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The identification of the primordial germ cells in the male gonads of pheasant (Phasianus colchicus) embryos using histochemical and immunostaining techniques

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Abstract

The purpose of this research was to look for primordial germ cells in male gonads at various stages of the pheasant embryos. Primordial germ cells are cells that differentiate into sperm or oocyte and are important for the transmission of genetic information across generations. In this study, embryos were extracted in embryonic days 8-24. The primordial germ cells were recognized in paraffin and resin tissue sections on days 8 to 10 of embryogenesis owing to the specific properties of these cells, which included a large size, large nucleus and nucleolus, and little cytoplasm. The primordial germ cells in the testes were identified using histochemical and immunostaining techniques. Periodic Acid-Schiff method, immunohistochemistry using stage-specific embryonic antigen-1, alkaline phosphatase and toluidine blue staining were used to identify primordial germ cells. Germ cells were detected only in semithin sections at older ages (10-24 days). Moreover, the response of these cells was negative, at all ages, to alkaline phosphatase, Periodic Acid-Schiff staining and stage-specific embryonic antigen-1 reaction. The findings of this study revealed that primordial germ cells in the testicular tissue of pheasant embryos of all ages tested responded adversely to histochemical and immunohistochemical techniques. Also, this study showed that routine staining of hematoxylin and eosin and using semithin sections are suitable for the histological diagnosis of these cells.

Key words: Primordial germ cells, Immunohistochemistry, Alkaline phosphatase, Pheasant, Male gonads

Introduction

In the fields of growth biology and stem cell biology, bird embryos have become a popular model (Stern, 2005). Among birds, regarding pheasant, meat contains low fat and cholesterol and high content of fatty acids is essential; hence, it is considered a valuable bird in terms of economy (Adamski and Kuźniacka, 2006). Their reproductive organs have distinct features. Female gonads and accessory embryonic oviducts grow asymmetrically, while male gonads develop symmetrically (Smith and Sinclair, 2004). Pheasant embryonic gonadal development is comparable to that of other bird species. The process of generating embryonic gonads is known as "gonadogenesis" (Clinton and Haines, 2001). Gonadogenesis in vertebrates starts with the migration of primordial germ cells (PGCs) (Y Nakamura et al, 2007). PGCs

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emerge from the extraembryonic area (on the first day of pregnancy) and travel to the genital ridges, where they settle down (Y Nakamura et al, 2007). PGCs in mammals are produced from the endodermal cells of the hindgut's yolk sac (Bendel–Stenzel et al, 1998). Avian PGCs, on the other hand, start in the central zone of the blastodisc (Y Nakamura et al, 2007) and move anteriorly to the extraembryonic area known as the "germinal crescent" (Y Nakamura et al, 2007). PGCs penetrate blood vessels and extravasate from the endothelium of capillaries towards the genital ridges by amoeboid movement, (Y Nakamura et al, 2007) employing pseudopodia or filopodia-like processes (Gomperts et al, 1994). In the developing gonads, established PGCs with contributions from the coelomic epithelium and mesonephroi give birth to a variety of cell types (Carlon, 1985). PGCs and surrounding somatic cells induce the formation of bipotential (indifferent or undifferentiated) gonads in developing gonads (Carlon, 1985). Finally, sexually differentiated gonads seem to develop and generate functional gametes as adult gonads (Clinton and Haines, 2001). Genetic information is passed down via the generations through PGCs which are considered the immortal cells (Kim and Han, 2018). Therefore, the study and identification of PGCs to preserve genetic resources to be used in basic biological research are considered an important goal (Yoshiaki Nakamura et al, 2013). Another importance of PGCs is due to the chance of obtaining male germ cells from transferring female ones to male embryo (Tagami et al, 1997). Considering the signals from their condition, PGCs are differentiated into gametes of each sex (Wylie, 1999). PGCs with large size and large spherical nuclei are identified using histochemical markers, including Periodic Acid Schiff (PAS), resulting in glycogen or immunohistochemical staining in the cell surface antigen (Ginsburg and Eyal-Giladi, 1986). One of the main markers to distinguish the pluripotent embryonic stem and associated cells is alkaline phosphatase (Štefková et al, 2015). As a cell surface glycoprotein, stage-specific embryonic antigen-1 (SSEA-1) can be utilized as another marker to detect PGCs in mammals and chickens (Yön Ertuğ and Akbulut, 2015). Therefore, to study PGCs, staining and special methods are needed to distinguish them from other embryonic cells.

Materials and Methods

Ethics

Ethical approval for this research was confirmed by the Ethics Committee of Ferdowsi University of Mashhad (IR.UM.REC.1399.136). All experiments were done based on relevant guidelines and regulations.

Separation of embryos and testes

Fertile pheasant (Phasianus colchicus) eggs were provided from a commercial farm. The eggs were incubated at a temperature of 37 °C and a relative humidity of 60%. An incubator rotated the eggs every 60 minutes to prevent the embryo from adhering to the eggshell. In the same vein, the goal of this research was to find out what traits PGCs have on embryonic days (ED) 8, 13, 17, and 24 (day of hatching). At each ED, after separating the embryo from the yolk, it was washed with a fixator and dissected under a stereomicroscope and the testes were removed. The removed testes were then fixed in 4% paraformaldehyde (PFA) for 24 hours. Other samples of testes were fixed in a Gender solution for 8 hours and then washed with 80% ethanol. The removed tissues were later dehydrated in ethanol (70%, 80%, 90%, and 100%) and purified xylene. They, then, were fixed in paraffin (Merck, Germany) and cut by a rotating microtome (Leica RM 2145; Germany) (cross-sections, 5 µm). Also, samples were taken to prepare semithin resin sections (size approx.1mm) and fixed in 2%

glutaraldehyde in 1M buffer cacodylate to embed epoxy resin. After histochemical and immunohistochemical staining, the micrographs were taken with a BX51 light microscope (Olympus; Japan) equipped with a camera (DP12, Olympus, Japan).

Immunohistochemical staining

The paraffin was removed from the tissue slices using xylene and rehydrated in an ethanol series that had previously been graded. It was then rinsed in phosphate buffered saline (PBS). To limit endogenous peroxidase activity, sections were treated with 0.3 percent H2O2 in PBS at room temperature for 15 minutes. They were then rinsed in PBS before being blocked in 10 percent bovine serum albumin (BSA/PBS) to reduce non-specific antibody binding. The slides were then treated at 4°C overnight with the primary antibody, stage-specific embryonic antigen-1(SSEA-1) (1:300; R&D; USA). The secondary antibody, donkey anti-mouse immunoglobulin M (IgM), was incubated for 1 hour at room temperature with HRP (1:500, Jackson ImmunoResearch Inc. USA). The slides were cleaned three times with PBS before being incubated in a dark, humid environment for 5 minutes with diaminobenzidine (DAB). Later, the sections were counterstained with hematoxylin. Ultimately dehydrated, cleaned with xylene, and mounted with Entellan (Merck, Germany). To incubate the samples as a negative control, only a secondary antibody was included (by omitting the primary antibody).

Alkaline phosphatase staining

To eliminate all the paraffin from the tissue samples, the solvent xylene was utilized. The segments were then rehydrated using the ethanol series. The alkaline phosphatase (ALP)-treated sections were substrate at room temperature for 30 minutes in a dark and humid chamber. Deionized water was used to clean the slides. Eventually, the sections were dehydrated and mounted with Entellan. Alkaline phosphatase substrate consisted of 33 µl stock 5% Bromo-4-Chloro-3-indoly-phosphate (BCIP) (Thermo Scientific, USA) in 100% Dimethylformamide (DMF), 66 µl stock 5% Nitro-blue tetrazolium chloride (NBT) (Thermo scientific; USA) in 70% DMF (Merck; Germany), and 10 ml of buffer substrate. The buffer substrate consisted of a PH of 9.5, with 10 mM NaCl (Merck; Germany), 5 mM Magnesium chloride (Mgcl2) (Merck; Germany) and 100 mM (hydroxymethyl) aminomethane (TRIS) (Sigma Aldrich-TM, USA).

Histological assessment

For the histological observation, paraffin samples were stained using hematoxylin and eosin (H&E) and PAS (Merk, Germany) to detect the glycogen granules in the cytoplasm of PGCs (Suvarna, Layton, and Bancroft, 2018).

Also, samples were used to make semithin resin sections (1µm thickness) that were fixed in 2% glutaraldehyde in 1 M buffer cacodylate before being embedded in epoxy resin.

The glutaraldehyde-fixed specimens were refixed in a 1 M cacodylate buffer with 1% osmium tetroxide solution. Before being soaked in epoxy resin (TAAB Laboratories Equipment Ltd, UK) and cut into 1-µm sections, the samples were dehydrated using the progressive lowering temperature (PLT) process.

The lumen of the tubules and the cells around the lumen were next examined using toluidine blue staining (Kuo, 2008).

Results

In 8-24-day-old embryos, dirty-white testes were seen on the ventral surface of mesonephros kidneys. The testes inclined and were tightly spaced along the body's median plane from the cranial border to the midportion of the kidneys (Figure 1f). In 8-day-old embryo, PGCs were easily detected by examining the slides of paraffin and resin

sections stained by H&E and toluidine blue due to their shape (Figures 1A and 1D). Germ cells can be distinguished from other cells with larger cells and nucleus and clearer cytoplasm. At this stage, the interaction with PAS was negative or very weak (Figure 1C). The histological samples of the rectum of rabbit were used as a positive control for PAS stain (Figure 5E). SSEA-1 and alkaline phosphatase reactions were negative (Figure 1B and 1E). The histological samples of chick liver at one-day age were used as a negative control for the ALP enzyme, by exposing the sample to a temperature of 60 °C for an hour without staining the nucleus (Figure 5C). The liver from the same stage was used as a positive control for the ALP enzyme (Figure 5D).

In 13 and 17 days old, in anti-SSEA-1 antibody-stained slides, in all phases, PGCs were negative (Figures 2B and 3B). Primordial germ cells could be differentiated from other cells in the gonads with their specific morphology even in slides which did not have positive staining with SSEA-1 antibody. The nucleus of these cells was round and very large, occupying most of the cytoplasmic space. The samples of gonad from chick embryo at stage 28 Hamburger and Hamilton (H&H) were used as a positive control for SSEA-1 antibody; germ cells in this stage positively reacted with SSEA1 at the end of staining (Figure 5B). The same stage was used as a negative control (Figure 5A), by omitting the primary antibody, and the research specimens were only incubated using the secondary antibody.

In the embryonic days 13 and 17, PGCs negatively reacted with the alkaline phosphatase enzyme (Figure 2E and 3E). In all the three embryonic stages, the primordial germ cells showed a negative reaction to PAS stain (Figures 2C, 3C and 4B). At 24 days of age, the primordial germ cells were differentiated into spermatogonia progenitor cells, Leydig and Sertoli progenitor cells (Figure 4).

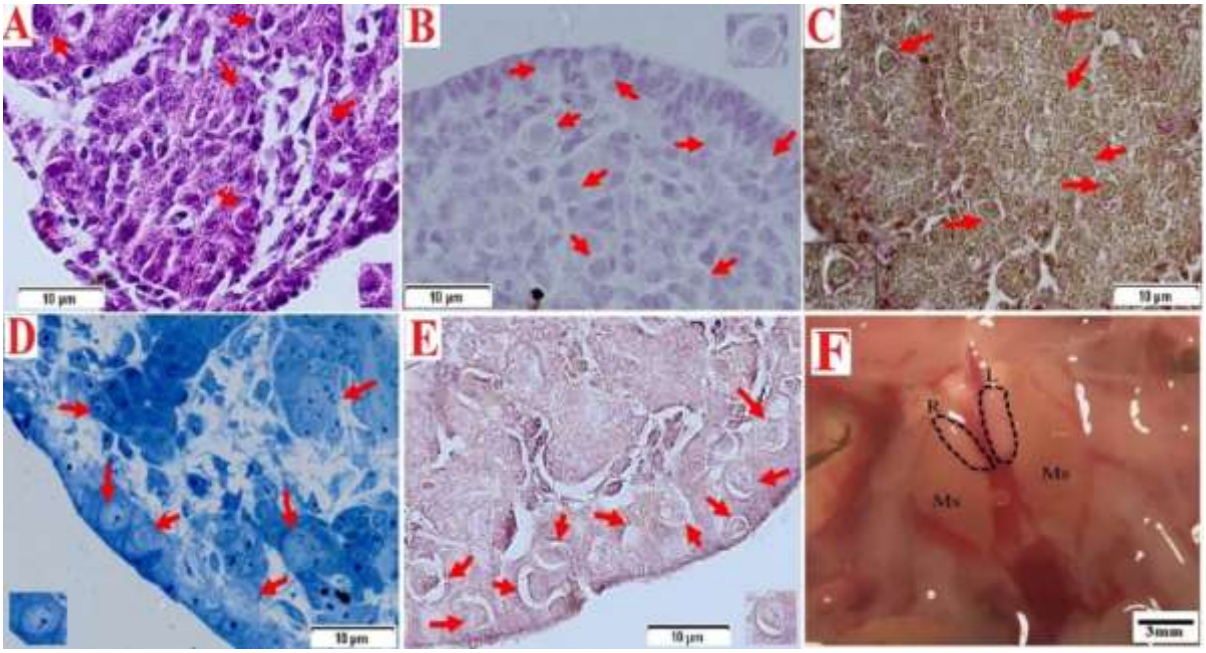

Figure 1. Identifying the pheasant PGCs in the testes tissue at the 8-day-old embryo. The PGCs (red arrows) were identified by A) H&E, B) SSEA-1 antibody, C) PAS stain, D) Toluidine blue stain and E) Alkaline phosphatase enzyme stain. F) Ventral view of pheasant embryo testes. Testes (dashed lines) are located in the ventral surface of mesonephros kidneys (Ms). Note that they are inclined toward the median plane of the body and are located close to each other. R: right, L: left.

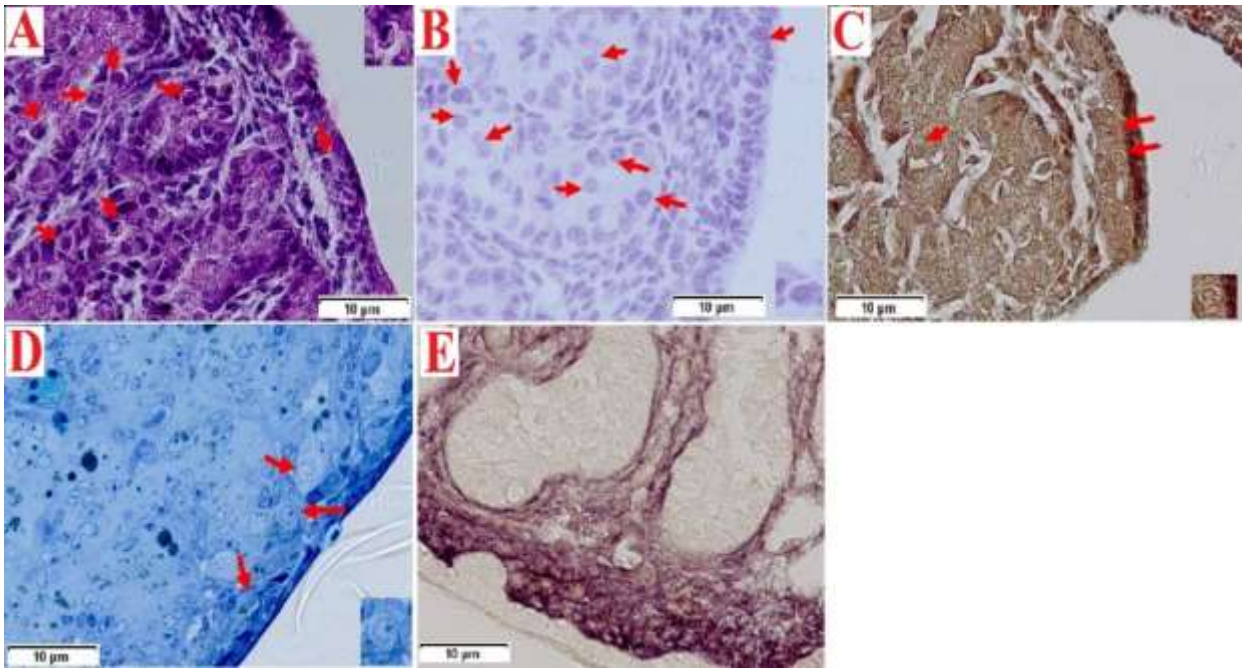

Figure 2. Identifying the pheasant PGCs in the testes tissue at the 13-day-old embryo. The PGCs (red arrows) were identified by A) H&E, B) SSEA-1 antibody, C) PAS stain, D) Toluidine blue stain and E) Alkaline phosphatase enzyme stain (PGCs did not differentiate from other cells in this color).

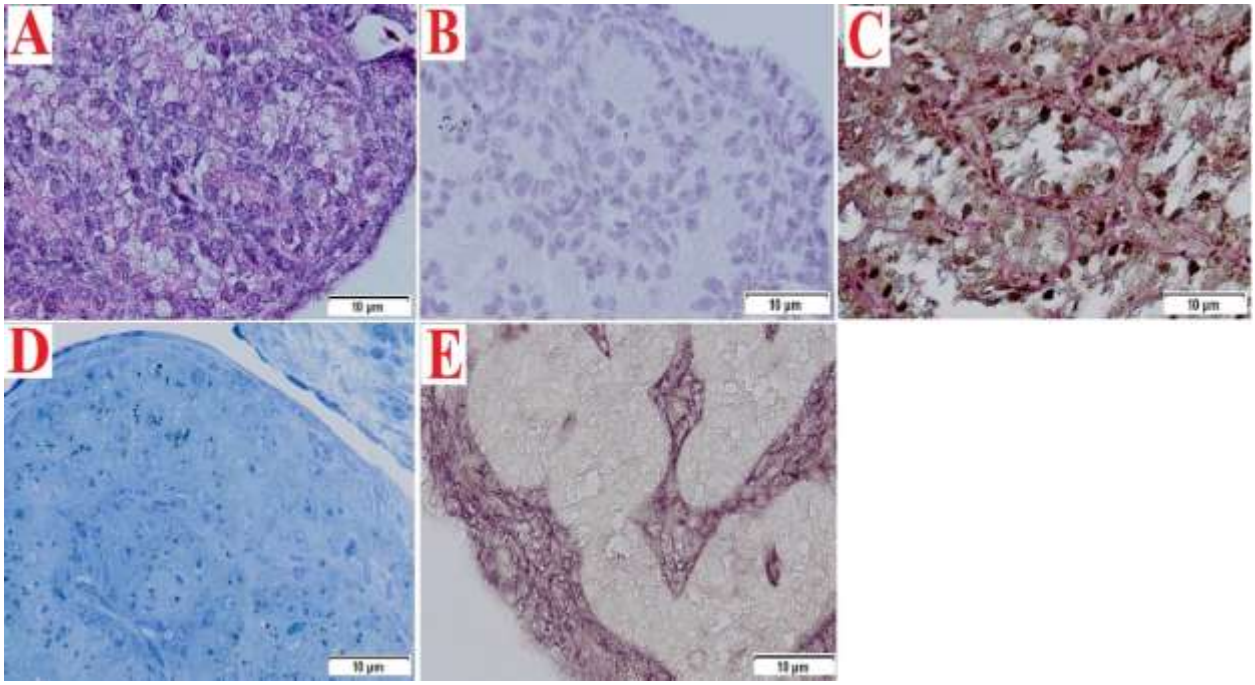

Figure 3. Pheasant embryo testes at 17 days. A) H&E stain, B) SSEA-1 antibody stain, C) PAS stain, D) Toluidine blue stain and E) Alkaline phosphatase enzyme stain. Primordial germ cells were not observed in any of the slides.

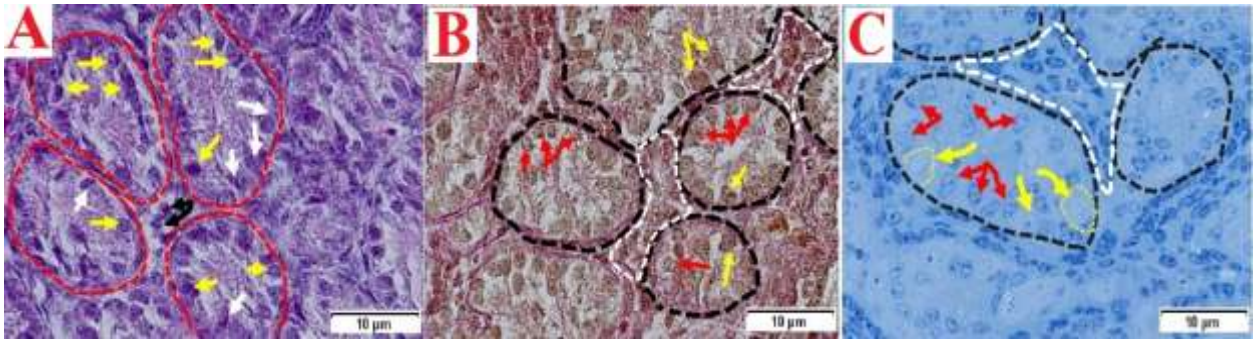

Figure 4. Pheasant embryo testes at 24 days. A) H&E stain. Lumen of primary seminiferous tubules marked by broken lines. Spermatogonia progenitor cells marked by yellow arrows. Sertoli progenitor cells marked by white arrows. Leydig progenitor cells marked by black arrows. B) PAS stain. Lumen of primary seminiferous tubules marked by broken lines. Spermatogonia progenitor cells marked by red arrows. Sertoli progenitor cells marked by yellow arrows. Leydig progenitor cells marked by marked by white broken lines. C) Toluidine blue stain. Lumen of primary seminiferous tubules marked by broken lines. Spermatogonia progenitor cells marked by red arrows. Sertoli progenitor cells marked by yellow arrows. Leydig progenitor cells marked by white broken lines. Primordial germ cells were not observed in any of the slides.

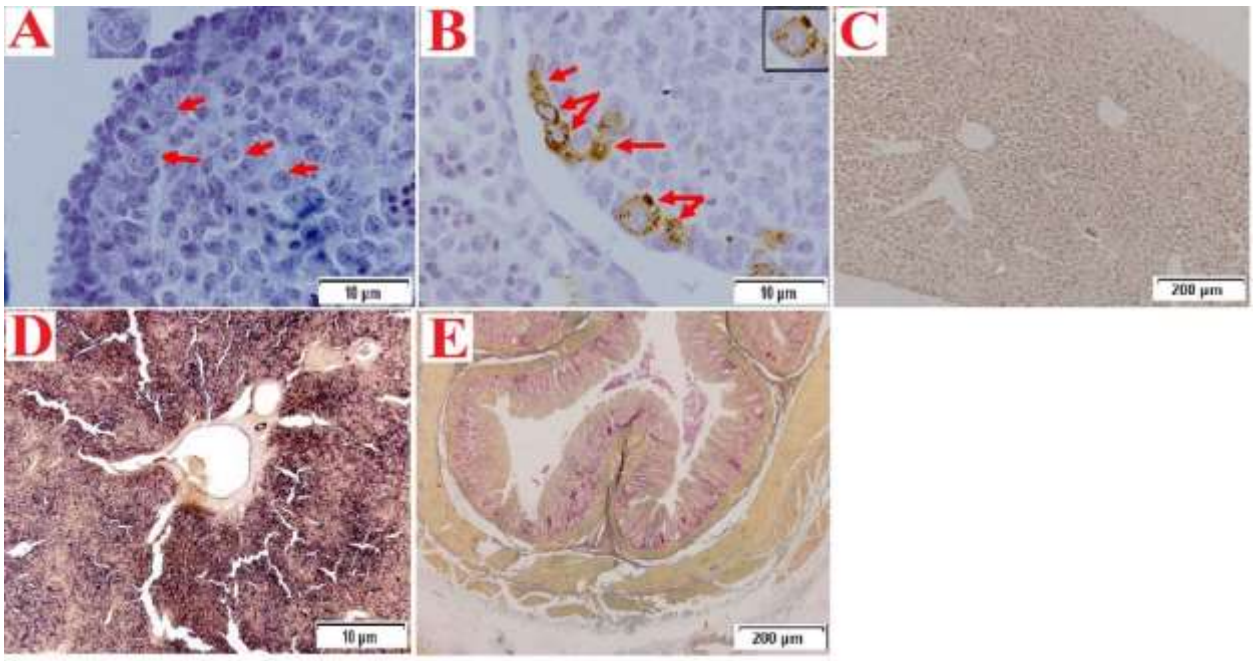

Figure 5. A) SSEA-1 negative control of chick embryo. PGCs (red arrows). B) SSEA-1 positive control of chick embryo. The samples of gonad from chick embryo at stage 28 Hamburger and Hamilton (H&H) were used as a positive control for SSEA-1 antibody, germ cells in this stage reacted positively with SSEA1 at the end of staining. PGCs (red arrows). C) Chick liver in one-day age were used as a negative control for the ALP enzyme. D) Chick liver in one-day age were used as a positive control for the ALP enzyme. E) Rabbit rectum were used as a positive control for PAS stain.

Discussion

We could not use H&H phases owing to variation in embryonic development between Pheasant and fowl (Hamburger and Hamilton, 1992). The results obtained in the first research phase revealed that the emergence of germ cells in the testes of 8-day-old pheasant embryos could be easily observed by staining with H&E and toluidine blue.

Studying PGCs needs special stains or antibodies to detect these cells, and PAS is a histochemical marker to differentiate PGCs from adjacent somatic cells (Chojnacka-Puchta, et al, 2012;

Macdonald, Glover, Taylor, Sang, and McGrew, 2010). Fujimoto et al. (1976) claimed that PGCs contained abundant glycogen by PAS reaction (Fujimoto et al, 1976). England and Matsumura (1993) confirmed that PGCs in chick are detected in the germinal crescent using PAS stain (England and Matsumura, 1993). Furthermore, we made the decision to use PAS staining to recognize the pheasant PGCs and then confirm it with specific antibody. Our study proved that PAS staining is a suitable technique to detect PGCs in pheasant embryos in paraffin sections of the testes only up to 8 days of age. We further supposed PAS-positive content of PGCs to have a dramatic downward trend from day 8 to day 13 so that on days 13, 17, and 24 of the embryos, no positive dye reaction was observed in the germ cells.

Hassanzadeh et al. (2019) claimed that PAS stain is an appropriate technique to recognize PGCs in paraffin sections in the gonad of ostrich embryo. The results of our study are not consistent with this study. Jung et al. (2005) identified the chick germ cells using PAS and anti-SSEA-1 staining. According to the observations of Fujimoto et al. (1976) PGCs are PAS positive, and somatic cells are negative because germ cells have high glycogen which contradicts this study's results because of the low glycogen content in the cytoplasm of germ cells in the pheasant testes. These PAS-colored cells were very weak or negative. This study is contradictory to the study in 2013 (Naeemipour and Bassami, 2013). Since PGCs are migratory cells, the migration of cells may lead to a change in the cellular components, including carbohydrates, and this may be one of the reasons why the cells do not show a positive reaction with PAS (Yoshinaga et al, 1992).

SSEA-1 is a cell surface antigen utilized as a marker for stem cell differentiation (Jung et al. 2005). Park and Han identified PGCs in chicken embryos using SSEA-1 and EMA-1(Park and Han, 2000).

There are many controversies surrounding SSEA1 marker, some reports consider SSEA1 as a PGCs marker; however, the others consider it as a pluripotency marker (Ghasemi et al, 2015). Fox et al. (1981) reported that PGCs in mouse embryos are easily detected by monoclonal antibodies, including SSEA-1 However, SSEA-1 pheasant embryo was negative in PGCs at all ages.

The pheasant PGCs were SSEA-1 negative for all stages. The present results disagree with Yu et al. (2019) who claimed that for SSEA-1, the purified PGCs of Chinese Meiling chicken was positive.

SSEA-1 was not observed in neither of the cleavage stages of bovine embryo nor in tissue sections of the genital ridge (Chojnacka-Puchta et al. 2012). In Turkey embryo, primordial germ cells migrating towards the genital ridge did not express SSEA-1 epitope (D'Costa and Petitte, 2004). Results of this study are in line with those mentioned above; PGCs in pheasants are unable to express SSEA-1 epitopes and by helping this method, PGCs were not identified. It indicated the possibility that the epitope was lost or masked in some way. It may suggest that pheasant PGC has different characteristics than other birds PGC (Wade et al, 2014).

Nonoyama et al. (2021) claimed that embryonic stem cells, pluripotent and undifferentiated stem cells in the human umbilical cord, have a positive reaction to the ALP enzyme and the expression of this enzyme in these cells indicates the existence of differentiation in the cells.

This results also showed that the primordial germ cells in the testes of pheasant embryos were ALP negative, which is consistent with the results of research in turkey embryos (Hosseini et al, 2020) and studies in chickens (Jung et al. 2005). However, the results contradict with Swarts (1982) and Naeimipour and Basami's (2013) study on chickens which may be because of the changes in the

metabolic activity of PGCs or changes in ALP enzyme synthesis.

In conclusion, the results showed that PGCs negatively reacted in all stages of study using the alkaline phsphatase and SSEA1 which is probably due to the differences in the amount of glycogen granule stores in the cytoplasm of primary germ cells or lack of carbohydrates at the cell surface. PGC pheasants' failure to detect SSEA-1 expression may be due to the lack of SSEA-1 expression in pheasants.

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Conflict of interest

The authors declare that they have no conflict of interest.

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شناسایی سلول‌های زایای اولیه در گنادهای نر رویان‌های قرقاول (*Phasianus colchicus*) با استفاده از تکنیک‌های هیستوشیمیایی و رنگ‌آمیزی ایمنی

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چکیده

هدف از این تحقیق شناسایی سلول‌های زایای اولیه در گنادهای نر در مراحل مختلف رویانی قرقاول بود. سلول‌های زایای اولیه سلول‌هایی هستند که به اسپرم یا اووسیت تمایز می‌یابند که برای انتقال اطلاعات ژنتیکی بین نسل‌ها مهم هستند. در این مطالعه، رویان‌ها در روزهای ۲۴-۸ جنینی استخراج شدند. سلول‌های زایای اولیه در مقاطع بافتی پارافینی و رزینی در روزهای ۸ تا ۱۰ تکوین رویانی به دلیل داشتن ویژگی‌های خاص این سلول‌ها شناسایی شدند شامل: اندازه بزرگ سلولی، داشتن هسته و هستک بزرگ و سیتوپلاسم اندک. سلول‌های زایای اولیه بیضه با استفاده از تکنیک‌های هیستوشیمیایی و رنگ‌آمیزی ایمنی شناسایی شدند. روش پریودیک اسید-شیف (PAS)، ایمونوهیستوشیمی با استفاده از آنتی‌ژن جنینی اختصاصی مرحله ۱ (SSEA-1)، رنگ‌آمیزی آلکالین فسفاتاز و رنگ‌آمیزی تولوئیدن بلو برای شناسایی سلول‌های زایای اولیه استفاده شدند. سلول‌های زایا در سنین بالاتر (۲۴-۱۰ روزگی) تنها از طریق برش‌های نیمه نازک قابل شناسایی بودند. علاوه بر این، واکنش این سلول‌ها در تمام سنین به رنگ‌آمیزی‌های آلکالین فسفاتاز، پریودیک اسید-شیف و آنتی‌ژن اختصاصی-۱ جنینی منفی بود. یافته‌های این مطالعه نشان داد که سلول‌های زایای اولیه در بافت بیضه رویان قرقاول در تمام سنین مورد مطالعه به تکنیک‌های هیستوشیمیایی و ایمونوهیستوشیمی واکنش منفی نشان دادند. همچنین این مطالعه نشان داد رنگ‌آمیزی معمول هماتوکسیلین و ائوزین و استفاده از مقاطع نیمه نازک برای تشخیص بافت‌شناسی این سلول‌ها مناسب هستند.

کلمات کلیدی: سلول‌های زایای اولیه، ایمونوهیستوشیمی، فسفاتاز قلیائی، قرقاول، گناد نر

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