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Microscopic Morphology and Apoptosis of Ovarian Tissue after Cryopreservation using a Vitrification Method in Post-Hatching Turkey Poults, *Meleagris gallopavo*

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The objectives of this study were to examine morphological changes of oogonia and primordial follicles in the ovaries of turkey poults within the first week after hatching, and to evaluate the effect of cryopreservation on histology and apoptosis of these immature ovaries. Ovaries from poults at Day 1, Day 3, Day 5 and Day 7 post-hatch were cryopreserved using a modified vitrification method. The histology of oogonia and primordial follicles in fresh and cryopreserved tissue was assessed, and the apoptosis of tissue in different age groups was identified using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The mean oogonium diameter in fresh tissue increased from 11.9±1.3 μm (Day 1) to 15.2±2.7 μm (Day 7) within the first week; however, oogonia in cryopreserved tissue from Day 3 and Day 7 ovaries were smaller than those in fresh tissue ($P<0.05$). Formation of primordial follicles was observed as early as Day 5. For Day 7 ovaries, follicles in cryopreserved tissue were smaller than those in fresh tissue; this was also true for oocyte diameter ($P<0.05$). Apoptosis was most frequent in Day 1 fresh tissue, which was reduced as the poults aged. The frequency of apoptosis in cryopreserved tissue was comparable among age groups. This study provides the first documentation of morphological changes in the turkey ovary within the first week post-hatching. Results suggest that oogonia and primordial follicles that are smaller in size could be more resistant to the damage caused by cryopreservation. Of the ages assessed in this study, it is concluded that 3 days of age appears optimal for recovery of donor ovaries for cryopreservation, taking the advance of reduced cryoinjury and ease of tissue handling at this age.

Key words: apoptosis, folliculogenesis, ovary, poults, vitrification

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Introduction

Poultry researchers and the industry have faced a massive loss of poultry genetic diversity in the past few decades, largely due to the high cost of maintaining live flocks (Fulton and Delany, 2003; Silversides *et al*., 2012; Liu *et al*., 2013a). Cryopreservation and subsequent functional recovery of germplasm allows long-term availability of genetic resources and reduces the cost (Silversides *et al*., 2012) and therefore can be a feasible alternative to maintaining live flocks. It is critical to maintain female germplasm in poultry species in order to preserve the genetic information carried by the W chromosome and mitochondrial DNA (Silversides and Liu, 2012; Benesova and Trefil, 2016).

Techniques available to mammals, such as cryobanking of oocytes or embryos, have limited applicability for birds because of the structure of the avian egg (Rothchild, 2003; Hagedorn, 2006). Alternatively, the ovarian tissue can be cryopreserved and recovered by orthotopic transplantation, which has been achieved in Japanese quail and chickens (Liu *et al*., 2010; 2013b, c) and used to preserve specialized lines in these species (Silversides *et al*., 2013). The essentiality of extrapolating this model to the domestic turkey, another important poultry species and model organism (Moore and Siopes, 2004; Aslam *et al*., 2012), appears obvious.

For all poultry species studied to date, the age of the donor birds ranged from day-old (chicken) to week-old (quail) (Liu *et al*., 2010; 2013b, c), taking advantage of the potentially superior cryosurvival of immature oocytes compared to mature oocytes (Woods *et al*., 2004). The effect of vitrification procedures on cryosurvival of ovarian tissue at different developmental stages in turkeys is not known. In previous

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studies in quail, the efficiency of ovarian cryopreservation was evaluated by tissue histology (Liu *et al*., 2010; 2012). Fundamental information regarding the microscopic morphology of post-hatching ovarian tissue, which is essential for evaluation of cryoinjury, has not been well documented in turkeys, with only two reports available. Burke and Henry (1999) reported histology of day-old turkey ovarian tissue as control to describe the effect of *in ovo* injection of an aromatase inhibitor prior to incubation on ovarian development of genetic female turkeys. Koba *et al*. (2008) described the macroscopic morphology of day-old ovaries in turkeys but not histology. The normal histology of ovarian tissue in poults during the first week of development remains poorly understood. While programmed cell death, or apoptosis, induced by cryopreservation of ovarian tissue has been evaluated by immunohistochemistry for mammalian species (Martinez-Madrid *et al*., 2007; Mazoochi *et al*., 2008; Merdassi *et al*., 2011), this approach has not been used with avian ovaries. The objectives of the present study were to: 1) examine the morphological changes of oogoina and primordial follicles in the ovarian tissue of turkey poults within the first week after hatching; and 2) evaluate the effect of a vitrification method on the histology and apoptosis of ovaries from turkey poults at different ages.

Materials and Methods

Birds, Chemicals and Tissue Preparation

Turkey poults were derived from a commercial line (Hybrid Turkey, Kitchener, Ontario, Canada) housed at the Beltsville Agricultural Research Center (Long *et al*., 2014). After hatching, the poults were housed in a floor pen with feed and water available *ad libitum*. The research protocol was approved by the Institutional Animal Care and Use Committee following principles described by the American Association for Laboratory Animal Science. All chemicals were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA) unless otherwise indicated.

The day of hatch was defined as Day 1. Ovarian tissue was obtained from four age groups: Day 1, Day 3, Day 5 and Day 7. Immediately after euthanasia, the left ovary was recovered using a pair of fine forceps (Fine Science Tools, Foster City, CA, USA) and immersed in Dulbecco's phosphate buffered saline (DPBS) on ice. The surrounding connective tissue was then gently removed from the ovary. The ovaries that were assigned for cryopreservation were transferred to a handling medium (HM) consisting of DPBS with 20% (v/v) fetal bovine serum (Liu *et al*., 2013b, c) and held on ice during transport to the lab for further treatment.

Vitrification and Warming Procedures

A vitrification method that has been used in Japanese quail (Liu *et al*., 2013b) and chickens (Liu *et al*., 2013c) was adapted to ovaries from post-hatching turkey pouts with minimal modifications. Ovaries were put into fresh HM at room temperature (RT) and four ovaries (from four different poults) were transfixed on an acupuncture needle (J-type, Size No. 2 (0.18)×30 mm, SEIRIN-America, Weymouth, MA, USA), using a dissecting microscope to minimize damage from excessive tissue handling. Transfixed ovaries were first submerged in an equilibration solution (HM plus 7.5% (v/v) dimethyl sulfoxide and 7.5% (v/v) ethylene glycol) for 10 min at RT. Ovaries were then transferred and submerged in a vitrification solution (HM plus 15% (v/v) dimethyl sulfoxide, 15% (v/v) ethylene glycol and 0.5 M sucrose) for 2 min at RT. The ovaries were blotted briefly with a piece of gauze and immediately plunged into liquid nitrogen to facilitate ultra-rapid cooling. Under liquid nitrogen, each needle was then inserted into a pre-cooled, 2-m*l* straw in with one end pre-sealed with two sealing balls (Minitube, Delavan, WI, USA). Another sealing ball was used to seal the other end of the straw. Sealed straws were stored in liquid nitrogen for 15 to 18 months before warming and fixation.

For warming, the straws were cut open at one end while immersed in liquid nitrogen. The needles were removed from the straws and directly immersed in HM containing 1 M sucrose for 5 min at RT. The needles were then sequentially submerged in 0.5 M sucrose HM, and 0.25 M sucrose HM solutions, and then HM without sucrose for 5 min in each solution. After removing from the needles, ovaries were suspended in fresh HM on ice subsequent to fixation for either histological examination or assessment of apoptosis.

Histological Examination

Fresh (n=3) and vitrified (n=3) ovaries from each age group, were fixed in Bouin solution (Ricca Chemical Company, Arlington, TX, USA) overnight, for a total of 24 ovaries. Fixed tissue was sent to an independent third party, Histoserv, Inc. (Germantown, MD, USA), embedded in paraffin, sectioned (5-μm serial sections; 200 μm apart), and stained with hematoxylin and eosin (Catalog number: ST001A). Images were captured and examined using a digital camera (Nikon DIGITAL SIGHT SD-2M, Nikon Instruments Inc., Melville, NY, USA) mounted on a microscope (ZEISS AXIOSKOP, Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). All measurements were conducted with the software NIS-Elements BR 3.2 (Nikon Instruments Inc., Melville, NY, USA).

Oogonia with the following characteristics were considered for measurement (Greenfield, 1966; Rothchild, 2003): (I) large enough to be distinguished from adjacent somatic cells; (II) visible nucleus with the presence of a juxtanuclear specialized ooplasm; and (III) intact plasma membrane. Oocytes with these characteristics that were completely enveloped by a defined follicular epithelium and clear basement membrane were considered as primary oocytes within a primordial follicle.

For each ovary, eight to twelve oogonia and all identified primary oocytes per section were examined. The shortest and the longest diameters of each sampled oogonium or primary oocyte were measured, and the average was defined as the diameter of the oogonium or primary oocyte. For each identified primordial follicle, the shortest and the longest diameters of the follicle were measured and the average was defined as the diameter of the follicle. One half of the difference between the diameters of oocyte and follicle was

defined as the depth of the follicular epithelium.

Assessment of Apoptosis using TUNEL Assay

For each combination of treatment (cryopreservation or fresh control) and age (Day 1, Day 3, Day 5 and Day 7) groups, three ovaries were fixed in 10% Neutral Buffered Formalin (Thermo Scientific™ Shandon™ Formal-Fixx™, Richard-Allan Scientific, Kalamazoo, MI, USA) overnight and subsequently stored in 70% EtOH at RT. Apoptosis was assessed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay combined with basic hematoxylin and eosin staining, which was performed by Histoserve, Inc. (Germantown, MD, USA, Catalog number: A2010). The procedure used to detect apoptotic cells was based on the standard labeling of fragmented DNA with digitoxin-nucleotides and the immunodetection of the labeled nuclei using anti-digitoxin and fuchisin.

The presence and abundance of apoptotic cells was estimated using quantitative image analysis procedures (Elsasser *et al*., 2007; Connor *et al*., 2012) for TUNEL-positive cells. TUNEL-positive cells appeared red to reddish-purple while normal cells were hematoxylin blue. Three separate images of each ovary were captured using an Olympus DP-70 CCD camera mounted on an Olympus BX-40 microscope. For each day of age, a composite of the three images was formed containing one fresh tissue, one cryopreserved tissue and one fresh image used as a standardizing control across all days and tissue preparation formats. A standardized quantification protocol to identify TUNEL-positive cells was developed and used to establish the *.RBG pixel identification algorithm as used by the analysis program (Image-Pro Premier Ver. 9.1, Media Cybernetics, Rockville, MD). Apoptotic cells were further discriminated by implementing a size gating where apoptotic cells were characteristically smaller than normal nuclei with condensed nuclear material. For each composite image and to maximize continuity across the images, the total pixels per cell of the standard control image were adjusted to differ between all composite images by less than one percent. With that optimization established, the positive pixel identification criteria were applied to each of the images. Data consisted of percent positive cells, mean number of positive pixels per cell and pixel cluster size (area) for positive cells.

Statistics

All statistical analyses were performed using SAS 9.4 TS Level 1M1 X64_7 PRO Platform (SAS Institute Inc., Cary, NC, USA). Data were presented as means±SD. The GLM procedure was used to analyze the variance in the diameter of oogonium and the mean percentage of TUNEL-positive cells among age groups. The TTEST procedure was used to compare the diameter of oogonium between fresh and cryopreserved ovarian tissue for each age group and the morphology of follicles (diameter of oocyte, diameter of follicle, and depth of follicular epithelium) in Day 7 tissue. The hypothesis of a linear relationship between the depth of follicular epithelium and the diameter of follicle was tested with the REG procedure. Statements of statistical significance were based on $P \leq 0.05$.

Results

Histology of Fresh and Cryopreserved Ovarian Tissue

Oogonia were distributed mostly in clusters beneath the surface epithelium of the ovarian cortex (Fig. 1A–H). In general, the morphology of oogonia in cryopreserved tissue was similar to that of fresh tissue, although somatic cells with condensely-stained nuclei and less-stained cell plasma were evident in cryopreserved tissue (Fig. 1 B, D, F and H). Mean oogonia diameters for each ovary age and tissue type (fresh and cryopreserved) are shown in Fig. 2. In fresh tissue, the diameter of oogonia in Day 1 ovaries (11.9±1.3 μm) was the smallest among all the age groups ($P \leq 0.05$). Oogonia of Day 3 (13.7±1.5 μm) ovaries were comparable to those of Day 5 (14.4±1.7 μm) ($P > 0.05$) but were smaller ($P \leq 0.05$) than those of Day 7 (15.2±2.7 μm). There was no difference ($P > 0.05$) between Day 5 and Day 7 in terms of the diameter of oogonia. For cryopreserved tissue, however, no difference ($P > 0.05$) was observed in the diameter of oogonia between Day 1 (12.2±0.8 μm) and Day 3 (11.8±1.2 μm), and both were smaller ($P \leq 0.05$) than that of Day 5 (14.1±1.6 μm) and Day 7 (13.9±1.8 μm) tissue. In addition, the diameter of oogonia was greater in fresh tissue than in cryopreserved tissue for Day 3 and Day 7 ovaries ($P < 0.05$, Fig. 2).

In Day 5 ovaries, a few oogonia were observed to be separated from the clusters by a small number of granulosa cells (Fig. 3, panels A and B), indicating the initiation of folliculogenesis. Primordial follicles with better defined structures were seen in the Day 7 tissue (Fig. 3, panels C and D). As shown in Table 1, the mean oocyte and follicle diameter was smaller in cryopreserved tissue than in fresh tissue ($P = 0.0019$ and 0.0078, respectively); no difference was seen in depth of follicular epithelium ($P > 0.05$). A linear relationship between depth of follicular epithelium and diameter of follicle was detected, regardless of whether the tissue was fresh ($P < 0.0001$, R-Square = 0.7651) or cryopreserved ($P = 0.0223$, R-Square = 0.7665). Overall, fewer follicles were observed in cryopreserved tissue ($n = 6$) than fresh tissue ($n = 17$) from Day 7 ovaries.

Apoptosis in Fresh and Cryopreserved Ovarian Tissue

As shown in Fig. 4, TUNEL-positive cells showed condensely stained nuclei, which occurred in both germ cells and somatic cells. A considerable number of cells with intermediate staining also were present. Comparing the primordial follicles in Day 7 fresh and cryopreserved tissue (panels G and H), loss of cell contents was evident in the oocyte and granulosa cells after cryopreservation. The frequency of apoptosis in fresh and cryopreserved ovarian tissue of post-hatching turkey poults is shown in Table 2. For fresh tissue, the percentage of apoptotic cells in Day 1 ovaries was comparable to that of Day 3 ovaries ($P > 0.05$), but significantly greater than that of Day 5 and Day 7 ovaries ($P \leq 0.05$). In contrast, no significant difference was detected among the four age groups for cryopreserved tissue.

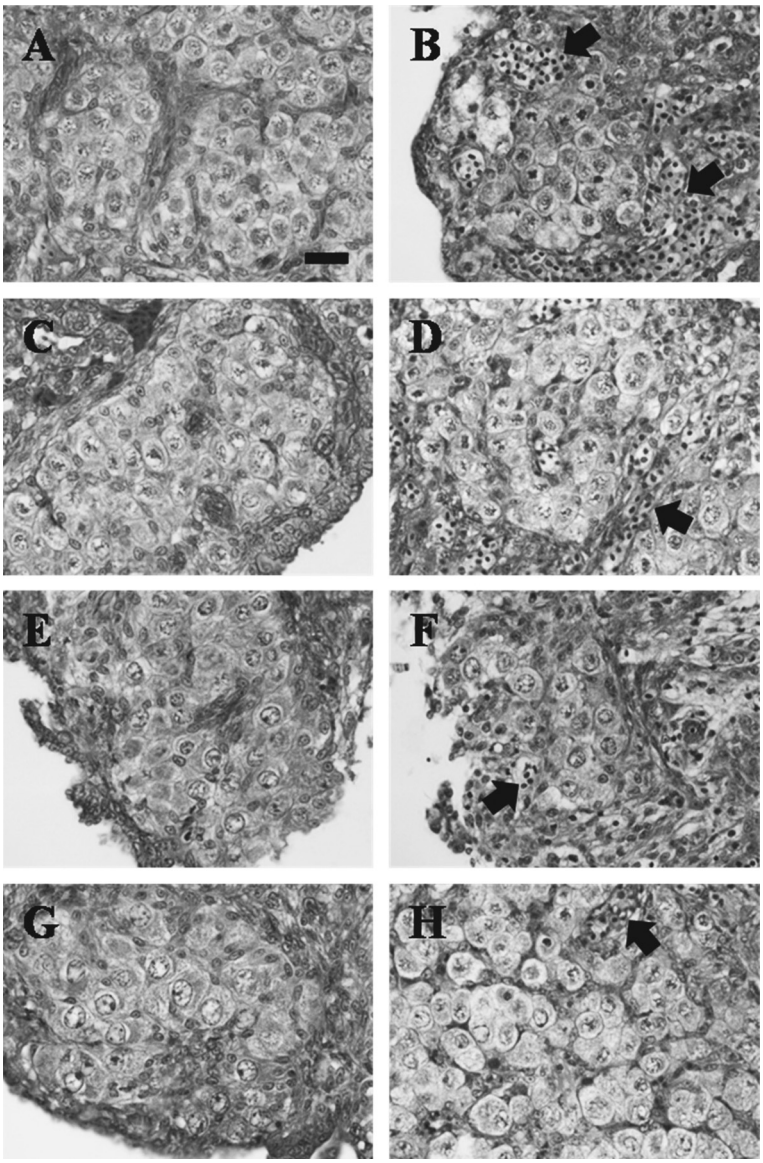

Fig. 1. **Histology of oogonia in fresh and cryopreserved ovarian tissue.** Panels A, C, E, G represent fresh tissue in the age groups of Day 1, Day 3, Day 5 and Day 7, respectively. Panels B, D, F, H represent cryopreserved tissue in the age groups of Day 1, Day 3, Day 5 and Day 7, respectively. Arrows indicate cells with condensely-stained nuclei and less-stained cell plasma in cryopreserved tissue. Bar＝20 μm.

Discussion

These observations provide fundamental information regarding morphological change in the ovarian tissue of turkey poults within the first week after hatching, of which our knowledge has been fragmentary at best. Shortly after hatching, oogonia in turkey poults were generally small (＜ 15 μm in diameter). Within five days after hatching, oogonia around 20 μm in diameter became enveloped with granulosa cells to form primordial follicles. The increased depth of the follicular epithelium is in accordance with the increased size of the follicle at the early stage of folliculogenesis. This general pattern is consistent with earlier reports in chickens, in which the onset of follicular development is between the fourth and the seventh day after hatching in oogonia above 20 μm in diameter, and larger follicles tend to have thicker follicular epithelium at the early stage of folliculogenesis (Hughes, 1963; Greenfield, 1966; Gilbert *et al*., 1980; González-Morán, 2011).

The general pattern of diameter of oogonia changed in

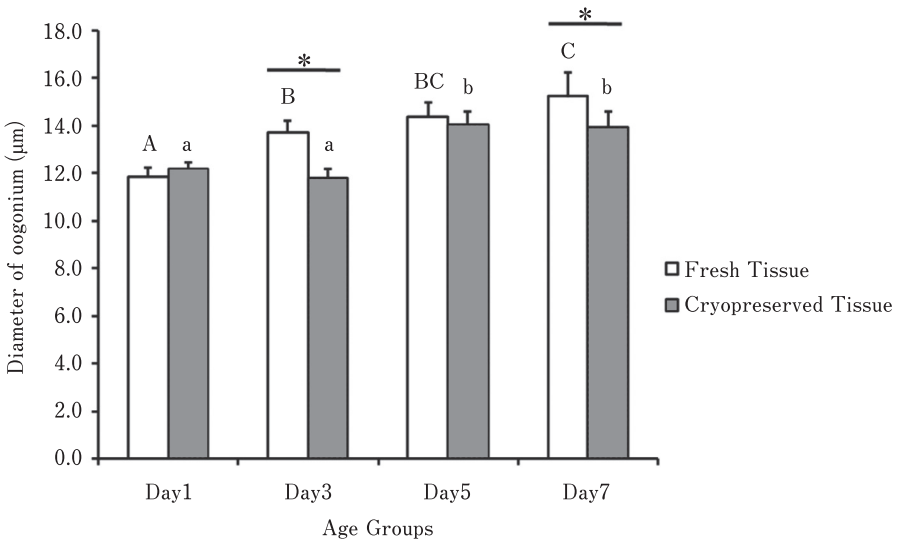

Fig. 2. **Diameter of oogonium in fresh and cryopreserved ovarian tissue of turkey poults at different ages.** Error bars equal to 95% Confidence Interval. Asterisk (*) indicates significant difference between fresh and cryopreserved tissue within an age group, $P \leq 0.05$. A–C: Means of fresh tissue with no common superscript differ significantly, $P \leq 0.05$. a–b: Means of cryopreserved tissue with no common superscript differ significantly, $P \leq 0.05$.

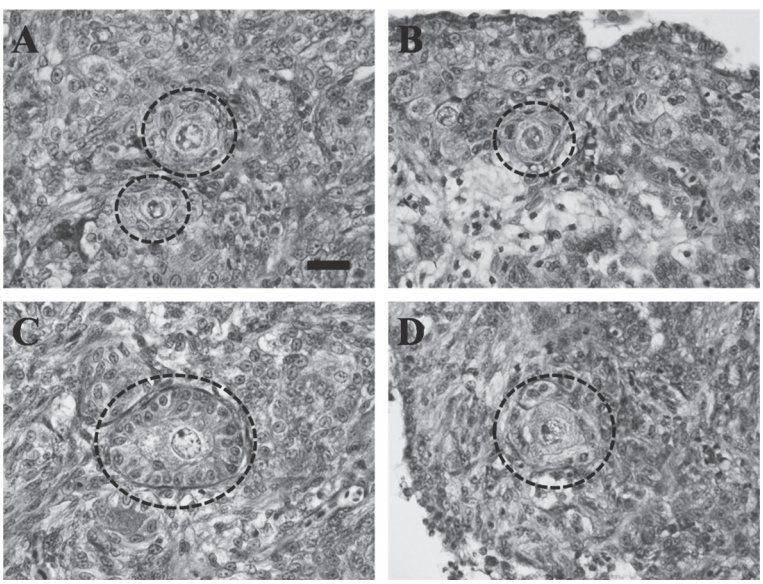

Fig. 3. **Histology of fresh (panels A and C) and cryopreserved (panels B and D) ovarian tissue.** Circles in panels A and B depict oogonia in Day 5 ovaries that are surrounded by a few granulosa cells but no basement membrane. Primordial follicles in Day 7 ovaries are indicated by circles in panels C and D. Bar=20 μm.

Table 1. **Microscopic morphology of follicles in post-hatching turkey poults at the age of Day 7**

Type of tissue	Follicles sampled	Diameter of oocyte (μm)	Diameter of follicle (μm)	Depth of follicular epithelium (μm)
Fresh	17	24.5±2.8[a]	40.9±5.5[a]	8.2±2.0
Cryopreserved	6	19.9±2.4[b]	33.5±4.5[b]	6.8±1.5

[a, b] Values in a column with different superscripts are different ($P<0.05$).

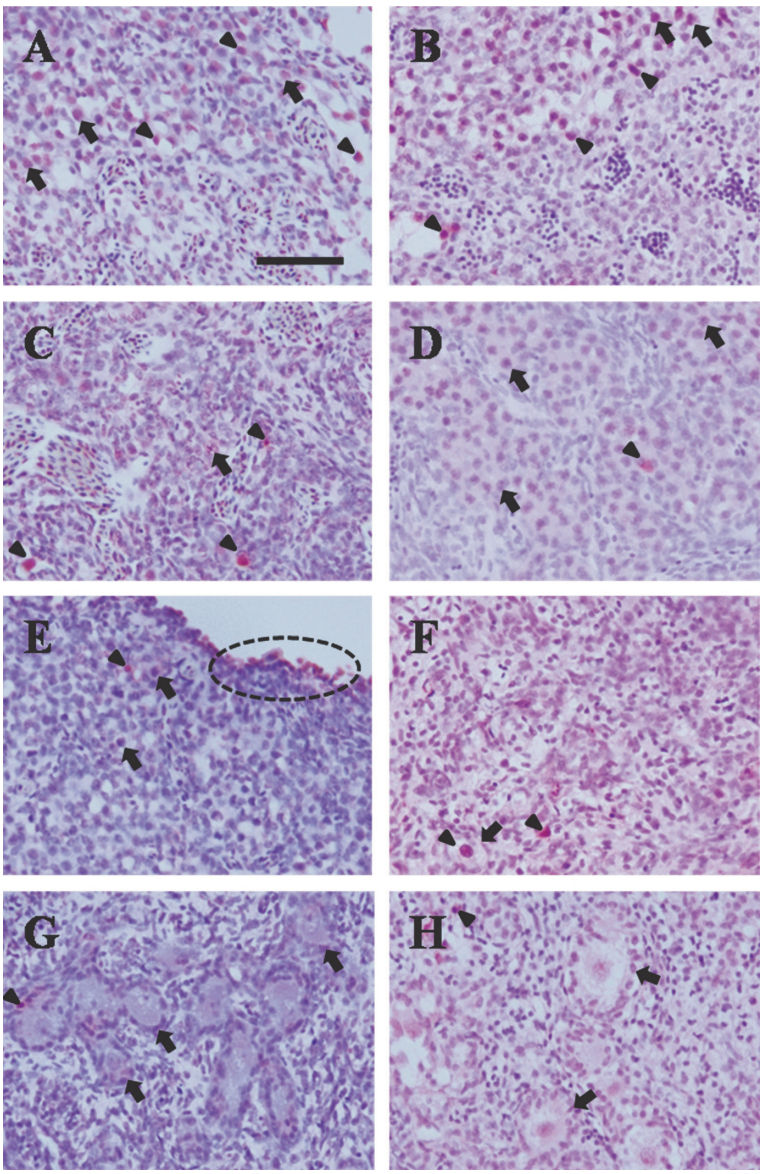

Fig. 4. **TUNEL staining of fresh and cryopreserved ovarian tissue.** Panels A, C, E, G represent fresh tissue in the age groups of Day 1, Day 3, Day 5 and Day 7, respectively. Panels B, D, F, H represent cryopreserved tissue in the age groups of Day 1, Day 3, Day 5 and Day 7, respectively. Examples of TUNEL-positive (apoptotic) cells are indicated by arrow heads. Arrows point to representative oogonia (panels A–F) or primordial follicles (panels G and H). Note that areas showing edge effect (such as circled in panel E) were excluded in the analysis. Bar=50 μm.

Table 2. **Frequencies of apoptosis in fresh and cryopreserved ovarian tissue of post-hatching turkey poults**

Tissue type	Age groups	No. images sampled	Total no. cells examined	Percentage of apoptotic cells (%)
Fresh	Day 1	26	92943	4.4±3.2[a]
	Day 3	27	102741	3.8±2.5[ab]
	Day 5	24	73570	2.8±2.8[b]
	Day 7	22	78187	2.5±2.2[b]
Cryopreserved	Day 1	19	82268	3.5±2.2
	Day 3	26	68451	2.3±3.1
	Day 5	24	68431	2.8±2.6
	Day 7	24	73687	3.5±3.4

[a, b] Within fresh tissue only, values with different superscripts are different ($P \leq 0.05$).

cryopreserved tissue, in which oogonia in Day 3 and Day 7 groups tended to be smaller than their fresh counterparts. Note that because only those oogonia that satisfied the morphological criteria were counted for measurement, it is likely that these two periods were correlated with rapid growth of oogonia but that the large ones were damaged during the cooling and warming process and thus did not contribute to the data collection. This hypothesis can be verified in future research with a larger sample size of ovaries from Day 3 and Day 7 poults using probes that specifically identify cell growth.

Primordial follicles with complete structures in cryopreserved tissue were generally less abundant than in fresh tissue at the age of Day 7, and their morphology is more comparable to those in Day 5 tissue, indicating damage to the relatively large and more developed follicles. This can be supported by the fact that both follicles and oocytes in cryopreserved tissue were smaller compared to those in fresh tissue. Noticeably, there was no difference in the depth of follicular epithelium, which means it is the primary oocytes that were vulnerable to cooling and warming process at this age.

Apoptosis in the fresh tissue was the most frequent at the age of Day 1, and the frequency was reduced as the poults aged. This trend was partially reversed in cryopreserved tissue, which suggests that apoptosis in cryopreserved tissue was induced by mechanisms other than the cooling and warming process itself. It should be noted that the high frequency of cells with intermediate staining led to difficulties in developing the algorithm for quantification and characterization during image analysis. Yoshimura and Nishikori (2004) reported in Japanese quail that oocytes in younger chicks were more prone to apoptosis than in older chicks during normal development. Studies in mammals also showed that apoptosis of ovarian cells was regulated through interactions of different cell populations (Aitken *et al*., 2011; Matsuda *et al*., 2012). Quantification of apoptosis in ovaries of post-hatching birds has not been achieved in other avian species. The current study was the first attempt in turkeys but was limited in scale and the availability of techniques. Refinement of assays (Krysko *et al*., 2008; Galluzzi *et al*., 2009) that allow subpopulation and quantification of cell death in ovarian tissue of post-hatching birds will be an important venue for future research.

Taken together, these observations provide insight for determining the optimal age of donors for ovarian tissue cryopreservation in turkeys. It has been demonstrated in mammals that small follicles are the most tolerant to cryopreservation (Shaw *et al*., 2000), which may be attributed to the small size and relatively less specialized morphology (Paris *et al*., 2004). This likely holds true in birds based on the results of histological examination in the current study. Although statistical significance in the cryopreserved tissue among age groups was not seen in terms of apoptosis, younger tissue before onset of folliculogenesis tended to be more resistant to cryopreservation-induced apoptosis. Causes for this relate to the fact that avian folliculogenesis is characteristic of hormone-controlled yolk deposition (i.e., vitellogenesis, Rothchild, 2003), yielding follicles with large size and specialized structures that are vulnerable to biochemical and biophysical stress caused by cryopreservation (Hagedorn, 2006). In addition, older donors may have a limited reserve of primordial follicles (although this is challenged by Johnson *et al*., 2004), resulting in limited fertility recovery (Liu *et al*., 2015). Therefore, in the situations when donors are relatively abundant such as in domestic species, it is preferable to have young donors. Our results concur, in that ovaries from Day 5 and Day 7 poults appear to be more sensitive to cryopreservation, most likely resulting from the advanced morphology of the developing oocytes and/or follicles.

It was striking that even the fresh ovarian tissue from Day 1 poults exhibited a high percentage of apoptotic cells. This suggests that, while less morphologically advanced, ovaries from newly-hatched poults are delicate and prone to a degree of apoptosis through intrinsic selective mechanisms, even when not subjected to cryopreservation. Thus, during future trials to evaluate and optimize the functionality of cryopreserved ovarian tissue, it would be difficult to delineate whether cell death is caused by cryopreservation procedures or intrinsic mechanisms. We conclude that, of the ages examined, Day 3-old poults are likely at the optimal age to

serve as ovarian donors for cryobanking female germplasm in turkeys. It should be addressed that the ultimate efficiency of ovarian cryopreservation is demonstrated by the production of live offspring, which is dependent on the somatic and germ cells that (1) initially survive cooling and warming procedures and (2) subsequently survive the stress related to the *in vitro* or *in vivo* functional recovery trials, in order to recruit the resting primordial follicles (Liu *et al*., 2013a; Silber, 2016). We therefore propose that the recovery of cryopreserved ovaries from Day 3-old poults be further investigated by tissue culture and/or ovarian transplantation.

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