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# Isolation, Purification and Culture of Spermatogonia in Chicken

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**Abstract:** To isolate, purify and culture spermotogonia from chicken testicular tissues, a procedure of enzymatic digestion and percoll density centrifugation was adopted for the single cell suspension to obtain purified spermatogonia. The results showed that, using the same purification method, the purity of spermatogonia gained from 6 days old chicken embryo was more than from 13, 15 and 19 days of age; adhesion purification step led to a harvest of 82% of total spermatogonia, which was 15.6% higher than that of direct isolation method; the adhesion time and survival time of spermotogonia before percoll density gradient centrifugation was earlier and longer than after precoll density gradient centrifugation.

**Key words:** Chicken, spermatogonia, isolation, purification, culture

## INTRODUCTION

Spermatogonial Stem Cells (SSCs) are the only stem cells in the body that can be recognized and studied at the cellular level with respect to proliferation, differentiation and the regulation of these activities. Moreover, spermatogonia are also the only carrier cells of transmitting genetic materials from parents to offspring. In the microenvironment of seminiferous tubules, SSCs can renew and differentiate into filial cells like embryonic stem cells (Brinster and Zimmermann, 1994). In the 1970s, the feature of serpmatogonia was known better. Bellve *et al*. (1977) first used BSA to isolate spermatogonia from 8 days old mice and obtain a 90% purity fraction of type A spermatogonia. Bucci *et al*. (1986) used enzymatic digestion to get 76% purity of spermatogoina from rat testis. But these methods either consumed a large number of experimental animals or result in insufficient purity of spermatogonia for experiment analyses. Through the development of cellular biology technology during several decades, the technology of isolation and purification of spermatogonia have greater break through. Fariborz *et al*. (2000) reported that 80% purity of spermatogonia was gained from bovine testis using percoll gradient centrifugation and adhesion purification after an enzymatic digestion. Zhang *et al*. (2000a) and Yu *et al*. (2002) used respectively percoll gradient centrifugation and BSA velocity sedimentation separation to isolate mice and Changbai boar testis cells and obtained 68.8 and 94.2% purity of spermotogonia, respectively. The recorded literature report only in mammalian and reports related the isolation and purification of chicken spermatogonia are still rare. The aim of this study was to explore the suitable time of chicken spermatogonia isolation, methods, purity and the possibility of *in vitro* culture.

## MATERIALS AND METHODS

**Experiment materials:** Fertilized eggs were obtained from the Poultry Institute, Chinese Academy of Agriculture Sciences and were incubated at 38°C and 70% relative humidity.

**Media and reagents:** Collagenase (Type I, Sangon), Hyaluronidase (Sangon), 0.05% Trypsin-EDTA (Sigma), Dulbecco's Moditied Eagle Medium (DMEM, Gibco), Percoll (Sigma), Fetal bovine serum (Four seasons green in Hangzhou,China), Phosphate-Buffered Saline (PBS), all other reagents were obtained from local suppliers at the highest purity available.

**Preparation of percoll gradients:** About 90% of percoll media was made, according to the proportion of percoll: PBS = 9: 1 and Dulbecco's Moditied Eagle Medium (DMEM) culture media was added to compound different percoll gradient media, then gently aspirate every gradient into 10 mL tubes according to the proper concentration from high to low (Table 1) to make up a discontinuous gradient layers that will be used in following steps.

**Preparation of cell suspension:** To collect testis, 15 days old chicken embryo, 19 days old chicken embryo, 6 days old chicken and 13 days old chicken were prepared. After decapsulation, the testis were cut into

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Table 1: Concentration and components of percoll gradients

| Concentration percoll (%) | Volume of percoll gradients (μL) | 90% percoll (μL) | DMEM culture of medium (μL) |
|---|---|---|---|
| 19 | 1500 | 317 | 1183 |
| 27 | 1500 | 450 | 1050 |
| 35 | 1500 | 583 | 917 |
| 43 | 1500 | 717 | 783 |

small pieces (1 $mm^3$ or so) and suspended in the dish with $ca^{2+}$ and $mg^{2+}$ free PBS. Gently blowing it with a pipette for 1-2 min and remove the supernatant after play it 5 min these pieces were then dispersed with 1 mg $mL^{-1}$ collagenase I in the condition of 37°C and 5% $CO_2$. Removing the supernatant, 0.25% trypsin was also added and digestion for 4-6 min in the same condition as collagenase I. After digestion with trypsin, Fetal Bovine Serum (FBS) was added. Cell were dispersed with a pipette and then filtered through a 25 μm screens. The filtrate (cell suspension) was centrifuged at 100 g for 5 min and resuspended in 1.5 mL DMEM containing 10% FBS.

**Tackling of cell suspension:** Two ways were employed to handle the single cell suspension.

**Method 1:** Single cell suspension, approximately, $3\times10^5$ cells $mL^{-1}$ were cultured to a hole of cultural plate (sign: A) and returned to the incubator with 38.5°C and 5% $CO_2$. After culturing 24 h, removed the old DMEM and washed the cultural plate with PBS, then added fresh DMEM into the hole. Randomly choosing three views under the stereoscope, counted the number of all cells and round spermatogonia and computed the means and station error. Then, continued to culture *in vitro* and observed growing feature of spermatogoina.

**Method 2:** To purity spermatogonia from testis cell suspension, testis cell suspension was slowly added to the top layer of percoll gradient. After centrifugations at 350 g for 25 min, cells of every band were, respectively collected by puncturing the sides with several greased of 25 gauge needle and slowly withdrawed them into several 5 mL tubes. Then added PBS into these tubes to dilute percoll media and removed supernatant after centrifugation at 100 g for 5 min. The cell pellet was resuspended in 3 mL DMEM and prepared to be used in following steps.

**Purification of spermatogonia:** Testis cell suspension (approximately, $3\times10^5$ cells $mL^{-1}$) gained from percoll gradients centrifugation was equivalently divided into 2 groups and respectively cultured in two holes of cultural plate with 0.1% gelatin coat (sign: B and C). Then, returned to the incubator to culture at 38.5°C and 5% $CO_2$ and observed cells' adhesion condition at different time. When somatic cells started to attach and spermatogonia remain suspension (about culture 5 h) in sign C hole of cultural plate, carefully aspirated the cultural media and unattached cells and cultured in another hole of cultural plate. After continuing to culture 24 h, removed old DMEM and washed the cultural plate with PBS and then added fresh DMEM into the hole. Randomly choosing three views under the stereoscope, counted the number of all cells and round spermatogonia and computed the means and station error. Then continued to culture *in vitro* and observed growing feature of spermatogoina. Cell suspension cultured in sign B hole had not any tackle and continued to culture *in vitro* 24-36 h, counting the number of all cells and round spermatogonia and computing their means and station error as was described by sign C.

**Computation of spermatogonial purify:**

$$\text{Percentage of spermatogonia} = \frac{\text{The number of spermatogonia}}{\text{The number of all cells}} \times 100$$

**Statistical analyses:** Values were presented as the mean and standard error of mean. Significant differences were obtained using the t-test and LSD test, performed by SPSS11.5 software.

## RESULTS

**Isolation of spermatogonia in different hatching day:** This experiment compared the purity of spermatogonia obtained from four different time periods. Every experiment was repeated 4-5 times and the means and station error were computed (Table 2).

Only 31.6% average purity of spermatogonia was gained before percoll gradient centrifugation, but the purity of spermatogonia was up to 57.6% when single cell suspension was centrifugal elutriation of percoll gradients, 26% higher ($p<0.01$) than previous percoll centrifugation. Comparing the purity of spermatogonia obtained from different periods, we found that 33.8% purity of spermatogonia was obtained from 6 days old chicken before percoll centrifugation, which was 2.6, 4.3 and 3.1% higher than of 15 days old chicken embryo, 19 days old chicken embryo and 13 days old chicken respectively and the difference among the four stages were also significantly different ($p<0.05$); After percoll centrifugation, the purity of spermatogonia obtained from

Table 2: Isolation effect of spermatogonia in different hatching days

| | | | | After isolation of percoll (19-35%) | |
|---|---|---|---|---|---|
| Hatching days | The number of all cells | The number of spermatogonia | The purity of spermatogonia | The number of spermatogonia | The purity of spermatogonia |
| 15 days old chicken embryo | 687.9±23.7 | 214.6±13.2 | 31.2[a] | 158.6±12.2 | 51.7[A] |
| 19 days old chicken embryo | 607.8±41.8 | 179.3±31.1 | 29.5[b] | 148.3±21.0 | 56.2[B] |
| 6 days old chicken | 660.7±33.0 | 223.3±21.3 | 33.8[c] | 174.6±18.7 | 61.9[C] |
| 13 days old chicken | 624.4±19.6 | 191.7±20.0 | 30.7[d] | 151.2±14.5 | 59.4[D] |
| Average | 639.9±29.5 | 202.2±21.4 | 31.6[e] | 158.2±16.6 | 57.6[E] |

In the same columns, differences among numbers containing the different letters in the superscripts are significant ($p<0.05$)

Table 3: Comparison different methods of spermatogonia collection

| Groups | Method of isolation | No. repeats | Total number of cells | Number of spermatogonia | Percentage of spermatogonia |
|---|---|---|---|---|---|
| A | Enzymatic digestion | 10 | 223.3±21.3 | 75.5±24.3 | 75.5/223.3 (33.8)[a] |
| B | Enzymatic Percoll | 8 | 218.1±25.3 | 145.1±21.9 | 145.1/218.1 (66.4)[b] |
| C | Enzymatic Percoll adhesion | 10 | 215.0±18.8 | 176.2±16.6 | 176.2/215.0(82.0)[c] |

In the same columns, differences among numbers containing the different letters in the superscripts are significant ($p<0.01$)

6-day-old chicken was 2.5, 10.2 and 4.2% higher ($p<0.05$) than of 13 days old chicken, 15 days old chicken embryo and 19 days old chicken embryo, respectively. Based on the above analysis, we found that the purity of spermatogonia obtained from 6 days old chicken was significantly higher than those of embryonic periods and 13 days old chicken.

**Comparing the isolating effects of spermatogonia by different methods:** To compare the purity of spermatogonia obtained from three different groups (as A, B and C), this experiment used 6-day-old chicken testis as experimental materials. After every group repeated 8-10 times, means and station error were also computed (Table 3).

Through the tackling of these three groups, the average purity of spermatogonia obtained from 6 days old chicken was up to 33.8, 66.4 and 82.0%, respectively and the purity of spermatogonia gained after the tackle of percoll centrifugation and adhesion purification was the highest in the three groups and was 15.6 and 48.2% higher than of A, B groups. The purity of spermatogonia in the B groups, which was gained after the tackle of only percoll centrifugation, was 32.6% higher ($p<0.01$) than of A groups.

**The feature of spermatogonia cultured *in vitro*:** Comparing the growing feature of spermatogonia cultured *in vitro* in the condition of no CEF feed layer existed, single cell suspension that was not centrifugal elutriation of percoll gradients cultured *in vitro* for 5 h, most of somatic cells attached to flask, while only a few spermatogonia were attached. Furthermore, attached spermatogonia were also easy to come off when gently shaking the flask. After culturing 24 h, most of viable cells including spermatogonia were attached. Sertoli cells started to spread and form many different shapes. But spermatogonia that were randomly dispersed on sertoli cells layer still remained round (Fig 1a). After 48 h of culture, round spermatogonia dispersed on sertoli cells layer develop further into chains of two, four or eight A spermatogonia, which were connected together through an intercellular bridge (Fig 1b). After 72 h of culture, four and more spermatogonia, which linked in line increased (Fig 1c, d). At the same time, the number of spermatogonia sited on sertoli cells layer reduced, but the number of sertoli cells in the culture system did not become bigger. After 4 days of culture, the number of spermatogonia sited on sertoli cells layer continued to decrease. After 5 days of culture, only a few spermatogonia dispersed on sertoli cells layer and most of them shed into the culture medium, which about 60-70% of spermatogonia in the cultural medium were still living by the staining of Trypan Blue.

Cell suspension obtained from percoll gradient centrifugation cultured *in vitro* for 6-7 h, most of somatic cells were attached, but attached spermatogonia were very few; After 24 h of culture, nearly all viable cells attached to the flask and most of somatic cells already spread to form many different shapes, but spermatogonia still remained round and randomly sited on sertoli cells layer; After 48 h. of culture , all somatic cells already spread to form a bigger irregular shape, but spermatogonia still remained round and sited on sertoli cells layer; After 72 h of culture, round spermatogonia started to proliferate slowly and two, four and more spermatogonia linked in line in the cultural system; After 4 days of culture, most of spermatogonia shed into the cultural medium, which about 50% of spermatogonia in the culture medium were living by the staining of Trypan Blue.

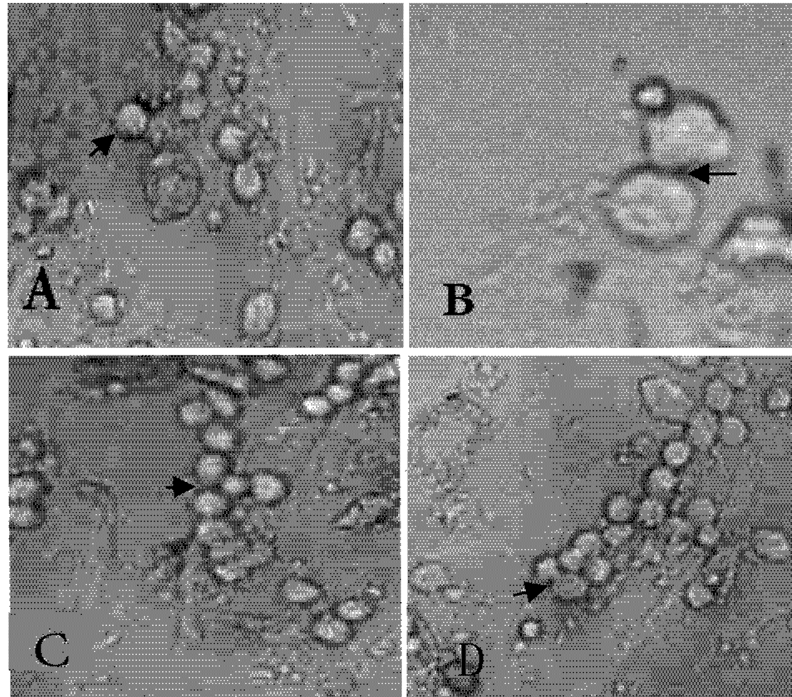


Fig. 1: The appearance of spermatogonia cultured *in vivo* before percoll isolation, a): Culture 24 h later, round spermatogonia randomly dispersing on sertoli cells (arrow was spermatogonia, ×250), b): Spermatogonia connected by bridge-cytoplasm (arrow was bridge-cytoplasm, ×400), c, d): Culture 72 h later, two, four or much spermatogonia were linked in line (arrow was somatic cells, ×250)

## DISCUSSION

The appropriate time to collect spermatogonia in chicken: Our initial experiment demonstrated that in the early days of testis forming (about 15 days old hatching time), only gonocytes and young sertoli cells existed in the seminiferous tubules and the number of spermatogonia were far less (Li *et al*., 2002; Zhang *et al*., 2000b). Following the extending of time (about 6 days old chicken), most of gonocytes began to differentiate to form SSCs and the number of spermatogonia in the tubules was also markedly increasing; following furtherly extending of time (about 14 days old chicken), many of SSCs started to differentiate into spermatogonia and the number of spermatogonia was also far more than of 6 days old chicken. Because of the slow proliferation of spermatogonia and the increasingly growing in number of sertoli cells, the purity of spermatogonia obtained from 14 days old chicken were relatively lower and it was more difficult to isolate and purity them. So to compare the number and purity of spermatogonia in different time periods, 15 days old chicken embryo, 19 days old chicken embryo, 6 days old chicken and 13 days old chicken were prepared in the experiment. Possible reason for a highest purity from 6 days old chicken was the slow proliferation of spermatogonia and the increasingly growing in number of somatic cells.

Affecting isolation and purification of spermatogonia: Percoll is a silica sol with non-dialysable PVP coating. The low osmolality and viscosity can result in rapid formation of gradients and particle separation. Percoll is also impermeable to biological membranes and is harmless to cells. Also, it can form stable gradient bands. So percoll was generally used to isolate spermatozoa of many animals by some scholars (Tang *et al*., 1992). However, separating early spermatogenic cells was only reported in mammalian. This study first tried to isolates spermatogonia of cock by using percoll gradient centrifugation. Result showed that every bands of percoll gradient contained, more or less, different number of spermatogonia, but most of the spermatogonia were found in bands of 19-27 and 27-35% of percoll gradient. So in this experiment, we merged the two gradients into a gradient (19-35%) and found that 66.4% purity of spermatogonia was obtained.

To obtain spermatogonia of high purity, some factors such as the choice of separation materials and the attached velocity of testis cells had to be taken into account. In the preliminary experiments, the attached time

of testis cells would be prolonged, while extending digestion time. Possible reason was that, the exoplasm of cells were destroyed when dissociated testis cells using an enzymatic digestion and the extracellular configuration of glycoprotein with higher molecular weight was also damaged, fully destroying the integrity of membranes. Though, cells still alive, they could not attach to flask or lower the attached velocity. Moreover, violently blowing with a pipette can also destroy the bridge-cytoplasm of differentiating spermatogonia, which reduces the viability of spermatogonia and extends the attached time of spermatogonia. So, it was inevitable for the adhesion and purification of spermatogonia to control the degree of enzymatic digestion and mechanically blowing. This experiment utilized the biological feature that the velocity of different cells attaching to flask was different to spermatogonia isolated from testis cell suspension. To further purify spermatogonia, a method of adhesion purification was employed and 82% purity of spermatogonia was also gained. The result was unanimous to the one gained by Fariborz *et al*. (2000) and Izadyar *et al*. (2002). So, we thought that the method of percoll gradient centrifugation and adhesion purification after enzymatic digestion was suitable to separate cock spermatogonia and can be used in many ordinary laboratories.

**The behavior of spermatogonia cultured *in vitro*:** When, there was no CEF feeder layer, most of spermatogonia cultured *in vitro* appeared to degenerate within a week whether, percoll gradient centrifugation was adopted or not. Only, a few spermatogonia can withstand the harmful external environment better and randomly disperse on sertoli cells layer. The possible reasons were that following the prolong of *in vitro* culture time, the microenvironment in culture system was not suitable to the continuous differentiation of spermatogonia and led to the degeneration of spermatogonia, much glycoprotein and polymorphism might exist in the surface of spermatogonia membrane, which might participate in the process of adhesion. Further, continuous differentiation would cause structural change of membrane, resulting in the drop of attaching ability of spermatogonia. Although, spermatogonia were living, they could not attach firmer and easy to come off, causing the disappearance of some spermatogonia. The experiment also found that spermatogonia obtained from percoll gradient centrifugation had a shorter life-span and a longer adhesion time when they were cultured *in vitro*. The possible reason: a great deal of somatic cells (such as myoid and sertoli cells) existed in the single cells suspension before percoll centrifugation and these somatic cells can release all kind of factors needed by spermatogonia growing *in vitro* (Amy *et al*., 2000). Moreover, these factors can also restrain differentiation of spermatogonia but accelerate proliferation of spermatogonia. When, single cell suspension was treated using centrifugal elutriation of percoll gradients, most of somatic cells were removed and high purity of spermatogonia was gained. For lack of the nursing of somatic cells and CEF feeder layer, spermatogonia only depended on themselves to fulfill the process of attaching to flask, which extended the adhension time of spermatogonia and shortened its life span.

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