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A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality

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Abstract

The optimization of cryopreservation extenders is a fundamental issue for adequately performing germplasm banking on wild species. We have tested two glycerol concentrations (4 and 8%), and three extender osmolalities (320, 380 and 430 mOsm/kg; before adding cryoprotectants), for cryopreservation of epididymal and ejaculated sperm samples from Iberian red deer. All the extenders were based on Tes–Tris and fructose (for osmolality adjustment), and complemented with 20% egg yolk. Epididymal and ejaculated sperm samples were obtained from the cauda epididymis (post-mortem) and using electroejaculation, respectively. Samples were diluted 1:1 with each extender and equilibrated for 2 h at 5 °C. Then, they were diluted down to 100×10^6 sperm/mL and frozen at -20 °C/min. Post-thawed samples were assessed for motility (CASA), HOS test, proportion of swollen (osmotically challenged) cells in the untreated sample, viability and acrosomal status. For epididymal samples, 8% glycerol rendered a slightly higher proportion of intact acrosomes on viable spermatozoa than 4%; regarding extender osmolality, 380 and 430 mOsm/kg rendered higher motility results, and the 430 mOsm/kg yielded the lowest proportion of swollen spermatozoa. For ejaculated samples, 4% glycerol yielded more viable spermatozoa than 8%; for extender osmolality, 320 mOsm/kg rendered the highest percentages of progressively motile and viable spermatozoa, although 380 mOsm/kg extender was not significantly different. These results show that sample source influence extender suitability, and that extenders should be isoosmotic or rather slightly hyperosmotic. Future studies should test multiple glycerol concentrations and extender osmolalities in order to adjust them to these kinds of sample.

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Keywords: Sperm cryopreservation; Epididymis; Electroejaculation; Extender; Red deer

1. Introduction

The composition of semen extenders is well known to affect the outcome of spermatozoa cryopreservation [1]. Not only cryoprotectants, but also sugars, buffer systems and other additives interact during the freezing-

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thawing process, often in a very specific manner, thus a lot of formulations have been proposed for different species [2,3]. In the case of wild species, available extenders are frequently adapted from others from domestic species, with little or no modifications. However, due to the importance of species differences, it is important to study each case in order to refine these extenders, to obtain optimal results.

There are few comparative studies on extenders for cervid semen [4,5], despite of the interest of these species, not only because of their ecological value, but also as trophies and farm animals [6], especially in the case of the red deer (*Cervus elaphus*). The creation of germplasm banks would greatly improve their management, both for economical and conservationist purposes, and many successful experiences on semen cryopreservation and insemination have been reported [7–13], using extenders usually adapted from formulations for bull or ram semen, but more detailed studies are scarce.

Among cryoprotectants, glycerol is considered as the most effective for red deer, such as in other ruminants. Nevertheless, an optimal concentration has not been defined yet, since comparative studies were generally carried out using extenders varying on many parameters (buffer, sugars, glycerol concentration, etc), thus making difficult to take decisions about the most adequate glycerol concentration. Reported concentrations ranged from 5 to 10% [7–13], but results seem to depend rather on other components or on the followed protocols than on glycerol concentration. Thus, extenders with the same glycerol concentration have frequently yielded different results, and vice versa [5].

Other important aspect in extender composition is the osmolality due to its impermeant components. During freezing and thawing, spermatozoa undergo dehydration and rehydration, which have an important impact in sperm endurance to cryopreservation, because dehydration decreases the risk of intracellular ice crystal formation (although excessive dehydration is detrimental too) [14–16]. Thus, adjusting the osmolality of the extender is of great importance, since it would influence the amount of intracellular water during the different steps of cryopreservation, which has been an issue subjected to study on many papers [17–22]. In general, it has been found that spermatozoa from different species respond differently to changes in the osmolality of the surrounding media, and that the osmolality of the extender is an important factor for the outcome of the cryopreservation [21]. However, these studies have been limited to several domestic species and human spermatozoa, and, taking into account the

variability found, it is not possible to merely extrapolate these results to other species.

Currently, there are few studies dealing with the cryopreservation and utilization of epididymal samples (obtained post-mortem) from red deer [10,13,23–25], despite of the value of these kinds of samples for germplasm banking of wild animals [26]. Although the use of extenders adapted from other ruminants have rendered good results, it would be highly desirable to optimize them in order to attain a good availability of specific extenders.

In this study, we have tested the effect of two glycerol concentrations and three extender osmolalities (previously to cryoprotectant addition) in the post-thawing quality of red deer spermatozoa, obtained by electroejaculation and by post-mortem recovery. This work must be considered within a more general plan to develop optimized extenders for cryopreservation of sperm from wild ruminants, in order to utilize them in the setting-up of germplasm banks. Besides to this practical approach, we are carrying out experiments to study basic aspects of sperm cryobiology, which may be helpful to understand and develop efficient cryopreservation protocols, not only for this species, but also for others.

2. Materials and methods

2.1. Animals

Epididymal samples were collected from 38 Iberian red deer (*C. elaphus hispanicus*, Helzheimer 1909) harvested in the game reserves of Ancares, Mampodre and Picos de Europa (León, Spain) and in several private hunting reserves of the region of Cáceres (Spain). All the animals were adults and lived in a free-ranging regime. Harvest plans followed Spanish Harvest Regulation, Law 4/96 of Castilla y León and Law 19/01 of Extremadura, which conforms to European Union Regulation. Furthermore, species and number of individuals that can be hunted, as well as the exact periods of the year when hunting can take place, are reviewed each year by the Annual Hunting Regulation of the respective regions.

Electroejaculation samples were obtained from 9 mature Iberian red deer stags. Animals (2–5 year old; 100–150 kg) were housed in a half-freedom regime at the University of Castilla-La Mancha (ETSIA, Albacete, Spain), maintained under natural daylength conditions at a latitude of 38°57'N. During the experiment they lived in an artificial meadow of 6500 m² composed of *Festuca arundinacea* (52.4%),

143
144 *Dactylis glomerata* (28.6%), *Medicago sativa* (14.3%)
145 and *Trifolium repens* (4.8%). Fodder was based on
146 barley, alfalfa and oats, and animals had ad libitum
147 access to cereal straw and water. Animal handling was
148 performed in accordance with the Spanish Animal
149 Protection Regulation, RD223/1998, which conforms to
150 European Union Regulation 86/609 and adheres to
151 guidelines established in the Guide for Care and Use of
152 Laboratory Animals as adopted and promulgated by the
153 American Society of Andrology.

154 In both cases, samples were collected during the
155 breeding season (autumn).

156 2.2. Post-mortem sperm recovery

157 Scrotum, including testicles and epididymes, was
158 removed from the carcass and refrigerated down to 5 °C
159 as soon as possible. Date and time of death, collection
160 and refrigeration were noted and attached to the
161 corresponding sample. Refrigerated genitalia were sent
162 to our laboratory at the Veterinary Clinic Hospital of the
163 University of León (Spain). Post-mortem time ranged
164 between 10 and 20 h, which we considered acceptable
165 for maintaining sperm quality [27].

166 Sample manipulation was carried out in a walk-in
167 fridge (5 °C). Testicles with epididymes and vas
168 deferens attached were isolated from the scrotum
169 and other tissues. Epididymes were dissected free from
170 the testicles, and cleaned of connective tissue. To avoid
171 blood contamination, superficial blood vessels were
172 previously cut and their contents wiped out. Sperma-
173 tozoa were collected making several incisions on the
174 cauda epididymis with a surgical blade, and taking the
175 liquid emerging from the cut tubules with the aid of the
176 blade. Osmolality of each sample was measured using a
177 cryoscopic osmometer (Osmomat-030, GonotecTM;
178 Berlin).

179 2.3. Electroejaculation procedure

180 Previously to electroejaculation, stags were
181 restrained and anaesthetised with intravenous injec-
182 tion of xylacine and ketamine (Rompun[®] 2% and
183 Imalgene 1000[®]). Rectum was cleaned from faeces
184 and the prepucial area was shaved and washed with
185 physiological saline serum. Electroejaculation was
186 carried out using a 3-electrode probe (250 mm
187 × 30 mm) connected to a power source which allowed
188 voltage and amperage control. Ejaculation occurred at
189 average values of 4.5 V and 90 mA (average time to
190 ejaculation: 3 min 44 s). Anesthesia was reverted with
191 yohimbine 0.9%. Osmolality of ejaculates was

191 measured in the same manner than for post-mortem
192 samples. 193

194 2.4. Preparation of the extenders

195 All chemicals were acquired from Sigma (Madrid,
196 Spain). The extenders were based in a Tes–Tris
197 buffering system, using fructose for adjusting osmol-
198 ality and complemented with egg yolk and glycerol as
199 cryoprotectants [28]. In order to prevent variations due
200 to egg yolk, it was obtained from fresh hen eggs in
201 aseptic conditions, and all the yolks were pooled. All the
202 extenders used for each experiment were prepared the
203 same day and using the same egg yolk pool.

204 For the comparison of glycerol concentrations on
205 epididymal samples, two extenders were prepared as
206 follows: a buffered solution (Tes 231.1 mmol/L, Tris
207 77.1 mmol/L and fructose 121.9 mmol/L in milli-Q
208 water; pH 7.2 and 430 mOsm/kg) was prepared and
209 double-filtered through a cellulose acetate membrane
210 (0.22 μm pore); then, it was complemented with 15%
211 egg yolk and centrifugated (3000 × g, 30 min),
212 discarding the sediment. Finally, we added 4 or 8%
213 glycerol (final concentration), depending on the variant,
214 which were called G4 and G8, respectively. For the
215 experiment on electroejaculated samples, the same
216 procedure was followed, but we added 16.9 mmol/L of
217 fructose, so the osmolality of the solution before adding
218 glycerol and egg yolk was 320 mOsm/kg.

219 The extenders used in the study of extender
220 osmolalities were prepared similarly. In this case,
221 fructose concentration was adjusted to obtain an
222 osmolality (before adding egg yolk and glycerol) of
223 320 mOsm/kg (16.9 mmol/L), 380 mOsm/kg (74.7
224 mmol/L) or 430 mOsm/kg (121.9 mmol/L). Then, the
225 solution was complemented with egg yolk (15%) and
226 glycerol, as described above. Glycerol concentration
227 was 4% for electroejaculated samples and 8% for
228 epididymal samples. The three extenders were called
229 E320, E380 and E430, accordingly to the adjusted
230 osmolalities.

231 2.5. Cryopreservation protocol

232 Samples were diluted 1:1 in the corresponding
233 extender. This dilution was carried out at 5 °C for
234 epididymal samples (the same temperature they arrived
235 at our laboratory) and at ambient temperature for
236 electroejaculated samples. The tubes containing the
237 later were then put in glasses containing 100 mL of
238 water at ambient temperature and transferred to 5 °C, so
239 temperature decreased smoothly. After resting 2 h at

239 5 °C, the sample was further diluted with the same
 240 extender down to 100×10^6 sperm/mL and packed in
 241 0.25 mL French straws. Freezing was carried out using
 242 a programmable biofreezer (Planner MRII[®]), at
 243 -20 °C/min down to -100 °C, and then transferred
 244 to liquid nitrogen containers. Thawing was performed
 245 by dropping the straws in water at 65 °C for 6 s.
 246

2.6. Motility analysis

247 Thawed samples were diluted down to $10\text{--}20 \times$
 248 10^6 spermatozoa/mL in PBS (pH 7.5, 320 mOsm/kg). A
 249 $5 \mu\text{L}$ drop was examined with a phase contrast
 250 microscope (Nikon Labophot-2; negative contrast
 251 optics), on a warming stage at 37 °C. We used a CASA
 252 system to analyze motility (Motility Analyzer v. 7.4 G,
 253 Hamilton-Thorne Research[™]), collecting the follow-
 254 ing parameters for further analysis: total motility (%;
 255 TM), progressive motility (%; PM), average path
 256 velocity ($\mu\text{m/s}$; VAP) and linearity (%; LIN). Detailed
 257 explanation of these descriptors of sperm movement has
 258 been provided elsewhere [29]. At least 5 fields and 200
 259 cells were recorded at $\times 200$, using an image acquisition
 260 rate of 25 frames/s and an acquisition time of 0.8 s.
 261 Progressive motility was defined as the percentage of
 262 total spermatozoa with VAP $> 25 \mu\text{m/s}$ and straightness
 263 (STR) $> 80\%$.
 264

2.7. HOS test

265 The functional integrity of the sperm plasma
 266 membrane was evaluated using the hypoosmotic
 267 swelling test (HOS test). Five microliters of sample
 268 were diluted in $500 \mu\text{L}$ of a hypoosmotic sodium citrate
 269 solution (100 mOsm/kg). After 18 min at room tem-
 270 perature, samples were fixed with a drop of glutar-
 271 aldehyde solution. Responsiveness to the test was
 272 determined by counting 100 cells with a phase-contrast
 273 microscope ($\times 400$). The percentage of positive cells
 274 (those with a swollen flagelle) was recorded for each
 275 sample [30].
 276

2.8. Assessment of osmotically challenged spermatozoa

277 We determined the percentage of spermatozoa with
 278 swollen tails (osmotically challenged) in the untreated
 279 samples ($5 \mu\text{L}$ of thawed sample fixated in $500 \mu\text{L}$
 280 of the glutaraldehyde solution), in order to assess if there
 281 were spermatozoa with signs of osmotic stress in the
 282 samples (OSM).
 283
 284

2.9. Evaluation of sperm viability and acrosomal status

285 Samples (pre-freezing and post-thawed) were diluted
 286 in buffered media (1:100, same composition that the one
 287 used for motility analysis), and stained with propidium
 288 iodide (PI; $25 \mu\text{g/L}$) and PNA lectin conjugated with
 289 FITC ($1 \mu\text{g/mL}$). After 10 min, the samples were
 290 analyzed using a FACSCalibur flow cytometer (Becton
 291 Dickinson, Franklin Lakes, NJ). We used a 15 mW
 292 argon-laser that provided an excitation wavelength of
 293 488 nm , using the FL3 photodetector channel to read
 294 the red emission light of PI (650 nm long pass filter), and the
 295 FL1 photodetector channel to read the green emission
 296 light of FITC ($530/30 \text{ nm}$ band pass filter). At least 10,000
 297 events (spermatozoa detected, after discarding debris)
 298 were acquired. The fluorescent technique allows to
 299 distinguish among four sperm populations: red (non-
 300 viable, acrosome intact), red-green (non-viable, acro-
 301 some damaged), green (viable, acrosome damaged) and
 302 non-stained (viable; acrosome intact) spermatozoa [31].
 303 For data analysis, we used the percentage of viable
 304 spermatozoa (green + non-stained cells; VIAB) and the
 305 proportion of viable spermatozoa with intact acrosomes
 306 respect to the total percentage of viable spermatozoa
 307 (non-stained/viable $\times 100$; ACR).
 308
 309

2.10. Statistical analysis

310 Statistical analysis were carried out using the SAS/
 311 STAT[™] program (SAS Inc., Cary, NC). Previously, we
 312 transformed our data using arc sine (percentages) or
 313 decimal logarithm. Treatment comparison (glycerol
 314 concentration or extender osmolality) was performed by
 315 ANOVA, with sample and treatment (glycerol concen-
 316 tration or extender osmolality) as factors of variation.
 317 Treatments (whenever this factor was significant) were
 318 compared using Tukey–Kramer multiple comparison
 319 for adjusted means.
 320

3. Results

3.1. Epididymal samples

321 Of the 38 post-mortem samples, 12 were used for
 322 comparing glycerol concentrations and 26 for the
 323 extender osmolality experiment. Osmolality of epidi-
 324 dymal samples was $387 \pm 27 \text{ mOsm/kg}$ (mean \pm S.D.),
 325 which is in agreement with a previous study on red deer
 326 epididymal samples [27].
 327
 328

329 Almost no differences were detected among samples
 330 frozen using 4 or 8% glycerol. As Table 1 shows, neither

Table 1

Post-thawing results for the two glycerol concentrations (G4: 4%; G8: 8%) assayed on epididymal sample (adjusted means and S.E.M.)

Parameter	G4	G8
TM (%)	47.08 ± 7.34	48.25 ± 9.66
PM (%)	17.50 ± 3.75	16.83 ± 4.38
VAP (µm/s)	45.58 ± 1.74	43.30 ± 2.05
LIN (%)	52.17 ± 1.30	48.67 ± 1.54
HOS test (%)	54.55 ± 1.80	55.91 ± 1.80
OSM (%)	7.44 ± 0.63	6.33 ± 0.63
VIAB (%)	56.30 ± 2.50	55.00 ± 3.15
ACR (%)	93.14 ± 1.57 a	95.56 ± 1.69 b

TM: total motility; PM: progressive motility; OSM: spermatozoa with a swollen tail; VIAB: sperm viability; ACR: % of acrosome-intact sperm within VIAB. Different letters indicate that columns differ $P < 0.05$.

Table 3

Post-thawing results for the two glycerol concentrations (G4: 4%; G8: 8%) assayed on electroejaculated samples (adjusted means and S.E.M.)

Parameter	G4	G8
TM (%)	63.75 ± 5.81	52.63 ± 5.81
PM (%)	42.50 ± 3.19	36.00 ± 3.19
VAP (µm/s)	60.36 ± 2.07	53.50 ± 1.82
LIN (%)	76.41 ± 6.46	65.13 ± 5.69
HOS test (%)	27.38 ± 1.72	26.38 ± 1.72
OSM (%)	0.38 ± 0.08	0.25 ± 0.08
VIAB (%)	45.13 ± 1.84 a	38.25 ± 1.83 b
ACR (%)	99.06 ± 0.33	99.25 ± 0.33

TM: total motility; PM: progressive motility; OSM: spermatozoa with a swollen tail; VIAB: sperm viability; ACR: % of acrosome-intact sperm within VIAB. Different letters indicate that columns differ $P < 0.05$.

330 motility nor viability were significantly affected by this
331 factor. Only acrosomal status seemed to be better
332 preserved when the 8% concentration was used,
333 although the difference was minimal.

334 The comparison between the three osmolalities of
335 the extenders showed that total motility of post-thawed
336 sperm was significantly lower when using E320
337 (Table 2). Besides, there was a decreasing proportion
338 of spermatozoa with swollen tails (OSM, osmotically
339 stressed) with increasing extender osmolality. Results
340 for other parameters were very similar in the three
341 extenders.
342

3.2. Electroejaculated samples

343 A total of 32 electroejaculations were carried out,
344 using 8 samples for comparing glycerol concentrations
345 and 24 for the extender osmolality experiment.
346

Table 2

Post-thawing results for the three extender osmolalities (E320: 320 mOsm/kg; E380: 380 mOsm/kg; E430: 430 mOsm/kg) assayed on epididymal samples (adjusted means and S.E.M.)

Parameter	E320	E380	E430
TM (%)	41.90 ± 2.20 a	50.47 ± 2.02 b	50.68 ± 2.13 b
PM (%)	13.35 ± 1.07	16.48 ± 0.98	16.77 ± 1.04
VAP (µm/s)	45.21 ± 1.26	43.08 ± 1.17	44.09 ± 1.18
LIN (%)	49.33 ± 0.96	48.03 ± 0.89	48.27 ± 0.90
HOS test (%)	54.09 ± 1.26	52.00 ± 1.21	52.09 ± 1.25
OSM (%)	10.57 ± 0.42 a	9.34 ± 0.30 b	7.21 ± 0.42 c
VIAB (%)	51.92 ± 1.47	52.92 ± 1.41	53.18 ± 1.47
ACR (%)	96.66 ± 0.56	98.09 ± 0.53	98.57 ± 0.56

TM: total motility; PM: progressive motility; OSM: spermatozoa with a swollen tail; VIAB: sperm viability; ACR: % of acrosome-intact sperm within VIAB. Different letters indicate that columns differ $P < 0.05$.

346 Osmolality of electroejaculated samples was
347 336 ± 44 mOsm/kg (mean ± S.D.).
348

349 As in the case of the epididymal samples, there was
350 little difference between the results obtained using 4 or
351 8% glycerol for cryopreservation (Table 3). However, in
352 this case 4% glycerol rendered higher mean results, but
353 significance was only reached for viability.

354 Table 4 shows results for the extender osmolality
355 comparison. In this case, E320 rendered significantly
356 higher results for progressive motility and sperm
357 viability than E430. There were no significant
358 differences among E380, which yielded intermediate
359 values, and the other two extenders.

4. Discussion

360 In this study, we have noticed some interesting
361 differences regarding extender suitability for red deer
362

Table 4

Post-thawing results for the three extender osmolalities (E320: 320 mOsm/kg; E380: 380 mOsm/kg; E430: 430 mOsm/kg) assayed on electroejaculated samples (adjusted means and S.E.M.)

Parameter	E320	E380	E430
TM (%)	47.96 ± 2.39	44.77 ± 2.39	42.24 ± 2.48
PM (%)	31.19 ± 1.67 a	29.52 ± 1.67 a,b	26.16 ± 1.73 b
VAP (µm/s)	59.92 ± 1.94	60.50 ± 1.97	57.89 ± 2.05
LIN (%)	56.77 ± 5.33	64.16 ± 5.39	70.64 ± 5.61
HOS test (%)	22.57 ± 0.74	21.57 ± 0.74	21.32 ± 0.74
OSM (%)	0.54 ± 0.17	0.33 ± 0.17	0.29 ± 0.16
VIAB (%)	49.15 ± 1.46 a	44.15 ± 1.46 a,b	42.87 ± 1.46 b
ACR (%)	98.83 ± 0.49	98.80 ± 0.49	97.65 ± 0.49

TM: total motility; PM: progressive motility; OSM: spermatozoa with a swollen tail; VIAB: sperm viability; ACR: % of acrosome-intact sperm within VIAB. Different letters indicate that columns differ $P < 0.05$.

sperm cryopreservation, depending on sample source (ejaculate or epididymis). We took into account the osmolality of the epididymal and ejaculated samples that we had obtained in previous studies [27,32,33] for the design of this experiment. Therefore, we carried out firstly the comparison of glycerol concentrations using for each kind of sample the extender we considered more adequate (E320 for electroejaculated samples and E430 for epididymal samples). Once we had an estimation of the most suitable glycerol concentration (4% for electroejaculated samples and 8% for epididymal samples), we prepared the extenders for the comparison of extender osmolalities, using the corresponding glycerol concentration.

The few differences found in the comparison of the two glycerol concentrations indicated that red deer spermatozoa have a broad margin of tolerance to glycerol. These results are in accordance to published data on red deer semen, both from epididymal and ejaculated sources, which report successful cryopreservation using a wide range of glycerol concentrations (from 4 to 10%) [7,10,12,13,25]. Recently, Cheng et al. [5], working with electroejaculated semen from *Cervus nippon* and *Cervus unicolor*, tested five different diluents with glycerol concentrations ranging 5–8%, and their results suggested that the efficacy of each extender was rather due to the presence of specific components and their interactions, than to glycerol concentration alone.

In this study we have analyzed two extenders varying solely in glycerol concentration, in order to isolate this factor. It seems that, effectively, epididymal spermatozoa from red deer can be cryopreserved almost equally in 4 and 8% glycerol, the later being preferred attending to its slightly better acrosomal protection. However, considering electroejaculated samples, 4% would be the recommended choice, since it yielded higher sperm viability. Taking into account the importance of this parameter, low glycerol concentrations may be adequate in formulations similar to ours. Nevertheless, as the study of Cheng et al. [5] suggests, glycerol effects may be modulated by other components of the extender, making necessary test not only a wider range of glycerol concentrations, but also its effect when used in different kind of extenders.

The other factor we studied, extender osmolality due to non-permeant components, is of great importance on sperm cryopreservation. For epididymal samples, extender E320 rendered the lowest post-thawing motility (TM) and the higher proportion of spermatozoa with a swollen tail (because of osmotic stress). This osmolality is much lower than the usual values for

epididymal samples (387 mOsm/kg on average in this study). On the other hand, E380 and E430 yielded significantly higher total motility, and E430 yielded the lowest percentage of swollen spermatozoa. Therefore, a high extender osmolality would be preferred when cryopreserving epididymal samples, even higher than the osmolality of the samples. Regarding the proportion of swollen spermatozoa, we have to consider that the female genital tract comprises a series of challenges for the spermatozoa, the first one consisting in the osmotic changes undergone by spermatozoa when depositing the thawed sample in the vagina or uterus [34]. Therefore, extenders that submit spermatozoa to unnecessary osmotic stress during the cryopreservation process should be avoided, since that stress may eventually reflect on a decreased fertilizing ability.

On the other hand, considering ejaculated samples, our results indicated that E320 would be the most adequate extender of the three tested. This result is logical considering the average osmolality of our electroejaculated samples (336 mOsm/kg). This would have made E430 too hyperosmotic for our samples, reflecting on the significantly lower results for progressive motility and viability when comparing with E320.

These results show that extender composition should be modified accordingly to the source of the sample, either ejaculated or epididymary (higher osmolality), and that better results might be achieved using an extender with an osmolality similar to that expected of the sample. Nevertheless, the good results obtained with E430 on epididymal samples and the acceptable ones with E380 on electroejaculated samples suggest that moderately hyperosmotic extenders might render even better results than isoosmotic ones, as other authors have proposed. Indeed, Liu et al. [21] found that increasing osmolality protected better bull spermatozoa during cryopreservation, provided that a rapid cooling rate was used. The explanation given by these authors was that a higher osmolality allowed a faster dehydration compatible with the cooling rate. Tested media ranged from 220 to 455 mOsm/kg, and, in general, good results were obtained for 260–380 mOsm/kg (depending on extender composition). Besides, Aisen et al. [17], working with ram semen, found that increasing extender osmolality improved cryopreservation results, but only to certain limit (up to a 100 mOsm/kg increment), and that it reflected into better lambing results.

We have observed a decreasing trend on some quality parameters of electroejaculated semen with increasing extender osmolality. However, the fact that

there were no significant differences between E320 and E380, and the findings of those authors, support the hypothesis that intermediate osmolalities may be more suitable than isoosmotic extenders. Besides, epididymal samples could be more resilient to hyperosmotic extenders and to the subsequent dehydration during the cryopreservation process, thus benefiting from these extenders without undergoing detrimental effects.

The findings of this study have a practical application considering the lack of comparative studies on extenders for cryopreservation of red deer semen. We have found that cryopreservation of epididymal and ejaculated sperm would require of different glycerol concentrations (8 and 4%, respectively). However, differences between the two concentrations were low, and at least epididymal samples seemed to have a high tolerance to glycerol. Considering osmolality of the extender, our results indicate that it is necessary to take into account the osmolality of the sample and its source, in order to design the extender accordingly. Isoosmotic extenders would render good results, however moderately hyperosmotic ones may be a better choice for cryopreservation. Considering the importance of extender design on the success of sperm banking, future studies should include testing of multiple glycerol concentrations and of hyperosmotic extenders for cryopreservation of these samples, adjusting these parameters according to sample source and characteristics.

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