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High Catalase Content of Rabbit Semen Appears to be Inherited

ROBERT H. FOOTE AND ELIZABETH HARE

From the Department of Animal Science, Cornell University, Ithaca, New York.

ABSTRACT: Reactive oxygen species, as hydrogen peroxide, can be damaging to sperm. Most species have little protective catalase in their semen, but rabbit semen contains substantial amounts of catalase. The objective of the present study was to characterize a substantial number of Dutch rabbits for seminal content of catalase and determine whether differences were inherited. Usually, 4 or more semen samples were analyzed per male. Catalase was measured by a gasometric procedure. In Experiment 1, the correlation between duplicate determinations was $r = .99$, and between 2 sets of semen samples from 55 males it was $r = .95$. There was a significant difference ($P < .05$) among pairs of males from 6 litters. In Experiment 2, semen from each of 11 males collected at an interval of 1 year contained an average of 13 and 12 units of catalase per mL of semen in consecutive years. The correlation between pairs of samples was $r = .85$. This indicated that the condition was permanent, and possibly genetically controlled. Experiment 3 analyzed the catalase content of semen from 7 sires and 32 sons. The heritability of seminal catalase concentration was 0.48. These studies indicate that rabbit seminal plasma is high in catalase, and that a substantial portion of the differences among males are under genetic control.

Key words: Sperm, seminal plasma, heritability.

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Catalase is present in all aerobic cells (Deisseroth and Dounce, 1970) where its primary function is to catalyze the formation of water and molecular oxygen from hydrogen peroxide. Catalase is associated with intracellular microbodies (de Duve, 1983), so it is not surprising that with the minimal cytoplasmic content of sperm cells, catalase concentration is low in the semen of many animals (White, 1959; Foote, 1962a; Mann, 1964).

The development of artificial insemination resulted in exposure of sperm to aerobic conditions during semen collection, evaluation, and preservation. Under these conditions bull sperm were demonstrated to produce H_2O_2 (Tosic and Walton, 1950). The addition of catalase to bull semen stored at 5°C improved survival of sperm exposed to air or O_2 (VanDemark et al, 1949; Tosic and Walton, 1950), or to agitation and light (Foote, 1967). Catalase also prevented a reduction in oocyte penetration of bull sperm in the presence of reactive oxygen species (Blondin et al, 1997). Oxidative reactions can damage sperm (Delamirande and Gagnon, 1995; Purohit et al, 1999), including sperm DNA (Aitken et al, 1998; Shen et al, 1999), and this may be a factor in in vitro fertilization and intracytoplasmic sperm injection. The addition of catalase to rabbit sperm stored at 5°C had no effect on sperm survival (Foote, 1962a), presumably because rabbit semen had sufficiently high endogenous catalase to prevent peroxidation.

Different forms of catalase, or lack of it, in blood and tissues in several species have been reported to be inherited (Allison et al, 1957; Feinstein et al, 1968; Deisseroth and Dounce, 1970). Also, in a preliminary report, Foote (1962a) found that differences in seminal catalase among 8 pairs of males from different litters of rabbits were greater than the variation within litters. This suggested that genetics, some common early postnatal environment, or both had an effect on the concentration of catalase in rabbit semen.

Considering the ubiquitous nature of catalase, its importance in protecting cells from peroxidation, the scarcity of information on seminal catalase in the literature, and the unusually high concentration of catalase in rabbit semen compared to semen of other mammals, we examined catalase in rabbit semen when a substantial group of males were set aside in our colony for other studies (Foote, 1999). The objectives were to characterize male rabbits for catalase activity in semen, to determine if differences were repeatable over time, and to determine if quantitative differences in seminal catalase appeared to be inherited.

Materials and Methods

Animals, and Semen Collection

Dutch-belted male and female rabbits were raised in our colony. They were maintained on a constant 12-hour dark-light cycle,

Correspondence to: Dr Robert H. Foote, Department of Animal Science, 204 Morrison Hall, Cornell University, Ithaca, NY 14853-4801 (e-mail: dgb1@cornell.edu).

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fed a complete pelleted ration that provided normal growth and maintenance of adult body weight, and fresh water was supplied ad libitum. Other details are described for each experiment.

Adult males were trained to serve an artificial vagina (Brederman et al, 1964) and semen was collected at 3- to 4-day intervals. Semen was collected essentially simultaneously over a period of a few weeks from all males within experiments. Each semen sample was examined for volume of ejaculate, gross appearance, absence of urine, and microscopically for general morphology and motility of sperm and absence of leukocytes. All samples that were free from contamination and that had a reasonable concentration of motile sperm ($\geq 100 \times 10^6$ sperm/mL) were retained to assess catalase activity.

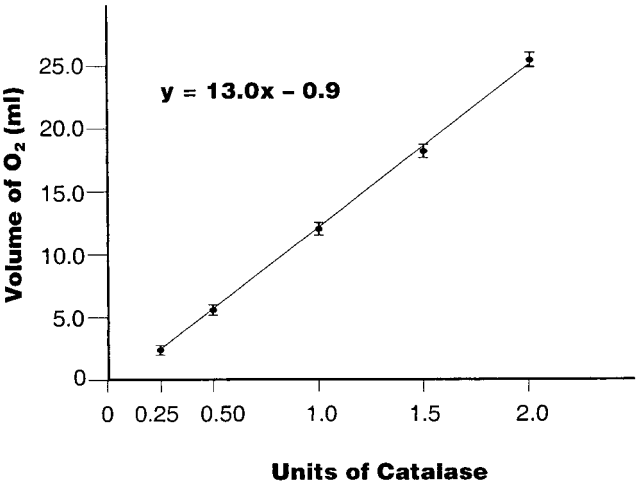
Establishing a Procedure for Quantifying Seminal Catalase

Catalase concentration in semen and blood was based on a gasometric procedure (Maehly and Chance, 1954) to avoid any turbidity problems that might arise in a spectrophotometric determination because of granules in rabbit semen. A fresh phosphate buffered solution (pH 7.0) containing 1% H_2O_2 was prepared daily. A small volume of semen was placed in a boat that floated on the top of 14 mL of a solution of H_2O_2 that had been prepared in a 50-mL Erlenmeyer flask and placed on a platform shaker. The system was closed and the boat was tipped over into the peroxide solution by starting the shaker at time zero.

The amount of H_2O_2 placed in the flask was determined on the basis of an earlier study (Foote, 1962a) and tested again. The amount was sufficient so that not more than 80% of the H_2O_2 was used to assay samples that contained the most catalase. The O_2 expelled during the reaction forced water out of a closed side vessel into a graduated collection vessel. The volume of O_2 released (water displaced) was recorded after 5, 10, and 15 minutes. Some O_2 was always released between 5 and 10 minutes, but a plateau was often reached after 10 minutes; therefore, the volume of water displaced after 10 minutes was used in the statistical analysis as a measure of O_2 released. All samples were run in duplicate and any that did not agree within ±10% were rerun.

Twice-crystallized lyophilized beef liver catalase (C-100, Sigma Chemical Company, St Louis, Mo) was used as the standard. The results are expressed as units of catalase in terms of this standard, with 1 unit approximately equal to 10 μg of catalase. The standard catalase and catalase in semen were inactivated at 55°C when tested in temperatures that ranged from 40°C to 60°C. When these 2 sources of heat-inactivated catalases were added to a solution of H_2O_2, no gas was released. This is consistent with the report by White (1959) who also found that sodium azide, sodium cyanide, and sodium fluoride added to rabbit semen completely inhibited the breakdown of H_2O_2.

A catalase standard curve was prepared using 0.25, 0.50, 1.0, 1.5, and 2.0 units of catalase. The standards were prepared 19 times. Results are illustrated in the Figure. The individual runs were very consistent. With each set of unknowns, a standard with 1 unit of catalase was included, and the result agreed with the standard curve within ±10%. Also, a control with no catalase was included, and zero to 0.2 mL of gas was evolved. Because this was below or at the lower limit of the sensitivity of the assay (0.1 unit of catalase), no correction was made.



Catalase standard curve showing the regression line with 95% confidence limits (n = 19). In the regression equation, Y is the volume of O_2 released associated with the catalase activity, X.

Whole rabbit semen was used. After centrifugation, the supernatant contained about 70% of the catalase and the pellet of granules and sperm contained about 30%. After recentrifugation, the washed pellet contained less than 10% of the catalase. Because the catalase was found in the plasma, whole semen was used without further processing. A previous study (Foote, 1962a) also indicated that catalase appeared in the seminal plasma portion.

Rabbit semen in volumes of 0.025, 0.05, and 0.1 mL were tested, and 0.05 mL usually produced results within the range of the standard curve. When the catalase content of semen was so high that the result fell outside the limits of the standard curve, 0.025 mL of semen was used for the retest. Likewise, for samples with little catalase, 0.1 mL of semen was used when reruns were necessary. This procedure was followed throughout all experiments.

Experiment 1

Experiment 1 was a study of 55 males from which at least 2 semen samples were assayed for catalase. Twenty males were retained for further study for possible use in a progeny test of inheritance of catalase concentration in semen. Attempts to collect semen from these 20 males were performed 10 to 12 times over a period of 5 weeks, and 17 of the males repeatedly ejaculated good-quality semen. Two males often urinated in the semen, and 1 produced aspermic ejaculates. These males were discarded from the study. Twelve of the males were pairs from 6 litters. Semen from all successful collections was analyzed for catalase contest.

Experiment 2

This study examined the catalase content of semen ejaculated by males when they were approximately 1 and 2 years of age. Eleven males from which semen had been collected in Experiment 1 were still available 1 year later. Four semen samples from each

buck were collected over a period of 2 weeks in successive years and the buck means for the 2 years were correlated.

Experiment 3

Experiment 3 was a sire-progeny comparison of the catalase content of semen to determine whether this trait might be inherited. We planned to compare semen from 8 sires with that from their progeny. Semen from 10 males (2 extra males) was collected and each sample was used to inseminate at least 2 females, with the plan to produce at least 2 male offspring per litter. From these male offspring, at least 2 semen samples per male was to be collected after they reached sexual maturity. After eliminating infertile inseminations, mortality of young males before attaining sexual maturity, fewer than 2 males in a litter, and so on, there were 6 males that produced 28 male progeny in 2 litters per male, and another male produced 1 male and 3 males, respectively, in litters from 2 females. To increase the number of semen samples for analysis, 4 samples were collected and analyzed for catalase from each sire and their male progeny during the same time period.

To estimate heritability of catalase in semen, variance components of additive genetic effects, litter effects, and both permanent and temporary environmental effects were estimated by a derivative-free restricted maximum likelihood (DFREML) approach. The multiple trait derivative-free restricted maximum likelihood (MTDFREML) software package by Boldman et al (1995) was used. This set of programs uses the downhill simplex method described by Nelder and Mead (1965). The model for the analysis of these data contained terms for animal additive genetic effects, permanent environmental effects, and litter effects. A matrix of relationships of each individual to all the other animals in the pedigree was included in this analysis to account for the covariance of records for related animals. Sires and dams of rabbits with records were added to the relationship matrix to complete the pedigrees. This model provided an unbiased estimate of heritability. The covariance between 2 different litter effects was assumed to be zero, as was the covariance between permanent environmental effects for different individuals with records.

Analysis of Variance

Rabbits and samples of semen were considered to be random and duplicate determinations to be fixed in the analysis of variance, using the GLM model version 7 (SAS, 1999). Agreement between duplicate determinations were so high that only averages of the duplicates were used in analyses. In subsets of data, in which males from the same and different litters were compared, the total variance was partitioned into, among, and within litters. Differences were considered to be statistically significant if $P \leq .05$.

Results

Experiment 1

Duplicate determinations of catalase in semen resulted in a correlation of +.99 ($P < .05$). The means ± SEM of catalase in the first 2 semen collections from 55 males were 18.7 ± 1.9 and 17.8 ± 2.2. The correlation between these 2 sets of semen samples was +.95 ($P < .05$). Thus, there was considerable uniformity within animals and duplicate assays of samples.

A total 178 semen samples were collected from 17 males out of the 20 males that were originally assigned. The means ± standard deviations for the 17 males, listed in ascending order of catalase per mL of semen, were 6 ± 1, 8 ± 2, 10 ± 3, 11 ± 1, 11 ± 2, 12 ± 2, 12 ± 3, 13 ± 3, 13 ± 4, 14 ± 3, 15 ± 4, 16 ± 4, 17 ± 4, 17 ± 8, 22 ± 4, 32 ± 8, and 41 ± 7. One of the original males that had been excluded because it produced aspermic ejaculates had a normal concentration of catalase in its seminal plasma. Autopsy revealed bilateral occlusions of the vas deferens. Sperm were aspirated from the cauda epididymides, and 25 normal young were born following insemination of 4 does. Several vasectomized males that had been kept in the colony also had typical catalase concentrations of 10 to 18 units of catalase per mL of seminal fluid.

Twelve bucks were pairs from 6 litters. The analysis of variance comparing among-litter against within-litter variances indicated that there were differences in catalase concentrations of semen from males in different litters ($P < .05$).

Table 1. *Catalase content (mean ± standard deviation) of semen collected from rabbit bucks when 1 and 2 years of age*

Buck No.	Catalase, U/mL of Semen*	
	Year 1	Year 2
1	6 ± 1.0	4 ± 0.9
2	15 ± 5.0	14 ± 3.3
3	12 ± 1.3	8 ± 2.7
4	9 ± 0.8	9 ± 3.0
5	12 ± 2.4	12 ± 3.6
6	15 ± 4.0	14 ± 3.0
7	22 ± 2.5	19 ± 4.3
8	17 ± 1.9	16 ± 4.1
9	17 ± 3.5	17 ± 6.3
10	14 ± 1.7	8 ± 2.4
11	11 ± 1.8	9 ± 1.5

* $r = .85$ (2 df, $P = .01$ for $r \geq .74$).

Experiment 2

A comparison of the catalase content averaged over 4 semen collections in 11 males when they were 1 and 2 years of age appears in Table 1. The correlation coefficient of .85 between the 2 sets of data was significant, $P < .05$.

Experiment 3

The catalase content in the semen from 7 sires and 32 male progeny is summarized in Table 2. There were sub-

Table 2. *Catalase in semen of sires and their progeny*

Sire No.	Sire Semen Catalase*	Catalase in Semen From Progeny, U/mL					
		Males in Litter 1			Males in Litter 2		
		1	2	3	1	2	3
1	3.7	4.5	16.6	4.4	5.3	17.1	7.0
2	9.3	15.0	. . .	. . .	23.8	9.0	18.1
3	11.8	9.5	10.0	. . .	20.7	8.8	19.6
4	12.7	6.6	16.0	. . .	18.1	12.4	. . .
5	31.9	24.2	11.7	36.3	44.4	15.3	. . .
6	13.9	10.4	18.4	. . .	28.6	13.4	. . .
7	17.4	26.8	13.9	. . .	11.4	30.6	. . .

* Semen catalase is based on the mean of 4 samples per male rabbit.

stantial differences among the sires, but relative uniformity among samples from the same sire. For example, the 4 values for the low catalase content in sire number 1 were 3.1, 3.1, 5.1, and 3.5. The 4 values for sire number 5, which had the highest concentration of catalase per mL of semen, were 34.0, 30.1, 38.2, and 26.3.

The variances for effects in the model used to estimate heritability are shown in Table 3. The variance due to litter effects was essentially zero. The permanent environmental effect was greater than the temporary environmental effect. The additive genetic variance of 45.45, as a proportion of the total phenotypic variance of 94.65, resulted in an estimated heritability of 0.48 for seminal catalase concentration. Repeatability of observations for seminal catalase concentration measured on the same rabbit was estimated to be 0.8.

Table 3. *Variances for catalase concentration in rabbit semen*

Variance Component	Value
Additive genetic variance	45.45
Litter variance	0.0013
Permanent environmental variance	34.83
Temporary environmental variance	14.37
Total phenotypic variance	94.65

Discussion

The normal functioning of mammalian cells is dependent upon maintaining an intricate balance between the production and elimination of reactive oxygen species (Deisseroth and Dounce, 1970; Mantha et al, 1993; Ghanaml et al, 1998). The increase in oxidative modification of proteins with aging has been associated with oxygen-free radical generating systems. This increase can be inhibited by catalase (Stadtman, 1992), and genetic manipulation to overexpress catalase in *Drosophila* increased life span by one-third (Orr and Sohal, 1994).

Low concentrations of reactive oxygen species, including H_2O_2, can promote capacitation, leading to the acrosome reaction (Griveau et al, 1994). No reports were found in the literature on antioxidant enzymes in rabbit oviduct fluid. Substantial accumulations of reactive oxygen species can negatively affect reproductive function and affect sperm cells in a variety of ways (Delamirande and Gagnon, 1995; Aitken et al, 1998; Shen et al, 1999), with peroxidative damage to sperm DNA and the plasma membrane.

Catalase is found in the cytoplasm of cells, but sperm cells, which are essentially devoid of cytoplasmic components, contain little if any catalase (White, 1959; Foote, 1962a; Mann, 1964). Sperm cells are extremely sensitive to H_2O_2 and its toxicity can be prevented by the addition of catalase (MacLeod, 1943; Tosic and Walton, 1950). For example, rabbit sperm removed from their seminal plasma are sensitive to the highly toxic effects of H_2O_2, but in their own seminal plasma they are about 100 times as resistant as bull spermatozoa (White, 1959). This is consistent with the high endogenous content of catalase in rabbit semen and low content in bull semen. Furthermore, the motility of rabbit sperm held under oxidative conditions is not improved by the addition of catalase (Foote, 1962a), in contrast to bull sperm (VanDemark et al, 1949; Foote, 1967).

In rabbits, Sertoli cells are a substantial source of catalase (Ihrig et al, 1974), but the catalase in semen appears to come from the accessory sex glands, which are complex in rabbits (Holtz and Foote, 1978). The prostate and proprostate both contain granular secretions, but the chemistry of these granules has not been determined. The secretions of the vesicular and bulbourethral glands are relatively agranular (Holtz and Foote, 1978).

Vasectomized rabbits maintained in our colony had a normal concentration of seminal catalase, and 1 male with blockage of the vas deferens produced seminal fluid that contained 13 units of catalase per mL. Although epididymal fluids were not routinely assayed, this suggests that epididymal fluid was not a substantial source of catalase.

The results of Experiment 1, based upon 55 males, indicated that there was a considerable range in seminal catalase concentrations and a high repeatability between 2 series of semen collections. These results are consistent with and extend those previously reported (Foote, 1962a). With 1 unit of catalase equivalent to approximately 10 μg of catalase in the present studies, catalase values for the 17 males ranged from 60 to 410 μg/mL of semen. The catalase content of semen was similar when 11 of these males were ejaculated 1 year later (Experiment 2), indicating that catalase concentration in semen was permanent.

The similarity of seminal catalase concentrations among bucks within litters versus bucks in different litters led to the heritability study in Experiment 3. The heritability estimate of 0.48 is relatively high in relationship to the heritability of many traits associated with reproduc-

tion. Because this study is the first one we found on the inheritance of catalase in semen in any species, there are no published landmarks for comparisons. Further studies are needed to extend this estimate of high heritability. However, the present group of animals were utilized in another planned study (Foote, 1999), and additional studies would be most valuable if they were conducted on a colony of rabbits unrelated to ours.

The significance of the presence of catalase in rabbit semen is an enigma. H_2O_2 can promote capacitation (Griveau et al, 1994) and it is possible that catalase assists in preventing premature capacitation during the 10 to 12 hours between mating and ovulation. Most species in which seminal catalase has been examined have minimal amounts, unless it is contaminated with leukocytes or bacteria (Aitken et al, 1998). For example, ram, bull, and boar semen were reported to contain 3.7, 3.1, and 0.2 μg of catalase per mL of semen, respectively, compared with 267 μg for rabbit (Foote, 1962a). Under normal mating conditions, sperm are not exposed to substantial concentrations of free O_2, when catalase could be beneficial. However, during laboratory manipulations associated with the processing of sperm for various in vitro fertilization and insemination procedures (MacLeod, 1943; VanDemark et al, 1949; Tosic and Walton, 1950; Foote, 1962b, 1967; Blondin et al, 1997; Aitken et al, 1998), protection of sperm against H_2O_2 by the addition of catalase may be useful, particularly because semen from most species contains negligible amounts of catalase.

In conclusion, these studies demonstrate that catalase content of rabbit seminal fluid is higher than in other mammals studied, and that the concentrations are relatively uniform over extended periods of time. The estimated heritability value of 0.48 for seminal catalase concentration indicates that there is a substantial genetic component contributing to differences among males.

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