PHYSIOLOGY AND REPRODUCTION

Concentration and total number of circulating primordial germ cells in Green-legged Partridgelike chicken embryos

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ABSTRACT The Green-legged Partridgelike fowl is an old Polish indigenous breed of chicken. Primordial germ cells (**PGCs**) are one of the best sources of precursor cells that can be used for the conservation and proliferation of the endangered breeds of bird. Initially, the chicken PGCs colonize at the anterior extraembryonic region called "germinal crescent," and after the establishment of blood vascular circulation, they temporally circulate via the embryonic blood vascular system along with embryonic blood cells. They further colonize at the microcapillary networks of both right and left future gonadal regions. Subsequently, they migrate interstitially to reach gonadal anlages, where they begin to differentiate and eventually develop into the future ova or sperm. The basic knowledge regarding the concentration and the total number of circulating PGCs (**cPGCs**) throughout their circulating phase in the early embryonic stages is crucial for providing an insight into the mechanisms by which they circulate and colonize at the capillary networks of left and right future gonadal regions in each developmental stage. The present study aims to determine the most efficient developmental stage that is suitable to collect cPGCs. The concentration of cPGCs was directly measured, and total volume of embryonic blood was calculated based on the concentration of PKH26-stained embryonic blood cells which were injected 10 min before the blood sampling process in the same embryo during each stage of embryonic development from stage 13 Hamburger and Hamilton (**HH**; Hamburger and Hamilton, 1951) to 16 HH. Analysis of whole embryonic bloodstream revealed that at stage 14 HH of embryonic development, peak total number of cPGCs (386.3 cells/μL) and peak concentration of cPGCs (18.6 cells/μL) were observed. Later, there was a decrease in concentration, suggesting that the cPGCs might be trapped gradually by the capillary networks at the future gonadal regions after stage 15 HH.

Key words: circulating primordial germ cell, embryonic blood volume, green-legged Partridgelike chicken, endangered chicken breed

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INTRODUCTION

Green-legged Partridgelike (**GP**), native Polish chicken breed, became recognized as a breed at the end of nineteenth century. GP was a dual-purpose chicken, reared locally in central-eastern Poland as a free-range chicken. GP chickens can adapt well to extreme environmental conditions and hence are considered to be more healthy and disease resistant than other breeds (Cywa-Benko, 2002; Witkowski et al., 2009). This breed could provide a tough competition to its counterparts because of the peculiar characteristics such as hardiness, low susceptibility to unfavorable conditions, and well-developed maternal traits. In addition, immense activity and ability to use green area make the GP chick an ideal breed for free-range keeping. Both the meat and eggs of this bird are characterized by an exceptional taste.

Previous research suggests that GP can be an excellent model for academic and scientific research aimed, for example, at understanding the inheritance pattern and interaction pathways of genes underlying admixed phenotypes (Siwek et al., 2013). GP is thought to be a remarkable avian model for investigating the genetic background of immune responses, especially taking into consideration potentially elevated levels of

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immunity in the GP breed (Siwek et al., 2010, 2012; Sławińska et al., 2011), as well as for studying the possibility of stimulating the chicken immune system (Sławińska et al., 2014). Our investigations also suggest that GP chicken can be used to study several other approaches. The GP embryo has been a good source of embryonic cells (Sawicka et al., 2015) and hence has been extensively used for biotechnological manipulations (Bednarczyk et al., 2002; Siwek et al., 2010).

Presently, there still exists a small unregistered population of these birds, thanks to the interest of small stock breeders and to those who keep them for fancy purposes. There are 2 major GP populations that are closely related with approximately 600 females and 70 males in each group. The animals in the conservation program were kept in a flock without subjecting to any selection process for more than 50 generations. Maintaining live birds requires considerable financial investment; furthermore, the flocks are exposed to loss of genetic variability as a result of selection and genetic drift. Therefore, the in situ method should be supported by the ex situ conservation strategy (Sawicka et al., 2011). According to the current state of knowledge (Chojnacka-Puchta et al., 2012; Glover and McGrew, 2012), the ex situ method of avian biodiversity preservation program involves a strategy that explores the properties and possibilities of manipulating the PGCs. Moreover, PGCs have been generally used to produce transgenic chickens by a nonviral cell-based method (see the article by Bednarczyk et al., 2018, for related references) and for application in avian biotechnology (Xie et al., 2019).

Chicken PGCs represent the germline stem cells that first arise in hyperblast at the central disk region of area pellucida at stage X of embryo (Ginsburg and Eyal-Giladi, 1986). They translocate toward the anterior from the embryo proper (so-called germinal crescent region) and simultaneously undergo morphological reconstruction to form a mesodermal layer (Swift, 1914; Ginsburg and Eyal-Giladi, 1986). Just after the establishment of blood vascular circulation, they circulate temporarily via the bloodstream. PGCs colonize near the gonadal anlage and migrate interstitially into the gonadal anlage, where they finally differentiate into functional gametes (either sperm in male or ova in female).

In the circulating phase, PGCs could be quite easily isolated from the embryonic blood samples (Kuwana, 1993). However, one of the problems with the PGCs collection of embryonic blood is the low number of cells found, especially in local breed of chicken (Kostaman et al., 2013). In fact, the data with regard to the concentration of cPGCs at different stages of chicken development, essential to use them effectively, are ambiguous.

In the previous studies, high variability in cPGC numbers as well as in cPGC concentrations in embryonic bloodstream in the different breeds of chicken and at different stages of chicken embryogenesis (from 13 Hamburger and Hamilton (**HH**)–16 HH; Hamburger and Hamilton, 1951) has been reported (Al-Thani and Simkiss, 1991, 1992; Tajima et al., 1999; Kuwana et al., 2006; Xie et al., 2019). In contrast, de Melo Bernardo et al. (2012) concluded that from stage 5 HH, when the PGCs are present in the germinal crescent, until stage 19 HH, when the PGCs have reached the genital ridges, the number of PGCs remained constant.

Thus, the present study aims to investigate the dynamics of circulating PGCs during the process of embryonic development and determine the most suitable and efficient developmental stage for their collection. This information might be crucial to understand the biology of circulating PGCs. Moreover, the results of the research are expected to be basic information for determining the appropriate stage for the isolation of circulating PGCs.

MATERIALS AND METHODS

Preparation of Chicken Embryos

Fertilized eggs of GP chicks were supplied by Zofia i Gracjan Skórniccy—Hodowla Kur Zielononóżek (Duszniki, Poland). They were incubated at 37.8°C for 48–54 h from the moment of setting eggs into the incubator, to obtain embryos of stages 13 to 16 HH (Hamburger and Hamilton, 1951).

Labeling of Embryonic Blood Cells

Embryonic blood cells were collected from embryos of stages 16 HH and washed 2 times with nonserum medium (Opti-MEM; Gibco Invitrogen Co., Grand Island, NY). The cells were labeled with PKH26 fluorescent dye (Z-PKH26-GL; Zynaxis, Malvern, PA) according to the manufacturer's instructions. After the staining process, the cells were washed twice with KAv-1 medium (Kuwana, 1993), which consists of 5% fetal bovine serum (Gibco Invitrogen Co., Grand Island, NY) and 5% chicken serum (Sigma-Aldrich, Poznan, Poland).

Measurement of Embryonic Blood Volume Through Stages 13 to 16

The eggs of the GP birds were broken; the embryos were not isolated from the yolk and collected in 100-mm plastic Petri dishes; and 1-μL cell suspension of PKH26-labeled blood cells was injected into each embryo via the dorsal aorta by using fine glass micropipettes (Figure 1A). The fine micropipette consists of a microcapillary needle (borosilicate glass, #B-100–75–10; Sutter Instrument, Co., Ltd., Novato, CA) which is connected to a micropipette puller (P-97/IVF; Sutter Instrument, Co., Ltd.).

All embryos injected with 1 μL of labeled blood cell suspension were further incubated at 38°C for 10 min to maintain the blood circulation, and then 1 μL of the embryonic blood was drawn from the dorsal aorta of the embryos. In the cases where hemorrhage was noted after injection, such embryos were rejected. As shown in Figure 1B, the total embryonic blood volume of

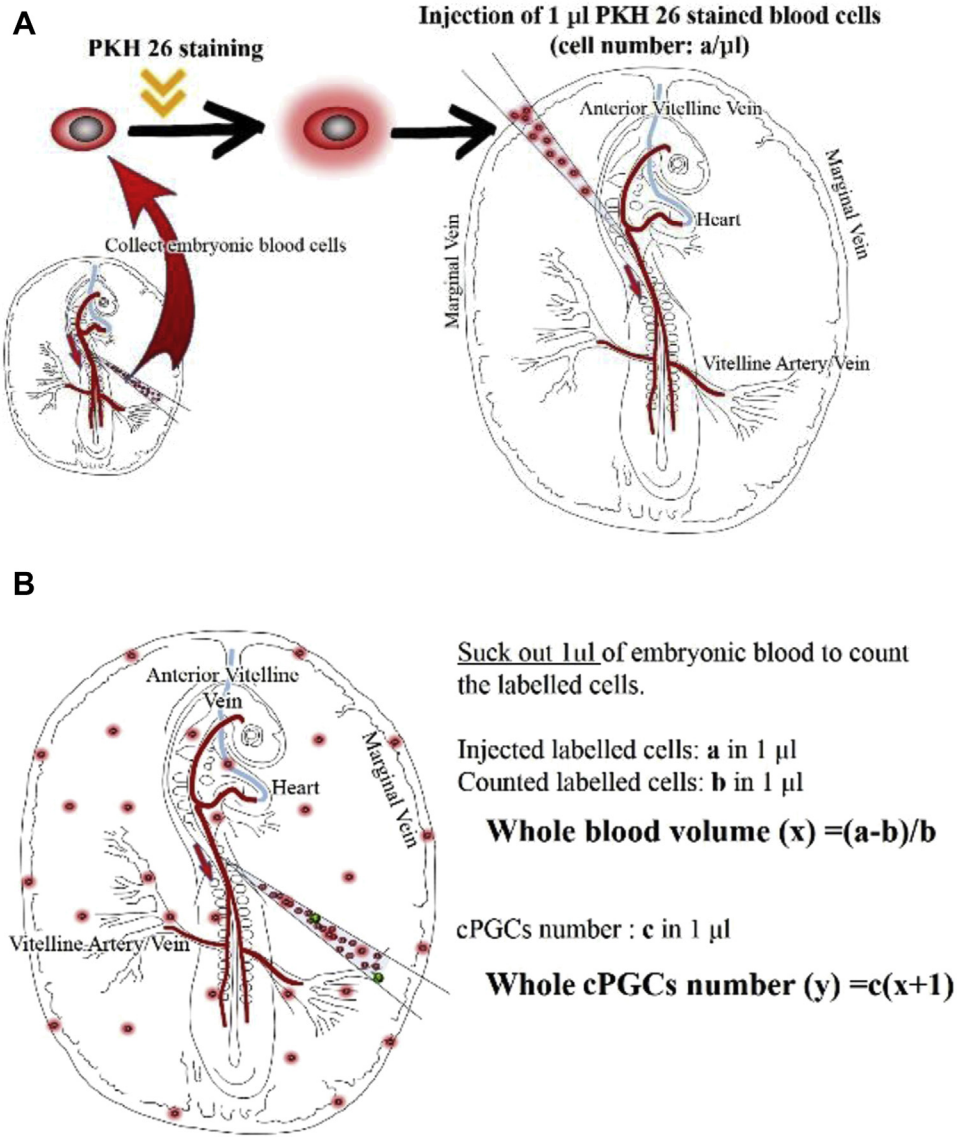

Figure 1. Protocol for the measurement of whole embryonic blood volume and cPGCs number at various developmental stages.

each embryo was obtained by using the formula (a–b)/b (where "a" represents the number of labeled cells injected in 1 µL of cell suspension, and "b" represents the number of labeled cells counted in 1 µL of drawn blood sample).

All the PKH26-positive cells in the embryonic blood samples were counted under a fluorescent inverted microscope (Axiovert 40 VFL; Zeiss, Germany).

Measurement of Number of cPGCs Through Stages 13 to 16

For the measurement of the number of cPGCs, the embryonic blood samples collected from stages 13 to 16 HH were prepared by following the same protocol as described for the measurement of embryonic blood volume. After incubation for 10 min at 38°C after the injection of labeled cells, 1 µL of embryonic blood was drawn out to count the number of cPGCs and PKH26-positive blood cells in each developmental stage (sample number at stages 13, 14, 15, and 16 HH were found to be 12, 15, 17, and 20, respectively).

The cPGCs were counted under an inverted phase-contrast microscope (Axiovert 40 VFL; Zeiss, Germany). The cPGCs can be easily identified by their large size (12–16 µm in diameter), compared to embryonic blood cells (around 8 µm in diameter) at the same developmental stage, cytoplasm rich in yolk granules, and presence of spherical large-sized nuclei.

The total number of cPGCs in each developmental stage was obtained by using the formula c(x + 1) (where c represents the number of cPGCs counted per 1 µL of blood, and x is the calculated whole embryonic blood volume).

RESULTS

The total volume of embryonic blood was calculated based on the number of PKH26-positive cells at each evaluated stage of embryogenesis, and the total number

of cPGCs in 1 μL of embryonic blood at each developmental stage was also calculated based on the results obtained for embryonic blood volume.

Blood Volume and Number of PGCs Through Stages 13 to 16 HH

Embryonic blood volume of GP chicks was measured at stages 13 to 16 HH, and the changes observed in the whole embryonic blood volume over time are shown in Figure 2. The mean blood volumes were found to be 8.1, 20.8, 11.4, and 17.5 μL at stages 13, 14, 15, and 16 HH, respectively.

The number of PGCs in 1 μL of embryonic blood (concentration of cPGCs) was determined at stages 13 to 16 HH. As shown in Figure 3, the peak concentration of cPGCs per 1 μL of embryonic blood was observed at stage 14 HH (18.6 PGCs/μL).

The total number of cPGCs in the embryonic blood at each embryonic stage was calculated based on the number of PGCs present per μL of sample and calculated total volume of embryonic blood. Figure 4 shows the result of the calculated number of total cPGCs, which were found to be 111.0, 386.3, 145.2, and 131.9 through stages 13 to 16 HH, respectively. The peak number of cPGCs in the whole embryonic bloodstream was obtained at stage 14 HH (386.3 PGCs/μL).

DISCUSSION

Generally, chicken embryos work as excellent experimental models and yield specific data that are difficult to obtain in other species. In particular, chicken PGCs have significant potential to be used as a model to carry out different studies in vertebrates (Farzaneh et al., 2016; Trefil et al., 2017; Han and Park, 2018). According to Al-Thani and Simkiss (1991), when the fertilized eggs of Rhode Island Red chickens were incubated at 37.5°C, peak concentration of the cPGCs in the embryonic blood was observed at stage 16 HH. Comparison of the number of cPGCs at different temperatures (36°C and 38°C) showed that the peak flux occurred at stage 15 HH of development at 36°C, which indicates an early developmental stage, whereas the peak was observed at stage 16 HH when the embryos were incubated at 38°C. However, Tajima et al. (1999) reported that the concentration of cPGCs in White Rock chickens was found to be the highest at stage 14 HH and decreased thereafter. Zhao et al. (2003) reported in inbred stock of Rhode Island Red chickens that 3 variation patterns, for number of cPGCs per 1 μL of sample, could be determined in different individuals at 38°C, with peaks at stages 13, 14, and 15 HH, respectively, and not at stage 16 HH. According to de Melo Bernardo et al. (2012), the migration of PGCs from the germinal crescent region into the embryonic bloodstream occurs continuously from stages 11 HH to 16 HH, and the major peak is observed from stages 12 HH to 13 HH.

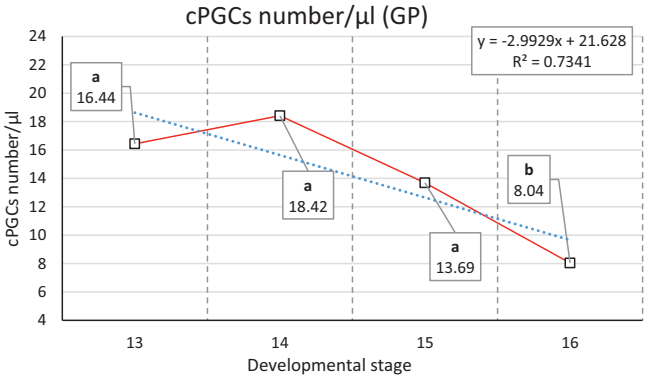

Figure 3. cPGCs number in 1 μL of embryonic blood.

Most of the previous studies used common chicken breeds, for example, Rhode Island Red (Al-Thani and Simkiss, 1991, 1992; Zhao et al., 2003), White Leghorn (de Melo Bernardo et al., 2012), White Rock (Tajima et al., 1999), and so on. However, in the case of inbred lines or endangered breeds, the number of total PGCs seems to be lower than that observed for commercial chicken breeds.

In the present study, the peak concentration of cPGCs of GP was found to be 18.6/μL, which is less than the peak concentrations of both noninbred line (66.9/μL at stage 14 HH; Tajima et al., 1999) and inbred line (26.8/μL at stage 14 HH; Zhao et al., 2003). In contrast, a threatened indigenous fowl (Kureko Dori) found in Japan has only 12.1 cPGCs/μL at stage 14 HH

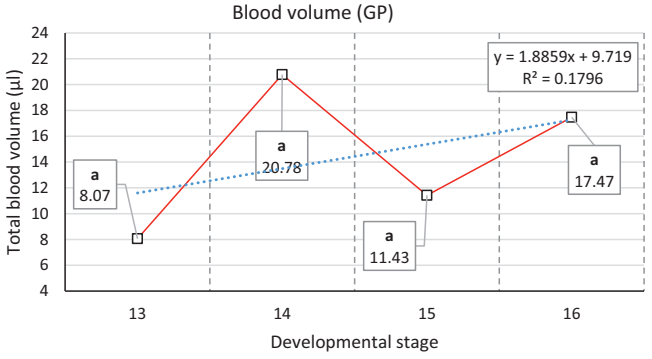

Figure 2. Changes of embryonic blood volume in Green-legged Partridgelike chicken.

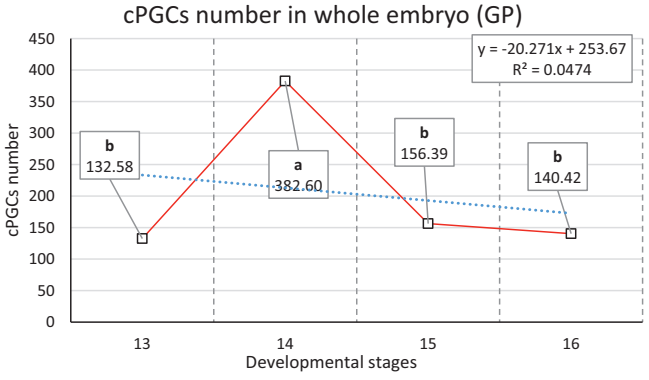

Figure 4. cPGCs number in whole embryo.

(Kuwana et al., 2006). Kuwana et al. (2006) suggested a big variation in the PGC concentration even within the same breed, and this might be the topic of research in future.

However, it should be noted that in the present study, the concentrations of cPGCs were directly measured. The total volumes of embryonic blood were calculated based on the concentrations of PKH26-stained embryonic blood cells which were injected 10 min before the blood sampling procedure in the same embryos through stages 13 to 16 HH in GP chickens, and both peak concentration and peak total number of cPGCs were observed at the same stage of development (stage 14 HH). The total number of cPGCs in the whole embryo is determined from the concentration of cPGCs and the blood volume. Thus, for manipulating the PGCs of GP chickens, it is recommended that they be collected from the embryos that are in stage 14 HH of development, and the number of cells estimated to be present at this stage is about 380. Then, there was a decrease in concentration, suggesting that the circulating PGCs might be trapped gradually by the capillary networks at the future gonadal regions after stage 15 HH.This finding is confirmed by our earlier study (Szczerba et al., 2019), which revealed that the blood flow in the future gonadal regions does not develop until the end of stage 14 HH because there is no functional outflow vein. In the early half of stage 15 HH, posterior vitelline vein rapidly develops on the left side of the gonadal region, and the vascular system at the gonadal regions receives its outflow vein. The present study might be the first report to measure, by the direct method, based on the concentrations of PKH26-stained embryonic blood cells, the total number of cPGCs throughout the circulating phase of PGCs in domestic endangered chicken breed.

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DISCLOSURES

The authors did not provide a conflict of interest statement.

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