Research Paper Veterinary Science

Monthly and Seasonality Variation in Freezability of Semen and Associations Between Freezability of Semen With Age and Testicular Dimension of Holstein Bulls Born in Iraq

Kreem Iwaid AL-Badry	Department of surgery and obstetrics, College of Veterinary Medicine, University of Baghdad.
Sadeq jaafer Zalzala	Department of surgery and obstetrics, College of Veterinary Medicine, University of Baghdad.
Faris Faisel Ibrahim	Ministry of Agriculture, Department of Artificial Insemination Center, Baghdad- Iraq.
Kamil Fayyad Hurish	Ministry of Agriculture, Department of Artificial Insemination Center, Baghdad- Iraq.

ABSTRACT

Cryopreservation in liquid nitrogen (where the temperature is(–196 C◦) is a technique that makes long-term storage of spermatozoa possible. This is of high practical importance for breeding programs in domestic animals, and the technique is also used to maintain the genetic diversity and establishment of gene-banks the freezability of spermatozoa ranges according to individual, and to the conditions of semen collection, its age and season when the semen collection is carried out in the same bull. Therefore, the objectives of the present study were to determine the associations between freezability of semen and testis dimensions were it is very important to improve Artificial insemination in Iraq whether through (BBSE) or limitation the ages , months, seasons, or testis dimensions, which produce the good freezability of semen. This study was carried out at artificial insemination center- Iraq on (14) Holstein bulls born in Iraq .Bulls were classified into three groups according to their age, semen collection by artificial vagina during 12 months (four season), in addition to testis dimensions were calculated for all bulls and during this year, in each collection individual motility evaluated before and after freezing in liquid nitrogen 48 hr . Results shows that individual motility of spermatozoa before and after freezing for all bulls to a greater extent differences from month , season to another, and from age to another and correlation between freezability of semen with age and testicular dimensions such as testis dimensions and scrotal circumference, it means that freezability of semen is poor except during hot months or season ,and extremely good in others, also results study revealed that freezability positively correlation with age and testicular dimensions.

KEYWORDS freezability of semen, associations, age, month, season, testicular dimension, Holstein bulls, Iraq.

Introduction

Protocols for BBSE developed by the Artificial insemination center in Iraq are widely used for routine evaluation of breeding bulls .In these protocols, physical soundness, the integrity of the reproductive tract, and semen evaluation are assessed and the bull classified as a satisfactory, questionable, or unsatisfactory potential breeder. Among the traits assessed during a breeding soundness evaluation, scrotal circumference (SC) and semen quality endpoints (such as motility and morphology) are highly correlated with fertility (Arteaga, 2001).The prevalence and importance of factors affecting semen quality and breeding soundness (Barth, and Waldner ,2002), there is a paucity of information regarding the association of breed, age, and scrotal circumference on the prevalence of sperm defects in beef bulls(Parkinson,2004). Selection of sires should be based on results of a Breeding Soundness Evaluation (BSE), the capacity for mount, testicular development and semen quality, with special attention on the abnormal spermiogram associated to sexual immaturity, should be included. Testicular development of bulls, during the post-weaning period, is associated to the age and breed of the animals, environmental conditions, and nutritional regime. A positive correlation between testicular development and semen quality has been documented through numerous studies (Bailey et al 1996, and Coe 1999). In another hand successful outcome of AI in cattle depends on a number of factors including improvement semen quality and freezability of bull semen in resultant improving AI (Haugana ***et al***., 2005),in addition to cryopreservation in liquid nitrogen (where the temperature is –196 ◦C) is a technique that makes long-term storage of spermatozoa possible. This is of high practical importance for breeding programs in domestic animals, and the technique is also used to maintain the genetic diversity and establishment of gene-banks (Jalme ***et al***., 2003, and Shivaji ***et al***., 2003) , the freezability of spermatozoa ranges according to individual, and in the same bull it ranges according to the conditions of semen collection, its age and season when the semen collection is carried out (Yamashita, 2004).Therefore, the objectives of the present study were to determine the associations between freezability of semen with age and testis dimensions were very important to improve Artificial insemination in Iraq whether through (BBSE) or limitation the ages , months, seasons, or testis dimensions, were which produce the good freezability of semen.

Materials& Methods

Experimental Bulls: This study was carried out at artificial insemination center of Abou-Ghareeb western of Baghdad, on (14) Holstein bulls born in Iraq. All bulls were kept under identical conditions of management, feeding and watering throughout the study period which lasts from December 2014 until November 2015 .Bulls were classified into three groups according to their age **7** (14-16 months), **3** (19-21 months) and **4**(23 months). Semen collected from all bulls by using the artificial vagina method, once a week during 12 months was classification in four seasons (autumn, spring, summer and winter). Immediately after collection, every ejaculation was examined according to the routine procedures performed in Artificial insemination center in Iraq implicate evaluation volume of ejaculate, mass and individual motility of sperms. Ejaculates were individual motility of spermatozoa equal or more than 50 % diluted in Tris–egg yolk (TEY) ex-

tender , the all diluters semen were transferred from water bath (37C°) into the cold cabinet, and in a controlled manner, allow to reach the stabilize degree 5C° in about 1-1.5 hour, the diluted semen is held at this temperature (5 C°) for 4 hours equilibration time, which calculated when the degree of water in the beaker reached 5°C and during this time package dilution semen in straws (0.25 ml) and evaluated individual motility before freezing and after storage 48hr in liquid nitrogen(thawing straws in water bath 37C° for 30 sec).

Testes dimensions and scrotal circumference measurements

Bulls were restrained in a squeeze chute and the scrotal content was held in the ventral scrotum from the cranial side of the scrotum .Testis dimensions(length of right and left testis) and scrotal circumference was measured in centimeters using measuring tape (Lane Manufacturing Co., Denver, CO, USA) as described by (Foote, 1969).

Statistical Analysis

The Statistical Analysis System- SAS (2012) was used to effect of different factors in study parameters. (Duncan, 1955) multiple range tests were used to significant compare between means in this study. Estimate of correlation coefficients between some parameters study

Results & Discussion

Results in tables (1, 2) shows that individual motility of spermatozoa after freezing for all bulls to a greater extent differences from month, season to another (Fig 1,2). Many authors found that some semen characteristics affected with this monthly variation, and some had none, also there are no successive modern studies in this aspect, since these anciently years have an effect on the reproductive efficiency of bulls (AL- Badry 1998; and Violeta, ***et al***., 2010). In addition to study have approached that there are a significant differences in the traits for months to other, this is may be referred to the changes in the external environmental factors such as photoperiod that changes according months (Noakes ***et al***., 2001, Andraba ***et al***., 2002 , 17-Chacon and Rodriguez –Martinez, 2002), but another studies have not observed any effect of month on the semen characteristics of bulls (Jainudeen and Hafez, 2000,and Eric ***et al.,*** 2010) this is my attribute to the difference in breeds and environmental condition of two country. Resent study revealed that months(June, July and August) summer season which were affected negatively on freezability of semen ,this is may be attribute to height temperature of this month's more than another's, (Bearden ***et al***., 2004), and since with years progress it should be makes an environmental changes whether in temperature aspect or humidity or photoperiod all of these has role in effecting the reproductive performance of the animals in general and AI in specific (Noakes ***et al***., 2001;and Carlsen ***et al***., 2003), the hot weather with a high humidity during summer makes the activity of the thyroid and the metabolism low resulting in the reduction of testosterone secretion, which lowers the sexual desire and causes the reduction of the fertility(Shioya, 2004,and Corcuera ***et al***., 2002)who reported higher variation and lower semen quality in hot months than in the other seasons.

Table (1) Effect of month in individual motility after freezing in Holstein bulls born in Iraq. (Mean ± SE)

Dec.	Jan.	Feb.	March	April	May	June	July	August	September	October	November
55.07 ± 0.91 b	56.07 ± 0.88 b	54.73 ± 1.02 b	59.64 ± 0.83 a	58.32 ± 0.49 a	59.69 ± 0.75 a	44.05 ± 0.96 c	43.66 ± 0.85 c	42.94 ± 0.63 c	55.09 ± 0.72 b	57.77 ± 1.06 b	55.08 ± 0.80 b

Different small letters significant at (p<0.05). **Dec=December**, **Jan= January, Feb=February.**

Table(2) Effect of season in individual motility after freezing in Holstein bulls born in Iraq. (Mean ± SE).

Winter	Spring	Summer	Autumn
56.95 ± 0.56 b	59.22 ± 0.41 a	44.55 ± 0.49 c	54.64 ± 0.53 b

Different small letters significant at (p<0.05).

Hot season is not the ideal season for the collection of semen from Friesland bulls (Vilakazi and Webb ,2004), also Holstein are animals of the more effected with heat stress(Garcia- Peniche ***et al.,*** 2005) . in addition to results of present study agree with (AL-Badry, 2012), which reported that changes in some semen parameters depression had taken place in hot months significantly ,and the heat quality either by its effect on the mechanical activity which is responsible for maintaining animal body temperature (Marai, 2010) or by its effect on disturbing sperms production and maturation(Cholami ***et al***., 2011) or by its indirect effect produced from its effect on energy balance in the body (Rensis ***et al***., 2003), this leads to decrease sperm motility and sperm concentration (Bilby, 2009, and Cholami ***et al***., 2011) and also causes a significant increase in sperms abnormalities (Noaks ***et al.***, 2001,and Carlsen, 2003).Also study have approached that there are a significant differences in the freezability of semen for months to other, this is may be referred to the changes in the external environmental factors such as temperature (Carlsen ***et al***., 2003) .Moreover result study in table (3)revealed that individual motility of spermatozoa after freezing for all bulls differences from age to another (Fig 3) and results in table (4) included the correlation between age ,testicular dimensions such as testis dimensions or scrotal circumference with freezability

Table (3) Effect of age group in individual motility after freezing in Holstein bulls born in Iraq (Mean ±SE)

	Bulls age		
	(14-16) month	Group2 (19-21) month	Group3 (23) month
individual motility after freezing	50.28 ± 0.50 c	55.13 ± 0.84 b	58.18 ± 0.63 a

Different small letters significant at (p<0.05).

Table (4) Correlation between age, month, season and testicular dimensions with freezability of semen for Holstein bulls born in Iraq.

	Variable			
	Age	Month	Season	Testicular dimensions
Correlation coefficient (r)	0.63 *	0.74**	0.52 *	0.42 *

*Significant at (p<0.05).

**Significant at (p<0.01).

Fig (1) Effect of month in individual motility after freezing in Holstein bulls born in Iraq.

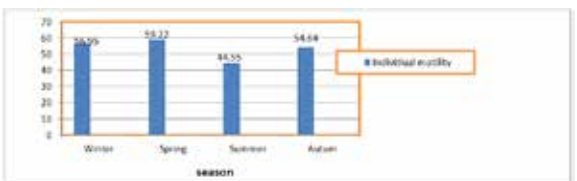

Fig (2) Effect of season in individual motility after freezing in Holstein bulls born in Iraq.

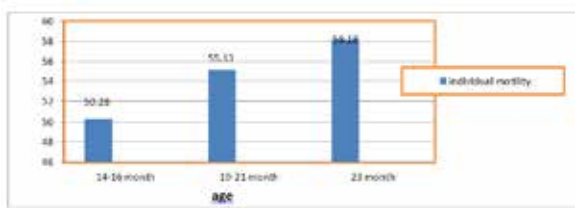

Fig (3) Effect of age group in individual motility after freezing in Holstein bulls born in Iraq.

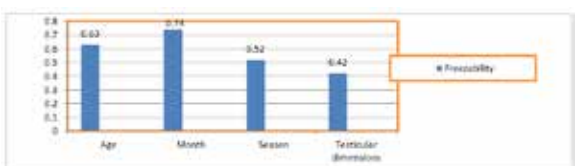

Fig (4) Correlation between age, month, season and testicular dimensions with freezability of semen for Holstein bulls born in Iraq.

of semen for bulls (Fig 4). (Arteaga ***et al.*** 2001) reported that sperm concentration and number of normal spermatozoids increased lineally between 11 and 15 months of age, and that the percentage of proximal droplets diminished significantly as the animals aged. Pre pubertal beef bulls exhibit low sperm count and motility, besides a high number of abnormal spermatozoa. The relationship among age, scrotal circumference and semen quality in ***Bos taurus*** and ***Bos indicus*** bulls has also been documented (Coe 1999). Likewise (Amann ***et al.*** 2000, and Thundathil ***et al.*** 2001) indicated that the incidence of proximal droplet in the semen of young bulls tended to diminish as the animals reach sexual maturity. In bull calves testicular tissue weighs 20 g at 36 weeks and 150 g at 81 weeks of age (Aponte ***et al.***, 2005). Bigger testicles are capable of producing more sperm cells compared with smaller testicles (Senger, 2003).Because capacity of a beef bull to produce sperm cells is mainly associated with the size of its testicles (Hansen, 2006). A positive correlation between testicular development and semen quality has been documented through studies of (Coe, 1999,and Arteaga ***et al***., 2001). The bulls with larger testes have been known to give larger volume of semen, larger sperm motility and percentage of normal spermatozoa (Ott ***et al***., 1981). Moreover testicular development of bulls, during the post-weaning period, is associated to the age and breed of the animals, environmental conditions, and nutritional regime (Vásquez and Arango, 2002).The Western Canadian Association of Bovine Practitioners recommended a minimum SC of 29 cm in Limousin, 30 cm in Hereford and Shorthorn, 31 cm in Charolais and Angus, and 32 cm in Simmental and Gelbvieh bulls at 12 mo of age . (Kasari ***et al***., 1996) suggested higher minimums (32 to 33 cm) should be used in breeds such as the Simmental, Angus, and Maine-Anjou. (Hopkins and Spitzer ,1997) recommended that all yearling bulls intended for breeding should have a minimum SC of 30 cm, The SC increased as age advanced, regardless of breed, and at above 26 month of age SC was > 42 cm in Hereford and > 41 cm in Angus, Simmental, Charolais, and Gelbvieh bulls. Conversely, it was > 38 cm in Limousin bulls older than 26 month of age (Ajitkumar ***et al.,*** 2011). (Hoognboezem& Swanepoel, 2000) Scrotal circumference has shown moderate to high heritability and high genetic correlation with other testicular measurements and semen quality traits in Bos índicus (Quirino, 1999), and reproductive potential of the bull (Unanian ***et al***., 2000). In addition to the scrotal circumference has been the most widely used and studied criterion in screening programs as a measure indicative of morphological and physiological characteristics of gonads and quantitative and qualitative characteristics of semen in cattle (Forni & Albuquerque, 2004), and the associations between sperm abnormalities and SC (Ajitkumar ***et al*** .,2011). Scrotal circumference, the most easily obtainable measure of a bull's ability to produce adequate numbers of spermatozoa, is highly correlated with testicular volume and semen quality but had a negative linear regression with the incidence of primary sperm defects (Kastelic ***et al***., 2001).Scrotal circumferences are positively correlated with the age of the animal (Brito ***et al***., 2002).The minimal acceptable scrotal circumference increases with age. A young bull with a Scrotal Circumference (SC) measurement that is less than the minimum required would usually be classed as "Deferred" as that measure could improve with age**(** Higdon ***et al.,***2000).On the other hand, it has been shown that bulls with a small SC at a year of age did not catch up over time and still had small SC measurements at 2 year of age. Therefore, final decisions based on SC could be made by the time bulls are 12 month old (Albert and Leonardo, 2004).Limousin bulls had the lowest SC, regardless of age. Conversely, Simmental bulls had the highest SC in 11 to 13 month and 19 to 26 month old animals, whereas Angus and Hereford bulls had the highest SC in 13.5 to 18 month and > 26 month age groups (Ajitkumar ***et al.,*** 2011).which is one of the body parts and there is significant correlation between the dimensions of the body and testis dimension and between scrotal circumferences with testis dimension (sarder, 2005).

Conclusion:

Individual motility of spermatozoa after freezing for all bulls to a greater extent differences from month and season to another. Moreover result study revealed that individual motility of spermatozoa after freezing for all bulls differences from age to another and correlation between age ,month, season and testicular dimensions such as testis dimensions and scrotal circumference with freezability of semen for Holstein bulls born in Iraq.

Acknowledgements

This study was carried out in the Artificial Insemination Center, Iraq (AI Center). The authors would like to thank every member in the AI Center, especially to the dear Dr.Kateg Ehthrir and Wafa'a Yeedam Lateef for their cooperation and afferent me indefinite facilities along experimental period in order to be performed conveniently.

REFERENCES

1- Ajitkumar, M.; Herman, B.; Randy, W.; John, K. and Jacob T. (2011). Associations between sperm abnormalities, breed, age and scrotal circumference in beef bulls. Can. J. Vet. Res., 75 (4): 241–247. | 2- AL-Badry, K. I. (1998).Factors influencing the reproductive efficiency of foreign bulls born in Iraq. Biol. Iraqi. J., (17): 85-95. | 3- AL-Badry, K.I.; Ibrahim, F.F. and Rajab, B.A.(2012). The effect of Foot and Mouth disease reproductive performance of Holstein bulls in Artificial Insemination Center of Iraq. Iraq. J. Vet. Med.,(36): 1-7. | 4- Albert, D. and Leonardo, F. (2004). Pubertal development of Bostaurus bulls. Large Anim. Vet. Rounds, 4 (4):17-48. | 5- Amann, R. P., Seidel, G. E. Jr. and Mortimer, R.G. (2000). Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. Theriogenology 54:1499-1515. || 6- Andrabi, S.; Naheed, S.; Khan, L. and Ullah N. (2002). Semen characteristics of crossbreed (Friesian × Sahiwal) bulls at Livestock Research Center Station National. Agriculture Research Center, Islamabad. Pak Vet J. 22(4): 181-187. | 7- Aponte, P.M.; de Rooij, D. G. and Bastidas, P.E. (2005). Testicular development in Brahman bulls. Theriogenology. (64): 1440-1455. | 8- Arteaga, A.; Baracaldo, M. and Barth, A.D. (2001)The proportion of beef bulls in western Canada with mature spermiograms at 11 to 15 months of age. Can Vet J.;42:783–787. | 9- Bailey, T. L.; Monkey, D.; Hudson, R. S.; Wolfe, D. F.; Carson, R. L. and Riddell, M. G. (1996) Testicular shape and its relationship to sperm production in mature Holstein bulls. Theriogenology 46:881-887. | 10- Barth, A.D. and Waldner, C.L. (2002). Factors affecting breeding soundness classification of beef bulls examined at the Western College of Veterinary Medicine. Can Vet J.;43:274–284. | 11- Bearden, H.J.; Fuquay J.W. and Willard S.T. (2004). Applied Animal Reproduction. Sixth Edition. New Jersey. Pearson Prentice Hall. behaviour of Zebu bulls in the humid tropics of Costa Rica: single versus multiple-sire groups. Anim. Reprod. Sci., (64): 139-148. | 12- Bilby, T.R. and Jordan, E.R.(2009). Reproduction Program Changes to Improve summer Fertility. Irymatters.org April 2009 | 13- Brito, L.F.;Silva, A.D.; Rodrigues, L.H.; Vieira, F.V.; Deragon, A.G. &Kastelic J.P.(2002). Effects of age and genetic group on characteristic of scrotum, testis and testicular vascular cone and on sperm production and semen quality of AI bulls in Brazil Theriogenology. (58):1175-1186. | 14- Carlsen, E.; Anderson, A.M.and Petersen, J.H.(2003). History of febrile illness and variation in semen quality. Hum Reprod; 18(10): 2089-2092. | 15- Chacon, J.; Pérez, E.and Rodiguez-Martinez, H.(2002). Seasonal variations in testicular consistency, scrotal circumference and spermiogramme parameters of extensively reared Brahman (Bos indicus) bulls in the tropics. Theriogenology 58, 41–50. | 16- Cholami, H.; Chamani, M.; Towhidi, A. and Fazeli, M.H.(2011). Improvement of Semen Quality in Holstein Bulls during Heat Stress by Dietary Supplementation of Omega-3 Fatty Acids. International Journal of Fertility and Sterility. 4; (4), Jan - Mar: 160-167. | 17- Coe, P. H. (1999). Associations among age, scrotal circumference and proportion of morphologically normal spermatozoa in young beef bulls during an initial breeding soundness examination. Journal of American Veterinary Medical Association 214:1664-1667. | 18- Corcuera, B.D.; Hernandez-Gil, R.; De Alba Romeo, C. & Martin-Rillo, S. (2002). Relationship of the environmental temperature and in boar facilities with seminal quality. Livest.. Prod. Sci. (71): 55-62. | 19- Duncan, D.B. (1955). Multiple Rang and Multiple F-test. Biometrics. 11: 4-42. | 20- Eric, W., Swanson, and Herman, H.A. (2010). Seasonal variation in semen quality of some Miss our dairy bulls. J. Dairy .Sci.27:303-310. | 21- Forni, S.; Albuquerque, L.G.(2004). Avaliação de características biométricas de testículos de bovinos Nelore. In: Simposio Da Sociedade BRASILEIRA De Melhoramento Animal, Pirassununga. Anais. Pirassununga: Sociedade Brasileira de Melhoramento Animal. Pp: 110-121. | 22- Foote, R.H. (1969). Research techniques to study reproductive physiology in the male. Techniques and Procedures in Animal Science Research, American Society of Animal Science, Champaign 111 pp. 81. | 23- Garcia-Peniche, T.B.; Cassell, B.G.; Pearson, R.E.and Misztal, I. (2005). Comparisons of Holsteins with Brown Swiss and Jersey cows on the same farm for age at first calving and first calving interval. J. Dairy Sci. 88, 790–796. | 24- Hansen, G. R. (2006). University of Florida – IFAS Extension. Available at http://edis.ifas.ufl.edu/AN153. |||| 25- Haugana,T.; Reksen,O.; Gr¨ohn,Y.T.; Kommisrud,E.; Ropstad,E.and Sehested,E.(2005). Seasonal effects of semen collection and artificial insemination on dairy cow conception. Animal Reproduction Science. 90: 57–71. | 26- Higdon, H.L.; Spitzer, J.C.; Hopkins, F.M.and Bridges, W.C.(2000). Outcomes of breeding soundness evaluation of 2898 yearling bulls subjected to different classification systems. Theriogenology.;53:1321–1332. | 27- Hoogenboezem, J.& Swanepoel, F. (2000). Zootechnological aspect of bull fertility Animal Genetic Resource Iowa. State University Press. | 28- Hopkins, F.M.and Spitzer, J.C. The new Society for Theriogenology breeding soundness evaluation system. Vet Clin North Am Food Anim Pract. 1997;13:283–293. | 29- Jainudeen, M.R.and Hafez, E.S.E. (2000). Cattle and Buffalo.In: Hafez, E.S.E., Hafez, B (Eds.), Reproduction in Farm Animals, 7th ed. Lippincott Williams and Wilkins Philadelphia, PA. | 30- Jalme, M.S.; Lecoq, R.; Seigneurin, F.; Blesbois, E.and Plouzeau, E. (2003). Cryopreservation of semen from endangered pheasants: the first step towards a cryobank for endangered avian species. Theriogenology. 59: 875–888. | 31- Kasari, T.R.; Wikse, S.E.and Jones R.(1996). Use of yearling bulls in beef cattle operations. Part 1. Economic analysis and fertility assessment. Comp Cont Educ Pract Vet.;(18):1244–1253. | 32- Kastelic, J.P.; Cook, R.B.; Pierson, R.A.and Coulter, G.H. (2001). Relationships among scrotal and testicular characteristics, sperm production and seminal quality in 129 beef bulls. Can J Vet Res. 2001;65:111–115. | 33- Marai, I.F.M.and Haeeb, A.A.M.(2010). Buffalo's biological functions as affected by heat stress- A review. Livestock Science. 127(2): 89-109 | 34- Noakes, D.E.; Parkinson, T.J. and England, G.C.W.(2001).Arthurs.Veternary Reproduction and Obstetrics .Eight Edition.Sunders.Elsevier Limited. | 35- Ott, R.S.; Cates, W.F.; Nicholson, H.H.; Crow, G.H.and Janzen ED. (1981).Testicular development in record-of-performance bulls. Proc Ann Mtg Soc Theriogenology:16-30. | 36- Parkinson, T.J. (2004) Evaluation of fertility and infertility in natural service bulls. Vet J.; 168:215–229. | 37- Quirino, C.R. (1999). Heredabilidades e correlações genéticas entre medições testiculares, características seminais e libido em touros Nelore. Doctoral Dissertation. Universidade Federal de Minas Gerais. Belo Horizonte, Brazil. 104 pp. | 38- Rensis, D. F. and Scaramuzzi, R.J.(2003). Heat stress and seasonal effects on reproduction in the dairy cow-a review.Theriogenology. 60(6): 1139-1151. | 39- Sarder,M.J.U. (2005).Scrotal circumfication variation on semen characteristics of A.I bulls .J.Anim. Vet.Advan.4(3):335-340. | 40- SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA. | 41- Senger, P.L. (2003). Reproductive behavior. In: Senger PL (ed.), Pathways to Pregnancy and Parturition. Current Concepts Inc., Washington State University Research and Technology Park, Pullman, WA, USA, pp. 240-265. | 42- Shioya, Y.(2004). Artificial Insemination Manual For Cattle –Revised Edition- Japan Livestock Technology Association. page 10.Semen characteristics were mostly affected by the environmental factors. Volume and concentration were more affected by season. | 43- Shivaji, S.; Kholkute, S.D.; Gaur, A.; Umapathy, G.; Singh, A.; Sontakke, S.; Shailaja, K.; Reddy, A.; Monika, S.; Sivaram, V.; Jyotsna, B.; Bala, S.; Ahmed, M.S.; Bala, Chandrashekar, B.V.N.; Prakash, S.and Singh, L. (2003). Conservation of wild animals by assisted reproduction and molecular marker technology. Indian J. Exp. Biol. 41, 710–723. | 44- Thundathil, J., Palasz, A.T., Barth, A.D. and Mapletoft, R. J. (2001). The use of in vitro fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmatic droplets. Animal Reproduction Science 65:181-192. | 45- Unanian, M.M.; Silva, A.E.D.F.and Mcmanus, C.(2000) . Características biométricas testiculares para avaliação de touros zebuínos da raça Nelore. Revista Brasileira de Zootecnia, v.29, p.136-144. | 46- Vásquez, L. and Arango, J. (2002). Relationship between corporal and testicular measurements in peripuberal Brahman bulls. Livestock Research for Rural Development 14. | 47- Vilakazi, D.M. and Webb,E.C. (2004). Effect of age and season on sperm morphology of Friesland bulls at an artificial insemination centre in South Africa. South African Journal of Animal Science . 34 (1):62-69. | 48- Violeta, I .; Mojeti, A.; Mircu, C.; Roman, C.; Ghiurca, D. and Casalean, H.C.(2010). The influence of some environmental factors and age on semen production of Fleckvieh bulls.Lucrari- Stiiniifice Medicina Veterinara Vol.Xlii (2) .Timisoara-Rominia.56. | 49- Yamashita ,Y.(2004). Artificial Insemination Manual For Cattle Japan Livestock Technology Association Revised Edition |