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ORIGINAL ARTICLE

Degenerative action on mice and rat testes of polyspermine and its complexes with RNase A

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Summary
A significant aspermatogenic activity, ascertained by microscopic studies of seminiferous tubules and interstitial tissue, and by the observation of the entrance of immunity and fibrocytic cells in mice injected with polyspermine (PS) or polyspermine conjugated to monomeric or dimeric RNase A (PS-RNase A or PS-dimeric RNase A, respectively), was found either in mice injected or in non-injected testes. Polyspermine and its complexes with RNase A destroyed all spermatogenic and intertestitial tissue, including Leydic cells, as well as their ability to secrete testosterone. The total loss of spermatogenic activity in injected testes is irreversible because spermatogonia cells also were destroyed. The injection of PS into both mice testes determined the total degeneration of testicle tissue in 50% of injected testes. The second half of testes was also partly degenerated, and if they were re-injected, almost all testes were fully destroyed. PS-dimeric RNase A injected once into both testicles produced a stronger degeneration and also the interruption of testosterone secretion in comparison with the effects due to injection of mice with PS or PS-RNase A. In all mice treated with these substances, as well as in rats in which PS was injected twice into their testes, we detected polymorfonucleates, monocytes, plasma cells, lymphocytes and fibrocytic cells. Antibodies against PS, PS-RNase A or PS-dimeric RNase A did not influence the aspermatogenic activity. Animals in which a repeated intra-peritoneal injection was carried out did not lose body mass and remained in good condition, with the exception of mice injected with spermine.

Keywords: aspermatogenesis – cell degeneration – testes – spermine – polyspermine – polyspermine-RNase A

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INTRODUCTION

The crystals that form in human semen left standing were identified as the phosphate of an organic base, which was named spermine by Schreiner in 1878 (Schreiner 1878). The synthesis of the substance and its structure were established by Dudley et al. (1927) in 1927. Since that time spermine and its crystalline derivatives, secreted mainly in the

human and animal prostate, have been used in the forensic detection of seminal stains (Mann and Lutwak-Mann, 1981). Spermine as a polyamine belongs to the family of low molecular weight biogenic amines considered to be essential constituents of all mammalian cells. Spermine and other polyamines influence cellular processes at cell stages from gene transcription to protein synthesis, stabilize nucleic acid structure, and are of central importance in the regulation of cell growth and differentiation (Pegg 1986, Seiler et al. 1996).

In 1977 Wang and Moore (1977) described the preparation of an RNase A derivative, called polyspermine-RNase, by binding a chain of polyspermine (PS), which in their best preparation consisted of eight spermine residues, to a lysine residue of an RNase A molecule through the bifunctional reagent dimethyl suberimidate. The spermine residues had in turn been linked one to another by the same reagent, to form polyspermine. These experiments were performed within the hypothesis that the number (and location) of basic charges in the ribonuclease molecule may be an important variable for ribonuclease activity towards double-stranded RNA and DNA:RNA hybrids (Libonati et al. 1975a,b; Libonati and Gotte 2004). Polyspermine-RNase showed indeed to have acquired the ability to remarkably degrade these substrates, confirming the importance of positive charges for the ribonuclease function (Wang and Moore 1977).

If polyspermine accumulates in the cell, toxic effects can occur because of its oxidation by polyamine oxidases (Lindsay and Wallace 1999). Polyamine oxidases contribute to the regulation of the levels of polyamines, catalyzing their oxidative deamination. The oxidation products, hydrogen peroxide, aldehydes and acrolein (generated by aminoaldehyde), have been implicated in programmed cell death, induction of cytotoxicity and inhibition of cell division (Lindsay and Wallace 1999, Agostinelli et al. 2004).

Polyspermine is also cytotoxic *in vitro* against tumour cell lines and inhibits the growth of xenograft tumours at low concentration (Yatin et al. 1999).

In studying the antitumour effects of polyspermine and polyspermine conjugated to monomeric or dimeric RNase A and their side effects in mice (Poučková et al., in press), we also found strong aspermatogenesis in the testicles of mice treated with those substances. This observation led us to a detailed study of the action of these substances on spermatogenesis in mice, and of the action of polyspermine also in rats. Based on the importance of basic charges for ribonucleolytic activity, our idea is now to see if the binding of polyspermine to monomeric RNase A or to cross-linked dimeric RNase A (as the simplest example of oligomeric RNase A), with the consequent remarkable increase of the basicity of the protein moiety, could enhance the anti-testicle activity of the complexes, besides the enzymatic activity on double-stranded RNA, which is already known. The cross-linked RNase A dimer, notwithstanding its slightly lower basicity compared to that of an aggregated dimer (due to the consumption of at least one lysine residue per monomer in the cross-linking reaction with dimethyl suberimidate), has the advantage of escaping interaction with the cellular RNase inhibitor, and of being stable, whereas the aggregated oligomers are meta-stable (Libonati and Gotte 2004). To prepare the covalently linked RNase A dimer we followed the procedure outlined by Wang et al. (1976), and already used by some of us to prepare cross-linked dimers and trimers of RNase A (Gotte et al. 1997).

MATERIALS AND METHODS

Preparation of cross-linked dimers of RNase A

To prepare the covalently linked dimer, we followed the procedure outlined by Wang et al. (1976). 40 mg of RNase A (Type XII-A, purchased from Sigma Chem. Co.) were dissolved in 0.64 ml of 0.2M sodium phosphate buffer, pH 8.0. One mg of solid dimethyl suberimidate (Sigma-Aldrich, Prague, Czech Rep.) was added in small aliquots to this solution, with stirring. The pH of the solution was adjusted to 8.0 and maintained with 1M NaOH, and the reaction time was 10 minutes at room temperature, with stirring. The reaction was stopped by adding 0.64 ml of 0.2M ammonium acetate. The product was then purified and isolated by gel filtration through a Superdex 75 GL 10/300 column in a FPLC system (GE-Healthcare, Munich, Germany), equilibrated with 0.2M sodium phosphate buffer, pH 6.7. Flow rate, 0.1 to 0.2 ml/min. The material was concentrated with Millipore Centricon YM-3, 3000 MW cut-off, the solvent was changed to remove dissolved salts, and the sample finally lyophilized.

Preparation of polyspermine

120 μmoles of spermine (41.78 mg; Sigma-Aldrich, Prague, Czech Rep.) were dissolved in 2 ml of 0.2M triethanolamine, pH 9.7. To this solution, under stirring, small aliquots of solid dimethyl suberimidate (124 μmol, 33.88 mg) were added, controlling and adjusting the pH to 9.7 with 1M NaOH. The reaction time was 1 minute at room temperature (Wang and Moore 1977).

Preparation of (cross-linked) polyspermine-RNase A and polyspermine-dimeric RNase A

We cross-linked polyspermine to monomeric RNase A or to its previously cross-linked dimer

(Gotte et al. 1997) following the method described by Wang and Moore (1977) to prepare polyspermine-ribonuclease. After the two steps of the previous preparation were completed, 1.1 μmoles of monomeric or dimeric RNase A, dissolved in 2 ml of 0.1M sodium phosphate buffer, pH 9.0, were added to the solution containing 120 μmol polyspermine and 124 μmol dimethyl suberimidate (see above). The reaction time, with stirring, was five minutes. The reaction was stopped by adding 1.5 ml of 2M ammonium acetate. The two procedures described here are similar to the method outlined by Wang and Moore (1977). The products of the mixture were then purified and isolated by gel filtration through a Superdex 75 GL 10/300 column in a FPLC system (GE-Healthcare, Munich, Germany) equilibrated with 0.2M sodium phosphate buffer, pH 6.7. Flow rate, 0.1 ml/min. The sample was concentrated and lyophilized as described above.

Spermatogenic toxicity in mice and rats

The aspermatogenic effect of polyspermine (PS), polyspermine conjugated to monomeric RNase A (PS-RNase A) or dimeric RNase A (PS-dimeric RNase A) and their components, spermine and RNase A, was determined by procedures described previously (Matoušek 1994). Adult male ICR mice were injected with 100 μg of each substance to be tested (which was dissolved in phosphate buffered saline − PBS) into their left testis or into both testes. Wistar rats were injected into both testes with 0.57 mg of PS. The injected testes and separated epididymis were excised and histologically examined. Destructive effects on the testes were detected by the decrease in the width of spermatogenic layers and the diameter of seminiferous tubules. The degeneration was also studied by spermatozoa presence or absence in epidydimis tubules and by the histological structure of testes and epididymises (Matoušek 1994). The microscopic observation of spermatogenic and interstitial tissues, and the invasion of immunite and fibrocytic cells into testicles was the main method followed in studying the processes of testicle degeneration. Moreover, in order to investigate the antigenicity (production of antibodies) of all substances used in this study, 100 μg of each substance was administered to the mice intraperitoneally once a week over 5 weeks. The degenerative effects on the testes of these mice were detected as described above.

Immunogenicity determination

The immunogenicity (production of antibodies) of all polyspermine substances was determined as described previously (Souček et al. 1996). A noncompetitive ELISA test was performed. Microtiter plate wells were coated with 25 μg of all studied substances. After washing the plates, the antibody from mice treated with the above mentioned substances, and control sera from mice injected with PBS were serially diluted in wells and incubated at 37 °C for two hours. Sw-AM-Px (swine anti-mice IgG with peroxide) conjugate 1:1000 was added, and after 20 min of incubation with the tetramethylbenzidine substrate solution, the reaction was stopped with the addition of 2.0M H_2SO_4. The antibody reaction was measured spectrophotometrically at 450 nm. The tests were defined as positive when the optical density of the serum tested was found to be at least three standard errors of the mean (S.E.M.) higher than that of control mice injected with PBS.

Histology

All animals injected with the studied substances were subjected to excision of testes. Small pieces of testicle tissue were then fixed in Bouin solution for histological examination. The fixed samples of tissues were embedded in paraffin blocks. Tissues slides (5 μm) were cut and stained with hematoxylin-eosin.

Testosterone radioimmunoassay

The concentration of testosterone in blood serum was assayed by using a Testosterone Direct Radioimmunoassay kit (Immunotech, Marseille, France). The cross-reactivity of the anti-testosterone antibody was the following: testosterone, 100%; 5α-dihydrotestosterone, 10%; 11β-hydroxytestosterone, 2%; 4-androstenedione, 0,6%; 19-nortestosterone, 5%; and methyltestosterone, 2%. The cross-reactivity with other steroids was <0.03%. Radioimmuno-assays were performed according to kit instructions. Briefly, standard, control, or plasma samples (50 μl) were dispensed into antibody-coated tubes. After the addition of a solution (500 μl) of ^{125}I-labelled testosterone tracer, the tubes were incubated for three hours at 37 °C. The liquid in the tubes was then removed by aspiration, and in each tube the radioactivity was determinated with a gamma radiation counter (Berthold Multi Gamma 2104, Berthold, Germany). The detection limit of the assay was 0.029 ng/ml, and the coefficient of variance within an assay was 3.83%.

Statistical analysis

The results are presented as mean ± SEM. The data were analyzed statistically using Fisher´s *t*-test at the significance level $2\alpha=0.05$.

Ethics

All reported experiments adhered to ethical standards and were approved by the institutional committee (approval no. 3/04); all researchers handling experimental animals possess certificates from the Central Committee for Animal Welfare.

RESULTS

Aspermatogenic effect of a single injection in the left testis of mice of spermine, polyspermine or polyspermine conjugated to RNase A

The aspermatogenic activity, determined by measuring the width and diameter of seminiferous tubules of mice injected with 100 μg of polyspermine or polyspermine conjugated to RNase A, occurred to a significant extent not only in the injected testicles but also (see the width of aspermatogenic layers) in non-injected testes. Spermine was also assayed. It evoked aspermatogenesis only in injected testicles and to a lower extent compared to the other substances (Table 1). Polyspermine and its conjugate with dimeric RNase A destroyed all spermatogenic and interstitial tissue, including Leydic cells (Fig. 1).

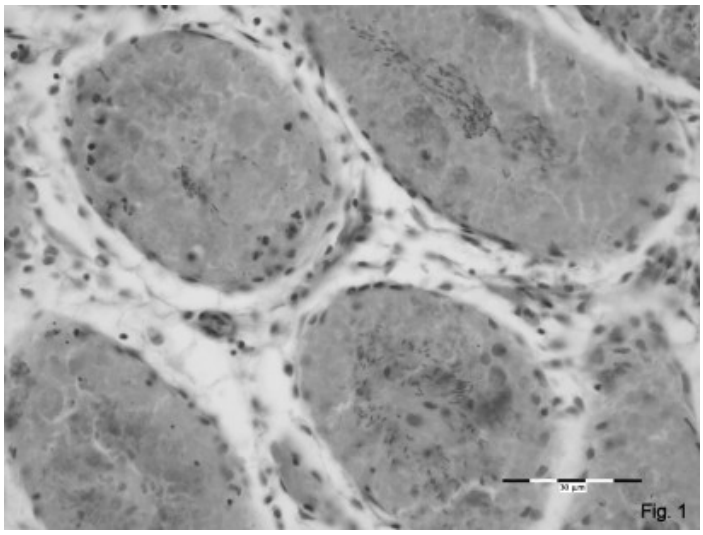

Fig. 1. **Effect of polyspermine conjugated to dimeric RNase A (PS-dimeric RNase A) injected once into both mice testes.** Testicle tissue of both testes was fully degenerated including Leydic cells in the interstitial tissue. Spermatogonia vacuolysed and lost their mitotic activity. The testicular tissue was gradually fully destroyed in all tubules, which lack any original testicular tissue cells. The necrotized structure was invaded by polymorphonucleates, monocytes, plasma cells and lymphocytes. In the whole testicle tissue the number of fibroblasts and fibrocysts increased and was fully ligamented.

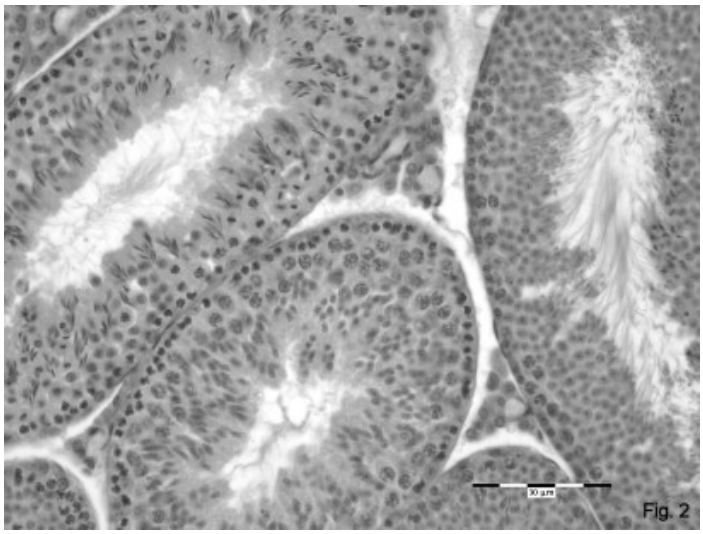

Fig. 2. **PBS injected twice into the left testicles of mice.** See the normal spermatogenesis, and even interstitial tissue of Leydic cells (control).

The total spermatogenic cell loss in injected testes was irreversible because all the spermatogonia cells were also destroyed. The effect was not so dramatic in non-injected testes, where spermatogenic and spermatogonia cells were not fully degenerated. No toxic changes were noticed in testicles of control mice and rats injected intratesticularly with PBS (Fig. 2).

Spermatogenesis of mice and rats injected into both testicles with polyspermine or polyspermine conjugated to RNase A

Injections of polyspermine into both testes (100 μg per testicle) of 13 mice evoked the total degeneration of both testes in only 50% (14 testicles) of treated mice (Table 2). The histological picture resembles that of Fig. 1. The second half of testes presented only a partial degeneration (Table 2, Fig. 3).)

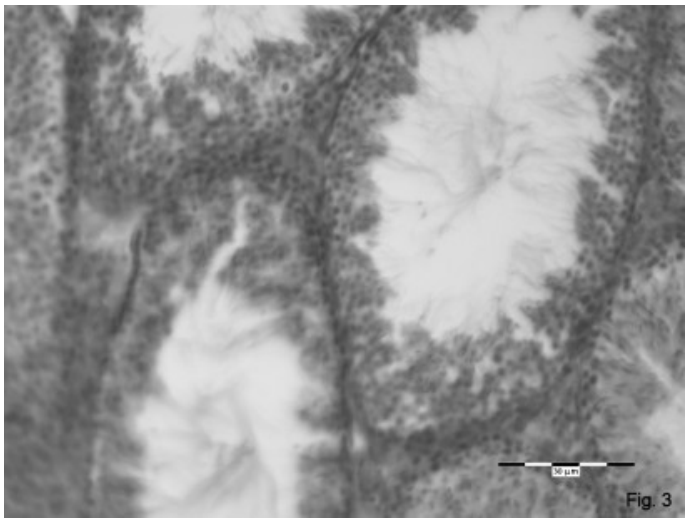

Fig. 3. **Effect of polyspermine conjugated to monomeric RNase A (PS-RNase A) injected five times intraperitoneally in mice.** Both testicles were partly degenerated. Secondary spermatocytes, spermatides and spermatozoa were lost from the seminiferous tubules of testicle tissue. Also spermatogonia and primary spermatocytes show initial degeneration. Interstitial tissue with Leydic cells is still present.

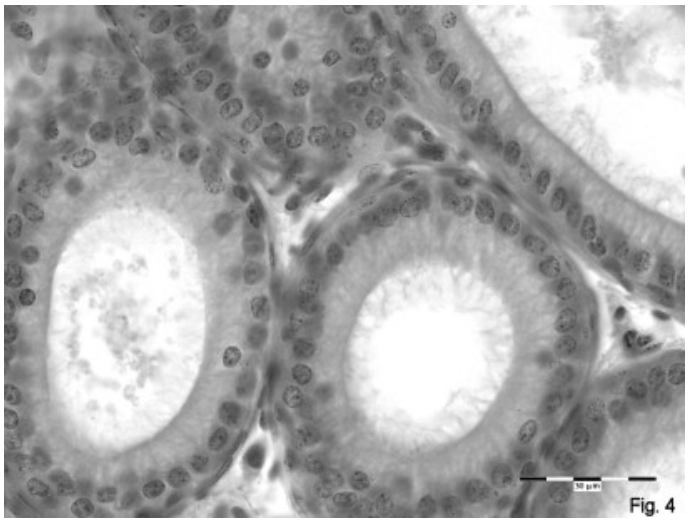

Fig. 4. **Effect of PS-dimeric RNase A on the heads of mouse epididymis of mice injected once in both testes.** Sperm was absent in epididymis tubules, which means that no spermatozoa were produced in the spermatogenic tissue. Epididymis tissue was not degenerated.

Table 1. **Aspermatogenic effect in mice after injection into their left testes of spermine, polyspermine, RNase A and polyspermine conjugated to RNase A (PS-RNase A).** Controls: mice and rats injected with PBS.

Substances injected	No. of mice/rats	Index mass of testes ± SEM		Width of aspermatogenic layers ± SEM in µm		Diameter of seminiferous tubules ± SEM in µm	
		Injected testes	Non-injected Testes	Injected testes	Non-injected testes	Injected testes	Non-injected testes
PBS	5	44 ± 3	45 ± 3	63 ± 5	64 ± 5	165 ± 5	164 ± 7
Spermine	5	53 ± 12	48 ± 6	51 ± 3^{+}	62 ± 8	158 ± 11	164 ± 6
Polyspermine	5	23 ± 5	49 ± 3	0^{+}	40 ± 3^{++}	123 ± 12^{+}	159 ± 9
RNase A	5	43 ± 5	45 ± 6	65 ± 4	67 ± 4	163 ± 6	160 ± 4
PS–RNase A	5	22 ± 4	49 ± 5	5 ± 6^{++}	41 ± 5^{++}	101 ± 13^{+}	158 ± 6
PBS (rats)	3	52 ± 9	50 ± 7	53 ± 8	56 ± 9	160 ± 5	162 ± 6

$^{+}$statistically significant

Table 2. **Spermatogenesis of mice and rats injected (inj.) and re-injected (re-inj.) into both testes with polyspermine (PS), PS conjugated to RNase A (PS-RNase A) and PS conjugated to dimeric RNase A (PS-Dimeric RNase A).**

Substances injected (no. of mice/rats)	No of testes		Index mass of testes (± SEM)		Width of spermatogenic layers ±SEM in μm		Diameter of seminiferou tubules ±SEM in μm	
	fully	partly	fully	partly	fully	partly	fully	partly
	degenerated		Degenerated		degenerated		degenerated	
Mice								
PBS – control (8)	no degeneration (see PBS – control in Table 1)							
PS inj. (13)	14	12	$22 \pm 12^{+}$	$44 \pm 7^{+}$	0^{+}	$18 \pm 10^{++}$	$118 \pm 11^{+}$	$137 \pm 14^{+}$
PS re-inj. (9)	15	3	$15 \pm 6^{+}$	$35 \pm 14^{+}$	0^{++}	$13 \pm 11^{+}$	$44 \pm 9^{++}$	$74 \pm 12^{+}$
PS-dimeric RNase A (8)	10	6	0^{++}	$10 \pm 11^{+}$	0^{++}	$28 \pm 3^{+}$	0^{++}	$138 \pm 18^{+}$
PS-RNase A re-inj. (3)	5	1	$5 \pm 3^{+}$	$15 \pm 0^{+}$	0^{++}	$16 \pm 0^{+}$	0^{++}	$51 \pm 0^{+}$
Rats								
PBS – control (3)	no degeneration (see PBS control in Table 1)							
PS re-inj. (3)	6	0	$10 \pm 7^{+}$	-	0^{+}	-	0^{+}	-

Symbols as in Table 1

Table 3. **Testosterone level in serum (μg/ml) of mice and rats injected and re-injected with polyspermine (PS), polyspermine conjugated to monomeric RNase A (PS-RNase A) and polyspermine conjugated to dimeric RNase A (PS-dimeric RNase A)**

Substances injected	No. of animals	Testosterone in serum μg/ml ± SEM
Mice		
PBS (control)	3	13.16 ± 6
PS (once injected)	6	$1.90 \pm 1.18^{+}$
PS (twice injected)	6	$0{,}42 \pm 0.06^{+}$
PS–RNase A (twice injected)	3	$0.22 \pm 0.09^{+}$
PS-dimeric RNase A (once injected)	7	$0.86 \pm 0.6^{+}$
Rats		
PBS (control)	3	2.91 ± 0.80
PS (three times injected)	3	$0.90 \pm 0.25^{+}$

Symbols as in Table 1

Table 4. **Aspermatogenic effect in mice after intraperitoneal injections of spermine, polyspermine, RNase A, and RNase A conjugated to polyspermine (PS-RNase A**). Control: mice injected with PBS.

Substances injected	No of mice	Index mass of testes ± SEM	Width of aspermatogenic layers ± SEM in μm	Diameter of seminiferous tubules ± SEM in μm
PBS (control)	5	96 ± 5	64 ± 5	165 ± 8
Spermine	5	97 ± 4	41 ± 5^{+}	146 ± 8^{+}
Polyspermine	4	90 ± 6	38 ± 6^{+}	151 ± 3^{+}
RNase A	5	93 ± 6	67 ± 12	162 ± 7
PS–RNase A	5	95 ± 6	37 ± 5^{+}	160 ± 14

Symbols as in Table 1

Table 5. **Titre of antibodies against PS-RNase A and its components in mice injected five times with 100 μg of each substance.**

Substances injected (μg)	No. of Mice	Titre of antibodies with PS–RNase A antigens			
		Spermine	Polyspermine	RNase A	PS - RNase
PBS (control)	5	0	0	0	0
Spermine	5	80−320	640−1.280	0−10	640−1.280
Polyspermine	4	320−640	2.560−5.120	80−160	1.280−2.560
RNase A	5	0	0	320−640	1.280−2.560
PS–RNase A	5	640−1.280	1.280−2.560	1.280−2.560	2.560−5.120

Table 6. **Body mass of mice injected one time into both testes and five times intraperitoneally with 100 μg of spermine, polyspermine, RNase A and polyspermine conjugated to RNase A (PS-RNase A)**

Mice injected intratesticularly				Mice injected intraperitoneally			
Substances	No. of mice	Body mass in g		Substances	No. of mice	Body mass in g	
		before injections	after injections			before injections	after injections
PBS (control)	5	25 ± 1	27 ± 2	PBS	5	23 ± 1	26 ± 1
Spermine	5	27 ± 1	27 ± 1	Spermine	5	28 ± 1	24 ± 2[+]
Polyspermine	5	26 ± 2	27 ± 1	Polyspermine	5	29 ± 2	28 ± 2
RNase A	5	27 ± 1	28 ± 1	RNase A	5	25 ± 1	27 ± 1
PS-RNase A	5	23 ± 2	24 ± 1	PS – RNase A	5	29 ± 2	30 ± 1

Symbols as in Table 1

This partial degeneration was characterized by a chaotic situation of spermatogenic cells in seminiferous tubules, cessation of spermatogenesis and destruction of tubular structures. Polyspermine conjugated to dimeric RNase A (PS-dimeric RNase A) injected only once into both testicles of 8 mice evoked cessation of hormonal secretion and a degeneration stronger than that observed after injection with PS or PS conjugated to monomeric RNase A (PS-RNase A) (Tables 2 and 3). The fully necrotized seminiferous tubules and the interstitial tissue between them were invaded by polymorphonucleates, monocytes, plasma cells and lymphocytes. In most tubules the spermatogenic cells were totally absent and substituted by fibroblasts and fibrocysts (Fig. 1).

Testes partly degenerated were re-injected with the same amount (100 μg) of PS or PS-RNase A about 30 days after the first injection, and sacrificed after further 30 days. The data relative to the degeneration of the testes of these re-injected mice are shown in Table 2. Of the eighteen testicles from nine mice re-injected with PS, fifteen were fully destroyed and three appeared to be only partly degenerated. A similar re-injection of three rats with 1400 μg into one testicle determined the total destruction of all 6 testicles (Table 2), and remarkably lowered the testosterone level in mice serum (Table 3). In three mice re-injected with PS-RNase A the spermatogenic epithelium was reduced, and the level of testosterone significantly decreased (Tables 2 and 3).

In all fully degenerated testicles the spermatogonia vacuolyzed and lost their mitotic activity. Sertoli and Leydic cells were destroyed together with spermatogenic cells (Fig. 1), and sperm was absent in mouse epididymis tubuli. No damage, however, could be observed in the epididymal tissue (Fig. 4). The testosterone level was also significantly low in all mice and rats in which both testes were degenerated (Table 3).

Aspermatogenic effects in mice injected intraperitoneally with some of the substances under study

Spermine, polyspermine and PS-RNase A evoked a significant reduction of the width of aspermatogenic layers, and only minimally influenced the diameter of the seminiferous tubules (Table 4). Actually, this parameter was practically unaffected by PS-RNase A.

Immunogenicity of polyspermine and polyspermine derivatives (production of antibodies)

The immnunogenicity of polyspermine or polyspermine conjugated to RNase A, expressed in titre of antibodies in mice serum, showed a little higher titre in comparison with spermine and RNase A in reactions with homologous antigens. However, this situation does not occur for antispermine antibodies against polyspermine or polyspermine conjugated to RNase A (PS-RNase) (Table 5). The antibodies against all polyspermine-RNase A derivatives did not influence the aspermatogenic effects (data not shown).

Body mass of mice injected intratesticularly and intraperitoneally with the substances under study

A single injection of polyspermine, PS-RNase A or PS-dimeric RNase A into both testes of mice did not determine loss of body mass. However, in mice injected five times intraperitoneally with spermine a significant loss of mass was observed. Polyspermine and polyspermine-RNase A derivatives were not so toxic to mice (Table 6).

DISCUSSION

Shortly after Wang and Moore's (1977) description of the bovine ribonuclease A derivative covalently linked to polyspermine, Mann and Lutwak-Mann (1981) described and discussed the effect of spermine and polyspermine on the reproductive function in male animals and man. Spermine administered in man usually displayed its inhibitory effects on cell metabolism through the spermine oxidation products formed by the action of diamine oxidases. The products of spermine oxidation strongly inhibit sperm fructolysis (Mann and Lutwak-Mann 1981). However, the precise role of spermine in male reproductive organs is still debated. For this reason, it is difficult to determine exactly the strong anti-testicle effects of this polyamine.

After finding the almost total degeneration of testes in mice that had been injected with polyspermine into their left testicles in order to study its aspermatogenetic action, we decided to inject both mice testes with polyspermine, and see what could happen. A study focused on the way to block in animals the reproductive and hormonal activity of both testes could be biologically meaningful. In the whole picture of testis degeneration the necrotic effect that polyspermine or polyspermine conjugated to monomeric or dimeric RNase A are able to evoke, as well as the rapid attack on testicle tissue by immunity cells, fibroblasts and fibrocytes, will end with the ligamentation of testicles and of all their functions. Polyspermine alone or conjugated to RNase A may be important for the biological removal of testicle tissue in some animal species, to suppress their aggressivity and smell of meat.

As for the antiproliferative activity of spermine and polyspermine, we found that both substances have a very strong anti-proliferative activity on various tumour cell lines (Pouckova et al., in press). We have also seen that polyspermine, after its

conjugation to the RNase A monomer and in particular to the RNase A dimer, displayed a higher anti-proliferative activity than polyspermine alone. This result was also proved in previous experiments performed with polyspermine linked to dimeric RNase A to test the immunosuppressive effect of these substances on MLC reaction testing. Besides the effective degeneration of spermatogenesis, the gradual and total destruction of Leydic cells and of their ability to synthesize testosterone can be useful for further studies in animals and plants.

As for the biological action of the polyspermine-RNase A derivatives reported in this work, and for its interpretation, it is worth pointing out that for oligomeric RNase A there is a parallelism between enzymatic activity towards double-stranded RNA and biological actions. This fact has been tentatively explained considering that both effects might be dependent on the positive charges present on the RNase A molecule (Libonati and Gotte 2004). On one hand, the increased number or density of positive charges of an RNase A oligomer would favor the splitting of dsRNA thanks to a previous destabilization of the secondary structure of the nucleic acid (Libonati et al. 1976, Libonati and Beintema 1977, Sorrentino and Libonati 1994, Libonati and Sorrentino 2001) in the terms proposed by Jensen and von Hippel (1976). The destabilization would indeed produce, within the double-helix, single-stranded RNA segments available, as such, to attack by the ribonuclease. On the other hand, the increased basicity of an RNase A could favor its adsorption to, and crossing through, the cell membrane, and, once inside the cell, enhance its enzymatic action towards all vital RNA secondary structures present in the cell, like double-helical regions of rRNA, tRNA and mRNA (Matoušek et al. 2003, Libonati and Gotte, 2004). Also transient but important DNA:RNA hybrid structures, like those occurring during the priming of DNA synthesis by RNA or during the synthesis of mRNA, as well as the polyadenylate tail of mRNA could become good targets (Libonati and Gotte 2004). These events, which have been hypothesized as the reason for the biological actions of oligomeric RNase A, could even more occur with the polyspermine-RNase A derivatives, and therefore justify the biological effects of these substances observed in the present work.

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