

《Research Note》

Effects of Supplementation with Dried Neem Leaf Extract on Lipid Peroxidation and Antioxidant Enzyme mRNA Expression in the Pectoralis Major Muscle of Broiler Chickens

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This study was conducted to evaluate the effects of dietary supplementation of dried neem (*Azadirachta indica*) leaf extract (DNE) on lipid peroxidation and the expression of genes encoding mRNAs in antioxidant enzymes in the pectoralis major muscle of chickens. A total of 24 male broiler chickens (ROSS308) were divided into three groups ($n = 8$) at 21 days of age. The control group of chickens was fed a basal diet, and the remaining two groups of chickens were fed a basal diet supplemented with DNE at a concentration of 0.5% or 2.0% until 35 days of age. Growth performance (body weight, weight gain, feed intake, and feed conversion ratio) and tissue weights did not differ among the three groups. The 2.0% DNE-supplemented diet decreased the muscle malondialdehyde content, a marker of lipid peroxidation, and drip loss compared to the control chickens. In addition, the expression of genes encoding mRNAs of antioxidant enzymes (i.e., Cu/Zn-superoxide dismutase, Mn-superoxide dismutase, glutathione peroxidase 7, and catalase) were higher in the pectoralis major muscle of chickens fed the 2.0% DNE-supplemented diet than in the control chickens. Therefore, DNE supplementation increased the expression of genes encoding mRNAs in antioxidant enzymes and reduced lipid peroxidation and drip loss in the pectoralis major muscle of broiler chickens.

Key words: antioxidant enzymes, *Azadirachta indica*, broiler chicken, lipid peroxidation, neem

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Introduction

The appearance of poultry meat is critical for the initial selection of the product by consumers and final product satisfaction (Fletcher, 2002). Lipid peroxidation is the oxidative degradation of lipids and is initiated in the highly unsaturated phospholipid fraction of the subcellular membranes of meat (Gray and Pearson, 1987). Chicken muscle

has a high polyunsaturated fatty acid content, making it more sensitive to oxidative deterioration during storage (Wilson *et al.*, 1976; Igene and Pearson, 1979; Grashorn, 2007). The oxidation of these lipids in chicken meat results in a rancid odor, off-flavor development, drip loss, discoloration, loss of nutritional value, decrease in shelf life, and the accumulation of toxic compounds that may be detrimental to the health of consumers (Richards *et al.*, 2002; Mapiye *et al.*, 2012). Numerous studies have demonstrated that feeding ingredients with high antioxidant activity results in the reduction of lipid peroxidation in the skeletal muscles of chickens (Surai *et al.*, 2018; Bottje, 2019; Maffuz and Piao, 2019; Saeed *et al.*, 2019).

Neem (*Azadirachta indica*) is a tree widely used in traditional medicine (Saleem *et al.*, 2018), with its leaves containing high concentrations of protein, carbohydrates, and dietary fiber. The leaves also contain relatively high fat and

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mineral content (Esonu *et al.*, 2005; Bonsu *et al.*, 2012). In chickens, feeding diets containing 2.5%–7.5% of neem leaves negatively affected growth performance because of the high crude fiber content (Udedibie and Opara, 1998; Bonsu *et al.*, 2012; Ubua *et al.*, 2019). Nnenna and Okey (2013) reported that dried neem leaf extract (DNE) could be added to chicken feed without negatively affecting the growth performance or altering the biochemical parameters in the blood. Furthermore, neem leaves are rich in phenolic compounds such as gallic acid and ferulic acid (Singh *et al.*, 2005; Shewale and Rathod, 2018), which exert high antioxidant activity (Prakash *et al.*, 2007; Heyman *et al.*, 2017).

In the present study, we evaluated the effects of feeding DNE at 0.5% and 2.0% of the diet on growth performance, drip loss, muscle lipid peroxidation levels, and the expression of genes of the encoding mRNAs in antioxidant enzymes of broiler chickens.

Materials and Methods

Preparation and Determination of Chemical Composition, Total Polyphenol Content, and Free Radical Scavenging Activity (FRSA) of DNE

Neem leaves were suspended and extracted with four times the amount of water of neem leaves and shaken at 80°C for 5 h. The extracts were dried under a vacuum for 24 h.

The chemical compositions of neem leaves and DNE were determined for moisture, fat, ash, and crude fiber, in triplicate, in accordance with the Association of Official Agricultural Chemists procedures (AOAC, 1990) and are displayed in Table 1. Nitrogen was determined using an NC analyzer (JM1000CN; J-Science Lab Co., Kyoto, Japan), and the percentage of nitrogen was converted to crude protein by multiplying by 6.25.

A total of 50 mg of neem leaves and DNE were homogenized with 1 mL of water and incubated at 80°C for 30 min. The homogenate was then centrifuged at 20,000×g for 5 min, and the supernatant was used to determine the total polyphenol content and FRSA.

The Folin–Ciocalteu method was used to evaluate the total polyphenol content according to the method described by Anesini *et al.* (2008). Briefly, 100 μL of the supernatant was mixed with 100 μL of 10% sodium carbonate, 50 μL of

Folin–Ciocalteu reagent, and 750 μL of distilled water. After incubation for 60 min at room temperature, absorption was measured at 700 nm. Gallic acid was used as a standard, and the data were expressed as g/g ingredient.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method was used to evaluate FRSA according to the method described by Nara *et al.* (2006). Briefly, 200 μL of the supernatant was mixed with 200 μL of 0.2 M 2-(N-morpholino) ethane sulfonic acid buffer, 200 μL of ethanol, and 200 μL of 1.2 M DPPH. After incubation for 20 min at room temperature, the absorbance was measured at 517 nm. Trolox (Cayman Chemical Company, MI, USA) was used as a control standard, and the data were expressed as mmol of Trolox equivalent/g ingredient.

Animals and Experimental Design

All experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of Kagoshima University (approval number A18010). A total of 24 male ROSS308 broiler chicks (*Gallus gallus domesticus*) at 0-day-old were provided by a commercial hatchery (Kumiai Hina Center, Kagoshima, Japan). The chicks were housed in an electrically heated battery brooder in a temperature-controlled room at 30°C, and the thermostat was turned down 1°C every 2 days. The temperature-controlled room was maintained at 25°C on and after 10 days of age. A continuous lighting program (23 h light and 1 h dark) was applied. Chicks were provided with water and a commercial starter diet (23% crude protein and 3,081 kcal/kg metabolizable energy; Nichiwa Sangyou Company, Kagoshima, Japan) *ad libitum* until 21 days of age. Then, chicks with similar body weights were selected and individually housed in wire-bottomed aluminum cages (40×50×60 cm) in a temperature-controlled room at 25°C. All chicks were allowed free access to a semi-purified corn/soybean meal diet (the basal diet described in Table 2) and water. At 21 days of age, the chicks were randomly allocated to receive one of three experimental diets (eight birds per diet): control group (basal diet), basal diet and DNE supplemented at 0.5%, and basal diet and DNE supplemented at 2.0%. Body weight and feed intake were measured to calculate the feed conversion ratio. At 35 days of age, all chickens were weighed and euthanized by cervical dislocation following carbon dioxide anesthesia.

Table 1. Chemical composition, total polyphenol content, free radical scavenging activity (FRSA) and dried neem leaf extract (DNE)

| | Neem leaves | DNE |
|--|-------------|--------|
| Chemical composition (%) | | |
| Moisture | 9.50 | 4.33 |
| Crude protein | 13.94 | 7.59 |
| Ether extract | 4.51 | 2.99 |
| Crude fiber | 12.29 | 0.25 |
| Crude ash | 8.09 | 18.34 |
| Nitrogen free extract | 51.67 | 66.50 |
| Total polyphenol content (mg/g ingredient) | 2.74 | 10.83 |
| FRSA (mmol eq.trolox/g ingredient) | 102.77 | 269.78 |

FRSA, free radical scavenging activity; DNE, dried neem leaf extract.

Table 2. Composition and analysis of the basal diet

| Ingredients (g/100 g) | |
|---|-------|
| Corn meal | 57.90 |
| Soybean meal | 34.00 |
| Corn oil | 4.30 |
| CaCO ₃ | 0.66 |
| CaHPO ₄ | 2.00 |
| NaCl | 0.50 |
| DL-Methionine | 0.14 |
| Mineral and vitamin premix ¹ | 0.50 |
| Calculated analysis | |
| Crude protein (%) | 20.0 |
| Metabolizable energy (Mcal/kg) | 3.1 |

¹Content per kg of the vitamin and mineral premix: vitamin A 90 mg, vitamin D3 1 mg, DL-alpha-tocopherol acetate 2000 mg, vitamin K3 229 mg, thiamin nitrate 444 mg, riboflavin 720 mg, calcium d-pantothenate 2174 mg, nicotinamide 7000 mg, pyridoxine hydrochloride 700 mg, biotin 30 mg, folic acid 110 mg, cyanocobalamine 2 mg, calcium iodinate 108 mg, MgO 198,991 mg, MnSO₄ 32,985 mg, ZnSO₄ 19,753 mg, FeSO₄ 43,523 mg, CuSO₄ 4019 mg, and choline chloride 299,608 mg.

The right half of the pectoralis major muscle was used to determine drip loss, and a portion of the remaining half was snap-frozen in liquid nitrogen and stored at -80°C . Blood samples were collected and immediately centrifuged to separate the plasma. The plasma was frozen and stored at -20°C until subsequent analysis.

Determination of Skeletal Muscle Drip Loss

Drip loss was measured based on the method described by Berri *et al.* (2008). The pectoralis major muscle was weighed immediately after dissection, placed in a plastic bag, and stored at 4°C for 24 h. The pectoralis major muscle was then wiped and weighed again. The difference in weight corresponded to the drip loss and was expressed as a percentage of the initial muscle weight.

Determination of Muscle Malondialdehyde (MDA) Concentration

MDA is one of the most frequently used indicators of lipid peroxidation. To evaluate the lipid peroxidation levels in the skeletal muscle of chickens, MDA concentrations were determined colorimetrically as a 2-thiobarbituric acid reactive substance according to the method described by Ohkawa *et al.* (1979). Briefly, 0.3 g of the pectoralis major muscle was weighed and homogenized in 1 mL of 1.15% KCl and centrifuged at $20,000\times g$ for 5 min, and the supernatant was collected. A sample of $80\mu\text{L}$ of the homogenate was mixed with $80\mu\text{L}$ of 8.1% SDS, $220\mu\text{L}$ of 20% acetic acid (pH 3.5), and $300\mu\text{L}$ of 0.8% 2-thiobarbituric acid. After vortexing, the samples were incubated at 95°C for 1 h and then transferred to ice. The samples were mixed by vortexing and centrifugation at $20,000\times g$ for 5 min after adding 1 mL of butanol-pyridine 15:1 (v/v). The absorbance of the supernatant, comprising the butanol-pyridine layer, was measured by excitation at 535 nm and emission at 585 nm.

RNA Extraction, Reverse Transcription, and Quantitative Real-time Polymerase Chain Reaction (PCR)

The skeletal muscle tissue was homogenized in ISOGEN II (Nippon Gene, Tokyo, Japan), and 60 ng samples of the purified total RNA were reverse transcribed using the PrimeScript RT Reagent Kit (RR036A; Takara, Shiga, Japan). Real-time PCR was performed as previously described (El-Deep *et al.*, 2016). The following primers were used: Cu/Zn-superoxide dismutase (SOD), 5'-AGGGGGTCATC-CACTTCC-3' and 5'-CCCATTTGTGTTGTCTCCAA-3'; Mn-SOD, 5'-CGCTGGCAAAGGTGATGTT-3' and 5'-CTCCTTTAGGCTCCCCTCCT-3'; glutathione peroxidase 7 (GPX7), 5'-TTGTAAACATCAGGGGCAA-3' and 5'-TGGGCCAAGATCTTTCTGTAA-3'; catalase, 5'-GGGG-AGCTGTTTACTGCAAG-3' and 5'-CTTCCATTGGCTA-TGGCATT-3'; and ribosomal protein S17 (RPS17), 5'-GCGGGTGATCATCGAGAAGT-3' and 5'-GCGCTTGT-TGGTGTGGAAGT-3' (Saneyasu *et al.*, 2011; El-Deep *et al.*, 2016). Each sample was run in duplicate with no template or negative RT controls in each plate. Efficiencies and R^2 were assessed using a five-point cDNA serial dilution: PCRs were highly specific and reproducible ($0.96 < R^2 < 1.05$) and all primer pairs had equivalent PCR efficiencies (from 98% to 105%). The melting curves revealed a single peak for all primer pairs. The coefficient of variation was 7%–11%. Amplification, dissociation curves, and gene expression analysis were performed using the Dissociation Curves software (Applied Biosystems). Because there were no significant differences in the cycle threshold values of RPS17 among the groups, the RPS17 level was used as an internal standard. The gene expression results were expressed relative to the values of the control chickens.

Statistical Analysis

All data are presented as mean \pm standard error of the mean. Differences between the groups were tested using Dunnett's multiple comparison test. Correlation and regression analyses were performed on individual values using a general linear model procedure. These analyses were performed using R software (R Core Team, 2019). Statistical significance was set at $P < 0.05$ for all comparisons.

Results and Discussion

Table 1 displays the chemical composition, total polyphenol content, and FRSA of neem leaves and DNE. DNE contained lower amounts of crude protein, ether extract, and crude fiber, and higher amounts of crude ash and nitrogen free extract than neem leaves. The total polyphenol content of DNE was 4.0-fold higher than that of neem leaves. Consequently, DNE had a 2.6-fold higher FRSA than neem leaves, with DNE containing more polyphenolic compounds than neem leaves. The polyphenol content of DNE was almost equal to that of green tea (Takasugi *et al.*, 2008).

Dietary supplementation of neem leaves has been reported to negatively affect the growth performance of broiler chickens (i.e., body weight gain and feed conversion ratio) (Bonsu *et al.*, 2012; Ubua *et al.*, 2019). Alternatively, one study demonstrated that dietary supplementation with 0.6%

Table 3. Effects of dietary supplementation with dried neem leaf extract (DNE) on growth performance, tissue weights, muscle malondialdehyde (MDA) concentration and drip loss of broiler chickens

| | Control diet | DNEN | |
|--|------------------|-----------------|-----------------|
| | | 0.5% | 2.0% |
| Growth performance | | | |
| Final body weight (g) | 1586.41 ± 71.69 | 1567.14 ± 52.02 | 1588.79 ± 54.94 |
| Body weight gain (g) | 745.34 ± 85.40 | 727.09 ± 50.68 | 769.24 ± 68.44 |
| Feed intake (g) | 1457.58 ± 128.51 | 1374.88 ± 67.68 | 1447.27 ± 58.81 |
| Feed conversion ratio | 2.01 ± 0.10 | 1.91 ± 0.06 | 1.92 ± 0.12 |
| Tissue weights | | | |
| Pectoralis major muscle (g) | 296.89 ± 14.72 | 304.30 ± 11.25 | 305.63 ± 13.11 |
| Leg muscles (g) | 299.61 ± 16.35 | 303.20 ± 14.62 | 288.31 ± 14.44 |
| Liver (g) | 32.49 ± 1.81 | 33.93 ± 1.89 | 36.08 ± 1.98 |
| Heart (g) | 8.58 ± 0.62 | 8.77 ± 0.51 | 8.29 ± 0.29 |
| Abdominal fat tissue (g) | 5.65 ± 1.87 | 4.31 ± 0.75 | 4.42 ± 0.67 |
| Muscle MDA concentration and drip loss | | | |
| Muscle MDA concentration (nmol MDA / g tissue) | 22.93 ± 2.33 | 18.96 ± 1.36 | 14.04 ± 1.10* |
| Drip Loss (%) | 5.14 ± 0.18 | 4.82 ± 0.20 | 4.15 ± 0.21* |

Results are expressed as mean ± standard error of the mean (SEM) ($n=8$). *, $P<0.05$. DNE, dried neem leaf extract; MDA, malondialdehyde.

water extract of neem leaves did not affect the growth performance or carcass weight of broiler chickens (Shaahu *et al.*, 2020). In agreement with this observation, dietary supplementation of 0.5% and 2.0% of DNE did not affect the final body weight, body weight gain, feed intake, and feed conversion ratio of broiler chickens in the present study (Table 3), although the calculated crude protein and metabolizable energy of the DNE-supplemented diet were 19.94%, 3.08 Mcal/kg and 19.91%, 3.04 Mcal/kg, respectively. Dietary supplementation with DNE did not affect the weights of the pectoral muscle, leg muscles, heart, liver, or abdominal fat tissue (Table 3). Therefore, DNE had no negative effects on the growth performance of broiler chickens when supplemented within the range of 0.5%-2.0%.

In the present study, we examined the effects of dietary supplementation with DNE on the MDA concentration in the skeletal muscle of broiler chickens. Dietary supplementation with 0.5% DNE decreased MDA concentration in the skeletal muscle of broiler chickens ($P=0.15$), and dietary supplementation with 2.0% DNE significantly decreased MDA concentration (Table 3). The muscle drip loss was lower in chickens supplemented with 2.0% DNE than that of the control chickens. Furthermore, the present study identified a positive correlation ($r=0.653$, $P<0.001$) between the MDA concentration and drip loss. These results suggest that dietary supplementation with DNE reduced lipid peroxidation in skeletal muscle and muscle drip loss in chickens.

Neem leaves are rich in phenolic compounds such as gallic acid and ferulic acid (Singh *et al.*, 2005; Shewale and Rathod, 2018), which exert high antioxidant activity (Prakash *et al.*, 2007; Heyman *et al.*, 2017). In rats and humans, gallic acid and ferulic acid are rapidly absorbed by the intestine and detected in the plasma (Lafay and Gil-

Izquierdo, 2008). In addition, dietary supplemented phenolic compounds, including gallic acid and ferulic acid, have been detected in the plasma and thigh meat of chickens (Muñoz-González *et al.*, 2019). Therefore, the higher total polyphenol content and FRSA of DNE might decrease the muscle MDA concentration in chickens.

Changes in the expression of genes encoding antioxidant enzymes might promote antioxidant activity. Previous studies have demonstrated a positive correlation between gene expression and antioxidant enzymatic activity in rats (Tiedge *et al.*, 1997). In the present study, dietary supplementation with DNE increased the expression of genes encoding antioxidant enzymes (i.e., Cu/Zn-SOD, Mn-SOD, GPX7, and catalase) in the skeletal muscle of chickens (Fig. 1). These increased gene expression levels, modified by dietary supplementation with DNE, might reduce lipid peroxidation and drip loss in the skeletal muscle of broiler chickens.

Furthermore, these antioxidant enzymes contain trace elements, including Zn, Mn, Cu, Fe, and Se (Hopkins and Tudhope, 1973; Nève, 1991; Kocyigit *et al.*, 2001). These trace elements regulate the expression of genes encoding antioxidant enzymes in the heart, liver, and plasma of chickens (Bai *et al.*, 2014; El-Deep *et al.*, 2016; Jarosz *et al.*, 2018). Otache and Agbajor (2017) reported that neem leaves are rich in trace elements, and DNE contains more crude ash than neem leaves (Table 1). Therefore, the contents of these trace elements in DNE are equal to or greater than neem leaves and might increase their gene expression levels in the skeletal muscle of broiler chickens.

In conclusion, DNE contained high levels of total polyphenols and had high FRSA. A diet supplemented with 2.0% DNE increased the expression of genes encoding

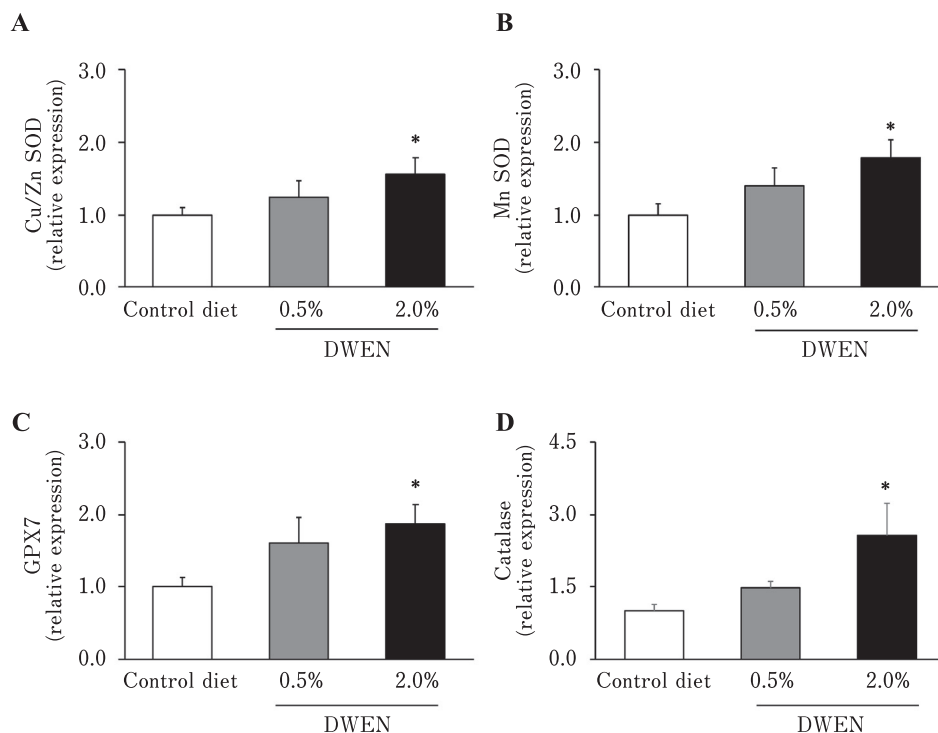


Fig. 1. Effects of dietary supplementation with DNE on the genes Cu/Zn-SOD (A), Mn-SOD (B), GPX7 (C), and catalase (D) in the pectoralis major muscle of broiler chicks. Results of the quantitative real-time PCR are normalized to RPS17 mRNA levels and expressed relative to the mean value of control chickens. Values are expressed as the mean \pm standard error of the mean ($n=8$ per treatment) *, $P<0.05$ (vs control chickens), DNE: dried neem leaf extract; SOD: superoxide dismutase; GPX: glutathione peroxidase; RPS17: ribosomal protein S17.

mRNAs in antioxidant enzymes and reduced lipid peroxidation and drip loss of the skeletal muscle in broiler chickens.

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Conflicts of Interest

The authors declare no conflicts of interest.

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