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A review on semen extenders and additives used in cattle and buffalo bull semen preservation

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Abstract
In the scenario of artificial breeding in cattle and buffalo, the superior quality male germplasm from elite bulls is exploited to maximum possible extent by artificially inseminating large number of cows and buffaloes merely from a single ejaculate. For this purpose, a good quality semen extender is required which plays an indispensable role in improving the post thaw semen quality in terms of sperm viability, motility, plasma membrane and acrosomal integrity (PMAI), mitochondrial membrane potential (MMP), sperm kinematics etc. Various semen additives are incorporated during extension of semen before preservation which imparts anti-oxidant and sperm membrane stabilization properties to improve post-thaw semen quality. The objective of this review is to disseminate the knowledge about the use of various types of semen extenders and additives in bull semen preservation to improve the extent of consuming superior male germplasm.

Keywords: Semen extender, semen additives, male germplasm, bull, semen preservation, sperm

1. Introduction
The most widely used technique for artificial breeding of cattle and buffalo requires a perfect medium to extend and preserve the semen ejaculates from elite bulls for the sake of exploitation of superior quality male germplasm to the maximum possible extent. Various components which combine to make semen extender are such that they possess all those properties which protract the longevity of spermatozoa in extended form during harsh ambient conditions and cryopreservation. A plenty of insults faced by spermatozoa during storage envisaged osmotic changes, pH fluctuations, energy depletion during metabolism, cold shock and cryo-damages during freezing-thawing procedures. During cryopreservation, cholesterol to phospholipid ratio of sperm bio-membranes gets disturbed mainly due to cholesterol efflux and generation of numerous reactive oxygen species (ROS). All these disturbances directly compromise spermatozoa fertility. Therefore, a combination of good quality semen extender and additives must be used in such a way that fertility of spermatozoa can be retained outstandingly during semen preservation.

2. Semen Extender
Semen 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. It must encompasses these properties namely isotonic (280-310 mOsm/kg), buffering capacity (regulate pH), cold shock protection, energy source (sperm metabolism), control microbial contamination, protection during freezing and thawing and capable of preserving spermatozoa fertility. Depending upon the period of post collected semen usage for A.I., preservation of semen is done in two forms, viz., liquid form (3-5 days) and frozen form (years). Semen Storage at refrigeration temperature becomes possible since the discovery of egg yolk as beneficial additive and phosphate as primary buffer [1]. Later on, use of citrate as buffer enhance sperm survival period at 5 °C [2]. Besides egg yolk, homogenized whole milk, fresh or reconstituted skim milk and coconut milk are also used for preservation of fertility of spermatozoa. Among various buffers (Tris, TES, MES, HEPES, PIPES, MOPS and BES), Tris based diluents are used widely for liquid/frozen semen storage. Tris and citrate are most common constituents in extenders used for freezing bovine semen. Bicarbonates and sodium citrate are other temperature instable buffers used and in contrast to these Tris, TES, MOPS and Hepes are more stable at high temperature and other

environmental conditions [3]. Different types of extenders has been developed keeping the objective to maintain maximum fertility of spermatozoa during storage. Coconut milk extender [4], CUE [5, 6], IVT [7] and CAPROGEN® [5, 8] are extenders which are used for liquid semen storage at ambient temperature (18-24 °C).

Table 1: Additives to extenders (ambient temperature)

Additive	Mechanism	Effect on fertility	Reference
Glycine (1%)	unknown	2.1%	[5]
Caproic acid	Maintain membrane integrity & fluidity	1.3%	[9]
Catalase	Scavenge free radicles	Increase % of non- return rate (NRR) at 49 days	[10]

Use of milk as semen extender was first done by Koelliker in 1856 [11]. Milk contains Lactenin which has spermicidal properties (toxic to spermatozoa) should be removed by heat treatment before it is used as extender. Foote (1978) [6] reported similar results in terms of fertility when both milk based and egg yolk citrate extender were compared. Lactose in milk is non-permeating and prevents intracellular crystallization by creating osmotic pressure outside the cell. 10% whole milk or skimmed milk along with 7% glycerol and antibiotics has been used widely for freezing semen. Now a day, its use is restricted due to difficulty in semen evaluation under microscope.

2.1 Various components used in semen extender are

2.1.1 Egg yolk

It is non-penetrating cryoprotectant used during cryopreservation which contains phosphatidylcholine (lecithin), phosholipids, lipid extracts, lipoprotein fractions and specific lipoproteins which provides protection from cold shock [12]. Phospholipid moiety of a LDL fraction provides protection against cold shock [13]. The quantity of egg yolk in frozen bovine semen extenders commonly used is 15-30% (v/v) [14]. Use of 1% and 2.5% egg yolk in tris based extender for preservation of buffalo semen at 5 °C shows best sperm survival rates during 72 hours period [15].

2.2 Ions

For maintaining osmolarity zwitterionic buffers, amino acids, α-keto acids and a combination of salts and carbohydrates are added in extenders [16, 17].

2.3 Cryoprotectants

Polge *et al.* [18] discover glycerol as cryoprotective agent (CPA). Breakthrough in the history of semen preservation was happened with the discovery of glycerol as cryoprotectant for frozen semen storage. Egg yolk glycerol extender with Tris was first developed in 1963 for both fresh and frozen semen [19]. Tris-yolk-glycerol, Tris-fructose-yolk-glycerol and Citrate-yolk-fructose-glycerol are most widely used Tris and citrate based extenders for freezing bovine semen Penetrating. CPA like glycerol, PEG, EG and DMSO prevents concentration effect (solution effect) of extracellular media. Glycerol (7%) is most widely used CPA for bull spermatozoa with citrate–yolk and Tris–egg yolk extenders [20].

2.4 Sugars and ployols

Semen contains fructose as a source of energy. In egg-yolk based extenders glucose is available from egg yolk as an energy source. Sugars provide appropriate osmoticum and act as CPA [17]. Sugars like Xylose, fructose, glucose, galactose, maltose, sucrose and raffinose are used effectively for cryopreservation of bull semen [21]. Polyols such as glycerol and other sugars form H-bond with polar head groups of membrane lipids and replace water from hydrated polar head groups thereby stabilize the membrane during phase transition [22].

2.5 Antimicrobials

E. coli and *Salmonella spp.* are common contaminant in semen [3]. *Cl. pyogenes* and *P. aurogenosa* can be transmitted via cryopreserved semen. *B. abortus, C. fetus, T. fetus, L. pomona, M. bovis* and *Mycobacterium Spp*. are source of venereal infections [23]. *E. coli, Staphylococcus, Streptococcus, Pseudomonus, Haemophilus, Salmonella, Avian influenza, Campylobacter, Listeria and Mycoplasma* can be transmitted by egg yolk [24]. According to WHO (2003) and OIE semen extender components from animal source should be microorganism free. First standard diluent containing Penicillin G, Streptomycin and Polymixim-B was Cornell extender [25]. In semen extenders which are being used for buffalo semen preservation, inclusion of a combination of penicillin and neomycin show best results as compared to combination of penicillin and streptomycin as far as their anti-microbial properties are concerned [26].

Table 2: Combination of anti-microbial drugs used in semen extenders

Antimicrobial	Dose	Reference
Penicillin and streptomycin	1 gram per liter	[25]
Ceftiofur, Apramycin and Aminoglycosides	0.2 gram per liter	[3]
Linco-spectin+ tylosin +Gentamycin (Mycoplasma and Bacterial spp.)	300/600 µg +100 µg+500 µg/ml	[27, 28]

2.6 Chemically defined and animal protein free extenders

Focusing on the problems of disease transmission through use of various animal protein sources in semen extenders and to standardize the components in semen extender, various chemically defined semen extender such as powdered coconut water based– ACP-111®, Tris based– Tris concentrate — Gibco BRL®, commercial egg yolk based media– Botu-Bov®, BullXcell®, Bovidyl®, Triladyl® and skim milk based– Laciphos® are commercially available.

Soy bean lecithin based semen extender is primarily obtained from plant source. Lecithin in various cryoprotectants takes care of sperm plasma membrane by restoring phospholipids which get lost during extension, cooling and cryopreservation and protect viability of cell [29].

Animal protein free commercial diluent used for semen preservation are Biocephos plus®, Bioxcell® and AndroMed® [30, 31].

3. Semen Additives

During cryopreservation, the biological membranes of spermatozoa are primarily affected site which ultimately results in their demise. A large number of spermatozoa

become non-viable and those which remain alive develop cryocapacitation, DNA fragmentation and abnormal acrosomes. These adventitious changes lead to sudden decline in sperm fertility. During cooling and freezing of spermatozoa, cholesterol to phospholipid ratio of sperm bio-membranes get disturbed mainly due to cholesterol efflux and numerous reactive oxygen species (ROS) are produced. Various additives has been used till now which imparts antioxidant properties during semen extension and preservation are Regucalcin, Curcumin, Sodium pyruvate, Glutathione, Astaxanthin, Virgin coconut oil, Epidermal growth factor, Oviductal proteins, Diospyros kaki, Lycopene, Coenzyme Q10, Silymarin, Melatonin etc.

Membrane stabilizers such as Cholesterol- loaded cyclodextrins, Soy –lecithin, Docosahexanoic acid etc. has been recently used to improve post-thaw semen quality which protects sperm PM from destabilization during cooling and freezing.

Table 3: Various additives used during semen extension for chilled and frozen semen storage

Additive	Extender	MOA	Effect	Reference
Cysteine (0.2%) (Buffalo semen) (4-7 °C) (96 hrs)	Citric-whey extender	-SH group of cysteine destroy lactanin (spermicidal toxin)	Increase motility (45%) and non-eosinophilic spermatozoa (51%)	[32]
Iodixanol (2.5 %) (Cattle semen)	Tris -egg yolk	Non-penetrating cryoprotectant (altering the ice crystal formation by removal of water from the solution at lower temperatures)	Increase progressive motility (27.33%), viability (85.33%), PMAI (59.94%)	[33]
Cholesterol- loaded cyclodextrins (5mM) (Buffalo semen)	Tris-Egg Yolk-Glucose	Restore lipid profile of sperm plasma membrane	Increase post thaw motility (63.33%), viability index (150.00), improves stability of spermatozoa Plasma membrane – reflected by decrease in seminal plasma ALP (18.67), ALT (18.67) and AST activities (28.67) (IU/L)	[34]
Soy-lecithin (25%) (Buffalo semen) (5 °C) (72hr)	Soya milk based-extender	Lecithin protects plasma membrane by restoring phospholipids	Enhances sperm membrane (48.3%) and acrosome integrity (93.3%), viability (48.3%) and motility (43.9%)	[35]
Cholesterol- loaded cyclodextrins (3mg/ml) (Buffalo semen)	BullXcell®	Stabilizing plasma membrane by restoring cholesterol:phospholipid	Reduce sperm cryocapacitation – CTC pattern B (51.3%), tyrosine phosphorylated pattern EA (5.6%)	[36]
Docosahexanoic acid (3ng/ml) (Brangus–Simmental cross-bred semen)	BioXcell®	Increases cryogenic tolerance and maintains physiological properties of the lipid bilayer	Increase in sperm motility (48.94%), normal Morphology (70.63%), viability (73.42%), acrosome integrity (75.68%) and membrane Integrity (74.84%)	[37]
Nanoparticles like Tio2, Multi Walled Carbon Nano Tube- Gold (MWCNT-Au) and others Ni, Fe2O3 or Fe3O2 and Au	-----	Bind to DNA in the nucleus. When passed through strong magnetic field separates X and Y spermatozoa	-------	[38, 39]
Coconut water (Buffalo semen)	CEBRAN-l diluter	Indole-3-acetic acid (IAA) – inhibits enzyme PLA2	Increase motility (38.8%), viability (61%) and less acrosomal damage (10.4%)	[40]
Regucalcin (40 µg/ml) (Buffalo semen)	Tris-citric acid-fructose-egg yolk-glycerol	Ca^{2+} homeostasis and antioxidant property, stimulate gluconolactonase enzyme	Increase in post-thaw progressive motility (50.6%), acrosome integrity (75.6%) and ZP binding (191.9)	[41]
Curcumin (diferuoyl methane) (1.5 mM) (Buffalo semen)	Tris-citric acid extender	Radical trapping-antioxidant (H-atom transfer from CH_2 group at the centre of the heptadione link along with that of its phenolic –OH group	High progressive motility (23.27%), rapid velocity (31.53%) and other secondary motion characteristics, high plasma membrane integrity (30.47%) and viable spermatozoa with intact acrosome (61.87%)	[42]
Sodium pyruvate (5mM) (Simmental bull semen)	Triladyl®	H_2O_2 scavenger	Higher levels of rapidly motile sperm (32.29%), plasma membrane and acrosome intact sperm (53.32%) and high MMP (59.94%) and lower % of DNA fragmentation (9.27%)	[43]
GSH (2.0 mM) (Nilli-Ravi buffalo semen)	Tris-citric acid	Enzymatic antioxidant property	Increase sperm motility (56.7%), viability (89%), plasma membrane integrity (88.7%) and acrosomal integrity (94%)	[44]
GSH (0.5mM) (Cattle semen)	Egg yolk- tris glycerol	Enzymatic antioxidant (catalyse reduction of H2O2)	Decrease in CTC pattern B (37.23%) and AR (1%) and % of	[45]

			DNA fragmentation (9%) and increase in % of spermatozoa having high MMP (69.3%)	
Astaxanthin (2µM) (Karan Fries semen) (5 °C) (72 hrs)	Tris egg yolk citric acid fructose	Antioxidant property	Increase progressive motility (72.5%), % of live sperm (80.92%) and reduce levels of catalase (14.43U/ml) and SOD (30 U/ml)	[46]
Soy lecithin (1.5 %) with 2% virgin coconut oil (VCO) (4 °C) (72hr)	Tris-based extender (egg yolk free)	VCO contains antioxidants such as tocotrienol, polyphenols, and tocopherols	improves sperm viability (64.83%), acrosome integrity (75.5%), morphology (97.96%), membrane integrity (62.29%) and lipid peroxidation status- MDA (23 nmol/sample)	[47]
Epidermal growth factor (100 ng/mL) (Buffalo semen)	Bioxcell®	Inhibit the propagation of the peroxidative chain reaction	Decrease in AST (34.99), ALP (24.05), ACP (0.98) and LDH (42.3), TBARS (1.08 nmol/ml) and MDA (17.92 nmol/ml) (decrease in enzyme leakage and lipid peroxidation)	[48]
Oviductal proteins (NLIP) (1mg/ml) (Buffalo semen)	Tris-egg yolk-citrate	Non luteal isthmic oviductal proteins (NLIP), catalase in oviductal secretions	Increase in motility (51.25%), viability (69.75%) and acrosomal integrity (75.25%)	[49]
Diospyros kaki (Persimmon fruit) (Cattle semen) (for chilled and frozen semen)	Tris-Citrate-Fructose egg yolk	Antioxidant property (due to high level of carotenoids, flavanoids and polyphenols)	Increase post thaw sperm motility (57%), higher conception rate (66.67%)	[50]
Lycopene (1.5 mmol/l) (natural carotenoid) (Bovine semen)	Triladyl®	Scavenger of singlet oxygen ($^1O_2$) and other ROS	High progressive motility (43.01%) and improved secondary motion characteristics, plasma membrane stability (84.90%), acrosomal Integrity (85.20%), mitochondrial activity (147.50%), low intracellular superoxide generation (42.58%) and MDA (2.43 µmol/g protein)	[51]
Vitamin E+C (1mg+5mM/ml) (Karan Fries Semen)	Tris-citric acid-Fructose egg yolk	Non-enzymatic antioxidants	Post thaw Lower total sperm abnormalities (17.36%) and increase HOST % (42.35%)	[52]
Vitamin B12 (2.5mg/ml) (Bovine semen)	Tris-Citrate-Fructose egg yolk	Prevent the generation of oxygen radicals	Improve sperm motility (53.67%) and high levels of oxidative defense enzymes (IU/ml) – SOD (1.63), CAT (3.25), GSH-Px (102.36) and GSH (24.12)	[53]
Alpha- tocopherol (4.8mM) (Holstein bull semen)	Bioxcell®	Scavenge ROS	Increase motility (75.9%) and velocity parameters, sperm viability (76.1%), decrease MDA (6.1 nmol/ml) and H2O2 (3.2 nmol/ml)	[54]
Coenzyme Q10 (ubiquinone) (30 µM) (Cattle and Buffalo semen)	Tris-egg yolk	Prevents LPO and DNA fragmentation (lipid soluble antioxidant and scavenge free radicals)	Increase post thaw motility (65.8%), livability (68.3%), plasma membrane (67.9%) and acrosome integrity (22.9% damage acrosome), lowers sperm abnormalities (22.7%) with reduced levels of AST (25.1 IU/l)and ALT (21.7 IU/l)	[55]
Silymarin (Silybum marianum) (0.18 & 0.36 mg/ml) (Cattle semen) (for chilled and frozen semen)	Tris-Citrate-Fructose egg yolk	Antioxidant property, stimulate rRNA and antioxidant enzymes like SOD and GSH-Px	Increase post thaw motility (47.5%), viable spermatozoa (83.83%) and intact spermatozoa (70%), decrease in abnormal spermatozoa (18.67%)	[56]
Melatonin (2.0 and 3.0 mM) (Bovine semen)	Citrate- egg yolk	Sequester and neutralize free radicals, electron donation, stimulate GSH-Px, catalase, and SOD	Increase post thaw sperm motility (30.1%), viability (69.8%), plasma membrane integrity (68.3%), normal sperm (93.1%), and antioxidant enzymes activity)	[57]

4. Conclusion

Different types of extenders has been developed keeping the objective to maintain maximum fertility of spermatozoa during storage. Tris-yolk-glycerol, Tris-fructose-yolk-glycerol and Citrate-yolk-fructose-glycerol are most widely used Tris and citrate based extenders for freezing bovine semen. By incorporating various types of additives in semen extenders for cryopreservation, significant improvement is reported in

post thaw sperm quality in terms of increased % motility, viability, PMAI, MMP and reduced DNA fragmentation, capacitation like changes reflected by less CTC pattern B and AR, Tyrosine Phosphorylated pattern A, E and AE.

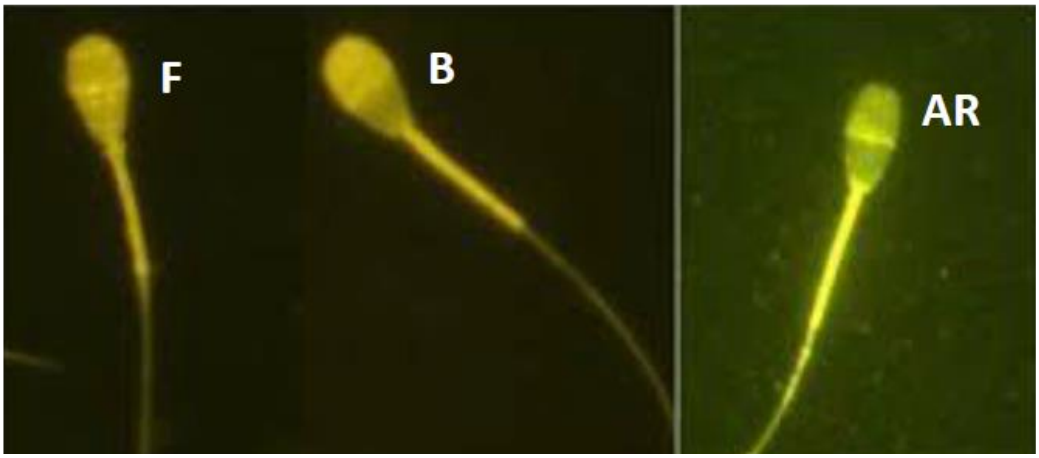

Fig 1: Chlortetracycline (CTC) assay F- pattern showing intact acrosome and non-capacitated ; B- pattern showing intact acrosome and capacitated; AR- pattern showing lost acrosome, capacitated, and acrosome reacted.[58]

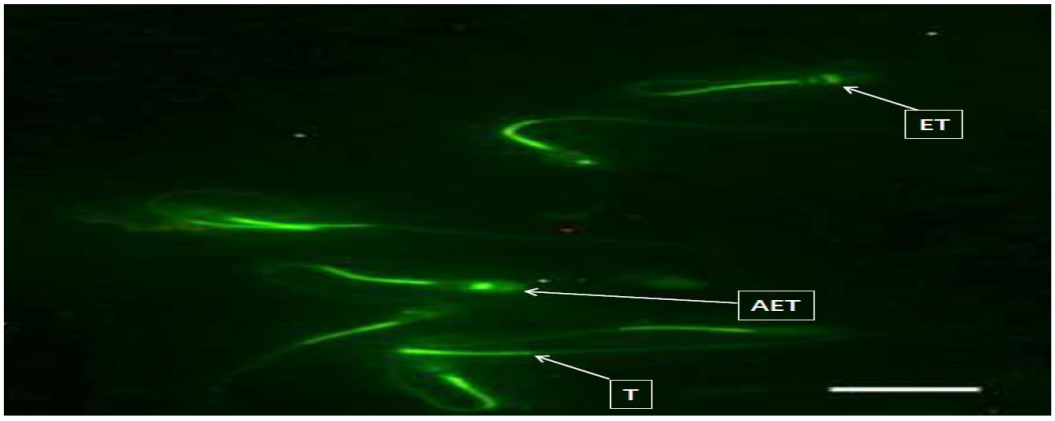

Fig 2: Tyrosine phosphorylation patterns: ET- Phosphorylated equatorial sub segment and tail; T- Phosphorylated tail and AET- Phosphorylated acrosomal area, equatorial sub segment and tail. [45]

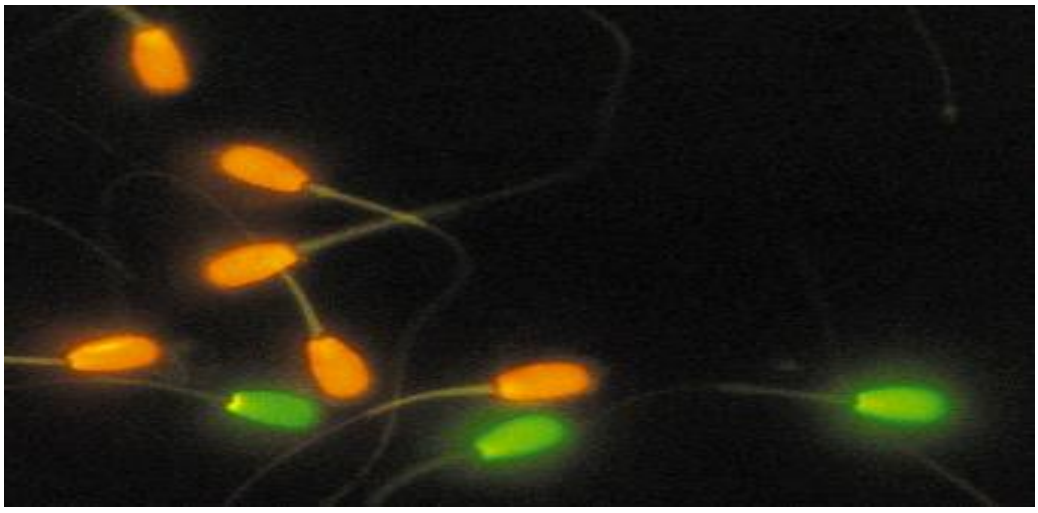

Fig 3: SYBR-14 stains viable sperm green and Propidium Iodide stains dead sperm as red. [59]

5. References

1. Phillips PH. Preservation of bull semen. The Journal of Biological Chemistry. 1939; 130:415.
2. Willett EL, Salisbury GW. Effect of various diluters, cooling rate, temperature of storage and some other factors, on the livability of spermatozoa in stored samples of bull semen, 1942.
3. Gadea J. Semen extenders used in the artificial inseminarion of swine. Spanish Journal of Agricultural Research. 2003; 1(2):17-27.
4. Chairussyuhur A, Sánchez-Partida LG, Maddocks S, Setchell BP. Quail yolk and coconut extract in diluents for storage of ram semen at 30C and 5C. In Proceedings of the 25th Annual Conference of the Australian Society for Reproductive Biology, 1993.
5. Shannon P. The effect of diluents containing glycine, and glycine and glycerol, on the fertility of diluted bovine semen. New Zealand Journal of Agricultural Research.

1964; 7(3):357-363.
6. Foote RJ. Extenders and extension of unfrozen semen. In: Salisbury GL, Van Demark NL, Lodge JR. Physiology of reproduction and artificial insemination of cattle. Edn 2, WH Freeman, San Francisco, CA. 1978, 442-493.
7. Bartlett FD, Van Demark NL. Effect of Diluent Composition on Survival and Fertility of Bovine Spermatozoa Stored in Carbonated Diluents1. Journal of Dairy Science. 1962; 45(3):360-367.
8. Shannon P. Contribution of seminal plasma, sperm numbers, and gas phase to dilution effects of bovine spermatozoa. Journal of Dairy Science. 1965; 48(10):1357-1361.
9. Shannon P. Effect of additions of volatile fatty acids on the viability and fertility of diluted bovine semen. Nature. 1962; 196(4860):1225.
10. Shannon P. Advances in semen dilution. In Proceedings of New Zealand Society of Animal Production. 1968; 28:23-31.
11. Salisbury GW, Van Demark NL. Diluents and extension of semen. In Physiology of reproduction and artificial insemination of cattle. Edn 1, WH Freeman, San Francisco, CA 1961, 412-435.
12. Mayer DT, Lasley JF. The factor in egg yolk affecting the resistance, storage potentialities, and fertilizing capacity of mammalian spermatozoa. Journal of Animal Science. 1945; 4(3):261-269.
13. Pace MM, Graham EF. Components in Egg Yolk which Protect Bovine Spermatozoa during Freezing1. Journal of Animal Science. 1974; 39(6):1144-1149.
14. Van Demark NL, Miller WJ, Kinney Jr WC, Rodriguez C, Friedman ME. Preservation of bull semen at sub-zero temperatures. Bulletin (University of Illinois (Urbana-Champaign campus). Agricultural Experiment Station); 1957, 621.
15. Sahni KL, Mohan G. Effect of various levels of yolk on viability of buffalo semen at 37°, 5° and-196 °C. In Proceedings of II World Buffalo Congress held in India during 12-16 December 1988 (volume III). Physiology and reproduction. Indian Society of Buffalo Development and Indian Council of Agricultural Research. 1990, 63-65.
16. Miller WJ, VanDemark NL. The influence of glycerol level, various temperature aspects and certain other factors on the survival of bull spermatozoa at sub-zero temperatures. Journal of Dairy Science. 1954; 37(1):45-51.
17. Watson PF. The preservation of semen in mammals. Oxford Reviews of Reproductive Biology. 1979; 1:283-350.
18. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 1949; 164(4172):666.
19. Davis IS, Bratton RW, Foote RH. Livability of bovine spermatozoa at 5, − 25, and− 85 °C in tris-buffered and citrate-buffered yolk-glycerol extenders. Journal of Dairy Science. 1963; 46(4):333-336.
20. Pickett BW, Berndtson WE. Preservation of bovine spermatozoa by freezing in straws: a review. Journal of Dairy Science. 1974; 57(11):1287-1301.
21. Nagase H. Deep-freezing bull semen in concentrated pellet form. I. Factors affecting survival of spermatozoa. In Proceedings of the 4th International Congress on Animal Reproduction and Artificial Insemination. 1964; 3:410-415.
22. Woelders H. Fundamentals and recent development in cryopreservation of bull and boar semen. Veterinary Quarterly. 1997; 19(3):135-138.
23. Michajilov NN. Sperm dilution in the milk. The Czechoslovak Veterinary Magazine. (Jan. 10, 1950). Abstracts Journal of the American Veterinary Medical Association. 1950; 117:337.
24. Thibier M, Guerin B. Hygienic aspects of storage and use of semen for artificial insemination. Animal Reproduction Science. 2000; 62(1-3):233-251.
25. Foote RH, Bratton RW. The Fertility of Bovine Semen Cooled with and without the Addition of Citrate-Sulfanilamide-Yolk Extender1. Journal of Dairy Science. 1949; 32(10):856-861.
26. Aleem M, Chaudhry RA, Naeem-Ullah K, Atta-Ur-Rehman R, Ahmad R. Occurrence of pathogenic bacteria in buffalo semen. Buffalo Journal. 1990; 6(1):93-98.
27. Lein DH. The current role of ureaplasma, mycoplasma, haemophilus somnus in bovine reproductive disorders. In Proceedings of the 11th Technical Conference on AI and Reproduction, 1986.
28. Shin S. The control of mycoplasmas, Campylobacter fetus, *Haemophilus somnus* in frozen bovine semen. In Proceedings of the 11th NAAB technical conference on AI and reproduction. 1986, 33-38.
29. Layek SS, Mohanty TK, Kumaresan A, Parks JE. Cryopreservation of bull semen: evolution from egg yolk based to soybean based extenders. Animal Reproduction Science. 2016; 172:1-9.
30. 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 AI Vets. Sixth Meeting. Edinburgh, Scotland, 1994.
31. Muller-Schlosser F. Advances with AndroMed® bull semen extender. Minitube; Sperm notes. 2005, 07.
32. Singh NP, Manik RS, Raina VS. Effect of cysteine fortification on preservability of buffalo semen in milk whey extenders. Theriogenology. 1989; 32(6):979-986.
33. Chuawongboon P, Sirisathien S, Pongpeng J, Sakhong D, Nagai T, Vongpralub T. Effects of supplementation of iodixanol to semen extender on quality and fertilization ability of frozen–thawed Thai native bull sperm. Animal Science Journal. 2017; 88(9):1311-1320.
34. Ezz MA, Montasser AE, Hussein M, Eldesouky A, Badr M, Hegab AE *et al.* The effect of cholesterol loaded cyclodextrins on post-thawing quality of buffalo semen in relation to sperm DNA damage and ultrastructure. Reproductive Biology. 2017; 17(1):42-50.
35. Singh AK, Singh VK, Narwade BM, Mohanty TK, Atreja SK. Comparative Quality Assessment of Buffalo (*Bubalus bubalis*) Semen Chilled (5 °C) in Egg Yolk-and Soya Milk–Based Extenders. Reproduction in Domestic Animals. 2012; 47(4):596-600.
36. Longobardi V, Albero G, De Canditiis C, Salzano A, Natale A, Balestrieri A *et al.* Cholesterol-loaded cyclodextrins prevent cryocapacitation damages in buffalo (*Bubalus bubalis*) cryopreserved sperm. Theriogenology. 2017; 89:359-364.
37. Kaka A, Haron W, Yusoff R, Yimer N, Khumran AM, Sarsaifi K *et al.* Effect of docosahexanoic acid on quality of frozen–thawed bull semen in BioXcell extender. Reproduction, Fertility and Development. 2017; 29(3):490-495.
38. Song M, Zhang R, Wang X. Nano-titanium dioxide

enhanced biosensing of the interaction of dacarbazine with DNA and DNA bases. Materials Letters. 2006; 60(17-18):2143-2147.
39. Zhang R, Wang X. Selective enhanced electrochemical response of DNA bases on carbon nanotube–gold nanocomposites modified gold electrode. Physica status solidi (a). 2010; 207(10):2263-2268.
40. Vale WG, Silva AOA, Sousa JS, Ribeiro HFL, de SOUZA HEM, Nahum BS *et al.* Preliminary report on the use of coconut water (*Cocos nucifera*) as a diluter of buffalo semen. In Embrapa Amazônia Oriental-Artigo em anais de congresso (ALICE). In: World Buffalo Congress, 5. 1997. Caserta Italy. Proceedings. Caserta Italy: [sn]. 1997.
41. Pillai H, Parmar MS, Shende AM, Thomas J, Sharma GT, Ghosh SK *et al.* Effect of supplementation of recombinant Regucalcin in extender on cryopreservation of spermatozoa of water buffalo (*Bubalus bubalis*). Molecular Reproduction and Development. 2017; 84(11):1133-1139.
42. Shah SAH, Andrabi SMH, Qureshi IZ. Freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of curcumin (diferuoyl methane) in semen extender. Andrologia. 2017; 49(8):12713.
43. Korkmaz F, Malama E, Siuda M, Leiding C, Bollwein H. Effects of sodium pyruvate on viability, synthesis of reactive oxygen species, lipid peroxidation and DNA integrity of cryopreserved bovine sperm. Animal Reproduction Science. 2017; 185:18-27.
44. Ansari MS, Rakha BA, Ullah N, Andrabi SMH, Iqbal S, Khalid M *et al.* Effect of exogenous glutathione in extender on the freezability of Nili-Ravi buffalo (*Bubalus bubalis*) bull spermatozoa. Animal Science Papers and Reports. 2010; 28:235-244.
45. Shah N, Singh V, Yadav HP, Verma M, Chauhan DS, Saxena A. Effect of reduced glutathione supplementation in semen extender on tyrosine phosphorylation and apoptosis like changes in frozen thawed Hariana bull spermatozoa. Animal Reproduction Science. 2017; 182:111-122.
46. Soren S, Singh SV, Kumar S. Effect of Astaxanthin Supplementation on Semen (Karan Fries Bulls) Storage at 5 C. International Journal of Current Microbiology and Applied Sciences. 2017; 6(5):23-28.
47. Tarig AA, Wahid H, Rosnina Y, Yimer N, Goh YM, Baiee FH. Effect of different concentrations of egg yolk and virgin coconut oil in tris-based extenders on chilled and frozen-thawed bull semen. Animal Reproduction Science. 2017; 182:21-27.
48. Kandiel MM, El-Khawagah AR, Mahmoud KG. Effect of epidermal growth factor on buffalo frozen spermatozoa biometry and metabolic activity. Asian Pacific Journal of Reproduction. 2017; 6(1):43.
49. Kumaresan A, Ansari MR, Garg A. Modulation of post-thaw sperm functions with oviductal proteins in buffaloes. Animal Reproduction Science. 2005; 90(1-2):73-84.
50. El-Sheshtawy RI, El-Nattat WS. Effect of Diospyros kaki enriched extender on cattle bull sperm parameters and conception rate. Asian Pacific Journal of Reproduction. 2017; 6(3):128.
51. Tvrda E, Mackovich A, Greifova H, Hashim F, Lukac N. Antioxidant effects of lycopene on bovine sperm survival and oxidative profile following cryopreservation. Veterinarni Medicina. 2017; 62(8):429-436.
52. Rao TKS, Mohanty TK, Bhakat M. Assessment of antioxidants for preservation of crossbred bull semen in Tris based extender. Indian Journal of Animal Research. 2017; 51(6):993-997.
53. Hu JH, Tian WQ, Zhao XL, Zan LS, Xin YP, Li QW. The cryoprotective effects of vitamin B12 supplementation on bovine semen quality. Reproduction in Domestic Animals. 2011; 46(1):66-73.
54. Motemani M, Chamani M, Sharafi M, Masoudi R. Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryopreservation of bull semen. Spanish Journal of Agricultural Research. 2017; 15(1):0401.
55. Saeed AM, El-Nagar HA, Wafa WM, Hussein YS. Effect of Coenzyme Q10 as an Antioxidant Added to Semen Extender during Cryopreservation of Buffalo and Cattle Semen. Journal of Animal and Poultry Production, Mansoura University. 2016; 7(11):403-408.
56. El-Sheshtawy RI, El-Nattat WS. Impact of Silymarin enriched semen extender on bull sperm preservability. Asian Pacific Journal of Reproduction. 2017; 6(2):81.
57. Ashrafi I, Kohram H, Ardabili FF. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Animal Reproduction Science. 2013; 139(1-4):25-30.
58. Kato Y, Nagao Y. Changes in sperm motility and capacitation induce chromosomal aberration of the bovine embryo following intracytoplasmic sperm injection. PloS one. 2015; 10(6):0129-285.
59. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction. 1995; 53(2):276-284.