Effect of *in ovo* ghrelin administration on subsequent serum insulin and glucose levels in newly-hatched chicks

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ABSTRACT: Ghrelin is a regulatory peptide in glucose homeostasis in animal species. Its effect in the avian embryo is unclear. The aim of this study was to investigate the effects of *in ovo* ghrelin administration on serum glucose and insulin levels of hatched chicks. A total of 250 fertilized eggs were divided into 5 groups; group T1 as control (without injection), group T2 (*in ovo* injected with 50 ng/egg ghrelin on day 5), group T3 (*in ovo* injected with 100 ng/egg ghrelin on day 5), group T4 (*in ovo* injected with 50 ng/egg ghrelin on day 10) and group T5 (*in ovo* injected with 100 ng/egg ghrelin on day 10). After hatching, serum insulin and glucose concentrations were determined. Group T4 and T5 showed significantly higher serum insulin levels (0.43 and 0.60 ng/ml, respectively) compared with T1, T2 and T3 (0.09, 0.10, and 0.23 ng/ml, respectively) in hatched chicks. Glucose concentrations have not been affected by *in ovo* administered ghrelin in all injected groups. It seems that embryonic β-cells were stimulated to secrete a considerable level of insulin in response to *in ovo* ghrelin in the late embryonic life. The observed stability of glucose rate suggests the incidence of insulin resistance at hatching time or in newly hatched chicks from *in ovo* ghrelin administered eggs on day 10.

Keywords: chicken; embryo; ghrelin; *in ovo* injection; insulin

Since the identification of ghrelin in mammals by Kojima et al. (1999), so many relative studies have been conducted on mammalian species until now. Mammalian ghrelin has a regulatory role in glucose homeostasis via the modulation of insulin secretion (Adeghate and Ponery, 2002; Ahima, 2006; Castańeda et al., 2010). The administration of ghrelin increases blood glucose (Dezaki et al., 2004) and decreases plasma insulin levels in humans and rodents (Broglio et al., 2001; Dezaki et al., 2004). In recent years some different results have been published. For example, Granata (2008) reported both acylated and non-acylated ghrelin stimulated glucose-induced insulin secretion by β-cells. Also, Lee et al. (2002) observed that the intravenous administration of ghrelin stimulated insulin secretion in free-feeding rats. *In vitro* studies have also shown that ghrelin increases insulin secretion from isolated pancreatic islets (Adeghate and Ponery, 2002; Date et al., 2002). The intraperitoneal injection of insulin lowered blood glucose levels in the ghrelin-administered and control mice (Dezaki et al., 2004), suggesting that the hyperglycaemic effect of ghrelin was due neither to the ability of ghrelin to release GH

nor to the induction of insulin resistance, but it was primarily caused by a reduction of plasma insulin levels. Close to birth, ghrelin-producing cells begin to localize at the periphery of developing embryonic islets and remain visible in marginal areas of the islets in the pancreases of neonates (Hayashida et al., 2002). Genetic studies indicated that ghrelin might regulate the development of β-cells in the foetal endocrine pancreas (Prado et al., 2004; Xu et al., 2008). In Chanoine and Wong (2004), GHS-R1a (ghrelin receptor) mRNA was present in all foetal pancreas samples and the embryonic pancreas was considered as a major ghrelin source. All these findings were detected in mammals, and they indicated possible roles of embryonic ghrelin in insulin-glucose balance. It seems that no information about the glycaemic action of ghrelin in avian species is available.

In avian species, Kaiya et al. (2002) isolated ghrelin peptide from the proventriculus of chicken for the first time. The peptide structure of chicken ghrelin has 26 amino acids with 54% of similarities to rat ghrelin. Currently, ghrelin has been identified in albumen and yolk of fertile chicken egg (Yoshimura et al., 2009). Also, ghrelin mRNA and ghrelin expression have been identified in chicken pancreatic cells (Richards et al., 2006). Chicken ghrelin has endocrine roles in the secretion of GH and corticosterone (Kaiya et al., 2002). The aim of the present study was to investigate the effect of *in ovo* administration of exogenous ghrelin on insulin and glucose levels in newly-hatched chicks.

MATERIAL AND METHODS

In ovo injection procedure

In this experiment 250 fertilized eggs were collected from a commercial breeder flock (Ross 308). The eggs were divided into five experimental groups; control T1 (without injection), group T2 (*in ovo* injected with 50 ng/egg ghrelin on day 5), group T3 (*in ovo* injected with 100 ng/egg ghrelin on day 5), group T4 (*in ovo* injected with 50 ng/egg ghrelin on day 10) and group T5 (*in ovo* injected with 100 ng per egg ghrelin on day 10). All groups were incubated in normal incubation conditions (37.8°C and 60% RH). Rat ghrelin was purchased from Sigma-Aldrich® (St. Luis, USA), dissolved in 1% acetic acid solvent and proposed concentrations of ghrelin were prepared. On day 5 of incubation, *in ovo* injection was conducted (T2 and T3). On day 10, the same *in ovo* injection procedures were done for group T4 and T5. The *in ovo* injection was done in a sanitary room at a temperature of 37°C. Before injection, egg shells were marked with a marker for the identification of air cell position and optimum injection point. In this experiment, 22G needles were used for *in albumen* injection. After hatching, blood samples were collected from chicks, immediately following the chick decapitation. Blood samples were centrifuged and serum was obtained for determination of the insulin level by an electrochemiluminescence immunoassay method on a Modular Analytics E170 analyser (Mathew et al., 2005) and glucose concentration with an Alcyon 300 autoanalyser (Abbott laboratories, Abbott Park, USA) and its commercial kits.

Statistical analyses

Data on 15 individual samples from 15 hatched chicks from each group (in total 75 samples) were analysed by SAS software (Ver. 9.1) and the differences between groups were evaluated by Duncan's Multiple Range Test, $P < 0.05$.

RESULTS AND DISCUSSION

Serum insulin and glucose levels in five experimental groups are presented in Table 1. The serum insulin level was high in group T4 and T5 in comparison with T1, T2 or T3. The serum glucose level did not show a significant difference between experimental groups.

Ghrelin mRNA expression was observed mainly in the proventriculus of hatching chickens. Also, a higher number of ghrelin-ex cells (ghrelin-producing cells) were identified in the gastrointestinal tract of hatching chickens, especially in the proventriculus (Wada et al., 2003), which indicated pre-hatching ghrelin production in chickens. Yamato et al. (2005) reported ghrelin gene expression and production in the neonatal chick proventriculus and ghrelin acylation on day 2.5 after hatching.

In chickens, insulin begins to be secreted from β-cells on day 4 of embryonic life, and the secretion rate could considerably increase from day 12 to hatching day (Bellairs and Osmond, 2005). Insulin content of the embryonic pancreas rises to peak during days 14–20 of incubation with subsequent falling at hatching time, but the blood glucose level of chickens is constant in pre-hatching, hatching

Table 1. Insulin and glucose concentrations in newly-hatched chicks after *in ovo* injection of exogenous ghrelin

Experimental groups	Injected dosage (ng)	Injection day (incubation day)	Serum insulin concentration (ng/ml)	Serum glucose concentration (mg/dl)
T1	–	–	0.09 ± 0.01[a]	294.5 ± 6[a]
T2	50	5	0.10 ± 0.01[a]	250.3 ± 9[a]
T3	100	5	0.23 ± 0.03[ab]	296.0 ± 10[a]
T4	50	10	0.66 ± 0.01[c]	211.0 ± 1[a]
T5	100	10	0.60 ± 0.03[c]	244.0 ± 9[a]

[abc]different letters show significant difference; n = 15

or post-hatching period (Langslow, 1975). Glucose was found to readily enter the cells of the heart of 5–6 day chicken embryo, in which the uptake of 2-deoxyglucose was stimulated by physiological levels of insulin as early as 5–6 days (Kutchai et al., 1977). It has been shown that insulin has a considerable role in chicken early-embryonic morphogenesis with expression changes in the involved genes (Patwardhan et al., 2004).

In Lamosová et al. (2003), after the *in ovo* administration of exogenous rat leptin (0.1 or 1 μg/ egg) to quail eggs on day 5 and 10, the concentration of glucose in the plasma did not show any significant differences and the level of glucose was in the same range during the whole experimental period. In the present study, similar results were observed for the glucose level following *in ovo* administration of ghrelin on day10. It seems that ghrelin, similarly like leptin, has a considerable role in glucose homeostasis in neonatal chickens and this effect in mammals was reviewed by Castańeda et al. (2010). Observed insulin rose in chicks hatched from T4 or T5 (0.66 and 0.60 ng/ml) suggesting that GHS-R1a was expressed in the chicken embryonic pancreas. This finding was also in accordance with Richards et al. (2006), who reported autocrine/paracrine effects of ghrelin in the chicken pancreas. But because of the low activity of β-cells on day 4–12 (Bellairs and Osmond, 2005), ghrelin administration in T2 or T3 (on day 5) could not affect insulin secretion as injection on day 5.

Ghrelin administration on embryonic day 5 or day 10 in different dosages did not cause any change in the glucose concentration of hatched chicks. These findings, together with serum insulin and glucose levels, indicate that the chicken embryo at late embryonic life, near to hatching, can maintain the blood glucose concentration, even in the hyperinsulinaemic condition induced by ghrelin administration. It suggests the incidence of insulin resistance at hatching time or in newly hatched chicks from *in ovo* ghrelin administered to eggs, for stability of the blood glucose level. This effect is similar to that of ghrelin in the occurrence of insulin resistance in humans (Vestergaard et al., 2008).

In summary, *in ovo* ghrelin administration on day 10 of embryonic life could increase the peripheral insulin level in newly hatched chicks, but ghrelin administration on day 5 had a minor effect on the insulin level while blood glucose levels did not change by ghrelin administration on day 5 or day 10 of incubation. Findings of the occurrence of insulin resistance in chicks are similar to human studies. Further studies on chicken embryonic GHS-R1a, its mRNA expression, chicken pancreatic ghrelin and its autocrine/paracrine effects are necessary for further elucidation of the chicken ghrelin puzzle.

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