View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

CROATICA CHEMICA ACTA
CCACAA **81** (1) 169–176 (2008)

ISSN-0011-1643
CCA-3227
Original Scientific Paper

Hydrolysis of Extracellular Adenine Nucleotides by Human Spermatozoa: Regulatory Role of Ectonucleotidases in Sperm Function

Pavle Romac,[a] Tihana Žanić Grubišić,[b,*] Davor Ježek,[c] Marijana Vučić,[a] and Ognjen Čulić[d]

[a]*University Clinic for Diabetes, Endocrinology and Metabolic Diseases Vuk Vrhovac, School of Medicine, University of Zagreb, Dugi dol 4a, HR-10000 Zagreb, Croatia*

[b]*Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Ante Kovačića 1, HR-10000 Zagreb, Croatia*

[c]*Department of Histology and Embryology, School of Medicine, University of Zagreb, Šalata 3, HR-10000 Zagreb, Croatia*

[d]*GlaxoSmithKline – Research Institute Ltd., Prilaz baruna Filipovića 29, HR-10000 Zagreb, Croatia*

RECEIVED FEBRUARY 28, 2007; REVISED SEPTEMBER 18, 2007; ACCEPTED OCTOBER 5, 2007

Evidence is presented for the existence of ectonucleotidases on the membrane of intact human spermatozoa. Enzymes hydrolyze extracellular ATP, ADP and AMP and hence could be described as ecto-NTPDase and ecto-5'-nucleotidase. Suramin, Cibacron 3GA and DIDS, well known ecto-NTPDase inhibitors, caused inhibition of the observed enzyme activity. Enzymatic hydrolysis of ATP, ADP and AMP follows simple Michaelis-Menten kinetics with similar enzyme affinity for all three substrates (K_m for ATP, ADP and AMP were (0.395 ± 0.027), (0.401 ± 0.031), (0.517 ± 0.038) mmole dm^{-3}, respectively). Influence of extracellular ATP, AMP, adenosine and cAMP on the parameters of sperm velocity and acrosome reaction was also examined. In normozoospermic samples, ATP and cAMP induced an increase in the amplitude of lateral head displacement and number of acrosomally reacted cells, but not in sperm velocity. However, adenosine and AMP enhanced sperm velocity, without influencing the acrosome reaction. These results show that ATP and adenosine regulate sperm motility parameters in different ways.

Keywords
spermatozoa
ecto-NTPDase
5'-nucleotidase
capacitation

INTRODUCTION

Mammalian spermatozoa are not able to fertilize oocyte until they complete capacitation within the female reproductive tract. Capacitation occurs over hours and proceeds through a number of processes that regulate alterations in flagellar motility, capacity to fuse with oocyte, and initiation of the acrosome reaction (AR). Biochemical characteristics involve ultrastructural changes, modification of membrane lipid composition, changes in sperm surface proteins distribution, and in enzyme activities, increased permeability to ions, phosphorylation of proteins on tyrosine, serine and threonine residues, or double phosphorylation of Thr-Glu-Tyr motif on specific fibrous proteins.[1–4] Phosphorylation appears to be regulated through activation of mitogen activated protein kinases

* Author to whom correspondence should be addressed. (E-mail: tihana_zanic@yahoo.co.uk)

(MAPK), extracellular signal regulated kinases (ERK), receptor type protein tirosine kinases (PTK), protein kinase A (PKA) and protein kinase C (PKC).[5] The endpoint of *in vitro/in vivo* capacitation is activation of sperm surface receptors for binding to the specific complementary ligands on zona pellucida, fusion of the sperm plasma membrane with the underlying outer acrosomal membrane and exocytosis of acrosomal contents. Adenosine (Ado) and Ca^{2+} are considered to be modulators of this process.[6] However, it has been shown that human sperm capacitation induced by physiological or pharmacological agents is regulated through very different signal transduction pathways, which might impose some limitations on the clinical application of experimental data.[5]

Sperm acrosome reaction (AR) is an exocytotic process by which lytic enzymes released from sperm acrosome digest zona pellucida so that spermatozoa can reach and fertilize the oocyte.[7] AR takes place after fusion between the acrosome and the overlying plasma membrane and involves Ca^{2+} influx, actin polimerization, a rise in intracellular pH, activation of phospholipases, kinases and G-proteins. AR occurs within minutes, cannot be reversed once it is induced and can be triggered *in vitro* by different inducers such as progesteron, calcium ionofores, lysophosphatidylcholine and ATP.[8,9,10] While protein phosphorylation is regarded as the hallmark of capacitation, the role of protein kinases and phosphatases has not been well documented in human AR. However, Ligouri *et al.* have shown activation of PKA, PKC, PKT and ERK signalling pathways in AR in humans.[2]

It has been demonstrated that ATP_e is a rapid and potent activator of the acrosome exocytosis in bovine and in human spermatozoa.[11,12] Activation is connected with stimulation of purinergic P_2 receptors.[13,14] On the other hand, extracellular adenosine (Ado_e), the nucleoside produced by adenine nucleotide dephosphorylation, can produce changes in sperm motility parameters through activation of Ado receptors.[15] Mammalian spermatozoa possess two kinds of Ado receptors, stimulatory-A_2 and inhibitory-A_1.[16] Ado_e is able to elicit biphasic responses in mammalian spermatozoa: stimulatory-A_2 in uncapacitated cells, and inhibitory-A_1 in capacitated cells.[17] A_2 receptors are responsible for Ado-mediated enhancement of sperm motility, cAMP production, and protein phosphorylation.[7] Capacitation and AR are triggered by diverse effectors and involve different signal transduction pathways, but adenine nucleotides appear to be involved in both processes. Therefore, correct balance of the extracellular concentration of ATP, ADP and Ado might be of great importance for the fertilization capacity of a sperm cell.

Ectonucleotidases are enzymes capable of hydrolyzing extracellular nucleotides; thus they effectively control the level of particular nucleotides at the cell surface. Members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family are principal ectonucleotidases. NTPDases hydrolyze both extracellular nucleoside-triphosphates and nucleoside-diphosphates and they vary in catalytic properties. Four out of eight members of this family, namely NTPDase1, NTPDase2, NTPDase3 and NTPDase8, appear relevant to the control of P_2 receptor signalling since they are located at the surface of the plasma membrane and have the active site oriented towards extracellular space.[18] The most relevant substrates for these enzymes, ATP, ADP, UTP and UDP, initiate a number of cellular responses *via* selective activation of the ionotropic P_2X and metabotropic P_2Y receptors.[19] Complete dephosphorylation of nucleotides is achieved by ecto-5'nucleotidase.[20] Since ecto-5'NT hydrolyzes AMP and liberates Ado, it has been suggested that this enzyme acts as a supplier of Ado. The presence of ecto-nucleotidases has been found on the membranes of bovine and equine spermatozoa, but has not been recorded on human spermatozoa.[21]

The objective of the present study was to investigate whether enzymes capable of degrading extracellular ATP, ADP, and AMP were present on the membrane of human spermatozoa. In order to examine the physiological relevance of the changes in extracellular concentration of nucleotides, we have tested the influence of ATP_e, AMP_e, $dbcAMP_e$ and Ado_e on the human sperm motility parameters and the acrosome reaction.

EXPERIMENTAL

Materials

Ado, ATP, ADP, AMP, dibutyryl cyclic AMP (dbcAMP), HEPES, oligomycin, ouabain, levamisol, TBA (tetrabutylammonium hydrogensulfate) and sodium dodecyl sulphate were all purchased from Sigma (St. Luis, MO, USA). KH_2PO4 and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Spectrophotometric kit for inorganic phosphate detection was purchased from Trace (Perth, Australia) Chromatographic column was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Patients and Samples

– Sperm Preparation and Analysis

Human semen with normal sperm characteristics according to WHO criteria (volume ≈ 2 cm^{-3}, concentration >(20×10^6) cells cm^{-3}, >50 % general motility and >25 % progressive motility) was collected by masturbation from healthy donors after 3 days of sexual abstinence (n = 40).[22] All experiments were performed on the specimens that remained after routine clinical analysis. After complete liquefaction at 37 °C, motile spermatozoa were selected by centrifugation at 800 g for 10 min through a two-step Percoll density gradient (90 % vol. ratio and 40 % vol. ratio, Sperm Prep). The bottom layer containing the motile sperm fraction was washed twice at 800 g for 10 min with 2 cm^{-3} of universal IVF me-

dium (Medicult, Yillinge, Danemark), suspended at the concentration 10 × 10^6 cells cm^{-3} and samples were distributed into 200 µl aliquots. The washing procedure was repeated and the presence of leukocytes in the sperm suspension was detected by peroxidase staining. Only pure sperm suspensions were used for the experiments.

For the computer assisted motility assessment aliquots were incubated in Universal IVF medium (Medicult, Yillinge, Denmark) with or without tested nucleotides and nucleosides at 37 °C and 5 % CO_2, as stated in the legend to Tables.

– Evaluation of Sperm Count and Motility

Sperm count and motility were assessed after video recording of ≈ 10 fields for every sample, in the Macler chamber at 37 °C. For each sample at least 400 spermatozoa were tracked for 1 s and analysed with Hamilton-Thorne IVOS 10 CASA system (Beverly, MA, USA). Motility parameters included curvilinear (VCL), straight-line (VSL) and average path (VAP) velocities and amplitude of lateral head displacement (ALH). Sperm cell viability and membrane integrity were assessed by performing hypoosmotic swelling test (HOS).[23]

– Evaluation of Acrosome Reaction

Sperm suspensions were incubated for 2 hours in a humidified atmosphere (5 % CO_2) in IVF medium and challenged with ATP, AMP, Ado or dbcAMP prior to evaluation of acrosome reaction (Spermac staining method developed for human spermatozoa). Briefly, sperm samples (100 µl aliquots), previously prepared on density gradient, were smeared on a glass slide and allowed to air dry for 5 min at the room temperature, and then fixed in the formalin solution provided in the Spermac kit (Stain Enterprises, Ondersteport, South Africa). Each slide with fixed sperm was stained as described before.[24] For each sperm smear the fraction of sperm with intact acrosomes was calculated. Morphologically abnormal sperm and sperm that lacked red colour counter stain (inadequately stained) were not counted.

Biochemical Characterisation of the Membrane Bound ATP and ADP Hydrolysing Activity

For the measurements of the membrane bound ATP and ADP hydrolysing activity the integrity of sperm cell membrane was confirmed by the hypoosmotic swelling test and only the samples with hypoosmotic swelling over 90 % were used.[23] Selected samples were washed twice with the reaction buffer (50 mmol dm^{-3} HEPES-Tris buffer (pH = 7.3), 5 mmol dm^{-3} glucose, 5 mmol dm^{-3} $MgCl_2$, 38 mmol dm^{-3} NaCl, supplemented with inhibitors of other non-specific phosphatases: 1 mmol dm^{-3} ouabain, 5 µg cm^{-3} oligomycin, 2 mmol dm^{-3} levamisole) and 3.5 % bovine serum albumine. The ATP and ADP hydrolysing activities were assayed by measuring concentration of ADP and AMP produced by enzymatic hydrolysis of the substrates, ATP and ADP, respectively. Calculation of kinetic parameters was performed using initial substrate concentration between 0.125–2.00 mmol dm^{-3}.

– Enzyme Assay for the ATP and ADP Hydrolysis

Assays was performed in 1000 µl of reaction medium containing reaction buffer and appropriate substrate (2.0 mmol dm^{-3} ATP or 2.0 mmol dm^{-3} ADP, respectively) and 2 × 10^6 spermatozoa. Reaction medium, without the sample, was preincubated for 20 min at 37 °C and reaction was initiated by addition of sperm sample. Controls contained equivalent amount of buffer. After 60 min of incubation 100 µl of reaction mixture was analysed for the adenine nucleotide content. ATP, ADP, and AMP concentrations were determined by high pressure liquid chromatography (HPLC).

HPLC was performed with a »Aekta Purifier« system equipped with autosampler A-900 (Pharmacia, Uppsala, Sweden) using Nova-Pack C_{18}, 3.9 × 300 mm, 4 µm particle size column (Waters Milford, MA, USA). For the determination of ATP, ADP and AMP concentration columns were equilibrated with the mobile phase (buffer A: KH_2PO_4, 15 mmol dm^{-3} with 10 mmol dm^{-3} TBA, pH = 5.4). A stepwise gradient, at a constant flow rate (0.8 cm^{-3} /min), was designed with a solution B (70 % methanol), as follows: step 1 (10.6 min, B: 0 to 40 %), step 2 (7.24 min, B: 40 %), step 3 (8.7 min, B: 40 to 0 %). Elution peaks were monitored at 254 nm, and data were processed by appropriate software (»Unicorn«, Pharmacia, Uppsala, Sweden).

Biochemical Characterisation of the Membrane Bound AMP Hydrolysing Activity

Assay was performed in 1000 µl of reaction medium containing reaction buffer (50 mmol dm^{-3} HEPES-Tris buffer (pH = 7.3), 5 mmol dm^{-3} glucose, 5 mmol dm^{-3} $MgCl_2$, 38 mmol dm^{-3} NaCl, supplemented with inhibitors of other non-specific phosphatases: 1 mmol dm^{-3} ouabain, 5 µg cm^{-3} oligomycin, 20 mmol dm^{-3} sodium tartarate, 2 mmol dm^{-3} levamisole), 2.0 mmol dm^{-3} AMP as substrate and 2 × 10^6 spermatozoa. Reaction mixture, without the sample was preincubated for 5 min at 37 °C and reaction was initiated by addition 200 µl of sample. Controls contained equivalent amount of buffer. After 60 min of incubation at 37 °C reaction was terminated by adding 100 µl of 10 % sodium dodecylsulphate. The AMP hydrolysing activity was determined by measuring concentration of inorganic phosphate produced during hydrolysis of AMP, as previously described.[25]

Statistical Analysis

Data were evaluated using GraphPadPrism software version 3.0 (GraphPad Software, Inc., San Diego, USA). Means were compared using standard *t*-test, and $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Membrane Bound ATP, ADP and AMP Hydrolyzing Activity of Human Spermatozoa

The rates of catabolism of extracellular ATP, ADP and AMP were initially studied by incubating 2 mmol dm^{-3}

TABLE I. Membrane bound ATP, ADP and AMP hydrolysing activity of human spermatozoa [(a)]

Substrate	Activity
	nmol min^{-1} per 10^6 spermatozoa
ATP	20.57 ± 6.66
ADP	18.22 ± 3.32
AMP	362.26 ± 44.99

[(a)] Concentration of ATP, ADP and AMP were 2.0 mmol dm^{-3}. Samples were incubated 60 min in humidified atmosphere at 37 °C. Results are expressed as mean ± SD (n = 10).

TABLE II. Kinetic constants for the membrane bound ATP, ADP and AMP hydrolysing activity of human spermatozoa [(a)]

Substrate	K_m	V_{max}
	mmol dm^{-3}	nmol min^{-1} per 10^6 spermatozoa
ATP	0.395 ± 0.027	21.18 ± 1.94
ADP	0.401 ± 0.031	21.85 ± 2.43
AMP	0.517 ± 0.038	430.1 ± 23.61

[(a)] Enzyme activity of human spermatozoa was measured in the substrate concentration range of 0.125–2.00 mmol dm^{-3} for ATP, ADP and AMP, respectively. Kinetic constants were calculated from Eadee-Hofstee plots of the experimental data. Results are presented as mean ± SD (n = 10), for each experimental point.

substrate with intact human spermatozoa. ATP_e, ADP_e and AMP_e were all metabolized in the presence of intact human spermatozoa. Figure 1 shows a typical chromatogram for the evaluation of ATPase activity. The ATP_e concentration significantly decreased, and concentrations of ADP_e and, to a smaller extent, of AMP_e increased after 60 min of incubation with the sample. A similar diagram with AMP_e as the main product was obtained when 2 mmol dm^{-3} ADP_e was used as a substrate (results not shown).

The observed ectonucleotidase activities were linear up to 2×10^7 spermatozoa and up to 100 min of incubation. We selected samples of 2×10^6 spermatozoa and 60 min as the incubation time for further experiments. The enzymatic activity was dependent on the presence of divalent cations – Ca^{2+} and Mg^{2+}. In the presence of 2 mmol dm^{-3} EDTA and 2 mmol dm^{-3} EGTA to chelate divalent cations, no activity could be detected (data not shown) Since the activity with Mg^{2+} was higher than with Ca^{2+}, 2 mmol dm^{-3} Mg^{2+} was always present in the reaction mixture. Normal sperm samples hydrolyzed ATP_e, ADP_e and AMP_e with an average activity, as shown in Table I.

Kinetic constants for ATP_e, ADP_e and AMP_e hydrolysis were derived from the experiments where enzyme activity was measured as the function of the substrate concentration in a range of 0.125 to 2.0 mmol dm^{-3}. Enzymatic hydrolysis of ATP_e, ADP_e and AMP_e followed simple Michaelis-Menten kinetics (Table II). Kinetic constants

TABLE III. Effect of inhibitors on sperm ATP hydrolysing activity [(a)]

Inhibitor	Concentration	Activity / %
Ouabain	3 mmol dm^{-3}	96 ± 5
Olygomicin	5 mg dm^{-3}	98 ± 4
Na-tartarate	20 mmol dm^{-3}	95 ± 3
Levamisole	3 mmol dm^{-3}	99 ± 6
Suramine	50 μmol dm^{-3}	47 ± 6
Cibacron 3GA	40 μmol dm^{-3}	34 ± 4
DIDS	40 μmol dm^{-3}	71 ± 5

[(a)] Enzyme activity was measured with 2.5 mmol dm^{-3} ATP as substrate. Samples were incubated 60 min in humidified atmosphere at 37 °C. Control activity (with no inhibitor added) was (21.9 ± 4.32) nmol min^{-1} ADP per 10^6 spermatozoa. Results are expressed with respect to control activity that was taken as 100 (mean ± SD; n = 4).

were calculated from the Eady-Hofstee plot of experimental data. Similar enzyme affinities were determined for all the three substrates tested. It was found that K_m for ATP_e, ADP_e and AMP_e were (0.395 ± 0.027), (0.401 ± 0.031) and (0.517 ± 0.038) mmol dm^{-3}, respectively. However, maximal velocities V_{max} for ATP_e, ADP_e and AMP_e were different: (21.186 ± 1.94), (21.85 ± 2.43) and (430.1 ± 23.6) nmol min^{-1} per 10^6 spermatozoa, respectively. The V_{max}/K_m ratio reveals that AMP_e hydrolysis showed a seventeen-fold higher efficiency than the hydrolysis of ATP_e, or ADP_e.

ATP_e hydrolysis was further characterized by testing the susceptibility to various inhibitors. A series of inhibitors were added to the reaction mixture and the rate of ATP_e hydrolysis was monitored. Ouabain, olygomycin, sodium tartarate and levamisole had no effect on the ATP_e hydrolyzing activity, indicating that the enzyme measured was not Na^+/K^+-ATPase, mitochondrial H^+-ATPase, acid or alkaline phosphatase, respectively. On the other hand, suramin, Cibacron 3GA and DIDS caused inhibition of enzyme activity, as shown in Table III. Inhibition produced by Cibacron 3GA was higher compared to the inhibition of suramin and DIDS. The observed adenine nucleotide hydrolyzing activity found on the membrane of intact spermatozoa could directly produce significant changes in the concentrations of ATP_e, ADP_e, AMP_e and Ado_e on the extracellular side of the sperm-cell membrane, and thus induce changes in sperm motility and the acrosome reaction.

Effects of Extracellular ATP, AMP, Adenosine and cAMP on Sperm Motility and Acrosome Reaction

In the subsequent series of experiments, sperm samples were incubated with 2 mol dm^{-3} ATP_e, AMP_e or Ado_e for 120 min. For each treatment, mobility analysis was performed with an IVOS CASA instrument and the acrosome reaction was evaluated by Spermac staining, as

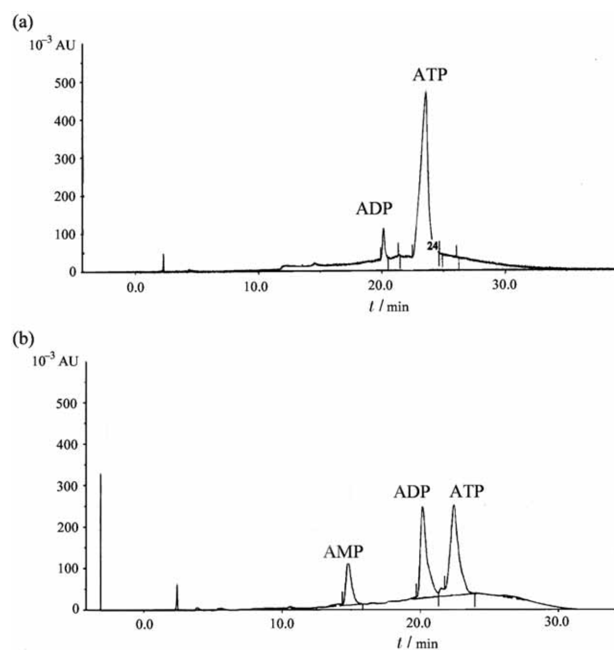

Figure 1. Chromatographic diagram of adenine nucleotides content before (a) and after (b) incubation with intact human spermatozoa. Sperm samples (2×10^6 intact spermatozoa) were incubated in 1000 μl reaction mixture (50 mmol dm^{-3} HEPES-Tris buffer (pH = 7.3), 5 mmol dm^{-3} glucose, 5 mmol dm^{-3} $MgCl_2$, 38 mmol dm^{-3} NaCl, 1 mmol dm^{-3} ouabain, 5 mg ml^{-1} oligomycin, 2 mmol dm^{-3} levamisole and 3.5 % bovine serum albumin) with 2 mmol dm^{-3} ATP at 37 °C for 60 min. Reaction mixture was preincubated for 20 min, and hydrolysis was initiated by addition of sample. At the indicated time point 100 μl of the reaction mixture was withdrawn, filtered through RC 0.2 μm Corning filters and analysed for the adenine nucleotide content by use of HPLC. Adenine nucleotide content is expressed in relative units.

already described. We assumed that ATP_e and Ado_e might stimulate respective receptors and influence the physiological response. Incubation with 0.1 mmol dm^{-3} $dbcAMP_e$ was applied in order to produce activation of the PKA signalling pathway. Since $dbcAMP_e$ is able to penetrate an intact cell membrane, it could be used for intracellular activation of PKA and phosphorylation of the target proteins.[26] It has been well established that PKA plays a central role in sperm capacitation, motility and acrosome reaction, so strong activation of the system was expected in this experiment.[1,27]

The results showed that ATP_e stimulated a rise in ALH values and produced an increase in sperm bending, typical of hyperactivation (Table IV). However, ATP_e was not efficient in increasing sperm velocity. We found that ATP_e stimulated nearly twice as many spermatozoa for the AR compared to the control non-stimulated samples (Table IV). Ado_e and AMP_e behaved in a similar manner and produced an increase in curvilinear sperm velocity (VCL), with no increase in VAP or VSL velocity parameters, nor in the number fraction of acrosome reacted spermatozoa (Table IV). In the experiments where $dbcAMP_e$ was applied, a clear stimulation of ALH, VCL and AR was observed, as expected. These results show that extracellular ATP and Ado have a different impact on spermatozoa and that enzymes involved in the regulation of Ado_e and ATP_e concentration might play a significant physiological role in the function of sperm cells.

DISCUSSION

Hydrolysis of extracellular ATP, ADP and AMP in the presence of intact human spermatozoa was demonstrated in the present study. The enzyme activity was membrane associated. All experiments were performed with spermatozoa that had been tested for membrane integrity; thus only enzymes located on the outer side of sperm membrane could react with the substrates in the medium. The rate of ATP_e and ADP_e hydrolysis was divalent cation dependent. Inhibitors of V-type and P-type ATPases, and of acid and alkaline phosphatases did not inhibit the observed adenine nucleotide hydrolysis (Table II). However, ATP_e and ADP_e hydrolyzing activity of human spermatozoa were both inhibited by Cibacron 3GA, suramin and DIDS, well-known inhibitors of ecto-NTPDases.[28,29] Ac-

TABLE IV. Influence of adenosine AMP, dbcAMP and ATP on sperm motility parameters: average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and acrosome reaction (AR) in normozoospermic samples [(a)]

	VAP / μm s^{-1}	VSL / μm s^{-1}	VCL / μm s^{-1}	ALH / μm	AR / %
Control	83.5 ± 6.3	64.2 ± 3.9	139.6 ± 7.8	4.8 ± 0.36	12.44 ± 2.8
Adenosine	86.4 ± 4.5	69.5 ± 3.8	(162.4 ± 7.3)[(b)]	4.7 ± 0.32	11.73 ± 2.4
AMP	85.6 ± 4.9	67.8 ± 4.1	(161.6 ± 8.4)[(b)]	4.6 ± 0.35	11.31 ± 2.2
dbcAMP	85.1 ± 7.3	67.6 ± 3.9	(158.4 ± 6.8)[(b)]	(5.9 ± 0.41)[(b)]	(23.19 ± 4.5)[(b)]
ATP	82.2 ± 7.8	63.6 ± 4.6	141.3 ± 5.9	(5.7 ± 0.27)[(b)]	(26.92 ± 4.2)[(b)]

[(a)] Samples were incubated for 120 min at 37 °C in Universal IVF medium containing either 2 mmol dm^{-3} adenosine, 2 mmol dm^{-3} AMP, 0.1 mmol dm^{-3} dbcAMP, 2 mmol dm^{-3} ATP, or 2 mmol dm^{-3} ATP. Results are expressed as mean ± SD of 40 individual samples and counted 200 sperm per sample.

[(b)] Significant differences *versus* control, non-treated samples ($P < 0.05$).

cording to the criteria defined by Plesner, the enzymes described might be considered as ectonucleotidases.[30] Membrane localization with the active site oriented towards the extracellular side, sensitivity to specific ecto-ATPase inhibitors and capacity to hydrolyze ATP_e and ADP_e suggested that the observed enzyme activity might belong to a family of ecto-NTPDases.[18,31] The enzymes of human spermatozoa had properties generally attributed to ecto-NTPDases seen in other specie.[32–35] Some characteristics of the observed enzyme activity suggest that it could be attributed to ecto-NTPDase 1, because it equally well hydrolyzed ATP_e and ADP_e. During the hydrolysis of ATP_e we could determine ADP_e and AMP_e as products (Figure 1). The results show that ATP_e and ADP_e hydrolysis followed simple Michaelis-Menten kinetics with similar enzyme affinities for both substrates.

The hydrolysis of extracellular AMP was also detected on the membrane of human spermatozoa (Tables I and II). This enzyme exhibited similar affinity for the substrate as it was found for ecto-ATPDase. However, V_{max} of ecto-5'NT was much higher than in ATP_e and ADP_e hydrolysis (Table II). The significant difference between V_{max} for the AMP_e and V_{max} for the ATP_e/ADP_e hydrolysis most probably reflects the higher amount of ecto-5'NT. The V_{max}/K_m ratio revealed that ecto-5'NT had much higher efficiency than ecto-ATPase or ecto-ADPase. Most probably, this enzyme is similar to the previously described ecto-5'NT.[21,37] Konrad *et al.* clearly showed the presence of ecto-5'NT and production of Ado_e in the male genital tract.[20] Based on their results, they suggested that spermal ecto-5'NT might be involved in the interactions between spermatozoa and seminal fluid and could be considered a scavenger of cell membrane components and a supplier of Ado.

The present study has shown that ATP_e and Ado_e stimulate sperm motility in different ways. ATP_e produced hyperactivation and increased the number of spermatozoa showing spontaneous AR, but ATP_e did not increase sperm velocity (Table IV). On the other hand, Ado_e, the product of the complete ATP_e hydrolytic cascade, stimulated the sperm velocity parameter VCL (Table IV). Similar results were obtained by other authors.[17] In our previous work, we showed that Ado_e could increase the activity of dyneine ATPase of asthenozoospermic samples. Ado_e could not stimulate sperm hyperactivation, since there was no change in ALH values. Ado_e did not stimulate sperm cells for AR.[38] This is in agreement with the results of Fraser and Adeoya-Osiguwa.[17] The effects of AMP_e were very similar to those of Ado_e. Since it has been shown that AMP_e could not bind to Ado receptors, it is plausible to assume that the effects result from Ado_e that was produced from AMP_e in the reaction catalyzed by ecto-5' nucleotidase. Extracellular adenosine production by concerted action of ecto-ATPDase and ecto-5'NT from ATP and ADP as substrates was already shown for the male genital tract, brush border enzymes in rat kidney, and was different in guinea pigs.[20,25,39] When we used $dbcAMP_e$, the cell membrane penetrable analogue of cAMP, we observed changes in the progressive motility parameter VCL similar to the Ado_e effects. On the other hand, $dbcAMP_e$ changed ALH movement and stimulated AR, as seen in ATP_e stimulation. Most probably, the concentration of $dbcAMP_e$ and the time of exposure applied in our experiments were high and long enough (100 μmol dm^{-3} and 120 min) to stimulate both effects.

It has been shown before that ATP_e has profound effects on sperm cells in a broad range of concentrations (50 μmol dm^{-3} to 5 mmol $dm^{-3)}$ and that the effects can be rapidly observed. The concentration of 5 mmol dm^{-3} was not deleterious to the spermatozoa. Some other cells might show apoptosis even at a concentration as low as 300 μmol dm^{-3}.[12] The results of this work show that 2 mmol dm^{-3} ATP_e produced changes in the type of sperm movement (ALH), but did not influence the progressive motility parameters VAP, VSL, VCL. The progressive movement changed into a star-like movement typical of hyperactivated cells. ATP_e stimulated spermatozoa for the AR (Table IV).

Findings relating to the regulatory role of Ado_e and ATP_e in spermatozoa *in vitro* raise the question whether there is any relevance of these effects *in vivo*. Urner and Sakkes have shown that approximately 50 % spermatozoa remain non-phosphorylated during capacitation in humans.[2] This subpopulation of spermatozoa from the ejaculate most probably exhibit different susceptibility to phosphorylation. Defects in phosphorylation have been implicated in certain types of infertility in males, since increased proportion of spermatozoa that are unable to undergo phosphorylation and achieve capacitation would significantly reduce fertilization capacity, especially in asthenozoospermic samples.

In the female genital tract (where increases in ATP concentration at the time of ovulation have been recorded), ATP_e hydrolysis could be performed by the action of sperm ecto-NTPDase. It appears essential that ATP_e stimulation does not occur prematurely, so the extracellular ATP needs to be eliminated in the vicinity of P_2 receptors. In the seminal plasma, high activity of acid and alkaline phosphatase efficiently hydrolyzes ATP_e, producing Ado_e which stimulates the first phase of capacitation. In this phase, motility should be increased but hyperactivation and acrosome reaction need to be inhibited. This could be achieved through the action of Ado_e. In the later phase, hyperactivation and acrosome reaction are required for successful fertilization, which should be supported by the ATP_e stimulation.

In conclusion, the results of this study show that extracellular ATP and adenosine influence the sperm function. These effects could be regulated through the controlled hydrolysis of ATP_e, ADP_e and AMP_e by the ectoNTPDases

described in this paper. With more adenosine that promotes motility and less ATP available, spermatozoa are maintained in the condition characteristic of the early stages of capacitation. We expect that pharmacological manipulation that might result in an increase of Ado_e concentration could be considered in the IVF program, especially in the cases where phosphorylation defects might be expected.

Acknowledgements. – This work has been supported by the Ministry of Science, Education and Sports of the Republic of Croatia (Project 006311).

REFERENCES

1. F. Urner and D. Sakkes, *Reproduction* **125** (2003) 17-26.
2. L. Ligouri, E. de Lamirande, A. Minelli, and C. Gagnon, *Mol. Hum. Reprod.* **11** (2005) 211–221.
3. J. Zhang, J. Wu, R. Huo, Y. Mao, Y. Lu, X. Guo, J. Liu, Z. Zhou, X. Huang, and J. Sha, *Mol. Hum. Reprod.* **9** (2007) 633–639.
4. S. Shivaji, V. Kumar, K. Mitra, and K. N. Jha, *Soc. Reprod. Fertil.* **63** (2007) Suppl., 295–312.
5. J. Thundathil, E. de Lamirande, and C. Gagnon, *Mol. Hum. Reprod.* **8** (2002) 811–816.
6. C. Allegruci, L. Ligouri, I. Mezzasoma, and A. Minelli, *Mol. Genet. Metab.* **71** (2000) 381–386.
7. P. E. Visconti, H. Galantino Homer, G. D. Moore, J. L. Bailey, X. Ning, M. Fornes, and G. Kopf, *J. Androl.* **19** (1998) 242–248.
8. C. N. Tomes, *Soc. Reprod. Fertil.* **65** (2007) Suppl., 275–291.
9. P. B. Seshagiri, D. Mariappa, and R. H. Aladakatti, *Soc. Reprod. Fertil.* **63** (2007) Suppl., 313–325.
10. I. M. Aparicio, M. J. Bragado, M. C. Gil, M. Garcia-Herreros, L. Gonzalez-Fernandez, J. A. Tapia, and L. J. Garcia-Marin, *Mol. Reprod. Dev.* **74** (2007) 1035–42.
11. A. Luria, S. Rubinstein, Y. Lax, and H. Breitbart, *Biol. Reprod.* **66** (2002) 429–437.
12. C. Foresta, M. Rossato, P. Chiozzi, and F. Di Virgilio, *Am. J. Physiol.* **270** (1996) C1709–C1714.
13. G. Burnstock, *Ciba Found. Symp.* **198** (1992) 112–128.
14. F. Kukulski, J. Sevigny, and M. Komoszynski, *Brain Res.* **1030** (2004) 49–56.
15. J. Linden, *Annu. Rev. Pharmacol. Toxicol.* **41** (2001) 775–787.
16. A. Minelli, C. Allegrucci, P. Piomboni, R. Mannucci, C. Lluis, and R. Franco, *J. Histochem. Cytochem.* **48** (2000) 1163–1171.
17. L. R. Fraser and S. Adeoya Osiguwa, *Mol. Reprod. Dev.* **53** (1999) 459–471.
18. H. Zimmermann, in: M. P. Abbracchio and M. Williams (Eds.), *Handbook of Experimental Pharmacology. Purinergic and Pyrimidinergic Signalling*, *Ectonucleotidases*, Springer Verlag, Heidelberg, 2001, pp. 209–250.
19. M. Picher, J. Sevigny, P. D'Orleans-Juste, and A. R. Beaudoin, *Biochem. Pharmacol.* **11** (1996) 1453–1460.
20. L. Konrad, P. Schiemann, H. Renneberg, G. Wennemuth, C. Fini, and G. Aumuller, *Biol. Reprod.* **59** (1998) 190–196.
21. A. Minelli, M. Moroni, D. Trinari, and I. Mezzasoma, *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* **117** (1997) 531–534.
22. World Health Organization, *Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction*, 3rd ed., Press Syndicate of the University of Cambridge, Cambridge, 1993, pp. 56–58.
23. R. S. Jeyendran, H. H. Van der Ven, Perez-Pelaez, and M. B. G. Crabo, L. J. D. Zaneveld, *J. Reprod. Fertil.* **70** (1984) 219–228.
24. P. J. Chan, J. U. Corselli, J. D. Jacobson, W. C. Patton, and A. King, *Fertil. Steril.* **72** (1999) 124–128.
25. O. Culic, I. Sabolic, and T. Zanic-Grubisic, *Biochem. Biophys. Acta.* **1030** (1990) 143–151.
26. P. R. Manna, Y. Jo, and D. M. Stocco, *J. Endocrinol*. **193** (2007) 53–63.
27. D. A. Harrison, D. W. Carr, and S. Meizel, *Biol. Reprod.* **62** (2000) 811–820.
28. F. P. Gendron, O. Benrezzak, B. W. Krugh, Q. Kong, G. A. Weisman, and A. R. Beaudoin, *Curr. Drug Targets* **3** (2002) 229–245.
29. L. Savegnago, C. W. Noguiera, R. Fachinetto, and J. B. T. Rocha, *Cell. Biol. Int.* **29** (2005) 559–566.
30. L. Plesner, *Int. Rev. Cytol.* **158** (1995) 141–214.
31. T. L. Kirley, P. A. Crawford, and T. M. Smith, *Purinergic Signal.* **2** (2006) 379–389.
32. M. Fausther, J. Lecka, F. Kukulski, S. A. Lévesque, J. Pelletier, H. Zimmermann, J. A. Dranoff, and J. Sévigny, *Am. J. Physiol., Gastrointest. Liver Physiol*. **292** (2007) G785–G795.
33. P. Ghildyal, and, D. Palani *J. Physiol.* **575** (2006) 469–480.
34. J. G. Stout, and T. L. Kirley, *J. Biochem. Biophys. Methods* **29** (1994) 61–75.
35. G. C. Majumder, *Biochem. J.* **195** (1981) 103–110.
36. P. J. Schiemann, M. Aliante, G. Wennemuth, C. Fini, and G. Aumuller, *Histochemistry* **101** (1994) 253–262.
39. P. Romac, T. Žanić-Grubišić, O. Čulić, and P. Cvitković, *Hum. Reprod.* **9** (1994) 1474–1478.
40. S. T. Mihaylova-Todorova, L. D. Todorov, and D. P. Westfall, *J. Pharmacol. Exp. Ther.* **302** (2002) 992–1001.

SAŽETAK

Hidroliza ekstracelularnih adeninskih nukleotida djelovanjem enzima humanih spermija: uloga ektonukleotidaza u regulaciji funkcije spermija

Pavle Romac, Tihana Žanić Grubišić, Davor Ježek, Marijana Vučić i Ognjen Čulić

U radu su prikazani rezultati koji potvrđuju aktivnost ektonukleotidaza na membranama ljudskih spermija. Ovi enzimi kataliziraju hidrolizu izvanstaničnog ATP-a, ADP-a i AMP-a i mogu se opisati kao ekto-NTPD-aze i ekto-5'-nukleotidaze. Enzimska aktivnost je podložna inhibiciji s, do sada dobro opisanim, inhibitorima ekto-NTPD-aza kao što su suramin, cibacron 3GA i DIDS. Enzimska hidroliza ATP-a, ADP-a i AMP-a slijedi Michaelis-Mentenovu kinetiku sa sličnim afinitetom za sva 3 supstrata (K_m za ATP, ADP i AMP bile su (0,395 ± 0,027), (0,401 ± 0,031), (0,517 ± 0,038) mmol dm^{-3}). Također je ispitan utjecaj izvanstaničnih ATP-a, AMP-a, adenozina i dbcAMP-a na parametre koji opisuju brzinu kretanja spermija i akrosomsku reakciju. U uzorcima normozoospermije ATP i dbcAMP su izazvali porast amplitude lateralnog pokretanja glave spermija i broja spermija koji su pokazivali akrosomsku reakciju, ali nisu izazvali povećanje brzine kretanja. Adenozin i AMP su, naprotiv, izazvali povećanje brzine kretanja, a bez utjecaja na akrosomsku reakciju. Ovi rezultati pokazuju da ATP i adenozin reguliraju parametre pokretljivosti spermija na različit način.