Scientific Research Publishing

Open Journal of Animal Sciences, 2018, 8, 184-190
http://www.scirp.org/journal/ojas
ISSN Online: 2161-7627
ISSN Print: 2161-7597

In Vitro Culture of Shell Gland Epithelial Cells in Japanese Quail (*Coturnix coturnix japonica*)

Osamu Kai, Kaoru Mineo

Department of Animal Science and Resources, College of Bioresource Sciences, Nihon University, Fujisawa, Japan
Email: kai.osamu@nihon-u.ac.jp

How to cite this paper: Kai, O. and Mineo, K. (2018) *In Vitro* Culture of Shell Gland Epithelial Cells in Japanese Quail (*Coturnix coturnix japonica*). *Open Journal of Animal Sciences*, **8**, 184-190.
https://doi.org/10.4236/ojas.2018.82013

Received: March 23, 2018
Accepted: April 20, 2018
Published: April 24, 2018

Abstract

To investigate the secretion of protoporphyrin IX (PpIX), superficial eggshell pigment, from shell gland cells of Japanese quail, the epithelial cells of the gland were collected and isolated for cultivation *in vitro*. An analysis of a peak for PpIX in the cells was performed using a fluorescence microplate reader. The measurement showed that PpIX has a peak of excitation wavelength at 410 nm and emission wavelength at 606 nm in the culture medium (HamF12 + 4% HCl). Volumes of PpIX in the medium after 4 hour culture of the cells were measured with a microplate reader using filter set of excitation wavelength 400/30nm and emission wavelength 620/40nm. However the cells did not secrete significantly PpIX during 4 hour incubation in this culture system, addition of quail plasma to the medium resulted in significantly higher secretion. A cultivation system in this study is able to use for the study on the mechanism of the secretion of eggshell pigment, PpIX from Japanese quail shell gland epithelial cells.

Keywords

Japanese Quail, Shell Gland Epithelial Cells, *In Vitro* Culture, Protoporphyrin, Fluorescence Microplate Reader

1. Introduction

Avian colored eggshells contain some bio-pigments, porphyrins [1] [2] [3] [4] [5]. The wild-type Japanese quail eggshell pigments contain both biliverdin (oocyan) and ooporphyrin and the pigments were identified by spectrophotometric analysis [6]. Kennedy and Vevers [2] surveyed the shell pigments of 108 avian species and reported the pigments present in quail eggshells are protoporphyrin IX and biliverdin IX. The superficial pigment is accumulated by the shell gland epithelial cells in the stage of calcification [7] [8] and secreted into the egg shell

surface 3.5-2hours before oviposition [6] [9] [10]. The ovulatory process, possibly the preovulatory surge of gonadotropin(s) or progesterone, and subsequent stimulation of the oviduct by the egg were associated with the accumulation of the pigment in the cells [11]. There have, however, been very few reports investigating the secretion of the protoporphyrin IX (PpIX). Soh and Koga [12] [13] and Soh *et al.* [14] examined the secretion of the porphyrin from the quail shell gland cells. Prostaglandin F2α and arachidonic acid injected intrauterinely 6 h before the expected oviposition time induced the secretion of the porphyrin within 30 min after the injection [13]. In order to investigate further the mechanisms on the secretion from the shell gland cells, in vivo system would be hampered by many factors.

As far as we know, there has not been reported about in vitro culture system on the secretion of porphyrins using shell gland epithelial cells. Therefore, the aim of this study is to establish *in vitro* culture system on PpIX secretion by shell gland epithelial cells in Japanese quail.

2. Materials and Methods

2.1. Experimental Birds

Japanese quail, 10 or more weeks of old and laying regular sequences, were used in this study. Birds were individually caged in windowless environmental-controlled room under a light regimen of 14h light and 10h dark. Food and water were available ad libitum. The oviposition times of each quail were automatically recorded (Egg Counter, O'Hara & Co., Ltd. Tokyo). Quail laying on repeated regular clutch were selected for the present experiment and were used 5 hour before estimated oviposition. All experimental procedures were conducted in accordance with the guidelines for animal experiments, College of Bioresource Science, Nihon University.

2.2. Fluorescence Spectrum of Protoporphyrin in the Epithelial Cells of the Shell Gland

In order to identify the spectrum of protoporphyrin in the epithelial cell using a spectrofluorometer (Synergy Mx Monochromator-Based Multi-Mode Microplate Reader, BioTek Instrument Inc., VT, USA), the cells were isolated from the gland. Immediately after blood collection, the shell gland was removed and placed in culture medium, HamF12 "Daigo" (Nissui Pharmaceutical, Tokyo, Japan) and the epithelial cell layer was scratched with a glass slide. The cell layers were dissolved in 4% Hydrochloric Acid (20% HCl, Wako Pure Chemical Industries, Osaka, Japan) before the supernatant solution were vortexed for extraction of porphyrins. Then, the supernatant solutions were collected after centrifugation (2000 G, 5 min, 4˚C) and measured by the spectrofluorometer. Protoporphyrin IX disodium salt (PpIX-2Na; Sigma-Aldrich, St. Louis, USA) was dissolved in the same solution and served as the control for the spectrum.

2.3. Isolation of Shell Gland Epithelial Cells

The cell layer was isolated from the tissue in HamF12 with $CaCl_2$ 5 mM, and then treated with 0.001% collagenase (Wako Pure Chemical Industries). The mixture was cultured in a shaking water bath for 10 min at 37˚C. The cells were centrifuged (30 G, 5 min, 4˚C). The precipitated cells were resuspended in HamF12 with $CaCl_2$ 5 mM and the supernatant of resuspension solution was collected. This was repeated before the supernatant was clear. Then the collected supernatants were centrifuged (30 G, 5 min, 4˚C) once more. The supernatants of the centrifuged solution were used for further experiments.

2.4. Culture of Shell Gland Epithelial Cells

The isolated epithelial cells were counted and adjusted to 2×10^6 cell/ml by HamF12 without $CaCl_2$, and placed at 2×10^5 cell/well in a 96 well culture plate (#3860-096, Iwaki, Tokyo, Japan). Cells were cultured in an incubator (5% CO_2, 37˚C) for 4 h with 10 µl mixed plasma taken from normal quail or with the same volume of the medium as the control. Cell viability was assessed by the trypan blue method. After the incubation, supernatants of the cells were collected and mixed with 20% hydrochloric acid (finally 4% HCl). The fluorescence of the solutions was determined with a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek Instrument Inc.) using a 400 nm, 30 nm bandwidth excitation filter and a 620 nm, 40 nm band width emission filter.

The data were analyzed by Tukey-Kramer's HSD multiple range test. The values were determined to be significant at $P < 0.05$.

3. Results

3.1. Spectroscopic Features of PpIX in Cells

Spectral scans from 350 nm to 440 nm of excitation light demonstrated that the extracted solution of the shell gland epithelial cells had a peak at 410 nm in HamF12 medium + 4% HCl (**Figure 1(a)**). Spectral scans from 560 nm to 640 nm of fluorescent emission light showed that the extracted solution had a peak at 606 nm using excitation wavelength of 410 nm in the same medium (**Figure 1(b)**). The control, PpIX-2Na had the same pattern on the spectral scanning (data not shown).

3.2. Culture of Shell Gland Epithelial Cells

The isolated shell gland epithelial cells were cultured for 4 hour by using HamF12 medium without $CaCl_2$ or with mixed quail plasma. Many of the cells after 4 hour culture looked like substantially normal in shape as well as the cells at starting the culture. Moreover, the viability of the cells after 4hour incubation did not differ to 0 hour group, a range from 77.9% to 81.6% for four groups (data not shown).

Figure 2 showed that the fluorescent values of the supernatants were the same level between 0 hour and 4 hour culture groups in the plasma-free control

group. However, the value of the group in which 10 μl quail plasma was added in the culture medium were significantly higher after 4 hour incubation than that of 0 hour group.

4. Discussion

Some studies on differential measurement of porphyrin group have been done using the fluorescence spectrometrical method. Polin [15] reported that protoporphyrin from hen's egg shell had an absorption peak at 410 μm in 3N HCl. In

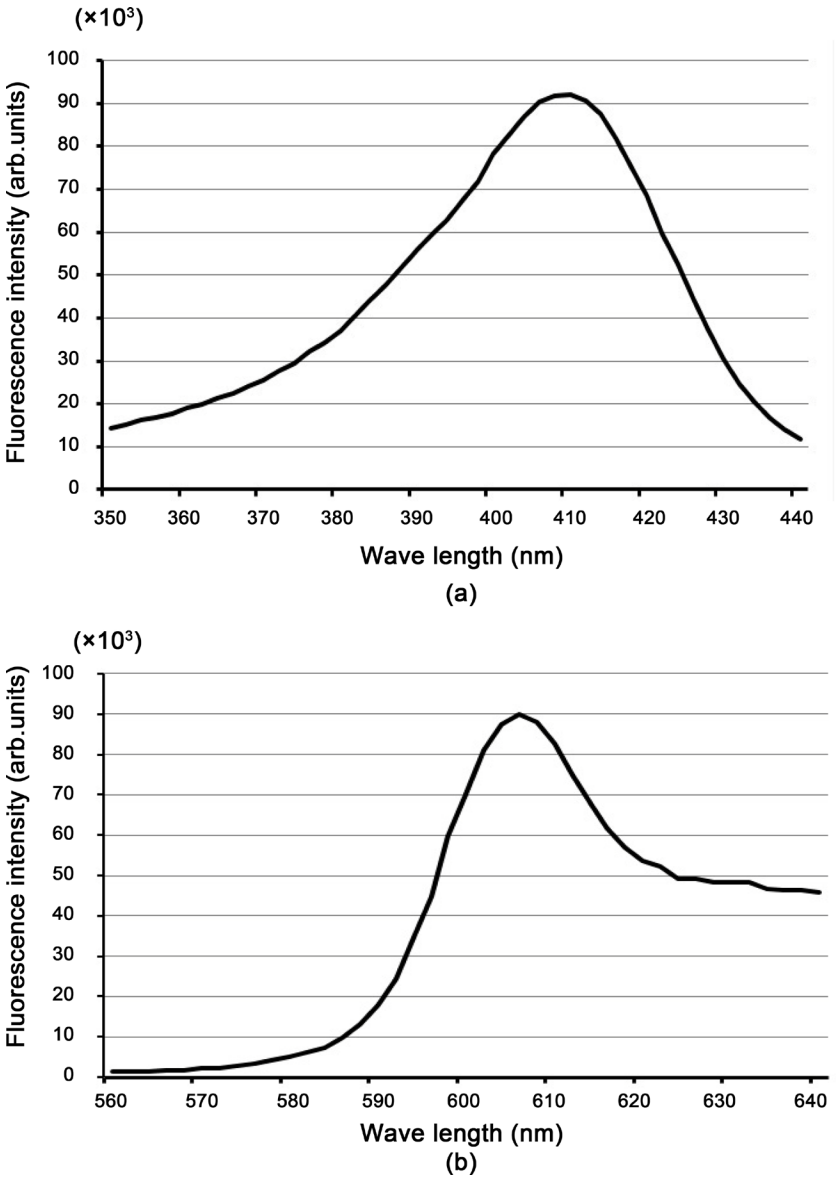

Figure 1. (a) Spectral scans from 350 nm to 440 nm of excitation light to the extracted solution of the shell gland epithelial cells. Protoporphyrin IX (PpIX) in the cells had a peak at 410 nm in HamF12 medium + 4% HCl. (b) Spectral scans from 560 nm to 640 nm of fluorescent emission light to the extracted solution of the shell gland epithelial cells. Protoporphyrin IX (PpIX) in the cells had a peak at 606 nm in HamF12 medium + 4% HCl.

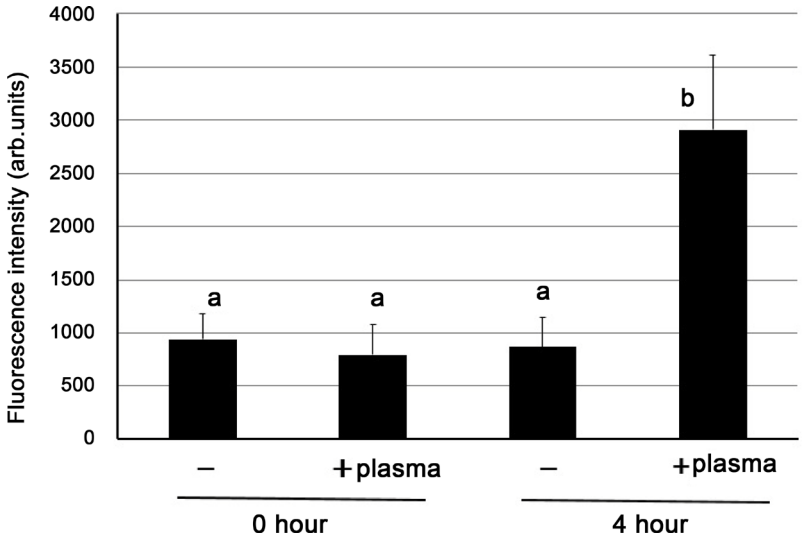

Figure 2. The fluorescent values of PpIX in the culture medium of the shell gland epithelial cells. The plasma-free controls of 0 hour and 4 hour culture group showed the same level. However, addition of 10 µl quail plasma in the culture medium resulted in significantly higher level after 4 hour incubation than those of plasma-free control and of 0 hour group. Values are expressed as mean ± SEM (n = 4). Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer's HSD multiple range test).

Japanese quail, Poole [6] identified spectrophotometrically 3N HCl extracts of the uterine tissue having a main peak at 410 mµ. Tamura *et al.* [7] also reported optical density of methyl-esterified pigments of uterine tissue at 400 - 405 mµ in HCl-methanol solution. According to Poole [6], since the absorption peak of the uterine tissue shifted from 410 to 415 mµ in methanol-HCl solution, the peak seems to shift in some mµ depends on the solution for the extraction. The optical density in the measurement of protoporphyrin also differs depending on the researcher, namely 410 mµ [6], 406 - 408 mµ [16]. Recently, suitable excitation wavelength and fluorescent wavelength for the measurement of protoporphyrin were reported from the scanning spectrum by a spectrofluorophotometer. Miksik *et al.* [17] measured it using the condition at 405 ex/620 em nm and Leonzio *et al.* [18] did at 410 ex/605 em nm. The present fluorescence spectrometrical study showed that a peak of the excitation wavelength of the pigments in the cells was range at 410 nm and fluorescent wavelength was 606 nm using excitation wavelength of 410 nm. On the basis of the results, fluorescence in this experiment was measured with a fluorescent spectrometer using a filter pair; 400 nm/30 nm bandwidth excitation filter and a 620 nm/40 nm band width emission filter, which are available to get from the supplier.

In this experiment, 4 hour-incubation time was taken for the experiment, in which PpIX would be secrete into the medium. Since porphyrin deposition on the egg shell is a short time event before 3.5 - 2 hours of oviposition and for about 30 minutes in Japanese quail [6] [9], the 4 hour-incubation time would be suitable for secretion of PpIX by uterine epithelial cells *in vitro*.

Addition of quail plasma in medium resulted in higher value of PpIX in su-

pernatant of the medium. The result suggests that unknown factors in the plasma could secrete PpIX to the cells. PpIX secretion from shell gland epithelial cells *in vivo* was suggested that prostaglandin F2*α* injected induced pigmentation [13] and indomethacin inhibit [12]. Therefore, prostaglandin F2*α* may be one of the factors for participation of porphyrin secretion from the cells. Plasma has contained various matters, prostaglandin F2*α* was one of them.

To our knowledge, the results presented here represent the first observation of secretion of PpIX *in vitro* and the system would be suitable for the secretion mechanisms *in vitro* experiment.

5. Conclusion

In conclusion, the results of the study showed the isolated shell gland epithelial cells of Japanese quail were cultured in vitro properly at least 4 hours. Moreover, released protoporphyrin IX from the cells were measured using a spectrofluorometer equipped a filter pair; 400 nm/30 nm bandwidth excitation filter and a 620 nm/40 nm bandwidth emission filter. In addition, this system would be suitable for the secretion mechanisms of protoporphyrin IX *in vitro* experiment.

Acknowledgements

The authors thank Mr. Takayuki Inoue, Central Scientific Commerce, Inc. for technical assistance in getting the spectrum of PpIX.

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

[1] Romanoff, A.L. and Romanoff, A.J. (1949) The Avian Egg. Wiley Co., New York.

[2] Kennedy, G.Y. and Vevers, H.G. (1976) A Survey of Avian Eggshell Pigments. *Comparative Biochemistry and Physiology*, **55B**, 117-123. https://doi.org/10.1016/0305-0491(76)90183-8

[3] Lang, M.R. and Wells, J.W. (1987) A Review of Eggshell Pigmentation. *World's Poultry Science*, **43**, 238-246. https://doi.org/10.1079/WPS19870016

[4] Liu, H.-C. and Cheng, W.T.-K. (2010) Egg Shell Pigmentation: A Review. *Journal of Chinese Society of Animal Science*, **39**, 75-89.

[5] Samiullah, S., Roberts, J.R. and Chousalkar, K. (2015) Eggshell Color in Brown-Egg Laying Hens—A Review. *Poultry Science*, **94**, 2566-2575. https://doi.org/10.3382/ps/pev202

[6] Poole, H.K. (1965) Spectrophotometric Identification of Eggshell Pigments and Timing of Superficial Pigment Deposition in the Japanese Quail. *Proceedings of the Society for Experimental Biology and Medicine*, **119**, 547-551. https://doi.org/10.3181/00379727-119-30234

[7] Tamura, T., Fujii, S., Kunisaki, H. and Yamane, M. (1965) Histological Observations on the Quail Oviduct; with Reference to Pigment (Porphyrin) in the Uterus.

Journal of the Faculty of Fisheries and Animal Husbandry, Hiroshima University, **6**, 37-57.

[8] Tamura, T. and Fujii, S. (1966) Histological Observations on the Quail Oviduct; on the Secretions in the Mucous Epithelium of the Uterus. *Journal of the Faculty of Fisheries and Animal Husbandry, Hiroshima University*, **6**, 357-371.

[9] Woodard, A.E. and Mather, F.B. (1964) The Timing of Ovulation, Movement of the Ovum through the Oviduct, Pigmentation and Shell Deposition in Japanese Quail (*Coturnix coturnix japonica*). *Poultry Science*, **43**, 1427-1432. https://doi.org/10.3382/ps.0431427

[10] Tanaka, K., Imai, T. and Koga, O. (1977) Superficial Pigmentation of Egg Shell in Japanese Quail, *Coturnix coturnix japonica*. *Japanese Poultry Science*, **14**, 229-231. (In Japanese). https://doi.org/10.2141/jpsa.14.229

[11] Soh, T., Koga, O. and Tanaka, K. (1989) Involvement of Ovulation Mechanism(s) in the Accumulation of Pigment in the Shell Gland of the Japanese Quail (*Coturnix coturnix japonica*). *Poultry Science*, **68**, 1156-1158. https://doi.org/10.3382/ps.0681156

[12] Soh, T. and Koga, O. (1999) The Effect of Phosphorus and Prostaglanglandins on the Secretion of Pigment from the Epithelium of the Shell Gland in Japanese Quail. *British Poultry Science*, **40**, 131-134. https://doi.org/10.1080/00071669987962

[13] Soh, T. and Koga, O. (1999) The Sensitivity of the Shell Gland to Prostaglandin in Quail; on the Secretion of Pigment from the Mucous Epithelium. *Animal Science Journal*, **70**, 170-173. https://doi.org/10.2508/chikusan.70.170

[14] Soh, T., Fujihara, N. and Koga, O. (2000) The Effect of Indomethacin on the Superficial Pigmentation of Japanese Quail Eggeshell. *Japanese Poultry Science*, **37**, 171-174. https://doi.org/10.2141/jpsa.37.171

[15] Polin, D. (1957) Formation of Porphyrin from Delta-Aminolevulenic Acid by Uterine and Liver Tissue from Laying Hens. *Proceedings of the Society for Experimental Biology and Medicine*, **94**, 276-279. https://doi.org/10.3181/00379727-94-22919

[16] Baird, T., Solomon, S.E. and Tedstone, D.R. (1975) Localisation and Characterization of Eggshell Porphyrins in Several Avian Species. *British Poultry Science*, **16**, 201-208. https://doi.org/10.1080/00071667508416177

[17] Miksik, I., Holan, V. and Deyl, Z. (1994) Quantification and Variability of Eggshell Pigment Content. *Comparative Biochemistry and Physiology*, **109A**, 769-772. https://doi.org/10.1016/0300-9629(94)90220-8

[18] Leonzio, C., Fossi, M.C. and Casini, S. (1996) Porphyrins as Biomarkers of Methylmercury and PCB Exposure in Experimental Quail. *Bulletin of Environmental Contamination and Toxicology*, **56**, 244-250. https://doi.org/10.1007/s001289900037