Institute of Experimental Morphology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 12
Sofia • 2007

Ultrastructural Characteristics of Germ Cell Apoptosis in Adult Rats after Treatment with Ethane Dimethanesulfonate (EDS)

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EDS selectively and temporary destroys Leydig cells in the adult rat testis and thus suppresses serum and intratesticular testosterone levels. Testosterone withdrawal results in marked increase of germ cell apoptosis and disturbance of spermatogenesis. We aimed to identify more precisely the apoptosis of germ cells at ultrastructural level. The present study provides detailed description of different phases of apoptotic process. We distinguished the specific appearance of apoptotic features in different germ cell types, spermatogonia, spermatocytes and round spermatids. The specificity of apoptotic manifestation involved the manner of chromatin clump condensation and localisation as well as pattern of cellular organelles disintegration. In situ detection of DNA fragmentation and ultrastructural investigations in tandem with biochemical studies on spontaneous and induced apoptosis, open new perspectives for understanding the importance of germ cell death in normal and pathological events in the spermatogenesis.

Key words: EDS, apoptosis, spermatogenesis, electron microscopy.

Introduction

Apoptosis is an active cellular process of gene-directed self-destruction. In vivo apoptosis is detected primarily in proliferating tissues such as the epithelium of the adrenal cortex, the germinal centers of lymph nodes, and the seminiferous epithelium of the testis [11]. Germ cells at different stages of the spermatogenic cycle possess their own physiological features and respond differently to exogenous stimuli. To date, much data are available showing that germ cell apoptosis is found spontaneously [1, 4] and also it can be induced by exposure to hyperthermia [16, 7], chemotherapeutic drugs [13], radiation [5] and chemical agents such as ethane dimethanesulfonate (EDS). EDS is known to destroy selectively Leydig cells in the testis and thus suppressed serum and intratesticular testosterone to undetectable levels [7].

Adult mammalian spermatogenesis is a testosterone-dependent process. Despite decades of studies, however, the mechanism (s) by which testosterone regulates spermatoge-

nesis remains uncertain. It has been shown that cell loss by apoptosis occurs normally during spermatogenesis [6, 9]. Recent studies have shown that testosterone withdrawal from the rat testis results in increased germ cell apoptosis [12, 18, 19], suggesting that testosterone may function as a cell survival factor, in some way protecting germ cell from apoptotic death.

The molecular mechanism by which testosterone does so, however, has not yet been elucidated. The regulation of apoptosis is dependent upon specific gene production. Bcl-2 multigene family is known to includes antiapoptotic genes Bcl-2, Bcl-x (promote cell survival by inhibiting apoptosis) and pro-apoptotic genes Bax, Bad, Bak (induce cell death by blocking the ability of Bcl-2 to inhibit apoptosis. An alternative and more rapid way for programmed cell death is mediated by Fas pathway. Fas lygand is a transmembrane protein that can initiate apoptosis by binding to Fas-receptor expressing cells activating caspase enzyme cascade [21]. Ever since it was proposed as a model of cell death [8, 13], apoptosis has generally been visualized by its morphological feature. In this study, rat testes exposed to EDS treatment were examined histologically and by TUNEL method (terminal transferase-mediated digoxigenin-11-dUTP nick end labeling) as well as by electron microscopy. The examination was undertaken to identify more precisely the different stages of apoptotic process at ultrastructural level.

Materials and Methods

Adult male Wistar rats received a single intraperitoneal injection of EDS at dose of 75 mg/kg body weight. The animals were killed on days 1, 3, 7, and 21 after treatment. One testis was fixed in Bouin's solution, embedded in paraffin, and examined by light microscope. In situ assay of apoptosis: apoptotic cells were detected by using terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) method that resulted in a high degree of specificity and low background staining [14] and the quantitative assessment of apoptosis was performed according method of W o o l v e r i d g e et al. [21]. Electron microscopy: testicular fragments were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, 1% osmium tetraoxide and embedded in Durcupan. Electron micrographs were made on an Opton EM 109.

Results

As we previously have shown [2] first signs of seminiferous epithelium regression were manifested three days post EDS treatment by marked increase in frequency of apoptotic germ cells identified by specific TUNEL method. The highest values of parameters for quantification of germ cell apoptosis (the number of apoptotic cells per tubule; the percentage of tubules with apoptosis and the apoptotic index) were found by 7 day after EDS which corresponded to the lowest plasma level of testosterone.

Electron microscopy confirmed the apoptotic nature of germ cell degeneration - at various phases of destruction apoptotic cells were surrounded by healthy neighbours. Ultrastructural observations revealed a unique set of degenerating germ cells, which were characterised by intense chromatine condensation, the shrinkage of cellular components and fragmentation of nuclear materials. Apoptotic cell death is characterised by nuclear and cytoplasmic condensation and usually affects single cells or small groups of cells in an asynchronous fashion. According to these features, the process of apoptosis of germ cells could be divided into three discrete stages: early, intermediate and late according to A l l a n et al. [1]. Apoptotic cells in the basal compartment of seminiferous tubules were

spermatogonia. We were also able to find the three stages of apoptosis (Fig. 1 A, B, C). In early stage of apoptosis the cheterochromatin was condensed and arranged in several sharply defined clumps which abutted against the nuclear membrane. The cytoplasm exhibited many vacuoles and dilated cisternae of the endoplasmic reticulum. The mitochondrial destruction also can be observed. Further, in the intermediate stage a massive clump of chromatin was present in the centre of nucleus and the cytoplasmic organelles were degenerated and as a result the whole cell profoundly diminished in size compared with intact spermatogonia (Fig. 1B). Finally, further disruption of the nuclear and cellular remnants was recognised and apoptotic bodies were formed (Fig. 1C).

More advanced germ cell types, spermatocytes and round spermatids also underwent apoptosis and they passed through the same stages of apoptotic process. The nucleus of apoptotic pachytene spermatocyte contained increasing amounts of chromatin. A dilatation of perinuclear space, cytoplasm vacuolisation and destruction of cellular organelles were observed (Fig. 2A). Final stages of apoptosis showed extensive shrinkage of nucleus and cytoplasm (Fig. 2B). The early apoptosis of round spermatids showed small clumps of cheterochromatin in the nucleus which were not seen in intact cells (Fig. 3A). In the later phase of apoptosis, the amount of chromatin increased and shrinkage of the cell with the complete condensation of the chromatin were appeared (Fig. 3B). In latest phases of apoptosis it is not possible to recognise the defined germ cell types and stages of differentiation.

Discussion

Testosterone withdrawal induced by EDS is a useful model for the in vivo investigation the possible role of apoptosis in the control of spermatogenesis, a phenomenon which can be considered as a result of an intriguing integration among cell proliferation, cell differentiation and cell death [20].

Although some biochemical features of apoptosis are now available, the in situ identification of DNA fragmentation by TUNEL technique and electron microscopy are the most reliable methods for detection of apoptosis.

The present study provides detailed description of different phases of apoptotic process. It was confirmed the apoptotic nature of dying cells from different stages of germ cell differentiation–spermatogonia, pachytene spermatocyte and round spermatids. We were able to visualise the common apoptotic stages described by A l l a n et al. [1], K o j i et al. [10]. Moreover, we distinguished the specific appearance of apoptotic features in different germ cell types, spermatogonia, spermatocytes and round spermatids. The specificity of apoptotic manifestation involved the manner of chromatin clump condensation and localisation as well as pattern of cellular organelles desintegration.

Several studies have indicated that spermatocytes and spermatids that died spontaneously or were killed by cytotoxic agents did not appear to be apoptotic [17]. In contrast, other authors based largely on the TUNEL methods have reported that cell death by apoptosis can be induced in spermatocytes or spermatids by hormonal changes or hyperthermia [3, 15]. The apoptosis could be triggered by many signals including the Fas and Fas ligand system and/ Bcl-2 family in the developing and adult testis [12, 18, 21]. N a n d i et al. [12] showed that EDS injection induced Fas-mediated germ cell apoptosis as a result of androgen ablation indicating an important role for testosterone in germ cell survival via suppression of Fas.

Recently S h o w et al. [17] proposed an alternative hypothesis about mechanism of spermatids death, so called anoikis involving loss of the attachment ability to the Sertoli cell (attachment-dependent cell from basal membrane) in contrast to apoptosis of less matured germ cells (spermatogonia and spermatocytes) induced by testosterone withdrawal.

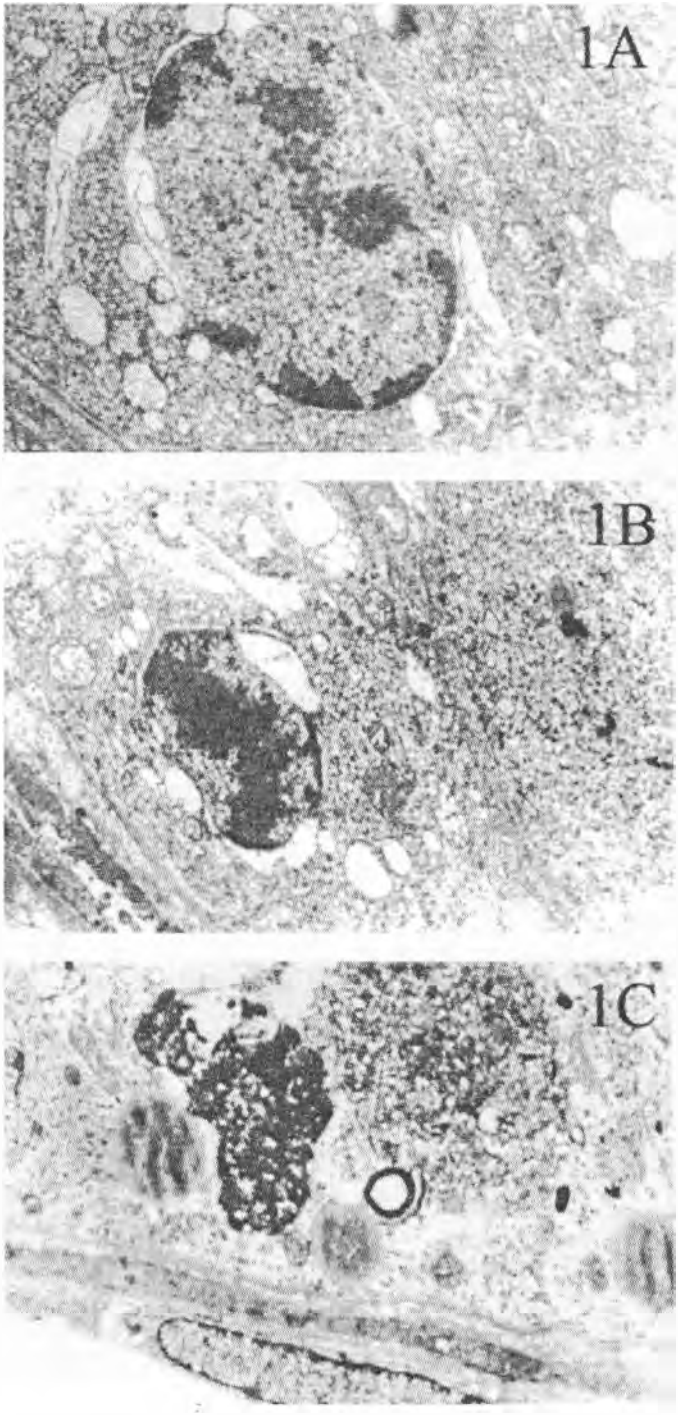

Fig. 1. Electron micrographs of spermatogonia at different stages of apoptosis (× 7000)
A — spermatogonium at early stage of apoptosis; B — an intermediate stage of apoptotic spermatogonium; C — late apoptotic stage with formation of apoptotic bodies

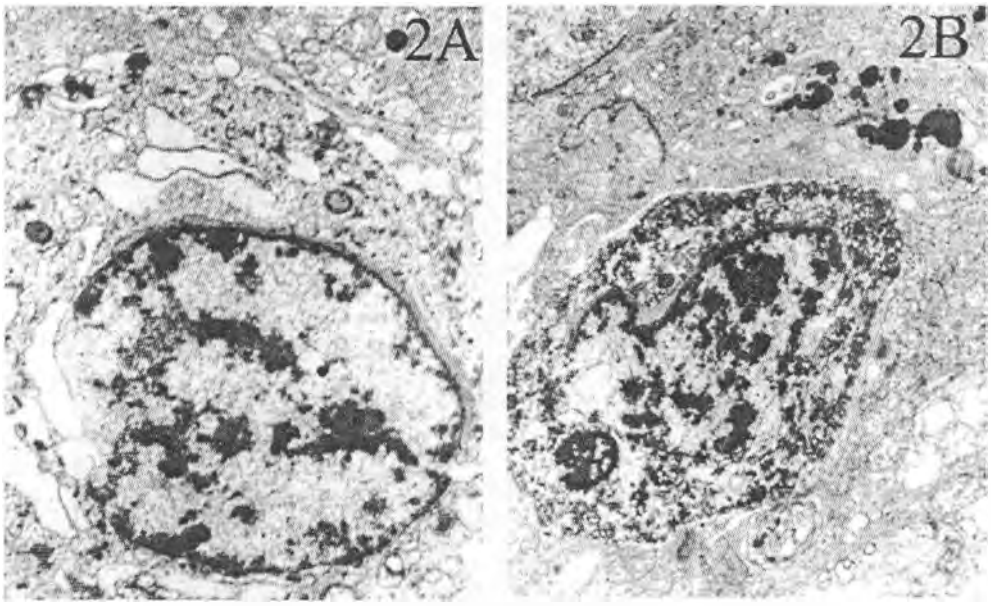

Fig. 2. Electron micrographs of spermatocytes at different stages of apoptosis (× 7000) pachytene spermatocytes in intermediate (A) and more advanced (B) state of apoptosis

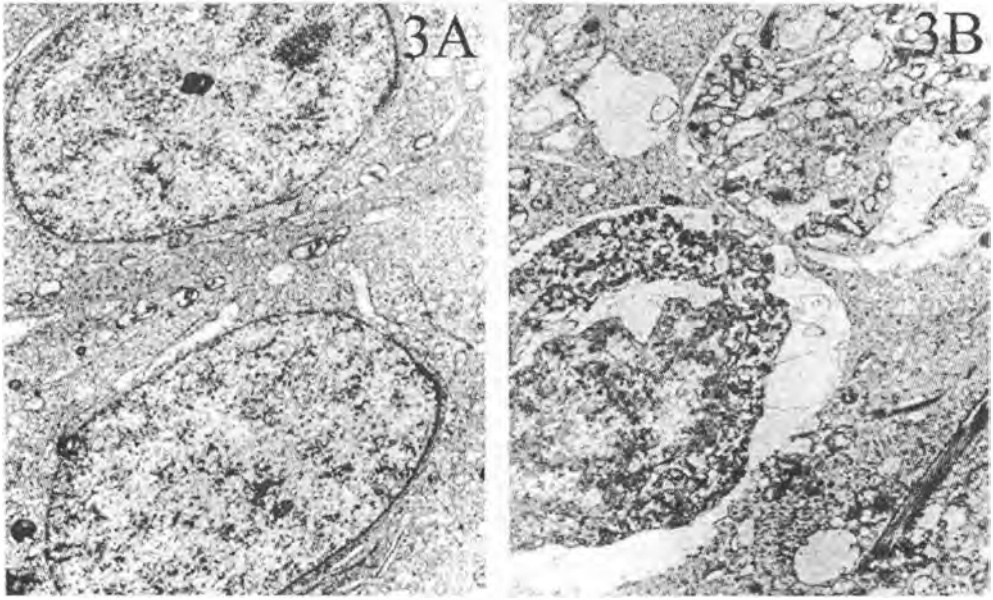

Fig. 3. Electron micrographs of round spermatids (× 4400)
A — two intact round spermatids; B — two spermatids in late stage of apoptosis

In situ detection of DNA fragmentation and ultrastructural investigations in tandem with biochemical studies on spontaneous and induced apoptosis, open new perspectives for understanding the importance of germ cell death in normal and pathological events in the spermatogenesis. Apoptosis which happens selectively to certain spermatogenic cells maintains normal spermatogenesis. However, unbalanced apoptosis could lead to spermatogenic dysfunction and infertility. Thus, a thorough understanding of the apoptosis mechanism might uncover the causes for many testicular failures and help to find efficient strategies against these defects.

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