Evaluation of Genistein Mediated Growth, Metabolic and Anti-Inflammatory Responses in Broilers

Muhammad Farooq Iqbal[1,2], Yu-Heng Luo[1], Malik Muhammad Hashim[3,4] and Wei-Yun Zhu[1]

[1]*Laboratory of Gastrointestinal Microbiology, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, China*
[2] *Faculty of Veterinary and Animal Sciences, PMAS Arid Agriculture University, Rawalpindi, Pakistan*
[3] *College of Food Science and Technology, Nanjing Agricultural University, Nanjing, China*
[4] *Department of Food Science and Technology, Gomal University, Dera Ismail Khan, Pakistan*

Abstract.- Flavonoids, a group of polyphenolic compounds found mainly in fruits and vegetables, are one of the widely researched non-antibiotic feed additives in animal nutrition. The objective of this study was to determine the effect of genistein on growth, metabolism and endocrine parameters in meat type chickens. A total of 384 day-old chicks, randomly divided into 4 groups, received a common basal diet with genistein added at 0 (control), 2.5, 5 and 10 mg/kg feed. Results showed no significant difference in body weight and FCR between controls and treated birds. Total cholesterol was generally lower for genistein treated groups while plasma glucose appeared to lower only with 2.5 mg/kg dose of genistein. Plasma triiodothyronine remained unaffected while insulin like growth factor (IGF-1R) gene expression, plasma thyroxine and glucagon exhibited age and dose dependent response generally in the positive direction after genistein supplementation. Prostaglandin E2 (PGE_2) increased in birds fed 10 mg/kg on day 21, whereas decreased in birds fed 2.5 and 5 mg/kg on day 42. Leukotriene B4 (LTB4) also reduced in 2.5 and 5 mg/kg fed groups on both experiment days. Hepatic inducible nitric oxide synthase (iNOS) gene expression augmented with all doses of genistein on day 21 and with 10 mg/kg on day 42. However, iNOS mRNA level was down regulated with 2.5 and 5 mg/kg on day 42. The genistein inclusion at 2.5 and 5 mg/kg of feed resulted in increased cyclooxygenase-2 (COX-2) gene expression on day 21. These results conclude that genistein may be applied as an alternative to antibiotic feed additives for broiler chickens.

Keywords: Anti-inflammatory response, broiler, genistein, metabolic responses, T3 and T4, prostaglandins, leukotriene, nitric oxide synthase, cyclooxygenase-2.

INTRODUCTION

Improving human health by nutritional manipulation is one of the current and important topics of health research. The use of nutritional strategies to improve human health combines both animal and food science. Consumption of meat containing high amounts of polyunsaturated fatty acids has increased greatly in the last decade due to nutritionists' recommendations (Mielnik *et al.*, 2006). However, a high degree of polyunsaturation accelerates oxidative processes leading to deterioration in meat flavor, color, texture and nutritional value. Supplementation of diets with flavonoids has shown to be effective in reducing lipid oxidation, improving meat color, and the consequent obtaining of meat products with

extended shelf-life (Tang, 2001; Jiang *et al.*, 2007).

Another concern about the animal origin food is the use of antibiotic as feed additives. Development of antibiotic-resistant bacteria has prompted researchers to investigate various methods of maintaining and improving animal health and performance in the absence of antibiotic feed additives (Brenes and Roura, 2010). In recent years, new additives of plant origin have been offered to animal producers as an alternative to antibiotic feed additives. For some of them, beneficial effects on health and productivity have been reported (Windisch *et al.*, 2008; khan *et al.*, 2010). Flavonoids are an important group of secondary metabolites which are synthesized by plants. They exhibit certain protective activities in humans as well as in animals including lowering the risk of cardiovascular diseases, preventing cancer and inhibiting tumor growth, suppressing inflammation and reducing the development of cataracts (Yang *et al.*, 2009). The compounds inhibit the infectivity of

pathogenic bacteria, toxins produced by some of these bacteria, and pathogenic and phytopathogenic viruses and fungi (Friedman, 2007).

Isoflavones may play a role in affecting growth and carcass composition of animals because of structural and functional similarity to natural estrogens (Zand *et al*., 2000). The effects of genistein in farm animals might be different as phytochemical studies with animal models yield results relevant only to the specific animal model tested (Dillard and German, 2000). Furthermore, the mechanism by which genistein exerts its health beneficial effects have not been well studied, especially *in vivo*. Therefore, present study was undertaken to investigate the effect of genistein on growth, metabolic and endocrinal indices in broiler chickens. In addition, anti-inflammatory activity was also investigated.

MATERIALS AND METHODS

Animals and treatments

Three hundred and eighty four, a day old mixed-sex meat-type broiler (Arbor Acres, market age 42 d), were divided into 4 treatment groups, each having 6 replicates (16 birds in each replicate), and received diet supplemented with either 0 (control), 2.5, 5 or 10 mg genistein/kg of diet *ad libitum*. The mash feed (Table I) based on soybean corn were prepared according to National Research Council procedure (NRC, 1994). Genistein was purchased from a commercial company (Sigma, New York, USA). Genistein was first mixed with a small amount of feed prior to incorporation into the final feed. All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) approved protocol.

Sample collection

On day 21 and 42 of age, birds were deprived off feed for 12 h and weighed just prior to slaughtering. Cumulative weight gain, feed intake and feed conversion ratio (FCR) were estimated. After weighing, six birds from each replicate were sacrificed and blood samples were taken into heparinized plastic tubes. Within 10 min after sampling, the blood plasma was separated by centrifugation at 2,000 r/min, and stored at -20°C

until assayed for selected parameters. Liver samples were removed and immediately frozen in liquid nitrogen and stored in a freezer at −80°C for subsequent extraction of total RNA.

Table I.- **Formulation and proximate analysis of starter (1-20 days) and finisher (21-42 days) diets.**

Ingredient	Starter (%)	Finisher (%)
Corn	59.1	64.3
Soybean meal	30.6	24.3
Corn gluten meal	3.8	4.5
Palm Oil	1.7	2.5
Limestone	1.31	1.23
Dicalciumphosphate	1.77	1.58
Lysine	0.15	0.16
Methionine	0.15	0.1
Vitamin-mineral premix*	1	1
Salt	0.42	0.33
Total	100	100
Proximate analysis		
ME (MJ/kg)	12.27	12.77
Crude protein (%)	21.2	19.3
Fat (%)	3.89	4.3
Crude fiber (%)	4.25	4.75
Ash (%)	6.45	5.99
Ca (%)	1.0	0.91
AP (%)	0.43	0.38
Lys (%)	1.08	0.95
Met (%)	0.50	0.43
Met + Cys (%)	0.82	0.73

*Supplied per kilogram of feed: vitamin A , 8,000 IU; DL-α-topherol acetate, 12.0 IU; cholecalciferol, 1,600 IU; vitamin K3, 3.5 mg; vitamin B12, 17 μg; riboflavin, 5.0 mg; thiamin, 2.0 mg; D-pantothenic acid, 10 mg; niacinamide, 40 mg; folic acid, 0.92 mg; pyridoxine, 3.50 mg; biotin, 90 μg; choline chloride, 210 mg; Zn, 80 mg; Se, 0.16 mg; Mn, 100 mg; Fe, 70 mg; I, 0.35 mg.

For the determination of PGE_2 and LTB_4 release in the whole blood, individual blood samples were challenged with calcium ionophore A23187 (30μM final concentration; Sigma, New York, USA) diluted in dimethyl sulfoxide (DMSO) for 30 min at 37°C. DMSO controls (No. A23187) was used as blanks. The samples were centrifuged (12,000 x g, 2 min) and the supernatant plasma was removed. Plasma samples were stored at -20°C until assayed.

RNA extraction and Real-Time PCR

Frozen liver samples were defrosted and total

Table II.-   Primers used in the real-time PCR analysis of chicken genes.

Target gene	Orientation	Primer sequence (5′→3′)	Reference
IGF-1R	Forward	GGCCTGCCGCAATTACTACTA	Tosca *et al.* (2008)
	Reverse	CGCCAGCCCTCAAACTTGT	
iNOS	Forward	GGCAGCAGCGTCTCTATGACTTG	Abdul-Careem *et al.* (2007)
	Reverse	GACTTTAGGCTGCCCAGGTTG	
COX-2	Forward	TCCACCAACAGTGAAGGACACTCA	Scott and Oweens (2008)
	Reverse	ATCCCACTCTGGATGCTCCTGTTT	
β-actin	Forward	CAACACAGTGCTGTCTGGTGG	Abdul-Careem *et al.* (2007)
	Reverse	ATCGTACTCCTGCTTGCTGAT	

Table III.-   Effects of dietary genistein supplementation on weight gain, FCR, plasma glucose and cholesterol concentrations in chickens at 21 and 42 days of age.

Age (days)	Parameters*	Genistein Level (mg/kg)			
		0	2.5	5	10
21	Weight gain	609±15	583±21	583±10	605±26
	Feed consumption	785.6±19	740.4±12	769.6±22	768.3±17
	FCR	1.29±0.03	1.27±0.01	1.32±0.02	1.27±0.02
	Glucose	11.1±0.13	10.4±0.42	10.8±0.56	12.7±0.28
	Cholesterol	3.43±0.10[a]	3.06±0.10[b]	3.47±0.34[a]	3.53±0.14[a]
42	Weight gain	1922±61	1889±49	2034±53	1954±24
	Feed consumption	3075.2±55	3041.3±67	3396.8±34	3067.8±45
	FCR	1.60±0.05	1.61±0.04	1.67±0.04	1.57±0.02
	Glucose	10.95±0.7[bc]	9.6±0.28[c]	10.75±0.7[bc]	11.95±0.17[a]
	Cholesterol	3.27±0.09[a]	2.23±0.08[b]	1.92±0.06[c]	1.78±0.11[c]

[a,b,c]: Mean values in a row not sharing a superscript are different at $P<0.05$. Results are presented as means ± SD

*g is unit for feed consumption, mmol/l is unit for glucose and cholesterol, pg/ml is unit for glucagon while gram (g) is unit for weight gain

RNA was isolated using the TRIzol reagent (Invitrogen, Japan) according to the manufacturer's protocol. The RNA integrity was assessed via agarose gel electrophoresis. RNA concentration and purity was determined spectrophotometrically using A260 and A280 measurements in a photometer (Eppendorf Biophotometer, Hamburg, Germany). Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Reverse transcription of total RNA (600 ng) was carried out by using Oligo (dT)18 primers (SuperScript™ First-Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Singleplex relative Real Time PCR was performed using an iCyclerMyiQ™ single color Real Time PCR detection system (BioRad, USA). Reactions were performed in a 20 μl volume reaction mixture containing 1 μl forward primer (300 nM), 1 μl reverse primer (300 nM), 4.5 μl water, 1 μl cDNA and 12.5 μl of SYBR®*Premix Ex Taq*™ (Perfect Real Time) (TaKaRa, Dalian) including TaKaRa *Ex Taq*™ HS and SYBR® Green I, dNTP and buffer. Polymerase chain reaction oligonucleotide primers specific for chicken insulin like growth factor type-1 receptor (IGF-1R), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and β-actin genes are listed in Table II. Thermal cycling parameters consisted of initial denaturation (95°C, 5 min), followed by 40

cycles of denaturation (95°C, 45 s), primer annealing (64°C for iNOS, 60°C for IGF-1R and β-actin, 55°C for COX-2, 30 s), and primer extension (72°C, 45 s). Detection of the fluorescent product was carried out at the end of the 72°C extension period (2 min). All reactions were run with a negative control and subjected to melting curve analysis. The 2 µl cDNA of each treatment were mixed together to prepare relative standards. Amplification, detection, and data analysis were performed with an iCycler IQ real-time detection system (Bio-Rad Laboratories). The resultant value was expressed relative to β-actin (control gene).

Laboratory analysis

Plasma samples were thawed at room temperature for laboratory analyses. Plasma glucose and cholesterol concentrations were measured using biochemical analyzer (Olympus AU-800). Blood concentrations of glucagon, triiodothyronine and thyroxine were measured using commercial radioimmunoassay kits (Adlitteram Diagnostic Laboratories, USA). Briefly, 50 µL of 1:1 diluted samples and 50 µL of respective anti-goat captured antibodies were added to plate wells and the plates were incubated at 37°C for one hour. Then, the plates were washed in three cycles and 80 µL of enzyme (Streptavidin-Peroxidase) was added to each well. The plates were incubated again at 37°C for 30 min. After incubation, plates were washed three times with ELISA wash buffer. 50 µL of substrate solutions (A and B) were pipetted into the plate wells. Plates were incubated at 37 °C for 10 min to develop color. Then, 50 µL of stop solution (H_2SO_4) were added to each well to stop the reaction and the plates were read for absorbance in a spectrophotometer at 450 nm. Using the standard curves, the levels of required substances were determined. The lowest limit of sensitivity for glucagon, triiodothyronine and thyroxine was 1.0 pg/mL, 0.1 nmol/L and 1.70 nmol/L, respectively. All samples in the trials were run in one batch to minimize assay variations.

Statistical analysis

The effect of genistein treatment on different parameters was evaluated by one-way ANOVA using SPSS 13.0 software (SSPS Inc., IL, USA).

Differences between means were determined by Duncan New Multiple Range Test (MRT) and P-<0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Feed intake, body weight gain and feed conversion ratio (FCR)

The results regarding body weight and FCR are given in Table III. Feed intake, body weight gain, and FCR of the birds during 6 week feeding period were not affected by the genistein intake. The effects of isoflavones on weight gain, feed intake, and feed efficiency in animals are somewhat inconsistent. Wilhelms *et al.* (2006) reported that synthetic soybean isoflavone (ISF) supplemented into the diet at 1 and 5% did not influence growth performance of the Japanese quail. Payne *et al.* (2001) reported that higher ISF levels in diet decreased FCR but did not affect average daily gain or feed intake in broilers. In ovariectomized mice the isoflavone genistein had non-significant effect on body weight or food intake (Naaz *et al.*, 2003). Similarly, Greiner *et al.* (2001a,b) found that soybean genistein (200 mg/kg) and daidzein (200 or 400 mg/kg) could improve growth in virally challenged pigs. Dietary soy isoflavone was also shown to lower food intake in female rats (Kishida *et al.*, 2008). These variations may be partially explained by the fact that effects of these compounds are dependent on dose, duration of use and individual metabolism. For example, Jiang *et al.* (2007) showed that dietary supplementations with 10 or 20 mg ISF/kg increased weight gain of birds significantly while 40 or 80 mg ISF/kg did not show any significant change in weight gain. The FCR of 43 to 63 day old chicks was also significantly improved by the supplemental ISF at 10 mg level. These discrepancies may also be attributed to the chemical structure, age and species specific responses of these compounds (Arora *et al.*, 1998).

Plasma glucose

The effects of genistein on plasma glucose are summarized in Table III. There was non-significant (P<0.05) effect of genistein on plasma glucose as compared to control chickens (21 days old). However, on day 42, genistein at 2.5 mg/kg

appeared to lower plasma glucose in the chickens included in the study. The same parameter was non-significant statistically when compared to control group.

The studies on the effects of genistein on glucose metabolism are lacking. However, few studies suggested that soy foods may have beneficial effect on obesity and diabetes (Bhathena and Velasquez, 2002). Similarly, Taha and Wasif (1996) reported that in alloxan diabetic hypercholesterolemic rats, soy flour added to whole durum meal lowered the elevated plasma glucose concentration. Some other studied indicated no effects of soy protein or soy isoflavones on plasma glucose levels on obesity or diabetes (Carroll, 1991; Morita *et al.*, 1997; Crouse *et al.*, 1999). Thus, the effect of isoflavones on glucose metabolism is unclear. Our study indicated the age specific effects of genistein on plasma glucose, however, further studies are required to confirm our findings.

Plasma cholesterol

Data regarding the cholesterol levels in different study groups are presented in Table III. In present study, cholesterol was not significantly (P<0.05) different between control and genistein treated chickens on 21 days except that it was low in birds supplemented with 2.5 mg/kg dose of genistein. However, there was significant decrease in cholesterol level in genistein supplemented groups as compared to control at 42 days of age. It was shaped at the level of 3.27 mmol/L in the control group, while in groups treated with genistein– from 1.78 to 2.23 mmol/L. A dose dependent increasing trend was observed for gnestein supplemented birds.

Isoflavones have a common diphenolic structure that resembles the potent synthetic estrogen diethylstilbesterol and hexestrol (Tham *et al.*, 1998). Just as estrogens lower LDL cholesterol levels and increase HDL levels, it has been proposed that isoflavones may have a similar effect (Potter, 1995). Many studies in normal and hypercholesterolemic human subjects confirm the reduction in plasma total and non-HDL cholesterol without significant effect on HDL cholesterol and triglycerides by soy protein (Anderson *et al.*, 1998; Bosello *et al.*, 1998; Hermansen *et al.*, 2001).

Animal experiments have indicated that the serum lipid-lowering properties of soy protein are related primarily to the presence of isoflavones (Anthony *et al.*, 1998; Kirk *et al.*, 1998). In contrast, Greaves *et al.* (1999) were unable to achieve the plasma lipid-lowering effects of soy protein when the amount of isoflavones contained in soy protein was added to a diet containing casein and lactalbumin. These results indicate that the cholesterol lowering effects of soy protein are yet unclear. However, isoflavone mixture containing genistein, daidzein, and glycetin reduced plasma cholesterol in rats indicating the potential of isoflavones in controlling cholesterol level (Ali *et al.*, 2004). Similarly, Yilmaz *et al.* (2008) reported that isoflavones supplementation reduced the amount of cholesterol in the liver and muscle tissue of quail. Our findings also provide direct evidence of the relationship between genistein and plasma cholesterol level. As high plasma cholesterol level is associated with an increased risk of cardiovascular diseases (Mastropaolo *et al.*, 2001; Iversen *et al.*, 2009), so lowering cholesterol contents may lower broiler mortality.

Expression of IGF-1R mRNA

As shown in Figure 1, relative abundance of liver IGF-1R mRNA expression was augmented significantly (P<0.05) in the low (2.5 mg/kg) and medium dose (5 mg/kg) groups on day 21, whereas the high dose (10 mg/kg) group expressed significantly higher IGF-IR on day 42 compared to control. Overall, a dose dependent decreasing trend on 21 days and increasing trend on 42 days was observed for genistein.
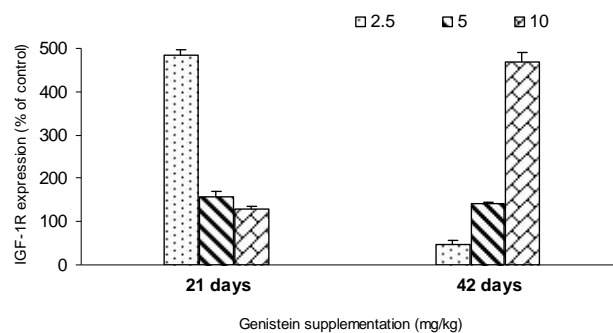

Fig. 1. Relative values for hepatic IGF-I mRNA expression in broilers at 21 and 42 days of age.

Although, not much data is available regarding the changes of gene expression for IGF-I in birds, particularly in liver, induced by the flavonoids, however, Zhao *et al.* (2004) reported that hepatic type-1 IGF receptor (IGF-1R) mRNA expression was suppressed markedly in the daidzein-treated group at hatching, but this suppression proved to be temporary, as at 4 weeks of age, expression levels of all investigated genes were restored. In Shaoxing ducks, daidzein supplementation at 3 mg/kg did not increase serum levels IGF-1 significantly, while 5 mg/kg daidzein supplementation significantly increased IGF-1, suggesting a dose-dependent effect (Zhou *et al.*, 2002). In the study of Ni *et al.* (2007) decreased IGF-IR mRNA expression in shell glands of daidzein treated hens was reported. The detailed mechanism underlying the flavonoids action on IGF receptor is worthy of further investigation.

Plasma triiodothyronine and thyroxine

The essential differences in thyroxine (T4) concentration in the blood plasma have been shown in growing broiler chickens (Table IV). The genistein treatment increased the plasma levels of thyroxine in broilers on 42 days and also in 2.5 and 5 mg/kg fed groups on 21days of age in comparison to control. Genistein presented dose dependent effects with maximum value for genistein supplementation were limited to an increase in total thyroxine but no modification of total triiodothyronine (T3) were observed.

These results are in parallel with the previous observations that supplemental flavonoids and isoflavones markedly increased T4 level in female rats while effect on total T3 concentrations was not significant (van der Heide *et al.*, 2003; Xiao *et al.*, 2004). Balmir (1996) observed elevated T4 levels in isoflavones treated male rats. However, our findings did not corroborate some previous findings on the antithyroid role of flavonoids (Davis *et al.*, 1983; Divi and Doerge, 1996). Synthetic flavonoids were also found to interfere with the thyroid hormone system. Effects of long-term treatment with F21388 (3-methyl-4,6-dihydroxy-3,5-dibromoflavone) were obtained by Schroder-van der Elst *et al.* (1991) in which plasma T4 levels decreased dramatically from ± 55 to ± 5 nmol/L while plasma T3 levels decreased about 20%.

In the present study, T3 concentrations were not affected after genistein treatment during the whole experimental period which is not in line with previous investigations indicated that elevated levels of T3 in the blood of isoflavone supplemented hens and quails (Meng *et al.*, 2001; Ke *et al.*, 2002).

These discrepancies in the effect of flavonoids on thyroid hormones may be partially interpreted to the age and species specific effects of these compounds observed in various studies (Meng *et al.*, 2001; Xiao *et al.*, 2004; Zhengkang *et al.*, 2006). Nevertheless, further studies are required to improve our understanding of the *in vivo* biological effects of flavonoids on thyroid hormone system.

Plasma glucagon

The effects of genistein on plasma glucagon are presented in Table IV. Low (2.5 mg/kg) and medium dose (5 mg/kg) groups did not show any significant difference from control group at 21 days while significant increase in plasma glucagon was recorded for high dose (10 mg/kg) group. On day 42, significant elevation in plasma glucagon was found for all genistein supplemented chicken in a dose dependent manner.

The data on the effect of flavonoids on plasma glucagon levels are lacking, although glucagon appears to be the dominant pancreatic hormone in birds compared with mammals. Glucagon has been shown to maintain "chronic hyperglycemic" blood glucose levels in birds that are 2 to 3 times greater than in most mammals (Hazelwood, 2000). Previously, Shin *et al.* (2000) reported that soybean protein intake had tendencies to raise the concentrations of plasma glucagon and glucagon-like peptide-1, and glucagon/insulin ratio in chicks. Our results depicted a dose and age specific relationship between glucagon and genistein supplementation.

Plasma PGE2 and LTB4

The effects of genistein supplementation on plasma LTB4 and PGE2 are presented in Fig. 2(A,B). On day 21, the mean plasma LTB4 level of genistein treated chickens was not significantly different from control group except the 10 mg/kg fed group in which LTB4 was elevated significantly.

Table IV.-   Effects of dietary genistein supplementation on plasma T3, T4 and glucagon concentrations in broilers.

Age (days)	Parameters*	Genistein Level (mg/kg)			
		0	2.5	5	10
21	T3	0.78±0.05	0.73±0.16	0.93±0.22	1.05±0.19
	T4	29.21±2.74[b]	46±2.02[a]	45.10±2.78[a]	30.85±4.32[b]
	Glucagon	131±15.65[b]	131.5±7.12[b]	133±13.43[b]	277±14.14[a]
42	T3	0.79±0.14	1.01±0.15	0.97±0.20	0.81±0.06
	T4	28.41±2.64[b]	43.08±2.12[a]	45.73±1.90[a]	48.16±4.71[a]
	Glucagon	133±12.12[c]	174±7.77[b]	182±13.43[b]	228±14.94[a]

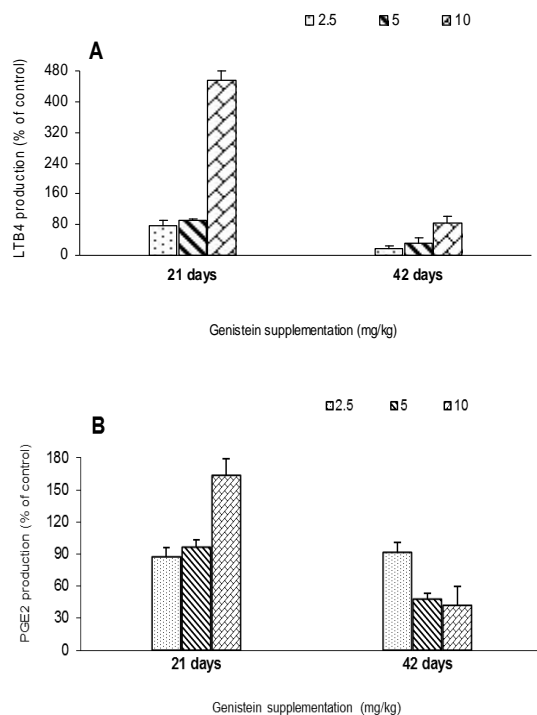* pg/ml is unit for glucagon while nmol/l is unit for T3 and T4

Fig. 2. Effects of dietary genistein supplementation on plasma LTB4 and PGE2 concentrations in broilers.

When LTB4 concentrations were measured in calcium ionophore stimulated plasma taken at 42 day old chickens from the groups receiving treatment with genistein, a considerably reduced (P<0.05) concentration of plasma LTB4 was seen compared to control group except the 10 mg/kg fed group where LTB4 remained unchanged. The PGE2 production increased greatly in high dose (10 mg/kg) group at day 21 but it did not differ (P<0.05) from control in 2.5 and 5 mg/kg fed groups. A reverse response was observed on day 42 with significant increase with low dose (2.5 mg/kg) group while medium (5 mg/kg) and high (10 mg/kg) doses inhibited the PGE2 production as compared to control group.

Several attempts have been made to explore the isoflavones showing significant inhibition of these pro-inflammatory enzyme activities. Takano-Ishikawa *et al.* (2006) demonstrated that genistein and daidzein inhibited PGE2 production in the LPS-stimulated macrophage, but the data indicated that genistein was markedly more active than daidzein. Kim *et al.* (1999) reported that tectoreginin and tectoridin inhibited the TPA-stimulated production of PGE2. Inhibition of LTB4 by soy protein was reported in rat peritoneal exudates cells (Yamada *et al.*, 1999). The present investigation clearly showed the age and dose dependent response to genistein supplementation in eosconoid production.

iNOS and COX-2 expression

The effect of genistein on iNOS and Cox-2 mRNA expression is presented in Figure 3(A,B). The iNOS expression was up-regulated at day 21 and down-regulated at day 42 in a dose dependent manner. For COX-2, Genistein supplementation also resulted in a dose dependent response. On day 21, low dose (2.5 mg/kg) up regulated the COX-2 expression as compared to control group while a significant decline in the expression of COX-2 was observed in birds fed 10 mg/kg. The response was reversed on day 42 with significantly down-regulation with low dose (2.5 mg/kg) and up-

□2.5   ◙5   ▨10

A

iNOS expression (% of control)

180
150
120
90
60
30
0

21 days   42 days

Genistein supplementation (mg/kg)

□ 2.5   ◙ 5   □ 10

B

COX-2 expression (% of control)

160
120
80
40
0

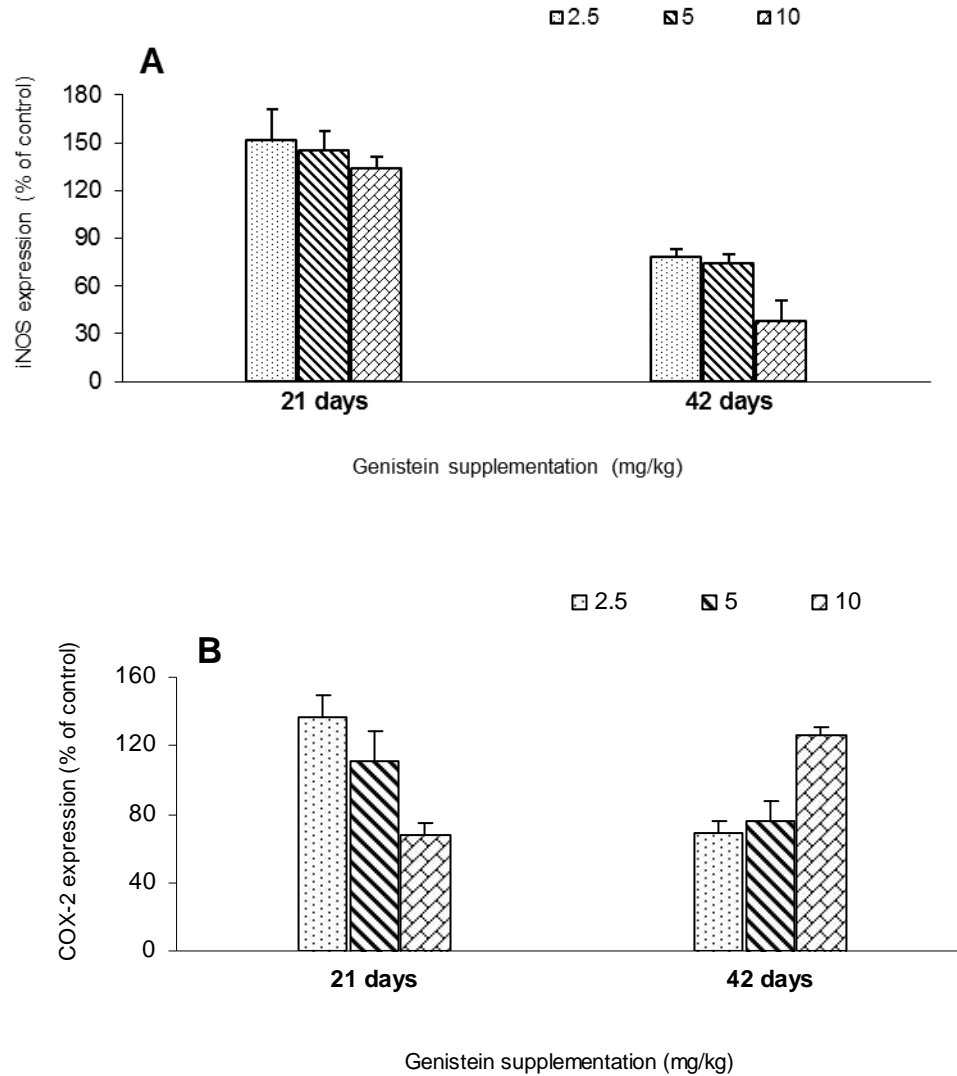21 days   42 days

Genistein supplementation (mg/kg)

Fig. 3. Relative values for hepatic iNOS and COX-2 mRNA expression in broilers at 21 and 42 days of age.

regulation with high dose (10 mg/kg). The gene expression of COX-2 with medium dose (5 mg/kg) did not differ (P<0.05) from control at any stage during the experiment period. Overall, a dose dependent decreasing or increasing trend was observed in genistein supplementation.

Some promising results for isoflavones were also found in this field in different *in vitro* experiments. Study conducted by Ishikawa *et al.* (2006) showed that apigenin and genistein suppressed COX-2 protein expression significantly in rat peritoneal macrophages, without any suppressive effect for COX-1. Hamalainen *et al.* (2007) reported that Isoflavones daidzein and genistein inhibited LPS-induced iNOS expression and NO production in a dose-dependent manner. The possible suggested mechanism is the activation of peroxisome proliferator-activated receptor (PPAR)-γ by isflavones which mediates the suppression of the promoters of COX-2 and iNOS

(Liang *et al.*, 2001). However, studies showing isoflavones effects *in vitro* have typically used isoflavones concentrations higher than would be obtained *in vivo*, thus, it is unclear whether similar effects occur at physiologic concentrations of isoflavones *in vivo*. This is the first *in vivo* study to show that genistein modulates iNOS and COX-2 expression in birds in a concentration and age-dependent manner.

In summary, dietary genistein supplementation should be considered further as an alternative non- antibiotics feed additive. The *in vivo* beneficial effects of genistein are attributed in part to the suppression of inflammatory parameters and up-regulation of metabolic parameters in birds. Furthermore, it is elucidated from the present study that genistein will be more effective if consumed in proper dose at proper age.

ACKNOWLEDGEMENTS

REFERENCES

ABDUL-CAREEM, M., HUNTER, B., PARVIZI, P., HAGHIGHI, H.R., THANTHRIGE-DON, N. AND SHARIF, S., 2007. Cytokine gene expression patterns associated with immunization against Marek's disease in chickens. *Vaccine,* **25:** 424–432.

ALI, A.A., VELASQUEZ, M.T., HANSEN, C.T., MOHAMED, A.I. AND BHATHENA, S.J., 2004. Effects of soybean isoflavones, probiotics, and their interactions on lipid metabolism and endocrine system in an animal model of obesity and diabetes. *J. Nutr. Biochem.,* **15**: 583-590.

ANDERSON, J.W., BLAKE, J.E., TURNER, J. AND SMITH, B.M., 1998. Effects of soy protein on renal function and proteinuria in patients with type 2 diabetes. *Am. J. clin. Nutr.,* **68**: 1347S–1353S.

ANTHONY, M.S., CLARKSON, T.B. AND WILLIAMS, J.K., 1998. Effects of soy isoflavones on atherosclerosis: Potential mechanisms. *Am. J. clin. Nutr.,* **68**: 1390S-1393S.

ARORA, A., NAIR, M.G. AND STRASBURG, G.M., 1998. Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radic. Biol. Med.,* **24**: 1355-1363.

BALMIR, F., STAACK, R., JEFFREY, E., JIMENEZ, M.D., WANG, L. AND POTTER, S.M., 1996. An extract of soy flour influences serum cholesterol and thyroid hormones in rats and hamsters. *J. Nutr.,* **126**: 3046-3053.

BHATHENA, S.J. AND VELASQUEZ, M.T., 2002. Beneficial role of dietary phytoestrogens in obesity and diabetes. *Am. J. clin. Nutr.,* **76**: 1191-1201.

BOSELLO, O., COMINACINI, L. AND ZOCCA, I., 1998. Short- and long-term effects of hypocaloric diets containing proteins of different sources on plasma lipids and apoproteins of obese subjects. *Ann. Nutr. Metab.,* **32**: 206–214.

BRENES, A. AND ROURA, E., 2010. Essential oils in poultry nutrition: main effects and modes of action. *Ani. Feed Sci. Tech.,* 158: 1-14.

CARROLL, K.K., 1991. Review of clinical studies on cholesterol-lowering response to soy protein. *J. Am. Diet. Assoc.,* **91**: 820-827.

CROUSE, J.R., MORGAN, T., TERRY, J.G., ELLIS, G., VITOLONS, M. AND BURKE, G.L., 1999. A randomised trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins. *Arch. Intern. Med.,* **159**: 2070–2076.

DAVIS, F.B., MIDDLETON, JR. E., DAVIS, P.J. AND BLAS, S.D., 1983. Inhibition by quercetin of thyroid hormone stimulation in vitro of human red blood cell ca2+-atpase activity. *Cell Calcium,* **4**: 71-81.

DILLARD, C. J. AND GERMAN, J. B., 2000. Phytochemicals: nutraceuticals and human health. *J. Sci. Fd. Agric.*, **80**: 1744-1756.

DIVI, R.L. AND DOERGE, D.R., 1996. Inhibition of thyroid peroxidase by dietary flavonoids. *Chem. Res. Toxicol.,* **9**: 16-23.

FRIEDMAN, M., 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Mol. Nutr. Fd. Res.,* **51**: 116-134.

GREAVES, K.A., PARKS, J.S., WILLIAMS, J.K. AND WAGNER, J.D., 1999. Intact dietary soy protein, but not adding an isoflavone-rich soy extract to casein, improves plasma lipids in ovariectomized cynomolgus monkeys. *J. Nutr.,* **129**: 1585-1592.

GREINER, L.L., STAHLY, T.S. AND STABEL, T.J., 2001a. The effect of dietary soy daidzein on pig growth and viral replication during a viral challenge. *J. Anim. Sci.,* **79**: 3113-3119.

GREINER, L.L., STAHLY, T.S. AND STABEL, T.J., 2001b. The effect of dietary soy genistein on pig growth and viral replication during a viral challenge. *J. Anim. Sci.,* **79**: 1272-1279.

HAMALAINEN, M., NIEMINEN, R., VUORELA, P., HEINONEN, M. AND MOILANEN, E., 2007. Anti-inflammatory effects of flavonoids: Genistein,

kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediat. Inflamm.,* **2007**: 1-10.

HAZELWOOD, R.L., 2000. Pancreas. In: *Sturkie's avian physiology* (ed. G.C. Whittow), ed. 5th, Academic Press, San Diego, CA, pp. 539-555.

HERMANSEN, K., SONDERGAARD, M. AND HOIE, L., 2001. Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects. *Diabetes Care,* **24**: 228–233.

IVERSEN, A., JENSEN, J., SCHARLING, H. AND SCHNOHR, P., 2009. Hypercholesterolaemia and risk of coronary heart disease in the elderly: Impact of age: The copenhagen city heart study. *Eur. J. Intern. Med.,* **20**: 139-144.

JIANG, Z.Y., JIANG, S.Q., LIN, Y.C., XI, P.B., YU, D.Q. AND WU, T.X., 2007. Effects of soybean isoflavone on growth performance, meat quality, and antioxidation in male broilers. *Poult. Sci.,* **86**: 1356 - 1362.

KE, Y.Y., WANG, G.J. AND HAN, Z.K., 2002. Effects of daidzein on the performance and serum hormone concentrations in the laying quails. *Acta Vet. Zootech. Sin.,* **33**: 243–246.

KHAN, M. T. J., AHMAD, K., ALVI, M. N., AMIN, N. U., MANSOOR, B., SAEED, M. A., KHAN, F. Z. AND JAMSHAID, M., 2010. Antibacterial and irritant activities of organic solvent extracts of *Agave americana* Linn., *Albizzia lebbek* Benth., *Achryranthes aspera* Linn., and *Abutilon indicum* Linn.—A preliminary investigation. *Pakistan J. Zool.*, **42:** 93-97.

KIM, Y.P., YAMADA, M., LIM, S.S., LEE, S.H., RYU, N., SHIN, K.H. AND OHUCHI, K., 1999. Inhibition by tectorigenin and tectoridin of prostaglandin e2 production and cyclooxygenase-2 induction in rat peritoneal macrophages. *Biochim. biophys. Acta,* **1438**: 399-407.

KIRK, E.A., SUTHERLAND, P., WANG, S.A., CHAIT, A. AND LEBOEUF, R.C., 1998. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J. Nutr.,* **128**: 954-959.

KISHIDA, T., MIZUSHIGE, T., OHTSU, Y., ISHIKAWA, S., NAGAMOTO, M., IZUMI, T., OBATA, A. AND EBIHARA, K., 2008. Dietary soy isoflavone-aglycone lowers food intake in female rats with and without ovariectomy. *Obesity (Silver Spring),* **16**: 290-297.

LIANG, Y.C., TSAI, S.H., TSAI, D.C., LIN-SHIAU, S.Y. AND LIN, J.K., 2001. Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-gamma by flavonoids in mouse macrophages. *FEBS Lett.,* **496**: 12-18.

MASTROPAOLO, W., MATHENY, A.J. AND LANG, C.A., 2001. Plasma cholesterol concentrations in twin children: Estimates of genetic and environmental influences. *Clin. Chem.,* **47**: 771.

MENG, T., HAN, Z.K. AND WANG, G.J., 2001. *Effect of diet supplemented with daidzein on laying chicken performance and serum estradiol levels.* Proc. The 7th National Academic Conference of the Animal Physio-Biochemistry, Zhengzhou (China).

MIELNIK, M.B., OLSEN, E., VOGT, G., ADELINE, D. AND SKREDE, G., 2006. Grape seed extract as antioxidant in cooked, cold stored turkey meat. *LWT Food Sci. Tech.*, 39: 191-198.

MORITA, T., OH-HASHI, A., TAKEI, K., IKAI, M., KASAOKA, S. AND KIRIYAMA, S., 1997. Cholesterol-lowering effects of soybean, potato and rice proteins depend on their low methionine contents in rats fed a cholesterol-free purified diet. *J. Nutr.,* **127**: 470–477.

NAAZ, A., YELLAYI, S., ZAKROCZYMSKI, M.A., BUNICK, D., DOERGE, D.R., LUBAHN, D.B., HELFERICH, W.G. AND COOKE, P.S., 2003. The soy isoflavone genistein decreases adipose deposition in mice. *Endocrinology,* **144**: 3315-3320.

NATIONAL RESEARCH COUNCIL, 1994. *Nutrients requirements of broilers.* National Academy Press. Washington D.C, U.S.A.

NI, Y., ZHU, Q., ZHOU, Z., GROSSMANN, R., CHEN, J. AND ZHAO, R., 2007. Effect of dietary daidzein on egg production, shell quality, and gene expression of er-alpha, gh-r, and igf-ir in shell glands of laying hens. *J. Agric. Fd. Chem.,* **55**: 6997-7001.

PAYNE, R.L., BIDNER, T.D., SOUTHERN, L.L. AND MCMILLIN, K.W., 2001. Dietary effects of soy isoflavones on growth and carcass traits of commercial broilers. *Poult. Sci.,* **80**: 1201-1207.

POTTER, S.M., 1995. Overview of proposed mechanisms for the hypocholesterolemic effect of soy. *J. Nutr.,* **125**: 606S –611S.

SCHRODER-VAN DER ELST, J.P., VAN DER HEIDE, D. AND KOHRLE, J., 1991. *In vivo* effects of flavonoid emd 21388 on thyroid hormone secretion and metabolism in rats. *Am. J. Physiol.,* **261**: E227-232.

SHIN, H., HIRONAO, K., SANAE, I., AYA, Y. AND TOORU, M., 2000. Mechanism of elevation in plasma glucagon concentration by soybean protein intake, with reference to changes in the concentration of plasma glucagon-like peptide-1. *Soy Prot. Res.,* **3**: 122-127.

SCOTT, T. AND OWEENS, M.D., 2008. Thrombocytes respond to lipopolysaccharide through Toll-like receptor-4, and MAP kinase and NF- B pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E2. *Mol. Immunol.*, **45:** 1001–1008.

TAHA, S.A. AND WASIF, M.M., 1996. Hypoglycemic effect

and protein nutritive quality of soy and methionine supplemented whole durum pasta products. *Nahrung,* **40**: 281–287.

TAKANO-ISHIKAWA, Y., GOTO, M. AND YAMAKI, K., 2006. Structure-activity relations of inhibitory effects of various flavonoids on lipopolysaccharide-induced prostaglandin e2 production in rat peritoneal macrophages: Comparison between subclasses of flavonoids. *Phytomedicin,* **13**: 310-317.

TANG, S.Z., KERRY, J.P., SHEEHAN, D., BUCKLEY, D.J. AND MORRISSEY, P.A., 2001. Antioxidative effect of dietary tea catechins on lipid oxidation of long-term frozen stored chicken meat. *Meat Sci.,* **57**: 331-336.

THAM, D.M., GARDNER, C.D. AND HASKELL, W.L., 1998. Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metab.,* **83**: 2223-2235.

TOSCA, L., CHABROLLE, C., CROCHET, S., TESSERAUD, S. AND DUPONT, J., 2008. IGF-1 receptor signaling pathways and effects of AMPK activation on IGF-1-induced progesterone secretion in hen granulosa cells. *Domes. Anim. Endocrinol.,* **34**: 204-216.

VAN DER HEIDE, D., KASTELIJN, J. AND SCHRODER-VAN DER ELST, J.P., 2003. Flavonoids and thyroid disease. *Biofactors,* **19**: 113-119.

WILHELMS, K.W., SCANES, C.G. AND ANDERSON, L.L., 2006. Lack of estrogenic or antiestrogenic actions of soy isoflavones in an avian model: The japanese quail. *Poult. Sci.,* **85**: 1885-1889.

WINDISCH, W., SCHEDLE, K., PLITZNER, C. AND KROISMAYR, A., 2008. Use of phytogenic products as feed additives for swine and poultry. *J. Anim. Sci.,* **86**: E140-E148.

XIAO, C.W., L'ABBE, M.R., GILANI, G.S., COOKE, G.M., CURRAN, I.H. AND PAPADEMETRIOU, S.A., 2004. Dietary soy protein isolate and isoflavones modulate hepatic thyroid hormone receptors in rats. *J. Nutr.,* **134**: 743-749.

YAMADA, K., SHOJI, K., MORI, M., UEYAMA, T., MATSUO, N., OKA, S., NISHIYAMA, K. AND SUGANO, M., 1999. Structure-activity relationship of polyphenols on inhibition of chemical mediator release from rat peritoneal exudate cells. *In Vitro Cell. Dev. Biol. Anim.,* **35**: 169-174.

YANG, C.S., LAMBERT, J.D. AND SANG, S., 2009. Antioxidative and anti-carcinogenic activities of tea polyphenols. *Arch. Toxicol.,* **83**: 11-21.

YILMAZ, O., GUVENC, M., CETINTAS, B., TUZCU, M., DAYANGAC, A. AND SAHIN, K., 2008. Effects of isoflavones supplementation on cholesterol and fatty acid levels of muscle and liver tissues of quail. *J. Anim. Vet. Adv.,* **7**: 1444-1449.

ZAND, R.S., JENKINS, D.J. AND DIAMANDIS, E.P., 2000. Steroid hormone activity of flavonoids and related compounds. *Breast Cancer Res. Treat.,* **62**:35-49.

ZHAO, R., WANG, Y., ZHOU, Y., NI, Y., LU, L., GROSSMANN, R. AND CHEN, J., 2004. Dietary daidzein influences laying performance of ducks (*Anas platyrhynchos*) and early post-hatch growth of their hatchlings by modulating gene expression. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.,* **138**: 459-466.

ZHENGKANG, H., WANG, G., YAO, W. AND ZHU, W.Y., 2006. Isoflavonic phytoestrogens--new prebiotics for farm animals: A review on research in china. *Curr. Iss. Intest. Microbiol.,* **7**: 53-60.

ZHOU, Y.C., ZHAO, R.Q., LU, L.Z., CHEN, W.H. AND CHEN, J., 2002. Effect of daidzein on egg laying performance and hormone levels in serum of shaoxing ducks during the late stage of egg production cycle. *J. Nanjing Agric. Univ.,* **25**: 73–76.