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1 **Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen-**
2 **thawed stallion spermatozoa.**
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12

Abstract

Alkaline phosphatase (AP) has been studied in several situations to elucidate its role in reproductive biology of the male from different mammalian species; at present its role in horse sperm physiology is not clear. The aim of the present work was to measure AP activity in seminal plasma and sperm extracts from freshly ejaculated as well as in frozen-thawed stallion spermatozoa and to verify whether relationship exists between AP activity and sperm quality parameters.

Our data on 40 freshly ejaculated samples from 10 different stallions demonstrate that the main source of AP activity is seminal plasma, while sperm extracts contribution is very low. In addition we demonstrated that AP activity at physiological pH (7.0) is significantly lower than that observed at pH 8.0, including the optimal AP pH (pH 10.0). AP did not exert any effect on sperm oocyte interaction assessed by heterologous oocyte binding assay. Additionally, we observed a thermal stability of seminal plasma AP, concluding it is similar to that of bone isoforms. Positive correlations were found between seminal plasma AP activity and sperm concentration, while a negative correlation was present between both spermatozoa extracts and seminal plasma AP activity and seminal plasma protein content.

A significant decrease in sperm extract AP activity was found in frozen thawed samples compared with freshly ejaculated ones (n=21), concomitantly with the decrease in sperm quality parameters. The positive correlation between seminal plasma AP activity measured at pH 10 and viability of frozen–thawed spermatozoa suggests that seminal plasma AP activity could be utilized as an additional predictive parameter for stallion sperm freezability.

In conclusion, we provide some insights in AP activity in both seminal plasma and sperm extracts and describe a decrease in AP after freezing and thawing.

Keywords

Horse sperm
Alkaline phosphatase
Seminal plasma
Sperm extracts
Freezing and thawing

1. Introduction

47
48 Alkaline Phosphatase (AP) is an enzyme that catalyzes the detachment of phosphate groups from
49 several substrates [1]; it is present in male genital tract fluids and its activity has been revealed in
50 semen from various mammalian species [2]. The presence of AP in male genital secretions suggests
51 it could have a role in mammalian reproduction and in particular in sperm metabolism [3], even if a
52 definite, clear conclusion has not been reachedso far..
53 In bull seminal plasma the activity of the enzyme has been correlated to fertility and sperm
54 concentration [4], while in stallion it resulted a promising tool to determine whether ejaculation
55 failure is due to either azoospermia or a blockage along the genital tract[5] and it was linked to sperm
56 quality[6].
57 Other recent studies on AP in stallion seminal plasma furnish data on the different levels of activity
58 in separated semen fractions [7], as well as on its possible role as an indicator of sperm longevity in
59 fractionated semen [8].
60 The above-mentioned studies evaluated the activity in seminal plasma after removing spermatozoa,
61 or in the whole ejaculate, thus indirectly giving information on AP activity of the sperm cells [5]. In
62 a recent study on boar semen [9] we measured the activity of AP from spermatozoa as well as the
63 functional change it undergoes after sperm capacitation; the overall conclusion was that it could play
64 a role in the control of sperm function. There are no data on AP activity in sperm extracts after
65 freezing-thawing process, excepting forthose on frozen ram spermatozoa by Salamon and Maxwell
66 [10] who reported a loss in phosphatase activity after freezing and thawing, thus indicating that the
67 enzyme activity could be used as an additional parameter for detecting cryoinjuries.
68 Based on the questions raised by the aforementioned studies, the present work was aimed at:
69 describing AP kinematic properties (thermal inactivation test) in stallion seminal plasma.
70
71 determining the possible correlations between AP and sperm quality parameters and sperm function
72 as well as its possible predictive role in freezability of stallion semen.
73
74
75 **2. Materials and methods**
76
77 The experiment was approved by the Ethic-scientific Committee of Alma Mater Studiorum,
78 University of Bologna
79 All the reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise
80 specified.

81

82 *2.1. Semen collection and preparation*

83

84 Forty ejaculates from 10 fertile stallions (9 trotter breed and one Connemara), aging from 5 to 25
85 years, were sampled. Stallions were housed individually and semen was sampled between February
86 and April 2014 by an artificial vagina (Missouri model) equipped with a disposable liner with an in
87 line filter; stallions were collected on a phantom and in presence of an oestrous mare.
88 Semen concentration was evaluated using a hemocytometer chamber (Thoma).
89 Two aliquots of semen were kept undiluted: the first one was used to obtain seminal plasma, the
90 second one to obtain spermatozoa for alkaline phosphatase activity assay. A third aliquot of semen
91 was diluted in Kenney extender at a concentration of $40x10^6$ spermatozoa /mL for semen quality
92 analysis (sperm motility, viability, mitochondrial membrane integrity).
93 Twenty-one ejaculates, collected in the same period of time from four of the aforementioned stallions,
94 were used for analysis of AP activity in frozen-thawed spermatozoa in comparison with fresh ones.
95 Part of the ejaculates was immediately diluted in Kenney extender for subsequent freezing procedure,
96 while part was kept undiluted to obtain seminal plasma and spermatozoa for AP activity
97 determination.

98

99 *2.2. AP activity in seminal plasma, freshly ejaculated and frozen-thawed spermatozoa*

100

101 Protein concentration in seminal plasma and sperm extracts was measured by Bradford's method [11]
102 using bovine serum albumin as the protein standard.
103 Seminal plasma was obtained by two subsequent centrifugations of the undiluted sperm aliquot at
104 12,000 x g for 15 min at 4°C. The resultant supernatant was observed at the microscope to check the
105 absence of either spermatozoa or their fragments.
106 Sperm cells ($2x10^9$ spz/mL) (both freshly ejaculated and frozen-thawed) were washed twice in PBS
107 (900 x g for 3 min at RT) Subsequently the sperm pellet was sonicated in PBS and subsequently
108 centrifuged at 12,000 x g for 15 min at 4°C; finally the pellet was discarded and the supernatant
109 analyzed for AP activity.
110 The enzyme activity was assayed as described in [9]. Briefly, AP activity was measured by a
111 spectrophotometric assay that monitors the absorbance change at 405 nm as para-
112 nitrophenylphosphate (pNPP, colourless) is converted to para-nitrophenol (yellow). AP was assayed
113 in 50 mM TrisHCl buffer, at 25°C at different pH: 7.0, 8.0 and 10.0. Fifty µL of seminal plasma or
114 sperm extracts were used and the absorbance at 405 nm was measured during 1 min and 30 min

115 respectively before the addition of pNPP to evaluate the non enzymatic variations of absorbance; no
116 interferences were recorded in any case. In the case of seminal plasma analyzed at pH 10.0, it was
117 diluted 1:10 in Tris buffer. The reaction was started by the addition of 10 mM pNPP and each
118 measurement was run in duplicate and averaged. Enzyme activity was expressed as specific activity,
119 nmol/min/mg of protein.
120 In order to better characterize the seminal plasma isoform, AP thermo-inhibition was carried out at
121 60°C, as described by [15] with some modifications. The samples were kept at 60°C for 0'', 15'',
122 30'', 1', 1'15'', 1'30'', 1'45'', 2', 2'30'', 3' then placed in ice and the activity was measured within
123 30'' at pH 8.0. The same assay was also performed on the thermo-stable AP from bovine intestinal
124 mucosa as a control.
125
126 *2.3. Evaluation of sperm viability, motility and mitochondrial activity*
127
128 Twenty-five µL of semen were incubated with 2 µL of a 300 µM solution of propidium iodide (PI),
129 and 2 µL of a 10 µM solution of SYBR green-14, obtained from the live/dead sperm viability kit
130 (Molecular Probes, Inc., Eugene, OR, USA) for 5 min at 37 °C in the dark. Ten µL of the stained
131 suspensions were placed on clean microscope slides, carefully overlaid with coverslips, and at least
132 200 spermatozoa per sample were observed under a Nikon Eclipse E 600 epifluorescence microscope
133 (Nikon Europe BV, Badhoeverdop, The Netherlands). Spermatozoa stained with SYBR-14 but not
134 with PI were considered as viable (SYBR-14- PI-). Spermatozoa both SYBR-14+ and PI+ and those
135 SYBR-14–/PI+ were considered with damaged membranes or dead.
136 For each sample, an aliquot (25 µL) of semen was incubated with 2 µL of a 300 µM propidium iodide
137 (PI) stock solution, 2 µL of a 10 µM SYBR green-14 stock solution, both obtained from the live/dead
138 sperm viability kit (Molecular Probes, Inc.) and 2 µL of a 150 µM JC-1 solution, for 20 min at 37°C
139 in the dark. Ten µL of the sperm suspension were then placed on a slide, and at least 200 spermatozoa
140 per sample were scored using the above described microscope. Spermatozoa stained with SYBR
141 green-14 and not stained with PI were considered as viable. Spermatozoa SYBR positive and PI
142 positive and those SYBR negative / PI positive were considered as cells with non-intact membrane
143 or dead. JC-1 monomers emit a green fluorescence in mitochondria with low membrane potential,
144 while emitting a bright red-orange fluorescence in case of multimer formation (J-aggregates) in
145 mitochondria with high membrane potential. When an orange fluorescence was present in the mid
146 piece, live spermatozoa were considered to have functional active mitochondria (SYBR+/PI-/JC-1+).
147 Motility was measured using a computer-assisted sperm analysis system (CASA, Hamilton Thorne,
148 IVOS Ver. 12, standard equine set up). Thousand-1000 cells were evaluated on each sample diluted

149 to $30x10^6$ sperm/mL using a fixed height Leja Chamber SC 20-01-04-B,(Leja, The Netherlands).
150 Sperm motility endpoints assessed were: percent of total motile spermatozoa (TM), percent of
151 progressive spermatozoa (PM), curvilinear velocity (VCL) and mean velocity (VAP). The setting
152 parameters of the program were the followings: frames per second 60, number of frames 45, threshold
153 path velocity 30 microns/sec, straightness threshold 50.
154
155 *2.4. Effect of AP on heterologous oocyte binding*
156
157 Porcine oocytes maturation was performed as reported by [12]. Briefly, ovaries were obtained from
158 pre-pubertal gilts at a local abbatoir and transported to the laboratory within 1 h. Cumulus–oocyte
159 complexes (COCs) were aspirated from 4 to 6 mm follicles using a 18 gauge needle attached to a 10
160 mL disposable syringe . Under a stereomicroscope, intact COCs were selected and transferred into a
161 petri dish (35 mm, Nunclon, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4%
162 BSA. After three washes in NCSU 37 supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10
163 ng/mL epidermal growth factor (EGF), 50 mM ß-mercaptoethanol and 10% porcine follicular fluid
164 (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µL
165 of the same medium per well and cultured at 39 ◦C in a humidified atmosphere of 5% CO2/7% O2 in
166 air. For the first 22 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP,
167 10 IU/mL, eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon,
168 Intervet). For the last 22 h COCs were transferred to fresh maturation medium.
169 Heterologous oocyte binding assay was conducted as described by [13]. Briefly, semen collected
170 from three of the above described stallions was washed twice in modified Tyrode's medium pH 7.4
171 for 2.5 min at 800 x g; the pellet was resuspended in the same medium at $1x10^6$ spermatozoa /mL and
172 aliquots of 500 µl were capacitated in presence or absence of AP (1.25 or 2.5 IU/mL) for 1 h at 38.5°
173 C in 95% humidity and 5% CO_2 atmosphere. Subsequently, 50 matured denuded oocytes were added
174 to each well. After 1 h of co-incubation at 38.5° C in 95% humidity and 5% CO2 in air, the oocytes
175 were washed three times in PBS 0.4% BSA with a wide bore glass pipette, fixed in 4%
176 paraformaldehyde for 15 min at room temperature in the dark and stained with 8,9 µM Hoechst
177 33342. Cells were washed twice in PBS, and individually placed in droplets of Vectashield (Vector
178 Laboratories, Burlingame, CA, USA) on a slide, and covered with a coverslip. The number of
179 spermatozoa attached to each oocyte was assessed by using the above described microscope and was
180 expressed as mean number of spermatozoa per oocyte.
181
182 *2.5. Sperm cryopreservation*

183
184 After dilution in Kenney extender, semen was centrifuged in glass conical tube at 600 g for 20', the
185 supernatant partially removed and the sperm pellet resupended in Heitland's extender supplemented
186 with 3% egg yolk and 3% glycerol [14] to a final concentration of 150x10^6 sperm/mL. Resupended
187 semen was loaded in 0.5 mL straws, kept at +4°C for two h and then frozen on liquid nitrogen vapors
188 for 20'6 cm above the liquid nitrogen level.
189
190 *2.6. Statistical analysis*
191
192 Data were analyzed using R version 3.0.3. (Copyright © 2014, The R Foundation for Statistical
193 Computing) and significance was set at $p<0.05$ unless otherwise specified.
194 Data were assayed for normal distribution using Shapiro-Wilk test; in case of non-normal distribution,
195 data were handled by a log transformation to match normality. Subsequently an ANOVA test was
196 used to assess differences between the AP activity at pH 7.0, 8.0 and 10.0. The paired samples t test
197 was used for comparison of the sperm quality parameters and AP activity in pre and post thawing
198 samples.
199 To test correlation between sperm quality parameters and AP activity, Spearman's rank test was used.
200 As for the oocyte binding assay, data were tested by a general mixed effects model, setting the
201 treatment as a fixed effect and the horse as a random effect .Linear regression was used to examine
202 relationship between AP activity in seminal plasma and post-thawing sperm viability [22].
203 Results are expressed as mean ± standard deviation.
204
205
206 **3. Results**
207
208 Sperm concentration, motility (total motility TM, progressive motility PM, path velocity VAP,
209 curvilinear velocity VCL), viability and mitochondrial membrane integrity as well as seminal plasma
210 and sperm extracts protein concentration are reported in Table 1.
211 Sperm quality parameters from each stallion are presented in supplementary file 1 (S1).
212 AP activity in both seminal plasma and sperm extracts is significantly different depending onthe
213 three analyzed pH. A significant increase is evident from pH 7.0 to 8.0 ($p<0.001$) as well as from pH
214 8.0 to pH 10.0 ($p<0.001$), as reported in Table 2.
215 Different AP activities in both seminal plasma and sperm extract were found among stallions, and
216 data from single subjects are resumed in supplementary file 1 (S1).

217 The thermal inhibition test, carried out on seminal plasma AP activity, showed a decrease due to heat
218 exposure in a time dependent manner, while the activity of the intestinal AP isoform was not affected
219 by incubation at 60 °C. In fact, seminal plasma AP lost 45% activity after 1 min and 15 sec at 60°C,
220 reaching the 80% after 1 min and 45 sec and was completely inactivated after 3 min. (Figure 1).
221 AP activity in frozen-thawed sperm extracts significantly decreased ($p<0.01$ for pH 7.0 and 8.0 and
222 $p<0.05$ for pH 10 measurements) in comparison with the relative freshly ejaculated sample. Results
223 on AP activity are resumed in Table 2.
224 AP activity in sperm extracts from freshly ejaculates and frozen-thawed spermatozoa from each
225 stallion are presented in supplementary file 2 (S2).
226 Sperm quality parameters significantly changed after freezing (Table 3). Motility parameters (TM,
227 PM, VAP and VCL) significantly ($p<0.01$) decreased after freezing and the same trend was evident
228 in viability and mitochondrial activity ($p<0.01$); on the contrary, sperm protein content was not
229 affected by the freezing process.
230 Results on sperm quality parameters from each stallion are presented in Supplementary file 2 (S2).
231 The oocyte binding assay did not show any difference between control group and those treated with
232 different AP concentrations (1.25 and 2.5 IU/mL).
233 The control group scored 13.85±14.4 sperm bound per oocyte, while AP 1.25 scored 15.17±13.04
234 and AP 2.5 14.44±14.26.
235 Spearman's correlations between AP activity in seminal plasma and sperm extracts and sperm quality
236 parameters in freshly ejaculated spermatozoa are resumed in Table 4.
237 Seminal plasma AP activities measured at pH 7.0, 8.0 and 10.0 were positively correlated. In addition,
238 sperm extracts AP activity measured at pH 7.0, 8.0 and 10.0 showed positive correlation. AP activity
239 in seminal plasma was positively correlated with sperm concentration and negatively with seminal
240 plasma protein concentration.
241 Table 5 resumes the correlation between AP activity in sperm extracts and sperm quality parameters
242 in frozen-thawed stallion spermatozoa. While in fresh sperm extracts no correlation was found
243 between motility and AP activity from sperm extracts, in frozen-thawed samples positive correlations
244 between AP activity in sperm extracts at pH 7.0 and 10.0 and total motility and progressive motility
245 were recorded.
246 Correlations between freshly ejaculated stallion sperm AP activity (in seminal plasma and sperm
247 extracts) and sperm quality parameters and frozen-thawed ones are presented in Supplementary file
248 3 (S3).
249 AP activity in seminal plasma measured at pH 10.0 and 7.0, as well as total motility and progressive
250 motility in freshly ejaculated spermatozoa were correlated with sperm viability after thawing.

251 Multiple regression generated a statistical model with only one explanatory variable (AP activity in
252 seminal plasma measured at pH 10.0) explaining 26.5% (Adjusted R^2) of the variation in sperm
253 viability after thawing.
254
255 **4. Discussion**
256
257 This study was aimed at determining alkaline phosphatase activity in stallion seminal plasma and
258 sperm extracts, and at defining AP relationship with some sperm quality parameters.
259 Our results are reported as specific activity, expressed as nmoles of substrate transformed in 1 min
260 for milligram of protein in the sample. This is, in our opinion, the best way to express the activity, as
261 it permits to delineate the substrate consumption in relation to the effective protein content of the
262 sample, that can considerably differ among both stallions (see supplementary file S1) (inter subject
263 variability) and samples from the same subject (intra subject variability). Therefore, this measurement
264 is standardized and is not affected by the different concentrations of protein (and enzyme) in the
265 ejaculate.
266 To better define the properties of seminal plasma AP, we performed a thermal inhibition test, as the
267 enzyme's isoforms show significantly different sensitiveness to heat. AP in horse is easily and rapidly
268 inhibited by heat, as reported in Figure 1. Recently, we observed that AP from boar seminal plasma
269 is quite more resistant, losing 100% activity only after 10 min at 60°C [9]; on the basis of these data
270 and those reported for boar and humans [15,16], we could infer that seminal plasma AP in stallion is
271 very similar to bone isoforms.
272 This is the first report that approached the study of AP activity respecting the physiological
273 environment of stallion semen. The enzyme activity at pH 7.0 is very low, in both seminal plasma
274 and sperm extracts. This fact seems to be conflicting with other reports [5, 6], stating that AP activity
275 in stallion semen is very high. Anyway, our data on AP activity reveal that it parallels the increase
276 of pH, thus confirming that, at least at pH 8.0 and 10.0, AP activity in stallion semen is very high.
277 The intriguing question risen from these data regards the role of AP in seminal plasma and sperm
278 extracts of physiological stallion semen pH (near 7.0). Turned and McDonnell [5] studied the activity
279 of the enzyme in extracts from testis, epididymis and ampullae, as well as from ampullary fluid and
280 epididymal fluid. The highest activity was found in fluids from both epididymis and ampullae, thus
281 letting to hypothesize that the enzyme is secreted in these compartments; in particular, epididymal
282 fluid activity is 50 fold the mean activity registered in seminal plasma.
283 In a previous work we hypothesized that AP could play a role in maintaining porcine spermatozoa
284 quiescent, preventing a premature capacitation [9]. In horse sperm, on the basis of ours and other's

285 [5] data, AP likely plays a role on spermatozoa metabolism and activity during their transit from
286 testicles to the ampullae, while sperm dilution in seminal plasma dramatically reduces the enzyme
287 activity and therefore its possible involvement in sperm metabolism. It should also be stressed that
288 spermatozoa, during natural mating, are diluted in seminal plasma for a brief period of time, after
289 which they enter the female genital tract. In this region, the environment is completely different and
290 spermatozoa gradually lose seminal plasma to begin the capacitation process that lead to sperm-
291 oocyte fusion and fertilization [17]. This process, in the mare, takes from 0.5 to 4 h.
292 To reinforce this hypothesis, the heterologous oocyte binding assay showed no difference between
293 untreated spermatozoa under capacitating condition and spermatozoa treated with AP at two different
294 doses. We may infer that the addition of AP to a capacitating medium with a neutral pH (7.4) is not
295 effective in modifying heterologous oocyte binding, considered a good parameter to assess sperm
296 capacitation in stallion [13].
297 AP activity of seminal plasma is significantly higher than that of sperm extracts (at all pH tested,
298 7.0, 8.0 and 10.0), with a minimum mean ratio respectively of 5:1 at pH 10.0 and a maximum of 9:1
299 at pH 8.0. This is not surprising: other Authors [5] showed that the contribution of spermatozoa to
300 the whole ejaculate's activity is very low if compared to that of seminal plasma. Those researchers,
301 anyway, obtained an indirect indication of AP activity from spermatozoa, as they subtracted the
302 activity of unprocessed semen samples to that of seminal plasma, while in our study we pointed out
303 the effective AP activity from sperm cell extracts
304 The role of AP attached to sperm surface is more intriguing: in boar spermatozoa we demonstrated a
305 significant decrease in AP activity after in vitro capacitation [9]. It should be highlighted that AP
306 activity at physiological pH in boar (8.0) is similar to that observed in this study in stallion at the
307 same pH that is significantly higher than that registered at pH 7.0.In that study we demonstrated that
308 AP exerts a down regulatory activity in tyrosine protein phosphorylation during capacitation [9]. This
309 action is intriguing, as under capacitating conditions, boar semen protein phosphorylation increases
310 significantly, and it lets us to hypothesize that AP should be hashed down to permit capacitation in
311 pig [9]. We do not have any experimental evidence that AP plays a role in stallion sperm capacitation,
312 as it is deducible from heterologous oocyte binding, and its role (if any) during sperm transit in the
313 male genital tract does not seem to be important. Recently, other Authors [18] showed that stallion
314 spermatozoa acquire capacitation by interacting with tubal epithelial cells: in particular, those Authors
315 demonstrated that tyrosine phosphorylation of spermatozoa is positively correlated to the increase in
316 intracellular pH, strictly associated with the microenvironment induced by epithelial cells.
317 Nevertheless, further studies are necessary to determine the possible role of AP during the
318 capacitation process.

319
320 .
321 There is a great range of AP activity in both seminal plasma and sperm extracts; we found significant
322 differences among horses in AP seminal plasma activity at pH 7.0 and 8.0, while no difference was
323 recorded either at pH 10.0 or in AP activity from sperm extracts (see supplementary file S1). The
324 same trend between AP activity in seminal plasma and sperm extracts has been observed in boar
325 [9]. The individual variation is well known in horse and pig, thus it is not surprising that mean values
326 of our samples are quite dispersed.
327
328 The relationship between sperm quality parameters and AP activity was investigated by correlation
329 test. As already described by Pesch and colleagues [6], we found a positive correlation between
330 seminal plasma AP activity and sperm concentration. A high sperm concentration results from a lower
331 secretion of seminal plasma by the accessory glands and, therefore, it is concomitant with a lower
332 dilution of epididymal and testicle secretions which are rich of AP [5]. On the other hand, in our
333 study, sperm extracts AP activity does not correlate with sperm concentration. We registered a
334 negative correlation between seminal plasma protein concentration and AP activity in both seminal
335 plasma and sperm extracts, excepting for sperm extracts activity at pH 7.0. These findings are difficult
336 to interpret and further studies are necessary to understand if they could be related to AP origin
337 (secretion from ampullae and epididymis). No correlations between AP activity in seminal plasma
338 and/or sperm extracts and viability were found, while a negative correlation between AP activity in
339 seminal plasma and sperm extracts, measured at pH 10.0, and the two sperm velocity parameters,
340 VCL and VAP, was recorded. This finding maybe deserves further insights, as it seems to indicate
341 that high AP activity is related with lower velocity of the spermatozoa, but evidence of a similar effect
342 at physiological pH is still lacking, thus letting the question open.
343 The second part of our work aimed at determining the possible involvement of AP activity in
344 cryopreservation of stallion sperm, with particular attention to sperm cryo-damage. Our results on
345 sperm quality parameters (motility, viability and mitochondrial activity) are consistent with those
346 reported by others [19; 20], thus delineating a significant loss in sperm quality after freezing and
347 thawing procedure.
348 We demonstrated that AP activity in sperm extracts significantly decreases after freezing and
349 thawing; similar results were reported for ram spermatozoa by Salamond and Maxwell [10], who
350 observed that the phosphatase activity of ram semen after freezing and thawing decreases
351 significantly. However, those Authors did not specify whether it was alkaline or acid one. Alkaline
352 phosphatase activity is related to protein dephosphorylation and it possibly plays an important role in

353 regulating this process, at least in pig [9]. Evidence exists that tyrosine phosphorylation is activated
354 after freezing and thawing of stallion spermatozoa and that the response of stallion sperm to
355 capacitation stimuli is different after freezing and thawing [21]. In addition, tyrosine phosphorylation
356 is one of the aspects of the so called cryo-capacitation [19], and its increase after freezing and thawing
357 may be due to AP inhibition. The intimate mechanism of AP inactivation is still unclear: can be the
358 inactivation a consequence of the membrane damage that lead to a loss of enzyme from the sperm
359 surface? Cryoelution of surface proteins?
360 As for the correlation between frozen-thawed sperm quality parameters and AP activity of relative
361 sperm extracts, only measurements at pH 10.0 and 7.0 (but not at pH 8.0) showed some correlations.
362 A strong positive correlation between AP activity (at pH 7.0 and 10.0) and TM and PM is evident;
363 this correlation is not present in the freshly ejaculated spermatozoa. We may suppose that this change
364 is due to a perturbation of both motility parameters and AP activity because of the freezing-thawing
365 process.
366 We also correlated AP activity in freshly ejaculated cells with sperm quality parameters of the relative
367 frozen sample (presented in supplementary file S3). This correlation could possibly be useful for
368 predicting sperm freezability based on AP activity in seminal plasma. The multiple linear regression
369 showed that a part of the variability in sperm viability after thawing could be related to AP activity
370 measured at pH 10, and that this parameter could be used for predicting, at least in part, the
371 freezability of stallion semen. The linear relationship, in fact, links the amount of AP activity
372 (measured at pH 10) in seminal plasma of the fresh ejaculate with the viability of post-thawing
373 spermatozoa indicating a direct proportionality between the parameters.
374 In conclusion, our data demonstrate for the first time the presence of AP on stallion sperm surface
375 and describe the enzyme activity in both seminal plasma and sperm extracts at different pH.
376 Measurements at pH 7.0 indicates that AP in the ejaculate is not very active, letting thus unknown its
377 role in the ejaculate. In contrast, the activity on sperm surface, when reaching the utero tubal junction,
378 could be enhanced by the higher pH in this part of the female genital tract. In addition, we found some
379 interesting correlations between AP and sperm quality parameters. Finally, we observed a decrease
380 of AP activity after freezing-thawing process, which parallels that of both viability and sperm
381 motility.
382

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384

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389

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469 Figure 1. Thermal inactivation of seminal plasma AP activity.

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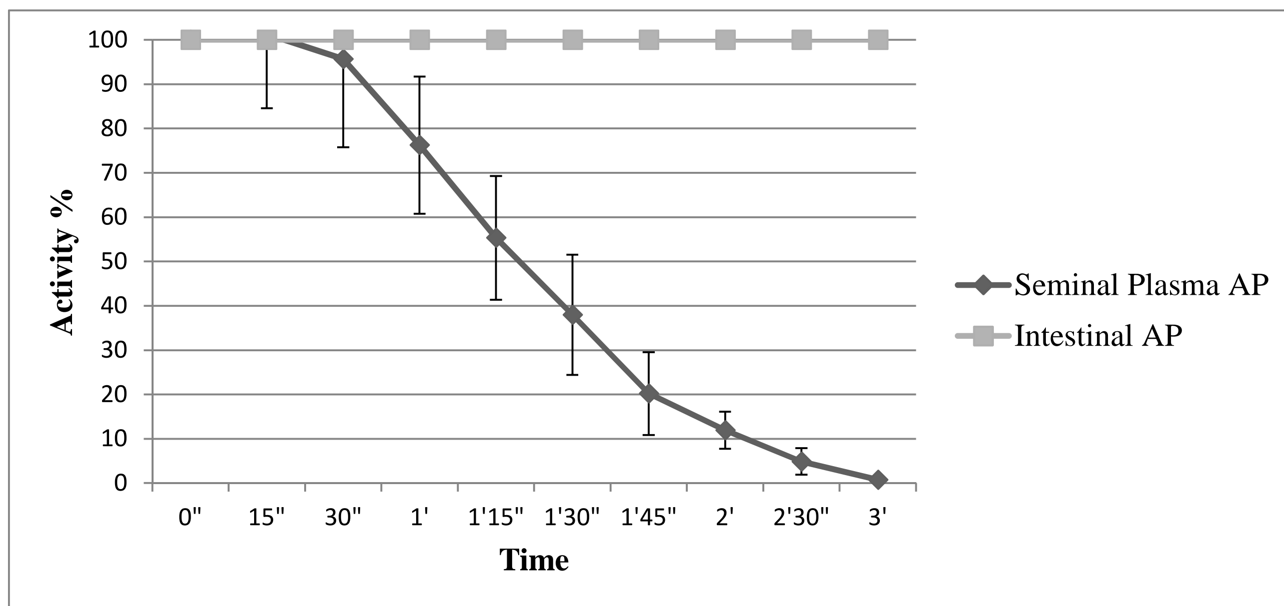

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473 AP activity was inhibited by heat (60°C) for different time periods. Error bars represent 1 SD.

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493 Table 1. Sperm quality parameters in freshly ejaculated spermatozoa. Results are expressed as mean
494 ± SD (n=40).
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496

Parameter	mean± SD
Concentration (10^6/mL)	197±125
Total Motility %	69.3±20.0
Progressive motility %	45.0±12.7
Average velocity (VAP) (µm/sec)	102.0±22.5
Curvilinear velocity (VCL) (µm/sec)	190.1±41.8
% Viability	71.4±13.8
Concentration (10^6/mL)	197±125
Seminal plasma protein (mg/mL)	12.4±10.4
Sperm extracts protein (mg/mL)	2.3±1.0

497
498 Table 2. AP activity in seminal plasma and spermatozoa extract at different pH.
499

	Specific activity (nmol/min/µg protein)		
	pH 7.0	pH 8.0	pH 10.0
AP activity Seminal plasma (n=40)	11.1±10.5^{a}	68.2±48.5^{b}	503.2±419.4^{c}
AP activity Sperm extracts (n=40)	2.7±2.3^{a}	7.5±6.9^{b}	102.9±78.7^{c}
AP activity pre-freezing (n=21)	2.6±2.0*	6.8±4.7*	58.3±46.2$^{\#}$
AP activity after freezing/thawing (n=21)	0.9±0.6*	3.0±1.6*	19.9±11.6$^{\#}$

500
501 Data are expressed as mean ± SD. Lowercase letters superscript indicate significant differences for
502 $p<0.001$. * indicates significant differences within a column for $p<0.01$; # indicates significant
503 differences within a column for $p<0.05$.
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510 Table 4. Sperm quality parameters in freshly ejaculated and frozen/thawed spermatozoa.
511

Parameter	Freshly ejaculated sperm	Frozen/thawed sperm
TM	73.8±18.2	28.8±15.5*
PM	47.4±11.2	22.5±13.4*
VAP	107.1±16.2	88.2±14.6*
VCL	195.9±30.0	157.9±25.5*
% JC1 positive live	63.1±13.9	31.8±12.5*
% Viability	74.8±12.6	41.1±13.1*
Protein sperm extracts (mg/mL)	2.6±1.1	2.2±1.1

512
513 Data are expressed as mean ± sd; n=21. * indicate significant difference in row for $p<0.001$.
514

515 Table 5. Spearman's correlations between sperm quality parameters and AP activity in seminal plasma and sperm extracts.

516

	AP_P7	AP_P8	AP_P10	AP_S7	AP_S8	AP_S10	Conc	PlProt	SPProt	TM	PM	VAP	VCL	Live	Livepos
AP_P7	1														
AP_P8	0.95*	1													
AP_P10	0.90*	0.88*	1												
AP_S7	0.14	0.17	0.27	1											
AP_S8	0.49*	0.51*	0.47*	0.74*	1										
AP_S10	0.37	0.49#	0.37	0.60*	0.91*	1									
Conc	0.59*	0.48*	0.53*	-0.05	0.01	-0.02	1								
PlProt	-0.56*	-0.60*	-0.50*	-0.13	-0.53*	-0.65*	0.18	1							
SPProt	0.49#	-0.25	-0.33	-0.09	-0.14	-0.61*	0.05	0.33#	1						
TM	-0.29	0.08	0.19	0.07	0.04	-0.27	0.31	0.33	0.10	1					
PM	0.19	0.35	0.50#	0.14	0.19	-0.21	0.33	0.03	-0.02	0.83*	1				
VAP	-0.33	-0.33	-0.42#	-0.27	-0.50	-0.59*	0.13	0.52*	0.17	0.41#	0.30	1			
VCL	-0.46#	-0.36	-0.52*	-0.25	-0.53#	-0.54#	0.14	0.49*	0.19	0.16	-0.04	0.89	1		
Live	0.20	0.18	0.18	-0.24	0.03	-0.11	0.10	0.12	-0.04	0.63*	0.61*	0.29	0.02	1	
Livepos	-0.09	-0.08	-0.12	-0.25	-0.26	-0.46#	0.01	0.40#	0.04	0.48*	0.38#	0.39#	0.26	0.60*	1

517

518 Data represent Spearman's rho coefficient; * indicates $p<0.01$; # indicates $p<0.05$.

519 Parameters are reported as follows: AP_P7 AP activity in seminal plasma pH7; AP_P8 AP activity in seminal plasma pH8; AP_P10 AP activity in
520 seminal plasma pH10; AP_S7 AP activity in sperm extracts pH7; AP_S8 AP activity in sperm extracts pH8; AP_S10 AP activity in sperm extracts
521 pH10; Conc: sperm concentration; PlProt: Seminal plasma proteins; SPProt: sperm extracts protein; TM: total motility; PM: progressive motility;
522 VAP: average path velocity; VCL: curvlinear velocity; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane
523 activity.

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Table 6. Spearman's correlation between AP activity in sperm extracts and sperm quality parameters in frozen/thawed spermatozoa.

	AP_S7	AP_S8	AP_S10	Conc	SPProt	TM	PM	VAP	VCL	Live	Livepos
AP_S7	1										
AP_S8	-0,0992	1									
AP_S10	0,5767#	0,3299	1								
Conc	-0,1808	-0,037	-0,0515	1							
SPProt	0,2149	-0,6305*	-0,0283	0,0455	1						
TM	0,5717#	-0,1129	0,5988#	0,2861	0,1482	1					
PM	0,5994*	-0,1334	0,6102*	0,2861	0,17	0,9928*	1				
VAP	0,1948	-0,1817	0,1214	0,1215	0,4448#	0,5629*	0,5672*	1			
VCL	0,0847	-0,0234	-0,0368	-0,0468	0,3012	0,3225	0,3108	0,8912*	1		
Live	0,0785	-0,0208	-0,0221	0,3494	-0,1535	0,6665*	0,658*	0,3852	0,2442	1	
Livepos	0,2841	-0,1326	0,2966	0,0091	-0,0338	0,7666*	0,7581*	0,5138#	0,3455	0,6753*	1

Data represent Spearman's rho coefficient; * indicate $p<0.01$; # indicate $p<0.05$.

Parameters are reported as follow: AP_S7 AP activity in sperm extracts pH7; AP_S8 AP activity in sperm extracts pH8; AP_S10 AP activity in sperm extracts pH10; Conc: sperm concentration; SPProt: sperm extracts protein; TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvlinear velocity; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane activity.

538 S1. AP activity in seminal plasma and spermatozoa extract and sperm quality parameters in freshly ejaculated stallion spermatozoa.

Horse	n	AP_P7	AP_P8	AP_P10	AP_S7	AP_S8	AP_S10	PlProt	SPProt	TM	PM	VAP	VCL	Live	Livepos
1	1	15.7±NA	49.7±NA	291.2±NA	NaN	5.2±NA	NaN	11.6±NA	3.0±NA	NaN	NaN	NaN	NaN	41.2±NA	41.2±NA
2	2	53.3±NA	218.6±NA	1181.4±NA	2.2±2.8	7.1±3.8	102.7±69.2	4.7±NA	2.4±0.7	9.0± NA	6.0±NA	70.0± NA	170.3± NA	60.8±28.1	14.5±14.0
3	8	6.2±3.4	33.5±15.23	204.8±128.3	1.7±0.8	3.81.2±	49.6±43.5	19.4±13.1	2.5±1.3	77.0±7.2	47.0±4.6	121.4±14.7	223.6±23.3	67.0±10.3	62.2±11.3
4	2	16.9±0.7	81.0±4.16	334.2±135.9	1.4±1.6	10.9±2.7	144.6±53.0	5.5±1.0	1.5±0.5	71.0± NA	55.0±NA	98.9± NA	162.0± NA	82.8±2.0	73.7±14.8
5	9	9.7±8.9	47.3±32.84	481.5±455.2	2.6±1.3	5.6±5.1	76.5±53.6	19.5±13.0	2.6±1.1	75.5±10.3	46.0±8.2	103.3±19.0	195.8±42.2	79.2±9.0	71.0±7.8
6	6	23.0±13.3	107.8±53.28	746.0±405.4	3.6±3.3	10.3±9.5	87.1±88.8	8.5±2.8	2.1±1.2	88.3±3.9	58.2±1.2	112.9±10.6	197.2±23.6	80.7±8.7	70.5±8.6
7	2	17.5±1.4	85.6±19.59	578.0±328.7	8.6±NA	19.7±22.0	183.0±198.3	6.2±1.3	2.0±1.1	66.0± NA	52.0±NA	70.0± NA	139.2± NA	80.5±3.3	72.6±3.7
8	2	29.3±3.1	127.0±6.92	1077.3±604.3	2.3±3.1	8.3±6.3	95.9±77.1	9.1±0.2	2.3±0.5	65.0± NA	43.0±NA	78.0± NA	134.4± NA	76.0±12.5	71.0±19.5
9	6	9.7±7.2	56.9±42.35	410.7±467.8	1.6±1.2	5.7±2.6	170.8± NA	5.6±1.2	2.0±0.8	45.6±13.2	31.4±7.6	99.4±8.8	194.2±30.1	56.2±10.1	46.9±10.8
10	2	8.0±1.0	62.3±27.73	171.3±72.9	3.3±NA	12.4±3.5	180.3±25.0	5.48±0.4	2.6±0.9	53.0± NA	39.0±NA	33.8± NA	68.3± NA	72.1±0.1	47.3±10.3

539

540 Parameters are reported as follow: n: number of ejaculates; AP_S7 AP activity in sperm extracts pH7; AP_S8 AP activity in sperm extracts pH8;
541 AP_S10 AP activity in sperm extracts pH10; Conc: sperm concentration; PlProt: Seminal plasma proteins; SPProt: sperm extracts protein; TM: total
542 motility; PM: progressive motility; VAP: average path velocity; VCL: curvlinear velocity; Live: % live spermatozoa; Livepos: % live spermatozoa
543 with high mitochondrial membrane activity.
544
545

546 S2. AP activity and sperm quality parameters in freshly ejaculated and frozen/thawed spermatozoa.

547

Horse	n	AP_S7	AP_S8	AP_S10	SPProt	TM	PM	VAP	VCL	Live	Livepos
3	4	2.65±1.85	6.24±5.96	33.98±17.82	3.09±0.97	79.25±5.91	49.00±1.41	120.55±16.77	219.95±25.30	75.38±3.30	61.06±12.58
3FT	4	1.01±0.31	2.17±0.85	15.52±4.35	3.25±1.22	30.25±7.93	23.25±5.56	103.10±9.64	190.80±10.58	39.73±5.36	35.14±3.11
5	6	2.32±0.85	6.38±3.00	52.10±42.02	2.98±1.15	79.00±7.01	48.83±7.47	98.78±19.45	180.18±34.21	83.28±2.28	71.45±6.11
5FT	6	0.81±0.54	2.59±0.70	17.46±3.86	2.65±0.70	32.17±16.19	24.00±13.43	95.42±12.45	166.33±23.12	44.43±15.43	30.77±13.66
6	6	3.22±3.06	8.26±6.98	62.58±41.45	2.18±1.20	88.33±3.93	58.17±1.17	112.92±10.61	197.15±23.62	80.68±8.68	70.53±8.63
6FT	6	1.14±0.78	3.05±1.64	26.43±17.83	2.05±1.09	39.33±13.32	33.00±11.78	85.03±6.14	144.67±12.53	48.79±13.71	38.21±13.45
9	5	1.62±1.24	5.94±2.87	170.83±17.82	2.07±0.91	45.60±13.20	31.40±7.57	99.40±8.85	194.20±30.06	57.00±11.09	45.82±11.69
9FT	5	0.44±0.52	4.07±2.46	12.22±4.35	1.19±0.64	11.00±4.58	7.40±4.51	71.32±9.23	137.24±17.55	29.15±4.53	22.74±11.56

548

549 Parameters are reported as follow: n: number of ejaculates; AP_S7 AP activity in sperm extracts pH7; AP_S8 AP activity in sperm extracts pH8;
550 AP_S10 AP activity in sperm extracts pH10; Conc: sperm concentration; PlProt: Seminal plasma proteins; SPProt: sperm extracts protein; TM: total
551 motility; PM: progressive motility; VAP: average path velocity; VCL: curvlinear velocity; Live: % live spermatozoa; Livepos: % live spermatozoa
552 with high mitochondrial membrane activity.

553

554

S3. Spearman's correlation between AP activity and sperm quality parameters in freshly ejaculated and frozen/thawed spermatozoa.

	AP_P7	AP_P8	AP_P10	AP_S7	AP_S7F	AP_S8	AP_S8F	AP_S10	AP_S10F	Conc	ConcF	PlProt	SPProt	SPProtF	TM	TMF	PM	PMF	VAP	VAPF	VCL	VCLF	Live	LiveF	Lipos	LiposF
AP_P7	1																									
AP_P8	0.99	1																								
AP_P10	0.94	0.94	1																							
AP_S7	0.49	0.45	0.38	1																						
AP_S7F	-0.09	-0.10	-0.11	-0.19	1																					
AP_S8	0.63	0.59	0.55	0.77	-0.03	1																				
AP_S8F	0.40	0.45	0.34	0.27	-0.10	0.44	1																			
AP_S10	0.62	0.60	0.6	0.3	-0.30	0.60	0.50	1																		
AP_S10F	0.07	0.04	0.10	-0.11	0.58	0.20	0.33	0.16	1																	
Conc	0.63	0.59	0.56	0.39	0.02	0.33	0.12	0.43	0.12	1																
ConcF	0.60	0.55	0.55	0.56	-0.18	0.51	-0.04	0.09	-0.05	0.64	1															
PlProt	-0.60	-0.70	-0.63	-0.07	0.15	-0.34	-0.37	-0.44	0.07	0.001	-0.02	1														
SPProt	-0.47	-0.50	-0.56	-0.01	0.11	-0.21	-0.15	-0.64	-0.05	-0.12	0.12	0.58	1													
SPProtF	-0.59	-0.63	-0.59	-0.15	0.21	-0.40	-0.63	-0.65	-0.03	-0.07	0.05	0.80	0.51	1												
TM	0.33	0.19	0.28	0.47	0.27	0.25	-0.31	-0.07	0.43	0.44	0.60	0.25	-0.06	0.36	1											
TMF	0.32	0.21	0.33	0.18	0.57	0.12	-0.11	0.004	0.60	0.47	0.29	0.28	-0.17	0.15	0.70	1										
PM	0.53	0.36	0.55	0.30	0.03	0.12	-0.02	0.22	0.30	0.54	0.48	0.13	-0.19	0.13	0.80	0.69	1									
PMF	0.31	0.22	0.34	0.18	0.60	0.12	-0.13	-0.01	0.61	0.49	0.29	0.26	-0.21	0.17	0.71	0.99	0.68	1								
VAP	-0.22	-0.27	-0.34	-0.04	0.40	-0.27	-0.21	-0.31	0.10	0.15	-0.15	0.38	0.11	0.38	0.36	0.49	0.36	0.46	1							
VAPF	-0.14	-0.23	-0.09	0.29	0.19	0.14	-0.18	-0.33	0.12	0.12	0.12	0.60	0.10	0.44	0.40	0.56	0.31	0.57	0.33	1						
VCL	-0.35	-0.30	-0.46	-0.01	0.41	-0.19	-0.07	-0.35	0.11	0.079	-0.23	0.29	0.23	0.26	0.10	0.28	-0.01	0.25	0.87	0.16	1					
VCLF	-0.24	-0.31	-0.16	0.22	0.08	0.16	-0.02	-0.25	-0.04	-0.01	-0.05	0.53	0.12	0.30	0.12	0.32	0.05	0.31	0.36	0.89	0.32	1				
Live	0.16	0.03	0.16	-0.06	0.45	0.002	-0.24	0.37	0.46	0.21	0.21	0.35	-0.10	0.28	0.53	0.68	0.52	0.67	0.22	0.32	-0.04	0.08	1			
LiveF	0.51	0.37	0.55	0.44	0.08	0.28	-0.02	0.05	-0.02	0.26	0.35	-0.01	-0.35	-0.2	0.58	0.67	0.63	0.66	0.23	0.39	0.01	0.24	0.49	1		

Liveposs	-0.22	-0.30	-0.26	-0.29	0.41	-0.27	-0.17	-0.44	0.53	0.01	0.12	0.55	0.37	0.50	0.45	0.47	0.40	0.46	0.33	0.34	0.13	0.17	0.61	0.19	1	
LIvepo sF	0.15	0.08	0.16	0.14	0.28	-0.01	-0.13	-0.24	0.30	0.18	0.01	0.09	-0.26	-0.0	0.45	0.77	0.44	0.76	0.48	0.51	0.32	0.35	0.32	0.68	0.34	1

Data represent Spearman's rho coefficient; yellow underline indicate $p<0.01$; green underline indicate $p<0.05$.

Parameters are reported as follow: AP_P7 AP activity in seminal plasma pH7; AP_P8 AP activity in seminal plasma pH8; AP_P10 AP activity in seminal plasma pH10; AP_S7 AP activity in sperm extracts pH7; AP_S8 AP activity in sperm extracts pH8; AP_S10 AP activity in sperm extracts pH10; SPProt: sperm extracts protein; TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvlinear velocity; Live: % live spermatozoa; Lipos: % live spermatozoa with high mitochondrial membrane activity. "F" letter following the parameters name refers to frozen/thawed spermatozoa.