

Review Article

Semen Extenders and Artificial Insemination in Ruminants

Fawad Ur Rehman^a, Chunquo Zhao^a, Muhammad Ali Shah^{a,b}, Muhammad Subhan Qureshi^b, Xuemei Wang^{a*}
^aState key Laboratory of Bioelectronics, School of Biological Sciences and Medical Engineering, Southeast University, Nanjing 210096, China

^bDepartment of Livestock Management, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan

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, polymixin 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

Received June 05, 2013; **Revised** August 08, 2013; **Accepted** August 13, 2013

***Corresponding author:** Xuemei Wang; E-mail: xuewang@seu.edu.cn

To Cite this manuscript: Rehman F, Zhao C, Shah MA, Qureshi MS, Wang X. Semen extenders and artificial insemination in Ruminants. Veterinaria 2013; 1: 1-8.

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 °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 °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

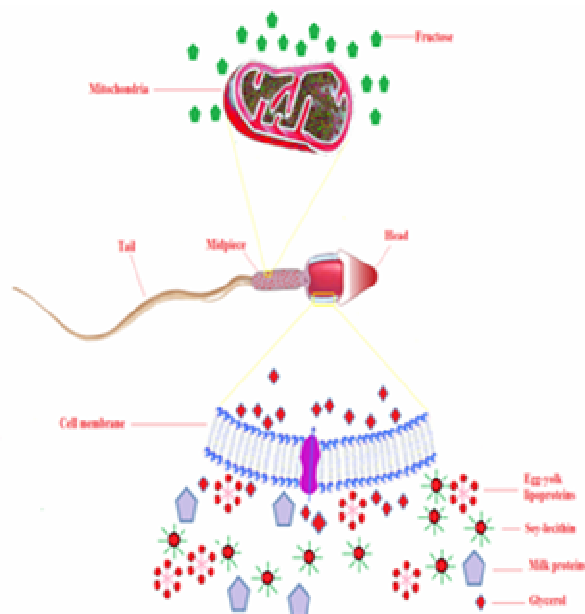


Fig. 1: Role of various components in semen extenders

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].

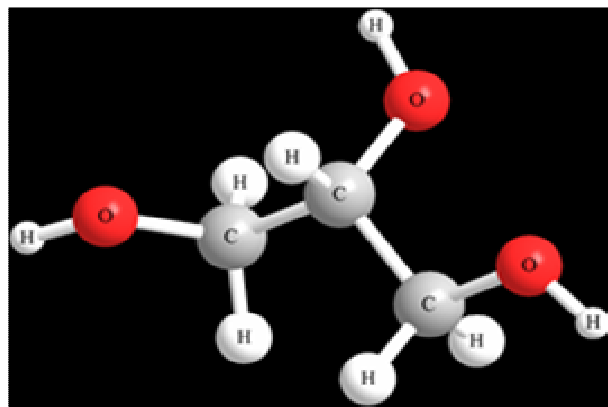


Fig. 2: Glycerol structural formulae

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 intracellular 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 °C to -196 °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].

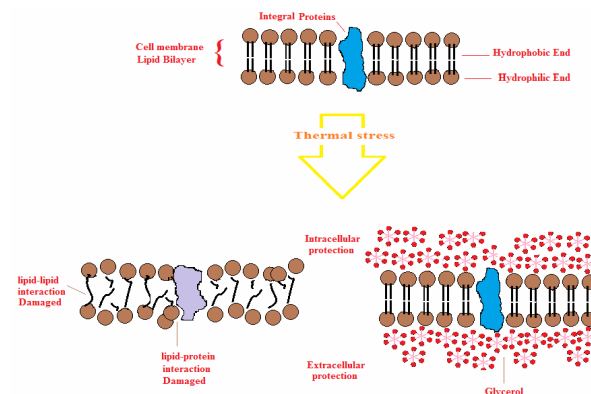


Fig. 3: Thermal stress effect on cell membrane

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].

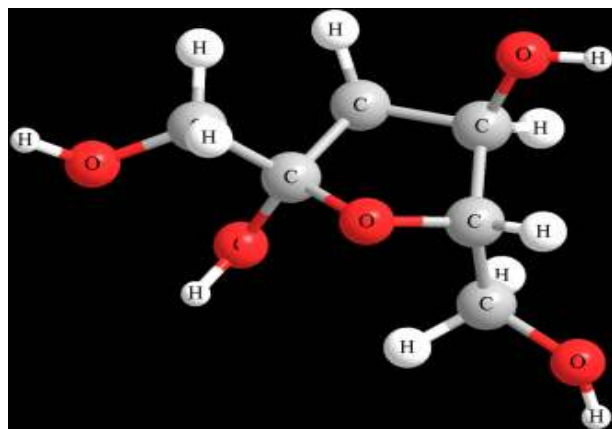


Fig. 4: Fructose structural formulae

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. pregnenolone 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.

decreased pH. The decreased pH will reduce cellular activities 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].

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].

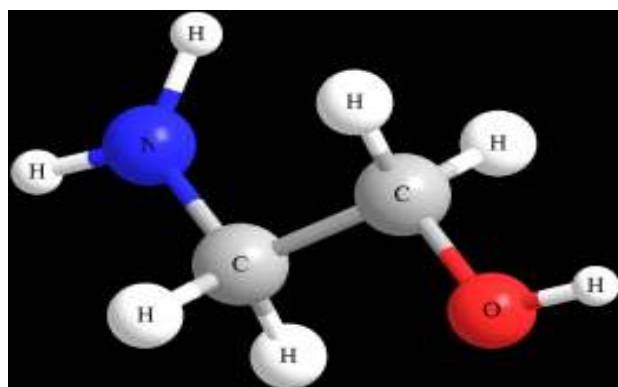


Fig. 5: Tris structural formulae

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

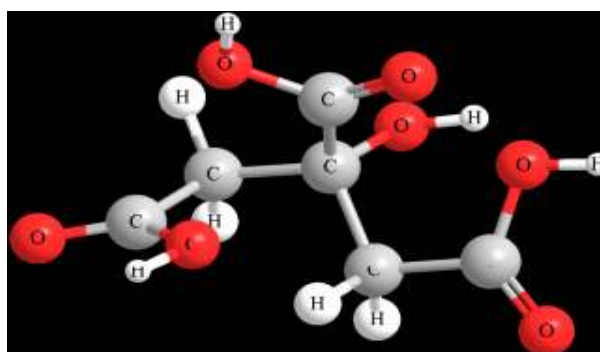


Fig. 6: Citric acid structural formulae

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°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 cytometry 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 TiO₂ [79] and Multi Walled Carbon Nano Tube- Gold (MWCNT-Au) [80] etc. Use of the aforementioned and some other magnetic nanoparticles (Ni, Fe₂O₃ or Fe₃O₂ 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.

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