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Review Article

Semen Extenders and Artificial Insemination in Ruminants

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Abstract

Artificial insemination is one of the important tools for genetic improvement in modern dairy breeding practices and it is only possible with efficient extender. Extender has a vital role in the preservation of sperm cell and its quality parameters such as viability, motility, acrosome and membrane integrity etc. Semen extenders contain buffering system to maintain pH of the medium (Tris, sodium phosphate, citric acid), cryoshock preservatives (glycerin, egg yolk, soy-lecithin, milk), provide energy (fructose) and guarantee the microbial free environment i.e. antibiotics (streptomycin, penicillin, polymyxin B). Here, in this review we will focus on the effects of different extender ingredients used for dilution of semen in ruminants to achieve Artificial insemination (AI).

Key Words: Semen extenders, Artificial insemination, Ruminants, Genetic improvement

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Introduction

The manual deposition of sperm cell in the sexually receptive female is termed as Artificial Insemination (AI) [1]. Naturally in plant cross breeding takes place due to insects, one of the excellent preexisting examples for AI. In animals the story begins with stealing the semen from the enemy horses by the ancient Arab tribes for breeding of their horses [2]. However the invention of the microscope by Leeuwenhoek in 1678 [3], let the first human eye to see sperm cell. Ivanovo [4] reported the first successful insemination in domestic farm animals after the early discovery by spallanzani in 1784 [5] in dogs. Danish scientist, Sorensen in 1940 [6] was the first to develop semen straw for artificial insemination and Amantea in 1914 [2] was the first to develop artificial vagina [7].

Sperm cell is the only factor for determining the genotypic sex of the progeny i.e. male or female. Different factors, including osmotic pressure, physico-chemical stresses and freeze-thaw temperature variations affects the semen quality parameters such as viability, motility and membrane integrity [8]. These different stressors will generate Reactive Oxygen Species (ROS) and lipid peroxidation of the cell membrane, which will ultimately affect the spermatozoa [9].

Extender or diluent is a chemical medium used for preservation, extension and protection of sperm cells against various shocks during processing, storage and transportation used for artificial insemination. Good extender should provide energy for metabolic activities with in sperm cell; maintain osmotic pressure and pH of the medium [10]. Extender also keeps a check on the contamination of the medium to protect semen from microbial growth. Different semen extenders provide sufficient nutrition in the form of fructose sugar to sperm cells during storage. It also prevents sperm cells against cryoshocks during cold storage at extreme temperature (-196 ^{o}C) in liquid nitrogen [2]. Moreover, liquid extended semen produces a higher conception rate with a relatively less number of sperm cells [11]. Semen extender is the only medium which enables us to exploit the reproductive potential of male animal more efficiently, with almost no venereal diseases. Commercially both egg-yolk based (Triladyl®, BULLXcell®, Bovidyl®) and soy-lecithin based (AndroMed®, Bioxcell®) extenders are available, also called as two steps and one step extenders, respectively.

Yolk-phosphate buffered extender was the first reported semen extender in USA [12]; later with the addition of sodium citrate in yolk phosphate buffer increased the survival of sperm cell up to three days at 5 ^{o}C and increased the sperm visibility by dissolving the fat globules [2]. Moreover the dilution of semen also decreases the concentration of K^{+} ions [13], proteins and other constituents in cell membrane.

Currently, two different kinds of semen extenders are in vogue, i.e. animal source lipoprotein based extenders [14, 15] and plant lipoprotein source based extenders [16, 17]. The animal source is mostly egg

yolk based and plant source is soybean lecithin based extender, both are commercially available. The latter one is developed recently with more benefits i.e. free of transboundary diseases and good conception rate. The major constituents of different semen extender are as follows

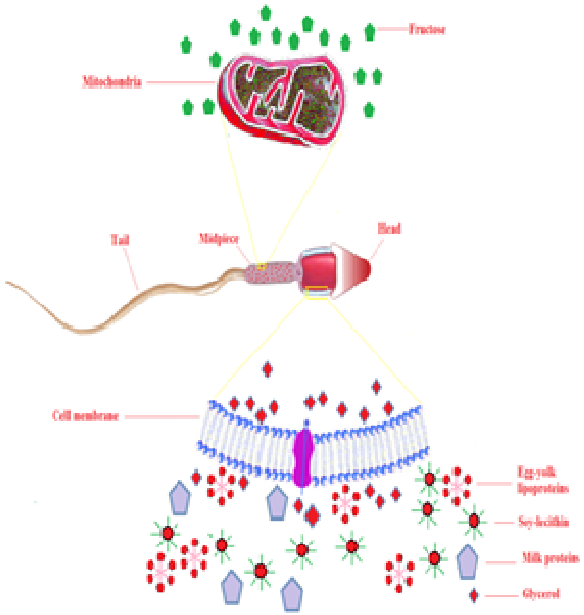

Fig. 1: Role of various components in semen extenders

Glycerol

To avoid cryoinjuries to sperm cells, different cryo protective agents have been used. Glycerol (Fig 2) is classified as a penetrating cryoprotectant and most common used for dilution of semen worldwide [18-20].

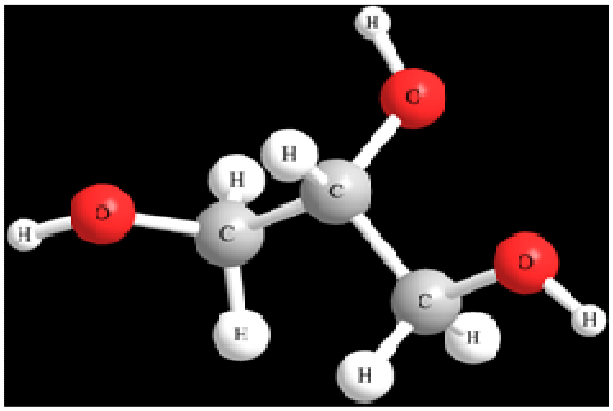

Fig. 2: Glycerol structural formulae

Glycerol cryo-protective effect was accidentally found by Polge et al, in 1949 [21], while performing experiments on the chicken spermatozoa. This discovery not only gave good cryoprotection to sperm cell, but also increased the post thaw quality of spermatozoa. It was found about 15 percent increase in the post thaw quality of semen was observed after addition of glycerol to the egg-yolk based extender [22]. Glycerol as conventional cryoprotectant prevents the intra-cellular crystallization; however, it affects the fertilizing ability of the spermatozoa by an increase in the transient osmotic pressure prior to equilibration around the cell membrane that will increase osmotic pressure and affecting the semen quality parameters [23-26]. A sudden decrease in temperature from +5 ^{o}C to -196 ^{o}C decreases the membrane integrity and increase permeability [27], this shock is protected by glycerol in addition to egg yolk lipoproteins. It has been suggested that thermal changes disturb the lipid to lipid and lipid to protein association within the cell membrane which ultimately leads to loss of function (Fig. 3) [28].

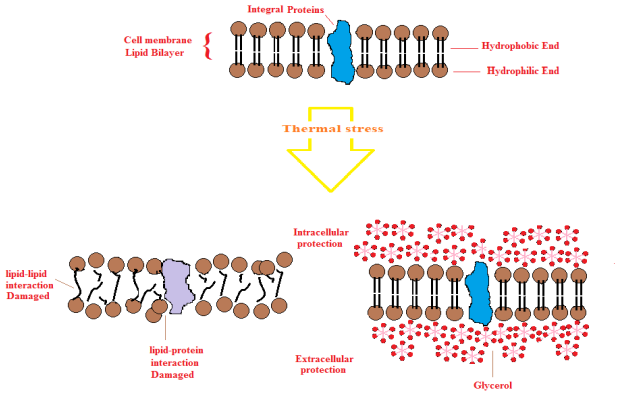

Fig. 3: Thermal stress effect on cell membrane

Energy Source

It is well known that every living organism needs energy for metabolism at the cellular level. In sperm cell, mitochondria are located in the neck region called midpiece and are responsible for the movement of sperm tail or flagellum that helps in sperm cell movement [29, 30]. The ATPs are generated in spermatozoa via oxidative phosphorylation process within the mitochondria [31, 32]. Semen naturally contains fructose (Fig. 4) as energy source and during extension egg yolk containing glucose and other compounds are also utilized by spermatozoa [33].

Different kinds of sugars glucose, trehalose, ribose, raffinose, saccharose, galactose has been used so far [34, 35]. Although in ruminants, still fructose sugar based extenders [14, 36, 37] are used worldwide, with less detrimental effects as compared to the others. Increased concentration of trehalose as an energy source in the ram semen extender increased the post thaw motility, recovery rates, thermal resistance and acrosomal integrity of spermatozoa [38].

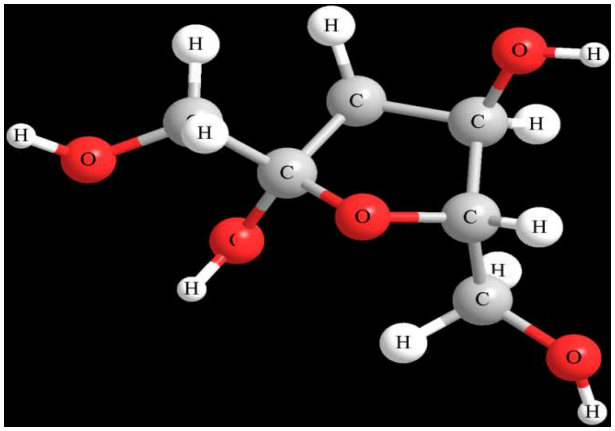

Fig. 4: Fructose structural formulae

Milk sources

Lactose is one of the important milk components that cannot diffuse across the cell plasma membrane, thus helps in creating osmotic pressure around the cell and prevents intra cellular crystallization [39]. Seminal proteins binding is reduced by milk casein, also decrease damage to cell membrane lipid and improve the sperm motility and viability [40].

A series of research work published in the mid of 20th century on the milk containing extenders for cryopreservation along with glycerol i.e. milk-glycerol based extender, made revolution in the cryopreservation of semen [41-43].

There is evidence that egg yolk based extenders decreased the acrosomal integrity in the goat spermatozoa [38]. There are some factors present in the accessory sex gland secretion in semen resulting in harmful effects on the sperm quality [53]. Egg yolk contain a considerable amount of steroids i.e. pregnelenone and progesterone, they induce an acrosomal reaction in the sperm cells that results in high risk of microbial contamination, drug residues and hormonally active substances in the egg yolk extended semen [54].

Egg yolk

Egg yolk is still used as primary non penetrating cryoprotectant in semen extenders. Different percentages are used for cryopreservation process so far. However, 20 percent Egg yolk is still used as a standard in most of the underdeveloped countries as it is cheap and readily available [44]. Philips in 1939, [45] was the first to report egg yolk use in the diluents, with a ratio of 1:1 to phosphate buffered solution (v/v) and become more popular [46]. It was reported that 4 % (v/v) addition of egg yolk produced satisfactory results for semen quality parameters [47]. In egg yolk, Low Density Lipids (LDL) abundance is considered a main cryoprotective agent as it adheres to the cell membrane and protects the cell from cryoshocks [48, 49]. The LDLs are composed of 17-60 nm spherical molecules, with lipid and triglycerides in the core surrounded by protein and phospholipid thin layers. The LDL contains 11-17% proteins and 83-89% lipids [50]. The addition of egg yolk increased post thaw motility by solubilizing the cell membrane lipids and binds to the sperm [51], but such a high amount increased the risk of microbial contamination resulting in metritis and transboundary diseases [52].

Soy-lecithin

Since last two decades, researchers have substituted the animal protein source with plant protein sources to avoid resulting diseases and keep the biosecurity from trans-boundary diseases. This concept lead to the development of animal protein free commercial diluent named as Biocephos plus® and AndroMed® [55,56]. Soybean lecithin based extender has been suggested as a replacement of egg yolk as animal source diluent [57, 58]. Commercially different soy-lecithin based extenders are available, among them AndroMed (Minitube, Tiefenbach Germany) and Biociphos (IMV Technologies, L'Aigle France) are very popular [59].

Lecithin (present in different cryoprotectants) protects the plasma membrane by restoring phospholipid that is lost due to heat and protects the cell viability [60]. Soy-lecithin is a very good alternative to phospholipids present in egg yolk for semen cryopreservation [61]. A total of 25% soy-lecithin based extenders increased the sperm membrane and acrosome integrity, live percentage, and motility in bovines [62]. In ovine 1-2 percent (w/v) soy-lecithin was considered as a good

replacement source for semen extension [63, 64]. In a recent study Kasimanickam et al [36] compared soy-lecithin based extender with milk containing conventional diluents and found that mitochondrial membrane potential was increased in soy-lecithin based extender. Moreover, DNA fragment index and sperm motility were also improved in soy-lecithin diluent as compare to milk based extenders.

Table 1. Extenders for semen processing

Extender type	Egg yolk (ml)	Glycerol (ml)	Fructose (gm)	Glucose	Tris (gm)	Citric acid (gm)	Sodium citrate (gm)	Skim Milk (ml)	Lactose	Ref
I	200	60	2	-	30.30	16.64	-	-	-	UP
II	200	70	8	-	-	--	-	-	48	[1]
III	200	50	5		36.3	19.9	-	-	-	[83]
IV	200	50	-	5	-	-	23.7	-	-	[82]
V	200	50	-	-	-	-	-	750	-	[84,85]
VI	200	50	-	7.74	33.19	16.704	-	-	-	[62]

UP = unpublished

Buffer

Hydroxymethyle aminomethane (Tris) (Fig. 5) and citric acid (Fig. 6) are very common used buffers in various types of diluents used for ruminant semen. Tris containing egg yolk glycerol extender was developed in 1963 and become most popular for both fresh and frozen semen [65, 66]. Earlier phosphate buffer was used with extender but due to limited visibility under the microscope its use was limited [46].

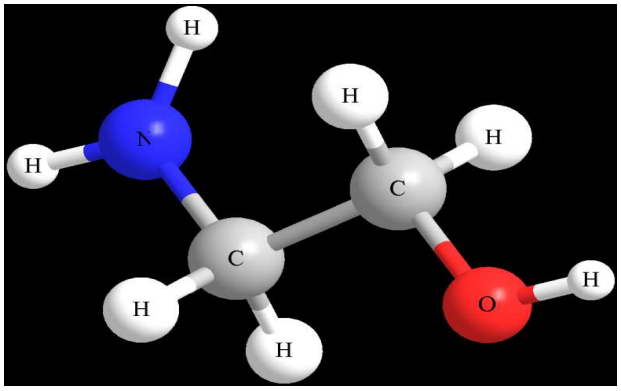

Fig. 5: Tris structural formulae

In bull the semen pH is slightly acidic ranging from 6.6-6.9 [67]. During processing of semen for cryopreservation, cellular metabolic activities are increased that leads to production of lactic acid [68] and some other acids and the extracellular environment become more acidic resulting in decreased pH. The decreased pH will reduce cellular activates within spermatozoa and storage life of semen.

The simplest buffers used are bicarbonates and sodium citrate. However, they are not temperature stable as compared to Tris, TES, MOPS, Hepes, which are more stable at high temperature and other different environmental conditions [35].

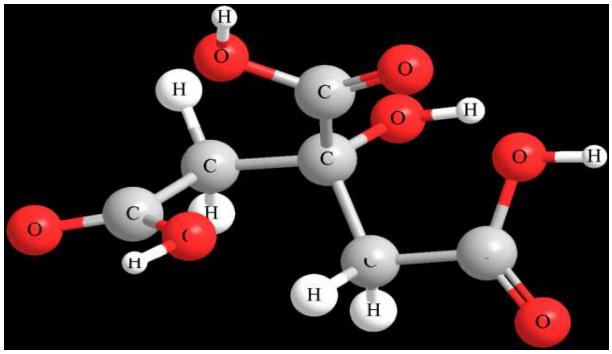

Fig. 6: Citric acid structural formulae

Antimicrobials

Various antibiotic ingredients have been used so far, to keep check on the microbial contamination in extenders. Generally the fresh semen from the physically fit bull is free from microorganisms. However, during semen collection via artificial vagina, presence of sugar (fructose) in extender and room temperature (20^{o}C) during processing are the promoting factors for bacterial growth. The most common contaminants are Gram positive bacteria along with *E. coli* and *Salmonella spp* [35]. Some other Species like *Clostridium pyogenes* and *Pseudomonas aurogenosa* are considered as potential threats to be transferred via cryopreserved semen. The other contagious bacteria include *Brucella abortus*, *Vibrio fetus*, *Trichomonas fetus*, *Leptospira pomona*, *Mycoplasma bovis* and *Mycobacterium Spp* [41].

Contamination with bacteria decreases the nutrients available for sperm cells leading to decreased pH that affects the motility and viability of spermatozoa. Similarly, fungi produce different kinds of endo- and exo-toxins that affect the sperm viability during preservation of semen. According to the recommendation of World Health Organization (WHO) 2003 and Office International des Epizooties (OIE), semen extender components from animal source should be free from all kinds of microorganisms [69].

The control of venereal disease along with cryoshock protection, improved the fertility by 15 % [2]. Cornel extender was the first standard diluent to have Penicillin G (Fig. 7), Streptomycin, and Polymixim-B [22] as an antimicrobial agent for several years approved by the National Association of Animal Breeders (NAAB). This recipe prevented most of the venereal disease and decreased the early embryonic death. Microbial contamination decreased sperm motility, acrosome integrity and pH of semen [70]. Similarly, some of the antibiotics used to exert a severe effect on the sperm cell mitochondria resulting in depression on the progressive motility as mitochondria plays vital role in the tail movements and overall energy required for cellular activities [71].

Since the first recipe of cornel extender (1950) penicillin and streptomycin are still used at the rate of one gram per liter. Some other antimicrobials i.e. *Ceftiofur*, *Apramycin* and *Aminoglycosides* such as *Gentamycin*, *Kenamycin*, *Neomycin* at the rate of 0.2 grams per liter are used in different semen extenders [35]. Combination of linco-spectin (300/600 µg), tylosin 100 µg, Gentamycin 500 µm showed good results against mycoplasma and other bacterial spp. [72, 73]. Most of commercially available soy-lecithin based extenders contain *Gentamycin, Lincomycine, Tylosin* and *Spectinomycin* as standard antibiotics.

Other additional supplementations

To decrease the concentration of sperm cell in the processed semen caproic acid and catalase along with 5 percent egg yolk were added to standard extender that could support the moderate ambient temperature of New Zealand [2]. The membrane permeability and its constituents have been effected by different diluents affecting the sperm viability, however, the addition of Bovine serum albumin to extenders increases the motility by protecting the cell membrane [74] and also improved the post thaw fertility rates [75]. Similarly, ethylene glycol and dimethyl sulphoxide were also used as additional penetrating cryoprotectant in different semen extenders [34].

Naturally semen is provided with balanced pro and anti-oxidative enzymes i.e. super oxide dismutase and catalase, but after dilution those become less effective and more reactive oxygen species (ROS) are spontaneously generated. Balanced ROS plays vital role in the chromatin condensation, membrane remodeling and intracellular pathways activation [76], however, increased ROS level will damage the cell membrane and disturb the intracellular pathways. Therefore, supplementation of extender with antioxidants plays a key role in semen extenders and has been found most effective in various species including Dog semen [77].

Future perspectives

Improved semen quality can be achieved via better semen extension medium under optimized conditions. Diluents supplemented with other than conventional ingredients may be more effective. Exploring the potential of diluents for latest technologies viz Sperm sexing, embryo transfer and prolongation the life of sperm cell for more than 48 hours, are the need of modern AI.

Sperm sexing is the next generation AI technology. It has been achieved via recently reported interferometry and flow cytometery techniques with 60 to 66 percent accuracy rate [78]. So far the development in the field of sperm cell sorting is very admirable, but expensive and not in reach of the small farmers. Moreover, still some scientists believe that during sexing the radiation (LASER beam) passed through the sperm cell can damage the cell DNA and can exert some teratogenic effects in the new born or destroy desired genes. Recently some researchers successfully attached nanoparticles to DNA within the cell nucleus, such as TiO_2 [79] and Multi Walled Carbon Nano Tube- Gold (MWCNT-Au) [80] etc. Use of the aforementioned and some other magnetic nanoparticles (Ni, Fe_2O_3 or Fe_3O_2 etc) or nanoclusters (Au) in semen extenders can be a good source to improve the sperm quality and strengthen the desired X or Y chromosome bearing sperm cell. We already know that X chromosome bearing sperm cell has 3.8 percent more DNA than Y bearing sperm cells [81]. Therefore, if these magnetic nanoparticles bearing sperm cells passed through the strong magnetic field they may show

some deviation to separate X from Y chromosome bearing sperm or may get strength with in the female genital organs without prior treatment during AI.

References

[1] Rehman, FU. Substitution of animal protein source with plant protein in semen extenders of various cattle breeds. Thesis submitted to department of Livestock Management, the University of Agriculture, Peshawar Pakistan 2012.

[2] Foote RH. The history of artificial insemination: Selected notes and notables. J Anim Science 2002; 80: 1-10.

[3] Leeuwenhoek A. De natis e` semine genitali animalculis. R. Soc. (Lond.) Philos. Trans 1678; 12:1040–43.

[4] Ivanoff EI. On the use of artificial insemination for zootechnical purposes in Russia. J Agric Sci 1922;12:244–56.

[5] Spallanzani L. Dissertations relative to the natural history of animals and vegetables. Trans. by T. Beddoes in Dissertations Relative to the Natural History of Animals and Vegetables. 1784;2:195–99.

[6] Sørensen E. Insemination with gelatinized semen in paraffined cellophane tubes [in Danish]. Medlernsbl Danske Dyrlaegeforen 1940;23:166–69.

[7] Perry EJ. The Artificial Insemination of Farm Animals. 4th ed. Rutgers University Press, New Brunswick, NJ 1968.

[8] Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl 1992;13:232–41.

[9] Wang Y, Sharma RK, Agarwal A. Effect of cryopreservation and sperm concentration on lipid peroxidation in human semen. Urology 1997;50:409–13.

[10] Salamon S, Maxwell WM. Storage of ram semen. Anim Reprod Sci 2000;62:77–111.

[11] Anzar M, Farooq U, Mirza MA, Shahab M, Ahmad. N. Factors affecting the effciency of artificial insemination in cattle and buffalo in Punjab. Pak Vet J 2003; 23: 106–113.

[12] Phillips PH, Lardy HA. A yolk-buffr pablum fo the preservation of bull semen. J Dairy Sci. 1940;23:399-404.

[13] Harrison RAP, Dott HM, Foster GC. Effect o fionic strength, serum albumin and other macro molecules on the maintenance of motility and the surface of mammalian spermatozoa in a simple medium. J Reprod Fert 1978;52:65-73.

[14] Kasimanickam R, Kasimanickam V, Tibracy A, Pelzer K. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4°C. Small Rum Res 2011;99:208-13.

[15] Khan, RU, Rahman ZU, Javed I, Muhammad F. Effect of vitamins, probiotics and protein on semen traits in post-molt male broiler breeders. Anim Rep Sci 2012;135:85-90.

[16] Rehman FU, Qureshi MS, Khan RU. Effect of Soybean Based Extenders on Sperm Parameters of Holstein-Friesian Bull During Liquid Storage at 4ºC Pakistan J Zool 2014; 46(1) 185-89.

[17] Gil J, Lundhein N, Soderquist L, Martines-Rodriguez H. Influence of extender, temperature, and addition glycerol on postthaw sperm parameters in ram semen. Theriogenol 2003;59:1241–55.

[18] Holt WV. Basic Aspects of Frozen Storage Of Semen. Anim Reprod Sci 2000;62:13–22.

[19] Curry MR. Cryopreservation of semen from domestic livestock. Rev Reprod 2000;5(1):46-52.

[20] Evans G, Maxwell W. Salamon's artificial insemination of sheep and goats. Butterworth, Sydney 1987; p 194.

[21] Polge C, Smith AU, Parkes AS. Revival of spermatozoa after verification and dehydration at low temperatures. Nat (Lond.) 1949;164:666.

[22] Foote RH, Bratton RW. The fertility of bovine semen cooled with and without the addition of citrate-sulfanilamide yolk extender. J Dairy Sci 1949;32:856–61

[23] Pommer AC, Rutllant J, Meyers SA. The role of osmotic resistance on equine spermatozoal function. Theriogenol 2002;58:1373–84.

[24] Graham EF, Rajamannan AH, Schmehl MKL, Maki-Laurila M, Bower RE. Preliminary Reports On Procedure And Rationale For Freezing Boar Semen. AI Digest 1971;19:12.

[25] Tselutin K, Seigneurin F, Blesbois E. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poult Sci 1999;78:586–90.

[26] Demick DS, Voss JL, Pickett BW. Effect of cooling, storage, glycerolization and spermatozoal numbers on equine fertility. J Anim Sci 1976;43:633.

[27] Rehman, FU, Quresi MS., Rehman SU, Khan I, Majid HA, Aftab M. Conventional semen processing technique and breeding soundness of various cattle breed bulls used in sub-tropical conditions. Sci Let 2013;1(1):13003e.

[28] Hammerstedt RH, Crichton EG, Watson PF. Comparative approach to sperm cryopreservation: does cell shape and size influence cryosurvival? Proc Soc Theriogenol 1992;8-11.

[29] Bahr GF, Engler WF. Considerations of volume, mass, DNA, and arrangement of mitochondria in the midpiece of bull spermatozoa. Exp Cell Res 1970;60:338-40.

[30] Alcivar A, Hake L, Millette C. Mitochondrial gene expression in male germ cells of the mouse. Dev Biol. 1989;135:263-71.

[31] Manfredi G, Thyagarajan D, Papadopoulo LC, Pallotti F, Schon EA. The fate of human sperm-derived mtDNA in somatic cells. Am J Hum Genet 1997;61:953-60.

[32] Lamirande ED, Gagnon C. Reactive oxygen species and human spermatozoa.II. Depletion of adenosine triphosphate plays an important role in the inhibition of spermatozoa motility. J Androl 1992;13:379-86.

[33] Salisbury GW, VanDemark NL, Lodge JR. Extenders and extension of unfrozen semen. In: Physiology of reproduction and artificial insemination in cattle. San Francisco: W. H. Freeman and Co, 1978:442-93.
[34] Evans G, Maxwell WMC. Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Sydney 1987.
[35] Gadea J. Review; semen extenders used in artificial insemination of swine. Spanish J Agri 2003; 1(2):17-27.
[36] Kasimanickam R, Kasimanickam V, Tibary A, Pelzer K. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4oC. Small Rumin Res J 2011;99:208– 13.
[37] Akhter S, Ansari MS, Rakha BA, Ullah N, Andrabi SMH, Khalid M. In Vitro Evaluation of Liquid-stored Buffalo Semen at 5o C Diluted in Soya Lecithin Based Extender (Bioxcell®, Tris-Citric Egg Yolk, Skim Milk and Egg Yolk-Citrate Extenders. Reprod Dom Anim 2011;46:45–49.
[38] Aboagla EM, Terada T. Trehalose enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol Reprod 2003;69:1245–50.
[39] Barbas JP. Mascarenhas ÆRD. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 2009;10:49–62
[40] Bergeron A, Brindle Y, Blondin P, Manjunath P. Milk caseins decrease the binding of the major bovine seminal plasma proteins to sperm and prevent lipid loss from the sperm membrane during sperm storage. Biol Reprod 2007;77:120–26.
[41] Michajilov NN. Sperm dilution in the milk. The Czecho- Slovak Vet Mag J Am Vet Med Assoc 1950; 117:337.
[42] Thacker DL, Almquist JO. Diluters for bovine semen. I. Fertility and motility of bovine spermatozoa in boiled milk. J Dairy Sci 1953;36:173–180.
[43] O'Dell WT, Almquist JO. Freezing bovine semen. I. Techniques for freezing bovine spermatozoa in milk diluents. J Dairy Sci 1957;40:1534–41.
[44] Almquist JO, Wickersham EW. Diluents for bovine semen. XII. Fertility and motility of spermatozoa in skim milk with various levels of glycerol and methods of glycerolization. J Dairy Sci 1962;45:782–87.
[45] Ahmad K, Foote RH. Postthaw survival and fertility of frozen bull spermatozoa treated with antibiotics and detergent. J dairy sci 1986;69:535-41.
[46] Phillips PH. The preservation of bull semen. J Biol Chem 1939;130:15.
[47] Salisbury G, Fuller H, Willett E. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. J Dairy Sci. 1941;24:905-10.
[48] Smith L, Berndston W, Unal M, Picket W. Influence of percent egg yolk during cooling and freezing on survival of bovine spermatozoa. J Dairy Sci 1979;62:1297-1303.
[49] Foulkes J. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. J Reprod Fertil 1977;49:277-84.
[50] 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. Cryobiol 1987;24:42-52.
[51] Moussa M, Martinet V, Trimeche A, Tainturier D, Anton M. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenol 2002;57:1695-1706.
[52] Watson PF. The role of lipid and protein in the protection of ram spermatozoa at 5 C by egg yolk lipoprotein. J Reprod Fertil 1981;2:337–40.
[53] Ritar AJ, Salamon S. Effects of seminal plasma and of its removal and of egg yolk in the diluent on the survival of fresh and frozen–thawed spermatozoa of the angora goat. Aust J Biol Sc 1982;35:305–12.
[54] Muller-Schlosser F. Advances with AndroMed® bull semen extender. Minitube; Sperm notes 2005;07.
[55] Muller-Schlosser F, Hinsch E, Bohm J, Schill WB, Hinsch KD. Investigations on egg-yolk free diluenting medium for the cryopreservation of bull spermatozoa. Tieraztl Prax 1995;23:363–366.
[56] Bousseau S, Brillard JP. In vitro and in vivo results of fertility in cattle, following inseminations performed with semen diluted and frozen in a diluent free of animal origin products (Biociphos Plus). In: European A.I. Vets. Sixth Meeting. Edinburgh, Scotland; 1994.
[57] Aires VA, Hinsch KD, Müller-Schlösser F, Bogner K, Müller-Schlösser S, Hinsch E. In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenol 2003;60:269-79.
[58] Aurich C, Seeber P, Muller-Schlosser F, Comparison of different extenders with defined protein composition for storage of stallion spermatozoa at 5^{o} C. Reprod Dom Anim 2007;42:445–48.
[59] Bousseau, S, Brillard JP, Marquant-Le Guienne B, Guorin 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. Theriogenol 1998;50:699-706.
[60] Futino D, Mendes M, Matos W, Mondadori R., Lucci C. Glycerol, methyl-formamide and dimethyl-formamide in canine semen cryopreservation. Reprod Domest Anim 2010;45:214–20.
[61] Campbell MK, Farrel SO. Lipídeos e proteínas estão associados a membranas biológicas. In: Campbell, M.K., Farrel, S.O. (Eds.), Bioquímica., 5th ed. Thomson Learning, São Paulo 2007, p202.
[62] Singh AK, Singh VK, Narwade BM., Mohanty TK. Atreja SK. 2012. Comparative quality assessment of buffalo (Bubalus bubalis) semen chilled (5ºC) in egg yolk- and soya milk–based extenders. Rep Dom Anim 2012;47:590-600.
[63] 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. Theriogenol 2010;74:663–71.
[64] Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, et al. In vitro comparison of egg yolk-based and soybean lecithin-

based extenders for cryopreservation of ram semen. Theriogenol 2010;73:480–87.

[65] Foote RH. Artificial Insemination to Cloning: Tracing 50 Years of Research. Published by the author, Ithaca, New York, 1998.

[66] Iritani A. Problems of freezing spermatozoa of different species. In: Proc. 9thInt. Congr. Anim. Reprod. Artif. Insemin., Madrid, Spain 1980;1:115–31.

[67] Qureshi MS. Reproductive physiology of domestic animals. First Edition. HEC printing press Islamabad, Pakistan 2011.

[68] Rigau T, Piedrafita J, Reverter J, Canal M, Rodriguez-Gil JE. The rate of L-lactate production: a Feasible parameter for the fresh diluted boar semen quality analysis. Anim Repro Sci 1996;43:161-72.

[69] Marco-Jime´Nez F, Puchades S, Moce E, Viudes-De-Cartro MP, Vicente JS, Rodriguez M. Use of powdered egg yolk vs fresh egg yolk for the cryopreservation of ovine semen. Reprod Dom Anim 2004;39: 438–41.

[70] Althouse GC, Kuster CE, Clark SG, Weisiger RM, Field investigation of bacterial contaminants and their effects on the porcine semen. Theriogenol 2000;53:1167-76.

[71] Ahmad K, Foote RH, Kaproth M. Antibiotics for bull semen frozen in milk and egg yolk extenders. J Dairy Sci 1987;70:2439-43.

[72] Lein DH. The current role of ureaplasma, mycoplasma, haemophilus somnus in bovine reproductive disorders. Paper presented at: Proc 11th Tech Conf on AI and Reprod; Wisconsin 1986.

[73] Shin SJ. The control of mycoplasmas, ureaplasmas, campylobacter fetus, haemophilus somnus in frozen bovine semen. Paper presented at: Proc 11th Tech Conf on AI and Reprod; Wisconsin 1986.

[74] Waberski D, Weitze KF, Rath D, Sallmann HP. Effect of bovine serum albumin and zwitterionic buffers on stored liquid bore semen. Zuchthygiene 1989;24:128-133.

[75] Waberski D, Meding S,Dirksen G. Fertility of long term stored bore semen; influence of extenders (Androhep and Kiev), storage time and plasma droplet in the semen. Animal reprod sci 1994;36:145-51.

[76] Pons-Rejraji H, Sion B, Saez F, Brugnon F, Janny L, Grizard G. Role of reactive oxygen species (ROS) on human spermatozoa and male infertility. Gynecol Obstet Fertil 2009;37:529– 35.

[77] Beccaglia M, Anastasi P, Chigioni S, Luvoni GC. Tris-Lecithin Extender Supplemented With Antioxidant Catalase for Chilling of Canine Semen. Reprod Dom Anim 2009;44(2):345–49.

[78] van Munster EB. Interferometry in Flow to Sort Unstained X- and Y-Chromosome-Bearing Bull Spermatozoa. Cytometry 2002;47:192–99.

[79] Song M, Zhang R, Wang X. Nano-titanium dioxide enhanced biosensing of the interaction of dacarbazine with DNA and DNA bases. Mat Let 2006; 60:2143–47.

[80] Zhang R, Wang X. Selective enhanced electrochemical response of DNA bases on carbon nanotube–gold nanocomposites modified gold electrode. Phys Status Solidi A 2010; 207(10):2263–68.

[81] Van Munster EB, Stap J, Hoebe R, te Meerman GJ, Aten JA. Difference in volume of X- and Y-chromosome bearing bovine sperm heads matches difference in DNA content. Cytometry 1999;35:125–28.

[82] Bohlooli S, Cedden F, PishJang J3, Razzaghzadeh S, Bozoğlu S. The Effectof Different Extenders on Post-thaw Sperm Viability, Motility and Membrane Integrity in cryopreserved semen of ZandiRam. J Basic Appl Sci Res 2012;2(2):1120-23.

[83] Salamon, S, Maxwell WMC. Storage of Ram Semen. Anim Reprod Sci 2000: 62:77-111.

[84] Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiol 1993: 30, 423–431.

[85] Lorton SP, Sullivan JJ, Bean B, Kaproth M, Kellgren H, Marshall C. A new antibiotic combination for frozen bovine semen. 3. Evaluation of fertility. Theriogenol 1988:29,609–614.