Kafkas Univ Vet Fak Derg
17 (6): 949-952, 2011
DOI:10.9775/kvfd.2011.4773

RESEARCH ARTICLE

Changes in the Differential Leukocyte Count in Newly Hatched Chicks Following *In Ovo* Ghrelin Administration

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Makale Kodu (Article Code): KVFD-2011-4773

Summary

The aim of this study was to investigation on effects of *in ovo* administration of ghrelin on leukocytes count and heterophil/lymphocyte (H/L) ratio in hatched chicks. 250 fertile eggs were divided to 5 groups; control or group 1 (without injection), group 2 (*in ovo* injected with 50 ng ghrelin at day 5), group 3 (*in ovo* injected with 100 ng ghrelin at day 5), group 4 (*in ovo* injected with 50 ng ghrelin at day 10) and group 5 (*in ovo* injected with 100 ng ghrelin at day 10). After hatching and blood sampling, lymphocyte, heterophil, monocyte, eosinophil and basophil count and H/L ratio were determined. The differential count of leukocytes was done observing 100 white blood cells, after staining the glass slides containing the spread blood. It was observed that lymphocyte count was more in control group (62%) in comparison with injected groups 2, 3, 4 or 5: 60, 56, 58 and 53.6% respectively). *In ovo* ghrelin injection at day-10 caused increases heterophil, monocyte, and eosinophil and basophil number in comparison with control group, that higher dosage (100 ng) caused significant elevation. It was concluded that in ovo ghrelin administration during incubation especially at day-10 and in 100 ng dosage can changes differential leukocyte count with increase heterophil, basophil and eosinophil counts and H/L ratio and decreases lymphocytes.

Keywords: *Differential leukocyte count, Ghrelin, Hematopoiesis, Incubation, In ovo injection*

In Ovo Ghrelin Enjeksiyonunun Yeni Çıkan Civcivlerin Lökosit Sayıları Üzerine Etkisi

Özet

Bu çalışmanın amacı *in ovo* ghrelin enjeksiyonunun yeni çıkan civcivlerin lokosit sayısı ve heterofil/lenfosit (H/L) oranı üzerine etkilerinin incelemesidir. Toplam 250 embriyolu yumurta 5 guruba ayrıldı; kontrol veya grup 1 (enjeksiyon uygulanmayan), grup 2 (5. günde *in ovo* 50 ng ghrelin enjekte edilen), grup 3 (5. günde *in ovo* 100 ng ghrelin enjekte edilen), grup 4 (10. günde *in ovo* 50 ng ghrelin enjekte edilen) ve grup 5 (10. günde *in ovo* 100 ng ghrelin enjekte edilen). Kuluçka çıkım sonrası kan örneklerinde lenfosit, heterofil, monosit, eozinofil ve bazofil sayıları ile H/L oranı belirlendi. Lökosit tipleri sayıları (lökosit formülü) kan frotisinin boyanmasından sonra 100 beyaz kan hücresi gözlemlenerek gerçekleştirildi. Lökosit sayısı kontrol grubunda (%62) enjeksiyon uygulanan grup 2, 3, 4 ve 5 ile (sırası %60, 56, 58 ve 53.6 oranlarında) kıyaslandığında yüksek bulundu. Onuncu günde yüksek dozda (100 ng) *in ovo* ghrelin enjeksiyonu, kontrol grupla kıyaslandığında heterofil, monosit, eozinofil ve bazofil sayılarının artmasına neden oldu. Sonuç olarak, inkubasyon sırasıda özellikle 10. günde ve 100 ng dozda *in ovo* ghrelin uygulaması, heterofil, bazofil ve eozinofil sayılarını ve H/L oranını arttırarak ve lenfosit sayısını azaltarak lökosit formülünü değiştirmektedir.

Anahtar sözcükler: *Lökosit tipleri sayıları (lökosit formülü), Ghrelin, Hematopoezis, Inkübasyon, In ovo enjeksiyon*

INTRODUCTION

From identification of ghrelin [1] to now, so many bio-functions were suggested for this peptide, such as GH-releasing [2], osteogenesis [3,4], food intake and energy balance [5,6], endocrine/paracrine roles in pancreas [7-8], and

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hematopoiesis [9,10]. Peptide structure of chicken ghrelin has 26 amino acids with 54% similarity to rat ghrelin [11]. Ghrelin has been identified in albumen and yolk of fertilized chicken egg by Yoshimura *et al*.[12] that it creates backgrounds for new studies on developmental or embryonic roles of chicken ghrelin. Many aspects of avian ghrelin are not clear and researches are focusing on similarity and differences of ghrelin functions between avian and mammals [11]. In this subject, GH-releasing effect after intracerebroventricular (ICV) - injection of human ghrelin [13], prolactin-releasing [14] and hematopoietic effects [15] follow *in ovo* administration of rat ghrelin have been documented.

Leukocytes differentiation and activity is affected by endocrine hormones, for example glucocorticoids have an important role in mammalian T cell selection and have similar effect on avian B cell [16]. Immune-regulation effects of testosterone, estradiol and other androgen on avian leukocyte counts and their activities have been reported [17]. It is proposed that ghrelin as a new member of endocrine system with hematopoietic effects may has considerable effects on leukocytes count. Taati *et al*.[10] showed that *icv*- injection of ghrelin to laboratory rat can has hematopoietic effect with increasing hematocrit, Hb, Red blood cell number and also neutrophil and basophil counts. In our past report [15] *in ovo* ghrelin administration at day-10 of incubation could increase Red blood cells, hematocrit percentage and hemoglobin concentration in hatched chicks. With attention to developmental function of ghrelin [18], its hematopoietic effects in mammals [10] and chicken [15] and finally based on identification of maternal or *in ovo* ghrelin [12], aim of this study was to investigation on effects of *in ovo* administration of ghrelin on leukocytes (lymphocyte, heterophil, monocyte, eosinophil and basophil) counts and H/L ratio in hatched chicks.

MATERIAL and METHODS

Experimental Groups

In this experiment, 250 eggs were obtained from commercial broiler breeder (Ross 308 strain) farm. the eggs were divided into five experimental groups; group1 or eggs without any injection (control), group 2 or eggs *in ovo* injected with 50 ng ghrelin at embryonic day-5, group 3 or eggs *in ovo* injected with 100 ng ghrelin at embryonic day-5, group 4 or eggs *in ovo* injected with 50ng ghrelin at embryonic day-10, and group 5 or eggs *in ovo* injected with 100 ng ghrelin at embryonic day-10 *(Table1)*.

In Ovo Injection Procedure

Lyophilized exogenous Rat ghrelin (Sigma-Aldrich®, USA) was solved in to 1% acetic acid solution (according to Sigma brochure) and proposed concentrations of ghrelin were prepared, qua each *in ovo* injected 0.5 ml solution was include 50 ng (group 2 or 4) or 100 ng (group 3 or 5) ghrelin. Next, solutions were transferred to incubator and warmed to 37°C for avoiding to any thermal stress for embryos. For *in ovo* injection, all of eggs were candled for identification of embryo, air cell, albumen position and optimum injection point that is remarked on egg shell surface. 22 g needles were used for *in albumen* injection. After Injection, pores were covered with special plastic agglutinate according to Iranian Razi Institute© recommendations. Similar *in ovo* experiments were done for all of injected groups on day-5 or -10 *(Table1)*.

At end of incubation, blood samples from each group were collected following chick decapitation. The whole blood samples were diluted (1:100). After this procedure, the leukocytes counting (n/µL) was done using Neubauer chamber. The differential count of leukocytes was determined via microscopic observing 100 white blood cells, after staining with May Grunwald-Giemsa. Differential counts were counted on each smear and identified according to Campbel [20].

Statistical Analysis

Data obtained by 15 individual samples from 15 hatched-chicks for each group were analyzed with SAS software (Ver.9.1) and the differences between groups were evaluated with Duncan multiple range test, $P<0.05$.

Table1. *In ovo injected point, dosages and volumes for experimental groups*
Tablo1. *Deney gruplarında in ovo enjeksiyon noktası, dozu ve miktarı*

Experimental Groups	Injected Dosage of Ghrelin (ng)	Injected Volume of Solvent (1% acetic acid)	Injection Day (Incubation Day)	Injection Site
T1	0	0	-	-
T2	50	0.5 ml	5	albumen *picked end of egg*
T3	100	0.5 ml	5	albumen *picked end of egg*
T4	50	0.5 ml	10	albumen *wide end of egg*
T5	100	0.5 ml	10	albumen *wide end of egg*

Blood samples and leukocytes counting

RESULTS

According to *Table 2, in ovo* ghrelin administration caused significant differences for all subunit of leukocytes in hatching chicks. Lymphocyte count was higher in control group (62%) in comparison with injected groups (2, 3, 4 or 5). Ghrelin administrated groups had significantly more monocyte. *In ovo* ghrelin injection at day-10 caused increases heterophil, monocyte, and eosinophil and basophil number in comparison with control group, that higher dosage (100 ng) caused significant elevation for mentioned measures when it compared with control group *(Table2)*. Administration of 50 ng ghrelin at day-5 (group 2) caused significantly lowers eosinophil and basophil rates, but ghrelin in higher dose (group 3) had opposite effect on eosinophil number (elevation) *(Table2)*. H/L (heterphile/lymphocyte) was in maximum number in group 5 or 100 ng/day-10 (0.66 in comparison with 0.47, 0.60, 0.64 and 0.56 for group1, 2, 3 and T4). In other word all of *in ovo* injected groups had higher H/L ratio when compared with control (group 1) *(Table2)*.

Narin and Çetin [21], reported that lymphocytes were increased in ghrelin injected rats but monocytes, eosinophil, and basophils didn't have any considerable change after ghrelin injection. They had suggested that rodent ghrelin had lymphopoietic effect, but in other side Taati *et al*.[10] showed that leukocytes counts in rats at 5 or 15 days after ghrelin injection did not has significant change and lymphocytes were decreased (minor) at 5- or 15- days post-ghrelin injection, that was different to Narin and Çetin [21] findings. Results of present study for lymphocyte (increases in *in ovo* ghrlein administrated groups) *(Table 2)* is according to Taati *et al*.[10] and apposite to Narin and Çetin [21] reports in mammalian model. Monocytes had increases in group 2, 3, 4 or 5 that *in ovo* ghrelin in both 5- or 10-day of incubation caused monocyte increases. *In ovo* administration of 100 ng ghrelin at day-5 or -10 caused significant eosinophil elevations, but 50 ng could not have considerable effect. Basophil count was more in group 5 (100 ng/day-10) in comparison with other groups. Our observations for monocyte, eosinophil and basophil were in agreement to Taati *et al*.[10] observations in rat. An earlier

Table 2. *Lymphocyte, heterophil, monocyte, eosinophil, basophil rates and H/L ratio in hatched chicks in experimental groups*
Tablo 2. *Deney gruplarında yeni çıkan civcivlerin heterofil, lenfosit, monosit, eozinofil, bazofil ve H/L oranı*

Experimental Groups	Heterophil	Lymphocyte	Monocyte	Eosinophil	Basophil	H/L
T1	29.3 ^{b}	62 ^{a}	1.3 ^{e}	2.7 ^{bc}	1.0 ^{b}	0.47 ^{c}
T2	36.0 ^{a}	60 ^{b}	2.0 ^{d}	2.0 ^{c}	0.0 ^{c}	0.60 ^{ab}
T3	36.3 ^{a}	56 ^{d}	3.0 ^{c}	4.0 ^{a}	1.0 ^{b}	0.64 ^{a}
T4	33.0 ^{ab}	58 ^{c}	5.0 ^{a}	3.0 ^{b}	1.0 ^{b}	0.56 ^{b}
T5	36.0 ^{a}	53.6 ^{e}	4.0 ^{b}	4.0 ^{a}	2.0 ^{a}	0.66 ^{a}
P value	0.0094	<.0001	<.0001	0.0029	0.0046	0.0005
SEM	0.837	0.538	0.149	0.298	0.258	0.020

Different letters (a, b, c, d or e) show significant difference between groups

DISCUSSION

Avian leukocytes are present in the blood only transiently (generally 12-20 h). After this relatively short period they leave the circulation and migrate into the tissues, where they perform their specialist functions. Monocytes constitute approximately 5-10% of peripheral blood leukocytes but this number may vary significantly between different chicken lines. In over all kinds of leukocytes had functional and special acts, for example lymphocytes are essential for generating immune responses and retaining memory of previous exposure to an antigen [17]. Avian heterophils act in acute inflammatory response with its highly phagocytic specify and accumulate in inflamed tissue [20]. Also, lymphocytes play such a key role in protection against infection and in tumor rejection. Monocytes, heterophils, basophils and eosinophil are categorized as inflammatory leukocytes that have considerable inflammatory response [17].

study [22] showed that *in ovo* inoculation of pathogens in early embryonic life had strength immune defense and change leukocyte count in hatched chicks. Pires *et al*.[23] stated that post-hatch fasting caused lymphocyte reduction, that this report may has indirect reason for lymphocyte reduction follow *in ovo* ghrelin administration, in other word, ghrelin is a potential orexigenic peptide [24] and according to Pires *et al*.[23] reports, *in ovo* ghrelin induced orexigenic condition for chicks could lower lymphocyte and increase heterophil, basophil and eosinophil counts and H/L ratio that is according to Pires e*t al*.[23] reports for fasting chicks. H/L (heterophil/ lymphocyte) is an important factor in avian leukocyte counts discussion and it can increase in feed restriction condition [23,25]. In our past study [15] increases in Red blood cells, hematocrit and hemoglobin have been observed by *in ovo* administration of 100 ng ghrelin at day-10, that in present study same ghrelin treatment caused leukocyte increases.

It was concluded that *in ovo* ghrelin administration

during incubation especially at day-10 and in 100ng dosage can changes differential leukocyte count with increase heterophil, basophil and eosinophil counts and H/L ratio and decreases lymphocytes. May because of orexigenic nature of ghrelin, lymphocytes as part of immune systems had stimulated following *in ovo* ghrelin administration. Also our findings support hematopoietic acts for chicken ghrelin that it was documented for mammalian ghrelin. Further studies are necessary for further clearness of ghrelin role in avian immune system and leukocytes count (at embryonic and post-embryonic life).

Acknowledgment

Authors are thankful to Islamic Azad University for financial support.

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