Effects on brown bear (*Ursus arctos*) spermatozoa freezability of different extender and dilution ratios used for prefreezing centrifugation

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Abstract

The objective of this study was to determine how the extender and dilution ratio used during centrifugation affect bear spermatozoa quality before and after freezing-thawing. Semen was collected from 15 brown bears by electroejaculation. In Experiment 1, semen was divided into five aliquots and diluted using one of the following extenders: Tris-Citric-Glucose (TCG), Tris-Citric-Glucose-3% BSA, Tris-Citric-Glucose-1 % egg yolk or CaninePro. In Experiment 2, semen was divided into five aliquots and diluted 1:1, 1:4, 1:8 or 1:16 (semen:extender) with Tris-Citric-Glucose. In both experiments, one aliquot was left undiluted and it was used as a control. All the aliquots were centrifuged at 600×g for 6 min and frozen. Samples were analysed post-thawing for motility (CASA) and, by flow citometry, for viability (YO-PRO-1), acrosomal status (PNA-FITC/PI) and mitochondrial status (JC-1). CaninePro rendered the highest motility respect to the undiluted control (Total motility: 53.1% vs. 38.5%), and CaninePro and TCG increased significantly the percentage of viable and acrosome-intact spermatozoa (CaninePro, 43.2 and 43.4 respectively, vs. 39.4). In Experiment 2, dilution 1:4 yielded the highest value of total motility (78.8 vs. 67.2) and proportion of spermatozoa with intact membrane and acrosome (64.5 vs. 54.4). In general, diluting 1:4 or 1:8 brown bear semen prior to centrifugation improved the motility and acrosome status of the thawed spermatozoa.

Keywords: brown bear, spermatozoa, centrifugation, extender, dilution.

1. Introduction

Brown bears are not catalogued as threatened worldwide because large populations exist in Russia, Canada, Alaska and some regions of Europe (IUCN 2007). However, in regions of Southern Europe and Central and Southern there are only very small populations, which are isolated and highly vulnerable. The Cantabrian brown bear (*Ursus arctos*) is the last autochthonous bear population in the Iberian Peninsula, and may constitute the last pure breed of brown bear in the world. This population is currently split into two small nuclei (around 100 specimens) in the Northern mountain ranges of Spain. Thus, the brown bear is considered at risk of extinction by Spanish law (Real Decreto 439/1990), and the European Union has classified it as endangered (Council Directive 92/43/EEC, annexes II and IV). Therefore, inbreeding and loss of genetic variability are a likely hazard. Genetic resource banks could help to reverse that situation, allowing the storage of gametes and embryos for long periods, eventually applying this material in recovery programs using assisted reproduction techniques. Adapting artificial reproductive techniques to the brown bear should be a priority in the recovery plans for this population (Anel et al., 2008). Centrifugation of spermatozoa is necessary prior to cryopreservation in many species for reaching an adequate sperm concentration and cleaning urine-contaminated samples. It is known that in carnivores electroejaculation yields low sperm concentration (Ishikawa et al, 2001). Indeed, our previous experience has shown that most brown bear ejaculates do not achieve a concentration high enough for the cryopreservation protocol (unpublished data) and thus packaging process by centrifugation is necessary. A consequence of centrifugation is the removal of seminal plasma and their potential effects on semen quality. Although some studies in several species have shown the beneficial effects of

63 seminal plasma addition on spermatozoa during processing and cryopreservation (deer:
64 Martinez-Pastor et al, 2006; ram: Ollero et al, 1997), others have reported deleterious
65 effects (dog: Rota et al, 2006; goat: Pellicer-Rubio et al, 1997; ram: Ritar and Salamon,
66 1982; stallion: Carver and Ball, 2002 Sieme et al, 2004; bull: Way et al, 2000).
67 Physiological contamination of ejaculates with urine occurs frequently in many species
68 (horse, Althouse et al, 1989; man, Chris et al, 2004). Some species such as the bear
69 (Kojima et al, 2001; Chen et al., 2007; Anel et al., 2008) may yield semen contaminated by
70 the urine during electroejaculation. An usual method for counteracting the noxious effect
71 of urine contamination is to add large volumes of extender to the contaminated sample and
72 centrifuge it immediately, removing the supernatant and substituting it with clean medium
73 (Makler et al, 1981, Kim et al, 1998).
74 However, centrifugation might induce negative effects on spermatozoa due to mechanical
75 stress. These effects appear to be species-specific. Thus, spermatozoa from the rat
76 (Cardullo and Cone, 1986), human (Ng et al, 1990; Alvarez et al, 1993; Aitken and
77 Clarson 1988) and mouse (Katkov and Mazur, 1998) are especially influenced by
78 centrifugal forces, but spermatozoa from other species, such as the bull and the stallion, do
79 not seem to be affected by centrifugation (Picket et al, 1975; Katkov and Ostashko, 1996;
80 Crockett et al, 2001). Therefore, adjusting the centrifugation procedure is necessary to
81 properly process the bear semen, taking into account any negative effect on sperm quality.
82 To our knowledge, no studies have reported the effect of different centrifugation protocols
83 on the quality of brown bear spermatozoa. Thus, the aims of this study were (1) to test
84 several diluents seeking the highest protection for spermatozoa during centrifugation, and
85 (2) to assess the effects of different dilution ratios prior to centrifugation.
86

87 **2. Materials and Methods**

2.1. Materials

Chemicals were of at least Reagent grade and were acquired from Sigma (Madrid, Spain), except YO-PRO-1 and JC-1 (Invitrogen, Barcelona, Spain).

2.2. Animals and sample collection

2.2.1. Brown bear

Semen samples from 15 adult bears were obtained by electroejaculation during the breeding season (end of April to early July) in 2007 (first experiment) and 2008 (second experiment). The animals were housed in a half-freedom regime in Cabárceno Park (Cantabria, Spain; 43º 21' N, 3º 50' W; altitude: 143 m), and fed on a diet based on chicken meat, bread and fruits. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

The animals were immobilized by zolazepam HCl and tiletamine HCl (Zoletil 100®; Virbac, Carros, France) 7 mg/kg and ketamine (Imalgene 1000®; Rhone-Mérieux, Lyon, France) 2 mg/kg applied by teleanaesthesia. They were monitored for the duration of the anaesthesia. After immobilization, the pubic region was cleaned, the penis was washed with sterile physiological saline and the rectum was emptied of faeces. Electroejaculation was carried out with a PT Electronics® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, in average). The bladder was catheterized

during semen collection to prevent urine contamination. Different fractions were collected into different sterile tubes.

2.3. Experimental design

Twenty brown bear ejaculates were used in the first experiment and eleven brown bear ejaculates in the second experiment.

2.3.1. Experiment 1

We evaluated the protective effects of 4 extenders during centrifugation of brown bear semen before cryopreservation and after thawing. Semen aliquots were diluted using a commercial extender (CaninePro, Minitüb, Tiefenbach, Germany) and three extenders prepared in our laboratory, described in Table 1: TCG, TCG-BSA and TCG-yolk. Immediately after collection, each ejaculate was divided into five aliquots. Four of the aliquots were diluted with the same volume of each extender, and the other was left undiluted and used as the control. The five aliquots were centrifuged at 600×g for 6 min in 15-mL glass centrifuge tubes and the supernatant was removed. The samples were resuspended in TTF extender and cryopreserved.

2.3.2. Experiment 2

In this experiment, the effects of different dilution ratios on the centrifugation of brown bear semen were evaluated before and after cryopreservation. Immediately after collection,

each ejaculate was divided into five aliquots. Four of the aliquots were diluted 1:1, 1:4, 1:8 or 1:16 (semen:extender) with TCG (chosen after evaluating media performance in the previous experiment) and the other was left undiluted and used as the control. The five aliquots were centrifuged at 600×g during 6 min in 15-mL glass centrifuge tubes and the supernatant was removed. The samples were resuspended in TTF extender and cryopreserved.

2.4. Cryopreservation of spermatozoa

The post-centrifugation semen pellets were resuspended, at 20 ºC, with the same volume of TTF extender, based in Anel et al. (2003), and modified according to Anel et al., (2010) [TES solution (300 mOsm/kg) and Tris solution (300 mOsm/kg) mixed to pH 7.1, with 4% final volume of D-fructose solution (300 mOsm/kg), 8% glycerol, 20% egg yolk, 2% EDTA and 1% Equex Paste (Minitüb, Tiefenbach, Germany)]. Tubes with diluted samples (at 4% glycerol) were put in glasses containing 100 mL of water at room temperature, and transferred to a refrigerator at 5ºC, thus the temperature decreased slowly to 5ºC (70–80 min). Next, a second 1:1 dilution was made at 5ºC, using the same freezing extender with 12% glycerol, in order to reach a final glycerol concentration of 8%. The sample was adjusted to obtain a final concentration of 100×10^6 spermatozoa/mL by adding TTF extender with 8% glycerol. After equilibration for 1 h at 5ºC, semen was packaged into 0.25 mL plastic straws, and the samples were frozen in a programmable biofreezer (Kryo 10-16 III PlanerTM) at -20ºC/min down to -100ºC, and then transferred to liquid nitrogen containers. The cryopreserved samples were stored in liquid nitrogen for a minimum of one week. Thawing was performed by dropping the straws in water at 65ºC for 6 s, and the semen was expelled into a test tube at room temperature before evaluation.

2.5. Semen evaluation

Samples were evaluated before and after freezing/thawing. The kinematic parameters were assessed by a computer assisted semen motility analysis system (Integrated Semen Analyser System; Proiser, Barcelona, Spain). Samples were diluted (10–20 $\times 10^6$ cells/mL) in a 1% egg yolk buffer (HEPES 20 mm/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7; 320 mOsm/kg), and warmed on a 37.5 ºC plate for 5 min. Then, a 5-µl sperm sample was placed in a Makler counting cell chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase contrast microscope (x10) with a warmed stage (38ºC). The standard parameter settings were as follows: 25 frames/s; 5 to 80 μm^2 for head area; VCL > 10 µm/s to classify a spermatozoon as motile. At least 5 fields or 200 spermatozoa were saved and analysed afterwards. Reported parameters were total motility (TM), progressive motility (PM; defined as VCL>25 and STR>80), curvilinear velocity (VCL, µm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, µm). The parameters cited here have been defined elsewhere (Mortimer et al, 1988).

Sperm viability, acrosomal status and mitochondrial status were analysed according to García-Macías et al. (2007). Sperm viability was assessed with YO-PRO-1 and propidium iodide (PI). Sperm samples were diluted with PBS down to 5 $\times 10^6$ spermatozoa/mL, with 2.5 µM YO-PRO-1 and 10 µM PI. The tubes were incubated at 37ºC for 10 minutes until analysis by flow citometry. The percentage of unstained cells for IP (viable cells without membrane damage) and YO-PRO-1 (non-apoptotic cells with normal membrane permeability) was recorded and termed viable and non-apoptotic spermatozoa.

For acrosomal status, we used the double stain PNA-FITC/PI. Sperm samples were diluted in PBS (5×10^6 spermatozoa/mL), with 10 µM PI and 10 µg/mL PNA-FITC. The tubes were incubated at 37ºC for 10 minutes until analysis by flow citometry. The percentage of viable cells (IP unstained) with an intact acrosome (FITC-PNA unstained) was recorded and termed membrane and acrosome intact spermatozoa.

JC-1 is a green-fluorescence monomeric probe that accumulates in the mitochondria and forms orange fluorescence aggregates if the mitochondrial membrane potential is high, identifying spermatozoa with functional mitochondria. Samples were diluted in PBS (5×10^6 spermatozoa/mL) with 6.8 µM JC-1. The tubes were incubated at 37ºC for 30 minutes until analysis by flow citometry.

For flow citometry, evaluations were carried out using a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with standard optics and an argon-ion laser, tuned at 488 nm and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). In all the cases we assessed 10 000 events per sample, with a flow rate of 200 cells/s.

2.6. Statistical analysis

The R statistical environment (R, 2009) was used for analysing data (results are shown as means and standard errors). To analyse the effects of dilution extender and dilution ratio on pre-freezing and post-thawing sperm parameters, we used linear mixed-effects models. Freezability was also studied, calculating a recovery ratio as the quotient among the post-thawing and pre-freezing result for each parameter. Each fixed effect (dilution extender or dilution ratio) was studied and the Control treatment was used as the intercept of the

model, obtaining the mean effects of the treatments respect to the mean Control values. The ejaculate was included in the models as a random effect.

3. Results

The fresh semen samples of the brown bear selected for this experiment yielded a volume of 3.6 ± 1.2 mL and a sperm concentration of 256.3 ± 98.3 $\times 10^6$ spermatozoa/mL (mean±SD). The average quality of fresh semen was TM: 68.0 % ± 4.9, PM: 34.2 % ± 3.8 and sperm viability: 69.0 % ± 5.1 (Mean ± S.E.M).

3.1. Experiment 1: Effect of centrifugation media on sperm quality

Before freezing, centrifuged diluted samples had higher values of motility than control undiluted samples (Table 2). In particular, CaninePro samples increased significantly TM (71.9 %), VCL (105.5 µm/s) and ALH (4.5 µm) compared with the control (62.7%, 91.2 µm/s and 3.9 µm, respectively).

In general, diluting semen samples with any medium prior to centrifugation improved post-thawing quality of spermatozoa in comparison with the undiluted control, especially motility (Table 2). Samples diluted with CaninePro yielded a higher post-thawing TM than the control (14.6±4.0 increase; $P<0.001$). VCL was higher for CaninePro (90.0 µm/s, $P=0.002$), TCG-BSA (87.3 µm/s, $P=0.028$) and TCG (85.8 µm/s, $P=0.011$) than the control (76.7 µm/s). ALH was about 0.6 µm higher than the control when the samples were diluted with CaninePro ($P=0.004$), and about 0.4 µm higher using TCG ($P=0.023$).

Prefreezing analysis by flow citometry (Table 3) did not show differences among extenders and the control regarding the percentage of viable non-apoptotic spermatozoa (YO-PRO-

1–/PI–), percentage of membrane and acrosome-intact spermatozoa (PNA–/PI–) or mitochondrial status (JC-1 orange). The flow citometry analysis of post-thawing samples (Table 3) did not show differences in the percentage of viable non-apoptotic spermatozoa (YO-PRO-1–/PI–), but CaninePro (43.2 %, P=0.034) and TCG (43.4 %, P=0.023) significantly improved the percentage of viable and acrosome-intact spermatozoa respect to the undiluted control (39.4 %). Mitochondrial status did not significantly change among treatments.

Sperm freezability analysis (post-thawing/pre-freezing ratios; Figure 1) showed that the TM ratio of CaninePro was significantly higher than the control (P=0.005). Ratios for other motility and viability parameters did not show differences among extenders, except for LIN. The recovery of this parameter for TCG was significantly lower than for the control.

3.2. Experiment 2: Effect of dilution ratios before centrifugation on sperm quality

In prefreezing samples, motility parameters, except TM, were significantly modified for all dilution ratios (Table 4). All dilutions ratios significantly decreased PM and LIN, but increased VCL and ALH. Post-thawing sperm motility was affected by the dilution ratio prior to centrifugation (Table 4). VCL increased significantly in all the dilutions in comparison with the control values (from 88.7 µm/s in 1:1 to 96.6 µm/s in 1:8 compared to 77.2), whereas LIN decreased and ALH increased significantly for all dilutions ($P<0.01$), with the exception of 1:1 dilution. TM was significantly improved only in the 1:4 dilution (P=0.033). The PM of the spermatozoa diluted 1:16 was significantly reduced in comparison with the control (-5.7%, P=0.042).

Pre-freezing flow citometry analysis (Table 5) showed that 1:16 dilution ratio significantly decreased the percentage of viable spermatozoa respect to control (72.9 vs. 79.8, P=0.017). Prefreezing results did not show differences among extenders and the control regarding the percentage of membrane and acrosome-intact spermatozoa or mitochondrial status. The post-thawing percentage of viable cells with intact acrosome was significantly higher in all diluted samples than in control, achieving higher results for 1:4 (64.5 % vs. 54.4 %).
The recovery rates of non-apoptotic viable spermatozoa increased significantly (Figure 2) with the dilution rate, (1:4= +8.1±3.6; 1:8= +8.0±3.6; 1:16= +9.1±3.6) respect to control (69.7±3.5 %). The recovery ratio for PM was higher for dilution 1:1 (+45.9±14.0, P=0.002) and dilution 1:8 (+32.1±14.0, P=0.027) than the control (80.1±14.0 %). VCL recovery was lower for the 1:16 dilution rate (-15.6±6.6, P=0.023) than the control (96.9±5.5 μm/s).

4. Discussion

Centrifugation is a critical step in the cryopreservation protocol when it is necessary to increase sperm concentration or ameliorate urine contamination. We have noticed elevated individual variability among semen samples from different bears, which may influence the results of semen centrifugation (unpublished data). Nevertheless, we have also found an overall positive effect of centrifugation on the samples prior to cryopreservation.
Considering the results obtained in this study, brown bear could be classified among the centrifugation-resilient species, such as stallion (Brinsko et al, 2000; Sieme et al, 2004; Waite et al, 2008), rat (Varisli et al, 2009) and boar (Carvajal et al, 2004; Matas et al, 2007). Rijsselaere et al. (2002) showed that centrifugation of canine sperm increased post-thaw progressive motility and viability. In addition, we have shown that diluting brown bear semen prior to centrifugation was advantageous to sperm quality. Shäfer-Somi et al.

(2006), in their assays in dog spermatozoa, concluded that sperm dilution prior to centrifugation exerted a positive effect, but that the procedure required special diluents. The choice of the extender used for diluting the semen before centrifugation hardly affected post-thaw sperm quality. In the brown bear, the commercial extender CaninePro and our TCG medium improved the general sperm quality in comparison with the undiluted control. Thus, although TCG could not improve motility as much as CaninePro, it might be an adequate choice for further research on bear semen. The composition of commercial media is not always known, hampering the interpretation of certain results. Therefore, having a diluent of known composition and which equivalent results to commercial media is a benefit taking into account future studies.

Different studies on electroejaculation of bears have indicated an elevated incidence of urospermia, with the consequent loss of semen quality (Okano et al, 2004b; Chen et al, 2007; Kojima et al, 2001; Anel et al, 2008). Thus, we considered that developing a protocol for washing sperm samples was critical for the success of a sperm recovery plan in the brown bear. The 1:4 dilution improved post-thawing results for motility, while not having deleterious effects in others parameters, but future research should clarify whether the observed modification in the kinetic pattern of motility would be detrimental when applying these spermatozoa in a reproductive procedure. Conversely, the highest dilution ratio, 1:16, decreased progressive motility after thawing. Similar results are observed in stallion (Pickett et al., 1975), and, therefore, these authors recommended seminal dilution before centrifugation at 1:4 and 1:8 dilution ratios.

In conclusion, the effect of diluting brown bear semen before centrifugation on post-thawing quality was positive overall. Based on our results, both CaninePro and TCG could be good options as extender to brown bear semen, but TCG might be preferable for further

research, since it has a known composition. The optimal dilution ratio of brown bear semen before centrifugation seems to be 1:4.

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420

FIGURE LEGENDS

Figure 1.

Means of post-thawing recovery ratio (post-thawing/pre-freezing, in %) of total motility (TM, %), curvilinear velocity (VCL, µm/s), linearity velocity (LIN, %) and viability (YO-PRO-1–, %) observed in different extender. CP: CaninePro; TCG: base extender; TCG-B: extender with BSA; TCG-y: extender with egg yolk. Asterisks indicate differences of the given extender with the control (* $P<0.05$).

Figure 2.

Means of post-thawing recovery ratios (post-thawing/pre-freezing, in %) of total motility (TM, %), curvilinear velocity (VCL, µm/s), linearity velocity (LIN, %) and viability (YO-PRO-1–, %) observed in different dilution ratio. Asterisks indicate differences of the given ratio with the control (* $P<0.05$).

435

436 Table 1. Composition of the self-made centrifugation extenders used in this study.

	TCG	TCG-BSA	TCG-yolk
TRIS	200 mM	200 mM	200 mM
Glucose	70 mM	70 mM	70 mM
Citric acid	63 mM	63 mM	63 mM
Benzylpenicillin	1000 IU/ml	1000 IU/ml	1000 IU/ml
Dihidrostreptomicin	1 mg/ml	1 mg/ml	1 mg/ml
BSA		3%	
Egg yolk			1%

437

Table 2

Effect of different centrifugation extenders (Mean ± S.E.M.) on sperm motility of thawed semen.

		TM	PM	VCL	LIN	ALH
Pre-freezing						
	Control	62,7 ± 4,0	20,5 ± 3,2	91,2 ± 7,8	41,3 ± 2,2	3,9 ± 0,3
	CaninePro	71,9 ± 4,1*	21,4 ± 3,7	105,5 ± 5,1**	39,9 ± 2,3	4,5 ± 0,2**
	TCG	68,1 ± 4,0	22,1 ± 2,4	101,2 ± 6,2*	43,2 ± 2,3	4,2 ± 0,3
	TCG-BSA	68,3 ± 3,9	19,4 ± 2,7	95,4 ± 5,7	40,6 ± 2,5	4,0 ± 0,2
	TCG-yolk	69,5 ± 4,1	18,6 ± 2,5	101,7 ± 6,9*	39,7 ± 2,0	4,3 ± 0,3*
Post-thawing						
	Control	38,5 ± 4,9	10,8 ± 2,1	76,7 ± 5,5	35,0 ± 0,9	3,6 ± 0,2
	CaninePro	53,1 ± 3,4***	12,4 ± 2,0	90,0 ± 6,2**	35,3 ± 1,0	4,2 ± 0,3**
	TCG	46,1 ± 4,2	8,8 ± 1,4	87,3 ± 5,7*	33,3 ± 0,8	4,1 ± 0,2*
	TCG-BSA	43,5 ± 3,9	9,0 ± 1,6	85,8 ± 5,9*	34,0 ± 0,9	4,0 ± 0,2
	TCG- yolk	46,7 ± 4,3	9,2 ± 1,5	84,9 ± 6,0	34,1 ± 1,0	4,0 ± 0,3

TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (µm/s); LIN: linearity of the curvilinear trajectory (%); ALH: amplitude of lateral head displacement (µm). TCG: base extender; TCG-BSA: extender with BSA; TCG-yolk: extender with egg yolk.

* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate differences between the given treatments and the control within each species.

Table 3

Effect of different centrifugation extenders (Mean ± S.E.M.) on sperm viability (YO-PRO-1/PI), acrosomal status (PNA-FITC/PI) and mitochondrial status (JC-1) of thawed semen.

		YO-PRO-1–	PI–/PNA–	JC-1+
Pre-freezing				
	Control	75,1 ± 4,1	72,7 ± 2,2	38,5 ± 5,9
	CaninePro	72,3 ± 4,3	71,3 ± 3,0	34,6 ± 5,9
	TCG	74,4 ± 4,3	72,4 ± 2,4	37,5 ± 6,0
	TCG-BSA	71,6 ± 4,3	71,1 ± 2,7	36,6 ± 6,2
	TCG-yolk	74,2 ± 3,7	70,8 ± 2,6	30,3 ± 5,6
Post-thawing				
	Control	42,6 ± 4,7	39,4 ± 4,0	33,5 ± 5,4
	CaninePro	41,1 ± 5,0	43,2 ± 4,2*	34,0 ± 4,8
	TCG	46,4 ± 5,3	43,4 ± 3,9*	29,5 ± 4,6
	TCG-BSA	43,3 ± 6,1	40,8 ± 4,7	31,0 ± 4,3
	TCG-yolk	44,3 ± 5,6	38,0 ± 4,2	33,9 ± 4,8

YO-PRO-1–: percentage of viable, non-apoptotic, spermatozoa, PI–/PNA–: percentage of cells with intact membrane and acrosome, JC-1+: percentage of spermatozoa with high mitochondrial membrane potential. TCG: base extender; TCG-BSA: extender with BSA; TCG-yolk: extender with egg yolk.

* $P<0.05$ indicates differences between the given treatments and the control.

462
463 Table 4
464 Effect of different dilution ratios prior to centrifugation (Mean ± S.E.M.) on sperm motility
465 parameters of thawed semen.
466

		TM	PM	VCL	LIN	ALH
Pre-freezing						
	Control	70,6 ± 6,8	37,6 ± 4,5	83,2 ± 6,5	45,8 ± 2,4	3,2 ± 0,3
	1:1	78,7 ± 5,5	27,9 ± 3,8**	107,7 ± 7,2**	35,7 ± 1,4***	4,4 ± 0,4**
	1:4	79,2 ± 5,8	29,4 ± 3,4**	112,6 ± 7,0***	34,4 ± 1,7***	4,7 ± 0,4***
	1:8	70,1 ± 8,5	29,3 ± 4,9**	113,6 ± 9,5***	34,1 ± 1,4***	4,9 ± 0,5***
	1:16	66,1 ± 8,3	28,7 ± 4,2**	116,7 ± 8,3***	34,3 ± 1,0***	5,1 ± 0,4***
Post-thawing						
	Control	67,2 ± 6,5	27,6 ± 3,4	77,2 ± 4,5	36,1 ± 1,5	3,5 ± 0,2
	1:1	75,3 ± 6,1	30,3 ± 3,1	88,7 ± 3,6*	35,3 ± 2,1	3,9 ± 0,2
	1:4	78,8 ± 6,4*	28,3 ± 2,3	93,1 ± 3,6**	32,0 ± 0,7**	4,4 ± 0,2**
	1:8	70,1 ± 7,7	25,0 ± 3,4	96,6 ± 5,0**	31,4 ± 1,0***	4,5 ± 0,2***
	1:16	65,4 ± 8,0	21,8 ± 3,0*	94,0 ± 6,8**	30,6 ± 0,7***	4,4 ± 0,3***

467 TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (µm/s);
468 LIN: linearity of the curvilinear trajectory (%); ALH: amplitude of lateral head
469 displacement (µm).
470 * P<0.05, ** P<0.01 and *** P<0.001 indicate differences between the given treatments
471 and the control.
472

Table 5

Effect of different dilution ratios prior to centrifugation (Mean ± S.E.M.) on sperm viability (YO-PRO-1/PI), acrosomal status (PNA-FITC/PI) and mitochondrial status (JC-1) of thawed semen.

		YO-PRO-1–	PI–/PNA–	JC-1+
Pre-freezing				
	Control	79,8 ± 2,0	73,1 ± 2,7	32,0 ± 4,9
	1:1	80,6 ± 2,9	78,8 ± 2,3	27,9 ± 5,4
	1:4	79,6 ± 2,8	78,1 ± 2,8	27,9 ± 6,5
	1:8	75,9 ± 4,7	74,4 ± 4,1	26,9 ± 4,3
	1:16	72,9 ± 4,9*	71,9 ± 4,3	28,2 ± 4,6
Post-thawing				
	Control	55,5 ± 2,7	54,4 ± 2,7	37,0 ± 4,9
	1:1	60,7 ± 3,0	62,9 ± 1,7*	35,0 ± 4,4
	1:4	61,3 ± 2,8	64,5 ± 1,6**	29,7 ± 3,0
	1:8	59,2 ± 4,0	61,9 ± 4,1*	30,7 ± 4,6
	1:16	56,7 ± 3,9	61,7 ± 3,9*	35,3 ± 4,1

YO-PRO-1–: percentage of viable, non-apoptotic, spermatozoa, PI–/PNA–: percentage of cells with intact membrane and acrosome, JC-1+: percentage of spermatozoa with high mitochondrial membrane potential.

* $P<0.05$ and ** $P<0.01$ indicate differences between the given treatments and the control.

Figure 1.

Means of post-thawing recovery ratio (post-thawing/pre-freezing, in %) of total motility (TM, %), curvilinear velocity (VCL, µm/s), linearity velocity (LIN, %) and viability (YO-PRO-1–, %) observed in different extender. CP: CaninePro; TCG: base extender; TCG-B: extender with BSA; TCG-y: extender with egg yolk. Asterisks indicate differences of the given extender with the control (* P<0.05).

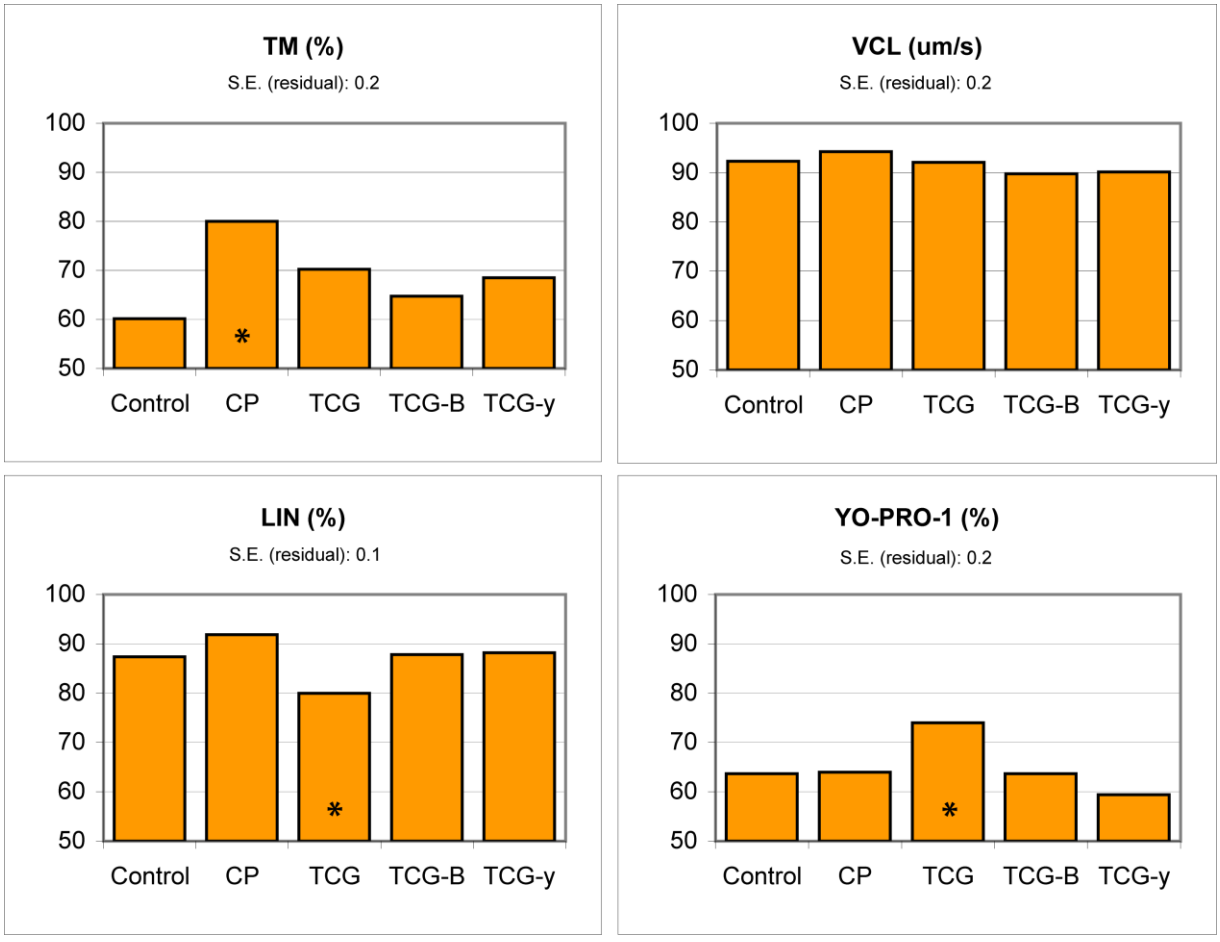

Figure 2.

Means of post-thawing recovery ratios (post-thawing/pre-freezing, in %) of total motility (TM, %), curvilinear velocity (VCL, µm/s), linearity velocity (LIN, %) and viability (YO-PRO-1–, %) observed in different dilution ratio. Asterisks indicate differences of the given ratio with the control (* P<0.05).