

Analysis of different cell death processes of prepubertal rat oocytes in vitro

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Abstract The processes of cell death were studied in vitro in populations of oocytes isolated from prepubertal rats. In order to identify apoptosis, the externalized phosphatidylserine was recognized with Annexin-V coupled to FITC and the fragmentation of DNA was demonstrated by means of electrophoresis. Oocytes were tested for autophagy by means of the incorporation of monodansylcadaverine and monitoring Lc3-I/Lc3-II by western blot. The expression of mRNA marker genes of autophagy and of apoptosis was studied by means of RT-PCR in pure populations of oocytes. Some oocytes expressed at least one of the following markers: caspase-3, lamp1 and Lc3. Some oocytes were positive to Annexin-V or to monodansylcadaverine. However, most of them were simultaneously positive to both markers. The relative frequency of oocytes simultaneously positive to markers of apoptosis and autophagy did not change in the different ages studied. The transformation of Lc3-I in Lc3-II was present in all populations of oocytes studied. The mRNAs for caspase-3, lamp1 and Lc3 were present in all populations of oocytes analyzed. Our results demonstrate that oocytes of rats from

new born to prepubertal age are eliminated by means of three different cell death processes: apoptosis, autophagy and a mixed event in which both routes to cell death participate in the same cell.

Keywords Apoptosis · Autophagy · Annexin-V · DNA-fragmentation · Lc3 · Monodansylcadaverine

Introduction

The oocytes located in the primordial follicles, present at birth in the ovary of the female mammal, are the ensemble of germinal cells available during the reproductive period [1]. Programmed cell death plays an important role in the homeostasis of the ovary. In each ovarian cycle a certain number of primary follicles mature to the preovulatory stage. The follicles not selected for ovulation undergo atresia. Until now apoptosis is believed to be the only cell death process involved in atresia [2]. Nevertheless, in the goose and quail three processes of cell death coexist. One of them is apoptosis and the other two have been characterized as autophagy and necrosis [3, 4]. Since these findings, autophagy and necrosis are considered as non-apoptotic programmed cell death processes.

The morphological features most frequently used to detect cells in the process of apoptotic death are: DNA fragmentation, externalization of phosphatidylserine on the cell membrane, and the formation of apoptotic bodies [5].

Autophagic cell death is accompanied by an important reorganization of membranes and vesicles and by an increase in lysosomal activity [6, 7]. Some type I transmembrane glycoproteins such as lamp1 and lamp2 are associated only with lysosomal membrane [8]; this feature allows the immunolocalization of lysosomes. Autophagic vesicles as

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well as lysosomes have an acid content due to a V-type H⁺-ATPase [9]. The weak bases that can go through the membranes are concentrated in acid compartments where they are protonated [10]. This mechanism can also trap various lysosomotropic substances, among which is monodansylcadaverine. Monodansylcadaverine is weakly basic and autofluorescent and is used as a marker of autophagic vacuoles in vivo [11]. Monodansylcadaverine is an inhibitor of transglutaminase [12] and of endocytosis [13], and stimulates the synthesis of some phospholipids such as phosphatidylinositol [14].

Monodansylcadaverine is an autofluorescent molecule used as a marker of autophagic vacuoles. It was found in autophagic vacuoles but not in lysosomes or endosomes [11]. The intensity of its immunolocalization signal increases in cells undergoing autophagy such as cells deprived of aminoacids [15] or treated with rapamycin [16]. The genes involved in autophagy were first described in yeast with alterations in the process of autophagy [6, 17, 18]. These genes related to autophagy are known as Atg. Orthologous are found in vertebrates, including mammals, among them *Homo sapiens* [19]. There are two key molecular systems in autophagy: the Atg12–Atg5 and the Atg8 (Lc3)–phosphatidylethanolamine. The microtubule-associate protein 1 light chain 3 (Lc3) is the mammalian ortholog of Atg8 in yeast [20]. It is located in mature autophagosomes and may be used as a marker of these structures [21]. Lc3 is processed by Atg4 to the form Lc3-I [22]. Lc3-I is cytosolic and undergoes a series of ubiquitylation-like reactions [23], becoming Lc3-II which is conjugated with phosphatidylethanolamine in order to localize autophagosome membrane. The amount of Lc3-II is correlated with the number of autophagosomes. This property makes it useful as an indicator of the formation of autophagosomes [22].

Cell death in the ovary has been studied using in vivo and in vitro procedures in different species [24–30]. These studies mainly deal with the processes of granulosa cell death and contributed a large number of facts for the analysis of these processes. Most evidence indicates that apoptosis is the main process of granulosa cell death during follicular atresia [31, 32]; however in ovaries of birds it was found that autophagy and necrosis also participate in cell disposal [3, 4]. Oocyte death during atresia was also attributed to apoptosis in several animals [33–38].

Previous studies using in situ procedures demonstrated that in prepubertal rats, the apoptosis and the autophagic participation are simultaneously present in the population of oocytes in the cell death process [39]. Furthermore, these processes coexist in same dying oocyte [40].

Using cytochemical and molecular methods, the present work explores the processes of the cell death of oocytes of prepubertal rats in an ex vivo system. Interestingly, the results show that oocyte disposal is by apoptosis,

autophagy or by a process involving a mixture of features of both events, indicating that the processes of cell death previously described in vivo [40] are conserved in vitro.

The study of the processes of cell death of the oocytes is especially important because they are involved in the transmission of genes to the next generation and altered information may cause detrimental evolutionary effects in the species. Furthermore, oncology is the basic information of cell death frequently used. The origin of tumors and their development may be due to flaws in the processes of cell death that allow the survival of altered cells, which may accumulate genetic defects affecting their proliferation and differentiation [41]. Several molecular strategies have been developed to attack damaged cells which do not undergo apoptosis because they may proliferate in a disorderly manner. An interesting proposal is the design of antagonists to members of Bcl-2 family interfering with the interactions between members of this family, which may be used synergistically with various cytotoxic agents [42]. The increase in the knowledge of the process of apoptosis allowed the proposition of possible therapeutic conducts for cancer treatment, such as the use of pro-apoptotic transcription factor p53 [reviewed in 43]. Another strategy against cancer cells is the induction of an autophagy-like process of cell death as in the treatment of breast cancer cells MCF-7 in which the antiestrogen-binding site ligands induce an active cell death with the characteristics of autophagy [44]. It is important to better know the different mechanisms of cell death that may be operative in each cellular type to design treatments for different illnesses related with tumoral or degenerative processes. The aim of the present study is to lend support by means of in vitro studies to previous in situ observations of our group showing that oocytes may be removed by a process sharing features of apoptotic and autophagic traits, which in different cytophysiological conditions may be differently modulated.

Materials and methods

In the manipulation of the animals we followed the ethical guidelines as recommended in the Guide for Care and Use of Laboratory Animals [45].

Oocyte isolation and culture

The ovaries of *Wistar* rats of 1, 5, 19, and 28 day old, were disaggregated in 0.1% trypsin (GIBCO; Grand Island, NY) in calcium and magnesium free solution for 15 min at 37°C. The ovarian cells were incubated at 37°C in an atmosphere containing 5% CO₂ for 24 h on 35 mm culture plates (Nunc; Denmark), with DMEM + GlutaMAX

(GIBCO; Grand Island, NY), supplemented with 0.1% albumin (SIGMA Chemical Co; St. Louis, MO), and 4% fetal bovine serum (GIBCO; Auckland, NZ). The cells were incubated during 24 h to allow the granulosa cells to attach to the dish bottom. The oocytes were identified by their spherical shape, and unattached localization, and were collected with an inverted microscope (Nikon Eclipse TE-2000-U) provided with a micromanipulator (Nikon Narishige IM-9B).

Germinal cells identification

To ensure oocytes were present in the cellular fraction, the protein VASA was immunodetected. This protein is present in germinal cells exclusively [46]. The cellular fraction was placed in a slide, and fixed in methacarn (acetic acid 10%, methanol 60% and chloroform 30%), during 20 min at 4°C. After washing in phosphate-buffered saline (PBS), the cells were incubated with anti-VASA, 1/500 (provided by Dr. Edgar Zenteno Galindo), for 18 h at 4°C. The primary antibody was revealed with an anti-rabbit immunoglobulin coupled to fluorescein (ZYMED Invitrogen; Carlsbad, CA) for 1 h in darkness at room temperature, then counterstained with DAPI (SIGMA Chemical Co; St. Louis, MO) to evaluate DNA distribution.

Annexin-V assay

The apoptotic oocytes were identified by fluorescence microscopy using the Annexin-V/FITC Apoptosis Detection Kit (SIGMA Chemical Co; St. Louis, MO) according to the manufacturer's instructions. 5 µl of Annexin-V/FITC and 10 µl of propidium iodide to each cellular fraction were added. Then the preparation was incubated at room temperature, protected from light, for exactly 10 min. The cells in early apoptotic process were stained by the Annexin-V/FITC alone. The live cells did not show staining by either propidium iodide or Annexin-V/FITC. The analysis of these preparations was carried out in a Leica TCS SP5 confocal microscope.

Monodansylcadaverine assay

To identify lysosomes and autophagic vacuoles *in vivo*, monodansylcadaverine, an autofluorescent compound was incorporated to the oocytes' cytoplasm. The cells were incubated in 100 mM monodansylcadaverine (SIGMA Chemical Co; St. Louis, MO) in DMEM + GlutaMAX medium for 15 min at 37°C in darkness. The excess of monodansylcadaverine was eliminated, and the oocytes were observed in the confocal microscope. Fluorescence

intensity measurement was made with the GNU Image Manipulation Program 2.2 (Gimp 2.2).

Western blot analysis

To evaluate the Lc3 I-Lc3 II conversion, Western blotting analyses were performed. Oocytes cultured for 24 h were collected, rinsed twice in PBS, and resuspended in lysis buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl, 0.1% SDS, 1 mM PMSF, 0.5% sodium deoxycholate, and 1% Nonidet P-40) supplemented with protease Inhibitor Cocktail Complete (Roche, Mannheim, Germany) during 10–15 min.

The protein content in the lysates was measured by Bradford Assay: 100 µg total proteins were boiled for 15 min and loaded on a 12% SDS-PAGE. Protein was transferred to polyvinylidene fluoride (PVDF) membranes, which were incubated 1 h at room temperature in blocking buffer. Then the membranes were incubated with anti-Lc3 rabbit polyclonal antibody 1:500 (Affinity BioReagents; Rockford, IL), and with anti-actin mouse monoclonal antibody 1:1000 (Sigma, USA), overnight at 4°C. Membranes were washed three times, for 10 min each, in 0.05 M Tris, 0.15 M NaCl, 0.1% Tween 20, pH 7.5 (TBS-T). Then the proteins were tagged by incubation with peroxidase-conjugated secondary antibody (Jackson, Newmarket, UK) 1:10,000 in blocking buffer during 1 h at room temperature. Membranes were washed three times for 10 min each, in TBS-T, and specific labeling was detected with chemiluminescent Horse Radish Peroxidase (HRP) substrate (Immobilon Western, Millipore Co, USA), according to the manufacturer's instructions. The membranes were exposed to film (Hyperfilm Amersham Biosciences, UK) to develop the blots.

Detection of DNA fragmentation by electrophoresis

In order to detect the presence of a ladder pattern of DNA degradation during apoptotic process of cell death, an electrophoresis of oocytes DNA was performed. DNA was obtained with DNazol (Invitrogen; Carlsbad, CA) from isolated oocytes, following the manufacturer's instructions. DNA electrophoresis was carried out in a 2% agarose gel, visualized by ethidium bromide (J. T. Baker; Phillipsburg, NJ). The 100 bp DNA ladder (Fermentas Inc; Burlington, ON) was used.

Analysis of mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR)

In order to evidence the gene expression of caspase-3, lamp1, and Lc3 mRNA in isolated oocytes, a RT-PCR was used. Total RNA was prepared using Trizol (Invitrogen; Carlsbad, CA), following the manufacturer's instructions.

Table 1 Sequences of primers used in RT-PCR analysis

Gen	GenBank	Length bp	Sequence 5'–3'
<i>Caspase 3</i>	NM_001100778	209	Fwd GCCTGTCCTGGATAAGACCA Rev TTGACTCAGAAGCCGAAGGT
<i>lamp1</i>	NM_012857	245	Fwd AGGATCAACCTTCCCAACT Rev CACCTTCAGGGTCACCAACT
<i>Lc3</i>	NM_134394	240	Fwd TGGCCCTGAAATACGAAGTC Rev GGCAGTAGTCGCCTCTGAAG
<i>β-Actin</i>	NM_031144	520	Fwd GTATGCCTCTGGTCGTACCA Rev CTCTGCATCCTGTCAGCAA

RT-PCR analysis was performed in a Mastercycler gradient (Eppendorf, Germany). For cDNA synthesis Oligo dT (Applied Biosystem; Foster City, CA) was used as a primer, following the condition for the Transcriptor Reverse Transcriptase (Roche; Mannheim, Germany). PCR contained 1× Taq polymerase buffer, 0.2 mM each of dNTPs, 0.5 µl of Taq Polymerase (Roche Applied Science; New Jersey), and 20 µM of appropriated primers (Table 1). The conditions of the PCR were: 2 min of initial denaturation at 94°C for 32 cycles (94°C for 45 s, 61°C for 45 s, 72°C for 45 s). For *β-actin* an annealing temperature of 60°C was used.

Amplified PCR products were resolved in 1% agarose gel electrophoresis, stained with ethidium bromide (J. T. Baker; Phillipsburg, NJ). Negative controls were provided omitting reverse transcriptase from the cDNA synthesis reaction.

Immunodetection of caspase-3, lamp1 and Lc3 in oocytes

Oocytes were fixed in 4% paraformaldehyde for 15 min and washed 4 times for 15 min in PBS. The cells were incubated overnight at 4°C with the corresponding antibody dilution: caspase-3 1:50 (Affinity BioReagents; Rockford, IL); lamp1 1:100 (Abcam, Cambridge, UK), and Lc3 1:200 (Affinity BioReagents; Rockford, IL). After washing, the oocytes were incubated in anti-rabbit-Alexa Fluor 594 (Invitrogen; Carlsbad, CA) for 2 h at room temperature and stained with DAPI to evidence the nucleus.

Electron microscopy

To study the ultrastructural morphology of the isolated oocytes and evaluate the presence, abundance and structural features of the autophagosomes, we used the classical methods for the preparation of biological material for its study with electron microscopy. The purified cellular fractions were fixed in 2% glutaraldehyde and 4% paraformaldehyde. Then the samples were embedded in Epon.

Ultrathin sections were stained with uranyl acetate and lead citrate. The preparations were studied in a Jeol 1010 electron microscopy provided with a digital camera Hamamatsu.

Results

We washed the culture dishes with culture medium in order to eliminate the detached and the dead cells. Then we harvested the oocyte-associated granulosa cells which were attached to the plate. This process permits working with living oocytes only. The viability of the oocytes in the collected fraction is around 95% evaluated with trypan blue.

The morphological analysis of ovarian cell population in cultures of rats of different ages reveals that spherical cells with large cytoplasmic volume, the oocytes, do not attach to the surface of the culture vessel. On the other hand, the somatic cells do adhere to the surface of the culture vessel (Fig. 1). In cultures of samples obtained from 1 day old rats all the oocytes were small and very similar in size. In cultures of cells of 5 day old rats, there are oocytes of different sizes and a larger number of somatic cells were present, corresponding with the existence of follicles at this age. In cultures of cells obtained from 19 and 28 day old rats, somatic cells form a monolayer in the culture dish and oocytes of different sizes are found.

Due to the low number of somatic cells obtained from each ovary of 1 day old rats, it was necessary to use at least 14 ovaries in each essay to be sure that there would be enough somatic cells to form a monolayer to which the oocytes may attach. In the ovaries of rats 5 or more days old there are follicles in different stages of development, thus the number of somatic cells are enough to make a monolayer to which the oocytes may attach. This method allowed us to obtain a large amount of oocytes, enough to carry out different biochemical and molecular proofs in order to identify autophagic or apoptotic processes of cell death.

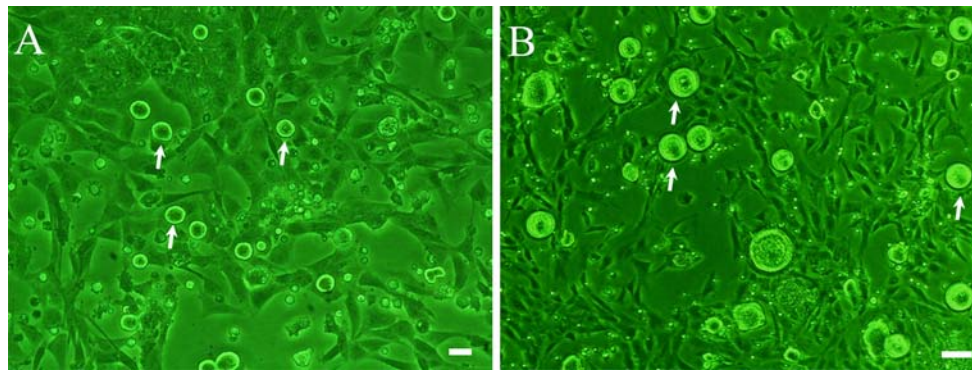


Fig. 1 Rat oocytes and somatic cells in culture. **a** 5 day old rats. **b** 19 day old rats. The oocytes are large spherical cells. Most of them can be recognized by a bright ring due to their spherical form and

their high refraction index (*arrows*). Somatic cells of different shapes are attached to the bottom of the plate. Some of them form a monolayer. *Scale bars a* 20 μm ; *b* 50 μm

To make sure that the cells we were studying were oocytes we detected VASA protein, specific to germinal cells [46]. The cytoplasmic green fluorescent labelling of the cells shows that the cellular fractions obtained are oocytes (Fig. 2).

Apoptosis marker

To evaluate the presence of oocytes undergoing apoptotic death, we used the method of the incorporation of Annexin-V labeled with FITC, which binds to the phosphatidylserine

located on the external side of the cell membrane of the isolated oocytes.

Supra-vital propidium iodide labeling was used to identify dying cells with damaged cell membrane. The externalization of phosphatidylserine was found in some of the oocytes in all ages studied. Oocytes positive to both markers were considered just dead cells and were not taken into account in this study, as damaged cell membrane allows Annexin-V to bind phosphatidylserine at the internal side of the open membrane. Furthermore, apoptosis does not produce rupture of the cell membrane. Those

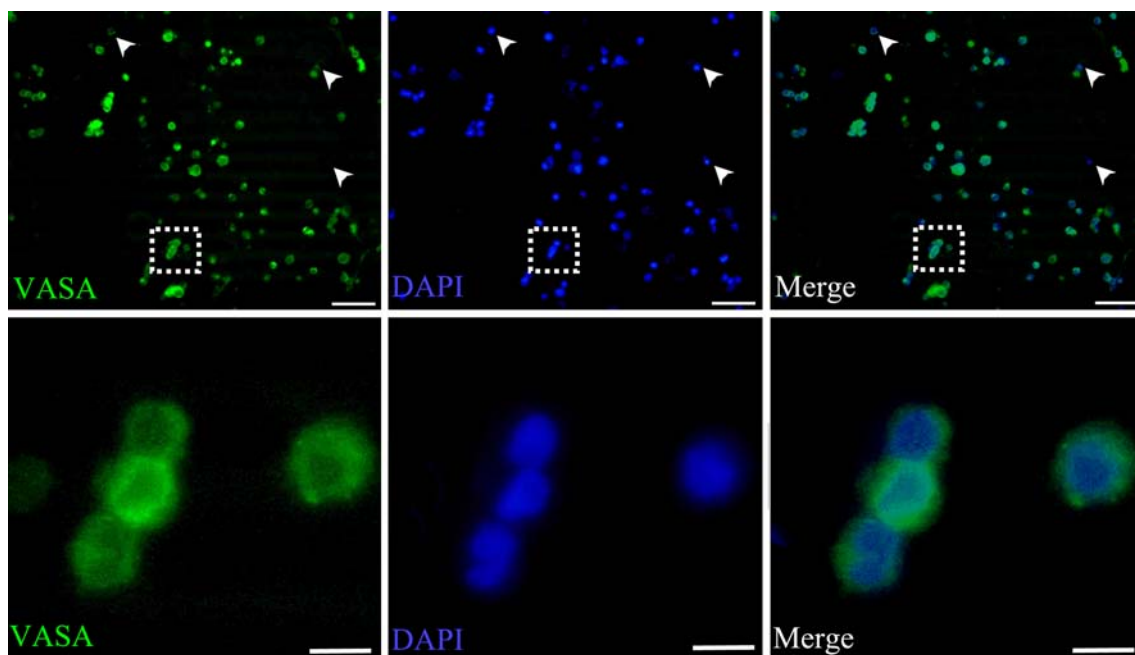


Fig. 2 Oocytes from 5 day old rats. Cellular fraction of isolated oocytes. VASA protein immunolocalization. The up image is a low power magnification of a cell population showing numerous VASA-positive cells. The *arrow heads* show a few cells negative to the

immunodetection. Higher magnification of the framed region in figures. The label is present only in the cytoplasm the isolated oocytes. *Scale bars low magnification* 100 μm ; *high magnification* 20 μm

oocytes positive to Annexin-V and negative to propidium iodide were considered to be at the initial stages of the process of apoptotic cell death. The relative frequency of oocytes positive to Annexin-V changed according to the age of the animals. They are most frequent in 1 day old rats (diplotene oocytes) and their frequency is minimal in 5 day old animals (diakinesis oocytes), indicating changes in the frequency of the initial signs of the apoptotic process in these populations of oocytes (Fig. 3).

DNA fragmentation

Another method to evaluate presence of apoptosis in populations of isolated oocytes is the DNA electrophoresis. The study of the DNA of oocytes by means of electrophoresis in agarose gels, showed the well known fragmentation in multiples of 180–200 base pairs, typical of the apoptotic process (Fig. 4). The samples added to each well were measured by total volume of DNA obtained in each population of oocytes, thus this method is strictly qualitative; the volume of the DNA was of 10 μ l for each sample. Interestingly, the oocytes of 5 day old rats are not fragmented to a detectable extent. This coincides with the low frequency of externalization of phosphatidylserine in the population of oocytes of 5 day old rats. Both are signs of different stages of the apoptotic process. These results show that even if the process of apoptosis exists in the oocyte oocytes of 5 day old rats, its frequency is very low.

Autophagy marker

One of the markers frequently used to evaluate the process of autophagy is the incorporation of monodansylcadaverine to lysosomes and autophagosomes.

The difference of the intensity of labelling of monodansylcadaverine between normal cells and those in process of autophagic cell death is large enough to allow us to identify the cells in the process of autophagic death. The blue fluorescence indicates the cytoplasmic regions in which the monodansylcadaverine is present. The large increase of the fluorescent signal can be easily seen at different magnifications (Fig. 5).

Interestingly, the intensity of the incorporation of monodansylcadaverine in lysosomes and autophagolysosomes varies between the labeling of small regions of the cytoplasm to the fluorescence of the entire cytoplasm of isolated oocytes of rats of different ages (Fig. 5b). Oocytes in the process of cell death incorporate more than twice the monodansylcadaverine than normal ones, as shown by fluorescence intensity quantification (Graph 1). The relative frequency of oocytes with increased monodansylcadaverine labeling is similar in all ages studied. No significant differences were found using ANDEVA test.

Lc3 Western blot

Another marker used to detect the autophagy is the conversion of the microtubule-associated protein Light chain 3 (Lc3) from the form Lc3-I to the form Lc3-II. This conversion may be detected by means of immunoblot analysis. Lc3-II is involved in forming autophagosomes [23] and thus it is a reliable indicator of the process of autophagy. In the population of isolated oocytes of prepubertal rats the conversion of protein Lc3-I to Lc3-II was clearly identified indicating an intense process of formation of autophagosomes (Fig. 6). These results coincide with those obtained with monodansylcadaverine, which evidence high lysosomal and autophagosomal activity.

Both markers in the same cell

In order to determine if both processes of cell death coexist in some of the cells, we carried out two reactions in the same cells: incorporation of Annexin-V (marker of apoptosis) and incorporation monodansylcadaverine (marker of autophagy). The results show that cells may be positive to both reactions, positive only to one marker or negative to both (Fig. 7), indicating that the elimination of the oocytes is carried out by processes of cell death in which apoptosis and autophagy are implicated in the same cell or only one process. The frequency of oocytes with double labeling does not change significantly with the size of the oocyte or with the age of the animal.

Analysis of mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR)

The presence of mRNAs of caspase-3 expressed during apoptosis, and lamp1 and Lc3 expressed in autophagy was studied by means of reverse transcription and DNA amplification. The PCR products were purified and sequenced in order to verify that they corresponded to the sequences of the mentioned genes. The results indicate that the three genes were expressed in oocytes of all ages studied (Fig. 8).

Lc3, lamp1 and caspase-3 protein immunolocalization

The evaluation of the presence of the proteins corresponding to the mRNAs analyzed by RT-PCR was carried out by means of immunolocalization at light microscope level. Lamp1, Lc3 and active caspase-3 were found in oocytes of rats of different ages. These findings demonstrate that the oocytes not only transcribe these genes but also translate the messengers in mature proteins that can be detected by the antibodies (Fig. 9).

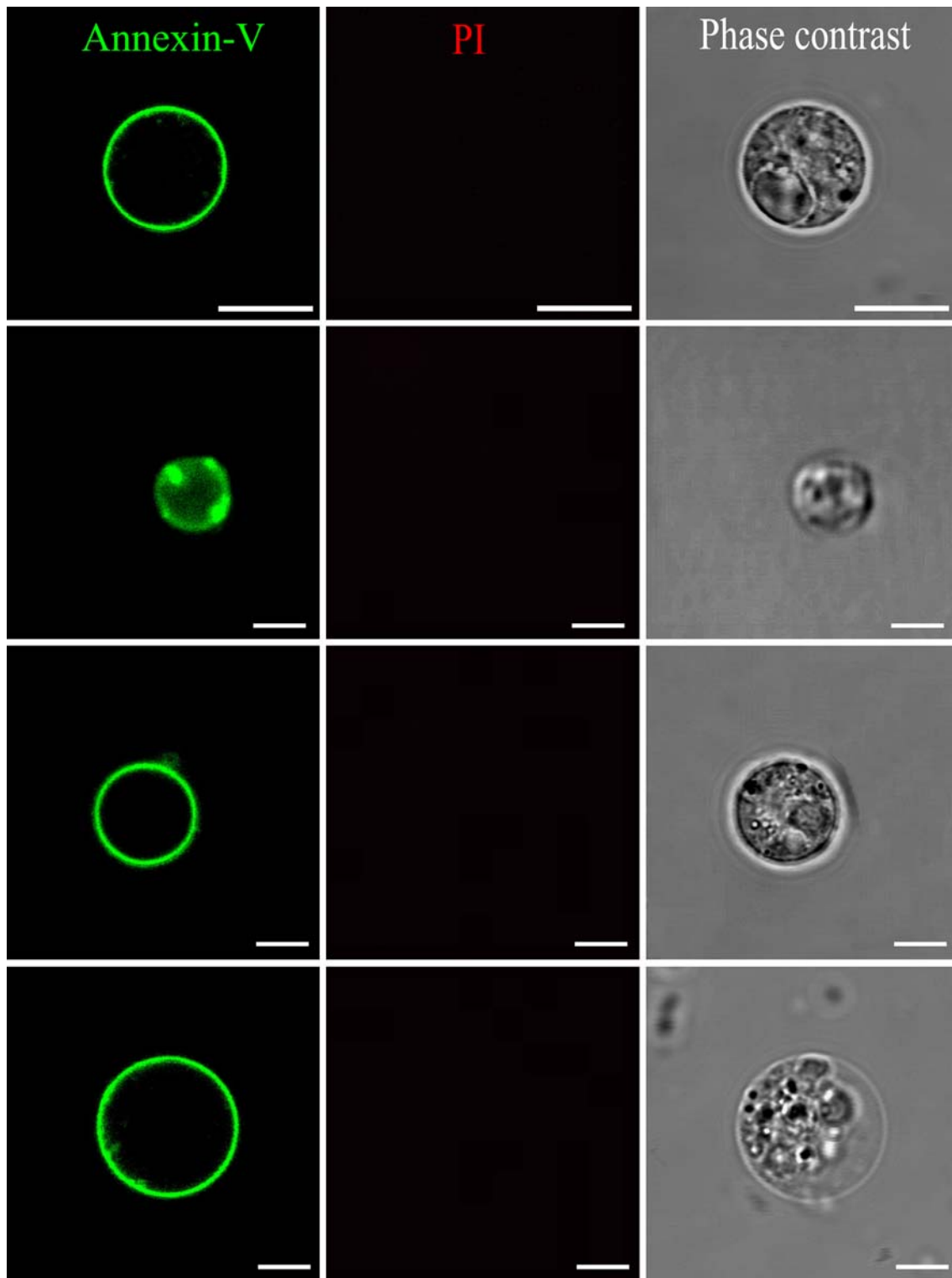
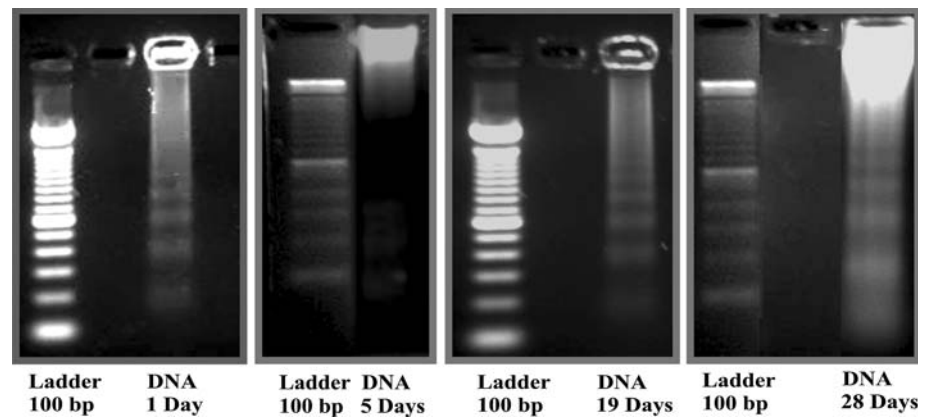


Fig. 3 High magnification view of the detection of externalized phosphatidylserine by means of Annexin-V coupled to FITC in oocytes of rats of different ages. The *green* fluorescence indicates the externalization of phosphatidylserine; a sign of the apoptotic cell

death process. All oocytes were negative to the incorporation of propidium iodide indicating that their membrane was intact. *Scale bars* 20 μ m

Fig. 4 DNA electrophoresis in agarose 2% gel with ethidium bromide. DNA was obtained from oocytes of 1, 5, 19 and 28 day old rats. Fragmentation of DNA was present in the oocytes of 1, 19 and 28 day old rats. The DNA of the oocytes of 5 day old rats was not fragmented



Electron microscopy

The ultrastructural study of numerous oocytes of rats of different ages allowed us to corroborate the presence of abundant autophagosomes containing decaying cytoplasmic structures (Fig. 10). Some autophagosomes contain membranous bodies, mitochondria in the process of degradation, and other non recognisable cytoplasmic remains (Fig. 10).

Discussion

Apoptosis is the most studied process of programmed cell death. It is mainly characterized by membrane blebbing, fragmentation of DNA, structural preservation of organelles [47], and activation of caspases [5]. These proteases coordinate the dismantlement and elimination of target cells. Several studies demonstrate the participation of apoptosis in cell death in the ovary of embryonic [48] or adult mammals [49–51].

In the present work we have developed a procedure to obtain pure populations of oocytes isolated from the ovaries of Wistar rats of the same stock used in previous works [39, 40]. The ages of the rats used in the present study are some of the ages employed in our *in vivo* study [40], so the previous results can be compared with the present ones.

In the present study we analyzed the processes of elimination of oocytes in different stages of follicle development, as previous works demonstrated that the processes of oocyte disposal are not related to the stage of the follicular development [39, 40].

We used biochemical and molecular methods devised to be performed on isolated cells or cell fractions to detect specific signs of apoptosis and autophagy.

The present morphological observations as well as those reported in a previous work of our group [39] demonstrate the presence of numerous primordial follicles in 1 day old rats. This feature causes a low amount of somatic cells in

the cell culture. The interactions oocyte-granulosa cells are very important in the determination of follicular development versus follicular atresia [52]. The oocytes isolated in our study are surrounded by granulosa cells allowing the oocyte-granulosa cells complex to join the somatic cell monolayer in the culture dish.

The studies on oocyte processes of death carried out *in situ* are significantly less than those on granulosa cells or on thecal cells, which are far more abundant in tissue sections. In the present work we designed a method to obtain a large number of oocytes which may be studied using methods that can only be performed on living cells *in vitro*, as the incorporation of Annexin-V to the externalized phosphatidylserine and the incorporation of monodansylcadaverine to lysosomes and autophagosomes. We also could use protein and DNA electrophoresis. Using pure populations of oocytes we could carried out the amplification of the mRNAs of proteins participating in the processes of apoptosis or autophagy in different levels of execution. We could also demonstrate markers of both processes of cell death in the same cell.

One of the earliest signs of apoptosis is the loss of asymmetry of the cell membrane. During this process phosphatidylserine is exposed to the exterior of the membrane, while the membrane integrity remains unchallenged [53, 54]. This exposition of phosphatidylserine is present not only in mammals but also in insects and even in plants [55]. Surface exposed phosphatidylserine can be detected by its affinity for Annexin-V, a phospholipid binding protein. Exposed phosphatidylserine functions as a tag for specific recognition by macrophages. FITC labelled Annexin-V permitted the measuring of phosphatidylserine externalized by oocytes undergoing apoptosis [56–59]. Annexin-V is not able to bind to normal cells since the molecule can not penetrate the phospholipid bilayer. However, in dead cells the inner leaflet of the membrane is available for binding the extrinsically applied Annexin-V, since the integrity of the plasma membrane is lost. To discriminate between dead and apoptotic cells, a DNA

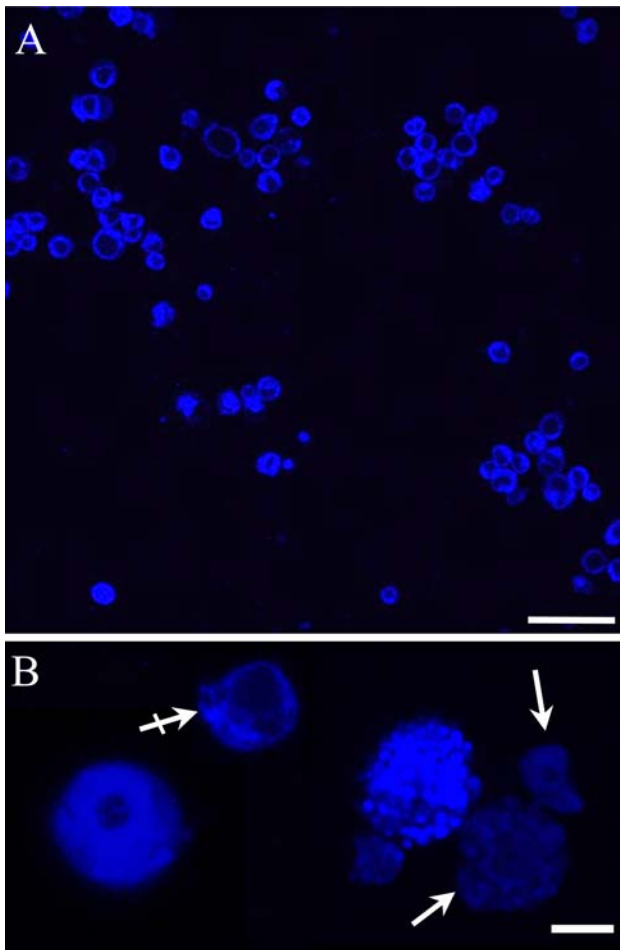


Fig. 5 Incorporation of monodansylcadaverine to lysosomes and autophagosomes in the cytoplasm of oocytes of rats of different ages. The *blue* fluorescence of the cytoplasm of the oocytes shows that lysosomes and autophagosomes have incorporated monodansylcadaverine, which is a fluorescent molecule. The dark zone in the cell corresponds to the nuclear space. All oocytes contain lysosomes, normal oocytes are weakly labeled, and oocytes in process of cell death are frequently heavily labeled. **a** Low power view. **b** The *arrows* point to the normal oocytes with a low level of labeling. Highly labeled oocytes contain numerous lysosomes and autophagosomes and are probably in autophagic process of cell death. The cytoplasm of the oocyte pointed out by a crossed arrow is almost homogeneously labeled indicating a dispersion of the lysosomes content. It is probably in the final stage of the process of cell death. Scale bars **a** 75 μm and **b** 20 μm

stain, such as propidium iodide, that cannot cross the normal cell membrane, can be used simultaneously. In this way normal, apoptotic and dead cells can be discriminated on the basis of double fluorescent labelling with FITC labelled Annexin-V and propidium iodide.

In this work numerous populations of oocytes of rat of different ages were positive to Annexin-V and negative to propidium iodide, indicating that they were undergoing an apoptotic process of cell death. The absence of oocytes

positive to propidium iodide shows that necrosis is not involved in the oocyte disposal.

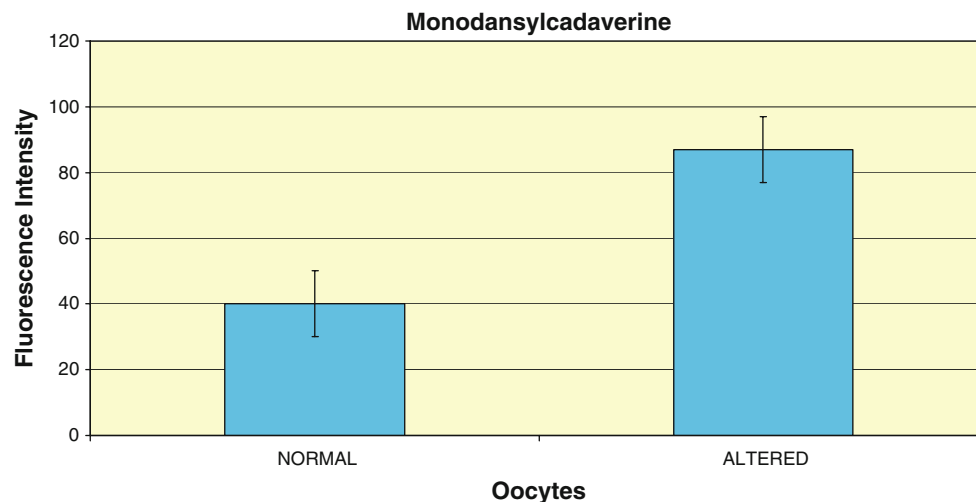
In a study carried out in mouse ovaries, active caspase-3 was found in oocytes and granulosa cells of atretic follicles [60]. Active caspase-3 as well as DNA fragmentation was found in follicular cells of morphologically normal antral follicles and also in follicles in early stages of atresia [61]. The detection of active caspase-3 and TUNEL are two methods frequently used to study apoptosis in different mammalian species, including humans, in which granulosa cells undergoing cell death positive to caspase-3 was observed. However, few granulosa cells are TUNEL positive in human samples [62].

In this work the comparison of the frequency of oocytes positive to Annexin-V and the amount of DNA fragmentation evaluated by electrophoresis shows that both markers of the apoptotic process are frequent in oocytes in the process of cell death of 1, 19 and 28 day old rats. On the other hand, no DNA fragmentation and scant Annexin-V labelling were found in decaying oocytes of 5 day old rats. These findings suggest that the process of elimination of these oocytes is not mainly due to apoptosis.

Autophagy is an ancestral process of survival; nevertheless the process may increase and radically change its function, leading to cell death. Autophagy is highly regulated by a mechanism that mainly involves genes first identified in yeast studies. A variety of essential components of autophagic machinery, such as Atg proteins, are phylogenetically highly conserved, and several mammalian counterparts, such as Atg5 and Atg7, have been reported [21, 63, 64]. Atg8 (homologous of mammalian Lc3) participates in the process of autophagy. Lc3 conjugates with phosphatidylethanolamine; this is an essential step for the formation of the autophagosome membrane. The formation of autophagosomes is necessary for the process of autophagy [23, 65, 66].

The strategies designed to study the process of autophagy in vitro involve the use of monodansylcadaverine, a substance that incorporates into lysosomes and autophagosomes [11, 67]. As lysosomes are present in normal cells, the increase of the labelling beyond its normal level indicates the presence of an autophagic process. Altered oocytes with higher than normal levels of incorporation of monodansylcadaverine were present at all ages studied. No significant differences in the frequency of oocytes positive to monodansylcadaverine were found in oocytes of rats of different ages. These results show that autophagy is a widespread process of oocyte disposal in prepubertal rats.

The evaluation of the participation of apoptosis and autophagy in the elimination of the same oocyte using monodansylcadaverine and Annexin V at the sametime showed that oocytes simultaneously positive to both markers are present at all ages studied. The relative frequency of oocytes simultaneously positive to markers of



Graph 1 The quantitation of fluorescence intensity (Gimp 2.2 program) of the oocytes demonstrates a significant increase of the incorporation of monodansylcadaverine in altered oocytes over

structurally normal ones and thus the increase in lysosomes and autophagosomes in altered oocytes

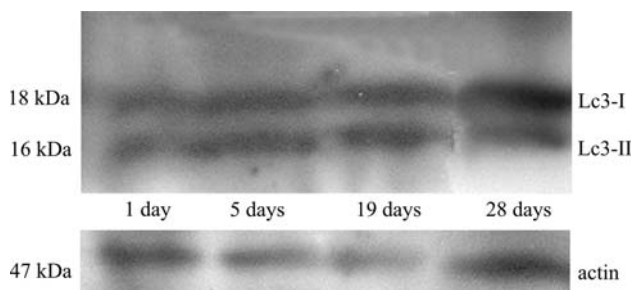


Fig. 6 Analysis of the production of Lc3-I and Lc3-II during the process of autophagy in oocytes isolated from rats of different ages. The production of the both forms of Lc3 indicates the increase in autophagosomes formation which suggests that the process of oocyte death is at least in part due to autophagy

apoptosis and autophagy does not change significantly in the different ages studied. These results allow us to propose that the process of oocyte death may use different combinations of a proteolytic mechanism mediated by caspases leading to DNA fragmentation without compaction of the chromatin and autophagic processes mediated by autophagolysosomes to destroy the cytoplasm of the cell.

In this work, new techniques that could not have been developed in an *in situ* environment were designed. These methods allow us to evidence new signs of the cell death process in oocytes and the evaluation of biochemical and molecular profiles expected to be related to autophagy and apoptosis in isolated rat oocytes. The cells of each culture were examined for the presence of the autophagic-related mRNAs (Lc3 and lamp1) and the presence of an apoptosis-related mRNA (caspase-3) by means of RT-PCR analysis. These studies demonstrated that

mRNAs coding for pro-apoptotic and pro-autophagic proteins are transcribed in isolated oocytes populations. The probable increase in the caspase-3 mRNA in the 28 day old rat population may be due to the large increase in size of the cytoplasm of these oocytes. However, our RT-PCR is only semi-quantitative.

Immunolocalizations showed that the proteins corresponding to mRNAs coding for lamp1, Lc3 and caspase-3 are present in altered oocytes. At all ages studied some oocytes expressed at least one of these markers. The use of western blotting to analyze the expression of the protein Lc3, allowed us to observe the conversion of Lc3-I in Lc3-II, indicating the production of new autophagosomes. These data support the view the process of autophagy participates in the process of death of numerous oocytes. This view is reinforced by our ultrastructural observations of the presence of abundant autophagosomes in dying oocytes.

According to McClallan et al. [68] a large proportion of rat oocytes are in pachytene between 16.5 days post coitus and the day of birth. Most of the oocytes of 1 day old rats are in early diplotene. The frequency of diplotene in the population of oocytes diminish drastically between 1 and 3 day old rats [68]; so the oocytes of 5 day old rat as thus used in our work are in diakinesis. These drastic changes may be related to different phases of the meiotic process (“Discussion”). It is interesting to note that apoptosis is the most frequent process of disposal of early diplotene oocytes in 1 day old rats, while its frequency is very low in diakinesis oocytes of 5 day old rats. These results suggest that these differences in oocyte process of death may be related to flaws in the meiotic process shortly after birth, as has been suggested by Ghafari et al. [69].

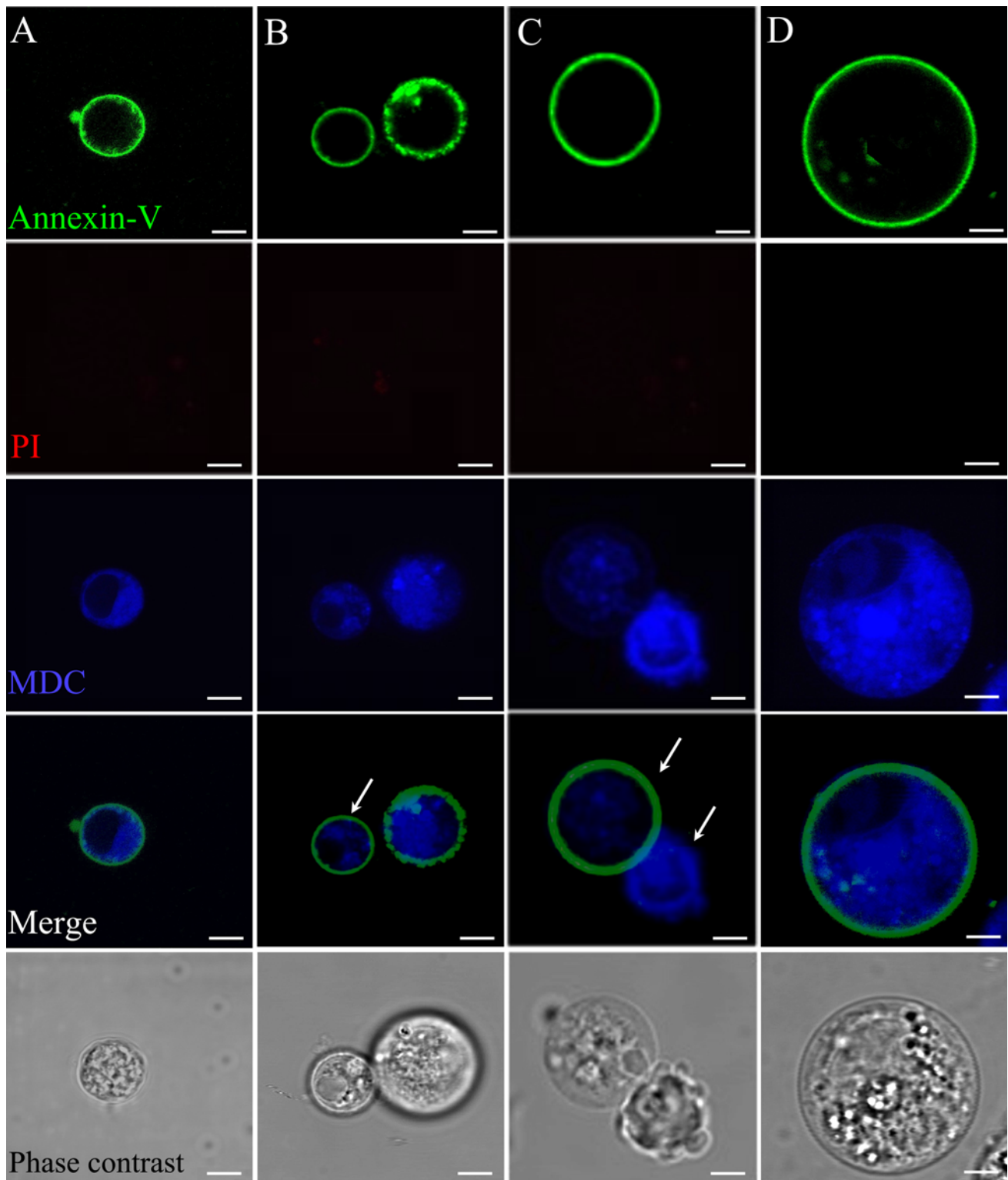


Fig. 7 Simultaneous incorporation of Annexin-V and monodansylcadaverine in the same cells. The *green* fluorescence indicates the presence of Annexin-V and *blue* fluorescence reveals monodansylcadaverine. Most oocytes are positive to Annexin-V and also show an increased signal of monodansylcadaverine (MDC). However, some of them are positive to only one reaction (*arrows*). Images merged show

the coincidences of both markers in the same cells. All the cells are negative to propidium iodide (PI); a test indicating that the membrane of these cells was intact. Columns: **a** oocytes 1 day old; **b** 5 day old oocytes; **c** 19 day old oocytes, and **d** 28 day old oocytes. *Scale bars* 20 μ m

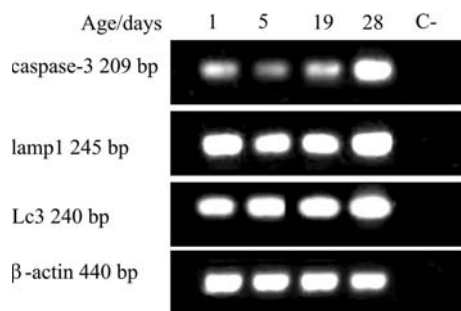


Fig. 8 Presence of mRNA of caspase-3, lamp1 and Lc3 in isolated oocytes. Total RNA was extracted and amplified by RT-PCR. In each reaction β -actin mRNA was amplified as internal control. C negative control. All the mRNAs analyzed are present in oocytes of rats of all ages studied. This indicates that genes participating in apoptosis and autophagy are expressed simultaneously in the population of dying oocytes

The presence of markers for apoptosis and autophagy in the same oocyte generates two possible interpretations of development of cell death processes:

- The first possibility is that both processes, autophagy and apoptosis, are activated from the beginning. However, only one of these processes is involved in the final disposal. The autophagy may be initiated when the process of apoptosis cannot be achieved [70].
- The second possibility is that both processes of cell death are activated simultaneously at the beginning and both participate in the disposal of the oocyte.

It has been shown that the processes of autophagic cell death and apoptotic cell death may cross-talk in various cell types under experimental manipulations [revised in

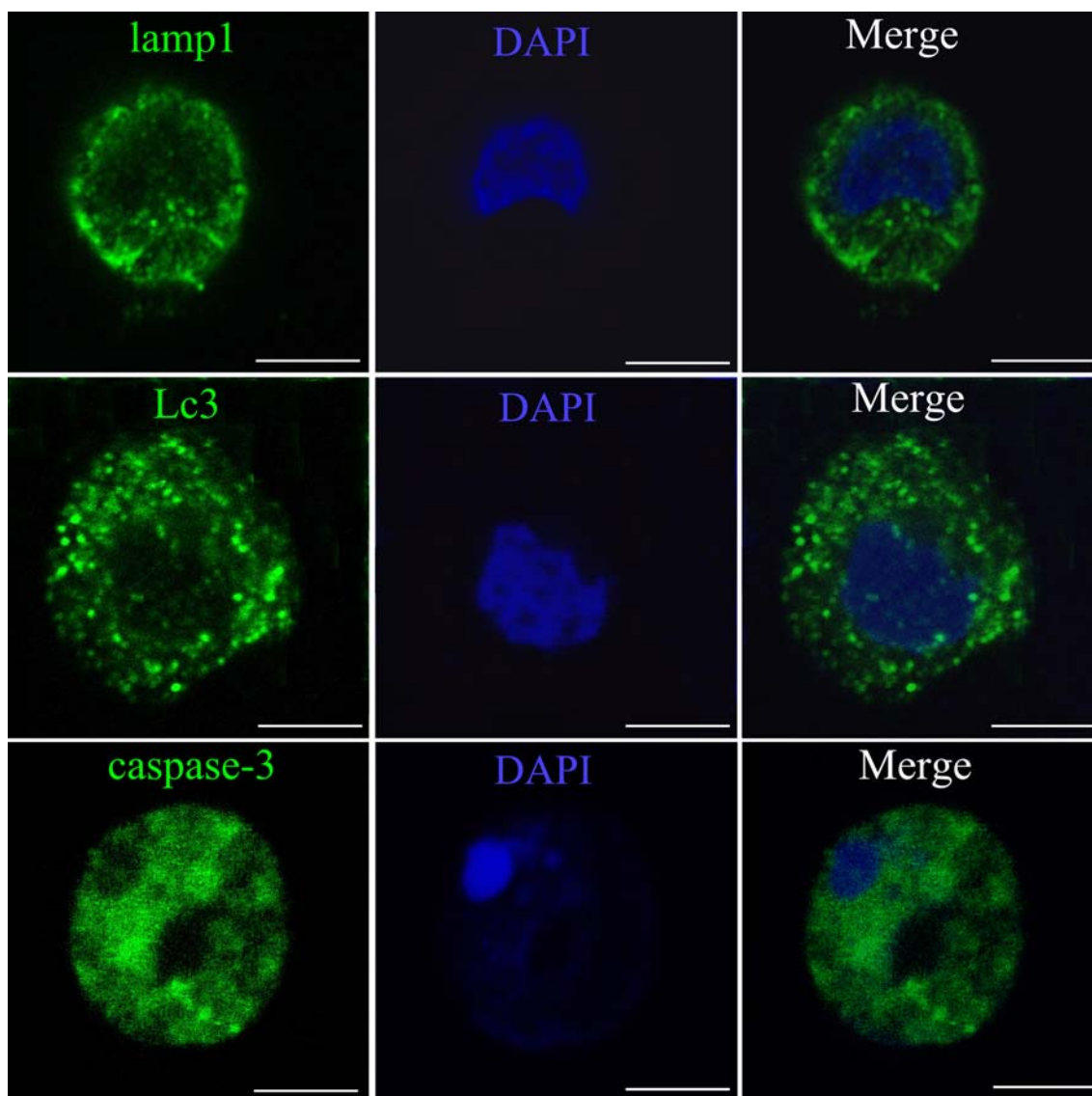


Fig. 9 Immunodetection of active caspase-3, lamp1 and Lc3 in isolated oocytes. The cytoplasm of the oocytes are intensively labeled by anti-lamp1, anti-Lc3, and anti-caspase-3. DAPI staining shows the nuclei. Scale bars 10 μ m

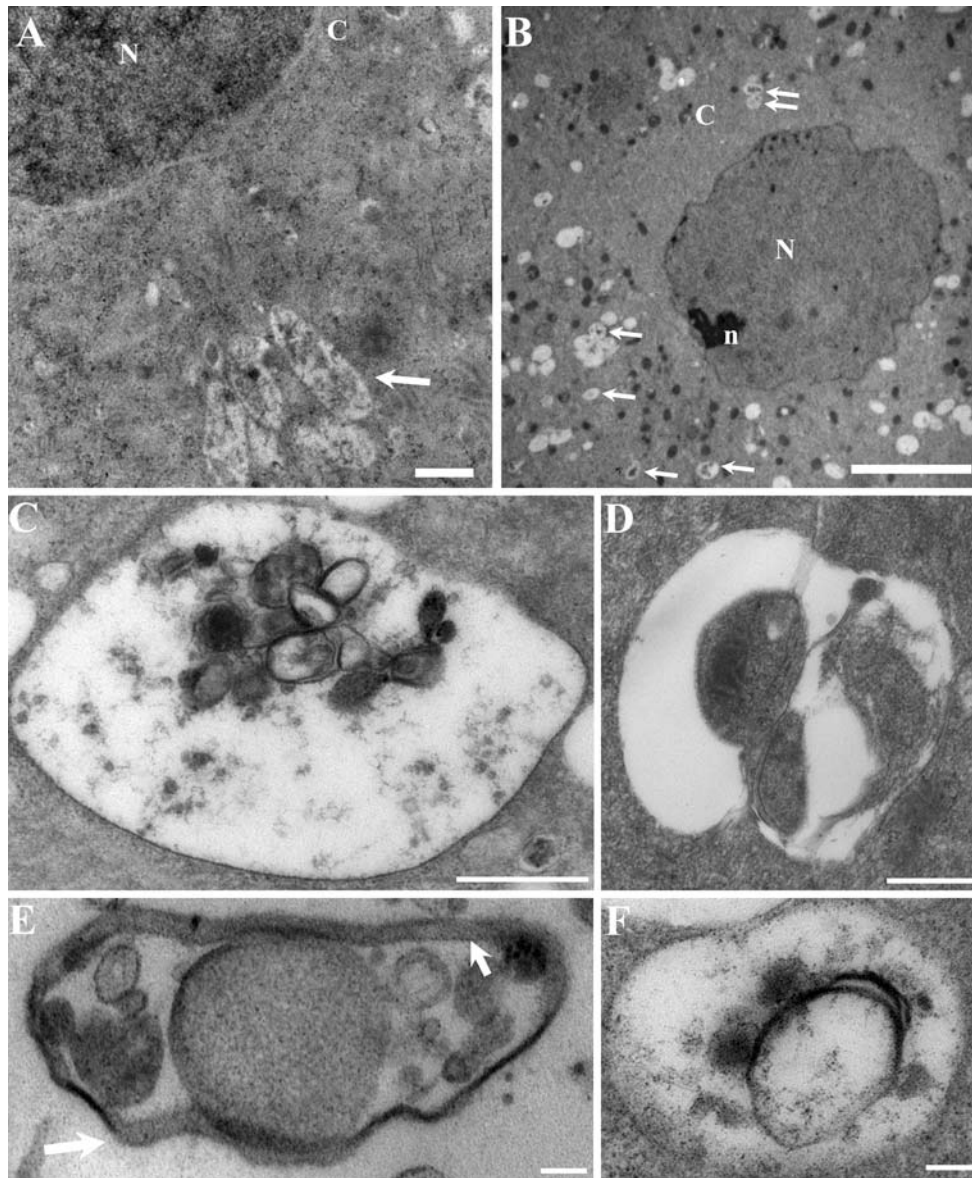


Fig. 10 Electron microscopic images of isolated oocytes. Autophagic vesicles (*arrows*) are numerous in the cytoplasm of rats of different ages. Most of these vesicles are autophagolysosomes containing cytoplasmic debris (**a**, **b**). **c–f** Electron microscopic images of autophagic vesicles in the cytoplasm of oocytes of rats of different ages containing the rest of membranes and membranous organelles in

different degrees of degradation. **e** A section thicker than standard sections for EM, the membrane of an autophagolysosome and those of some internal vesicles can be clearly seen (*arrows*). *N* nucleus, *n* nucleolus, *C* cytoplasm. Osmium tetroxide postfixation. Contrasted with uranyl acetate and lead citrate. *Scale bars a* 500 nm; **b** 2 μ m; **c**, **d** 500 nm; **e**, **f** 100 nm

71]. In the present study we use normal animals without any manipulation. Thus our results contribute with new evidence to the knowledge of oocyte cell death of prepubertal rats adding evidences of a process sharing apoptotic and autophagic traits. Recent evidences indicate that some apoptotic features as PUMA and Bax induce the Lc3 puncta formation typically autophagic as well as apoptosis. It was also shown that the protein BH3-only PUMA may induce autophagy and apoptosis at the same time. The PUMA induction of autophagy requires the presence of

Bax or Bak [72]. The pro-autophagy protein Beclin-1 has a BH-3 domain [73, 74] and interacts with BCL2 allowing the regulation of autophagic and apoptotic processes of cell death [73]. The proapoptotic BH3-only proteins, BNIP3 and Bad may induce autophagy [75, 76].

The presence of the domain BH3 in the pro-autophagic protein Beclin-1 and also in proapoptotic proteins suggest, that these proteins may activate autophagy by means of the same stimulus they activate the intrinsic route of apoptosis.

The Atg gene which was considered to be specifically involved in the autophagic formation of autophagosomes through an ubiquitin-like conjugation system now also seems to be an important mediator of apoptosis. Some death stimuli may cleave Atg 5 in a product which may stimulate apoptosis mediated by mitochondria [77].

A correlation between follicular atresia and the proapoptotic protein Bax was found in the rat ovary indicating the participation of this protein in the cell disposal in this organ [78]. It was proposed that the autophagy-apoptosis relationship constitutes a more efficient cell degradation system [77]. The volume of the oocytes is significantly larger than somatic cells. It is possibly that the combined degradation process may be efficient in the elimination of a large cytoplasmic content of only one cell.

The experimental data obtained in the present work interestingly show that the oocytes of prepubertal rats explanted *in vitro* maintain the same mechanisms of cell death as those found *in situ*: apoptosis, autophagy and a mixed procedure in which markers of both coexist [40]. It is important to note that the processes found in the present research are natural events. Experimental studies showed that the inhibition of the process of autophagy increases apoptosis and that the blocking of apoptosis results in a programmed cell death similar to autophagy. In some stages of these processes the cells may have features of apoptosis and autophagy simultaneously [79, 80]. Interestingly this work confirms that the two better characterized processes of cell death, apoptosis and autophagy function together in the elimination of prepubertal rat oocytes.

A better understanding of the molecular processes implicated in the elimination of the oocytes may provide important information in the studies of the problems of fertility in human and other mammals. Furthermore, the evidence of a co-participation may provide an important tool to stimulate the elimination of pathological cells, as cancer cells.

The ensemble of all the results of the present work demonstrates the coexistence of the processes of apoptosis and autophagy in the same cell. These results strengthen our previous data obtained *in situ* [40], where the observations demonstrated the presence of cytoplasmic clear vesicles, absence of apoptotic bodies, acid phosphatase activity, active caspase-3 and an increased signal of lamp1 coexistence in the cytoplasm of the oocytes in process of death, indicating a frequent simultaneous presence of proteins characteristic of apoptosis and autophagy. The same stock of rats was used for both studies.

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