Inhibition of human and ovine acrosomal enzymes by tannic acid *in vitro*

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The effect of tannic acid, a common flavonoid, on the acrosin and plasminogen activator activity and plasmin activity of human and ram spermatozoa was evaluated. Acrosin and plasminogen activator activity were determined by spectrophotometry using the chromogenic substrates N- α -benzoyl-DL-arginine para-nitroanilide-HCl (BAPNA) and H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide-2HCl (S-2251), respectively. In extracts from both human and ovine acrosomes, the activities of acrosin and plasminogen activators were susceptible to tannic acid inhibition. The inhibitory effect of tannic acid was observed at concentrations $> 50 \mu\text{mol l}^{-1}$ in a dose-dependent manner. In additional experiments, low concentrations of

tannic acid significantly inhibited tissue-type plasminogen activator, urokinase-type plasminogen activator and plasmin activity in a concentration-dependent manner over the range $0.25\text{--}200 \mu\text{mol l}^{-1}$. Tannic acid reduced the motility of ram spermatozoa at a concentration of $1000 \mu\text{mol l}^{-1}$ after 2 and 3 h co-incubation with spermatozoa. The motility of human spermatozoa remained unchanged over the range $0.1\text{--}1000 \mu\text{mol l}^{-1}$ during 3 h co-incubation. These results indicate that tannic acid inhibited the activity of both acrosin and plasminogen activator and indicates a possible mechanism by which flavonoids exert their antifertility effects.

Introduction

Acrosomal enzymes appear to have an essential role in induction of the acrosome reaction, as well as in sperm binding to and penetration through the layers surrounding oocytes (Yanagimachi, 1994). The most widely studied acrosomal enzyme is the endoproteinase acrosin (EC 3.4.21.10). Acrosin is a sperm acrosomal serine proteinase that is involved in the early stages of fertilization: the acrosome reaction (Lui and Meizel, 1979; Tesarik *et al.*, 1988; De Jonge *et al.*, 1989), recognition and binding of spermatozoa to the zona pellucida (Saling, 1981; Jones, 1991; Urch and Patel, 1991) and penetration of the zona pellucida by spermatozoa (Kennedy *et al.*, 1983; Schill, 1991).

Spermatozoa contain a number of other proteinases besides acrosin with less well-documented roles in reproduction. Plasminogen activators, as well as plasminogen activator inhibitors and plasmin inhibitors, have been found in ejaculated spermatozoa from various species (humans, bulls, rams, bucks, boars and stallions) (Smokovitis *et al.*, 1987). There are at least two types of plasminogen

activators in mammals: the tissue-type plasminogen activator (t-PA) and the urokinase-type plasminogen activator (u-PA), which convert the abundant extracellular zymogen plasminogen to plasmin. Plasmin is the active proteinase that stimulates fibrinolysis and proteolysis. The plasminogen activators and their inhibitors are localized to the outer acrosomal membrane and plasma membrane of human and boar spermatozoa (Smokovitis *et al.*, 1992a). In addition, plasminogen activator activity varies with breed and season (Rekkas *et al.*, 1993) and is influenced by chromosomal anomalies (Smokovitis *et al.*, 1992b) and hormones (Rekkas *et al.*, 1991; Tsantarliotou, 1997) in rams. During the acrosome reaction plasminogen activators are released in human, boar, bull and ram spermatozoa (Taitzoglou *et al.*, 1996).

It has been estimated that humans consume approximately 1 g mixed flavonoids per day (Hertog *et al.*, 1993). There is much evidence from human epidemiological and animal studies that there are beneficial effects associated with the consumption of polyphenols (Bravo, 1998; Chung *et al.*, 1998). It has been demonstrated recently that polyphenols, such as flavonoids and their glycosides, are present in human plasma at concentrations between 0.5 and 1.6 mol l^{-1} (Paganga and Rice-Evans, 1997). Tannins are plant polyphenols comprising a heterologous group of

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compounds. Tannic acid is a common flavonoid found in tea, coffee, red wine and immature fruits, and it has also been used as a food additive. Tannic acid is reported to have potent inhibitory effects on the activity of hyaluronidase extracted from cynomolgus monkey spermatozoa (Li *et al.*, 1997). Another plant-derived polyphenolic compound, gossypol, has long been the focus of research efforts due to its antifertility effects in males (Shi and Friend, 1985; Srivastava *et al.*, 1989). The results of a recent study indicated that gossypol has dose-dependent inhibitory effects on several rabbit acrosomal enzymes (Yuan *et al.*, 1995). In addition, gossypol was found to inhibit the activities of human and ovine acrosomal plasminogen activators (Taitzoglou *et al.*, 1999).

The aim of the present study was to determine the effects of tannic acid on the activities of acrosin and plasminogen activator extracted from human and ram spermatozoa and on pure t-PA, u-PA and plasmin preparations to elucidate further the role of acrosin and plasminogen activator and the plasmin system in fertilization.

Materials and Methods

Chemicals

The plasminogens used were isolated from human and ovine blood plasma by affinity chromatography on lysine-sepharose 4B (Sigma, Deisenhofen) as described by Deutsch and Mertz (1970). Tannic acid (molecular weight 1701.23) was obtained from Sigma; immediately before use it was dissolved in Tris-HCl buffer (0.1 mol l⁻¹; pH 7.4) to the appropriate concentration. The substrate for plasmin was H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide-2HCl (S-2251) (Chromogenix, Mölntal). Plasmin from pig blood (Sigma), two-chain t-PA (Biopool, Umeå) and purified human u-PA (Serono, Rome) were used for the inhibition assays and to draw the standard curves. All other chemicals were of the highest purity available.

Assessment of sperm motility

Before studying the effects of tannic acid on acrosin and plasminogen activator activity from human and ram spermatozoa, the effects of tannic acid on sperm motility were determined. In brief, washed semen samples were diluted with modified Biggers, Whitten and Whittingham (BWW) medium different for each species (Biggers *et al.*, 1971; Taitzoglou *et al.*, 1996) into 5 ml plastic centrifuge tubes and incubated at 37°C, in 5% CO₂ in air. The percentage motility of the spermatozoa was evaluated visually at 1 h intervals by placing a drop of each sperm suspension onto a prewarmed microscope slide with prewarmed coverslip and observing at × 200 magnification with a temperature-controlled (37°C) phase contrast microscope. At least four fields were evaluated and 200 spermatozoa were counted in each sample. The final concentrations of tannic acid were 0, 0.1, 1.0, 10.0, 100.0 and 1000.0 µmol l⁻¹.

Preparation of acrosomal extracts for plasminogen activator activity assay

Men. Semen was obtained from healthy donors ($n = 5$) with normal parameters of sperm density, motility and morphology (WHO, 1992). The samples were allowed to liquefy for 30 min at room temperature before processing. Seminal plasma was removed by layering semen over 11% Ficoll in NaCl (0.12 mol l⁻¹) containing Hepes (0.025 mol l⁻¹), pH 7.4 and centrifuged at 1000 *g* for 10 min at room temperature (Kennedy *et al.*, 1989). The seminal plasma and Ficoll supernatant were removed and the sperm pellet was resuspended in 1 ml Tris-HCl (0.1 mol l⁻¹), pH 7.4 and centrifuged again at 500 *g* for 2 min. Finally, the sperm pellet was extracted with a mixture of 4 volumes of Tris-HCl (0.1 mol l⁻¹) and 1 volume of potassium thiocyanate (0.5 mol l⁻¹), pH 7.4, to a concentration of 5×10^7 spermatozoa ml⁻¹ (Smokovitis *et al.*, 1987). The supernatants were collected and stored at -70°C until assayed.

Rams. Ejaculates from Friesian rams ($n = 10$) (National Agricultural Research Foundation, Thessaloniki, Greece) were collected once a week using an artificial vagina. Any sample with < 60% motile spermatozoa was discarded. Pooled semen was centrifuged each time at 900 *g* for 15 min at 22°C to sediment the spermatozoa. The spermatozoa acrosomal extract was prepared as described by Srivastava (1973) and Srivastava *et al.* (1974). In brief, washed spermatozoa were stirred first with MgCl₂ (0.05 mol l⁻¹) in Tris maleate (0.05 mol l⁻¹) buffer, pH 6.1, at 2–4°C for 30 min and centrifuged at 900 *g* for 15 min. After the MgCl₂ treatment the spermatozoa were incubated with 0.1% (v/v) hyamine 2389 and 0.1% (v/v) Triton X-100 in Tris-maleate buffer and the detergent extracts were precipitated with three volumes of ethanol (-15°C) dialysed against glass distilled water and stored at -70°C until assayed.

Plasminogen activator activity assay

Plasminogen activator activity was determined by spectrophotometry using the chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide-2HCl) as described in detail by Smokovitis *et al.* (1987). In brief, a total volume of 500 µl mixture consisting of 200 µl substrate S-2251 (final concentration 0.73 mmol l⁻¹), 100 µl plasminogen solution (final concentration 0.5 casein units ml⁻¹), 100 µl Tris-HCl buffer (0.1 mol l⁻¹), pH 7.4, and 100 µl acrosomal extract was incubated for 30 min at 37°C. The reaction was terminated by adding 1000 µl 50% (v/v) acetic acid. The absorbance was read at 405 nm.

In separate experiments, plasminogen activator activity and plasmin activity were evaluated in pure enzyme preparations. For this purpose, the 100 µl acrosomal extract used in the plasminogen activator activity assay was substituted with 100 µl of a solution containing 5 iu t-PA, 50 miu u-PA or 20 µl of a solution containing 2 miu plasmin. No plasminogen was added for determination of plasmin activity.

Plasminogen activator activity and plasmin activity were expressed in terms of t-PA (iu), u-PA (miu) and plasmin (miu) by referring the net absorbance to standard curves which were drawn using 1–50 iu t-PA, 10–500 miu u-PA or 0.5–10 miu plasmin, respectively.

Acrosin assay

Acrosin activity was determined in human and ram spermatozoa by spectrophotometry (Kennedy *et al.*, 1989) using the chromogenic substrate BAPNA (N- α -benzoyl-DL-arginine para-nitroanilide-HCl; Sigma). In brief, a small volume of each ejaculate was washed with Ficoll solution; human spermatozoa were washed twice. After washing, a 100 μ l volume (0.12 mol NaCl l⁻¹, 0.025 mol Hepes l⁻¹, pH 7.4) containing 5.0×10^6 human spermatozoa or 5.0×10^5 ram spermatozoa was obtained from each sample; 100 μ l benzamidine (Sigma) solution (0.5 mol l⁻¹) was added to control tubes and 1 ml BAPNA solution was added to all tubes after vortexing. The chromogenic substrate solution was prepared by dissolving BAPNA in dimethylsulphoxide (DMSO) to a final concentration of 23 mmol l⁻¹. The BAPNA–DMSO solution was diluted nine times with an extender (0.01% (v/v) Triton X-100 in 0.055 mol Hepes l⁻¹, 0.055 mol NaCl l⁻¹ (pH 8.0)). The tubes were incubated at 37°C for 90 min (Mack and Zaneveld, 1987). After incubation, 100 μ l benzamidine solution was added to the test tubes and all the tubes were centrifuged at 1000 g for 15 min. The supernatants were collected and the absorbance was read at 410 nm. The difference between control and test tubes was adopted as the net absorbance. Acrosin activity was expressed as μ iu 10⁻⁶ spermatozoa.

Studies of inhibition

Enzyme inhibition studies were performed by preincubating a range of tannic acid concentrations with samples at 37°C for 10 min. Each experiment was repeated at least five times. Incubations were run in duplicate.

Statistical analysis

Statistical analysis was performed by Student's *t* test. The level of significance was *P* < 0.05.

Results

Effect of tannic acid on sperm motility

Before examining the effect of tannic acid on sperm acrosomal enzymes it was necessary to determine the effect of this compound on sperm motility. Sperm motility was examined at 1 h intervals during 3 h co-incubation with tannic acid. It was found that 0.1–1000 μ mol tannic acid l⁻¹ did not affect the motility of human spermatozoa, whereas 1000 μ mol tannic acid l⁻¹ significantly inhibited the motility of ram spermatozoa after 2 and 3 h incubation (Table 1).

Effect of tannic acid on acrosomal plasminogen activator activity

The plasminogen activator activity of both human and ovine acrosomal extracts was susceptible to tannic acid inhibition. The inhibitory effect of tannic acid was observed at concentrations > 50 μ mol l⁻¹. Plasminogen activator activity decreased in a concentration-dependent manner: a 50% reduction (IC₅₀) in the plasminogen activator activity of human and ovine extracts occurred at 100 and 200 μ mol tannic acid l⁻¹, respectively. Plasminogen activator activity in human extracts (38 iu t-PA 10⁻⁸ spermatozoa) and in ovine extracts (1588 iu t-PA 10⁻⁸ spermatozoa) was inhibited almost completely by tannic acid at concentrations of 500 and 700 μ mol l⁻¹, respectively (Table 2).

Effect of tannic acid on acrosin activity

Tannic acid inhibited acrosin activity in both human and ovine acrosomal extracts, at concentrations as low as 50 μ mol l⁻¹ (Table 3). The sensitivity of acrosin to inhibition by tannic acid was highest at 50–400 μ mol tannic

Table 1. Percentage motility of human and ovine spermatozoa after 0, 1, 2 and 3 h incubation with tannic acid

Tannic acid (μ mol l ⁻¹)	Human spermatozoa				Ovine spermatozoa			
	Duration of incubation (h)				Duration of incubation (h)			
	0	1	2	3	0	1	2	3
0	81.25 $\pm$ 4.15	71.25 $\pm$ 6.50	66.25 $\pm$ 6.50	61.25 $\pm$ 4.15	77.50 $\pm$ 4.33	70.00 $\pm$ 3.54	63.75 $\pm$ 2.17	63.75 $\pm$ 4.15
0.1	78.75 $\pm$ 2.15	70.00 $\pm$ 6.12	65.00 $\pm$ 3.54	60.00 $\pm$ 6.12	76.25 $\pm$ 4.14	70.00 $\pm$ 6.12	62.50 $\pm$ 2.50	63.75 $\pm$ 2.17
1	78.75 $\pm$ 2.15	68.75 $\pm$ 5.45	65.00 $\pm$ 6.12	60.00 $\pm$ 7.08	76.25 $\pm$ 6.50	68.75 $\pm$ 2.17	61.25 $\pm$ 4.15	61.25 $\pm$ 5.45
10	77.50 $\pm$ 2.50	67.50 $\pm$ 5.59	65.00 $\pm$ 7.90	57.50 $\pm$ 8.29	75.00 $\pm$ 6.12	66.25 $\pm$ 4.15	61.25 $\pm$ 2.17	58.75 $\pm$ 2.17
100	77.50 $\pm$ 4.33	65.00 $\pm$ 3.54	63.75 $\pm$ 6.50	56.25 $\pm$ 5.45	75.00 $\pm$ 5.00	66.25 $\pm$ 2.16	60.00 $\pm$ 3.54	57.50 $\pm$ 4.33
1000	75.00 $\pm$ 6.12	63.75 $\pm$ 4.15	62.50 $\pm$ 4.33	55.00 $\pm$ 6.12	73.75 $\pm$ 5.45	65.00 $\pm$ 3.54	43.75 $\pm$ 2.17 ^a	22.50 $\pm$ 2.50 ^a

Values are mean percentage motility $\pm$ sd (*n* = 4).
^aValues with a superscript are significantly different from the other values within that column (*P* < 0.001).

Table 2. Inhibitory effects of tannic acid on the activity of tissue-type plasminogen activator extracted from human and ram spermatozoa

Tannic acid ($\mu\text{mol l}^{-1}$)	Human		Ram	
	Residual activity (%)	Tissue-type plasminogen activator (iu 10^{-8} spermatozoa)	Residual activity (%)	Tissue-type plasminogen activator (iu 10^{-8} spermatozoa)
0	100	38.00 ± 2.74	100	1588.00 ± 63.21
50	77.40 ± 7.02^a	29.41 ± 2.67^a	85.50 ± 4.70^a	1356.15 ± 74.14^a
100	52.80 ± 6.80^a	20.06 ± 2.58^a	63.40 ± 6.27^a	1006.79 ± 99.55^a
200	35.60 ± 7.23^a	13.53 ± 2.75^a	48.20 ± 5.26^a	765.42 ± 83.58^a
300	21.60 ± 5.99^a	8.21 ± 2.16^a	38.80 ± 4.35^a	616.14 ± 68.67^a
400	14.00 ± 3.54^a	5.32 ± 1.34^a	29.80 ± 3.35^a	473.22 ± 53.14^a
500	5.20 ± 1.64^a	1.98 ± 0.62^a	24.60 ± 2.70^a	390.65 ± 42.90^a
600	–	–	20.20 ± 1.92^a	320.78 ± 30.55^a
700	–	–	9.20 ± 1.52^a	152.45 ± 24.08^a

–: total inhibition.
Values are mean $\pm$ SD ($n = 5$).
^aValues with a superscript are significantly different from control value within each column ($P \leq 0.05$).

Table 3. Inhibitory effects of tannic acid on the activity of acrosin extracted from human and ram spermatozoa

Tannic acid ($\mu\text{mol l}^{-1}$)	Human		Ram	
	Residual activity (%)	Acrosin ($\mu\text{iu } 10^{-6}$ spermatozoa)	Residual activity (%)	Acrosin ($\mu\text{iu } 10^{-6}$ spermatozoa)
0	100	79.46 ± 5.21	100	1139.00 ± 58.18
50	76.80 ± 4.38^a	60.91 ± 3.45^a	80.00 ± 5.00^a	911.20 ± 56.95^a
75	68.60 ± 2.51^a	54.51 ± 1.99^a	71.20 ± 3.35^a	810.97 ± 38.12^a
100	61.20 ± 3.03^a	48.63 ± 2.41^a	60.80 ± 1.48^a	692.51 ± 16.89^a
150	52.40 ± 1.95^a	41.63 ± 1.55^a	48.00 ± 2.55^a	546.72 ± 29.04^a
200	44.60 ± 3.65^a	35.44 ± 2.90^a	40.80 ± 1.48^a	462.72 ± 16.89^a
250	38.60 ± 2.30^a	30.67 ± 1.83^a	34.00 ± 2.92^a	387.26 ± 33.21^a
300	25.00 ± 3.81^a	19.87 ± 3.03^a	27.20 ± 1.48^a	309.81 ± 16.86^a
400	8.80 ± 2.17^a	6.99 ± 1.72^a	13.80 ± 2.86^a	157.18 ± 32.62^a

Values are mean $\pm$ SD ($n = 5$).
^aValues with a superscript are significantly different from the control value in that column ($P \leq 0.02$).

acid l^{-1} . The IC_{50} for acrosin in human and ovine extracts was $150 \mu\text{mol l}^{-1}$. Acrosin in human extracts ($79.46 \mu\text{iu } 10^{-6}$ spermatozoa) and in ovine acrosomal extracts ($1139 \mu\text{iu } 10^{-6}$ spermatozoa) was inhibited almost completely by $400 \mu\text{mol tannic acid l}^{-1}$ (Table 3).

Effect of tannic acid on pure enzyme activity

In additional experiments, plasmin (2 mIU), t-PA (5 iU) and u-PA (50 mIU) were added to solutions containing 0.25–200 $\mu\text{mol tannic acid l}^{-1}$ and assayed for residual activity. The IC_{50} of all three enzymes was $1 \mu\text{mol l}^{-1}$. Plasmin activity was reduced by 92% at 200 $\mu\text{mol tannic acid l}^{-1}$, whereas t-PA and u-PA activator activities were reduced by 93 and 95%, respectively, at 100 $\mu\text{mol tannic acid l}^{-1}$ (Table 4).

Discussion

Flavonoids are a group of naturally occurring low molecular weight benzo[r]pyrone derivatives that are ubiquitous in plants (Havsteen, 1983). Although their mechanism of action is not entirely clear, the flavonoids act as competitive enzyme inhibitors (Kuppusamy *et al.*, 1990). Considering the numerous benzene rings and hydroxyl groups present in the structure of tannic acid, it is possible that the inhibitory effects of tannic acid may result from conformational changes or denaturation of the enzymes caused by hydrogen bonding or hydrophobic interactions between the enzymes and tannic acid.

The present data demonstrate clearly that tannic acid has potent inhibitory effects on acrosin activity and plasminogen activator activity in human and ovine acrosomal

Table 4. Inhibitory effects of tannic acid on plasmin, tissue-type plasminogen activator and urokinase-type plasminogen activator activity in pure enzyme preparations

Tannic acid (μmol l ⁻¹)	Plasmin		Tissue-type plasminogen activator		Urokinase-type plasminogen activator	
	Residual activity (%)	miu	Residual activity (%)	iu	Residual activity (%)	miu
0	100	2.00	100	5.00	100	50.00
0.25	75.40 ± 3.84 ^a	1.42 ± 0.09 ^a	80.60 ± 6.31 ^a	4.03 ± 0.32 ^a	84.40 ± 4.45 ^a	42.20 ± 2.22 ^a
0.50	58.80 ± 4.76 ^a	1.18 ± 0.10 ^a	65.20 ± 5.81 ^a	3.26 ± 0.29 ^a	60.00 ± 7.21 ^a	30.00 ± 3.61 ^a
1.0	50.40 ± 4.04 ^a	1.01 ± 0.08 ^a	51.80 ± 5.26 ^a	2.59 ± 0.26 ^a	49.60 ± 3.97 ^a	24.80 ± 1.99 ^a
5.0	42.20 ± 4.14 ^a	0.84 ± 0.08 ^a	32.80 ± 5.72 ^a	1.64 ± 0.29 ^a	41.60 ± 3.43 ^a	20.80 ± 1.72 ^a
10.0	35.20 ± 2.39 ^a	0.70 ± 0.05 ^a	20.60 ± 7.54 ^a	1.03 ± 0.38 ^a	33.20 ± 3.11 ^a	16.60 ± 1.56 ^a
50.0	27.40 ± 2.89 ^a	0.55 ± 0.06 ^a	10.40 ± 1.52 ^a	0.52 ± 0.08 ^a	16.60 ± 4.34 ^a	8.30 ± 2.17 ^a
100.0	20.80 ± 2.39 ^a	0.42 ± 0.05 ^a	6.80 ± 1.79 ^a	0.34 ± 0.09 ^a	4.60 ± 1.14 ^a	2.30 ± 0.57 ^a
200.0	7.80 ± 1.48 ^a	0.16 ± 0.03 ^a	–	–	–	–

–: total inhibition.
Values are mean ± SD (n = 5).
^aValues with a superscript are significantly different from the control value within that column (P ≤ 0.02).

extracts. The inhibition is concentration-dependent. This is believed to be the first report of tannic acid-induced inhibition of these acrosomal enzymes and provides a new explanation for flavonoid-induced male infertility.

Newly ovulated mammalian ova are embedded in a mass of the cumulus oophorus, corona and zona pellucida. Spermatozoa must pass through these barriers at the time of fertilization. The acrosome contains several hydrolytic enzymes that are necessary for penetration of the spermatozoon through these investments (Yanagimachi, 1994). The involvement of proteinases at different stages of fertilization seems increasingly likely as the molecular events of fertilization are progressively being elucidated.

It is generally accepted that the activity of acrosin, a unique serine proteinase associated with the acrosome of mammalian spermatozoa, is essential for fertilization (Meizel, 1984; Katz *et al.*, 1987; De Jonge *et al.*, 1989; Schill, 1991). The essential role of acrosin in fertilization is indicated by the ability of various proteinase inhibitors to block fertilization or to impair sperm functions involved in fertilization (Saling, 1981; Dravland and Meizel, 1982; Van der Ven *et al.*, 1985; De Jonge *et al.*, 1989).

In addition to acrosin, the well-characterized gamete proteinases, plasminogen activators, are likely candidates for catalysing proteolysis during fertilization. The membrane localization of plasminogen activators in spermatozoa and the release of plasminogen activators during acrosome reaction indicate that these proteolytic enzymes could participate in fertilization (Smokovitis *et al.*, 1992a; Taitzoglou *et al.*, 1996). Plasmin, the active enzyme of the plasminogen activation system, is also a trypsin-like proteinase that can cleave and degrade a wide spectrum of substrates (Danø *et al.*, 1985). Indeed, previous results indicated that *in vitro* fertilization of mouse eggs was in-

hibited by antibodies that inhibit the catalytic activity of plasmin and that addition of plasminogen to the fertilization medium increased the yield of fertilized eggs (Huarte *et al.*, 1993).

The primary mechanism for the contraceptive effect of flavonoids appears to be inhibition of several acrosomal enzymes (Yuan *et al.*, 1995; Taitzoglou *et al.*, 1999). Some flavonoids inhibit the activity of hyaluronidase from bovine testes (Kuppusamy *et al.*, 1990), as well as from snake, scorpion and bee venom (Kuppusamy and Das, 1991). Recently, Li *et al.* (1997) demonstrated that tannic acid inhibited the activity of hyaluronidase extracted from monkey spermatozoa in a concentration-dependent manner over the range 50–200 μmol l⁻¹.

Sperm motility and high velocity are important for sperm binding to and penetration into the zona pellucida; immotile spermatozoa cannot bind to or penetrate through the zona pellucida (Liu and Baker, 1993; Yanagimachi, 1994). Many of the common polyphenolic compounds that are naturally present in abundance in plant extracts exert inhibitory effects on mammalian sperm motility *in vitro*. These polyphenolic compounds could be another cause of the antifertility effects of crude plant extracts; it is possible that augmentation of the generation of superoxide radicals by polyphenols may inhibit sperm motility *in vitro* (Kumar *et al.*, 1989). Indeed, co-incubation of monkey spermatozoa with tannic acid significantly inhibited percentage motility (Li *et al.*, 1997). In contrast, in the present study the percentage motility of ram spermatozoa was reduced significantly only at the highest concentration tested. This discrepancy may be attributable to species differences. In addition, plasmin added to bovine semen affected sperm motility; the highest increase in sperm motility was observed after addition of 5 ng plasmin ml⁻¹ (Smokovitis *et al.*, 1987).

In the present study, tannic acid inhibited plasminogen activator activity in pure enzymatic preparations with IC₅₀ of 1 µmol l⁻¹ for plasmin, t-PA and u-PA. In acrosomal extracts, the IC₅₀ of tannic acid was 100 µmol l⁻¹ for human spermatozoa and 200 µmol l⁻¹ for ram spermatozoa. As tannic acid inhibits a large number of enzymes, the discrepancy observed between pure preparations and acrosomal extracts might be due to the fact that tannic acid affects other sperm factors beside the plasminogen activator and plasmin systems when mixed with acrosomal extracts.

Two-step Ficoll separation was necessary during isolation of human spermatozoa for the acrosin assay as simple Ficoll separation did not remove acrosin inhibitors completely. This finding was in accordance with those of Breden *et al.* (1996).

As sperm acrosin does have a role in multiple phases of mammalian fertilization and plasminogen activators and the plasmin system are involved in several stages of fertilization, flavonoids such as tannic acid will be useful tools for assessing the involvement of acrosomal enzymes in these gamete functions.

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