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≪Research Note≫

Identification of Ghrelin in Fertilized Eggs of Chicken

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The aim of this study was to determine whether ghrelin was present in the internal contents of eggs. Ghrelin in the internal contents of fertilized eggs incubated for 0 to 5 days was measured by a time-resolved fluoro-immunoassay. Ghrelin was identified in both yolk and albumen of the fresh fertilized eggs before incubation with a higher concentration in the yolk than albumen. The concentration in the whole fertilized egg internal contents (mixture of yolk, albumen and embryo) did not show significant changes during 5 days of incubation. These results suggest that ghrelin is contained in the internal contents of fertilized eggs, which may affect the functions of embryonic cells during early stage of development in chickens.

Key words: chicken, fertilized egg, ghrelin

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Introduction

The chicken ghrelin, a 26-amino acid peptide, has a unique structure that the active form is acylated with n-octanoic acid at Ser3 residue. It interacts with the growth hormone secretagogue receptor (GHS-R), which consists of two variants of GHS-R1a and GHS-R1aV, to exert multiple biological effects (Kaiya *et al.,* 2007). Ghrelin stimulates growth hormone release in both chickens and mammals (Ahmed and Harvey, 2002; Kaiya *et al.,* 2002). Central ghrelin acts as an inhibitor for food intake in birds, whereas it acts as stimulator in mammals (Shousha *et al.,* 2005; Geelissen *et al.,* 2006; Saito *et al.,* 2005). Ghrelin is synthesized in the proventriculus, gastrointestinal tissues and brain in chicken (Chen *et al.,* 2007). Recently, it has been also reported that mRNAs of ghrelin are expressed in the chicken ovarian follicles (Sirotkin *et al.,* 2006), and that ghrelin may be involved in the local regulation of ovarian cell functions such as proliferation, apoptosis and progesterone secretion (Sirotkin *et al.,* 2007, 2008). We found that the ghrelin mRNA and protein were expressed in the epithelial cells in the cephalic magnum of Japanese quail, and the ghrelin immunoreactivity in the epithelial cells was decreased after egg passing through the magnum (Yoshimura *et al.,* 2005). Although these reports suggest that ghrelin is synthesized in the ovary and oviduct, it remains unknown whether ghrelin is present in the egg internal contents.

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Ghar *et al.* (2004) reported that chicken embryos express GHS-R mRNA, which was low during embryonic day (ED) 0 to ED 4, followed by an increase on ED5, and it remained constant to ED 17. In mammals, active-form of ghrelin was present in fetal and maternal circulation, which may regulate the energy supply and development in the utero (Yokota *et al.,* 2005). Nakahara *et al.* (2006) reported that maternal ghrelin was transferred to fetal circulation and GHS-R was expressed in various fetal tissues such as skin, bone and intestine of rats. They also showed that chronic treatment of mothers with ghrelin resulted in a significant increase in birth weight, and suggested that maternal ghrelin may regulate fetal development during the stage of pregnancy in rats. Harison *et al.* (2007) reported that both ghrelin and GHS-R1a were expressed in ovine placenta, and their expression pattern was in accordance with fetal development. This finding suggests that the ghrelin system may play a role in feto-placental development. Furthermore, ghrelin enhanced blastocyst formation of porcine embryos (Zhang *et al.,* 2007). All of these findings suggest that maternal ghrelin may affect embryonic development in mammals.

If ghrelin that was synthesized in the ovary and oviduct is transmitted to the fertilized eggs, the ghrelin may participate in the regulation of development of embryos in birds as suggested in mammals. The aim of this study was to determine the presence of ghrelin in the yolk and albumen of chicken fertilized eggs.

Materials and Methods

Experimental Animals

White Leghorn hens regularly laying 5 or more eggs in

a sequence were kept in individual cages under a light regimen of 14-h light and 10-h dark, and provided with feed and water *ad libitum*. Ten birds were weekly inseminated with 0.05 mL of undiluted fresh semen to obtain fertilized eggs as described by Das *et al.* (2007). The fertilized eggs were incubated at 37.5℃ in a humidified air condition for 5 days using an incubator (model P-008, Showa Furanki Ltd., Saitama, Japan).

Time-resolved Fluoro-immunoassay (TR-FIA) of Ghrelin in Eggs

The ghrelin concentrations in fertilized eggs before and after incubation for 5 days (from day 0 to day 5) were measured by a competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic rat ghrelin and polystyrene microtiter strips (Nunc-Immuno Modules, Nalge Nunc Int., Japan) coated with anti-rabbit γ-globulin as described by Sugino *et al.* (2002). Yolk and albumen of eggs before incubation were separately homogenized to measure the concentration of ghrelin in them, whereas all the internal contents (yolk, albumen and embryo) were homogenized together using a Polytron homogenizer (Kinematica AG, Switzerland). Five eggs were used for each measurement. Homogenized sample (1 mL) was treated with 1 mL of 1 M acetic acid (pH 2.0), and the protein fractions were precipitated by adding 4 mL acetone. After centrifugation, supernatant was evaporated and resuspended in an assay buffer (50 mM Tris-HCl, 140 mM NaCl, 0.5% BSA, 0.05% γ-globulins, 0.00078% DTPA, 0.05% sodium azide, and 0.01% Tween 40, pH 7.8) containing 10 KIU/mL aprotinin. Ghrelin (3 μg/100 μL 10 mM bicarbonate saline, pH 8.5) was labeled with Eu according to the manufacturer's instructions (PerkinElmer, Inc, USA). Each well was incubated overnight with a rabbit anti-ghrelin serum (([Cys12]-rat ghrelin [1–11]) polyclonal antiserum; Hosoda *et al.,* 2000) diluted at 1: 2,000,000. After washing the ghrelin antibody out, a serial diluted synthetic chicken ghrelin standard (0.01–10 ng/mL) or extracted samples dissolved in an assay buffer (100 μL/well) was added to the wells and incubated overnight. Then, Eu-labeled ghrelin (ca. 50 pg/100 μL) was added to all wells, and incubated for 2 h. After washing six times, 100 μL of the enhancement solution (PerkinElmer, Inc, USA) was added to each well and fluorescence was measured with a time-resolved fluorometer (Multilabel Counter, 1420 ALVO, PerkinElmer, Inc, USA). Ghrelin concentrations of all samples were determined in the same assay. The recovery rate was obtained using homogenized samples (1 mL) exogenously added with or without 10 ng synthetic chicken ghrelin. Extraction and measurement of ghrelin amount was performed in a same manner as described above. The recovery value of added ghrelin was obtained from the difference between the values of samples added with and without ghrelin, and then the recovery rate was calculated against the exogenously added amount of ghrelin. The mean recovery of ghrelin from extracted sample was 88.3%. The intra-assay of coefficients of variation was 4.6%. Least detectable dose and IC_{50} in this assay system were 0.012 and 0.463 ng/mL, respectively.

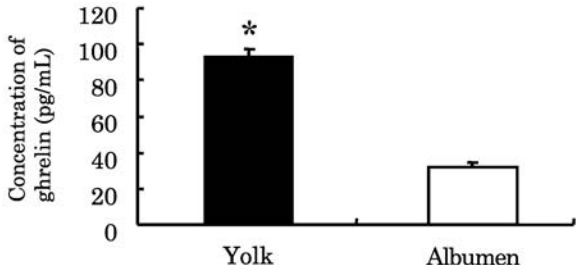

Fig. 1. **Ghrelin concentration in yolk and albumen of non-incubated fresh fertilized eggs.** Values are mean±SE (n =5). * Significantly different between yolk and albumen.

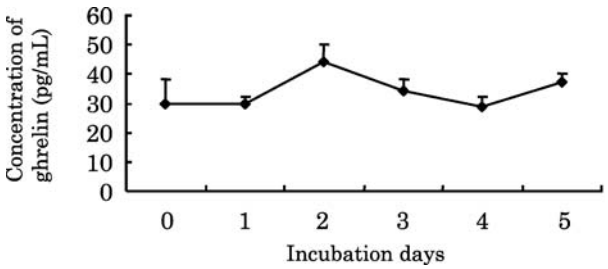

Fig. 2. **Changes in the ghrelin concentration in the internal contents of fertilized eggs (yolk, albumen and embryo) from day 0 (before incubation) to day 5.** Values are mean ±SE (n=5).

Statistical Analysis

Ghrelin concentrations were expressed as mean±SE (n =5). Significance of differences between yolk and albumen was examined by Student's t-test, whereas those among different incubations days were examined using one-way ANOVA followed by Duncan's multiple t-test. Differences were considered significant when P value was less than 0.05.

Results and Discussion

Ghrelin was identified in both yolk and albumen of the fresh fertilized eggs before incubation, and the concentration was significantly greater in the yolk than albumen (Fig. 1). Changes in the ghrelin concentration in the internal contents of whole fertilized egg (mixture of yolk, albumen and embryo) from day 0 to day 5 of incubation are shown in Fig. 2. Ghrelin concentration did not change significantly during 5 days of incubation.

This is the first report to show the presence of ghrelin in both yolk and albumen. Sirotkin *et al.* (2006) reported the expression of genes encoding ghrelin in the ovarian follicular cells in laying hens. The surface epithelium of the infundibulum and cephalic magnum contained immunoreactive ghrelin in Japanese quail (Yoshimura *et al.,* 2005). Thus we assume that ghrelin in the eggs was synthesized in the ovary and oviduct and transmitted to the yolk and albumen.

Gahr *et al.* (2004) reported that GHS-R expression was identified in the chicken embryo, although it was low on day 0 of incubation, and remained at the same level through day 4, and increased about 2.5-fold on day 5. The ghrelin concentration in whole internal contents in fertilized eggs was unchanged for 5 days of incubation (Fig. 2). The ghrelin in the egg may interact with the GHS-R expressed in the embryonic cells. In mammals, maternal ghrelin may regulate the blastocyst formation (Zhang *et al.,* 2007), feto-placental development (Harrison *et al.,* 2007) and fetal development (Nakahara *et al.,* 2006). The development of embryonic cells may be affected by ghrelin also in birds as suggested in mammals.

In conclusion, we have shown the presence of ghrelin in the egg internal contents. The endogenous maternal ghrelin may interact with their receptors of embryonic cells at early stage of development in chickens.

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References

Ahmed S and Harvey S. Ghrelin: a hypothalamic GH-releasing factor in domestic fowl (*Gallus domesticus*). Journal of Endocrinology, 172: 117–125. 2002.

Chen LL, Jiang QY, Zhu XT, Shu G, Bin YF, Wang XQ, Gao P and Zhang YL. Ghrelin ligand-receptor mRNA expression in hypothalamus, proventriculus and liver of chicken (*Gallus gallus domesticus*): studies on ontogeny and feeding condition. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology, 147: 893–902. 2007.

Das SC, Isobe N and Yoshimura Y. Changes in the mRNA expressions of sperm transforming growth factor β-isoforms and their receptors with reference to fertility during in vitro storage of fowl semen. Journal of Poultry Science, 44: 189–197. 2007.

Gahr SA, Kocamis H, Richter JJ and Killefer J. The effects of in ovo rhIGF-I administration on expression of the growth hormone secretagogue receptor (GHS-R) during chicken embryonic development. Growth, Development, and Aging, 68: 3–10. 2004.

Geelissen SM, Swennen Q, Geyten SV, Kühn ER, Kaiya H, Kangawa K, Decuypere E, Buyse J and Darras VM. Peripheral ghrelin reduces food intake and respiratory quotient in chicken. Domestic Animal Endocrinology, 30: 108–116. 2006.

Harrison JL, Adam CL, Brown YA, Wallace JM, Aitken RP, Lea RG and Miller DW. An immunohistochemical study of the localization and developmental expression of ghrelin and its functional receptor in the ovine placenta. Reproductive Biology and Endocrinology, 5: 25. 2007.

Hosoda H, Kojima M, Matsuo H and Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochemical and Biophysical Research Communications, 279: 909–913. 2000.

Kaiya H, Darras VM and Kangawa K. Ghrelin in birds: its structure, distribution and function. Journal of Poultry Science, 44: 1–18. 2007.

Kaiya H, van der Geyten S, Kojima M, Hosoda H, Kitajima Y, Matsumoto M, Geelissen S, Darras VM and Kangawa K. Chicken ghrelin: purification, cDNA cloning, and biological activity. Endocrinology, 143: 3454–3463. 2002.

Nakahara K, Nakagawa M, Baba Y, Sato M, Toshinai K, Date Y, Nakazato M, Kojima M, Miyazato M, Kaiya H, Hosoda H, Kangawa K and Murakami N. Maternal ghrelin plays an important role in rat fetal development during pregnancy. Endocrinology, 147: 1333–1342. 2006.

Saito ES, Kaiya H, Tachibana T, Tomonaga S, Denbow DM, Kangawa K and Furuse M. Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. Regulatory Peptides, 125: 201–208. 2005.

Shousha S, Nakahara K, Kojima M, Miyazato M, Hosoda H, Kangawa K and Murakami N. Different effects of peripheral and central ghrelin on regulation of food intake in the Japanese quail. General and Comparative Endocrinology, 141: 178–183. 2005.

Sirotkin AV, Grossmann R, María-Peon MT, Roa J, Tena-Sempere M and Klein S. Novel expression and functional role of ghrelin in chicken ovary. Molecular and Cellular Endocrinology, 257–258: 15–25. 2006.

Sirotkin AV and Grossmann R. The role of ghrelin and some intracellular mechanisms in controlling the secretory activity of chicken ovarian cells. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology, 147: 239–246. 2007.

Sirotkin AV and Grossmann R. Effects of ghrelin and its analogues on chicken ovarian granulosa cells. Domestic Animal Endocrinology, 34: 125–134. 2008.

Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K and Terashima Y. A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. Biochemical and Biophysical Research Communications, 295: 255–260. 2002.

Yokota I, Kitamura S, Hosoda H, Kotani Y and Kangawa K. Concentration of the n-octanoylated active form of ghrelin in fetal and neonatal circulation. Endocrine Journal, 52: 271–276. 2005.

Yoshimura Y, Nagano K, Subedi K and Kaiya H. Identification of immunoreactive ghrelin and its mRNA in the oviduct of laying Japanese quail, *Coturnix japonica.* Journal of Poultry Science, 42: 291–300. 2005.

Zhang K, Wei H-X, Zhang Y-H, Wang S-H, Li Y, Dai Y-P and Li N. Effects of ghrelin on *in vitro* development of porcine *in vitro* fertilized and parthenogenetic embryos. Journal of Reproduction and Development, 53: 647–653. 2007.