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Recent Advances in Artificial Insemination (AI) in Horses: Stallion Management, Processing and Preservation of Semen and Insemination Techniques

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ABSTRACT

The efficiency of artificial insemination (AI) for horses remains unsatisfactory. It is mainly because each process of AI causes a detrimental effect on semen quality. To sustain quality of semen properly, several factors including libido of stallions and sperm damage during sperm processing and preservation should be considered. Stallions with decent libido produce a high ratio of sperm to seminal plasma in their ejaculates, which is the ideal semen composition for maintaining sperm quality. Thus, to maximize the fertility rate upon AI, stallions should be appropriately managed to enhance their libido. Seminal plasma should have a positive effect on horse fertility in the case of natural breeding, whereas the effects of seminal plasma on both sperm viability and quality in the context of AI remain controversial. Centrifugation of semen is performed during semen processing to remove seminal plasma and to isolate fine quality sperm from semen. However, the centrifugation process can also result in sperm loss and damage. To solve this problem, several different centrifugation techniques such as Cushion Fluid along with dual and single Androcoll-E^{TM} were developed to minimize loss of sperm and to damage at the bottom of the pellet. Most recently, a new technique without centrifugation was developed with the purpose of separating sperm from semen. AI techniques have been advanced to deliver sperm to optimal region of female reproductive tract at perfect timing. Recombinant equine luteinizing hormone (reLH) and low dose insemination techniques have been developed to maximize both fertility rate and the efficiency of AI. Horse breeders should consider that the entire AI procedure should be optimized for each stallion due to variation in individual horses for a uniformed AI protocol.

(Key words : artificial insemination; stallion; cooling semen; freezing/thawed semen; horses)

INTRODUCTION

Major horse breed registries, such as the Jockey Club (Thoroughbred registry), the standard Jack and Jennet Registry of America and the America Miniature Horses Association, do not accept registration of horses produced by artificial insemination (AI). Further, in countries where thoroughbred horse racing constitutes almost the entire horse industry, AI is not actively used. In contrast, in many developed countries AI of horses is commonly used. To select a stallion, the most important parameter is its performance at competition. Unlike other domestic animals such as bulls or boars, in horse breeding industry, fertility is not usually considered as an important parameter for selection of sires. Thus, it is common for stallions to show fertility problems, which cause financial loss across the horse breeding industry (Aurich, 2008). As a solution, assisted reproduction techniques have become commonplace in the horse industry, with AI used to improve the fertility. However, additional studies are needed to improve the efficiency of AI because its proper application can improve the fertility rates of stallions. AI of horses can be performed using fresh, cooled, or frozen semen. In Europe and North America, AI using cooled semen is preferable over that using fresh semen due to its longevity (Morrell, 2011). Although there are many advantages in using frozen-thawed semen, freezing of semen is not frequently performed due to its detrimental effects on fertility rate (Morrell, 2011).

The main processes of horse AI include 1) semen collection, 2) semen processing, 3) semen preservation, 4) induction of ovulation and 5) insemination. Each step is critical to enhance the success of AI. In this review, recent studies conducted on the stabilization of sperm at each stage of the AI process are discussed.

FACTORS TO CONSIDER FOR SPERM QUALITY DURING SEMEN COLLECTION

1. Libido of Stallions

Libido is an important factor in determining the quality of se-

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men during collection of semen. The libido of the stallion affects on both time from initiation of sexual excitement to ejaculation and the number of mounts until ejaculation (Aurich, 2008). Stallions with high libido mount less frequently and ejaculate sooner compared to stallions with low libido. Further, both the number of mounts and the duration of ejaculation many change the volume and concentration of ejaculate. Specifically, stallions that ejaculate more quickly with less frequent mounting ejaculate semen of lower volume and higher concentration. Thus, management of libido is critical to improve the conditions of semen and ultimately increasing the fertility rate of horses subjected to AI. The reaction time of stallions is characterized as the time from first visual contact with the mare to initiation of copulation. Reaction time is affected by various factors, including season, age, training status and stallion management (Papa *et al.*, 2011; Pillet *et al.*, 2011). Pickett and coworkers (1970) reported that reaction time of stallions in August and January were 206 and 819 seconds, respectively. Age and training status are also related to the reaction time of stallions (Pickett *et al.*, 1970). Compared to young and untrained horses, older and experienced horses showed significantly short reaction time (Pickett *et al.*, 1970). Further, too frequent breeding or seminal collection may induce sexual satiation, including lengthened reaction time and young horses are more acceptable changes (Pickett, 1993).

2. Seminal Plasma

Seminal plasma is the fluid portion of semen primarily secreted from both the epididymis and the accessory gland before and during semen ejaculation (Kareskoski and Katila, 2008). Seminal plasma is composed of various compounds, including proteins, ions, amino acids, monosaccharides, lipid, polyamines, prostaglandins and steroid hormones (Kareskoski and Katila, 2008). Although the substances within seminal plasma appear to be beneficial for sperm during natural breeding, research have demonstrated their adverse effects on sperm during cooling and freezing (Brinsko *et al.*, 2000). The effect of seminal plasma on sperm remains controversial mainly due to variations in seminal plasma composition obtained from individual stallions (Aurich, 2008). Several studies have demonstrated that removal of seminal plasma has a positive effect on sperm motility and integrity during cooling and freezing. In a previous study, centrifugation and partial removal of seminal plasma (approximately 90%) following cooled storage for 48 hours were shown to increase the proportion of progressively motile spermatozoa in semen obtained from stallions (Brinsko *et al.*, 2000). A proportion of seminal plasma of about $\leq$5% was shown to have a positive effect on sperm maintenance compared to samples containing higher proportions of seminal plasma (10～30%) for both cooled storage and cryopreservation (Alghamdi *et al.*, 2002; Moore *et al.*, 2005). Complete removal of seminal plasma had a positive effect on sperm motility and DNA integrity compared to sperm stored with 20% seminal plasma for 48 hours in an equitainer (Love *et al.*, 2005). Daily centrifugation and resuspension with a fresh extender containing 10% seminal plasma increased the longevity of sperm for cooled sperm (Love *et al.*, 2012). However, previous experiments demonstrated that the effect of seminal plasma on semen varies among stallions (Aurich, 2008; Brinsko *et al.*, 2000; Padilla and Foote, 1991) due to variable composition of seminal plasma and the sperm plasma membrane.

The reproductive status of a stallion appears to be an important parameter in determining the effect of seminal plasma on sperm motility. The advantage of seminal plasma depletion appears to be more pronounced in stallions whose ejaculates display poor tolerance during cooling and storage (Brinsko *et al.*, 2000). Thus, the tolerance of ejaculate to cooling and storage should be evaluated prior to setting up the protocol.

Although previous studies have shown similar results, it is difficult to determine the effect of seminal plasma on sperm because different ejaculates from the same stallion can vary depending on several factors, including collection procedure, collector, nutrition, collecting place and season. Therefore, experiments with a reasonable number of stallions and consistent collecting procedures should be conducted to determine the effect of seminal plasma on sperm during cooling and storage.

3. Centrifugation to Separate Seminal Plasma

Centrifugation is commonly used to separate seminal plasma from ejaculate. The speed of centrifugation should be adjusted to minimize sperm loss, but vigorous centrifugation may result in low quality of sperm (Aurich, 2008). The recommended centrifugation protocol for separating seminal plasma from semen is a centrifugal force of 400～600×g for 10～15 min, which results in approximately 25% of spermatozoa in the supernatant (Loomis, 2006). Ferrer and coworkers (2012) demonstrated that the survival rate of sperm after centrifugation is dependent upon several different factors, including centrifugal force, duration, column height, sperm concentration and centrifugation medium. Sperm loss was minimized when volume of semen was less than 20 ml during centrifugation (Ferrer *et al.*, 2012). In contrast, a volume of 40 ml at a concentration of 25～50×10^6 spermatozoa/ml in a milk-based

medium is recommended when semen is centrifuged at 900×g for 10 min (Ferrer *et al.*, 2012). Thus, the result of this study indicated that volume of semen should be adjusted based on the speed of centrifugation.

Previously, a technique employing a solution of high specific density (Cushion Fluid, Minitüb) was developed to protect spermatozoa during high-speed centrifugation. The solution protects semen by avoiding compaction at the bottom of the tube during centrifugation at 1,000×g for 20 min. More than 90% of spermatozoa can be recovered using this procedure. Bliss and coworkers (2012) demonstrated that approximately 1 ml of cushion fluid in 50 ml conical-bottom centrifuge tubes is optimal for the centrifugation of up to 3×10^9 sperm.

Ramires and coworkers (2013) developed a new technique, a filter composed of a synthetic hydrophilic membrane (Sperm Filter, BotuPharma, Botucatu, Brazil), to separate seminal plasma from semen without centrifugation. The Sperm Filter system allows 90～95% of seminal plasma to pass through due to the size of pores combined with capillary action. Sperms retained in the filter can be recovered by washing with an extender. In a comparison of efficiency between the filter separation method and centrifugation (at 600×g for 10 min), no significant difference in terms of kinetic parameters or the plasma membrane integrity of frozen/thawed semen was noticed. However, a significantly higher number of sperm was recovered by filter separation (89.4±7.4%) compared with centrifugation (80.9±5.5%). This technique may be useful for horse breeding farms where centrifugation is not available. However, the process should be carried out with extra care as the semen sample is exposed to the environment longer. Further, the efficiency of this new technique on pregnancy rate has not been compared with other separation techniques. Therefore, the pregnancy rates of various methods need to be compared to determine the efficiency of the technique.

4. Centrifugation to Improve Sperm Quality

After semen collection, ejaculate is centrifuged to remove seminal plasma and to separate high quality sperm. Colloid (Androcoll-E™, SLU; patent applied for), which is silane-coated silica colloid in a buffered salt solution, was used as a separation material with two separate protocols. For dual densities, extended semen at a concentration of 100×10^6 spermatozoa/ml was layered onto 2 ml each of high and low gradients (total of 4 ml) in a centrifuge tube. The sperm pellet at the bottom can be obtained after centrifugation at 300×g for 20 min (Morrell *et al.*, 2009). In a single density protocol, 3～4 ml of extended semen at a concentration of 100×10^6 spermatozoa/ml was layered on top of Androcoll-E and centrifuged at 300×g for 20 min to isolate sperm (Morrell *et al.*, 2010). Morrell and coworkers (2009) compared the efficiency of these two methods and reported that both dual gradient and single layer centrifugation can improve the proportion of morphologically normal sperm up to 9.5% and 10.5%, respectively. Morrell and coworkers (2009) also demonstrated that both dual gradient or single gradient centrifugation improve sperm motility and chromatin integrity. In another study, the single density protocol was shown to significantly improve sperm motility and viability compared to centrifugation at 30×g for 20 min without any gradient (Morrell *et al.*, 2010). Hoogewijs and coworkers (2011) compared the single density gradient protocol to Cushion Fluid centrifugation in terms of their effects on the quality of frozen/thawed semen. Although centrifugation with a single density gradient reduced the yield of sperm compared to cushioned centrifugation, both semen quality prior to cryopreservation as well as progressive motility of post-thawed semen were significantly improved (Hoogewijs *et al.*, 2011). The result of this study suggests that Androcoll-E may be used to improve the rate of pregnancy of breeding mares inseminated with cryopreserved semen. Centrifugation of semen with a double-layered iodixanol density gradient (16% and 30%) has been used for sperm separation (Stuhtmann *et al.*, 2012). Specifically, centrifuged sperm in the bottom 30% iodixanol layer showed a greater percentage of morphologically normal sperm and progressively motile sperm after 72 hours of cold storage (Stuhtmann *et al.*, 2012). Compared to sperm obtained by ordinary centrifugation, semen recovered from centrifugation with the iodixanol density gradient showed significantly higher viability, progressive motility and intact sperm after cryopreservation (Stuhtmann *et al.*, 2012).

PROPER SPERM STORAGE

1. Cooling Semen

Sperm should be cooled or frozen to store for longer than 12 hours (Aurich, 2008; Lo *et al.*, 2002). The semen cooling procedure should be performed properly as sperm is very sensitive to temperature changes and the success rate of pregnancy with cooled semen is determined based on the efficiency of the cooling procedure (Brinsko *et al.*, 2011b). Cooling rate is the critical factor resulting in detrimental effects on sperm during the cooling procedure. Cold shock is characterized as sperm with abnormal swimming patterns, lack of motility, membrane perturbations and signs of oxidative stress (Amann and Pickett, 1987; Aurich, 2005,

2008; Brinsko *et al.*, 2011b; Moran *et al.*, 1992). A rapid change in temperature from 18 to 8℃ is critical and can induce cold shock damage in sperm. A previous study showed that the most desirable cooling rate is less than 0.3℃ per minute (Amann and Pickett, 1987). Whereas, recent study showed that a rate from 0.05℃ and 0.17℃ per minute is optimal cooling rate (Brinsko *et al.*, 2011b). Semen can be cooled at appropriate cooling rate using commercially available containers. Most commercial semen containers can be used without any detrimental effect on semen (Aurich, 2008). However, semen stored longer than 30 hours or under severe weather conditions can be affected by the quality of the container (Aurich, 2008). Brinsko and coworkers (2000) compared changes in semen quality during cooling among commercial semen containers, including Equitainer Ⅰ, Equitainer Ⅱ, Equitainer Ⅲ, ExpectaFoal, Bio-Flite, Lane STS and Equine Express. Motility rate, progressive motility rate and the curvilinear velocity of semen stored in these containers were significantly affected by ambient temperature. The only exception was ExpectaFoal, which was kept below 4℃ when the highest ambient temperature was at 37℃ for 24 hours (Brinsko *et al.*, 2000). DNA fragmentation occurs earlier in semen from sub-fertile stallions compared to semen from fertile ones, suggesting that tolerance of sperm to cooling condition appears to be dependent on the reproductive status of stallions (Lo *et al.*, 2002). Thus, breeders should evaluate whether or not stallions are good or poor cooler prior to ship their semen.

2. Freezing Semen

Application of the uniform semen freezing protocol to all stallions is challenging as the efficiency of each semen-freezing method varies for each stallion. Amann and Pickett (1987) indicated that optimization of the extender (s), cooling rate and warming rate may be necessary for each individual horse rather than using the same protocol for all stallions. The main reason for variation is that semen freezability of stallions is not the factor in selecting stallions, unlike other domestic animals (Loomis and Graham, 2008). For this reason, the protocols were optimized to each horse, resulting in the development of several semen-freezing protocols. To optimize freezing conditions for each horse, split-ejaculate test-freeze procedures were performed at many commercial semen-freezing laboratories. This procedures are performed to test the effects of several factors, including extenders, centrifugation techniques, cooling rates and package type, on the quality of frozen-thawed semen split from the same ejaculate (Loomis and Graham, 2008). The split-ejaculate test-freeze process is efficient for customizing the procedure to each stallion (Loomis and Graham, 2008). The stallion semen-freezing process adopted by many practitioners involves 1) dilution of semen with 37℃ with an extender containing egg-yolk, 2) cool down to 4℃ over 4 hours, 3) addition of freezing extender, followed by packaging of semen at 4℃ and 4) freezing to −140℃ and storage in liquid nitrogen. The new protocol with modification of centrifugation (to remove seminal plasma) and addition of the freezing extender containing a cryprotectant such as glycerol are performed at 22℃ rather than 4℃ showed a significantly increase in efficiency of AI. The new protocol showed that 84.6% of 322 stallions produced spermatozoa with ≥30% post-thaw progressive motility (Loomis and Graham, 2008).

In a previous study, the replacement of egg yolk with soybean lecithin showed similar post-thaw semen parameters, but the fertility was significantly reduced with soybean lecithin (Papa *et al.*, 2011). The result of this study suggests that the fertility rate is not always correlated with the quality of post-thaw sperm. Thus, optimization of the stallion semen-freezing method should be performed based on the fertility rate rather than the result from post-thaw semen quality. As a cryoprotectant agent, hen egg yolk is widely used in semen-freezing extenders. However, egg yolk constitutes a potential risk of contamination by bacteria, which could be the source of endotoxins. Further, egg yolk may introduce exotic diseases from imported egg-based products (Pillet *et al.*, 2011). Other disadvantages in using egg yolk as a cryoprotectant is that egg yolk need to be manually isolated prior to its addition to the extender and the composition of egg yolk is not consistent, which may cause variation in the fertility rate. As an alternative source to natural egg yolk, egg yolk plasma, gamma irradiation-sterilized egg yolk, has been developed and tested for its efficiency (Pillet *et al.*, 2011). Compared to natural egg yolk, frozen-thawed semen with egg yolk plasma showed no significant difference in semen parameter and fertility rate (69% vs 60%). A ready-to-use semen freezing extender, INRA-Freeze (IMV-Technologies, Frances), was developed using egg yolk plasma.

ADVANCED AI TECHNIQUE FOR HORSES

1. Use of an Ovulatory Agent, reLH

To improve the fertility rate of AI with fresh, cooled, or frozen semen, several types of hormone agents have been developed with the purpose of inducing ovulation in mares. Agents that have been developed include hCG, native GnRH, Deslorelin (Ovuplant, GnRH-agonist), Buserelin (GnRH analogue), equine pituitary ex-

tracts and equine chorionic gonadotropin (eCG or PMSG). However, none of these agents are reliable because of inconsistent of functions and stability. As an alternative, single chain reLH was developed and its efficacy tested *in vitro* and *in vivo* (Jablonka-Shariff *et al.*, 2007). Intravenous injection of reLH into stallions stimulated testosterone production. Also *in vitro* study showed that treatment with reLH stimulated testosterone production of cultured Leydig cells. These studies suggest that reLH is as effective as endogenous eLH (Jablonka-Shariff *et al.*, 2007). Yoon and coworkers (2007) conducted a study to evaluate the efficacy of reLH on induction of ovulation in mares. Mares were treated with different doses of reLH when the size of their follicle reached at 35 mm in diameter and were monitored until their time of ovulation. Nine of 10 (90%) mares treated with 0.75 mg of reLH ovulated within 48 hours after injection. In the same study, 16 of 20 (80%) mares treated with hCG (2,500 IU) ovulated within 48 hours after injection. Although this study showed that reLH can induce ovulation within 48 h, further research needs to investigate the effect of reLH on improving the fertility rate of mares subjected to AI. These results were used to develop a new ovulatory agent for mares called EquiLH (AspenBio Pharma).

2. Low Dose Insemination

As a universal standard, a minimum of 500 and 1,000 million progressively motile sperm are inseminated in fresh and cooled semen, respectively. In the case of freeze-thawed stallion sperm, at least 200～250 million progressively motile sperm should be inseminated (Brinsko *et al.*, 2003; Samper and Plough, 2010). Following this protocol, diluted semen is deposited into the uterine body. In horse breeding, when fertility rate is consistent, mares should be bred with a low dose of semen, which may lead to reduced prices of semen as well as uterine inflammatory reaction post-breeding (Samper and Plough, 2010). Further, low dose insemination increases the possibility of breeding mares with sex-sorted or epidermal semen, which are always limited in volume (Samper and Plough, 2010). In low semen insemination, rectally guided deep-horn insemination (RI) and hysteroscopic insemination (HI) have been developed (Brinsko *et al.*, 2011a). To perform RI, a 65 cm long flexible pipette (Minitube, Tieffenback, Germany) with a 3 ml syringe attachment (containing 2 ml of air) is placed inside the uterus (Brinsko *et al.*, 2000). After placing the pipette into the uterine body, the inseminator's hand inside the rectum guides the pipette tip towards the end of the uterine horn near the oviductal papilla, after which the semen is deposited at the tip of the uterine horn. The procedure for HI is performed using a pre-warmed 1.2 m or 1.6 m flexible videoendoscope (Samper and Plough, 2010). By monitoring the placement of the endoscope, the breeder's hand inside the rectum can guide the endoscope to the oviductal papilla. Once the endoscope reaches the oviductal papilla, semen loaded in the delivery system (Low Dose Insemination Setl Mila International Inc., Erlanger, KY, USA) is deposited (Brinsko *et al.*, 2003). Using these techniques, it was previously shown that the number of sperm was reduced to 1 to 20 million and the fertility rate was shown to be similar compared to normal insemination techniques (Sieme *et al.*, 2004). The pregnancy rate did not significantly differ between mares inseminated with 14 million motile frozen-thawed sperm into the uterine body and mares bred hysteroscopically in the oviductal papilla with the same number of sperm (67% vs 64%). However, the HI technique with 3 million sperm yielded a significantly higher pregnancy rate compared to that of insemination into the uterine body (45% vs 15%). The efficiency of low dose insemination depends on the reproductive status of mares. Samper and Plough (2010) demonstrated that the fertility rate can be significantly reduced when low dose insemination is applied to mares with a history of reproductive problems. Although breeders use less semen to breed horses, there are disadvantages in using these AI techniques, including 1) high risk of trauma to both the uterus and rectum, 2) highly technical procedures with high difficulty, 3) intensive labor compared to insemination into the uterine body, 4) additional expenses related to equipment such as endoscopes and 5) sedation procedure. Despite such disadvantages, these techniques can be used to extend the applicability of frozen-thawed stallion semen in the horse breeding industry.

CONCLUSIONS

AI is a powerful tool to enhance the breeding efficiency of horses. However, the pregnancy rate using AI is not satisfactory. The results of recent studies reviewed in this paper have shown that advanced techniques yield improvement of sperm quality and/or pregnancy rate. It is critical to aware variation of individual horses using a uniform AI protocol. Thus, pre-evaluation of stallion semen through the entire AI procedure with fine recording system should be performed to optimize the protocol. As reduction of sperm quality occurs in each step of the AI process, additional studies are warranted to discover the mechanism through which sperm quality is reduced, which may allow the replacement of materials and protocols during AI with fresh, cooled, or frozen semen.

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REFERENCES

Alghamdi AS, Troedsson MH, Xue JL and Crabo BG. 2002. Effect of seminal plasma concentration and various extenders on postthaw motility and glass wool-Sephadex filtration of cryopreserved stallion semen. Am. J. Vet. Res. 63:880-885.

Amann RP and Pickett BW. 1987. Principle of cryopreservation and a review of cryopreservation of stallion spermatozoa. J. of Equine Vet. Sci. 7:145-173.

Aurich C. 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim. Reprod. Sci. 89: 65-75.

Aurich C. 2008. Recent advances in cooled-semen technology. Anim. Reprod. Sci. 107:268-275.

Bliss SB, Voge JL, Hayden SS, Teague SR, Brinsko SP, Love CC, Blanchard TL and Varner DD. 2012. The impact of cushioned centrifugation protocols on semen quality of stallions. Theriogenology 77:1232-1239.

Brinsko SP, Blanchard TL, Varner DD, Schumacher J, Love CC, Hinrichs K and Hartman DL. 2011a. Manual of Equine Reproduction. 3rd ed, Mosby Elsevier, Missouri. pp. 160-175.

Brinsko SP, Blanchard TL, Varner DD, Schumacher J, Love CC, Hinrichs K and Hartman DL. 2011b. Manual of Equine Reproduction. 3rd ed, Mosby Elsevier, Missouri. pp. 207-227.

Brinsko SP, Crockett EC and Squires EL. 2000. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 54:129-136.

Brinsko SP, Rigby SL, Lindsey AC, Blanchard TL, Love CC and Varner DD. 2003. Pregnancy rates in mares following hysteroscopic or transrectally-guided insemination with low sperm numbers at the utero-tubal papilla. Theriogenology 59:1001-1009.

Brinsko SP, Rowan KR, Varner DD and Blanchard TL. 2000. Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. Theriogenology 53:1641-1655.

Ferrer MS, Lyle SK, Eilts BE, Eljarrah AH and Paccamonti DL. 2012. Factors affecting sperm recovery rates and survival after centrifugation of equine semen. Theriogenology 78:1814-1823.

Hoogewijs M, Morrell J, Van Soom A, Govaere J, Johannisson A, Piepers S, De Schauwer C, De Kruif A and De Vliegher S. 2011. Sperm selection using single layer centrifugation prior to cryopreservation can increase thawed sperm quality in stallions. Equine Vet. J. Suppl. 43 Suppl 40:35-41.

Jablonka-Shariff A, Roser JF, Bousfield GR, Wolfe MW, Sibley LE, Colgin M and Boime I. 2007. Expression and bioactivity of a single chain recombinant equine luteinizing hormone (reLH). Theriogenology 67:311-320.

Kareskoski M and Katila T. 2008. Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. Anim. Reprod. Sci. 107:249-256.

Lo CC, Thompson JA, Lowry VK and Varner DD. 2002. Effect of storage time and temperature on stallion sperm DNA and fertility. Theriogenology 57:1135-1142.

Loomis PR and Graham JK. 2008. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim. Reprod. Sci. 105:119-128.

Loomis PR. 2006. Advanced methods for handling and preparation of stallion semen. Vet. Clin. North. Am. Equine Pract. 22: 663-676.

Love CC, Blanchard TL, Varner DD, Brinsko SP, Voge J, Bliss S, Sudderth K, Teague S and LaCaze K. 2012. Effect of daily semen centrifugation and resuspension on the longevity of equine sperm quality following cooled storage. Theriogenology 77:1911-1917.

Love CC, Brinsko SP, Rigby SL, Thompson JA, Blanchard TL and Varner DD. 2005. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology 63:1584-1591.

Moore AI, Squires EL and Graham JK. 2005. Effect of seminal plasma on the cryopreservation of equine spermatozoa. Theriogenology 63:2372-2381.

Moran DM, Jasko DJ, Squires EL and Amann RP. 1992. Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. Theriogenology 38:999-1012.

Morrell J. 2011. Artificial Insemination: Current and Future Trends. Artificial Insemination in Farm Animals.

Morrell JM, Johannisson A, Dalin AM and Rodriguez-Martinez H. 2009. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reprod. Domest. Anim. 44:512-

517.

Morrell JM, Rodriguez-Martinez H and Johannisson A. 2010. Single layer centrifugation of stallion spermatozoa improves sperm quality compared with sperm washing. Reprod. Biomed. Online 21:429-436.

Padilla AW and Foote RH. 1991. Extender and centrifugation effects on the motility patterns of slow-cooled stallion spermatozoa. J. Anim. Sci. 69:3308-3313.

Papa FO, Felicio GB, Melo-Ona CM, Alvarenga MA, De Vita B, Trinque C, Puoli-Filho JN and Dell'Aqua JA, Jr. 2011. Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. Anim. Reprod. Sci. 129:73-77.

Pickett BW, Faulkner LC and Sutherland TM. 1970. Effect of month and stallion on seminal characteristics and sexual behavior. J. Anim. Sci. 31:713-728.

Pickett BW. 1993. Equine Reproduction. Williams & Wikins, Media, PA, USA. pp. 809-820.

Pillet E, Duchamp G, Batellier F, Beaumal V, Anton M, Desherces S, Schmitt E and Magistrini M. 2011. Egg yolk plasma can replace egg yolk in stallion freezing extenders. Theriogenology 75:105-114.

Ramires Neto C, Monteiro GA, Soares RF, Pedrazzi C, Dell'aqua JA, Jr., Papa FO, Castro-Chaves MM and Alvarenga MA. 2013. New seminal plasma removal method for freezing stallion semen. Theriogenology 79:1120-1123.

Samper JC and Plough T. 2010. Techniques for the insemination of low doses of stallion sperm. Reprod. Domest. Anim. 45 Suppl 2:35-39.

Sieme H, Bonk A, Hamann H, Klug E and Katila T. 2004. Effects of different artificial insemination techniques and sperm doses on fertility of normal mares and mares with abnormal reproductive history. Theriogenology 62:915-928.

Stuhtmann G, Oldenhof H, Peters P, Klewitz J, Martinsson G and Sieme H. 2012. Iodixanol density gradient centrifugation for selecting stallion sperm for cold storage and cryopreservation. Anim. Reprod. Sci. 133:184-190.

Yoon MJ, Boime I, Colgin M, Niswender KD, King SS, Alvarenga M, Jablonka-Shariff A, Pearl CA and Roser JF. 2007. The efficacy of a single chain recombinant equine luteinizing hormone (reLH) in mares: induction of ovulation, hormone profiles and inter-ovulatory intervals. Domest. Anim. Endocrinol. 33:470-479.

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