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Influence of Dietary Metformin on the Growth Performance and Plasma Concentrations of Amino Acids and Advanced Glycation End Products in Two Types of Chickens

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Glycation is a non-enzymatic reaction inducing the bonding of glucose to amino acids and proteins. Glycated amino acids are not useful for protein synthesis, suggesting that glycation reduces the utilization of amino acids. Metformin (MF) is well known as a therapeutic drug for type II diabetes that inhibits glycation. It is possible that treatment with MF raises the utilization of amino acids by the inhibition of glycation, thereby improving the growth performance of chickens. In the present study, therefore, we investigated the influence of dietary MF on the growth performance, and plasma concentrations of free amino acids and N^{ϵ}-(Carboxymethyl)lysine (CML), which is an advanced glycation end product, in layer (Experiment 1) and broiler (Experiment 2) chickens. From 7 d of age, chicks were allowed free access to one of the experimental diets containing MF at 3 supplementation levels (0, 150, and 300 mg/kg diet) for 14 days. Body weight and feed intake were measured every week. At the end of the experiments, blood and breast muscle (M. pectoralis major) were collected for further analysis. Dietary MF did not affect weight gain, feed intake, or feed efficiency in both layer and broiler chickens. Dietary MF at the level of 150 mg/kg diet increased breast muscle weight in both layer and broiler chickens. Dietary MF increased plasma concentrations of branched chain amino acids and decreased concentrations of CML in layer chickens, although it did not affect plasma concentrations of glucose. The present study suggested that dietary MF might have the potency to increase breast muscle weight of layer chickens with an increment in plasma concentrations of branched-chain amino acids.

Key words: amino acids, broiler, N^{ϵ}-(Carboxymethyl)lysine, Glycation, layer, metformin

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Introduction

Glycation is a non-enzymatic reaction causing condensation between the carbonyl group of glucose and the amino group of proteins and amino acids. Glycation of proteins leads to the formation of a Schiff base, which rearranges to more stable Amadori products. Amadori products undergo further complex reactions such as cross-linking, oxidation, dehydration, and condensation to form advanced glycation end products (AGEs), which have a causative role in the development of diabetes complications represented by nephropathy, neuropathy, and retinopathy (Makita *et al*., 1994; Koschinsky *et al*., 1997; Brownlee, 2005).

Metformin (MF), which is one of the biguanide drugs, has been widely used as a first-line treatment for type II diabetes. Biguanides are composed of two guanidino groups combined with each other. Guanidino derivatives, including MF, have anti-hyperglycemic effects by inhibiting hepatic gluconeogenesis and improving insulin sensitivity (Rena *et al*., 2013; Foretz *et al*., 2014). It is well known that MF not only decreases blood glucose levels but also inhibits glycation, which is a chemical reaction easily carried out in diabetic patients (Rahbar *et al*., 2000; Beisswenger and Ruggiero-Lopez, 2003; Chakraborty *et al*., 2011). It was also reported that therapeutic MF reduced risk of diabetic complications (UK Prospective Diabetes Study (UKPDS) Group, 1998).

Chickens are known to be hyperglycemic animals, and their blood glucose level is over 200 mg/dL (Hazelwood and Lorenz, 1959). Because chickens have high blood glucose levels compared to mammals, their amino acids might be more easily converted to glycated amino acids. In our pre-

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vious study, it was revealed that approximately 10% of tryptophan in chicken plasma was glycated (Makino *et al*., 2015a). We also reported that glycated amino acids lost the capability to synthesize peptide bonds with other amino acids *in vitro* since these compounds lack the α-amino group (Makino *et al*., 2015b). These results suggest that dietary MF acts as an anti-glycation agent and could enhance the bioavailability of amino acids *in vivo*.

Therefore, the purpose of this study was to investigate the influence of dietary MF on the growth performance and plasma concentrations of free and glycated amino acids in two types of chickens.

Materials and Methods

Animals and Experimental Procedure

In Experiment 1, 50 1-day-old male layer chickens (White Leghorn, Julia) were purchased from a local hatchery (Ninobe Hatchery, Kagawa, Japan). A commercial chicken mash diet (240 g crude protein/kg, 12.8 MJ/kg metabolizable energy; Toyohashi Feed Mills Co. Ltd, Aichi, Japan) and water were available *ad libitum* to the chickens. Chickens were transferred to the experimental cage at least 2 d before the experiment in order for them to become accustomed to the experimental environment. The commercial diet was then changed to the control diet (Table 1). Before starting dietary treatments, body weight was measured and the chickens were distributed individually into experimental groups so that the average body weight was as uniform as possible. Twenty-four birds were selected and divided into 3 groups of 8 birds each. From 7 d of age, the chickens had free access to one of the experimental diets containing MF at 3 supplementation levels (0, 150, and 300 mg/kg diet) for 14 days. Body weight and feed intake were measured on days 7 and 14 of the experiment. At the end of the experiment, blood samples were collected by heart puncture from chickens anesthetized with isoflurane and transferred to heparin-containing micro-tubes. Blood samples were centrifuged at 3,000×g for 20 min at 4℃ to separate the plasma. Plasma samples were stored at −80℃ until analysis. After blood sampling, the right pectoralis major muscles (M. pectoralis major) were weighed and frozen in liquid nitrogen, and then stored at −80℃ until analysis.

In Experiment 2, 50 1-day-old male broiler chickens (Chunky, Ross308) were purchased from a local hatchery (Mori Breeding Farm Co. Ltd.). A commercial chicken mash diet (220 g crude protein/kg, 13.0 MJ/kg metabolizable energy; JA Nishinihon Kumiai Shiryou Corporation, Hyogo, Japan) and water were available *ad libitum* to the chickens. All other procedures were the same as those described in Experiment 1, except for the compositions of the experimental diets (Table 1).

This study was approved by the Committee of Animal Care and Use in Ehime University (No. 08011-10).

Sample Preparation for Analysis of Amino Acids, N^{ε}-(Carboxymethyl)lysine (CML), and MF

Tissue samples of the pectoralis major muscle were homogenized using a bead mill (TissueLyser LT, Qiagen, Germany) with an oscillation frequency of 50 Hz for 1 min. Homogenized tissues in Dulbecco's phosphate buffered saline were centrifuged at 14,000×g for 5 min at 4℃, and the supernatant was collected and stored at −80℃ until analysis. Before applying ultra performance liquid chromatography (UPLC), 100 μL of plasma or supernatant from the tissue samples was mixed with 50 μL of stable isotope labeled amino acid mixture solution (APDSTAG Wako Amino Acids Internal Standard Mixture Solution, Wako, Osaka, Japan) as an internal standard for amino acids and CML, and 400 ng aminoguanidine as an internal standard for MF. For deproteinization, an equal volume of 4 mmol/L perchloric acid was mixed with the sample and stored on ice for 10 min. Then, the samples were centrifuged at 14,000×g for 5 min at 4℃. The supernatant was passed through a 0.22

Table 1. **Composition of the control diet for layers and broilers**[a]

Ingredients	Layer (g/kg)	Broiler (g/kg)
ISP[b]	210.5	262.6
L-Methionine	0.8	2.2
L-Cystine	0.9	3.4
L-Threonine	0.4	0.0
Cornstarch	498.0	539.7
Cellulose	194.9	100.6
Corn oil	30.0	27.0
Vitamin mixture	2.0	2.0
Mineral mixture	60.0	60.0
Choline chloride	1.5	1.5
Inositol	1.0	1.0
Crude protein (CP)	18%	23%
Metabolizable energy (ME) (kcal/kg)	2850	3200

[a] Composition of diets was calculated according to National Research Council (1994).
[b] ISP is isolated soybean protein which contains 855 g CP/kg

Table 2. **Overview of *m/z* values for the analysis of amino acids, N^{ε}-(carboxymethyl) lysine, metformin, and their internal standards**

ID	Compounds	*m/z*	Internal standards	*m/z*
1	Alanine	90	Alanine-$^{13}C_3$	93
2	Arginine	175	Lysine-$^{13}C_6$, $^{15}N_2$	182
3	Asparagine	134	Asparagine-$^{13}C_4$, $^{15}N_2$	137
4	Aspartic Acid	134	Aspartic acid-2, 3, 3-d_3	137
5	Cystine	241	Cystine-3, 3', 3'-d_6	245
6	Glutamic Acid	148	Glutamic acid-$^{13}C_5$, ^{15}N	154
7	Glutamine	147	Glutamine-$^{13}C_5$, $^{15}N_2$	154
8	Glycine	76	Glycine-$^{13}C_5$, ^{15}N	79
9	Histidine	156	Histidine-$^{13}C_6$, $^{15}N_3$	165
10	Isoleucine	132	Isoleucine-$^{13}C_6$, ^{15}N	139
11	Leucine	132	Leucine-5, 5, 5-d3	135
12	Lysine	147	Lysine-^{13}C, $^{15}N_2$	155
13	Methionine	150	Mehionine-$^{13}C_5$, ^{15}N	156
14	Phenylalanine	166	Phenylalanine-$^{13}C_9$, ^{15}N	176
15	Proline	116	Proline-$^{13}C_5$, ^{15}N	122
16	Serine	106	Serine-$^{13}C_3$, ^{15}N	110
17	Threonine	120	Threonine-$^{13}C_4$	124
18	Tryptophan	205	Tryptophan-$^{13}C_{11}$, $^{15}N_2$	218
19	Tyrosine	182	Tyrosine (Ring-$^{13}C_6$)	188
20	Valine	118	Valine-$^{13}C_5$, $^{15}N_2$	124
21	Anserine	241	Histidine-$^{13}C_6$, $^{15}N_3$	165
22	Carnosine	227	Histidine-$^{13}C_6$, $^{15}N_3$	165
23	Citrulline	176	Citrulline-4, 4, 5, 5-d_4	180
24	Cystathionine	223	Citrulline-4, 4, 5, 5-d_4	180
25	Hydroxylysine	163	Ornithine-$^{13}C_5$	138
26	Hydroxyproline	132	Proline-$^{13}C_5$, ^{15}N	122
27	Monoethanolamine	62	Monoethanolamine-1, 1, 2, 2-d_4	66
28	N^{τ}-Methylhistidine	170	N^{τ}-methyl-d_3-histidine	173
29	N^{π}-Methylhistidine	170	N^{τ}-methyl-d_3-histidine	173
30	Ornithine	133	Ornitine-$^{13}C_5$	138
31	Sarcosine	90	Alanine-$^{13}C_3$	93
32	α-Amino butyric Acid	104	Glutamic acid-$^{13}C_5$, ^{15}N	154
33	α-Aminoadipic Acid	162	Alanine-$^{13}C_3$	93
34	β-Alanine	90	Alanine-$^{13}C_3$	93
35	γ-Aminobutyric Acid	104	Alanine-$^{13}C_3$	93
36	N^{ε}-(Carboxymethyl)lysine	205>84	Lysine-^{13}C, $^{15}N_2$	155
37	Metformin	130>60	Aminoguanidine	75>45

μm membrane filter.

Measurement of Amino Acids, CML, and MF in Plasma and Tissues

Separation of amino acids, CML, and MF was performed by UPLC (ACQUITY, Waters Corporation, Milford, MA, USA). The mobile phase A was 20% acetonitrile and 100 mM ammonium formate, while mobile phase B consisted of acetonitrile containing 0.3% formic acid. The flow rate was 600 μL/min. The sample injection volume was 10 μL. The temperature of the column (2.1×100 mm; Intrada Amino Acid, Imtakt Corporation., Kyoto, Japan) was set at 37℃. Samples were quantified using mass spectrometry (ACQUITY TQD, Waters Corporation) with electrospray ionization. Amino acids were measured in positive ion mode using single ion monitoring, and CML and MF were measured using multiple reaction monitoring (Table 2).

Measurement of Plasma Glucose

Measurement of plasma glucose was carried out using a commercial kit (Glucose CII test, Wako, Osaka, Japan) according to the manufacturer's instructions.

Statistical Analysis

All data are presented as mean±standard error (SE). Statistical analysis of growth performance was performed by repeated two-way analysis of variance (ANOVA), and other data analysis was performed by one-way ANOVA and Tukey's HSD test for multiple comparisons ($P<0.05$) using the General Linear Model Procedures of SAS (SAS/STAT version 9.4) (SAS Institute, 2012).

Results

Growth Performance and Tissue Weight

Body weight gain, feed intake, and feed efficiency of layer and broiler chickens fed experimental diets with various MF

Table 3. **Body weight gain, feed intake, and feed efficiency of layer and broiler chickens fed a diet with various metformin concentrations**

Metformin (mg/kg)	Week	Experiment 1 (Layer)			Experiment 2 (Broiler)		
		Body weight gain (g)	Feed intake (g)	Feed efficiency (%)	Body weight gain (g)	Feed intake (g)	Feed efficiency (%)
0	0–1	46.4±2.4	109.3±2.6	42.5±2.2	163.0±8.8	216.4±8.6	75.1±1.4
	1–2	40.9±3.5	124.4±4.4	32.8±2.8	174.4±12.8	321.4±14.0	54.3±3.4
150	0–1	53.8±2.5	117.3±3.5	45.8±1.6	162.6±7.9	210.2±7.0	77.1±1.7
	1–2	44.1±3.5	140.1±5.1	31.5±1.9	200.8±18.6	347.9±20.0	57.9±4.6
300	0–1	46.0±1.7	105.8±2.8	43.5±1.4	139.6±5.8	198.7±4.7	70.2±2.0
	1–2	50.4±5.0	144.5±17.2	36.1±3.6	156.9±16.0	302.7±29.5	51.9±3.7
Analysis of variance (probability)							
Metformin		0.21	0.37	0.66	0.06	0.27	0.13
Week		0.19	<0.01	<0.01	<0.05	<0.01	<0.01
Metformin x Week		0.11	0.26	0.36	0.48	0.48	0.91

Values are given as mean±SE. The number of chickens used in each treatment group was 8.

Table 4. **Breast muscle (right M. *pectoralis major*) weight of layer and broiler chickens fed a diet with various metformin concentrations**

Metformin (mg/kg)	Experiment 1 (Layer)	Experiment 2 (Broiler)
	Breast muscle weight (g)	Breast muscle weight (g)
0	4.7±0.2[b]	35.2±1.6[b]
150	5.6±0.2[a]	41.6±1.8[a]
300	5.3±0.3[ab]	32.8±1.8[b]

[a, b] Means not sharing a common superscript letter in the same column were significantly different (Tukey's HSD test, $P<0.05$). Values are given as mean±SE. The number of chickens used in each treatment group was 8.

levels are shown in Table 3. Body weight gain, feed intake, and feed efficiency of layer chickens were not affected by dietary MF. Although feed intake and feed efficiency of broiler chickens were also not affected, body weight gain tended to be decreased ($P=0.06$) after feeding with 300 mg MF/kg diet.

Breast muscle (M. pectoralis major) weights of the chickens fed on diets including MF are shown in Table 4. Pectoralis major muscle weights of both layer and broiler chickens in the 150 mg/kg MF group were heavier than those in the control group.

Plasma Concentrations of MF, glucose, CML, and Free Amino Acids

MF concentrations in the plasma and breast muscle of layer and broiler chickens are shown in Table 5. MF levels in the plasma and breast muscle of both layer and broiler chickens were increased by dietary MF supplementation.

Plasma concentrations of glucose and CML in layer and broiler chickens are shown in Table 6. Plasma glucose concentration was not affected by dietary MF in both layer and broiler chickens. The CML levels in the plasma of layer chickens decreased as the dietary MF content increased. However, the plasma levels of CML in broiler chickens were not affected by dietary MF supplementation.

Free amino acid profiles in the plasma of layer chickens fed diets supplemented with MF are shown in Table 7. For amino acids, which can be a precursor of body proteins, dietary MF supplementation at levels of 150 and 300 mg/kg diet significantly increased the plasma concentrations of alanine, isoleucine, leucine, and valine in layer chickens. A significant increment of aspartic acid and glutamic acid was observed only in the MF 300 mg/kg diet group. The plasma concentration of N^{τ}-methylhistidine increased with the increment in dietary MF levels.

Free amino acid profiles in the plasma of broiler chickens fed diets supplemented with MF are shown in Table 8. Except for β-alanine, dietary MF supplementation did not affect plasma amino acid concentrations.

Discussion

It has been reported that MF decreases feed intake in mammals and broilers (Rouru *et al*., 1992; Lee and Morley, 1998). Ashwell and McMurty (2003) showed that oral administration of a single dose of 300 mg MF/kg body weight reduced feed intake, and chronic intake of MF in drinking water also reduced feed intake and body weight gain in broilers. As shown in Table 3, although dietary MF did not affect both body weight gain and feed intake in layer chickens, body weight gain of broiler chickens tended to be decreased ($P=0.06$) by feeding on a diet including 300 mg MF/kg diet. It is considered that dietary MF might affect growth performance in broilers but not in layers at an optimal amount of dietary MF.

Avian species, including chickens, have high blood glucose levels which are approximately two to three times higher than healthy humans. This feature implies that the

Table 5. **Metformin concentration in the plasma, breast muscle (right M. *pectoralis major*), and liver of layer and broiler chickens fed a diet with various metformin concentrations**

Metformin (mg/kg)	Experiment 1 (Layer)		Experiment 2 (Broiler)	
	Plasma (μmol/L)	Breast muscle (nmol/g tissue)	Plasma (μmol/L)	Breast muscle (nmol/g tissue)
0	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
150	0.9±0.0^{b}	1.8±0.8^{a}	17.9±7.0^{ab}	0.8±0.3^{b}
300	1.7±0.0^{a}	2.0±0.6^{a}	35.2±8.4^{a}	1.7±0.3^{a}

$^{a-c}$ Means in the same column not sharing a common superscript letter were significantly different (Tukey's HSD test, $P<0.05$). Values are given as mean±SE. The number of chickens used in each treatment group was 8.

Table 6. **Glucose and N^{ε}-(carboxymethyl)lysine concentrations in the plasma of layer and broiler chickens fed a diet with various metformin concentrations**

Metformin (mg/kg)	Experiment 1 (Layer)		Experiment 2 (Broiler)	
	Glucose (mg/100 mL)	N^{ε}-(Carboxymethyl)lysine (ng/mL)	Glucose (mg/100 mL)	N^{ε}-(Carboxymethyl)lysine (ng/mL)
0	265.8±7.7	0.43±0.03^{a}	331.2±22.6	23.2±5.8
150	274.0±8.0	0.36±0.02^{ab}	360.1±36.5	26.3±6.6
300	278.6±9.5	0.33±0.03^{b}	310.4±15.7	19.7±4.1

$^{a, b}$ Means not sharing a common superscript letter in the same column were significantly different (Tukey's HSD test, $P<0.05$). Values are given as mean±SE. The number of chickens used in each treatment group was 8.

utilization of amino acids would be easily inhibited by glycation in avian species. In our previous study, it was revealed that approximately 10% of tryptophan in chicken plasma was glycated (Makino *et al*., 2015a) and that these compounds lost the capability to synthesize peptide bonds with other amino acids because of the lack of the α-amino group in the glycated amino acids (Makino *et al*., 2015b). In the present study, we expected the positive effect of dietary MF, which is an anti-diabetes drug, to inhibit glycation and to improve the growth performance of chickens. As shown in Table 4, the weight of breast muscle in both layers and broilers fed the diet with 150 mg/kg MF were heavier than those of the control group. Increment in tissue weight is caused by protein deposition, which can be expressed as the difference between protein synthesis and protein degradation. As shown in Table 7, the plasma concentration of N^{τ}-methylhistidine, which is posttranslationally modified histidine found in muscular protein and is known as an index of protein degradation (Young and Munro, 1978), was increased in layer chickens with elevated dietary MF contents. It has been reported that MF increases gene expression and protein levels of myostatin in the C2C12 myotube cell line (Das *et al*., 2012). Myostatin is known as an inhibitor of myogenesis. Protein degradation was promoted in the soleus of Wister rats treated with myostatin (Manfredi *et al*., 2017). These findings suggest that MF feeding would enhance protein degradation of skeletal muscle. The fact that the weight of breast muscle rose despite the increase in muscle protein degradation caused by dietary MF suggests that muscle protein synthesis increased more than protein degradation. As shown in Table 7, dietary MF increased the plasma concentrations of leucine and isoleucine in layer chickens. Recently, it has been reported that MF suppresses branched-chain amino acid (BCAA) catabolic enzyme expression in C2C12 myotubes (Rivera *et al*., 2020). Leucine and isoleucine are known as BCAAs which have a role in promoting protein synthesis (Alvestrand *et al*., 1990; Nair *et al*., 1992; Blomstrand *et al*., 1997). It was reported that leucine serves as a substrate to directly activate the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway (Dodd and Tee, 2012). These results suggest that MF might raise muscle weight due to the increment in plasma concentrations of leucine and isoleucine, stimulating protein synthesis. On the other hand, there were no significant differences observed in the plasma concentrations of leucine and isoleucine among the groups of broiler chickens. It is unclear whether the mechanisms of breast muscle mass increment in broiler chickens are the same as in layer chickens. It does not seem that MF directly increases protein accumulation in skeletal muscle, because MF contributes to AMP-activated protein kinase (AMPK) activation in the skeletal muscle (Zhou *et al*., 2001), and AMPK activation leads to diminished protein synthesis and promotes protein degradation (Kjøbsted *et al*., 2018). The concentration of BCAAs might increase in skeletal muscle, although only plasma amino acids were measured in this study. Since MF suppressed BCAA catabolic enzyme mRNA expression in myotubes (Rivera *et al*., 2020), it is possible that BCAA

Table 7. **The profile of free amino acids in the plasma of layers fed a diet with various metformin concentrations**

Amino acids (μmol/L)	Metformin (mg/kg)		
	0	150	300
Essential amino acids			
Arginine	84.0±6.9	109.7±8.7	100.4±6.8
Histidine	140.6±11.6	135.6±7.9	163.1±13.9
Isoleucine	118.5±6.9^{b}	151.0±8.2^{a}	159.2±10.9^{a}
Leucine	165.8±7.9^{b}	195.9±7.0^{a}	194.0±7.9^{a}
Lysine	293.7±23.2	286.0±14.1	313.1±15.7
Methionine	51.4±3.5	60.1±5.8	62.0±6.0
Phenylalanine	105.1±3.7	111.1±2.4	110.0±4.5
Threonine	340.6±21.6	314.3±5.8	336.1±10.2
Tryptophan	72.9±3.0	74.7±3.7	71.4±4.2
Valine	234.8±12.9	276.3±10.9	274.0±13.3
Nonessential amino acids			
Alanine	370.4±20.3^{b}	467.8±18.7^{a}	440.8±17.5^{a}
Asparagine	498.0±40.1	666.3±49.3	673.2±68.6
Aspartic acid	25.5±2.8^{b}	27.1±4.6^{b}	46.0±6.2^{a}
Cystine	29.6±3.4	32.0±4.9	37.1±2.6
Glutamine	637.7±33.2	595.8±27.7	646.0±22.1
Glutamic acid	150.6±10.8^{b}	163.6±9.7^{b}	203.2±11.6^{a}
Glycine	392.4±24.2	405.5±20.0	414.3±21.2
Proline	405.7±42.3	477.1±32.7	534.7±35.2
Serine	498.8±42.4	507.4±18.5	462.1±19.5
Tyrosine	120.4±6.2	125.2±4.8	133.3±8.7
α-Aminoadipic acid	5.3±1.2^{b}	7.5±0.5^{ab}	10.9±1.3^{a}
α-Aminobutyric acid	7.4±2.6	6.2±0.8	8.5±2.9
Amino ethanol	7.0±0.5	7.0±0.4	8.3±0.4
Anserine	9.2±0.5^{b}	10.2±0.7^{ab}	12.6±1.1^{a}
β-Alanine	13.3±1.7	12.1±1.0	12.0±1.0
Carnosine	10.4±0.6^{b}	11.0±0.4^{ab}	12.7±0.5^{a}
Citrulline	6.9±0.7	11.5±2.2	11.6±2.1
Cystathionine	8.0±1.6	7.5±1.3	11.7±2.7
Hydroxylysine	2.7±0.7	3.5±0.6	3.6±0.6
Hydroxyproline	132.1±12.2^{b}	294.7±27.3^{a}	330.5±34.2^{a}
Ornithine	57.8±8.1	63.5±7.7	67.5±6.2
Sarcosine	1.7±0.3	1.6±0.2	1.7±0.3
N^{π}-Methylhistidine	24.5±2.6	26.1±2.7	29.0±3.6
N^{τ}-Methylhistidine	9.8±1.2^{b}	15.2±1.2^{ab}	18.0±1.7^{a}

$^{a, b}$ Means not sharing a common superscript letter in the same row were significantly different (Tukey's HSD test, $P<0.05$). Values are given as mean±SE. The number of chickens used in each treatment group was 8. ND; not detected.

levels increase in the skeletal muscle. Therefore, the increment of muscle protein synthesis by BCAAs could partially explain the increase in muscle weight in broilers.

As shown in Table 5, dietary MF increased MF levels in the plasma and the breast muscle of both layer and broiler chickens. This result reveals that dietary MF is successfully absorbed from the gastrointestinal tract and elevates plasma and muscular concentrations of MF in chickens. Interestingly, plasma concentrations of MF in broilers were dozens of times higher than those in layers. As the feed intake of broilers was higher than that of layers (Table 3), the difference in MF intake caused by the different feed intake would be partially involved in the difference in plasma MF concentration between broilers and layers. Another possible explanation is that the rate of MF elimination might be different between layer and broiler chickens. MF in blood circulation is transferred to tissues via organic cation transporters (OCTs), and further excreted from the kidney into the urine via multidrug and toxin extrusion protein (MATE) (Gong *et al*., 2012). If the broiler chickens excrete less MF in the urine because the levels of OCT and/or MATE in the broilers are lower than in the layer chickens, that might be the reason for the high MF levels in the plasma of broilers. However, the explanations for the difference in plasma MF levels between layer and broiler chickens are limited due to the lack of knowledge about MF metabolism in birds.

Despite dietary MF being absorbed by the chickens, the plasma glucose level was not changed in both layer and

Table 8. **The profile of free amino acids in the plasma of broilers fed a diet with various metformin concentrations**

Amino acids (μmol/L)	Metformin (mg/kg)		
	0	150	300
Essential amino acids			
Arginine	217.2±17.0	202.9±14.6	170.0±10.3
Histidine	128.2±14.6	105.4±7.6	127.1±8.5
Isoleucine	174.6±21.2	149.3±18.5	139.3±9.3
Leucine	176.9±10.9	153.9±7.5	158.1±7.4
Lysine	227.8±12.4	217.4±8.3	236.7±5.1
Methionine	62.4±5.0	61.1±4.8	58.2±5.5
Phenylalanine	124.7±8.1	118.8±4.1	126.0±5.9
Threonine	244.8±16.9	229.0±22.1	256.3±15.2
Tryptophan	129.7±13.0	132.1±16.5	117.1±9.5
Valine	247.3±27.6	214.4±30.7	209.4±10.6
Nonessential amino acids			
Alanine	401.3±32.6	387.4±19.7	416.7±21.5
Asparagine	962.7±201.9	1146.0±195.8	889.9±125.3
Aspartic acid	62.0±8.1	46.1±10.5	73.7±13.3
Cystine	66.2±5.0	57.7±5.7	59.0±5.2
Glutamine	828.2±53.3	973.7±128.6	783.0±49.4
Glutamic acid	168.2±21.1	158.1±17.3	134.0±13.2
Glycine	322.3±26.6	422.4±85.7	320.8±28.6
Proline	284.2±13.8	257.5±17.3	268.8±19.7
Serine	304.0±25.1	394.2±84.4	414.3±56.7
Tyrosine	170.3±13.7	170.7±14.3	146.0±10.3
α-Aminoadipic acid	4.2±0.3	4.7±1.1	4.6±0.3
α-Aminobutyric acid	8.9±2.0	7.3±0.6	8.0±0.8
Amino ethanol	10.4±1.2	11.3±1.5	10.8±0.9
Anserine	11.2±1.9	12.0±1.7	10.9±1.2
β-Alanine	35.3±4.3[a]	27.4±5.4[ab]	18.5±3.4[b]
Carnosine	22.3±2.4	27.6±1.7	22.7±3.5
Citrulline	9.5±1.1	8.9±0.8	10.5±1.0
Cystathionine	34.4±10.0	32.4±6.7	25.4±3.9
Hydroxylysine	2.0±0.3	2.2±0.6	2.3±0.4
Hydroxyproline	39.7±4.3	46.0±1.8	39.2±5.5
Ornithine	101.3±12.2	82.8±8.5	103.2±6.3
Sarcosine	2.7±0.6	2.0±0.3	1.8±0.5
N^{π}-Methylhistidine	13.3±1.3	13.5±1.1	15.5±0.8
N^{τ}-Methylhistidine	9.4±1.4	8.1±1.2	12.0±1.7

[a, b] Means in the same row not sharing a common superscript letter were significantly different (Tukey's HSD test, $P<0.05$). Values are given as mean±SE. The number of chickens used in each treatment group was 8. ND; not detected.

broiler chickens (Table 6). MF has great properties for improving insulin sensitivity and reducing blood glucose levels. A possible explanation for these conflicting results is that homeostasis is maintained, because a blood glucose level of around 300 mg/100 mL is the normal condition for chickens. For instance, oral administration of MF to chickens has a temporary hypoglycemic effect, but their blood glucose levels then return to pre-dose levels (Ashwell and McMurty, 2003). It was reported that oral administration of MF to healthy SD rats had no effect on blood glucose levels (Zhou *et al*., 2001).

MF inhibits the production of AGEs by reacting with α-dicarbonyl compounds, forming triazepinone-derivatives (Ruggiero-Lopez *et al*., 1999). In the present study, CML was measured as an index of AGE production. While the plasma concentration of CML in layer chickens was reduced by elevating dietary MF contents (Table 6), the plasma CML concentration in broiler chickens was not affected by dietary MF. In addition, the plasma levels of CML in broilers were approximately one hundred times higher than those in layers. This result indicates that the metabolism of CML might be different in layer and broiler chickens. However, studies of AGE metabolism in chickens have not been conducted, thus this issue needs to be investigated in the future. Although AGE formation decreased with increased dietary MF, suggesting that MF might be involved in suppression of glycation, it has not been clarified whether or not dietary MF would successfully inhibit the formation of glycated amino

acids. This issue should be investigated in the future. Even if inhibiting glycation does prove to promote meat production in chickens, it is not practical to use MF as a feed additive. In the future, we will need to search for other feed materials that have anti-glycation properties. Some compounds known to have anti-glycation activity include pyridoxamine (Onorato *et al*., 2000), thiamine (Babaei-Jadidi *et al*., 2003), citric acid (Nagai *et al*., 2010), and quercetin (Li *et al*., 2014). Various plants have been also shown to have anti-glycation properties (Sadowska-Bartosz and Bartosz, 2015; Asgharpour *et al*., 2019), and some natural products could likely be added to the diet. For example, thiamin hydrochloride is a vitamin approved for use as a feed additive. Pyridoxine hydrochloride, a precursor to pyridoxamine, is also allowed. Adding these additives and plants with anti-glycation properties to the feed might provide MF-like effects.

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Conflicts of Interest

The authors declare no conflict of interest.

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