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An investigation of equine sperm quality following cryopreservation at low sperm concentration and repeated freeze-thawing

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ABSTRACT

Stallion spermatozoa are typically cryopreserved at 200 to 300 million sperm/ml; however recent advances such as intracytoplasmic sperm injection (ICSI) requires only one spermatozoon, wasting many, after thawing a whole straw. Cryopreserving at concentrations less than the current standard or refreezing thawed spermatozoa could maximise the use of genetically valuable animals and reduce waste. This investigation aimed to identify if lowering the sperm concentration for cryopreservation affected post-thaw quality after one and two freeze-thaw cycles. Nine ejaculates were collected from three fertile, 'good freezer' stallions (post-thaw motility ≥35%) for experiment 1. Each ejaculate was split into 8 treatments: 5, 10, 20, 50, 100, 200, 300, 400 million sperm/ml and cryopreserved. Post-thaw: motility, viability, acrosome integrity and oxidative stress were assessed. Experiment 2, straws from experiment 1 (300 million sperm/ml) were thawed, diluted to 20 million sperm/ml or left undiluted (control) and refrozen. Post-thaw motility and viability were assessed. In experiment 1 sperm concentration did not affect post-thaw total motility (TM), progressive motility (PM) or viability at 50 to 400 million sperm/ml ($P>0.05$). Whilst sperm concentrations of 5 to 20 million/ml did differ (post-thaw TM and PM). Both refreezing and reducing spermatozoa concentration, decreased TM, PM and viability ($P<0.05$) after two freeze-thaw cycles. These results suggest cryopreserving at sperm concentrations as low as 50 million/ml maintains spermatozoa quality in good freezer stallions. Spermatozoa maintained some motility and viability when initially cryopreserved at 20 million sperm/ml and after two freeze-thaw cycles but research should investigate more optimal conditions.

Keywords

Stallion; Spermatozoa; Cryopreservation; Low concentration; Refreeze

1. Introduction

From the first discovery of sperm cells in the 1670's, by Antonie Van Leeuwenhoek [1] through to intracytoplasmic sperm injection (ICSI) and sexing technologies used today [2,3]. Assisted reproductive technologies (ART's) have shaped animal breeding programmes, allowing multiple progenies from a single ejaculate and successful pregnancies from sub or infertile individuals [4]. Today, the use of artificial insemination (AI) for bovine [5], porcine [6] and equine breeding programmes, is common practice [7].

Sperm cryopreservation allows preservation of valuable genetics and worldwide transportation due to infinite longevity if stored correctly [8]. Another benefit of sperm cryopreservation is improved biosecurity, since eliminating natural mating reduces the spread of sexually transmitted diseases such as equine viral arteritis (EVA) and contagious equine metritis (CEM) [9]. Although the use of cryopreserved semen in equine breeding is common, typically sperm quality after thawing is still low in the equid, with post-thaw motility averaging 35% [10]. Stallions are categorised as 'good' or 'poor' 'freezers', where semen quality post-thaw is above ('good' freezer) or below ('poor' freezer) the threshold of 35% motile [11]. Further research is required to develop the ideal conditions for cryopreservation to optimise post-thaw quality and fertility for both good and poor freezer stallions.

For the insemination of chilled semen, the optimal insemination dose for the equid (500 million progressively motile sperm cells) [12] has been challenged over the last couple of decades to meet high demand for semen from individual stallions and economic gain from splitting one ejaculate for multiple mares [12,13]. Studies have shown that much smaller doses (1-10 million sperm cells) of chilled semen have yielded pregnancies with 60-75% conception rates [14].

In comparison to chilled semen, currently there is no standard insemination dose for cryopreserved spermatozoa in the equid. In general, straws (0.5 ml) are packaged with 200 to 300 million sperm cells/ml [13]. However, studies have shown that much lower total numbers of motile spermatozoa (14 million) can achieve acceptable pregnancy rates (64-67%) [15]. In addition, total motility of spermatozoa was significantly higher when cryopreserved at 100

million/ml (47.8%, 48.9%) compared to 400 million/ml (31.4%, 28.4%), in both stallions [16] and donkeys [17] respectively. However, Heitland et al. [18] found no significant difference in total motility of equine spermatozoa cryopreserved at 20, 200 or 400 million/ml.

Taken together these studies suggest that cryopreserving spermatozoa at concentrations below 200 million sperm/ml, could improve post-thaw quality and with it reduce wastage and increase the number of straws produced per ejaculate. However, with only a limited number of published studies more research is needed to determine the optimum dose of frozen-thawed equine semen.

Alongside the increased use of ICSI within equine breeding programmes there is a proportional need to avoid wasting spermatozoa especially when considering preserving critically endangered breeds [19]. Whilst spermatozoa are usually cryopreserved at a concentration of 200-300 million cells/ml, ICSI requires only one individual sperm cell to be injected directly into an oocyte [20]. Therefore, many spermatozoa are wasted by thawing a whole straw. Refreezing semen has the potential to overcome this. Some success has been shown with spermatozoa cryopreserved at 200 million sperm/ml in the equid, retaining 15% progressive motility following 8 freeze-thaw cycles (thawed at 22°C, 30 seconds) [21]. Conversely after 3 freeze-thaw cycles (thawed at 37°C, 30 seconds) progressive motility was just 3.3% when sperm were cryopreserved at 20 million/ml [11].

Currently there is limited published research into the post-thaw quality of cryopreserved spermatozoa at concentrations <100 million/ml or the effects of refreezing of spermatozoa at these low concentrations. Also, the lack of standardisation of conditions between studies and differences in the methods used and concentration of cryopreserved sperm makes comparisons between studies difficult. Therefore, this investigation aimed to determine the effects of cryopreserving equine spermatozoa from ‘good freezer’ stallions at much lower (5 to 20 million sperm/ml) and a broader range (5 to 400 million sperm/ml) of sperm concentrations than other published studies on sperm quality post-thaw, to identify the optimum spermatozoa concentration (Experiment 1). The aim of the second study was to

investigate the feasibility of refreezing equine spermatozoa from 'good freezer' stallions (Experiment 2).

2. Materials and Methods

This investigation was conducted at a UK based commercial equine stud farm from October 2020 to April 2021 and approved (project number: 0254-202002-PGMRES) by the ethics committee at Harper Adams University, Shropshire, UK.

2.1. Animals and semen collection

Semen collections were taken from three stallions (a Welsh Section A, a Belgium Warmblood and an Andalusian). The stallions were aged between 5-20 years, trained, and regularly used for semen collection. One week prior to starting the investigation, at least three semen collections were taken from each of the three stallions to ensure adequate sperm quality (≥ 70% motile). Ejaculates were collected from the stallions using a dummy mare and a Missouri artificial vagina with an inline filter and bottle kept at 45-50°C. All ejaculates used in this investigation were ≥ 70% motile in the raw state and all stallions had been previously proven to have spermatozoa with post-thaw motility of ≥35% ('good freezer stallions'). All stallions were stabled in individual stables and had access to *ad libitum* water and hay, were fed a diet of alfalfa pro fibre and slow-release energy mix (Spillers, UK), although the ration size offered did differ according to age, size and workload of each stallion.

2.2. Initial Semen Assessment

Volume (ml), concentration (sperm cells/ml), motility (%) and viability (%) were recorded for each ejaculate immediately after collection. The ejaculates (n=9) contained ≥ 70% motile sperm cells, ≥ 70% viablility and ≥ 8 billion sperm cells per ejaculate.

The volume of each ejaculate was measured and recorded using scales (1 ml = 1 g). Motility was assessed by eye at x 400 magnification with a light microscope using a 10 µl droplet of

136 the raw ejaculate on a warm stage, pre-warmed slide (37°C) and covered with a 22 x 22 mm
137 coverslip. To avoid technician bias, the same person assessed and processed all samples
138 throughout the investigation. Concentration and viability were assessed using a
139 NucleoCounter® SP-100™ as described by Morrell et al. [22].
140
141 *2.3. Experiment 1: Effect of sperm concentration on post-thaw quality*
142 Three ejaculates were collected each from the three stallions over a 4-week period
143 (*n*=9 ejaculates). Each ejaculate was split into 8 treatments: 5, 10, 20, 50, 100, 200, 300, 400
144 million sperm cells/ml (F5, F10, F20, F50, F100, F200, F300, F400 respectively), by dilution
145 in cryo-diluent (ethylenediaminetetraacetic acid (EDTA) lactose extender) containing 3% N-N
146 dimethylformamide (DMF) and 20% egg yolk. Straws (*n*=10 per treatment) were then all
147 cryopreserved, as described in section 2.3.1. Following thawing; quality assessments were
148 made for motility, viability, oxidative stress and acrosomal status (see section 2.4).
149
150 *2.3.1. Cryopreservation*
151 After initial assessments, the ejaculates were extended 1:1 with INRA 96 (IMV
152 Technologies, France) warmed to 37°C. Otiprep™ (2 ml; Abbott Rapid Diagnostics Ltd, UK)
153 at 37°C was added before centrifugation (20 minutes at 1000 x *g*), similarly to Saragusty et al.
154 [23]. After centrifugation the spermatozoa were removed using a blunt aspiration needle and
155 2-part syringe, the supernatant and Otiprep™ were discarded. The spermatozoa were re-
156 extended to F5, F10, F20, F50, F100, F200, F300, F400 with cryo-diluent (37°C). Straws (0.5
157 ml, Minitube, Germany) were then filled using an IMV MRS1 Dual straw filling machine (IMV
158 Technologies, France). The straws were placed onto racks and chilled at 4°C for 25 minutes,
159 as described by Clulow et al. [24] and Dias Maziero et al. [25]. After chilling, the straws were
160 placed into an Ice Cube 14S, automatic freezer (Minitube, Germany). The temperature was
161 reduced from 4°C to -143°C in approximately 9.5 minutes at a rate of: -7°C/minute for 2
162 minutes, -11°C/minute for 6.5 minutes, -60°C/minute for 1 minute. The straws were then

plunged directly into liquid nitrogen (-196°C). All straws were stored submerged in liquid nitrogen until analysis.

2.3.2. Thawing

Immediately, prior to analysis, straws were removed from liquid nitrogen and placed into a water bath at 37°C for 30 seconds [26]. The straws were then dried and cut at one end and emptied into a 5 ml glass test tube for assessment.

2.4. Post-Thaw Assessments

2.4.1. Computer-Assisted Sperm Analysis (CASA)

Although many published studies in the equid use a CASA system for motility analysis, there is limited consistency between studies as to the diluent and concentration used for assessments [16,27,28]. Therefore, a preliminary study was undertaken to determine the diluent most appropriate for equine semen. Straws from 6 randomly selected stallions previously frozen at 300 million sperm/ml were thawed (37°C, 30 seconds) and diluted to 100 million sperm/ml and 20 million sperm/ml in Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich, Gillingham, UK), INRA 96 or cryo-diluent (see section 2.4.1). A 6 µl droplet was placed onto a microscope slide (37°C) with a 22 x 22mm coverslip on a CASA system (HTM-IVOS 14) for analysis. A minimum of 300 sperm cells were counted from at least 5 frames and total and progressive motility were recorded. The settings for the CASA are described in Table 1.

The preliminary study determined that the concentration of spermatozoa did not affect their total and progressive motility $P>0.05$, but that diluent did ($P<0.05$). At a concentration 20 million/ml, sperm diluted in DPBS had significantly lower total and progressive motility (30.0% and 23.7%, respectively) than sperm diluted in either cryo-diluent (55.7%, 46.8%) or INRA 96 (51.5%, 44.8%), whilst motility of sperm diluted with cryo-diluent and INRA 96 did not differ significantly from each other. Similarly, at 100 million sperm/ml total and progressive motility (38.5%, 29.7%) were lower ($P<0.05$) when sperm cells were diluted in DPBS, in comparison

190 to the cryo-diluent (56.3%, 46.3%) or INRA 96 (52.3%, 44.0%). Therefore, it was decided that
191 after thawing, spermatozoa should be diluted to 20 million/ml in the cryo-diluent used in the
192 present study. This enabled nearly all the treatments used for experiment 1 and 2 to be diluted
193 to the same concentration (20 million sperm/ml) for motility assessment (except for F5, F10
194 and F20) to ensure more consistency.

195 For both experiment 1 and 2, measurements made using the CASA were: total motility
196 (TM), progressive motility (PM), average path velocity (VAP) (μm/s), progressive velocity
197 (VSL) (μm/s), track speed (VCL) (μm/s), lateral amplitude (ALH) (μm), beat frequency (BCF)
198 (Hz), straightness (STR) (%), linearity (LIN) (%), percentage of rapid, medium, slow and static
199 cells (Table 1).

200

201 *2.4.2. Viability*

202 Viability was assessed as a percentage of intact cell membranes using a
203 NucleoCounter® SP-100™ as described by Morrell et al. [22]. The NucleoCounter® SP-100™
204 uses propidium iodide to identify sperm cells with a non-intact membrane by binding to DNA
205 within the sperm.

206

207 *2.4.3. Acrosome Integrity- FITC-PNA*

208 Acrosome integrity was assessed using fluorescein isothiocyanate-conjugated peanut
209 agglutinin (FITC-PNA; Sigma-Aldrich, Gillingham, UK) adapted from the methods of Wu et al.
210 [29] and Yeste et al. [30]. FITC-PNA binds to proteins on the acrosomal membrane if exposed
211 and fluoresces green, indicating a non-intact acrosome.

212 Following thawing, the semen samples were washed by diluting 1:1 in DPBS and
213 centrifuged at 300 x *g* for 3 minutes. The supernatant was discarded and the original volume
214 of DPBS was added and washing was repeated. Then the semen samples were diluted to 25
215 million sperm/ml in DPBS and 2.5 μl of FITC-PNA (200 μg/ml) was added to 47.5 μl of the
216 diluted sample to give a final FITC-PNA concentration of 10 μg/ml. The sample was then

incubated in the dark for 5 minutes at 37°C, fixed with 2 µl of formaldehyde (10%) and left in the dark for a further 5 minutes at room temperature. A 10 µl droplet was assessed with a 22 x 22 coverslip using a Zeiss Axiostar plus microscope with a HBO 50 fluorescent lamp (filter set 09, emission 450-490 nm), at 1000 x magnification under oil immersion. Positioning the fluorescent microscope in one field, the spermatozoa with non-intact acrosomes (green) were counted first and then the total number of spermatozoa were counted in the same field under phase-contrast, to calculate the percentage of spermatozoa with non-intact acrosomes. A total of 200 spermatozoa were assessed for each sample.

2.4.4. Oxidative Stress

Oxidative stress was assessed by the presence of reactive oxygen species (ROS) using Nitro Blue tetrazolium (NBT). The method used was as described by Esfandiari et al. [31]. NBT is converted to blue formazan crystals by superoxide ions (ROS) present within the spermatozoa which can be seen under light microscopy. The sperm cells were counted and categorised by the percentage of formazan present in the head of the sperm cell (0%, <50%, >50% or 100%), as described by Saleh and Agarwal [32]. A total of 200 individual spermatozoa were assessed in each sample.

2.5. Experiment 2: Effect of re-freezing on sperm quality assessments

Based on the findings of Experiment 1, F300 numerically showed the best quality spermatozoa post-thaw and F20 was the lowest sperm concentration which showed statistically similar TM and PM post-thaw compared to F50, F100, F200 and F400 therefore F300 was selected for thawing and refreezing to 20 million sperm/ml in experiment 2. F300 straws (*n*=36; *n=4* from each of the nine ejaculates collected) were thawed and the volume divided, one half (1 ml) was diluted to 20 million sperm/ml (R20) with cryo-diluent and the other half remained at 300 million sperm/ml. The remaining 1 ml was not diluted and used as a control (R300). The straws were then cryopreserved as described in section 2.3.1 and stored

submerged in liquid nitrogen (-196°C) until analysis. After a second thaw, sperm quality was assessed using motility and viability, as described above.

2.6. Statistical Analysis

Genstat version 18 was used for statistical analysis. The data were assessed for distribution (Shapiro-Wilk) and transformed were necessary (square root and log_{10} transformations) to achieve normality and presented as back transformed data. A 2-way Analysis of Variance (ANOVA) revealed there was no interaction between stallion and ejaculate (P>0.05). Therefore, a one-way ANOVA was used to analyse the data (*TM, PM, VAP, VSL, VCL, ALH, BCF, STR, LIN, percentage of rapid, medium, slow and static cells, viability, percentage of Formazan in the sperm head and acrosome integrity*) between the treatments (F5, F10, F20, F50, F100, F200, F300, F400) generated in experiment 1 with stallion as the blocking factor. Tukey's post-hoc tests were performed to determine whether differences between treatments were significant. For experiment 2, a repeated-measures ANOVA was used with a blocking factor of ejaculate within stallion to determine the differences between the once (F300) and twice frozen ejaculates (R300 and R20). Post-hoc, least-significant difference tests were used to determine significant differences between treatments (significance level 5%).

3. Results

3.1. Semen Characteristics

Within the fresh ejaculates, there were no differences in sperm parameters between stallions or ejaculates (*P*>0.05). The means ± SEM of these parameters were as follows: volume 24.0 ± 3.38 ml, concentration 595.9 ± 50.70 million sperm/ml, motility 78.3 ± 1.18 % and viability 80.9 ± 1.67 %.

3.2. Experiment 1

Spermatozoa concentration at cryopreservation did affect motility of spermatozoa post-thaw (P<0.05) (Table 2). TM, PM and percentage of rapid sperm cells were all significantly higher for treatments F50, F100, F200, F300, F400 than F5 and F10 (P<0.05) but did not differ from each other (Table 2). Also, TM and PM for F20 were no different (P>0.05) than F50, F100, F200 or F400. Numerically, treatment F300 had the highest value for TM, PM, VAP, VSL, VCL, BCF, LIN and percentage of rapid sperm cells with significantly higher TM, PM and percentage of rapid cells than F20. ALH, BCF, STR, LIN, percentage of medium velocity and slow velocity sperm cells were not significantly different between the treatments.

The concentration that spermatozoa were cryopreserved did affect (P<0.05) post-thaw viability (Table 3). Sperm exposed to treatments F50, F200 and F400 had a significantly higher viability than those in treatments F5, F10 and F20. Similarly, to the results for TM and PM there was no significant difference in viability of sperm between each of the treatments F5, F10 and F20 or each of F50, F100, F200, F300 and F400.

The oxidative stress results (Table 3) showed that the number of spermatozoa with 0% formazan in treatment F20 was higher (P<0.05) than F300, but no other treatment differences were found. The percentage of cells with <50% formazan decreased as sperm concentration at freezing increased (P<0.05). There was a significantly higher number of spermatozoa displaying <50% formazan from treatment F5 compared to F100. In comparison there was a significantly lower percentage of sperm cells displaying >50% Formazan at F5 compared to F100, F200, F300, F400. However, oxidative stress did not differ (P>0.05) between treatments F50, F100, F200, F300 and F400. Furthermore, the number of spermatozoa showing 100% formazan in the sperm head did not significantly differ between treatments.

The percentage of sperm cells with an intact acrosome was also affected by treatment (P<0.05), Table 3. Sperm in treatment F200, F300 and F400 did not differ from each other but did have a higher percentage of intact acrosomes (P<0.05) than F5, F10 and F20 (which also did not differ from each other).

Numerically treatment F300 preserved sperm cell quality better than the other treatments. Furthermore, cryopreserving at ≤10 million sperm/ml was more damaging;

298 therefore, it was chosen that straws from treatment F300 would be thawed and refrozen at
299 concentrations of 300 million and 20 million sperm/ml for experiment 2.
300
301 *3.3. Experiment 2*

302 Refreezing (R20 and R300) reduced (P<0.05) TM, PM, VAP, VSL, VCL, BCF, LIN,
303 percentage of rapid sperm cells, viability of spermatozoa and significantly increased
304 percentage of static cells in comparison to once frozen spermatozoa (Table 4). The
305 concentration of spermatozoa at refreezing also affected sperm quality. TM, PM, BCF,
306 percentage of rapid cells and viability were significantly lower for treatment R20 than R300
307 and percentage of static cells was significantly higher at R20 than R300.

308

309 **4. Discussion**

310 In this investigation individual stallion and ejaculate number had no effect on sperm
311 quality post-thaw. This is likely to be due to all three stallions having proven fertility and
312 produce sperm with proven suitability for freezing (post-thaw motility ≥35%). This is also
313 reflected in the low variability in the results. However, as shown in many investigations,
314 individual stallion can greatly affect post-thaw sperm quality [33,34].

315 In the present study TM and PM of the spermatozoa at concentrations between 10 to
316 400 million/ml all surpassed the equine industry standard (≥35% motile) post-thaw [10,35].
317 Further investigations should be undertaken using semen from 'poor freezer' stallions to
318 identify if the results are reproducible. Sperm concentrations of 50-400 million/ml at
319 cryopreservation had no significant effect on motility parameters: TM, PM, VSL, VCL
320 percentage of rapid and static cells. Numerically F300 had the highest TM, PM, VAP, VSL,
321 VCL, BCF, LIN, percentage of rapid cells and the lowest percentage of static cells, however
322 the statistics revealed limited significances compared to the other treatments. TM and PM for
323 F20 were no different (P>0.05) than F50, F100, F200 or F400 but did show significant
324 differences compared to F300. Similarly, to this present investigation in stallions, Heitland et
325 al. [18] found no significant difference in TM (51%, 52%, 50%) when cryopreserving at 20,

200 and 400 million sperm/ml. Also, in rams D'Alessandro et al. [36] found no significant difference between the post-thaw motility of spermatozoa frozen at 50, 100, 200 and 400 million sperm/ml. In contrast, Nascimento et al. [37] and Gomez-Arrones et al. [38] both found that cryopreserving spermatozoa at 100 million/ml produced significantly higher TM (47.8% and 48.9%) than at 400 million/ml (31.4% and 28.4%) in the stallion. Differences in the findings of these studies could be due to differences in the cryo-diluent used. Nascimento et al. [37] used glycerol (1%) and DMF (4%) in BotuCrio, while Gomez-Arrones et al. [38] used a modified INRA (4% DMF and 2.5% egg yolk) to cryopreserve semen from donkeys, whilst in the present investigation of equine sperm quality, the cryo-diluent contained 3% DMF and 20% egg yolk. The type and volume of cryoprotectant is known to affect spermatozoa quality [39,40]. Differences between the findings of the studies could also be due to the differences in the CASA settings used to assess sperm motility. Nascimento et al. (2008) used the VAP cut off for progressive cells at 70 μm/s and VAP cut off at 20 μm/s compared to 35 μm/s and 25 μm/s respectively for the present investigation. Currently there are no standard settings for using the CASA system in the equid, which can significantly affect results and make study to study comparisons difficult [41]. Furthermore, there are limited studies for comparison of the effects of cryopreserving stallion's semen at low sperm concentrations (<20 million sperm/ml). To the authors knowledge the study presented in this paper analyses the effects of a much wider range of spermatozoa concentrations than other published studies.

Similarly to the motility parameters assessed in experiment 1, viability was reduced where sperm were cryopreserved at lower concentrations (5 to 20 million sperm/ml). However, at concentrations above 50 million sperm/ml there was no effect of freezing concentration, which is in agreement with the study of Contri et al. [42] where viability was also found not to differ significantly between spermatozoa cryopreserved at 100, 250 and 500 million/ml in donkeys. This is in contrast to the findings of Nascimento et al. [37] who report decreased viability (43.5% vs 40.1% vs 34.9%) with increasing concentration (100 to 200 to 400 million sperm/ml) of equine sperm.

Nascimento et al. [37] suggested that motility and viability were maintained more successfully at lower spermatozoa concentrations (100 million/ml) due to the spermatozoa's increased exposure to the protective components of the cryo-diluent. Whilst cryopreserving at lower spermatozoa concentrations does increase exposure of spermatozoa to the cryo-diluent this also increases exposure to the cryoprotectant, which may have been detrimental to spermatozoa at the lowest concentrations used in the present study. In support of this Hoffmann et al. [43] found that equine spermatozoa cryopreserved using 4% DMF had numerically lower motility and viability than when 2-3% DMF was used. In the present investigation the cryo-diluent was used at 3% DMF and not adjusted for each sperm dilution. Therefore, at the lower concentrations of spermatozoa exposure to the cryoprotectant was higher. As the percentage of cryoprotectant increases so does the osmolality of the cryo-diluent. It has been found that an osmolality of 450 to 895 mOsm kg-1 is damaging to spermatozoa [44,45]. The osmolarity of cryo-diluent containing 3% DMF (as in the present investigation) falls within this range. The action of the cryoprotectant during cryopreservation is to increase permeability of the sperm cell membrane to allow water to move out of the cell but if the osmolarity of the solution is too high, the spermatozoa can shrink beyond their limit causing lysis and cell death decreasing viability and motility [44,46,47]. The process of diluting sperm cells could have also affected the motility and viability results in the present investigation. Studies have previously reported loss of motility and viability due to the physical dilution of spermatozoa ≤10 million/ml, in chilled stallion [48,49] and bull semen [50]. During artificial processing of equine spermatozoa, the seminal plasma (SP) is removed through centrifugation, this loss includes components such as decapacitation factors, in turn causing premature capacitation and detrimental membrane surface changes rendering the spermatozoa unviable. These protective compounds are reduced further through dilution of spermatozoa [49,51–53]. Furthermore, in this investigation the sperm dilution occurred quickly (approximately 2-5 seconds) to ensure that all treatments were cryopreserved at the same time. However, it should be further investigated if the speed and/or volume of cryo-diluent used for sperm dilution was the cause of the differences seen post-thaw sperm quality. In

some species a dropwise addition of the cryo-diluent is required to avoid osmotic shock from the cryoprotectant (ovine) [54]. Commonly in stallion semen processing a dropwise addition of the cryo-diluent is not required. However, some studies have investigated a 2-step dilution process, where the cryoprotectant is added only in the second step, to minimise osmotic shock from the cryoprotectant in dog [55] bull [56] and stallion [57] semen.

Oxidative stress was measured in experiment 1 by the presence of formazan in the head of the spermatozoa converted from NBT by ROS. The results of experiment 1 suggests that decreasing the concentration of spermatozoa for cryopreservation could minimise oxidative stress, although results did not reveal many significant differences. The increase of ROS due to cryopreservation and the presence of dead spermatozoa causes oxidative damage through the dysregulation of redox reactions within the spermatozoa [44,58,59]. This is further promoted when increasing the concentration of cryopreserved spermatozoa, as seen in this investigation, by increasing the number of live metabolising spermatozoa as well as dead cells. Redox dysregulation causes damage to spermatozoa via multiple pathways including lipid peroxidation producing lipid hydrogen peroxides, oxidation of nucleotides causing DNA damage and reduced motility, all rendering the spermatozoa unviable [59]. There are limited studies which assess oxidative stress on equine spermatozoa in relation to spermatozoa concentration. However, similarly to the present investigation, bull spermatozoa packaged at a lower concentration (4 million sperm/ml) have been shown to have lower oxidative stress levels than at a higher concentration (20 million sperm/ml), in chilled semen [60]. In cryopreserved pig semen it has also been seen that lipid peroxidation reduced as concentration of spermatozoa was reduced from 800 to 100 million/ml [61]. In both studies it was suggested that increasing the concentration of spermatozoa increased the number of cells exposed to oxidative stress and subsequently the number of dead sperm cells and ROS produced [60,61]. It has also been seen in the equid, that the production of ROS is positively correlated with TM (r =0.9) [58]. The more motile the spermatozoa, the greater the metabolism and thus the production of more ROS. In the present study at higher spermatozoa concentrations at cryopreservation (50 to 400 million/ml) spermatozoa generally had higher

motility and oxidative stress levels which could be linked. Although, in contrast to Gibb et al. [58] Akbarinejad et al. [62] found no correlation between ROS production and TM or PM in stallions.

In experiment 1, fewer intact acrosomes were found at concentrations ≤20 million sperm/ml. Similarly, in bulls, cryopreserving spermatozoa at 10 million sperm/ml reduced the proportion with intact acrosomes compared with 60 million sperm/ml [63]. However, at concentrations above 200 million/ml in the present investigation the percentage of intact acrosomes did not differ. Likewise in donkeys there was no significant difference in acrosome integrity between sperm concentrations of 100, 250, 500, 750 and 1000 million/ml [42].

Cryopreservation damages spermatozoa through various pathways; oxidative stress unbalances redox regulation within the spermatozoa and increase ROS such as H_2O_2 which can trigger the acrosome reaction, reducing fertilisation ability [59,64]. It is not entirely clear why at lower concentrations (5 to 20 million sperm/ml) there was more acrosome damage seen in the present investigation. Similarly, to motility and viability reduction, it has been suggested that increasing the dilution of spermatozoa can induce more capacitation like changes (such as the acrosome reaction) [48–50,63]. The speed of dilution (equilibration) could also have negatively affected acrosome integrity in this investigation [55].

The present study has demonstrated that cryopreserving ejaculates at concentrations as low as 20 million sperm/ml has a limited effect on sperm quality. Although the present investigation did not determine pregnancy rates, other studies have found that inseminating as few as 3 to 14 million motile spermatozoa have yielded pregnancies (37%, 67%) [65,66]. Therefore, cryopreserving ejaculates at 20 million sperm/ml for highly desired or genetically valuable individuals for use within ICSI programmes could be feasible and would be beneficial to reduce waste. Furthermore, in the present investigation, little difference was seen in motility, viability, oxidative stress and acrosome integrity post-thaw between cryopreserving spermatozoa at 50 to 400 million/ml. These results suggest that reducing spermatozoa concentrations for cryopreservation from the current industry standard (200 to 300 million/ml) to as low as 50 million/ml would increase the number of straws produced per ejaculate with

limited compromise of post-thaw quality. However, the total number of motile spermatozoa inseminated should not be forgotten. Currently, there is no standard [13], but it has been found that increasing the total number of motile spermatozoa inseminated (above 600 million) can increase pregnancy rates from 54.5 to 88.2% [67]. However, Sieme et al. [68] found no difference in pregnancy rate when 100 million or 800 million sperm cells were inseminated. Further investigation should identify if pregnancy rates are acceptable using cryopreserved spermatozoa at concentrations lower than the current 200 to 300 million/ml and the effect of total sperm numbers inseminated.

Motility results from experiment 2 show that it is possible to refreeze equine spermatozoa and still maintain some motility. However, the motility of the sperm after refreezing were all below the minimum industry standard (>35% motile) for use in AI [69]. It should be noted that in the present investigation the stallions used were known 'good' freezers and further investigation is required for stallions with poor suitability for freezing. Despite differences in freezing media and methods, the TM (23.3%) and PM (17.2%) found in experiment 2 (20 million sperm/ml) are comparable to Sielhorst et al. [11] where PM was 24% and Gonzalez-Castro et al. [70] 22.3% and 15.1% (TM and PM, respectively) after refreezing. This is to be expected as both Sielhorst et al. [11] and Gonzalez-Castro et al. [70] refroze the spermatozoa at 40 and 20 million/ml respectively which was similar to the lower concentration used in the present investigation (20 million sperm/ml). [69]Currently there is limited available data on the use of both motile and non-motile twice frozen spermatozoa via ICSI or conventional AI in the equid and should be further investigated to improve success. One study has shown limited success in blastocyst development (13%) following ICSI using twice frozen non-motile spermatozoa which was significantly lower than using twice frozen motile spermatozoa in the stallion (23%) [71]. With regards to establishing pregnancies, very low rates (10%) have previously been achieved using twice frozen spermatozoa (40 million sperm/ml) following AI in the equid [11].

The results from experiment 2 also found that viability decreased following both dilution and refreezing. Similarly, Sielhorst et al. [11] and Leisinger et al. [21] found that viability

decreased following more than one freeze-thaw cycle. Although experiment 2, showed a similar decrease in sperm viability following one and two freeze-thaw cycles compared to the study of Sielhorst et al. [11] (29.6% to 14.9%) the values were higher in the present investigation. This difference maybe due to Sielhorst et al. [11] using a dual stain to categorise viable spermatozoa as having both acrosome and plasma membrane intact, while in the present study viable sperm were classified as membrane intact. Cryopreservation is known to reduce spermatozoa's quality post-thaw due to the osmotic and oxidative stress on the cells [44,46,59]. Therefore, it is not unexpected that subjecting spermatozoa to cryopreservation two (or more) times, further damages the cells [11,70]. To the authors knowledge currently there are no other published studies which investigate the effect of the concentration of spermatozoa at refreezing on subsequent viability. However the results of experiment 2 are comparable with experiment 1, where the lower the concentration of spermatozoa at cryopreservation decreased motility and viability post-thaw. Therefore, it is suggested that this decrease is caused by the change in osmolarity after two cycles of dilution and cryopreservation [45,56].

While the present studies have utilised a range of parameters to examine the effects of the concentration at cryopreservation and re-freezing on sperm quality, other published studies have utilised additional measures, such as DNA integrity [11,21]. This has been shown to affected by repeated freeze-thawing in some [21], but not all studies [11].

Whilst motility and viability were assessed in both Experiment 1 and 2, it should be noted that this alone does not guarantee successful fertilisation. Therefore, it is recommended that further investigations are undertaken to assess the ability of sperm cells to fertilise oocytes following repeated freeze-thawing.

5. Conclusion

The findings of the present study suggest that cryopreserving the spermatozoa of stallions classed as 'good freezers' at concentrations as low as 50 million/ml has limited damaging effects on post-thaw quality. This has the potential to increase the number of straws

494 produced for insemination compared to the current standard, provided acceptable pregnancy
495 rates can be achieved. The present investigation also found that refrozen stallion spermatozoa
496 maintained some post-thaw motility. However, sperm motility alone is not a guarantee of
497 successful embryo production. Thus, on the basis of the present findings further studies are
498 required to determine whether low sperm concentration at cryopreservation and refreezing
499 can be considered viable techniques for use with ART's.
500

501 **CRediT authorship contribution statement**

502 **B. Morse-Wolfe:** Conceptualization, investigation, methodology, formal analysis, writing-
503 original draft, review & editing. **C. Kershaw:** Supervision, writing- review & editing. **E. Bleach:**
504 Supervision, writing- review & editing.
505

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509 **Declaration of competing interest**

510 The authors declare the following financial interests/personal relationships which may be
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512 Bethany Morse-Wolfe reports equipment and supplies were provided by Stallion AI Services.
513 Bethany Morse-Wolfe reports a relationship (at the time of the investigation) with Stallion AI
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765 Table 1. The Computer-Assisted Sperm Analysis (CASA) settings used in the analysis of
766 sperm cells.
767

Parameter	Setting
Frames per second	60 Hz
Number of frames	30
Minimum contrast	40
Minimum cell size	5 pixels
VAP cut-off for static cells	25 µm/s
Lower VSL cut-off for static cells	12 µm/s
VAP cut-off for progressive cells	35 µm/s
Straightness	50%
Temperature	37°C

768
769 VAP: average path velocity, VSL: progressive velocity.

770

771 Table 2. Mean (± SEM) sperm motility parameters measured by Computer-Assisted Sperm
772 Analysis (CASA) post-thaw following cryopreservation at 5, 10, 20, 50, 100, 200, 300 and 400
773 million sperm/ml.

Motility Characteristic	Treatment F5	F10	F20	F50	F100	F200	F300	F400
TM (%)	25.2 ± 2.6 ^{a}	38.6 ± 3.3 ^{b}	53.7 ± 2.5 ^{c}	54.8 ± 2.2 ^{cd}	55.4 ± 0.7 ^{cd}	58.7 ±2.6 ^{cd}	66.1 ±3.2 ^{d}	57.3 ± 3.7 ^{cd}
PM (%)	21.0 ± 2.2 ^{a}	32.3 ± 3.2 ^{a}	44.2 ± 2.4 ^{b}	47.6 ± 2.1 ^{bc}	46.8 ± 0.8 ^{bc}	51.2 ± 2.3 ^{bc}	57.0 ± 3.2 ^{c}	49.7 ± 3.8 ^{bc}
VAP (um/s)	84.9 ± 2.1 ^{a}	93.7 ± 2.4 ^{abc}	93.7 ± 2.6 ^{abc}	96.7 ± 2.8 ^{bc}	93.6 ± 3.4 ^{abc}	98.8 ± 3.4 ^{bc}	105.2 ± 4.4 ^{c}	93.1 ± 3.5 ^{ab}
VSL (um/s)	64.3 ± 2.0 ^{a}	71.6 ± 2.3 ^{ab}	72.7 ± 1.7 ^{ab}	74.8 ± 2.1 ^{ab}	71.7 ± 2.4 ^{ab}	78.0 ± 2.9 ^{b}	83.7 ± 4.3 ^{b}	74.6 ± 4.2 ^{ab}
VCL (um/s)	167.7 ± 5.1 ^{a}	180.5 ± 3.3 ^{ab}	180.8 ± 6.8 ^{ab}	183.1 ± 4.2 ^{ab}	178.5 ±4.2 ^{ab}	184.5 ± 4.3 ^{b}	185.1 ± 4.0 ^{b}	172.0 ± 2.4 ^{ab}
ALH (um)	6.9 ± 0.2	6.9 ± 0.2	7.1 ± 0.3	7.0 ± 0.2	7.2 ± 0.2	6.9 ± 0.2	6.8 ± 0.2	6.6 ± 0.2
BCF (Hz)	36.1 ± 1.2	37.1 ± 1.0	37.5 ± 0.8	38.5 ± 0.9	36.7 ± 1.1	38.9 ± 0.9	39.7 ± 1.3	38.5 ± 1.4
STR (%)	77.0 ± 1.3	78.1 ± 1.2	78.9 ± 1.6	78.1 ± 1.0	77.3 ± 0.9	79.4 ± 1.3	79.1 ± 1.3	79.9 ± 1.8
LIN (%)	42.1 ± 1.3	42.9 ± 1.1	43.4 ± 1.5	42.9 ± 0.9	42.3 ± 0.9	44.3 ± 1.5	46.2 ± 1.9	45.0 ± 2.0
Rapid (%)	23.0 ± 2.4 ^{a}	35.0 ± 4.0 ^{a}	49.0 ± 2.6 ^{b}	52.0 ± 2.1 ^{bc}	52.0 ± 0.7 ^{bc}	56.2 ± 2.6 ^{bc}	63.0 ± 3.3 ^{c}	54.2 ± 3.7 ^{bc}
Medium (%)	2.4 ± 0.4	2.6 ± 0.3	4.9 ± 1.4	2.8 ± 0.4	3.6 ± 0.5	2.3 ± 0.3	3.4 ± 0.6	2.8 ± 0.2
Slow (%)	27.6 ± 5.0	28.2 ± 4.5	27.9 ± 3.7	25.4 ± 3.3	23.2 ± 4.4	25.6 ± 3.8	22.9 ± 2.8	22.9 ± 2.3
Static (%)	46.9 ± 6.7 ^{c}	34.0 ± 5.8 ^{bc}	18.4 ± 4.1 ^{ab}	19.7 ± 4.0 ^{ab}	21.4 ± 4.6 ^{ab}	16.0 ± 3.9 ^{ab}	10.8 ± 2.2 ^{a}	20.0 ± 3.1 ^{ab}

774 a, b, c, d Different superscripts within rows differ significantly ($P < 0.05$).
775 F5, F10, F20, F50, F100, F200, F300, F400: 5, 10, 20, 50, 100, 200, 300, 400 million sperm cells/ml respectively. TM: total
776 motility, PM: progressive motility, VAP: average path velocity, VSL: progressive velocity, VCL: track speed, ALH: lateral
777 amplitude, BCF: beat frequency, STR: straightness, LIN: linearity, velocity distribution for rapid, medium, slow and static (%).
778 *n*= 2 straws from each treatment from each of the 9 ejaculates collected were thawed for assessment.
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Table 2. Mean (± SEM) percentage of viable sperm, oxidative stress (% Formazan) and percentage of sperm with intact acrosomes post-thaw following cryopreservation of sperm at 5, 10, 20, 50, 100, 200, 300 and 400 million/ml.

Sperm Characteristic (%)	Treatment F5	F10	F20	F50	F100	F200	F300	F400
Viability	53.2 ± 3.1^{a}	52.8 ± 3.3^{a}	53.0 ± 3.4^{a}	63.9 ± 2.8^{b}	61.9 ± 3.2^{ab}	62.7 ± 2.0^{b}	61.0 ± 2.8^{ab}	64.6 ± 2.8^{b}
0% Formazan	6.3 ± 1.1^{ab}	6.7 ± 0.7^{ab}	7.8 ± 1.5^{b}	6.1 ± 0.9^{ab}	4.6 ± 0.9^{ab}	3.2 ± 0.6^{ab}	3.0 ± 0.8^{a}	3.6 ± 1.2^{ab}
<50% Formazan	73.8 ± 0.9^{b}	69.2 ± 4.8^{ab}	66.6 ± 1.7^{ab}	65.6 ± 3.7^{ab}	55.4 ± 6.8^{a}	64.7 ± 4.1^{ab}	62.8 ± 4.4^{ab}	61.0 ± 4.1^{ab}
>50% Formazan	16.1 ± 1.6^{a}	19.0 ± 3.3^{ab}	22.6 ± 2.5^{abc}	25.6 ± 4.2^{abc}	36.6 ± 6.8^{c}	29.8 ± 3.9^{bc}	30.7 ± 4.2^{bc}	31.2 ± 3.5^{bc}
100% Formazan	3.8 ± 0.5	5.1 ± 1.4	3.1 ± 0.5	2.8 ± 0.3	3.4 ± 1.0	2.3 ± 0.4	3.6 ± 0.4	4.2 ± 0.8
Acrosome Intact	42.4 ± 2.2^{de}	39.3 ± 1.7^{e}	41.8 ± 1.9^{de}	46.0 ± 2.0^{cde}	47.7 ± 2.5^{bcd}	52.4 ± 2.8^{abc}	55.3 ± 1.5^{ab}	56.8 ± 1.7^{a}

a, b, c, d, e Different superscripts within rows differ significantly ($P < 0.05$).
F5, F10, F20, F50, F100, F200, F300, F400: 5, 10, 20, 50, 100, 200, 300, 400 million sperm cells/ml respectively.
Formazan %: percentage of Formazan present in the sperm head.
n= 2 straws from each treatment from each of the 9 ejaculates collected were thawed for assessment.

Table 3. Mean ± SEM of sperm motility and viability following refreezing at 300 million or 20 million sperm per ml.

Sperm Characteristic	Treatments F300	R300	R20
TM (%)	66.1 ±3.2 ^{a}	29.9 ± 2.4 ^{b}	23.3 ± 1.8 ^{c}
PM (%)	57.0 ± 3.2 ^{a}	23.1 ± 2.4 ^{b}	17.2 ± 1.5 ^{c}
VAP (um/s)	105.2 ± 4.4 ^{a}	75.9 ± 2.6 ^{b}	76.5 ± 2.5 ^{b}
VSL (um/s)	83.7 ± 4.3 ^{a}	56.0 ± 2.3 ^{b}	56.9 ± 1.9 ^{b}
VCL (um/s)	185.1 ± 4.0 ^{a}	155.0 ± 4.9 ^{b}	155.3 ± 5.6 ^{b}
ALH (um)	6.8 ± 0.2	7.1 ± 0.2	6.7 ± 0.2
BCF (Hz)	39.7 ± 1.3 ^{a}	32.5 ± 0.7 ^{b}	35.2 ± 0.8 ^{c}
STR (%)	79.1 ± 1.3	75.8 ± 1.1	76.4 ± 1.4
LIN (%)	46.2 ± 1.9 ^{a}	40.6 ± 1.9 ^{b}	41.6 ± 1.9 ^{b}
Rapid (%)	63.0 ± 3.3 ^{a}	26.6 ± 2.4 ^{b}	20.1 ± 1.7 ^{c}
Medium (%)	3.4 ± 0.6	3.3 ± 0.3	3.1 ± 0.7
Slow (%)	22.9 ± 2.8	36.0 ± 6.6	23.7 ± 6.7
Static (%)	10.8 ± 2.2 ^{a}	34.6 ± 7.0 ^{b}	53.0 ± 7.0 ^{c}
Viability (%)	61.0 ± 2.8 ^{a}	45.6 ± 2.7 ^{b}	34.7 ± 3.2 ^{c}

a, b, c Different superscripts within rows differ significantly ($P < 0.05$).
TM: total motility, PM: progressive motility, VAP: average path velocity, VSL: progressive velocity, VCL: track speed, ALH: lateral amplitude, BCF: beat frequency, STR: straightness, LIN: linearity, velocity distribution for rapid, medium, slow and static (%).
F300: concentration 300 million sperm cells/ml freeze 1, R300: concentration 300 million sperm cells/ml freeze 2 and R20: concentration 20 million sperm cells/ml freeze 2.