SYNTHETIC ENZYME INHIBITORS AS ANTIFERTILITY AGENTS

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1. Introduction

The acrosome of the mammalian spermatozoon is a cap over the anterior end of the sperm head containing digestive enzymes that the sperm uses to penetrate the investing layers of the ovum. One of these enzymes has characteristics similar to both plasmin and trypsin, and has been assigned the name acrosin [1–5]. The function of acrosin is to enable the fertilizing sperm to penetrate the zona pellucida of the ovum by digesting a passage through this structure. Soybean and lima bean trypsin inhibitors block both the enzyme activity of acrosin [5], and ferilization *in vitro*, using sperm that have achieved the capacity in the female tract to penetrate ova, i.e. capacitated sperm [6]. Of greater practical significance is the observation that in the acrosomes of freshly ejaculated sperm, acrosin activity is inhibited by a trypsin inhibitor from the seminal plasma which later is removed in the female tract [2, 7]. Pancreatic trypsin inhibitor and a seminal vesicle trypsin inhibitor inhibit acrosin and fertilization *in vivo* if the capacitated sperm are first treated with these inhibitors before insemination into the oviducts [4]. All the natural trypsin inhibitors are polypeptides and bind reversibly to the proteolytic enzymes they inhibit. It has been shown, in fact, that seminal plasma trypsin inhibitor is removed from acrosin in the rabbit uterus [2]. This observation led us to study inhibitors that irreversibly react with the active site of certain proteolytic enzymes similar to acrosin, with the object of permanently blocking fertilization during the *in vivo* life of the sperm in the female. If such inhibition of acrosin could be achieved while the enzyme was still in the acrosome of freshly ejaculated sperm, it is possible that the inhibitors would be effective systemic or virginal contraceptives in the female, and systemic contraceptives in the male. We have demonstrated that TLCK (tosyl-L-lysine chromomethyl ketone) but not TPCK (tosylphenylalanine chloromethyl ketone) prevents fertilization of capacitated sperm. NPGB (nitrophenyl *p*-guanidinobenzoate) and TLCK but not EPGB (ethyl *p*-guanidinobenzoate) significantly decrease fertility on incubation with ejaculated sperm. We further show that TLCK is effective as a vaginal contraceptive in the rabbit, an animal in which present vaginal products designed for human use are ineffective [8].

2. Materials and methods

TLCK was obtained from Sigma Chem. Co., TPCK from Mann Res. Co. and NPGB and EPGB were kindly provided by Dr. Elliot Shaw, Brookhaven National Laboratories. Each inhibitor was dissolved in Krebs–Ringer phosphate buffer (KRP) at pH 7.0 before addition to sperm. NPGB was dissolved in 2% dimethyl sulfoxide (Fisher Chem. Co.) in KRP. Mature, New Zeeland white rabbits were isolated for at least 18 days before use. Usually four rabbits were used per group. Three types of experiments were performed:

a) Capacitated spermatozoa were recovered from the uteri of rabbits 12 hr after breeding, washed in KRP and treated with TLCK or TPCK for 30 min at 37°. Excess inhibitor was removed by sedimenting the sperm at 700 *g*. The sperm were resuspended in KRP and tested for fertility by insemination of 5×10^4 sperm in 0.05 ml into oviducts of ovulated rabbits 12.5 hr after administration of human chorionic gonadotrophin (HCG, Spencer Mead). The contralateral oviducts were inseminated with untreated capacitated sperm. The rabbits were killed 24 hr

later, the oviducts flushed with KRP and the ova examined for fertilization by presence of uniform cleavage [9].

b) Ejaculated rabbit spermatozoa were obtained by artificial vagina, washed and treated with TLCK, NPGB or EPGB for 30 min at 37°. Excess inhibitor was removed, the sperm resuspended in KRP and tested for fertility by surgical insemination of 2.5×10^6 sperm in 0.25 ml into one uterus of a rabbit. The contralateral uterus was ligated at the cervical end to prevent sperm migration, and untreated, ejaculated sperm were added to it. HCG was administered to the rabbits at the time of surgery. The rabbits were killed 36 hr later, the oviducts flushed and the ova observed for fertilization.

c) TLCK (in KRP) was thoroughly mixed with an equal volume of K–Y sterile lubricant (Johnson and Johnson). Five ml of the mixture was deposited into the vagina using a syringe and a plastic tube. The rabbits were bred within 5 min of deposition. The rabbits were killed 36 hr later, the oviducts flushed and the ova observed for cleavage.

3. Results and discussion

High levels of the synthetic inhibitors killed the spermatozoa but the amounts used in these experiments had no visible effect on motility. In KRP both treated and control sperm maintained motility for 8 hr *in vitro*.

TPCK had virtually no antifertility effect on capacitated spermatozoa whereas TLCK completely inhibited fertilization (table 1). This corresponds with the effect of these inhibitors on acrosin, since TPCK does not inhibit this enzyme and TLCK has as great an inhibitory effect on acrosin as it has on pancreatic trypsin [5]. The antifertility activity of TLCK results from the inhibition of acrosin by specifically alkylating histidine in the active site of enzymes similar to acrosin [10]. Alkylation did not occur to an extent elsewhere sufficient to impair motility of the sperm.

TLCK and NPGB but not EPGB decreased fertility of ejaculated sperm (table 2). These results again correlate with the effect of these inhibitors on proteolytic enzymes like acrosin, since NPGB and TLCK react approximately 1000 times faster with trypsin-like enzymes than does EPGB [10]. The 30-min incubation period was probably not long enough for EPGB to react with the acrosin in the sperm acrosome or EPGB penetrated the acrosome less effectively.

Using the same amount of inhibitor (10 μg) TLCK was a more effective antifertility agent than NPGB and was therefore used for vaginal experiments. Table 3 shows that TLCK at a level of 3 mg/ml completely prevents fertilization under practical conditions.

From these data we can conclude that synthetic trypsin inhibitors successfully prevent fertilization *in vivo*. They accomplish this most likely through irreversible inhibition of acrosin, an acrosomal protease that enables sperm to penetrate the zona pellucida of the ovum. These inhibitors may well be of practical importance as antifertility agents either if used as a vaginal contraceptive alone or with other known vaginal contraceptive ingredients.

Table 1
Effects of TLCK and TPCK on the fertilizing ability of capacitated rabbit spermatozoa.

Inhibitor	Treatment of sperm			Control	
	Amount per 10^5 sperm (μg)	No. of ova	Percent fertilization	No. of ova	Percent fertilization
TLCK	5	13	15.6	10	100
TLCK	15	16	0	16	87.5
TPCK	15	7	86	6	100

The inhibitor was incubated with 2.5×10^5 sperm in 0.5 ml. Fertilization experiments were performed using 5×10^4 sperm (see text). Control sperm were treated the same except that no inhibitor was added.

Table 2
Effects of TLCK, EPGB and NPGB on the fertilizing ability of ejaculated rabbit spermatozoa.

Inhibitor	Treatment of sperm			Control	
	Amount per 10^5 sperm (μg)	No. of ova	Percent fertilization	No. of ova	Percent fertilization
TLCK	3	20	9	14	100
TLCK	10	25	0	19	100
EPBG	10	13	100	17	100
NPBG	10	9	21	10	100

Each incubation mixture consisted of 100 times the indicated amount of inhibitor and 10^7 sperm in 10 ml. The treated sperm (2.5×10^6) were inseminated into non-ligated uteri (see text). Control sperm were treated the same as the test sperm except in the absence of inhibitor and were inseminated into ligated uteri.

Table 3
Antifertility effect of TLCK in the vagina.

TLCK (mg/ml)	Rabbits	No. of ova	Percent fertilization
0	5	37	100
1	5	34	38.3
2	4	32	6.2
3	5	28	0

The TLCK was dissolved in 2.5 ml KRP, mixed with 2.5 ml K–Y sterile lubricant and deposited in the vagina 5 min before breeding.

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