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Modification of Hypo-Osmotic Swelling Test to Evaluate the Integrity of Stallion Sperm Plasma Membrane

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Abstract: The objectives of this study were to determine the changes in the functional integrity of the sperm plasma membrane in a total of 30 semen samples collected from five mature light breed stallions (6 each) and evaluated by using the following assays. Sperm cell progressive motility (PM), computer-aided spermatozoal analysis system (CASA), supravital stain (E, eosin-negrosin stain), standard hypo-osmotic sucrose solution (HOS, 100 mOsm), formalized hypo-osmotic sucrose solution (F-HOS, 2.85% formaldehyde) and combined standard HOS and supravital stain (HOS/E). The results revealed that, the percentage of the progressive motile spermatozoa was lower than the corresponding HOS positive percentage (HOS+%). Furthermore, the HOS+% was lower than the corresponding eosin negative percentage (E-%). Adding formaldehyde to the HOS raised the HOS+% to become close to the E-% and both positively correlated. Combined HOS test with supravital stain decreased the HOS+/E-% to the level of PM% and both positively correlated. Incubation of the sperm cells for 24 hours in formalized HOS or combined HOS with supravital staining strongly correlated to the standard HOS and with most of the conventional semen parameters. In conclusion, the modified HOS assays can improve the reliability of the HOS test in the raw stallion semen.

Key words: Stallion, Sperm cell, Hypo-osmotic solution test, HOS, Supravital stain

INTRODUCTION

The conventional semen analysis represented by sperm cell concentration, motility, morphology and vitality are still used for predicting the fertility of sperm in most of animal and human insemination laboratories. These tests separately or in combination failed to correlate with fertility in equine reproduction [1,2]. The functional tests which assess membrane function and integrity may be more accurate predictor of *in vitro* or *in vivo* fertilization than other methods. The findings of Choudry *et al.* [3] and Parlevliet *et al.* [4], using zona or oocyte binding assays for ram and stallion sperm, support this suggestion. However, such assays are relatively complicated to perform and are not suitable for routine use. The functional integrity of the sperm plasma membrane can also be measured by simple assays such as determination of motility, resistance against hypo-osmotic media or different vital staining methods. Because motility is certainly a quick and easy way to evaluate spermatozoal characteristics, the majority of studies on functional integrity of the plasma membrane still use the motility of the spermatozoa as its major marker. Despite the relevance of the membrane integrity for the motility of spermatozoa, motility largely depends on energy production originating from the mitochondrial compartment of the sperm mid-piece [5]. Therefore, loss of motility is not only related to the membrane integrity but also to mitochondrial dysfunction. Regarding stallions, motility characteristics are poorly correlated with fertility [6-8]. In cases in which sperm motility is good, live/dead stains typically provide little additional information, since sperm motility is highly correlated with sperm viability in stallions [9,10]. The plasma membrane of spermatozoa is heterogeneous and consists of five specific domains: the acrosome, equatorial segment, basal, mid-piece and tail [11]. A number of specialized dyes and fluorescent markers designed to distinguish between viable and nonviable sperm are available [12]. Eosin-nigrosin is used to evaluate membrane alterations due to cell injury or death and has been shown to be as accurate at assessing sperm viability as some fluorescent methods [13]. Some authors considered the HOS test as an alternative to viability staining and it may have, in addition to determining

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whether or not the cell membrane is intact, also indicate whether or not the membrane is osmotically active [12]. Other authors distinguished between vital stains and HOS by each of these tests evaluates different areas of the sperm membrane [14]. Jeyendran *et al*. [15] were the first to use HOS in the evaluation of plasma membrane integrity in human spermatozoa. Available data from several human studies indicate that, sperm curling in response to the HOS is better endpoint for assessing the ability of spermatozoa to capacitate and effect fertilization than other semen analysis assays [15-17]. A good correlation between the ability of human sperm to swell in a HOS and the results of the zona-free hamster ovum penetration assays has been reported [15,17,19]. They also noted that in case of sub-fertility the HOS positive cells scored less than 60%. In contrast to those encouraging results, Chan and coworkers [20] found an insignificant correlation between the HOS test and hamster ovum penetration assay and they assumed that, each test evaluates different qualities of the sperm. The HOS has been validated for equine semen by Neild *et al.* [21] and Nie and Wenzel [22] however, its use in studies on equine semen quality is still limited. Currently, the best means of determining the fertility of a stallion ejaculate is by diagnosing pregnancy outcome in mares bred with the evaluated spermatozoa. The result obtained by Neild *et al*. [23] showed stallions with low HOS swelling scores (<40%) to be of doubtful fertility. They added that, the HOS test was not correlated with percentage of pregnancy but tend to correlate with the number of services per pregnancy. Nie *et al*. [1] reported that, the total number of spermatozoa, the absolute number of progressively motile, the morphologically normal nor the hypo-osmotic swelling positive spermatozoa inseminated, was not closely associated with pregnancy outcome.

The objectives of this study were to 1) evaluate the sperm cell membrane integrity in the raw stallion semen by determination of motility, resistance against HOS and by vital staining with eosin-nigrosin stain.2) Modifications of HOS test to improve its sensitivity or applicability, either by adding formaldehyde or by combined HOS with eosin staining.

MATERIALS AND METHODS

Five stallions of mature light-horse breed, ranging in age from 3-15 years and weighing 400-550 kg, were individually housed in small paddocks during the project. Each was fed with costal Bermuda grass-hay and a commercial concentrate ration (12% protein) for maintenance of body condition. In the morning, the assigned stallion was subjected for collection on a phantom using a Missouri model artificial vagina. Gel free semen samples were collected from each stallion 6 times for a total of 30 ejaculates during the project. Raw semen samples were examined for ejaculate volume, spermatozoal concentration, motility, morphology, vitality and HOS parameters. Spermatozoal concentration was determined using a densimeter (Animal Reproduction Systems, Chino, CA). Motility parameters were evaluated within minutes of collection by using a computer-aided spermatozoal analysis system (CASA; HTM-C, Hamilton Thorne Research, Beverly, MA). A 4.5µl drop of semen was placed on a pre-warmed (37°C) glass slide and covered with a 22 X 22 mm cover slip. Slide temperature was maintained with a stage warmer during evaluation. A minimum of five fields and 500 spermatozoa were analyzed from each sample using the CASA system. Sperm morphology and vitality were evaluated by using an eosin-nigrosin morphology stain (Lane Mfg, Denver, CO). Smears of equal volumes of semen and stain were air dried on glass slides. Slides were examined for spermatozoal morphology and vitality using bright-field microscopy (1000X). One hundred Spermatozoa were classified into normal or abnormal morphology and stained (unvital/dead) or unstained (vital/alive). HOS test procedure described by Nie and Wenzel [22] was used to determine the percentage of HOS positive cells in each sample. A 100 µl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed 100 mOsm sucrose solution (1.712g sucrose dissolved in 50 mL of sterile, de-ionized water). The mixture was incubated at 37°C for 60 minutes in a 1.5 ml micro-centrifuge tube. Following incubation, a small drop of sample was placed on a microscope slide and cover-slipped for evaluation. Samples were examined using phase contrast microscopy (400X) to evaluate 100 spermatozoa for evidence of swelling and curling changes (HOS+). The swelling phenomena of sperm tail irrespective of the types of tail coiling, type b to g described by Jeyendran *et al*. [15]. Formalized hypo-osmotic solution test (F-HOS) was prepared by mixing 200 µl of 37 % formaldehyde to 1.0 ml of HOS solution before adding the 100 µl of the semen sample to give final formaldehyde concentration of 2.85%. The F-HOS sperm samples were evaluated after one hour of incubation at 37°C (F-HOS/1hr) and reevaluate after 24 hours (F-HOS/24hr) of incubation as described above. A combined HOS-Eosin test (HOS/E) was performed to

the sperm samples. After one-hour of incubation in the stander HOS solution, smears of eosin-nigrosin stain were prepared as mentioned before. Slides were examined for spermatozoa with HOS+ using bright-field microscopy (1000X). One hundred spermatozoa were counted and classified into four categories. Type I: tail swollen and head white (HOS+/E-). Type II: tail non-swollen and head white (HOS-/E-). Type III: tail swollen and head red (HOS+/E+). Type IV: tail non-swollen and head red (HOS-/E+). All data were statistically analyzed using Stat View 5.0 software program (SAS Institute, Wangen, Switzerland). The statistical significance was assessed using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Correlation coefficients between different assays and between each assay with conventional semen analysis are performed.

RESULTS

Figure 1 demonstrates the means of different conventional parameters of the raw semen samples for each stallion and the overall average. The correlation coefficients between these parameters are presented in Table 1.

Table 2 illustrates the average of different types of the combined HOS-E assay. Type I is the group of viable spermatozoa with intact membrane (HOS+/E-). Types II and III are transitional states showing membrane defects either in the head or in the tail and Type IV is the group of non-viable sperm with membrane defects in both the head and tail (HOS-/E+). The percentage of type I cells is used for sample evaluation for sperm cell membrane integrity.

Table 1: Correlation coefficient between different conventional semen analyses

	Concentration 10^9/ml	Total motility %	Progressive motility %	Vitality (E-) %	Normal morphology %
Ejaculate volume/ml	-0.45**	-0.62**	-0.10	-0.28	-0.49**
Concentration 10^9/ml	--	0.16	0.53**	-0.13	0.03
Total motility %	--	--	0.04	0.36*	0.36*
Progressive motility %	--	--	--	0.19	0.03
Vitality (E-) %	--	--	--	--	0.35*

* Significant at P < 0.05. ** Significant at P < 0.01

Table 2: Different types of membranes integrity by using combined HOS-E assay (Mean ± MSE)

	Type I (HOS+/E-)	Type II (HOS+/E+)	Type III (HOS-/E-)	Type IV (HOS-/E+)
Stallion 1	68.67±3.35	8.67±1.41	3.33±0.61	19.33±2.22
Stallion 2	47.33±9.90	8.33±1.67	1.50±0.76	42.83±9.48
Stallion 3	45.67±5.21	15.17±2.79	0.50±0.34	38.67±3.68
Stallion 4	48.83±5.61	14.67±4.40	6.67±0.80	29.83±3.41
Stallion 5	68.00±1.63	9.83±1.45	1.33±0.49	20.83±0.48
Average	57.37±2.46	11.33±1.22	2.67±0.49	28.63±1.82

Type I: Tail swollen and head white (HOS+/E-), Type II: Tail non-swollen and head white (HOS-/E-),
Type III: Tail swollen and head red (HOS+/E+), Type IV: Tail non-swollen and head red (HOS-/E+)

Table 3: Correlation coefficient between different HOS assays

	F-HOS/1hr	F-HOS/24hr	Type I (HOS+/E-)
HOS/1hr	0.63**	0.43**	0.49**
F-HOS/1hr	--	0.18	0.25
F-HOS/24hr	--	--	0.50**

HOS = un-formalized standard HOS, F-HOS = formalized HOS, 1hr = one hour incubation, 24hr = 24 hour incubation, Type I (HOS+/E-), is the group of viable spermatozoa with intact membrane
** Significant at P<0.01

Table 4: Correlation coefficient between different HOS assays and conventional semen analysis

	HOS/1hr	F-HOS/1hr	F-HOS/24hr	HOS+/E-
Ejaculate volume	-0.40*	-0.23	-0.40*	-0.56**
Concentration	0.55**	0.30*	0.45*	0.59**
Total motility	0.17	0.14	0.38*	0.48**
Progressive Motility	0.71**	0.60**	0.28	0.53**
Vitality (E-)	0.20	0.32*	0.37*	0.08
Normal morphology	0.12	0.39*	0.30*	0.30*

** Significant at P< 0.01, * Significant at P< 0.05

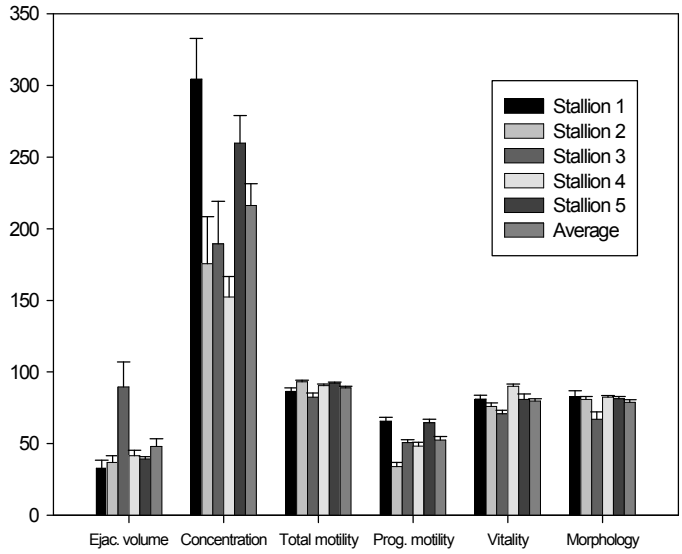

Fig. 1: Convention semen analysis of raw semen in diffrent stallions (Mean±MSE)

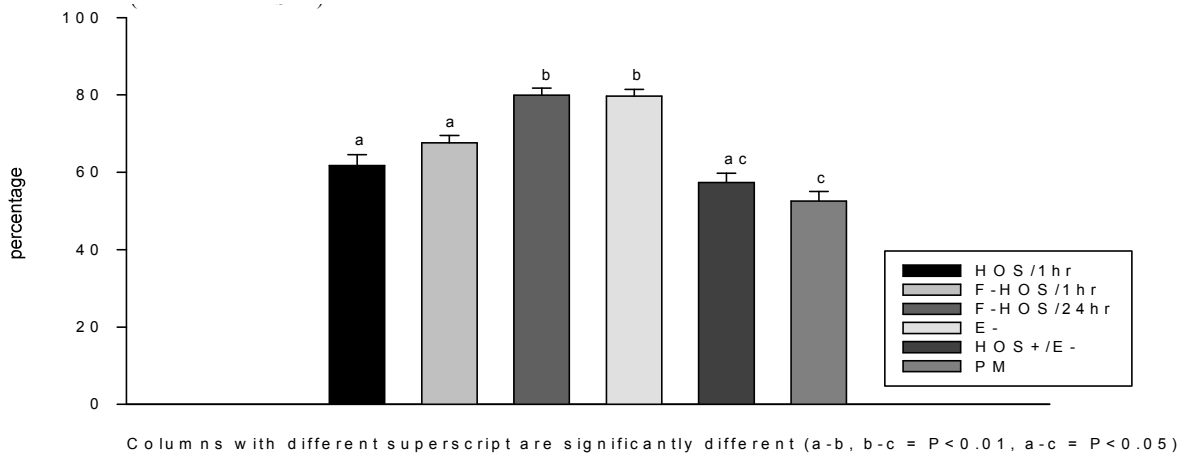

Fig. 2: Evaliation of cell membranc integrity by using different assays

Figure 2 presents the average of the percentage of cell membrane integrity evaluated by progressive motility, eosin negative staining and HOS+ in different assays (HOS, F-HOS, HOS/E). The correlation coefficients between the different HOS assays are listed in Table 3 and the correlation coefficient between these different HOS tests and the different conventional semen analysis of the raw semen are tabulate in Table 4.

DISCUSSION

Investigators tend to compare the outcome of new sperm evaluation assays with conventional sperm analysis. If a high correlation is found, this is used as an argument for the validity of the new test. In case of a poor correlation is found they suggested that each of these tests evaluates different qualities of the sperm. According to the current results, the progressive motility was strongly correlated ($P < 0.01$) with sperm cell concentration. While the total motility, the vitality and the normal morphology had moderately correlation to each other ($P < 0.05$). Love *et al.* [9] and Brinsko *et al.* [10] reported a high correlation between motility and vitality and they suggested that, live/dead stains provide little additional information in cases in which sperm motility is good. HOS test may have more to offer than straight viability testing in that the HOS test, in addition to determine whether or not the cell membrane is intact, also may indicate whether or not the membrane is osmotically active. According to Nie and Wanzel [22], incubation of

stallion's sperm cells for one hour in hypo-osmotic 100 mOsm sucrose solution leads to a high percentage of HOS+ cells in comparison with other hypo-osmotic solutions. The present results show that, in the same HOS solution, the percentage of HOS+ sperm is negatively correlated with the ejaculate volume. This is may be due to the increase in the seminal plasma may lead to a harmful effect on the cell membrane integrity and therefore, decrease in the HOS+ reading. Ejaculate volume is also negatively correlated with sperm cell concentration, total motility and normal morphology. Centrifugation of the sperm-rich fractions of the stallion's ejaculate becomes a valuable technique, especially before cooling or freezing, since it improves the post-thaw motility and sperm membrane integrity [25]. The percentage of HOS+ cells in the standard HOS assay is strongly ($r = 0.71$, $P < 0.01$) correlated with sperm cell progressive motility. These results agree with that reported in stallion [21], human [26,27] and dog [28,29].

The percentage of sperm with intact membrane represented by HOS+ is significantly lower than that represented by percentage of eosin negative cell. This phenomenon has more than one explanation. However, some authors distinguished between vital stains and HOS test by each of these tests evaluates different qualities or regions of the sperm membrane [14], other authors explained it by the fact that, some of the sperm cells may have a nonfunctional membrane that is still capable of preventing the eosin molecules from entering the cell [19], but it is weak and fragile that may not express the increase in the pressure of the influx of water and their flagellae will remain unchanged under the light microscopic examination [12]. Staining of sperm cells after one-hour incubation in standard HOS (HOS/E assay) non-significantly decrease the percentage of HOS+ cells but significantly drops the eosin negative percentage. This is may be due to the eosin molecules can go through the weak fragile membranes that are ruptured in response to the influx of water.

In a trial to determine the effect of adding formaldehyde to the HOS solution, our unexpected results revealed that, overnight incubation in a F-HOS significantly increases the percentage of HOS+ sperm, which becomes close to the percentage of eosin negative cells. This may suggest that adding formaldehyde to the HOS solution may fix the cells with weak fragile membranes and convert it to intact osmotically active ones. The positive correlation between both eosin negative and F-HOS+ supports this suggestion. In a previous work with the rat semen, adding formaldehyde to the isotonic solution do not affect on the sperm cell morphology [30]. The percentage of both HOS and HOS/E assays are poorly correlated to the eosin negative percentage and strongly correlated to each other. These results confirm the fact that HOS assay evaluate the plasma membrane integrity by different qualities than the eosin staining as the supravital stains evaluate the physical damage of the membrane while the HOS test also evaluates the biochemical activity of the membrane [14].

It could be concluded that, modification of HOS test by adding formaldehyde to the hypo-osmotic (100mOsm) sucrose solution or combined the HOS test with eosin-negrosin stain can improve the reliability of the test in the raw stallion semen.

REFERENCES

1. Nie, G.J., J.G. Wenzel and K.E. Johnson, 2002. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. Animal Reproduction Science, 69: 211-222.
2. Kuisma, P., M. Andersson, E. Koskinen and T. Katila, 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. Acta Veterinaria Scandinavica, 48:14.
3. Choudry, T.M., T. Berger and M. Dalby, 1995. *In-vitro* fertility evaluations of Cryopreserved ram semen and its correlation with relative in-vitro fertility. Theriogenology, 43: 1195-1200.
4. Parlevliet, J.M., A.R. Fazeli, W. Steenweg, M.M. Bevers and B. Colenbrander, 1994. The effect of storage methods on the sperm zona binding in the horse. 6th Int. Symp. Equine Reproduction, Caxambu, Brazil, Programme and miniposters, pp: 171-172.
5. Ruiz-Pesini, E., C. Diez, A.C. Lapena, A. Perez-Martos, J. Montoya, E. Alvarez, J. Arena and M.J. Lopez-Perez, 1998. Correlation of sperm motility with mitochondrial enzymatic activity. Clinical Chemistry, 44: 1616-1620.
6. Doesett, K.F. and W.A. Pattie, 1982. Characteristics and fertility of stallion semen. Journal of Reproduction and Fertility Supplement, 32: 1-8.
7. Jasko, D.J., 1992. Evaluation of stallion semen. Veterinary Clinnics of North America Equine Practice, 8: 129-148.
8. Jasko, D.J., T.V. Little, D.H. Lein and R.H. Foote, 1992. Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987–1988). Journal of American Veterinary Medicine Association. 200: 979-985.

9. Love, C.C., J.A. Thompson and S.P. Brinsko, 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology, 60: 1127-1138.
10. Brinsko, S.P., T.L. Blanchard and S.L. Rigby, 2003. Effects of dead spermatozoa on motion characteristics and membrane integrity of live spermatozoa in fresh and cooled-stored equine semen. Theriogenology, 59: 735-742.
11. Ladha, S., 1998. Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. Journal of Member. Biology, 165: 1-10.
12. Turner, M. Regina, 2005. Current techniques for evaluation of stallion fertility. Clinical Techniques in Equine Practice, 4: 257-268.
13. Merkies, K., T. Chenier and C. Plante, 2000. Assessment of stallion spermatozoa viability by flow cytometry and light microscope analysis. Theriogenology, 54: 1215-1224.
14. Zhu, W.J. and X.G. Liu, 2000. Cryodamage to plasma membrane integrity in head and tail regions of human sperm. Asian Journal of Andrology, 2: 135-138.
15. Jeyendran, R.S., H.H. Van der Ven, M. Perez-Pelaez, B.G. Carbo 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. Journal of reproduction and fertility, 70: 219-228.
16. Jeyendran, R.S., H.H. Van der Ven, S.P. Rachagan, M. Perez-Pelaez and L.J. Zaneveld, 1989. Semen quality and in-vitro fertilization. Australian and New Zealand Journal of Obstetrics and Gynecology, 29: 168-172.
17. Van Der Ven, H.H., R.S. Jeyendran, S. Al-Hasani, M. Perez- Pelaez, K. Diedrich and L.J.D. Zaneveld, 1986. Correlation between human sperm swelling in hypo-osmotic medium (Hypo-osmotic Swelling test) and in-vitro fertilization. Journal of Andrology, 7: 190-201.
18. Takahashi, K., A. Vchida and M. Kitao, 1990. Hypo-osmotic swelling test of sperm. Archives of Andrology, 25: 225-242.
19. Vigano, P., C. Brignate C. Gonfiantini, N. Doldi and M. Busacca, 1990. Which is the best test to evaluate the integrity of sperm plasma membrane? Acta Europaea Fertilitatis, 21(5): 231-234.
20. Chan, S.Y.W., E.J. Fox and M.M.C. Chan, 1985. The relationship between the human sperm hypo-osmotic swelling test, routine semen analysis and the human sperm zona-free hamster ovum penetration assay. Fertility and Sterility, 44: 668.
21. Neild, D., G. Chaves, M. Flores, N. Mora, M. Beconi and A. Aguero, 1999. Hypo-osmotic Test in equine spermatozoa. Theriogenology, 51: 721-727.
22. Nie, G.J. and J.G.W. Wenzel, 2001. Adaptation of the hypo-osmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. Theriogenology, 55: 1005-1018.
23. Neild, D.M., M.G. Chaves, M. Flores, M.H. Miragaya, E. Gonzalez and A. Aquero, 2000. The HOS test and its relationship to fertility in the stallion. Andrologia, 32: 351-315.
24. Chan, P.J., D.R. Tredway, J. Coreselli, C.S. Pang and B.C. Su, 1991 Combined supravital staining and hypo-osmotic swelling test. Human Reproduction, 6(8): 1115-1118.
25. Sieme, H., T. Katila and E. Klug, 2004. Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. Theriogenology, 61: 769-784.
26. Jeyendran, R.S., H.H. Van Der Ven and L.J.D. Zaneveld, 1992. The hypo-osmotic swelling text: An update. Archives of Andrology, 29: 105-116.
27. Van den Saffele, J., L. Vermeulen, F. Schoojans and F.H. Comhaire, 1992. Evaluation of the hypo-osmotic swelling test in relation with advanced methods of semen analysis. Andrologia, 24: 213-217.
28. Kumi-Diaka, J. and G. Badtram, 1994. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa. In vitro bioassay for canine spermatozoa. In vitro bioassay for canine semen for canine semen. Theriogenology, 41: 1355-1366.
29. Rodriquez-Gil, S.E. and T. Rigan, 1996. Effects of ouabain in the response to osmotic changes in dog and boar spermatozoa. Theriogenology, 45: 873-888.
30. Tayama, K., H. Fujita, H. Takahashi, A. Nagasawa, N. Yano, K. Yuzawa and A. Ogata, 2006 Measuring mouse sperm parameters using a particle counter and sperm quality analyzer: A simple and inexpensive method. Reproductive Toxicology, 22: 92-101.