Comparison of Two Commercial Extenders for Refrigerated Stallion Semen: Effects on Sperm Quality over 48 h

Refrigerated Stallion Semen Extenders

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Summary

Artificial insemination in horses relies on effective semen preservation to maintain sperm function during storage and transport, particularly for refrigerated semen used in national and international shipment within 24 to 48 h. This study compared the effect of two commercial extenders, INRA 96® and Equiplus®, on the quality of refrigerated stallion semen. Semen from ten stallions of proven fertility was collected and diluted to a final concentration of 25 × 10⁶ spermatozoa/mL in each extender. Samples were evaluated at 0, 24, and 48 h for total motility (TM) and progressive motility (PM) using computer-assisted sperm analysis (CASA), and for functional parameters using flow cytometry, including viability, mitochondrial membrane potential (MMP) and acrosome status. Sperm motility decreased over time after dilution with both extenders; however, TM and PM were significantly higher in samples diluted with INRA 96® at 48 h ($p < 0.05$), with no significant differences at 0 or 24 h ($p > 0.05$). Sperm viability declined during storage but remained significantly higher in samples diluted with INRA 96® at 48 h ($p < 0.05$). No significant differences were observed in MMP between extenders at any time point ($p > 0.05$). Acrosome damage differed between extenders, being significantly higher in samples diluted with Equiplus® at 48 h ($p < 0.05$), with no significant changes over time within each extender ($p > 0.05$). These results suggest that INRA 96® may be associated with improved preservation of motility and viability, and reduced acrosome damage during refrigerated storage for up to 48 h.

Keywords: stallion semen, semen extender, INRA 96®, Equiplus®, refrigeration, sperm quality

Resumo

A inseminação artificial em equinos depende de uma preservação eficaz do sémen para manter a funcionalidade dos espermatozoides durante o armazenamento e o transporte, particularmente no caso do sémen refrigerado utilizado em transportes nacionais e internacionais num período de 24 a 48 horas. Este estudo comparou o efeito de dois diluentes comerciais, INRA 96® e Equiplus®, na qualidade do sémen refrigerado de garanhões. O sémen de dez garanhões de fertilidade comprovada foi colhido e diluído até uma concentração final de 25 × 10⁶ espermatozoides/mL em cada um dos diluentes. As amostras foram avaliadas às 0, 24 e 48 horas quanto à motilidade total (MT) e à motilidade progressiva (MP), recorrendo à análise computorizada do sémen (CASA), bem como quanto a parâmetros funcionais determinados por citometria de fluxo, incluindo viabilidade espermática, potencial da membrana mitocondrial (PMM) e integridade do acrossoma. A motilidade espermática diminuiu ao longo do tempo após a diluição com ambos os diluentes; no entanto, a MT e a MP foram significativamente superiores nas amostras diluídas com INRA 96® às 48 horas ($p < 0,05$), não se verificando diferenças significativas às 0 ou 24 horas ($p > 0,05$). A viabilidade espermática diminuiu durante o período de armazenamento, mas manteve-se significativamente superior nas amostras diluídas com INRA 96® às 48 horas ($p < 0,05$). Não foram observadas diferenças significativas no potencial da membrana mitocondrial entre os dois diluentes em nenhum dos momentos de avaliação ($p > 0,05$). Os danos no acrossoma diferiram entre os diluentes, sendo significativamente superiores nas amostras diluídas com Equiplus® às 48 horas ($p < 0,05$), sem alterações significativas ao longo do tempo dentro de cada diluente ($p > 0,05$). Estes resultados sugerem que o INRA 96® poderá proporcionar uma melhor

preservação da motilidade e da viabilidade espermática, bem como uma menor ocorrência de danos no acrossoma durante a conservação do sémen refrigerado por um período até 48 horas.

Palavras-chave: sémen de garanhão; diluente de sémen; INRA 96®; Equiplus®; refrigeração; qualidade espermática.

Introduction

Artificial insemination has become a cornerstone of modern equine reproduction, allowing genetic improvement, wider dissemination of desirable traits and improved biosecurity through reduced animal movement (Oliveira et al., 2019; Aurich, 2012). Therefore, the collection, evaluation and preservation of stallion semen have assumed greater importance in both clinical and commercial settings. However, the reproductive efficiency of a stallion is not always directly correlated with its athletic or genetic value, making semen handling and preservation strategies critical to ensure optimal fertility outcomes (Munroe & Weese, 2011).

Accurate assessment of semen quality relies on a combination of conventional and advanced techniques. Traditional parameters such as motility, concentration and morphology remain fundamental, but modern approaches including computer-assisted sperm analysis (CASA) and flow cytometry provide deeper insights into sperm function and fertilizing potential (Battut et al., 2016; Peña et al., 2016; Mortimer, 2000). These techniques enable the evaluation of key functional attributes such as mitochondrial activity, plasma membrane integrity and acrosomal status.

Following collection, semen must be appropriately processed and preserved to maintain sperm viability. Semen extenders play a central role in this process by protecting spermatozoa against thermal shock, providing energy substrates, stabilizing cell membranes and inhibiting microbial growth (Hernández-Avilés et al., 2020; Loomis & Graham, 2008). The effectiveness of semen preservation is influenced by several factors, including storage temperature, duration and, importantly, the composition of the extender used.

Refrigerated semen is widely used in equine practice due to its practicality and relatively good fertility outcomes when used within 24 to 48 hours after collection (Dascanio & McCue, 2021; Aurich, 2008). However, sperm survival during storage is highly variable and depends not only on the extender but also on individual stallion characteristics (Ferrer et al., 2020; Varner et al., 2015). Therefore, the selection of an appropriate extender is essential to maximize semen quality and fertility potential.

Among the commercially available extenders, INRA 96® and Equiplus® are commonly used in equine reproduction (Rečková et al., 2022; LeFrapper et al., 2010). Despite their widespread application, comparative data on their performance under practical refrigerated conditions remain limited.

The aim of this study was to compare the effects of two commercial extenders (INRA 96® and Equiplus®) on stallion semen quality during refrigerated storage at 5 ºC. Samples were evaluated at 0, 24 and 48 hours for total and progressive motility, as well as for functional sperm parameters assessed by flow cytometry, including viability, mitochondrial membrane potential and acrosome status.

Materials and Methods

Animals and Experimental Design

The study was conducted using ten stallions of proven fertility housed at the Animal Selection and Reproduction Center (CENSYRA, Badajoz, Spain). Animals ranged in age from 4 to 19 years and included several breeds (Purebred Spanish, Arabian, Anglo-Arabian, Spanish Sport Horse crossbred and Dutch Warmblood) during the 2022 breeding season. Each stallion provided three ejaculates collected on non-consecutive days to minimize the effect of collection frequency. The ejaculates were treated as independent samples.

Semen Collection and Processing

Ejaculates were obtained using a pre-warmed (45 ºC) and lubricated INRA model artificial vagina (IMV, L'Aigle, France) with an inline filter to eliminate the gel fraction. Semen was immediately transported to the laboratory for evaluation and processing. All procedures were performed under standard management conditions routinely applied at the facility and complied with national regulations on animal welfare.

Immediately after ejaculation, semen was transported to the laboratory under controlled temperature conditions to avoid thermal shock. Ejaculates were weighed to determine volume and maintained at 37 ºC prior to analysis. Sperm concentration was assessed using a hemocytometer (Mackler® chamber). Samples were then divided into two aliquots and diluted with two commercial extenders: INRA 96® and Equiplus®.

Dilutions were performed to achieve a final concentration of 25 × 10^6 spermatozoa/mL. Aliquots corresponding to each extender were prepared for each ejaculate and treated as paired samples. After initial evaluation (0 h), samples were maintained at room temperature for 10–15 minutes to allow gradual cooling and subsequently stored at 5 ºC in darkness to minimize photodamage and oxidative stress. Evaluations were conducted at three time points: immediately after dilution (0 h), after 24 hours and after 48 hours of storage. Before each evaluation, samples were rewarmed to 37 ºC for 5 minutes.

Motility Assessment

Sperm motility was evaluated using a computer-assisted sperm analysis system (ISAS®). For each sample, five microscopic fields were recorded, and the highest and lowest values were excluded to reduce variability. The final result corresponded to the mean of the remaining three fields. Both total motility (TM) and progressive motility (PM) were recorded.

Flow Cytometry Analysis

Flow cytometry was performed on samples from five stallions at 0 and 48 hours to assess sperm functional parameters. Mitochondrial membrane potential was evaluated using the JC-1 probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), which distinguishes between high and low mitochondrial activity. Acrosome status was assessed using peanut agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC) in combination with propidium iodide (PI), with PNA-positive spermatozoa considered to have damaged acrosomes.

Protocols, including probe concentrations and incubation conditions, were conducted according to previously described methodologies (Robles & Martínez-Pastor, 2016; Peña et al., 2016). Analyses were limited to 0 and 48 hours due to logistical and methodological constraints, including the complexity of sample processing and limited availability of equipment and reagents.

Statistical Analysis

Data were tested for normality using the Shapiro–Wilk test and are presented as mean ± standard error of the mean (SEM). Comparisons between extenders and time points were performed using analysis of variance (one way ANOVA) followed by Tukey's post-hoc test. Statistical significance was set at $p < 0.05$. All analyses were conducted using SigmaPlot software (version 12.3, Systat Software, Chicago, IL, USA).

Ethical Statement

All procedures were carried out in accordance with standard animal care practices and institutional guidelines.

Results

At 0 h, no significant differences were observed between extenders for either total motility (TM) or progressive motility (PM) ($p > 0.05$) (Table 1).

At 24 h, both TM and PM decreased in samples diluted with INRA 96® and Equiplus®. However, no significant differences were detected between extenders at this time point ($p > 0.05$) (Table 1).

At 48 h, TM and PM were significantly higher in samples diluted with INRA 96® compared to Equiplus® ($p < 0.05$). A significant time-dependent decrease in motility (0–48 h) was observed within each extender for both TM and PM ($p < 0.05$), as indicated by different superscript letters in Table 1.

Sperm viability decreased over time in both extenders, with a significant reduction observed between 0 and 48 h ($p < 0.05$). At 48 h, viability remained significantly higher in samples diluted with INRA 96® compared to Equiplus® ($p < 0.05$). No significant differences were detected between extenders in mitochondrial membrane potential (MMP) at either time point ($p > 0.05$). In contrast, acrosome damage was significantly higher in samples diluted with Equiplus® at both 0 and 48 h ($p < 0.05$), with no significant changes over time within each extender ($p > 0.05$) (Table 2).

Table 1. Mean (± SEM) total motility (TM) and progressive motility (PM) of stallion semen diluted with INRA 96® and Equiplus® at 0, 24, and 48 hours. Different superscript letters indicate significant differences ($p < 0.05$) between time points and extenders (two-way ANOVA followed by Tukey's post-hoc test).

Storage time (h)	Extender	n	TM (%) Mean ± SEM	PM (%) Mean ± SEM
0 h	INRA 96®	30	78.03 ± 2.21^{a}	61.23 ± 3.75^{a}
	Equiplus®	30	75.10 ± 2.11^{a}	56.03 ± 1.52^{a}
24 h	INRA 96®	30	69.07 ± 2.17^{b}	56.07 ± 2.05^{ab}
	Equiplus®	30	62.23 ± 2.59^{c}	52.33 ± 2.20^{b}
48 h	INRA 96®	30	55.60 ± 3.44^{d}	43.43 ± 3.21^{c}
	Equiplus®	30	46.87 ± 3.21^{e}	37.40 ± 2.63^{d}

Discussion

The present study shows that the choice of extender has a significant impact on the preservation of stallion semen quality during refrigerated storage. Overall, INRA 96® demonstrated superior performance compared to Equiplus®, particularly in maintaining sperm motility and viability after prolonged storage.

Table 2. Flow cytometry parameters (mean ± SEM) including sperm viability, sperm showing High mitochondrial membrane potential (hMMP) and Acrosome damage (% PNA-positive spermatozoa) in samples diluted with INRA 96® and Equiplus® at 0 and 48 hours. Significant differences were indicated with different letters $p < 0.05$.

Storage time Hours (h)	Extender	n	Sperm hMMP (%) Mean ± SEM	Sperm viability (%) Mean ± SEM	Acrosome damage (% PNA-positive spermatozoa) Mean ± SEM
0 h	INRA 96®	5	60.05 ± 6.61	69.26 ± 3.08 [a]	9.62 ± 1.66[a]
	Equiplus®	5	67.33 ± 3.21	65.57 ± 2.24 [a]	20.23 ± 1.75[b]
48 h	INRA 96®	5	49.90 ± 4.83	67.52 ± 2.49[a]	3.06 ± 1.08[a]
	Equiplus®	5	47.87 ± 4.96	52.14 ± 3.29[b]	15.92 ± 3.56[b]

A progressive decline in both total and progressive motility was observed in all samples, which is consistent with previous findings on refrigerated equine semen (Dascanio & McCue, 2021; Hernández-Avilés et al., 2020). The significantly higher motility observed in INRA 96® at 48 h ($p < 0.05$) suggests a greater protective capacity of this extender against storage-induced damage under refrigeration conditions. This improved preservation of motility may be related to the composition of INRA 96®, which includes components that contribute to membrane stabilization and provide adequate energy substrates to sustain sperm metabolism during storage (Hernández-Avilés et al., 2020). Differences among extenders in their ability to preserve sperm function during cooling have been previously reported and are known to influence fertility outcomes (Ferrer et al., 2020). These findings are particularly relevant under field conditions, where cooled semen is routinely transported over long distances and storage time directly impacts fertility outcomes.

Flow cytometry has proven to be a robust and reliable tool for the multiparametric assessment of sperm function, allowing simultaneous evaluation of key indicators such as membrane integrity, mitochondrial activity and acrosomal status (Umirbaeva et al., 2024). The absence of significant differences in mitochondrial membrane potential ($p > 0.05$) suggests that both extenders similarly preserved mitochondrial function, which is closely associated with sperm motility and energy production (Peña et al., 2016).

In contrast, sperm viability was significantly higher in samples diluted with INRA 96® at 48 h ($p < 0.05$), indicating better preservation of plasma membrane integrity. Membrane integrity is a key determinant of sperm survival and fertilizing capacity (Battut et al., 2016).

Acrosome damage was significantly higher in Equiplus® samples at both time points ($p < 0.05$), indicating greater structural compromise of the acrosome in this extender. The acrosome plays a crucial role in oocyte penetration, and its integrity is essential for successful fertilization; therefore, increased acrosomal damage may negatively affect fertilizing potential (Peña et al., 2016; Battut et al., 2016).

Individual variability among stallions is an important factor influencing semen preservation, as previously described in equine reproduction studies (Ferrer et al., 2020). Although multiple ejaculates per stallion were analyzed, this variability should be considered when interpreting the results.

Some limitations of this study include the relatively small number of stallions evaluated by flow cytometry and the absence of intermediate (24 h) data for these parameters, which limits the assessment of temporal changes in sperm functional characteristics. In addition, the lack of in vivo fertility data prevents direct extrapolation of laboratory findings to reproductive outcomes. Future studies should aim to correlate in vitro parameters with fertility results and explore strategies to further improve extender performance.

Overall, the results of this study support that extender selection is a key factor in equine reproductive management and that INRA 96® may provide improved preservation of sperm quality during refrigerated storage.

Conclusions

Extender selection significantly influences the preservation of stallion semen quality during refrigerated storage. Under the conditions evaluated, INRA 96® showed superior performance compared with Equiplus®, particularly in maintaining motility

and viability and reducing acrosome damage after 48 h at 5 °C. These results suggest that INRA 96® may represent a more suitable option for short-term refrigerated semen storage.

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