TOXIC EFFECTS OF AROMATIC AMINO ACIDS ON THE LIVABILITY OF BULL SPERMATOZOA

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Abstract

The addition of L-tyrosine, L-tryptophan, or L-phenylalanine at concentrations as low as 0·5 mM reduced the livability of sperm incubated in Caprogen at 37°C. The toxicity of each amino acid increased with concentration, but L-phenylalanine was most toxic. Catalase (4·5 μg/ml) significantly increased livabilities but did not completely eliminate the toxic effect of L-phenylalanine or L-tryptophan. The D-amino acids were not toxic. Dialysing egg yolk prior to its incorporation into semen extenders improved sperm livability but the inclusion of catalase produced a significantly greater response. The beneficial effect of catalase was most pronounced at low sperm concentrations (5 million sperm per millilitre). The results suggest that the previously reported dilution effect may be due to peroxide produced from aromatic amino acids.

I. INTRODUCTION

The presence of an amino acid oxidase in bull semen was originally reported by Tosic and Walton (1950). This enzyme produces hydrogen peroxide and ammonia by the oxidative deamination of phenylalanine, tyrosine, or tryptophan. Shannon and Curson (1972) found that this enzyme appears to be bound in an inactive form to motile sperm, but following their death it is released and activated, thereby reducing the *in vitro* livability of the living sperm because of the production of hydrogen peroxide.

The following series of experiments was designed to study the toxic action of these aromatic amino acids on bull spermatozoa.

II. MATERIALS AND METHODS

Semen was obtained from four mature Jersey bulls. A 3-ml sample from each ejaculate was initially diluted in 22 ml of Caprogen (Shannon 1965) containing 20% egg yolk and at 32°C. Following cooling to ambient temperature, aliquots from each primary dilution were added to 5 ml of Caprogen containing 5% egg yolk and appropriate experimental additives to give a final concentration of 10 million sperm per millilitre. The samples were incubated at 37°C and *in vitro* livability assessed from motility estimates at relevant intervals (Shannon 1965). Most experiments were repeated so that two livability estimates were obtained for sperm from each bull with each treatment.

Analyses of variance were initially used to determine the significance of treatment differences and specific comparisons tested by orthogonal contrasts or by the Newman–Keul's ranking method (Winer 1962).

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Analar grade chemicals were used in preparing diluents. The beef liver catalase prepared at the Newstead Artificial Breeding Centre was added where applicable to give a final concentration of 4·5 μg/ml.

III. Results

(a) *Amino Acid Concentration*

The L-forms of tyrosine, tryptophan, and phenylalanine were used separately at concentrations of 5, 2, and 0·5 mM with and without catalase. Their addition significantly reduced livability in the absence of catalase, the effect becoming more pronounced with increasing amino acid concentration ($P < 0·01$) (Table 1). The addition of catalase increased livability ($P < 0·01$), although it did not completely eliminate the depressive effect of tryptophan or phenylalanine ($P < 0·01$) (Table 1).

TABLE 1

EFFECT ON SPERM LIVABILITY OF ADDING THE L-FORM OF TYROSINE, TRYPTOPHAN, OR PHENYLALANINE TO CAPROGEN, IN THE ABSENCE OR PRESENCE OF CATALASE

Amino acid	Catalase (4·5 μg/ml)	Livability (hr) at L-amino acid concen. (mM) of:				S.E.
		0	0·5	2·0	5·0	
Tyrosine	—	52·8	49·2	37·9	24·2	5·1
Tryptophan	—	54·7	46·7	39·1	29·7	4·0
Phenylalanine	—	60·6	36·5	21·0	18·0	2·6
Tyrosine	+	92·5	100·5	92·2	86·0	4·2
Tryptophan	+	94·1	85·7	72·2	63·0	3·9
Phenylalanine	+	107·1	91·7	77·2	75·6	6·3

(b) *Amino Acid Form*

Tyrosine, tryptophan, and phenylalanine were compared simultaneously, the L-form of each at 0·5 mM concentration and the D-form of each at 2·0 mM concentration. In the absence of catalase L-phenylalanine was more toxic than L-tryptophan or L-tyrosine ($P < 0·01$). Whereas the addition of L-tyrosine was not toxic in the presence of catalase, this enzyme did not eliminate the toxicity of L-phenylalanine or L-tryptophan ($P < 0·05$) (Table 2).

Adding the D-form of the respective amino acids did not reduce livability (Table 2). Inclusion of D-tyrosine (2 mM concn.) did not increase livability in the presence of L-tyrosine (0·5 mM concn.).

(c) *Egg Yolk Dialysis*

20 ml of fresh egg yolk was dialysed at 5°C for 20 hr against 2 litres of a solution of 2% trisodium citrate dihydrate. This treatment of the egg yolk prior to its incorporation in Caprogen only improved livability in the absence of catalase ($P < 0·01$) (Table 3). The results obtained when L-tyrosine, L-tryptophan, or L-phenylalanine was added (0·5 mM concn.) have not been tabulated as they were similar to those recorded in Table 1 irrespective of whether dialysed or undialysed egg yolk was used.

(*d*) *Hydrogen Peroxide Addition*

The L-amino acid oxidase in semen reacts with aromatic amino acids to produce hydrogen peroxide and ammonium ions. The relative peroxide toxicity was tested by adding hydrogen peroxide at concentrations ranging from 0·005 to 0·2 mM, both with and without catalase. Hydrogen peroxide was highly toxic to sperm only in the absence of catalase [Fig. 1(*a*)]. Although its addition reduced the livability of semen samples from all four bulls, there was a significant bull × treatment interaction, indicating that the toxicity of hydrogen peroxide is greater to sperm from some bulls.

TABLE 2

EFFECT ON SPERM LIVABILITY OF ADDING THE D-FORM (2·0 mM) OR L-FORM (0·5 mM) OF TYROSINE, TRYPTOPHAN, OR PHENYLALANINE TO CAPROGEN, IN THE PRESENCE OR ABSENCE OF CATALASE

Amino acid	Livability (hr)		Amino acid	Livability (hr)	
	No catalase	With catalase (4·5 μg/ml)		No catalase	With catalase (4·5 μg/ml)
Nil	49·6	91·2	Nil	57·5	103·7
D-Tyrosine	51·9	89·2	L-Tyrosine	52·2	102·2
D-Tryptophan	50·7	85·2	L-Tryptophan	49·2	94·0
D-Phenylalanine	53·2	88·0	L-Phenylalanine	40·5	91·6
S.E.	2·1	3·5	S.E.	1·7	3·1

TABLE 3

EFFECT OF USING DIALYSED EGG YOLK ON SPERM LIVABILITY IN PRESENCE OR ABSENCE OF CATALASE

Egg yolk	Catalase (4·5 μg/ml)	Livability ± S.E. (hr)
Dialysed	−	68·7 ± 4·7
Undialysed	−	53·8 ± 3·1
Dialysed	+	103·8 ± 3·6
Undialysed	+	99·5 ± 3·8

(*e*) *Ammonium Citrate Addition*

Anhydrous triammonium citrate was added at concentrations ranging from 0·004 to 0·169 g per 100 ml of Caprogen but only one series of samples was incubated with catalase. An appropriate compensatory reduction in trisodium citrate dihydrate concentration was made. Livability was only reduced if the triammonium citrate concentration was at least 0·04 g per 100 ml of Caprogen. Although the inclusion of catalase increased livability it did not affect the harmful action of the triammonium citrate at these higher concentrations [Fig. 1(*b*)].

(*f*) *Sperm Concentration*

The results for the five previous experiments were based on the livabilities of samples incubated at a final concentration of 10 million sperm per millilitre in Caprogen containing 0·3 g of glucose per 100 ml. A comparison was made between concentrations of 5, 10, 15, and 20 million sperm per millilitre in Caprogen containing

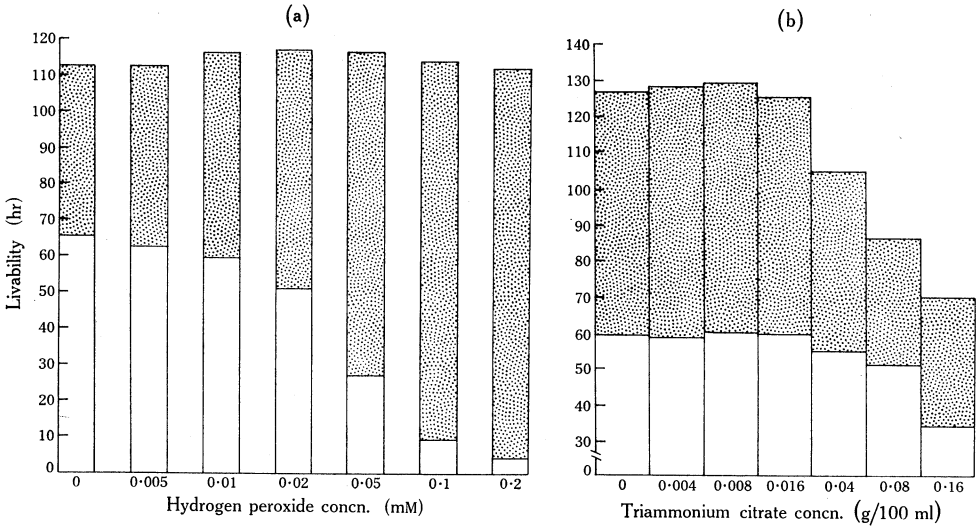

Fig. 1.—Effect of adding hydrogen peroxide (*a*) and triammonium citrate (*b*) on *in vitro* sperm livability in Caprogen without (open rectangles) or with (stippled rectangles) catalase at a concentration of 4·5 μg/ml.

0·15 g of glucose per 100 ml. In the absence of catalase, a significant decline in livability with sperm concentrations below 15 million sperm per millilitre was apparent ($P < 0·01$) (Table 4). This decline was reversed following the addition of catalase

TABLE 4

EFFECT OF VARYING SPERM CONCENTRATION ON LIVABILITY IN PRESENCE OR ABSENCE OF CATALASE

No. of sperm per millilitre	Livability (hr)	
	No catalase	With catalase (4·5 μg/ml)
5×10^6	40·0	119·0
10×10^6	60·4	121·7
15×10^6	80·1	—
20×10^6	82·4	106·0
S.E.	4·7	1·9

as the average livability of samples incubated at a concentration of 20 million sperm per millilitre was significantly lower than for samples at 5 and 10 million sperm per millilitre ($P < 0·01$) (Table 4).

IV. Discussion

The toxicity of hydrogen peroxide observed by Tosic and Walton (1950) and Wales, White, and Lamond (1959) has been confirmed in our experiments. Its production is apparently enhanced by high dilution rates possibly because of the increased concentration of either oxygen or suitable amino acid substrate per unit sperm (Table 4). Therefore the amino acid oxidase identified by Tosic and Walton (1950) may be of greater significance relative to the maintenance of sperm motility and conception rates at high dilution rates and when sperm are stored at ambient temperature. Shannon and Curson (1972) have shown that the peroxide-producing capacity of a semen sample is directly related to the number of dead sperm, and since all samples of bull semen contain some dead sperm a better understanding of the properties of this enzyme appears desirable.

Our results (Table 1) confirm that the addition of low concentrations of L-tryosine, L-tryptophan, or L-phenylalanine reduces the livability of sperm when incubated at 37°C (Tosic and Walton 1950). The toxicity differs between the amino acids but increases with increasing amino acid concentration. White (1954) did not find that these amino acids were toxic even though considerably higher concentrations were used. In that study samples were held at room temperature and not incubated at 37°C. In addition, livabilities at low sperm concentrations were much less than we recorded and there may have been insufficient time for significant peroxide production. Vandemark, Bratton, and Foote (1950) also recorded no improvement in livability following the addition of catalase but samples were stored at 5°C.

Shannon and Curson (1972) have shown that the amino acid oxidase exerts its influence as an extracellular enzyme, and the results in Figure 1(*b*) show that catalase can completely eliminate the toxic effect of added hydrogen peroxide. The toxicity of L-tyrosine is primarily due to peroxide production as the addition of catalase eliminated toxicity at all concentrations (Table 1). In contrast, catalase did not eliminate the toxicity of either L-phenylalanine or L-tryptophan (Table 1); this may be due to differences in their suitability as enzyme substrate or the formation of other end-products of variable toxicity. Certainly the increase in livability with the addition of catalase is specifically due to its enzymic activity, as there is no response with heat-inactivated catalase (Shannon, personal communication).

The equation suggested by Tosic and Walton (1950) for the reaction catalysed by the amino-acid oxidase is:

$$RCH_2CH(NH)_2COOH + O_2 + H_2O \rightarrow R.CH_2CO.COOH + H_2O_2 + NH_3,$$

where R can only be phenyl, *p*-hydroxyphenyl, or indolyl groups. The results in Figure 1(*b*) show that if the triammonium citrate concentration is equal to or greater than 0·04 g per 100 ml, *in vitro* livability at 37°C is reduced and this effect is proportionately greater in the presence of catalase. A concentration of 0·04 g triammonium citrate per 100 ml would be equivalent to 5 mM ammonium ion concentration if the salt completely dissociated. This concentration is not likely to be reached but the inability of catalase to completely eliminate the toxicity of L-phenylalanine and L-tryptophan even at the lowest concentration of 0·5 mM (Table 1), suggests an intrinsic difference in the toxicity of the amino acid or carboxylic acid formed following deamination.

The reductions in livability recorded by Shannon and Curson (1972) following the addition of 5 million dead sperm/ml of incubated sample were 19·6 and 2 hr in the

absence or presence of catalase respectively. The reduction of 19·6 hr is comparable to that which we obtained by the addition of 0·5 mM L-phenylalanine or 5 mM of either L-tryptophan or L-tyrosine (Table 1). But since catalase virtually eliminated the dead sperm effect it is probable that the usual substrate in normally incubated samples is L-tyrosine.

Our results confirm the observation of Tosic and Walton (1950) that reduction of free amino acid concentration by preliminary dialysis of the egg yolk significantly increases livability (53·0 *v.* 68·7 hr) (Table 3). Because this increase is not as great as that obtained by the addition of catalase (99·5 hr), amino acid oxidase substrate may be produced by proteolytic degradation of the egg yolk; however, unpublished work in our laboratory shows that heating egg yolk to 55°C for 30 min prior to its incorporation in the diluent produces only a small increase in livability. It is unlikely that these amino acids are derived from the semen as the levels of the three amino acids in question are particularly low in bull semen (Sexton, Amann, and Flipse 1971). Wales, Scott, and White (1961) reported that the proteolytic activity of bovine semen and seminal plasma is weak, although some free amino acids may be produced during incubation (Bhargava, Bishop, and Work 1959).

Tosic and Walton (1950) inferred that the amino acid oxidase was L-specific although no pure D-forms were tested. Our results show that the D-forms of tyrosine, tryptophan, and phenylalanine are not toxic (Table 2); D-tyrosine does not apparently act as a competitive inhibitor of the enzyme if L-tyrosine is present.

Maximal fertility with highly diluted semen and low sperm doses has only been obtained in New Zealand by using nitrogen-saturated diluents which contain catalase (Shannon 1968). The success of these advances appear to be the result of minimizing the toxic effect of the peroxide produced by the L-amino acid oxidase. Certainly with implementation of these procedures, any dilution effect on bovine sperm is not reflected in lower conception at a concentration of 3 million sperm per millilitre and 0·5 ml insemination dose. Reducing the egg yolk concentration of the diluent reduces the availability of enzyme substrate (Shannon and Curson 1972) and allows dilution to 1 million sperm/ml (New Zealand Dairy Board Farm Production Division 1970). Other alternatives would be to use non-protein or less degradable protein additives in preparing semen extenders. With high dilution rates in ambient temperature extenders, the effect of the L-amino acid oxidase must be minimized by enzyme inhibition, by enzymatic degradation of the peroxide, or by protecting the sperm from the lethal effect of the peroxide.

V. References

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