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Ultrastructure of apoptotic T lymphocytes and thymic epithelial cells in early postnatal pig thymus

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Abstract: The thymus is a primary lymphoid organ that provides the microenvironment required for the development of T lymphocytes. Thymic epithelial cells (TECs) are among the most critical components in the thymic microenvironment supporting thymocyte selection and maturation. Apoptosis is primarily a physiologic process that plays a major role during the development of T lymphocytes. The first purpose of this study was to examine morphological changes in apoptotic thymocytes by transmission electron microscope in piglets. The second purpose was to reveal the morphological typology of TECs in piglets. Tissue samples of the thymus were taken from healthy Yorkshire race pigs of two different age groups (7 days and 1 month). Ultrastructurally, apoptotic lymphocytes showed similar morphologies, but they also had differences in distribution. In both groups a high density of apoptotic and proliferating thymocytes was found in the cortex. The rate of apoptotic thymocytes in the cortex and medulla increased with age, respectively. It was observed that the TECs had various morphological subtypes in both the cortex and the medulla. A similar cellular distribution pattern of TECs was seen in both groups. It was concluded that the morphological diversity in the TECs may be related to the form–function relationship and the metabolic process.

Key words: Pig, ultrastructure, thymus, T lymphocyte, thymic epithelial cells

1. Introduction

Due to physiological, anatomical, and developmental similarities to humans, researchers have been increasingly using the pig as a model of choice for research designed to develop new treatments (1). In particular, the immune system of pigs is very similar to its human counterpart in terms of anatomy, organization, and response (2). This makes the pig more advantageous than other experimental animals for various purposes such as studying specific disease models, xenotransplantation, and usage as a large animal model for educational and research purposes. Moreover, it is clear that results obtained from pig-oriented studies have higher applicability and reliability for humans compared with other animal models (3).

The thymus of the pig is a highly specialized primary lymphoid organ for T lymphocyte maturation. During the first third of gestation, this organ is colonized by T lymphocyte precursors, originating from the hematopoietic stem cells within the bone marrow (4). T lymphocytes are highly specific cells that contribute to the management and regulation of the immune system in mammals for functions such as resistance against diseases and cellular immunity (5). Thymic epithelial cells (TECs) form a stromal network to essentially shape the T lymphocyte repertoire in the thymus. Based on their location, these cells are divided into cortical and medullary TECs, which are phenotypically and functionally different. TECs play a crucial role in thymocyte positive and negative selections. Mutual interactions between T lymphocytes and TECs are needed for the development of a fully functioning parenchyma in thymus (6).

Apoptosis is a type of cell death that plays a role in several physiological and pathological processes. It is a key regulator in achieving tissue homeostasis by providing a balance between cell proliferation and cell death (7). In the primary lymphoid organs, and especially in the thymus, apoptosis is required for lymphocyte development and homeostasis. T lymphocytes are removed by apoptosis during their developmental processes (8). In other words, approximately 95% of ineffective T lymphocytes or those that have the potential of reacting against their own tissues are eliminated by apoptosis before they enter the blood circulation. In this context, apoptosis has an important role in controlling the lymphocyte population in both the thymus and peripheral circulation, and in achieving central and peripheral tolerance (9).

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As is known, development of the immune system has various developmental differences among different mammal species. As a result of pigs having an epitheliochorial placenta, fetal pigs continue their growth in a completely germ-free environment. This situation provides great ease and benefits in various ways while examining the steps of immune system development in newly born piglets (10). Nevertheless, studies on lymphocyte biology in pigs are mostly conducted with lymphoid organs obtained from pigs that have reached the age of slaughter (11). Pigs at this stage has a lower chance of providing reliable information for researchers due to the diseases they have experienced, antigen accumulations, and the stress they experience prior to slaughtering. Moreover, many researchers work only on lymphocytes in peripheral circulation (12). Today, there is still very little known about T lymphocyte morphology and physiology in postnatal piglets. In recent years, the number of phenotypic and molecular studies related to the pig thymus has been increasing. However, the number of studies on the morphological characteristics of the thymus is small (13).

The aim of this study was to describe the morphology of the apoptosis process taking place in the thymus, which has an important role in the immune system of postnatal piglets, on the level of fine structures, and to morphologically classify TECs, which have a significant role in shaping the microenvironment in the development of T lymphocytes.

2. Materials and methods

2.1. Animal material

A total of 14 healthy Yorkshire pigs used as material in the study were supplied by the Teaching and Research Farm of the Faculty of Veterinary Medicine at Ankara University. The pigs were divided into two age groups as 7 and 30 days old and their thymuses were collected right after euthanasia by intravenous injection of a 26% solution of sodium pentobarbital. For all procedures, approval number 2012-1/2012-4/2012-1-5 was received from the Local Ethics Board of Animal Experimentation at Ankara University.

2.2. Light microscopy

Thymus tissue samples were fixed for 24 h in 10% buffered formalin solution (pH 7.2–7.4). The tissues, which were left in a running water bath for 24 h to remove the formalin, were left in each of 70%, 80%, and 96% alcohol for 1 h to achieve dehydration. This was followed by three 1 h applications of absolute alcohol. To prevent the tissues from hardening and to polish them three 12 h applications of xylol were done. The tissue samples were then blocked in Paraplast using a paraffin dispenser and stored at room temperature. Mallory's modified triple staining method was used on the cross-sections with thickness of 4 μm obtained from the paraffin blocks by microtome. General histological analyses of the tissues were made using this staining. All findings were analyzed by light microscope (Leica DM 2500) and imaged (Leica DFC 450).

2.3. Transmission electron microscopy

Tissue samples collected from each pig were initially fixed in glutaraldehyde-paraformaldehyde, washed in cacodylate buffer, and fixed for the second time in 2% osmic acid. They were then blocked in Araldite M by passing them through alcohols in different concentration and propylene oxide. After marking the needed region on the examined semithin cross-sections, cross-sections of 300–400 Å in thickness were taken. After these cross-sections were turned into grids, they were passed through uranyl acetate and lead citrate stains (14) and examined with a Philips CM 100 transmission electron microscope. The study by De Waal and Rademakers (15) was taken as a basis for defining epithelial cells morphologically. The electron density of the nucleus and cytoplasm was used as the main criterion to distinguish cells from each other. In addition to "pale" and "dark" cells, other cells showing intermediate characteristics along this spectrum were defined as "middle" cells. The shape of the nucleus was used to distinguish pale cells (round nucleus) and other cells (irregularly shaped nucleus). Dark cells were distinguished from middle cells by their electron-dense ground substances.

3. Results

A completely developed thymus was observed in both groups in examinations with the light microscope. It was seen that the capsule surrounding the lobes was wide; septa separating the organ into lobules were noticeable. There was a dense and spread collection of cells in the general parenchyma of the thymus; the cortex and medulla were easily separated. The corticomedullar junction between the cortex and medulla was noticeable in both groups (Figure 1A).

In examinations by transmission electron microscope, the T lymphocytes in both groups were tightly packed, and much more densely so in the cortex. Lymphocyte membranes were in contact in many places. The shapes of T lymphocytes were round, elliptic, or polygonal. The T lymphocytes had very large nuclei with just a little ring-shaped cytoplasm. The cortex also had lymphocytes with smaller nuclei. Chromatin was dense around the nuclear membrane in the nucleus with electron-dense appearance. The cytoplasm was poor in terms of organelles. It was noticeable that some lymphocytes became larger and the chromatin of their nuclei was apparent. These lymphocytes observed in the cortex in noticeable large amounts were in different stages of mitosis (Figure 1B).

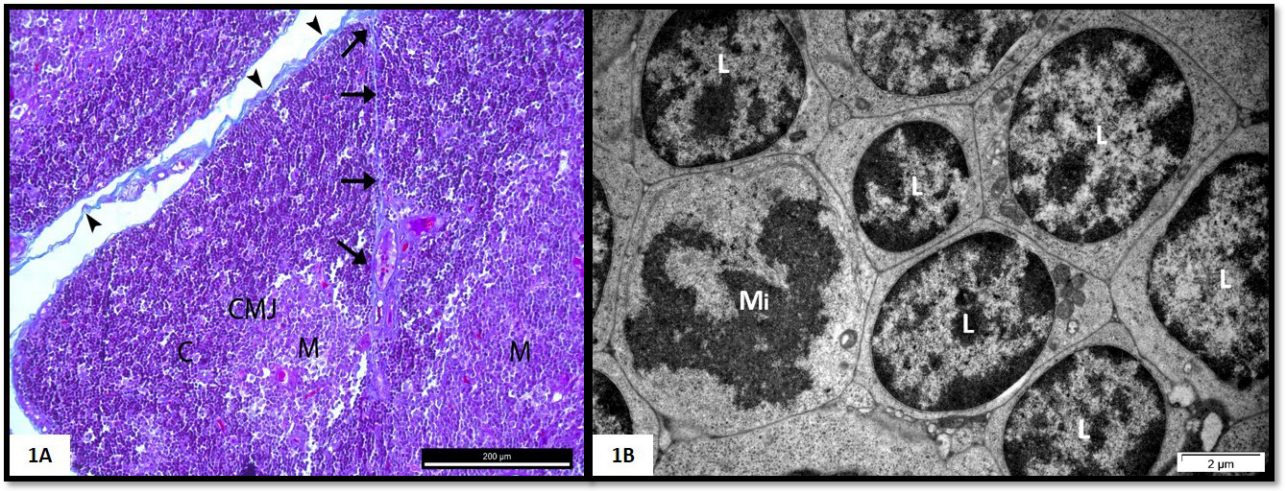

Figure 1. A) General histological features of pig thymus at day 7. Thymic capsule (arrowheads) and septa (arrows), cortex (C), medulla (M) and corticomedullar junction (CMJ). B) General ultrastructure of T lymphocytes (L) and a mitotic T lymphocyte (Mi).

TECs found in the cortex were seen to have differences in terms of morphology and placement. It was possible to divide these cells into four groups. The first group of cells was located so as to form an external epithelium barrier in subcapsular and perivascular areas. These cells were observed to be rich in tonofilaments and have varying degrees of electron density (Figure 2A). The second group of cells was scattered throughout the cortex, while being located more densely in the outer cortex. These cells had pale cytoplasm and nuclei with low electron density. In addition to the round, euchromatic nuclei of the cells, their highly developed cytoplasmic organelles were noticeable, whereas tonofilaments were rarely seen (Figure 2B). Cells in the third group were seen more in the inner cortex and had an irregular distribution. The nucleus had an irregular shape in these cells with a denser (middle) cytoplasmic and nuclear electron density (Figure 2C). The fourth group of cells was very electron-dense (dark) and located in the inner parts of the cortex. The most frequently observed TECs in the cortex were pale cells (Figure 2D).

It was seen that the TECs in the medulla also had varying characteristics. The first group of TECs consisted of TECs with morphology similar to that of those in the cortex, located around the capillaries (Figure 3A). The other three groups of TECs were distinguishable with their varying cytoplasmic and nuclear electron density, like their counterparts in the cortex (Figures 3B and 3C). The most notable among these cells were the Hassall corpuscles, with their dense cytoplasmic tonofibrils (Figure 3D). Hassall corpuscles were developed in both groups.

In the observations made by examining all cross-sections, it was found that the most frequently encountered TECs spread through the general parenchyma were cells with pale appearance in terms of electron density. In contrast, dark cells were seen less frequently. The number of dark cells that were easily noticeable with their irregularly shaped electron-dense nuclei was very low, respectively.

In both groups, in the early stage of apoptosis, it was seen that connections of the lymphocytes with neighboring lymphocytes were weakened, and they became smaller by volume and cell surfaces became more spherical (Figure 4A). In this stage, while the apoptotic lymphocytes were smaller than their neighbors, they preserved the integrity of their cell membranes and the organelles in the cytosol got closer to each other and appeared denser. As in the cell membrane, organelles in the cytosol was found to exhibit a denser appearance and preserve their integrity (Figure 4B).

In apoptotic lymphocytes in both sections of the parenchyma, chromatin condensation and karyorrhexis were observed as basic morphological evidence of apoptosis. It was found that the nucleus condensation in the periphery started right from the bottom of the nuclear membrane and went on to form a shape of a horseshoe or a ring (Figure 4C).

In the following stages, clustering formation known as cell blebbing was seen in the cell membrane (Figure 4D). Additionally, apoptotic bodies with varying sizes consisted of tightly packed organelles and cytoplasm, with or without a nuclear fragment inside (Figure 4E). It was observed that large apoptotic bodies were generally filled with condensed chromatin, while smaller ones were filled with cytoplasmic pieces. The apoptotic bodies accumulated frequently in the intracellular area in masses, but they were mostly phagocyted by macrophages and TECs (Figure 4F).

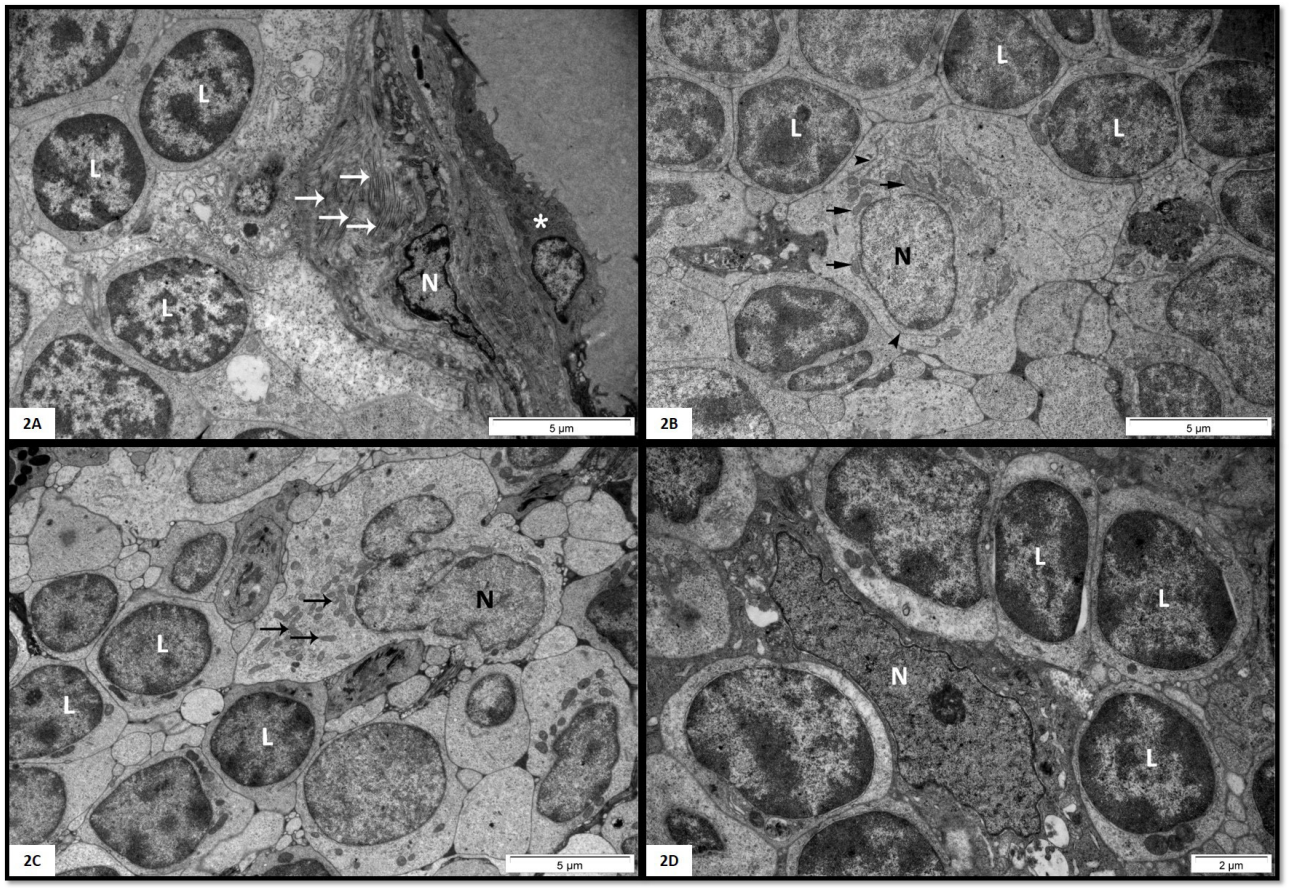

Figure 2. A) Subcapsular TEC with elongated cytoplasmic extensions bordering a capillary (asterisk). Nucleus (N), prominent tonofilaments (arrows), T lymphocytes (L). B) Pale TEC with voluminous cytoplasm. Nucleus (N), mitochondria (arrows), rough endoplasmic reticulum (arrowheads), T lymphocytes (L). C) Intermediate TEC with moderate cytoplasmic and nuclear density. Nucleus (N), mitochondria (arrows). D) Dark TEC. Nucleus (N).

4. Discussion

Apoptosis is known as a physiological process that takes part in the ontogeny and involution of the thymus and the elimination of autoreactive T lymphocytes. Despite the many biological and molecular indicators for determining apoptosis, the morphological changes taking place in this process are still the golden standard (8,16). Another known fact is that TECs support the proliferation and selection processes of T lymphocyte progenitors by the means of lymphostromal interaction (17,18). Today researchers use different molecular techniques to understand the mechanisms that drive TEC differentiation and function (6). However, prior studies did not identify morphological characteristics of TECs by transmission electron microscopy. In this study, as far as we know, we have ultrastructurally revealed for the first time the apoptotic T lymphocyte morphology in the piglet thymus during the early postnatal period and the morphological classification of TECs, which have a key role along with apoptosis in the development process of T lymphocytes.

Many physiological processes require a balance between cell proliferation and apoptosis, which is one of the most important physiological and genetic mechanisms affecting the development process of T lymphocytes in the thymus. In studies on the thymus of humans (19), rats (20), horses (21), ducks (22), and chickens (23), it was found that the increase in the number of apoptotic lymphocytes is directly proportional with age. Studies focused on cell proliferation showed that proliferation rate decreased with age (11,20,22–24). In our study, because the age groups of the piglets were close to each other, no considerable amount of quantitative difference was found in terms of balance between apoptosis and mitosis. While examinations were made on the morphology of apoptotic thymocytes and epithelial cells in our study, it was seen in general that the thymocytes with mitotic morphology were apparently much denser in the cortex than in the medulla. In studies that investigated cell proliferation in rat (20) and

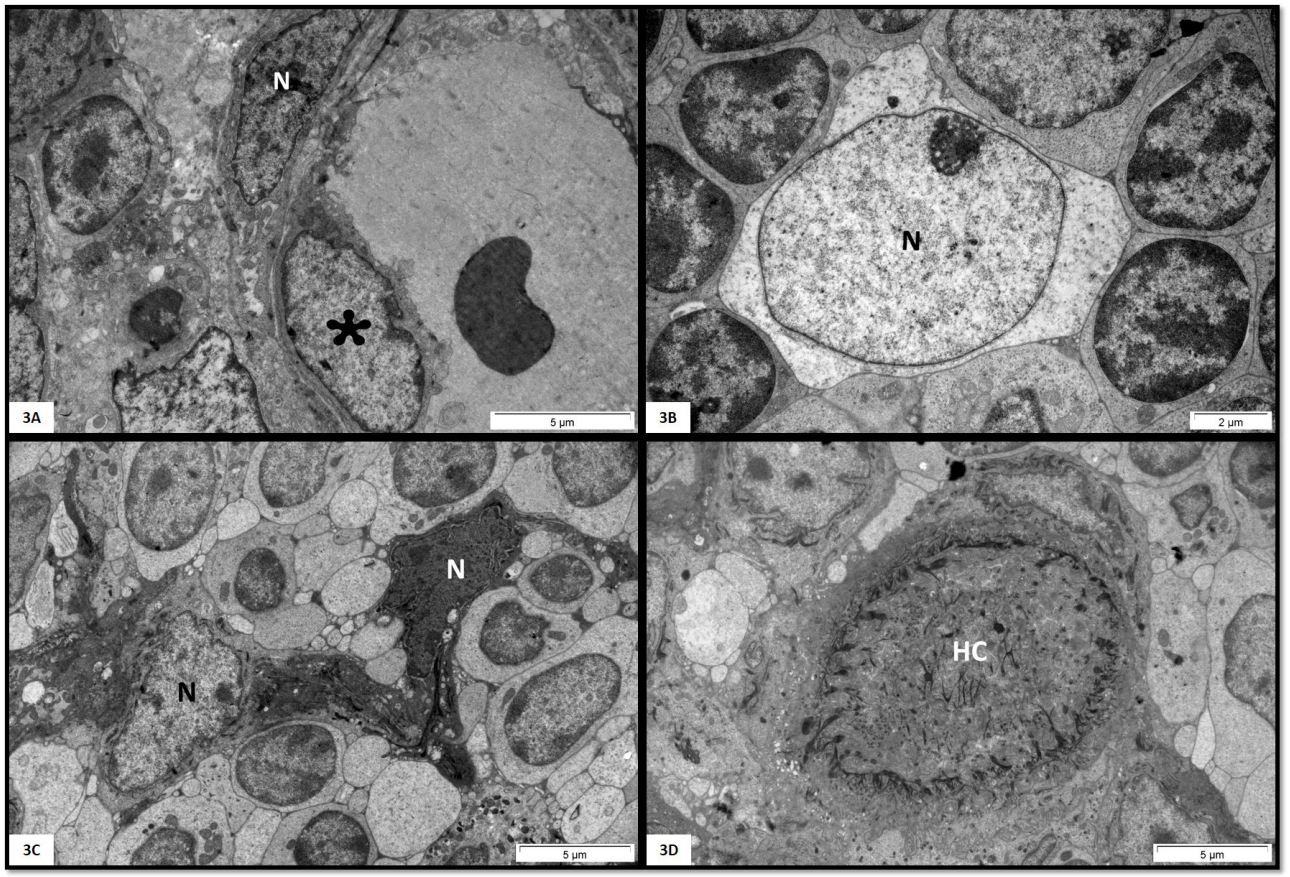

Figure 3. A) Perivascular TEC bordering a medullar capillary. Nucleus (N), capillary endothelial cell (asterisk). B) Pale medullary TEC. Nucleus (N). C) Medullary intermediate TEC (on the left) and dark TEC (on the right). Nucleus (N). D) Hassall corpuscle (HC).

duck (22) thymuses, as in our study, it was found that cell proliferation was more in the cortex than in the medulla in all age groups and these values were determined to be statistically significant. This result supports the hypothesis that in the normal developmental process of T lymphocytes most of the young ones are located in the cortex. Anatomically, thymocytes in different development stages are distributed in the parenchyma of the thymus in varying densities. As opposed to the result of studies on various mammals reporting that apoptotic thymocytes are concentrated in the corticomedullar junction (24–26), in our study we did not observe a concentration of apoptotic cells in this region on the level of fine structures. We think this was caused by the fact that positive and negative selection mostly takes place in other sections of the parenchyma.

It was shown light-microscopically that TECs in mammalian species, which are the most important group of cells forming the thymic stroma, exhibit a heterogeneous morphology (27). Furthermore, the diversity of these cells was demonstrated on the ultrastructural level in humans (28), mice (29), and rats (15). In line with the results we obtained in our study, it was concluded that this method that we used to classify epithelial heterogeneity in the pig thymus, which is used for classification in other species, is also suitable for this species. However, we think it should not be ignored that differences may arise based on species-specific staining character variations in classifications to be made using phenotypical indicators. At the same time, it should be noted that transmission electron microscopy, which enables the examination of cell morphology at the ultrastructural level and yields detailed and valuable findings, is still considered to be the gold standard. According to the classification criteria that we used in our study, the epithelium cells in the parenchyma of the thymus classified as pale, middle, and dark may be interpreted as steps of the differentiation process or indicators of functional activity. Moreover, this morphological variation may have resulted from a metabolic and physiological process (cell cycle) that the cells experience from the pale to the dark stage. In this cellular cycle, pale cells are defined as cells with high metabolic activity, while dark cells may be defined as cells which started to degenerate. Considering the microenvironment of the thymus and

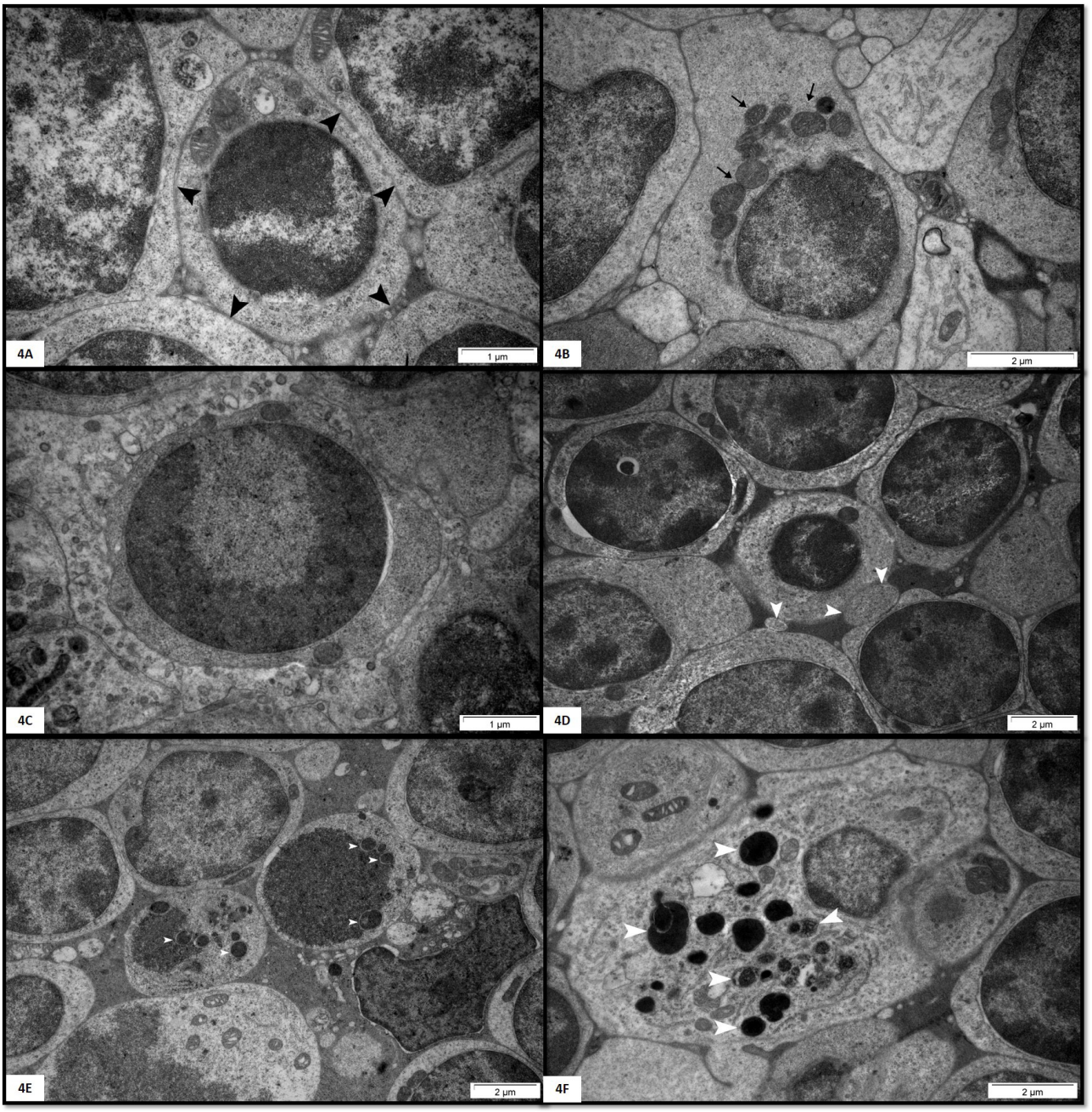

Figure 4. A) Early stages of apoptosis in a spherical T lymphocyte. Cellular membrane (arrowheads). B) A dense view of organelles (arrows) in apoptotic T lymphocyte. C) Horseshoe-shaped chromatin. D) Cell blebbing (arrowheads). E) Apoptotic bodies (arrowheads). F) Apoptotic bodies (arrowheads) phagocyted by a medullary TEC.

the form–function relationship, this situation may be explained as the process of TECs that complete their contribution to the development of thymocytes going into a silence or aging stage. Nevertheless, in a study in which the effects of immunotoxic substances on the thymus were investigated (30), it was demonstrated that, in connection to the increase in the number of dark TECs and the elimination of thymocytes, TECs also collapsed.

Consequently, our study demonstrated the morphological heterogeneity of TECs forming the microenvironment in the thymus, which is one of the most important actors of the immune system, and the morphological changes experienced by apoptotic T lymphocytes in piglets. Undoubtedly the alterations in cellular parameters will become a basis for the development of specific markers for microscopy and molecular

techniques to understand apoptosis on a molecular level. It is thus always recommendable to study several parameters at a time, which provides a multidimensional view of the advancing apoptotic process. Therefore, morphological studies simultaneously conducted with rapidly advancing molecular studies will make it easier to understand the mechanism of apoptosis and will also yield fruitful results for treatment approaches. It is very important to investigate the development of the immune system in fetal life and newborns and to explain its mechanisms. Our study will contribute to the understanding of the ways of regulation in apoptotic cell death mechanisms and the explanation of cellular changes in the developmental process in the thymus, and we think it will also support the elucidation of the role of apoptosis in the immunopathogenesis of infectious pig diseases, as well as treatment approaches.

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