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TRENDS IN RABBIT INSEMINATION EXTENDERS FOR FRESH AND FROZEN SEMEN. A REVIEW

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Abstract: Artificial insemination (AI) has become a popular technique in rabbit farms worldwide. This report discusses the progress made on semen extenders used in rabbit AI, setting out the latest innovations. Fresh and frozen semen have different requirements, so the extender composition will vary depending on the type of semen used. We discuss the endocrine supplementation of extenders for ovulation induction, the use of active molecules as an alternative to conventional antibiotics and the extenders developed for rabbit sperm cryopreservation.

Key Words: artificial insemination, rabbit, extender composition.

INTRODUCTION

Artificial insemination (AI) is a highly efficient assisted reproductive technology that has become common practice in rabbit farms. This technique has improved rabbit breeding, facilitating the development of new reproductive management systems, using the semen from genetically selected lines and, in some cases, contributing to improve the health of farms. Semen is actually a combination of mature sperm and fluids from the accessory glands, the seminal plasma, which contributes to the safe environment for sperm maturation and viability. Seminal plasma provides enough energy to maintain the high metabolic rate required for sperm transport through the female's reproductive tract to the fertilisation site (Muiño-Blanco *et al.*, 2008; Rodríguez-Martínez *et al.*, 2011; Bromfield, 2016). The protein composition of mammalian seminal plasma varies among species and has essential effects on sperm function (Rodríguez-Martínez *et al.*, 2011). Even though seminal plasma contains hundreds of proteins, their roles are not fully understood in rabbits. Seminal plasma has a positive effect in maintaining sperm motility and viability during in vitro storage (Castellini *et al.*, 2000). Hence, the composition of AI extender is of utmost importance, since it must compensate for the dilution of seminal plasma components and provide the necessary conditions to maintain the sperm's fertilising capacity. The aim of this paper is to review the trends in the development of new extenders for AI in rabbits, both for fresh and frozen semen.

Extenders for fresh semen

There are different commercial extenders for AI in rabbits; however, due to commercial interests, their composition is unknown. A basic extender for AI is composed of an organic buffer such as tris(hydroxymethyl)aminomethane (Tris), TES (Tris-etheylenediaminetetraacetic acid-sodium dodecyl sulfate) or sodium citrate, to maintain the pH (6.8 to 7.2),

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sugars such as glucose or fructose as an energy source to maintain motion competence, solutes to adjust pH and osmotic pressure to 300 mOsm/kg and antibiotics to prevent bacterial growth. A temperature range of 15 to 18°C is adequate for rabbit semen storage for up to 48 h (International Rabbit Reproduction Group, 2005). In addition, in order to reduce metabolic demand for semen to preserve quality parameters during storage, gelatine supplementation of rabbit extenders has been successfully used at 15°C (Nagy *et al.*, 2002; López-Gatius *et al.*, 2005) and 5°C (Rosato and Iaffaldano, 2011), which could facilitate dosage and transport of AI doses.

Endocrine supplement of extenders for ovulation induction.

Rabbit is an induced ovulator species. The induction of ovulation in rabbits through coitus has not been able to be associated only with the presence of type b nerve growth factor (b-NFG) as in other species of induced ovulators. Its concentration is relatively low, and it seems that adequate stimulation of the does is necessary to induce the ovulation successfully (Rebollar *et al.*, 2012; Dal Bosco *et al.*, 2011; Viudes-de-Castro *et al.*, 2017; Maranesi *et al.*, 2018). That is why even today it is necessary to use gonadotropin-releasing hormone (GnRH) analogues to induce ovulation when practising AI in this species. Administration of GnRH analogues could be done intramuscularly, subcutaneously, intravenously or intravaginally. Concern for the welfare of farm animals has led to an increase in the number of works related to the development of extenders for rabbit AI with GnRH analogues in their composition over the last two decades (Quintela *et al.*, 2004, 2009; Viudes-de-Castro *et al.*, 2007, 2014a; Vicente *et al.*, 2008, 2011; Zapletal and Pavlik 2008; Ondruška *et al.*, 2008; Zhang and Qin, 2012; Dal Bosco *et al.*, 2014; Gogol, 2016; Casares-Crespo *et al.*, 2016a; 2018a, 2018b; Munari *et al.*, 2019). The abundant blood supply and large surface area of the rabbit doe vaginal mucosa allow rapid absorption of low molecular weight drugs (Jitendra *et al.*, 2011; Gupta *et al.*, 2011). Intravaginal application of GnRH avoids its intramuscular application, making it a welfare-orientated method that has clear advantages, as it is a non-invasive route that causes less treatment distress and reduces both farmers' labour and handling time. Unfortunately, analysis of the rabbit seminal plasma proteome revealed that 50% of the proteins identified were related to catalytic activity (Casares-Crespo *et al.*, 2018a), and among them, the aminopeptidase B is an enzyme with a transcendental role in the rabbit AI when GnRH is added to the extender, as it is capable of degrading analogues of GnRH when present. Therefore, the success of intravaginal application of GnRH will depend on the proteolytic enzymes present in the seminal plasma (Vicente *et al.*, 2011; Viudes-de-Castro *et al.*, 2014a) and the quantity and type of GnRH analogue used. Hence, to achieve ovulation induction rates similar to those obtained by intramuscular application, the concentration of GnRH present in the extender must be much higher. Therefore, solutions must be sought that allow us to improve the administration of GnRH by vaginal route. The use of protease inhibitors, membrane absorption enhancers, mucoadhesive polymers and/or new transport systems, such as nanoparticles, are different methods that can optimise the intravaginal administration of proteins and peptides.

Thus, it has been observed that it is possible to protect the hormone from enzymatic degradation using a broad mix of protease inhibitors in the AI rabbit extender, although this could negatively affect prolificacy (Casares-Crespo *et al.*, 2016a). Subsequently, it was observed that extender supplementation with bestatin and ethylenediaminetetraacetic acid (EDTA) inhibited part of the seminal plasma aminopeptidase activity and did not affect rabbit seminal quality or reproductive performance (Casares-Crespo *et al.*, 2018c). The selection of suitable inhibitors of aminopeptidase activity is therefore decisive in the development of GnRH supplemented extenders for rabbit AI.

If the vaginal route is used, encapsulation of the analogue can protect the hormone from degradation, allowing greater absorption at vaginal mucosa level. Nanoparticles of biodegradable polymers have been extensively studied over the last few decades in pharmaceutical research for controlled drug delivery. The main advantage of using nanoparticles is their ability to load molecules and enhance their transport across mucosal surfaces (Trapani *et al.*, 2010). Recently, incorporation of buserelin acetate into nanoparticles was achieved to study their effect on rabbit semen quality, showing enhanced acrosome integrity and no effect on motility, viability and membrane functionality (Fernández-Serrano *et al.*, 2017). Furthermore, when the encapsulated GnRH was used in extenders supplemented with EDTA and bestatin, the poor stability of the GnRH analogue in the presence of seminal aminopeptidases can be overcome, allowing a reduction of the GnRH concentration in the extender without affecting the reproductive performance of female rabbits (Casares-Crespo *et al.*, 2018b).

On the other hand, the presence of b-NGF, a highly conserved protein, has been observed in the seminal plasma of several livestock species (Harper *et al.*, 1982; Kershaw-Young *et al.*, 2012; Ratto *et al.*, 2012; Druart *et al.*, 2013;

Casares-Crespo *et al*., 2018c; Maranesi *et al*., 2018). This protein is an endocrine or hormonal-like substance (Bradshaw *et al*., 2017) with a well-established ovulatory and luteotropic effect in camelids (Adams *et al*., 2016; Paiva *et al*., 2022). The abundance of b-NGF in camelid seminal plasma and the effects of seminal plasma on ovarian function strongly support the idea of an endocrine mode of action, which explains the male's influence on the female's hypothalamus-pituitary-gonadal axis (Ratto *et al*., 2012). In rabbits, it has been established that b-NGF plays an important role in rabbit reproduction. However, there is no clear evidence of the specific role of b-NGF in ovulation induction (Mattioli *et al*., 2021). Maranesi *et al.* (2018) suggest two complementary mechanisms in rabbit ovulation induction: a nervous pathway where the neuroendocrine reflex provoked by vaginal stimuli during natural mating or insemination is reinforced by a paracrine mechanism driven by some ovulation-inducing factor, in addition to b-NGF, present in the seminal plasma. Recent research explores the use of exogenous b-NGF in rabbit semen extender to induce ovulation. Sánchez-Rodríguez *et al.* (2020) showed a positive effect of b-NGF addition on the main sperm quality parameters and when the seminal dose was supplemented with 1 mg/mL of recombinant b-NGF, 60% of females were induced to ovulate, compared to 100% of females intramuscularly treated with GnRH. The efficacy of b-NGF, when added to semen extender, seems to be related to the abundance and distribution of their receptors (Castellini *et al*., 2022). Further studies should be carried out on AI extenders supplemented with b-NGF to improve their ovulation induction results.

Active molecule supplementation of extenders for antibiotic replacement.

Even though most of the bacteria found in semen are non-pathogenic, they can negatively influence sperm quality and longevity if present in high concentrations (Moreti *et al*., 2009; Ubeda *et al*., 2013; Marco-Jiménez *et al*., 2020). Bacterial contamination is of particular relevance in rabbit AI, where most of AI is performed with fresh or refrigerated semen stored for no longer than 48 h. Nevertheless, the excessive use of antibiotics worldwide has increased antibiotic resistance, and nowadays it is one of the biggest concerns in food security and global health (Da Costa *et al*., 2013; European Food Safety Authority, 2020). In this context, efforts need to be made to replace conventional antibiotics in the animal production industry.

One approach to overcoming bacterial growth is the inclusion of active molecules in the extender. A wide range of molecules present antibacterial activity, such as EDTA, (Finnegan and Percival, 2014), chitosan (Rabea *et al*., 2003; Saharia and Másson, 2017), nanoparticles (Rudramurthy *et al*., 2016), peptides (Bahar and Ren., 2013; Mahlapuu *et al*., 2016) and aminopeptidase inhibitors (Dickneite *et al*., 1985; Correa *et al*., 2017), among others. Recently, in rabbit semen stored at 15°C, it has been observed that the addition of molecules such as EDTA and bestatin to the insemination extender was able to control bacterial growth for up to 72 hours after recovery, whereas an antibiotic cocktail lost its efficacy after 24 h (Viudes-de-Castro *et al*., 2021a). This suggested that both molecules could be an alternative to conventional antibiotics in insemination extenders for this species. Further studies involving more molecules with antimicrobial growth effect should be conducted in future in order to find new alternatives to the use of antibiotics in rabbit insemination extenders.

Extenders for frozen semen

Although most rabbit inseminations are carried out with fresh semen stored for short periods of time, cryopreservation is an excellent way to store sperm for a long period of time and may facilitate operations at insemination centres by reducing the need for sperm collection at specific times. In addition, cryopreservation allows the conservation of genetically superior animals, transgenic animals, or breeds in danger of extinction.

Cryopreservation is known to contribute, both during the freezing and thawing processes, to the deleterious effects on structure and functions of spermatozoa, resulting in an undetermined number of spermatozoa more sensitive to osmotic stress, with a short lifespan or having sublethal dysfunctions (Watson, 2000). There are, therefore, relatively small numbers of viable spermatozoa that are fully functional in the population. The integrity of sperm membranes is necessary for spermatozoa to remain functional during storage in the female reproductive tract and penetration of the oocyte (Holt, 2000).

In recent years, several studies have been done in order to improve the quality and fertility of frozen rabbit semen. The effectiveness of rabbit semen cryopreservation is dependent on different factors such as cryopreservation protocol,

cryoprotective agents used, genetic line of males, number of spermatozoa in insemination dose and those aspects associated with the individual per se, probably related to the proteome of both the seminal plasma and the sperm membrane (Mocé *et al*., 2014, 2015; Viudes-de-Castro *et al*., 2014b; Casares-Crespo *et al*., 2018c, 2019). Hence, improvement of freezing results can be obtained by optimising the cryopreservation protocol or extender composition. Therefore, different experiments tested whether extenders in freezing and thawing protocols could extend the fertile life span of frozen-thawed sperm. There have been technical improvements in the protocols used in rabbit semen during in recent years (Mocé *et al*., 2014, 2015; Di Lorio *et al*., 2018). However, despite the advances achieved, the cryopreservation process drastically reduces the number of viable and motile spermatozoa after thawing.

Numerous studies have been performed to study the effect of freezing extender composition on sperm cryopreservation (Mocé and Vicente 2009; Nishijima *et al*., 2021; Kubovicova *et al*., 2022). Cryoprotectants are used to minimise the damage caused by ice crystal formation in cells during the freezing-thawing process. Most rabbit cryopreservation extenders include Me_2SO (DMSO) or acetamide as a penetrating cryoprotectant, but these can cause membrane injury due to osmotic shock at the time of their addition, so it is common to use them in combination with non-permeating compounds, such as lactose, sucrose, raffinose or trehalose (Mocé and Vicente 2009; Nishijima *et al*., 2021). On the other hand, the presence of substances of animal origin such as egg yolk or bovine serum albumin (BSA) is still common in freezing media (Rosato and Iaffaldano, 2013; Hall *et al*., 2017). However, the risk of contamination with pathogens of animal origin, in addition to the inherent variability of products of animal origin depending on the batch used, make their use controversial (Gil *et al*., 2003; Marco-Jiménez *et al*., 2004). Therefore, one of the challenges when formulating freezing extenders is to create a chemically defined extender without any components of animal origin.

Antioxidant supplementation of freezing extenders

Recently, different works have focused on the supplementation of cryopreservation extender with substances with antioxidant activity (Zhu *et al.,* 2015, 2017a, 2017b, 2019; Abdelnour *et al.* 2020, 2022; Fadl *et al.,* 2021), always showing in vitro results in terms of sperm quality traits, but it is difficult to establish a correlation between the seminal quality parameters and their response in terms of reproductive performance. Assessment of seminal characteristics includes the analysis of sperm motility, viability and membrane injury. Considering that only sperm maintaining an intact acrosome have the capacity for fertilisation of an oocyte, this seminal variable could be very important in fertility outcomes when frozen-thawed semen is used for AI. Motility analyses are often assisted by computer software (CASA systems) that can measure several motion traits of spermatozoa and determine different sperm subpopulations based on motion characteristics that are associated with the physiological status of spermatozoa, such as hyperactivation (Amann and Waberski, 2014). However, an inherent characteristic of rabbit semen is the high presence of particles of different sizes (Castellini, 2008), which makes it difficult to assess motility using CASA systems, which means that high dilution rates are normally used (1:20 to 1:80) to evaluate the kinetic characteristics. Considering the great decrease in motility characteristics in rabbit semen when dilutions greater than 1:10 are used (Castellini *et al*., 2000) and the mechanical stress suffered by spermatozoa during the freezing and thawing processes, the motility results observed with CASA systems probably do not reflect the real quality of the ejaculate after cryopreservation. On the other hand, it has been difficult to evaluate associations between values for seminal quality variables and quantitative response in terms of fertility and prolificacy. Many of the methods used for semen assessment for assisted reproduction are only of limited value for fertility prediction, as there are a multitude of factors and interactions that affect semen quality (Rodríguez-Martínez, 2003). Thus, the prediction of fertility in cryopreserved sperm remains unresolved and fertility potential of frozen-thawed semen must be confirmed *in vivo*.

Unfortunately, most of the experiments conducted to improve the results of rabbit semen cryopreservation extenders were laboratory evaluations of seminal characteristics or small-scale experiments with very few females inseminated. The reproductive potential of cryopreserved semen must be demonstrated by AI, since in the case of frozen semen there is no correlation between the quality characteristics usually examined and its real fertility.

Synthetic polymer supplementation of freezing extenders

Non-permeable synthetic polymers used in freezing extenders in combination with permeable cryoprotectants can improve semen extender properties. In this way, recent approaches using non-permeable synthetic polymers

with large molecular weight such as Ficoll (Kuliková *et al.*, 2014) or Dextran (Viudes-de-Castro *et al.*, 2021b) on a cryopreservation extender with DMSO and sucrose have shown that the presence of these macromolecules provided additional membrane protection, and in both cases, the reproductive performance observed was similar to that obtained with fresh semen. These results are encouraging and supplementation of freezing extenders with synthetic polymers could represent an effective breakthrough in AI with frozen semen in this species. Further studies involving synthetic polymers should be conducted in the future in order to generalise reproductive outcomes at farm level.

CONCLUSION

In recent years there has been great interest in the development and improvement of AI extenders in rabbits. Numerous works have been performed both in fresh and cryopreserved semen. Supplementation with peptides or proteins to induce ovulation in the female, the removal of the usual antibiotics from extender composition or the improvement on development of synthetic frozen extenders are topics that arouse great interest in the scientific community. It is crucial to continue testing different extenders for fresh and cryopreserved semen. However, it is necessary that these studies address the effect on in vivo fertility, as the correlation between the different parameters of semen quality and the fertility obtained after insemination is low, especially with frozen semen.

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