Original Article

Apoptosis, Autophagy, and Necrosis in Murine Embryonic Gonadal Ridges and Neonatal Ovaries: An Animal Model

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Abstract

Background: In mammalian ovaries, loss of over two-thirds of germ cells happens due to cell death. Nonetheless, the exact mechanism of cell death has yet to be determined. The present basic practical study was designed to detect 3 types of programmed cell death, namely apoptosis, autophagy, and necrosis, in murine embryonic gonadal ridges and neonatal ovaries.

Methods: Twenty gonadal ridges and ovaries from female mouse embryos 13.5 days post coitum and newborn mice 1 day postnatal were collected. The TUNEL assay was performed to evaluate apoptosis. The interplay of autophagy was evaluated by immunohistochemistry for beclin-1. Necrotic cell death was analyzed by propidium iodide (PI) staining. The count and percentage of the labeled oocytes in the gonadal ridges and ovaries were evaluated and compared using the independent *t* test and one-way ANOVA. A P value less than 0.05 was considered statistically significant.

Results: We detected TUNEL-positive reaction in the embryonic germ cells and in the small and large oocytes of the neonatal ovaries. The germ cells and small oocytes reacted to beclin-1. PI absorption was detected in the embryonic germ cells and the large oocytes of the neonatal ovaries, but not in the small oocytes. The percentage of the TUNEL-positive and PI-labeled oocytes in the gonadal ridges was significantly higher than that in the neonatal ovaries ($P<0.01$ and $P=0.01$). In the neonatal ovaries, the percentage of the beclin-1-labeled oocytes was significantly higher than that in the embryonic phase ($P<0.01$).

Conclusion: We showed that all 3 types of programmed cell death, namely apoptosis, autophagy, and necrosis, accounted for embryonic and neonatal germ-cell loss. Our observations demonstrated a potential role for necrosis, particularly in the embryonic gonadal ridge in comparison to the neonatal ovary, in mice.

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Keywords ● Apoptosis ● Autophagy ● Necrosis ● Mice ● Ovary

What's Known

- In mammalian ovaries, massive germ-cell death occurs during fetal and neonatal periods. It is known that 2 types of programmed cell death, namely apoptosis and autophagy, play the main role in germ-cell death.
- Recently, necrosis has been considered a type of programmed cell death that has not been investigated yet in mice as an important animal model

What's New

- The current study showed that 3 types of programmed cell death, namely apoptosis, autophagy, and necrosis, were involved in embryonic and neonatal germ-cell loss in mice.
- Specifically, we showed that necrosis, along with apoptosis and autophagy, had a role in germ-cell death.

Introduction

The exact mechanism of mammalian female germ-cell death is not well understood as yet.[1] There are 3 types of programmed cell death (PCD): apoptosis, autophagy, and necrosis.[2] During development, PCD is responsible for eliminating unnecessary or

damaged cells. Inappropriate or absence of cell death can prove fatal to organisms; it should, therefore, be carefully monitored and regulated.[3] Kerr was the first investigator to introduce the term "apoptosis".[4] Apoptosis occurs through intrinsic and extrinsic pathways. The extrinsic pathway is activated by receptor families such as the tumor necrosis factor and fatty acid synthase, which are stimulated by pre-apoptotic ligands.[5] The intrinsic pathway eliminates cells that are deprived of vital growth factors or cells whose homeostasis has been disturbed.[5] Apoptosis detection methods are based on the evaluation of the morphological and biochemical changes that happen in apoptotic cells. Morphological changes are examined by light and electron microscopies. Identification of the subcellular changes by electron microscopy can be considered the gold standard.[6] Enzymes such as cysteine proteases called "caspases" play an important role in apoptosis. Caspases lead to morphological changes in apoptotic cells by breaking the main components of the cells.[7]

In non-apoptotic cell death, the morphological and biochemical characteristics of apoptosis are absent.[8] Autophagic cell death is associated with the formation of double-membrane structures that surround the autophagic materials or autophagosomes; they will subsequently merge with lysosomes.[9] Autophagic cell death specifically is an essential mechanism in the first hours after delivery, when the neonate tends to experience intense changes when feeding from the mother's breast milk as opposed to the mother's circulatory system.[10]

Recently, necrosis has been considered a type of PCD.[11] In the absence of essential apoptotic effectors, the Bcl2 homologous antagonist/killer (BAK), and the Bcl2-associated X protein (BAX), the cell's DNA is damaged. Moreover, necrotic cell features such as organelle swelling, increase the cytoplasmic levels of reactive oxygen species and calcium, decrease the cytoplasmic levels of ATP, activate cathepsin protease, and eventually tear organelles and cell membranes.[11, 12]

The first peak of germ-cell death happens on embryonic days 13.5 to 15.5, when oogonia stop their mitosis and enter meiotic division.[13] The second peak coincides with nest breakdown, concurrent with primordial follicle formation in the time period between embryonic days 17.5 and postnatal day 1.[14] At birth, the mouse ovary contains a large number of germ-cell syncytia. Meiosis in oocyte nuclei ceases at the diplotene stage of prophase I, during which they connect to one another by cytoplasmic bridges. Syncytia are surrounded by somatic cells, including pre-granulosa and stromal mesenchymal cells.[15]

Apoptotic cell death has been demonstrated as the main mechanism of germ-cell loss in the perinatal period. However, in the postnatal period, another form of PCD other than apoptosis has been suggested to mediate germ-cell death.[16] In the neonatal rat ovary, autophagic cell death has been reported as an alternative mechanism for cell death in primordial follicle atresia.[17] Different types of cell death have been reported to occur in the ovaries of various species in different developmental stages.[1,10,17,18] During the gonadal development, there is a variety of non-apoptotic PCD, which is an unknown aspect of this process.[8]

Accordingly, we sought to detect the role of each type of cell death in the critical stages (13.5 days post coitum and 1 day postnatal)[13] of the development of the gonad, when the abundance of cell death occurs. As a novel point, we investigated the incidence of necrosis in comparison with apoptosis and autophagy in the mouse species. Evaluation of cell death in the female germ line and identification of the proportion of each type of cell death can provide a basis for the provision of new methods to delay menopause time.

Necrosis is a potential type of cell death that has not been investigated yet in mice as an important animal model. With regard to the existing contradictory results concerning the type of cell death, further studies are needed to evaluate the role of necrosis in the mouse ovary. In the current study, we evaluated the occurrence of 3 types of cell death in murine embryonic gonadal ridges and neonatal ovaries.

Materials and Methods

Animals

All the experiments and animal handling were done according to the guidelines approved by the Ethics Committee of Shiraz University of Medical Sciences.[19] To mate the animals, we kept 2 female and 1 male BALB/c mice in each cage at a temperature of 22±1 °C and a 12:12 light/dark cycle with free access to food and water. Vaginal plug was checked on the morning after mating. The presence of vaginal plug was considered 0.5 day post coitum.

Tissue Collection and Preparation

Twenty murine gonadal ridges from female embryos 13.5 days post coitum and 20 neonatal ovaries 1 day postnatal were fixed in 10% buffer formalin. Thereafter, the ovaries and gonads were dehydrated in gradually increasing ethanol, cleared in xylene, and embedded in paraffin wax. The samples were sectioned at 5-μ thickness.

The deparaffinized sections were stained with hematoxylin and eosin. The diameters of the small and large oocytes were estimated by stereo lite software, designed ad hoc at our lab.

TUNEL Assay

Apoptosis in the gonadal ridges and ovaries was detected using TACS TdT DAB Kit (R&D, USA, and Catalog #4810-30-K) according to the manufacturer's instruction. Briefly, the paraffin-embedded sections were rehydrated and treated with proteinase K for 30 minutes at 37°C, followed by blocking endogenous peroxidase with a quenching solution for 5 minutes at room temperature. The tissue samples were incubated with the labeling reaction mix (Tdt dNTP mix and Tdt enzyme) for 60 minutes at 37°C. The samples were incubated with the Streptavidin-HRP solution for 10 minutes at 37 °C. Thereafter, incubation was done with diaminobenzidine for 7 minutes at room temperature and finally the samples were counterstained with methyl green.

Immunohistochemistry

Autophagy was investigated by using an anti-beclin 1 primary antibody (Abcam, USA, ab62557). Five fresh gonadal ridges from the embryos and 5 fresh neonatal ovaries were embedded in the optimal cutting temperature compound, sectioned at 5-µ thickness using cryomicrotome (Leica, USA, CM1950), and then stored at -20°C for immunohistochemistry assessments. The sections were thawed for 1 hour at room temperature. Fixation was done with acetone for 15 minutes. Blocking of nonspecific binding sites was done using phosphate-buffered saline, which contained 1% bovine serum albumin and 1% goat serum, for 1 hour at room temperature. The anti-beclin-1 primary antibody was diluted 1:50 and incubated at 4°C overnight. An Alexa Fluor 488-conjugated secondary antibody (Abcam, USA, ab150105) was added to each sample at 1:500 dilution for 1 hour. The primary antibody was replaced with the dilution medium in the negative control. Purkinje cells and their processes in the cerebellum were used as the positive control.

Propidium Iodide Staining

Necrosis was evaluated using propidium iodide (PI) (Sigma, USA, P4170). To prepare live gonadal ridges and ovaries at the time of staining (n=10), we kept them in the Dulbecco Minimum Essential Medium (DMEM, Gibco, 11995), which contained 1.5 µM/mL PI at 37°C and 5% CO_2 in a humidified incubator for 30 minutes in the dark. The PI solution was removed and the tissues were washed 3 times in the DMEM. The samples were mounted on a glass slide with mineral oil and observed under a fluorescent microscope. Immediately after photographs were taken, the newborn ovaries were embedded in the optimal cutting temperature compound and sectioned at 5-µ thickness with cryomicrotome to evaluate the samples with less thickness. The sections were counterstained with Hoechst (Sigma Aldrich, Germany, 33342) and examined with a fluorescent microscope.

Morphometric Analyses

Five ovaries from 5 different mice were analyzed. The percentages of the oocytes labeled with TUNEL, beclin-1, and PI were estimated by randomly selecting 3 sections from the fetal and neonatal mouse ovaries and the percentage of the labeled-cells was evaluated in 10 fields of each section. In each field, the labeled oocytes were counted relative to the entire oocytes, and then the percentage of the labeled oocytes was estimated.

Statistical Analysis

Differences in the mean value of the percentages of the TUNEL-, beclin-1, and PI-positive oocytes were analyzed between the fetal and neonatal phases using the independent *t*-test. Additionally, the distribution of each cell-death type was analyzed in each stage using one-way ANOVA, with statistical significance assigned at a P value less than 0.05. SPSS for Windows, version 16, was utilized.

Results

Morphological Features of the Embryonic Gonadal Ridges and the Neonatal Ovaries of the Mice

At 13.5 days post coitum, germ cells accumulated at the gonadal regions of the embryonic urogenital ridges. Somatic and germ cells dispersed throughout the gonadal ridges, and follicular arrangement was not observed (figure 1).

On the first day after birth, the deep cortical areas of the ovaries contained large oocytes with a mean value of 14.28±0.69 µm in diameter. They were surrounded by follicular cells to form primordial follicles. Germ-cell syncytia presented in the superficial cortical areas (figure 2A).These syncytia were arranged in the form of cord-like structures surrounded by the ovarian somatic cells (figure 2B).The mean value of the diameters of the small oocytes was 9.39±0.76 µm, and they had formed syncytia by connecting with the cytoplasmic bridges (figure 2B). The cells that constituted the ovarian medulla had the same morphological

features as the stromal mesenchymal cells. A number of germ cells seemed to be close to the ovarian surface epithelium and were shaded from the surface (figure 2C).

Apoptosis

The TUNEL assay was used for apoptosis evaluation. In the embryonic gonadal ridges, TUNEL-positive germ cells were detected (figure 3A). In the neonatal ovaries, apoptosis was detected in both large and small oocytes (figure 3B).

Autophagy

Autophagic vacuoles were shown as green fluorescent dot-like structures spreading within the cytoplasm of the germ cells in the embryonic gonadal ridges (figure 4). In the neonates, the expression of beclin-1 in the primordial follicles was limited to the somatic follicular cells, and large oocytes did not react to the anti-beclin-1 antibody. The syncytia of the small oocytes also contained the beclin-1 protein (figure 5).

Necrosis

Necrotic cells were detected in the ovaries based on the absorption of PI. This red fluorescent molecule is capable of penetrating into cells that have lost membrane integrity, including necrotic cells. In the urogenital ridges of the embryos, necrotic cell death was seen in both mesonephric regions and gonadal ridges. Necrosis was observed in both germ and somatic cells (figure 6). Necrotic cell death was shown in both cortical and medullary regions of the neonatal ovaries (figure 7A). In the medullary regions, necrosis was prevalent, especially in the stromal cells. After sectioning from the cortical regions of the ovaries, the cells that had undergone necrosis were detected. The large oocytes surrounded by follicular cells were also labeled with PI (figure 7B). In contrast, the somatic cells failed to absorb PI. The small oocytes not surrounded by follicular cells were not labeled with PI (figure 7B).

Percentage of Programmed Cell Death in the Embryonic Gonadal Ridges and the Neonatal Ovaries of the Mice

The mean value of the percentages of the oocytes labeled with TUNNEL, beclin-1, and PI in the embryonic and neonatal mouse ovaries is presented in figure 8 and table 1. In the embryonic gonadal ridges, the percentage of the TUNEL-positive oocytes (16.53%±0.32) was significantly higher than that of the beclin-1-labeled cells (10.16%±0.42) (P<0.01) or the PI-labeled cells (8.70%±0.82) (P<0.01). In the neonatal ovaries, the mean value of

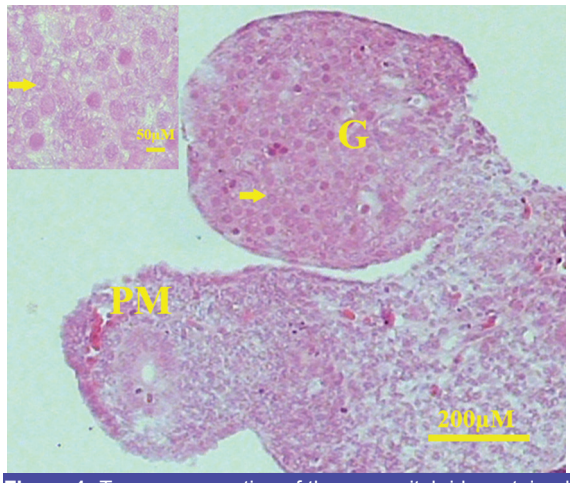

Figure 1: Transverse section of the urogenital ridge, stained with hematoxylin and eosin, on embryonic day 13.5. The arrow shows the germ cell. G: Gonad; PM: Paramesonephros.

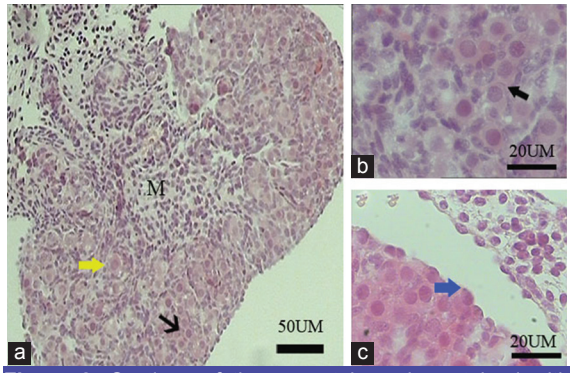

Figure 2: Sections of the neonatal ovaries, stained with hematoxylin and eosin. (A) Small oocytes (black arrow) in the outer cortical region and large oocytes (yellow arrow) in the inner cortical region can be detected. (B) Cytoplasmic bridge (black arrow) connects the small oocytes to one another. (C) Oocyte (blue arrow) at the surface epithelium near the bursa. M: Medulla.

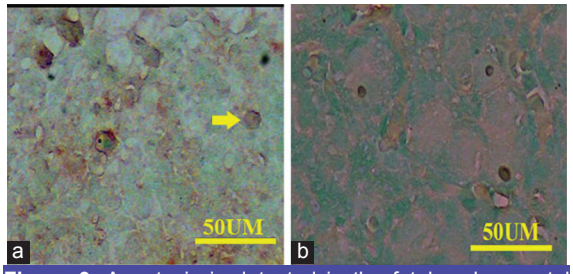

Figure 3: Apoptosis is detected in the fetal and neonatal ovaries. (A) TUNNEL-positive (brown) germ cells (arrow) on embryonic day 13.5. (B) TUNNEL assay also reveals that apoptosis has happened in both small and large (brown) oocytes in the neonatal ovary.

the percentages of the oocytes labeled with beclin-1 (14.82%±0.81) was significantly higher than that of the TUNEL-positive (10.38%±0.54) (P<0.01) or PI-labeled (4.64%±0.7) (P<0.01) oocytes. The proportion of the TUNEL-positive oocytes (16.53%±0.32) in the gonadal ridges was significantly higher than that happening in the

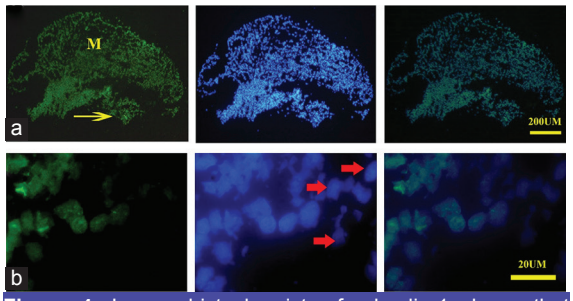

Figure 4: Immunohistochemistry for beclin-1 shows that autophagy has happened in the gonad. The nuclei are stained with Hoechst. (A) Longitudinal sections of the urogenital ridge on embryonic day 13.5 show the gonadal region (arrow) and mesonephros (M). (B) In the gonadal region, reaction to beclin-1 is prominent in the germ cells. In some germ cells, beclin-1 protein has not been expressed (arrow).

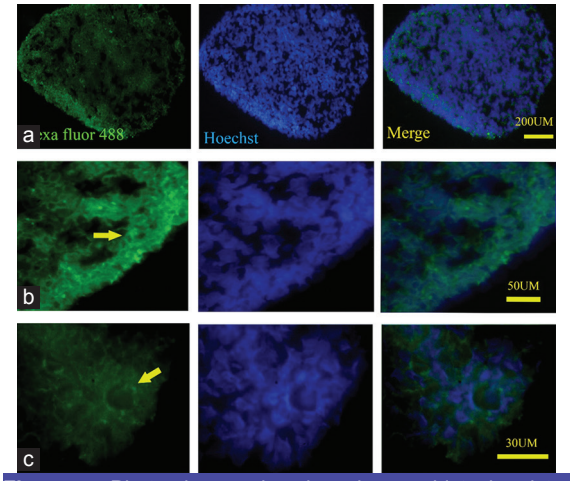

Figure 5: Photomicrographs show immunohistochemistry for beclin-1. (A) Lower magnification of the neonate ovary shows that beclin-1-positive cells are prominent in the cortical region of the newborn ovaries. (B) Higher magnification of the cortical region shows that small oocytes are labeled for beclin-1 (arrow). (C) Follicular cells surrounding the large oocytes have reacted to the beclin-1 antibody (arrow).

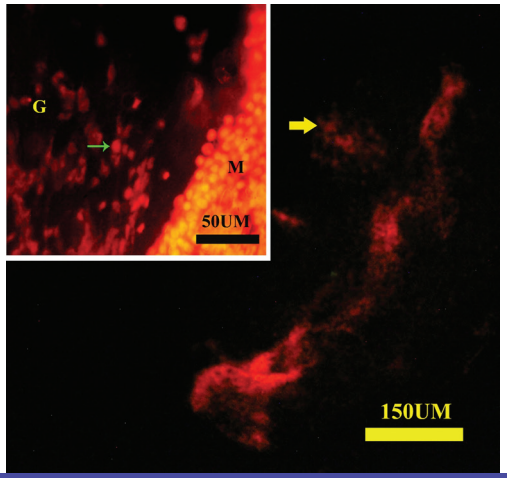

Figure 6: Whole-mount staining of the necrotic cell death by PI absorption in the urogenital ridge. The gonad (yellow arrow) is located near the mesonephros on embryonic day 13.5. Higher magnification shows PI absorption in the germ cells (green arrow). PI: Propidium iodide; G: Gonad.

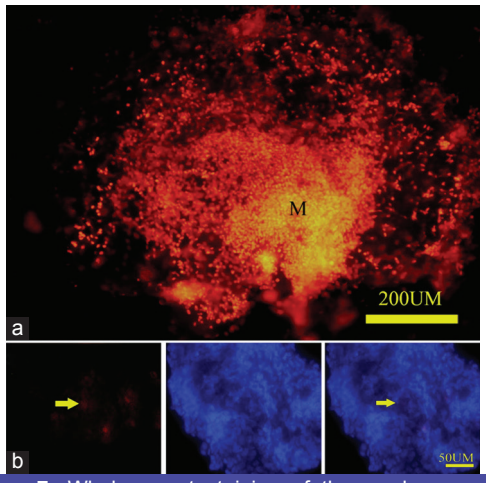

Figure 7: Whole-mount staining of the newborn ovaries reveals areas of PI absorption in the cortical and medullary regions. (A) Necrosis has happened mainly in the medullary region. (B) Sections of the ovary pre-exposed to PI are sectioned and counter stained with Hoechst. Necrosis is shown in the large oocytes (arrow). PI, Propidium iodide; M: Medulla.

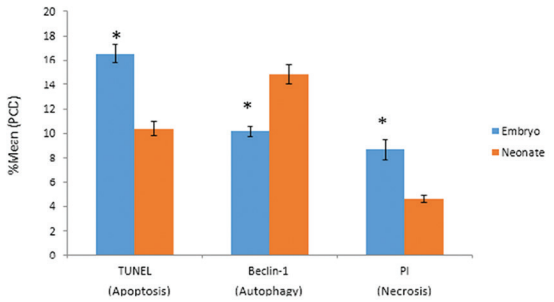

Figure 8: Mean percentage of the oocytes labeled with TUNNEL (apoptosis), beclin-1 (autophagy), and PI (necrosis) in the embryonic gonadal ridges and the neonatal ovaries of the mice. *Significant differences with the neonatal group (P<0.01 TUNNEL, P<0.01 beclin-1, and P=0.01 PI). PI: Propidium iodide; PCD: Programmed cell death.

neonatal ovaries (10.38%±0.54) (P<0.01). In the neonatal ovaries, the percentage of the beclin-1-labeled oocytes (14.82%±0.81) was significantly higher than that in the embryonic phase (10.16%±0.42) (P<0.01). The mean percentage of the PI-labeled oocytes in the embryonic group (8.70%±0.82) was significantly higher than that in the neonatal ovaries (4.64%±0.7) (P=0.01).

Discussion

The current study showed that all 3 types of cell death had roles in embryonic germ-cell and oocyte death. We observed that apoptosis was the main type of germ-cell loss in the perinatal mouse ovary. This result is in agreement with the findings reported by Lobascio et al.[20] However, there is no general agreement in this regard.[8]

Continuous germ-cell death during fetal and neonatal periods leads to the loss of two-thirds

Table 1: Mean percentage of the oocytes labeled with TUNNEL, beclin-1, and PI in the embryonic gonadal ridges and the neonatal ovaries of the mice

Groups	TUNNEL-positive Oocytes (mean%±SE) (total number of oocytes: labeled oocytes)	Beclin-1-labeled Oocytes (mean%±SE) (total number of oocytes: labeled oocytes)	PI-labeled Oocytes (mean%±SE) (total number of oocytes: labeled oocytes)
Embryonic gonadal ridges	16.53±0.32 (507:83)	10.16±0.41 (404:41)	8.70±0.82 (354:31)
Neonatal ovaries	10.38±0.54* (453:47)	14.82±0.81* (364:54)	4.64±0.31* (282:13)

*Significant differences with the embryonic group (P<0.01 TUNNEL, P<0.01 beclin-1, and *P*=0.01 PI), PI: Propidium iodide

of the cells.[14] In the embryonic and neonatal periods, apoptosis is thought to be an important mechanism of germ-cell death and oocyte attrition. Apoptotic cell death has been reported to happen in both embryonic germ cells and neonatal oocytes during nest breakdown in mice.[1, 20] In mammalian ovaries, apoptosis plays an important role in the process of primordial follicle formation.[13] It is well known that apoptosis can occur in fetal and newborn mouse oocytes at meiotic prophase I.[21] Apoptosis is probably one of the multiple mechanisms that participate in germ-cell loss.[14] Germ-cell division is incomplete and leads to the formation of germ-cell clusters. Intercellular bridges have been previously investigated in mice, rats, rabbits, hamsters, and humans via electron microscopy.[22] These cytoplasmic bridges are similar to the ring channels in the drosophila ovary. In drosophilae, only 1 germ cell will become oocyte in the bunch. While in mice, there is a threefold decrease in the number of germ cells. There is still no clear explanation for this massive cell death.[23] Nevertheless, as the drosophila germ cells connect to one another via cytoplasmic bridges, it is likely that caspase-mediated apoptotic cell death in the ovary threatens the survival of the other germ cells in the syncytium due to the diffusion of the apoptotic mediators.[24] The other types of cell death may exert a more important impact than what was thought previously. Indeed, in the present study we found that both autophagy and necrosis, along with apoptosis, were involved in germ-cell death.

Although our results demonstrated that beclin-1, as an autophagic marker, was expressed in both embryonic germ cells and newborn small oocytes, beclin-1 is dominant in newborn ovaries and it seems that it is the main cause of germ-cell loss. In this regard, Rodrigues et al.[1] showed that a 43% reduction in the number of follicles in their study occurred during the time period between embryonic day 19.5 and postnatal day 2, while apoptosis happened in a small number of the cells and autophagic marker expression showed a significant increase.

Autophagy has also been previously reported to occur in fetal and neonatal mouse ovaries.[10,17,20] The occurrence of PCD in some oocytes is an essential event in the process of breaking oocyte nest and the formation of primordial follicles.[25] Our findings suggested the involvement of autophagy in oocyte nest breakdown. Autophagy can be regarded as a mechanism involved in both survival[26] and cell death.[27] Maintaining a balance between apoptotic cell death and cell survival has been suggested as a possible survival mechanism of autophagy for germ cells.[26] In the first hours after birth, the newborn is exposed to the extrauterine environment and also maternal hormone and trophic factor withdrawal,[1,28] which is a source of stress for germ cells.

Deprivation of maternal hormones such as estrogen may play a role in oocyte death during nest breakdown. Steroid hormones also have a role in regulating cell death in multitude tissues. For instance, estrogen prevents the death of granulosa cells in the ovarian tissue, while it leads to cell death in the nervous system. Exposing mouse neonates to phytoestrogen genistein retains cytoplasmic bridges even 4 days after birth. Therefore, exposing neonate oocytes to estrogen compounds stops nest breakdown and prevents oocyte death.[29]

Autophagy is an essential way for cells to respond to cellular stress.[28] If autophagy fails to prevent cell stress, apoptosis occurs.[30] In the present study, beclin-1 was expressed just in the follicular cells, whereas the oocytes were beclin-1 negative. Whereas we found that autophagy had occurred in the follicular cells rather than in the large oocytes, another study showed that adult murine oocytes in primordial follicles expressed beclin-1 protein.[10] This contradiction is probably related to the different stages of oocyte development.

We found necrosis in the fetal germ and somatic cells and also in the large neonatal oocytes. Our observation demonstrated that necrosis in the mouse ovaries was a more important mechanism for germ-cell death in the perinatal period than in the postnatal period. In drosophilae, necrosis has been reported in ovarian nurse cells.[12] Necrotic cell

death activates the inflammatory response,[31] which probably acts as an alarm system in confronting toxic agents such as drugs and harmful substances consumed by the mother.[32] In the neonatal ovary, the medullary region contains strong PI-positive cells, which may play an important role in stromal cell death. Necrosis has been suggested to maintain tissue homeostasis. For instance, necrosis has been shown to involve tissue homeostasis in the epiphyseal plate and the intestinal epithelium.[33,34] Choi et al.[35] used the Lucifer yellow fluorescent tracer to evaluate necrotic areas in the neonatal mouse ovary. The living cell membrane was impermeable to this tracer. In this method, Choi and coworkers employed a confocal laser scanning microscope to evaluate the necrotic areas at different levels of the organ. We described a method based on the use of PI and fluorescent microscopy in order to study necrotic cell death in the ovary in a mechanism similar to that when using Lucifer yellow. Moreover, necrosis in germ cells may be involved in the regulation of germ-cell populations. Molecular studies are needed to understand how these diverse cell-death types work together, particularly in relation to the role of necrosis in the ovary. We suggest future studies for the evaluation of the ultrastructural characteristics of germ-cell death and confirmation of the accuracy of the 3 types of cell death. Transmission electron microscopy is very helpful as a gold standard for the assessment of cell death. What can reduce the prominent limitation of the current study is the application of additional methods such as flow cytometry to confirm apoptotic and necrotic cell death.

Conclusion

The current study presented evidence of the role of 3 types of PCD, namely apoptosis, autophagy, and necrosis, in germ-cell death in murine embryonic gonadal ridges and neonatal ovaries. Our results demonstrated that apoptosis was the main type of germ-cell loss in the perinatal ovaries, whereas autophagy was dominant in the newborn ovaries. Specifically, we showed that the role played by necrosis in germ-cell death was probably as pivotal as the role played by apoptosis and autophagy. Furthermore, we demonstrated that necrosis was a more important mechanism for germ-cell death in the ovaries in the perinatal period than in the postnatal period. Be that as it may, further molecular research is needed to precisely identify the synergy between the different types of cell death in the female germ line.

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Conflict of Interest: None declared.

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