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Peak Abundance and Developmental Dynamics of Circulating Primordial Germ Cells in Cemani Chicken (*Gallus gallus domesticus*) Embryo

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ABSTRACT

The Cemani chicken is an Indonesian poultry germplasm and an important biological model for studying germ cell migration. Research is needed to address the limited knowledge of primordial germ cells (PGCs) dynamics to support conservation. This study aimed to identify the peak abundance of circulating PGCs (cPGCs) in the embryonic circulation of Cemani chickens and to analyse the migration of donor cPGCs into recipient embryos. Two hundred fertile eggs were used (100 donor and 100 recipient embryos), divided into five treatments (T1 = Stage 14, T2 = Stage 15, T3 = Stage 16, T4 = Stage 17, and T5 = Stage 18) and then assigned according to their stages. cPGCs were collected at different developmental stages by isolating blood samples from the dorsal aorta of embryos using a micropipette. Blood samples were placed into tubes containing 1,000 μL of phosphate-buffered saline (PBS, without calcium and magnesium), and cPGCs were purified using a gradient method with Nycodenz. For migration analysis, purified cPGCs were labeled with PKH26, and 1 μL of the solution was injected into each recipient embryo, which was then incubated for 6.5-7 days. Embryos at stage 15 showed the highest average number of cPGCs (25.53 ± 11.13 cells/μL; $P < 0.05$), while migration efficiency was highest at stages 15 and 16. These results indicate that stage 15 provides the optimal concentration for isolating cPGCs and for germ cell migration studies in Cemani chickens. Further studies are needed to elucidate the molecular mechanisms underlying cPGCs directed migration and colonization within the recipient gonadal environment.

1. INTRODUCTION

Multipotent stem cells known as primordial germ cells (PGCs) can give rise to either oogonia or spermatogonia [1]. These PGCs play a crucial role as progenitor cells, transmitting essential genetic information to subsequent generations [2]. When it comes to chicken PGCs, they exhibit distinctive developmental characteristics, beginning in the epiblast and subsequently migrating toward the germinal crescent [3]. These cells circulate through the circulatory system, eventually settling in the germinal ridges and colonising the developing gonadal anlage [4, 5]. Although PGCs can be readily isolated from the embryonic bloodstream, it is important to note that their numbers tend to be limited during the circulation period [6]. Therefore, it is imperative to consider this limitation while conducting further investigations and experiments.

Previous investigations have demonstrated that the quantity and concentration of circulating PGCs (cPGCs) in chick embryos vary significantly, with notable differences observed at various stages of embryogenesis and among embryos [7, 8]. De Melo Bernardo et al. [4] conducted a different study that, in striking contrast, found a remarkable consistency in the number of cPGCs during stages 5 to 19. Stages 5 to 19 include the time from cPGCs presence at the germinal crescent to their arrival at the vaginal ridge.

Cemani chicken is an Indonesian breed of chicken that originated in Temanggung, Central Java (Figure 1) [9]. Cemani chicken, especially in the Javanese Islands, is not only used in ancient ceremonies but also as traditional medicine for cardiovascular and respiratory illnesses [10]. Furthermore, Cemani chickens play a significant role in Bali, as they are used in cockfighting, a long-standing custom ingrained in the island's culture [11]. In addition, the Cemani chicken is recognised as an attractive breed in Europe, lending a touch of grace and beauty to poultry fans [12]. Cemani chicken has secured its status as a highly sought-after and appreciated breed due to its numerous applications and widespread recognition. Due to its cultural and economic value, the Cemani chicken has potential as a biological model for the study of germ cell migration and reproductive biotechnology applications. However, information on the temporal dynamics of PGCs in Cemani embryos is still limited, making this research crucial to fill this knowledge gap and support conservation and utilisation efforts for local germplasm. Furthermore, studies on the peak abundance and developmental dynamics of PGCs in Cemani embryos are expected to provide a scientific basis for more efficient germ cell isolation.

Figure 1. Cemani chickens

Based on the above information, this study aims to identify the peak period of PGCs abundance in the circulating blood of Cemani chicken embryos during early development and to analyse the migration of germ cells to the recipient. Thus, this study provides a scientific basis for more efficient cPGCs isolation strategies and contributes not only to a fundamental understanding of developmental biology but also has practical relevance in reproductive biotechnology and genetic conservation.

2. MATERIAL AND METHODS

2.1 Chicken embryo preparation

This study used 200 fertile Cemani chicken eggs, including 100 designated for donor embryos and 100 for recipient embryos. All eggs were maintained at the Indonesian Animal Husbandry Research Institute (IRIAP), Ciawi, Bogor. The eggs were incubated in a portable incubator (Biotype model P-008B) at 37.8°C and 60% relative humidity. Embryos were categorized by developmental stage (Table 1) and assigned to five treatments. These stages correspond to an incubation period of 50-69 hours, as previously established by Hamburger and Hamilton [13]. The average yield of cPGCs per embryo was calculated using the following formula:

$$\text{Average cPGCs} = \frac{\text{Total number of cPGCs isolated at a given stage}}{\text{Number of embryos sampled at that stage}}$$

2.2 Isolation and purification of donor cPGCs

Once the embryos reached stages 14–18, the eggshell was carefully removed and the contents transferred to a sterile Petri dish, ensuring that the yolk remained intact. As shown in Figure 2, blood samples from donor embryos were collected from the dorsal aorta using a micropipette (Drummond Scientific Co., PA, USA) under a stereomicroscope (Olympus SZ30, Japan). The collected blood was transferred into tubes containing 1,000 µL of PBS without calcium and magnesium (PBS-). Purification was then performed by gradient centrifugation with Nycodenz, with minor modifications to the methodology described by Kostaman et al. [14].

Table 1. The developmental stage of chicken embryos is based on incubation time

Stage	Embryo Object	Embryo Age (hours)	Description
14		50–53 hours	At this stage, 22 pairs of somites, arches, and fissures initiated their formation, while the amniotic cavity extended its coverage to encompass seven to ten somites.
15		50–55 hours	The amniotic membrane can cover somite pairs up to 14. At that point, the cleft, the third branchial arch, and the optic cup of the eye have completely developed.
16		51–56 hours	The wing and tail buds become visible during this time, and the amniotic membrane envelops up to 18 pairs of somites.
17		52–64 hours	The amniotic fluid surrounds the whole embryo, except for somites 26-36. During this developmental stage, nasal pits form.
18		65–69 hours	The process of allantois formation commences. Comparatively, the leg buds exhibit greater size in relation to the wing buds, and the amniotic membrane completes its closure.

Figure 2. Embryonic blood sampling from the dorsal aorta using a micropipette under a stereomicroscope

2.3 The number of cPGCs donors, labelling, and injection into the recipient

Quantification of cPGCs was performed on donor embryo blood samples, whereas PKH26 labeling and injection were used to trace the migration and colonization of donor cPGCs in recipient embryos. Purified cPGCs cells from donors were labelled with the cellular dye PKH26 Red Fluorescent (Mini 26-1 KT, Sigma-Aldrich) according to the manufacturer's instructions. Before injection, 100 recipient eggs were prepared and divided into groups according to embryonic developmental stages 14-18 (20 eggs per group). Then, a small hole was made in the blunt part of each recipient egg until the embryos were visible (Figure 3). A total of 1 μL of donor cPGCs was injected into the dorsal aorta of each recipient embryo using a micropipette. The opening hole was sealed with parafilm, and the embryos were incubated until 6.5-7 days of age to observe the migration of donor cPGCs. Donor cPGCs in recipient embryos were subsequently identified based on PKH26 fluorescence signals under a fluorescence microscope [15].

Figure 3. Injection of labelled donor cPGCs into recipient embryos

2.4 Assessment of PKH26-labelled donor cPGCs migration

After 6.5-7 days of incubation, each recipient embryo was collected at its developmental stage and placed into a Petri dish filled with PBS(-). To assess cPGCs migration, the gonads of recipient embryos attached to the mesonephros were carefully excised with tweezers (Figure 4), mounted on a glass slide, covered with a coverslip, and then examined under both light and fluorescent microscope to detect PKH26-labelled donor cPGCs. Only PKH26-positive cells located within the gonadal ridge region were considered as successfully migrated donor cPGCs.

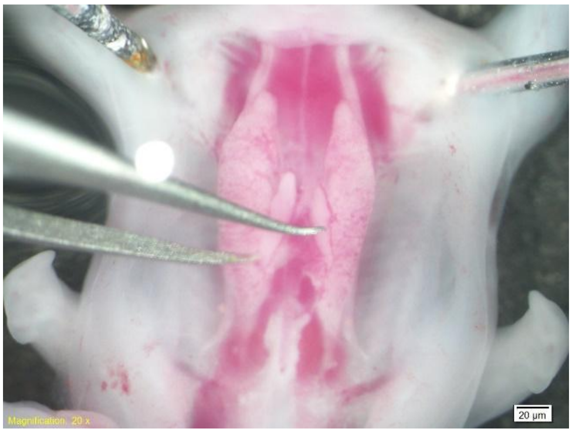

Figure 4. Macroscopic view of paired gonads (indicated by tweezers) in a chicken embryo at 6.5–7 days of incubation

2.5 Statistical analysis

Standard one-way analysis of variance (ANOVA) was used to compare the average number of cPGCs. Significant differences between means were identified using Duncan's test. A 5% significance level was used for all statistical analyses. Meanwhile, donor cPGCs migration in the recipient embryos' gonads was assessed descriptively.

3. RESULTS AND DISCUSSION

3.1 Collection of cPGCs

The collection of cPGCs occurs at stages 14-18, encompassing an incubation period of 55-69 hours. Within the embryo's circulatory system, two distinct cell types exist. cPGCs are distinguished by a larger diameter (ranging from 12 to 16 mm) than red blood cells (approximately 8 mm) at the corresponding stage. This size difference is a key feature used to identify and isolate cPGCs during embryonic development [16]. In addition, collecting cPGCs from the bloodstream of adult chickens is impossible because cPGCs have differentiated into precursor cells that generate egg cells or sperm.

The morphology of cPGCs in Cemani chickens is readily identifiable, as they are round with a textured surface, rendering them larger than red blood cells when observed under a microscope at 20× magnification (Figure 5) [17]. The cPGCs of Cemani chickens closely resemble those reported in previous studies [14, 18] for Gaok and Chinese Meiling chickens, respectively. In addition to their size, cPGCs usually have numerous small cytoplasmic spikes projecting from the cell surface and an abundance of refractive granules in the cytoplasm.

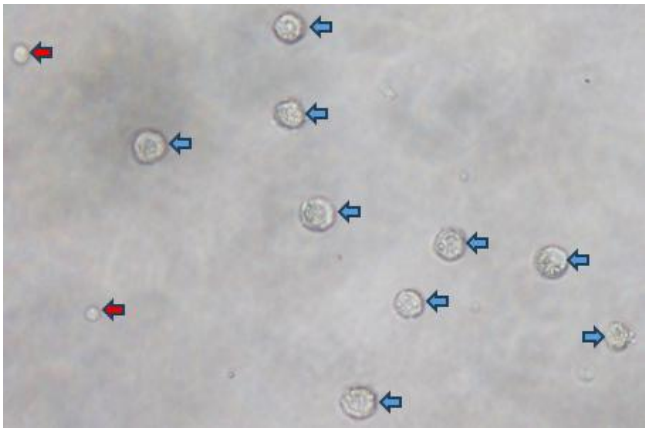

Figure 5. Morphology of circulating primordial germ cells (cPGCs) from Cemani chicken embryos under light microscopy (20×)
Blue arrows are cPGCs, and red arrows are other cells.

Cytoplasmic spikes are believed to be involved in cell adhesion and migration, which are crucial for the movement of cPGCs to the developing gonads. Refractive granules indicate high metabolic activity and the presence of specific proteins and organelles that are essential for the development and differentiation of germ cells [19]. Cemani chickens may have more varied core characteristics, whereas commercial chickens may have more uniform cores due to selective breeding for certain traits.

3.2 Number of cPGCs in the embryo's blood circulation

Optimal collection of cPGCs was observed when PGC harvesting occurred at stage 15 of embryonic development (Figure 6). Notable differences were observed when cPGCs were harvested at different stages ($p < 0.05$). A disparity in embryonic development was demonstrated between stages 14, 16, 17, and 18 ($p < 0.05$). These findings suggest that the quantity of cPGCs can be influenced by embryonic development.

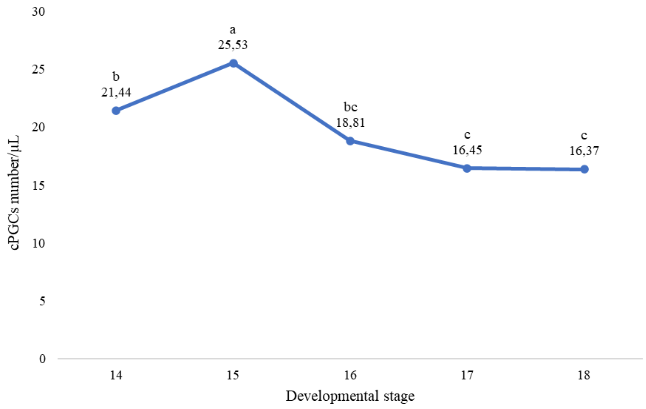

Figure 6. Average cPGCs yield per embryo in Cemani chickens

The average number of cPGCs varies across stages of embryonic development. At stage 15, cPGCs can actively migrate through the bloodstream, and their number subsequently declines beyond that stage. This investigation has substantiated that cPGCs in Cemani chickens decrease after embryos reach stages 16, 17, and 18. With this reduction at this stage, they are likely to have exited the bloodstream, as they exist only briefly. Subsequently, they aggregate into colonies within the germinal ridges. Consequently, when obtaining blood containing cPGCs, collection was suboptimal, likely due to challenges in isolating cPGCs and variations in their number across different developmental stages [20]. Comparable findings were observed in White Leghorn, Kureko Dori, Barred Plymouth Rock, and Silkie chickens [21-24], where it was ascertained that as the embryo ages, the number of cPGCs that can be isolated diminishes.

The average number of cPGCs in Cemani chicken embryos peaked around stage 15 (25.53 ± 11.13 cells/µL) because this stage provides an optimal window for isolating and collecting cPGCs, as they are abundant and accessible in the embryo's blood vessels. Stage 15 is considered safe for isolation and preservation [24]; a similar result was observed in Gaok embryos [14]. Conversely, in Green-legged Partridge-like chick embryos, the peak occurred during stage 14 (18.42 cells/µL) [17]. A prior study [4] reported that cPGCs continuously migrate from the germinal crescent to the embryo's bloodstream (stages 11–16), which explains the differences in results. Cemani chickens might have cPGCs that are more dispersed throughout embryonic development, while commercial chickens may exhibit a more concentrated distribution due to selective breeding for reproductive efficiency.

Embryonic development may influence differences in PGCs characteristics, as reported by Dunislawska et al. [25], who found significant differences in PGCs characteristics on day 8 of embryonic development, with faster rates in Green-legged Partridge-like chickens than in White Leghorns. Genetic factors and migration efficiency are additional factors that influence PGCs characteristics, as genetic differences can affect the efficiency and success of this migration, ultimately shaping germline formation [26]. This is reinforced by Tagami et al. [26], who found that genetic factors play a significant role in PGCs migration and PGCs number [27].

The findings reveal a contrast in chick embryos, specifically in the Green-Legged Partridge, which reaches its peak at stage 14 (18.42 cells/µL) [28]. This discrepancy could be attributed to the breed of chickens, as the cPGCs in native chickens are reported to be fewer than those in commercial chickens [28]. Numerous investigations conducted thus far have employed purebred chickens, for instance, the White Leghorn, among others [29]. Furthermore, cPGCs are influenced by egg production performance, as highlighted by the study [30] in relation to laying and broiler hens. The weight of the egg also influences the number of cPGCs; a larger egg weight results in more cPGCs. This correlation is supported by a regression analysis of egg weight, which reveals a strong association between duck egg weight and the number of cPGCs, as observed by the researcher [31]. It turns out that not only female factors, but also the male factor that provides sperm influences the total number of cPGCs in different individuals [27].

From all that has been mentioned before, the factors that influence the success of cPGCs isolation are: (a) stage of embryonic development, as the concentration of cPGCs in the blood varies with embryonic development [20, 28]; (b) breed variations, perhaps within the same breed of chicken there may be significant variations in cPGCs concentrations [28]; (c) isolation method, the cPGCs isolation method greatly influences the success of cPGCs isolation [20, 28]; (d) purification and quantity, the ability to obtain cPGCs with high purity from blood is influenced by the success of isolation [20]; and (e) genetic, the total number of cPGCs may be lower in local or endangered chickens compared to commercial breeds [28].

3.3 Injection and migration cPGCs

Gonad migration efficiency in Cemani chicken embryos varies across five developmental stages (stages 14–18). The highest success rates were observed at stages 15 and 16 (each at 85%), followed by stage 17 (80%), stage 14 (75%), with a marked decline at stage 18 (65%) (Table 2). The consistent outcomes at stages 15–16 indicate that this developmental window is an optimal time for tracking cPGCs migration in Cemani chickens.

Table 2. Efficiency of cell migration in the embryo gonad recipient, using PKH26 labelling at stages 14-18

Stage	Number of Embryos Examined	Gonad Positive PKH26	Percentage of Migration Success (%)
14	20	15	75
15	20	17	85
16	20	17	85
17	20	16	80
18	20	13	65

PGCs undergo a complex migration to reach the developing gonad, as this migration is critical and governed by intricate interactions between somatic cells and the surrounding microenvironment [32], making early tracking essential for understanding PGCs migration. In avian species, PGCs utilize the bloodstream for migration and subsequently extravasate to the gonadal ridge, where further colonization occurs [33].

The reduction in efficiency observed at stage 18 is likely attributable to increased gonadal tissue complexity and potential microstructural changes, which may hinder label penetration or reduce target visibility. Consistent with this, Furuta et al. [34] reported that PGCs can successfully migrate to the gonads of recipient embryos around stages 13–16.

In addition, the study by Tagami et al. [26] emphasized that molecular intervention and germ cell tracking are most effective when performed before stage 18, as subsequent gonadal tissue reorganisation may affect label distribution. Therefore, these findings further support that stages 15–17 represent an ideal window for PGCs migration-tracking studies and genetic interventions in local chicken embryos, such as Cemani. Accordingly, the migration and proliferation capacities of cPGCs in the recipient gonads appear to be highly variable.

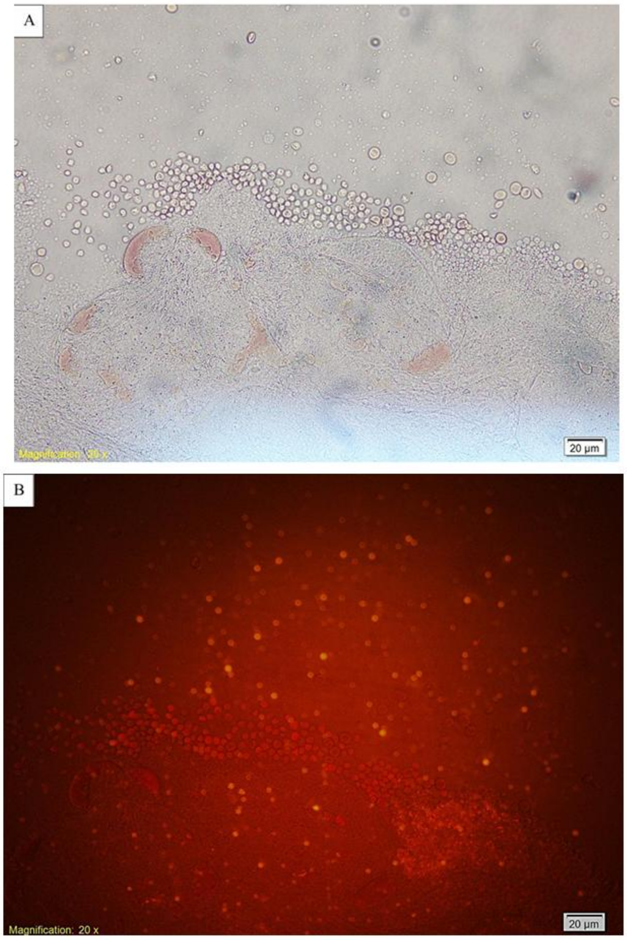

Figure 7. Detection of PKH26-labelled cPGCs in the gonads of recipient embryos, observed under (A) a light microscope and (B) a fluorescence microscope, at 20× magnification

To assess migration, donor cPGCs were labeled with PKH26 before transferring to recipient embryos. Figure 7 illustrates PKH26-labelled cPGCs in the gonads of recipient embryos at the post-injection stage. Figure 7(A) presents a 20× magnification light microscopy image, where the gonadal cellular structure is clearly visible but without fluorescence. In contrast, Figure 7(B) shows a 20× fluorescent microscopy image, featuring bright red dots throughout the gonad tissue. These fluorescent dots confirm the presence of cPGCs, successfully tagged with PKH26, at a scale of 20 µm.

Fluorescent detection of PKH26-labelled cPGCs in the recipient embryos' gonads confirmed that the isolated and labelled cells successfully migrated and colonized the target site. The presence of a bright red signal under fluorescent microscopy (Figure 7(B)) indicated that the PKH26 labelling method was effective for tracking cPGC distribution.

PKH26 is a lipophilic fluorochrome that intercalates into the cell membranes and has been widely used for cell tracking in various biological systems [35]. Its effectiveness in migration and cell colonization studies has been demonstrated across multiple models, making it a reliable tool for long-term cell labeling [36].

The absence of a detectable signal in the light microscopy image (Figure 7(A)) further confirms that fluorescent detection is required for the specific identification of labelled cells. This finding supports the efficiency of the injection and labelling technique employed in cPGCs migration and colonization studies and highlights their potential for further applications in germline engineering and avian genetic conservation.

The successful detection further indicates that the observation timing and sampling locations were appropriately aligned with the physiologically established migration pathway of cPGCs. Accordingly, this approach can be integrated into germ cell tracking protocols for advanced studies investigating the migration competence and colonization capacity of donor cells.

In avian reproductive biology, studying primordial germ cells is crucial. Examining the occurrence and concentration of PGCs in Cemani chickens is of utmost importance, as it helps understand their reproductive capabilities and supports conservation efforts.

4. CONCLUSION

cPGCs in the Cemani chicken are predominantly observed at embryonic stage 15. This stage represents the peak abundance of cPGCs in the embryonic bloodstream, optimal for germ cell isolation and utilisation for cell migration. Cell labelling with PKH26 could provide a foundational framework for investigating cell migration from donor to recipient embryos. Nevertheless, additional investigation is needed to explore quantitative methods, including specific antibody detection, as well as the underlying mechanisms of cell migration and intracellular interactions.

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