ISSN 2347-6893

COMBINED METHOD FOR THE EVALUATION OF THE INITIAL AND LATE PHASES OF THE APOPTOSIS PROCESS IN PREPUBERTAL RAT TESTES

Escobar ML, Echeverría OM, Ortiz R, Márquez HG, Juárez S, Vázquez-Nin GH
Laboratorio de Microscopía Electrónica. Departamento de Biología Celular. Facultad de Ciencias. Universidad Nacional Autónoma de México (UNAM),04510, México, D F. vazqueznin@ciencias.unam..mx

ABSTRACT

Apoptosis is a physiological process that maintains tissue homeostasis. This study used testes from pre-pubertal Wistar rats to evaluate the initial and late phases of germ cell alterations. We used toluidine blue staining and immunodetection of active caspase-3 in semi-thin sections, as well as electron microscopy techniques to determine the ultrastructural modifications that correspond to initial or advanced cellular damage. Our qualitative results from optical observations showed that the general stains permit identification of the advanced stages of apoptosis; however, the more sensitive techniques of immunolocalization as well as the electron microscopy also revealed the initial phases of apoptosis. The ultrastructural evidence not only confirms the observations made with the optical techniques, but also allowed the identification of the earliest morphological sign of cell elimination as nuclear envelope and mitochondrial dilatation. Our observations indicate that the combined use of the techniques of light and electron microscopy permits better identification of the initial alterations of the process of cell death in the testes.

Keywords

apoptosis, testes, Wistar, active caspase-3, toluidine blue stain.

Academic Discipline And Sub-Disciplines

Cell Biology, Ultrastructure, Light microscopy

SUBJECT CLASSIFICATION

Microscopy Subject Classification

TYPE (METHOD/APPROACH)

Light and electron microscopy techniques

ISSN 2347-6893

INTRODUCTION

The major morphological characteristics of apoptosis, or type I programmed cell death, include cellular contraction, DNA fragmentation, and the formation of apoptotic bodies (Kerr et al., 1972). The apoptosis process has been studied extensively and its biochemical characteristics are well known. It is characterized by the activation of a family of proteases named caspases, which cleave their substrates at specific aspartate residues (Cohen, 1997; Earnshaw et al., 1999; Fuentes-Prior and Salvesen, 2004; Thornberry and Lazebnik, 1998). These proteases are divided into initiators (such as -8 and -9) and executers (-3, -6 and -7) (Danial and Korsmeyer, 2004), with the latter being responsible for the morphological changes that occur in cells undergoing apoptosis. It has been shown that caspase-3 is the major effector caspase responsible for the cleavage of several substrates (Fischer et al., 2003).

The testes are made up of complex structures named seminiferous tubules, which enclose the germinal cells. Inside the seminiferous tubules the cells are organized concentrically. The spermatogonia are located at the periphery of the tubule near the basement membrane and constitute the stem cells that will produce the primary spermatocytes which, through a series of steps of meiosis and cellular differentiation, will become mature spermatozoa (Russell et al., 1990; Sharpe, 1994). Earlier studies conducted with mammal testes have indicated the presence of continuous cellular renovation; a process that has been defined as spontaneous in various phases of germ cell development (Clermont, 1962). Several studies have shown germ cell death during the spermatogenesis process (Hikim et al., 1995; Mori et al., 1997). Characteristics of the apoptotic process have been identified in the final phases of the event, where both highly-compacted chromatin and cellular shrinkage are evident. This study was carried out to evaluate the initial and late morphological events related to the cell elimination that occurs in pre-pubertal Wistar rats, using light microscopy, immunohistochemical analysis and ultrastructural techniques.

MATERIAL AND METHODS

Forty-eight prepubertal male Wistar rats were kept under a 12-h light-dark cycle with water and food ad libitum. All animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Testis tissue processing

Testes were fixed in two different fixers and then embedded in two different plastic resins.

a) Hydrophilic embedding. Testes were fixed in 4% paraformaldehyde (Aldrich Chemical; Milwaukee, WS) in phosphate-buffered saline (PBS) at pH 7.2 for 2 hours at 4°C and then embedded in Lowicryl K4M (Polysciences, Inc.; Washington, PA).

b) Hydrophobic embedding. Testes were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences; Hatfield, PA) in PBS at pH 7.2 for 2 hours at room temperature. After rinsing in the same buffer, tissues were post-fixed in 1% osmium tetroxide (OsO_4) in PBS at pH 7.2 for 1 hour. Testes were then embedded in Epon (Embed-812, Electron Microscopy Science; Hatfield, PA).

Toluidine blue staining

Semi-thin sections (approx. 750 nm) of testes embedded in Epon resin were stained with toluidine blue. Sections were examined under a Nikon Eclipse E600 Microscope and images were recorded with a Nikon Digital DXM1200F Camera.

Active caspase-3 immunodetection

The testes embedded in Lowicryl K4M were serially sectioned. Sections approximately 750 nm thick from each testis were aligned on glass microscope slides covered with poli-l-lisyne (Sigma, St Louis, MO). The sections were treated with 10% H_2O_2 (Merck; Darmstadt, Germany) in phosphate-buffered saline (PBS) at pH 7.2 for 30 min to allow antibody-tissue interaction. After washing the slides with PBS, they were incubated with anti-active caspase-3 (Sigma, St Louis, MO) at a 1:100 dilution in PBS for 18 h at 4°C. Negative controls were made by omitting the primary antibody. Next, the slides were washed with PBS and incubated for 1 h under darkness at room temperature with anti-rabbit immunoglobulin coupled to fluorescein-5-isothiocyanate (FITC-isomer 1) (Ex max 492 nm; Em max 520 nm). Semi-thin sections were counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Ex 364 nm; Em 454 nm). Immunoassays were evaluated under a Nikon Eclipse E600 Microscope and images were recorded with a Nikon Digital DXM1200F Camera.

Electron microscopy assays

Semi-thin sections from the testes post-fixed with OsO_4 and embedded in Epon resin were obtained and stained with toluidine blue to highlight the areas of interest. Ultra-thin sections were mounted on grids and contrasted with 4% uranyl acetate and 0.4% lead citrate. Sections were examined under a JEOL 1010 electron microscope operated at 100 KV. Digital images were taken with a Hamamatsu camera.

RESULTS

Toluidine blue staining

The semi-thin sections allowed us to define the morphological characteristics of the seminiferous epithelium. Different high degrees of alteration in the damaged cells were identified.

ISSN 2347-6893

The seminiferous tubules from pre-pubertal rats consisted of several cell layers, and these tubules showed a central lumen of different sizes. Different cellular associations were observed in these organisms, some of which were similar to different stages of the spermatogenic cycle described for adult rats. Diverse cellular types were present, including type A spermatogonia, and primary spermatocytes in the zygotene and late pachytene phases (figure 1a). This particular cellular association is similar to that of stage XII in adult rats. Type B spermatogonia and spermatocytes transitioning from the preleptotene towards the pachytene phase were present in some of the seminiferous tubules (figure 1b). This association is similar to stages VI to VIII of the spermatogenic cycle in adult rats.

Other tubules revealed the presence of type A spermatogonia and spermatocytes in the leptotene and mid-pachytene phases (figure 1c). In this type of tubule, apoptotic bodies were present at the basal region of the epithelium in a cellular association that coincided with stage X in adults.

In a few seminiferous tubules, type A spermatogonia and early pachytene spermatocytes were abundant. In this cellular association, the type A spermatogonia were in the mitotic metaphase (figure 1d). These tubules were classified in stages I to VI of the spermatogenic cycle. At this age, the cytoplasm of the type B spermatogonia and preleptotene spermatocytes had vesicles, and those cells were surrounded by cytoplasmic prolongations of the Sertoli cells.

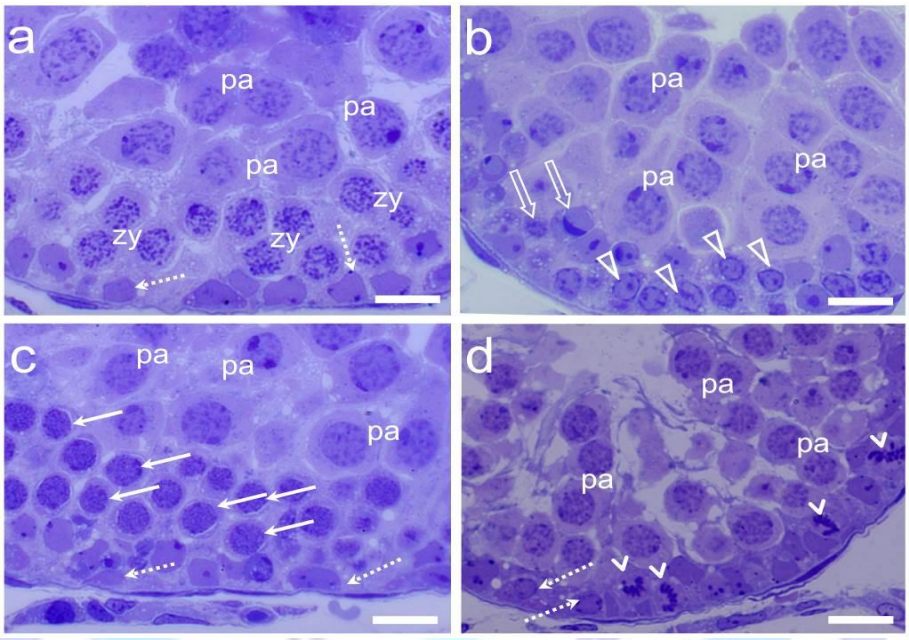

Fig 1: Semi-thin sections of seminiferous tubules stained with toluidine blue. a) Type A spermatogonia (dotted arrows) and spermatocytes in the zygotene (zy) and pachytene (pa) phases are present. b) Type B spermatogonia (empty arrowheads) and pachytene spermatocytes (pa). There are some apoptotic bodies in the basal region (empty arrows). c) Type A spermatogonia (dotted arrows), with leptotene spermatocytes present (arrows), and pachytene spermatocytes (pa). d) Type A spermatogonia (dotted arrows) and early-pachytene spermatocytes (pa). Mitotic type A spermatogonias (arrowheads). Scale bars 10 microns

We found type A spermatogonia and primary spermatocytes in the zygotene meiotic phase, accompanied by apoptotic bodies on the basal layer of the seminiferous tubules (figure 2). Likewise, when type A spermatogonia and early pachytene spermatocytes were present, the spermatocytes often had highly compacted chromatin that evidenced apoptotic alterations. The presence of preleptotene and early pachytene spermatocytes was frequently accompanied by cellular debris inside the lumen.

Some tubules consisted of type A spermatogonia, primary early-pachytene spermatocytes, and round spermatids (figure 3a). This kind of cellular association corresponds to stages I to III of the spermatogenic cycle of the adult rat. Type A spermatogonia and primary spermatocytes in the zygotene and late pachytene phases were observed in some tubules, in an association that corresponds to stages XI to XII (figure 3b). Some tubules evidenced altered primary spermatocytes with highly-compacted cytoplasm and chromatin (figure 4).

ISSN 2347-6893

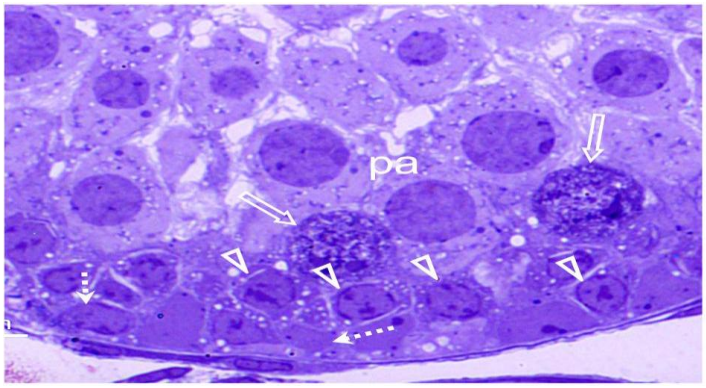

Fig 2: Semi-thin sections stained with toluidine blue from a seminiferous tubule. Several germ cells showing features of apoptotic cell death (empty arrows). Type A (dotted arrows) and type B spermatogonia (empty arrowhead). Spermatocytes have highly-compacted chromatin (empty arrows). pa: pachytene spermatocytes. Scale bar 10 microns

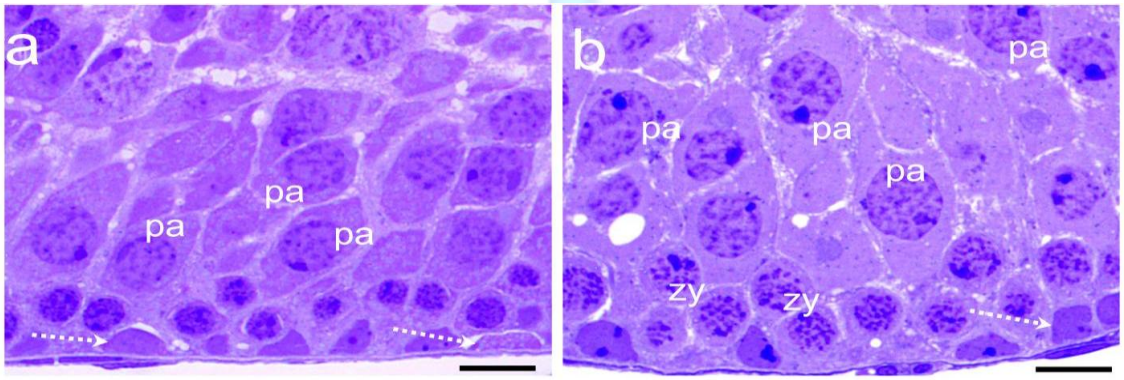

Fig 3: Semi-thin sections of a seminiferous tubule stained with toluidine blue. a) Type A spermatogonia (dotted arrows), primary spermatocytes in the early pachytene (pa) phase. b) Type A spermatogonia (dotted arrow), zygotene (zy) and late pachytene (pa) spermatocytes. Scale bars 10 microns

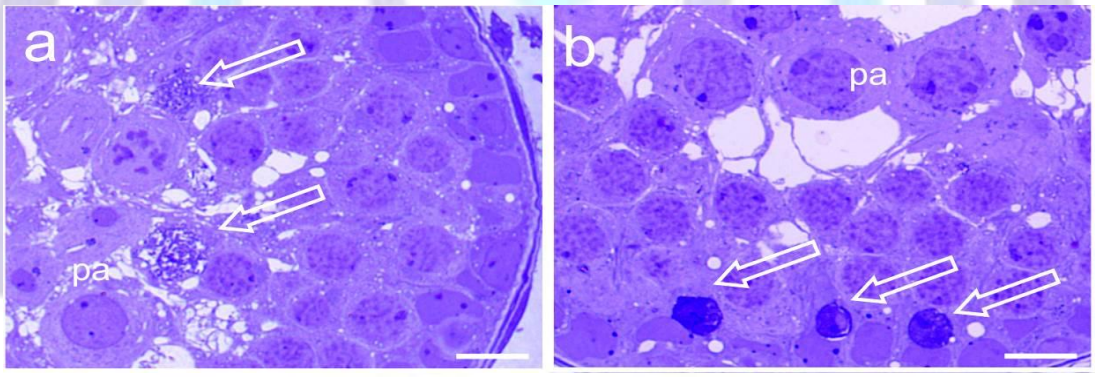

Fig 4: Section of a seminiferous tubule stained with toluidine blue. a) The distribution of cells in the seminiferous epithelium allows the identification of altered cells (empty arrow), Pachytene cells (pa) are detached from their neighbors (empty arrows). b) Several altered cell displays morphological characteristics evidencing an advanced apoptotic process (empty arrows). Scale bars 10 microns

Electron microscopy observations

The ultrastructural characteristics of the interior of the seminiferous tubules indicate the presence of different cellular associations that correspond to the optical interpretations observed. These ultrastructural findings revealed a series of tubules with normal and altered cells with different degree of alterations.

Low magnification allowed us to identify the organization of the germinal epithelium, where it was possible to identify Sertoli cells, type A and type B spermatogonia, as well as primary spermatocytes (figure 5a). Some tubules presented highly-compacted cells that reflect the advanced apoptosis process in which the cytoplasm and nuclear space are contracted with clumps of compacted chromatin (figure 5b). These cellular types correspond to those that were easily identified in preparations with toluidine blue stain.

ISSN 2347-6893

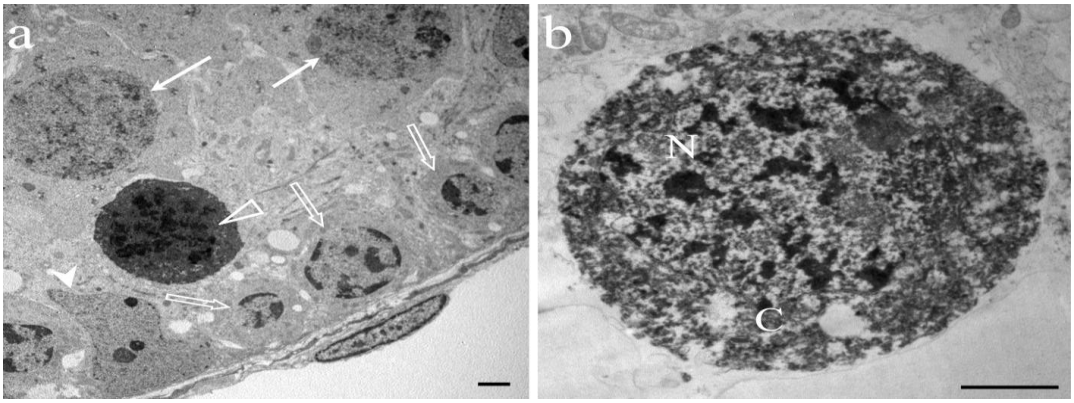

Fig 5: Ultrathin section of a seminiferous tubule. a) The distribution of cells in the seminiferous epithelium allows the identification of type B spermatogonia (empty arrow), Sertoli cells (arrowhead) attached to the basal membrane, and primary spermatocytes (arrows). An altered cell displays morphological characteristics that correspond to an advanced apoptotic process (empty arrowhead). b) High magnification of an apoptotic cell with highly-compacted chromatin inside the nucleus (N) and contracted cytoplasm (C). Scale bars: a -2 microns; b - 500 nanometers

Certain tubules exhibited cells with ultrastructural characteristics that showed normal cytoplasm. These normal germinal cells had a cellular arrangement that included Sertoli cells, and were completely joined such that they showed close unions among neighboring cells (figure 6a). Other seminiferous tubules had Sertoli cells, primary spermatocytes, and several cytoplasmic prolongations of the Sertoli cells located in the lumen (figure 6a). The cells positioned towards the lumen showed slight alterations that were not perceptible under light microscopy observation. The alterations included mitochondrial dilatation in the spermatocytes (figure 6b) but not in the Sertoli cells, which presented the normal mitochondrial cristae and membrane structure, as well as an elongated shape. Some primary spermatocyte had a highly-degraded cytoplasm (figure 6c).The non-normal mitochondria were identified by dilated cristae (figure 6d).

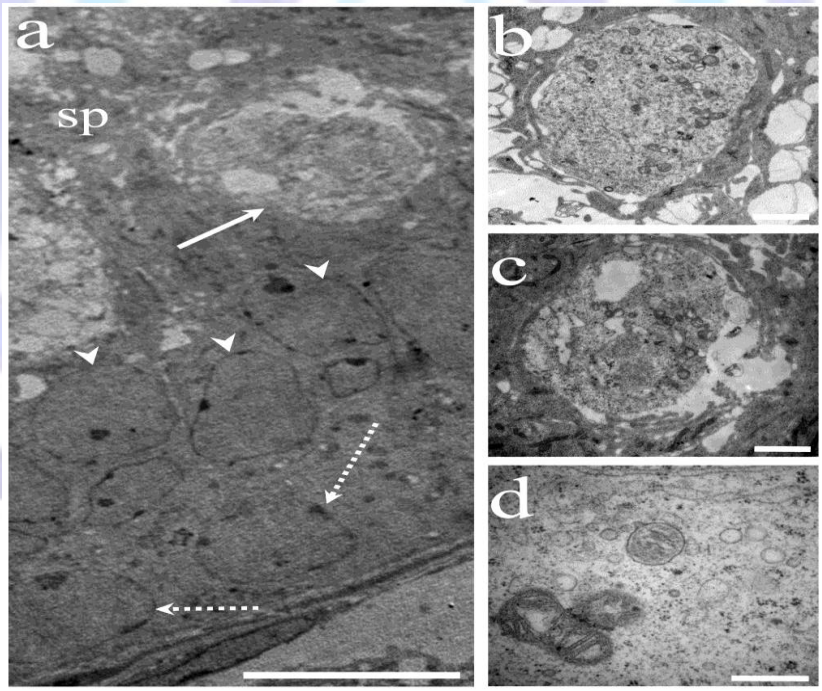

Fig 6: Ultrathin section of a seminiferous tubule. a) Low magnification shows the presence of Sertoli cells (arrowhead), type A spermatogonia (dotted arrow), and altered spermatocyte (arrow). In the lumen, several cytoplasmic prolongations from the Sertoli cells are present (sp). b) The primary spermatocyte is highly-altered; the mitochondria are greatly-dilated. c) Primary spermatocyte in an advanced degree of degeneration with highly-degraded cytoplasm. d) High magnification shows that mitochondria with crest-swelling are very frequent. Scale bars: a -5 microns; b and c -2 microns; d -500 nanometers

It was possible to identify different cellular types of altered cells, from type A and type B spermatogonia to pachytene spermatocytes. Some of the alterations observed in the type A and type B spermatogonia consisted of nuclear envelope dilatation, evidenced by the detachment of the external membrane of the nuclear envelope from the inner one. Also, some zygotene spermatocytes presented such alterations as a nuclear envelope and dilated mitochondria (figure 7).

ISSN 2347-6893

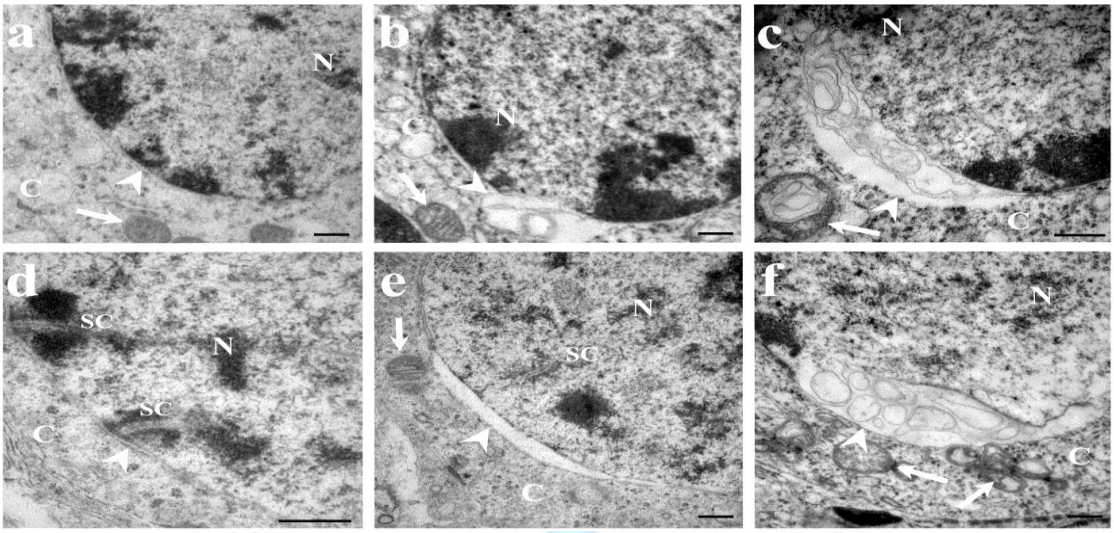

Fig 7: High magnification of altered cells. a) Normal Type A spermatogonia. In the periphery of the nucleus there are clumps of normal compact chromatin; the nuclear envelope is not dilated (arrowhead). The mitochondria have normal cristae (arrows). b) and c) Type A spermatogonia with altered nuclear envelopes; the external membrane (arrowhead) is detached from the inner one. There is an initial swelling of the mitochondria (arrow); in c) the separation of both membranes of the nuclear envelope is more evident (arrowhead) as is mitochondrial swelling (arrow). d) Normal pachytene spermatocyte. Several synaptonemal complexes can be seen (sc), the nuclear envelope is conserved and the cytoplasmic compartment is normal. e) and f) An altered primary spermatocyte with the nuclear envelope dilated (arrowheads). In f) the mitochondria are highly-dilated (arrows). N: nucleus; C: cytoplasm. Scale bars: 500 nanometers

Immunodetection of active caspase-3

Active caspase-3 immunodetection allowed us to identify the initial biochemical changes that take place during the apoptosis process in the seminiferous tubules of pre-pubertal rats. The immunodetection processes also permitted identification of the cells that are present at the beginning of cell death, though the morphological changes were revealed and identified by toluidine blue staining.

We found that the primary spermatocytes were positive to immunolocalization (figure 8). In some tubules, the pachytene spermatocytes near the lumen of the tubule were positive to active caspase-3 immunodetection, while others with a similar position and morphology were not.

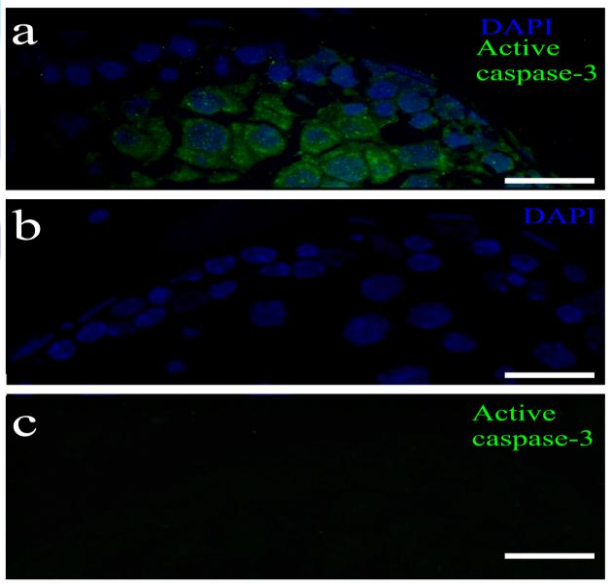

Fig 8: Immunolocalization of active caspase-3 in seminiferous tubules. The blue fluorescence corresponds to DAPI staining of DNA. Green fluorescence corresponds to active caspase-3 immunodetection. a) Seminiferous tubule showing pachytene spermatocytes positive for detection of active caspase-3. b) and c) Illustrate the negative controls of immunodetection of seminiferous tubule. b) DAPI-staining evidences several nuclei in the seminiferous tubule; and c) is the image of the negative control in the absence of the primary antibody. Scale bars: 20 microns

DISCUSSION

Apoptotic cell death is an event responsible for maintaining cellular homeostasis in numerous tissues. In mammal testes, apoptosis plays an important role during cell maturation and renewal (Huckins, 1978; Kerr et al., 1979; Allan et al., 1992; Tapanainen et al., 1993). Several studies have demonstrated the apoptosis process during the loss of germ cells in the seminiferous epithelium, but that observations illustrate the advanced phases of apoptosis, including DNA fragmentation, highly-compacted chromatin, and cytoplasmic shrinking (Allan et al., 1992; Billig et al., 1995; Stumpp et al., 2004). In this study, we used toluidine blue staining, active caspase-3 immunodetection, and transmission electron microscopy to identify the initial and advanced characteristics of cellular elimination in the seminiferous epithelium pre-pubertal Wistar rats.

Toluidine blue staining made it possible to evaluate the tissular organization present in the seminiferous tubules of different-aged rats. We were able to identify several types of cellular associations with arrangements similar to those described in adult organisms. The seminiferous tubules from rats had a cellular distribution that evoked the different stages of the spermatogenic cycle in adult males, including stages I to VI, VI to VII, stage X, and stage XII of the spermatogenic cycle of mature males (Hermo et al., 2010; Leblond and Clermont, 1952). In these seminiferous tubules, some pachytene cells were highly-altered with greatly-compacted chromatin.

Staining with toluidine blue also allowed us to identify such morphological characteristics as highly-compacted cells revealed under intense staining. These cells were sometimes detached from their neighbors and corresponded to pachytene spermatocytes. Using this technique we were able to identify the more advanced cellular alterations that pertain to the classic apoptosis process.

Apoptosis is a highly-regulated process in which proteases called caspases are the principal actors. The morphological patterns of apoptosis are associated with the activities of caspases in their active form. Caspases exist inside cells as latent zymogens divided into two broad groups known as initiator caspases (such as -8, -9 -10), and executer caspases (-3, -6, -7) (Los et al., 1999). Activation of the executer caspases results in the degradation of several intracellular substrates which cause the morphological changes that characterize apoptosis (Jänicke et al. 1998; Zhen et al. 1998; Woo et al., 1998). The presence of active caspase-3 indicates the onset of the process of cell death. Initial biochemical activation is not detectable in the form of evident alterations even though the cell is undergoing an elimination process. These characteristics make active caspase-3 detection a useful method for identifying the initial cellular alterations that eventually lead to cell death, even before the definitive features of apoptosis become manifest. After this morphological characterization and identification of highly altered cells with the toluidine blue staining, we used active caspase-3 immunodetection to evaluate the correspondence between the morphological alterations and the activation of pro-apoptotic proteases. However, several of the labeled cells showed an apparently normal morphology that indicated the beginning of the process of cell death, before the apoptotic characteristics were evident.

Using the technique of toluidine blue staining, on several occasions we observed cellular populations that showed an apparently normal morphology; however, when the same sample was analyzed using the biochemical procedures of immunodetection, they proved to be positive to active caspase-3. This difference may have arisen primarily because the cells were in different stages of apoptosis. This means that the cells that were positive to immunodetection were in the initial phase of apoptosis, but could not be identified with toluidine blue staining because they were not yet sufficiently altered.

The cells that were positive to the immunodetection of active caspase-3 accomplished using ultrastructural observations corroborate that the initial alterations corresponded to the onset of cell death in the cells that were positive to active caspase-3. Our observations indicate that the classic mitochondrial ultrastructure of two membranes with cristae had different degrees of dilatation, showing heavily-damaged mitochondria with dilated matrixes and fragmented cristae. It is well known that mitochondria are the main intracellular targets involved in apoptotic cell death (Condello et al., 2013). The ultrastructural analyses offer significant advantages, because they make it possible to evaluate modifications of the cellular organelles, such as early changes in the mitochondria and the nucleus. The electron microscopy techniques are considered a sensible method to visualize either early or late morphological changes at high ultrastructural level (White and Cinti, 2004). The combined use of these two techniques demonstrates that a large number of apparently normal cells were actually altered.

The present study contributes to the design of strategies for the study and characterization of the processes of cell death.

ACKNOWLEDGMENTS

This work was supported by the grants CONACYT 180526 and PAPIIT IN212912. The authors would like to thank Ernestina Ubaldo Pérez for technical assistance. They also thank Paul C. Kersey Johnson for reviewing the English word usage and grammar.

REFERENCES

[1] Allan, D.J., Harmon, B.V., Roberts, S.A. 1992. Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. Cell Proliferation, 25:241-250.

[2] Billig, H., Furuta, I., Rivier, C., Tapanainen, J., Parvinen, M., Hsueh, A.J. 1995. Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology, 136(1):5-12. doi: http://dx.doi.org/10.1210/endo.136.1.7828558

[3] Clermont, Y. 1962. Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. American Journal of Anatomy, 111–129.

[4] Cohen, G.M. 1997. Caspases: the executioners of apoptosis. Biochem J, 326:1-16.

[5] Condello, M., Caraglia, M., Catellano, M., Arancia, G., Meschini S. 2013. Structural and functional alterations of cellular components as revealed by electron microscopy. Microsc Res Tech, 76(10):1057-1069. doi: 10.1002/jemt.22266

[6] Danial, N.N., Korsmeyer, S.J. 2004. Cell death: critical control points. Cell, 116:205-219. doi: 10.1016/S0092-8674(04)00046-7

[7] Earnshaw, W.C., Martins, L.M., Kaufmann, S.H. 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem, 68:383-424. doi: 10.1146/annurev.biochem.68.1.383

[8] Fischer, U., Jänicke, R.U., Schulze-Osthoff, K. 2003. Many cuts to ruin: a comprehensive update of caspase substrates Cell Death and Differentiation, 10:76-100. doi:10.1038/sj.cdd.4401160

[9] Fuentes-Prior, P., Salvesen, G.S. 2004. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J, 384:201-232. doi:10.1042/BJ20041142

[10] Hermo, L., Pelletier, R., Cyr, D., Smith, C. 2010. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. Microsc Res Tech, 73(4):241-278. doi: 10.1002/jemt.20787

[11] Hikim, A.P., Wang, C., Leung, A., Swerdloff, R.S. 1995. Involvement of apoptosis in the induction of germ cell degeneration in adult rats after gonadotropin-releasing hormone antagonist treatment. Endocrinology, 136:2770-2775. doi: http://dx.doi.org/10.1210/endo.136.6.7750502

[12] Huckins, C. 1978. The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. Anat Rec, 190:905-926.

[13] Jänicke, R.U., Sprengart, M.L., Wati, M.R., Porter, A.G. 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem, 273(16):9357-9360. doi: 10.1074/jbc.273.16.9357

[14] Kerr, J.B., Rich, K.A., de Kretser, D.M. 1979. Effects of experimental cryptorchidism on the ultrastructure and function of the Sertoli cell and peritubular tissue of the rat testis. Biol Reprod, 21: 823-838.

[15] Kerr, J.F., Wyllie, A.H., Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer, 26(4):239-257.

[16] Leblond, C.P., Clermont, Y. 1952. Definition of the Stages of the Cycle of the Seminiferous Epithelium in the Rat. Ann N Y Acad Sci, 55(4):548-573. doi: 10.1111/j.1749-6632.1952.tb26576.x

[17] Los, M., Wesselbourg, S., Schulze-Osthoff, K. 1999. The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity, 10:629-639. doi: 10.1016/S1074-7613(00)80062-X

[18] Mori, C., Nakamura, N., Dix, D.J., Fujioka, M., Nakagawa, S., Shiota, K., Eddy, E.M. 1997. Morphological analysis of germ cell apoptosis during postnatal testis development in normal and Hsp70-2 knockout mice. Dev Dyn, 208:125-136. doi:10.1002/(SICI)1097-0177(199701)208:1<125::AID-AJA12>3.0.CO;2-5

[19] Russell, L.D., Etllin, R.A., Sinha Hikim, A.P., Clegg, E.D.1990. Histological and Histopathological Evaluation of the Testis. Cache River Press, Clearwater, Florida.

[20] Sharpe, R.M. 1994. Regulation of spermatogenesis. In: The Physiology of Reproduction pp 1363-1434 Eds E Knobil and JD Neill. Raven Press, New York.

[21] Stumpp, T., Sasso-cerri, E., Freymüller, E., Miraglia, S.M. 2004. Apoptosis and testicular alterations in albino rats treated with etoposide during the prepubertal phase. The Anat Record Part A, 279A:611-622. doi: 10.1002/ar.a.20045

[22] Tapanainen, J.S., Tilly, J.L., Vihko, K.K., Hsueh, A.J.W. 1993. Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. Mol Endocrinol, 7:643-650. doi: http://dx.doi.org/10.1210/mend.7.5.8316250

[23] Thornberry, N.A., Lazebnik, Y. 1998. Caspases: enemies within. Science, 281:1312-1316. doi: 10.1126/science.281.5381.1312

[24] White, M.K., Cinti, C. 2004. A morphologic approach to detect apoptosis based on electron microscopy. Methods Mol Biol, 285:105-111.

[25] Woo, M., Hakem, R., Soengas, M.S., Duncan, G.S., Shahinian, A., Kägi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S.A., Senaldi, G., Howard, T., Lowe, S.W., Mak, T.W. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev, 12(6):806-819.

[26] Zheng, T.S., Schlosser, S.F., Dao, T., Hingorani, R., Crispe, I.N., Boyer, J.L., Flavell, R.A. 1998. Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis in vivo. Proc Natl Acad Sci U S A, 95(23):13618-13623. doi: 10.1073/pnas.95.23.13618