

Diet Supplementation of Silymarin Increased the Antioxidantive Capacity in Cumene Hydroperoxide-Challenged Ducks

¹Dan Yi, ¹Cong Wang, ¹Danfeng Sun, ¹Yongqing Hou, ¹Binying Ding,
¹Lei Wang and ²Joshua Gong

¹Hubei Key Laboratory of Animal Nutrition and Feed Science,
Wuhan Polytechnic University, 430023 Wuhan, China

²Guelph Food Research Centre, Agriculture and Agri-Food Canada,
Guelph, Ontario, Canada

Abstract: This study was conducted to investigate the efficacy of Silymarin (SIL) as antioxidant against Cumene Hydroperoxide (CH) induced oxidative damage in liver and intestinal mucosa of ducks. One hundred and sixty male Cherry Valley ducks (28 days old) were randomly allotted to control, CH, CH + 100 SIL (100 mg kg⁻¹ diet) and CH + 200 SIL (200 mg kg⁻¹ diet) groups. At the age of 42 days, ducks in CH, CH + 100 SIL and CH + 200 SIL groups received intraperitoneal administration of CH (30 mg kg⁻¹ BW) whereas ducks in the control group received the same volume of sterile saline for 5 consecutive days. At the age of 50 days, the blood samples were collected and ducks were killed to obtain liver and intestine for analysis. Compared with the control group, CH challenge increased the levels of plasma Glutamic-Pyruvic Transaminase (GPT), Alkaline Phosphatase (AKP), hepatic and Mucosal Malondialdehyde (MDA) (p<0.05) but reduced the levels of Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione S-Transferase (GST) in liver (p<0.05). CH-treated ducks exhibited a higher crypt depth (p<0.05) and a lower concentration of protein, RNA, DNA and the activities of sucrase and maltase in intestine mucosa (p<0.05). These adverse effects of CH were attenuated (p<0.05) by supplementation of SIL, particularly at the dosage of 200 mg kg⁻¹ diet. These data suggest that dietary supplement with 200 mg kg⁻¹ SIL alleviates hepatic oxidative injury and improves intestinal absorptive capacity in CH-challenged ducks.

Key words: Silymarin, antioxidantive capacity, cumene hydroperoxide, liver, intestinal mucosa, ducks

INTRODUCTION

Oxidative stress commonly occurs when the concentrations of reactive oxygen species generated exceed the antioxidant capability of animal tissues or cells (Pradeep *et al.*, 2007; Wu *et al.*, 2010). It causes the oxidative modification of cellular macromolecules (such as protein and DNA), cell death by apoptosis or necrosis and structural tissue damage (Lykkesfeldt and Svendsen, 2007; Yu *et al.*, 2010), resulting in cellular injury and dysfunctions and thus animal productivity (Mates *et al.*, 1999). In broilers, oxidative stress may occur as a result of nutrition including the contamination of feed with fungal toxins (Gong *et al.*, 2011), high ambient temperatures and several pathological conditions such as pulmonary hypertension, ascites and coccidiosis (Voljc *et al.*, 2011).

Oxidative damage may be diminished by antioxidant defence mechanisms that protect animal cells or tissues

against oxidants and repair systems that prevent the accumulation of oxidative radicals. Endogenous enzymatic antioxidants like Catalase (CAT), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GSH-Px) play pivotal role in scavenging these free radicals and protect the tissues and cells from oxidative damage. Non-enzymatic antioxidants such as Glutathione (GSH), Vitamin E and C have also been used to protect tissues from oxidative radicals and enhance cell survival by stimulating endogenous enzymes (Pradeep *et al.*, 2007). Therefore, dietary supplementation of non-enzymatic antioxidants is the most preferred and practical ways to improve the animal antioxidantive defense against the reactive oxygen species.

Several synthetic antioxidants are available but a growing trend has been targeted towards the use of natural products (polyphenols, flavonoids, carotenes and lycopenes) as antioxidants (Kiruthiga *et al.*, 2007). Of these, silymarin is ubiquitous group of flavonoids present

in most plants which can be isolated from the fruits and seeds of the milk thistle (*Silymarin marianum*) (Nijveldt *et al.*, 2001). Silymarin consists of 4 flavonolignans like silibinin (its main, active component), isosilibinin, silidiani and silichristin (Saller *et al.*, 2001). It is widely used for the treatment of various diseases like cirrhosis, chronic hepatitis and diseases associated with alcohol consumption and environmental toxin exposure (Kren and Walterov, 2005). Earlier studies have shown that the antioxidant and free radical scavenging activity of silymarin could be attributed to the number and position of hydroxyl groups in its molecule (Farkas *et al.*, 2004). In addition, silymarin is accepted as a safe herbal product, since no health hazards or side effects have been reported in conjunction with proper administration of dosages (Toklu *et al.*, 2007).

However, little is known the efficacy of silymarin on the poultry production under the condition of disadvantage factors (immune stress and high temperature). This study was conducted to investigate the effects of silymarin on oxidative status and intestinal absorptive function in ducks challenged with cumene hydroperoxide.

MATERIALS AND METHODS

Birds and diets: One hundred and sixty of 28 days old male Cherry Valley ducks (BW, 1.76±0.01 kg) were randomly allotted into 4 dietary treatment groups (4 replicates/group and 10 ducks/replicate): control (ducks fed the basal diet and receiving intraperitoneal administration of sterile saline); CH (ducks fed the basal diet and receiving intraperitoneal administration of cumene hydroperoxide); CH + 100 mg kg⁻¹ SIL (ducks fed the basal diet supplemented with 100 mg kg⁻¹ silymarin and receiving intraperitoneal administration of CH); CH + 200 mg kg⁻¹ SIL (ducks fed the basal diet supplemented with 200 mg kg⁻¹ silymarin and receiving intraperitoneal administration of CH). The dosage of 100 and 200 mg kg⁻¹ was chosen based on its antioxidant properties. The antioxidant activity of silymarin was evaluated by the 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical assay (Iwashima *et al.*, 2005). The details of basal diet were shown in Table 1 and SIL was purchased from commercial company (80%; Hetian Biotech, Hangzhou, China). The birds were housed in stainless steel cages and had free access to feed and water with 24 h of light exposure. At 42 days of age, ducks in the CH group and SIL group were interaperitoneally injected with CH (Sigma chemical; dissolved in sterile saline; 30 mg kg⁻¹ BW) whereas ducks in the control group received intraperitoneal administration of the same volume of

Table 1: Composition and nutrient levels of the basal diets (dry basis)

Ingredients	Values (g kg ⁻¹)
Corn	621.30
Soybean meal	195.10
Bran	58.00
Rapeseed meal	50.00
Cottonseed meal	30.00
Soybean oil	7.10
Limestone	13.00
Dicalcium phosphate	9.20
Salt	3.90
DL-methionine	0.90
Mold inhibitor	1.50
Premix ¹	10.00
Nutrient levels²	
Metabolism energy (MJ kg ⁻¹)	11.84
Crude protein	174.50
Total lysine	8.30
Total methionine	3.60
Calcium	8.00
Total phosphorus	6.20

¹The premix supplied the following per kg of complete feed: retinyl palmitate, 25 mg; cholecalciferol, 62.5 µg; DL-α-tocopheryl acetate, 30 mg; menadione sodium bisulfite, 2.65 mg; thiamin, 2 mg; riboflavin, 6 mg; pantothenic acid, 12 mg; pyridoxine, 2 mg; cyanocobalamin, 25 µg; biotin, 32.5 µg; folic acid, 1.25 mg; nicotinic acid, 50 mg; copper, 8 mg; iron, 80 mg; zinc, 75 mg; manganese, 100 mg; selenium, 0.15 mg; iodine, 350 µg. ²Calculated value

sterile saline at 9:00 am for 5 consecutive days. At 50 days of age, the blood samples were collected and ducks were killed under anesthesia with injection of pentobarbital sodium (40 mg kg⁻¹ BW) to obtain liver and intestine for biochemical analysis. The animal use protocol for this research was approved by the Animal Care and Use Committee of Hubei province (Yongqing *et al.*, 2011).

Blood sample collection and analysis: At 50 days of age, three ducks from each replication were selected for sampling blood from the wing vein. Blood sample were centrifuged at 3000×g for 10 min at 4°C to obtain plasma. Plasma was stored at -80°C until to analysis (Deng *et al.*, 2009). Glutamic-Pyruvic Transaminase (GPT), Glutamic-Oxaloacetic Transaminase (GOT) and Alkaline Phosphatase (AKP) in plasma were determined with automatic biochemical analyzer (Model 7020, Hitachi, Japan) according to the instructions from manufacture's manual.

Intestinal sample collection: At 50 days of age, three ducks from each replication were randomly selected and killed through anesthesia with injection of pentobarbital sodium (40 mg kg⁻¹ BW). The whole intestinal tract was removed and placed on an ice-chilled stainless steel tray according to Hou *et al.* (2010) and Yin *et al.* (2001). The 5, 10 and 10 cm segments from the duodenum (the midpoint), jejunum (midpoint) and ileum (10 cm proximal to the ileocecal junction) were taken. The 5 cm intestinal

segments were flushed gently with ice-cold Phosphate-Buffered Saline (PBS, pH 7.4) and then placed in 10% fresh, chilled formalin solution for histological measurements. The 10 cm intestinal segments were opened longitudinally and the contents were flushed with ice-cold PBS. Mucosa was collected by scraping using a sterile glass microscope slide, rapidly frozen in liquid nitrogen and stored at -80°C until analyses (Kang *et al.*, 2008, 2010).

Intestinal morphology: After a 24 h fixation, the tissue samples were removed from the formalin solution and embedded in paraffin wax. The segments were sliced into $4\ \mu\text{m}$ crosscut sections using a microtome (American Optical Co., Scientific Instrument Div., Buffalo, NY, USA) and stained with hematoxylin and eosin. Morphometric measurements were performed with a light microscope with a computer-assisted morphometric system (BioScan Optimetric, BioScan Inc., Edmonds, WA, USA). Measurements were taken after examining in ten, well-oriented villi and crypts from each intestinal tissue section of a duck (one slide from each duck). The villus height (the distance from the villus tip to crypt mouth) and the associated crypt depth (the distance from the crypt mouth to base) were measured according to the method of Touchette *et al.* (2002).

Measurements of liver oxidative status: At the end of experimental period, ducks were sacrificed and the liver was excised, washed in ice cold saline and blotted to dryness. A 1% homogenate of the liver tissue was prepared in Tris-HCl buffer ($0.1\ \text{mol L}^{-1}$; pH 7.4), centrifuged at 1000 rpm for 10 min at 4°C to remove the cell debris. Malondialdehyde (MDA) in liver was determined by thiobarbituric acid reaction as described by Ohkawa *et al.* (1979), Superoxide Dismutase (SOD) according to Sun *et al.* (1989) and Catalase (CAT) according to the method of Prasad *et al.* (1992). The activity of Glutathione S-Transferase (GST) in the liver tissue was estimated with the method of Habig *et al.* (1974). The activity of Glutathione Peroxidase (GSH-Px) in the liver was estimated using the method of Lawrence and Burk (1976). Liver protein content was estimated as described by Lowry *et al.* (1951).

Measurements of sucrase and maltase activities: The activities of sucrase [EC 3.2.1.48] and maltase [EC 3.2.1.20] in jejunal mucosa were determined according to the method of Dahlqvist (1964). In brief, intestinal mucosal homogenates ($100\ \mu\text{L}$) were incubated at 37°C with $100\ \mu\text{L}$ of $0.056\ \text{mol L}^{-1}$ sugar (sucrose or maltose) dissolved in $0.1\ \text{mol L}^{-1}$ maleate buffer (pH 6.5). After 60 min of

incubation, $0.8\ \text{mL}$ of distilled water was added into the tube and then the enzymic reaction was immediately interrupted by the immersion of the tube in boiling water for 2 min; the tube was then cooled in tap water. At the same time a standard series was incubated, containing 0, 100, 300 and $500\ \mu\text{L}$ of the standard glucose solution (these tubes contain 0, 10, 30 and $50\ \mu\text{g}$, respectively, of glucose), distilled water to the combined volume of 500 and $300\ \mu\text{L}$ of the TGO (Tris-Glucose Oxidase) reagent. Absorbance was measured at 420 nm with a spectrophotometer. Enzyme activities were presented as the amount of enzyme hydrolyzing 1 mol of substrate per min.

Measurements of mucosal protein, DNA and RNA: Frozen jejunal mucosa samples (about 0.1 g) were powdered under liquid nitrogen using a mortar and pestle and then homogenized with a tissue homogenizer in 1 mL of ice-cold PBS-EDTA buffer ($0.05\ \text{mol L}^{-1}\ \text{Na}_3\text{PO}_4$, $2.0\ \text{mol L}^{-1}\ \text{NaCl}$, $2\ \text{mmol L}^{-1}\ \text{EDTA}$, pH 7.4). Protein concentration of small-intestinal mucosal homogenates was measured as described by Lowry *et al.* (1951) and Tan *et al.* (2010) using reagents from BioRad Laboratories (Hercules, CA, USA) and bovine serum albumin as the standard.

The extraction of mucosal RNA was processed according to the method of Fleck and Munro (1962). In brief, frozen intestinal mucosa were powdered and homogenized in ice-cold saline solution (85%). About 2 mL of a cold homogenate of mucosal sample were transferred to a centrifuge tube and 1 mL ice cold $0.6\ \text{mol L}^{-1}\ \text{HClO}_4$ added. After mixing the solution and standing 10 min at 0°C , the precipitate was centrifuged down and washed twice with 2 mL $0.2\ \text{mol L}^{-1}$ cold HClO_4 . Excess acid was removed and $2\ \text{mL}^{-1}\ 0.3\ \text{mol L}^{-1}\ \text{KOH}$ added and then was incubated at 37°C for 1 h. The alkaline solution was cooled in ice and 1 mL cold $1.2\ \text{mol L}^{-1}\ \text{HClO}_4$ added. After centrifugation of the mixture, the supernatant (the acid-soluble RNA fraction) were made up to a suitable volume for absorbancy measurement at the wavelength of 260 and 232 nm to determine the quantity of mucosal RNA (Fleck and Begg, 1965). However, the residual part (the precipitate) of the mixture was collected to extraction and determination of DNA in mucosa (Ceriotti, 1955). Briefly, the precipitate was dissolved in 4 mL cold $1\ \text{mol L}^{-1}\ \text{HClO}_4$ solution and boiled for 10 min and then cooled in ice. After centrifugation of the mixture, the supernatant was made up to a suitable volume for absorbancy measurement at the wavelength of 260, 280 and 320 nm to determine the quantity of mucosal DNA.

Statistical analysis Results are expressed as means with SEM and analyzed by one-way Analysis of Variance (ANOVA) in the SPSS system (SPSS, Chicago, IL). The significance of differences among different groups was evaluated by Tukey-Kramer multiple comparisons test. Overall differences between dietary treatment means were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Body weight: At the end of experiment, the average body weight of ducks did not differ among the control, CH, CH + 100 SIL and CH + 200 SIL groups (CON: 3.22 kg, CH: 3.15 kg, CH + 100 SIL: 3.17 kg, CH + 200 SIL: 3.28 kg; SEM = 0.053; $p = 0.889$), suggesting no treatment effect.

Blood biochemical parameters: Compared with the control group, CH ducks exhibited an increase in plasma activity of GPT ($p < 0.01$) and AKP ($p = 0.082$). In comparison with CH ducks, dietary supplementation of 100 or 200 mg kg^{-1} SIL reduced the activity of GPT ($p < 0.01$) and AKP ($p < 0.05$) (Table 2). There was no difference in activity of GOT among different treatment groups ($p = 0.224$).

The oxidative status of liver and intestinal mucosa: Data for liver and intestinal mucosal oxidative status are shown in Table 3. The MDA levels of liver and mucosa were significantly elevated ($p = 0.060$) in CH ducks compared to control ducks. Diet supplemented with 100 or 200 mg kg^{-1} SIL reduced the concentration of MDA in the mucosa ($p < 0.001$) but not affect in liver ($p > 0.05$). A

significant decrease in the activities of CAT ($p = 0.022$) and GST ($p = 0.034$) in the liver was noted after single dose administration of CH. However, upon feeding of 100 or 200 mg kg^{-1} SIL, the activity of CAT was significantly reversed to normalcy. Compared to the control groups, CH-challenged ducks reduced the activities of SOD and GSH-Px in intestinal mucosa ($p < 0.001$). The diet supplemented with 200 mg kg^{-1} SIL elevated the activity of SOD ($p < 0.05$) and the diet supplemented with 100 or 200 mg kg^{-1} SIL increased the activity of GSH-Px in intestinal mucosa ($p < 0.05$) in comparison with the CH group.

Intestinal morphology: The data for intestinal morphology of control and experimental ducks are shown in Table 4. Compared with control ducks, CH ducks exhibited an increase in crypt depth in duodenum ($p = 0.006$) and ileum ($p < 0.001$) and a decrease in the ratio of villus height to crypt depth in duodenum ($p = 0.044$), jejunum ($p = 0.084$) and ileum ($p = 0.015$). Moreover, the villus height in jejunum was significantly decreased in CH ducks compared to the control ducks. In comparison with the CH ducks, dietary supplementation of 100 or 200 mg kg^{-1} SIL increased the villus height of jejunum and the ratio of villus height to crypt depth in duodenum, jejunum and ileum ($p < 0.05$). However, the diet supplemented with SIL did not affect crypt depth and villus height of duodenum and ileum ($p > 0.05$).

Activities of mucosal sucrase and maltase: The changes in the activities of sucrase and maltase in intestinal mucosa are shown in Table 5. The activities of sucrase

Table 2: Effects of dietary SIL on blood biochemical parameters of ducks after CH challenge

Items	Control	CH	CH + 100 SIL	CH + 200 SIL	SEM	p-value
GPT (U L ⁻¹)	23.10 ^b	25.50 ^a	19.88 ^c	18.11 ^c	0.476	<0.001
GOT (U L ⁻¹)	8.89	8.60	8.33	9.31	0.192	0.224
AKP (mmol L ⁻¹)	417.90 ^{ab}	490.50 ^a	434.31 ^{ab}	408.00 ^b	12.334	0.082

Table 3: Effects of dietary SIL on concentrations of MDA and activities of SOD, CAT, GST and GSH-Px in liver and intestinal mucosa of ducks after CH challenge

Items	Control	CH	CH + 100 SIL	CH + 200 SIL	SEM	p-value
Liver						
MDA (nmol mg ⁻¹ ptn)	1.29 ^b	1.80 ^{ab}	1.92 ^a	1.73 ^{ab}	0.997	0.060
SOD (U mg ⁻¹ ptn)	158.68	132.05	163.83	160.86	5.792	0.311
CAT (U mg ⁻¹ ptn)	54.23 ^a	52.28 ^b	53.38 ^{ab}	54.86 ^a	0.303	0.022
GST (U mg ⁻¹ ptn)	22.38 ^a	20.59 ^b	19.94 ^b	20.43 ^b	0.293	0.034
GSH-Px (U mg ⁻¹ ptn)	96.35	95.04	93.19	95.53	0.490	0.106
Mucosa						
MDA (nmol mg ⁻¹ ptn)	1.89 ^b	4.72 ^a	1.91 ^b	1.82 ^b	0.181	<0.001
SOD (U mg ⁻¹ ptn)	31.86 ^a	22.68 ^b	24.31 ^b	29.16 ^a	0.834	<0.001
GSH-Px (U mg ⁻¹ ptn)	4.27 ^{bc}	2.65 ^c	8.69 ^a	5.43 ^b	0.470	<0.001

Control = Ducks fed the basal diet and injected with sterile saline; CH = Ducks fed the basal diet and challenged with cumene hydroperoxide; CH + 100 SIL = Ducks fed the basal diet supplemented with 100 mg kg^{-1} silymarin and challenged with CH; CH + 200 SIL = Ducks fed the basal diet supplemented with 200 mg kg^{-1} silymarin and challenged with CH. Values within a row with different letters differ ($p < 0.05$) $n = 8$

and maltase were decreased ($p < 0.05$) in intestinal mucosa of CH ducks compared with the control groups. However, the diet supplemented with 100 mg kg⁻¹ or 200 mg kg⁻¹ SIL could reverse the activity of sucrase to normalcy ($p = 0.012$).

Mucosal protein, RNA and DNA: The concentrations of protein, RNA and DNA of jejunal mucosa are shown in Table 6. CH treatment reduced concentrations of protein, RNA, DNA and the ratio of RNA/DNA compared to the control ducks ($p < 0.05$). Compared with CH-treated ducks, dietary supplementation of 200 mg kg⁻¹ SIL increased the protein, RNA, DNA and the ratio of RNA/DNA in jejunum ($p < 0.01$). Cumene hydroperoxide is a strong non-polar oxidizing agent which has been used as a model

compound to assess the effect of oxidative stress on various biological systems (Shimura *et al.*, 1985; Taffe *et al.*, 1987; Ayala *et al.*, 1996). CH was regarded as an intracellular source of reactive oxygen intermediates leading to membrane damage, cell lysis, organ necrosis and tumor promotion (Ayala *et al.*, 1996). The present study was undertaken to investigate the effects of dietary silymarin on oxidative status and intestinal morphology in ducks under the condition of oxidative stress mediated by CH.

In the present study, body weight of ducks slightly decreased in response to CH challenge whereas dietary supplementation of 200 mg kg⁻¹ silymarin could restore the body weight to normalcy. CH has been suggested to cause the generation of reactive oxygen species resulting in oxidative stress and cellular injury. As liver is the site of CH metabolism, the production of reactive oxygen species may be responsible for cell injury and liver damage (Ayala *et al.*, 1996). In this study, CH administered to ducks lead to a marked increase in the activities of blood GPT and AKP which is indicative of hepatocellular damage. This might be a result of the release of these enzymes from cytoplasm, into blood circulation rapidly after rupture of the plasma membrane and cellular damage. GPT, GOT and AKP in blood are the most sensitive markers employed in the diagnosis of hepatic damage because they are cytoplasmic in location and hence released into the circulation after cellular injury (Wroblewski, 1959; Wilkinson, 1970). Treatment with silymarin, particularly at the dose of 200 mg kg⁻¹, dramatically attenuated the increase in plasma activities of the above marker enzymes in CH-treated ducks. This indicates that silymarin tended to be a potential hepatoprotective agent for maintaining the integrity of the plasma membrane and suppressing the leakage of enzymes through membrane. It might be responsible for the restoration in the activities of these enzymes during administration of silymarin. The results consisted with the results of Pradeep *et al.* (2007) who have reported that 50 mg kg⁻¹ silymarin orally for 30 days significantly reversed the diethylnitrosamine induced alterations in serum GPT and GOT.

It has been reported that the status of lipid peroxidation as well as altered levels of certain

Table 4: Effects of SIL on the intestinal mucosal morphology of ducks after CH challenge

Items	Control	CH	CH + 100		SEM	p-value
			SIL	SIL		
Villus height (µm)						
Duodenum	240.02	228.55	231.27	237.72	4.878	0.869
Jejunum	202.75 ^a	177.58 ^b	214.46 ^a	223.11 ^a	4.373	0.006
Ileum	213.37	202.48	235.01	230.51	6.007	0.277
Crypt depth (µm)						
Duodenum	36.78 ^b	42.79 ^a	44.36 ^a	46.62 ^a	0.925	0.006
Jejunum	37.32 ^b	38.39 ^b	43.54 ^a	42.81 ^a	0.756	0.006
Ileum	42.44 ^b	54.51 ^a	51.42 ^a	53.49 ^a	0.910	<0.001
Villus height/crypt depth						
Duodenum	6.28 ^a	5.38 ^b	5.27 ^b	5.08 ^b	0.142	0.044
Jejunum	5.45 ^a	4.64 ^b	5.07 ^{ab}	5.40 ^a	0.110	0.084
Ileum	5.01 ^a	3.82 ^b	4.70 ^a	4.33 ^{ab}	0.124	0.015

Control = Ducks fed the basal diet and injected with sterile saline; CH = Ducks fed the basal diet and challenged with cumene hydroperoxide; CH + 100 SIL = Ducks fed the basal diet supplemented with 100 mg kg⁻¹ silymarin and challenged with CH; CH + 200 SIL = Ducks fed the basal diet supplemented with 200 mg kg⁻¹ silymarin and challenged with CH. Values within a row with different letters differ ($p < 0.05$) n = 8

Table 5: Effects of dietary SIL on the activities of sucrase and maltase in intestinal mucosa of ducks after CH challenge

Items (U mg ⁻¹ ptn)	Control	CH	CH+100		SEM	p-value
			SIL	SIL		
Sucrase	1.64 ^a	0.85 ^b	2.06 ^a	1.64 ^a	0.137	0.012
Maltase	10.81 ^a	7.21 ^b	5.99 ^b	4.98 ^b	0.474	<0.001

Control = Ducks fed the basal diet and injected with sterile saline; CH = Ducks fed the basal diet and challenged with cumene hydroperoxide; CH + 100 SIL = Ducks fed the basal diet supplemented with 100 mg kg⁻¹ silymarin and challenged with CH; CH + 200 SIL = Ducks fed the basal diet supplemented with 200 mg kg⁻¹ silymarin and challenged with CH. Ptn: Protein. Values within a row with different letters differ ($p < 0.05$) n = 8

Table 6: Effects of dietary SIL on concentrations of protein, RNA and DNA in the jejunal mucosa of ducks after CH challenge

Items	Control	CH	CH + 100 SIL	CH + 200 SIL	SEM	p-value
Protein (mg g ⁻¹ mucosa)	79.70 ^b	69.46 ^c	67.84 ^c	90.89 ^a	2.223	<0.001
RNA (mg g ⁻¹ mucosa)	1.65 ^{ab}	1.35 ^b	1.36 ^b	2.04 ^a	0.080	0.002
DNA (mg g ⁻¹ mucosa)	0.29 ^a	0.17 ^b	0.13 ^b	0.26 ^a	0.026	<0.001
RNA/DNA	10.20 ^a	4.49 ^b	9.71 ^a	9.83 ^a	0.523	<0.001

Control = Ducks fed the basal diet and injected with sterile saline; CH = Ducks fed the basal diet and challenged with cumene hydroperoxide; CH + 100 SIL = Ducks fed the basal diet supplemented with 100 mg kg⁻¹ silymarin and challenged with CH; CH + 200 SIL = Ducks fed the basal diet supplemented with 200 mg kg⁻¹ silymarin and challenged with CH. Values within a row with different letters differ ($p < 0.05$) n = 8

endogenous radical scavengers was taken as direct evidence for oxidative stress (Khan, 2006). Free radical scavenging enzymes such as SOD, CAT and GSH-Px protected the tissue or cell from oxidative stress. These enzymes acted mutually and constitute the enzymic antioxidative defense mechanism against reactive oxygen species (Bhattacharjee and Sil, 2006; Pradeep *et al.*, 2007). The second line antioxidant enzyme, GST was also important in detoxifying the lipid peroxidation products (Kiruthiga *et al.*, 2007). In the present study, the reduction in activities of CAT, SOD and GST in ducks after CH challenge may be due to excessive utilization of these enzymes in scavenging the free radicals generated during the metabolism of CH. This was further enhanced by an increase in the concentrations of liver MDA. Several studies have reported similar elevation in the levels of MDA and decrease in the activities of SOD and GSH (Koster *et al.*, 1985; Gebhardt, 1997). Although, not affecting the level of MDA, administration of silymarin at the doses of 200 mg kg⁻¹ could restore the activities of both SOD and CAT and thereby scavenged the accumulation of excessive free radicals and prevented further damage to membrane lipids. Earlier studies have reported that silymarin could act as a scavenger of the free radicals and influence enzyme systems associated with glutathione and superoxide dismutase (Valenzuela *et al.*, 1989; Letteron *et al.*, 1990). The studies are consistent with the studies of Pradeep *et al.* (2007) and Kiruthiga *et al.* (2007). Therefore, the antioxidative property of silymarin might have sparing effect on the activities of the endogenous antioxidant against the free radicals.

The intestine constitutes the key barrier against mucosal penetration of wide array of toxic antigens and molecules such as bacteria and bacterial by-products, food compounds and other proinflammatory agents (Banan *et al.*, 2000). Under the condition of oxidative stress, intestinal mucosal redox balance was perturbed and thereby led to accumulation of lipid hydroperoxides which can promote oxygen radical generation (Aw, 1999). In the present study, CH-treated ducks exhibited a significant decrease in the activities of SOD and GSH-Px in mucosa. This result was substantiated by the lipid peroxidation parameters in CH ducks which showed an increase in MDA and conjugated diene levels, indicative of oxidative stress. It was reported that oxidative stress in the intestine may develop either by the generation of oxygen species in the mucosa or possible inducers generated in the livers and transported to the intestinal lumen through bile when liver was damaged (Ramachandran *et al.*, 2002). This was confirmed by the identification of free radicals in the bile (Knecht *et al.*,

1995) and superoxide production stimulated by deoxycholate in colonic mucosal scrapings or crypt epithelium (Craven *et al.*, 1986). Therefore in this study, oxidative stress in the intestine may have developed by inducer in the liver and transported to the tract through the bile after CH challenge.

There is little information regarding to the effects of oxidative stress induced by CH on the intestinal morphology and mucosal protein synthesis. Oxidative stress might influence functional damage to the intestine by interfering with normal cellular migration along the crypt-villus axis and compromising brush border membrane function (Ramachandran *et al.*, 2002). In the present study, CH-treat ducks had a lower villus height and a higher crypt depth than the control ducks, indicating that oxidative stress might exert unprofitable effects on mucosal cellular migration from crypt to the tip of villus. Treatment with 100 or 200 mg kg⁻¹ silymarin could increase the villus height but did not affect the crypt depth in intestine of CH ducks. This might be due to the diminished of mucosal free radicals and lipid hydroperoxides by the activities of SOD and GSH-Px which was restored by dietary silymarin. This speculation was substantiated by the observation of decreased levels of MDA in CH + SIL groups. Results could be explained by the speculation in term of the increased activities of sucrase and maltase in birds of CH + SIL groups. On the other hand, it was shown that reactive oxygen species produced cellular injury and necrosis via several mechanisms including membrane lipid peroxidation, protein denaturation and DNA damage in mucosa (Ramachandran *et al.*, 2002).

Therefore, reactive oxygen species might inhibit the enterocyte proliferation, affect the inherent cell turnover and reduce the total number of enterocytes in intestinal mucosa which contributed to the changes of intestinal morphology. Silymarin could maintain the redox state of the cell and regulate DNA, RNA and protein synthesis and even regulation of the cell cycle by increasing the content of nonprotein thiols (Soto *et al.*, 2010). In 200 mg kg⁻¹ silymarin group, elevation of mucosal DNA, RNA and protein in jejunum are characteristic of tissues undergoing increased cell proliferation or repair in intestine of ducks treated with CH. The results are in consistent with the studies of Sonnenbichler *et al.* (1999) and Magliulo *et al.* (1973) who have reported that silymarin could stimulate proliferation as well as protein and DNA synthesis in various cell lines. Hence, silymarin could reverse the adverse effects of CH on intestinal morphology to the normalcy either by increasing the activities of SOD and GSH-Px or by stimulating the enterocyte proliferation in mucosa.

CONCLUSION

This study shows that dietary supplementation with 200 mg kg⁻¹ silymarin can alleviate hepatic injury and improve intestinal absorptive capacity in CH-challenged ducks. The effects of silymarin appear to be associated with increased activity of antioxidant enzymes.

ACKNOWLEDGEMENTS

This research was jointly supported by National Agriculture Science and Technology fund projects of China (Grant No.: 2011GB2D100014), Hubei Provincial Research and Development Program (Grant No.: 2010BB023) and Natural Science Foundation of Hubei Province (Grant No.: 2011CDA051).

REFERENCES

- Aw, T.Y., 1999. Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. *Am. J. Clin. Nutr.*, 70: 557-565.
- Ayala, A., J. Parrado, M. Bougria and A. Machado, 1996. Effect of oxidative stress, produced by cumene hydroperoxide, on the various steps of protein synthesis. Modifications of elongation factor-2. *J. Biol. Chem.*, 271: 23105-23110.
- Banan, A., S. Choudhary, Y. Zhang, J.Z. Fields and A. Keshavarzian, 2000. Oxidant-induced intestinal barrier disruption and its prevention by growth factors in a human colonic cell line: Role of the microtubule cytoskeleton. *Free Radic. Biol. Med.*, 28: 727-738.
- Bhattacharjee, R. and P.C. Sil, 2006. The protein fraction of *Phyllanthus niruri* plays a protective role against acetaminophen induced hepatic disorder via its antioxidant properties. *Phytother. Res.*, 20: 595-601.
- Cerriotti, G., 1955. Determination of nucleic acids in animal tissues. *J. Biol. Chem.*, 214: 59-70.
- Craven, P.A., J. Pfanstiel and F.R. DeRubertis, 1986. Role of reactive oxygen in bile salt stimulation of colonic epithelial proliferation. *J. Clin. Invest.*, 77: 850-859.
- Dahlqvist, A., 1964. Method for assay of intestinal disaccharidases. *Anal. Biochem.*, 7: 18-25.
- Deng, D., K. Yao, W.Y. Chu, T.J. Li and R.L. Huang *et al.*, 2009. Impaired translation initiation activation and reduced protein synthesis in weaned piglets fed a low-protein diet. *J. Nutr. Biochem.*, 20: 544-552.
- Farkas, O., J. Jakus and K. Heberger, 2004. Quantitative structure-antioxidant activity relationships of flavonoid compounds. *Molecules*, 9: 1079-1088.
- Fleck, A. and D. Begg, 1965. The estimation of ribonucleic acid using ultraviolet absorption measurements. *Biochim. Biophys. Acta.*, 108: 333-339.
- Fleck, A. and H.N. Munro, 1962. The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. Biophys. Acta.*, 55: 571-583.
- Gebhardt, R., 1997. Antioxidative and protective properties of extracts from leaves of the artichoke (*Cynara scolymus* L.) against hydroperoxide-induced oxidative stress in cultured rat hepatocytes. *Toxicol. Applied Pharmacol.*, 144: 279-286.
- Gong, G.S.M., Y. Yin, R. Huang, Z. Ruan, T. Zhou and M. Xie, 2011. Occurrence of mycotoxins in feeds and feed ingredients in China. *J. Food Agric. Environ.*, 9: 163-167.
- Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.*, 249: 7130-7139.
- Hou, Y., L. Wang, B. Ding, Y. Liu and H. Zhu *et al.*, 2010. Dietary α -ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. *Amino Acids*, 39: 555-564.
- Iwashima, M., J. Mori, X. Ting, T. Matsunaga and K. Hayashi *et al.*, 2005. Antioxidant and antiviral activities of plastoquinones from the brown alga *Sargassum micracanthum* and a new chromene derivative converted from the plastoquinones. *Biol. Pharm. Bull.*, 28: 374-377.
- Kang, P., D. Toms, Y.L. Yin, Q. Cheung, J.H. Gong, K.D. Lange and J.L. Li, 2010. Epidermal growth factor-expressing *Lactococcus lactis* enhances intestinal development of early-weaned pigs. *J. Nutr.*, 140: 806-811.
- Kang, P., Y.L. Yin, Z. Ruan, J. Pan and Q. Hu *et al.*, 2008. Effect of replacement of lactose with partially hydrolysed rice syrup on small intestine development in weaned pigs from 7 to 21 days. *J. Sci. Food Agric.*, 88: 1932-1938.
- Khan, S.M., 2006. Protective effect of black tea extract on the levels of lipid peroxidation and antioxidant enzymes in liver of mice with pesticide-induced liver injury. *Cell Biochem. Funct.*, 24: 327-332.
- Kiruthiga, P.V., R.B. Shafreen, S.K. Pandian and K.P. Devi, 2007. Silymarin protection against major reactive oxygen species released by environmental toxins: Exogenous H₂O₂ exposure in erythrocytes. *Basic Clin. Pharmacol. Toxicol.*, 100: 414-419.

- Knecht, K.T., Y. Adachi, B.U. Bradford, Y. Iimuro, M. Kadiiska, Q.H. Xuang and R.G. Thurman, 1995. Free radical adducts in the bile of rats treated chronically with intragastric alcohol: Inhibition by destruction of Kupffer cells. *Mol. Pharmacol.*, 47: 1028-1034.
- Koster, J.F., R.G. Slee, C.E. Essed and H. Stam, 1985. Studies on cumene hydroperoxide-induced lipid peroxidation in the isolated perfused rat heart. *J. Mol. Cell Cardiol.*, 17: 701-708.
- Kren, V. and D. Walterov, 2005. Silybin and silymarin-new effects and applications. *Biomed. Papers*, 149: 29-41.
- Lawrence, R.A. and R.F. Burk, 1976. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem. Res. Commun.*, 71: 952-958.
- Letteron, P., G. Labbe, C. Degott, A. Berson and B. Fromenty *et al.*, 1990. Mechanism for the protective effects of silymarin against carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice: Evidence that silymarin acts both as an inhibitor of metabolic activation and as a chain-breaking antioxidant. *Biochem. Pharmacol.*, 39: 2027-2034.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randell, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Lykkesfeldt, J. and O. Svendsen, 2007. Oxidants and antioxidants in disease: Oxidative stress in farm animals. *Vet. J.*, 173: 502-511.
- Magliulo, E., P.G. Carosi, L. Minoli and S. Gorini, 1973. Studies on the regenerative capacity of the liver in rats subjected to partial hepatectomy and treated with silymarin. *Arzneimittelforschung*, 23: 161-167.
- Mates, J.M., C. Perez-Gomez and I.N. De Castro, 1999. Antioxidant enzymes and human diseases. *Clin. Biochem.*, 32: 595-603.
- Nijveldt, R.J., E. van Nood, D.E. von Hoorn, P.G. Boelens, K. van Norren and P.A. van Leeuwen, 2001. Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.*, 74: 418-425.
- Ohkawa, H., N. Ohishi and K. Yagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, 95: 351-358.
- Pradeep, K., C.V. Mohan, K. Gobianand and S. Karthikeyan, 2007. Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. *Eur. J. Pharmacol.*, 560: 110-116.
- Prasad, K., P. Lee, S.V. Mantha, J. Kalra, M. Prasad and J.B. Gupta, 1992. Detection of ischemia-reperfusion cardiac injury by cardiac muscle chemiluminescence. *Mol. Cell Biochem.*, 115: 49-58.
- Ramachandran, A., R. Prabhu, S. Thomas, J.B. Reddy, A. Pulimood and K.A. Balasubramanian, 2002. Intestinal mucosal alterations in experimental cirrhosis in the rat: Role of oxygen free radicals. *Hepatology*, 35: 622-629.
- Saller, R., R. Meier and R. Brignoli, 2001. The use of silymarin in the treatment of liver diseases. *Drugs*, 61: 2035-2063.
- Shimura, J., F. Shimura and N. Hosoya, 1985. Functional disability of rat splenocytes provoked to lipid peroxidation by cumene hydroperoxide. *Biochim. Biophys. Acta.*, 845: 43-47.
- Sonnenbichler, J., F. Scalera, I. Sonnenbichler and R. Weyhenmeyer, 1999. Stimulatory effects of silibinin and silicristin from the milk thistle *Silybum marianum* on kidney cells. *J. Pharmacol. Exp. Ther.*, 290: 1375-1383.
- Soto, C., J. Perez, V. Garcia, E. Uria, M. Vadillo and L. Raya, 2010. Effect of silymarin on kidneys of rats suffering from alloxan-induced diabetes mellitus. *Phytomedicine*, 17: 1090-1094.
- Sun, L., T.E. Peterson, M.L. McCormick, L.W. Oberley and J.W. Osborne, 1989. Improved superoxide dismutase assay for clinical use. *Clin. Chem.*, 35: 1265-1266.
- Taffe, B.G., N. Takahashi, T.W. Kensler and R.P. Mason, 1987. Generation of free radicals from organic hydroperoxide tumor promoters in isolated mouse keratinocytes. Formation of alkyl and alkoxy radicals from tert-butyl hydroperoxide and cumene hydroperoxide. *J. Biol. Chem.*, 262: 12143-12149.
- Tan, B.E., Y.L. Yin, X.F. Kong, P. Li and X.L. Li *et al.*, 2010. L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. *Amino Acids*, 38: 1227-1235.
- Toklu, H.Z., T. Tunaly-Akbay, G. Erkanly, M. Yuksel, F. Erc an and G. Sener, 2007. Silymarin, the antioxidant component of *Silybum marianum*, protects against burn-induced oxidative skin injury. *Burns*, 33: 908-916.
- Touchette, K.J., J.A. Carroll, G.L. Allee, R.L. Matteri, C.J. Dyer, L.A. Beausang and M.E. Zannelli, 2002. Effect of spray-dried plasma and lipopolysaccharide exposure on weaned pigs: I. Effects on the immune axis of weaned pigs. *J. Anim. Sci.*, 80: 494-501.
- Valenzuela, A., M. Aspillaga, S. Vial and R. Guerra, 1989. Selectivity of Silymarin on the increase of the glutathione content in different tissues of the rat. *Planta Med.*, 55: 420-422.

- Voljc, M., T. Frankic, A. Levart, M. Nemeč and J. Salobir, 2011. Evaluation of different vitamin E recommendations and bioactivity of α -tocopherol isomers in broiler nutrition by measuring oxidative stress *In vivo* and the oxidative stability of meat. *Poult. Sci.*, 90: 1478-1488.
- Wilkinson, J.H., 1970. Clinical significance of enzyme activity measurements. *Clin. Chem.*, 16: 882-890.
- Wroblewski, F., 1959. The clinical significance of transaminase activities of serum. *Am. J. Med.*, 27: 911-923.
- Wu, X., Z. Ruan, Y. Gao, Y. Yin and X. Zhou *et al.*, 2010. Dietary supplementation with L-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. *Amino Acids*, 39: 831-839.
- Yin, Y.L., S.K. Baidoo, H. Schulze and P.H. Simmins, 2001. Effect of supplementing diets containing hullless barley varieties having different levels of non-starch polysaccharides with α -glucanase and xylanase on the physiological status of gastrointestinal tract and nutrient digestibility of weaned pigs. *Livest. Prod. Sci.*, 71: 97-107.
- Yongqing, H., K. Yao, L. Wang, B. Ding and D. Fu *et al.*, 2011. Effects of α -ketoglutarate on energy status in the intestinal mucosa of weaned piglets chronically challenged with lipopolysaccharide. *British. J. Nut.*, 106: 357-363.
- Yu, J., P. Yin, J. Yin, F. Liu and X. Zhu *et al.*, 2010. Involvement of ERK1/2 signalling and growth related molecules expression in response to heat stress induced damage in rat jejunum and IEC cells. *Int. J. Hyperthermia*, 26: 538-555.