Alkaline phosphatase added to capacitating medium enhances horse sperm-zona pellucida binding

Published Version:
Alkaline phosphatase added to capacitating medium enhances horse sperm-zona pellucida binding / Bucci, Diego; Giaretta, Elisa; Merlo, Barbara; Iacono, Eleonora; Spinaci, Marcella; Gadani, Beatrice; Mari, Gaetano; Tamanini, Carlo; Galeati, Giovanna. - In: THERIOGENOLOGY. - ISSN 0093-691X. - ELETTRONICO. - 87:(2017), pp. 72-78. [10.1016/j.theriogenology.2016.08.003]

Availability:
This version is available at: https://hdl.handle.net/11585/586264 since: 2019-07-17

Published:
DOI: http://doi.org/10.1016/j.theriogenology.2016.08.003

Bucci D, Giaretta E, Merlo B, Iacono E, Spinaci M, Gadani B, Mari G, Tamanini C, Galeati G. Alkaline phosphatase added to capacitating medium enhances horse sperm-zona pellucida binding. Theriogenology 2017;87:72-78.

The final published version is available online at: https://doi.org/10.1016/j.theriogenology.2016.08.003

Diego Bucci[1*], Elisa Giaretta[1], Barbara Merlo[1], Eleonora Iacono[1], Marcella Spinaci[1], Beatrice Gadani[1], Gaetano Mari[1,2], Carlo Tamanini[1], Giovanna Galeati[1].
[1]DIMEVET, Department of Veterinary Medical Sciences, Via Tolara di Sopra, 50; 40064 Ozzano dell'Emilia, BO, Italy;
[2] AUB INFA National Institute of Artificial Insemination, Via Gandolfi 16, 40057 Cadriano, BO, Italy.

Alkaline phosphatase added to capacitating medium enhances horse sperm-zona pellucida binding

Abstract

Alkaline phosphatase (AP) is present in equine seminal plasma and spermatozoa, but its functional role is not fully understood yet. Being that sperm-oocyte interaction in equine species has been demonstrated to be enhanced at a slightly basic pH, this work aimed at verifying whether exogenous alkaline phosphatase exerts any role on stallion spermatozoa and sperm-oocyte interaction at different pHs (7.4; 8.0; 9.0).

Stallion spermatozoa were capacitated in Tyrode's medium at pH 7.4, 8.0 and 9.0 for 4 h at 38°C, 5% CO2 with 2.5 IU AP (AP group) or without AP (CAP group); viability with mitochondrial activity, motility and acrosome integrity were measured. In addition, a homologous binding assay was carried out: stallion spermatozoa were capacitated 1 h at 38°C, 5% CO2 with 2.5 IU AP (AP group) or without AP (CAP group). Oocytes were then added to sperm suspensions and co-incubated for 1 h.

Our results indicate that AP at pH 9.0 significantly increases the percentage of living cells with active mitochondria whereas it significantly reduces the percentage of acrosome-damaged cells at pH 8.0. No significant differences were registered in motility parameters. The homologous binding assay showed a strong effect of AP, that increased the number of sperm bound to the oocyte's zona pellucida at all pHs tested.

In conclusion, AP can induce some modifications on sperm membranes thus enhancing their capacity to bind to the zona pellucida of equine oocytes.

KEYWORDS

Alkaline phosphatase; pH; Capacitation; Equine spermatozoa; Zona-binding

HIGHLIGHTS

Alkaline phosphatase (AP) added to capacitating medium at different pH AP exerts positive effects on semen parameters after capacitation at elevated pH

AP enhances homologous oocyte binding in equine species

1. INTRODUCTION

The presence and the activity of alkaline phosphatase (AP) in horse semen have been widely demonstrated; in particular, some Authors [1;2] documented the importance of this enzyme for clinical purposes (determining ejaculation failure) as well as a factor linked to sperm quality.

Other researchers better defined the distribution of AP activity in the different fractions of ejaculated semen [3], highlighting a higher activity in the sperm rich fraction, compared with sperm poor or pre-spermatic fractions. These results, together with those by Turner and McDonnell [1], indicate that AP originates from epididymal and ampullary fluids. Kareskoski et al. [4] confirmed the high activity of AP in the sperm rich fraction and showed a positive correlation between AP activity and sperm concentration. In a recent study [5] we delineated the activity of AP also in pig spermatozoal extracts, showing that it is significantly lower if compared with the seminal plasma one. A similar result was obtained by Turner and McDonnell [1] who indirectly measured AP activity in spermatozoa. In addition, we demonstrated that AP activity is highly influenced by medium pH and that it could represent a parameter for predicting sperm quality after freezing[6]. Anyway, the role of the cell surface attached enzyme is still unclear. Different studies [5,7] showed that, in pig, sperm surface phosphatase could play a crucial role in sperm function, in particular in sperm capacitation, sperm-oocyte interaction and fertilization [5]. Other evidences of a possible involvement of AP in sperm function are reported by Glogowski et al. [8] who showed that AP could be inhibited by theophyllines which are enhancers of the capacitation process in pig.

Leemans and co-workers [9] demonstrated that the optimal condition for stallion sperm capacitation may be reached by increasing the environmental pH; oviductal epithelial cells may be responsible for this modification by secreting intracellular alkaline vesicles. In addition, Loux et al. [10] demonstrated that hyperactivation of horse sperm could be induced by increasing the environmental pH, even though this does not seem to be the main mechanism involved in stallion sperm capacitation.

Basing on the information from these studies, the aim of the present work was to determine the effect of exogenous alkaline phosphatase on:

- stallion sperm parameters (such as viability and mitochondrial activity, motility and acrosome integrity) under capacitating condition in different pH media;
- sperm-zona pellucida binding in media with different pH.

2. MATERIALS AND METHODS

All the reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

2.1. Experimental design

Thyrode's [11] modified medium at three different pHs (7.4, 8.0 and 9.0, pH adjusted by NaOH) was used as capacitating medium as reported by Bucci et al [12].

Three experimental groups were set up: freshly ejaculated spermatozoa washed and diluted in Tyrode's medium (F), capacitated spermatozoa (CAP), and capacitated spermatozoa in presence of 2.5 IU/mL of AP (from bovine intestinal mucosa) (AP 2.5 group).

The subsequent sets of parameters were assayed for each experimental group:

- Sperm viability and mitochondrial activity;
- Acrosome integrity;
- Sperm motility

In addition, a zona-sperm binding assay was set up incubating washed spermatozoa for 1 hour under capacitating condition with (AP group) or without (CAP group) 2.5 IU/mL alkaline phosphatase.

2.2. Semen collection and preparation

Ejaculates from four fertile stallions, aging 5 -25 years, were used. Stallions were housed individually at the National Institute of Artificial Insemination, University of Bologna . The ejaculates were collected with a Missouri artificial vagina equipped with a disposable liner and an inline filter to avoid eventual gelatinous fraction to be collected (Nasco, Fort Atkinson, WI, USA) on a phantom and in presence of an estrous mare.

Sperm concentration was evaluated using a hemocytometer chamber (Thoma).

Semen was diluted in Kenney [13] extender pH 6.8 at a final concentration of $30x10^6$ spermatozoa /mL and sent to the laboratory within 1h.

Diluted spermatozoa were washed twice (900 x g for 2 min) and resuspended in capacitating medium at three different pHs (7.4; 8.0 and 9.0); an aliquot was immediately analyzed for viability with mitochondrial activity, motility and acrosome integrity (F group). Another aliquot was incubated for 4 h at 38.5°C, 5%CO_2 in absence (CAP group) or in presence (AP group) of 2.5 IU AP. After incubation, viability and mitochondrial activity, motility and acrosome intactness were assayed; each assay was performed 8 times (twice for each stallion) for all different media (pH 7.4, 8.0 and 9.0)

2.3. Mitochondrial activity and viability and acrosome integrity assay

For each sample, an aliquot (25 μL) of semen was incubated with 2 μL of a 300 μM propidium iodide (PI) stock solution, 2 μL of a 10 μM SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc, Eugene, OR, USA) and 2 μL of a 150 μM JC-1 solution, for 20 min at 37°C in the dark. Ten μL of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using a Nikon Eclipse E 600 epifluorescence microscope.

Spermatozoa stained with SYBR green-14 and not stained with PI were considered as viable. Spermatozoa SYBR positive and PI positive and those SYBR negative / PI positive were considered as dead or with non-intact membrane. JC-1 monomers emit a green fluorescence in mitochondria with low membrane potential and a bright red-orange fluorescence in case of polimer formation (J-aggregates) when membrane potential is high. When an orange fluorescence was present in the mid piece, live spermatozoa were considered to have functional active mitochondria (SYBR+/PI-/JC-1+).

Acrosome integrity was assessed by a FITC-conjugated lectin from Pisum Sativum (FITC-PSA) which labeled acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended in ethanol 95% and fixed/permeabilized at 4°C for 30 min. Samples were dried in heated slides and incubated with FITC-PSA solution (5 μg PSA-FITC/1ml H_2O) for 15 min in darkness. After staining, samples were washed in PBS and mounted with Vectashield mounting medium with propidium iodide (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands). The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, while a partial or total absence of fluorescence was considered to indicate acrosome disruption and/or acrosome reaction.

2.4. Motility

Motility was measured by a computer-assisted sperm analysis system, using the open source Image J CASA plugin as described by Wilson-Leedy and Ingermann [15]. Sperm cells ($30x10^6$ sperm/mL) were evaluated using a fixed height Leja Chamber SC 20-01-04-B (Leja, The Netherlands). Sperm motility endpoints assessed were: percent of total motile spermatozoa (TM), percent of progressive spermatozoa (PM), curvilinear velocity (VCL) and mean velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), lateral head displacement (ALH) and wobble (WOB). The setting parameters of the program were the followings: frames per second 60, number of frames 45, threshold path velocity 30 microns/sec, straightness threshold 75. These settings were chosen on the basis of the Standard Operating Procedure of Italian Experimental Istitute "Lazzaro Spallanzani" (Law 403/2000) for stallion sperm analysis.

2.5. Equine oocytes maturation and homologous oocyte binding assay

2.5.1. Collection and culture of cumulus oocyte complexes

Horse ovaries were collected at a local abattoir and transported to the laboratory at 25°C in a thermos case (Cell Incubator, IMV Technologies Italy). Upon arrival 2-3 h later, the ovaries were dissected free from connective tissue, rinsed with 25°C tap water and transferred to 0.9% (w/v) saline supplemented with 0.1% (v/v) penicillin/streptomycin. The cumulus-oocyte complexes (COCs) were recovered by aspirating 5–30 mm follicles using a 19-gauge butterfly infusion set connected to a vacuum pump (about 100 mmHg; KNF S.r.l, Italy). The fluid containing the COCs was collected into 250 ml glass flasks (Duran Group, Germany) and filtered through a 65 μm mesh nylon filter (EmSafe, Minitube, Germany). COCs with at least 3–5 layers of cumulus investment were classified as compact (having a tight, complete compact cumulus with a distinct, smooth hillock), expanded (having a granular or expanded cumulus), or denuded (having a partial cumulus or only corona radiata) [16]; all types of COCs were used for this study. For IVM, groups of 25–30 COCs were cultured for 26 h in 500 μl maturation medium in four-well plates (Scientific Plastic Labware, EuroClone, Italy) at 38.5°C in a humidified atmosphere of 5% CO2 in air. Maturation medium consists in the Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; Gibco) , ITS (insulin, transferrin, sodium selenite) supplement, 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1, 10IU/mL equine chorionic gonadotropin (Folligon, Intervet, Italy), and 10 IU/mL human chorionic gonadotropin (Corulon, Intervet).

At the end of the maturation period, the oocytes were denuded by gentle repeated pipetting in maturation medium.

2.5.2. Homologous sperm-zona pellucida binding assay

Homologous binding assay was carried out as described in [6,14] with some modifications. Briefly, spermatozoa were washed twice (900 x g for 2 min) and incubated in Tyrode's medium at three different pHs (7.4, 8.0 and 9.0) for 1 h at 38.5°C, 5% CO_2) in absence (CAP group) or in presence (AP group) of 2.5 IU AP as already reported [6; 14]. Subsequently, spermatozoa were washed in fresh Tyrode's medium without AP and resuspended in the same medium to obtain a concentration of 1.0 x 10^6 spermatozoa/mL (total) and placed in 500µL wells. Matured denuded oocytes were added to each well at a sperm suspension volume/oocyte ratio of 50 µL/oocyte. After 1 h of co-incubation, the oocytes were washed three times in PBS 0.4% BSA with a wide bore glass pipette to remove the excess and unbound. The oocytes were then fixed in 4% paraformaldehyde for 10 min at room temperature, washed in PBS and stained in the dark with 8.9 µM Hoechst 33342 for 10 min. Cells were washed twice in PBS, and individually placed in droplets of Vectashield (Vector Laboratories) on a slide, and covered with a coverslip. The number of spermatozoa attached to each oocyte was assessed by using a Nikon Eclipse E 600 epifluorescence microscope.

Homologous oocyte binding was repeated eight times (twice for each stallion). In total, 750 oocytes were used and divided as follows: 113 for CAP pH 7.4; 115 for AP pH 7.4; 118 for CAP pH 8.0; 106 for AP pH 8.0; 142 for CAP pH 9.0; 156 for AP pH 9.0.

2.6. Statistical analysis

Data were analyzed using R version 3.0.3. (Copyright © 2014, The R Foundation for Statistical Computing) [17] and significance was set at $p<0.05$ unless otherwise specified.

Results are expressed as mean ± standard deviation. Data were assayed for normal distribution using Shapiro-Wilk test; the Levene test for homogeneity of variance was carried out. Subsequently an ANOVA test was used to assess differences between treatments and pHs and their interaction. The Tukey Honest Significant Difference test was applied when due.

As for the quantification of the effect of AP on oocyte binding assay, a general linear model with Poisson distribution was set up.

3. RESULTS

3.1. Mitochondrial activity and acrosome integrity

The percentage of viable cells with active mitochondria is summarized in Figure 1. Capacitation, both with and without 2.5 IU of AP, significantly reduces viable cells with active mitochondria as compared to F groups ($p<0.05$).

At pH 9.0, a significant increase in viable cells with active mitochondria induced by the addition of AP to the capacitating medium was observed as compared to CAP group ($p<0.05$).

The different pHs did not induce any difference between CAP groups.

The percentages of viable cells with active mitochondria in F groups were 67.8 ± 7.3% , 67.2 ± 7.4% and 60.5 ± 10% at pH 7.4, 8.0 and 9.0 respectively; in CAP groups they were 45.9 ± 9.2%; 39.8 ± 8.3% and 38.1 ± 8.1% while in the AP 2.5 groups the percentages of spermatozoa with active mitochondria were 51.6 ± 9.2%; 47.5 ± 8.4% and 48.8 ± 7.1% for the three different pHs respectively.

3.2 Acrosome integrity

Capacitation in presence or absence of AP increased acrosome reacted cells in comparison with F group in each of the media at different pH (Fig. 2; $p<0.05$).

A significant reduction of acrosome reacted cells was evident at pH 8.0 in AP groups compared to CAP groups.

The percentages of acrosome reacted cells in F groups were 12.5 ± 8.6%, 17.9 ±9.4%; 15.4 ± 8.5% at pH 7.4, 8.0 and 9.0 respectively. In the CAP groups the percentages of acrosome reacted cells were 32.9 ± 6.5%; 32.5 ± 9.1%; 37.1 ± 8.4% at pH 7.4, 8.0 and 9.0 respectively.

Finally, the percentage of acrosome reacted cells in AP group were: 24.0 ± 6.3%; 29.0 ± 10.%3; 32.0 ± 8.9% at the three different pHs .

3.3. Sperm motility

Sperm motility parameters were not different between CAP and AP groups. Some parameters (total and progressive motility, straightness beat cross frequency and linear velocity, VSL) showed a significant difference between F group and capacitated one (in presence or absence of AP). The motility results are reported in Table 1.

3.4. Homologous oocytes binding

AP significantly increased ($p<0.05$) the mean number of attached spermatozoa at all the pHs tested; no significant difference was found between the different pHs (Fig. 3).

4. DISCUSSION

The role of alkaline phosphatase in sperm function is still unclear, particularly in stallion; this paper was aimed at determining the effect of exogenous AP on stallion sperm function under capacitating conditions.

We studied the effects of exogenous AP on viability, mitochondrial activity, motility and acrosome integrity after incubating spermatozoa for 4 h under capacitating condition at different pHs. Moreover, the effect of exogenous alkaline phosphatase on sperm-zona pellucida binding in media with different pH was evaluated. Our results on mitochondrial activity in living cells clearly show that the incubation in capacitating medium results in a decrease in the number of live cells with active mitochondria. This is not surprising, as the capacitating condition leads to a metabolic activation of the cell that could induce an energy resources depletion, membrane disruption and cell death [18]. Interestingly, no significant differences were recorded between sperm cells incubated at pH 7.4, 8.0 and 9.0; environmental pH does not therefore seem to interfere with capacitation-induced changes. However, AP reduces the percentage of dead spermatozoa after capacitation at pH 9.0. This trend is also evident considering acrosome integrity: the number of acrosome intact cells is higher in AP than in CAP group even if the difference is significant only at pH 8.0. Similar results have been observed in boar sperm [5]; in that species, however, AP added during capacitation does not affect cell viability, while it significantly reduces acrosome reacted cells Furthermore, AP added during capacitation tends to reduce the number of capacitated cells as well as tyrosine phosphorylation [5].

As above reported, an effect of the capacitation process is evident on some motility parameters (TMOT, PMOT, VSL, BCF, STR), irrespective to pH or treatment with AP. Other parameters as VAP, VCL, ALH, LIN and WOB do not change even after capacitation.

Alkaline phosphatase can play a role in pH-dependent sperm activation under capacitating condition; in that medium alkalinization highly enhances its activity. [6]. We demonstrated the enzyme is present on pig sperm surface; however, its activity is lower than that observed in seminal plasma [5]. Molecules present in seminal plasma could play a very different role from that exerted onto sperm surface: seminal plasma AP, in fact, can concur to prevent capacitation [5]. Anyway, it is reasonable to hypothesize that the enzyme could play a regulative role in the sperm-oocyte interaction: follicular fluid of pre-ovulatory follicles has been recently demonstrated to contain alkaline vesicles that stimulate hyperactivation and capacitation of stallion sperm [9,19]. This extracellular microenvironment with a slight alkaline pH (7.9, as reported by Leemans et al., [19]) is more favorable for AP activity.

It should also be highlighted that capacitation is a very complex process [20] that induces several changes of the sperm cell, involving membrane, motility as well as metabolism [21,22]. Some interesting studies [9, 23, 24] showed that equine spermatozoa undergo some capacitation- related changes in response to the alkalinization of the micro-environment, as it seems to occur in the oviduct at the time of ovulation. This modification in environmental pH leads to an increase of the intracellular pH (that, in species such as mouse and pig, could be achieved by adding bicarbonate to the capacitating medium [25]) with a subsequent activation of the spermatozoa. Finally, as reported by many Authors [22,26], mitochondria in stallion sperm seem to play a central role in modulating metabolism, and their preservation could be crucial for an optimal sperm function.

We did not observe significant changes in sperm-related parameters due to capacitating conditions and different pH, but the most interesting results are those related to sperm-ZP binding, which was clearly stimulated by AP, irrespective of pH. In a previous work on pig spermatozoa [5] we observed a strong inhibitory effect of AP on fertilization rate, with a consequent increase in normospermic zygotes; we hypothesized that AP could play a role in maintaining pig sperm quiescent and that it should be "hashed up" to permit sperm-oocyte interaction and fertilization.

Conversely, AP exerted a positive effect on stallion sperm-ZP interaction in that it highly stimulated sperm binding with zona pellucida after one hour of incubation under capacitating conditions, thus suggesting that AP could act at plasma membrane level.

This aspect deserves an insight. In a recent work on stallion sperm [6] we did not observe any effect of AP on sperm oocyte-ZP heterologous binding. It is therefore evident that the mechanism and/or the molecules involved in the enhancement of the binding capacity are species-specific. As reported by Mugnier et al., [27], porcine zonas are probably more selective and limit stallion spermatozoa binding; in addition, porcine zona protein composition has been demonstrated to be different from the equine one in terms of localization and isoforms [27]. Therefore, it is reasonable that the effect of AP has been reduced by these characteristics of porcine zona pellucida. Taken together, the overall results indicate that AP could improve some sperm parameters (viable sperm with active mitochondria at pH 9.0; acrosome integrity at pH 8.0) after 4 hours of incubation in capacitating condition and enhances cells ability to bind to the zona pellucida. It should be stressed that this effect is exerted only with horse oocytes, and that the enzyme acts specifically on some components of the outer membrane during sperm-oocyte interaction.

Acknowledgments

The Authors wish to thank Mrs Cinzia Cappannari for her precious technical support.

REFERENCES

[1] Turner RMO, McDonnell SM. Alkaline phosphatase in stallion semen: characterization and clinical applications. Theriogenology 2003;60:1–10.

[2] Pesch S, Bergmann M, Bostedt H. Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality. Theriogenology 2006;66:307–13. doi:10.1016/j.theriogenology.2005.11.015.

[3] Kareskoski A M, Reilas T, Sankari S, Andersson M, Güvenc K, Katila T. Alkaline and acid phosphatase, β-glucuronidase and electrolyte levels in fractionated stallion ejaculates. Reprod Domest Anim 2010;45:e369–74. doi:10.1111/j.1439-0531.2009.01579.x.

[4] Kareskoski M, Sankari S, Johannisson A, Kindahl H, Andersson M, Katila T. The association of the presence of seminal plasma and its components with sperm longevity in fractionated stallion ejaculates. Reprod Domest Anim 2011;46:1073–81. doi:10.1111/j.1439-0531.2011.01789.x.

[5] Bucci D, Isani G, Giaretta E, Spinaci M, Tamanini C, Ferlizza E, et al. Alkaline phosphatase in boar sperm function. Andrology 2014;2:100–6. doi:10.1111/j.2047-2927.2013.00159.x.

[6] Bucci D, Giaretta E, Spinaci M, Rizzato G, Isani G, Mislei B, et al. Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen-thawed stallion spermatozoa. Theriogenology 2015;85:288–95.e2. doi:10.1016/j.theriogenology.2015.09.007.

[7] Yi Y-J, Sutovsky M, Kennedy C, Sutovsky P. Identification of the inorganic pyrophosphate metabolizing, ATP substituting pathway in mammalian spermatozoa. PLoS One 2012;7:e34524. doi:10.1371/journal.pone.0034524.

[8] Glogowski J, Danforth DR, Ciereszko A. Inhibition of alkaline phosphatase activity of boar semen by pentoxifylline, caffeine, and theophylline. J Androl 2002;23:783–92.

[9] Leemans B, Gadella BM, Sostaric E, Nelis H, Stout TAE, Hoogewijs M, et al. Oviduct Binding and Elevated Environmental pH Induce Protein Tyrosine Phosphorylation in Stallion Spermatozoa 1. Biol Reprod 2014;9113:1–12. doi:10.1095/biolreprod.113.116418.

[10] Loux SC, Crawford KR, Ing NH, González-Fernández L, Macías-García B, Love CC, et al. CatSper and the relationship of hyperactivated motility to intracellular calcium and pH kinetics in equine sperm. Biol Reprod 2013;89:123. doi:10.1095/biolreprod.113.111708.

[11] Rathi R, Colenbrander B, Bevers MM, Gadella BM. Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod 2001;65:462–70.

[12] Bucci D., Isani G., Spinaci M., Tamanini C. Mari G., Zambelli D., Galeati G. Comparative immunolocalization of gluts 1, 2, 3 and 5 in boar, stallion and dog spermatozoa. Reprod Domest Anim, 2010; 45: 315-322.

[13] Kenney RM, Bergman RV, Cooper WL. Minimal contamination techniques and preliminary findings. Procannu Meet Am Assoc Equine Pract 1975;21:327–36.

[14] Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Peña FJ, Mari G, et al. Effect of sex sorting on stallion spermatozoa: heterologous oocyte binding, tyrosine phosphorylation and acrosome reaction assay. Anim Reprod Sci 2013;141:68–74.

[15] Wilson-Leedy JG, Ingermann RL. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 2007;67:661–72. doi:10.1016/j.theriogenology.2006.10.003.

[16] Hinrichs K, Schmidt a L, Friedman PP, Selgrath JP, Martin MG. In vitro maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. Biol Reprod 1993;48:363–70. doi:10.1095/biolreprod48.2.363.

[17] R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

[18] Gibb Z, Aitken RJ. The Impact of Sperm Metabolism during In Vitro Storage : The Stallion as a Model. BioMed Reseach Int 2016,: Article ID 9380609, 8 pages. doi:10.1155/2016/9380609

[19] Leemans B, Gadella BM, Stout TAE, Nelis H, Hoogewijs M, Van Soom A. An alkaline follicular fluid fraction induces capacitation and limited release of oviduct epithelium-bound stallion sperm. Reproduction 2015;150:193–208. doi:10.1530/REP-15-0178.

[20] Yanagimachi R. Mammalian fertilization, in Knobil E, Neil JD, et al (eds). The physiology of reproduction; 1. New York Raven Press, Ltd.; 2008 2008, pp 189–319.

[21] Bucci D, Galeati G, Tamanini C, Vallorani C, Rodriguez-Gil JE, Spinaci M. Effect of sex sorting on CTC staining, actin cytoskeleton and tyrosine phosphorylation in bull and boar spermatozoa. Theriogenology 2012;77:1206–16. doi:10.1016/j.theriogenology.2011.10.028.

[22] Gibb Z, Lambourne SR, Aitken RJ. The Paradoxical Relationship Between Stallion Fertility and Oxidative Stress. Biol Reprod 2014;91:1–10. doi:10.1095/biolreprod.114.118539.

[23] Gonzalez-Fernandez L, Macias-Garcia B, Velez IC, Varner DD, Hinrichs K. Calcium-calmodulin and pH regulate protein tyrosine phosphorylation in stallion sperm. Reproduction 2012;144:411–22. doi:10.1530/REP-12-0067.

[24] Macias-Garcia B, Gonzalez-Fernandez L, Loux SC, Rocha AM, Guimaraes T, Pena FJ, et al. Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reproduction 2015;149:87–99. doi:10.1530/REP-14-0457.

[25] Flesch FM, Brouwers JF, Nievelstein PF, Verkleij a J, van Golde LM, Colenbrander B, et al. Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. J Cell Sci 2001;114:3543–55.

[26] Ferrusola CO, Fernandez LG, Sandoval CS, Garcia BM, Martinez HR, Tapia JA, et al. Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. Theriogenology 2010;74:458–65. doi:10.1016/j.theriogenology.2010.02.029.

[27] Mugnier S, Dell'Aquila M, Pelaez J, Douet C, Ambruosi B, De Santis T, et al. New insights into the mechanisms of fertilization: comparison of the fertilization steps, composition, and structure of the zona pellucida between horses and pigs. Biol Reprod 2009;81:856–70. doi:10.1095/biolreprod.109.077651.

Table 1. Motility measures comparing treatments and pHs. Abbreviations: TMOT – total sperm motility; PMOT – Progressive sperm motility; VAP – average path velocity; VSL– straight line velocity; VCL – curvilinear velocity; ALH – amplitude of lateral head displacement; BCF– beat cross frequency; STR – straightness of track; LIN – linearity of track; WOB wobble. Data are reported as mean ± SD. Different superscripts represent significant difference for $p<0.05$ between treatments within the same pH.

	pH 7.4			pH 8.0			pH 9.0		
	F	CAP	AP	F	CAP	AP	F	CAP	AP
TMOT	74.70±10.92^{a}	9.26±3.35^{b}	14.99±8.38^{b}	73.05±11.86^{a}	12.42±5.83^{b}	16.09±9.36^{b}	74.16±11.90^{a}	19.05±16.46^{b}	22.89±7.37^{b}
PMOT	42.23±7.25^{a}	3.38±2.94^{b}	5.64±2.18^{b}	36.07±3.93^{a}	5.06±3.08^{b}	3.89±4.86^{b}	44.91±15.90^{a}	7.32±3.62^{b}	9.54±1.30^{b}
VAP	84.10±15.24	77.51±33.04	83.15±4.78	99.08±20.01	74.15±25.72	82.46±21.38	102.49±31.36	95.68±31.14	92.58±19.06
VSL	63.78±9.18^{a}	41.90±3.7 3^{b}	48.16±13.17^{b}	71.69±12.63^{a}	40.18±15.34^{b}	43.75±9.04^{b}	75.14±13.91^{a}	53.56±12.90^{b}	53.76±4.40^{b}
VCL	175.07±19.79	157.58±72.28	176.35±8.15	181.51±26.52	131.53±21.18	149.36±33.08	197.56±48.63	183.71±34.23	188.62±20.24
ALH	6.73±0.85	6.23±2.95	6.80±0.35	7.32±1011	5.33±1.20	6.08±1.35	7.79±1.97	7.29±2.06	6.95±1.38
BCF	28.78±1.61^{a}	24.52±1.25^{b}	25.10±1.29^{b}	28.38±1.69^{a}	24.94±1.95^{b}	24.00±1.67^{b}	30.40±0.30v	25.96±5.73^{b}	22.89±0.97^{b}
STR	74.33±2.52^{a}	63.00±2.02^{b}	62.0±2.01^{b}	73.25±3.95^{a}	63±3.56^{b}	60.25±6.66^{b}	75.00±7.00^{a}	67.67±11.24^{b}	64.33±8.50^{b}
LIN	36.67±2.51	33.33±13.87	32±8.54	40±4.69	37.25±6.70	34.75±5.91	39±2	37.33±14.43	36.33±3.06
WOB	48.00±5.29	49.67±3.79	48.33±4.04	54.25±5.43	57.25±13.05	56.50±9.11	51.67±3.06	52.00±17.78	49.33±5.77

Figure 1. Boxplot representing percentage of live stallion spermatozoa with active mitochondria depending on treatment (F, CAP, AP2.5) and pH: pH 7.4 (black boxes), pH 8.0 (dark grey boxes), and pH 9.0 (light grey boxes). Different superscripts represent significant difference for p<0.05 between treatments within the same pH.

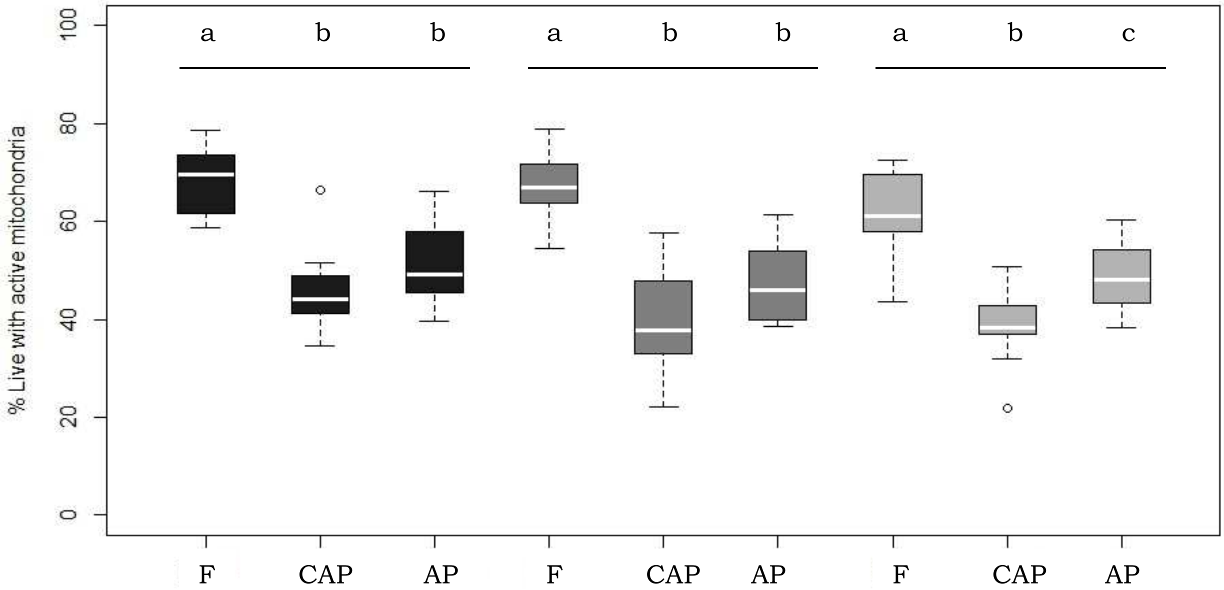

Figure 2. Boxplot representing percentage of acrosome reacted cells in the three groups (F, CAP, AP2.5) at different pH: pH 7.4 (black boxes), pH 8.0 (dark grey boxes), and pH 9.0 (light grey boxes). Different superscripts represent significant difference for p<0.05 between treatments within the same pH.

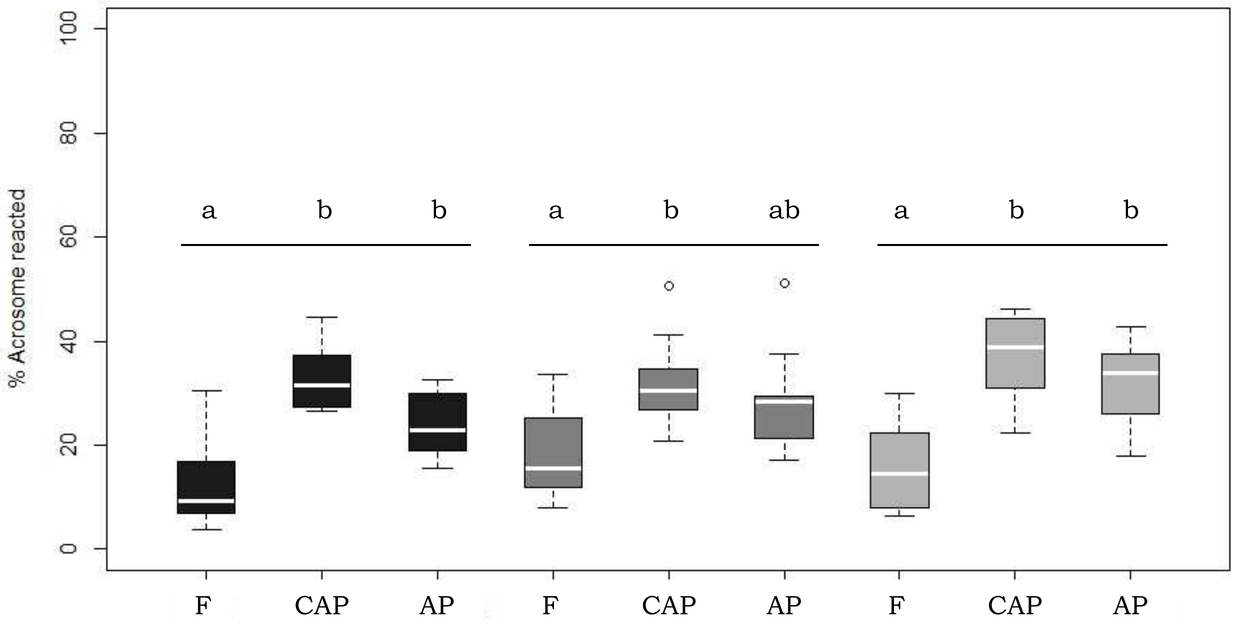

Figure 3. Boxplot representing the number of spermatozoa bound to the oocytes in the two groups (CAP, AP) at different pH: pH 7.4 (black boxes), pH 8.0 (dark grey boxes), and pH 9.0 (light grey boxes). Different letters represent significant difference for p<0.05 between treatments within the same pH.