Volume 293, number 1,2, 219–223 FEBS 10427 November 1991

Magainins affect respiratory control, membrane potential and motility of hamster spermatozoa

Anthony de Waal[1], Ana Vaz Gomes[1,2], Anne Mensink[1], J. Anton Grootegoed[3] and Hans V. Westerhoff[1,2]

[1]*Section of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands,* [2]*E.C. Slater Institute for Biochemical Research, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands* and [3]*Department of Endocrinology and Reproduction, Erasmus University Rotterdam, P.O. Box 1738, NL-3000 DR Rotterdam, The Netherlands*

Received 5 August 1991; revised version received 30 September 1991

The hypothesis was tested that the magainin peptides, known to compromise bacterial and mitochondrial energetics, are highly active against spermatozoa. A mixture of magainin A and PGLa (1:1) caused a 50% reduction in motility of hamster spermatozoa at 4 μg/ml total peptide concentration. All motility was lost at 8 μg/ml. At this concentration, respiratory control was released and respiration in the presence of uncoupler was inhibited. Uptake of the lipophilic cation tetraphenyl phosphonium was largely abolished by addition of magainin A and PGLa at this concentration, indicative of a decrease in the mitochondrial membrane potential. Magainin A and PGLa showed synergism with respect to release of respiratory control.

Magainin; Spermicidal; Sperm motility; Spermatid; Energetics; Peptide-based defense

1. INTRODUCTION

Magainins are cationic peptides with antibacterial activity, synthesized by granular glands in the skin of *Xenopus laevis* [1,2]. The 23-residue peptides magainin 1 and magainin 2, which differ only in 2 positions, were found by Zasloff in a search for broad-spectrum antibiotic substances in *Xenopus* skin [3]. The peptides are identical to the previously described [4] peptides PGS (magainin 2) and PGS Gly-10;Lys-22 (magainin 1). Magainin 1 and 2 were found to arise from a common precursor [3,5]. The 21-residue peptide PGLa [6] from *X. laevis* skin is here considered a magainin on the basis of its structure and bactericidal activity [7].

Magainins are able to adopt an amphiphilic α-helix structure in a hydrophobic environment [8–10]. Such a structure is believed to be common in membrane-active peptides [11,12]. A positive correlation was found between α-helix content and antimicrobial activity in a series of magainin analogues [13].

The probable mechanism of action of magainins is interference with membrane-linked free-energy transduction [14–16], by making the energy conserving membrane permeable to certain ions [17–19]. Presumably, this involves a multimer of magainins, since there is positive cooperativity in the concentration dependence of the effects of magainin 2 amide [14,16], and synergism between magainin 2 amide and PGLa [20,21].

Correspondence address: H.V. Westerhoff, Section of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands. Fax: (31) (20) 6172625.

Physical disruption of the membrane is apparently not part of the mechanism at the relevant concentrations, since the effects on respiration and membrane potential are reversible in *Escherichia coli* and mitochondria, probably due to proteolysis of the peptides [14,16].

An interesting feature of the magainins is that they are not generally toxic to eukaryotic cells, e.g. erythrocytes are not lysed [3,13] and peripheral blood lymphocytes remain viable [22] at concentrations toxic to sensitive cells. This makes the magainins and their analogues candidates for use as selective antibiotics against bacteria [3,13,23], fungi [13], trypanosomes [24], or malaria parasites [25]. Also, certain tumor cells may be specifically killed by magainin analogues [22]. Whether specificity is accomplished by differences in membrane composition, -charge or -potential, or proteolytic defense of the target cells remains to be elucidated. Interaction with specific receptor proteins seems unlikely, since all-D-magainin analogues are fully active [26,27].

Our hypothesis that magainins are toxic through interference with membrane-linked free-energy transduction [14–16], predicts that eukaryotic cells that depend strongly on the latter, should be highly sensitive to the peptides. In the spermatid stage of spermatogenesis in rats and hamsters, when glycolysis is not capable of producing all the required ATP [28,29], lactate oxidation is the primary source of free energy, thus making mitochondrial metabolism of great importance [30], and these cells a suitable test system for our hypothesis. In contrast with spermatids, spermatozoa make use of glycolysis to make much of the ATP they need, but lactate

oxidation may well be an important source of free energy in several species, including the rat [30].

Here we report that magainins do compromise mitochondrial free-energy transduction in hamster testicular spermatids and epididymal spermatozoa.

2. EXPERIMENTAL

2.1. *Peptides*

PGLa (H_2N-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-$CONH_2$) was a synthetic peptide [6,7], identical to the natural compound. It was a gift from Drs M. Zasloff and G. Bialey. Magainin A was a gift from Dr H.-C. Chen, and synthesized as described [13]. The sequence (H_2N-Ala-Ile-Gly-Lys -Phe-Leu-His-Ala-Ala-Lys-Lys-Phe-Ala-Lys-Ala-Phe-Val- Ala-Glu-Ile-Met-Asn-Ser-$CONH_2$) differs from magainin 2 at position 1 (Gly to β-Ala), 8 (Ser to Ala), 13 and 18 (Gly to Ala), and 23 (Magainin A is carboxy-amidated). Magainin A has an increased α-helix propensity [13], and is relatively insensitive to proteolysis [16].

2.2. *Solutions*

The standard incubation buffer, designated PBS-L, consisted of 1.47 mM KH_2PO_4, 6.47 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, 12 mM DL-lactate (sodium salt, Sigma), and 1 mg/ml bovine serum albumin fraction V (A-4503, Sigma). The pH was 7.0. Magainins were stored at −20°C as stock solutions of 20 mg/ml in water. Working solutions of 1 mg/ml and 0.1 mg/ml in water were kept on ice. 2,4-dinitrophenol (Eastman-Kodak) was kept as a 200 mM stock solution in dimethyl sulphoxide at room temperature. Fresh working solutions were made by dilution in water to 10 mM, and kept at room temperature. FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) from Sigma was stored as a 10 mM stock solution in ethanol at −20°C. Fresh working solutions (0.20 mM in ethanol) were kept at room temperature. All other chemicals were of the highest purity available.

2.3. *Respiration measurements*

Respiration was measured by monitoring oxygen depletion in a closed, stirred 180-μl vessel equipped with a Clark-type oxygen electrode. Temperature was maintained at 32°C. The incubation buffer was PBS-L. Solubility of oxygen was taken as 0.217 mM O_2 [31]. Oxygen depletion in the absence of cells (J_B) (typically 0.1–0.3 nmol/min, depending on the actual oxygen concentration, and the permeability of the electrode's membrane) was subtracted from all values. Relative respiratory rate was defined as respiration in the presence of peptide (J_P) divided by the coupled respiration (J_V). To correct for electron-transfer-inhibiting activity of the magainins, the relative respiratory rate was normalized to the maximal rate of FCCP-uncoupled respiration in the absence (J_M) and the presence (J_{MP}) of magainin. The corrected relative respiratory rate was calculated as: $(J_P/J_V) \times (J_M/J_{MP})$. A series of magainin concentrations was measured three times, and for each series a new J_M was measured. J_V was recorded for 5 min, then magainin was added, and J_P recorded for 2–3 min (i.e. until a linear part of 1 min duration was recorded). Subsequently, FCCP was added to record J_{MP}. The value of the relative respiratory rate after addition of uncoupler measured for whole cells will be lower than the actual respiratory control (state 3 respiration divided by state 4 respiration) of the mitochondria, due to cellular ATP consumption, e.g. for motility.

2.4. *Apparent trans-membrane electric potential*

The apparent membrane potential of spermatozoa in PBS-L was evaluated by following the uptake of TPP^+ (tetraphenyl phosphonium ion) by added cells, in a stirred 2-ml vessel equipped with a TPP^+-selective electrode [32] to measure the extracellular concentration. The signal was recorded as electrode response (E) in mV, which was linear to the logarithm of the extra-cellular concentration of the probe. The electrode response was calibrated for each single run by repeated doubling of the TPP^+ concentration. Temperature was maintained at 30°C. The apparent membrane potential was estimated from the electrode potential relative to the potential in the presence of uncoupler. The extracellular concentration of probe after complete de-energization did not reach the level before addition of the cells. This may be attributed to dilution, and binding of the probe to membranes and proteins. Cells previously de-energized by excess of magainins did take up or bind some TPP^+. This binding or uptake was not reversed by subsequent addition of excess 2,4-dinitrophenol, and was similar to that after pretreatment of the cells for 90 min at 60°C. In the absence of cells, addition of magainins did not influence the electrode response (i.e. TPP^+ binding to magainins, or an electrode artifact, did not occur). It should be noted that the membrane potential measured here is apparent only, because (i) the cells harbour more than a single compartment and (ii) energization dependent TPP^+ binding was not corrected for.

2.5. *Isolation of cells*

2.5.1. Isolation of spermatozoa.

Golden hamsters (*Mesocricetus auratus*) of 10–16 weeks were anaesthetised with diethyl ether, and killed by cervical dislocation. The caudal epididymides were removed, and cut through several times. Spermatozoa were allowed to flow out from the tubule segments into 5–10 ml PBS-L for about 15 min. Epididymal tissue was removed by filtration through two layers of 60-μm nylon net. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added. The cells were stored in a 15-ml polyethylene disposable tube, with the lid on. All procedures, including storage, were at room temperature. Typical yield was 2.5–5 × 10^8 spermatozoa per hamster.

2.5.2. Isolation of spermatids.

Spermatids were isolated from decapsulated, collagenase- and trypsin treated testes from Golden hamsters by sedimentation at unit gravity through an albumin stabilised column (STA-PUT) essentially as described [28]. Yield was around 2 × 10^7 round spermatids and 2 × 10^7 elongating spermatids per hamster.

2.6. *Motility measurements*

A tenfold dilution of the sperm suspension as described above (3.9 × 10^7 cells/ml) was prepared every hour. Of this dilution, 10 μl were further diluted into 90 μl buffer containing the indicated concentration of magainin. After 5 min at room temperature, motility was evaluated in a Zeiss microscope with dark field optics, using a 10× objective. Of each incubation, a 20-μl sample was applied diagonally to a slide, and covered with a coverslip of 24 × 32 mm. The slide was scanned twice, at 1/4 and 3/4 of the width of the coverslip. Any trace of motility of individual cells was scored as positive. The counts of motile cells and immotile cells were summed to give one value for motility percentage, based upon 100–300 cells. Two or three slides were counted from each incubation, and incubations were performed at least in duplicate. The incubations were performed in random order.

3. RESULTS

3.1. *Motility*

Spermatozoa became immotile within 5 min of incubation with a combination of magainin A and PGLa: half-maximal inhibition of motility occurred at 3–4 μg/ml total peptide, whereas at 8 μg/ml all spermatozoa were immotile (Fig. 1).

The amount of magainin needed to abolish motility of spermatozoa was relatively independent of the sperm concentration during incubation (not shown).

3.2. *Respiratory control*

Incubation with magainins enhanced the respiratory rate (Fig. 2), suggestive of uncoupling of respiration from oxidative phosphorylation. At the same concentrations of magainins that caused increase of respiration, the uptake of oxygen in the presence of excess uncoupler (Fig. 2) decreased, indicative of inhibition of electron transfer through the respiratory chain and confirming that magainins cause uncoupling rather than stimulation of respiration.

There was a sigmoidal relation between magainin concentration and increase in respiration (Fig. 2), suggesting positive cooperativity between magainin monomers (or magainin A-PGLa oligomers), as also observed for mitochondria [14] and cytochrome oxidase liposomes [16].

Release of respiratory control and inhibition of uncoupler-stimulated respiration, both half-maximal around 4 μg/ml magainin, correlated with loss of motility of the spermatozoa (Fig. 1).

It is evident from Table I that there was synergism between the action of magainin A and PGLa with respect to the increase of respiration. Half the amount of magainin A that by itself caused little increase of respiration, caused a much larger increase of respiration when added together with a nearly non-perturbing amount of PGLa. No significant synergism was observed with respect to the inhibition by magainin A of electron transport in the presence of uncoupler.

Another feature of the action of a mixture of magainin on spermatozoa, was a lag-period of 1–2 min after administration of the magainins (not shown).

A combination of magainin A + PGLa at 5.5 μg/ml also caused release of respiratory control in round and elongating spermatids (Table II), and a mixture of germinal cells (not shown).

Table I

Synergistic action of magainins on respiration of hamster epididymal spermatozoa

Addition				
Mag. A (μg/ml)	PGLa (μg/ml)	FCCP (μM)	Relative respiration[a]	Inhibition-corrected relative respiration[a]
-	-	1	11.3±0.4	
3.75	3.75	-	3.6±0.1	7.8±0.8
3.75	3.75	1	5.5±0.4	
7.5	-	-	1.3±0.1	2.5±0.3
7.5	-	1	6.2±0.3	
-	7.5	-	1.1±0.1	1.2±0.1
-	7.5	1	10.7±0.3	
-	15	-	1.0±0.0	1.1±0.0
-	15	-	9.1±0.5	

Results are the mean and SEM of two experiments, each performed in triplicate. The number of cells per incubation was 3.9×10^6, and 3.4×10^6, respectively.

[a]All values are expressed relative to the respiration without additions

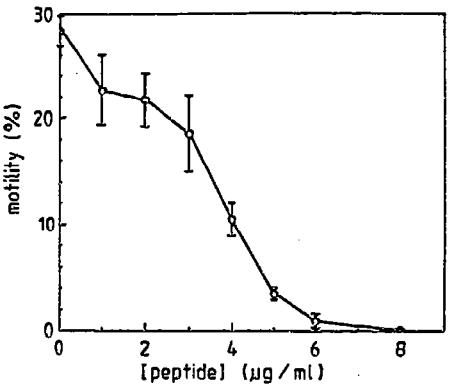

Fig. 1. Magainins compromise motility of hamster epididymal spermatozoa. Motility was scored as the percentage of cells that showed any motility after 5 min incubation at room temperature with equal amounts of magainin A + PGLa at the indicated total concentration. Values are given as mean ± SEM. Number of determinations was between 4 and 10

3.3. *Mitochondrial membrane potential*

We made use of the membrane permeant cation, tetraphenyl phosphonium, in conjunction with an ion-selective electrode, to monitor changes in trans-membrane electric potential. From Fig. 3 it follows that 2.5 μg/ml of a combination of magainin A + PGLa was capable of releasing about half of the actively accumulated TPP^+. If the TPP^+ release were solely due to a decrease in mitochondrial membrane potential, we would estimate that decrease to amount to approximately 40 mV. At 5 μg/ml of magainin A + PGLa the potential difference was further decreased by about 20 mV. Further addition of magainins to a final concentration of 15 μg/ml virtually abolished the membrane

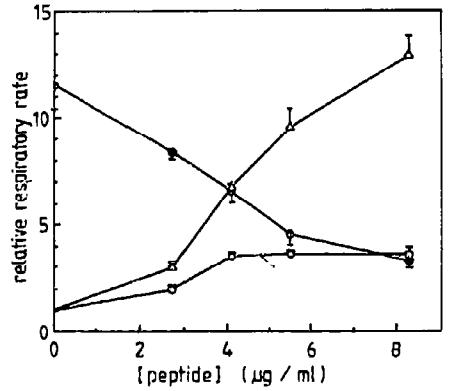

Fig. 2. Stimulation and inhibition of respiration of hamster epididymal spermatozoa by magainins. Spermatozoa were allowed to respire for 5 min in an oxygraph vessel with lactate as a substrate. A 1:1 mixture of magainin A and PGLa was added, and the highest value of subsequent respiration measured. After 2–3 min, 1 μM uncoupler (FCCP) was added, and the highest value of subsequent respiration measured. All values are expressed relative to the respiration without additions. Open circles: relative respiratory rate before addition of FCCP. Filled circles: relative respiratory rate after addition of FCCP. Triangles: relative respiratory rate corrected for inhibition of electron transfer. Error bars represent standard error of the mean (n=3)

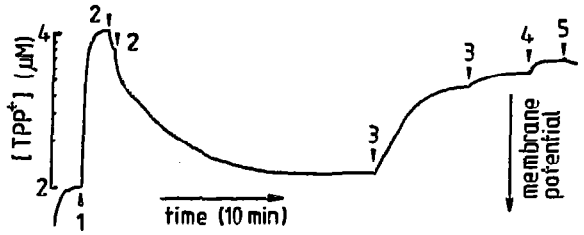

Fig. 3. Effect of magainins on apparent trans-membrane electric potential of hamster spermatozoa. Trans-membrane potential was monitored as the cellular ability to accumulate the membrane-permeable cation, tetraphenyl phosphonium (TPP^+). Downward deflection of the trace reflects a decrease in TPP^+ concentration in the extracellular space. Upward deflection is indicative of loss of actively accumulated TPP^+. Additions were: 1, from 2 to 4 μM TPP^+; 2, approx. 5×10^6 hamster epididymal spermatozoa in 0.2 ml; 3, 1.25 μg/ml magainin A + 1.25 μg/ml PGLa; 4, 5 μg/ml magainin A + 5 μg/ml PGLa; 5, 0.1 mM 2,4-dinitrophenol

potential, as judged from the absence of further release of TPP^+ when excess uncoupler was added.

4. DISCUSSION

Magainins have been shown to interfere with free-energy transduction in several systems: *Escherichia coli* [15,16,21], liposomes energized with cytochrome oxidase [16,21], and isolated rat liver mitochondria [14,15]. Interference takes place at the level of reduction of the proton-motive force. Judging from the release of respiratory control [15], dissipation of the proton-motive force (possibly by ion-channel formation [17–19]) is a major factor, although inhibition of respiration also occurs [14,15,21]. Here we show that magainins also affect free-energy transduction in testicular spermatids and epididymal spermatozoa (cf. [33]), at a concentration not different from concentrations toxic for other described target cells [3,7,13,19,23,33]. In spermatozoa, the release of respiratory control and loss of membrane potential seem to occur at the same concentration of magainins, at which the latter cause loss of motility. Hence, it is in line with the hypothesis that magainins interfere with free-energy transduction, to suppose that motility is lost through depletion of ATP from the flagellar dynein ATPase. However, it cannot be ruled out that other mechanisms underlie the loss of motility.

The experiments presented here are a first indication that the mechanism of action of magainins on one-compartment systems like bacteria, liposomes, or isolated mitochondria, is also relevant for certain eukaryotic cells. Our experiments demonstrate that mitochondrial free-energy metabolism is affected also in eukaryotic cells. Of course, we cannot exclude that the magainins have an additional effect on the plasma membrane (cf. [22]).

Although the effects on germ cells shown here should be taken into account in any future development of magainins as antibiotics, the possibility to introduce subtle modifications of their physicochemical properties makes the magainins a promising point of departure for the design of peptides with selective toxicity for prokaryotic or eukaryotic target cells.

Acknowledgements: The authors thank Dr Florence P. Hasseltine for helpful discussions, Jos Hoogerbrugge for isolation of spermatids, Dr Klaas Hellingwerf for help with the measurement of membrane potential, Dr Luitzen de Jong for making available the oxygen electrode, and Dr Jan Berden for critical reading of the manuscript and helpful suggestions. This investigation received financial support from the World Health Organization, Special Programme of Research, Development and Research Training in Human Reproduction (Project 88100). A.V.G. was supported by JNICT-Portugal, and H.V.W. by the Netherlands Organization for Scientific Research (NWO).

Table II

Effect of magainins on respiration of hamster testicular spermatids

Cells	Addition		Relative respiration[a]	Inhibition-corrected relative respiration[a]
	Mag. A/PGLa	Uncoupler		
Round spermatids	-	+	5.1 ± 0.7	
	+	-	1.7 ± 0.2	2.4 ± 0.1
	+	+	4.3 ± 0.5	
Elongating spermatids	-	+	4.5 ± 0.3	
	+	-	1.8 ± 0.3	2.6 ± 1.0
	+	+	2.8 ± 0.6	

Results are the mean and SEM of two experiments. In the first experiment, 4.7×10^6 round spermatids, and $4.9 \times$ elongating spermatids were incubated. Dinitrophenol was used as an uncoupler. In the second experiment, 2.8×10^6 round spermatids, and 2.2×10^6 elongating spermatids were incubated. FCCP was used as an uncoupler. The concentration of uncoupler was used that gave optimal respiration rate: 0.28 mM dinitrophenol or 1 μM FCCP. Magainin A and PGLa concentrations are 2.75 μg/ml each.

[a]All values are expressed relative to the respiration without additions

REFERENCES

[1] Berkowitz, B.A., Bevins, C.L. and Zasloff, M. (1990) Biochem. Pharmacol. 39, 625–629.
[2] Bevins, C.L. and Zasloff, M. (1990) Annu. Rev. Biochem. 59, 395–414.
[3] Zasloff, M. (1987) Proc. Natl. Acad. Sci. USA 84, 5449–5453.
[4] Giovanni, M.G., Poulter, L., Gibson, B.W. and Williams, D.H. (1987) Biochem. J. 243, 113–120.
[5] Terry, A.S., Poulter, L., Williams, D.H., Nutkins, J.C., Giovannini, M.G., Moore, C.H. and Gibson, B.W. (1988) J. Biol. Chem. 263, 5745–5751.
[6] Andreu, D., Aschauer, H., Kreil, G. and Merrifield, R.B. (1985) Eur. J. Biochem. 149, 531–535.
[7] Zasloff, M., Martin, B. and Chen, H.-C. (1988) Proc. Natl. Acad. Sci. USA 85, 910–913.
[8] Marion, D., Zasloff, M. and Bax, A. (1988) FEBS Lett. 227, 21–26.
[9] Matsuzaki, K., Harada, M., Handa, T., Funakoshi, S., Fujii, N., Yajima, H. and Miyajima, K. (1989) Biochim. Biophys. Acta 981, 130–134.
[10] Williams, R.W., Starman, R., Taylor, K.M., Gable, K., Beeler, T., Zasloff, M. and Covell, D. (1990) Biochemistry 29, 4490–4496.
[11] Kaiser, E.T. and Kézdy, F.J. (1984) Science 223, 249–255.
[12] Lee, S., Mihara, H., Aoyagi, H., Kato, T., Izumiya, N. and Yamasaki, N. (1986) Biochim. Biophys. Acta 862, 211–219.
[13] Chen, H.-C., Brown, J.H., Morell, J.L. and Huang, C.M. (1988) FEBS Lett. 236, 462–466.
[14] Westerhoff, H.V., Hendler, R.W., Zasloff, M. and Juretic, D. (1989) Biochim. Biophys. Acta 975, 361–369.
[15] Westerhoff, H.V., Juretic, D., Hendler, R.W. and Zasloff, M. (1989) Proc. Natl Acad. Sci. USA 86, 6597–6601.
[16] Juretic, D., Chen, H.-C., Brown, J.H., Morell, J.L., Hendler, R.W. and Westerhoff, H.V. (1989) FEBS Lett. 249, 219–223.
[17] Duclohier, H., Molle, G. and Spach, G. (1989) Biophys. J. 56, 1017–1021.
[18] Cruciani, R.A., Stanley, E.F., Zasloff, M., Lewis, D.L. and Barker, J.L. (1988) Biophys. J. 53, 9a.
[19] Cruciani, R.A., Barker, J.L., Raghunathan, G., Durell, S., Guy, R., Chen, H.-C. and Stanley, E. (1991) Biophys. J. 59, 372a.
[20] Juretic, D., Hendler, R.W., Zasloff, M. and Westerhoff, H.V. (1989) Biophys. J. 55, 572a.
[21] Juretic, D. (1990) Studia Biophysica 138, 79–86.
[22] Cruciani, R.A., Barker, J.L., Zasloff, M., Chen, H.-C. and Colamonici, O. (1991) Proc. Natl. Acad. Sci. USA 88, 3792–3796.
[23] Cuervo, J.H., Rodriguez, B. and Houghten, R.A. (1988) Peptide Res. 1, 81–86.
[24] Huang, C.M., Chen, H.-C. and Zierdt, C.H. (1990) Antimicrob. Agents Chemother. 34, 1824–1826.
[25] Gwadz, R.W., Kaslow, D., Lee, J.Y., Maloy, W.L., Zasloff, M. and Miller, L.H. (1989) Infect. Immun. 57, 2628–2633.
[26] Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G. and Merrifield, R.B. (1990) Proc. Natl. Acad. Sci. USA 87, 4761–4765.
[27] Bessalle, R., Kapitkovsky, A., Gorea, A., Shalit, I. and Fridkin, M. (1990) FEBS Lett. 274, 151–155.
[28] Grootegoed, J.A., Jansen, R. and van der Molen, H.J. (1986) J. Reprod. Fert. 77, 99–107.
[29] Mackenbach, P., Den Boer, P.J., de Jong, J.W. and Grootegoed, J. A. (1990) Reprod. Fert. Dev. 2, 145–152.
[30] Grootegoed, J.A. and Den Boer, P.J. (1989) in: Scientific basis of fertility regulation. Cellular and molecular events in spermiogenesis (D.W. Hamilton and G.M.H. Waites, eds) pp. 193–215, Cambridge University Press, Cambridge.
[31] Reynafarje, B., Costa, L.E., and Lehninger, A.L. (1985) Anal. Biochem. 145, 406–418.
[32] Kamo, N., Maratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membrane Biol. 49, 105–121.
[33] Edelstein, M.C., Gretz, J.E., Bauer, T.J., Fulgham, D.L., Alexander, N.J. and Archer, D.F. (1991) Fertil. Steril. 55, 647–649.