm curling types is illustrated in the following plates:



Different types of curling (A, B and C), representing the initial, intermediate and maximal curling of spermatozoa.

Statistical analysis:

A factorial design (8 x 6) was used to establish the osmolarity level (0, 50, 100, 150, 200, 300, 400 and 600 mOsm) and the incubation time (0, 5, 15, 30, 45 and 60 min). Also, a factorial design (2 x 6) to estimate percentage of shrunk spermatozoa with hyper-osmolarity levels (400 and 600 mOsm) at different incubation times (0, 5, 15, 30, 45 and 60 min) was applied.

The percentage values of curled/shrunk spermatozoa were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed values to percentages. Results were statistically analyzed according to Snedecor and Cochran (1982).

RESULTS

Effect of osmorality level:

The effect of osmolarity levels, regardless the incubation time, on the percentages of total curled spermatozoa and frequency distribution of different types of swelling were highly significant ($P<0.01$, Table 1).

Data in table (1) revealed significant ($P<0.05$) increase in percentage of total curled spermatozoa by decreasing the osmotic level from 0 up to 600 mOsm, showing the highest percentage of swelling with 0 mOsm solution and the lowest values with 600 mOsm solution. Such rise was paralleled with significant ($P<0.01$) increase in the frequency distribution of types B and C of curled spermatozoa and significant ($P<0.01$) increase in the frequency distribution of type A of curled spermatozoa.

Effect of incubation time:

Regardless osmolarity level, the percentage of total curled spermatozoa significantly ($P<0.05$) increased by increasing incubation time from 0 up to 30 min, thereafter it insignificantly increased to reach the maximal curling at 60 min incubation time (Table 2). This trend of increase in percentage of total curled spermatozoa was associated with significant ($P<0.05$) increase in frequency distribution of curling type B over 60 min incubation time. However, frequency distribution of spermatozoa with curling

type A insignificantly decreased and those with curling type C insignificantly increased by increasing incubation time from 0 to 60 min (Tables 2).

Table (1): Percentage of total and different types of curled spermatozoa of Holstein bulls as affected by osmolarity level.

Osmolarity level (mOsm)	Total curled spermatozoa (%)	Frequency distribution of curling (%)		
		Type A	Type B	Type C
0	74.9±1.3^{a}	21.3±0.5^{h}	35.5±0.2^{a}	43.2±0.4^{a}
50	69.6±1.3^{b}	26.8±0.5^{g}	32.8±0.3^{c}	40.4±0.3^{b}
100	62.6±1.3^{c}	32.3±0.5^{f}	29.1±0.3^{d}	38.6±0.4^{c}
150	55.5±1.4^{d}	38.1±0.6^{e}	29.9±0.7^{d}	32.0±0.7^{d}
200	47.3±1.4^{e}	45.7±0.5^{d}	34.3±0.3^{b}	20.0±0.4^{e}
300	46.5±1.4^{e}	49.5±0.3^{c}	34.2±0.3^{b}	16.3±0.2^{f}
400	18.0±0.4^{f}	91.1±2.7^{b}	8.9±1.1^{e}	00.0±0.0^{g}
600	13.1±0.5^{g}	96.4±2.1^{a}	3.6±0.99^{f}	00.0±0.0^{h}

a, b...h: Means having different superscripts in the same column are significantly different at P<0.01.

Table (2): Percentage of total and different types of curled spermatozoa of Holstein bulls as affected by incubation time.

Incubation time (min)	Total curled spermatozoa (%)	Frequency distribution of curling (%)		
		Type A	Type B	Type C
0	39.1±1.9^{c}	52.8±2.9	24.7±1.3^{b}	22.5±1.7
15	44.3±2.2^{b}	51.4±3.0	25.0±1.4^{b}	23.6±1.7
30	51.1±2.6^{a}	49.5±3.0	25.8±1.3^{ab}	24.7±1.8
45	53.4±2.7^{a}	48.9±3.0	26.6±1.4^{ab}	24.5±2.0
60	54.4±2.7^{a}	48.2±3.1	28.1±1.4^{a}	23.7±1.9

a, b and c: Means having different superscripts in the same column are significantly different at P<0.05.

Effect of interaction:

As affected by the interaction between osmolarity level and incubation time, percentage of total curling significantly ($P<0.05$) increased by decreasing osmolarity level from 200 down ward to 0 mOsm. At these levels of osmolarity, percentage of total curling significantly ($P<0.05$) increased when incubation time increased from 0 up to 30 min, thereafter, it showed slight increase by increasing incubation time up to 60 min. Another trend of increase ($P<0.05$) in total curling percentage was observed by decreasing osmolarity level from 600 down ward to 300 mOsm, being similar for all incubation times with hypo-osmotic levels (400 and 600 mOsm) and significantly ($P<0.05$) increased when incubation time increased from 0 up to 30 min with 300 mOsm. It is interest to note that there were insignificant changes in percentage of total curling between 200 and 300 mOsm under each incubation time. Generally, the highest percentage of observed total curling was recorded at 0 mOsm after 60 min incubation, while the lowest one was found at 600 mOsm (Fig. 1A).

Concerning the frequency distribution of different types of curling, it was obvious that distribution of curling type A showed significant ($P<0.05$) increase at all incubation times by increasing osmolarity level from 0 up to 400 mOsm, thereafter it showed in significant change from 400 to 600 mOsm. The differences among all incubation times at each osmolaity level were not significant (Fig. 1 B). The opposite was observed for the distribution of curling type C (Fig. 1D). On the other hand, the frequency distribution of curling type B was not affected significantly by incubation times and osmolarity level from 300 down ward to 0 mOsm, but showed significant ($P<0.05$) decrease by increasing osmolarity level from 300 up to 600 mOsm, with insignificant differences among incubation times at each osmolarity level (Fig.1 C). Generally, the highest response of spermatozoa to curl in both types B and C and the lowest distribution of curling type A were observed at 0 mOsm after 60 min incubation (Fig. 1 A-D).

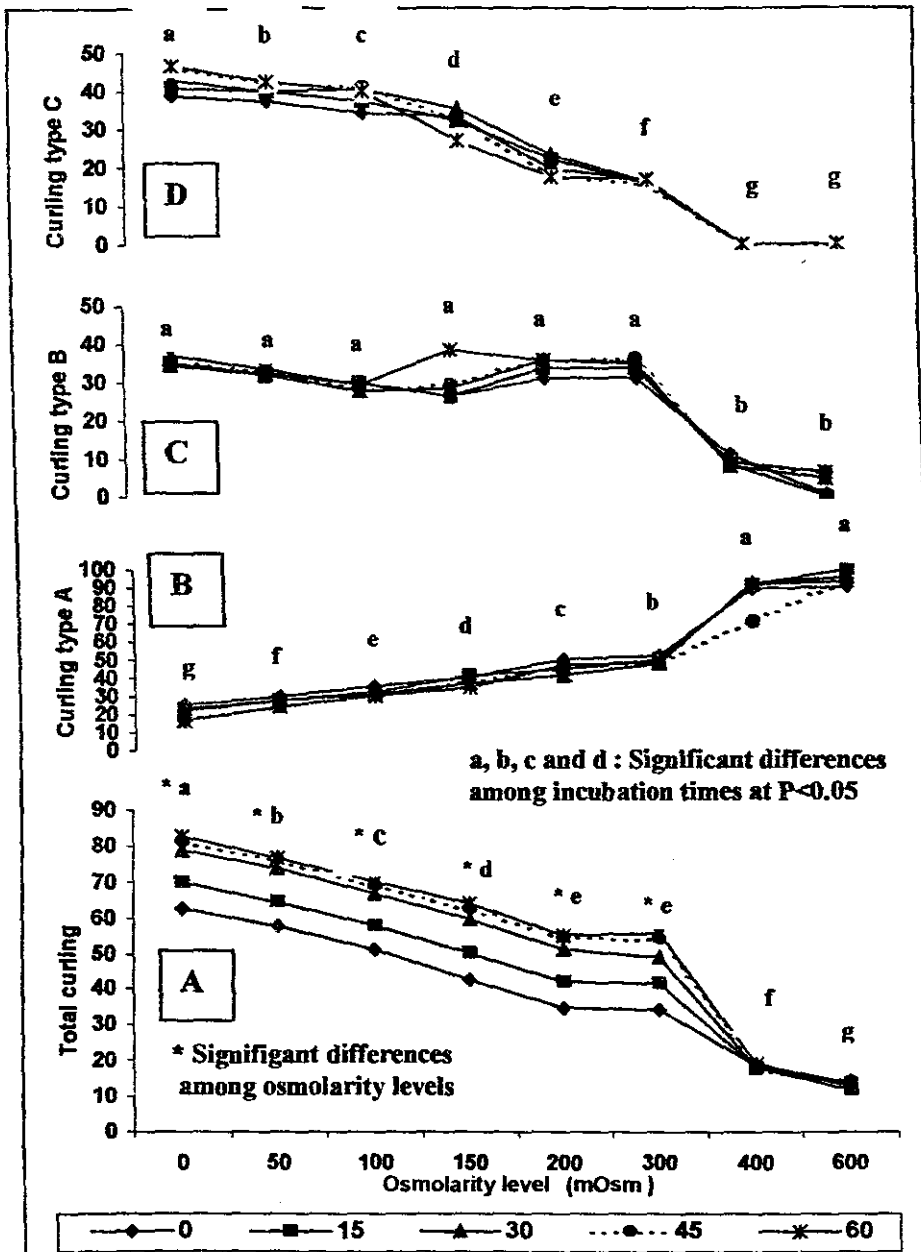


Fig. (1 A-D): Changes in percentage of total curled spermatozoa and frequency distribution of different types of curling (A, B and C) at different osmolarity levels and incubation times.

Shrunk spermatozoa:

Effect of hyper-osmorality level:

The effect of hyper-osmolarity level on the percentage of shrunk spermatozoa was significantly ($P<0.001$) higher at 600 than 400 mOsm, being in contrast to percentage of total curling which showed the opposite trend. This was reflected in significantly ($P<0.05$) higher total response of spermatozoa at 600 than 400 mOsm (Table 3).

It is of interest to not that the significant ($P<0.01$) increase in percentage of shrunk spermatozoa by increasing the osmotic level from 400 to 600 mOsm may be associated with increased water transportation from intracellular fluids to extracellular hyper-osmotic solution.

Table (3): Percentage of shrunk, curled and total responded spermatozoa of Holstein bulls as affected by hyper-osmolarity level and incubation time.

Item	Shrinking (%)	Curling (%)	Total response (%)
Hyper-osmolarity level (mOsm):			
400	41.4±1.5^{b}	18.0±0.4^{a}	59.4±1.5^{b}
600	50.6±1.6^{a}	13.1±0.5^{b}	63.7± 1.3^{a}
Incubation time (minute):			
0	31.1±1.4^{d}	15.7±2.1	46.8± 1.1^{e}
15	38.4±1.5^{c}	14.7±2.3	53.1±1.2^{d}
30	49.0±1.6^{b}	15.6±1.9	64.6± 1.2^{c}
45	54.7±1.0^{a}	15.5±2.1	70.2±1.1^{b}
60	56.8±1.5^{a}	16.2±1.6	73.0± 1.1^{a}

a, b...e: Means having different superscripts in the same column of each classification are significantly different at $P<0.01$ and $P<0.05$, respectively.

Effect of incubation time:

Percentage of total shrunk spermatozoa significantly ($P<0.05$) increased, while, percentage of curled spermatozoa did not differ significantly by increasing incubation time from 0 up to 45 min. This trend resulted in significant ($P<0.05$) increase in percentage of total response of spermatozoa (Table 3).

It is of interest to note that percentage of total shrunk spermatozoa showed the highest rate of increase between 15 and 30 min. incubation time.

Effect of interaction:

Percentage of shrunk spermatozoa showed significantly ($P<0.05$) gradual increase by increasing the incubation time up to 45 min, thereafter it did not significantly differ between 45 and 60 min incubation, it was significantly ($P<0.05$) higher at 600 than 400 mOsm level (Fig. 2)

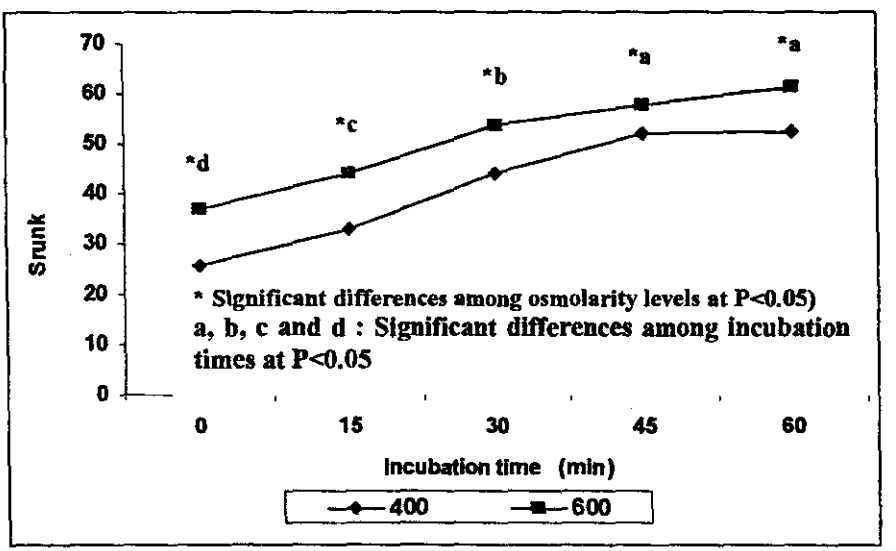


Fig. (2): Changes in percentage of shrunk spermatozoa at 400 and 600 mOsm levels after different incubation times.

DISSCUSION

The present study aimed to study the response of Friesian spermatozoa to different hypo- (0, 50, 100, 150, 200 and 300 mOsm) and hyper- (400 and 600 mOsm) osmotic levels with different incubation times (0, 15, 30, 45 and 60 min). Percentage of total curled spermatozoa showed significant ($P<0.05$) increase by decreasing the osmolarity level from 200 down ward to 0 mOsm. Similar trend

The obtained results of total curling between 50 and 300 mOsm revealed similar trend to that reported by Zaneveld and Jeyendran (1990); Correa and Zavos (1994) on bull spermatozoa, El-Kishk (2003) on buffalo spermatozoa, and Dandoush (2002) and El-Sherbieny (2004) on bull and buffalo spermatozoa. Such investigators significantly found that the maximal and minimal percentages of curled spermatozoa occurred with 50 and 200 mOsm solutions, respectively.

The significant increase in total curling by decreasing osmolarity level would imply normal membrane integrity of the reactive spermatozoa that is the ability of the sperm membrane to allow passage of water to establish equilibrium between the fluid components of the spermatozoa and the external environment (Drevius, 1972 and Jeyendran *et al.*, 1984). The later author speculated that sugars and electrolytes have different influences on the influx of water across the sperm membrane.

The trend of change in the frequency distribution of different types of curling indicated transformation of curled spermatozoa from type A to B and then to C by decreasing the osmolarity level from 200 to 0 mOsm. While at hyper-osmotic levels (400 and 600 mOsm), most curling process was in type A and none was in type C. This finding indicated the highest response of spermatozoa at 0 mOsm level (distilled water) in term of the highest percentage of total curling, the maximum curling of type B and C, and the minimum curling of type A.

The present results also showed insignificant change in percentage of total curled spermatozoa between 200 and 300 mOsm levels and the critical levels of osmolarity to occur pronounced changes in curling process was between 200 and 150 mOsm (Table 1). The minimal changes in percentage of total curled spermatozoa was reported between 200 and 300 mOsm by Correa and Zavos (1994) in bull and Vazquez *et al.* (1997) in boar spermatozoa. This suggested that this osmotic pressure is too low to induce a high incidence of swelling.

It is worthy noting that the pronounced decrease in percentage of total curled spermatozoa and frequency distributions of different types of curling at hyper-osmotic solutions (400 and 600 mOsm) were mainly related to the fact that most spermatozoa

were in shrinkage type. Moreover, the hyper-osmotic levels (more than 300 mOsm) are so high to induce curling in incubated spermatozoa. In other words, the main response of spermatozoa to hyper-osmotic solution is shrinkage process (El-Sherbieny, 2004), which will be discussed as independed results. This finding is considered as a slight reactivity of bull spermatozoa to hyper-osmotic solutions.

In the present study, total curled spermatozoa significantly ($P<0.05$) increased by increasing incubation time from 0 up to 30 min and insignificantly increased, thereafter. Increasing the incubation time of Holstein bull spermatozoa in different hypo-osmotic solutions up to 120 min, Dandoush (2002) found that the highest ($P<0.05$) response to HOS-t was detected at 120 min. This may suggest that bull spermatozoa exhibit higher response to HOS-t during the first 30 min and for a long incubation time (120 min).

The degree of curling is dependent on cellular water uptake (hypo-osmotic pressure of solution) per unit of time (incubation time). Hence, the differences in response to the hypo-osmolarity level may be related to variation in membrane integrity of spermatozoa (Jeyendran *et al.*, 1984). In agreement with the present results, Correa and Zavos (1994) reported that the highest reactivity of bull spermatozoa was observed after 30 min incubation. However, El-Sherbieny (2004) reported that the maximal percentage of curling of Holstein spermatozoa was recorded 45 min post-incubation. Such differences between the present results of bovine spermatozoa and that reported by the later author may be due to differences in the processing procedures, breed and/or diluents used in assessment of spermatozoa. In the present study lactose solution was used, while El-Sherbieny (2004) used fructose solution.

The increase in shrunk spermatozoa percentage by increasing osmolarity level from 300 up to 600 mOsm (Table 1) are in accordance with the trend reported on bull spermatozoa by Bredderman and Foote (1969) and Liu and Foote (1998), who indicated incidence of shrinkage of spermatozoa when transferred from an iso-osmotic solution to more than 500 mOsm. After exposure of spermatozoa to highly hypertonic solution, plasma membrane of the sperm cells has an ability to remain intact, with

some recovery of motile sperm after return to physiologic conditions, which may have implications for sperm cryo-preservation. Lack of motility in hypertonic media was not an absolute indicator of cell death and it is a factor to be considered when spermatozoa are evaluated, especially in association with cryo-preservation (Liu and Foote, 1998).

Based on the obtained results concerning the effect of hypo- and hyper-osmolarity levels, in was obvious that bull spermatozoa showed higher reactivity to curl than shrunk as affected by increasing incubation time.

The present study may suggest pre-freezing exposure of spermatozoa to hypertonic solutions to remove some of the water might facilitate more rapid freezing of spermatozoa without intracellular ice formation. The present results concerning the effect of osmolarity level and incubation time on curling of spermatozoa suggest that water test (0 mOsm) with 60 min incubation time is a sensitive and reproducible test for assessing the function integrity of Holstein spermatozoa under the experimental conditions of the present study.

REFERENCES

Bakst, M.R. (1980). Fertilizing capacity and morphology of fowl and turkey spermatozoa in hypotonic extender. J. Reprod. Fertil., 60: 121.

Bredderman, P.J. and R.H. Foote (1969). Volume of stressed bull spermatozoa and protoplasmic droplets and the relationship of cell size to motility and fertility. J. Animal. Sci. 28, 496.

Choudhry, T.M.; T. Berger and M. Dally (1995). *In vitro* fertility evaluation of cryopreserved ram semen and its correlation with relative '*in vitro*" fertility. Theriogenology, 43, 1195.

Correa, J.R. and P.M. Zavos (1994). The hypo-osmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology, 45, 351.

Dandoush, S.I.S. (2002). Comparative studies on methods of evaluating semen quality in farm animals. M. Sc. Thesis, Fac. of Agric., Mansoura Univ., Egypt.

Drevius, L.O. (1972). The permeability of bull spermatozoa to water, polyhydric alcohols and univalent anions and the

effects of the anions upon the kinetic activity of spermatozoa and sperm models. J. Reprod. Feril., 28, 41.

El-Kishk, M.A. (2003). Physiological studies on Farm animals. M. Sc.Thesis, Fac. of Agric., Mansoura Univ., Egypt.

El-Sherbieny (2004). Physiological studies on reproduction in farm animals. Ph. D. Thesis, Fac. of Agric., Mansoura Univ., Egypt.

Engel, S. and R. Petzoldt (1994). The spermatozoa volume as indicator of the plasma membrane integrity (modification of the hypoosmotic swelling test): I. Methods. Andrologia, 26, 309.

Jeyendran, R.S.; H.H. Van der Ven; M. Perez-Palaez; B.G. Crabo and L.J.D. Zaneveld (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil., 70,219.

Liu M. and R.H. Foote (1998). Bull sperm motility and membrane integrity in media varying in osmolarity. J. Dairy Sci. 81, 1868.

Peris, D.S.; A. Pena; J. Enric-Rodriguez-Gil and T. Rigau (2000). Ion-Mediated resistance to osmotic changes of ram spermatozoa: The role of a Miloride and ouabain. Theriogenology, 54, 1453.

Snedecor, G.W. and W. G. Cochran (1982). *Statistical Methods.* 7th ed. Iowa state. Univ. Press, Iwoa, U. S. A.

Vazquez, J. M.; E. A. Martinez P. Martinez; C. Garcia- Artiga and J. Roca (1997). Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenology, 47, 913.

Watson, P.F.; R. Funze; P. Camer. and R.H. Hammerstedt (1992). A comparison of critical osmolarity and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. J. Androl., 13, 131.

Zaneveld, L.J.D. and R.S. Jeyendran (1990). Hypo-osmotic swelling test. In: *Handbook of the Laboratory Diagnosis and Treatment of Infertility.* Keel B. A., Webster B.W. (Eds.). Boca Raton, CRC Press.

الملخص العربى

استجابة الحيوانات المنوية لطلائق الهولشتين لدرجات أسموزية منخفضة و مرتفعة وتحت أوقات تحضين مختلفة

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هدفت هذه الدراسة لتقييم استجابة الحيوانات المنوية لذكور الهولشتين لمستويات مرتفعة و منخفضة الأسموزية. أجريت هذه الدراسة على خمس طلائق هولشتين (سليمة صحياً وخالية من أي أمراض وناضجة جنسياً حيث تم جمع السائل المنوي لمدة عشرة أسابيع بمعدل قذفه أسبوعيا (٥٠ قذفه) و أجريت جميع الاختبارات على درجة حرارة ٣٧م°. ويمكن تلخيص النتائج فيما يلي:

أ- النسبة المئوية للحيوانات المنوية ملتوية الذيل في كل الأشكال انخفضت معنويا بانخفاض درجة الأسموزية من ٦٠٠ إلى صفر مل ازمول وكانت أعلي نسبة التواء علي مستوي صفر مل أزمول(٧٣,٩%) وأقل نسبة التواء عند مستوي ٦٠٠ مل أزمول (١٣,١%) وكانت الزيادة في نسبة الالتواء الكلى مرتبطة بزيادة معنوية في الحيوانات المنوية الملتوية شكلي (ب)، (ج) و انخفاض معنوي في الالتواء شكل (أ). زادت النسبة المئوية للإلتواءات الكلية بزيادة وقت التحضين ، وكانت الزيادة ملحوظة بعد ٣٠ دقيقة (٥١,١%) تحضين، بينما كان معدل الزيادة غير معنوي بعد ٦٠ دقيقة تحضين (٥٤,٤%) . كان هناك انخفاض ملحوظ في الالتواء شكل (أ) و زيادة في الالتواء شكل (جـ) حتى ٣٠ دقيقة وقت تحضين بينما كانت الزيادة معنوية في الالتواء شكل (ب) حتى ٦٠ دقيقة من التحضين .

ب- ارتفعت النسبة المئوية للحيوانات المنوية المنكمشة والنسبة المؤية للاستجابة الكلية عند مستوي أسموزية ٦٠٠ عن ٤٠٠ مل أزمول (٥٠,٦ و٦٣,٧ مقابل ٤١,٤ و ٥٩,٤%، على التوالى). كما زادت النسبة المئوية للحيوانات المنوية المنكمشة والنسبة المؤية للاستجابة الكلية معنوياً بزيادة وقت التحضين من صفر حتى ٤٥ دقيقة (٥٤,٧ و ٧٠,٢%، على التوالى) وكان أعلي معدل للزيادة بين ١٥ و ٣٠ دقيقة وبمعدل مرتفع معنوياً عند مستوى أسموزية ٦٠٠ عن ٤٠٠ مل أزمول لكل أوقات التحضين.

أوضحت النتائج وجود اختلافات في استجابة الحيوانات المنوية لذكور الهولشتين لاختبار الأسموزية والذي يعتبر مؤشر لسلامة الغشاء البلازمي للحيوانات المنوية وكانت أعلى استجابة التواء عند مستوى أسموزية صفر وأعلى درجة انكماش عند مستوى أسموزية ٦٠٠ مل أزمول، ويعتبر ذلك الاختبار مهم للحكم علي خصوبة الطلائق.