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Isolation and Characterization of Gonadal Primordial Germ Cells (gPGCs) of Turkey (*Meleagris gallopavo*) from 11-14 Days Old Embryos

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Primordial germ cells are the only cells in developing embryos with the potential to transmit genetic information to the next generation. The recent development of techniques for germline chimaeric production through germ cell transfer and its long-term culture without losing their germline transmission capability have provided important breakthroughs for the preservation of germplasm, transgenic bird production and the study of germ cell system. While efforts on the study of chicken germ cells had peaked, similar endeavour in turkey is lacking. In this study, a simple isolation method described in chicken for gPGCs was tested in turkey embryos. The results showed that gPGCs could be isolated/collected from 11-14 day old embryos of turkey. The highest initial discharged of germ cells was in the 1st hr of incubation with more than 60% purity rate. Discharged of germ cells appeared higher in the left gonad than right gonad. The gPGCs of turkey embryos are nucleated almond shaped cells or oval-shaped cells with tapered ends. The results of this study can be a starting point on further researches about the gPGCs of turkey embryos. Moreover, it can used for future studies related to production of transgenic birds, hybrid avian species, and chimeras etc., Future experimentations should focus on other factors that can affect the viability and survivability of gPGCs in vitro, these factors can be attributed to morphological, cytological and chemical characteristics of the cells.

Key words: Primordial germ cells, turkey, embryo, purity

Introduction

In the Philippines, turkey species that can be found are smaller than imported breeds with a mean weight of 7-8 kg at its mature age of 7-8 months

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(Bureau of Animal Industry, 2014). They are mostly raised in the backyard for meat purposes rather than egg production with other poultry species. Although this species are very prolific and can adapt well to varying environmental conditions (Yassin *et al*., 2013), its farming remained underdeveloped due to several factors such as the availability of other poultry products (eg., such as chicken and duck) which are less expensive, unfamiliarity of turkey meat in most Filipino gatherings, and lack of facilities, equipment and information about the proper raising of turkeys. Unlike in the USA, improved genetic selection, technical advancement and better production management has made turkey to become an effective meat producer with a mean weight of 30.3 pounds (USDA, 2014). Therefore, improving the genetic morphology of turkey can produce a better product that can be a competitive commodity in the poultry industry. Modification of the genetic characteristics of livestock/poultry to produce a globally competitive species for market consumption has become a major part of a system in solving problems associated with the fast increasing human population that resulted in an increased demand for food. Some techniques have evolved to improve the genetic constitution of many animal species such as cloning, genetic engineering, production of genetically modified organisms (GMOs) and transgenic animals, chimeric production and others. Some procedures used somatic cells (Qiao *et al*., 2004; Mavilio and Ferrari, 2008) derived directly from the parent individual while others uses germ cells or primordial germ cells (Yasuda *et al.,* 1992; Tajima *et al.,* 2004).

During early embryonic development, primordial germ cells (PGCs) are formed. These are the only type of cell that can transmit all genomic information to the next generation. It also serves not only as germ line but also as stem cells (Park *et al*., 2014). A marked difference between mammalian and avian follicles is that, the PGCs of mammals are surrounded by extracellular matrix whilst are loosely packed in avians (Hummel *et al.,* 2004). This phenomena emphasizes the need to develop modified strategies for the isolation, characterization and culture of turkey PGCs which is the first step in producing morphologically competent species (Leghari *et al*., 2015). Isolated germ cells from turkeys possess a great potential in the preservation of the germ line of wild and domestic species that can be used to further develop its physical characteristics by exploring their genetic constitution. While there are appreciable involvement in the manipulation of early embryos using gPGCs of other poultry species to understand germ cell differentiation (Park *et al.,* 2014; Nakamura, 2016), similar informations concerning the gPGCs of turkey remained sparse/absent, hence this study. gPGCs isolation and characterization could serve as an excellent tool in the development of techniques for commercial applications, to further basic researches in the production of

transgenic birds, for genetic improvements and preservation of foundation stocks of turkey and other poultry species which are endangered under natural conditions (Tajima *et al.,* 1993).

Objectives: The purpose of this study was to isolate and characterize the gonadal primordial germ cells (gPGCs) of turkey. Specifically, this study determined:

1. the embryonic age that optimally released gPGCs
2. the incubation time that released the highest number of gPGCs
3. the morphological characteristics of the gPGCs
4. the number and purity of discharged gPGCs

Materials and methods

Source of fertilized eggs

Eggs from 5 hens (8 months old) confined with 2 toms/gobblers (mature male turkey) in a free-ranged set up were collected and placed in an incubator with provisions for required temperature (38.5℃) and relative humidity (50-60%) for embryonic development for 11, 12, 13 and 14 days. Tilting of eggs was done once a day. Candling method using a light bulb was utilized in checking the eggs fertility. Fertile eggs were identified with the presence of spider-like blood veins spreading out like a spider's legs.

Gonad collection from donor embryos

As the eggs reach the desired embryonic age for gonad collection, they were cracked open and the embryos in yolk were transferred to a sterile petri dish (Fig. 1). Then, the embryos were detached from the yolk and washed with PBS(-). The embryos were positioned in a dorsal recumbency and opened craniocaudally. The heart, gizzard, liver and gut were then removed exposing the mesonephros with the gonad (Fig. 2). The gonad was detached from the mesonephros using a needle attached to a 1-ml tuberculin syringe under a stereomicroscope (Fig.3-4). The isolated gonad (both left and right) were then transferred separately in a 4-well culture plate containing 500 µl PBS(-) and placed in an incubator maintained at 38.5℃ with gas atmosphere of 5% CO_2 and 4.9% O_2 for 1, 4, 7, 10 and 13 hours. The composition of PBS(-) was NaCl (8 gm), Na^2HPO^4 (1.15 gm), KCl (0.2 gm) and KH^2PO^4 (0.2 gm) in a 1 liter preparation using a nanopure water for dilution.

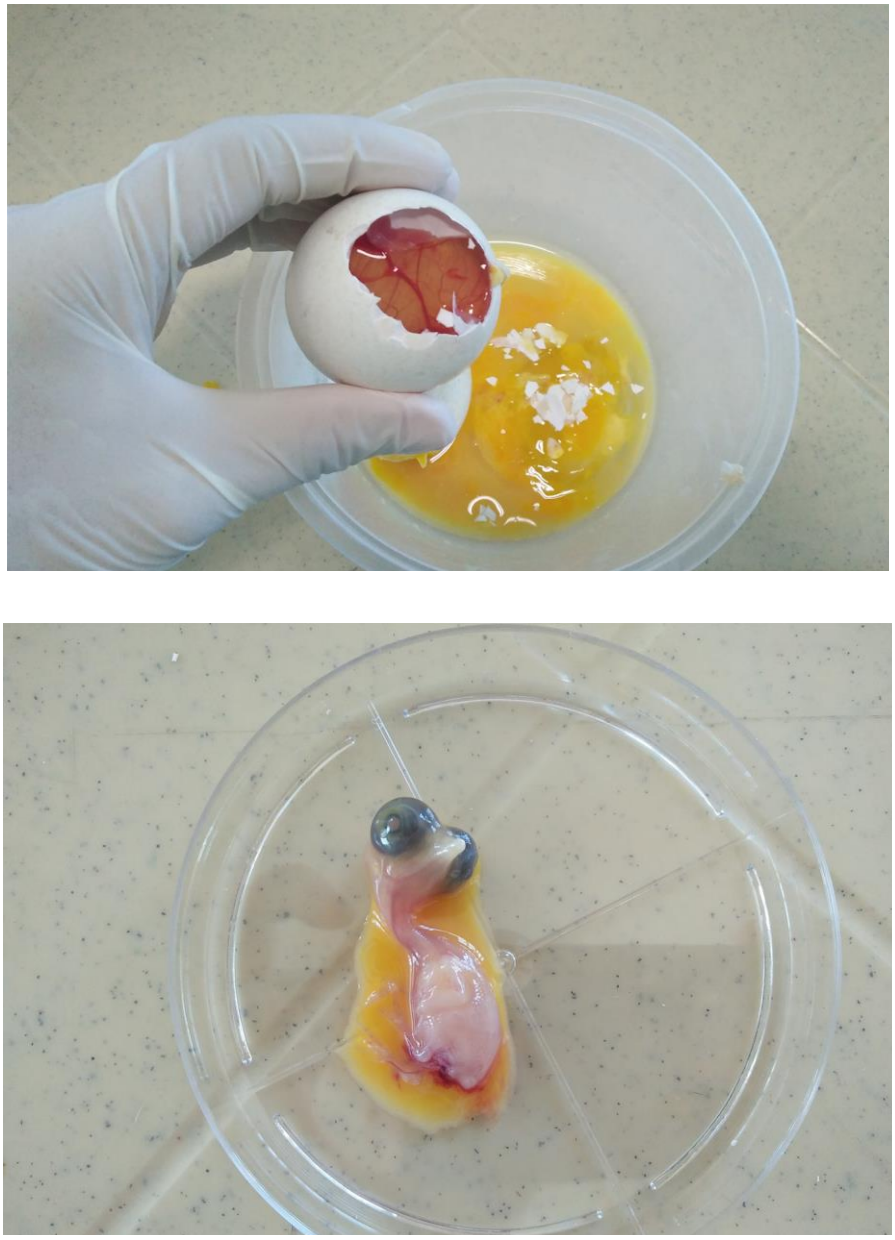

Fig. 1. Turkey embryo exposed and placed in a sterile dish.

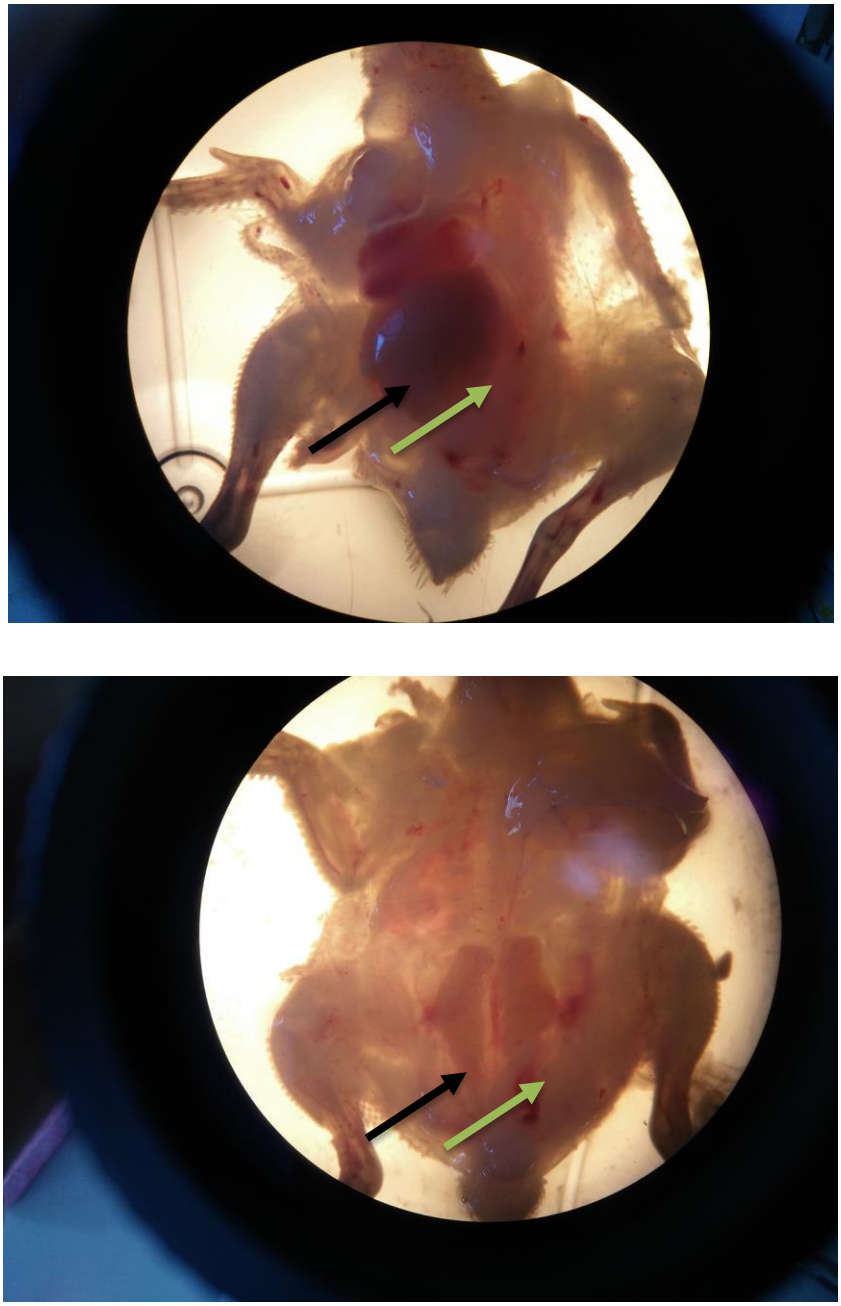

Fig. 2. The visceral organs of a 13 day- old embryo were exposed, removed and the mesonephros with the attached gonads were located on the retroperitoneal area (right gonads = black arrow; left gonads = green arrow) under a stereomicroscopre at 40x magnification.

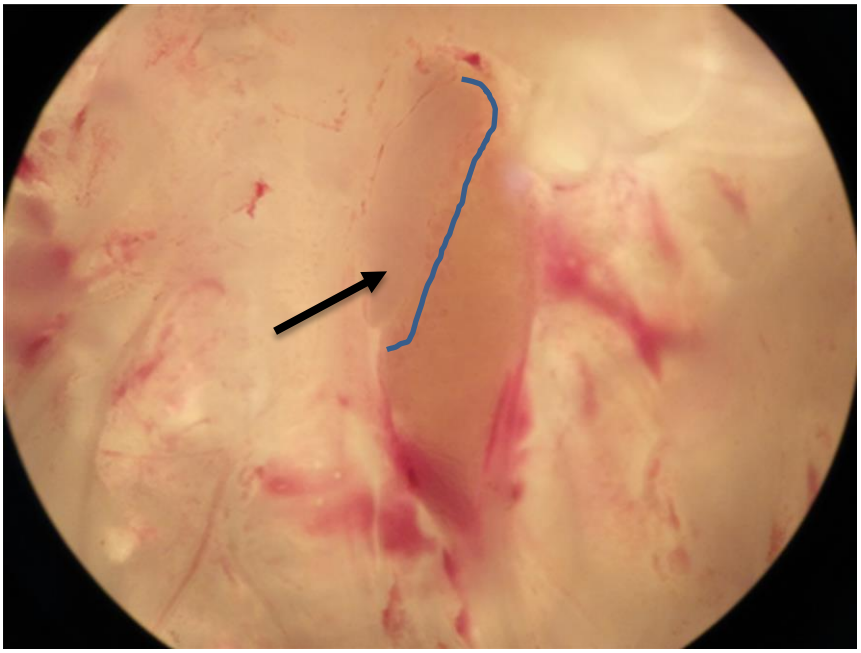

Fig. 3. Left gonad in the mesonephros of a 13- day old turkey embryo observed under stereomicroscope at 40x magnification. **(**Gonad = black arrow**)**

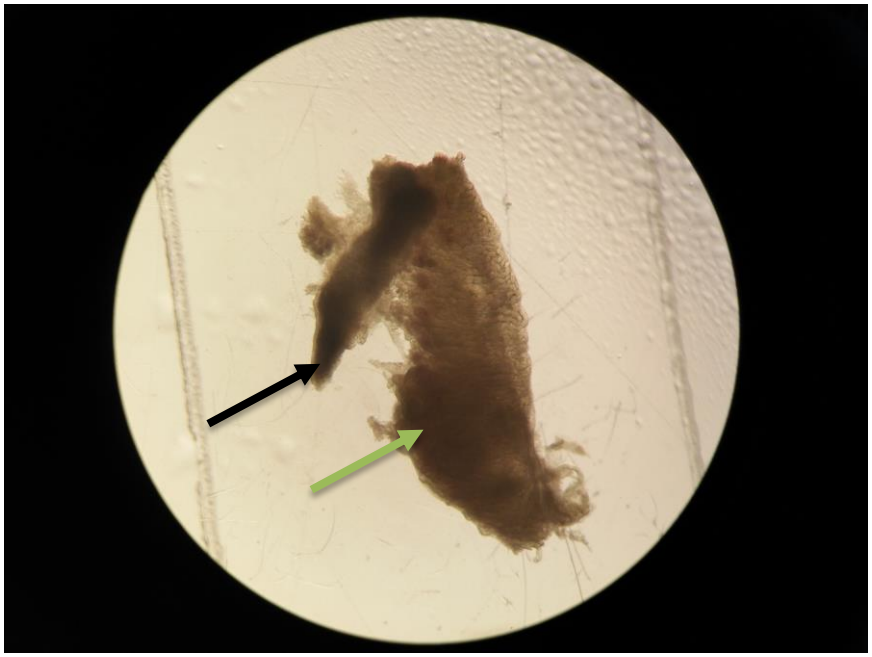

Fig. 4. Separated gonad from the mesonephros (Gonad = black arrow; Mesonephos = green arrow**)**

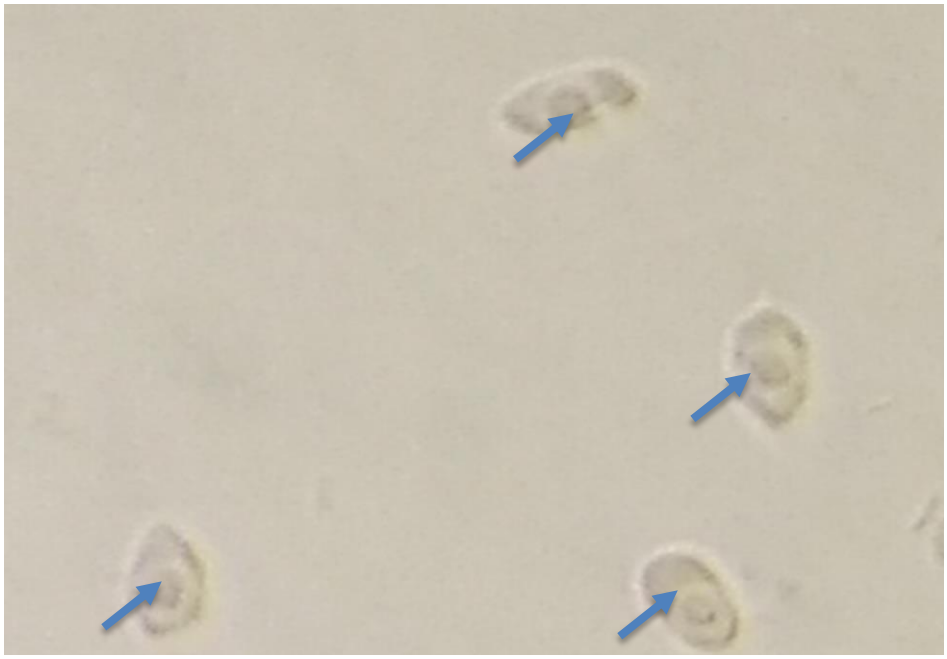

Fig 5. The gPGCs with almond shaped appearance and central nucleus (blue arrow) seen through an inverted microscope. 400x.

Isolation of gPGCs

After each incubation time point of culture, the gonads were removed from the well and transferred to adjacent well with PBS(-). The number of discharged gPGCs were counted/evaluated and the purity were observed. The purity of discharged gPGCs was defined as the ratio between the number of discharged gPGCs and the total number of discharged cells and expressed in percentage (Nakajima *et al.*, 2011). The morphological characteristics of gPGCs was evaluated under an inverted microscope Tx10i (Nikon Eclipse Ti, Japan). The sampling from each "donor age" level was repeated three times.

Statistical analysis

The data were presented as mean ± standard deviation of the three replicates and analyzed using the one-way ANOVA followed by Tukey's HSD.

Results

Some morphological characteristics of fertilized eggs used in the study is shown in Table 1. The size of the embryo is directly proportional to the age of the egg. The size of the left mesonephros with the gonad was higher than right mesonephros.

Table 1. Morphological characteristics of fertilized turkey eggs used in the study.

Age (day)	Weight (gm)	Size of Embryo (mm)	Size of Mesonephros (mm)	
			Right	Left
11-	72.4	37.0	3.8	4.0
12-	76.8	45.3	5.8	6.1
13-	80.8	51.7	7.9	8.1
14-	85.0	60.7	7.5	7.7

*Mean values from 3 replicates.

The mean number of discharged gPGCs from day 11- to 14- day-old embryos after incubation in PBS(-) was presented in Table 2. The highest number of discharged cells, regardless of embryonic stage from either left or right gonad was highest in the 1st hr of incubation. Also, the highest number of discharged germ cells was observed in 13 days old embryos (n=12,875 cells). The number of discharged germ cells decreases as the incubation time of gonad progresses. Also, it was observed that higher number of gPGCs were released from the left gonad than right gonad of turkey embryos throughout the incubation period. In chicken, it was shown that gPGCs colonized the left gonad (70%) more than right gonad (30%) (Zaccanti *et al.*, 1990; Nakamura *et al.*, 1988; Naito *et al.*, 2009). This is a result of asymmetrical development of the ovary that has been influenced by Pitx2 that leads to unequal ovarian development through up- or down regulation of retinoic-acid (RA)-synthesizing retinaldehyde dehydrogenases (Rdldh2), Ad4-binding protein/steroidogenic factor 1 (Ad4BP/SF-1), estrogen receptor α and cyclin D1 in chickens (Ishimaru *et al.*, 2008). Due to the active migration of gPGC to the left gonad in both sexes there is a greater concentration of gPGCs in the left side.

Table 2. Number of discharged gPGCs on right and left gonad of turkey embryos.

Age (days)	Incubation (hr)	Right Mean ± SD	Left Mean ± SD	Total no. of gPGCs
11	1	1095 ± 13.22[a]	1110.00 ± 15.00[a]	
	4	63.33 ± 12.58[b]	723.33 ± 12.58[b]	
	7	393 ± 12.58[c]	410.00 ± 15.00[c]	
	10	206.67 ± 7.63[d]	220.00 ± 15.00[d]	
	13	80.00 ± 18.02[e]	103.33 ± 7.63[e]	
	Total	**2438.33**	**2566.66**	**5,005**
12	1	1813.33 ± 123.42[a]	2015.00 ± 135.37[a]	
	4	1436.67 ± 63.31[b]	1441.67 ± 68.98[b]	
	7	708.33 ± 20.81[c]	718.33 ± 16.07[c]	
	10	270.00 ± 13.22[d]	283.33 ± 33.29[d]	
	13	103.33 ± 2.88[e]	105.00 ± 5.00[e]	
	Total	**4331.66**	**4563.33**	**8,895**
13	1	2560.00 ± 123.79[a]	2691.67 ± 67.88[a]	
	4	2100.00 ± 27.83[b]	2240.00 ± 130.76[b]	
	7	935.00 ± 31.22[c]	1015.00 ± 39.05[c]	
	10	395.00 ± 5.00[d]	420.00 ± 18.02[d]	
	13	250.00 ± 26.45[e]	268.33 ± 10.40[d]	
	Total	**6240.00**	**6635.00**	**12,875**
14	1	1623.33 ± 63.70[a]	1666.67 ± 5.77[a]	
	4	1266.67 ± 14.43[b]	1338.33 ± 16.07[b]	
	7	468.33 ± 5.77[c]	508.33 ± 2.88[c]	
	10	260.00 ± 5.00[d]	275.00 ± 5.00[d]	
	13	150.00 ± 5.00[e]	166.67 ± 15.27[e]	
	Total	**3768.33**	**3955.00**	**7,723**

[a,b,c,d,e] Values with different superscript differ significantly ($p<0.05$).

Discussion

The germ cells purity was defined as the proportion of gPGCs versus the total number of observed cells. At 1 hour of incubation it was almost 70% (Table 3). In chicken, highly pure (approximately 50% of total cells) and viable gonadal germ cells were recovered efficiently by simply culturing the embryonic gonads in PBS(-) at 37.8℃ for 0.5-1.5 hour. Although the underlying mechanism for the efficient discharged of gPGCs in PBS(-)

remained unclear, this simple method of isolating and collecting gPGCs from embryonic gonads will facilitate studies on germline chimera production (Nakajima *et al.,* 2011). gPGCs in chicken embryo has a large and spherical nucleus which is a remarkable morphologic characteristic of these cells that makes it readily distinguishable from other cells (Fujimoto *et al.,* 1976). In turkey embryos gPGCs were observed to be nucleated almond shaped cells with tapered ends. On the basis of examination of cytological characteristics derived from chicken embryos on the 4th to 8th day of incubation, it was shown that there were age dependent changes in the number of gPGCs (McDonald *et al.,* 2010). At this point of embryogenesis, the cells also showed subsequent partial loss of their morphological features. This significant variation on the morphological features of discharged gPGCs may result to decreased purity which can be attributed to the depletion of nutrients essential for maintaining the cellular metabolism of discharged gPGCs (Nakajima *et al.,* 2011).

Table 3. Percentage purity of discharged Gonadal germ cells on both gonads

Age (day)	Right	Left
11	62%	63%
12	69%	71%
13	75%	78%
14	65%	66%

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