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Alkaline phosphatase in stallion semen: characterization and clinical applications

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Abstract

Significant amounts of alkaline phosphatase (AP) activity have been found in semen plasma from numerous species. In species in which the majority of semen plasma AP (SPAP) activity originates from the epididymis and testicle, SPAP activity can be used clinically as a marker to differentiate testicular origin azoospermia or oligospermia from ejaculatory failure. Information on SPAP activity in stallions to date has been limited. In this study, a standard clinical chemistry analyzer was used to determine AP activity in pre-ejaculatory fluid and ejaculates from groups of normal stallions. Additionally, accessory glands, epididymides, testicles and other components of the urogenital tract of normal stallions were assayed to determine which tissues contain SPAP activity. The results indicated that levels of AP activity are low in pre-ejaculatory fluid, but significantly higher in ejaculatory fluid from normal stallions. Spermatozoa were not a significant source of SPAP activity. High levels of SPAP activity were found in the testes and epididymides. These findings suggest that SPAP activity is a candidate for a sperm-independent marker for ejaculation in the stallion. Finally, AP activity was determined in ejaculatory fluid from a stallion with bilaterally blocked ampullae, both before and after relief of the blockage. While the blockage was present, AP activity in ejaculatory fluid was low. However, following relief of the blockage, AP activity in ejaculatory fluid rose dramatically, thus suggesting that AP activity will be useful as an inexpensive, simple clinical assay for differentiating ejaculatory failure or excurrent duct blockages from testicular origin azoospermia and oligospermia.

Keywords: Stallion; Alkaline phosphatase; Ejaculate; Azoospermia; Oligospermia

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1. Introduction

Alkaline phosphatase (AP) is a dephosphorylating enzyme that is active in many tissues including bone, liver, kidney, intestine, lung and placenta. Variable levels of AP activity also have been reported in the semen plasma of men, dogs, toms, bulls, rabbits, rams, goats, buffalo, cocks, turkeys, boars and camels [1–9] where it is believed to be involved in sperm glycolytic reactions and fructose formation [2]. The site of production of semen plasma AP (SPAP) has been determined in some species. In dogs, the majority of SPAP is produced in the epididymis [10]. In the rabbit, the testicle, epididymis, vas deferens and ampulla all synthesize significant amounts of the enzyme [7,11,12]. In contrast, the majority of SPAP in bulls originates from the seminal vesicles and, to a lesser extent, from the testes and epididymides [13] while in men, the majority of SPAP activity originates from the prostate [14]. In species in which the majority of SPAP activity originates from the epididymis and testicle, SPAP activity can be used clinically as an ejaculatory marker to differentiate azoospermia or oligospermia from ejaculatory failure [15]. Little information is available on AP activity in the semen plasma of stallions. One early report suggested that a significant amount of SPAP activity was present in secretions from the ampullae [16]. Additionally, a preliminary study found high levels of AP activity in stallion testes and epididymides [17]. As part of an overall goal of developing better diagnostic and therapeutic approaches for stallions with ejaculatory problems, the aims of this study were: to determine if AP activity is present in the ejaculate of normal stallions and, if present, to determine its normal concentration and total activity; to determine which tissues of the stallion reproductive tract contain AP activity, and to determine if AP might be useful as a clinical marker for ejaculation in the stallion.

2. Materials and methods

2.1. Determination of presence and levels of AP activity in normal stallion ejaculates

Pre-ejaculatory fluid and ejaculate samples were obtained from nine horse and pony stallions actively involved in successful breeding programs. The stallions represented various breeds and ranged in age from 6 to 24 years. All stallions were classified as fertile based on breeding history, testicular palpation, testicular measurement and semen analysis (>60% progressively motile spermatozoa, >60% percent morphologically normal spermatozoa, total sperm numbers within normal limits for testicular size, total number of morphologically normal, progressively motile spermatozoa $>1 \times 10^9$ in each ejaculate and satisfactory longevity of motility at 4 °C [18,19]). Samples were obtained as part of routine semen collections for shipment during April–July of a single year. Following collection, each semen sample was filtered to remove the gel portion (Kleen-Test In-Line Milk Filters, Agway, Oxford, PA). Pre-ejaculatory fluid samples were collected either by manual stimulation of the erect glans penis prior to mounting or, in cases in which an unproductive mount occurred, directly into a collection bag attached to a Missouri Artificial Vagina (MAV). Ejaculate samples were collected routinely in a MAV while stallions were mounted on a dummy or on an ovariectomized mount mare. All pre-ejaculatory fluid

Table 1
Alkaline phosphatase (AP) activity in ejaculates and pre-ejaculatory fluid from normal stallions

Stallion ID	Ejaculate volume (ml)	AP activity in pre-ejaculatory fluid (IU/l)	AP activity in ejaculate (IU/l)	Total AP activity per ejaculate (IU)
1	70	18	26300	1841
2	70	16	48700	3409
3	50	28	11900	595
4	40	36	30400	1216
5	30	20	29700	891
6	100	33	26700	2670
7	Not available	90	6390	Not assessed
8	40	10	15397	616
9	40	29	22180	887
Mean (±S.D.)		29 (±22)	24185 (±12398)	1516 (±1039)

and ejaculate samples were stored at 4 °C for no longer than 24 h prior to being assayed for SPAP activity.

The concentration of SPAP activity (IU/l) was determined in pre-ejaculatory fluid and ejaculate samples for each stallion. The concentration of SPAP activity was measured using a Kodak Ektachem Clinical Chemistry Analyzer according to the manufacturer's recommendation (Eastman Kodak Company, Rochester, NY). Samples containing AP activity that exceeded the analyzer's dynamic range (>1500 IU/l) were diluted with 7% bovine serum albumin in reagent grade water and reanalyzed. The results then were multiplied by the dilution factor to obtain the original sample's AP activity. For eight of the nine stallions, total ejaculate AP activity (IU) was determined by multiplying the concentration of AP activity (IU/l) by the gel-free ejaculate volume (ml) and then dividing by 1000 (Table 1). The concentration of AP activity in pre-ejaculatory fluid samples was compared to that in gel-free ejaculate samples using the paired *t*-test.

2.2. Determination of the tissue of origin of SPAP activity

To rule out spermatozoa as the primary source of SPAP activity, ejaculates were collected from 11 fertile horse and pony stallions and the concentration of AP activity was compared between unprocessed ejaculates and sperm-free semen plasma. Ejaculates were collected in April and May of a single year using either a MAV while mounted on a dummy or an ovariectomized mount mare or manual stimulation while mounted on a dummy [20]. The stallions represented various breeds and ranged in age from 4 to 18 years. All ejaculates were classified as normal based on the criteria described previously. Half of each ejaculate was centrifuged at 16,000 × *g* for 20 min and the sperm-free semen plasma supernatant was removed from the sperm pellet. The other half of each ejaculate was left unprocessed. Unprocessed ejaculates and paired sperm-free semen plasma were stored at 4 °C for no more than 12 h before being assayed for AP activity. For 9 of the 11 stallions, total AP activity was determined in sperm-free semen plasma and in the unprocessed ejaculate by multiplying the appropriate AP concentration (IU/l) by 1/2 of the total ejaculate volume (ml) and dividing by 1000. Total ejaculate AP activity (IU) was

Table 2
Alkaline phosphatase (AP) activity in paired sperm-free seminal plasma and unprocessed ejaculates from normal stallions

Stallion ID	Ejaculate volume (ml)	AP activity in sperm free seminal plasma (IU/l)	AP activity in unprocessed ejaculate (IU/l)	Total AP activity per ejaculate (IU)
2	40	24112	22180	887
3	40	14363	15397	616
12	20	18141	17313	346
13	18	20217	20556	370
14	15	19370	19530	293
15	40	8074	7993	320
16	20	14294	16047	321
17	Not available	1640	3574	Not assessed
18	37	20733	19132	708
19	Not available	19870	21236	Not assessed
20	105	6183	6913	726
Mean (±S.D.)		15182 (±7075)	15443 (±6391)	509 (±233)

determined by multiplying the concentration of AP activity in the unprocessed ejaculate (IU/l) by the original total ejaculate volume (ml) and dividing by 1000 (Table 2).

The concentration of AP activity in unprocessed ejaculates was compared to that in sperm-free semen plasma using the paired *t*-test. Concentration of and total AP activity in unprocessed ejaculates were compared between the stallions in this group and the stallions listed in Table 1 using the unpaired *t*-test.

Normal tissue samples from testicle ($n = 4$), epididymal tail ($n = 4$) and ductus deferens ($n = 5$) were obtained from horse stallions following routine castration. Normal tissue samples from ampulla, seminal vesicle, prostate, bulbourethral gland and urethra were obtained from horse and pony stallions following euthanasia ($n = 5$ for each tissue type). All tissues were stored at -20 °C until processing. Before processing, tissue was thawed at room temperature. A section of each tissue weighing between 125 and 140 mg was homogenized manually in a glass tissue grinder in 3 ml of 0.1% Triton X-100. The resulting fluid was centrifuged at 16,000 × *g* for 20 min. The supernatant was removed from the pelleted cellular debris and assayed for the concentration of AP activity as described previously. Secretions were aspirated or manually expressed from the lumina of the epididymal tail ($n = 4$), ampulla ($n = 3$) and seminal vesicle ($n = 4$) of normal horse and pony stallions following euthanasia. Samples were stored at 4 °C for no more than 12 h. The concentration of AP activity was determined as described above. Significant differences in mean concentration of AP activity among the various tissue and luminal fluid samples were determined using ANOVA with the Tukey–Kramer HSD Correction.

2.3. Determination of AP activity in a stallion with blocked ampullae

An 11-year-old Morgan stallion presented to our clinic for evaluation of a sudden onset of infertility and azoospermia. This stallion was diagnosed with bilaterally blocked ampullae using the criteria established by Love et al. [21] and based on response to

treatment. The stallion's testicles were subjectively soft on palpation and total scrotal width was 7.8 cm (slightly less than the width of 8.0 cm recommended by the Society for Theriogenology for classification as a Satisfactory Prospective Breeder [18]). Ultrasonographic examination of the testicles revealed no abnormalities. Pre-ejaculatory fluid and ejaculate samples were collected prior to relief of the blockage and an ejaculate sample was obtained immediately following relief of the blockage. The concentration of AP activity was determined in each sample as described above.

3. Results

3.1. Alkaline phosphatase activity in pre-ejaculatory fluid and ejaculate samples from normal stallions

The concentration of AP activity in ejaculate samples was significantly higher than AP activity in pre-ejaculatory fluid samples ($P < 0.001$, Table 1). Mean concentration of AP activity in normal stallion pre-ejaculatory fluid was 29 ± 22 IU/l while that in ejaculate samples was $24{,}185 \pm 12{,}398$ IU/l. The range of AP activity concentration in pre-ejaculatory fluid samples was 10–90 IU/l while that in ejaculate samples was 6390–48,700 IU/l. Mean total AP activity in normal ejaculates was 1516 ± 1039 IU. The range of total AP activity in normal ejaculates was 595–3409 IU.

3.2. Alkaline phosphatase activity in unprocessed ejaculates and sperm-free semen plasma from normal stallions

Mean concentration of AP activity in the 11 unprocessed ejaculates from this group was $15{,}443 \pm 6391$ IU/l. Values ranged from 3574 to 22,180 IU/l. Mean concentration of AP activity in sperm-free semen plasma from the stallions in this group was $15{,}182 \pm 7075$ IU/l. These values ranged from 1640 to 24,112 IU/l. The concentration of AP activity in unprocessed ejaculate samples was not significantly different from that in sperm-free semen plasma ($P = 0.5$, Table 2). Similarly, total AP activity in unprocessed ejaculate samples and sperm-free semen plasma did not differ significantly ($P = 0.97$, data not shown). The mean total ejaculate AP activity was 509 ± 233 IU.

Concentration of AP activity in the unprocessed ejaculates from the stallions in this group was not significantly different from that in the ejaculates from the nine stallions in the group reported in Table 1 ($P > 0.05$). However, total ejaculate AP activity was significantly lower in this second group of stallions compared to the stallions listed in Table 1 ($P < 0.05$).

3.3. Alkaline phosphatase activity in tissue and luminal fluid samples from the reproductive tracts of normal stallions

Mean AP activities in tissue and luminal fluid samples from normal stallion reproductive tracts are shown in Table 3. Extremely high levels of AP activity were identified in luminal fluid from the epididymal tail (mean AP activity $557{,}350 \pm 277{,}066$ IU/l). Although AP

Table 3
Alkaline phosphatase (AP) activity in tissue and fluid samples from normal stallions

Tissue	Mean AP activity (IU/l)	±S.D.
Bulbourethral gland tissue	48	32
Prostatic tissue	17	9
Seminal vesicular gland tissue	40	40
Ampullary tissue	1137	1189
Ductus deferens tissue	220	107
Urethral tissue	40	22
Testicular tissue	3585	906
Epididymal tail tissue	6157	1906
Seminal vesicular gland fluid	1292	1209
Ampullary fluid	35724	40070
Epididymal tail fluid	557350	277066

activity in ampullary lumen fluid was still high (mean $35,723 \pm 40,079$ IU/l) compared to semen vesicular fluid (mean 1292 ± 1209 IU/l), it was noticeably lower than that in the lumen of the epididymal tail.

Alkaline phosphatase activity in testis and epididymal tail tissue (mean concentration of AP activity 3585 ± 906 and 6157 ± 1906 IU/l, respectively) was significantly higher than AP activity in the other reproductive tract tissues ($P < 0.05$).

3.4. Alkaline phosphatase activity in ejaculate samples from a stallion with blocked ampullae

Alkaline phosphatase activity was 18 IU/l in one pre-ejaculate and one ejaculate sample collected from a stallion with bilaterally blocked ampullae. These values are similar to the AP activity in normal pre-ejaculatory fluid and are much lower than the AP activity in normal ejaculates. Following relief of the blockage (as evidenced by superphysiologic sperm numbers and a high percentage of detached heads), the concentration of AP activity in the ejaculate rose to 4855 IU/l (total AP activity in the ejaculate was 97 IU).

4. Discussion

4.1. High levels of AP activity are present in the normal stallion ejaculate

Biochemical analysis of the ejaculates of normal stallions reveals very high levels of AP activity. These values are significantly higher than those found in pre-ejaculatory fluid or than those reported for normal horse serum (normal serum AP activity range for our lab is 109–315 IU/l). SPAP activity was determined in two separate groups of normal stallions. SPAP concentration was not significantly different between these two groups ($P > 0.05$). However, total AP activity was significantly different between the two groups ($P < 0.05$). This difference is most likely due to lower ejaculate volumes in the stallions in Group 2 (7 of the 11 stallions in Group 2 were ponies, while only two of the nine stallions in Group 1 were ponies). Total AP activity is a function of both concentration and volume and, since

pony stallions typically have lower ejaculate volumes than horse stallions [20], it might be expected that total AP activity in pony stallion ejaculates would be lower than that in horse stallion ejaculates.

4.2. The major source of SPAP activity is not spermatozoa

An experiment was designed to determine how much of the SPAP activity was contributed by sperm cells. AP activity in unprocessed ejaculates did not differ significantly from AP activity in sperm-free semen plasma, indicating that the vast majority of AP activity in the stallion ejaculate originates from the semen plasma and not from the spermatozoa. This is consistent with studies in men in which AP activity in isolated spermatozoa was very low compared to activity in semen plasma [22].

4.3. The highest tissue levels of AP activity in the stallion reproductive tract are found in the epididymis and the testicle

Our data indicate that in normal stallions AP activity levels in the testis and epididymis are significantly higher than in other reproductive tract tissues. Additionally, our data suggest that AP activity is concentrated to extremely high levels in the lumen of the epididymal tail. After leaving the epididymal tail, AP activity is diluted to ejaculate levels probably as a result of the addition of secretions from the accessory glands. These findings are similar to those found in dogs and rabbits [7,10] but differ from findings in men [2] and bulls [13]. Because the highest levels of AP activity apparently originate from the testicle and epididymis in stallions, SPAP activity may be a useful marker for the presence of epididymal or testicular secretions (i.e., a marker for ejaculation). Because spermatozoa do not contribute significantly to SPAP activity, SPAP activity can be used as an ejaculatory marker which is independent of the presence or number of spermatozoa.

4.4. Bilateral blockage of the ampullae resulted in baseline SPAP activity

In one stallion with bilaterally blocked ampullae, AP activity was similar in pre-ejaculatory fluid and ejaculate samples and was similar to AP activity in pre-ejaculatory fluid from normal stallions. After resolution of the blockage, SPAP concentration rose to levels similar to those found in ejaculatory fluid of normal stallions. The change in SPAP activity from baseline during the blockage (when contributions from the testicles and epididymides were prevented from reaching the ejaculate) to levels similar to those in the ejaculates of normal stallions following relief of the blockage (when contributions from the testicles and epididymides were contained in the ejaculate) strongly supports the hypothesis that the testicles and epididymides are the major sources of SPAP activity in the stallion. This is consistent with our findings that the highest levels of AP activity in the reproductive tracts of normal stallions are found in the testicles and epididymides. Taken together, this information suggests that measurement of SPAP activity in stallions with azoospermia will be very useful in differentiating stallions with azoospermia resulting from ampullary blockage (good prognosis for future fertility) and those with azoospermia resulting from testicular abnormalities (poor prognosis for future fertility).

It should be noted that even after relief of the blockage, total SPAP activity in the blocked stallion (97 IU) remained lower than those values reported for normal stallions (range 595–3409 IU). Nonetheless, it remained significantly higher than total SPAP activity in pre-ejaculatory fluid (data not shown). In this regard, even after complete resolution of the blockage, semen parameters for this stallion were well below those values required for classification as a Satisfactory Prospective Breeder [18]. SPAP levels were not available for subsequent ejaculates. Due to marginal semen quality and problems with recurrent ampullary blockage, this stallion was gelded at the owner's request. Histopathologic examination of the testicles revealed mild to moderate testicular degeneration. Since in normal stallions the testicles contain high levels of AP activity, it is possible that the testicular pathology present in this stallion may have resulted in a reduction in SPAP activity, even after relief of the blockage. In support of this hypothesis, we have recently examined SPAP activity in several stallions with testicular pathologies. In these stallions, preliminary data suggests that SPAP activity remains significantly elevated above pre-ejaculate levels ($P < 0.001$) but is consistently lower than SPAP activity in normal stallions ($P < 0.01$) [23].

4.5. Seminal plasma alkaline phosphatase as a clinical marker for ejaculation

These results expand on previously reported data [17] and, together with the ready availability of inexpensive clinical assays for AP activity, suggest that SPAP activity will be useful clinically as a marker for ejaculation in stallions. SPAP activity may be useful, for example, for differentiating true azoospermia (high SPAP, no spermatozoa) from ejaculation failure (low SPAP, no spermatozoa) or azoospermia resulting from ampullary blockage (low SPAP, no spermatozoa). In the future, it will be important to examine SPAP activity in stallions with testicular origin azoospermia and oligospermia. Since the testis is a significant source of SPAP activity, it is possible that testicular pathology resulting in low sperm numbers also could result in reductions in SPAP activity. This may have been the case for the stallion with blocked ampullae in this report. Additionally, it has been shown in bulls that production of SPAP is, at least in part, androgen dependent [24]. If the same is true in stallions, then hormonal aberrations also could result in secondary reductions in SPAP. In spite of this, our preliminary data suggests that, even in stallions with testicular abnormalities, SPAP activity remains significantly higher than levels found in pre-ejaculatory fluid.

4.6. Normal values for AP in pre-ejaculatory and ejaculatory fluid

The work reported here defines a starting point for the clinical use of SPAP activity as a marker for ejaculation in stallions. Based on the population of normal horse and pony stallions described in this manuscript, initial ranges for normal pre-ejaculatory fluid and ejaculate AP activities are:

- AP concentration in pre-ejaculatory fluid ($n = 9$): 10–90 IU/l.
- AP concentration in ejaculate ($n = 20$): 1640–48,700 IU/l.
- Total AP in ejaculate ($n = 17$): 293–3409 U.

From these values, it is likely that AP concentrations under 100 IU/l in an unprocessed sample indicate either that ejaculation did not occur (i.e., pre-ejaculatory fluid, ejaculatory dysfunction, false mount) or that epididymal and testicular secretions are not present in the ejaculate (i.e., ampullary blockage). Conversely, AP concentrations over 1000 IU/l or total AP activity over 200 IU indicates that complete ejaculation occurred (i.e., the sample contains epididymal and testicular secretions). Because of the large standard deviations observed in AP concentrations in ejaculates from normal stallions, evaluation of total AP activity rather than AP concentration may prove to be more useful once the influence of stallion size and ejaculate volume is better defined.

The clinical significance of AP concentrations greater than 100 IU/l and less than 1000 IU/l is not yet known. If values within this range are obtained, it may be beneficial to repeat the assay on additional samples to better establish AP concentration. Further studies on subfertile and infertile stallions are warranted as it is possible that AP concentrations between 100 and 1000 IU/l may be indicative of hormonal abnormalities or testicular and epididymal pathology.

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