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Short Contribution

Long-term culture of avian embryonic cells *in vitro*

TAKASHI KUWANA[1,2*], KOICHIRO HASHIMOTO[3], AKIRA NAKANISHI[3], YOSHIAKI YASUDA[4], ATSUSHI TAJIMA[5], and MITSURU NAITO[6]

[1]"Inheritance and Variation", Precursory Research for Embryonic Science and Technology, Research Development Corporation of Japan, [2]Pathology Section, National Institute for Minamata Disease, Kumamoto, [3]Laboratory of Developmental Biology, Meiji Institute of Health Science, Kanagawa, [4]Department of Anatomy, Kumamoto University School of Medicine, Kumamoto, [5]Institute of Agriculture and Forestry, University of Tsukuba, Ibaraki and [6]National Institute of Animal Industry, Ibaraki, Japan

ABSTRACT The pH of the embryonic blood, one of the most important environmental factors for embryonic cells, was found to range from 8.1 to 8.5 in chick embryos until 108 h after incubation. Based on these results, the culture medium adjusted to pH 8.0 was used to culture embryonic chick and quail cells. They were easily subcultured for a long period of time at pH 8.0. This pH culture condition may have wide application for manipulating embryonic cells or tissues and establishing cell lines from avian embryos.

KEY WORDS: *embryonic blood, pH, long-term culture method, chick and quail*

Avian PGCs might be a good vehicle to introduce foreign genes into the next generation because PGCs are germ-line cells. Avian PGCs segregate from the hypoblast of the so-called germinal crescent region, and accumulate between the endoderm and ectoderm during stages 4 to 8 (staging of Hamburger and Hamilton, 1951). They begin to circulate via bloodstream along with the establishment of embryonic blood circulation till stage 10. After leaving the blood circulation from the capillaries near the GE, they migrate interstitially, as do mammalian PGCs, and finally penetrate the developing gonad by 2.5 days of incubation (Swift, 1914; Fujimoto *et al.*, 1976). In the circulating phase, PGCs could be easily isolated from embryonic blood samples. Moreover, by transplantation of these isolated PGCs into blood vessels of other embryos we could effectively produce PGC chimeras and obtain offspring derived from donor PGCs (Kuwana, 1993). Avian PGCs may be the best candidate as a vehicle to produce transgenic birds.

To introduce foreign genes and select transfected PGCs it would be essential to culture them *in vitro*. In cell culture of chick and quail, the primary culture is generally possible. In fact, it is not so difficult to obtain a cell population originating from adult tissues using a common culture medium at pH from 7.2 to 7.4 (Novero and Asem, 1993). Although cells proliferate rapidly during the initial period of subculture, their proliferation gradually decreases and stops within a few weeks. In the case of embryonic cells, however, such long-term subculture has been difficult. The embryonic cells could be subcultured using a medium with chick embryo extracts at pH 7.2 to 7.4 (Watanabe *et al.*, 1989) but in chick or quail, there has been no report to our knowledge on subculture of cells originating from young embryos before 3 days of incubation. Moreover, the development of subculture methods may succeed in establishing avian normal cell lines.

Though we have tried many culture mediums for chick or quail embryonic cells, the long-term subculture has not been performed at the standard pH range. The pH of the fluid around cells *in vivo* was finally realized to be one of the important factors in their culture. In the present study, we measured in chick and quail embryos the pH of embryonic blood, the yolk next to the embryo and the albumen as the environment of the embryonic cells.

Transition in pH as environment of embryonic chick cells

Embryonic blood

Embryonic chick blood circulation begins at stage 10 and one can extract 2 μl of blood only after stage 12. The transition in the blood pH is shown in Table 1 and Figure 1. The pH at stage 12 is 8.4 and then ranged from 7.7 to 8.4 throughout the embryonic period. The blood pH at stages 12 to 18 ranged from 8.1 to 8.5 (average 8.3).

Yolk

The pH of the yolk next to the embryo was measured after stage 1 till embryos were 6 days-old (stage 25). The pH value of the yolk just behind the embryo indicated an acidic value till 35 h after incubation and then increased gradually till stage 25 (108 h of incubation time).

Abbreviations used in this paper: PGCs, primordial germ cells; GE, germinal epithelium; KAv-1 medium, Kuwana's modified avian culture medium-1; 2-ME, 2-mercaptoethanol; EPPS, N-2-hydroxethylpiperazine-N'-3-propanesulfonic acid; FBS, fetal bovine serum; CS, chick serum.

***Address for reprints:** Pathology Section, National Institute for Minamata Disease, 4058-18 Hama, Minamata, Kumamoto 867, Japan. FAX: 81-966-61-1145. e-mail: kuwana@infobears.or.jp

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Albumen

Unincubated fertilized egg albumen had a pH of approximately 9.3, then decreased gradually. Throughout stages 12 to 25, the pH of embryonic blood consistently ranged between that of the yolk and that of the albumen. The transition in pH in quail also showed a result similar to that in chick (data not shown).

Establishment of subculture conditions for embryonic cells in birds

In the present study, the pH of the culture medium was adjusted to 8.0 in air. Cells proliferated well in this culture condition, and 6 kinds of cells, which we attempted to subculture for a long-term, were subcultured for over 3 months (20 passages) except liver and primordial germ cells. Finally, all the subcultured cells were kept in liquid nitrogen at the respective number of passages shown in Table 2.

TABLE 1

TRANSITION OF pH AS ENVIRONMENT OF EMBRYONIC CELLS

Incubation Time (h)	Stage	Sample No.	Blood pH (S.D.)	Yolk pH (S.D.)	Albumen pH (S.D.)
0	1	8	-	6.08 (0.17)	9.26 (0.07)
29	7	4	-	6.92 (0.60)	9.63 (0.21)
32	9	4	-	6.73 (0.36)	9.60 (0.14)
35	10	3	-	6.80 (0.10)	9.70 (0.17)
38	10+	1	-	7.20 (-)	9.50 (-)
45	11	1	-	7.00 (-)	9.60 (-)
47	13	7	8.39 (0.09)	6.08 (0.21)	9.62 (0.12)
52	14	9	8.30 (0.13)	6.17 (0.27)	9.56 (0.10)
53	15	3	8.27 (0.15)	6.17 (0.21)	9.50 (0.10)
54	16	2	8.45 (0.07)	7.15 (0.35)	9.65 (0.07)
58	17	3	8.43 (0.05)	7.63 (1.11)	9.53 (0.06)
67	18	7	8.44 (0.07)	7.89 (0.43)	9.40 (0.22)
108	25	10	8.15 (0.11)	7.93 (0.14)	8.14 (0.07)
144	29	10	7.89 (0.11)	7.95 (0.14)	8.03 (0.15)
264	37	10	7.79 (0.07)	7.04 (0.74)	-

The pH levels in column are represented in mean values with standard deviations of each developmental stage.

In preliminary experiments, we attempted to culture the chick cells of the germinal crescent region at stage 3-5 with KAv-1 adjusted to pH 7.2 and KAv-1 adjusted to pH 8.0. At pH 8.0, the cells had been subcultured over 3 months as described above. In contrast, when the pH of the culture medium was adjusted to 7.2, the same cells had died at the 10th and 14th passage (Fig. 2).

Viability of PGCs

When 100 PGCs were cultured at pH 7.4, their viability was 5.75% after 4 h and 3.75% after 24 h, respectively. When 100 PGCs were cultured at pH 8.0, their viability was 95% after 4 h and 18.25% after 24 h, respectively (Table 3). In the present experiments, PGCs were considered dead cells when their periphery was not demarcated, bubbling occurred on their surfaces or they were taken to be completely destroyed. Additionally, the viability of PGCs was reconfirmed by the absorption test of trypan blue.

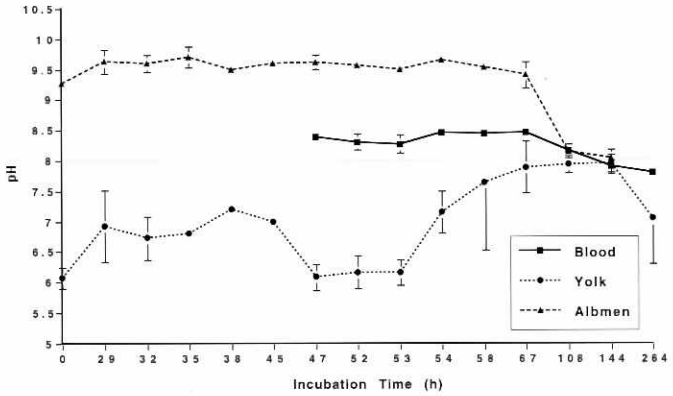

Fig. 1. Transition of pH as environment of embryonic cells. *These graphs indicate changes in pH levels of blood, yolk, and albumen as shown in Table 1. ■ indicates the pH level of embryonic blood; ● indicates the pH level of yolk just under the embryo; ▲ indicates the pH level of albumen just over the embryo at each developmental stage.*

There are no established conditions to culture avian cells for a long time. The present study indicates that the pH of embryonic blood as the environment of the embryonic cells was higher than that of the medium conventionally used for cell culture (7.2 to 7.4). Additionally, the cells from all tested tissues except liver and PGCs could be subcultured at pH 8.0. In the case of PGCs and liver, cells did not proliferate in our culture conditions.

In the present study, the high pH (8.0) of the culture medium as an environment was demonstrated to be essential for long term culture of avian embryonic cells. Although it was not clear why such high pH is essential for such long-term cultivation, embryonic blood pH seemed to be controlled by a balance between the pH of the

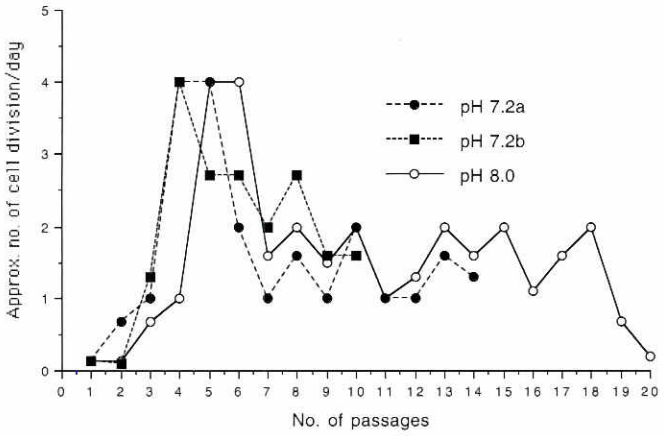

Fig. 2. Cell growth in the medium adjusted to pH 7.2 and 8.0. *Cells were obtained from the germinal crescent region of stage 3-5 chick embryos. The abscissa represents the number of passages. The ordinate represents the approximate number of cell divisions per day, which was calculated by dividing the reciprocal number of dilution rate at a passage by days till the next passage. In pH 7.2 a and b, all cells had died by passage 10 and 14, respectively. ○ indicates the cell dividing rate at pH 8.0; ■ and ● indicate those at pH 7.2.*

TABLE 2

KINDS OF CULTURED CELLS AND NUMBER OF PASSAGES IN CHICK AND QUAIL

Species	Embryonic stage	Tissues	No. of passages
Chick	1	Whole blastodisk	1
	3-4	Germinal crescent region*1	7
	3-5	Germinal crescent region	20**
	4-8	Germinal crescent region*1	18
	4-8	Whole embryo	13
	5	Whole embryo*1	17
	11	Presumptive gonadal region*1	7
	12	Presumptive gonadal region*1	8
	13	Whole embryo	4
	15	PGCs	0#
	16	Whole embryo*1	8
	17	Presumptive gonadal region	7
	17	Area vasculosa	7
	20	Whole embryo	4
	25	Male developing gonads*1	1
	25	Female developing gonads*1	1
	25	Male whole embryo*1	20**
	25	Female whole embryo*1	24**
	day-4 embryo	Developing gonads	24**
	day-5 embryo	Developing gonads	6
	day-7 embryo	Developing gonads	5
	day-7 embryo	Heart	5
	day-7 embryo	Liver	0#
Quail	3-5	Germinal crescent region	9
	11	Whole embryo*2	18
	11	Whole embryo*3	25**
	15	Whole embryo*2	10
	17	Presumptive gonadal region	8
	17	Area vasculosa	35**
	day-7 embryo	Developing gonads	12
	day-7 embryo	Heart	16
	day-7 embryo	Liver	0#
	hatching (male)	Testis	3
	hatching (male)	Heart	2
	hatching (male)	Liver	0#

Six kinds of cells (**) were subcultured with over 20 passages, and then kept in liquid nitrogen; the other cells were frozen in liquid nitrogen before 20 passages. Only in the liver and PGCs (#) they did not proliferate in our culture condition and died within 10 days. *1, SPF chick embryo. *2, L_2 inbred line; *3, H_2 inbred line quail embryo.

albumen (pH 9.4-9.65) and that of the yolk (pH 6.1-7.9), as shown in Table 1 and Figure 1. The pH of blood was over 8.0 till 108 h incubation (the end of early embryogenesis, Table 1). This result suggests that the high pH may play some role in the proliferation of undifferentiated cells in the early development of the avian embryo and that the decrease of pH may trigger the next developmental steps.

This pH condition for avian cell culture may make it possible to establish new cell lines from not only embryonic cells but also adult cells. Moreover, the viability of the PGCs *in vitro* improved greatly by using KAv-1 medium at pH 8.0. This suggests that a culture medium of pH 8.0 might have a wide application in the field of avian biotechnology.

Experimental Procedures

pH measurement

Fertilized eggs of White Leghorn and Japanese quail were incubated at 38.5°C and 60% relative humidity in a forced air incubator (P-800, Showa Incubator Lab., Japan) to obtain embryos at various developmental stages (Hamburger and Hamilton, 1951). The pH levels of embryonic blood, yolk and albumen as the environment of the embryonic cells were measured at various developmental stages.

Embryonic blood samples of 2 μl were collected from the embryos at stages 12, 13, 14, 15, 16, 17, 18 and 25, and from 6- and 11-day embryos and at hatching. Yolks located just behind the embryo and thin albumen were collected from the eggs from stage 1 until hatching. All specimens were collected using glass micro-capillaries which were rinsed 3 times in double distilled water before use. After collecting the specimens from the eggs, approximately 2 μl of each was immediately measured for pH by pH BOY-C1 (Shindengen, Japan).

Moreover, thin albumen and the yolk next to the embryonic disc from unincubated fertilized chick eggs were used for pH measurement.

Source of cultured cells

Chick: Germinal crescent region at stage 3-4
Germinal crescent region at stage 3-5
Germinal crescent region at stage 4-8 (SPF embryo)
Whole embryo at stage 4-8 (SPF embryo)
Whole embryo at stage 13
Primordial germ cells (PGCs) from stage 15 embryo
Presumptive gonadal region and vascular area at stage 17
Whole embryo at stage 20
Developing gonads of stage 25 male embryo (SPF embryo)
Developing gonads of stage 25 female embryo (SPF embryo)
Whole male embryo at stage 25 (SPF embryo)
Whole female embryo at stage 25 (SPF embryo)
Developing gonads of day-4 embryo (stage 24)
Developing gonads of day-5 embryo (stage 27)
Developing gonads, heart and liver of day-7 embryo (stage 31)

Quail: Germinal crescent region at stage 3-5 (stage 1-3 of Zacchei, 1961)
Whole embryonic body of stage 11 (L2 line)
Whole embryonic body of stage 11 (H2 line)
Presumptive gonadal region and area vasculosa at stage 17 (stage 14 of Zacchei)
Developing gonads, heart and liver of day-7 embryo (stage 31; stage 22 of Zacchei)
Testis, heart and liver of hatching (male) (stage 46; stage 33 of Zacchei)

SPF eggs of White Leghorn were obtained from Nisseiken Co., Ltd. (Yamanashi, Japan). Fresh quail L2 and H2 inbred line eggs (the 47th generation) (Takahashi *et al.*, 1984) were obtained from the animal center of the National Institute of Pollution.

Establishment of subculture conditions for embryonic avian cells

Cells from chick and quail embryos were cultured with KAv-1 medium; α-MEM (GIBCO BRL, USA) added with 1 mM D-glucose, $5x10^{-5}$ M 2-ME,

TABLE 3

VIABILITY OF PGCs IN DIFFERENT pH MEDIUMS

	0 h	4 h	24 h
pH 7.4 (n= 4)	100	5.75±0.96*	3.75±0.96*
pH 8.0 (n= 4)	100	95±2.16*	18.25±3.30*

*mean±standard deviation.

and 10 mM EPPS (Wako Chem., Japan) containing 5% of FBS (JRH Biosciences, USA) and CS (JRH Biosciences, USA). Medium pH was adjusted by Na_2CO_3 to the designated pH (8.0), similar to that of the embryonic blood in air.

Five ml of the KAv-1 medium in the plastic culture flask (No. 25102S, CORNING Co., Ltd., USA) was changed every second day, and the cells were subcultured when the cells proliferated so as to cover 90% of the culture area.

Viability of PGCs

Viability of PGCs was compared at two different pHs using KAv-1 medium. Chick PGCs were collected from embryonic blood at stage 13-15 according to Kuwana and Fujimoto (1984) using KAv-1 medium. Each 100 PGCs were cultured with KAv-1 medium at pH 7.4 or 8.4. PGCs were cultured in these conditions for 4 h or 24 h, and their viability was confirmed under phase contrast microscopy by morphological criteria and absorption test of trypan blue.

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