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Original Research Article

Effect of in-ovo injection of metformin on blood parameters and AMPK gene expression in liver of pre- and post-hatch broilers

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Abstract

Purpose: *To investigate the effect of metformin in-ovo injection on blood parameters and adenosine monophhosphate-activeed protein kinase (AMPK) gene expression in the liver of pre- and post-hatch broilers.*
Methods: *A total of 300 fertilized broiler breeders' eggs (Ross 308) were selected for injection of metformin. There were four treatment levels and four replicates. Each replicate contained 15 eggs. Treatment involved injecting a solution without metformin (physiological serum), injecting one ml of 2 mg/l metformin, injecting one ml of 4 mg/l metformin and injecting one ml of 6 mg/l metformin. The first experiment was conducted on one-day-old chicks and the second experiment on 42-day-old chicks. Blood parameters, including glucose, cholesterol, LDL (low density lipoprotein), HDL (high density lipoprotein) and VLDL (very low density lipoprotein) were examined in both the experiments. Gene expression was also tested separately.*
Results: *In the test on 1-day old chicks, the results of the effect of metformin on AMPK gene expression indicate that the treatment caused significantly modified the latter ($p < 0.05$). Furthermore, triglyceride, HDL, LDL and VLDL concentrations were significantly reduced by increasing concentration of metformin ($p < 0.05$). In the experiment on 42-day old chicks, the effect of metformin on the levels of the blood parameters was not significant ($p > 0.05$).*
Conclusion: *Metformin injection into one-day-old chicks activates lipid metabolism in the yolk sac of the embryo, resulting in the lipid depletion. However, there was no significant effect on AMPK gene expression and blood parameters in 42-day-old chicken due to the long interval between metformin injection (42 days).*

Keywords: *Metformin, AMPK gene expression, in-ovo injection, Broiler*

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INTRODUCTION

Metformin (N, N-Dimethylimidodicarbonimidic diamide) is a chemical drug with a molecular weight of 129. 16364 grams per mole and is presented as $C_4H_{11}N_5$. Metformin is one of the oldest and most widely used oral drugs for the treatment of type 2 diabetes. The mechanism of

action of metformin is such that it creates an obstruction in the complex 1 of mitochondrial respiratory chain (NADH-ubiquinone oxido-reductase). Metformin is a biguanide, like buformin and phenformin.

The main effect of metformin is reduced hepatic glucose production and increased cell sensitivity to peripheral insulin; thus it lowers blood sugar. Metformin reduced triglycerides [1], non-esterified fatty acid (NEFA) [2] and results in energy supply to the lipids and their oxidation by decreasing sensitivity to glucose. Its anti-diabetic activity occurs through AMPK activation, reduced hepatic glucose and increased glucose uptake in skeletal muscles. Metformin activates AMPK enzyme by *LKB1* gene [3].

AMPK is an enzyme that plays a key role in signaling insulin, the body's energy balance and glucose as well as lipid metabolism [4]. AMPK activation occurs by physiological stimulations such as muscle activity, physiological stress, secretion of hormones such as leptin and adiponectin, glucose reduction, tissue hypoxia, oxidative stress and osmotic shock. AMPK activation inhibits gluconeogenesis and lipogenesis and leads to energy production towards triglyceride oxidation and lipolysis. AMPK is activated by 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) and metformin [5] and then by inhibiting two key genes' expression by hepatic gluconeogenics called PEPCK (Phosphoenolpyruvate carboxykinase) and G6Pase (Glucose 6 phosphatase) it inhibits gluconeogenesis [6]. Another method of AMPK activation is AMP (Adenosine monophosphate) to ATP (Adenosine triphosphate) ratio so that it is activated by increasing AMP and decreasing ATP under stress conditions [7]. AMPK is a signal path for all reactions within the cells. This enzyme increases oxidation of fatty acids and produces energy. Many researchers have studied the effect of metformin on human and rats but few studies have been conducted on chickens and perhaps the *in-ovo* injection of metformin and analysis of metformin injection's effect on broilers has been addressed for the first time. Therefore, the aim of this study was to investigate the effect of metformin *in-ovo* injection on blood parameters and AMPK gene expression in the liver of pre-and post-hatch broilers.

EXPERIMENTAL

Experimental groups

This study was first conducted on chick embryo and given the limited resources there is no precise injection dosage. In order to ensure that the dosage would not be lethal for the chick embryo, two pretests were conducted to achieve the main and non-lethal dosage to chicken embryos. A total of 300 fertilized broilers' eggs (Ross 308) (breeder flock of age 57 weeks) were selected after candling the eggs and making sure about the fertilized eggs. They were weighed individually (average weight 59 ± 1 g). Each group consisted of four replications and each replication included 15 eggs and the treatment consisted of 60 fertilized eggs (a 60-member group of the embryonated egg was considered positive control without injection). Treatment involved injecting a solution without metformin (physiology serum as sham control), injecting one ml of 2 mg/l metformin, injecting one ml of 4 mg/l metformin and injecting one ml of 6 mg/l metformin. Metformin was prepared from a local pharmacy store.

Ethical approval

Approval was obtained for the animal studies from SRB of IAU (ref no. 2356002, 2016, 29.03), and international guidelines for animal studies were followed [9].

Injection process

To determine the exact location for injecting into the amniotic fluid, the blue Kumasi solution was injected from the broad side of the egg. On the 16^{th} day of incubation, all the eggs were candled prior to injection and analyzed in terms of fetal physiological conditions, fluidity of amniotic fluid and fetal motility. To determine the exact location for injection into the amniotic fluid, the blue Kumasi solution was injected from the broad side of the egg. To begin testing, the eggs were pierced by pins from the broad side and one ml of the solution was injected into the amniotic fluid of fertile eggs by a needle on a syringe (21). Syringes with 1m volume were used for injection. The injected part was disinfected by ethanol 70 % and blocked with liquid paraffin.

Incubation and hatching process

After injecting metformin, the eggs were transferred to the hatcher. Temperature and humidity of the hatcher (Petersime Model192, Manual) were 37.5 °C and 50 %, respectively. The chickens were removed after 508 h when only 5 % of the chicks was still not hatched [8].

Birds and diet

After hatching of 32 chicks, two chicks from each replicate and each treatment were transferred to

the Pasteur Institute of Iran for conducting experiments on gene expression and blood parameters (research unit located in the northern city of Amol). Also, 160 one-day-old chicks were randomly grouped and moved to the breeding hall following protocols of the Animal Care Committee of the Islamic Azad University (Science and Research Branch in Tehran, Iran). The chicks were reared in the breeding hall. The birds in each experimental group had free access to feed and safe water. Environmental conditions were the same for all the birds and there were 10 chicks per square meter. Lighting power was 30 to 40 lux and there were 23 h of light and 1 h of darkness. No medication and vaccination was used during the breeding period. The compositions of experimental diets (starter, grower and finisher) are presented in Table 1. Diets were based on UFFDA and nutrition guide of Ross 308.

Table 1: Composition of broiler diet

Ingredient (%)	Days 1–11	Days 12–24	Days 25–42
Corn	55.14	57.51	62.72
Soybean meal	39.4	36.43	30.98
Soybean oil	1.3	2.26	2.75
Di-calcium phosphate	1.55	1.44	1.3
Calcium carbonate	1.18	1.03	0.96
Mineral supplements	0.25	0.25	0.25
Vitamin supplements	0.25	0.25	0.25
DL-methionine	0.3	0.3	0.27
Lysine	0.24	0.18	0.19
Threonine	0.1	0.06	0.04
Salt	0.29	0.29	0.29
Chemical composition			
Metabolism energy (kcal/kg)	3016	3081	3105
Crude protein (%)	21.68	19.26	18.25

Composition of each kg of mineral supplement: 40000 mg manganese, 33880 mg zinc, 20000 mg iron, 4000 mg copper, 400 mg iodine, 80 mg selenium, and 100000 milligram choline chloride; The composition of each kg of vitamin supplement: 3600000 international units of vitamin A, 720 mg vitamin B_{1}, 2640 mg vitamin B_{2}, 4000 mg nicotinic acid, 1200 mg vitamin B_{6}, 400 mg folic acid, 6 mg vitamin B_{12}, 80000 International units of vitamin D_{3}, 7200 international units of vitamin E, 800 mg vitamin K_{3}, 40 mg biotin, and 100000 milligrams of antioxidants

Sampling

1st experiment

The weight of each egg and chick body weight as well as the body weight to the weight of the eggs ratio were determined. Hatchability of the fertilized eggs was determined by counting the number of hatched chicks in each group. For sampling the first stage, 32 one-day-old chicks (two chicks were chosen randomly from each replicate and each treatment) and blood sampling was performed. 1 ml of blood was collected from each chick and the chick's liver was removed. Blood samples were centrifuged at 1500 g for 10 min. The separated blood sera were frozen in vials at -20 °C. To measure the blood parameters (triglycerides, glucose, HDL, LDL and VLDL) Pars Azmoon commercial kits were used. *AMPK* gene expression stages were the same for the chickens and hens' livers.

2nd experiment

At the end of the breeding period, 32 hens and roosters, two pieces of each treatment and two replications were randomly selected and blood sampled. For carcass analysis, five pieces from each experimental group were randomly selected, weighed and killed by cervical dislocation. After filling, the carcass weight (without stomach) and the carcass components including weights of the liver, spleen and pancreas were measured after being removed. The livers were sent to the laboratory for gene expression tests.

AMPK gene expression studies

100 mg of liver samples with 5 ml phosphate buffered saline solution (PBS) with pH=4.7 were homogenized manually. After transferring the uniform liquid to Eppendorf, 1500×g centrifuge was done for 20 min at 4 °C. After centrifugation, the supernatant was collected with a swab and the lower transparent liquid was evacuated in ELISA kit wells by the sampler.

Standardization of kit

Standards included 5, 10, 20, 40, and mIU/L. Kit sensitivity in equivalent measure was 0.2 ng/ml. The steps and wavelength (450 nm) reading were performed according to the instructions by Shanghai Crystal day Biotech Co. (Table 2).

Table 2: *AMPK* gene standardization

L/mIU80	Standard No. 5	120 µl Standard thinners + 120 µl Original standard
L/mIU40	Standard No. 4	120 µl Standard thinners + 120 µl Standard No. 5
L/mIU20	Standard No. 3	120 µl Standard thinners + 120 µl Standard No. 4
L/mIU10	Standard No. 2	120 µl Standard thinners + 120 µl Standard No. 3
L/mIU5	Standard No. 1	120 µl Standard thinners + 120 µl Standard No. 2

Evaluation of biochemical parameters

Glucose, cholesterol, LDL, HDL and VLDL were examined in both the experiments. Blood samples of chickens were collected in tubes containing anticoagulant (EDTA) after killing them. The samples' plasma was separated after 5000×g centrifuging for five minutes and frozen inside the micro tubes at -20°C. Blood parameters were determined by autoanalyzer with Pars Azmoon commercial kits by enzymatic procedure and photometric principles.

Data analysis

The data were analyzed by SAS version 9.1 with completely randomized design of four treatment types and four replicates (Eq 1). Mean comparison was analyzed by Duncan's multiple range test at 5 % probability.

$Y_{ij}=\mu+T_i+e_{ij}$

where Y_{ij}= amount per view, μ = average population, T_i = treatment effect, and e_{ij} = effect of experimental error.

RESULTS

Gene expression (1st experiment)

Tests related to gene expression were conducted on the liver of 32 chick after isolation. There was a significant difference in the *AMPK* gene expression between treatment with concentrations of 2 and 4 ml ($p < 0.05$) (Table 3).

Table 3: Effect of metformin on *AMPK* gene expression related to the first experiment (n = 32)

AMPK concentration	Metformin dose
23.772[ab]	0 ml
25.932[a]	2 ml
20.186[b]	4 ml
23.768[ab]	6 ml
1.757	SEM

Note: [a-b] dissimilar letters in each column and each sub-group indicate significant difference ($p < 0.05$)

Second experiment gene expression

Tests related to gene expression were conducted on the liver of 32 chickens after isolation. There was no significant difference in the *AMPK* gene expression during treatment ($p > 0.05$) (Table 4). Table 4 presents the effect of metformin on *AMPK* gene expression related to the second experiment (average data of 32 chicks). In the first experiment, the regression coefficient was 0.9446 and the regression equation y = 0.0125x + 0.1326 while in the second experiment, they were 0.9985 and y = 0.0219x - 0.0881.

Table 4: Effect of metformin on *AMPK* gene expression related to the second experiment (mean data for 32 chicks)

AMPK concentration	Metformin dose
8.091	0 ml
10.937	2 ml
10.127	4 ml
12.558	6 ml
1.725	SEM

Note: [a-b] dissimilar letters in each column and each sub-group indicated significant differences ($p < 0.05$)

Serum biochemical profile

There was no significant difference in glucose concentration during treatment ($p > 0.05$). By increasing the concentrations of metformin, the chicken's triglycerides increased ($p < 0.05$). Cholesterol was not significantly different during treatment ($p > 0.05$). Chickens' LDL was increased with increasing concentrations of metformin ($p < 0.05$). Different concentrations of HDL and VLDL were observed by increasing concentrations of metformin (Table 5).

Table 5: Effect of metformin on serum biochemical parameters in the first experiment (n = 32)

Metformin	Glucose	Triglycerides	Cholesterol	LDL	HDL	VLDL
0 ml	276.7	91.6[b]	106.75[a]	49.695[b]	75.375[a]	18.32[a]
2 ml	270.72	86.951[b]	106.25[a]	51.89[b]	71.75[ab]	17.3903[b]
4 ml	284.5	88.79[b]	118[a]	66.133[a]	69.625[b]	17.758[ab]
6 ml	281.74	90.418[ab]	117.875[a]	61.209[ab]	74.75[a]	18.0835[ab]
SEM	13.77	1.17	4.148	4.2	1.66	0.22

Note: [a-b] dissimilar letters in each column and each sub-group indicated significant differences ($p < 0.05$)

In the second experiment, no significant difference was observed in blood parameters during treatment ($p > 0.05$) (Table 6).

DISCUSSION

In-ovo feeding is an important factor in improving intestinal growth of the fetus and newly hatched chicks. It also improves hatchability, growth, body and increases muscles weight. It reduces chick mortality after hatching. On fetal day 16 or 17, the amniotic fluid is used orally and with the arrival of nutrients to the intestines their function is improved; therefore, injecting nutrients into the amnion before hatching can be used as an external feed before the injection so that the injected nutrients are digested and absorbed in the intestinal tissues [10,11].

Table 6: Effect of metformin on serum biochemical parameters in the second experiment (n = 32)

Metformin dose	Glucose	Triglycerides	Cholesterol	LDL	HDL	VLDL
0 ml	203.750	89.625	100.875	88.75	77.5	17.92
2 ml	221	87.875	101.5	89.375	757.75	17.57
4 ml	216.75	87.875	98.75	91.429	78.42	17.2
6 ml	206.625	84	107.5	96.625	73.125	16.8
SEM	6.25	1.93	3.18	0.98	0.98	0.98

Note: a-b dissimilar letters in each column and each sub-group indicated significant differences (p <0.05)

AMPK is an enzyme that affects the energy balance of cells. With the activation of this enzyme, the signaling path from the hypothalamus to the target organs, including the brain, liver and muscles, is stimulated. Metformin activates protein kinase AMP in the hepatic cells; in this study, with increasing concentrations of metformin in the first experiment (chicken), *AMPK* gene expression was significant but it was not significant in the second experiment (chicken). Researchers found that the reduced Acetyl-CoA carboxylase activity oxidized the fatty acids and stopped lipogenic enzymes' expression [12]. Many studies have shown that AMPK disables Acetyl-CoA carboxylase and limits its activity in lipogenesis [13]. Zhou *et al* [12] demonstrated that AMPK inhibitor hinders the activity of metformin to prevent hepatic glucose production.

In the study by Ashwell and McMurtry [14], AMPK increased the reabsorption of glucose by the muscles. The duo's results were consistent with this study in that they found that metformin reduces triglyceride level in non-esterified fatty acid (NEFA). Decreasing sensitivity to glucose leads the source of energy to the lipids and disabling Acetyl-CoA carboxylase increases oxidation of fatty acids by reducing plasma triglycerides. Their research showed that within the first three hours of metformin intake, the feed intake, insulin, glucose and triglyceride concentrations reduced. But in this study, by increasing concentrations of injectable metformin during the first experiment, LDL concentration increased and triglyceride levels decreased significantly.

Results of the present study showed that in the second experiment, there were no significant changes in treatment for blood parameters (p >0.05). It is expected that by injecting metformin, after hatching and the beginning of breeding, especially in the first week, there is a significant reduction in infections caused by the yolk sac and umbilical cord due to the intake of yolk nutrients by embryos. In a study on diabetic rats, it was seen that by administering alloxan, an injection of metformin dose of 150 mg/kg reduced blood glucose, triglycerides, total cholesterol, LDL and VLDL and increased serum HDL significantly [15]. However, the results of this study showed that in the first experiment, by increasing concentrations of metformin, the levels of LDL increased and triglycerides, HDL and VLDL decreased.

During embryonic development, metformin adjusts fat metabolism pathways by activating the AMPK signaling pathway. Activation of the AMPK signaling pathway may increase ATGL protein expression, fatty acid β-oxidation, glucose transport and, thus, ATP generation [16]. Besides, lipolytic enzyme, metformin is also a potential AMPK activator [17]. The activation of AMPK phosphorylates ACC and then inhibits its activity; this results in a decrease in malonyl-CoA synthesis. The reduction of malonyl CoA may stimulate transportation of fatty acids into the mitochondria for β-oxidation via upregulation of the transporter of fatty acids, CPT1 [18]. Indeed, metformin lifted the phosphorylation of AMPK and ACC and the expression of CPT1, and it implies that metformin probably raises fatty acid β-oxidation and that is why the free fatty acid levels were decreased in a dose dependent manner both in the plasma and the liver. Here, significant linear relationships existed.

CONCLUSION

Metformin increases fatty acid oxidation of chick yolk and accelerates fetal use of this energy source by activating AMPK enzyme. This drug, when injected on day 16, increases fetal yolk consumption by the fetus. However, *AMPK* gene is not expressed in 42-day-old chickens due to the time lag between injection and the time of the test (42 days).

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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