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 Nε-(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, Nε-(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-
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 microtubes. Blood samples were centrifuged at 3,000×g for 20 min at 4°C to separate the plasma. Plasma samples were stored at −80°C 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°C 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 Nishinohon 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, N²-(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°C, and the supernatant was collected and stored at −80°C 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°C. The supernatant was passed through a 0.22

| Ingredients              | Layer (g/kg) | Broiler (g/kg) |
|--------------------------|--------------|----------------|
| ISPb                     | 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
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, Intakt Corporation, Kyoto, Japan) was set at 37°C. 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).

| ID | Compounds                     | m/z | Internal standards        | m/z |
|----|-------------------------------|-----|---------------------------|-----|
| 1  | Alanine                       | 90  | Alanine-13C3              | 93  |
| 2  | Arginine                      | 175 | Lysine-13C6, 15N2         | 182 |
| 3  | Asparagine                    | 134 | Asparagine-13C4, 15N2     | 137 |
| 4  | Aspartic Acid                 | 134 | Aspartic acid-2, 3, 3-d3  | 137 |
| 5  | Cystine                       | 241 | Cystine-3, 3', 3'-d6      | 245 |
| 6  | Glutamic Acid                 | 148 | Glutamic acid-13C5, 15N   | 154 |
| 7  | Glutamine                     | 147 | Glutamine-13C5, 15N2      | 154 |
| 8  | Glycine                       | 76  | Glycine-13C5, 15N         | 79  |
| 9  | Histidine                     | 156 | Histidine-13C6, 15N3      | 165 |
| 10 | Isoleucine                    | 132 | Isoleucine-13C6, 15N      | 139 |
| 11 | Leucine                       | 132 | Leucine-5, 5, 5-d3        | 135 |
| 12 | Lysine                        | 147 | Lysine-13C6, 15N2         | 155 |
| 13 | Methionine                    | 150 | Methionine-13C5, 15N      | 156 |
| 14 | Phenylalanine                 | 166 | Phenylalanine-13C9, 15N   | 176 |
| 15 | Proline                       | 116 | Proline-13C5, 15N         | 122 |
| 16 | Serine                        | 106 | Serine-13C3, 15N          | 110 |
| 17 | Threonine                     | 120 | Threonine-13C4            | 124 |
| 18 | Tryptophan                    | 205 | Tryptophan-13C11, 15N2    | 218 |
| 19 | Tyrosine                      | 182 | Tyrosine (Ring-13C6)      | 188 |
| 20 | Valine                        | 118 | Valine-13C6, 15N2         | 124 |
| 21 | Anserine                      | 241 | Histidine-13C6, 15N3      | 165 |
| 22 | Carnosine                     | 227 | Histidine-13C6, 15N3      | 165 |
| 23 | Citrulline                    | 176 | Citrulline-4, 4, 5, 5-d4  | 180 |
| 24 | Cystathionine                 | 223 | Citrulline-4, 4, 5, 5-d4  | 180 |
| 25 | Hydroxylysine                 | 163 | Ornithine-13C5            | 138 |
| 26 | Hydroxyproline                | 132 | Proline-13C5, 15N         | 122 |
| 27 | Monoethanolamine              | 62  | Monoethanolamine-1, 1, 2, 2-d4 | 66 |
| 28 | N’-Methylhistidine            | 170 | N’-methyl-di-histidine    | 173 |
| 29 | N’-Methylhistidine            | 170 | N’-methyl-di-histidine    | 173 |
| 30 | Ornithine                     | 133 | Ornithine-13C5            | 138 |
| 31 | Sarcosine                     | 90  | Alanine-13C3              | 93  |
| 32 | α-Amino butyric Acid          | 104 | Glutamic acid-13C5, 15N   | 154 |
| 33 | α-Aminoadipic Acid            | 162 | Alanine-13C3              | 93  |
| 34 | β-Alanine                     | 90  | Alanine-13C3              | 93  |
| 35 | γ-Aminobutyric Acid           | 104 | Alanine-13C3              | 93  |
| 36 | N’-(Carboxymethyl)lysine     | 205>84 | Lysine-13C, 15N2        | 155 |
| 37 | Metformin                     | 130>60 | Aminoguanidine            | 75>45 |

### 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
levels are shown in Table 3. Body weight gain, feed intake, and feed efficiency of layer and broiler 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\(^{\tau}\)-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 \(\beta\)-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
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, dietary MF increased the plasma concentrations of leucine and isoleucine in layer chickens. It does not seem that MF directly increases 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., 1992; Blomstrand 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

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, 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., 1992; Blomstrand 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 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±0.0b             | 0.0±0.0b               | 0.0±0.0b        | 0.0±0.0b                     |
| 150               | 0.9±0.7b             | 1.8±0.8a               | 17.9±7.0b       | 0.8±0.3b                     |
| 300               | 1.7±0.0a             | 2.0±0.6a               | 35.2±8.4a       | 1.7±0.3a                     |

*a,b* 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 Nε-(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)  | Nε-(Carboxymethyl)lysine (ng/mL) | Glucose (mg/100 mL) | Nε-(Carboxymethyl)lysine (ng/mL) |
| 0                 | 265.8±7.7            | 0.43±0.03a             | 331.2±22.6        | 23.2±5.8                      |
| 150               | 274.0±8.0            | 0.36±0.02ab            | 360.1±36.5        | 26.3±6.6                      |
| 300               | 278.6±9.5            | 0.33±0.03b             | 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.
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 because the levels of OCT and/or MATE in the in the urine because the levels of OCT and/or MATE in the

**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±6.9³       | 151.0±8.2⁴        | 159.2±10.9⁴ |
| Leucine                    | 165.8±7.9³       | 195.9±7.0⁴        | 194.0±7.9⁴ |
| 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 |
| Phenylation                 | 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       | 467.8±18.7         | 440.8±17.5³ |
| Asparagine                 | 498.0±40.1       | 666.3±49.3         | 673.2±68.6 |
| Aspartic acid              | 25.5±2.8³        | 27.1±4.6⁴         | 46.0±6.2⁴ |
| Cysteine                   | 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±10.8³      | 163.6±9.7⁴        | 203.2±11.6⁴ |
| 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-Aminoacidic acid         | 5.3±1.2³         | 7.5±0.5³           | 10.9±1.3³ |
| 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±0.5³         | 10.2±0.7³          | 12.6±1.1³ |
| β-Alanine                  | 13.3±1.7         | 12.1±1.0           | 12.0±1.0 |
| Carnosine                  | 10.4±0.6³        | 11.0±0.4³          | 12.7±0.5³ |
| 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       | 294.7±27.3³       | 330.5±34.2³ |
| 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 |
| N⁵-Methylhistidine         | 24.5±2.6         | 26.1±2.7           | 29.0±3.6 |
| N⁵-Methylhistidine         | 9.8±1.2³         | 15.2±1.2³          | 18.0±1.7³ |

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.
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.

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-Aminoacidic 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±4.3a        | 27.4±5.4ab | 18.5±3.4ab |
| 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   |
| Nα-Methylhistidine   | 13.3±1.3         | 13.5±1.1  | 15.5±0.8  |
| Nα-Methylhistidine   | 9.4±1.4          | 8.1±1.2   | 12.0±1.7  |

*p<0.05. Values are given as mean±SE. The number of chickens used in each treatment group was 8. ND; not detected.
 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.

References

Alvesson, E., Hagenfeldt, L., Merli, K., Oureshi, A. and Eriksson, L.S. 1990. Influence of leucine infusion on intracellular amino acids in humans. European Journal of Clinical Investigation, 20: 293–298.

Asgharpour, D.F., Ranjekesh, Z. and Goodarzi, M.T. 2003. A systematic review of antglycation medicinal plants. Diabetes and Metabolic Syndrome, 13: 1225–1229.

Ashwell, C.M. and McMurtry, J.P. 2019. Hypoglycemia and reduced feed intake in broiler chickens treated with metformin. Poultry Science, 82: 106–110.

Babaei-Jadidi, R., Karachalias, N., Ahmed, N., Battah, S. and Thornalley, P.J. 2003. Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. Diabetes, 52: 2110–2120.

Beisswenger, P. and Ruggiero-Lopez, D. 2003. Metformin inhibition of glycation processes. Diabetes and Metabolism, 29: 6895–68103.

Brownlee, M. 2005. The pathobiology of diabetic complications: a unifying mechanism. Diabetes, 54: 1615–1625.

Blomstrand, E., Hassme’n, P., Ek, S., Ekblom, B. and Newsholme, E.A. 1997. Influence of ingesting a solution of branched-chain amino acids on perceived exertion during exercise. Acta Physiologica Scandinavica, 159: 41–49.

Chakraborty, A., Chowdhury, S. and Bhattacharyya, M. 2001. Effect of metformin on oxidative stress, nitrosative stress and inflammatory biomarkers in type 2 diabetes patients. Diabetes Research and Clinical Practice, 93: 56–62.

Das, A.K., Yang, Q-Y., Fu, X., Liang, J-F., Duarte, M.S., Zhu, M-J., Trobridge, G.D. and Du, M. 2012. AMP-activated protein kinase stimulates myostatin expression in C2C12 cells. Biochemical and Biophysical Research Communications, 427: 36–40.

Dodd, K.M. and Tee, A.R. 2012. Leucine and mTORC1: a complex relationship. American Journal of Physiology: Endocrinology and Metabolism, 302: E1329–E1342.
Rivera ME, Lyon ES and Vaughan RA. Effect of metformin on myotube BCAA catabolism., Journal of Cellular Biochemistry, 121: 816–827. 2020

Rouru J, Haupponen R, Pesonen U and Koulu M. Subchronic treatment with metformin produces anorectic effect and reduces hyperinsulinemia in genetically obese Zucker rats. Life Sciences, 50: 1813–1820. 1992.

Ruggiero-Lopez D, Lecomte M, Moinet G, Patereau G, Lagarde M and Wiernsperger N. Reaction of metformin with dicarbonyl compounds. Possible implication in the inhibition of advanced glycation end product formation. Biochemical Pharmacology, 58: 1765–1773. 1999.

Sadowska-Bartosz I and Bartosz G. Prevention of protein glycation by natural compounds. Molecules, 20: 3309–3334. 2015.

Tachibana T, Sato M, Oikawa D and Furuse M. Involvement of CRF on the anorexic effect of GLP-1 in layer chicks. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 143: 112–117. 2006.

UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet, 352: 854–865. 1998.

Young VR and Munro HN. Ntau-methylhistidine (3-methylhistidine) and muscle protein turnover: an overview. Federation Proceedings, 37, 2291–2300. 1978.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ and Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action., Journal of Clinical Investigation, 108: 1167–1174. 2001.