CHARACTERIZATION BY CONVENTIONAL FLOW CYTOMETRIC PARAMETERS AND APOPTOTIC MARKERS OF GOAT SPERM UNDER THE ACTION OF ACROSINIC NATURAL INHIBITORS

Alexandrina RUGINA[1], Luminita TAKACS[1], Catalin IORDACHEL[1], Stela ZAMFIRESCU[3], Maria CALOIANU[2], Daniela BRATOSIN[1*]

[1] National Institute of Biological Science Research & Development (INSB), Bucharest, Romania
[2] University of Bucharest, Faculty of Biology, Romania
[3] University " Ovidius" of Constanta, Faculty of Biology, Romania

*** Correspondence:** Daniela BRATOSIN, National Institute for Biological Science Research and Development, Spl. Independentei nº 296, Bucharest, Romania, Tel/Fax: 40.21.2200881, E-mail: bratosind@yahoo.com
Received: march 2008; Published: may 2008

ABSTRACT. During fertilization, sperm utilizes a specific serine-dependent proteolytic enzyme, known as acrosin, to aid in penetrating the zona pellucida and which is also involved in the dispersal of the acrosomal matrix. Recently, flow cytometry has been extensively used to evaluate several different characteristics of sperm. The objectives of this study were to evaluate the action of acrosinic natural inhibitors on the sperm viability and on a possible cell death, correlated with the determination of acrosomal reaction. The sperm cells exposed to diferents concentrations of acrosinic natural inhibitors were evaluated by flow cytometry after 30 min. incubation endpoints. We investigated cellular viability (calcein-AM assay) and apoptosis (light scattering properties of sperma, study of cell death using annexin-V-FITC and propidium iodide double - labelling, mitochondrial function assessed using $DIOC_6$ and caspase-3 activity determination. Our results indicate that the percentage of viable cells and MFI of calcein fluorescence, on the one hand, and the percentage of apoptotic or necrotic cells, on the other hand, provide a rapid and ideally adapted methodology for screening and evaluation of acrosinic natural inhibitors action in sperm cells preservation and for assessing the functional status of cells.

Keywords: goat spermatozoa, *Allium* extracts, apoptosis, cell viability, flow cytometry

INTRODUCTION

The sperm acrosome reaction (AR) is a calcium dependent exocytolitic event required for mammalian fertilization. The acrosome reaction facilitates the penetration of zona pellucida by spermatozoa and the subsequent fusion of the sperm plasma membrane with the oocyte's oolemma. Ejaculated spermatozoa require a series of preparatory changes in order to undergo the acrosome reaction. These physiological changes are collectively termed capacitation and involve primary membrane modification [Gadella et al., 1995]. Capacitation occures either *in vivo,* during the passage of spermatozoa through the femele genital tract, or *in vitro,* during incubation of washed spermatozoa under proper conditions. *In vitro* studies have shown that the acrosome reaction can be initiated in preincubated spermatozoa either spontaneously or by various physiological (zona pellucida glycoproteins and follicular fluid) inducers [Yanagimachi 1994, Bruker and Lipford 1995]. However, a biologically efective acrosome reaction will depend on the presence and the activity of physiological inducers as well as the capacity of spermatozoa to respond to these inducers.

According to the current dogma, capacitated and acrosome-intact spermatozoa initiate binding to the zona pellucida [Yanagimachi R., 1994]. Although the zona pellucida is considered as the prime physiological inducer of acrosome reaction.

In mammals, binding of the spermatozoon to the zona pellucida, an extracellular layer surrounding the oocyte, initiates the acrosome reaction. When the sperm comes in contact with ZP3 in the zona pellucida, this reaction takes place, during which actin polymerizes to form the acrosomal process; also, the acrosomal vesicle fuses with the plasma membrane of the egg cell, and enzymes are released from the sperm that digest the zona pellucida and give access to the egg. This process releases the enzyme hyaluronidase, which digests the matrix of hyaluronic acid in the vestments surrounding the oocyte. Fusion between the sperm and oocyte plasma membranes follows, allowing the entry of the sperm nucleus, mitochondria, centriole and flagellum into the oocyte. Acrosin is present in mammalian spermatozoa [Morton, 1977] and has been identified as a specific serine-dependent proteolytic enzyme, with a substrate specificity and an inhibitor sensitivity similar to that of trypsin [Schleuning et al., 1976]. This is an important proteolytic enzyme capable of hydrolyzing the zona pellucida (ZP) in oocyte, and playing a vital role in the process of fertilization.

A sperm cell consists of several membrane compartments (i.e. plasma, acrosomal and mitochondrial membrane) and cell competancy

requires that each of these membrane compartments is intact.

Flow cytometry offers the possibility of analyzing thousand of cells in a very short time (<1 min) with precision and without the extensive preparation necessary for dried sperm smears. Several procedures have been used in vieu (a) to differentiate live and dead cells by the ability of an intact plasma membrane to prevent stain entry into the cell, (b) to evaluate the mitochondrial functions, and (c) to assess cell viability.

The *Allium* genus includes approximately 500 species, the most widely used of which are onions (*Allium cepa*), red onion, (*Allium cepa rubra*), garlic (*Allium sativum*), leeks (*Allium porrum*), chives (*Allium schoenoprasum*), and shallots (*Allium ascalonicum*). Anion (*Allium sp.)* have attracted particular attention of modern medicine because of its widespread health use around the world, and the cherished belief that it helps in maintaining good health warding off illnesses and providing more vigor. To date, many favorable experimental and clinical effects of garlic and onion preparations have been reported. These biological responses have been largely attributed to the following therapeutic effects: i) reduction of risk factors for cardiovascular diseases and cancer, ii) stimulation of immune function, iii) enhanced detoxification of foreign compound, iv) hepatoprotection, v) antimicrobial effect and vi) antioxidant effect [Schleuning et al., 1976]. Recently was reported the sperm immobilization activity of aqueous homogenate, of garlic and onion extracts [Chakrabarti et al., 2003].

The objectives of this study were to evaluate the action of acrosinic natural inhibitors obtained from bulb of *Allium cepa* and *Allium cepa rubra* on the sperm viability and on a possible cell death induction.

MATERIALS AND METHODS

Chemicals

Fluorescein-conjugated Annexin-V (Annexin-V-FITC), propidium iodide and HEPES buffer were purchased from Pharmingen (San Diego, USA). PhiPhiLux G1D2 (OncoImmunin) was furnished by Calbiochem (La Jolla, USA), and 3,3'-dihexyloxacarbocyanine – iodide (DiOC6) from Molecular Probes (Interchim, Montluçon, France). N-α- benzoyl-DL-arginine-p-nitroanilide (BAPNA) was obtained from Sigma.

Goat semen collection and cells treatment

Goat semen was kindly supplied by PALAS Constanta. The sperm cells were exposed to three different concentrations of onion extracts from *Allium cepa* (bulb) and *Allium cepa rubra* (bulb) containing acrosinic natural inhibitors which were identified by spectrophotometrical methods with N-α- benzoyl-DL-arginine-p-nitroanilide (BAPNA). The onion extract from *Allium cepa rubra* has an inhibitory activity of 36,257±0,0530 UI/min at 0.5g/ml protein concentration and 35,240±0.035 UI/min for the same protein concentration for *Allium cepa* extract. The sperm cells were incubated with three extract concentrations: 0.5g/ml, 0.25g/ml and 0.12g/ml for 30 min. After incubation the cells were evaluated by flow cytometry for cell death analysis and cell viability determination.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACScan cytometer (San Jose, USA) using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Cell death assays

Cell death was determined using an annexin-V-FITC/propidium iodide apoptosis kit. Annexin-V is a Ca2+- dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells, since viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. Cells that stain positive for annexin-V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both annexin-V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both annexin-V-FITC and PI are alive and not undergoing measurable apoptosis.

The spermatozoa were washed with PBS buffer pH 7.4 and the cells (2x105) were resuspended in 100 µl of 1x binding HEPES buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). 10 µl propidium iodide and 5 µl annexin-V-FITC were added and incubated for 30 min at room temperature in the dark. After adding 400 µl of 1X binding buffer, the suspension was analysed in the flow cytometer and gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (PI fluorescence). The light scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. All studies were performed at least three times with three replicates each time.

Mitochondrial membrane potential measurement

Changes of mitochondria membrane potential were assessed using the cationic lipophilic fluorochrome DiOC6. Cells were incubated at 37°C for 30 min in the presence of DiOC6 (40 µM in PBS buffer pH 7.4). All studies were performed at least three times with three replicates each time.

Caspase-3 activity determination

Caspase-3 activity was assessed using the fluorogenic peptide substrate PhiPhiLux G1D2 (green fluorescence). 106 cells were labeled with 50 µl of PhiPhiLux G1D2 (10 µM) substrate solutions following the manufacture's protocol Oncoimmunin. The suspensions were incubated in 5% CO2 at 37°C for 1 h. Cleavage of the peptide linker of PhiPhiLux G1D2 (sequence DEVD) results in fluorescence

detectable on the FL1 channel. After 2 washings with flow cytometer buffer, the cells were analyzed by flow cytometry. Experiments were carried out in triplicate.

Flow cytometric assay of cell viability using calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin et al., [Bratosin et al., 2005]. The membrane-permeable dye calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 µM in PBS buffer pH 7.4. The cells (4 x105 in 200 µl PBS buffer) were incubated with 10 µl calcein-AM working solution (final concentration in calcein-AM : 5 µM) for 45 min at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

RESULTS AND DISCUSSION

Light scattering properties of chondrocytes in osteoarthritis

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides informations about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents [Darzynkiewicz et al., 1997].

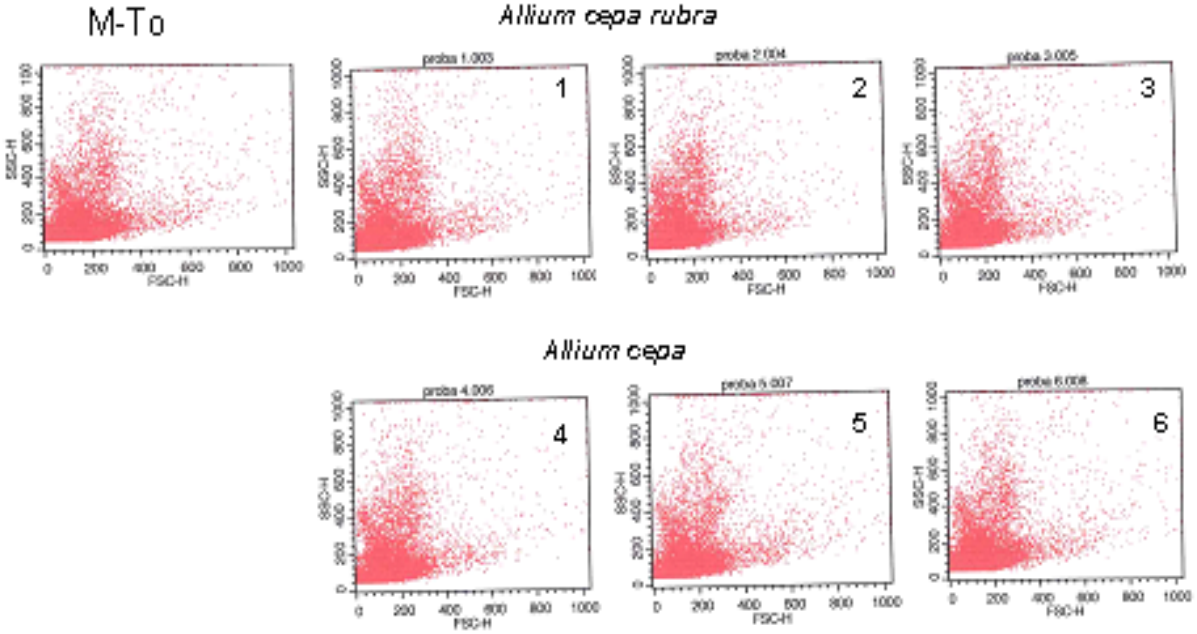

Fig.1 Comparative flow cytometric analysis of morphological cytograms of Goat semen untreated (M-To) and treated with three different concentrations (0,5g/mL, 0,25g/mL, 0,12g/mL) of onion extracts from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6) containing acrosinic natural inhibitors. Dot-plot analysis FSC/SSC of cells shape changes. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness). Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

Figure 1 shows that the morphological characteristics of sperm cells exposed to the three different concentrations of onion extracts from *Allium cepa (bulb)* and *Allium cepa rubra (bulb)* containing acrosinic natural inhibitors are not associated with cell shrinkage (decreased forward scatter and increased side scatter), one of the characteristic features of apoptosis, and the cytograms are practically identical to the control sample M-To.

Study of Goat semen death by Annexin-V-FITC / propidium iodide double – labelling

Goat semen untreated and treated with the three different concentrations of acrosinic natural inhibitors from *Allium cepa rubra* and *Allium cepa* were

analyzed by flow cytometry for phosphatidylserine (PS) exposure (Annexin-V labelling) and membrane permeabilization (propidium iodide labelling). Phosphatidylserine residues are exposed in the external leaflet of cell membrane early during the process of apoptosis whereas the uptake of propidium iodide indicates a disrupted cellular membrane integrity generally observed during late apoptosis and cell necrosis. Figure 2 shows comparative flow cytometric analyses of Annexin-V-FITC / propidium iodide double-stained of Goat semen untreated and treated with three different concentrations of onion extracts from *Allium cepa rubra* and *Allium cepa* containing acrosinic natural inhibitors.

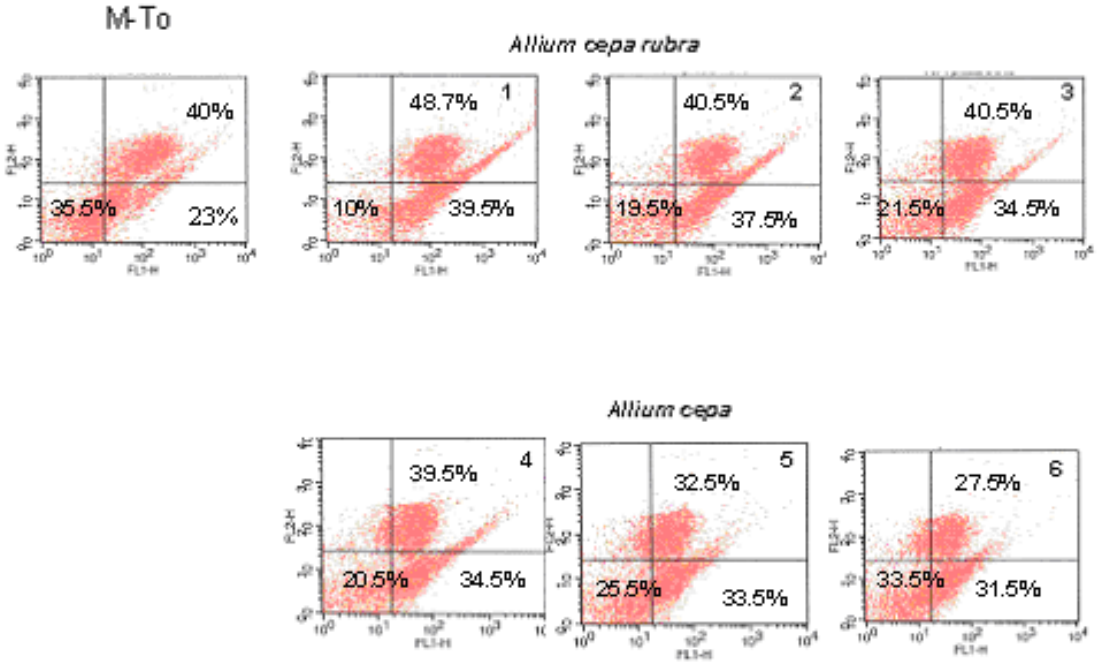

Fig. 2 Comparative flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained of Goat semen untreated (M-T0) and treated with three different concentrations (0,5g/mL , 0,25g/mL, 0,12g/mL) of onion extracts from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6) containing acrosinic natural inhibitors.. Abscissae: log scale green fluorescence intensity of annexine-V-FITC (FL-1). Ordinates: log scale red fluorescence intensity of propidium iodide (FL-2). Low left quadrant: viable cells (annexin-V and propidium iodide negative cells); low right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); upper right quadrant: dead cells (annexin-V and propidium iodide positive cells). % refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

For Goat semen treated with *Allium cepa rubra* the number of living cells (Annexin$^-$/PI$^-$) decreased drastically and proportionally with the concentration of Alium extract, from 35% ("normal" semen) to 21.5%, 19.5% and 10% respectively. The proportions of Annexin-V positive and PI negative cells (Annexin$^+$/PI$^-$) were significantly increased (39.5%) for semen treated contrary to 23 % for "normal" semen. The proportions of Annexin-V positive and PI positive cells (Annexin$^+$/PI$^+$) indicating late apoptosis or death cells was approximately 40% for "normal" semen and 49% for treated cells. In the samples treated with *Allium cepa* the death was also induced proportionally with the concentration of extract.

Mitochondrial membrane potential measurement

It is well admitted that mitochondria is a central sensor in nucleated apoptotic cells. This apoptosis displayed a loss in mitochondrial membrane potential ($\Delta\Psi m$). We then assessed whether changes in $\Delta\Psi m$ characterize death of Goat semen. As depicted in figure 3, the sperm cells incubated with the different concentrations of onion extracts from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6) alone showed a significant decrease of mitochondrial membrane potential (Peak M2) in comparison to untreated erythrocytes (M-To) and revealed that death of sperm cells is associated with an early depolarization of the mitochondria membrane potential.

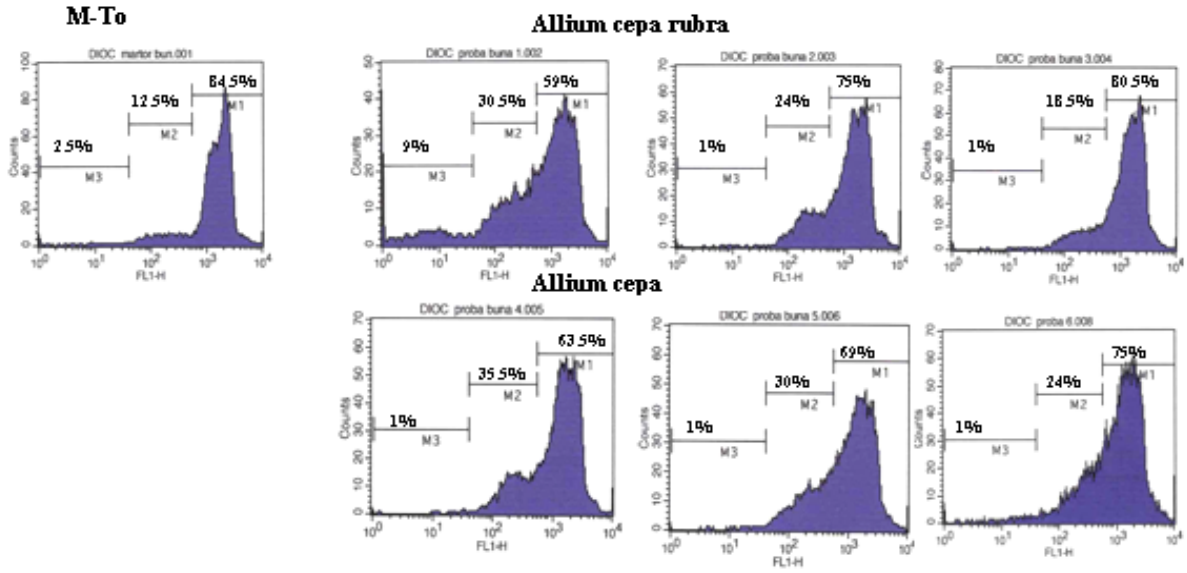

Fig. 3 Flow cytometry of mitochondrial transmembrane potential ($\Delta\Psi m$) of Goat semen untreated (M-T0) and treated with three different concentrations (0,5g/mL , 0,25g/mL, 0,12g/mL) of onion extracts from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6) containing acrosinic natural inhibitors. Abcissae: $DiOC_6$ fluorescence (FL1 mode). Ordinates: cell number. Numbering refers to the cell percentage of each cell population. Number of counted cells: 10 000

Caspase-3 activity determination

Current knowledge of apoptosis has put caspase activation at the center of the apoptotic machinery. Multiple apoptosis-inducing factors directly or indirectly activate the cascade of caspases which are the executioners of cell death sentence by catalyzing protein degradation so leading to cell death. In this viewpoint, we focused our experiment to the caspase-3, known as the executioner caspase of the so-called "nuclear apoptosis". Caspase-3 activation was assessed by flow cytometric analysis using the fluorogenic caspase-3 substrat PhiPhiLux G_1D_2. As shown in figure 4, a significant decrease of the fluorogenic substrat cleavage was detected in the sperm cells incubated with the different concentrations of onion extracts from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6). Interestingly, caspase-3-like activity was greatly decreased in sperm cells pretreated with onion extracts, indicating that onion extract also contain cistein inhibitors.

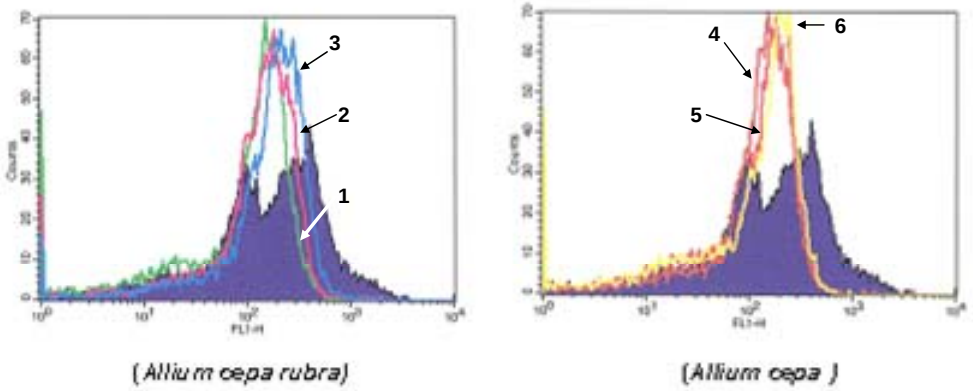

Fig. 4 Comparative flow cytometric histogram analysis of caspase-3 activity in Goat semen untreated (M-T0) and treated with three different concentrations (0,5g/mL , 0,25g/mL, 0,12g/mL) of onion extracts from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6) using PhiPhiLux G_1D_2 fluorescent substrat. Data are expressed as mean of fluorescence intensity (MFI) Abscissae: log scale green fluorescence intensity of PhiPhiLux G_1D_2 (FL-1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

Cell viability calcein-AM assay of Goat semen

We recently devised a new flow cytometric assay for the measurement of cell viability using calcein-AM [8]. The assay is based on the use of acetoxymethyl ester of calcein (calcein-AM), a fluorescein derivative and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes.

Application of this assay for analysing Goat semen untreated (M-To) and treated with three different concentrations of acrosinic natural inhibitors from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6) showed that two regions could be clearly and unambiguously defined: the region of fluorescent cells with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells (region M1) and the region of

nonfluorescent dead cells with damaged cell membranes (region M2).

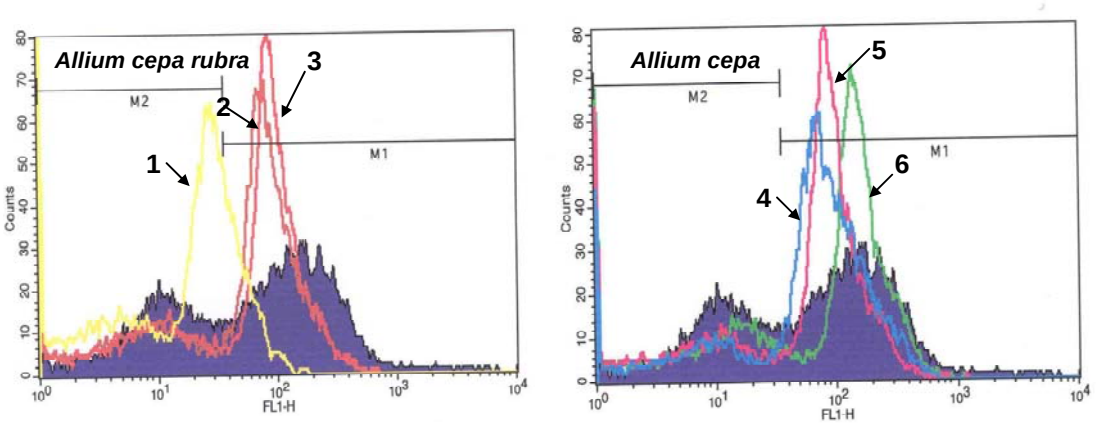

Fig. 5 Comparative flow cytometric histogram analysis of calcein-AM cell viability of Goat semen untreated (M-To) and treated with three different concentrations (0,5g/mL , 0,25g/mL, 0,12g/mL) of acrosinic natural inhibitors from *Allium cepa rubra* (1-3) and *Allium cepa* (4-6). M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Abscissae: log scale green fluorescence intensity of calceine (FL-1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

As shown in Fig. 5, calcein fluorescence of Goat semen untreated (M-To) and treated with three different concentrations (0,5g/mL, 0,25g/mL, 0,12g/mL) of acrosinic natural inhibitors from *Allium cepa rubra* (and *Allium cepa* (4-6) was lower than in M-To and we observed a good correlation with the concentration of acrosinic inhibitors.

In this regard, it is important to mention that we have previously demonstrated that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure [Bratosin et al., 2005].

CONCLUSIONS

For many years, scientists were tempted to develop assays that accurately predict the fertilizing capacity of a semen sample. This goal, however, has proven elusive and will most likely be very difficult to achieve, due to the complex nature of the problem. Part of the problem results from the many attributes that a spermatozoon must possess to fertilize an egg, and how laboratory assays can evaluate all of these attributes simultaneously. The percentage of motile sperm in a sample is most commonly used to evaluate semen quality. This assay, however, is not highly correlated with the fertilizing capacity of semen samples. One reason motion assays do not correlate well with fertility is that we are evaluating only one of many attributes that a sperm must possess to fertilize

an oocyte. One of the problems of measuring multiple sperm attributes is the time and cost required. Flow cytometry offers the possibility of analyzing a high number of sperm (10.000) in less than 1 minute and several papers have been written on sperm measured by flow cytometry [Evenson et al., 1982; Garner et al., 1986; Graham et al., 1990; Garner and Johnson, 1995; Graham, 2001]

Using flow cytometric assays, multiple sperm attributes, including cell viability, acrosomal integrity, mitochondrial function, can be measured simultaneously in sperm cells. In addition, the ability of sperm to undergo capacitation and the acrosome reaction, as well as the chromosomal integrity of sperm can be measured using flow cytometry.

The data presented here indicate that the extracts from *Allium cepa rubra* and *Allium cepa* may possess an acrosin inhibitory property that can be useful as a spermicidal agent. Our results indicate that the combination of specific fluorometric staining and flow cytometry, cell viability calcein-AM assay and the cell death analysis (the percentage of apoptotic or necrotic cells) respectively provide a rapid and ideally adapted methodology for screening and evaluation of acrosinic natural inhibitors action in sperm cells and for assessing the functional status of cells. Our studies are underway in this direction for obtaining a natural spermicidal product with contraceptive efficiency.

ACKNOWLEGEMENTS

This work was supported by the Romanian Ministry of Education and Research, (National Plan of Research-Development and Innovation, Program BIOSTAR). Authors are grateful with many thanks to Dr. Francis Goudaliez, Director of MacoPharma C°, Tourcoing, (France) for providing reagents and FACScan flow cytometer.

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Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii (Life Sciences Series), vol. 18, 2008

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