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Research Article

IDENTIFICATION OF PRIMORDIAL GERM CELLS IN EARLY PHEASANT (*PHASIANUS COLCHICUS*) EMBRYO

Entekhab Hameed Abed Al-Shuwaili [1,2], Azam Hosseini [1], Abolghasem Nabipour[1]

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ABSTRACT: The aim of this study is identification of primordial germ cells during their circulation in the blood stream and migration to the primary gonads in pheasant embryo. The blood was taken from stages 14-17 and genital ridge tissue was taken from stages 20-21 Hamburger & Hamilton. Primary gonads were acquired from stages 28-30 Hamburger & Hamilton. Primordial germ cells have been recognized by using histochemical and immunostaining methods. The current article specified that these cells are distinguishable based on their relatively large size, large nuclei and cytoplasm with defined boundaries. In blood smear, such cells were negative for alkaline phosphatase, while the circulating primordial germ cells were negative for stage-specific embryonic antigen-1 (SSEA-1). Further, the Periodic Acid-Schiff staining was negative or very weak. These cells showed a very weak or negative reaction to staining with Alcian blue. At stages 20-30 Hamburger & Hamilton, primitive germ cells might not be distinguished by alkaline phosphatase and SSEA-1 immunostaining. Alcian blue and Periodic Acid-Schiff staining was either negative or very weak in these cells. According to the results of this study, primordial germ cells in all stages showed very weak or negative reactions to different histochemical and immunostaining techniques. We recommend that the use of routine hematoxylin and eosin staining yields better results for primordial germ cells identification.

Keywords: Germ cells, Immunohistochemistry, Alkaline phosphatase, Identification.

INTRODUCTION

The cells that produce spermatogonia or oogonia are known as primordial germ cells (PGCs). These cells arise from the epiblast within the central area of the blastoderm (area pellucida). As the primitive streak forms, these cells migrate to the anterior extra-embryonic region known as the germinal crescent. Following vascularization, PGCs enter the bloodstream and travel to the genital ridges, where they settle and contribute to gonad formation [1, 2]. PGCs are germline stem cells and are beneficial in the genetic information transfer between generations [3, 4]. Depending upon signals from their circumstance, PGCs differentiate into gametes of either sex [5]. In the ostrich, turkey and chick embryo, the primordial germ cells have been recognized by large nuclei, large size, and cytoplasmic granules [6, 7, 8]. The cells were recognized by several methods such as: histochemical staining, immunohistochemistry and enzyme histochemistry [alkaline phosphatase (ALP)]. Periodic Acid-Schiff (PAS) staining is a standard technique for detecting polysaccharides, such as glycogen, in cells and tissues. Immunohistochemistry staining is used to detect cell-surface antigens in various tissues [8]. Also, ALP known as an important diagnostic technique that could be used for identification of embryonic stem cells and PGCs [9]. The antibody against stage-specific embryonic antigen (SSEA1) is used to recognize PGCs in different species [10, 11, 12]. Researchers found that PGCs can generate germline chimeric chickens in laboratory conditions. These cells migrate toward blood vessels at stage 11-12 H&H, peak at stage 14 H&H, and decline at stage 18H&H, making stage 12-17 H&H (45h-64h of incubation) the optimal time for their isolation. [2, 13]. Although pheasants are an important economical species [14], but there are very few studies on pheasant embryos. In order to study primordial germ cells, they require special stains and methods to be distinguished from other embryonic cells. PGCs derived from a variety of the embryonic tissues are characterized and

[1]*Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
[2]*Department of Biology, College of Education for Pure Science ibn Al-Haitham, University of Baghdad, Iraq*
**Corresponding Author. e-mail: nabipour@um.ac.ir*

examined afterwards by using a specific antibody, ALP and other histochemical techniques. Therefore, this study aimed to identify primordial germ cells in pheasant using histochemical and immunohistochemical techniques and to determine which method provides the most accurate detection.

MATERIALS AND METHODS

Fertile pheasant (*Phasianus colchicus*) eggs (28 eggs) were collected from commercial poultry farm. The eggs were placed in an incubator. During incubation, optimal conditions were maintained at 37˚C and 60% humidity, and the eggs were rotated at one-hour intervals.

Separation of embryos

At the end of the incubation period for each stage (stage 14-17, stage 20-21 and stage28-30 H&H), the eggs were removed from the incubator, and placed under a stereomicroscope. Then, the eggshell was cut at the location of the embryo and the embryonic stages were determined using the Hamburger and Hamilton (H&H) criteria [15]. At stage 14-17 H&H, with Using a fine heparinized glass needle, blood samples were obtained from extraembryonic vessels and, in some cases, the dorsal aorta (Monoject scientific, Ireland). After that, blood samples were mixed with phosphate buffer saline (PBS) to maintain an isotonic environment and to prepare a uniform cell suspension on the slide. The blood sample, previously diluted with PBS, placed on slides with adhesive coating (Poly-L-lysine). The blood smears were directly treated with paraformaldehyde solution (PFA), 4% in PBS. The slides were washed with PBS and allowed to dry at the room temperature. They have been stored in PBS at 4^{o}C for upcoming usage.

The stages 20-21 H&H (3.5-4 days of incubation) and stages 28-30 H&H (6-7 days of incubation) eggs were opened as described earlier. The embryos were taken from the yolk then embedded in 4% PFA for 24 h at 4^{o}C. Some embryos immediately immersed in Gender's fixative and fixed for 8 hours at room temperature. Gender's fixative (alcoholic bouin's fluid) was employed to more effectively preserve glycogen particles within the cells [16]. After fixation, the embryos were washed with 80% ethanol. The embryos were dehydrated in series of ascending ethanol, cleared in xylene and embedded in paraffin. 5 µm thick paraffin sections were obtained with a rotary microtome (Leica RM 2145; Germany). The sections were photographed on an BX-51 light microscope with digital Olympus camera (DP-12) after histochemical and immunohistochemical staining.

Immunohistochemical staining

For immunohistochemistry, paraffin sections were dewaxed, hydrated in an ethanol series and washed in phosphate buffered saline (PBS). The sections were incubated with 0.30% H_2O_2 in PBS at room temperature for 15min to block any endogenous peroxides. then, the slides formerly washed with PBS. A 10% bovine serum albumin in PBS was prepared. Tissue sections and blood smears were incubated in it in a humid chamber. Slides were incubated with the primary antibody SSEA-1 (R&D; US), followed by a donkey anti-mouse IgM secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch, US). Concentration 1:300 was used for the primary antibody overnight at 4^{o}C and the secondary antibody was used at a concentration of 1:500 for one hour at room temperature. After washing with PBS, peroxidase label was revealed by reaction with Diaminobenzidine (DAB) for 5 min at room temperature. The sections were counterstained with hematoxylin and mounted using Entellan (Merck; Germany). Negative controls were prepared by omitting the primary antibodies [7,17].

Alkaline phosphatase staining

Tissue sections were deparaffinized and hydrated by graded alcohol series. Sections and blood smears were incubated for 30 min at room temperature in ALP substrate solution (stock 5% BCIP in 100%. Dimethylformamide (33µl), stock 5% NBT in 70% Dimethylformamide (66µl) and 10ml of buffer substrate. Deionized water was utilized in order to wash the slides. Lastly, the sections were dehydrated and then mounted by using Entellan. BCIP: Bromo-4-Chloro-3-indolyphosphate (Thermo scientific; USA), NBT: Nitro-blue tetrazolium chloride (Thermo scientific; USA) and the buffer substrate consisted of 10 mM NaCl (Merck; Germany), 5 mM Magnesium chloride ($MgCl_2$) (Merck; Germany) and 100 mM hydroxymethyl aminomethane (Sigma Aldrich-TM; USA) with PH 9.5 [7,17].

Paraffin sections were stained with hematoxylin and eosin (H&E), PAS and Alcian blue stains. PAS and Alcian blue stains detect glycogen granules and acidic mucosubstances, respectively, in the cytoplasm [18].

RESULTS AND DISCUSSION

The pheasant PGCs at stages14-17 of H&H had a large and eccentric nucleus which occupied the majority of cytoplasm (Fig. 1A). These cells in blood slides were not stained with SSEA-1 antibody, and the reaction was negative (Fig. 1B, 1C), despite that, PGCs were easily diagnosed. The cells appeared spherical, containing a

large compact nucleus and a terminal site, and could be differentiated from light/pale blood cells (Fig. 1B). Some PGCs were imaged during division (Fig. 1D), and some appeared in clusters (Fig. 1E). PGCs did not react to ALP staining. These cells, after ALP staining, appeared clear, with a brown cytoplasm, with the nucleus lighter than the cytoplasm (Fig. 1F). Results of PAS (Fig. 1G) and Alcian blue staining (Fig. 1H) were also negative or weak, while the cytoplasm and nucleus showed blue and white color, respectively.

Detection of PGCs in genital ridge

During the formation of genital ridge, PGCs were observed in a migratory state. These cells were placed

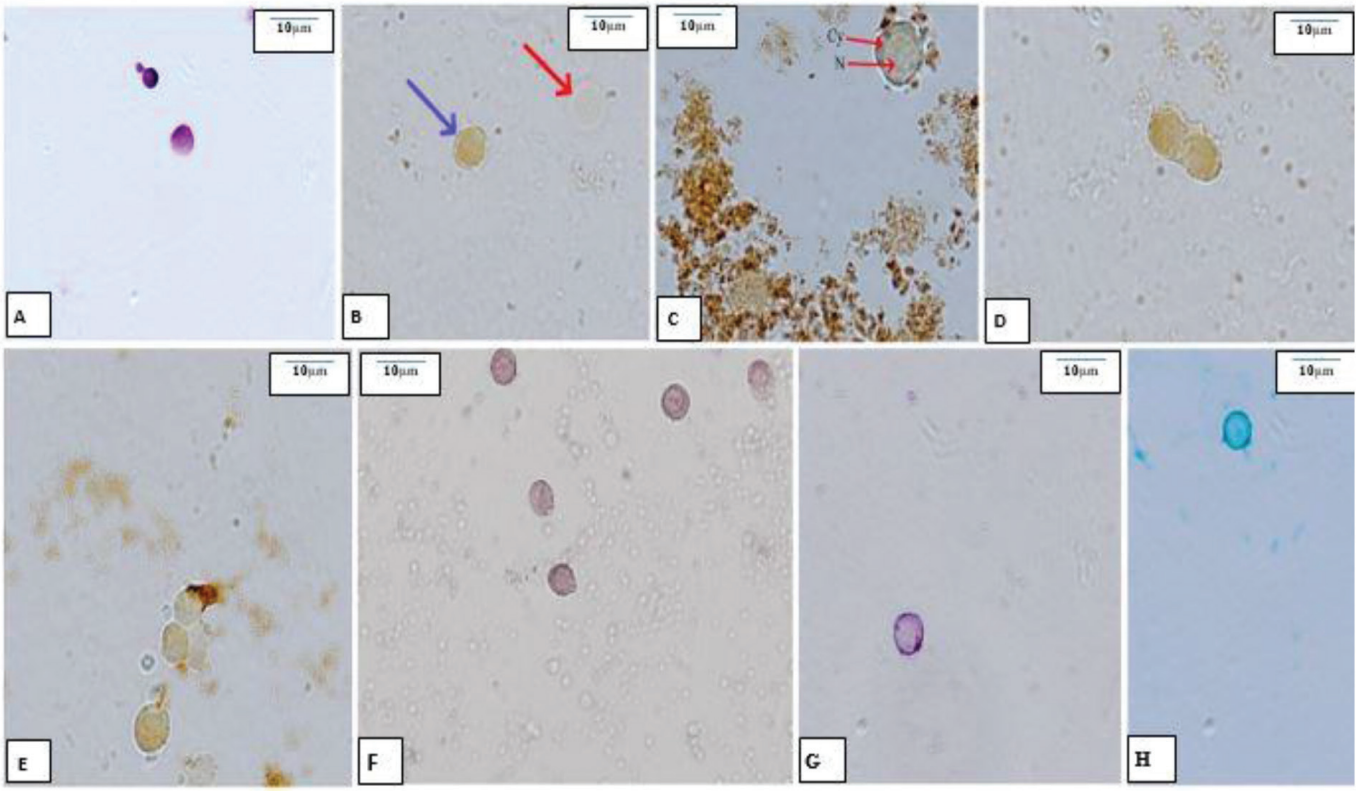

Fig. 1. Photomicrograph of blood smear from stage (14-17 H&H) pheasant embryo (1000×). A) PGCs stained with H&E, **B**) PGCs treated with SSEA1 antibody (blue arrow), blood cell (red arrow), **C**) PGCs stained with SSEA1 antibody demonstrate nucleus (N), cytoplasm (Cy), **D**) PGCs in division state, **E**) clusters of PGCs stained with SSEA1 antibody, **F**) ALP activity, **G**) PAS stain, **H**) Alcian blue stain.

inside the blood vessels of the embryo or attached to the vessel wall (Fig. 2A). Also, by using H&E, it was possible to distinguish the difference in shape between the primordial germ cells in the migratory state, which had dark pink nuclei (Fig. 2B), and white nuclei in the genital ridge in stage 20 H&H (Fig. 2C). The PGCs could easily be detected by examining the paraffin sections stained with H&E. These cells were larger than the mesenchymal cells, had a large white and compact nucleus occupying most of the cell volume, and a narrow cytoplasm appeared pink at both stages 20 (Fig. 2C) and 21 H&H (Fig. 2D). It was noticed that the genital ridge in stage 21 H&H was thicker than in stage 20 H&H, possibly due to the accumulation of PGCs at stage 21 H&H (Fig. 2D).

The majority of the primordial germ cells migrated through dorsal mesentery in order to reach the genital ridge. They could be observed using different staining methods. In this study, they were observed in tissue sections stained with hematoxylin and eosin (Fig. 3A), as well as SSEA-1 immunolabeling (Fig. 3B). Although the reaction was negative with SSEA1, the shape of the cells helped in their diagnosis. During migration toward and within the genital ridge, primordial germ cells displayed a negative reaction to SSEA-1 immunostaining at both 20 (Fig. 3C) and 21 H&H (3D) stages. At stages 20-21 H&H, the pheasant PGCs showed a negative reaction with the alkaline phosphatase enzyme (Fig. 3E and 3F). Histological specimen of liver from chick with one-day-old were

utilized as negative control for enzyme histochemistry method (ALP staining) through revealing them to a 60°C temperature for 1 hr without straining the nucleus (Fig. 3G). The liver from the similar stage was utilized as well for the ALP enzyme staining as a positive control (Fig. 3H). PGCs in pheasant embryos at 20-21 stages of H&H stained with Alcian blue (Fig. 3I and 3J) and PAS stains (Fig. 3K and 3L) showed a weakly positive or negative reaction. Histological samples of ovine trachea were utilized as a positive control for Alcian blue stain (Fig. 3M) and PAS stains (Fig. 3N).

Detection of PGCs in the primary gonad

At stages 28, 29 and 30 H&H, the gonads were formed on the ventromedial surface of the mesonephros. In the current work, in the transverse sections, the gonads formed two ovals or circular structures (Fig. 4A, 4B, and 4C). The PGCs settled in the area could be easily differentiated from gonad mesenchymal cells by their phenotypic characteristics mentioned in the previous stages. In H&E staining, the cells might be easily recognized by their white nucleus, big size, and pink cytoplasm (Fig. 4D, 4E, and 4F). In the slides stained with anti-SSEA1 antibody, the PGCs in all stages had been negative (Fig. 4G, 4H, and 4J), while only in some cases did, they react very weakly (Fig. 4G and 4I). These cells, had no positive staining with the SSEA-1 but, were easily distinguished by their morphology. PGCs were spherical, large size with large nucleus that lodging the majority of cytoplasm. A number of slides were prepared from chick embryo gonad samples and had been utilized as positive control for the SSEA1 antibody. Germ cells at this stage countered positively to SSEA-1 staining (Fig. 4K). The similar stage had also been utilized as negative control by neglecting primary antibody and using only the secondary antibody (Fig. 4L). The PGCs in stages 28, 29, and 30 H&H, were negative or weakly positive with PAS staining (Fig. 4M, 4N, and 4O). In the embryo of stages 28, 29, and 30 H&H, the PGCs reacted negatively with alkaline phosphatase enzyme (Fig. 4P, 4Q, & 4R). In entirely three embryonic stages, primordial germ cells displayed negative or very weak reaction with Alcian blue (Fig. 4S, 4T, and 4U).

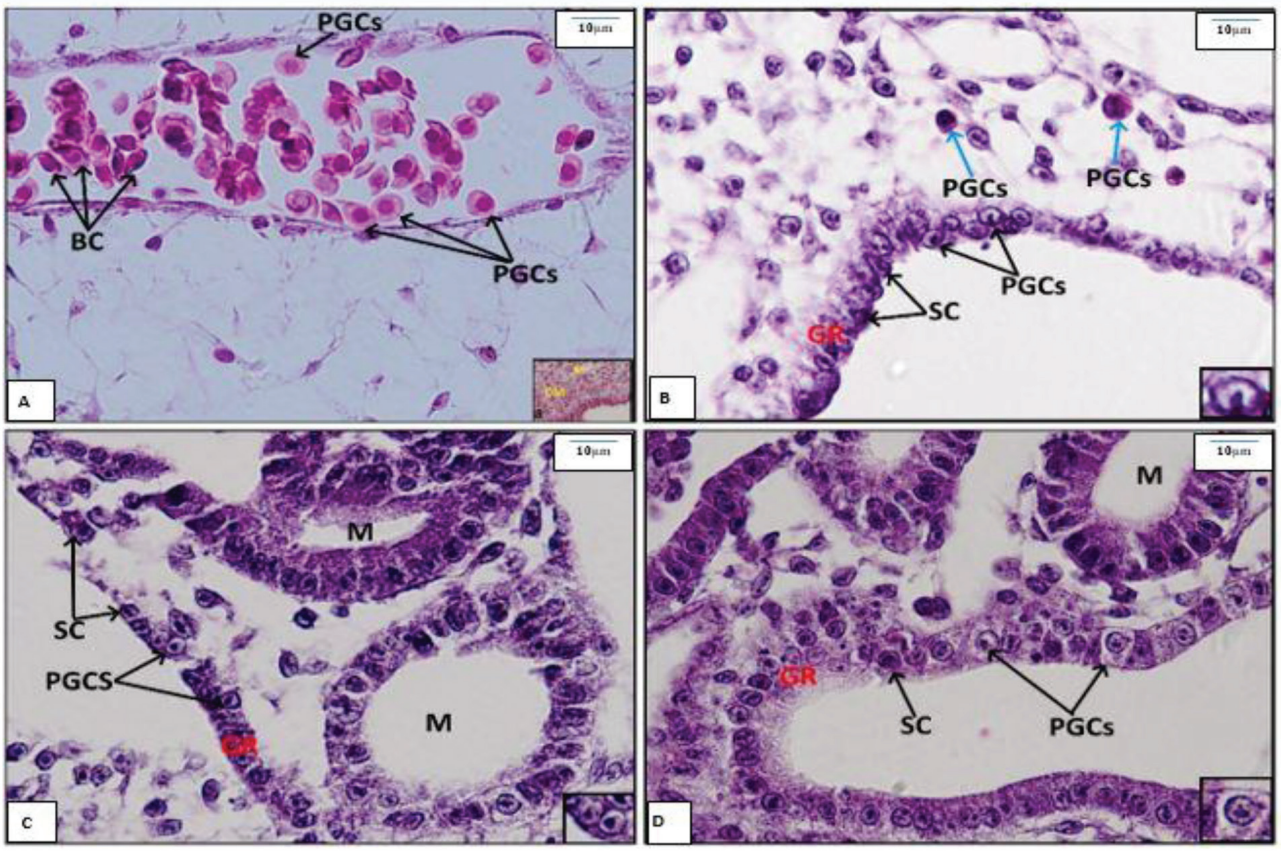

Fig. 2. Identifying the pheasant PGCs, migration toward the genital ridge at stage 20-21 H&H (1000×). A) PGCs in dorsal aorta and some of PGCs adhered to the wall of the dorsal aorta, or in dorsal mesentery (a). **B**) PGCs migrate from blood vessel to genital ridge (blue arrows), **C**) PGCs in genital ridge of stage 20 H&H, **D**) stage 21 H&H (D). Genital ridge (GR), primordial germ cell (PGCs), somatic cells (SC), mesonephros (M), dorsal mesentery (DM). H&E stain.

The transfer of PGCs between embryonic blood vessels represents a common approach for establishing germline chimeric [18, 19]. The chromosomal sex and germ cell competition's role in differentiating avian germ cells was studied by [20]. In the past, methods of freezing eggs were used to preserve embryos, which was an impractical method. At present, the freezing of primordial germ cells allows preserving the offspring of male and female animals [21]. The process by which primordial germ cells enter blood vessels is similar to that occurs during the inflammation and the movement of lymphocytes to the sites of inflammation and tumor [22, 23]. PGCs in birds have an epiblastic origin [1]. They migrate through blood vessels, enter the dorsal mesentery, and reach the genital ridges. At stage 17 H&H, they settle down in the genital ridges [8], where they finally differentiate into oogonia or spermatogonia [1, 2]. Germ cells can be observed in the bloodstream from stage 10 onward [24]. They are characterized by being migratory cells, the origin and migration of which are important topics that have attracted the attention of researchers [25]. The PGCs concentration in the bloodstream reaches its peak at stage 14 H&H and then begins to decline at stage 15 H&H because of the presence of PGCs in the network of capillaries that supply the gonads [26]. Histochemical and immunohistochemical staining facilitate cell detection, as PAS staining is widely recognized as an effective

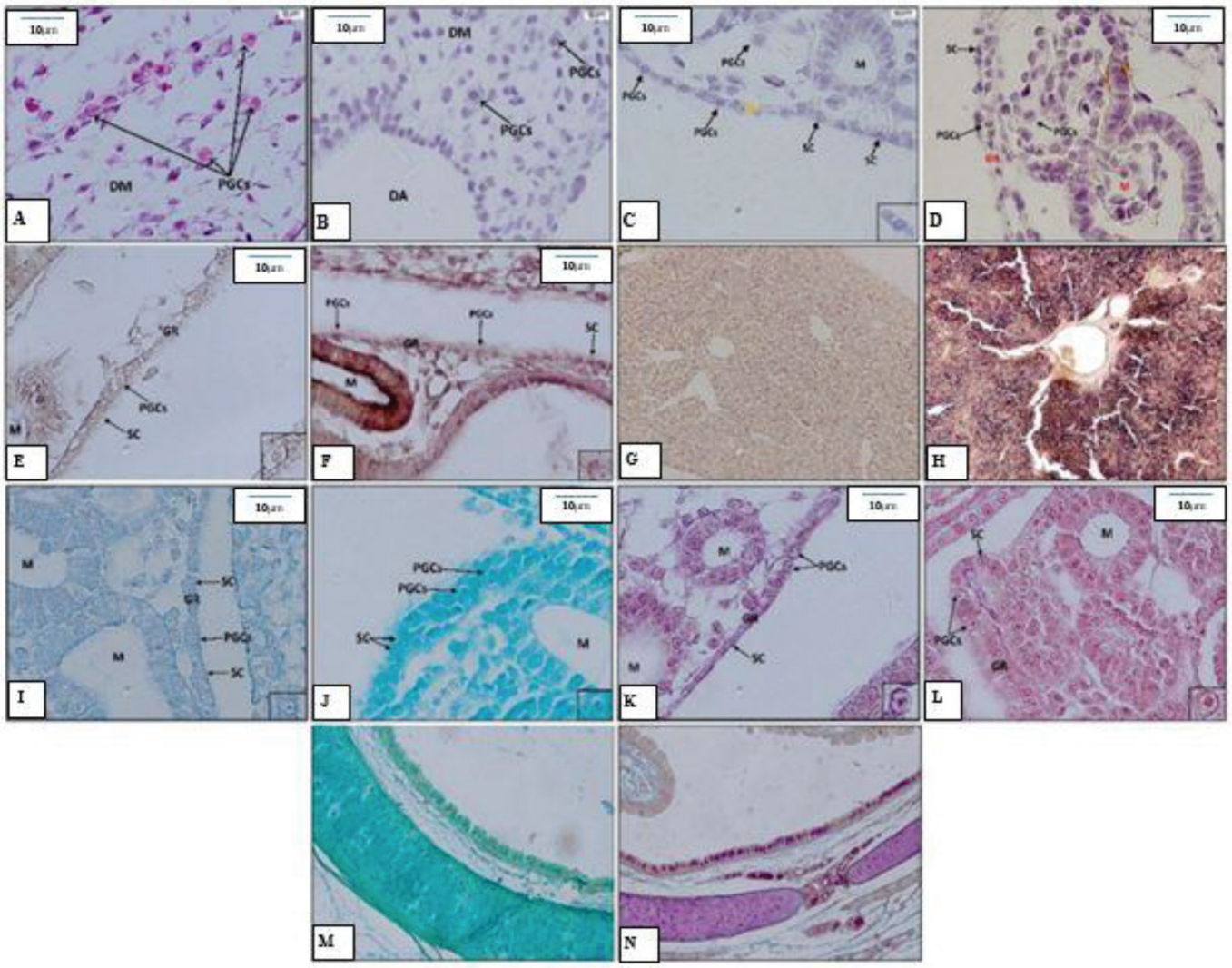

Fig. 3. Identifying the pheasant PGCs, migration toward the genital ridge at stage 20 (C, E, I, K), and stage 21 H&H (D, F, J, L). A) Some of the PGCs seen in dorsal mesentery by using H&E stain (1000×), **B**) SSEA-1 antibody (1000×), **C, D**) The PGCs were identified by SSEA-1 antibody (1000×), **E, F**) ALP activity (1000×), **G**) ALP negative control (100×), **H**) ALP positive control (400×), **I, J**) Alcian blue stain (1000×), **K, L**) PAS stain (1000×), **M**) Alcian blue control (400×), **N**) PAS stain control (100×). Primordial germ cells (PGCs), somatic cells (SC), dorsal mesentery (DM), dorsal aorta (DA), genital ridge (GR), mesonephros (M).

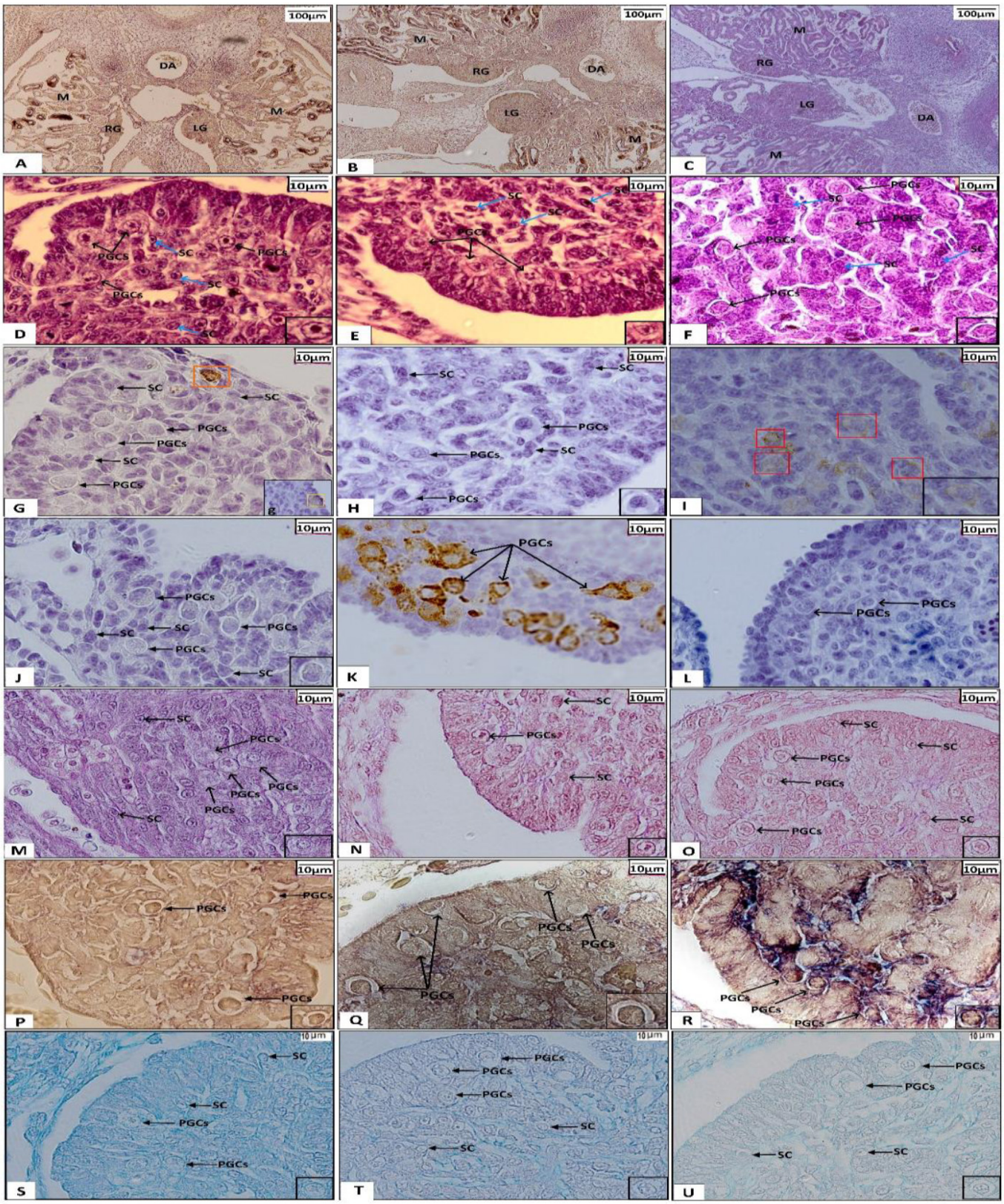

Fig. 4. Identifying the pheasant PGCs in the primary gonad. **A, B, C**) Site of gonads inside body in stages 28-30 H&H (100×), **D, E, F**) the PGCs were identified by H&E (1000×), **G, H, I, J**) SSEA-1 antibody (1000×), **M, N, O**) PAS stain (1000×), **P, Q, R**) ALP activity (1000×), **S, T, U**) Alcian blue stain (1000×), **K**) SSEA-1 positive control of chick embryo (1000×), **L**) SSEA-1 negative control of chick embryo (1000×). Primordial germ cells (PGCs), somatic cells(SC), PGCs weak reaction with SSEA-1in stage28-29 H&H (A, C), left gonad (LG), right gonad (RG), dorsal aorta (DA), mesonephros (M).

method for differentiating PGCs from surrounding cells [27]. The PGCs are demonstrated to contain abundant glycogen by the PAS reaction [24]. England and Matsumura showed the PGCs in chicks to be distinguished in the germinal crescent by the PAS stain [28]. Reported that PGCs in blood smear and genital ridge of turkey embryo were PAS-positive because the PGCs contain high glycogen granules in turkey embryo (*Meleagris gallopavo*) [7]. While chicken and turkey PGCs contain glycogen and can be detected with the PAS stain, pheasant cells have very little glycogen. This may indicate that the aldehyde groups or carbohydrates in pheasant PGCs are very low or negligible and therefore, the PAS method is less effective for identifying PGCs in pheasants [7, 29, 30]. At stages 20-21 H&H of the chick embryo, observed that PGCs were settled in the genital ridge and these cells have been localized within the mesenchyme [31]. The present study showed that PGCs in pheasant embryos from stage 20-21 H&H were found in genital ridge, as well as the dorsal mesentery and blood vessels. PAS stain is a promising approach to identifying PGCs in blood smears and paraffin slices of the primitive gonad of an ostrich embryo [6]. PGCs in the blood and genital ridge of chick embryos were investigated using the PAS stain by [13, 32]. The present study's findings in pheasant embryos differ from those reported in chick embryos. PAS staining and anti SSEA-1 antibodies are routinely employed to identify chicken PGCs [8, 24]. Somatic cells are PAS-negative, and germ cells are PAS-positive since PGCs have a high glycoprotein content. Further, PAS technique is utilized for demonstrating the neutral mucins [33]. In the present study, PGC reactivity to PAS has been too weak to clearly detect PGCs, due to the absence or decreased amount of glycogen in the cytoplasm of pheasant PGCs. The findings of this study are inconsistent with those reported by Naeemipour & Bassami, and contradict the results reported by Meyer [17, 34]. A histochemical method was developed for detecting proteoglycans and acidic carbohydrates using Alcian blue-silver staining by [35]. According to [11], the ovomucin-like protein (OLP), cell surface mucin, on migrating chick and rat PGCs enhances the cells broad migration to vaginal ridges by blocking adherence of PGCs to the walls of blood vessel and mesenchyme. Alcian blue has been used to demonstrate various kinds of acid glycoconjugates and sulfomucins [36]. Sulfomucins are a subclass of mucins characterized by a high content of sulfate groups and play an important role in histopathological evaluations. Alcian Blue selectively interacts with the sulfated moieties of sulfomucous secretions, enabling the identification of cells that produce these mucins in abundance. In addition, the combined application of Alcian Blue and PAS staining provides a valuable diagnostic approach for differentiating cell types and tissue structures based on their specific mucin secretion profiles [16, 35, 36]. In this study, an Alcian blue stain was used to prove if the cells contain mucosubstances sulfomucins in PGCs. The present study agrees with [37] but disagrees with [7]. Since these cells are migratory, cell migration could result in an alteration in cellular parts containing carbohydrates which may be a reason why cells show no positive reaction with PAS and Alcian blue. According to [38], once the PAS and Alcian blue stain were used, the germ and epithelial cells in the right ovary in chicken embryos showed more intense reaction than in the left ovary. The reason was attributed to the increase of materials in mucinous in the advanced stages of the formation of the gonads, playing a vital role in cell migration. SSEA-1 is antigenic epitope on the cell surface that is deployed as a specific antibody for stem cell detection [24]. Other researchers identified PGCs using SSEA-1 and EMA-1 [39]. There are numerous disagreements about the SSEA-1 marker; certain articles consider SSEA-1 a PGCs marker, while others view it a pluripotency marker. PGCs are easily recognized utilizing monoclonal antibody SSEA-1[40]. In this study, the SSEA-1 was negative in PGCs of a blood smear, the same result was detected by [41] and [6]. In the current

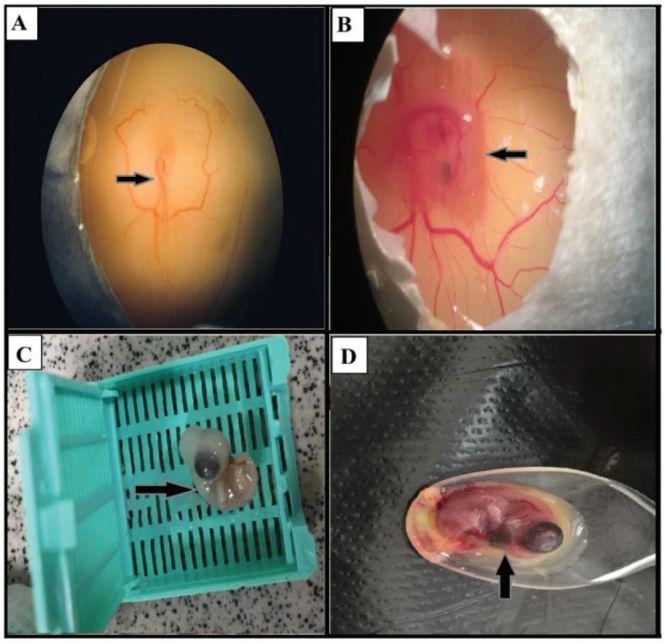

Fig. 5. Photograph of Pheasant embryos at four developmental stages. A) Pheasant embryo at stage 15 H&H, **B**) stage 21 H&H, **C**) stage 28 H&H, **D**) stage 30 H&H. Black arrows in all panels indicate the embryo.

research, the pheasant PGCs were SSEA-1 negative at stage 20-21 H&H and was very weak or negative in PGCs of gonad at stages 28, 29, and 30 H&H. These results disagree with previous studies [7, 42, 43]. Some researchers reported that, PGCs from the Chinese Meiling chicken were SSEA-1 positive. Previous studies show that, SSEA1 has not been detected during the cleavage and gonadal ridge stages in the bovine embryo [10]. In the turkey embryo, primordial germ cells that migrate to genital ridge at stage 20 H&H did not express SSEA1 epitope; however, an SSEA-1 positive reaction could be detected in PGCs of chick embryo after they settled in the gonad [44]. The current research is in line with those mentioned above in that the PGCs in pheasants are incapable of expressing the SSEA1 epitopes, and with the help of this method, PGCs are not identified, although a very weak reaction of cells appears in the 28th and 29th stage. The reasons behind the negative results of identifying PGCs with SSEA-1 in the pheasant are unclear. The absence of SSEA-1 marker expression in pheasant PGCs may indicate that these cells are intrinsically unable to express this marker, suggesting that their cell type dose not correspond to SSEA-1- positive profiles. This could be due to species-specific differences between chicken and pheasant. ALP enzyme expression appears in many cells, like the osteoblasts and odontoblasts, as well as in undifferentiated embryonic stem cells and pluripotent stem cells; its enzymatic activity indicates differentiation in cells [45]. The cytoplasm of PGCs differs from somatic cells in that alkaline phosphatase activity (enzymatic properties) is higher in PGCs [46]. In zebra fish, the PGCs show a high alkaline phosphatase activity in peripheral cytoplasm [47]. During passive and active migration phases, ALP has been shown to play a vital part in enzymatic activity [48]. In this article, in blood, the PGCs were not detected by ALP; a different finding was reported in turkey and chicken PGCs [7, 24]. In the genital ridges and primary gonads in the pheasant embryo, the PGCs did not react positively with the ALP enzyme. The findings in this study agree with those in the turkey and chicken embryo but inconsistent with the study of Swarts in chickens [7, 17, 48]. The reason may be the change in the metabolic activities of PGCs or the synthesis of alkaline phosphatase [48].

CONCLUSION

Primordial germ cells in blood smear showed a negative reaction with alkaline phosphatase, and circulating PGCs in the blood were negative for SSEA-1. The PAS staining has been negative or very weak. These cells showed a very weak or negative reaction to staining with Alcian blue. At stages20-30 H&H, primitive germ cells cannot be distinguished by alkaline phosphatase, and cannot be detected by SSEA-1 immunostaining. Those cells showed negative or very weak staining with Alcian blue and PAS. The results of this study showed that PGCs in all stages studied had very weak or negative reactions to different histochemical and immunostaining techniques. Conventional H&E staining provides a reliable method for the microscopic identification of these cells. Morphologically, pheasant PGCs resemble those of birds; however, species-specific differences led to negative reactions in histochemical and immunohistochemical staining.

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ETHICAL APPROVAL

All experiments were approved by the Ethics Committee of Ferdowsi University of Mashhad (IR. UM.REC.1399.136).

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