Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(4): 398-402

Received: 04-05-2019
Accepted: 06-06-2019

Manoj Kumar Yadav
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Gyanesh Kumar
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Atul Saxena
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Vikas Sachan
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Anuj Kumar
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Dilip Kumar Swain
Department of Veterinary Physiology, DUVASU, Mathura, Uttar Pradesh, India

Brijesh Kumar Yadav
Department of Veterinary Physiology, DUVASU, Mathura, Uttar Pradesh, India

Jitendra Agrawal
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Correspondence
Manoj kumar Yadav
Department of Veterinary Gynaecology & Obstetrics, DUVASU, Mathura, Uttar Pradesh, India

Effect of different concentrations of soyabean milk based extander on seminal attributes of cryopreserved semen

Manoj Kumar Yadav, Gyanesh Kumar, Atul Saxena, Vikas Sachan, Anuj Kumar, Dilip Kumar Swain, Brijesh Kumar Yadav and Jitendra Agrawal

Abstract
The present investigation was under taken on four Hariana bulls to study the effect of cryopreservation using Soya-milk extender. A total of 28 collections from four bulls (seven collections per bull) were utilized for the study. After initial evaluation, each semen sample was diluted in two different extenders viz. Tris egg yolk extender which acted as control and Soya-milk extender as experimental. Various sperm parameters were studied at post dilution, post equilibration and post-thaw stages. There was significant difference found for The progressive motility, live sperm percentage, Host Percenatage. Intact acrosomes was significantly higher for Tris egg yolk extender at post dilution, equilibration and post-thaw stages. In conclusion Tris egg yolk extender found to be superior than soyamilk extender.

Keywords: Hariana semen, cryopreservation, soya-milk, Tris egg-yolk

Introduction
Cryopreservation is the main essential tool for long-standing storage of semen and control of venereal diseases However, cryopreservation yields detrimental effects on post-thaw sperm quality and fertilization process [26]. Egg yolk is the most widely used cryoprotectant in the composition of cryopreservation extenders of mammalian spermatozoa; yet efforts have been made to find ways to substitute it due to the possibility of transporting pathogenic microorganisms, production of harmful metabolites and toxins [6, 3]. Growing concern over issues with egg yolk as a semen extender components motivated consideration of alternate plant based extenders that effectively maintain sperm viability and fertility while minimizing the risk of disease transmission. Plant based proteins have been used in semen extenders. Amongst plant based proteins, soya bean has been tested. Soya bean contains lecithin, a substitute for high molecular weight lipoprotein in egg yolk that can prevent or repair damage to the sperm plasma membrane during cryopreservation. Low density lipoproteins (LDL) extracted from egg yolk, gamma-irradiated egg yolk plasma, pasteurized powdered egg yolk or lecithin from non-animal source like soya were tested as a non-permeable cryoprotectant in extender for deep freezing of farm animals spermatozoa [28, 20]. Moreover, the use of non-animal origin chemically defined medium is the method of choice in assisted reproductive technology and semen cryopreservation [15, 23]. Recently, there are several studies indicating the valuable effects of soya bean lecithin for cryopreservation of sperm in Bull [1, 16]. Ram [10, 14] and Goat [32, 34]. Substitution of egg yolk with soya bean lecithin may reduce hygienic risks in extenders. The soya bean lecithin is a valuable plant-based phospholipids source that included in commercial extenders used for freezing mammalian sperm without clear levels and adjustments due to trade protection. Soya bean is a leguminous plant which has become a major source of dietary protein in domestic animals and humans. Soybean seeds contain an average of 36–38% protein, 30% carbohydrate, 19% oil, 9% crude fibre and 5% ash on a dry weight basis [7]. Lecithin generally refers to the entire phospholipid fraction. Lecithin can easily be extracted from soya bean chemically (using hexane) or mechanically (by grinding and extraction). The potential of soya bean based extenders for replacing egg yolk extenders has been investigated by several researchers [17, 12]. Most studies have tested the efficacy of commercially available soya bean lecithin based extenders [18, 6, 29, 4]. Very few studies using a crude soybean extract have been reported [24, 25, 35, 36].

Materials and Methods

The present study was conducted at Semen Biology Lab of Department of Gynaecology and Obstetrics situated at University Instructional Livestock Farm Complex (ILFC), College of Veterinary Science and A.H., U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalya Evam Go Anusandhan Sansthan (DUVASU), Mathura (U.P), INDIA during 2017-2018.

Experimental animals

Four healthy breeding Hariana bulls age 8.5 to 9.5 years and weight 450 to 500 kg maintained at Semen Biology Lab, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & A.H., DUVASU, Mathura, was utilized as semen donor for the present study.

Feeding and management of bulls

All the experimental bulls were kept in individual pens made up of brick and cement with concrete floor and asbestos roof. The bulls were being fed balance ration as per its availability at the farm. They were vaccinated against important contagious and infectious diseases. They were dewormed routinely.

Semen Collection

Semen from Hariana bulls was collected using artificial vagina (40 cm long and 6.5 cm in diameter). Collections were taken twice weekly between 08.00 to 09.00 hrs, in the before feeding. The practice of cleaning and washing of bull was routinely followed before collection. Immediately after collection, the tubes containing semen were placed in the water bath maintained at 37 ºC and samples were evaluated for various, macroscopic (volume, colour and consistency) and microscopic (mass activity, per cent initial motility, percent livability and per cent HOST responsive spermatozoa) semen characteristics according to the method described by [8, 22, 23, 33].

Sampling procedure

A total of 7 ejaculates each from four (4) bulls were collected. Thus the total number of ejaculates collected for the entire study is 28 (7×4=28).

Dilution of Semen

Semen samples which qualifies (>70% progressive motility) freezing criteria were divided in two equal aliquots. Aliquot-1 was used for study of the initial basic parameters and following fulfilling the criteria for cryopreservation the Aliquot-2 was subdivided into four parts, part one part of the semen was extended with basic extender (EYTG) and was considered as control sample. The other three parts were added with the basic extender however was without the egg yolk which was replaced with 5%, 15% and 25% soya milk (Treatments). The dilution rate was calculated to keep the final concentration of sperm as 80 x 106 sperms per ml. After the final dilution, each semen aliquot (control and treatment) was evaluated for post-dilution individual motility, sperm viability, acrosomal integrity, hypoosmotic swelling test (HOST).

Composition of TRIS buffer

Tris buffer (Tris hydroxymethyl) aminomethane 2- Amino-2-(hydroxymethyl) propane-1,3- diol 3.025 gm
Citric acid (monohydrate) 1.675 gm
Glucose (anhydrous) 0.750 gm
Penicillin G. Sodium 1 Lac unit
Dihydrostreptomycin sulfate 100 mg
Double glass distilled water upto 100 ml

(c) Soya milk preparation

Soya milk was prepared as per the method of [12]. Accordingly, 10 grams of Soya bean grains was washed, soaked in 100 ml distilled water and boiled for 30 min. After boiling, the water was discarded, the whole soybean grains washed again and finally cooled down with 50 ml distilled water containing 0.25% NaHCO3. The grains were then grinded in a blender for 5 min and the slurry cooled. Soymilk was extracted by filtration through a clean cotton cloth, centrifuged and boiled again for 10 minutes. The slurry was allowed again to cool down. Then, antibiotics were added at the rate of 0.005 g streptomycin/100 ml of the slurry. After all, the soya bean milk extender was ready for use.

(d) Incorporation of glycerol: @ 7% glycerol

(e) Incorporation of egg yolk: @ 20% (20 parts of egg yolk are mixed with 80 parts of buffer)

(f) Incorporation of soya milk: @ 5%, 15%, and 25% respectively (5 parts of soya milk mixed with 95 parts of buffer, 15 parts of soya milk mixed with 85 parts of buffer, 25 parts of soya milk mixed with 75 parts of buffer)

Cryopreservation procedure

The extended (Tris and soya milk) and semen samples were kept in water bath at 37 degree centigrade and following the dilution were filled in straws.

Filling and sealing of straws

French Top Bull mini straws (0.25 ml, 135 mm length and 2 mm diameter, IMV) of different colours were used. Automatic straw filling and sealing machine (IMV, France) was used for filling of semen into the straws and sealing it. Filling and sealing were done at room temperature.

Equilibration

The filled and sealed straws were kept on freezing racks and placed in cold handling cabinet at 4^0C for 5 hours.
Vapour freezing after completion of equilibration, the freezing racks along with straws were kept in the liquid nitrogen vapour freezing chamber of biological freezer (IMV, France). The temperature of semen straws reaches from 4^0C to -140^0C within 7 minutes.

Storage

The straws were transferred into goblets with the help of cryo gloved hand and the goblets were stored in separate canisters in the LN2 (-196^0C) in cryovessel. Cryovessel was always kept full by replenishing LN2 time to time.

Thawing of frozen semen

The frozen semen was thawed in thawing unit (IMV, France) maintained at a temperature of 37^0C and employing a time deuration of 45 sec.

Evaluation of Semen

Semen was evaluated for the following parameters.The sperm progressive motility was determined according to [27], live and

dead spermatozoa were evaluated using eosin negrosin mixture prepared as described by [18], plasma membrane integrity of spermatozoa was according to [22] and acrosome integrity was assessed using Geimsa stain according to Watson, Evaluation of capacitation status by Chlortetracycline assay (CTC Assay) in post–diluted, post–equilibrated and post–thawed semen.

Statistical Analysis

Data were statistically analyzed using statistical software (SPSS, version 18.0). One–way analysis of variance was used to test the significance of extenders on the studied traits [37]. Means of the significantly affected traits were separated by Duncan Multiple Range Test [11].

Results

(a) Sperm progressive motility

The progressive motility was significantly ($p<0.05$) higher in tris egg yolk based extender compared to any concentration of soya milk extender. Amongst the different concentration of soya milk, 15% soya milk based tris extender was significantly ($p<0.05$) superior compared to 5% and 25% soya milk.

Table 1: Effect of different concentration of soya milk on Sperm progressive motility at post dilution, pre-freeze and post thaw stages of cryopreservation (Mean±SEM=28).

Stages	EYTG	Tris glycerol-Soya-milk		
		5%	15%	25%
Post dilution	78.57 ± 1.05^{d}	70.00 ± 1.17^{b}	74.82 ± 0.97^{c}	63.04±1.16^{a}
Pre-freeze	72.14 ± 0.97^{d}	30.71 ±1.08^{b}	37.71 ± 0.94^{c}	24.82±1.07^{a}
Post thaw	46.11 ± 1.00^{d}	9.11 ± 0.63^{b}	13.21± 0.52^{c}	6.79 ± 0.46^{a}

Small letters (a, b, c, d) superscripts differed significantly within a row. This pattern has been followed in subsequent tables.

(b) Live sperm

The percentage of live spermatozoa was significantly ($p<0.05$) higher in tris egg yolk based extender compared to any concentration of soya milk extender. Amongst the different concentration of soya milk, 15% soya milk based tris extender was significantly ($p<0.05$) better compared to 5% and 25% soya milk.

Table 2: Effect of different concentration of soya milk on per cent live spermatozoa at post dilution, pre-freeze and post thaw stages of cryopreservation (Mean±SEM=28).

Stages	EYTG	Tris glycerol-Soya-milk		
		5%	15%	25%
Post dilution	85.54±0.96^{d}	77.21 ±0.89^{b}	81.57±0.82^{c}	71.68±0.87^{a}
Pre freeze	77.71±1.03^{d}	37.21±0.82^{b}	43.61±0.91^{c}	33.86±1.16^{a}
Post thaw	54.21±0.99^{c}	16.11±0.43^{a}	19.75±0.4^{b}	14.39±0.59^{a}

(c) HOST reactive spermatozoa

The HOST reactive spermatozoa was significantly ($p<0.05$) higher in tris egg yolk based extender compared to any concentration of soya milk extender. Amongst the different concentration of soya milk, 15% soya milk based tris extender was significantly ($p<0.05$) higher compared to 5% and 25% soya milk.

Table 3: Effect of different concentration of soya milk on HOST reactive spermatozoa at post dilution, pre-freeze and post thaw stages of cryopreservation (Mean±SEM=28).

Stages	EYTG	Tris glycerol-Soya-milk		
		5%	15%	25%
Post dilution	79.75±0.70^{c}	75.42±0.70^{a}	77.5±0.64^{b}	73.71±0.68^{a}
Pre freeze	74.21±0.69^{d}	47.96±0.50^{b}	52.10±0.34^{c}	45.64±0.79^{a}
Post thaw	63.96±0.52^{d}	38.46 ±0.54^{b}	42.39±0.41^{c}	35.32±0.58^{a}

(d) Spermatozoa with Intact acrosome (per cent)

The percentage of intact acrosome was significantly ($p<0.05$) higher in tris egg yolk based extender compared to any concentration of soya milk extender. Amongst the different concentration of soya milk, 15% soya milk based tris extender was significantly ($p<0.05$) superior compared to 5% and 25% soya milk.

Table 4: Effect of different concentration soya milk on per cent spermetozoa with intact acrosome at post-dilution, pre-freeze and post thaw stages of cryopreservation (Mean ± SEM=28).

Stages	EYTG	Tris glycerol-Soya-milk		
		5%	15%	25%
Post dilution	87.71±0.50^{c}	81.75±0.81^{a}	84.89±0.62^{b}	80.71±0.69^{a}
Pre freeze	82.75±0.62^{c}	74.82±0.62^{a}	79.00±0.63^{b}	73.39±0.63^{a}
Post thaw	77.54±0.55^{c}	54.14±0.69^{a}	60.82±0.48^{b}	52.68±0.55^{a}

Discussion

Individual sperm progressive motility is an important criterion of semen quality as it determine the success rate of the fertilization. In the present study, the progressively motile sperm percentage was significantly lower in treatment (soya milk supplemented extender) groups compare to control (egg yolk supplemented extender) at post dilution, pre-freeze and post thaw stages [21]. Used soya milk for ultralow freezing of Murrah bull semen. They observed significantly lower sperm motility in soya milk based extender at post dilution, prefreeze and post thaw stages [9]. Also reported similar results while using commercially available soya lecithin extender (Boxcell). These reports are confirming our findings however, the post thaw motility in our study was very low compare to the finding of these workers. The lower motility in our study could be due to higher concentration of soya lecithin as has been stated by [14]. Further, high viscosity may be the cause for low motility. Contrary to our findings, [36] observed no significant difference between egg yolk based and 25% soya milk based extender while cooling crossbred cattle semen at 5ºC or when freeze using standard protocol for preservation [13]. Used soya–lecithin in the concentration of 0.5%, 1% and 2% and found significant increase in post equilibration and post thaw motility in 0.5% soya milk based extender.

In the present study, the per cent live spermatozoa was significantly lower in treatment (soya milk supplemented extender) groups compare to control (egg yolk supplemented extender) at post dilution, pre-freeze and post thaw stages. Similar findings was also reported by [21] who observed higher percentage of live spermatozoa in egg yolk based extender compare to 25% soya milk extender for persevering buffalo bull semen. Contrary to our finding, various other reports suggest soya milk as better option than egg yolk. For liquid preservation (4ºC) of HF bull semen, [31] found 25%

soya milk comparable with egg yolk however, 50% soya milk was not fit for preservation [2]. Also found soya milk a better option than egg yolk for liquid preservation. Even at post thaw stage, many others reported soya milk extender better than the egg yolk [30, 35]. The available literature on soya bean suggests that it contain large amount of lipoprotein called soya lecithin which has got similarity with egg yolk lecithin. Higher concentration of soya lecithin which probably may interferes with the stains. The ability of spermatozoa to swell in the presence of hypoosmotic medium reflects normal water transport across the sperm membrane, which is a sign of normal membrane integrity and functional activity [22].

In the present study, the per cent HOST reactive spermatozoa was significantly lower in treatment (soya milk supplemented extender) groups compare to control (egg yolk supplemented extender) at post dilution, pre-freeze and post thaw stages [2]. Compared different extenders for liquid preservation of Nilli Ravi bull semen and concluded that plasma membrane integrity (HOST positive spermatozoa) remained similar in Bioxcell (commercial soya milk extender), milk and tris egg yolk extender. These worker in Nilli Ravi bull semen found that 10% soya milk was significantly superior in preserving plasma membrane integrity compared to 5%, 15% soya milk and control group [36]. Found 25% soya milk at par with the egg yolk extender both at liquid preservation as well as ultralow freezing in preserving crosssbred bull semen [31]. Found significantly higher HOST reactive spermatozoa in 50% concentration of soya milk for liquid preservation of HF bull semen, although, for other sperm parameters they found 25% soya milk as the best and significantly better than the egg yolk.

The intactness of the acrosome is the determining factor of fertilization which regulates the outcome of fertility. Most workers examined the acrosome abnormalities by using the Giemsa stain technique [5]. In the present study, the per cent spermatozoa with intact acrosome were significantly lower in treatment (soya milk supplemented extender) groups compare to control (egg yolk supplemented extender) at post dilution, pre-freeze and post thaw stages [36]. Reported 25% soya milk at par with egg yolk based extender in preserving the acrosomal integrity of spermatozoa both at liquid preservation as well as ultralow freezing [13]. Observed 0.5% soya lecithin significantly better for preserving the acrosomal integrity of spermatozoa both at pre freeze and post thaw stage while cryo-preserving buffalo bull semen [21]. While preserving Murrah bull semen did not find any significant difference in per cent intact acrosome in soya milk or egg yolk based extender.

Conclusions

Addition of soya milk by replacing the egg yolk in tris based extender does not have any beneficial effect on progressively motile spermatozoa, live spermatozoa, HOST reactive spermatozoa, acrosome intact spermatozoa percentage. Thus, egg yolk based extender proved significantly ($p<0.05$) superior based on the evaluated parameters. Based on these results, a further study is warrented to ellucidate these observed results which should include (1) effect on low soya milk concentration (2) fixing osmolarty of extenders and (3) simultaneous effect of commercially avilable soya milk.

References

1. Aires VA, Hinsch KD, Muller F, Bonger K, Mullerschlosser S, Hinsch E. *In vitro* and *in vivo* comparision of egg yolk based and soya bean based extenders for cryopreservation of bovine semen. Therio. 2003; 60:269-279.
2. Akhter S, Ansari MS, Rakha BA, Ullah N, Andrabi SMH, Khalid M. *In vitro* evaluation of liquid-stored Buffalo semen at 5 degree centigrade diluted in soya lecithin based extender (Bioxcell®) tris-citric eggyolk, skim milk and egg yolk-citrate extenders. Reprod. Domest. Anim. 2011; 46:45-49.
3. Althouse GC. Sanitary procedures for the production of extended semen. Reprod. Domest. Anim. 2008; 43:374-378.
4. Arifiantini RS, Yusuf TL. Developing of tris soy milk diluents for frisian holstein bull frozen semen. Hayati J. Bio sci. 2010; 17(2):91-94.
5. Bhosrekar MR, Mokashi SP, Purohit JR, Gokhale SB, Mangurkar BR. Effect of glycerolization and deep freezing on the levels and release of enzymes in buffalo semen in relation to initial semen attributes Proc. 4th Int. Buffalo Congress, Sao Paulo, Brazil. 1994; 3:465-467.
6. Bousseau S, Brillard JP, Guienne M, Guerin B, Camus A, Lechat M. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin-based diluents. Theriogenology. 1998; 50(5):699-706.
7. Brumm T, Hurburgh C. Jr. In: Quality of the soybean crop from the United States. American Soybean Association, St. Louis, MO changes in hariana bull spermatozoa during cryopreservation. M.V.Sc., Thesis, 2002.
8. Campbell RG, Hancock JC, Rothschild L. Counting live and dead bull spermatozoa. Mol. Reprod. Dev. 1953; 30:44-49.
9. Chaudhari DV, Dhami AJ, Hadiya KK, Patel JA. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (-196°C) of buffalo semen. Vet. World. 2015; 8(2):239-244.
10. De Paz P, Esteso MC, Alvarez M, Mata M, Chamorro CA, Anel L. Development of extender based on soybean lecithin for its application in liquid ram semen. Theriogenology. 2010; 74:663-671.
11. Duncan DB. Multiple range and multiple F-test Biometrics. 1955; 11:1-42.
12. ELkeraby FE, Osman KT, Ganah HB, Siefy EM. Soymilk-based extender for cryopreservation of bovine semen. Journal of Animal Poultry Production. 2010; 2:61-69.
13. ElSherbien MA. Impact of replacing egg yolk with lecithin on sperm characteristics, bacterial count and fertilizing ability of cryopreserved buffalo semen**.** Journal of animal and poultry production Mansoura University. 2014; 5(6):353-364.
14. Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L. *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology. 2010; 73:480-487.
15. Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K. Fertility after artificial insemination using a synthetic semen extender in sheep. J Reprod. Dev. 2008; 54:286-289.
16. Gonzales R, Rosales ABM, Perea CF, Velarde CJ, Soto BE, Palomares ABR. Conception rates using Brahman

bull semen frozen in milk based extender containing egg yolk or soybean lipids. A field study in a tropical environment. Reprod. Fertil. Dev. 2003; 16:170-171.
17. Graham JK, Foote RH. Effect of several lipids, fatty acyl chain length and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. Cryobiology. 1987; 24:42-52.
18. Hancock JL. A staining technique for the study of temperature shock in semen. Nature. 1951; 167:323.
19. Hinsch E, Hinsch KD, Boehm JG, Mueller SWB, Schloesser F. Functional parameters and fertilization success of Bovine semen cryopreserved in egg yolk free and egg yolk containing extenders. Reprod. Domest. Anim. 1997; 32:143-149.
20. Hu JH, Jiang ZL, Lv RK, Li QW, Zhang SS, Zan LS. The advantages of low-density lipoproteins in the cryopreservation of bull semen. Cryobiology. 2011; 62:83-87.
21. Hussain S, Kumar S, Iqbal M, Ahmed T. Effect of soya-milk based extender on the physico-morphological parameters of Murrah bull semen during cryopreservation. Theriogenology Insight. 2016; 6(2):97-101.
22. Jayendran RS, VanderVen HH, Perz PM, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journal of the society for Reprod. Fertil. 1984; 70:219-228.
23. Jeyendran R, Acosta V, Land S, Coulam C. Cryopreservation of human sperm in a lecithin-supplemented freezing medium. Fertil. Steril. 2008; 90:1263-1265.
24. Kasimanickam R, Asimanickam V, Tibracy A, Pelzer K. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4°C. Small Rumin. Res. 2011; 99:208-213.
25. Kasimanickam VR, Kasimanickam RK, Memon MA, Rogers HA. Effect of extenders on sperm mitochondrial membrane, plasma membrane and sperm kinetics during liquid storage of canine semen at 5◦C. Anim. Reprod. Sci. 2012; 136:139-145.
26. Lemma A. Effect of cryopreservation on sperm quality and fertility. In Manafi M, editor. Artificial insemination in farm animals. Croatia in Tech, 2011, 19-216.
27. Melrose DR, Laing JA. The characteristics of normal semen. ed. By J. A. laing Bailliere tindall and cassel. London, 1970.
28. Moussa M, Martinet V, Trimeche A, Tainturier D, Low MA. Density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology. 2002; 57:1695-1706.
29. Mui R, Fernandez M, Pena AI. Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 h. Reprod. Domest. Anim. 2007; 42:305-311.
30. Papa FO, Felício GB, Meloon CM, Alvarenga MA, Vita BD, Trinque C, *et al*. Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. Anim. Reprod. Sci. 2011; 129:73-77.
31. Rehman FU, Qureshi MS, Khan RU. Effect of Soybean Based Extenders on Sperm Parameters of Holstein Friesian Bull during Liquid Storage at 4ºC. Pak. J Zool. 2014; 46:185-189.
32. Roof DJ, Bowley S, Price LL, Matsas DJ. Comparison of two commercial extenders for cryopreservation of goat semen without sperm washing. Theriogenology. 2012; 77:412-420.
33. Salisbury GW, Van Demark NL, Lodge JR. Physiology of reproduction and artificial insemination in cattle, 1978, 639.
34. Salmani H, Towhidi A, Zhandi M, Bahreini M, Sharafi, M. *In vitro* assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. Cryobiology. 2014; 68:276-280.
35. Singh AK, Singh VK, Narwade BM, Mohanty TK, Atreja SK. Comparative quality assessment of buffalo (Bubalus bubalis) semen chilled (5ºC) in egg yolk- and soya milk–based extenders. Repod. Domest. Anim. 2012; 47:590-600.
36. Singh VK, Singh AK, Kumar R, Atreja SK. Development of soyamilk extender for semen cryopreservation of Karan Fries (crossbreedcattle). Cryo Letters. 2013; 34:52- 61.
37. Steel RGD, Torrien TH, Dieky DA. Principle and procedure of statics 3rd. Macgraw hill book co. Ing. New York, 1997.