Universiteit Utrecht

Refreezing equine semen for intracytoplasmic sperm injection

K.A.M. van Gerwen

Abstract

The use of cryopreserved semen in assisted reproduction in the horse is nowadays a standard technique. In case only few straws of semen are left, a careful choice has to be made on how to use the semen in the most efficient way. Intracytoplasmic sperm injection requires only few sperm per session. Thus the remainder of the sperm may be reprocessed for later use. Reprocessing straws, which means thawing, diluting and re-freezing could increase the efficiency in use of frozen equine sperm . Therefore, this study was conducted to evaluate the effects of high dilution rates and refreezing on motility and viability of frozen equine spermatozoa. Semen from seven stallions with an average motility of 74.3 ± 8.4 % in raw semen samples were used. The semen of each stallion was frozen under standardized conditions. In the first experiment, the effect of different dilution rates after thawing of frozen stallion semen on motility (CASA), viability and acrosome integrity (propidium iodide, PNA-FITC & PSA-FITC staining) was evaluated. High dilution rates after thawing had no influence on motility. Viability was on average 2% lower in the highest dilution (1:59, v:v) compared to the lowest dilution ratio (1:4, v:v). In experiment 2, high diluted semen (1:59) was refrozen in straws of different size (0.5ml and 0.25ml straws). Higher total motility was observed in the 0.5ml straws after refreezing. Independent of the straw size, refreezing reduced the number of motile and viable sperm. Exposure to glycerol during reprocessing lowered the number of viable cells. Finally, experiment 3 evaluated whether enrichment of viable sperm by density gradient centrifugation before refreezing improves the number of motile and viable spermatozoa after the second thawing. After centrifugation, the average loss of sperm was 96.5 %. No enrichment of motile and viable sperm was observed. After refreezing, all sperm were immotile. In conclusion, frozen/thawed equine sperm can be diluted to low sperm concentrations with only minor loss in sperm motility and viability. However, refreezing frozen/thawed stallion semen decreased motility and viability tremendously. It is recommended to do a fast processing and limit processing steps, as well as exposure time to glycerol to a minimum.

Introduction

Cryopreserved semen is an important tool in assisted reproduction and has facilitated the international use of semen. The use of frozen semen is especially useful for stallions with a busy competition schedule. Commercially produced semen is mostly packed into 0.5ml straws at a concentration between 100-300 x 10^6 sperm/ml. Multiple straws of semen are usually needed for one insemination dose (Choi et al., 2006). If a stallion is not able anymore to produce sperm, each straw of frozen semen is literally irreplaceable. In case, only few straws of semen are left, a careful choice needs to be made on how to use the semen efficiently for producing offspring. One option is to use the spermatozoa for intracytoplasmic sperm injection (ICSI). ICSI consists of fertilizing a metaphase II oocyte by direct injection of a single spermatozoon (Galli et al., 2014). Thus, the number of spermatozoa needed for each ICSI session is very small. Only few sperm from a commercially frozen straw are needed. Under commercial aspects, it is desirable to reprocess the remainder of sperm at low concentrations to have sperm available for multiple ICSI session.

Reprocessing straws, which means thawing, diluting and re-freezing, could increase the efficiency in use of sperm. In other species, including cattle and sheep, reprocessed spermatozoa have been used successfully to produce offspring (Arav et al., 2002; Evans et al., 2004). It has been shown that mouse sperm that became even nonviable through repeated freezing and thawing can fertilize mouse oocytes, with subsequent embryonic development after ICSI (Ahmadi , 1999). In the study of Choi et al., there was no significant decrease in blastocyst development after ICSI when motile spermatozoa from reprocessed stallion semen were used for sperm injection, indicating that the spermatozoa that survive reprocessing have normal function. Non-motile reprocessed spermatozoa were also able to initiate embryo development to the blastocyst stage, but the proportion of blastocyst achieved tended to be lower (Choi et al., 2006)

Current freezing protocols for stallions semen involve a two-step dilution procedure in which semen is first diluted with a primary extender, centrifuged and then diluted a second time prior to freezing in an extender containing a cryoprotectant Freezing extenders for cryopreservation of stallion sperm include skim milk, egg yolk and often glycerol as a cryoprotective agent. The mechanisms of the ways in which cryoprotective agents protect sperm during freezing and thawing are not clear (Oldenhof et al., 2013). They likely play a role in minimizing exposure to osmotic stress, stabilizing biomolecules and cellular structure, and limiting the damaging effects of reactive oxygen species (Woelders et al., 1997; Hoffman et al., 2011; Parks & Graham, 1992; Watson, 2000; Pena et al., 2011).

The freezing and thawing process has serious negative effects on spermatozoa, many of which result in sublethal damage to the cells, and subsequent reduction of fertility (Ricker et al.,2006). The plasma membrane is the first barrier to protect the sperm cell from the surrounding environment and, at the same time, it is the first site of freeze-thaw damage. Osmotic stress is a reason for freeze-thaw damage. The result of osmotic stress is imbalance in ion fluxes and membrane potential. This may lead to reduced motility and increase permeability of the plasma membrane (Ricker et al., 2006). During freezing, cells become dehydrated and shrink. In this respect, the freezing rate is an important step to progressively dehydrate the spermatozoa and maintain the cellular integrity and functionality (Parks & Graham, 1992). Thawing is associated with volume expansion. The shrinkage and swelling of cells are limited by their capacity to withstand such changes (Sieme et al.,2008). Long exposure to extenders with glycerol may result in toxic effect to spermatozoa and cause osmotic changes that affect the post-thawing viability (Alvarenga et al.,2005).

In order to obtain the best sperm population for freezing, a density gradient centrifugation (EquiPure) can be easily added to the normal semen freezing procedure (Gutierrez-Cepeda et al., 2011). With density gradient centrifugation, a sperm subpopulation will be selected from other particles by centrifugation through a density gradient solution. Removing dead or damaged sperm before refreezing can improve the post-thaw motility (Maxwell et al., 2007).

The aim of this study was to investigate the effect of high dilution rates, reprocessing and refreezing on motility and viability of cryopreserved equine spermatozoa.

Material and methods

General experimental design

Cryopreserved semen from one ejaculate of each of seven stallions with at least 50 % motile spermatozoa in the raw semen (74.3 ± 8.4 %) was available. Semen of all stallions was frozen at Utrecht University according to a routine procedure to standardize semen extender (mix of Spervital EVD and Ghent blue), final glycerol concentration (3 %) and freezing rate (start at 20˚C, -0.5˚C/min to 5˚C, -10,0˚C/min to -15˚C, -25˚C/min to -150˚C), respectively.

In total, three experiments were conducted. The specific aims were: 1) evaluating how different dilution rates after thawing of frozen stallion semen affect the motility and viability of the spermatozoa; 2) evaluating how refreezing of highly diluted semen in straws of different size (0.5 ml and 0.25 ml) affects the motility and viability; and 3) evaluating whether enrichment of viable sperm by density gradient centrifugation before refreezing improves the number of motile and viable spermatozoa after the second thawing.

Chemicals

All chemicals were of analytical grade. Propidium iodide was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), PNA-FITC and PSA FITC from EY Laboratories (San Mateo, CA, USA) and Hoechst 33342 was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), respectively. Equipure bottom layer was bought from Nidacon International (Mölndal, Sweden).

Experiment 1

Thawed semen was stepwise diluted to determine potential adverse effects on motility and viability (*supplemental Figure 1*). One 0.5 ml straw was thawed for 30 s at 37°C. The thawed semen was step-wise diluted with semen extender at room temperature at a ratio of 1:4, 1:9, 1:19, and 1:59 (v:v), respectively. The semen extender consisted of two parts of Spervital and three parts of Ghent red (without glycerol). The glycerol concentration was gradually lowered to levels ranging from 0.6 % (1:4 ratio) to 0.05 % (1:59). Sperm motility, viability and acrosome integrity in each sample were determined. Based on the results, a maximum dilution ratio was chosen for the following experiments.

Experiment 2

Thawed semen was refrozen in two different straw sizes (0.5 ml and 0.25 ml). Motility, viability and acrosome integrity after the second thawing were compared (*supplemental Figure 2*). Two 0.5 ml straws per stallion were thawed for 30 seconds at 37˚C. To determine the motility directly after the first thawing, 50µl of the semen was diluted 1:4 with a mixture of 80 µl Spervital and 120 µl Ghent red. The motility of this sample was analyzed immediately, i.e. without any incubation. 75 µl of this sample was mixed with fixative for later assessment of viability and acrosome integrity.

350 µl of the thawed semen was diluted with a mixture of 8.26 ml Spervital and 12.39ml Ghent blue to reach a dilution ration of 1:59 (final glycerol concentration: 3 %). Before the straws were filled, samples for assessing motility, viability and acrosome integrity were taken. Half of the volume was frozen in 0.5ml straws, and the other half in 0.25 ml straws.

Independent from the glycerol exposure, the time for semen processing may affect the semen quality. To assess this factor, 100 µl of the thawed semen was diluted (1:59) with a mixture of 2.36 ml Spervital and 3.54 ml Ghent red (final glycerol concentration: 0.05 %). This sample was held at room temperature and analyzed just after all filled straws had been transferred into the controllable freezer for refreezing.

After a minimum of 3 days, three 0.5 ml straws and three 0.25ml straws of refrozen semen from each stallion were thawed. For assessment of motility, viability and acrosome integrity from both straw types, three different approaches were used. The thawed samples were assessed directly after thawing.

Experiment 3

This experiment tested whether enrichment of viable sperm by density gradient centrifugation before refreezing improves the number of motile and viable spermatozoa after the second thawing (*supplemental Figure 3*). Two 0.5 ml straws per stallion were thawed for 30 seconds at 37˚C, pooled and split into two aliquots. One aliquot of 100µl was diluted 1:57 with 2.28ml Spervital and 3.41ml Ghent red. The sperm concentration of this dilution was calculated based on the concentration after the first freezing. The diluted semen was held at room temperature until further processing. Just before freezing, 0.21ml glycerol (87%) was added to reach a final concentration of 3%. And a dilution ration of 1:59 (c.f. *Supplemental Table 1*). Before the straws were filled, samples for assessing motility, viability and acrosome integrity were taken

The other aliquot (800 µl) was diluted 1+4 with Spervital and Ghent red (2:3, v:v) and layered on top of 3 ml Equipure bottom layer (Nidacon International, Mölndal, Sweden) and centrifuged for 20 min at 300g at

room temperature. The supernatant was removed down to 100µl. The concentration of the pellet was assessed with a Bürker-Türk counting chamber. After that, the pellet was diluted with semen extender (2 parts of Spervital and 3 parts Ghent red) to reach the same sperm concentration as in the sample that had been diluted at a ratio of 1:59 (c.f. *Supplemental Table 2)*. Immediately before filling the straws for freezing, glycerol was added to reach a final concentration of 3% and samples to measure motility, viability and acrosome integrity were taken.

After a minimum of three days, a straw from each processing line (Equipure centrifugation / 1:59 dilution) was thawed. Motility, viability and acrosome integrity were assessed directly after thawing.

Semen collection, freezing and thawing

After semen collection with the aid of an artificial vagina (model: Hannover), ejaculates were diluted 1:1 (v:v) in pre-warmed (38°C) semen extender (Spervital EVD, Toldijk, The Netherlands). Seminal plasma content was reduced by cushioned centrifugation with 1.5 ml Maxifreeze (IMV technologies, Leeuwarden, The Netherlands) in a 50 ml tube (1000 g, 15 minutes, room temperature). The resulting sperm pellet was diluted in a freezing extender to a concentration of approx. 300 x 10^6 spermatozoa per ml. The freezing extender consisted of Spervital and Ghent Blue diluter with glycerol (Ghent diluter, Merelbeke, Belgium). The semen extenders were used in a ratio of 2:3 (Spervital:Ghent).

Diluted semen was loaded into 0.5ml straws and frozen in a controlled rate freezer (Planer, Kryo 10 series III, United Kingdom). The controlled rate freezer was programmed as follows: start at 20˚C, -0.5˚C/min to 5˚C, -10,0˚C/min to -15˚C, -25˚C/min to -150˚C. Thereafter, samples were plunged in liquid nitrogen. Refreezing of spermatozoa was done with the same freezing curve as for the first freezing step.

Frozen samples were stored in liquid nitrogen until further processing or evaluation. For all experiments, straws were thawed in a water bath at 37°C for 30 seconds.

Computer-assisted semen analysis (CASA)

The CASA system was fitted with a 20-fold objective with cells being examined at 200 x magnification, a camera adapter (U-PMTVC, Olympus, Hamburg, Germany) and a camera with a resolution of 648 x 484 pixel (Pulnix TM-6760CL, JAI A/S, Glostrup, Denmark). The System was operated by SpermVision® software (Version 3.5.6, Minitüb, Germany) and equipped with an automated stage. For each sample, 12 successive fields in the centre of a droplet were recorded at a rate of 60 Hz per field, with a minimum of 400 sperm being recorded. In low concentrated samples several droplets were analysed. Sperm cell heads were recognized in the size range of 14 to 85 µm². The parameters assessed were the percentage of motile sperm (total motility), and the percentage of progressive motile sperm (progressive motility). For the group of progressively motile sperm the average values for straight line velocity (VSL), curved line velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), wobble (WOB = VAP/VCL), average amplitude of lateral head-displacement (ALH), and beat cross frequency (BCF) were included. A spermatozoon was considered to be motile when it met one of the following three definitions 1) average head orientation change (AOC) higher than 7° and BCF greater than 25Hz, 2) DSL greater than 3.5µm, VSL greater than 8µm/s and DSL greater than 15µm, 3) VAP greater than15µm. Subsequently, all motile cells were assessed for the following 7 criteria. If one of them was met, the cell was considered to be progressively motile: 1) DSL greater than 15µm, 2) BCF greater than 40Hz and radius greater than 5µm, 3) VSL greater than 20µm/s, 4) DCL greater or equal to 20µm, 5) STR greater than 0.3 and LIN greater than 0.2, 6) Radius greater than 10µm, 7) BCF greater than 30 and ALH greater than 0.85.

Unless otherwise stated, 200 to 250 µl of the samples was incubated for 5 minutes at 38 ˚C in a heating block before motility assessment to avoid any bias of low temperature on sperm motility. The microscope stage, as well as glassware and pipette tips were equilibrated at 38°C. After incubation, 8 µl of the semen sample were placed on a preheated slide with cover slip (20 mm x 20 mm, Menzel Glaser, Braunschweig, Germany) to prepare a fluid layer of approximately 20 µm height.

Assessment of sperm viability and acrosome integrity

Semen samples were diluted at a ratio of 3:1 (v:v) in a fixative (0.5% formaldehyde in a buffer of 55 mM NaH_2PO_4 110 mM Na_2HPO_4, 51.3 mM NaCl; pH 7.0) to prevent deterioration, until samples were stained and analyzed. For staining, propidium iodide (final concentration: 10 µg/ml), Hoechst 33342 (final concentration: 50 µg/ml), PNA-FITC and PSA-FITC (both 25 µg/ml final concentration) were added to the fixed samples. After incubation for 30 min at room temperature in the dark, samples were analyzed on a FACS Canto II flow cytometer (BD, Breda, The Netherlands). Dyes were excited by a 405 nm laser (30 mW) and a 488 nm laser (20 mW). Fluorescence signals were collected in a 450/50 BP filter (Hoechst 33342), a 530/30 BP filter (PNA-FITC and PSA-FITC), and a 585/42 BP filter (PI), respectively. In each measurement, data of 10,000

spermatozoa were collected at medium speed. Data were analyzed using FCS Express (version 3, De Novo Software, Glendale, CA, USA). Overlap between emission spectra was compensated post acquisition.

Hoechst 33342 stains all DNA-containing particles, i.e. mainly spermatozoa. This allowed to limit the analysis to DNA-containing events with forward and sideward light scatter characteristics of single spermatozoa, thereby excluding egg yolk particles from the analysis. Propidium iodide is a DNA-binding, fluorescent stain (red emission spectrum) which penetrates cells with defect plasma membranes. This allows to distinguish between live (PI-negative) and dead (PI-positive) spermatozoa. PNA-FITC and PSA-FITC are both lectins that specifically tag either the inner leaflet of the outer acrosomal membrane (PNA-FITC) or structures in the acrosomal matrix (PSA-FITC). A spermatozoon with a positive staining (green fluorescence) for one or both of the two lectins was considered as having a defect acrosome.

Statistics

All data were analyzed with the statistic program SPSS 22 (IBM, Amsterdam, The Netherlands). Parameters (motility, progressive motility, plasma membrane- and acrosome integrity) were tested for normal distribution (Shapiro-Wilk test) and, if necessary, transformed into logarithm to achieve normal distribution. In Experiment 1, the effect of an increasing dilution ratios on motility, progressive motility, plasma membrane- and acrosome integrity was tested with a one-factorial analysis of variance (ANOVA) for repeated measurements. All other comparisons were done with a Student's t-test for paired samples. Unless otherwise stated, data are presented as means and standard deviations. Differences were considered to be significant when their probability of occurring by chance was less than 5% ($P<0.05$).

Results

Experiment 1

Semen samples at 1:4 dilution were characterized by 38.3 ± 16.1% live and acrosome intact sperm (PI-, PNA-FITC and PSA-FITC negative; Table 1). This percentage decrease slightly to 34.3 ± 14.1% after 1:59 dilution ($P<0.05$). High dilution ratios had no influence on progressive motility ($P>0.05$). Total motility was lower after 1:19 dilution (45.9 ± 16.8%) when compared to a dilution of 1:4 (53.1 ± 19.6%; $P<0.05$). However, total motility for dilution ratios of 1:9 and 1:59 did not differ when compared to a dilution ratio of 1:4 (Table 1).

Experiment 2

The percentage of sperm with intact plasma and acrosomal membranes decreased from 37.4 ± 11.6% directly after thawing to 26.4 ± 5.9% after exposure to glycerol-containing extender ($P<0.05$; Table 2). There was no difference between sperm directly after thawing and sperm processed with glycerol free extender on the percentage of intact plasma and acrosomal membranes ($P>0.05$). The use of glycerol-free or glycerol-containing extender after thawing had no influence on motility ($P>0.05$).

Semen just before refreezing was characterized by 26.4 ± 5.9% live and acrosome intact sperm (PI-, PNA-FITC and PSA-FITC negative), 44.3 ± 13.6% total motility and 38.6 ± 4.8% progressive motility (Table 3). All parameters decreased after the second thawing (Table 3). A higher total motility was observed in 0.5ml straws (8.8 ± 5.1%) compared to 0.25 ml straws (7.0 ± 4.3%; $P<0.05$). Refreezing in different straw sizes had no influence on progressive motility and viability.

Experiment 3

No differences were observed in membrane intact sperm between semen directly after thawing, semen centrifuged with Equipure or semen diluted (1:59) and held at room temperature (Table 4). Total motility and progressive motility were lower for 1:59 diluted semen held at room temperature ($P<0.05$). No significant differences were observed between Equipure-processed semen before refreezing and the semen directly after thawing on motility and progressive motility ($P>0.05$).

Figure 1 is showing the loss of sperm using the Equipure density gradient. The total number of sperm cells decreased from 140.5 ± 33.5 $x10^6$ before centrifugation to 4.9 ± 2.6$x10^6$ after centrifugation. After refreezing and thawing the samples, no motile sperm were observed. There was only a small percentage of sperm left with intact plasma and acrosomal membranes (Table 4).

Table 1: Semen parameters at different dilution ratios after one freeze/thaw cycle (Experiment 1)

	1:4 dilution	**1:9 dilution**	**1:19 dilution**	**1:59 dilution**
Sperm concentration (*10^6 sperm/ml)	54.0±12.9	27.0±6.4	13.5±3.2	4.5±1.1
PI- & FITC-PNA- & FITC-PSA- neg. (%)	38.3 ± 16.1[a]	36.9 ± 15.1[a,b]	36.3 ±15.3[b]	34.3 ± 14.1[b]
Total motitity (%)	53.1 ± 19.6[a]	47.4 ± 16.7[a,b]	45.9 ± 16.8[b]	45.1 ± 19.6[a,b]
Progressive motility (%)	37.9 ± 15.0[a]	37.9 ± 14.4[a]	38.2 ± 14.2[a]	39.3 ± 17.8[a]
VAP (µm/s)	87.9 ± 7.2	92.9 ± 7.9	92.9 ± 8.4	102.6 ± 27.3
VCL (µm/s)	143.6 ± 14.8	149.7 ± 17.2	151.1 ± 17.8	154.7 ± 19.0
VSL (µm/s)	75.2 ± 4.9	82.0 ± 6.1	82.9 ± 6.0	83.8 ± 5.1
ALH (µm)	3.0 ± 0.6	2.8 ± 0.5	2.7 ± 0.5	2.8 ± 0.5
BCF (Hz)	42.5 ± 3.2	43.7 ± 2.4	44.8 ± 2.7	44.1 ± 2.4

VAP = average path velocity *VCL = curvilinear velocity* *VSL = straight line velocity*
ALH = amplitude of lateral head-displacement *BCF = beat cross frequency*

The percentage of sperm with intact plasma and acrosomal membranes (PI-, FITC-PNA- & FITC-PSA- negative) was determined by flow cytometry. Motility parameters were assessed by a CASA-system. All data are means and standard deviations. Different letters within a row indicate significant differences (P<0.05; n=7)

Table 2: Semen parameters after processing with or without exposure to glycerol (Experiment 2)

	Directly after 1st thawing	**After processing without glycerol**	**After processing with glycerol**
PI- & FITC-PNA- & FITC-PSA- neg. (%)	37.4 ± 11.6[a]	34.6 ± 10.2[a]	26.4 ± 5.9[b]
Total motility (%)	53.4 ± 17.6[a]	40.0 ± 11.3[b]	44.3 ± 13.6[b]
Progressive motility (%)	37.0 ± 4.0[a]	32.7 ± 4.2[a]	38.6 ± 4.8[a]
VAP (µm/s)	90.5 ± 8.5	100.9 ± 7.9	95.4 ± 9.1
VCL (µm/s)	148.5 ± 18.4	169.8 ± 21.0	162.4 ± 23.5
VSL (µm/s)	77.4 ± 5.5	87.6 ± 5.4	83.3 ± 5.9
ALH (µm)	3.0 ± 0.6	2.9 ± 0.7	3.0 ± 0.6
BCF (Hz)	42.3 ± 3.1	43.7 ± 2.2	43.1 ± 3.0

VAP = average path velocity *VCL = curvilinear velocity* *VSL = straight line velocity*
ALH = amplitude of lateral head-displacement *BCF = beat cross frequency*

The percentage of sperm with intact plasma and acrosomal membranes (PI-, FITC-PNA- & FITC-PSA- negative) was determined by flow cytometry. Motility parameters were assessed by a CASA-system. Samples were assessed directly after the first thawing and after processing of semen for refreezing. All data are means and standard deviations. Different letters within a row indicate significant differences (P<0.05; n=7).

Table 3: Semen parameters after refreezing sperm in straws of different sizes (Experiment 2)

	Before re-freezing	**After refreezing 0.5ml straws**	**After refreezing 0.25 ml straws**
PI- & FITC-PNA- & FITC-PSA-neg. (%)	26.4 ± 5.9[a]	12.4 ± 4.0[b]	12.7 ± 5.8[b]
Total motility (%)	44.3 ± 13.6[a]	8.8 ± 5.1[b]	7.0 ± 4.3[c]
Progressive motility (%)	38.6 ± 4.8[a]	6.0 ± 1.6[b]	4.6 ± 1.2[b]
VAP (µm/s)	95.5 ± 9.1	80.4 ± 3.0	112.6 ± 27.0
VCL (µm/s)	162.4 ± 23.5	147.1 ± 24.1	116.3 ± 66.5
VSL (µm/s)	83.3 ± 5.9	66.6 ± 5.8	69.8 ± 17.1
ALH (µm)	3.0 ± 0.6	3.1 ± 0.8	2.6 ± 0.2
BCF (Hz)	43.1 ± 3.0	37.8 ± 4.8	43.1 ± 2.7

VAP = average path velocity *VCL = curvilinear velocity* *VSL = straight line velocity*
ALH = amplitude of lateral head-displacement *BCF = beat cross frequency*

The percentage of sperm with intact plasma and acrosomal membranes (PI-, FITC-PNA- & FITC-PSA- negative) was determined by flow cytometry. Motility parameters were assessed by a CASA-system. All data are means and standard deviations. Different letters within a row indicate significant differences (P<0.05; n=7).

Table 4: Semen parameters before and after refreezing of Equipure-selected spermatozoa (Experiment 3)

	After 1st freeze/thaw cycle			**After 2nd freeze/thaw cycle**	
	Directly after 1st thawing	**1:59 diluted**	**after Equipure**	**1:59 diluted**	**Equipure**
PI- & FITC-PNA- & FITC-PSA- neg. (%)	32.1 ± 12.8[a]	25.1 ± 15.3[a]	32.3 ± 21.2[a]	7.8 ± 0.03	6.2 ± 0.03
Total motility (%)	42.3 ± 18.6[a]	7.9 ± 5.6[b]	35.9 ± 27.1[a]	0	0
Progressive motility (%)	29.4 ± 17.1[a]	4.2 ± 4.3[b]	26.0 ± 23.6[a]	0	0
VAP (µm/s)	100.1 ± 9.8	54.0 ± 1.2	66.0 ± 9.2	n/a	n/a
VCL (µm/s)	163.3 ± 17.6	99.9 ± 1.9	124.5 ± 23.9	n/a	n/a
VSL (µm/s)	86.2 ± 7.3	47.1 ± 4.0	54.8 ± 6.2	n/a	n/a
ALH (µm)	3.2 ± 0.3	2.3 ± 0.1	2.8 ± 0.7	n/a	n/a
BCF (Hz)	41.2 ± 2.1	38.7 ± 0.4	37.4 ± 3.3	n/a	n/a

VAP = average path velocity *VCL = curvilinear velocity* *VSL = straight line velocity*
ALH = amplitude of lateral head-displacement *BCF = beat cross frequency* *n/a = not applicable*

The percentage of sperm with intact plasma and acrosomal membranes (PI-, FITC-PNA- & FITC-PSA- negative) was determined by flow cytometry. Motility parameters were assessed by a CASA-system. Samples were assessed directly after the first thawing, after processing of semen for refreezing and after the second freeze/thaw cycle. All data are means and standard deviations. Different letters within a row indicate significant differences (P<0.05; n=7).

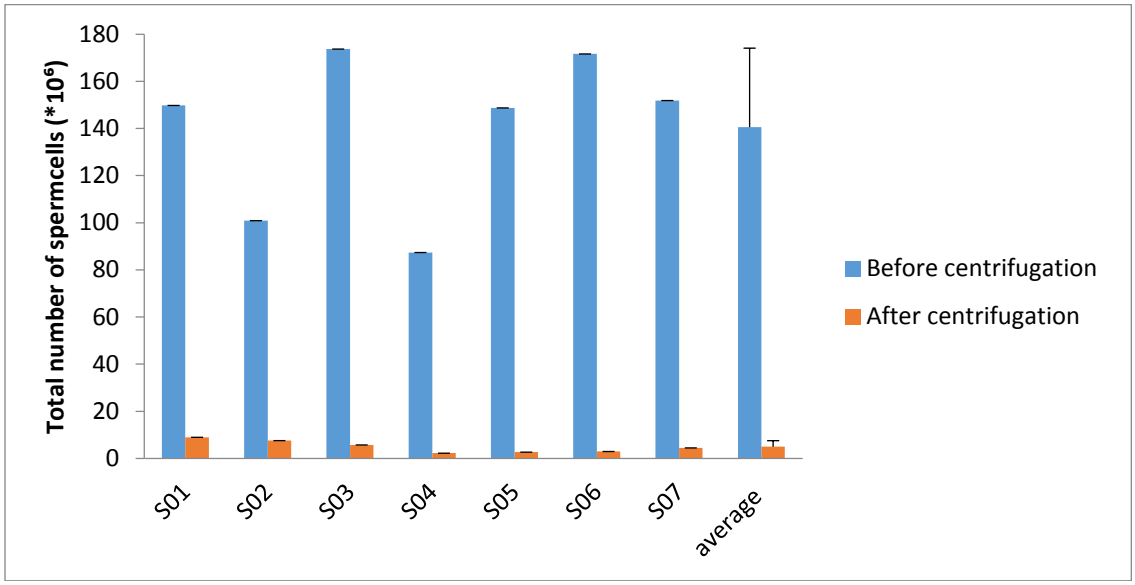


Figure 1: Recovery rate of sperm after centrifugation through an Equipure bottom layer (Experiment 3)
This figure is showing the sperm concentration of the individual stallions (S01-S07) before and after centrifugation through an Equipure bottom layer. The mean ± standard deviation before centrifugation are 140.5 ± 33.5 x10^6 sperm cells. The mean ± standard deviation after centrifugation are 4.9 ± 2.6x10^6 sperm cells. The average loss of sperm cells was 96.5%.

Discussion

In this study, the effect of high dilution rates, reprocessing and refreezing on motility and viability of cryopreserved equine spermatozoa was evaluated. The data from Experiment 1 demonstrate that a high dilution rate resulted only in a minor decrease in total motility and progressive motility. Similarly, Choi et al. reported that sperm diluted to concentrations of about $2x10^6$spermatozoa/ml was compatible with harvesting adequate numbers of motile, functional spermatozoa from the thawed sample to perform the ICSI procedure (Choi et al., 2005). For ram spermatozoa is described that high? dilution prior to freezing improved motility, viability and acrosome integrity (Leahy et al., 2010). We could not confirm this effect for stallion sperm. Our results rather indicated a slightly lower viability after 1:59 dilution compared to a dilution ratio of 1:4.

In experiment 2, the effect of reprocessing and refreezing in straws of different size (0.5ml and 0.25ml) on motility, progressive motility and viability was evaluated. Reprocessing and refreezing stallion semen, which means thawing, dilute and re-freeze could increase the efficiency use of sperm. The entire procedure would be expected to decrease the motility and viability of the spermatozoa (Ricker et al.,2006). In this study, exposure to an extender with glycerol reduced the percentage sperm with intact plasma and acrosomal membranes already before sperm were refrozen. Long exposure to glycerol may be toxic for spermatozoa and cause osmotic changes that affect the post-thawing viability (Alvarenga et al., 2000).

After refreezing, 0.5 ml straws provided slightly higher total motility. This is in contrast to previous studies in other species (give references). It is generally accepted that increasing the volume of straws is detrimental for motility due to a slower cooling at the centre of the straw (Weitze et al., 1987). However, Cordova et al. (2001), evaluated the effect of straw volume on motility of frozen boar semen and didn't find significant differences on motility between 0.5 ml and 5 ml straws.

Based on the results of experiment 2, the biggest loss of sperm motility and membrane integrity was observed after refreezing and thawing. The freezing rate is an important step to progressively dehydrate the spermatozoa and maintain the cellular integrity and functionality (Parks & Graham, 1992). Therefore, a alternative methods of refreezing, including different freezing-rates and extenders should be evaluated, to increase the motility and viability after refreezing. In other species, the resistance of sperm to freezing was improved by increasing the cholesterol-content of the plasma membrane (insert references). Similarly, increasing the cholesterol-content of the plasma membrane from stallion sperm prior to cryopreservation increased the survival rates of stallion sperm (Moore et al., 2005; Oliveira et al., 2010),. Previous studies indicate that the cholesterol/phospholipid ratio of the plasma membrane is a major determinant in plasma membrane fluidity and stability during cryopreservation (Darin-Bennett & White, 1977; Watson, 1981). Cholesterol reduces the transition temperature of membranes, and maintains them in a fluid-like state at reduced temperatures, thereby reducing the membrane damage that occurs at low temperatures (Amann & Pickett, 1987). Sperm from species that possess very high cholesterol/phospholipid ratios, such as human and rabbit sperm, do not experience this membrane damage when cooled.

In experiment 3, thawed sperm were centrifuged through a density gradient (Equipure) to enrich motile and viable sperm before refreezing. The protocol that was used in this study resulted in a high loss of sperm cells and no enrichment of motile or viable sperm. The low sperm recovery after density gradient centrifugation might be a result of the density of the Equipure bottom layer. Adjusting the density of the gradient might improve at least the recovery rate. Processing of sperm through the density gradient did not improve sperm survival after refreezing. One reason could be duration of semen preparation. After a straw was thawed, it took about 45 minutes before the processed semen was filled into straws and refrozen.

In conclusion, the results of this study demonstrate that frozen/thawed equine sperm can be diluted to low sperm concentrations with only minor loss in sperm motility and viability. However, refreezing frozen/thawed stallion semen decreased motility and viability tremendously irrespective of the straw size. It is recommended to do a fast processing and limit processing steps, as well as exposure time to glycerol at room temperature to a minimum. Future studies may focus on improving the resistance of frozen/thawed sperm to re-freezing.

References

Ahmadi A (1999). Developmental capacity of damaged spermatozoa. Hum. Reprod 1999; 14; 2279-2285

Alvarenga MA, Papa FO, Landim-alvarenga FC.(2005) Amides as cryoprotectants for freezing stallion semen: a review. Anim Reprod Sci 2005; 89; 105-113

Alvarez C, Gil L, González N, Olaciregui M, Luño V (2014). Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender. *Cryobiology*; 69, 144-148; doi: 10.1016/j.cryobiol.2014.06.008

Amann RP, Picket BW (1987). Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. *Equine Veterinary Science* 7, 145-173.

Arav A, Zeron Y, Shturman H, Gacitua H. (2002) Successful pregnancies in cows following double freezing of a large volume of semen. Reprod Nutr Dev;42, 583–586.

Choi YH, Love CC, Varner DD, Hinrichs K. (2006) Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology*. 1;65(4), 808-819.

Choi, Y. H., Okada, Y., Hochi, S., Braun, J., Sato, K., and Oguri, N. (1994). *In vitro* fertilization rate of horse oocytes with partially removed zonae. *Theriogenology* **42**, 795–802. doi:10.1016/0093-691X(94)90448-R

Choi YH, Love CC, Varner DD, Hinrichs K. (2006) Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology*. 1;65(4), 808-819

A Cordova, JF Perez, B Lleo , C Garcia Artiga and S Martin Rillo (2001) In vitro Fertilizing Capacity of Deep Frozen Boar Semen Packaged in 0.5 and 5 ml Straws. *Reprod Dom Anim* 36, 199-202

Darin-Bennet A, White IG. (1977) Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology 14, 466-470*

Evans G, Hollinshead FK, Maxwell WM. (2004) Preservation and artificial insemination of sexed semen in sheep. *Reprod Fertil Dev* 16, 455–464.

Galli C, Duchi R, Colleoni S, Lagutina I, Lazzari G (2014). Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology*; 81 , 138-151; doi: 10.1016/j.theriogenology.2013.09.008

Gutierrez-Cepeda L, Fernandez A, Crespo F (2011), Simple and economic colloidal centrifugation protocols may be incorporated into the clinical equine sperm processing procedure. *Animal Reproduction Science.* 124(1-2), 85-89. doi: 10.1016/j.anireprosci.2011.02.00

Hinrichs K, Choi YH, Norris JD, Love LB, Bedford-Guaus SJ, Hartman DL, Velez IC (2012). Evaluation of foal production following intracytoplasmic sperm injection and blastocyst culture of oocytes from ovaries collected immediately before euthanasia or after death of mares under field conditions. *Journal of the American Veterinary Medical Association* 241, 1070-1074; doi: 10.2460/javma.241.8.1070

Hoffman N, Oldenburg H, Morandini C, Rohn K, Sieme H (2011) Optimal concentrations of cryoprotective agents for semen from stallions that are classified good and poor for freezing. Anim Reprod Sci 2011; 125; 112-118

Lazzari G, Crotti G, Turini P, Duchi R, Mari G, Zavaglia G, et al (2002) Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology* 58, 709–712.

T. Leahy T, J.I. Martib,1, N. Mendozac, R. Pérez-Péc, T. Mui˜no-Blancoc, J.A. Cebrián-Pérezc, G. Evansa, W.M.C. Maxwella (2010) High pre-freezing dilution improves post-thaw function of ram spermatozoa Animal Reproduction Science 119 (2010) 137–146

Maxwell WM, Parrilla I, Caballero I, Garcia E, Roca J (2007), Retained functional intergrity of bull spermatozoa after double freezing and thawing using puresperm density gradient centrifugation. *Reproduction in Domestic Animals,* 42, 489-494

Moore I Amanda, Squiers L Edward, Graham K James (2005). Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. *Cryobiology* 51, 241-249

Olaciregui M, Gil L, Montón A, Luño V, Jerez RA, Martí JI (2014). Cryopreservation of epididymal stallion sperm. *Cryobiology* 68, 91-95; doi: 10.1016/j.cryobiol.2013.12.009

Oliveira CH, Vasconcelos AB, Souza FA, Martins-Filho OA et al.(2010). Cholesterol addition protects membrane intactness during cryopresercation of stallion sperm. *Animal reproduction Science 118, 194-200*

Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. Theriogenology 1992; 83; 209-222

Pickett BW, Voss JL, Squires EL, Vanderwall D, McCue PM, Bruemmer JE (2000) Collection, preparation and insemination of stallion semen. Bulletin no. 10. Fort Collins, CO: Animal Reproduction Biotechnology Laboratory, 94–95.

Pena FJ, Macias Garcia BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO (2011). Dissecting the molecular damge to stallion spermatozoa; the way to improve current cryopreservation protocols. Theriogenology 2011; 76; 1177-1186

Pugliesi G, Fürst R, Carvalho GR (2013). Impact of using a fast-freezing technique and different thawing protocols on viability and fertility of frozen equine spermatozoa. *Andrologia*, doi: 10.1111/and.12205

Ricker JV, Linfor JJ, Delfino WJ (2006), Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. *Biology of Reproduction,* 74, 359-365

Sessions-Bresnahan DR, Graham JK, Carnevale EM (2014). Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using in vitro fertilization, perivitelline, and intracytoplasmic sperm injections. *Theriogenology*; 82, 274-282; doi: 10.1016/j.theriogenology.2014.04.002

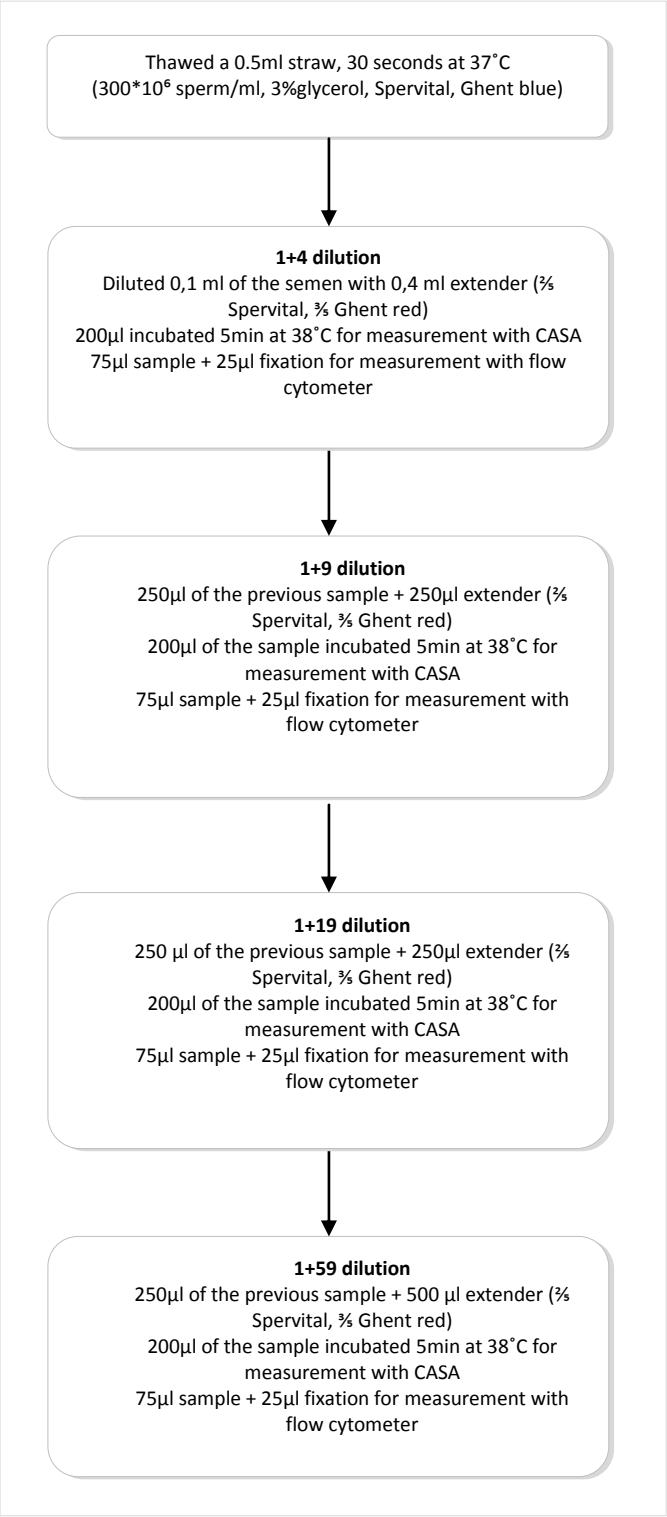
Watson PF (2000). The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000; 60-61; 481-492

Watson PF (1981). The effects of cold shock on sperm cell membranes. Academic Press, New York pp.189-218

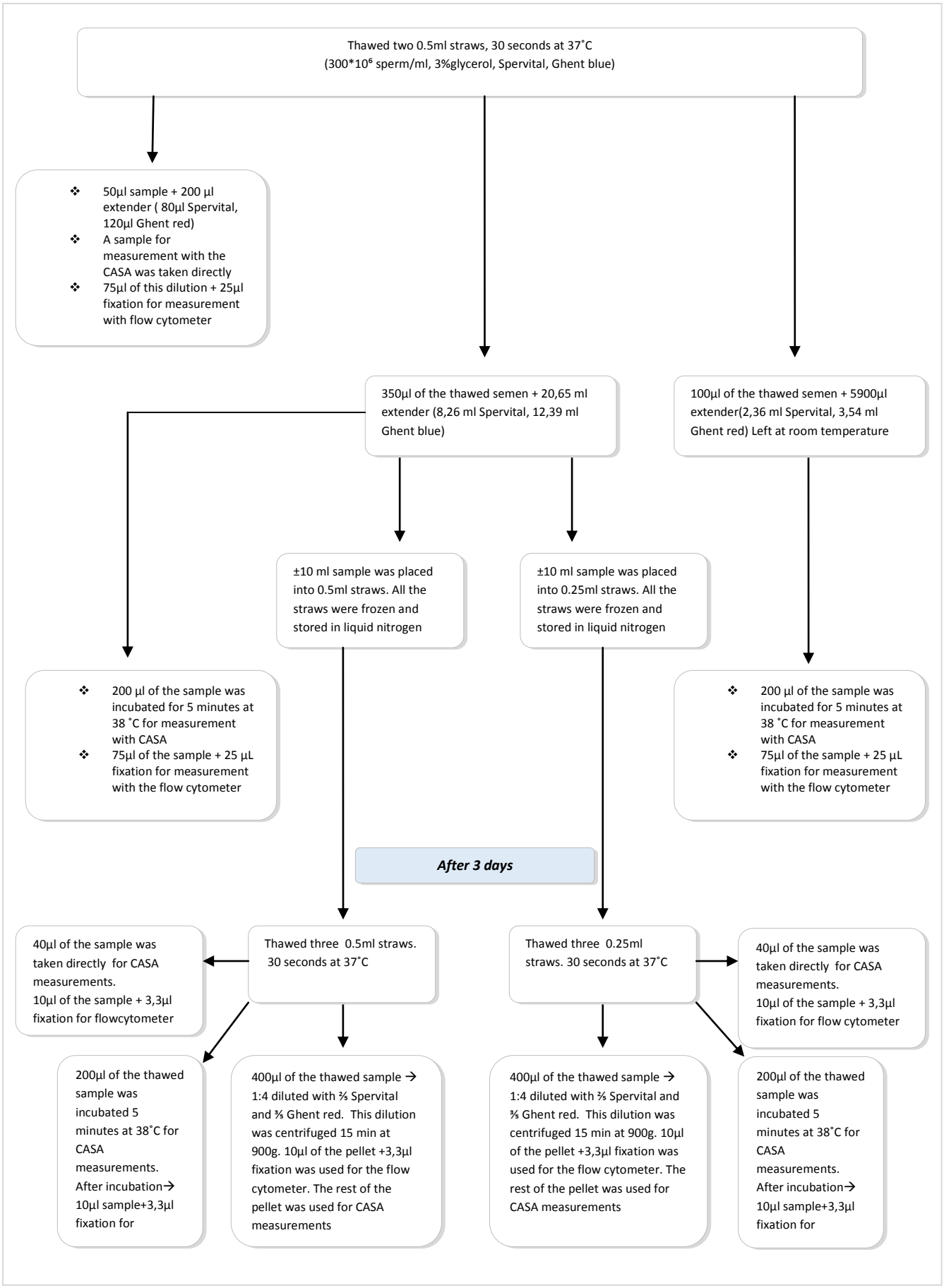
Weitze KF, Rath D, Baron G (1987) Deep freezing of boar semen in plastic straws. *Dtsch Tierarztl Wschr* 82, 261-267

Woelders H, Matthijs A, Enge B (1997) Effects of trehalose and sucrose osmolarity of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. Cryobiology 1997; 35; 93-105

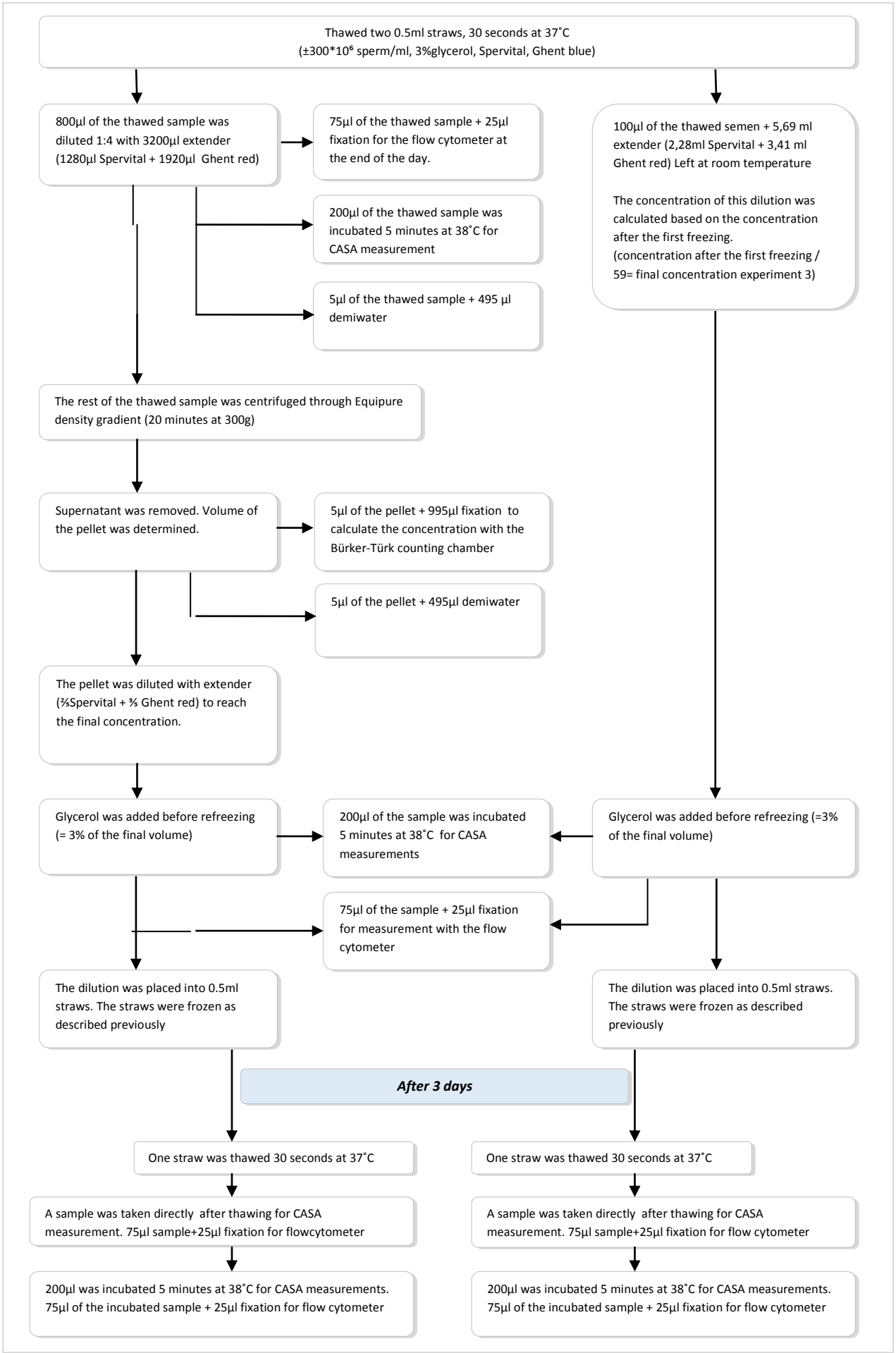
Supplement



Supplemental Figure 1: Work protocol for Experiment 1



Supplemental Figure 2: Work protocol for Experiment 2



Supplemental Figure 3:Work protocol for Experiment 3

Supplemental Table 1: Calculation of final glycerol concentration in Experiment 3

Calculation glycerol concentration 1:59 dilution
• 6 ml= total volume of the 1:59 dilution • 1:59 dilution → 0.05 % glycol was already presented in the dilution • Final concentration glycerol= 2.95% (3-0.05) of the total volume • 2.95% of 6ml= 0.18 ml glycerol • Concentration glycerol dilution in the lab was 87% (0.18/87)*100= 0.21 ml glycerol added

Supplemental Table 2: Exemplary calculation of the added amount extender to the pellet after using Equipure (Experiment 3)

Calculation of the amount extender after Equipure (Example)
• Concentration of the pellet= $100*10^6$ sperm/ml • Volume of the pellet= 300µl • The final reached concentration= $3.2*10^6$ sperm/ml • (100/3.3)*300= 9.4 ml final volume • 3% of the final volume glycerol= 0.28ml • Concentration glycerol dilution in the lab was 87% (0.28/87)*100= 0.32ml glycerol added • 9.4-(0.30 + 0.32)=8.78 ml extender added