Research Paper: Characterizing Primordial Germ Cells in the Turkey (*Meleagris Gallopavo*) Embryo

Azam Hosseini[1], Abolghasem Nabipour[1*], Hesam Dehghani[1,2]

1. Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
2. Stem Cell Biology and Regenerative Medicine Research Group, Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran.

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Citation Hosseini A, Nabipour A, Dehghani H. Characterizing Primordial Germ Cells in the Turkey (Meleagris Gallopavo) Embryo Anatomical Sciences. 2020; 17(2):63-72.

Article info:
Received: 03 Feb 2020
Accepted: 02 Jun 2020
Available Online: 01 Jul 2020

Keywords:
Germ cells, Turkeys, Embryology, Immunohistochemistry, Staining and labeling

ABSTRACT

Introduction: The current study aimed to identify and characterize primordial germ cells in the blood, genital ridge, and primary gonad in turkey. Besides, we described the histological characteristics of ovaries in the turkey embryo.

Methods: The embryos from stages 14 to 31 per Hamburger and Hamilton, were studied by the means of blood smear and serial sections from the whole-mount embryo. The primordial germ cells were identified by histochemical and immunostaining techniques.

Results: The present research results suggested that these cells could be identified by their remarkably large size, large nuclei, and granules in the cytoplasm. At stages 20-21 of Hamburger and Hamilton, the alkaline phosphatase reaction was negative in these cells. Furthermore, the primordial germ cells could not be labeled by stage-specific antigen-1 antibody in the primary gonad. We observed that these cells provided negative or poorly positive staining with Alcian blue solution in the migration phase. The presence of glycogen in the cytoplasm of the primordial germ cells was verified using periodic acid-Schiff and Best's Carmine methods. These cells were Best's Carmine positive; however, they contained reduced amounts of glycogen in the primary gonad.

Conclusion: The study findings demonstrated that the periodic acid-Schiff is the best method for identifying the turkey primordial germ cells in the blood and migration phase. Moreover, we reported the existence of histological differences between the right and left ovaries in the turkey embryo.

*** Corresponding Author:**
Abolghasem Nabipour
Address: *1. Veterinary Biomedical Science, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.*
Tel: *+98 (51) 38805649*
E-mail: *nabipour@um.ac.ir; gnabipour@gmail.com*

1. Introduction

Primordial Germ Cells (PGCs) are multipotent stem cells that give rise to spermatogonia or oogonia [1]. Swift was the first who described these cells [2]. The PGCs originate from the epiblast [3] and migrate to the germinal crescent region. These cells circulate in the bloodstream and settle at the germinal ridge and the primitive gonad [4, 5]. The PGCs are progenitor cells and carry genetic information to future generations [6-8]. Thus, these cells have been used to generate germline chimeras [9] and transgenic birds [9, 10]. Studies on identifying PGCs in birds have mostly been performed on the chick embryo [1, 11, 12]. Turkey is a commercially important species [13]; however, few studies have explored the PGCs and primary gonad in this bird. The current study aimed to identify turkey PGCs in the blood, genital ridge, and primary gonad using immunostaining and histochemical techniques. We demonstrated the most optimal method for identifying the turkey PGCs in the blood and migration phase. Finally, we observed the existence of histological differences between the right and left ovaries in the turkey embryo.

2. Materials and Methods

Fertile turkey (Meleagris gallopavo) eggs were obtained from a commercial hatchery. The collected eggs were incubated at 38°C and 65% relative humidity. The incubator was equipped with an automatic turning device that rotated the eggs once an hour. The embryos were staged according to the staging system of Hamburger and Hamilton (H&H) and stages 14-31 (3-9.5 days of incubation) were employed in this study.

The stages 14-16 of H&H (3-3.5 days of incubation) eggs were opened at the blunt end by a small scissor under a stereomicroscope. Blood was collected from the dorsal aorta or the extraembryonic vitelline veins applying a glass heparinized needle (Monoject Scientific; Ireland). Blood was diluted in Phosphate-Buffered Saline (PBS) (2: 20) and placed on poly l-lysine coated slides. The smears were immediately fixed with 4% Paraformaldehyde (PFA). Then, the slides were rinsed with PBS and dried at room temperature. These slides were maintained in PBS at 4°C for future use.

The eggs of 20-21 (4.5-5 days) and 29-31 (8.5-9.5 days) of H&H were opened as a previous stage. The embryos were removed from the yolk and washed with a fixative. They were fixed in 4% PFA at 4°C for 24h. Additionally, several embryos were fixed in gender fixative for 8h, followed by washing with 80% ethanol. Then, the embryos were dehydrated in an ethanol series (70%, 80%, 90%, 100%) and cleared in xylene. They were embedded in paraffin (Merck; Germany) and sectioned (transverse sections, 5-6µm) using a rotatory microtome (Leica RM 2145; Germany). After histochemical and immunohistochemical staining, microphotographs were taken by a BX51 light microscope (Olympus; Japan) equipped with a camera (DP 12, Olympus; Japan).

Paraffin was removed from tissue sections by xylene and rehydrated in graded ethanol series. After washing in PBS, to inhibit the endogenous peroxidase activity, sections were treated with 0.3% H_2O_2 in PBS for 15min at room temperature. Accordingly, they were washed in PBS; to minimize nonspecific binding of antibodies, the sections and blood smears were blocked in 10% Bovine Serum Albumin (BSA)/PBS. The slides were incubated with the primary antibody, Stage Specific Antigen-1(SSEA-1) (1: 300; R&D™; USA) overnight at 4°C.

The slides were washed in PBS and incubated with the secondary antibody, donkey anti-mouse Immunoglobulin M (IgM) conjugated to HRP (1:500, Jackson Immunoresearch™; USA) for 1h at room temperature. The slides were washed three times in PBS and incubated with Diaminobenzidine (DAB) for 5min in a dark humid chamber. The sections were counterstained with hematoxylin. The sections were eventually dehydrated, cleared in xylene, and mounted with Entellan (Merck™; Germany). As a negative control, the study samples were incubated only with secondary antibody (by omitting the primary antibody).

The solvent xylene was used to remove all paraffin from the tissue sections. Subsequently, the sections rehydrated in an ethanol series. The sections and blood smears covered with Alkaline Phosphatase (ALP) were substrates for 30min in a dark humid chamber at room temperature. The slides were washed in deionized water. The sections were finally dehydrated and mounted with Entellan. The ALP substrate contained 66 µL stock 5% Nitro-blue Tetrazolium Chloride (NBT) (Thermo Scientific™; USA) in 70% Dimethylformamide (DMF) (Merck™; Germany), 33 µL stock 5% 5-Bromo-4-Choloro-3-Indolyl Phosphate P-Toluidine salt (BCIP) (Thermo Scientific™; USA) in 100% DMF and 10mL of buffer substrate. The buffer substrate contained 100mM (hydroxymethyl) aminomethane (TRIS) (Sigma Aldrich-TM; USA), 10mM NaCl (Merck™; Germany) and 5mM Magnesium Chloride (MgCl2) (Merck™; Germany) with a PH of 9.5.

Paraffin sections were stained with hematoxylin and eosin, Periodic Acid-Schiff (PAS), Alcian blue, Masson's trichrome, and Best's carmine stains for histological observations. PAS detect the glycogen, glycoproteins, and natural mucins. However, Alcian blue stain detected the acidic mucins and mucosubstances, and the Best's carmine method only detected the glycogen [14].

3. Results

At stages 14-16 of H&H, the turkey PGCs observed in the blood smears were distinguished by large size, large nuclei, and cytoplasmic granules. In the turkey embryo, SSEA-1 labeled PGCs were found in blood smears (Figure 1A). These cells were identified using PAS (Figure 1C) and Alcian blue staining (Figure 1B). The ALP reaction was positive in the PGCs, particularly for cytoplasmic granules (Figure 1D).

At stages 20-21 of H&H, the turkey PGCs were characterized by staining with PAS (Figures 2C & I) and Best's carmine methods (Figure 2E). These cells were observed in the dorsal mesentery migrating toward the germinal ridge (Figure 2L). Furthermore, the presence of the PGCs in both stages was verified in immunohistochemical staining. These cells were characterized by the expression of the SSEA-1 epitope (Figures 2A & G). A few PGCs were observed that adhered to the endothelial cell layer of the blood vessels (Figures 2B, E & G). These germ cells were identified by large size, large spherical nuclei, and glycogen granules in the cytoplasm. Alcian blue staining and Masson's trichrome negative or weakly positive cells were observed in both stages (Figures 2F & K); ALP activity was not detected in these cells (Figure 2B & H).

By the eighth and ninth days of incubation in the whole mount embryo, primary gonad was observed on the ventromedial surface of the mesonephros (Figure 3R). The left ovary was divided into cortex and medulla and separated by a thin layer of connective tissue (Figure 4C). The cortical region has formed a minor component of the left ovary. This region consisted of germinal epithelium and PGCs (Figure 4C). The medullary region consisted of secondary sex cords, blood vessels, lacunar channels, and connective tissue (Figure 4C).

A thinner germinal epithelium was observed in the right ovary (Figure 4B). Similar to the left ovary, there were no secondary sex cords on the right side. Moreover, there were blood vessels filled with erythrocytes, lacunar channels, and connective tissue in the medulla of the right ovary (Figure 4B & D). At stage 31 (9.5d), the left ovary presented the same components as in the previous stages. In this stage, the inner medullary region of the right ovary was more porous than the previous stages (Figure 4B). In the primary gonad, the PGCs were observed in the cortical and medullary regions. This cell was round or oval with a large size and large prominent nucleus (Figure 4C).

The turkey PGCs in the primary gonad could not be identified using the SSEA-1 antibody (Figures 3A, B, & C). In the positive controls, the chick embryo contained the PGCs in the primary gonad; however, in the negative control sections, the PGCs expressed no SSEA-1 epitope (Figures 3P & Q). In the three stages (29-31 of H&H), the PGCs were easily identified by PAS reaction with a deep purplish-red stain. This was due to the presence of glycogen in the cytoplasm (Figures 3M, N, & O). At stage 29 (H&H), some of the PGCs were observed in the dorsal mesentery migrating to the primary gonad (Figure 3J). In the slides stained with histochemical Best's carmine technique, the PGCs were identified in the cortex of the primary gonad (Figures 3K & L).

At the 8.5d of incubation (stage 29 of H&H), the ALP reaction in the PGCs was negative (Figure 3G); however, in the stages 30-31 of H&H, the PGCs were identified by high ALP activity in the cortex region of the gonad (Figures 3H & I). Concerning the positive controls of ALP activity, a tissue sample from the liver of a 1-day-old chick was used (Figure 3S). For the negative controls, the sections were preheated at 60°C for 1h (Figure 3T). In the primary gonad, the PGCs presented a positive reaction with hematoxylin and eosin staining. These

Table 1. The mean number of PGCs in the gonads of turkey embryos

The Mean Number of PGCs in the Right Gonad	The Mean Number of PGCs in the Left Gonad	
3	11	Stage 29
4	14	Stage 30
6	16	Stage 31

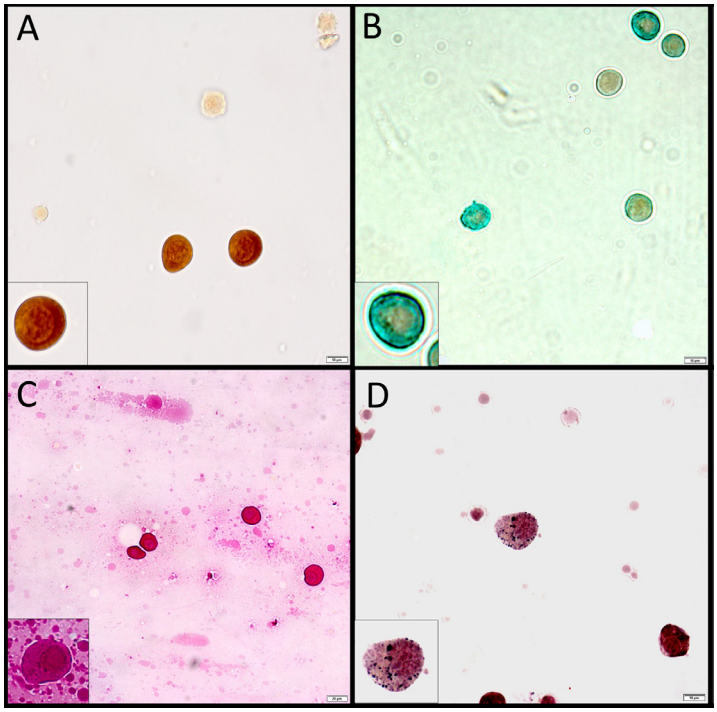

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Figure 1. Characterizing the turkey PGCs using the histochemical and immunohistochemistry methods

The turkey PGCs (stages 14-16 of H&H) were identified among blood cells using SSEA-1 antibody (A), Alcian blue (B), PAS (C), and ALP activity (D).

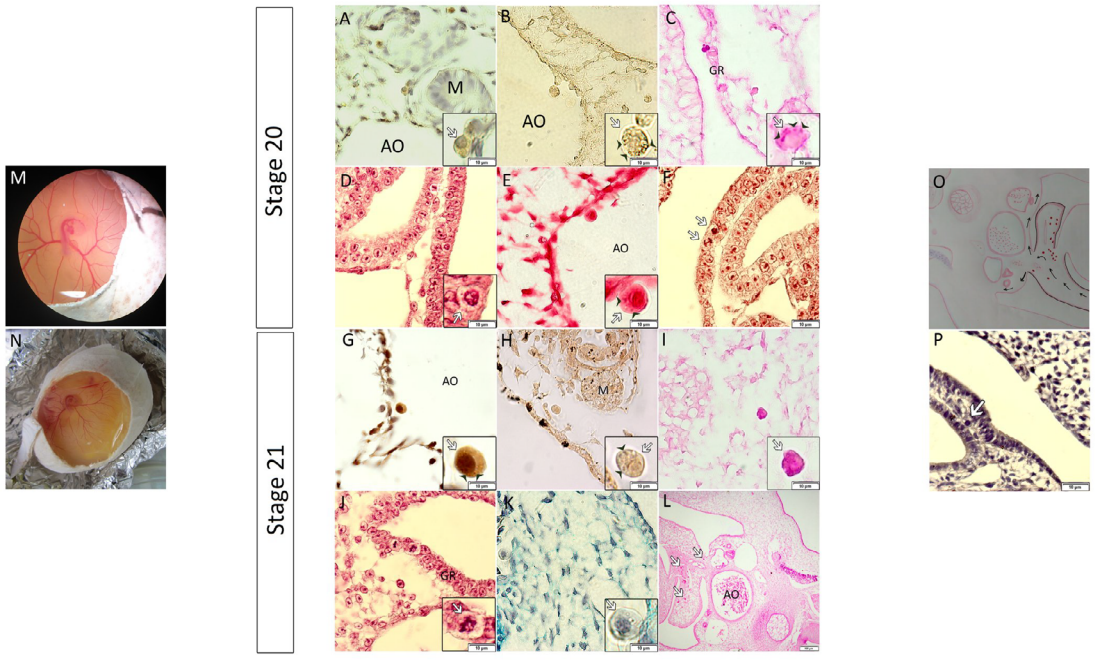

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Figure 2. Identifying the turkey PGCs, migration toward the genital ridge and coelomic epithelium at stages 20 of H&H (A, B, C, D, E, F) and 21 of H&H (G, H, I, J, K, L)

Some of the PGCs adhered to the wall of the intra embryonic vessels (B, E, G). The PGCs were identified by SSEA-1 antibody (A, G), PAS (C, I), Masson's Trichrome (F), and Best's carmine staining (E). These cells were not stained with ALP activity (B, H) and were poorly stained with Alcian blue staining (K). The PAS-positive germ cells could be identified in the dorsal mesentery; migrating towards the genital ridge (L), and schematic image of this stage (O) (arrows indicate PGCs). Turkey embryo at stages 20 (M) and 21 (N). The negative control of the turkey PGCs for immunostaining (P). Ao: dorsal aorta, M: Mesonephros, GR: Genital Ridge.

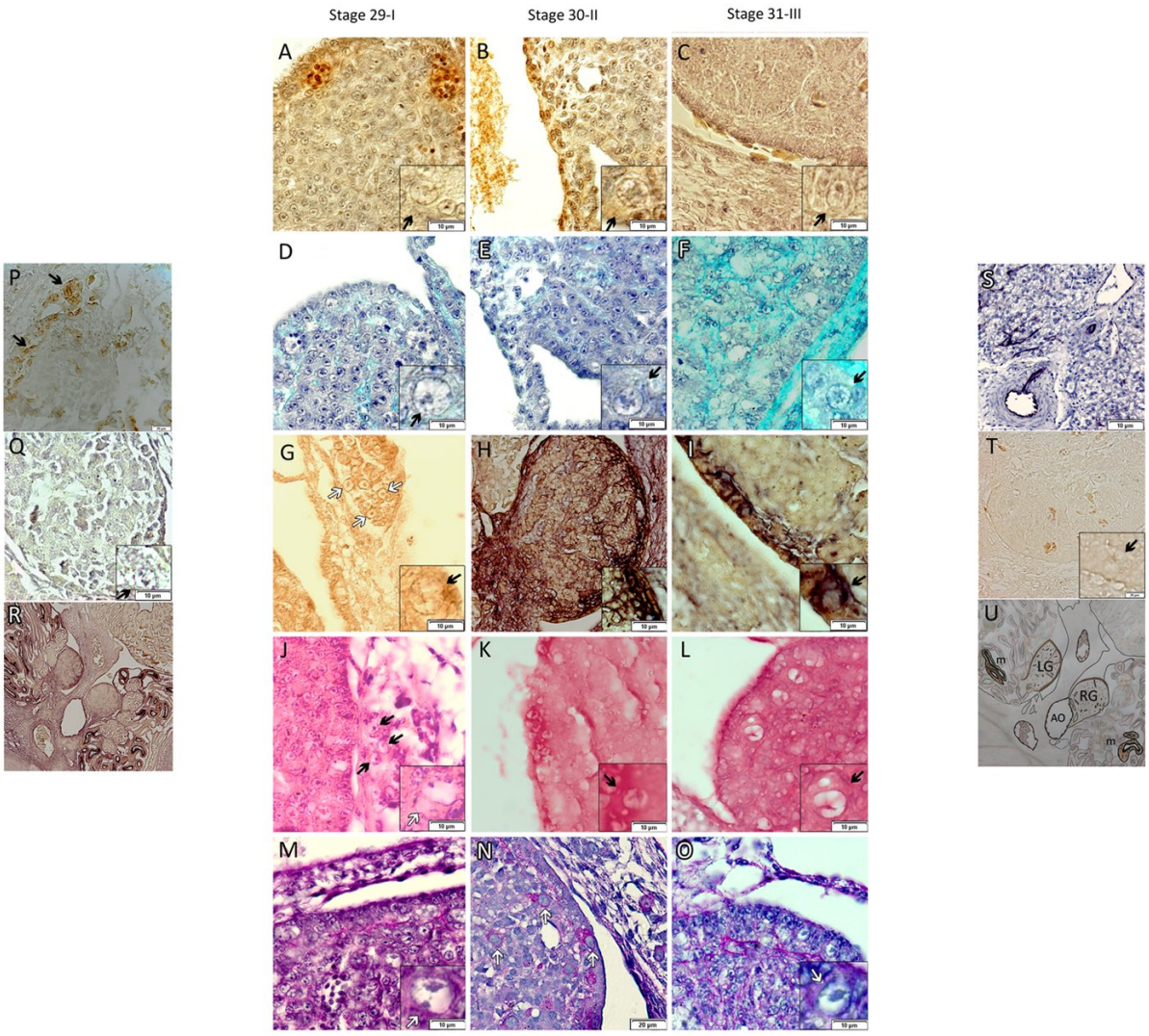

Figure 3. Identifying the turkey PGCs in the primary gonad

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The PGCs were identified by PAS (M, N, O), Best's carmine (L, K), as well as hematoxylin and eosin (J) staining; however, they did not express the SSEA-1 epitope (A, B, C) (arrows). These cells were also identified by ALP activity at stages 30-31 of H&H (H, I), but not at stage 29 of H&H (G) (arrows). The real (R) and schematic image for ALP activity in this stage (U). The turkey PGCs were poorly stained with the Alcian blue method (D, E, F). In the positive control for immunostaining, the PGCs were stained in the gonad at stage 28 of H&H in the chick embryo (P). In the negative controls, the chick PGCs did not express the SSEA-1 epitope (Q). The positive control for alkaline phosphatase (ALP) activity (by using NBT/BCIP assay) (S) and negative staining for ALP activity (by NBT/BCIP assay) on sections preheated at 60°C for 1h (T). Arrowhead: granules in the cytoplasm of PGCs, LG: left gonad, RG: right gonad, m: mesonephros tubule, AO: dorsal aorta.

cells were identified by the eosinophilic cytoplasm and a large nucleus (Figure 3J). Besides, the turkey PGCs were stained as negative or weakly positive using the Alcian blue staining (Figure 3D, E, & F).

In this study, 30 slides from 3 embryonic stages were subjected to cell (PGC) count. These obtained data suggested that the number of cells was dependent on the stage of embryonic development. At day 7 of incubation (HH stage 31), the number of cells was higher than that of the other 2 stages. In the left gonad, the number of cells was higher than that in the right gonad (Table 1).

4. Discussion

The PGCs are the only totipotent cells that could be found after gastrulation. These cells give rise to pluripotent stem cells, such as embryonic germ cells and embryonic cells in vitro [15]. In the chick embryo at stage 12 H&H, the PGCs, after passing through the germinal

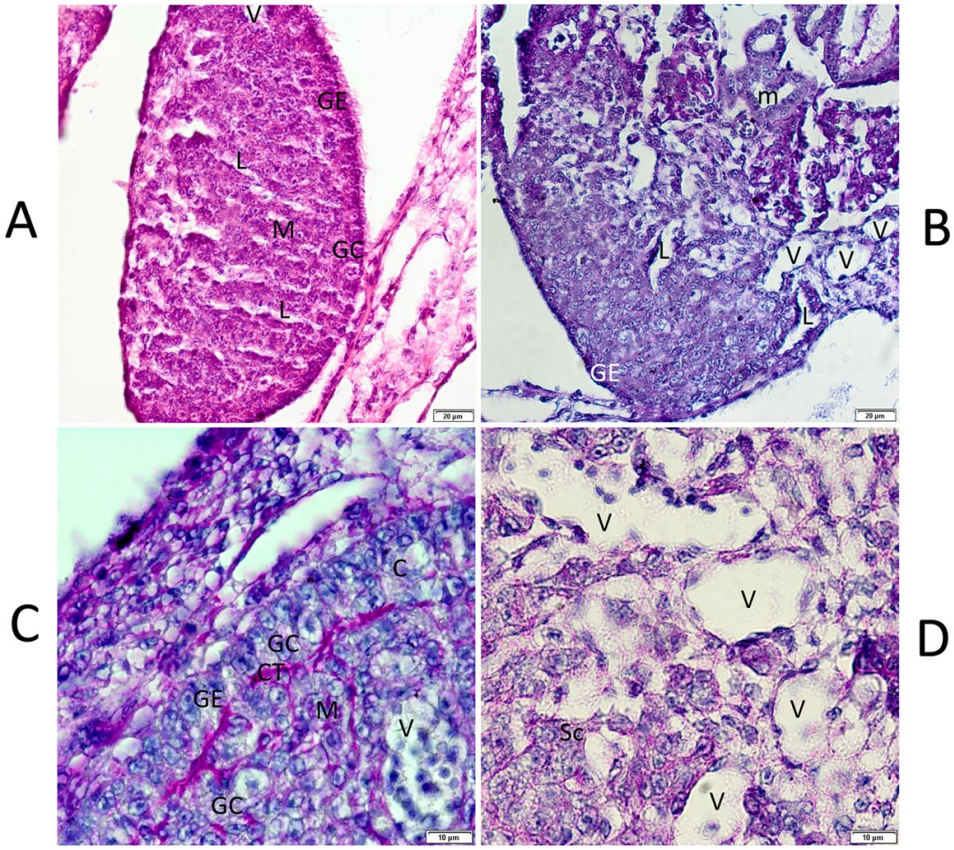

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Figure 4. The photomicrograph of the left ovary at stage 30 of H&H (A, C) and right ovary at stage 31 of H&H (B, D) in the turkey embryo

The high magnification of cortex in the left gonad (C). The high magnification of ovarian medulla (D). GE: germinal epithelium, GC: germ cells, SC: somatic cells, CT: connective tissue, M: medulla, V: blood vessels, L: lacunar channels, E: erythrocytes, m: mesonephric tubule, C: cortex. Hematoxylin & eosin (A), Periodic Acid-Schiff (B, C. D) staining.

crescent, enter the developing vascular system and circulate through the embryo [11]. At stages 16-17 of H&H, these cells exit the blood vessels, migrate through the dorsal mesentery, and enter the germinal epithelium (stage 20 of H&H) [16]. In this stage, the chemotactic factor for PGCs is released by the coelomic epithelium [17]. Finally, these cells colonize in the primary gonad [16]. The SSEA-1 antibody is a cell-surface glycoprotein that could be used as a marker for identifying PGCs in the chicks and mammals [12, 18-22].

As previously reported [23] the present study findings, the SSEA-1 was detected in the turkey blood PGCs. However, some reports could not find the SSEA-1 labeled blood PGCs [24]. In the present study, the turkey PGCs expressed the SSEA-1 at stages 20-21 of H&H, but not in the primary gonad. Similar findings were reported in the mouse embryo [25]. However, in the chick, PGCs were SSEA-1 positive after they colonized in the gonad [23]. This could be due to the lack of expression of this epitope in the turkey PGCs, once they colonize in the gonad. Such loss of antigenicity could also be related to the changes in the surface morphology migration phase [26]. This could also be connected to the different characteristics of turkey and chicken PGCs [24].

ALP is a metalo zinc enzyme that hydrolyzes the ster bond in animal and plant tissues [27, 28]. Studies have indicated that this enzyme could be used for the identification of PGCs in the chicken and mammals [29]. The histochemical localization of ALP activity has been based on the Gomori technique [30]. Moreover, other methods are available for detecting ALP activity [31, 32]. This histochemical technique was the golden standard to identify the PGCs in the mouse [33]. The positive reaction with ALP in the turkey PGCs in the blood agreed with the earlier studies in the zebrafish and chicken [11, 34]. Although the PGCs in the blood had ALP activity,

employing this method is only beneficial when accompanied by the study of the morphology of the cells [11].

When turkey germ cells exit blood vessels and begin to migrate toward the gonads, the enzyme activity could not be detected until the start of stage 30 (H8H) development. This negative reaction with ALP in the genital ridge is in contradiction with the previous study in the chick embryo [35]. Mac Gregor argued that the mouse PGCs in the genital ridge could not be identified using ALP staining [36]. Swartz's investigations (1982) have revealed that ALP activity is present in the gonad of the chick embryo. They reported changes in enzyme activity in the migration phase. One interpretation of our data is that the embryonic ALP gene is active during some stages of embryonic development.

In the mice, there are three isozymes for ALP, as follows: specific ALP, embryonic ALP, and intestinal ALP. By Reverse Transcription (RT)-PCR methods, embryonic ALP and tissue non-specific ALP isozymes were reported to be expressed in the normal mouse embryo [37, 38]. The presence of intestinal ALP was not verified [37]. In this study, in the control sections, we could not observe the residual ALP activity after the inactivation of tissue sections with heat. There seems to be an active embryonic ALP gene activity at stages 30-31 (H8H) in the turkey embryo. The role of ALP in the PGCs remains undiscovered; however, it could be involved in the proliferation and differentiation of these cells [39].

The PAS reaction is a histochemical marker for identifying PGCs [9, 10]. The PAS and Best's carmine methods are used for demonstrating glycogen [40, 41]. Glycogen, glycoproteins, glycolipids, and neutral mucins are distinguished by the PAS technique. The Best's carmine is a staining method to detect only glycogen [42-44]. In the present study, PAS-positive turkey PGCs were identified in blood smears by the presence of abundant glycogen in the cytoplasm. This finding was consistent with those of other reports [11, 23]. In the genital ridge, the presence of abundant glycogen provides a purplish-red (in the PAS method) or pinkish color (in Best's carmine method) to the cytoplasm. These results were similar to those of the reports in the chick and zebrafish embryos [45-48].

We suggest that the appearance of purplish-red and pinkish color in the cytoplasm is due to the variation in the carbohydrates in the turkey PGCs. These carbohydrates studied by lectins [49, 50] and various carbohydrate antigens in the cell surface, have been presented in the chick embryo [51, 52]. These carbohydrates significantly impact embryonic development [53]. Mucin is composed of a large group of high molecular weight glycol conjugates [54]. The acidic mucins and mucosubstances nonsulfated are detected with the Alcian blue technique [55, 56]. An Alcian blue method was used as a specific stain for the acidic group of carbohydrates [57]. It seems that the positive reaction of the PGCs with PAS and Alcian blue staining in the blood smears is due to the mixture of neutral-acidic mucins [56]. However, the reason for negative or poorly positive staining with Alcian blue staining in the migration phase remains unclear. These results could indicate changes in the carbohydrate molecules on the cell surface of the germ cells during development [50].

The incubation period of the turkey eggs is 28 days; in comparison with chick and quail, turkey has lag behind physiological development [58]. The morphological development of ovaries was documented in the chick and ostrich embryo [48, 59, 60]. In the chick embryo on the fourth and 5.5th days of incubation, the germinal epithelium in the gonad forms the primary sex cords [61]. Then, in the sixth and seventh days of incubation, the primary sex cords of the left ovary are break up and called secondary sex cords. The secondary sex cord contains somatic cells and germ cells [59]. We observed the secondary sex cords form the vast majority of the left ovary in the turkey embryo.

There seem to be differences in the size of the right and left ovaries; the right ovary was smaller than the left one. Additionally, the left ovary contained a thick germinal epithelium, unlike the right ovary. However, further studies are required in the turkey gonad to better characterize the functional and molecular context of this increased size. Scholars have explained differences in size between the right and left ovaries with the asymmetric development of gonads [62, 63]. An early asymmetric expression of PITX2 and Estrogen Receptor Alpha (ESR1) genes leads to the asymmetric ovarian development in the left gonad [63, 64]. Besides, retinoic-acid synthesizing enzymes were effective in asymmetric ovarian development in the right ovary [63]. We observed that the right cortex was thinner than the left one. The right ovary might have lost the ability of development during the gonadal phase [63].

5. Conclusion

The turkey PGCs observed in blood smears were distinguished by large size, large nuclei, and cytoplasmic granules. We observed that SSEA-1 antibody and PAS staining were desirable methods for identifying the PGCs in the genital ridge. Besides, turkey PGCs in the primary gonad could be properly identified using the his-

tochemical techniques, such as ALP activity and PAS staining. The PAS-positive turkey germ cells were observed in all stages. Therefore, we suggest this method as the best approach to identify these cells. Additionally, we demonstrated the histological differences between the right and left ovaries in the turkey embryo.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the University of Ferdowsi University of Mashhad (Code: ???).

Funding

This research was supported by the Research Council of the Ferdowsi University of Mashhad (Grant No.: 3.41149).

Authors' contributions

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflicts of interest.

Acknowledgments

This research was supported by the Research Council of the Ferdowsi University of Mashhad (Grant No.: 3.41149).

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