Catalase activity in equine semen

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Objective—To characterize the activity of catalase in equine semen.

Animals—15 stallions of known and unknown reproductive history.

Procedure—Seminal plasma was collected from raw equine semen by centrifugation, and samples of seminal plasma were frozen prior to assay for catalase activity. Tissue samples (n = 3 stallions) from the bulbourethral gland, prostate gland, vesicular gland, and testis were homogenized, and cauda epididymal fluid was collected for determination of catalase activity. Catalase activity was determined as an enzyme kinetic assay by the disappearance of H2O2 as measured by ultraviolet spectrophotometry.

Results—Catalase activity in equine seminal plasma was 989.3 ± 1678 U/ml (mean ± SEM), and the specific activity of catalase in equine seminal plasma was 98.7 ± 29.2 U/mg of protein. Specific activity of catalase in tissue homogenates was significantly higher in the prostate gland (954 ± 270 U/mg of protein) than in the ampulla (93 ± 5 U/mg of protein), bulbourethral gland (54 ± 11 U/mg of protein), vesicular gland (39 ± 3 U/mg of protein), cauda epididymal fluid (11 ± 3 U/mg protein), or tests (54 ± 6 U/mg of protein).

Conclusions and Clinical Relevance—Equine seminal plasma contains a high activity of catalase that is derived primarily from prostatic secretions. Procedures such as semen cryopreservation that remove most seminal plasma from semen may reduce the ability to scavenge H2O2 and thereby increase the susceptibility of spermatozoa to oxidative stress. (Am J Vet Res 2000;61:1026–1030)

Oxidative stress is an important factor in pathologic conditions of a number of cell systems. The role of reactive oxygen species (ROS) in the pathophysiological function of spermatozoa has been defined for many years. The production of ROS in semen derives from contaminating leukocytes and, although it has been shown that ROS from contaminating leukocytes accounts for most ROS production in semen, it appears that production in semen is counterbalanced by numerous enzyme systems and antioxidants that reduce the effect of ROS on spermatozoa. Scavenger enzymes in semen include glutathione peroxidase and reductase, superoxide dismutase, and catalase. Of these enzyme scavenger systems, it appears that the activity of catalase, which degrades H2O2, may be one of the most important. Several studies have characterized the activity of catalase in semen from domestic animals1,2 and humans.11,12,14,15 Footo1 found considerable species variation in the activity of catalase in seminal plasma from rabbit, ram, bull, and boar semen. Although Aurich et al16 examined the effect of addition of catalase to semen extenders for preservation of liquid stallion semen, no reports were found regarding the activity of native catalase in equine semen. The purpose of the study reported here was to characterize catalase activity in equine seminal plasma and in equine spermatozoa as well as to characterize the source of the enzyme within the male reproductive tract.

Materials and Methods

Semen collection and processing—Semenal plasma samples were collected from stallions (n = 15) admitted to the Veterinary Medical Teaching Hospital or from stallions housed at the Department of Animal Science or the Center for Equine Health, University of California, Davis. Semen was collected via an artificial vagina and filtered to remove gel and debris. After collection, aliquots of semen were held on ice prior to separation of seminal plasma. Raw semen was centrifuged (3,000 X g; 10 minutes; 5 C), and seminal plasma was separated by aspiration from the pellet within 30 minutes after semen collection. Seminal plasma was stored at –20 C prior to assay (results of preliminary experiments indicated that enzyme activity was stable at –20 C for at least 1 month). Additional aliquots of semen (held at 37 C) were evaluated for total and progressive motility, concentration, and morphologic characteristics of spermatozoa according to accepted procedures.17

Detergent extraction of spermatozoa membranes—Equine spermatozoa were separated from seminal plasma by either centrifugation (twice; 300 X g; 10 minutes) in a modified Tyrode albumin-lactate-pyruvate (TALP; without albumin)18 or separation across a 80:40% Percoll gradient followed by a single centrifugation in TALP.19 Spermatozoa were resuspended in TALP at a final concentration of 50 X 106 spermatozoa/ml. Spermatozoa were detergent extracted by

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incubation with 1% Triton X-100 (38.5°C; 60 minutes). Samples were centrifuged (2,000 X g; 10 minutes), and the supernatant was stored at –20°C until assayed for catalase activity and protein concentration.

Preparation of tissue homogenates—Testes, accessory sex glands (bulbourethral gland, prostate, vesicular gland, and ampulla), and cauda epididymal fluid were collected from 3 stallions postmortem and transported on ice to the laboratory. Tissues were rinsed in ice-cold PBS solution to remove excess blood and minced into 5-mm cubes. Tissues were homogenized in 50 mM NaH₂PO₄ using 3 ml/g of wet tissue (60 seconds homogenization on ice). Cell debris was removed via centrifugation (10,000 X g; 5°C; 20 minutes), and supernatants were removed and stored at –20°C until assayed for catalase activity and protein concentration. Cauda epididymal spermatozoa were collected, and spermatozoa were removed by centrifugation at 10,000 X g for 20 minutes at 5°C. The supernatant cauda epididymal fluid was removed and stored at –20°C until assayed for catalase activity and protein concentrations.

Determination of catalase activity—Catalase activity has been measured by a number of methods including decomposition of H₂O₂ and generation of O₂ according to the following reaction:

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

The enzymatic decomposition of H₂O₂ by catalase proceeds at a rapid rate and appears to be a first-order reaction during the initial period (0 to 30 seconds) at low concentrations of H₂O₂ (0.01 to 0.05 M). Catalase activity (1 U = catalolysis of 1 μM of H₂O₂/min) was determined as an enzyme kinetic assay by the disappearance of H₂O₂ as measured by ultraviolet spectrophotometry, using a molar absorption coefficient of 52 μM-cm for H₂O₂. Degradation of H₂O₂ by catalase was determined by the decline in absorbance at 240 nm of a solution of 10 mM H₂O₂ (adjusted to an absorbance of 0.50 to 0.55) in a 50 mM NaH₂PO₄ (pH 7.5) buffer. Results were standardized to specific activity per mg of protein, which was determined by the bichromatic method. Bovine liver catalase was used as a standard to confirm the activity of catalase in the assay.

Preliminary experiments indicated that catalase activity in equine seminal plasma was high, and small volumes (0.5 μl or less) of seminal plasma were required to avoid excess decomposition of H₂O₂ and formation of bubbles within the cuvette, which interfered with measurement of H₂O₂ decomposition. Therefore, seminal plasma samples were diluted 1:20 in 50 mM NaH₂PO₄ buffer prior to determination of catalase activity, and samples were assayed in triplicate (0.125, 0.25, and 0.5 μl seminal plasma per assay). Diluted seminal plasma samples were added to 10 mM H₂O₂ (1 ml final volume), mixed by inversion, and changes in H₂O₂ concentration were determined for 30 seconds at A₂₄₀.

**Inhibition of catalase**—Specificity of the assay for catalase on the basis of degradation of H₂O₂ was determined by incubation of seminal plasma (0.5 or 5 μl) with 10 mM H₂O₂ in phosphate buffer (1 ml) in the presence of the inhibitors 3-amino-1,2,4-triazole (aminotriazole; 5 or 25 mM) or sodium azide (0.5 or 1 mM). Samples were incubated at approximately 22°C for 10 minutes, and concentrations of H₂O₂ were determined by a fluorometric assay. Samples were assayed in duplicate. The experiment was repeated 3 times using a pooled seminal plasma sample produced by combining seminal plasma from ejaculates collected from 3 stallions. In a separate experiment, pooled seminal plasma samples (from 3 stallions) were heat inactivated (75°C; 5 minutes) prior to assay for catalase activity to determine whether catalase activity in equine seminal plasma was heat labile.

**Statistical analysis**—Data were analyzed by ANOVA, and posthoc comparisons were computed with Fisher protected least-significant difference. Pearson correlation coefficients were determined, and their significance was tested with Fisher r to z transformation. Differences were evaluated in catalase activity between ejaculates characterized as normal versus abnormal on the basis of seminal variables. Ejaculates were categorized as normal if progressive motility of spermatozoa was >58%, and the percentage of morphologically normal spermatozoa was >58%. Differences in catalase activity between normal and abnormal ejaculates were compared using a Mann-Whitney U test and by ANOVA. Data are expressed as mean ± SE. Differences were considered significant at P < 0.05.

**Results**

Catalase activity in seminal plasma—Catalase activity per milliliter of seminal plasma varied significantly (P < 0.001) among stallions (Table 1). There was a moderately strong and significant positive correlation between catalase activity per unit volume of seminal plasma and the percentage of spermatozoa with proximal cytoplasmic droplets (r = 0.58); however, specific activity of catalase was not correlated with percentage of proximal droplets. There was also a moderate negative correlation between specific activity of catalase and protein concentration in seminal plasma (r = –0.49) as well as with percentage of total and progressive motility of spermatozoa.

When catalase activity was compared between ejaculates characterized as normal versus abnormal,
there was no significant difference \( P = 0.21 \) in the activity of catalase per unit volume of seminal plasma (Table 1). Likewise, specific activity of catalase and protein concentration in seminal plasma was not significantly different between normal and abnormal ejaculates.

Catalase activity in detergent-extracted spermatozoa—Catalase activity was detected in detergent-extracted spermatozoa after separation from seminal plasma via centrifugation alone or centrifugation through a discontinuous Percoll gradient. The specific activity of catalase in seminal plasma was significantly less than that recovered from detergent-extracted spermatozoa recovered after either centrifugation in TALP \( P = 0.023 \) or through a Percoll gradient \( P = 0.001 \) (Fig 1). Specific activity of catalase did not differ between detergent-extracted spermatozoa recovered after centrifugation alone, compared with centrifugation through Percoll. Specific activity of catalase recovered from spermatozoa after either 2 washes in TALP or Percoll separation was 2.3 ± 0.4 U/10^6 spermatozoa or 1.9 ± 0.4 U/10^6 spermatozoa, respectively.

Catalase activity in tissue homogenates—The greatest activity of catalase in accessory sex glands was detected in the prostate gland. Specific activity of catalase was significantly \( P < 0.001 \) higher from prostate gland, compared with other accessory sex glands, cauda epididymal fluid, or testis homogenates (Fig 2).

Prostate gland activity for catalase was 954.5 ± 270.3 U/mg of protein.

Because of the high activity of catalase in equine seminal plasma, there was a significant effect of volume of seminal plasma assayed and decomposition of H\(_2\)O\(_2\) determined spectrophotometrically. Assay volumes with greater than 0.5 \( \mu \)l seminal plasma per ml of reaction substrate significantly reduced measured catalase activity (Fig 3). Therefore, assay results reported here used volumes of 0.125, 0.25, and 0.5 \( \mu \)l as triplicate determinations.

Inhibition of catalase activity—There was no significant effect of inhibitors, aminotriazole, or sodium azide on detection of H\(_2\)O\(_2\) in the Amplex Red assay (data not shown). The activity of catalase in seminal plasma, as determined by the degradation of H\(_2\)O\(_2\) determined spectrophotometrically. Assay volumes with greater than 0.5 \( \mu \)l seminal plasma per ml of reaction substrate significantly reduced measured catalase activity (Fig 3). Therefore, assay results reported here used volumes of 0.125, 0.25, and 0.5 \( \mu \)l as triplicate determinations.

Inhibition of catalase activity—There was no significant effect of inhibitors, aminotriazole, or sodium azide on detection of H\(_2\)O\(_2\) in the Amplex Red assay (data not shown). The activity of catalase in seminal plasma, as determined by the degradation of H\(_2\)O\(_2\) during a 10-minute incubation, was significantly \( P = 0.015 \) inhibited in the presence of 0.5 and 1 mM sodium azide but not by aminotriazole for either concentration of seminal plasma (Fig 4).

Discussion

We identified a high activity of catalase in equine seminal plasma. Results of previous studies related to
catalase activity in seminal plasma from either humans \(^1^{2,10}\) or animals \(^13\), indicates a considerable species variation in the activity of this enzyme in semen. Unfortunately, differences in assay methods for determination of catalase activity across studies make direct comparison difficult. The activity of seminal plasma catalase was rabbit > ram > bull > boar on the basis of work reported by Foote. \(^13\) Foote identified a high catalase activity in seminal plasma from rabbits that appeared roughly comparable with the activity reported in our study for stallion seminal plasma. The activity of catalase in equine seminal plasma determined in our study appears similar to that described for human seminal plasma. \(^14\) The importance of reported differences in the activity of catalase among mammals relative to the susceptibility of spermatozoa to oxidative stress is unclear.

There was also a significant stallion-to-stallion variation in the activity of catalase in seminal plasma; however, data were not available to evaluate fertility variables in relationship to this variation in catalase activity. When ejaculates were classified as normal or abnormal on the basis of progressive motility and morphologic characteristics of spermatozoa, there were no significant differences in either the activity of catalase or the specific activity of catalase in seminal plasma in normal versus abnormal ejaculates. There was a strong positive correlation between catalase activity in seminal plasma and the percentage of spermatozoa with proximal cytoplasmic droplets. The basis of this correlation is uncertain. Previous reports on human semen indicate a higher activity of seminal plasma catalase in oligospermic samples \(^13\) and in samples with a higher production of ROS, \(^14\) whereas results of other studies indicate a lower catalase activity in seminal plasma samples with teratozoospermia. \(^15\) Because the retention of the cytoplasmic droplet may enhance production of ROS by spermatozoa, \(^8\) it seems reasonable that the association of a high catalase activity with proximal droplets in equine semen is associated with a high production of ROS. This hypothesis requires testing in the horse.

The function of catalase in equine seminal plasma remains to be defined. Equine spermatozoa produce H\(_2\)O\(_2\) in vitro, and damaged spermatozoa produce higher quantities of H\(_2\)O\(_2\). \(^3\) Because H\(_2\)O\(_2\) appears to be the primary cytotoxic ROS in semen, \(^7\) it would appear that catalase plays a critical role in the protection of spermatozoa from damage caused by ROS in semen. Recent studies with human semen indicate that catalase activity is reduced in semen from infertile men. \(^13\) ROS production from defective spermatozoa is increased. \(^13\) Studies with human semen also indicate that processing of semen by removal of seminal plasma may increase the susceptibility of spermatozoa to damage by ROS caused by the removal of antioxidant systems. \(^7\) Because processing of equine semen samples for procedures such as semen cryopreservation typically requires removal of most if not all seminal plasma, such procedures may increase the detrimental effects of ROS to spermatozoa in such samples.

Removal of seminal plasma from equine semen did not, however, eliminate catalase activity associated with equine spermatozoa. There remained a high specific activity of catalase in detergent-extracted equine spermatozoa after separation by washing in TALP or by separation on a discontinuous Percoll gradient. It is unclear whether catalase associated with equine spermatozoa represents adsorbed catalase derived from prostatic contributions to seminal plasma or whether catalase activity in equine spermatozoa is found within the cytoplasmic compartment of the cell. Because spermatozoa lack peroxisomes, and because the specific activity of catalase in equine testsis and cauda epididymal fluid was low; it appears likely that much of the catalase activity in detergent-extracted spermatozoa represents adsorbed enzyme contributed by the prostate gland. However, the higher specific activity of catalase in detergent-extracted spermatozoa compared with seminal plasma suggests that not all of the catalase activity identified in spermatozoa originates from seminal plasma or that catalase preferentially associates with spermatozoa. This observation differs from that of Jeulin et al., \(^16\) who concluded that human spermatozoa contained little catalase, and adsorption from seminal plasma was unlikely to contribute to this activity. As in our study, Zini et al \(^14\) identified a higher activity of catalase associated with human spermatozoa than in seminal plasma.

Evaluation of accessory gland tissues, cauda epididymal fluid, and testsis indicated that the highest specific activity of catalase was found in the prostate gland of the stallion. Therefore, it appears likely that prostatic secretions are the major source of catalase activity in equine semen. This finding is similar to that reported by Jeulin et al. \(^14\) for humans and indicates that the prostate may be the primary source of catalase in seminal plasma.

Determination of catalase activity on the basis of catalobilism of H\(_2\)O\(_2\) has been used to evaluate the activity of this enzyme in semen and seminal plasma. \(^10\) In our study, determination of H\(_2\)O\(_2\) concentrations on the basis of ultraviolet spectrophotometry at 240 nm provided a rapid repeatable assay for the measurement of catalase activity in equine seminal plasma, provided that volumes of seminal plasma assayed in the 1-ml reaction volume of 10 mM H\(_2\)O\(_2\) did not exceed 0.5 \(\mu\)l. Volumes of seminal plasma exceeding 0.5 \(\mu\)l were associated with an artifactual reduction in catalase activity caused by the excess production of bubbles, which interfered with the assay result. Similar problems have been reported in using the decomposition of H\(_2\)O\(_2\) to measure catalase activity. \(^21\)

Results of our study and previous studies have associated decomposition of H\(_2\)O\(_2\) with the activity of catalase in seminal plasma; however, glutathione peroxidase \(^24\), \(^25\) or low molecular weight components of seminal plasma such as taurine or hypotaurine \(^16\) might also account for the disappearance of H\(_2\)O\(_2\) in this assay. In our study, 97% of the degradation of H\(_2\)O\(_2\) could be inhibited by the addition of 0.5 or 1 mM sodium azide; however, the addition of the catalase inhibitor, aminotriazole, did not result in a significant inhibition of H\(_2\)O\(_2\) degradation. Because catalase, but not glutathione peroxidase, is inhibited by sodium azide, it appears that most degradation of

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H₂O₂ in this assay was associated with catalase activity in seminal plasma. The lack of inhibition of catalase activity by aminotriazole may be caused by the requirement for H₂O₂ in the presence of aminotriazole to inhibit catalase. Rapid catabolism of H₂O₂ by catalase may prevent inhibition of the enzyme by aminotriazole.

In summary, equine seminal plasma contains a high activity of the antioxidant scavenger, catalase, which is derived primarily from prostatic secretions. The specific activity of catalase was higher in detergent-extracted spermatozoa than in seminal plasma, suggesting that catalase associates preferentially with spermatozoa. Although there was significant variation among stallions in the activity of catalase in seminal plasma, further studies are required to examine the associations, if any, between catalase activity in seminal plasma and fertility in stallions.

References


