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Research Article

Influence of In Ovo Leptin Injection into Yolk Sac on Embryo Development, Blood Biochemical Parameters and Lipid Metabolism of Broiler Chicks during Early Post-Hatching Period

Bülent CELLAK[1], Elif BABACANOĞLU[*2]

[1,2]Van Yuzuncu Yil University, Agriculture Faculty, Animal Science Department, 65080, Van, Turkey

[1]https://orcid.org/0000-0002-0936-9141, [2]https://orcid.org/0000-0002-6329-315X

*Corresponding author e-mail: elifbabacanoglu@yyu.edu.tr

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Abstract: The study aimed to investigate the effect of in ovo (IO) leptin injection on yolk sac leptin concentration, embryo development, blood lipid metabolism, and biochemical parameters of broiler chicks during the early post-hatching period. Hatching eggs were weighed and placed in an incubator with 100 eggs/4 replications/group. The groups were: non-injected group (C); and 3 injected groups with 100 µl of phosphate buffer solution (PBS); 0.5 µl leptin ($L_{0.5}$), and 1 µl leptin (L_1) in 100 µl of PBS. Pure leptin hormone dissolved in PBS was injected into the yolk sac on day 7 of incubation. Yolk/yolk sac leptin concentrations at the onset of incubation, on days 7 and 15 of incubation, and at hatching were determined. Embryo/chick development was determined at all ages. Serum leptin, tri-iodothyronine (T_3), thyroxine (T_4), creatine kinase, total protein, uric acid, and lipid profiles were measured at hatching and day 7 of chick age. The leptin level of the yolk was similar to the leptin level of the yolk sac at hatching. Leptin level of a yolk sac was higher on 7th day than at other ages. IO injection* age was significant for serum leptin level, which was similar to one-day chicks in the $L_{0.5}$ at day 7 of chick age. T_3, T_4, creatine kinase, uric acid, lipid profiles, and embryo/chick development did not change with IO leptin. Serum HDL level was higher in the leptin groups than C and PBS groups at hatching. IO leptin application affects blood lipid metabolism depending on dose level for male broiler chicks at the early post-hatch period, without affecting embryo/chick development.

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1. Introduction

Leptin hormone encoded by the obesity gene (Zhang et al., 2019) is produced by fat cells and is secreted into the bloodstream. Leptin can be synthesized in the hypothalamus, pituitary gland, skeletal muscle, adipose tissue, and especially the yolk sac and liver (Ashwell et al., 1999a; Hu et al., 2008; Rao et al., 2009; Hausman et al., 2012). The first report by Ashwell et al. (1999a) concluded that the expression of leptin hormone from the egg yolk sac begins after day 3 of embryo development. Ashwell et al. (1999b) proved the presence of leptin mRNA expression in the brain, bursa, heart, muscle, and spleen of chick embryos on day 5 of embryo development. This result is confirmed by the result of

Huang et al. (2008), who indicated that leptin level is higher in the hypothalamus on days 1 and 3 of incubation compared to day 11 of incubation, and it is higher in egg yolk on days 1 and 3 of incubation compared to the beginning of incubation. Leptin hormone has a functional role in embryogenesis because leptin mRNA expression in the yolk sac of the developing broiler embryo (Ashwell et al., 1999a) activates chicken growth hormone mRNA expression and thereby enhances lipid metabolism and muscle development (Murase et al., 2016). It was reported that leptin and leptin mRNA expression in the different tissues can be affected hepatic lipid metabolism in newly hatched broiler chicks (Hu et al., 2012; Yuan et al., 2017).

In ovo (IO) leptin administration, which is an easier and faster way to understand the effects of leptin transferred from female breeder to egg yolk (Babacanoğlu and Cellak, 2019), may be a helpful application for physiological parameters and development of broiler embryos. Several studies reported that IO administration of leptin influences embryonic development (Lamoŝova et al., 2003; Su et al., 2012), skeletal muscle growth in sex-specific manner (Liu et al., 2013), the expression of growth hormone in the hypothalamus (Yuan et al., 2017), embryonic metabolism (Cellak and Babacanoğlu, 2019), hepatic leptin secretion and lipid metabolism (Hu et al., 2012). To investigate the effect of maternal leptin, IO injection into broiler breeder eggs of 0.5 µg recombinant mouse leptin dissolved in 100 µl phosphate-buffered saline at the onset of incubation changed liver leptin secretion and lipid metabolism, revealing the role of maternal leptin (Hu et al., 2012).

Leptin hormone has many physiological effects, such as the metabolism of energy, lipid, and glucose, and the development of embryos by affecting growth hormone receptors, growth factors, and protein synthesis in muscle cells (Lamoŝova et al., 2001; Lamosová et al., 2003; Hu et al., 2008; Hu et al., 2012; Liu et al., 2013; Yuan et al., 2017). However, the exact mechanism of leptin hormone level in the yolk or yolk sac on growth and development and metabolisms of lipid is not yet known for broiler embryos/chicks. Hence, this study will provide information about the activation of maternal leptin by IO leptin injection into the yolk sac in broilers. The study aimed to investigate the effect of IO leptin injection on embryo development, serum lipid metabolism, and biochemical parameters during the early post-hatch period in broilers.

2. Materials and Methods

2.1. Experimental design

The study was carried out at the Research and Application Farm of Van Yüzüncü Yıl University and approved by Van Yüzüncü Yıl University Animal Care and Use Committee (Protocol no: 2017/10).

Total 425 hatching eggs obtained from broiler breeders of Ross 308 genotype at 45 weeks- old were used as material in the experiment. At the onset of incubation, egg quality traits were measured in 25 eggs. The rest of the 400 eggs were numbered and weighed, and 4 repeats in each group (25 eggs/repeat/group) were placed in the incubator with 100 eggs/group. Prior to being placed in the incubator, mean egg weight was measured as 63.43 ± 0.39 g, and egg weights for all groups ranged from 63.00 to 63.87 g. The incubator was pre-heated 8 hours before incubation at 27 °C. The experimental design was as follows: the group without injection was control (C), and injection groups were the 100 µl phosphate buffer solution group (PBS); 0.5 µg-leptin + 100 µl PBS group ($L_{0.5}$); and 1 µg-leptin + 100 µl PBS group (L_1). One mg pure leptin hormone (recombinant rat leptin, Peprotech, CAT: 400-21, USA) was dissolved in 1 ml PBS and prepared as 0.5 µg and 1 µg-leptin solutions for 100 eggs/group.

On the 6th hour of the 7th day of incubation (at 174th hour of the incubation), in ovo injection (IO) was administered. The injection site on the eggs taken from the different repetitions in each of the three injection groups was cleaned with 70% ethanol, and the egg was pierced with a 22 G needle so that the blunt tip would slope downwards. Prepared solutions of PBS, $L_{0.5}$ + PBS, and L_1 + PBS were injected directly into the egg yolk sac using a semi-automatic injector with a 26 G needle. According to the group doses, PBS 100 µl / 100 eggs; 0.5 µg-leptin + 100 µl PBS/100 eggs; and 1µg-leptin + 100µl PBS / 100 eggs were injected into the yolk sac, respectively; After the injection, the hole opened in the egg was closed with paraffin and the injected eggs were quickly placed into the incubator. At the 178th hour of incubation, the yolk sac was separated from 8 egg samples randomly selected from each group (including the non-injection group) and stored at -20 ° C until analysis of the leptin hormone level.

On the 12th day of incubation, unfertilized eggs identified by fertility control in each group were separated. On the 18th day of incubation, the embryonated eggs were transferred to the trays, and repeats of the groups were preserved. Female and male chicks hatched between 472 and 496 hours of incubation and were left in the hatcher for drying. The dried chicks were placed in a rearing unit with 3 replicates for each group after 3 hours of their hatching time. All the traits were measured by random selection of 8 male chicks from each group to eliminate the effect of sex at both ages (at hatching and 7 days-old).

2.2. Examined characteristics

2.2.1. Egg quality traits

Egg quality traits to eliminate the effects of IO administration were measured on 25 randomly selected eggs at the onset of incubation. All quality traits (daily egg water loss, eggshell conductance, pore number and diameter, egg weight, weights of eggshell, yolk and albumen, egg shape index, length and width of egg air cell, pH and heights of yolk and albumen, eggshell surface area, eggshell thickness) were measured as described by (Peebles and McDaniel, 2004; Babacanoğlu, 2018; Babacanoğlu et al., 2018). All examined egg quality traits are presented as average values.

2.2.2. Preparation of yolk and yolk sac extracts and ELISA analysis of yolk/yolk sac and serum leptin concentrations

At the beginning of the experiment, after determining egg quality characteristics, yolk samples from 8 eggs were kept at -20 ºC until leptin analysis. After IO injection, the leptin hormone level was determined by the ELISA method in the yolk sacs obtained from embryos in each group at the 12nd hour of the 7th day of incubation (180th hour of incubation), on the 15th day of incubation, and in the residual yolk sac obtained from day-old male chicks at hatching. Yolk/yolk sac and residual yolk sac samples, randomly selected from each group (8 samples/group/day), were subjected to the extraction process before analysis. Egg yolk, yolk sac, and residual yolk sac samples were weighed to approximately 20-30 mg. Potassium chloride (KCL) solution was added as 140 mmol, which was 9 times the sample weight. After KCL was added, samples were homogenized in the homogenizer for 1 minute, and the homogenates were centrifuged at 3750 rpm for 10 minutes at 4 ºC. The obtained supernatant was transferred to eppendorf tubes as 200 µl. One ml of KCL was added to each supernatant and vortexed for 1 minute. All samples were re-centrifuged at 3750 rpm for 15 minutes at 4 °C, and after this process, 200 µl sample volume was transferred to numbered eppendorf tubes and kept at -20 ºC until analysis (Von Engelhardt and Groothuis, 2005; Babacanoğlu et al., 2013).

Two leptin hormone kits from a commercial brand (Rel Assay Diagnostics Chicken LEPTIN ELISA kit, Turkey) were used. Leptin concentration was measured by ELISA based on enzymatic immuno-sorbent assay analysis at 450 nm wavelength on a reader (Biotek ELx800 ELISA reader, USA) with units washed and studying the double antibody sandwich method. Absorbances of the standards and samples were obtained from the ELISA reader. The regression equation of the standard curve was calculated with the OD value of the graph plotted according to the standard concentrations (Hau et al., 2001; Sunwoo et al., 2011; Babacanoğlu et al., 2013). According to this calculation, when the absorbance values were placed in the formula, the yolk/yolk sac and serum leptin hormone levels were determined.

2.2.3. Embryo, yolk sac, and organ weights

On the 15th day of incubation, the shells were removed from 10 eggs randomly selected from each group, and embryo weight was measured with the embryo separated from the yolk sac and dried with a paper towel. The weights of the liver, lung, heart, brain, bursa fabricius, spleen, proventriculus + gizzard, and breast muscle dissected from the embryo were determined. At hatching and day 7 of chick age, the same measurements were repeated for 8 male chicks randomly selected from each group in order to eliminate the effect of sex on all the characteristics.

2.2.4. Serum biochemical analysis

Leptin hormone level was in blood samples taken from the vein under the left wing of 8 male chicks randomly selected from each group at hatching using the ELISA method (Hau et al., 2001; Sunwoo et al., 2011; Babacanoğlu et al., 2013). Serum from the blood samples was placed in blood

tubes and centrifuged at 3750 rpm for 10 minutes and 4 C by using a SIGMA 3 30 K brand centrifuge device. Serum-free tri-iodothyronine (T_3) and thyroxine (T_4), low-density lipoprotein (LDL), high-density lipoprotein (HDL), very low-density lipoprotein (VLDL) triglyceride, creatine kinase, uric acid, cholesterol, and total protein were measured by Roche CREJ2 Cobas INTEGRA 400 plus and Cobass E 411-USA autoanalyzer devices and their commercial kits (Babacanoğlu et al., 2013; Babacanoğlu, 2018). Cholesterol concentration was measured at a wavelength of 512/659 nm by the enzymatic colorimetric method using 142 µl of serum sample and cholesterol kit (CHOL2 Kit). Total protein concentration was measured at a wavelength of 512/659 nm by the colorimetric method using 152 µl of serum sample and total protein kit (TP2 Kit). HDL concentration was measured at a wavelength of 583/659 nm by a homogeneous colorimetric method using 169.5 µl of serum sample and HDL kit (HDLC4 Kit). The uric acid concentration was measured at a wavelength of 552/659 nm by the enzymatic colorimetric method using 134 µl of serum sample and uric acid kit (UA2 Kit). Triglyceride concentration was measured at a wavelength of 512/659 nm by the enzymatic colorimetric method using 150 µl of serum sample and triglyceride kit (TRIGL Kit). Creatine kinase concentration was measured at 340/552 nm wavelength by UV test method using 12.75 µl of serum sample and creatine kinase kit (CK Kit). Free T_3 concentration was measured with an autoanalyzer device using a specific anti-T_3 antibody labeled with ruthenium complex from a T_3 kit (FT_3 Kit). Free T_4 concentration was measured with an autoanalyzer device using a specific anti-T_4 antibody labeled with ruthenium complex from a T_4 kit (FT_4 II Kit). LDL and VLDL concentrations were calculated with the following equations: LDL= Cholesterol – (HDL+ Triglyceride/5) and VLDL= Triglyceride/5.

2.2.5. Performance

Chicks fed with broiler starter feed until 7 days old were raised under standard rearing conditions. The results for all the performance features were presented in Table 4.

2.3. Statistical analysis

Data were performed by using ANOVA in the SAS package program (SAS, 2009), and the main effects (group, age) and their interactions were included in the GLM procedure. Tukey's HSD test was used to compare means. The significance test was used to compare treatment groups to the control for each age on embryo and organ development. The means of serum biochemical parameters and serum lipid profiles were analyzed for treatment groups, ages, and their interaction. If group*age interaction was detected for any of these features, the mean values of this interaction were also shown on the graph.

3. Results

3.1. Egg quality traits

Eggshell conductance, pore number, pore diameter, egg weight, egg shape index, yolk weight, albumen weight, eggshell weight, width and length of the air cell, heights of albumen and yolk, pH of yolk and albumen, eggshell surface area, eggshell thickness were 8.92 mg H2O d^{-1} $Torr^{-1}$, 26.65 and 22.06 µm, 65.54 g, 80.01%, 20.17 g, 33.80 g, 6.86 g, 12.48 mm, 20.94 mm, 6.04 mm, 6.47, 9.21, 76.09 cm^2, 33.01 µm, respectively.

3.2. Yolk/yolk sac leptin concentration

At the onset of incubation, yolk leptin concentration was 658.46±35.46 ng/ml. Leptin hormone levels decreased significantly in the IO leptin injection groups compared with C at the 10^{th} hour of the seventh day of incubation (P=0.014). At hatching, injection groups were not different from C, but the yolk sac leptin level of the $L_{0.5}$ group significantly increased on the 15^{th} day of incubation. The interaction between group and age for yolk sac leptin levels was significant (P=0.025). This interaction was due to the significant decrease in the yolk sac leptin level of the L_1 group compared to the $L_{0.5}$ and PBS groups on the 15^{th} day of incubation and all groups at other embryonic ages. The effect of age was significant on yolk/yolk sac leptin concentration (P<0.001), which was the reason for the interaction of group and age (P=0.025) (Figure 1).

3.3. Embryo and organ development

Yolk sac weight of the L_1 group was significantly decreased compared to the control at hatching (P=0.012). The weights of all examined organs and embryos did not change at all ages, but the interaction between IO leptin injection and embryo/chick age was significant for liver and intestine weights. These significant interactions were due to increasing age for liver and intestine weights, but IO groups did not differ at each age (Table 1).

3.4. Blood parameters

IO injection did not affect concentrations of serum leptin, uric acid, creatine kinase, total protein, T_3, and T_4, but chick age affected significantly serum leptin, creatine kinase, total protein, T_3, and T_4 concentrations, which were higher at 7 days-old than those at hatching, except for serum T_4 concentration (Table 2). The interaction between IO injection and chick age was significant for serum leptin concentration (Figure 2). Serum leptin level of chicks in the $L_{0.5}$ injection group at one-day old was similar to serum leptin levels of all groups at 7 days of age. Therefore, this interaction was due to the increased serum leptin level of chicks in the $L_{0.5}$ injection group at hatching (Figure 2). The significant interaction between IO treatment * chick age for total protein concentration was due to age (Figure 3).

Serum triglyceride, cholesterol, HDL cholesterol, LDL cholesterol, and VLDL concentrations did not change with IO injection (Table 3). Serum triglyceride, cholesterol, LDL cholesterol, and VLDL concentrations significantly decreased with increasing chick age (Table 3). Serum HDL cholesterol concentration had significant interaction between IO treatment * chick age. Serum HDL cholesterol concentration was higher in the leptin injection groups than C and PBS at hatching and in all groups at 7-day-old chicks (Figure 4).

3.5. Performance

The results for all features representing numerical averages are presented in Table 4. Feed intake was found to be lower in IO treatment groups compared to PBS and C groups. The feed conversion ratio was found to be lower in IO leptin groups than in C and PBS groups. Sex ratio was the highest for male chicks with 63% in the $L_{0.5}$ group, and this ratio for male chicks was 55% in the PBS group and 53% in the L_1 group. The highest mortality rate was 6.45% in the PBS group, while the lowest mortality rate was 2.5% in the L_1 group.

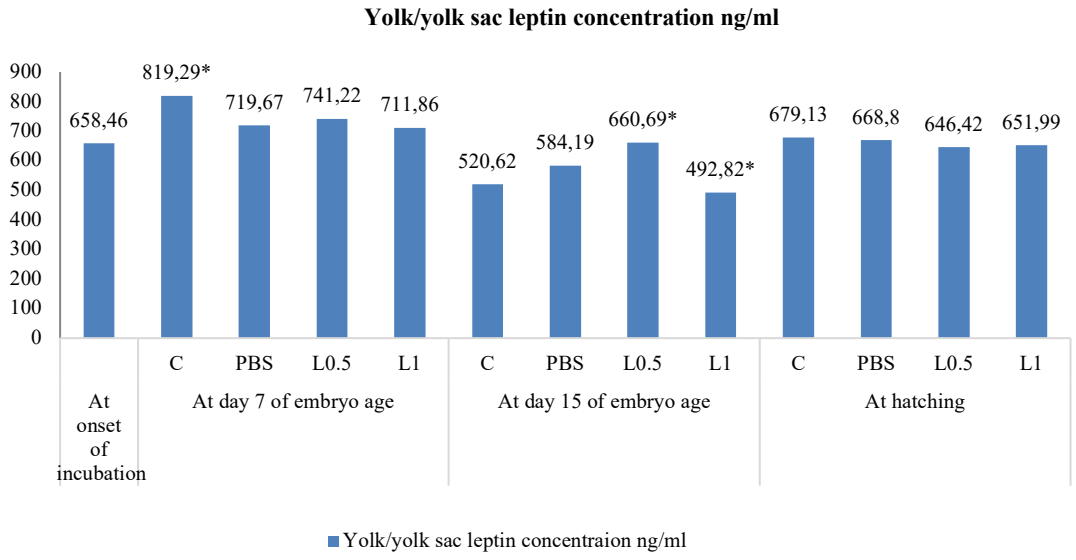

Figure 1. Interaction between IO treatment and chick age for the yolk/yolk sac leptin concentration (ng ml^{-1}).

*Means differ significantly at the same age ($P < 0.05$). C: control (non-injection group); PBS: 100 µl phosphate buffer solution; $L_{0.5}$: 0.5 µg leptin+100 µl PBS; L_1: 1 µg leptin+100 µl PBS.

Table 1. Effects of IO leptin injection, chick age, and IO leptin injection * chick age interaction on embryo and organ development

Age (day)	IO administration[1]	Embryo weight (g)	Yolk sac weight (g)	Liver weight (%)	Lung weight (%)	Heart Weight (%)
On day 15 of incubation	**C**	14.32	15.17	1.86^{b}	0.93^{b}	0.67^{b}
	PBS	14.01	14.79	2.40^{a}	1.22^{a}	0.83^{a}
	$L_{0.5}$	13.57	16.02	2.05^{ab}	1.06^{ab}	0.73^{ab}
	L_1	14.79	13.99	1.96^{b}	1.01^{ab}	0.71^{b}
	SEM	0.53	1.14	0.10	0.07	0.03
P value		0.440	0.684	0.006	0.052	0.045
At hatching	**C**	44.79	5.73^{a}	2.43	0.90	0.62
	PBS	44.57	4.38^{b}	2.67	1.05	0.63
	$L_{0.5}$	44.73	4.78^{ab}	2.76	1.09	0.69
	L_1	44.54	4.29^{b}	2.76	1.11	0.66
	SEM	1.09	0.45	0.13	0.06	0.02
P value		0.998	0.012	0.270	0.103	0.365
At 7 days old chick age	**C**	180.55	0.28	3.70	0.92	0.65
	PBS	179.92	0.40	3.41	0.93	0.68
	$L_{0.5}$	169.04	0.25	3.65	0.83	0.65
	L_1	176.34	0.45	3.66	0.84	0.64
	SEM	5.18	0.02	0.15	0.04	0.03
P value		0.128	0.716	0.562	0.196	0.884
Group*day		0.056	0.919	0.045	0.053	0.899

Table 1. The effects of IO leptin injection, chick age and IO leptin injection * chick age interaction on embryo and organ development (continued)

Age (day)	IO administration[1]	Intestine weight (%)	Proventriculus+ gizzard weight (%)	Pancreas Weight (%)	Breast Weight (%)
On day 15 of incubation	**C**	1.18^{b}	2.79	-	-
	PBS	1.78^{a}	3.12	-	-
	$L_{0.5}$	1.35^{ab}	3.11	-	-
	L_1	1.44^{ab}	2.97	-	-
	SEM	0.15	0.16	-	-
P value		0.051	0.458	-	-
At hatching	**C**	4.61	5.71	0.15	-
	PBS	4.74	6.97	0.14	-
	$L_{0.5}$	4.03	6.08	0.15	-
	L_1	5.23	6.75	0.16	-
	SEM	0.28	0.32	0.01	-
P value		0.052	0.168	0.779	-
At 7 days old chick age	**C**	10.40	3.86	0.46	9.84
	PBS	9.44	3.84	0.39	10.89
	$L_{0.5}$	10.31	4.02	0.48	9.63
	L_1	10.64	4.14	0.48	9.98
	SEM	0.38	0.19	0.03	0.31
P value		0.192	0.715	0.219	0.199
Group*day		0.049	0.513	0.381	-

[a,b] Means within a column with a different superscript differ significantly at $P<0.05$. The significance test is the comparison of treatment groups to the control for each age.
[1]C: control (non-injection group); PBS: 100 µl phosphate buffer solution; L0.5: 0.5 µg leptin+100 µl PBS; L1: 1 µg leptin+100 µl PBS.
SEM: Standard error mean.

Table 2. Effects of IO leptin injection, chick age, and IO leptin injection * chick age interaction on concentrations of serum leptin, uric acid, creatine kinase, total protein, T_3 and T_4

	Leptin ng ml^{-1}	**Uric acid** mg dl^{-1}	**Creatine Kinase** U l^{-1}	**Total protein** mg dl^{-1}	**T_3** pg ml^{-1}	**T_4** ng ml^{-1}
IO injection(group)[1]						
C	469.31	6.62	5221.37	1.95	8.91	5.44
PBS	448.10	5.88	5132.56	1.91	11.20	6.77
$L_{0.5}$	478.37	5.13	5332.01	1.83	10.53	6.66
L_1	511.35	6.10	5309.93	1.95	11.11	7.02
SEM	31.85	0.56	533.64	0.05	0.94	0.64
Age (days)						
0	388.86^{b}	6.02	3851.37^{b}	1.51^{b}	6.79^{b}	9.76^{a}
7	564.70^{a}	5.84	6646.56^{a}	2.31^{a}	14.09^{a}	3.18^{b}
SEM	21.80	0.40	533.64	0.03	0.66	0.72
P values						
Group	0.587	0.320	0.993	0.363	0.296	0.312
Age	<0.001	0.755	<0.001	<0.001	<0.001	<0.001
Group x age	0.045	0.847	0.274	0.017	0.596	0.255

[a-d] Means within a column with a different superscript differ significantly at P<0.05. The significance test is the comparison of treatment groups to the control for each age.
[1]C: control (non-injection group); PBS: 100 µl phosphate buffer solution; L0.5: 0.5 µg leptin+100 µl PBS; L1: 1 µg leptin+100 µl PBS.
SEM: Standard error mean

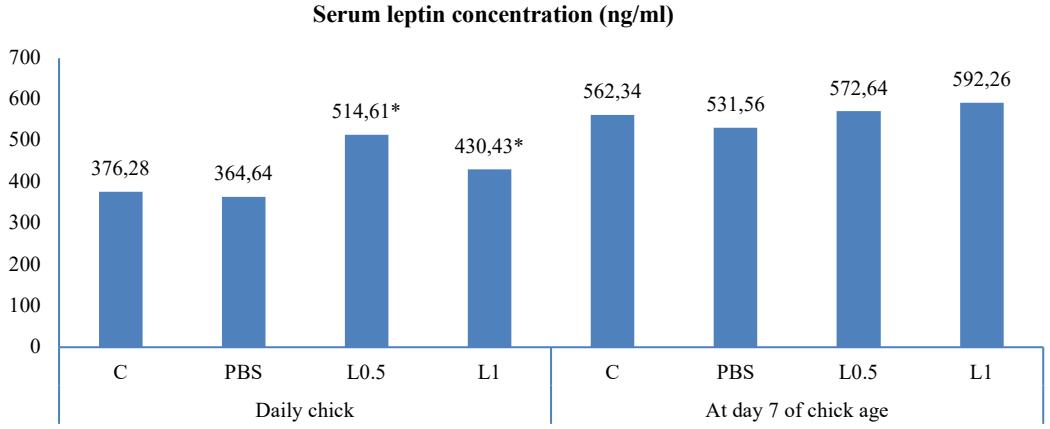

Figure 2. Interaction between IO treatment and chick age for the serum leptin concentration (ng ml^{-1}).

* Means differ significantly at the same age (P < 0.05). C: control (non-injection group); PBS: 100 µl phosphate buffer solution; $L_{0.5}$: 0.5 µg leptin+100 µl PBS; L_1: 1 µg leptin+100 µl PBS.

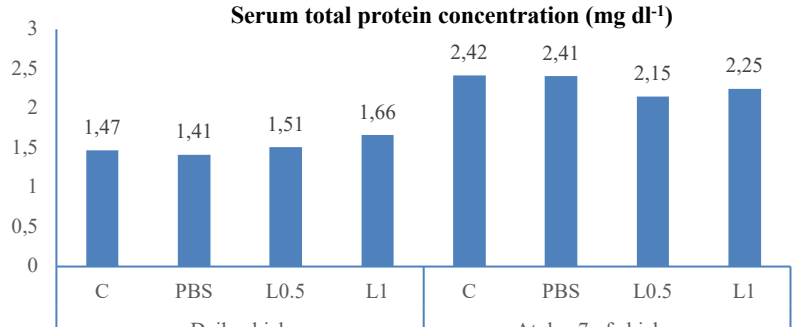

Figure 3. Interaction between IO treatment and chick age for serum total protein concentration (mg dl^{-1})

* Means differ significantly at the same age (P < 0.05). C: control (non-injection group); PBS: 100 µl phosphate buffer solution; $L_{0.5}$: 0.5 µg leptin+100 µl PBS; L_1: 1 µg leptin+100 µl PBS.

Table 3. Effects of IO leptin injection and age on serum lipid profiles (triglyceride, cholesterol, HDL cholesterol, LDL cholesterol, and VLDL) at hatching and 7 day chick age

	Triglyceride	Cholesterol	HDL	LDL	VLDL
IO injection (group)		mg dl^{-1}			
C	74.31	256.49	120.70	120.92	14.84
PBS	60.00	255.65	123.64	121.03	11.98
L0.5	68.00	241.05	127.77	99.69	13.58
L_1	61.50	258.14	125.23	120.62	12.28
SEM	5.72	11.86	4.42	10.85	1.14
Age (day)					
0	75.03[a]	336.78[a]	128.53	120.93[a]	14.98[a]
7	56.87[b]	168.88[b]	120.14	37.37[b]	11.36[b]
SEM	4.04	8.39	3.12	7.33	0.80
P-values					
Group	0.278	0.723	0.719	0.397	0.278
Age	0.001	0.001	0.063	0.001	0.025
Group x age	0.867	0.313	0.009	0.481	0.869

[a.b.c] Means within a column with a different superscript differ significantly at P<0.05.
C: control (non-injection group); PBS: 100 µl phosphate buffer solution; $L_{0.5}$: 0.5 µg leptin+100 µl PBS; L_1: 1 µg leptin+100 µl PBS.

Table 4. Averages for performance characteristics with IO leptin injection

	Daily feed intake	Feed conversion ratio	Sex ratio	Mortality
IO injection (group)	g	(g feed/g gain)	%	%
C	19.65	1.35	50	3.13
PBS	20.44	1.25	45	6.47
$L_{0.5}$	17.51	1.49	37	3.70
L_1	15.43	1.55	47	2.50

C: control (non-injection group); PBS: 100 µl phosphate buffer solution; $L_{0.5}$: 0.5 µg leptin+100 µl PBS; L_1: 1 µg leptin+100 µl PBS.

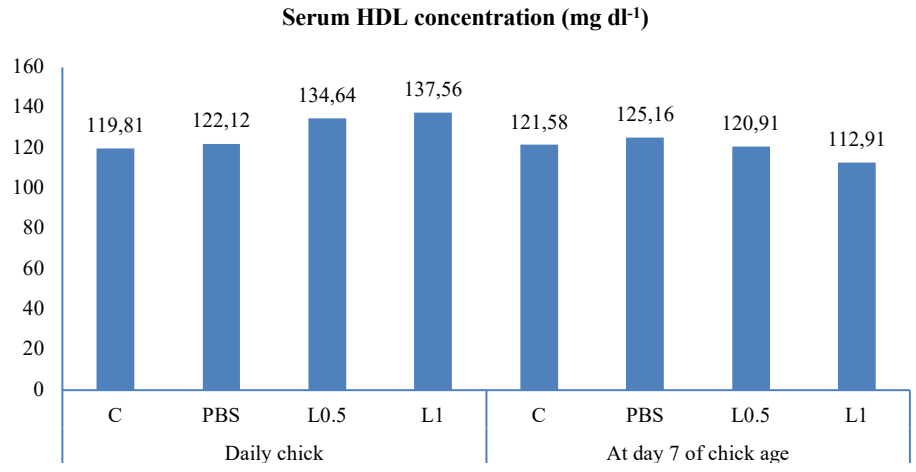

Figure 4. Interaction between IO treatment and chick age for serum HDL concentration (mg dl^{-1}).

* Means differ significantly at the same age (P < 0.05). C: control (non-injection group); PBS: 100 µl phosphate buffer solution; $L_{0.5}$: 0.5 µg leptin+100 µl PBS; L_1: 1 µg leptin+100 µl PBS

4. Discussion

This study investigated the effects of leptin hormone injected into the yolk sac of embryos on blood biochemical parameters, lipid metabolism, and development of the embryo. Leptin concentration in the yolk was determined as 658.46 ng/ml at the onset of incubation. This result indicates that leptin hormone is transferred to follicles through the plasma of the laying hen, demonstrating the maternal origin of leptin hormone in broiler breeders. Hu et al. (2012) reported that IO injection of 0.5 µg recombinant mouse leptin into broiler breeder eggs, revealing that the role of maternal leptin could change liver leptin secretion. These findings indicate the possible roles of *maternal leptin* in the *egg* on the development of an embryo. Moreover, Hu et al. (2008) determined the presence of a leptin-like immunoreactive substance in the egg yolk and albumen in broiler breeders. It was also reported that some practices related to maternal nutrition affect the storage of leptin in the egg (Hu et al., 2008; Rao et al., 2009). In this study, due to the presence of leptin hormone in egg yolk at the onset of incubation and transferral to the egg yolk from female breeders in previous studies, leptin hormone was injected into the yolk sac of the embryo by using the IO method on the 7th day of incubation. In our study, leptin hormone concentration (819.29 ng/ml) in the yolk sac of the control group on the 7th day of embryonic age revealed that leptin hormone is synthesized by chicken embryos. The presence of mRNA for leptin in the brain, bursa, heart, liver, muscle, and spleen of 5 day-old embryos (Ashwell et al., 1999b), and the presence of leptin hormone in the yolk sac of embryos in the first half of incubation indicates that this hormone is active and has a functional role during avian embryogenesis (Ashwell et al., 1999a). The result of this study indicates that egg yolk leptin concentration at onset of incubation was similar to yolk sac leptin concentration at hatching because newly hatched chick functionally utilized from leptin hormone. However, the yolk leptin level changed depending on embryonic age. Yolk sac leptin level was higher on the 7th day than on the 15th day and at hatching. Similar to the findings in our study, Huang et al. (2008) showed that egg yolk leptin level was higher on the 1st and 3rd days of incubation than on the 11th day of incubation. Huang et al. (2008) determined lower yolk sac leptin concentration at hatching and in one-day old chicks than 3 days-old chicks in two different strains of broilers. On the 15th day of embryonic age, the reduced yolk sac leptin level depends on the highest dose of leptin treatment can be a consequence of the influence of leptin gene expression at day 14 of embryonic age in this study.

Serum leptin level of 7 days-old chicks was higher than day-old chicks in the leptin injection groups, showing that serum leptin levels increased with increasing age. When the results of the interaction between age and IO application are examined, the blood serum leptin level changed and increased with age in the leptin-low dose treatment group during the early post-hatching period. Cassy et al. (2004) reported that the inhibitory effect of leptin on appetite regulation is an age dependent process in growing chicks. In the present experiment, higher serum leptin levels were determined at hatching when leptin was administered in a low dose-dependent manner than in a high dose-dependent manner in the first weeks of embryonic development. This result indicates that the leptin hormone might be changed in the early programming of appetite regulation in a dose-dependent manner because the leptin hormone may be affected by appetite regulation related to leptin dose and age of chicken (Chuang et al.,2020)

It was concluded that IO leptin injection had no effect on blood lipid profiles (serum triglyceride, cholesterol, LDL, and VLDL levels) at hatching and 7-day chick age, but the interaction of IO application and chick age on blood serum HDL level was significant. The reason for this interaction is due to the fact that there was a lower serum HDL level at day 7 of chick age than for day-old chicks after injecting 1 µg leptin. Decreased total lipid concentrations in the liver of embryos treated with leptin during incubation promote the possibility of higher lipid degradation in the first days of development in the post-hatching period (Lamosova et al., 2003). Elevated serum HDL concentration in newly-hatched chicks treated with leptin on day 7 of embryonic age may be due to affecting leptin synthesis and secretion, lipid metabolism, and mRNA expression in the liver, which is the major source of leptin in day-old broiler chicks (Hu et al. 2012).

It was reported that injection of leptin into eggs during incubation affects the levels of thyroid hormones in plasma (Mácajová et al. 2002), decreasing the T_3 level and increasing the T_4 level (Lamoŝova et al. 2003). However, IO leptin administration did not change the levels of thyroid hormones because blood lipid metabolism did not affect the liver metabolism of broiler chicks during the early

post-hatching development period in our study. In addition, leptin injection to the yolk sac of 7-day-old embryos did not affect the serum uric acid level after hatching, revealing that leptin had no effect on protein metabolism in a dose-dependent manner.

The embryo and chick weights did not change at each age examined, which may be due to the unchanged serum leptin level. ICV injection of different doses of leptin had no effect on chick weight (Mácãjová et al., 2003; Kuo et al., 2005), which is consistent with our results. While leptin application did not affect embryo and chick development, day-old chicks treated with 1 µg leptin utilized more nutrients from the yolk sac than controls. The reason for this result may also be increased digestive system efficiency due to the effect of 1 µg leptin dose in the first week after hatching because leptin plays an important role in the regulation of feed intake by inhibiting insulin secretion (Taouis et al., 2001). In this study, it was revealed that daily calculated feed intake decreased in the leptin dose groups. We can explain this result with the results of a study (Dridi et al., 2005) which reported that leptin affected the central nervous system and feed intake through selective hypothalamic neuro-peptides. Also, this result confirms that the inhibitory effect of leptin hormone on the regulation of feed intake in growing chicks is age-dependent (Cassy et al., 2004).

Conclusion

It was concluded that the leptin hormone not only originates from the maternal but it is also synthesized by the embryo. IO leptin application affects blood lipid metabolism through cholesterol metabolism of male chicks after hatching depending on leptin dose level and chick age in broilers, without affecting embryo and chick developments. These findings indicate that early programming of appetite regulation in leptin-treated embryos can alter broiler chicks in a dose-dependent manner during the early post-hatching period.

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