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Effects of two different cooling devices for testicles transport on stallion epididymal sperm quality

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Abstract

This study evaluates the effects of two cooling devices and temperature for testicles storage on epididymal sperm quality after 24h; different levels of seminal plasma (0 and 10%) were evaluated on sperm after recovering. Testicles from 6 stallions were recovered immediately after castration (2) or at the slaughterhouse (4); of the same animal one testicle was placed in Equitainer® (+8 C°), the other in a styrofoam box with ice (+3 C°). After 24h the temperature of parenchyma was measured, and testicles and epididymal were weighted. Sperm were flushed from the cauda epididymides with Kenney extender, total sperm number recorded and motility and viability evaluated immediately after flushing (T0) with or without 10% SP (G1 Eq0%, G2 Eq10%, G3 Ice 0%, G4 Ice 10%). Motility and viability were evaluated after 24h and 48h of storage at +4°C. Temperature of the parenchyma was lower in testicles stored in Ice compared to Equitainer® (3.2±0.6°C and 8.6±2.5°C respectively; $P<0.05$). Motility and viability at T0 were similar ($p>0.05$) in G1 and G3,while addition of SP after recovery significantly improved motility only in samples stored in Equitainer® (G2).

Viability was higher ($p<0.05$) in G2 than in G4.

At T24 and T48 no differences ($p>0.05$) in sperm quality were found between storage methods or samples with or without SP. In conclusion, equine testicles can be safely stored either at lower (+3°C) or higher (+8°C) temperature than +5°C. This can be useful, especially when testicles are shipped in a hot climate, where devices can't guarantee optimal refrigeration conditions

Keywords: epididymal sperm, stallion, testicle, storage

1. Introduction

Flushing of tail of the epididymis permits recovery and preservation of sperm from stallions that die prematurely, due to acute or chronic illness, as well as following routine castration.

The equine *cauda epididymis* contains a high number of spermatozoa with normal fertilizing ability and resistance to cold shock [1]. It has been demonstrated that epididymal sperm has similar progressive motility [2] and fertility [3] when compared to ejaculated sperm collected by artificial vagina.

Location of castration or recovery of testicles post mortem may be remote to the laboratory for sperm handling and preservation; therefore, transport or shipment of the testicles to the site of semen processing may require a long period of time (24-48 hours). In these cases, testicles and epididymis are generally sent refrigerated to the laboratory [4].

Previous studies have reported the effect of epididymal storage at +4-5°C following various periods of storage [3-8] In these studies the method of storage varied, but comparison between storage devices was not performed, as well as the temperature of the testicular parenchyma after storage was not recorded. Only James [6] stored one testicle on ice, at +4°C, and shipped it in a styrofoam cooler box for 24h before processing; the other one was stored at +4°C for 48h, 72h and 96h.

Brinsko [9], in a very detailed study, showed that stallion semen may be able to tolerate a wider range of cooling rates and storage temperatures than previously thought.

In addition, different authors already showed that temperatures between +4°C and +15°C are suitable for preserving the fertilizing capacity of stallion semen stored for 22h or more in INRA 96® extender [10], and more important that temperature between +8-10°C can be more suitable than +4°C for “bad cooler” stallions [11].

The motility of epididymal sperm is often low, probably due to the lack of exposure to activating factors present in the SP [12], and effect of SP on the quality of epididymal sperm is unclear as several studies have not shown any improvement in freezability or fertility with or without added SP [2,10,13]

Different studies investigated the amount of SP to added to epididymal sperm. Tiplady [14] showed that addition of SP marginally improve the initial total and progressive motility after equilibraton at +4°C, but they did not calculate the SP level they utilized because SP was used for flushing the *cauda epididymis*. Neuhauser [15], in a study about post-thaw addition of SP to epididymal sperm, showed that motility improved in a dose-dependent manner at concentration of 20% and 50%, but did not further increase at 80%. They concluded there seems to be a threshold level, above which there is no further improvement in most of sperm motion characteristics. Five to 20% SP was beneficial for sperm motion characteristics for up to 96 h of storage, and are usually recommended for preservation [15-23.

Based on these assumptions, the aims of this study were: 1) to evaluate motility and viability of epididymal sperm recovered after storage of testicles for 24 h at different temperatures, especially to evaluate if temperature higher than previously used can effect semen quality; for this purpose two different cooling environments (Equitainer® with one coolant can and ice) were used; 2) to evaluate sperm motility and viability immediately after recovering and after 24 and 48 h of refrigeration ; 3) to evaluate the effect of adding 10% seminal plasma..

2. Materials and methods

2.1 Samples collection

Testicles and epididymides from six healthy stallions of different breeds, between 3 and 5 years of age, were recovered immediately after castration (2) or at the slaughterhouse (4). There were no history of previous ejaculation and castration was performed because owners wanted to eliminate stallion behavior. The *vas deferens* was ligated and each testicle from one stallion was placed in a plastic bag and stored, one in Equitainer® (Hamilton-Thorne Research, Danvers, MA, USA), containing one frozen coolant can, the other one immersed in a styrofoam box with ice.

After 24 h of storage, the temperature of each testicle was measured by placing the probe (Hanna

Checktemp1, Hanna Instruments, Italy) into the testicular tissue. After carefully dissecting the connective tissue, epididymis and *vas deferens* were isolated from the testis and the weights of epididymis and testis were measured. A retrograde flushing of the tail of epididymis was performed using a cat urinary catheter (Arnolds 96 1.3mm OD 4f X 30.5 cm.) connected with an AI syringe (Luer; 20 ml Norm-Ject) containing 20 ml of Kenney extender [24].

2.2 Semen dilution and evaluation

Spermatozoa were recovered in a sterile 50 ml Falcon tube and the volume of recovered fluid was measured. A NucleoCounter® SP-100™ (Chemotec, Denmark) was used for evaluating sperm concentration and viability.

For concentration, 50 µl of the sample were diluted with 5000 µl of Reagent S100 (Chemometec, Denmark), aspirated in SP1™- Cassette (Chemometec, Denmark) and inserted in the NucleoCounter®.

For viability, a second sample diluted with 5000 µl of PBS was used to evaluate non-viable sperm; the percent of viable sperm was calculated using the following formula:

$$\frac{Concentration - Non - viable(\%)}{Concentration}$$

From each treatment (ice and Equitainer®), $300x10^6$ spermatozoa were diluted in 10 ml of Kenney extender ($30x10^6$/mL) with or without 10% seminal plasma (SP), and stored in a fridge at +4°C (Tube 10mL100x16PP, SARSTEDT, Australia).

For motility evaluation, at each time point (T0,T24 and T48) 1 mL was incubated at 37°C for 10 min, and a pre-warmed slide (Leja Standard Count 4 Chamber Slide 20 micron; Leja Products B.V., Nieuw Vennep, The Netherlands) was used for CASA evaluation (IVOS Vers.12, Hamilton Thorn Biosciences), with standard equine setup (Table 1). Sperm motility endpoints assessed were: total motility (TM); progressive motility (PM); curvilinear velocity (VCL); straight line velocity (VSL);

average path velocity (VAP); amplitude of lateral head displacement (ALH); straightness (STR); linearity (LIN) and beat cross frequency (BCF).

2.3 Harvesting of seminal plasma

Seminal plasma was obtained by centrifuging at 3000 g for 30 minutes semen collected from a fertile stallion using a Missouri artificial vagina with an inner liner and an in-line filter (Hamilton-Thorne Research, Danvers, MA, USA); the supernatant was aspirated and filtered (two in-line syringe filters, 5.0 μm and 1.2μm, Minisart Sterile®, Supelco). SP was stored in 1.0 mL aliquots at - 20 °C until needed for dilution.

2.4 Statistical analysis

In order to asses normal distribution of the data, the Shapiro-Wilk test was used. A paired t-test was used to compare weights, number of recovered sperm and testicular temperature. Effects of storage container and SP on motility and viability were analyzed using an ANOVA, and a Tukey HSD test was used for post hoc comparison (IBM SPSS Statistics, Milan, Italy). Significance was assessed at $P<0.05$.

3. Results

No differences were found in total sperm number (TSN) and testicular and epididymal weights ($P>0.05$) after testicular storage in ice and Equitainer® (Table 2).

After storage, the inner temperature of the testicle in ice was significantly lower than that stored in Equitainer® (3.2±0.6°C vs 8.6±2.5°C; $P<0.05$) (Table 2).

Motility and viability at T0 are showed in Table 3. Sperm motility values (TM, PM, VAP, LIN) were higher in G2 than G1 ($P<0.05$), but were similar to G3 ($P>0.05$).

Sperm from testes preserved in ice or Equitainer® and diluted without SP (G1-G3) showed similar values of sperm motility and viability at T0.

Probably due to the relatively low number of samples, no statistically significant differences were found ($P>0.05$) in motility values between the two storage devices.

At T0, sperm viability was higher in G2 than G4 ($P<0.05$); treatments without SP (G1-G3) were not different than either treatment with SP (G2-G4).

After 24 (T24) and 48 (T48) h of sperm refrigeration, no significant differences ($p>0.05$) in all the motility parameters evaluated were found between storage methods or between samples containing SP (Table 4 and 5). Longevity of sperm motility at +4°C was not affected by storage type and/or SP addition.

4. Discussion

The most effective technique to recover spermatozoa from *cauda epididymis* is the retrograde flushing of the *cauda* that allows the collection of over 15 billion sperm [25]; in our study the average TSN recovered from testicles stored in Equitainer® or ice was similar to that previously reported [14,25]. No differences were found in testicular and epididymal weights after 24 h of storage in ice and Equitainer® , in agreement with a previous study [6].

Different studies were performed for evaluating effects of different storing temperature on quality and fertility of ejaculated sperm. Brinsko [9], in a very detailed study, showed that stallion semen may be able to tolerate a wider range of cooling rates and storage temperatures than previously thought. Vidament [10] demonstrated that temperatures between +4°C and +15°C are suitable for preserving the fertilizing capacity of stallion semen stored for 22h or more in INRA 96 extender. Cuervo-Arango [11] reported that temperature between +8-+10°C can be more suitable for "bad cooler" stallions than +4°C. The same Author [26], in one study on the effect of storage temperature of equine transported semen on sperm quality, showed that storing semen between +15°C and +20°C, as compared to the standard temperatures +1°C and +8°C, resulted in similar pregnancy rates, sperm

quality and microbial growth.

Epididymal spermatozoa from many species have been successfully processed and frozen after 24 of storage at +4/+5°C: stallion [27], dog [28], goat [29], man [30], mice [31], monkey [32] and boar [33]. Different studies demonstrated that it is possible to refrigerate the testicles and epididymides for 24 h, before collecting sperm, without negative effects on motility and viability [3,7]. Bruemmer [5] and Guimaraes [4] processed one testicle-epididymis complex immediately after castration, and the other one after 24 h of storage at +5°C; a similar study has been performed by Vieira [8] but only epididymides were stored up to 96 hours at +4°C.

In all these studies testicles were stored refrigerated or at room temperature, but comparison between storage devices was not performed. Only James [6] stored one testicle on ice at +4°C and shipped it in a styrofoam cooler for 24h before processing it, but the temperature of the testicular parenchyma was not directly measured. In the present study, the testicular temperature was measured inserting a rigid probe into the parenchyma, giving precise information on the real storing conditions of the testis-epididymis complex.

Different types of containers are available for storing-shipping equine semen with different and variable inner temperatures. Douglas-Hamilton [34] developed the reusable plastic container Equitainer® to be used with two frozen cans, which is considered the gold standard and the most widely used container for transporting equine cooled semen. Monteiro [3,7] stored the epididymis for 24h at +5°C in a similar container (Botutainer®, Botupharma, Botucatu, Brazil) before recovering epididymal sperm; other Authors used insulated containers not clearly specified [35]. In the present study, the Equitainer® was used for the first time for storing testicles, and only one frozen coolant can was used for evaluating if slower cooling rate and storing temperature higher than +4°C of the testis-epididymis complex, compared to those in a styrofoam box with ice, could improve sperm quality after 24 h of storage. Qiaoxiang Dong [32], in an interesting study, showed that monkey epididymal sperm refrigerated at a temperature of +4 to +10°C on ice in a styrofoam box maintains an higher

motility than sperm refrigerated at +4°C in refrigerator or at 0°C in ice/water slurry. Our results indicate that semen quality is similar between ice (+3°C) and Equitainer® (+8°C) groups.

These findings do not support our hypothesis that, as for monkey epididymal sperm, slower cooling rate and storage temperature of the equine testicles higher than +4°C, as those obtained in Equitainer® with one coolant can, positively affect sperm quality.

Nevertheless, our data demonstrate that a temperature of +8-9°C is not detrimental to sperm quality; this information can be useful, especially when testicles need to be shipped in a hot climate, where devices can't guarantee optimal refrigeration conditions.

Seminal plasma (SP) is composed of enzymes, hormones and metabolites and it is essential in natural mating to transport and protect spermatozoa. The complete role of SP is still not entirely understood, although it is known to transport and sustain the initial motility of spermatozoa after ejaculation, even though it may have detrimental effects on sperm longevity with increasing time [36]. Motility of epididymal sperm is often low, probably due to the lack of exposure to activating factors present in the SP [12]. The effect of SP on epididymal sperm quality is unclear, as several studies did not show any improvement in freezability or fertility with or without the addition of SP [2,13,14], while other studies showed positive effects on fresh epididymal sperm [37,38].

Different Authors showed a positive effect of adding SP on motility and viability of epididymal sperm [12,14,35,38] and in a dose-dependent manner [15].

Previous studies investigated the optimal level of SP to be added to epididymal sperm, but their conclusions are contradictory [15,35]. Graham [39] investigated beneficial effect of SP on ram and bull epididymal spermatozoa. Other Authors showed a positive effects of adding SP to fresh epididymal sperm on pregnancy rates after routine artificial insemination [37].

Braun [19] tried to cool epididymal sperm with a skim milk glucose extender at 3 concentrations of SP (0%, 5% and 25%), concluding that SP had no effect at a concentration of 5%, but immediately increased motility of spermatozoa at 25% compared to 5%, and the stimulating effect of 25% SP in epididymal sperm was apparent in all stallions at 0 h and to a lesser extent at 24h and 48h**.**

In our study addition of 10% SP significantly improved motility, but not viability, only in samples recovered from testicles stored in Equitainer®, suggesting that sperm stored in epididymides at +8/9 C° are more susceptible to activating factors of SP than sperm stored in epididymides at +3 C°. Moreover, even if not statistically different, sperm motility had a positive trend after testicles storage in ice, regardless addition of SP.

5. Conclusions

Equine testicles can be safely stored for 24 h either at +3°C or +8/9°C, and both the shipping methods, ice and Equitainer®, are valuable for preserving sperm motility and viability without any improvement in sperm quality after testicles storage at higher temperatures than previously used.

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Authors contribution

All Authors contributed to the study design, samples collection, semen analysis and writing the paper.

Table 1 – Standard equine setup for CASA

Characteristic	Adjusted to
Frames per second	60
Number of frames	45
Minimum contrast	70 pixels
Minimum cell size	4 pixels
Straightness	75%
Minimum VAP to progressive cells	30 µm/s
VSL cutoff to slow cells	0.0 µm/s
VAP cutoff to slow cells	20 µm/s
Temperature	37°C

CASA, computer-assisted sperm analysis;
VAP, average path velocity;
VSL, straight-line velocity.

Table 2. Media and Standard deviation from testicular temperature, testicular and epididymal weights and total sperm recovered after storage in Equitainer® or ice for 24 h.

Storage group	Testicle weight (g)	Epididymis weight (g)	Testicle Temperature (°C)	Total Sperm Number ($x10^9$)
Equitainer®	215.3±67.7	46.4±10.3	8.6±2.5[a]	15.5±8.6
Ice	211.4±77.2	46.7±11.6	3.2±0.6[b]	11.2±6.6

a,b different letters in the same column $P<0.05$

Table 3. Motility and and Viability (VIAB) of horse epididymal spermatozoa after 24 h testicular storage (T0) (mean ± SD).

GROUP	TM(%)	PM(%)	VAP(µm/sec)	VSL(µm/sec)	VCL(µm/sec)	ALH(µm)	BCF(Hz)	STR(%)	LIN(%)	VIAB(%)
G1	59±20^{a}	25±17^{a}	72±20^{a}	50±19^{a}	161±36^{a}	8.3±0.8	47±2	58±11	27±6^{a}	73±11^{ab}
G2	88±6^{b}	55±17^{b}	104±15^{b}	78±17^{b}	206±22^{b}	8.1±0.3	47±2	70±8	36±6^{b}	75±10^{a}
G3	71±12^{ab}	39±16^{ab}	82±7^{ab}	61±9^{ab}	180±10^{ab}	8.0±0.5	45±2	67±6	32±4^{ab}	74±9^{ab}
G4	87±5^{b}	51.7±11.8^{b}	99.2±18.3^{b}	73.5±15.3^{ab}	197.9±25.7^{ab}	7.9±0.7	45.8±2.1	68.8±6.5	35.5±4.2^{b}	67.7±11^{b}

a,b different letters in the same column $P<0.05$

TM-total motility (%); PM-progressive motility (%);VAP-average path velocity (µm/sec); VSL-straight line velocity (µm/sec); VCL-curvilinear velocity (µm/sec); ALH-amplitude of lateral head displacement (µm); BCF-beat cross frequency (Hz); STR-straightness (%); LIN-linearity (%);VIAB- viability (%)

G1: Eq0%SP;

G2: Eq10%SP;

G3: Ice 0%SP;

G4:Ice10%SP.

Table 4. Motility and kinematic parameters (analysed by CASA) of horse epididymal spermatozoa after 24 h storage + 4C°(T24) (mean ± SD).

GROUP	TM(%)	PM(%)	VAP(µm/sec)	VSL(µm/sec)	VCL(µm/sec)	ALH(µm)	BCF(Hz)	STR(%)	LIN(%)
G1	69.2±11.0	33.8±17.9	62.5±6.8^{a,b}	42.1±8.4^{a,b}	153.0±13.0	8.0±0.5	41.8±3.6	62.4±9.8	26.2±4.1^{a,b}
G2	84.6±14.8	53.2±23.1	97.4±21.9^{a}	70.4±22.4^{a}	204.8±31.2	8.5±0.9	44.9±2.6	66.4±8.5	32.8±6.5^{a}
G3	63.0±13.4	25.6±12.7	60.8±18.3^{b}	39.4±11.4^{b}	150.0±35.9	8.2±0.8	41.5±4.1	58.8±4.1	24.4±2.5^{b}
G4	76.8±18.6	43.0±23.4	84.2±25.7^{a,b}	61.1±21.2^{a,b}	179.7±42.5	7.6±0.9	45.0±1.6	66.8±6.5	32.6±4.8^{a,b}

a,b different letters in the same column $P<0.05$

TM-total motility (%); PM-progressive motility (%);VAP-average path velocity (µm/sec); VSL-straight line velocity (µm/sec); VCL-curvilinear velocity (µm/sec); ALH-amplitude of lateral head displacement (µm); BCF-beat cross frequency (Hz); STR-straightness (%); LIN-linearity (%)

G1: Eq0%SP;

G2: Eq10%SP;

G3: Ice0%SP;

G4:Ice10%SP.

Table 5. Motility and kinematic parameters (analysed by CASA) of horse epididymal spermatozoa after 48 h storage + 4C°(T48) (mean ± SD).

GROUP	TM(%)	PM(%)	VAP(μm/sec)	VSL(μm/sec)	VCL(μm/sec)	ALH(μm)	BCF(Hz)	STR(%)	LIN(%)
G1	56.8±13.2	20.6±3.1	58.0±15.5	37.9±9.0	142.3±28.6	8.1±1.0	42.1±3.1	59.0±5.0	24.6±3.0
G2	78.6±15.7	46.4±26.0	92.0±24.4	66.0±26.4	197.2±39.4	8.2±1.0	44.3±2.2	66.6±11.2	32.2±7.6
G3	54.2±21.5	24.0±13.6	61.5±11.9	39.9±7.3	154.5±25.1	8.8±1.1	40.5±3.5	60.4±6.0	24.4±3.2
G4	58.8±29.5	31.0±26.6	73.8±28.3	52.8±24.8	166.2±45.1	7.8±0.9	45.2±1.5	63.6±9.0	29.2±7.2

TM-total motility (%); PM-progressive motility (%);VAP-average path velocity (μm/sec); VSL-straight line velocity (μm/sec); VCL-curvilinear velocity (μm/sec);ALH-amplitude of lateral head displacement (μm); BCF-beat cross frequency (Hz); STR-straightness (%); LIN-linearity (%)

G1: Eq0%SP;

G2: Eq10%SP;

G3: Ice0%SP;

G4:Ice10%SP.

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