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RESEARCH ARTICLE

Effect of *in ovo* Ghrelin Administration on Thyroid Hormones and Some of Serum Biochemical Parameters in Newly-hatched Chicks

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Summary

The aim of this study was to investigate the effect of *in ovo* ghrelin administration on serum thyroid hormones levels and serum total cholesterol, triglyceride, calcium (Ca), phosphate (P) concentrations and alkaline phosphatase (ALP) activity in newly-hatched chicks. Fertilized eggs were divided into 5 groups; group G1 as control (intact or without injection), group G2 (50 ng ghrelin/egg at day 5), group G3 (100 ng ghrelin/egg at day 5), group G4 (50 ng ghrelin/egg at day 10), and group G5 (100 ng ghrelin/egg at day 10). Data obtained from serum analysis showed that 100ng/egg ghrelin administration caused T_4 elevation, whereas there were no any significant changes when ghrelin was administrated in 50ng/ egg dosage. There was no any significant difference for T_3 level between experimental groups. Serum triglyceride concentration was lower in all injected groups than control group, but only the differences between control group and G2 and G4 groups were statistically significant ($P<0.01$). Serum Ca concentration was higher in G2 and G3 compared with control group. No significant differences were observed in total cholesterol, P concentrations and ALP activity among experimental groups. The present results indicate that appropriate timing of *in ovo* injection of ghrelin has considerable effects on serum Ca levels of newly-hatched chicks. Also, higher dosage of *in ovo* administrated ghrelin can elevate serum T_4 level.

Keywords: *Chicken, Ghrelin, in ovo administration, Thyroid hormones, Serum biochemical measures*

Yumurtadan Yeni Çıkmış Civcivlerin Tiroid Hormonları ile Bazı Serum Biyokimyasal Değerleri Üzerine *in ovo* Ghrelin Enjeksiyonunun Etkisi

Özet

Bu çalışmanın amacı; yumurtadan yeni çıkmış civcivlerde *in ovo* ghrelin uygulamasının serum tiroid hormonları seviyeleri ile serum total kolesterol, trigliserid, kalsiyum, fosfat konsantrasyonları ve alkalin fosfataz (ALP) aktivitesindeki etkilerinin araştırılmasıdır. Döllenmiş yumurtalar G1-G5 olmak üzere 5 gruba ayrıldılar; G1: kontrol (enjeksiyon uygulanmayan), G2: 5. günde 50 ng ghrelin/ yumurta enjeksiyonu, G3: 5. günde 100 ng ghrelin/yumurta enjeksiyonu, G4: 10. günde 50 ng ghrelin/yumurta enjeksiyonu, G5: 10. günde 100 ng ghrelin/yumurta enjeksiyonu. Serum analiz sonuçları 100 ng ghrelin/yumurta uygulamasının T_4 seviyesinde artışa neden olurken 50 ng ghrelin/yumurta uygulamasının herhangi bir değişikliğe neden olmadığını gösterdi. Deneme grupları arasında T_3 seviyesi bakımından herhangi bir değişikliğe rastlanmadı. Serum trigliserid konsantrasyonu kontrol grubu ile kıyaslandığında enjeksiyon uygulanan tüm gruplarda daha düşük olarak tespit edilmesine rağmen sadece G2 ve G4 grupları istatistiksel olarak fark göstermekteydi ($P<0.01$). Kontrol ile kıyaslandığında, G2 ve G3 gruplarında serum Ca konsantrasyonu daha yüksek olarak belirlendi. Total kolesterol, fosfat konsantrasyonu ve ALP aktivitesi yönünden deney grupları arasında bir fark gözlemlenmedi. Bu sonuçlar uygun zamanda *in ovo* ghrelin uygulamasının yumurtadan yeni çıkmış civcivlerde serum Ca miktarında önemli bir etkisinin olduğunu göstermektedir. Ayrıca, yüksek doz *in ovo* ghrelin uygulaması serum T_4 seviyesini artırabilir.

Anahtar sözcükler: *Tavuk, Ghrelin, in ovo uygulama, Tiroid hormonları, Serum biyokimyasal değerler*

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INTRODUCTION

Ghrelin is a multifunctional regulatory peptide that was discovered in the rat stomach by Kojima *et al*.[1]. Researches on avian ghrelin are less than those of mammalian ghrelin, but the findings have demonstrated a similarity on GH-releasing activity, and a difference in appetite regulation between mammals and birds, especially in chicken [2]. Chicken ghrelin includes 26 amino acids and is shorter than human or rat ghrelin with 28 amino acids [3]. *In vivo* and *in vitro* investigations showed that GH-releasing action of ghrelin has a dose-depended manner and is similar to growth hormone releasing hormone (GHRH) with a stimulatory effect on somatotrophs in chicken [4]. Regarding metabolic aspects of chicken ghrelin, anti-lipogenic effect of administrated ghrelin in neonatal broiler chicks has been reported by Buyse *et al*.[5].

Human ghrelin is expressed in fetal thyroid, but not in adult [6]. There is no available evidence for ghrelin expression in chicken thyroid. Onset of chicken thyroid activity is on second week of embryonic development. The first appearance of colloid droplets is on d 7 but it is not until about days 10-13 that the adenohypophysis thyroid interactions become established, while the growth of the embryo, as measured by body weight, skeletal size, muscle growth and growth of cartilages and bones, is greatly influenced by thyroid hormones in late embryonic development [7-9].

Khazali [10] with ventricle infusion of ghrelin in goats reported that ghrelin may increase the mean plasma concentration of T_3 and T_4. In human, the effect of ghrelin on hypothalamus-pituitary-thyroid axis and circulated T_4 was documented. In this regard, Kluge et al.[11] had stated, early fT4 increases following ghrelin infusion was possibly induced by direct action of ghrelin on the thyroid gland where ghrelin receptors have been identified. In other hand, a study conducted via intracerebroventricular administration (another injection method) of ghrelin had different result with reducing the plasma TSH and T_4 [12]. Regarding metabolic aspects of chicken ghrelin, anti-lipogenic effect of administrated ghrelin in neonatal broiler chicks has been reported by Buyse *et al*.[5].

In ovo ghrelin has been identified in albumen and yolk of fertile chicken egg [13] and its gene expression was observed during embryonic life, especially after 5-day of incubation [14]. Also, ghrelin mRNA and expression have been identified in follicles [15], pancreatic cells of chicken [16] and oviduct of quail [17].

Regardless to ghrelin's effects on plasma concentration of GH [3], Insulin [18] and prolactin [19], many effects of chicken ghrelin on serum biochemical measures such as plasma lipids, calcium (Ca) and phosphorus (P) concentrations are unclear. The aim of present study was investigate the effects of *in ovo* ghrelin administration on thyroid hormones level and some of serum indices include total cholesterol, triglyceride, Ca and P concentrations and alkaline phosphatase (ALP) activity in newly-hatched chicks.

MATERIAL and METHODS

In this study, 250 fertilized eggs were collected from commercial broiler breeder flock (Ross 308). The eggs were divided into five groups; group G1 (control or intact), group G2 (*in ovo* injected with 50 ng ghrelin/egg at day 5), group G3 (*in ovo* injected with 100 ng ghrelin/egg at day 5), group G4 (*in ovo* injected with 50 ng ghrelin/egg at day 10) and group G5 (*in ovo* injected with 100 ng ghrelin/egg at day 10). Eggs were incubated with normal hatchery (37.8°C and 60%: RH). The lyophilized Ghrelin was obtained from Sigma-Aldrich® (Rat Ghrelin - USA), dissolved in 1% acetic acid solvent and proposed concentrations of ghrelin were prepared. At day 5 and day 10 of incubation, *in ovo* injections were conducted in hygiene room at 37°C. Before injection, egg shells were marked with marker for identification of air cell position and detection of optimum injection point. At this experiment, 22G needles were used for safe *in albumin* injection. After hatching, the blood samples were collected from 75 hatched chicks (15 individual samples from 15 hatched-chicks for each group), immediately following chick decapitation. Blood samples were centrifuged and serum was obtained for determination of T_3 and T_4 level with Electrochemiluminescence immunoassay method on a Modular Analytics E170 analyzer [20], and serum biochemical analysis with Alcyon 300 auto analyzer (Abbott Park, IL., USA) and its commercial kits.

Data obtained by 15 individual samples from 15 hatched-chicks for each group (in total 75 samples) were analyzed (one-way analysis of variance) with SAS software (Ver. 9.1) by and the differences between groups were evaluated with Duncan multiple range test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Table 1 shows T_3 and T_4 concentrations in newly-hatched chicks following *in ovo* injection of exogenous ghrelin. In this study, ghrelin administration in 100 ng/egg caused T_4 elevation in G3 and G5 compared with control group, whereas there was no significant change when ghrelin was administered at 50 ng/egg dosage (G2 and G4). There was no any significant change for T_3 level between experimental groups. *Table 2* shows serum total cholesterol, triglyceride, Ca, P and ALP level in experimental groups following *in ovo* injection of exogenous ghrelin. Exogenous ghrelin administration (50 and 100 ng/egg) at embryonic day 5 or 10 has no significant effect on total cholesterol concentration. however, triglyceride level declined in G2 and G4 (57.3 and 70.6 mg/dl, respectively) compared with

Table1. *Serum T_3 and T_4 concentrations in newly-hatched chicks following in ovo injection of exogenous ghrelin*

Tablo 1. *in ovo ghrelin uygulanan yumurtadan yeni çıkmış civcivlerde serum T_3 ve Te_4 konsantrasyonları*

Experimental Groups	Injected Dosage (ng/egg)	Injection Day (Incubation Day)	T_3	T_4
G1	0	-	10.10	4.08[b]
G2	50	5	9.83	4.20[b]
G3	100	5	9.96	4.96[a]
G4	50	10	9.80	4.2[b]
G5	100	10	10.6	4.47[ab]
P value			0.0572	0.0303
SEM[1]			0.6899	0.5773

** Different letters (a, b or c) show significant difference, [1] SEM, based on pooled estimate of variance*

level of T3 in experimental groups *(Table 2)* may be indicator of high thyroid activity for releasing sufficient level of T_3 and T_4 for possible successful hatching process. Possible mechanism for increased plasma T_4 level following 100 ng ghrelin administration may be due to TRH-releasing effect of ghrelin as currently identified by Pekary and Sattin [24].

In present study, serum total cholesterol was not affected significantly by *in ovo* ghrelin administration although there were slight decline in G2, G3 and G4 groups *(Table 2)*. Triglyceride concentration was lower in G2, G3, G4 and G5 groups compared with control or solution injected groups *(Table 2)*. It has been reported that circulating human ghrelin can decrease blood lipids and it is a key factor for prevention of hyperlipidemia after dietary fat intake [25]. The high cholesterol or arthrosclerosis cause higher concentration of circulating ghrelin in comparison

Table 2. *Serum total cholesterol, triglyceride, Ca, P and ALP levels in in newly-hatched chicks following in ovo injection of exogenous ghrelin*

Tablo 2. *in ovo ghrelin uygulanan yumurtadan yeni çıkmış civcivlerde serum total kolesterol, trigiliserid, Ca, P ve ALP değerleri*

Experimental Groups	Injected Dosage (ng/egg)	Injection Day (Incubation Day)	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	Ca (mg/dl)	P (mg/dl)	ALP (U/l)
G1	0	-	577.7	[a] 120.0	10.1 [bc]	6.3	2166.7
G2	50	5	417.7	[b] 57.3	11.4 [ab]	5.7	2565.0
G3	100	5	514.1	[ab] 87.6	12.3 [a]	5.8	2328.7
G4	50	10	506.3	[b] 70.6	10.0 [bc]	4.9	2640.0
G5	100	10	618.0	[ab] 81.3	8.6 [c]	6.1	2903.3
P value			0.3597	0.0079	0.0029	0.0797	0.8732
SEM[1]			80.473	12.488	0.521	12.488	500.556

** Different letters (a, b or c) show significant difference, [1] SEM, based on pooled estimate of variance*

G1 (120 mg/dl). There were no significant differences in P concentrations and ALP activity rates among experimental groups. Ca concentration was significantly higher in G3 (100 ng ghrelin/egg injection at day 5: 12.3 mg/dl), and was slightly lower in G5 (100 ng ghrelin injection at day 1 0: 8.6 mg/dl) compared with G1 (10.1 mg/dl).

DISCUSSION

The reports about ghrelin effects on thyroid activity are different, due to experimental animal, dosages and duration. However, the effect was not investigated in chicken.

Park *et al.*[21] observed that Ghrelin enhances the proliferating effect of thyroid stimulating hormone in thyroid cells. Gjedde *et al.*[22], and Caminos *et al.*[23] reported that circulating ghrelin levels were increased in hypo-thyroid condition. Their finding that shows negative correlation of plasma ghrelin and T_4 levels are in contrast with findings of present study *(Table 1)*. In present study, ghrelin administration in high concentration (100 ng) caused subsequent elevation in T_4 level *(Table 1)* that this result is in according to Khazali [10] findings. In other hand, constant with healthy condition [26]. Buyse *et al.*[5] have reported that fatty acid synthesis decreased and there is anti-lipo-genic effect of ghrelin following ghrelin injection to chickens. Results of the present study are similar to the findings of Buyse *et al.*[5], and partially are in agreement with the report by Egecioglu *et al*.[25] on human. It is suggested that the ghrelin has a regulatory effect for lipid metabolism even in embryonic life of chickens.

The effect of ghrelin on serum Ca, P and ALP activity has not been studied in early embryonic development. In the present study *in ovo* administrated ghrelin had no significant effect on ALP activity *(Table 2)*. The role of ghrelin in ALP activity limited with some information obtained in mammalian species. Fukushima *et al*.[27] stated that ghrelin causes osteogenesis by stimulation of ALP. Maccarinelli *et al.*[28] observed high ALP activity in mice with injection of ghrelin. With attention to lack of findings in relation to the ghrelin acts in ALP activity in birds, the present data show that *in ovo* exogenous ghrelin administration had no effect on serum ALP activity in any of ghrelin-administrated groups. Our findings are different from the report of Maccarinelli *et al.*[28] on ghrelin function to ALP activity in mammalian model. G3 group (100 ng/egg ghrelin at day 5) had significantly higher Ca rate

than control group (G1) (P<0.01). However, there was no significant difference between control group and G2, G4 and G5 groups in terms of serum Ca concentrations when compared with control (T1) *(Table 2)*. Pérez-Castrillón *et al*.[29] declared that ghrelin has not any important role in Ca metabolism and serum Ca has not significant correlation with circulation ghrelin level in human. Increasing serum Ca in group G3 is opposite to ghrelin osteogenic effect and osteogenic-related Ca decreases [27,30]. *In ovo* administrated 100 ng/egg ghrelin at d 5 might stimulate uptake of *in ovo* Ca from extra-embryonic sources (yolk and eggshell), and it may divert to embryonic circulation [31] for cartilage and bone formation may occurred mainly eight day after incubation, such as tibia formation [32]. Similarly, serum P concentration had not any significant differences in any of experimental groups.

In ovo administration of 100 ng ghrelin/egg has considerable effect on serum T_4 and triglyceride levels, but minor effect on serum Ca levels, whereas it didn't affect serum total cholesterol, P and ALP of newly-hatched chickens. Further studies with different methods of *in ovo* injection and *in yolk* injection, also administration at different incubation days (pre-incubation or at third week of incubation) in further experiments can be useful to clarify an effect of maternal or *in ovo* ghrelin in avian species.

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