Antibiotics and other additives for semen extenders to enhance fertility

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Abstract

Semen extender formulations have been evolving for over 70 years and this evolution continues. Although milk was found to be suitable as a major component of equine semen extenders over 60 years ago, preparation of the extenders remained tedious and time consuming in part, due to the need to heat the milk before use. With the publication of the formulation for a nonfat, dried skim milk–glucose (NFDSM-Gluc) in the 1970s, a simple, convenient semen extender became available, prompting a dramatic expansion in the use of equine artificial insemination. This basic formulation of the NFDSM-Gluc extender is still in use today, with various modifications. More recent work has further identified the beneficial components of milk, which has led to more defined extender formulations. This paper briefly reviews the development of equine semen extenders and discusses the effect of including various additives such as antibiotics, cryoprotectants, and antioxidants on equine sperm survival and fertility.

Keywords: Stallion, semen extender, additives

Introduction

The search for an optimum semen extender formulation has been an ongoing quest almost from the inception of artificial insemination. Despite the fact that artificial insemination of mares is reported to have occurred much earlier than in cows and much of the early work in the field was done in the horse, the development of effective extenders for stallion semen was outpaced by the development of bull semen extenders.[1,2] This was due in part to the lack of demand for storage of stallion semen, the belief that stallion sperm lacked "the innate resistant factor necessary for storage" and the restrictive attitude of many breed registries toward artificial insemination.[1,3] While a thorough review of the development of equine semen extenders is beyond the scope of this manuscript, a brief review of early formulations should help set the stage for where we are today.

Over the years, numerous extender formulations have been employed in attempts to improve the survivability of stallion semen. Included in these formulations are various combinations and levels of: egg-yolk, sugars, buffers, citric acid, electrolytes, gelatin, glycerin, honey, milk products and even blood serum and follicular fluid.[1,2,3] The milk products have included mare's milk, sheep and goat milk, cow's milk, cream, skim milk, buttermilk, and nonfat dried milk solids (NFDSM).[1,2,3] One of the first extenders for stallion semen was a glucose-sulphate-peptone formulation,[4] which was later modified to a tartaric-glucose-peptone formulation.[5] Peptone is a water-soluble mixture of amino acids and peptides derived from the partial hydrolysis of protein. Although the source of the peptone used in those early experiments is not stated, it is interesting to note that the

peptone that can currently be obtained from Sigma-Aldrich (St. Louis, MO, USA) is derived from the enzymatic digestion of the milk protein, casein.

For many years, equine semen extender formulations were either the same or very similar to those used for bull semen. Results were mixed, sometimes being favorable and oftentimes less than satisfactory. After the discovery that adding egg yolk to a buffer improved the survival of bull sperm[6] and lessened its susceptibility to temperature shock,[7] a number of investigators incorporated egg yolk into equine semen extenders.[8,9,10] In 1949, Buĭko-Rogalevič reported that sperm motility was preserved for 8 to 13 days when stallion semen was diluted in an egg yolk-glucose extender compared to 2.5 days when diluted with glucose and that an 85.5% pregnancy rate was achieved with semen stored for 12 to 42 hours in this egg yolk-glucose extender.[9] In a series of experiments, Kühr obtained similar results with sperm survival increasing from 8.2 hours in undiluted semen to 100.8 hours in 7% glucose and to 290 hours when semen was diluted in 7% glucose + 5% egg yolk.[11] Other extender formulations based on successful bull semen extenders were far less satisfactory for stallion semen.[2]] Pace and Sullivan reported that the fertilizing capacity of equine semen was depressed almost immediately after mixing with hydrogen ion extenders.[12] Investigators from several laboratories found that even though various extender formulations could maintain sperm motility, the fertility of semen diluted in these extenders was poor.[3,11-14] The inferiority of these extenders is best exemplified by the fact that pregnancy rates were higher when similar numbers of sperm were inseminated using raw semen than with extended semen.[12,14,15] As a result, even up through the mid 1970s, it was recommended by some to use raw semen for equine artificial insemination unless the semen was to be stored or unless antibiotics

needed to be added to the semen because the stallion was shedding pathogenic bacteria.[3,14,16] When one examines the composition of the extenders used in many of those studies, it is likely that the glycerol they contained contributed to the poor fertility observed.

Milk-based extenders

Milk was used as an extender for stallion semen as early as the 1940s and boiled mare's milk was reported to yield more favorable results than sheep, goat and even cow's milk.[17] One of the major drawbacks of using fresh milk in semen extenders is the need to heat the milk to 92 to 95 °C for 10 minutes in order to inactivate lactenin, which is toxic to sperm.[18-20] Because of the heating and pasteurization involved in their manufacture, use of reconstituted dried milk products is thought to alleviate the need for heating when used in semen extenders. In the late 1950s and early 1960s, the Chinese established a very successful equine artificial insemination program involving 40 stallions and thousands of mares, using semen diluted in a powdered milk-based extender.[21] In comparative studies, Cheng reported that both maintenance of sperm motility and pregnancy rates were higher using the powdered milk extender when compared to fresh mare's milk or sugar-based (glucose or sucrose) extenders.[21,22] Following up on favorable results with bull semen in the late 1950s, workers at Texas A&M evaluated reconstituted buttermilk with glucose added (BMG) as an equine semen extender.[1] Although fertility was not examined, this BMG extender was found to be far superior to mare's milk, cow's milk and egg yolk-glucose extenders for preserving sperm motility for up to four days. The dried buttermilk was an "extra grade" product prepared by a company in Wisconsin and it may be that limited availability of this product precluded its

widespread use in semen extenders. However, non-fat dried skim milk had been readily available for years and once Kenney and co-workers[23] published the recipe for a non-fat dried milk solids-glucose extender (NFDMS-Gluc), this 'Kenney extender' as it is known, revolutionized equine artificial insemination in the western world. With the availability of a convenient, reliable semen extender, the use of artificial insemination in horses increased worldwide and the basic formula for this extender has remained virtually unchanged since its publication in 1975. Kenney-type extenders are available from a number of commercial sources, differing primarily in the type and level of antibiotic(s) added to the basic formulation.

Antibiotics

Inclusion of antibiotics in semen extenders is meant to reduce or eliminate bacterial growth in semen, especially when it is stored, and to help control post breeding endometritis. As with many other extender components, the incorporation of antibiotics was based on satisfactory methods employed with bull semen. However, it was found that the levels of antibiotics commonly used for bull semen extenders were toxic to stallion sperm.[1] Berry and Gazder reported that inclusion of 400 I.U./mL of penicillin and 1 mg/mL of streptomycin in their BMG extender was effective in controlling bacterial growth without adversely affecting sperm motility. The original Kenney extender contained either 1,500 I.U. of crystalline penicillin/mL and 1.5 mg of crystalline streptomycin/mL or 1 mg/mL of reagent grade gentamicin. Antibiotics commonly included alone or in combination in equine semen extenders today are: penicillin, streptomycin, polymixin-B, ticarcillin, timentin, gentamicin, and amikacin. Although less commonly used, ceftiofur[24] and pipericillin,[25] have also been shown to be safe and

effective antibiotics to include in equine semen extenders. For some very acidic antibiotics, eg. gentamicin and amikacin, buffers also need to be added to adjust pH and it is important to use reagent grade rather than injectable products because the preservatives in the latter can be toxic to sperm.

While sperm motility and fertility of stored stallion semen can generally be maintained or improved by extenders containing any of the antibiotics listed above, the choice of which antibiotic to include in the extender may be determined based on specific needs or circumstances. For some normal stallions, certain antibiotics appear to be more favorable than others for maintaining sperm motility in stored semen. Certainly, for stallions that are shedding specific pathogens into their semen, the choice of antibiotic to include in the extender should be based on the sensitivity pattern of the offending organism(s).

In the 1980s, Colorado State University entered into a licensing agreement with a commercial company to market a NFDSM-Glu extender. The formulation was essentially the same as the Kenney extender except that 1000 IU/mL of polymixin B sulfate replaced gentamicin sulfate as the antibiotic.[26] For a number of years, this extender (EZ –Mixin® original formula, Animal Reproduction Systems, Chino, CA, USA) was used extensively in the industry for both fresh and cooled-stored equine semen. Later, Colorado workers examined the effects of different antibiotics on motion characteristics in stored semen.[27] Reagent grade amikacin sulfate, ticarcillin disodium, gentamicin sulfate and polymixin B sulfate were added to a nonfat, dried, skim milk - glucose seminal extender at concentrations of 1000 or 2000 μg or IU/ml. They found that overall the addition of antibiotics to extender did not significantly improve motion

characteristics of sperm over control samples but that levels of gentamicin sulfate greater than 1000 μg /ml and polymixin B sulfate equal to or greater than 1000 IU/ml significantly reduced sperm motility. These workers concluded that genatmicin and polymixin B greater than or equal to these levels should be avoided in seminal extenders used for cooled semen. Texas A&M workers performed a similar series of experiments, but also evaluated the control of bacterial growth.[24] Results of this study demonstrated that semen stored in extender containing 1000 IU/mL of polymixin B sulfate resulted in the greatest reduction in sperm motion characteristics and the poorest control of bacterial growth. These workers determined that a NFDMS-Gluc extender containing potassium penicillin G (1000 IU/mL) and amikacin sulfate (1000 μg/mL) yielded the best combination of motility maintenance and control of bacterial growth. Individual stallion effects were also noted.

While not an antibiotic, the inclusion of the sugar mannose into semen extenders has been proposed by Illinois workers as an alternative to antibiotics for reducing post breeding bacterial endometritis.[28] Previous work from this laboratory has indicated that this sterioisomer of glucose was able to reduce the adherence of certain bacteria to endometrial tissue.[29-31] Replacing up to 37 mg/mL of glucose with mannose in NFDSM-Gluc semen extender did not affect the fertilizing capacity of sperm when immediate insemination was performed on reproductively healthy mares.[28] However, whether the inclusion of mannose in semen extenders can control bacterial growth in semen or maintain acceptable pregnancy rates with cooled-transported semen or in susceptible mares requires further study.

Variations on basic components

Texas A&M workers also developed another variation of the Kenney extender. This formulation not only contained the penicillin-amikacin combination but also reduced the level of glucose from 4.9 mg/mL to 2.65 mg/mL with the addition of sucrose at 4.0 mg/mL. This TAMU extender has proven to be an excellent extender for use in fresh, cool-stored breeding programs and also as a base extender for frozen semen after the addition of egg yolk and glycerol.

French workers developed a successful milk-based extender that has been widely used for frozen semen. In addition to sterilized skim milk, glucose and antibiotics, the base INRA 82 extender also contains lactose, raffinose, sodium citrate and potassium citrate to which egg yolk and glycerol are added prior to freezing.[32] More recently, studies which evaluated the effects of different milk fractions on sperm survival resulted in the development of a defined milk protein extender (INRA 96) for use with fresh and cooled semen.[33] In this extender, skim milk is replaced with the specific milk component; native phosphocaseinate (NPPC) in a Hank's salts solution supplemented with HEPES, glucose, lactose (HGLL) and BSA. While no difference was detected in sperm motility after 24 h storage of semen in either INRA 82 or INRA 96, fertility was higher for the semen stored in INRA 96.[33] This extender was also shown to be as efficient at preserving sperm motility and fertility when semen was stored at 15 ºC as when stored at 4 ºC.[34] This extender can also be used for freezing stallion semen. A fertility trial was conducted comparing INRA 82 and INRA 96 supplemented with egg yolk and glycerol. Although motility parameters were significantly higher in INRA 82 than in INRA 96, the

INRA96 extender significantly improved per-cycle pregnancy rates compared with INRA82 (71% versus 40%) in a total of 84 mare cycles.[35]

Japanese workers reported that the addition of 2% casein and 5% egg-yolk to a boar semen extender (Modena) resulted in superior sperm viability in cooled stored semen compared to Kenney extender.[36] Semen stored in this extender at 5 ºC resulted in 14 of 22 mares becoming pregnant within 72 h of storage and 3 of 4 mares becoming pregnant with semen stored within 96 to 120 hours. The problem with adding egg yolk to extenders is that it compromises the ability to accurately assess sperm motion characteristics if the extender is not clarified.

Workers in Austria, evaluated another defined milk protein extender (EquiPro®, Minitüb, Tiefenbach, Germany) containing caseinate, selected whey proteins, a range of different sugars and glycine.[37] Interestingly, casein and glycine were components of early extenders such as the CGH-27 extender described by Nishikawa in 1975.[13] After 48 and 72 hours of storage at 5 ºC semen stored in EquiPro® extender reportedly had significantly higher sperm motility than that stored in a Kenney extender. They also reported that centrifugation and removal of 90% of the seminal plasma, which is replaced by the defined milk protein extender, increased the longevity of sperm during storage.

Seminal plasma

The adverse effects of seminal plasma on the survival of equine sperm were recognized as early as the 1930s in the investigations of semen storage.[5,38,39] Many early investigations not only examined various extender formulations, but also optimal dilution ratios of semen in extender. More recently, Colorado workers demonstrated that when using milk-based extenders, complete removal of seminal plasma resulted in significant

reductions in the sperm motion characteristics of cooled equine semen whereas suspension of equine sperm in extenders containing 5 to 20% seminal plasma maintained motion characteristics for over 72 hours of cooled storage.[40,41] Subsequently, it has been widely recommended that dilution ratios of at least three to four parts extender to one part semen be used for cooled equine semen, so that the level of seminal plasma does not exceed 20 to 25% by volume and the sperm concentration remains between 25 x 10^6 and 50 x 10^6/mL. For some stallions, whose sperm do not tolerate the rigors of cooling and storage using simple dilution, centrifugation and partial removal of the seminal plasma to achieve even lower levels (≤ 10 to 12%, v:v) may be necessary to optimize sperm survival.[4] However there are other stallions whose seminal plasma is so toxic to their sperm that complete removal is necessary to avoid a rapid reduction in longevity. When complete removal of seminal plasma is required, alternatives to typical milk-based extenders must be employed.

Padilla and Foote[43] demonstrated that after centrifugation and complete removal of seminal plasma, the motility of cooled-stored equine sperm was greatly improved when resuspended in a Kenney's NFDSM-Gluc extender supplemented with a high-potassium modified Tyrode's medium (KMT). However when KMT extender was used in the presence of seminal plasma, motility was reduced, indicating an interaction between seminal plasma and the extender composition. Workers at Texas A&M confirmed these results, and went on further to demonstrate that fertility was maintained with 13 of 17 mares becoming pregnant using semen stored for 48 h in the KMT extender.[44] Other work from this laboratory demonstrated that both motility and DNA integrity were maintained in sperm from which seminal plasma was removed, followed

by resuspension in either Kenney extender or modified Kenney Tyrodes-type extender [45]. Other investigators have shown that motion characteristics and acrosomal integrity of sperm are maintained when stored 48 hours after seminal plasma removal and resuspension in a Kenney extender supplemented with commercially available phosphate buffered saline containing glucose and pyruvate.[46] Investigators from this laboratory also reported pregnancy rates of 75% (3/4) and 88% (22/25), when this extender was used with semen from two poor cooling stallions in a commercial cooled-transported semen program.[47]

The effects of seminal plasma are not always deleterious and appear to be stallion dependent. When semen from stallions that exhibited low post-thaw sperm motility (<20%) was supplemented with seminal plasma from stallions that produce semen with high post-thaw motility, greater numbers of spermatozoa survived cryopreservation.[48]

Cryoprotectants

The discovery in the 1930s that the addition of egg yolk to suitable buffers significantly increased the fertilizing capacity of stored sperm from a number of species resulted in the widespread use of artificial insemination in dairy cows.[6,49,50] Most equine freezing extenders consist of milk, egg yolk, glycerol, various sugars, and electrolytes. While chicken eggs are the most common source of yolk used in semen extenders, yolk from other species has been substituted with favorable results. One study demonstrated that sperm motility parameters were improved when stallion semen was frozen in lactose EDTA extender supplemented with duck egg yolk rather than chicken egg yolk .[51]

Glycerol has been one of the most widely used cryoprotectants for frozen semen. However, while a higher level of glycerol often yields better post-thaw sperm motility, higher glycerol levels are also contraceptive in the mare.[12,52,53] Levels of glycerol in early studies ranged from as low a 1% to as high as 10%. In fact, the first reported pregnancy using frozen-thawed epididymal stallion sperm was obtained using an extender containing 10% glycerol (glycerin).[54] Many equine semen freezing extenders currently contain approximately 4% glycerol, but some European studies suggest that a final glycerol concentration of 2 to 3.5% may be most appropriate for cryopreservation of equine semen.[55,56] However, INRA 96 with 6% glycerol was recently reported to improve survivability of cryopreserved equine sperm while not adversely affecting fertility.[57]

Because of the tremendous variability observed in the post-thaw motility and fertility of stallion semen frozen in conventional extenders, alternative cryoprotectants to glycerol have been investigated. In one study, the presence of glutamine at 50 mM was not sufficient to offset the need to use glycerol.[58] However, it was found that 50 mM glutamine added to a 2.5% glycerol medium significantly improved sperm motility compared to classical freezing medium containing 2.5% glycerol. These workers concluded that glutamine has a synergistic cryoprotective effect with glycerol on cryopreservation of stallion sperm, and suggested that glutamine acts at the extra-cellular level, independently of glycerol.

Recent studies have demonstrated that both methyl formamide and dimethyl formamide could protect stallion sperm from cryodamage as effectively as glycerol, and it was suggested that these cryoprotectants might provide an alternative for stallions that

have poor post-thaw sperm motility when frozen in glycerol.[59] A new freezing extender Botu-Crio® (Biotech Botucatu, Botucatu,Sao Paulo, Brazil) has recently been made commercially available. The main difference in Botu-Crio® compared to other freezing extenders, is the combination of glycerol and methyl formamide as the cryoprotectant. Fertility was assessed for good and poor freezing stallions in a retrospective analysis of 355 cycles of mares bred with semen frozen in a glucose–EDTA–lactose extender containing glycerol and on 98 mare cycles for semen frozen in Botu-Crio®.[60] While there was no difference in fertility in the good freezing group between extenders, fertility of the poor freezing group was significantly better for semen frozen in Botu-Crio®. It was concluded that the Botu-Crio® extender appears to improve the post-thaw quality and fertility of stallions with semen that is considered to have poor freezability.

Antioxidants

Oxidative damage to sperm during storage is thought to be a potential cause of the decline in motility and fertility. Endogenous lipase activity in seminal plasma was suggested to be a contributing factor in the adverse effects of seminal plasma on cooled stallion sperm.[61] Numerous antioxidants have been added to semen extenders, with varying results, in an effort to prevent damage to equine sperm by lipid peroxidation.[62-68] As with many other extender additives, much of the work with antioxidants has examined in vitro sperm characteristics rather than fertility.

The addition of taurine to several different extenders was reported to consistently result in better sperm motility after storage than non-taurine containing extenders.[62] Addition of ascorbic acid was found to increase the percentage of membrane intact sperm stored in a skim milk extender compared to controls.[65] In contrast, another study found

that the addition of the enzymatic scavenger catalase, or a variety of water-soluble or lipid-soluble antioxidants did not significantly improve the maintenance of sperm motility in semen stored at 5 °C in a NFDSM-Gluc extender.[64] The addition of 2 mM pyruvate to a skim milk extender was beneficial in maintaining sperm motility for semen stored for 48 hours, and based on embryo recovery rates, also tended to improve fertility.[69] Although lactate dehydrogenase activity was found to be correlated with sperm motility,[70] neither pyruvate nor lactate could protect sperm from a H_2O_2 challenge, and it was suggested that beneficial effects exerted by the addition of pyruvate or lactate to semen extenders were probably resulting from them acting as an energy source rather than as antioxidants.[67] Quercetin was recently reported to protect sperm from peroxidation after challenge with xanthine-xanthine-oxidase.[66] These authors also suggested that addition of quercetin to NFDSM-Gluc extender could reduce lipid peroxidation of sperm and thereby prevent premature capacitation of sperm while still allowing the sperm to capacitate and acrosome react after insemination.[68] However, this latter conclusion was drawn from the ability of sperm in quercetin treated semen extender to acrosome react after challenge with A23187, which is not very physiologic, and the authors rightly suggested that fertility trials should be performed to determine the effectiveness of quercetin on sperm storage.

The value of including additional antioxidants to semen extenders has been challenged by results of more recent experiments, which indicate that there is not a substantial increase in lipid peroxidation during semen storage and that peroxidative damage to sperm membranes is not the predominant cause of reduced semen quality.[71,72] Workers from this laboratory report that the inherent antioxidative activity in stallion

semen appears to prevent the formation of reactive oxygen species (ROS) and that the simple addition of extender increases this activity further.[71,72] Other workers have also suggested that although equine seminal plasma contains high superoxide dismutase-like activity, sperm themselves have limited glutathione peroxidase and superoxide dismutase-like activity.[73] They also suggest that the enzymatic antioxidant activity in equine sperm appears to be predominantly derived from seminal plasma adsorbed onto the sperm plasma membrane and that removal of seminal plasma during semen processing may increase oxidative stress in equine sperm. Brazilian investigators reported that lipid oxidation in the seminal plasma appeared to be a general indicator for sperm damage and suggested that both lipid and protein oxidation may aid in the identification of subfertile stallions, but only during the non-breeding season.[74] They also reported that ROS production levels did not appear to result in compromised sperm DNA integrity, which indicated to them that either the measurements were within physiological levels and/or that there is an efficient antioxidant activity in stallion sperm cells.[74]

Conclusions

The NFDSM-Gluc formulation, with slight variations on the basic components ranging from antibiotics to sugars, remains the mainstay of equine semen extenders. Inclusion of a variety of other components such as cryoprotectants and antioxidants has also been attempted, with mixed results. Differences in laboratory techniques and sample populations of stallions likely contributed to this disparity. Use of defined milk proteins has been the most recent major evolutionary step in the development of universally acceptable equine semen extenders and variations on this theme will continue to fuel further research.

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