Effect of sperm and glycerol concentration on epididymal sperm motility parameters after thawing

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Abstract

The aim of this study was to evaluate the effect of different glycerol concentrations and different sperm concentrations on stallion epididymal sperm motility indicators after thawing. For statistical analysis, 25 stallions were used. Collection of the epidydimal spermatozoa was performed as a retrograde flush of cauda epididymis and ductus deferens within 12 h post castration. After evaluation, the resuspended spermatozoa were centrifuged, the supernatant removed and the spermatozoa resuspended in Gent semen extender to get three different groups with different concentrations of sperm (250×10^6 in ml, 500×10^6 in ml, 1000×10^6 in ml) and different final glycerol concentrations (2%, 4%, and 6%). Therefore, 9 different samples were finally obtained from each stallion. The spermatozoa were packed and placed in a fridge (4 °C) for 2 h, then placed in liquid nitrogen vapour (−80 to −100 °C) and after 25 min plunged into the liquid nitrogen and stored at −196 °C for at least 5 days. The selected straws were individually thawed in a 38 °C water bath for 30 s prior to post-freezing analysis. Motility indicators were assessed at 0, 60, 120, and 180 min after thawing. Parametric test was used for analysis; the measured indicators were total motility, progressive motility, curvilinear velocity, straightness, and average-path velocity. In this study, the best results were reached in samples diluted into a concentration of $1{,}000 \times 10^6$ in ml, regardless of the concentrations of glycerol.

Cryopreservation, stallion, epididymal sperm, motility analysis

To preserve the genetic material of a stallion when semen collection or mating is impossible, harvesting of epididymal sperm is an option. Because the number of epididymal sperm is limited, the most efficient processing method is necessary to optimize fertility. Glycerol (VG) is used as a basic cryoprotectant for freezing ejaculated and epididymal sperm of stallions (Olaciregui et al. 2014). However, some studies have shown that VG concentrations higher than 2.5% are significantly harmful to sperm (Macías García et al. 2012) and that it leads to low-quality insemination doses. Most of the studies were performed at relatively low sperm concentrations of $100–200 \times 10^6$ sperm per ml (Macías García et al. 2012; Olaciregui et al. 2014; Neuhauser et al. 2019). Insemination doses prepared with these concentrations consist of several straws (usually 5–10) which is impractical in terms of storage space and handling during mare insemination. The study of Mráčková et al. (2017) showed that the concentration of VG and its harmfulness to sperm is closely related to the concentration of sperm used in the insemination dose for freezing, so it would be advantageous to use a higher concentration of sperm for freezing.

The quality of the insemination dose after thawing is most often assessed by the percentage of motile sperm (Barrier Battut et al. 2017). In routine evaluation of data from the measurement of post-thaw motility indicators using the computer-assisted sperm analysis (CASA), it is quite clear that sperm motility is usually directly related to egg fertilization. Therefore, the assessment of post-thaw insemination dose by CASA remains very important, especially because of its simplicity and availability.

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The main aim of this study was to verify the theory that the appropriate concentration of the cryoprotectant VG in the sperm freezing diluent affects the motility of epididymal sperm after thawing, depending on the concentration of sperm used in the freezing process.

Materials and Methods

Animals, semen collection, and semen processing

For this research, 25 stallions were used. All of them were between 2 and 12 years old, of varying breeds, clinically healthy and had both testicles descended in the scrotum.

The stallions were castrated under total intravenous general anaesthesia (xylazine, ketamine, diazepam, guaifenesin). The anaesthesia took place without any complications, recovery was spontaneous. The method of castration was closed with covered ductus deferens and sutures of the incision wounds.

After castration, ductus deferentes were closed, and testicles were washed with sterile saline and transferred to the laboratory. Collection of the epididymal spermatozoa was performed as a retrograde flush of cauda epididymis and ductus deferens within 12 h post castration. The retrograde flushing technique is considered the gold standard technique due to its high efficiency in sperm recovery and minimal blood contamination. This technique required careful removal of the surrounding tissue of the cauda epididymis and very gentle preparation of the ductus deferens to prevent damage to it. After this, a 14-gauge catheter was inserted in the proximal part of the ductus deferens and a pre-warmed (38 °C) centrifuge medium for equine semen (Minitübe International, Tiefenbach, Germany) was passed through the cauda epididymis and ductus deferens under gentle pressure. Spermatozoa were collected into a preheated beaker and merged from both epidydimes. The volume of the medium was 10–20 ml for each epididymis. After that, the syringe was filled with 20 ml of air, which was also gently forced through the structures to maximize the number of epididymal spermatozoa obtained.

Sample evaluation was done under a stereomicroscope and measured by CASA. Only epididymal flushings that, on first evaluation, had a beginning progressive motility higher than 10% (motility I_{1-9}) were selected for freezing. As all samples from all stallions fulfilled this requirement, a total of 25 epididymal flushings were used for statistical analysis. After evaluation, the epididymal spermatozoa were centrifuged at 3000 *g* for 10 min, the supernatant was removed, and the spermatozoa were slowly resuspended in a semen extender (Gent Extender, Minitübe International, Tiefenbach, Germany) to obtain different groups with different concentrations of sperm (250×10^6 in ml, 500×10^6 in ml, 1000×10^6 in ml) and different final VG concentrations (2%, 4%, and 6%). Final VG concentration in ml was achieved by replacing 20, 40 a 60 µl of the solution with 100% VG solution. Finally, 9 different samples were obtained from each stallion:

250×10^6 in ml, VG concentration 2%
250×10^6 in ml, VG concentration 4%
250×10^6 in ml, VG concentration 6%
500×10^6 in ml, VG concentration 2%
500×10^6 in ml, VG concentration 4%
500×10^6 in ml, VG concentration 6%
1000×10^6 in ml, VG concentration 2%
1000×10^6 in ml, VG concentration 4%
1000×10^6 in ml, VG concentration 6%

The motility in each sample was evaluated using a CASA system (motility II_{1-9}). The spermatozoa were packed into 0.5 ml straws and placed in the fridge for 2 h (4 °C). Then the straws were placed in liquid nitrogen vapour (−80 °C to −100 °C) and after 25 min plunged into liquid nitrogen and stored at −196 °C for at least 5 days.

Post-thaw semen evaluation

A number of straws were selected and individually thawed in a 38 °C water bath for 30 s before post-freezing analysis. The sperm motility of samples (n = 25) was evaluated immediately after thawing (motility III_{1-9}). The samples were then kept in a water bath at 38 °C and sperm motility was assessed at 60, 120, and 180 min (IV_{1-9}, V_{1-9}, VI_{1-9}) after thawing.

Statistical analysis

The following indicators were measured by CASA and evaluated: total motility (TM), progressive motility (PM), curvilinear velocity (VLC), average-path velocity (VAP) and straightness (STR). For these indicators, a large number of parametric *t*-tests were performed where each group was compared with all other groups. Using this statistical design, differences in TM, PM, VLC, VAP and STR values between groups with different VG concentrations and freezing times were estimated. The level of significance was set as $P < 0.05$.

Results

Total motility

Not surprisingly, the total sperm motility was confirmed to have significantly decreased after thawing for all analysed sample groups (for all sperm and VG concentrations) (all $P < 0.001$).

Our results indicate that 180 min after incubation, the highest total motility was found in samples with a sperm concentration of 1000×10^6 sperms/ml dilution. These samples, fixated in 4% VG, had significantly better total motility than all other samples with a dilution of 500×10^6/ml (all $P < 0.05$). For the TM indicator, the VG concentration apparently did not have as great effect as the concentration of sperm.

The dynamics of total sperm motility within 180 min after thawing are shown in Table 1.

Table 1. Mean values (%) of total motility before freezing and during incubation with the addition of 2, 4, and 6% glycerol in sperm concentrations of 250×10^6, 500×10^6, and 1000×10^6 in ml.

Duration (min)	Concentration 250×10^6 in ml			Concentration 500×10^6 in ml			Concentration 1000×10^6 in ml		
	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%
Before freezing	41.44	35.70	26.24	38.35	29.18	26.25	34.43	26.53	23.11
0	3.24	3.70	2.43	2.61	2.41	2.40	2.10	2.53	2.17
60	2.26	2.39	2.06	2.43	2.47	1.79	2.89	3.53	3.35
120	1.70	2.00	1.07	2.50	1.75	1.33	3.36	2.50	2.31
180	1.72	1.60	1.36	2.00	1.43	1.50	2.85	2.58	2.27

VG – glycerol

Progressive motility

Progressive motility of sperm after freezing and consequent thawing significantly decreased, regardless of the concentration of sperm and VG (all $P < 0.01$). Generally, motility decreased with time after thawing, except for samples with concentrations of 1000×10^6/ml with VG 4% and 6%, where a high peak of rising PM 60 min post thawing was seen. However, the only significant difference was found between samples 1000×10^6/VG 6% and sample sperm concentration of 250×10^6/ml with VG 6% ($T = 2.17$, $P = 0.05$). Overall, PM after 180 min was zero in almost all samples.

The dynamics of progressive sperm motility up to 180 min after thawing are shown in Table 2.

Table 2. Mean values (%) of progressive motility before freezing and during incubation with the addition of 2, 4, and 6% glycerol in sperm concentrations 250×10^6 in ml, 500×10^6 in ml, and 1000×10^6 in ml.

Duration (min)	Concentration 250×10^6 in ml			Concentration 500×10^6 in ml			Concentration 1000×10^6 in ml		
	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%
Before freezing	7.96	6.35	4.38	7.61	4.64	4.60	7.71	5.53	4.50
0	0.16	0.40	0.10	0.22	0.27	0.20	0.33	0.21	0.11
60	0.09	0.16	0.17	0.19	0.16	0.16	0.28	0.29	0.59
120	0.05	0.29	0.07	0.06	0.00	0.07	0.21	0.13	0.08
180	0.06	0.07	0.09	0.07	0.00	0.00	0.15	0.08	0.09

VG – glycerol

Curvilinear velocity, straightness, and average-path velocity

The highest values of VCL after 180 min of incubation were seen in samples with 6% VG, especially in samples with a sperm concentration of 250 and 1000×10^6/ml. A significant difference was also found between samples 500×10^6/ml with 4% VG and 500×10^6/ml with 6% VG ($T = 2.40$, $P = 0.03$). Overall, VG concentration apparently had

a greater effect than the dilution of sperm and with the dilution of 500 × 10^6/ml, the 4% VG concentration had better VCL. The mean VCL values during incubation are shown in Table 3.

Table 3. Mean values of curvilinear velocity (μm/s) before freezing and during incubation with the addition of 2, 4 a 6% glycerol in sperm concentrations 250 × 10^6 in ml, 500 × 10^6 in ml, and 1000 × 10^6 in ml.

Duration (min)	Concentration 250 × 10^6 in ml			Concentration 500 × 10^6 in ml			Concentration 1000 × 10^6 in ml		
	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%
Before freezing	101.76	98.51	96.44	98.72	94.55	91.98	97.91	97.46	93.46
0	72.41	79.56	72.68	72.11	71.29	77.01	59.41	76.37	74.85
60	69.58	73.56	63.12	63.71	74.04	76.32	57.03	73.98	65.07
120	69.76	66.44	65.19	69.44	71.86	69.29	64.24	70.77	70.10
180	70.51	64.93	83.14	62.83	75.61	62.60	65.67	75.29	92.15

VG – glycerol

Straightness (STR) of sperm changed considerably during 180 min of incubation post thawing. The samples that showed good results immediately after thawing decreased during incubation (Table 4). The best results 180 min after thawing were seen in the sample 1000 × 10^6/ml with 6% VG. The sample 250 × 10^6/ml with 2% VG showed significantly better results 180 min after thawing than 250 × 10^6/ml with 6% VG and 500 × 10^6/ml with 2% and 6% VG (all $P < 0.05$).

Table 4. Mean values of straightness (%) before freezing and during incubation with the addition of 2, 4 a 6% glycerol in sperm concentrations 250 × 10^6 in ml, 500 × 10^6 in ml, and 1000 × 10^6 in ml.

Duration (min)	Concentration 250 × 10^6 in ml			Concentration 500 × 10^6 in ml			Concentration 1000 × 10^6 in ml		
	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%
Before freezing	64.12	65.85	65.57	66.13	63.91	66.70	67.67	67.11	68.22
0	57.40	63.75	57.81	66.22	55.64	56.95	48.62	64.26	50.22
60	68.45	60.05	51.78	54.10	65.00	55.26	51.44	65.29	55.82
120	59.20	54.12	52.33	66.94	53.69	44.47	61.21	57.75	60.23
180	58.00	57.60	44.27	44.50	57.00	43.58	62.69	53.42	63.18

VG – glycerol

The average path velocity (VAP) was at its highest 180 min after incubation in the sample 1000 × 10^6/ml with 6% VG, and was significant only in the sample 500 × 10^6/ml with 6% VG ($T = 2.81$, $P = 0.02$). The dynamics of VAP are shown in Table 5.

Table 5. Mean values of average-path-velocity (μm/s) before freezing and during incubation with the addition of 2, 4, and 6% glycerol in sperm concentrations 250 × 10^6 in ml, 500 × 10^6 in ml, and 1000 × 10^6 in ml.

Duration (min)	Concentration 250 × 10^6 in ml			Concentration 500 × 10^6 in ml			Concentration 1000 × 10^6 in ml		
	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%	VG 2%	VG 4%	VG 6%
Before freezing	48.58	46.35	44.83	46.87	44.23	43.44	46.36	45.96	44.66
0	35.99	37.42	35.97	35.86	34.99	37.00	28.33	38.71	36.81
60	33.18	35.29	27.98	32.28	36.83	36.54	29.19	35.08	32.08
120	34.06	31.97	28.07	30.68	30.12	27.82	33.34	32.18	31.35
180	31.21	32.29	34.51	28.00	33.28	25.78	30.25	33.52	38.68

VG – glycerol

Discussion

In this study, VG was used as a basic cryoprotectant for freezing stallion epididymal sperm. Because VG causes injury to spermatozoa during the cryopreservation process (Fahy et al. 1990), its usage is usually limited to concentrations lower than 2.5% (Macías García et al. 2012). Other studies have shown that concentrations higher than 2.5% may have better results (Mráčková et al. 2017). Most of the studies were performed at relatively low sperm concentrations of 100–200 × 10^6 sperms per ml (Macías García et al. 2012; Olaciregui et al. 2014; Neuhauser et al. 2019). The previous study by Mráčková et al. (2017) showed that the VG concentration and its harmfulness to sperm is closely related to the concentration of sperm used in the insemination dose for freezing. In the same study, the authors found that the best VG concentration for freezing equine epididymal sperm was 4%. In this study, a higher concentration of sperm for freezing (250–1000 × 10^6 sperms per ml) was used with different VG concentrations to find out which combination of VG and sperm concentrations is beneficial for the sperm. It was decided to use VG concentrations of 2%, 4%, and 6%.

Fertile sperm are present in the oviduct within 3 h after insemination (Troedsson et al. 1998). That is the reason why the present study evaluated motion indicators of epididymal sperm at 180 min analysis post-thawing. Samples were evaluated at 60, 120 and 180 min of post-thaw incubation at different sperm and VG concentrations. This is quite different from other studies that evaluated motility indicators immediately after thawing (Macías García et al. 2012; Neuhauser et al. 2019) or 120 min after thawing (Olaciregui et al. 2014; Mráčková et al. 2017).

Cryopreservation of spermatozoa causes irreversible damage to the sperm and leads to a reduction of sperm motility, functional membrane integrity, and fertilizing ability (Bucak et al. 2009). The authors were mindful of this fact throughout the whole study when, previously, PM after 180 min was zero in almost all animals.

Our results indicate that 180 min after incubation, the highest total and progressive motility were in samples with a sperm concentration of 1000 × 10^6 sperms/ml dilution. In the TM and PM indicators, the VG concentration apparently does not have as great effect as the concentration of sperm.

Controversially, higher values of VCL after 180 min of incubation were recorded in samples with 6% VG, especially in samples with sperm concentrations of 250 and 1000 × 10^6/ml. The best values of STR and VAP after 180 min incubation were seen in the sample 1000 × 10^6/ml 6% VG.

We confirmed our hypothesis that the appropriate concentration of VG cryoprotectant in the sperm freezing diluent affects the epididymal sperm motility after thawing and depends on the sperm concentration used in freezing. In our study, the samples diluted to a concentration of 1000 × 10^6 per ml showed the best results in the evaluated motion indicators (TM, PM, VCL, STR, VAP) 180 min after thawing. The experiment was conducted under laboratory conditions and motion indicators were evaluated after 60, 120, and 180 min of incubation under the harmful influence of VG. For this reason, its effect on the fertilizing capacity of sperm needs to be investigated *in vivo*.

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