Interaction of the central Histaminergic and Melanocortin Systems on Leptin-induced Hypophagia in Neonatal Layer Chicken

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Abstract

The present study aimed to investigate the probable impact of the central histaminergic and central melanocortin systems on hypophagia induced by leptin in neonatal layer chickens. In experiment 1, control solution, 250 nmol of α-FMH, 10 µg of leptin and co-injection of α-FMH+leptin were injected into chickens through the intracerebroventricular (ICV) route. Experimental groups 2-8 were injected the same as experiment 1. However, chickens in experiments 2-8 received ICV injections of 300 nmol of chlorpheniramine (H₁ receptor antagonist), 82 nmol of famotidine (H₂ receptor antagonist), 300 nmol of thioperamide (H₃ receptor antagonist), 0.5 nmol of SHU9119 (M₃/M₄ receptors antagonist), 0.5 nmol of MCL0020 (M₄ receptor antagonist), 30 µg of astressin-B (CRF₁/CRF₂ receptors antagonist) and 30 µg of astressin2-B (CRF₂ receptor antagonist) instead of α-FMH, respectively. Food was provided for the birds instantly following the injection, and 30, 60 and 120 min after the injection, cumulative food intake (g) was measured. Our findings demonstrated that the ICV injection of leptin diminished food intake in neonatal chicken (P<0.05). The co-administration of the M₃/M₄ receptor antagonist+leptin significantly decreased the hypophagic effect of the leptin (P<0.05). A significant decrease was found in the hypophagic effect of leptin following the co-administration of M₄ receptor antagonist and leptin (P<0.05). Moreover, co-injecting the antagonists of CRF₁/CRF₂ receptors with leptin reduced the hypophagic effect of leptin significantly (P<0.05). The CRF₂ receptor antagonist co-injection with leptin led to a decrease in the hypophagic effect of leptin. According to the results of the current investigation, the hypophagic effect of leptin is mediated by the receptors of H₁, H₃, M₃/M₄, and CRF₁/CRF₂ in neonatal layer chicken.
Keywords: Histaminergic, Melanocortin, Leptin, Food intake, Neonatal layer chicken

Introduction

Central regulation of food intake has some differences between avian and mammalian species because of the regulatory impact of neurotransmitters and their receptors, in addition to the relevant nucleus (Zendehdel et al. 2020). Leptin is known as a peptide hormone secreted from the white adipose tissue with blood levels being correlated with body fat mass (Adeli et al. 2020). This hormone can cross the blood-brain barrier (Irving and Harvey, 2014) and exert an effect on diverse hypothalamic areas, including the amygdala, nucleus tractus solitaries, and arcuate nucleus (ARC) (Doherty et al. 2013). Leptin plays a role in regulating feeding and the feeling of satiety so that food intake diminished after the ICV injection of leptin at the doses of 2.5, 5, and 10 µg in broiler chickens (Adeli et al. 2020).

Central histaminergic neurons are located in the tuberomammillary nucleus (TMN) with axon projects branched to various brain areas (Rafiei et al. 2011). Brain histamine is of high importance in determining the feeding behavior. Consequently, histamine administration through the ICV route reduced food intake, while food intake was elevated under the influence of chlorpheniramine, as an antagonist of H₁ receptor, and alpha-fluoromethylhistidine (α-FMH), as a selective inhibitor of the histamine-synthesizing enzyme histidine decarboxylase (Rozov et al. 2014). Different physiological functions are mediated by the melanocortin system, namely grooming, thermoregulation, learning, and the regulation of energy balance. Although melanocortin receptors (MC₁R-MC₅R) has been recognized as the MC₃R and MC₅R subtypes account for central appetite regulation in the ARC, ventromedial hypothalamus, and periventricular nucleus (PVN) (Schneeberger et al. 2014). Studies have indicated that the ICV injection of the agonists of MC₃R/MC₅R receptors could reduce food intake in broiler chickens (Ahmadi et al. 2017).

Some studies in the past decades assessed the central and peripheral systems responsible for the regulation of appetite in avian (Zendehdel et al. 2020). Investigations that compared physiology showed variations in the pathways of food intake regulation among mammals and avian species (Richards 2003). Central leptin, histamine, and melanocortin systems were revealed to have interactions. Furthermore, previous investigations applied the histaminergic system for the leptin-induced suppression of food intake. However, this influence is suggested to be indirect because of the lack of leptin receptor expression in the TMN (Michael et al. 2020). Some authors reported that the anorectic effect of leptin was amplified by the H₁ receptors (Gloy et al. 2010). Leptin is the link between the peripheral energy stores
and proopiomelanocortin (POMC) signaling activity in the hypothalamus. It should be noted that all mammalian hypothalamic POMC neurons do not express leptin receptors, which indicates that a leptin-unrelated melanocortin signaling system may also exist. The diacetyl-α-MSH is acetylated to α-MSH, as a more active melanocortin. The latter reaction is facilitated by leptin (Guo et al. 2004). In spite of the interaction between leptin, histamine, and melanocortin, there is no report concerning their possible interactions in terms of food intake regulation in birds. With this background in mind and no report on layers, the present study aimed to evaluate the role of the central histaminergic and central melanocortin systems on hypophagia induced by leptin in neonatal layer chickens.

Material and Methods

Animals
This study was performed on 352 one-day-old layer chickens (Hy-Line), which were purchased from a local hatchery (Morghak Co., Iran). Birds were kept in stabilizing electrically-heated batteries at the temperature, relative humidity, and lighting/dark period of 32±1°C, 40%-50%, and 23:1, respectively (Olanrewaju et al. 2017). The subjects were kept at the mentioned conditions for 2 days as flocks followed by random allocation and transfer to individual cages. A commercial diet was provided with 21% crude protein and 2850 kcal/kg metabolizable energy during the course of study (Chineh Co., Iran) (Table 1). Birds had free access to food and fresh water. The subjects were food-deprived 3 h before injections. However, they still had free access to water. The ICV injections were performed at the age of 5 days.

Experimental Medications
The administered medications were leptin, α-FMH, chlorpheniramine, famotidine (i.e., H₂ receptor antagonist), thioperamide (i.e., H₃ receptor antagonist), SHU9119 (i.e., M₃/M₄ receptors antagonist), MCL0020 (i.e., M₄ receptor antagonist), astressin-B (i.e., CRF₁/CRF₂ receptors antagonist), astressin2-B (i.e., CRF₂ receptor antagonist), and Evans blue. All the medications were bought from Sigma-Aldrich (USA) and Tocris Co. (UK) and were dissolved in absolute dimethyl sulfoxide (DMSO). Afterwards, the medicines were diluted using 0.85% saline containing Evans blue at a ratio of 1/250 (0.4% DMSO). No cytotoxic effect was found for DMSO at this ratio (Blevins et al. 2002; Qi et al. 2008). The DMSO/saline mixture that had Evans blue was utilized for the control group.
ICV injections

The subjects were randomly assigned to nine experimental groups with four sub-groups (n=44). The birds were weighed and allocated into test groups based on their body weight (BW) as the mean BW of diverse treatment groups was the same. The ICV injections were performed once for each group by a microsyringe (Hamilton, Switzerland) without anesthesia according to the techniques of Davis et al. (1979) and Furuse et al. (1997). In summary, the chicken head was held applying an acrylic device with the bill holder at 45º. Calvarium was in a parallel position relative to table surface as described by Van Tienhoven and Juhasz (1962). Next, an orifice was made in a plate over the skull of the right lateral ventricle, through which a microsyringe was inserted. The needle tip perforated 4 mm under skull skin (Jonaidi and Noori, 2012) and the volume of all injections was 10 μL (Furuse et al. 1999). The animals in the control group received 10 μL of control solution (Furuse et al. 1999). It should be noted that the mentioned method does not cause physiological stress for newly hatched chickens (Saito et al. 2005). To identify injection accuracy at the end of experiments, decapitation was carried out. Accuracy of the injection site in the ventricle was confirmed based on the presence of Evans blue and the slicing of frozen brain tissues. All birds in each intervention group received injections. However, only the data of 11 birds in each group, which had dye in their lateral ventricle were analyzed. All test procedures were performed during 8-13:30.

Feeding Experiments

In experiment 1, the birds were injected with a control solution, 250 nmol of α-FMH, 10 μg of leptin, and a co-administration of α-FMH and leptin. The chickens in experimental experiment 2, received a control solution, 300 nmol of chlorpheniramine, 10 μg of leptin, and chlorpheniramine+leptin. Injections in experiment 3 were control solution, 82 nmol of famotidine, 10 μg of leptin, in addition to the co-injection of famotidine+leptin. The birds in experiment 4 received a control solution, 300 nmol of thioperamide, 10 μg of leptin, and thioperamide+leptin. In experiment 5, control solution, 0.5 nmol of SHU9119, 10 μg of leptin, and SHU9119+leptin was injected. In experiment 6, the subjects were injected with a control solution, 0.5 nmol of MCL0020, 10 μg of leptin and a co-administration of MCL0020+leptin. The birds in experiment 7 received ICV injections of control solution, 30 μg of astressin-B, 10 μg of leptin, and astressin-B+leptin. In experiment 8, chickens were injected with control solution, 30 μg of astressin2-B, 10 μg of leptin, and the co-injection of astressin2-B+leptin. Instantly following the injections, the birds were fed, and cumulative
food intake (g) was measured 30, 60 and 120 min after the injection. Food consumption (g) was calculated as the percent of body weight (g/100g BW) to minimize the influence of body weight on food intake. The latter doses of medications were determined based on the previous investigations (Ahmadi et al. 2017, 2019; Adeli et al. 2020; Zendehdel et al. 2020).

Statistical analysis
In the current study, eight experimental groups were designed. Each test group entailed four subgroups (I-IV). Only one injection was performed in each group. Cumulative food intake, stated as a percent of body weight, was analyzed for each intervention group using two-way repeated measures analysis of variance (ANOVA) by the following model:

\[
Y_{ijk} = \mu + \alpha_i + \beta_k + (\alpha\beta)_{jk} + \varepsilon_{ijk}, \quad \text{with} \quad \varepsilon_{ijk} \sim N(0, \sigma^2)
\]

Where \(Y_{ijk}\) represents the value of individual observation for valuables, \(\mu\) is the grand mean, \(\alpha_i\) denotes the treatment effect for the time, \(\beta_k\) refers to the treatment effect for the medications, \((\alpha\beta)_{jk}\) denotes the effect of time × medicine interaction, and \(\varepsilon_{ijk}\) represents error. All the data were statistically analyzed using the SPSS software version 16 (IBM, Chicago, IL., USA). The means were compared by the Tukey test (P<0.05) and descriptive statistics are presented as mean ± SEM (standard error of the mean).

Results
In experiment 1, hypophagia was observed after the ICV injection of 10 µg leptin, compared to the control group (P<0.05). Cumulative food intake was not affected by injecting 250 nmol of α-FMH, compared to the control group (P>0.05). Moreover, the hypophagic effect of leptin significantly diminished due to the co-administration of α-FMH+leptin (P<0.05) (Figure 1).

In experiment 2, hypophagia was found following the ICV injection of 10 µg of leptin, in comparison with the control group (P<0.05). Chlorpheniramine injection through the ICV route (300 nmol) did not influence cumulative food intake, compared to the control group (P>0.05). Co-injecting chlorpheniramine and leptin resulted in a significant decline in the hypophagic effect of leptin (P<0.05) (Figure 2).

In experiment 3, the ICV injection of 10 µg of leptin significantly reduced food intake, in comparison with the control group (P<0.05). The ICV injection of 82 nmol famotidine did not affect cumulative food intake in this group, compared to the control group (P>0.05).
Furthermore, co-injecting famotidine and leptin exerted no impact on hypophagia due to leptin in chickens (P>0.05) (Figure 3).

In experiment 4, 10 µg of leptin was injected through the ICV route causing hypophagic effect, in comparison with the control group (P<0.05). No significant alteration was observed cumulative in the food intake of this group following thioperamide injection (300 nmol) (P>0.05). However, the co-injection of thioperamide and leptin resulted in a significant reduction in the hypophagic effect of leptin (P<0.05) (Figure 4).

In experiment 5, 10 µg leptin was injected through the ICV route causing hypophagic effect, in comparison with the control group (P<0.05). No significant alteration was observed cumulative in the food intake of this group following thioperamide injection (300 nmol) (P>0.05). However, the co-injection of thioperamide and leptin resulted in a significant reduction in the hypophagic effect of leptin (P<0.05) (Figure 4).

In experiment 6, the ICV injection of leptin (10 µg) led to hypophagia, in comparison with the control group (P<0.05). Injecting 0.5 nmol MCL0020 did not affect food intake in this group, compared to the control group (P>0.05). The leptin-induced hypophagia decreased significantly after the injection of MCL0020 and leptin together (P<0.05) (Figure 6).

In experiment 7, food intake significantly diminished due to leptin ICV injection (P<0.05). However, food intake did not change significantly after the injection of 30 µg astressin-B (P>0.05). The leptin-induced hypophagic effect declined significantly after co-administring astressin-B+leptin (P<0.05) (Figure 7).

In birds in experiment 8, leptin ICV injection caused a significant decrease in their food intake, compared to the subjects in the control group (P<0.05). The injection of astressin-2B (30 µg) through the ICV route imposed no significant impact on food intake, in comparison with the control group (P>0.05). The injection of astressin-2B and leptin together significantly reduced the hypophagic effect of leptin (P<0.05) (Figure 8).

Discussion

A review of the literature revealed that the present investigation is the first study on the role of the central glutamatergic system in the hypophagic effect of melatonin in neonatal broiler chickens. Our findings showed that injecting leptin through the ICV route diminished food intake in neonatal chickens. Leptin, which is secreted by the adipose tissue, can pass across the blood-brain barrier and bind its receptor in the central nervous system (Morton et al. 2009). It has been reported that the ICV injection of leptin may reduce food intake in broilers
and Leghorns (Denbow et al. 2000), which is consistent with the results of the current study. However, in a study by Furuse et al. (1997), food consumption was not affected by the ICV injection of mouse leptin in neonatal Single-comb White Leghorn chickens. The genetic factors of animals, age, and the source of leptin might contribute to the mentioned varieties. The gene that encodes leptin in chicken can be cloned (Lamoová et al. 2003). Although the types of leptin produced by chickens and rodents are different, they can be highly similar with 95% identical amino acids (Hen et al. 2008).

The information concerning the effect of leptin on the CNS in birds is limited. Leptin in birds triggers the downstream signaling pathways through its receptor. However, the highest rate of the expression of their mRNA in distinct species takes place in the liver or brain (Wang et al. 2015). The sensitivity of broilers to peripheral leptin is lower than layer chickens (Richards and Proszkowiec-Weglarz, 2007). Feed consumption, basal metabolism, and energy expenditure are all higher in broilers, which could be attributed to the genetic alterations in the mechanisms responsible for controlling food intake. Broilers are believed to be less sensitive to the anorexigenic signals from peripheral tissues (Richards and Proszkowiec-Weglarz, 2007).

According to the findings of the current study, the co-administration of the antagonists of H₁ receptors with leptin reduced leptin-induced hypophagia. Moreover, the hypophagic effect of leptin declined as the result of co-injecting an antagonist of H₂ receptors with leptin. Hypothalamic neuronal histamine along with its H₁ receptor play role in forming the leptin-signaling pathway in the brain. These were demonstrated to influence food intake and uncouple protein mRNA expression leading to the regulation of body weight and adiposity in mice with diabetes (db/db) or obesity induced by diet (Masaki et al. 2003). It was observed that α-FMH had the potential to inhibit the food intake decrease induced by leptin in fasted rats. Leptin reduced the histamine content of the hypothalamus. On the other hand, it augments the ratio of telemethyl-histamine and histamine, which shows that leptin has the capacity to diminish the metabolism of HA. In addition, α-FMH suppressed CRH expression both at the basic condition and following induction by leptin. In contrast, it stimulated NPY expression in fasted rats. In conclusion, histamine contributes to the inhibition of food intake, which is caused by leptin. Histamine can be a mediator and directly activate and/or alter the histaminergic system metabolism. The histaminergic system might play a role permissively (Toftegaard et al. 2003).
Our findings revealed that administrating M₃/M₄ receptor antagonists with leptin caused a significant decline in leptin-induced hypophagia. The co-injection of an antagonist of the M₄ receptor with leptin significantly diminished the hypophagic effect of leptin. Co-administration of the antagonists of CRF₁/CRF₂ receptors+leptin led to a decline in the hypophagic effect of leptin. Injecting CRF₂ receptor antagonist with leptin reduced the hypophagic effect imposed by leptin. It has been suggested that the MC₄ receptor is of importance for signaling by leptin in rodents (Masaki et al. 2003). Our results were similar to the latter report. This receptor is known to be among the main pathways of leptin signaling in the hypothalamus (Satoh et al. 1998). Agouti protein may be over-expressed ectopically in Ay/a obese mice, which results in higher food intake and body weight due to the antagonistic impact of MC₄ receptor that causes notable obesity in the abdomen region and leptin-resistant diabetes (Halaas et al. 1997). Although leptin injection through the ICV route diminished food intake in wild-type mice, it did not happen in MC₄ receptor knockout obese or Ay/a obese mice (Masaki et al. 2003). The mentioned results demonstrate that the MC₄ receptors were involved in the regulation of leptin-induced hypophagia.

Leptin and the MC₄ receptor can severely elevate adiposity regardless of the type of diet consumed (Kenny et al. 2011). Overall, it is believed that MC₄ and H₁ receptors are involved in regulating energy homeostasis in the brain pathways downstream from the leptin action site. However, there is limited information about the relationship between neuronal histamine H₁ and MC₄ receptors (Masaki et al. 2003). Leptin has the potential to stimulate α-MSH, as well as M₃ and M₄ receptor antagonists. Moreover, it can inhibit Agouti-related peptide (AgRP), in addition to M₃ and M₄ receptor antagonists (Dridi et al. 2005). The CRH remarkably mediates the hypophagic effect of leptin (Uehara et al. 1998), which is in line with our findings and the results of Morimoto et al. (2000). The blockade of histamine synthesis by α-FMH inhibits CRH gene expression, which is stimulated by leptin in the PVN showing that the influence of leptin results from interaction with the histaminergic neurons (Toftegaard et al. 2003). Histaminergic fibers project to diverse discrete areas, such as the PVN, in which the leptin receptors are expressed (Toftegaard et al. 2003). Some authors suggest that leptin might stimulate CRH directly in the PVN. Furthermore, the inhibition of CRH expression due to histaminergic system blockade by α-FMH demonstrates that for CRH stimulation by leptin the activation of the neuronal histamine system is needed (permissive effect) (Toftegaard et al. 2003). Our findings indicated that H₁, H₃, M₃/M₄, and CRF₁/CRF₂ receptors mediate the hypophagic effect of leptin in neonatal layer chickens. Further studies are recommended for
clarifying the cellular and molecular signaling pathways that underlie the interactions between leptin, melanocortin, histamine systems, and food intake in neonatal chickens.

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Author's contribution
M. S: PhD student, experimental procedure, draft of paper
M. Z: supervisor, study design, revise of paper
B. V: supervisor
A. A: advisor

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Conflict of interest
Authors declare no conflict of interest

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**Fig. 1.** Effects of intracerebroventricular injection of control solution, α-FMH (250 nmol), leptin (10 µg) and co-injection of the α-FMH + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. α-FMH: alpha-fluoromethylhistidin. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time ($P < 0.05$).
**Fig. 2.** Effects of intracerebroventricular injection of control solution, chlorpheniramine (300 nmol), leptin (10 µg) and co-injection of the chlorpheniramine + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. Chlorpheniramine: H₁ receptor antagonist. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time (P < 0.05).

**Fig. 3.** Effects of intracerebroventricular injection of control solution, famotidine (82 nmol), leptin (10 µg) and co-injection of the famotidine + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. Famotidine: H₂ receptor antagonist. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time (P < 0.05).
Fig. 4. Effects of intracerebroventricular injection of control solution, thioperamide (300 nmol), leptin (10 µg) and co-injection of the thioperamide + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. Thioperamide: H₃ receptor antagonist. Data are expressed as mean ± SEM. Different letters (a, b and c) indicate significant differences between treatments at each time (P < 0.05).

Fig. 5. Effects of intracerebroventricular injection of control solution, SHU9119 (0.5 nmol), leptin (10 µg) and co-injection of the SHU9119 + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. SHU9119: M₃/M₄ receptors antagonist. Data are expressed as mean ± SEM. Different letters (a, b and c) indicate significant differences between treatments at each time (P < 0.05).
**Fig. 6.** Effects of intracerebroventricular injection of control solution, MCL0020 (0.5 nmol), leptin (10 µg) and co-injection of the MCL0020 + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. MCL0020: M₄ receptor antagonist. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time ($P < 0.05$).

**Fig. 7.** Effects of intracerebroventricular injection of control solution, astressin-B (30 µg), leptin (10 µg) and co-injection of the astressin-B + leptin on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. Astressin-B: CRF₁/CRF₂ receptors antagonist. Data are expressed as mean ± SEM. Different letters (a, b and c) indicate significant differences between treatments at each time ($P < 0.05$).
**Fig. 8.** Effects of intracerebroventricular injection of control solution, astressin2-B (CRF$_2$ receptor antagonist; 30 µg), leptin (10 µg) and co-injection of the astressin2-B + leptin (10 µg) on cumulative food intake (gr/100gr BW) in neonatal chicks. Control: normal saline. Astressin2-B: CRF$_2$ receptor antagonist. Data are expressed as mean ± SEM. Control: normal saline. Different letters (a and b) indicate significant differences between treatments at each time ($P < 0.05$).

**Table 1.** Ingredient and nutrient analysis of experimental diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>(%)</th>
<th>Nutrient analysis</th>
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<tbody>
<tr>
<td>Corn</td>
<td>52.85</td>
<td>ME, kcal/g</td>
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<tr>
<td>Soybean meal, 48% CP</td>
<td>37.57</td>
<td>Crude protein (%)</td>
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<tr>
<td>Wheat</td>
<td>5</td>
<td>Linoleic acid (%)</td>
</tr>
<tr>
<td>Gluten meal, 61% CP</td>
<td>2.50</td>
<td>Crude fiber (%)</td>
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<td>Wheat bran</td>
<td>2.47</td>
<td>Calcium (%)</td>
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<td>Di-calcium phosphate</td>
<td>1.92</td>
<td>Available phosphorus (%)</td>
</tr>
<tr>
<td>Oyster shell</td>
<td>1.23</td>
<td>Sodium (%)</td>
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<tr>
<td>Soybean oil</td>
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<td>Potassium (%)</td>
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<td>Vitamin premix</td>
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<tr>
<td>Acidifier</td>
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<td>Lysine (%)</td>
</tr>
<tr>
<td>Ingredient</td>
<td>ME (%)</td>
<td>Protein (%)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>DL-Methionine</td>
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<td>Methionine</td>
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<tr>
<td>Toxin binder</td>
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<td>Methionine + cystine</td>
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<td>L-Lysine HCl</td>
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<td>Threonine</td>
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<td>Vitamin D₃</td>
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<td>Tryptophan</td>
</tr>
<tr>
<td>Multi enzyme</td>
<td>0.05</td>
<td>Valine</td>
</tr>
</tbody>
</table>

**ME**: metabolisable energy, **CP**: crude protein, per kg of diet, the mineral supplement contains 35.2 g manganese from MnSO₄·H₂O; 22 g iron from FeSO₄·H₂O; 35.2 g zinc from ZnO; 4.4 g copper from CuSO₄·5H₂O; 0.68 g iodine from ethylene diamine dihydroiodide; 0.12 g selenium from Na₂SeO₃. The vitamin supplement contains 1.188 g of retinyl acetate; 0.033 g of dl-α-tocopheryl acetate; 8.84 g of tocopherol, 1.32 g of menadione, 0.88 g of thiamine, 2.64 g of riboflavin, 13.2 g of nicotinic acid, 4.4 g of pantothenic acid, 1.76 g of pyridoxin, 0.022 g of biotin, 0.36 g of folic acid, 1500 mg of choline chloride.