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Animal Reproduction Science 110 (2009) 293–305

ANIMAL REPRODUCTION SCIENCE

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Effect of hen's egg yolk on capacitation and acrosome reaction of diluted canine spermatozoa

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Received 22 October 2007; received in revised form 15 January 2008; accepted 18 January 2008
Available online 13 February 2008

Abstract

The aim of this study was to investigate the influence of progesterone, cholesterol and calcium (Ca^{2+}) in an egg-yolk-containing extender on capacitation and acrosome reactions (AR) of diluted canine spermatozoa during 4 days of cooled-storage. For this purpose, we first investigated the effect of supplementation of a Tris–citrate–fructose buffer (TCF) with progesterone in a final concentration of 0.1, 0.2 and 1.0 μg progesterone/ml TCF-diluted semen. We then compared the effects of TCF and the same buffer-containing 20% egg yolk (TCF–EY). In egg yolks and the TCF–EY, progesterone was measured by enzyme immunoassay, cholesterol by enzymatic colorimetry and Ca^{2+} by flame atomic absorption spectrophotometry. For both experiments, ejaculates from eight dogs were used. For the comparison of diluents, one ejaculate was divided and one half diluted with TCF, the other with TCF–EY. One half of each TCF- and TCF–EY-diluted sample was evaluated immediately (D1), the other after storage for 4 days at +4 °C (D4). In diluted semen, motility and viability were measured by a computer assisted sperm analyzer (CASA; Sperm Vision, Minitüb, Germany), capacitation and AR were evaluated with a modified chlortetracycline assay (CTC) and the AR additionally by flow cytometry. *Results*: Supplementation of progesterone revealed, that between D1 and D4, total and progressive motility decreased with all progesterone concentrations, while viability as well as percentage of capacitated and acrosome reacted spermatozoa stayed constant. Progesterone-, cholesterol- and Ca^{2+} concentrations in egg yolks were 524.8 ± 131.4 ng/g, 13.9 ± 2.03 mg/g and 1.27 ± 0.17 mg/g, respectively. In the TCF–EY-diluent, the respective values were 210.9 ng/g, 2.52 mg/g and 1.1 mg/g. In TCF–semen, at D1, motility and viability were significantly higher than in TCF–EY-samples ($p < 0.05$), however at D4, no significant differences were detectable. Further, in TCF–semen, percentages of spermatozoa with intact membranes decreased significantly ($p < 0.05$) and capacitated spermatozoa increased ($p < 0.05$), which was

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0378-4320/$ – see front matter
doi:10.1016/j.anireprosci.2008.01.022

not seen in TCF–EY-samples. In all samples, low percentages of AR were detected and after 4 days, the highest value of AR in TCF–EY-samples was 5.3% on average, as detected by flow cytometry. We therefore conclude that progesterone from egg yolk in routine extenders does not substantially influence semen longevity or AR of canine semen during cold-storage for 4 days. In contrary, egg yolk seems to prevent a significant increase in capacitated spermatozoa.

Keywords: Canine spermatozoa; Progesterone; Cholesterol; Calcium; Egg yolk; Acrosome reaction

1. Introduction

Egg yolk has become a common component of extenders for cooling and cryopreservation of semen from different species during the past 60 years. It has been shown that egg-yolk prevents spermatozoa cell damage during cooling and freezing and, in addition, has protective effects against detrimental environmental conditions such as changes in the pH and osmotic pressure or accumulation of harmful substances as, for example reactive oxygen species (Bogart and Mayer, 1950; Silva et al., 2002; Manjunath et al., 2002; Bergeron et al., 2004; Aboagla and Terada, 2004). The reason for this is a membrane protective capacity of still unknown egg-yolk components. One possible effect may be a specific interaction of the low-density lipoprotein (LDS) fraction with some major proteins of seminal plasma (Manjunath et al., 2002). This decreases the efflux of cholesterol and phospholipids from the spermatozoa membrane and thus prevents premature capacitation and subsequent acrosome reaction (AR; Bergeron et al., 2004).

Ejaculated spermatozoa must undergo some physiological changes before they are able to fertilize an oocyte. These changes are known as capacitation and AR, and enable spermatozoa to partially lyse and thereby penetrate the zona pellucida of the oocyte (Yanagimachi, 1988; Wu et al., 2005). Physiologically, capacitation and AR take place within the female reproductive tract stimulated by specific components of the follicular fluid but can also be induced in vitro (Rathi et al., 2003). Besides other molecules, progesterone from follicular fluid is known to physiologically induce the AR in several mammalian species such as human (Schuffner et al., 2002), boar (Wu et al., 2006), bull (Thérien and Manjunath, 2003; Lucoseviciute et al., 2004), stallion (Rathi et al., 2003) and dog (Sirivaidyapong et al., 2001; Wu et al., 2005; Cheng et al., 2005). In addition, other molecules like cholesterol (Cross, 1998) and calcium (Ca^{2+}; Fraser, 1987) are involved in related changes of the spermatozoa plasma membrane. Cholesterol efflux from the spermatozoa plasma membrane is required for capacitation (for review see Cross, 1998) and an important prerequisite for the subsequent AR. Calcium plays a key role in the process of the AR since Ca^{2+} influx and a subsequent increase in the intracellular Ca^{2+} concentration are crucial steps (Florman et al., 1998). If these changes already occur during cooled-storage or cryopreservation, viability of spermatozoa is impaired and fertilizing ability reduced. Under in vitro conditions, progesterone at certain concentrations is able to induce the AR (Thérien and Manjunath, 2003; Lucoseviciute et al., 2004; Wu et al., 2005).

Egg yolk has been shown to contain considerable concentrations of progesterone (Möstl et al., 2001), but it has not been investigated so far, to which extent the egg-yolk component might induce capacitation and AR in diluted semen. In the present study, this question is addressed by determination of the effects of an egg-yolk-containing extender on motility, longevity, capacitation and AR. Further, the concentrations of substances commonly believed to participate in the initiation and procedure of capacitation and AR, namely progesterone, cholesterol and Ca^{2+}, are measured

in individual egg yolks, a Tris–citrate–fructose buffer (TCF) and the same buffer-containing 20% egg yolk (TCF–EY).

2. Materials and methods

2.1. Semen collection and evaluation

For each experiment, semen was collected from eight clinically healthy beagle-dogs, aged 2–5 years, of proven fertility. Semen was obtained by digital manipulation and separated into the different fractions as described by Günzel (1986). Each fraction was collected in a prewarmed glass tube and only the spermatozoa rich part was used for further investigation. Immediately after collection, each ejaculate was evaluated for gross appearance and volume, estimated motility, spermatozoa concentration and pH (routine analysis). Motility of the native semen was estimated by optical microscopy at a magnification 200× (Optiphot-2, Nikon, Japan). Spermatozoa concentration was determined with a Thoma Chamber. Only ejaculates that met minimal quality requirements were used for further investigations.

2.2. Reagents

All chemicals were obtained from Sigma–Aldrich (Vienna, Austria; Hoechst 33258: H6024; glutaraldehyde: G6403; DABCO: D2522, chlortetracycline CTC: 94498; Cystein: 30095; polyvinylpyrollidone PVP: 81400; propidium iodide PI: 2.99 mM, P-4170; lectin from *Arachis hypogaea* (peanut), fluorescein isothiocyanate conjugate FITC-PNA 100 μg/ml Aqua dest.: L-7381 and water-soluble progesterone: P7556), except for DMSO (dimethyl sulphoxide reinst, 1.16743), which was obtained from Merck KGaA (Darmstadt, Germany) and protein free medium (DEPC treated water) obtained from Invitrogen (Carlsbad, CA, USA). The cholesterol-test was from Boehringer-Mannheim/R-Biopharm (Austria R-Biopharm AG, Vienna, Austria).

The two extenders used were a Tris–fructose–citrate–buffer (TCF) and the same Tris-buffer-containing 20% egg yolk (TCF–EY). Both extenders contained Tris (3.025 g), citric acid (1.7 g), fructose (1.25 g), streptomycin (0.1 g), dissolved in 100 ml distilled water, and benzyl penicillin (0.06 g in 0.3 ml distilled water; Linde-Forsberg, 2001). For the TCF–EY, 20% egg yolk was added from a mixture of 20 hens' eggs. For the TCF-extender and the TCF–EY-diluent, the pH was 6.8 and 6.2, the osmolarity 312 and 297 mOsm/l, respectively. From the pure egg yolk as well as from each extender, three aliquots were stored at −20 °C for measurement of progesterone, cholesterol and Ca^{2+}.

2.3. Experiment 1: supplementation of progesterone

This pilot experiment was conducted at the beginning of the project. All ejaculates ($n = 8$) were divided into four aliquots (A0–A3) and diluted 1:3 with TCF. A0, A1, A2 and A3 were then supplemented with 0, 0.1, 0.2 and 1.0 μg progesterone/ml, respectively. One half of each sample was stained and examined immediately (D1), while the other was kept at +4 °C until analysis on day 4 (D4). At D1 and D4, samples were evaluated for total and progressive motility (computer-assisted semen analysis, CASA, Sperm Vision, Minitüb, Germany), viability (CASA), velocity (CASA) and capacitation as well as acrosomal status (CTC).

2.4. Experiment 2: comparison of extenders with and without egg yolk

2.4.1. Experimental design

For the comparison of diluents, again eight ejaculates were collected, each sample was routinely analysed (see Section 2.1) and then divided into two aliquots and diluted 1:3 with TCF and TCF–EY, respectively. One half of each sample underwent immediate analysis (D1), whereas the other half was stored at +4 °C for 4 days and then analysed (D4). In diluted semen, at D1 and D4, motility and viability were measured by CASA, capacitation and acrosomal status were evaluated with a modified chlortetracycline assay (CTC, see below) and, in a second experiment, the acrosomal status additionally by flow cytometry (see below).

2.4.2. Determination of progesterone, calcium and cholesterol in individual egg yolks and extender

Progesterone concentration in the 20 egg yolks as well as in the one TCF–EY-sample prepared with 20% of the pooled egg yolks was determined by enzyme immunoassay (EIA) for progesterone and other 20-oxopregnanes as described by Schwarzenberger et al. (1996) and Möstl et al. (2001). The cholesterol concentrations in egg yolks as well as in the TCF and TCF–EY-extender was determined by an enzymatic colorimetric method (Cholesterol Colour Assay; No. 10139050035; Austria R-Biopharm AG, Vienna, Austria) according to the manufacturer's instructions and at the Institute for Nutrition, University of Veterinary Science, Vienna, Austria. The Ca^{2+} concentrations in individual egg yolks as well as in the different extenders were determined by flame atomic absorption spectrophotometer (AAS, PerkinElmer, Typ 3030 B, Überlingen, Germany).

2.4.3. CASA measurements

Motility, velocity and membrane integrity (viability) of diluted semen were evaluated using a CASA system (Sperm Vision, Minitüb, Tiefenbach, Germany) as described by Schäfer-Somi and Aurich (2007) and Pagl et al. (2006). The following motility parameters were determined: total motility (M); progressive motility (P); curvilinear velocity (VCL, μm/s), mean velocity (VAP, μm/s) and linear velocity (VSL, μm/s). Membrane integrity of spermatozoa was determined after staining with SYBR-14/PI (Schäfer-Somi and Aurich, 2007). Briefly, 3 μl of the staining solution were mixed with 100 μl of diluted semen and incubated for 10 min at room temperature. One drop of the stained semen sample was evaluated automatically at magnification 400× using a fluorescence microscope connected to the CASA (Olympus AX70, Olympus Optical Co. Ltd., Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). Spermatozoa concentration during analysis was 100×10^6 ml^{-1} and temperature of analysis was 37 °C.

2.4.4. Chlortetracyclin (CTC)-assay

For the CTC-assay, the staining protocol described by Rota et al. (1999) and Fraser (1995), modified by Hewitt (1997) was used. Briefly, from each native or diluted spermatozoa sample, 200 μl was taken, stained with 7 μl of a working solution and incubated for 2 min. After addition of 2 ml of a 2% polyvinylpyrrolidone (PVP) solution, the samples were centrifuged at $900 \times g$ for 5 min. The supernatant was immediately removed and the spermatozoa pellet resuspended in 45 μl CTC-solution for 20 s. Then 2 μl of a glutaraldehyd-solution (50%) was added. Following this, a 5 μl droplet of the stained sample was placed on a microscope slide (two droplets on each slide) and fixed with a 2 μl droplet of DAPCO (Sigma–Aldrich, D2522) to attenuate the fading of fluorescence. These two were mixed on the slide, the cover slip applied, and the excess fluid

removed. At last the cover slip was sealed on the slide using nail varnish. The samples were kept at +4 °C under dark conditions for 24 h and analysed by ultraviolet illumination. The CTC-patterns of the spermatozoa were assessed with a laser scanning microscope (LSM 510-Meta, Zeiss, Jena) at magnification 1000× using the filter cube I 2/3 for the CTC-patterns. On each smear, 100 cells were counted and three different patterns were found: uncapacitated and acrosome intact (F), capacitated and acrosome intact (B), capacitated and acrosome reacted (AR).

2.4.5. Flow cytometric measurements

For flow cytometric analyses (FACS) of processed semen, ejaculates from the same eight beagles were collected and quality assessed as described before. Each sample was then divided into two aliquots and diluted 1:3 with TCF and TCF–EY, respectively. One half of each diluted sample was stored at +4 °C for 4 days and then analysed (D4). The other half of the TCF- and TCF–EY diluted samples were left 1 h at room temperature, and then both samples were further diluted with the TCF-buffer to an end concentration of 3×10^6 ml^{-1}. These samples were labelled with 3 μl propidiumiodide (PI) and 5 μl FITC-PNA per 500 μl of diluted semen sample. The stained samples were incubated at room temperature and in darkness for 20 min. Measurements were performed with a flow cytometer (Becton Dickinson FACScan System, BD Austria GmbH, Schwechat, Austria). The cells were excited at 488 nm using an argon laser. FITC-PNA fluorescence was detected with a 520 nm band pass filter (FL-1) and PI fluorescence with a 610 nm band pass filter (FL-2), on logarithmic scales. The forward and side scatter data were measured in the linear mode. For each measurement, 10,000 cells were counted. The control settings for each day's series analyses were performed as described by Petrunkina et al. (2004). Each sample was measured in triplicate and the average values were calculated. With the combined staining it was possible to differentiate between four different groups of spermatozoa: dead, dead with AR, alive and alive with AR.

2.5. Statistical analysis

All statistical comparisons were performed using the SPSS Software® (Version 14 for Windows, SPSS Inc., Chicago, IL, USA). All data except the CTC values were normally distributed. One-way analysis of variance (procedure ANOVA) and Student's *t*-test were used to determine statistical differences between groups on 1 day and between different days, respectively. In case of CTC values, the non-parametric Friedman- and Wilcoxon-tests were used. All results are given as means ± S.D. For all tests, a *p*-value of <0.05 was considered statistically significant (Fig. 1).

3. Results

3.1. Experiment 1: supplementation of progesterone

Between D1 and D4, a significant decrease in the average total motility occurred only without progesterone supplementation (0 μg P_4/ml; $p < 0.05$) and in the average progressive motility with a progesterone supplementation of 0 and 1.0 μg P_4/ml ($p < 0.05$), while viability stayed constant with all progesterone concentrations (n.s.; see Fig. 2). Also, the velocity parameters VCL, VAP, VSL decreased significantly without, but not with progesterone supplementation (data not shown).

With all progesterone concentrations, the percentage of spermatozoa with intact plasma membranes (F) decreased between D1 and D4, whereas the percentage of capacitated sperm (B) increased (n.s., Fig. 3a and c). At all concentrations, low numbers of acrosome reacted

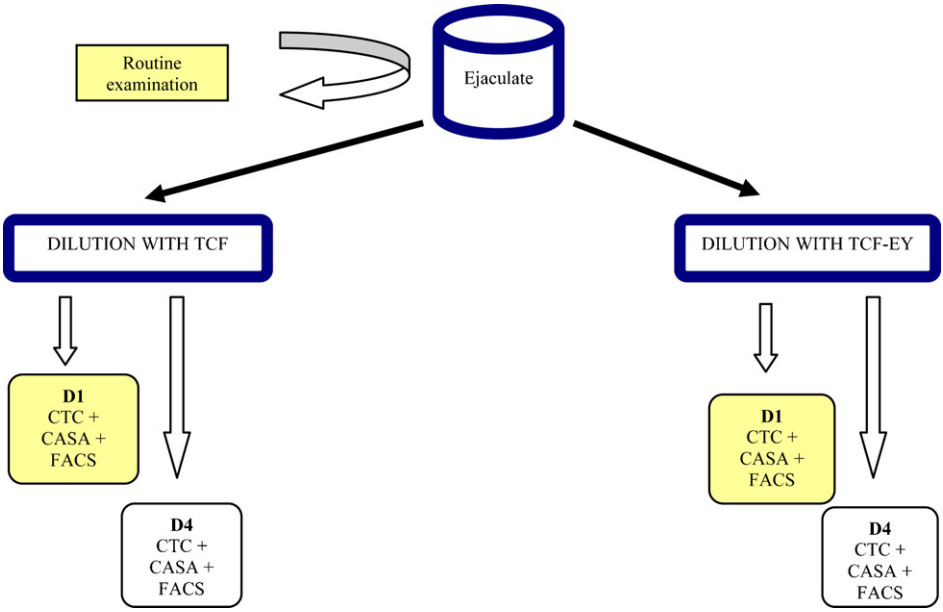

Fig. 1. Experimental design. D1: day of semen collection; D4: after 4 days of cooled-storage (+4 °C); TCF: diluted with Tris–citric acid–fructose buffer; TCF–EY: diluted with the same buffer-containing 20% egg yolk; CTC: chlortetracycline assay; CASA: computer assisted sperm analyser; FACS: fluorescence assisted cell sorter.

spermatozoa were detectable; a significant change between both days was only assessed with 1 μg progesterone/ml ($p < 0.05$; see Fig. 3c). The osmolarity of the TCF-diluted samples increased with increasing concentrations of progesterone (mOsm/kg: 0.1 μg progesterone/ml: 309.3 ± 1.2; 0.2 μg progesterone/ml: 314.7 ± 0.6; 1 μg progesterone/ml: 463.0 ± 2.0; $R = 99.95\%$, $p < 0.001$).

3.2. Experiment 2: comparison of extenders with and without egg yolk

3.2.1. Concentrations of progesterone, cholesterol and calcium in egg yolks and TCF–EY

In egg yolks ($n = 20$), the average Ca^{2+} concentration was 1.27 ± 0.17 mg/g (0.84–1.55 mg/g), the mean cholesterol content 13.9 ± 2.03 mg/g (18.18–11.18 mg/g) and the average progesterone concentration 524.8 ± 131.4 ng/g (395.9–906.6 ng/g). In the TCF–EY-diluent, the respective values were 210.9 ng progesterone/g, 2.52 mg cholesterol/g and 1.1 mg Ca^{2+}/g of TCF–EY.

3.2.2. Total motility, viability and velocity in extended semen

In TCF-diluted semen at D1, percentages of total motility, progressive motility and viability were significantly higher than in TCF–EY (78.2 ± 22.3 vs. 72.9 ± 28.3, 72.0 ± 30.0 vs. 61.3 ± 32.4 and 90.9 ± 5.7 vs. 88.6 ± 7.9, $p < 0.05$), whereas at D4, no significant differences were found (83.9 ± 8.5 vs. 88.6 ± 2.2, 77.9 ± 14.0 vs. 82.1 ± 2.6 and 89.3 ± 5.8 vs. 90.8 ± 2.9, n.s.).

Between D1 and D4, in TCF- and TCF–EY-samples, motility, progressive motility and viability did not differ significantly (Fig. 4). However, there was a marked increase in percentages of motility and progressive motility in TCF–EY samples (M: D1 72.9 ± 28.3, D4 88.6 ± 2.2; P: D1 61.3 ± 32.3, D4 82.1 ± 2.6 n.s., see Fig. 4a). The velocity and linearity parameters stayed approximately constant between D1 and D4 and did not differ significantly between the two extenders (n.s., data not shown).

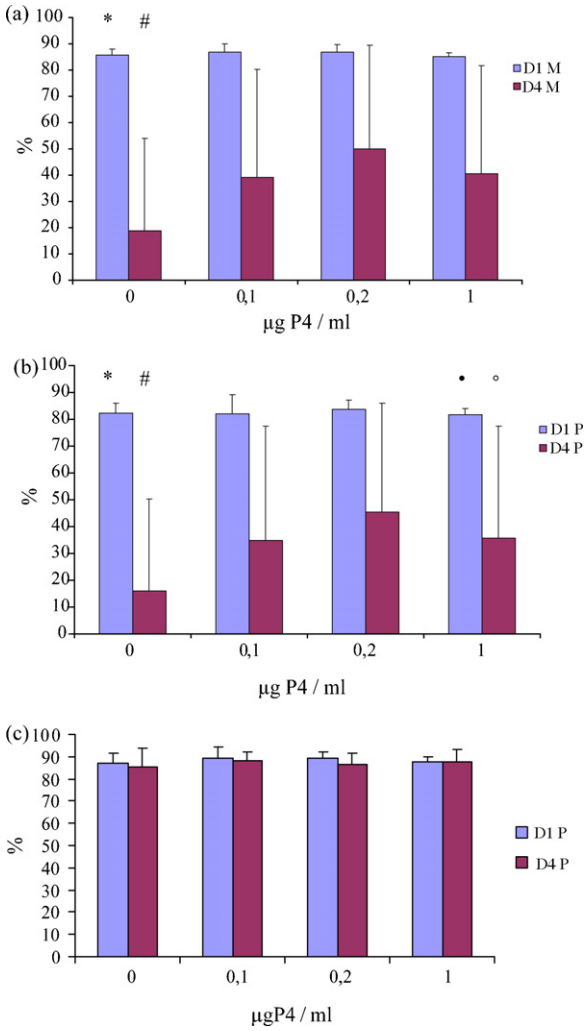

Fig. 2. Effect of progesterone supplementation on motility (a), progressive motility (b) and viability (c) during a 4 days cooled-storage period. M: motility; P: progressive motility; V: viability; P_4: progesterone; (*, #, • and °) different indices indicate a significant difference ($p < 0.05$).

3.2.3. CTC-measurements

With TCF, the percentage of spermatozoa with intact membranes (pattern F, see Section 2.4.4) decreased significantly from 42.1 ± 27.7 to 25.4 ± 18.5 ($p < 0.05$) and the percentage of capacitated spermatozoa (pattern B, see Section 2.4.4) increased significantly from 57.1 ± 26.7 to 74.5 ± 18.8 ($p < 0.05$) between D1 and D4. In TCF–EY-extended semen, neither the percentage of spermatozoa with intact membranes nor the percentage of capacitated spermatozoa changed significantly between D1 and D4 (F: $35.9 \pm 16.9\%$ on D1 vs. $32.6 \pm 18.3\%$ on D4; B: $63.3 \pm 17.0\%$ on D1 vs. $67.2 \pm 18.4\%$ on D4, n.s.).

In all samples, low percentages of spermatozoa with AR were detected. On D1, the percentage of acrosome-reacted spermatozoa in the TCF-diluted semen was $0.9 \pm 1.0\%$, in the TCF–EY-diluted semen $0.06 \pm 0.1\%$; while on D4 the values were 0.1 ± 0.3 vs. $0.07 \pm 0.1\%$ (n.s.).

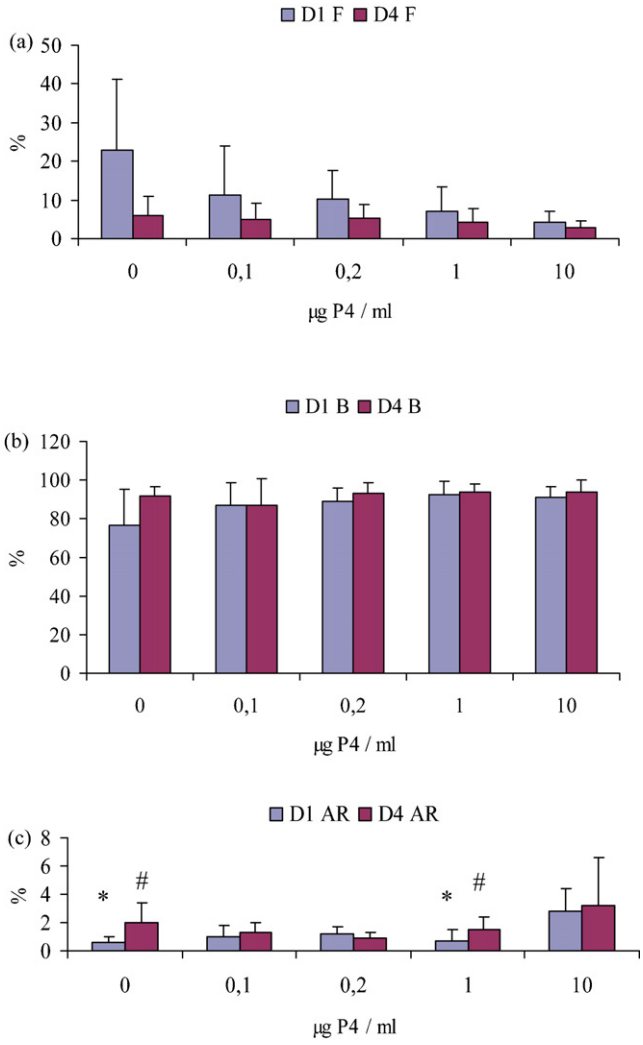

Fig. 3. Effect of progesterone supplementation on the acrosomal status of diluted semen during a 4 days cooled-storage period. (a) Pattern F, (b) pattern B and (c) pattern AR. D1: day of semen collection; D4: after 4 days of cooled-storage (+4 °C); CTC: chlortetracycline assay; F: uncapacitated and acrosome intact cells; B: capacitated and acrosome intact cells; AR: capacitated and acrosome reacted cells; P_4: progesterone; (* and #) different indices of naboured bars indicate a significant difference ($p < 0.05$).

3.2.4. Flow cytometric measurements

In TCF-samples, all groups of spermatozoa (dead, dead with AR, alive, alive with AR) stayed constant during the 4 days of cooled-storage. At D1 and D4, few alive spermatozoa with AR were assessed (D1: $1.6 \pm 0.7\%$ vs. D4: $1.8 \pm 0.6\%$, n.s.; Fig. 4a). The percentage of alive spermatozoa did not differ significantly from the viability (V) as measured by CASA, neither at D1 (V: 90.4 ± 4.0) nor at D4 (V: 88.7 ± 5.1).

In TCF–EY samples, the total percentages of dead and alive spermatozoa did not change significantly between D1 and D4. However, the percentages of both dead and alive spermatozoa

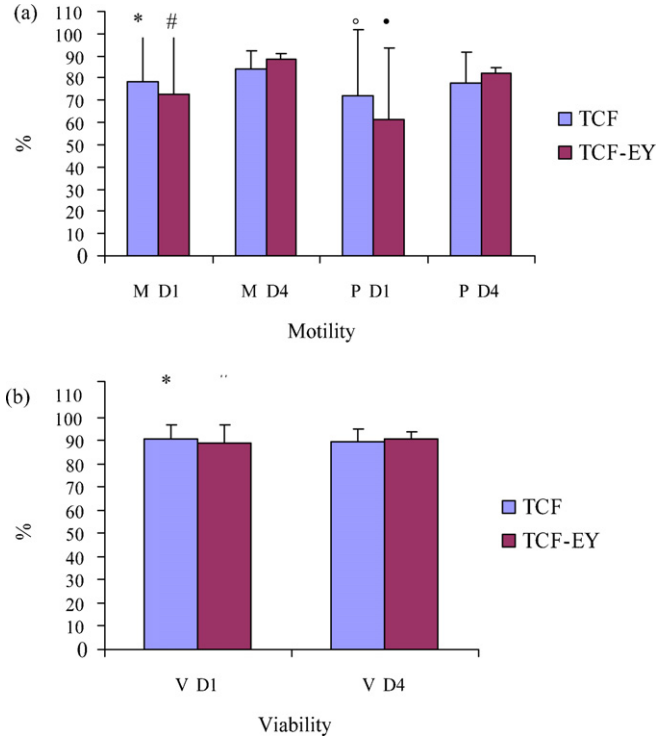

Fig. 4. Comparison of TCF- and TCF–EY-diluted semen during 4 days of cooled-storage. TCF: diluted with Tris–citric acid–fructose buffer; TCF–EY: diluted with the same buffer-containing 20% egg yolk; (a) motility and progressive motility; M D1: motility and P D1: progressive motility on the day of semen collection; M D4: motility and P D4: progressive motility 4 days after cooled-storage at 4 °C; (*, #, • and °) different indices indicate a significant difference ($p < 0.05$); (b) viability; V D1: viability on the day of semen collection; V D4: viability 4 days after cooled-storage at 4 °C; (* and #) different indices indicate a significant difference ($p < 0.05$).

with AR increased significantly (dead + AR: D1: 1.0 ± 0.9 vs. D4: 2.2 ± 1.6; $p < 0.05$; alive + AR: D1: 2.6 ± 1.9 vs. D4: 5.3 ± 1.6; $p < 0.05$; Fig. 4b). The percentage of alive spermatozoa did not differ significantly from the viability (V) as measured by CASA (D1, V: 91.8 ± 2.9; D4, V: 92.7 ± 2.1, n.s.) (Fig. 5).

Comparison between TCF and TCF–EY data revealed, that the percentage of dead spermatozoa was significantly higher in the TCF group, on both days investigated (D1 TCF vs. TCF–EY: 7.4 ± 3.2 vs. 3.6 ± 3.5, $p < 0.05$; D4 TCF vs. TCF–EY: 5.5 ± 1.8 vs. 2.4 ± 1.3, $p < 0.01$). In contrary, at day 4, the percentage of alive spermatozoa with AR was significantly higher in the TCF–EY samples (D4 TCF vs. TCF–EY: 1.8 ± 0.6 vs. 5.3 ± 2.6; $p < 0.01$).

4. Discussion

A variety of biological substances with different signalling pathways and molecules, such as progesterone, cholesterol and Ca^{2+} are involved in the capacitation and acrosome reaction (AR) of spermatozoa (for review see Flesch and Gadella, 2000; Gadella et al., 2001; Neild et al., 2005). Many of these molecules are also present in egg yolk, a frequently used component of canine semen extenders. We therefore investigated whether egg yolk as an extender component

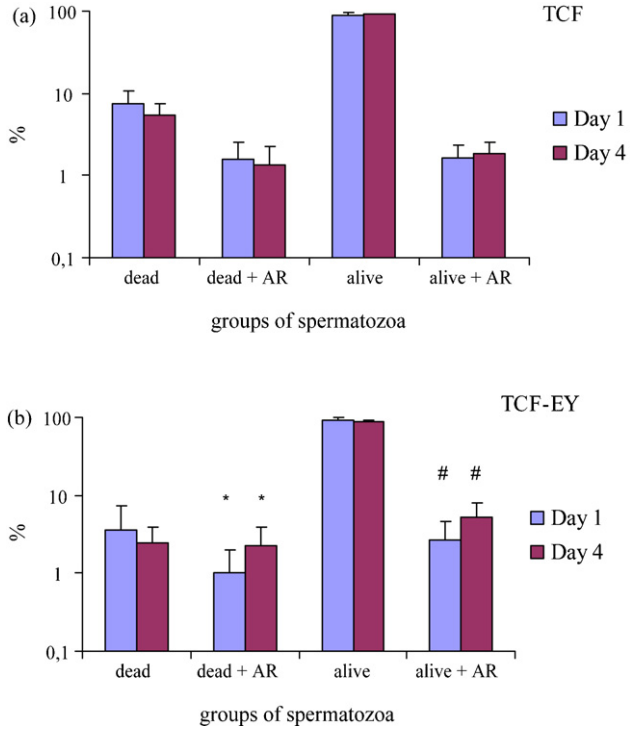

Fig. 5. Comparison of (a) TCF- and (b) TCF–EY-diluted semen during 4 days of cooled-storage—flow cytometric measurements. AR: acrosome reactions; TCF: diluted with Tris–citric acid–fructose buffer; TCF–EY: diluted with the same buffer-containing 20% egg yolk. (* and #) Equal indices indicate a significant difference ($p < 0.05$).

is involved in induction of the AR during cooled-storage. In the present study, we could clearly demonstrate that progesterone, cholesterol and Ca^{2+} in concentrations common in routinely used egg-yolk extenders (20%) do not substantially affect viability, longevity or the percentage of acrosome-reacted canine spermatozoa during cooled-storage for 4 days. In contrary, the egg yolk in the TCF–EY prevented a significant increase in capacitated cells during cooled-storage. The percentage of acrosome reacted spermatozoa stayed constant as did the percentage of capacitated spermatozoa. After 4 days, the average percentage of AR as detected by FACS was very low and an impact on fertility could not be expected.

In the present study, the progesterone concentration determined in the TCF–EY was much lower than the concentrations necessary to induce AR in mammalian spermatozoa in vitro (Sirivaidyapong et al., 2001; Cheng et al., 2005; Wu et al., 2005, 2006). However, supplementation of progesterone to the TCF diluent in low concentrations resulted in a constant decrease in total and progressive motility as well as an increase in capacitated spermatozoa, while viability and AR stayed constant. This is in agreement with studies on bovine spermatozoa where addition of progesterone enhanced capacitation but did not significantly affect plasma membrane lipid stability or AR (Lucoseviciute et al., 2004). However, it is important to mention that with the progesterone preparation used in the present and other cited studies (Schuffner et al., 2002; Thérien and Manjunath, 2003; Lucoseviciute et al., 2004), it is not possible to differentiate whether the in vitro changes were caused by progesterone itself, the sugar component (2-hydroxypropyl-β-cyclodextrin) or the increase in osmolarity. In the present study, the relatively low progesterone

concentrations measured in the TCF–EY extender did not significantly increase the percentage of AR in cooled stored canine semen, nor the percentage of capacitated cells. Together with all other results this might indicate that other components in the egg-yolk prevented the increase in capacitation thus rendering the TCF–EY advantageous in comparison to the TCF.

Calcium is known to be involved in membrane changes during capacitation and AR (Foresta et al., 1993). An increase in the intracellular Ca^{2+} concentration by an influx of extracellular Ca^{2+} through voltage-dependent ion channels is a crucial step for the induction of AR (Jagannathan et al., 2002; Gonzáles-Martinez et al., 2002). In the present study, relatively high Ca^{2+} concentrations in the egg-yolk-containing extender were determined. Even though these Ca^{2+} concentrations might have contributed to premature AR immediately after addition of TCF–EY, the percentages of AR stayed constantly low during the following 4 days, thus rectifying the addition of egg yolk to a diluent for short time preservation of canine semen.

For the assessment of capacitated and acrosome reacted spermatozoa a modified CTC-assay was used. This is a well-established method for this purpose (Fraser, 1995; Hewitt, 1997; Rota et al., 1999; Iguer-Ouada and Verstegen, 2001; Rodriguez et al., 2005). However, to confirm this result and to get more reliable data concerning the AR, we additionally used flow cytometry with FITC-PNA and PI-stained samples. Propidiumiodide binds to the DNA of cells with membrane lesions, thus staining dead cells, whereas FITC-PNA selectively binds to defective spermatozoa acrosomes (Rathi et al., 2001). Due to higher cell numbers, sensitivity and objectivity, flow cytometry is the better method for the detection of AR, whereas the CTC assay is in the first place useful for the additional detection of capacitated spermatozoa. This explains the slightly differing percentages of AR obtained with the different methods. However, in general, very low percentages of AR were detected and by means of CTC even less than with flow cytometry. This is in agreement with findings from Iguer-Ouada and Verstegen (2001), who detected comparable amounts of AR after 6 days of cooled-storage with a Tris–fructose extender-containing egg yolk.

5. Conclusion

Concentrations of progesterone, cholesterol and Ca^{2+} determined in a routinely used egg-yolk-containing extender did not induce a significant increase in capacitated and acrosome-reacted spermatozoa during cooled-storage of canine semen. On the contrary, a stabilizing effect of egg-yolk preventing a significant increase in capacitated spermatozoa could be confirmed. We therefore conclude that the addition of egg yolk to a Tris–citric–fructose buffer is advantageous for short-term preservation of canine semen.

Acknowledgements

The authors are grateful to Martin Finsterboeck (Institute for Nutrition, University for Veterinarian Science, Vienna) and Silvia Kluger (Centre for Artificial Insemination and Embryo Transfer, University for Veterinarian Science, Vienna) for laboratory assistance.

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