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AN OVERVIEW OF SEMEN EXTENDERS AND FUTURE CHALLENGES TO DEVELOPMENT OF ARTIFICIAL INSEMINATION IN CAMEL

M. Umer[1*], M. Jameel[2], A. Baseer[3], Z. Ahmed[4] and A. G. Ghotia[5]

[1]Department of Animal Reproduction, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Pakistan
[2]Livestock and Dairy Development Department, Balochistan, Quetta, Pakistan
[3]Department of Veterinary Microbiology, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Pakistan
[4]Veterinary research Institute, Livestock and Dairy Development Department, Balochistan, Quetta, Pakistan
[5]Department of Livestock Management, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Pakistan

ABSTRACT

Artificial insemination is an emerging technique to ensure productivity and rapid genetic improvement in camels. This technique is not well developed due to involving different challenging factors because of semen collection, viscosity, nature of ejaculates, dilution in stabilized semen extenders, processing and cryopreservation, challenging related to artificial insemination and low conception rate. Numerous bovine commercial and non-commercial extenders with slight modification have been utilized for camel semen cryopreservation with little success. The gelatinous nature of the camel semen is one of the major challenges for proper evaluation, dosage calculation and cryopreservation. Various techniques have been used to liquefy the semen within a short spin of time without compromising the quality of sperm cells. This review provides precise data that appears in the peer reviews journals collected most of information to highlight the different semen extenders, addition of different supplementation and essential additives in semen extenders, challenges factors regarding camel bull and camel cow involved in success of artificial insemination.

Keywords: artificial Insemination, camel, challenges, semen extenders

INTRODUCTION

Artificial insemination (AI) has been successfully developed to boost reproductive performance and productivity in various domestic animals. The camel is now thought to be a possible potential animal to meet the human needs for transportation, meat and milk production. In addition, reproductive biology of camel presents some remarkable challenges for researchers (Tibary *et al.*, 2007). Technology of AI has not advanced yet for production purpose in camels, various difficulties are still stands related to seminal attributes, preservation and insemination of semen in the animal (Ibraheem & Afsal, 2021). A significant unsolved issue for the handling and preservation of sperm is the viscous nature of camelid ejaculates (Morrell *et al.*, 2021). Moreover, 95 percent of AI is completed with preserved semen, despite the fact that AI requires fresh or well-preserved semen (Raheja *et al.*, 2018). Thus, semen must be preserved in a perfect medium to maintain its quality (Raheja *et al.*, 2018; Hernández-Avíles *et al.*, 2020). Because of this, semen extenders that are used to protect semen using chilling or cryopreservation must be developed and evaluated (Santos *et al.*, 2018). Furthermore, it has been noted that the

*Corresponding author: umersrig@gmail.com

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type of extender used to dilute semen is a crucial and efficient component for the successful storage and survival rates of both frozen and non-frozen spermatozoa (Paulenz *et al.*, 2002). Additionally, Tris-fructose extender was suggested to be better than lactose (Vyas *et al.*, 1998) and Bicephos (Deen *et al.*, 2004) for the preservation of camel semen at refrigerated temperature.

In Bactrian camels, AI is said to have been extremely successful, however, results in dromedary camels have been less encouraging (Arthur, 1992). A little success has been attained on inseminated trials in female camels in respect of conception rate using liquid stored semen, similar results were obtained in trials with frozen thawed semen (Tibary & Anouassi, 2018). All the pregnancies were established with a particular extender and no pregnancy could be established with frozen thawed semen (Skidmore, 2003). The reasons for poor success have not been determined but a major difficulty with AI in camel is to ensure ovulation in inseminated animals (Anouassi & Asnani, 1992; Arthur, 1992). Incidence of ovulation and pregnancy is significantly lower in female camels inseminated either with fresh undiluted or diluted semen alone than those obtained with AI following mating with vasectomised teaser (Skidmore, 2019).

A various problems associated with semen collection, bull sexual behaviour, and attributes of semen produced low volume, viscosity and low sperm concentration. In addition, limited knowledge is available to optimize the time of insemination, sperm dose and lack of technique related semen storage (Tibary & Anouassi, 1997; Skidmore *et al.*, 2013). Similarly, none of a single semen production unit (SPU) falls in the world for routine AI practices and semen cryopreservation in camel. For successful implementation of AI in camels, development of effective extender preparation for chilled and frozen semen, addition of appropriate supplementation, and overcome the challenges related to artificial insemination have often been a major constrains limiting use of AI in camel. Therefore, the current review carried out to discuss semen extenders and its supplements, camel bull and female challenges to successful application of AI in camel.

Extenders used in camel semen cryopreservation

In domestic animals, AI is a crucial tool for maximizing the use of genetically superior males to ensure rapid genetic advancement. However, the gelatinous nature of seminal plasma has made handling and collection of semen from camelids extremely difficult, making this technique less effective. (Skidmore *et al.*, 2018).

Semen extenders

The majority of semen extenders have been adapted from protocols of other species, which include antimicrobials, a buffering system, a source of protein (such as casein from milk or lipoprotein from egg yolks), and a source of energy that contains glucose or fructose (Sieme *et al.*, 1993), a maintained osmotic pressure system, addition of cryoprotectant agents and electrolyte (Figure 1). Extenders are used to dilute the semen ejaculate for preservation in perspective of artificial insemination. In addition, variety of extenders for cryopreservation of other species has been modified for dromedary as well as Bactrian camel (Tibary & Anouassi, 2018).

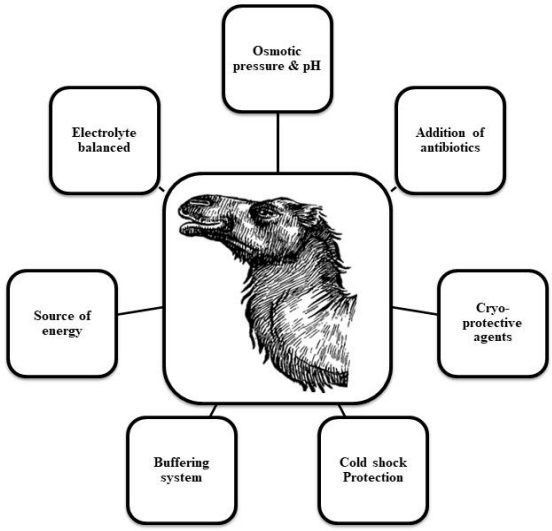

Figure 1. Potential qualifying qualities of camelid semen extender

According to earlier research, a good quality extender can be used to deep-freeze the semen of Bactrian and Dromedary camels using a stallion or boar method (Musa *et al.*, 1993). In addition, two extenders are used in semen cryopreservation, one for the cooling extender containing 80 ml of Lactose (11%) supplemented with 20 ml of Egg Yolk, and the second for the freezing extender containing 95.5 ml of cooling extender in addition of 6.0 ml of Glycerol supplemented with 1.5 ml of Orvus paste-Equex. The cooling extender is added immediately after

collection of semen in camel. Addition of freezing extender holds the cooling extender with glycerol (a cryo-protectant) and an emulsifying agent (Orvus-paste) that shows stabilization role in the sperm plasma membrane within ejaculate (Tibary and Anouassi, 2018).

During the post-thawing evaluation, the Triladyl significant demonstrated superior sperm vitality, DNA integrity, and plasma membrane integrity. Compared to other extenders like SHOTOR, Steridyl, AndroMed, and OPTIXcell, Triladyl demonstrated a higher sperm motility (38.63 ± 0.81%), progressive sperm motility at post-thaw in the dromedary camel (Swelum *et al.*, 2018). According to a study, a better semen extender for preserving Bactrian camel semen at 4°C is the Tris-based extender/SHOTOR diluent (Niasari-Naslaji *et al.*, 2005). In addition, this extender was also used for semen cryopreservation of camel by Niasari-Naslaji *et al.* (2006), further, it offers better-quality sperm after thawing. Overall, triladyl semen extender is also the best option for cryopreserving the semen of dromedary camels (Swelum *et al.*, 2018; Rahman *et al.*, 2023).

Further studies have shown that the sucrose-based sodium citrate-egg yolk extender offers the best defense against motility and acrosome integrity values after thawing (Zhao *et al.*, 1994). Mostly, semen extenders are generally contained 20% egg yolk and antibiotics (streptomycin and penicillin). In comparison, SHOTOR diluent performed better than commercially prepared IMV extender in cryopreservation of Bactrian camel semen (Niasari-Naslaji *et al.*, 2006). In contrast, the commercially prepared extenders including Equex®, SHOTOR® and Green Buffer® did not put significant effect of seminal parameters at post-thaw of Bactrian camel (Niasari-Naslaji, 2008). Finally, a variety of extenders i.e. Green Buffer® EY-glycerol, INRA + EY (Crichton *et al.*, 2015) and Tris-egg yolk-glycerol (Deen and Sahani, 2006) can be used in semen cryopreservation of camel. Increased viability of camel sperm was noted upon dilution with liquid Triladyl extender and 48 hours of storage at 4 or 15 °C (Al-Bulushi *et al.*, 2019a).

Extender effectiveness in colloids centrifugation technique

Colloid centrifugation is a technique used to separate the heterogeneous populations of cells particularly semen samples. Through assisted reproduction, it is also possible to obtain viable, functional sperm with intact chromatin (Morrell & Rodriguez-Martinez, 2016). Colloids are used to prepare the sperm for scaled-up assisted reproductive techniques including vitro-fertilization, cryopreservation and artificial insemination. According to findings of Fortune (2003), he applied silica particle in colloid centrifugation technique for improving the semen quality, separation of selected motile spermatozoa is done with normal morphology, intact membranes and stable DNA of camel sperm.. Colloids have been successfully used in various species to improve the sperm populations for cryopreservation and IVF. An experimentally study illustrated that the application of colloid centrifugation with single layer of centrifugation of spermatozoa in Green Buffer can protect the sperm motility in post thawed of dromedary camel cryopreserved semen (Malo *et al.*, 2018). Spermatozoa of alpaca preserved in Tris-citrate-fructose extender prepared by Single Layer Centrifugation (SLC) through a colloid. Highly motile sperm obtained with great plasma integrity through extracted by freezing and thawing of dromedary camel through pipetting and SLC (Morrell *et al.*, 2021). Pervious last decades, different suitable commercial and developed extenders has been developed to some extend for cryopreservation of camelid semen (Table 1).

Table 1. List of different developed suitable extenders with supplementations for camelid semen cryopreservation

References	Semen extemders	Supplementations cryo-protactants, outcomes and conclusion
Swelum *et al.* (2019)	Triladyl (Tris-based extender)	Triladyl is a commercial extender supplemented with egg yolk. It gives the best post thawing sperm quality. Commercially, it is a significant extender used for cryopreservation of dromedary camel semen.
Niasari-Naslaji *et al.* (2006)	SHOTOR diluent (Tris-based extender)	A suitable extender utilized for a short term preservation of semen of Bactrian camel having 330 mOsm/kg and pH of 6.9 at 4 °C during 24 hours. It showed increased progressive forward motility (PFM), plasma membrane integrity and % of live spermatozoa.
Malo *et al.* (2020b)	Green Buffer or INRA-96®	Ejaculates of dromedary camel semen liquefied in Tris-Citric acid-Fructose buffer and centrifuged through a colloid. The processed sperm chilled and cryopreserved in Green Buffer or INRA-96® containing 0 or 3% glycerol or ethylene glycol for 24 hours in presence of catalase (500 IU/mL). The dromedary sperm could be chilled and can be inseminated or cryopreserved. Presence of glycerol did not interfere during chilling and cryopreservation procedure. Catalase supplemented may help to maintain the motility of sperm at post thaw.

Rahman *et al.* (2023)	Triladyl (Tris-based extender)	Commercially, the Tris based extender supplemented with eggs yolk for cryopreservation of dromedary camel semen. Much higher viability and integrity of DNA, higher plasma membrane integrity, increases sperm motility, greater progressive sperm motility, and increased sperm quality were observed after semen cryopreservation at thawing.
El-Bahrawy (2010a)	Tris-Lactose 3% glycerolated extender (TLG)	Different concentration of enzyme (α-amylase) and 20% fresh egg-yolk added as supplementation. The α-amylase (mucolytic effects) liquefied the seminal plasma of dromedary camel prior to 4 hours of the freezing. It showed enhance the post-thaw forward motility and no significant detectable effects on acrosomal integrity and abnormalities.
Morrell *et al.* (2021)	Tris-citrate-fructose	Viscous alpaca semen ejaculates were liquefied by gentle pipetting in tris-citrate-fructose. Samples were prepared by Single Layer Centrifugation (SLC) through a colloid centrifugation. SLC samples retaining greater motility and plasma membrane integrity after freezing and thawing.
Morton *et al.* (2013)	INRA-96 (INRA)	Sperm membrane integrity and sperm viability was higher for INRA-96 (INRA) after liquid storage of dromedary semen. INRA is a suitable extender for liquid-storage of dromedary semen and gives higher pregnancy rate with 300 and 600 x 106motile sperm are recommended sperm concentration dose for AI dose with fresh and liquid-stored.
Crichton *et al.* (2015)	INRA-96	Supplemented with 20% egg yolk, 2.5% methyl formamide and cholesterol-loaded cyclodextrin (CLC). HEPES-buffered Tyrode's albumin lactate pyruvate (TALP) and papain used for liquefaction. Increased sperm motility, sperm progressive motility and acrosomal membrane integrity were observed in cryopreserved straws pre-treated with CLC at post-thawing. Exposure of CLC may be beneficial for dromedary camel sperm before cryopreservation.
Niasari-Naslaji *et al.* (2007)	SHOTOR diluent (a Tris-based extender)	SHOTOR diluent (29.9%), a Tris-based extender having 6% glycerol as cryo-protactant. It increases the values in preserving progressive motility, membrane integrity and sperm longevity at post thaw. SHOTOR diluent + 6% glycerol may be suitable extender for cryopreservation for Bactrian camel semen.
El-Nady *et al.* (2022)	Tris-extender	Supplemented with different concentrations of honey (0.0, 1.0, 2.5, or 5.0%) used for preservation of epididymal camel semen at 5º C for up to 48 hrs. Honey at levels 2.5% increases the sperm motility %, intact acrosome, and membrane integrity.
Zeidan *et al.* (2008)	Fructose-yolk-citrate (FYC) Lactose-yolk-citrate (LYC) Sucrose-yolk-citrate (SYC) Tris-yolk-fructose (TYF)	These four extenders were used in cryopreservation of dromedary camel semen. Increased values were recorded in the sperm motility%, sperm longevity at cryopreservation of camel semen at post thaw.

Additions of different components to semen extenders

Crichton *et al.* (2015) showed that addition of cholesterol loaded cyclodextrin (1.5 mg/ml) into the extender during the liquefaction process protects structural and functional damage and cryo-tolerance of dromedary sperm. The studies have shown that glycerol is a cryoprotectant that can be added to semen extenders such as in dromedary camel semen at various concentrations ranging from 3 to 7% (Morton *et al.*, 2010), it acts a critical role for the survival of spermatozoa and maintain its ability of fertilization.

Moreover, addition of higher concentrations of 6% is more harmful than lower than concentration of 3% (Morton *et al.*, 2010). In addition of glycerol or ethylene, researchers showed greater the sperm motility at post-thaw during cooling of dromedary camel semen (Malo *et al.*, 2020a). Furthermore, the addition of honey with different concentrations i.e. 1% and 2.5% in Tris-extender improve the sperm motility percentage, intact acrosome, and membrane integrity during storage at 5º C for up to 48 hours of dromedary camel semen (El-Nady *et al.*, 2022). El-Nady *et al.* (2022) showed that the adding of 2.5% honey in Tris-extender enhance the camel semen quality storage at 5º C for up to 48 hours. For Tris-Citric acid-Fructose buffer addition, researcher found that Tris-Citric acid-Fructose containing buffer improves the liquefaction of Dromedary camel ejaculates and enhance the quality of parameters for semen cryopreservation(Malo *et al.*, 2020a).

Adding of mucolytic/pro-teolytic enzymes

Furthermore, adding different mucolytic enzymes such as collagenase, α-chymotrypsin, trypsin, and amylase showed that the process of liquefying camel ejaculates could be sped up, though with varying degrees of success (Bravo *et al.*, 2000; Ghoneim *et al.*, 2010; El-Bahrawy *et al.*, 2015; Monaco *et al.*, 2016). Further researcher showed that the adding of collagenase at 0.5 to 1% provides speed up the process of liquefaction without affecting any major function of camel sperm. El-Bahrawy (2010b) showed that the additions of α-amylase and 20% fresh egg-yolk as supplemented in Tris-Lactose 3% glycerolated extender (TLG have no significant effect on acrosomal integrity and the sperm abnormalities. The researcher also found that α-amylase has beneficial effects on liquefaction and increases the forward motility of camel sperm at post-thaw.

Malo *et al.* (2020b) showed that addition of 3% glycerol or ethylene glycol and catalyse in Green Buffer or INRA-96® provide a good quality of semen extender for came semen cryopreservation. Further, researchers found that these additions may help in the sperm motility on post thawing during cooling. Furthermore, the presence of zinc and selenium nanoparticles (SeNPs or ZnONPs) to SHOTOR extenders may increase the progressive motility; vitality and integrity of sperm membrane, it also improves the cryo-tolerance of camel epididymal spermatozoa (Shahin *et al.*, 2020). Further, researchers added the α-amylase and 20% fresh egg-yolk added as supplemented in Tris-Lactose 3% glycerolated extender (TLG). These additions have no significant effect on acrosomal integrity and the abnormalities, however, increased post thaw recorded in forward motility of camel sperm (El-Bahrawy, 2010b). In the last, the most important is the lack of consistency in development and experimentation on cryopreservation of camel semen. (Tibary & Anouassi, 2018).

Artificial insemination challenges in camel

First the technique of AI reported by Elliot (1961) using frozen thawed semen. In the past decades, it was not clear the differences amongst species due to differences in seminal attributes, biological composition and functional properties. In addition, Better seminal attributes concentration and sperm concentration can be obtain in semen collected by artificial vagina from Bactrian camels as compared to dromedaries (Mosaferi *et al.*, 2005). A series of research on AI in camelids has been conducted and all concluded that the poor conception/pregnancy rate with frozen thawed semen (Tibary & Anouassi, 1997; Bravo *et al.*, 2000; Tibary, 2001; Adams *et al.*, 2009; Bravo *et al.*, 2013; Skidmore *et al.*, 2013). Acceptable rate of pregnancies have been achieved only in Bactrian camel using frozen thawed semen, however, discouraging findings have been reported in other species of camel (Chen *et al.*, 1984; Chen *et al.*, 1985; Xu *et al.*, 1985; Xu *et al.*, 1993). A more systematic and theoretical research required to optimize the success of AI with preserved semen excluding Bactrian camel (Tibary *et al.*, 2007). In addition, this failure is due to the lack of progress in the development of AI in camelid and particularly in the dromedary (Tibary & Anouassi, 2018).

Challenges related to camel bull

Conventionally, the semen has collected using either electro-ejaculator or artificial vaginal (Tibary *et al.*, 2014). Many researchers has not considered a valuable procedure of the semen collection through electro-ejaculator in camel (Al-Qarawi *et al.*, 2002). In contract, several semen ejaculates gives poor seminal attributes mainly azoospermic or oligozoospermic and contaminated with sand/debris from the environment collected through artificial vagina (Tibary & Anouassi, 1997).

Many researchers showed that the semen viscous nature did not provides precise assessment of motility, sperm concentration and morphology in camel (Tibary *et al.*, 2007). The viscous portion of seminal plasma represents approximately 85 to 90 % of camelid ejaculate.

Liquefaction of dromedary and Bactrian camel semen is time taking, decreases the semen quality and a challenging process in assisted reproduction (Malo *et al.*, 2020a). Various studies did not illustrated the exact role of viscosity nature of seminal plasma, however, it might be required due to sperm loss and its protection within the reproductive tract of camel cow (Kershaw-Young & Maxwell, 2012; Kershaw *et al.*, 2013). Minimum level about 10% is needed to maintain motility, acrosome integrity and viability in seminal plasma of alpaca (Kershaw-Young & Maxwell, 2011). Mostly, viscous nature of alpaca semen is related due to presence of apomucin, mucin 5AC and mucin 5B (MUC5B aka MG1) (Kershaw-Young & Maxwell, 2012).

Challenges related to camel cow

Failure of semen deposition due to its viscous nature and failure and high incidence of ovulation failure in camel are the major factors affecting the development of AI in the camel (Deen *et al.,* 2005). Besides that, various factors of camel bull, cow and others which provoke the challenges related to AI in camel shown in Figure 2. In addition, the standardized the time of insemination, and injection the hCG at responding pre-ovulatory size of follicle before insemination (Deen *et al.*, 2005).

Success of AI using liquid and frozen thawed semen

There are many challenges in camel semen cryopreservation, including increased viscosity and decreased motile sperm after semen ejaculation, in addition to the use of the proper semen extender (Shahin *et al.*, 2020). Moreover, epididymal spermatozoa provides a capable

substitute solution to overcome these problems and suitable for AI in camels (Shahin *et al.*, 2020).

Fresh or chilled dromedary semen has been used in a few documented AI trials, with doses of semen ranging from 8 to 300 million spermatozoa per insemination (Tibary *et al.*, 2007). Sperm concentration for AI per dose was 300 x 10^6 showed higher conception rate than 150 x 10^6 in camel. Further, a minimum dose of sperm concentration should be 600 x 10^6 is recommended for AI in camel stored semen at liquid form (Morton *et al.*, 2013).

Typically the time frame of AI is 24 hours following ovulation induction in camel cow (Tibary *et al.*, 2007). Promising conception rates after AI using frozen-thawed semen have only been observed in Bactrian camels (Chen *et al.*, 1984; Chen *et al.*, 1985; Zhao *et al.*, 1994). Limited data has existed about the fertility of frozen and thawed dromedary semen. There have been a few AI trials, but the conception rates are incredibly low. Further, it is challenging to determine the cause of this discrepancy between Bactrian and dromedary camels (Tibary & Anouassi, 1997). Notably, the unsatisfactory outcomes of AI with frozen-thawed semen also affect South American camelids, with the exception of a few incomplete reports (Vaughan *et al.*, 2003; Bravo *et al.*, 2013).

According to researchers, length of chilled storage was associated with a lower conception rate, as fresh extended and 24-hour chilled semen samples resulted in 32.7 and 32% of conception, respectively. Further, In the second season, out of 110 inseminations, two pregnancies (1.82 %) were obtained using cryopreserved semen (Ibraheem & Afsal, 2021).

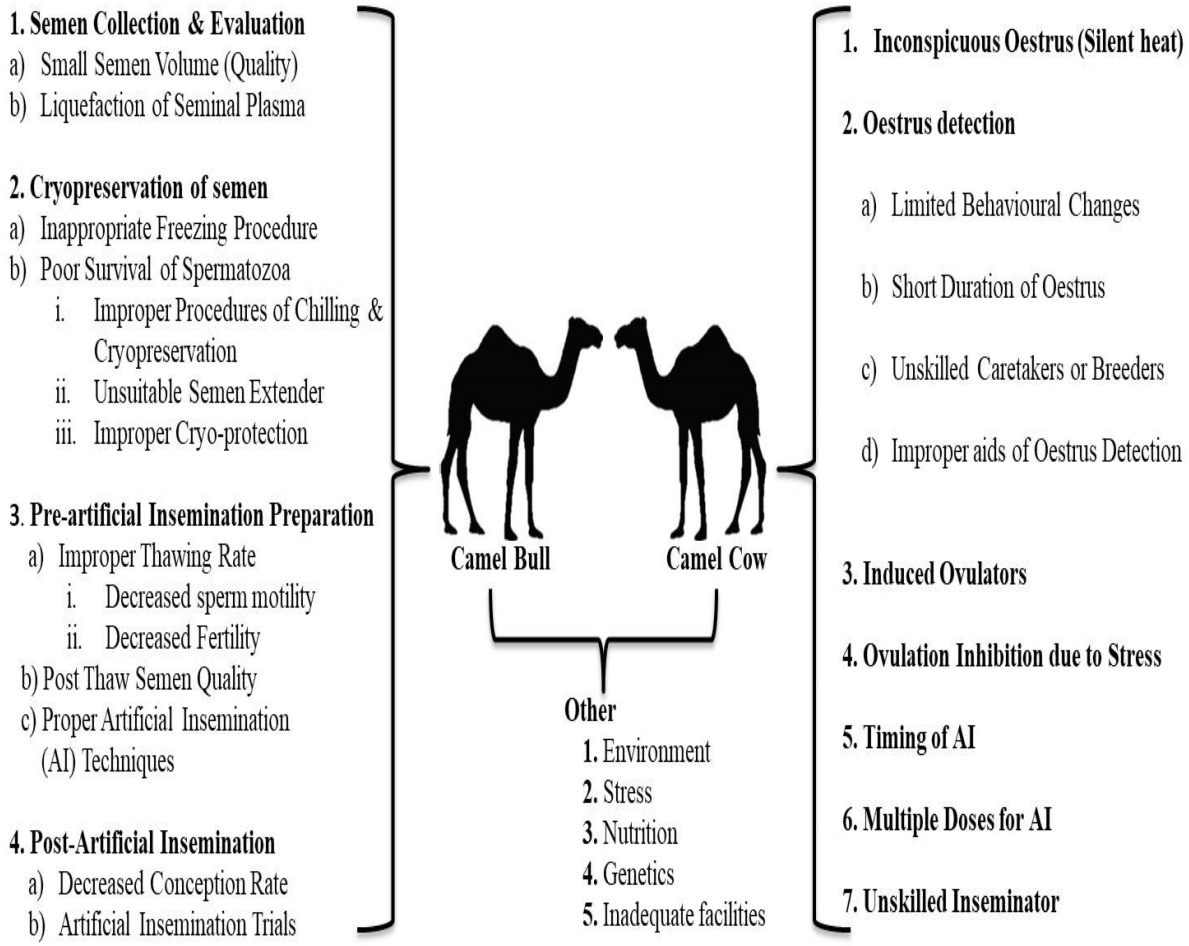

Figure 2. Potential challengs of artificial invesmination in camel

CONCLUSION

It is concluded that resilient challenges are preseent in cryopreservation of camel semen. Further, the cryopreservation of camelid semen is the confusing task for veterinarians and the researchers are facing main challenging problems including liquefaction of seminal plasma (viscous in nature), low concentration of spermatozoa and development of appropriate extenders. These factors overall affect the performance of diluted and cryopreserved sperm and decreases the rate of conception upon AI with cryopreserved semen. Further, the copious oestrus and induced ovulatory behaviour of camel cow remained an unresolved problem contributing the failure of rate of AI success in camels. Moreover, the challenges regarding the liquefaction of seminal plasma, evaluation and dilution of semen in a suitable extender, challenges related to successful of AI needs proper investigation to overcome the problem.

AUTHOR'S CONTRIBUION

M. Umer: Study Conception and draft writing.
M. Jameel: Manuscript writing and revision.
A. Baseer: Drafting the article.
Z. Ahmed: Criticsl revision of the article.
A. G. Ghotia: Proof reading.

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