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Effects of cryo-injury on progesterone receptor(s) of canine spermatozoa and its response to progesterone

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Abstract

The integrity of sperm progesterone (P4) receptor(s) and its response to steroid stimulation might be crucial for the maintenance of sperm fertilizing ability after cryopreservation. The aim of the current investigation was to study the effect of cryo-procedures on canine sperm P4 receptor(s). In addition, alteration of P4 receptor(s) at the molecular level and their functional integrity following cryo-procedures was evaluated. Fresh and frozen-thawed semen samples ($n = 6$ same dogs) after capacitation were treated with 10 μg/mL P4 to induce the acrosome reaction (AR, FITC-PNA staining). Parallel samples were treated with 50% canine seminal plasma (SP) prior to AR induction with P4. The percentages of AR in capacitated fresh and frozen-thawed semen samples after treatment with P4 were 31.0 ± 6.7 and $21.6 \pm 4.1\%$ ($P < 0.05$), respectively. The percentage of AR in fresh and frozen-thawed semen samples pretreated with SP and incubated with P4 was; 11.5 ± 4.8 and $16.5 \pm 2.0\%$ ($P < 0.05$), respectively. The incidence of the spontaneous AR ($P > 0.05$) in fresh

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and frozen-thawed semen samples at the onset (5.5 ± 2.2 and 6.1 ± 1.8%; respectively) and after a 2 h (9.6 ± 5.1 and 10.4 ± 2.7%; respectively) capacitation, avoiding P4 stimulation, were not different. The percentage of progesterone-BSA-FITC staining over the acrosomal region was 18.3 ± 10.3% in fresh semen, 36.0 ± 11.9% in capacitated ($P < 0.05$) and less than 5% in SP treated spermatozoa. This staining was barely visible in frozen-thawed spermatozoa regardless of capacitation status. In western blot analysis, mAb C262 recognized two bands (54 and 65 kDa). Digitonin treated fresh and frozen-thawed spermatozoa, labeled with [^{3}H]-progesterone, revealed that the P4 binding capacity decreased from 6.0 ± 4.4 in fresh to 3.0 ± 2.1 nM in frozen-thawed spermatozoa. In nearly all samples tested (except one) 65 kDa protein band decreased significantly after freeze-thaw procedures while the 54 kDa protein was increased. These results indicate that the reduced incidence of AR in response to P4 in frozen spermatozoa is possibly due to the conformational changes of P4 receptor(s) and/or reduced P4 receptor density derived from freezing injury.
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Keywords: Progesterone receptors; Acrosome reaction; Freezing injury

1. Introduction

Cryopreservation extends the availability of spermatozoa to be used for artificial insemination and in vitro fertilization programs; however, it results in an impaired fertility compared to fresh semen. Reduction of fertility is generally attributed to a decline of sperm motility and viability caused by cryo-preservation procedures. In addition the reduced functionality of frozen-thawed spermatozoa can be explained by damaged membranes as a result of cryo-injury [1,2].

Recently, researchers have demonstrated the presence of at least two different types of progesterone (P4) receptor(s) on the human [3] sperm plasma membrane. Similar putative receptor(s) have also been reported in other species (horse: [4]; canine: [5]). P4, a steroid hormone present at high concentration in follicular fluid and secreted by the granulosa cells at the time of ovulation, is a potent activator of sperm fertilizing ability [6]; which is manifested by modifications in the sperm membranes and functions, including hyperactivation, zona pellucida binding and the acrosome reaction (AR) [7,8]. Male subfertility as a result of sperm inability to undergo AR in response to P4 has been previously demonstrated [9,12–15]. Furthermore, other studies have shown that this defect (inability of sperm to undergo AR in response to P4) is highly correlated with the proportion of spermatozoa that fail to expose P4 receptors [10,11].

Up to now, little has been published concerning the fate of the membrane P4 receptor(s) after cryo-preservation. In human spermatozoa, cryo-preservation procedures reduced the sperm responsiveness to P4 in terms of [$Ca2^{+}$]i rise and plasma membrane depolarization. These inhibitory effects may result from modifications of the receptors for P4 [16]. On the other hand, McLaughlin and Ford [17] described that sperm cryopreservation procedures cause a substantial rise in the intracellular [$Ca2^{+}$]i and suggested that damage to the plasma membrane possibly result in the loss of the P4 receptor(s). These damages may contribute to the loss of fertility too. The integrity of sperm P4 receptor(s) and its response to steroid stimulation might be crucial factors for maintenance of sperm fertilizing ability after cryopreservation. The aim of the current investigation was to study the effect of

cryopreservation procedures on canine sperm P4 receptor(s). In addition, alteration of P4 receptor(s) at the molecular level and their functional integrity following cryo-procedures was evaluated.

2. Materials and methods

2.1. Semen preparation and cryopreservation

Healthy and proven fertile mongrel male dogs ($n = 6$), aged 2–4 years and weighing 10–15 kg were used throughout the study. Sperm-rich ejaculate fractions were obtained by digital manipulation and immediately transported to the laboratory in an insulated container. Semen samples with ≥70% motility were divided into two aliquots. One aliquot was subjected to a cryopreservation procedure for further experiments. Another aliquot was centrifuged at 700 × g for 30 min at 25 °C over a discontinuous density gradient, in which the sperm suspensions were layered on two layers of 2 mL of 30% and 2 mL of 70% Percoll [18]. The resulting sperm pellets were suspended in Canine Tris medium (CTM, referred to [19]) (50×10^6 sperm/mL) at 37 °C in humidified air saturated with 5% CO_2 for 2 h [19] to induce capacitation.

To cryo-preserve spermatozoa, semen was washed with Tris-buffer [20] (one part semen and two parts buffer) by centrifugation at 700 × g for 10 min at 25 °C to remove the seminal plasma. Subsequently, sperm pellets were suspended ($200–250 \times 10^6$ sperma-spermatozoa/mL) in Extender 1 [20] and incubated for 45 min at 5 °C in cotton insulated conical tube. Thereafter, the semen was further diluted 1:1 ($100–125 \times 10^6$ spermatozoa/mL) with Extender 2-E [20] and incubated for 15 min at 5 °C. The diluted semen was loaded into 0.25 mL straws, which were sealed, frozen in a programmable freezer (Planner Kyro 10, Middx, England) at the rate of −5 °C/min from 5 °C to −20 and −20 °C/min from −20 to −150 °C [20]. Subsequently, straws were stored in liquid nitrogen for 2 weeks before use. Straws were then thawed in a water bath at 37 °C for 1 min. The frozen-thawed spermatozoa were applied to the discontinuous density gradient (as described above) and capacitation was induced in this sample as mentioned above.

2.2. Induction of the acrosome reaction with P4

To investigate the effect of P4 on induction of AR in fresh and frozen spermatozoa, aliquots of capacitated spermatozoa each containing 500 μL of either fresh or frozen-thawed spermatozoa (obtained from the same individual, $n = 6$) were either treated with 10 μg/mL P4 or 0.1% (v/v) DMSO (vehicle only). These samples were incubated for 30 min in humidified air saturated with 5% CO_2 at 37 °C.

In a parallel experiment, to investigate the potential effect of seminal plasma on induction of AR by P4, fresh and frozen semen samples were diluted 1:1 with pooled canine seminal plasma (SP, $n = 3$ dogs). These samples were incubated for 15 min in humidified air saturated with 5% CO_2 at 37 °C. Thereafter these SP pre-treated semen samples were further treated with either 10 μg/mL P4 or 0.1% (v/v) DMSO (vehicle only) and incubated for 30 min in humidified air saturated with 5% CO_2 at 37 °C.

To assess the incidence of spontaneous AR in above experiments, samples were obtained from both fresh and frozen-thawed semen samples at the onset and after 2 h further incubation without P4 stimulation. The incidence of AR as well as percentage viability was assessed in these samples.

2.3. Assessment of the acrosome reaction

To assess sperm acrosomal status and viability, sperm suspensions were stained with ethidium homodimer-1 (EthD-1, Molecular Probes Inc., Eugene, OR USA) in CTM followed by FITC-PNA staining as previously described [4]. To estimate the proportion of AR, 200 live spermatozoa were assessed in randomly selected fields under an epifluorescence microscope (magnification: 500×) equipped with a DMU filter set (BH2-RFC; Olympus, Tokyo, Japan) [4].

2.4. Localization of P4 binding sites

2.4.1. Progesterone-BSA-FITC conjugates

Progesterone 3-(*o*-carboxymethyl)oxime:BSA coupled with fluorescein isothiocyanate (PBF conjugate) (Sigma Chemical Co., St. Louis, MO) were dissolved in PBS at a concentration of 1 mg/mL. At the onset and after a 2 h capacitation in CTM, fresh and frozen-thawed semen samples were treated as described previously [4]. Briefly, 225 μL sperm suspension was added to 25 μL of PBF conjugate solution, resulting in a final concentration of 100 μg conjugate/mL. After incubation for 5 min at 37 °C, spermatozoa were fixed in 250 μL of 4% paraformaldehyde and 1% glutaraldehyde in PBS. The sperm suspension was centrifuged at 600 × *g* for 3 min. After removal of supernatant, the fixed sperm cells were washed with 1 mL PBS and centrifuged to remove remaining aldehyde residues. After repeated washing, the sperm pellet was resuspended in 200 μL PBS. A volume of 10 μL sperm suspension was transferred to a glass slide and mounted with 5 μL antifade. Slides were kept in the dark until examination under an epifluorescence microscope.

2.4.2. Monoclonal antibody C262

The mouse monoclonal antibody (mAb, C262; Stress Gen, Victoria, BC, Canada) was raised against synthetic peptides corresponding to the C-terminal tail of the human nuclear P4 receptor representing the steroid-binding site. Localization of the P4 receptor(s) in canine spermatozoa was carried out by indirect immunofluorescence using mAb C262 and FITC-rabbit anti-mouse IgG (Zymed, San Francisco, CA, USA) as described by Cheng et al. [4].

2.4.3. Binding assay of [^{3}H]-progesterone

[^{3}H]-progesterone (specific activity 91 Ci/mM) was purchased from Amersham International (Amersham, UK). Uncapacitated fresh or frozen-thawed sperm samples ($n = 6$) were identically processed as follows: after Percoll gradient centrifugation, spermatozoa were divided into two aliquots. One aliquot was treated with 0.1% digitonin for 30 min at 4 °C, and the other was washed in PBS and received no digitonin treatment.

Washed and digitonin treated spermatozoa (20×10^6 sperm cells) were incubated in 10 nM [^{3}H]-progesterone with (non-specific binding) or without (total binding) 1 μM P4 for 1 h. The free radio-ligand and the spermatozoa-ligand complexes were separated by filtration though poly(ethleneimine)-coated glass-fiber filters. For the determination of retained radioactivity, the filter was equilibrated in a 3 mL scintillation cocktail. All binding data are given as specific binding, i.e. corrected for non-specific binding. Triplicate determination of each data point were performed; each experiment was repeated at least three times [21,22].

2.5. SDS-gel electrophoresis and western immunoblot analysis

To identify and characterize the P4 receptor proteins from fresh spermatozoa, the digitonin-treated sperm extract and pellet, containing 16×10^6 sperm/lane, were electrophoresed on 7.5% polyacrylamide-bisacrylamide gels [23,24]. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Sigma Chemical Co.). Transferred nitrocellulose was blocked for 3 h at room temperature in TTBS (0.05% Tween-20, 20 mmol/L Tris pH 7.4, and 500 mmol/L NaCl) containing 3% gelatin, then washed repeatedly in TTBS and incubated for 2 h in 1% gelatin-TTBS containing mAb C-262 (10 μg/mL). After washing, nitrocellulose was incubated with alkaline phosphatase-conjugated goat antimouse IgG (1:5000 in 1% gelatin-TTBS). After several washes in TTBS, reacted proteins were revealed with freshly prepared BCIP/NBT substrate solution [25]. In western blot analysis, transferred membrane was directly incubated with secondary antibody as control. For detection of P4 receptor proteins from frozen-thawed semen samples ($n = 5$), similar procedures were conduced and mAb C262 was applied as primary antibody.

2.6. Statistical analysis

Analysis of variance (general linear model procedure) was used to evaluate the effect of P4 on induction of the acrosome reaction. Significant differences between treatment means were determined by Duncan's multiple range tests (SAS, V6.12). In case of P4 binding assay, data was subjected to paired t-test to determine the difference between fresh and frozen-thawed spermatozoa in the same dogs. Values were presented as mean ± S.D. and considered statistically significant when $P < 0.05$.

3. Results

Immunofluorescent patterns of PBF and C262 mAb staining over the canine sperm acrosomal region are illustrated in Fig. 1(A and B). FITC-PNA staining patterns, indicating intact acrosomes are depicted in Fig. 1(C) and reacted acrosomes are depicted in Fig. 1(D).

The percentage of AR was $5.5 \pm 2.2\%$ in fresh semen, $9.6 \pm 5.1\%$ after a 2 h capacitation, compared to $31.0 \pm 6.7\%$ in capacitated spermatozoa in response to 10 μg/mL P4 ($P < 0.05$) and $11.5 \pm 4.8\%$ in capacitated spermatozoa treated with SP before

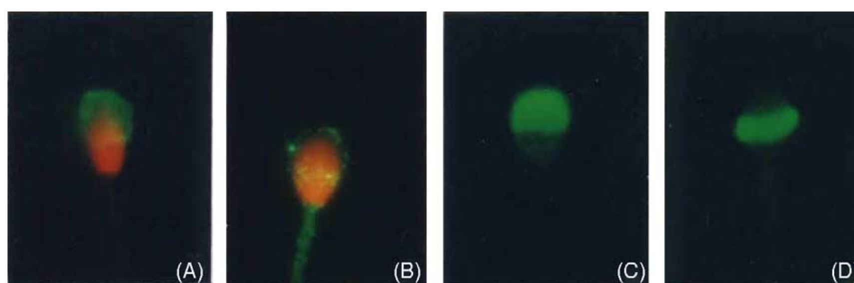

Fig. 1. Fluorescence pattern of canine sperm observed under an epifluorescence microscope. (A) Binding of P-BSA-FITC to a sperm displaying fluorescence over the acrosomal region of the head. (B) Localization of the progesterone receptor(s) on a frozen-thawed sperm by indirect immunofluorescence with mAb (C262) against progesterone receptor(s). (C) Binding of FITC-PNA to a sperm displaying fluorescence over the acrosomal region, indicating intact acrosome and (D) the equatorial region, indicating reacted acrosome. Red fluorescence was due to counterstain of ethidium homodimer (1250×).

incubation with P4 (Table 1). The percentage of AR in frozen-thawed semen samples of the same males was 6.1 ± 1.8, $10.4 \pm 2.7\%$ after capacitation, compared to $21.6 \pm 4.1\%$ in capacitated and P4 induced spermatozoa ($P < 0.05$) and $16.5 \pm 2.0\%$ in SP treated and P4 induced spermatozoa (Table 1). A significant difference in the incidence of AR was detected between fresh and frozen-thawed sperm following treatment with P4 ($P < 0.05$).

The percentage of fresh spermatozoa displaying PBF staining over the acrosomal region was $18.3 \pm 10.3\%$ in uncapacitated and $36.0 \pm 11.9\%$ in capacitated spermatozoa ($P < 0.05$) and less than 5% in spermatozoa treated with SP. Indirect immuno-labeling using monoclonal antibody C262 and PBF staining was barely visible in frozen-thawed spermatozoa regardless of capacitation process (Fig. 1(B)).

Digitonin treated uncapacitated fresh and frozen-thawed spermatozoa, labeled with $[^3H]$-progesterone, revealed that the progesterone binding capacity decreased from 6.0 ± 4.4 in fresh to 3.0 ± 2.1 nM in frozen-thawed spermatozoa ($P < 0.05$) (Fig. 2).

After SDS-gel electrophoresis, sperm pellet and extract proteins (16×10^6 sperm/lane) containing specific proteins were revealed by mAb C262. Two apparent protein bands were particularly identified to be 54 kDa (the major) and 65 kDa (the minor) (Fig. 3). No stained

Table 1
The percentage of acrosome reaction in live spermatozoa under different treatments

	0 h	2 h Capacitation	2 h + P4[a]	2 h + seminal plasma[b] + P4
Fresh	5.5 ± 2.2^a	9.6 ± 5.1^b	31.0 ± 6.7^c	11.5 ± 4.8^b
Frozen-thawed	6.1 ± 1.8^a	10.4 ± 2.7^b	21.6 ± 4.1^d	16.5 ± 2.0^e

Acrosome reaction in live spermatozoa was assessed using FITC-PNA staining combined with ethidium homodimer-1. Values with different letters indicate significant difference ($P < 0.05$). Mean ± S.D. of ejaculates from six fertile dogs are shown.

[a] Fresh or frozen-thawed spermatozoa were capacitated in CTM for 2 h and then induced for 30 min with 10 μg/mL progesterone (P4) at 37 °C in humidified air saturated with 5% CO_2.

[b] Sperm suspensions were pre-incubated (50%) with pooled canine seminal plasma (three dogs) for 15 min prior to P4 induction.

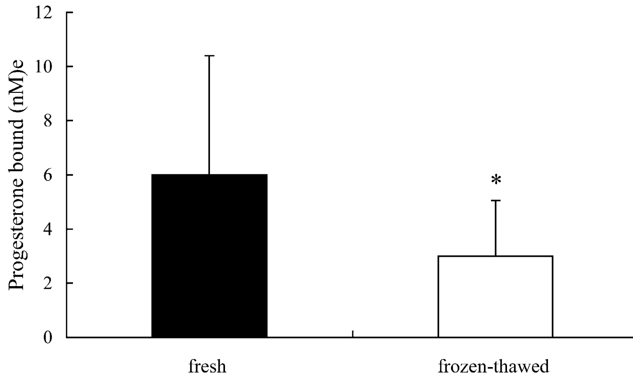

Fig. 2. Binding of [^{3}H]-progesterone to digitonin-treated fresh and frozen-thawed spermatozoa from the same dogs ($n = 6$). The progesterone binding capacity decreased significantly in frozen-thawed spermatozoa (paired *t*-test, $^*P < 0.05$). Values are means ± S.D. of triplicate determinations.

bands appeared in controls when the sperm extracts were only blotted with the alkaline phosphatase-conjugated antibody.

Changes of sperm protein pattern and P4 receptor proteins, resulting from the frozen-thawed process, are shown in Fig. 4. Indirect immuno-labeling revealed that the minor receptor protein (65 kDa) diminished significantly while the major protein (54 kDa) was increased substantially after freeze-thaw procedures. Similar patterns were detected in four sperm samples out of five.

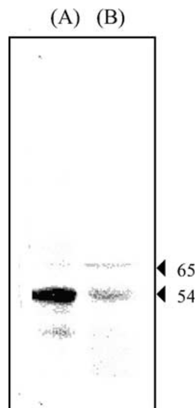

Fig. 3. Western blot analysis of digitonin-treated dog spermatozoa with monoclonal antibody C262 directed against C' terminal domains of the nuclear progesterone receptor. After 0.1% digitonin treatment, proteins of sperm extract (A) and pellet (B) (16×10^6 sperm/lane) were separated by 7.5% SDS-PAGE and transferred to PVDF membrane. The major proteins recognized by mAb C262 (10 μg/mL) combined with second antibody goat antimouse IgG mAb conjugated to alkaline phosphatase are approximately 54 kDa as the major band and 65 kDa as the minor band.

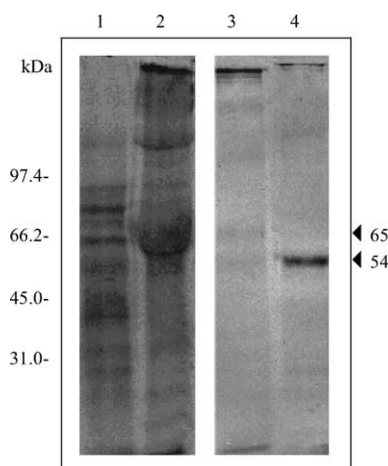

Fig. 4. Changes of sperm protein patterns and progesterone receptor proteins after freeze-thaw procedures. Digitonin-treated pre-(1,3) and post-(2,4) frozen-thawed sperm extract proteins were separated by 10% SDS-PAGE. Progesterone receptor proteins (3, 4) were revealed by mAb C262 (against C terminal domains of the genomic progesterone receptor) combined with second antibody goat antimouse IgG mAb conjugated to alkaline phosphatase. The components of sperm proteins were modified as observed on SDS-PAGE (1 vs. 2). This modification caused the diminution of 65 kDa protein as shown by mAb C262 immuno-staining (3 vs. 4). Similar results were observed in four sperm samples ($n = 5$). Molecular mass markers (M) are indicated on left side of the figure.

4. Discussion

This study clearly demonstrates that cryopreservation procedures reduced sperm responsiveness to P4 stimulation, in terms of the AR occurrence. These phenomena could be explained by the absence and/or modification of P4 receptor(s) on the frozen-thawed sperm surface, as evidenced by the decline of P4 binding capacity and weak staining of progesterone-BSA-FITC conjugate and mAb C262 of frozen-thawed spermatozoa. Loss and/or conformational changes of receptor(s) during the freezing process may therefore contribute to malfunction of sperm physiological behavior, leading to the loss of fertility. In accordance with our findings, previous study on calcium influx, an event proceeding to the AR, showed a substantial elevation of intracellular calcium in cryopreserved spermatozoa and half of preserved sperm samples failed to respond to P4 stimulation [16]. In addition, Rossato et al. [17] reported P4 induces plasma membrane depolarization in fresh spermatozoa that was absent in thawed spermatozoa. This membrane modification is a prerequisite step for ions fluxes and the subsequent AR [32]. It is therefore concluded that the reduced AR incidence derived from the freeze-thawing is caused by the inhibitory effect on intracellular calcium influx induced by P4 [16,17].

Western immunoblot analysis under reducing conditions with mAb C262 detected a single major immunoblot band of 54 kDa, a minor band at 65 kDa and several barely visible bands of lower apparent molecular mass. These detectable bands were observed in both sperm suspension and pellet preparations. The apparent molecular mass of the 54 kDa

protein is different from that of the A and B isoforms of the human intracellular P4 receptor, 94 and 120 kDa, respectively [3,26]. We speculate that the 54 kDa protein detected with mAb C262 is a canine sperm plasma membrane receptor. A previous report which demonstrated the inhibitory effect of the C262 on P4 induced AR, strongly suggested that the proteins detected with this mAb are involved in the biological effects of P4 [3]. The 65 kDa minor band may represent another (iso)form of the 54 kDa molecule or a second type of plasma membrane receptor. Thus, this might suggest the existence of two types of P4 receptors in canine spermatozoa. In fact, two putative P4 receptors, responsible for a rapid and a delayed calcium influx have been proposed in human spermatozoa [27]. It is probable that the two detected protein masses represent two different receptors, mediating different calcium transport patterns. Although Brewis et al. [28] studying calcium fluxes in canine spermatozoa reported a gradual but marked influx of calcium ions, which was sustained over 2 min. Sperm P4 receptor molecule masses have been reported in the human (54 and 57 kDa [29]; 50–52 and 46–48 kDa [3]). In nearly all frozen-thawed canine semen samples we investigated, the 65 kDa protein was barely detectable while the 54 kDa protein was abundant. We speculate that this is due to proteolysis/degradation of the 65 kDa protein during cryo-modifications.

The fact that a 2 h capacitation process increased the incidence of AR induced by P4, suggests that a modification of the receptor is necessary in response to the ligand stimulation. Nevertheless, one cannot exclude the effect of cytosolic changes (i.e. elevation of the intracellular $[Ca2^{+}]i$) and membrane modifications (i.e. increasing membrane fluidity), resulting from capacitation [32], that may contribute to sperm sensitivity to P4 as observed in our investigation and previous studies [4,5]. Sperm population that express P4 receptor are consistently larger than the population of spermatozoa within the same semen sample that are responsive to P4. In dogs, mature spermatozoa from cauda epididymis and freshly ejaculated spermatozoa demonstrate functional P4 receptor(s) while less mature spermatozoa from the testicle, caput, and corpus epididymis fail to demonstrate such receptor(s) [30]. Thus, the AR of dog spermatozoa can be induced by P4; however, its occurrence is dependent on the maturational stage of spermatozoa and subsequent sperm modifications during capacitation. It seems cryopreservation causes alterations in spermatozoa resembling premature capacitation, which accounts for the reduced longevity of spermatozoa and its readiness to undergo egg penetration (see review in [31]). Our results indicate that impaired sperm competence following cryopreservation is at least in part due to the reduced responsiveness to P4 stimulation.

A high proportion of dog spermatozoa from cauda epididymis show PBF staining, suggesting the existence of receptor(s) at the surface of spermatozoa at the time of ejaculation [30]. Some molecules originating from seminal plasma may bind to P4 receptor(s) when they encounter spermatozoa [4]. This may provide a mechanism of protection to maintain integrity of P4 receptor(s) from possible damage during transport in the female reproductive tract. This conjugating process seems to be reversible as evidenced by reduction in the rate of AR induced by P4 in spermatozoa treated with seminal plasma. Whether the identity of these protein ligands is the same as the so called ‘‘decapacitation factors’’ [32] needs to be further investigated.

In conclusion, these results indicate that a reduced AR responsiveness to P4 is evident in frozen-thawed spermatozoa and this might be due to the conformational changes of P4

receptor(s) and/or reduced P4 receptors' density deriving from freeze-thaw injury. The seminal plasma contains molecules that can functionally and morphologically mask the P4 receptor(s) and provide barriers to P4 stimulation. Further studies will be necessary to evaluate the effects of cryopreservation on sperm membrane structures and develop new strategies for freezing and thawing procedures that can better preserve the functional integrity of sperm P4 receptor(s). These modifications may enhance fertility of frozen-thawed spermatozoa.

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