Pre-protective effects of dietary chitosan supplementation against oxidative stress induced by diquat in weaned piglets

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Abstract

The protective effects of chitosan (CS) supplementations on oxidative stress induced by diquat in weaned piglets were investigated. A total of 36 crossbreed piglets with an average live body weight (BW) of 8.80 ± 0.53 kg were weaned at 28 ± 2 days and randomly divided into six dietary treatments ($n = 6$): control (basal diet), negative control (10 mg diquat/kg BW injected to piglets fed with basal diet), and basal diet treatments containing either 250, 500, 1000, or 2000 mg/kg of CS administered to piglets injected with 10 mg diquat/kg BW. The experiment conducted for 21 days which consisted of pre-starter period (14 days) and starter period (7 days). BW, feed intake, and fecal consistency were monitored. Blood samples were collected to determine antioxidative and immune parameters. CS supplementation improved the growth performance and decreased fecal score of piglets from days 1 to 14. Diquat also induced oxidative stress and inflammatory responses by decreasing the activities of antioxidant and regulating cytokines. But dietary CS alleviated these negative effects induced by diquat that showed decreasing serum concentrations of pro-inflammatory cytokines but increasing activities of antioxidant enzymes and anti-inflammatory cytokines. Results indicated that CS attenuated the oxidative stress of piglets caused by diquat injection.

Keywords Chitosan · Growth performance · Diarrhea · Antioxidant capacity · Cytokine

Introduction

After weaning, piglets are subjected to an increased susceptibility to gastrointestinal disorders, infections, and diarrhea. Additionally, weaning can induce oxidative stress and damage including growth retardation, disease, and even death (Zhu et al. 2012), causing serious economic losses. Under normal circumstances, reactive oxygen species (ROS) in the body are maintained homeostasis involving maintenance of signal transduction, gene expression, and activation of receptors (Kumar and Pandey 2015). However, excessive oxidative radicals are generally neutralized by the antioxidant system including non-enzymatic components, such as glutathione, selenium, vitamin E, and vitamin C, and a series of antioxidant

enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Imbalance in this protective mechanism can lead to the damage of DNA, proteins, and lipids (Durackova 2010). In addition, under hypoxic conditions, nitric oxide (NO) may also be produced during the respiratory chain reaction (Poyton et al., 2009), further leading to the production of reactive species such as malondialdehyde (MDA) and 4-hydroxynonenal (Hussain et al. 2003). Indeed, overproduction of ROS, such as H_2O_2 , and reactive nitrogen species (RNS), such as NO, could result in oxidative stress (Valko et al. 2007) and changes in the antioxidant defense capacity, including decreased activity of SOD, CAT, and GSH-Px (Han et al. 2009; Han et al. 2011). Moreover, oxidative stress may be deleterious and cause irreversible damage to cellular structure and functions, resulting in cell death by the necrotic and apoptotic processes, somatic mutations, and pre-neoplastic and neoplastic transformations (Wang et al., 2004).

In the past, post-weaning disorders were controlled using antibiotic growth promoters in weaned piglet diets (Williams et al. 2001). Currently, specific dietary interventions can offer a viable and practical alternative to antibiotics that alleviate physiological disorders after weaning. Supplementation of

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natural antioxidants, such as vitamins E (Bonnette et al. 1990) and C (Eicher et al. 2006), tea polyphenols (West et al. 2008), probiotics (Deng et al. 2010), and chitosan (CS) (Xiong et al. 2015), has been used to relieve stress in livestock husbandry. Chitosan is a natural polysaccharide with strong bioactivity, including immune-stimulant (Xiong et al. 2014), anti-inflammatory (Villiers et al. 2009), and antioxidant properties (Mendis et al. 2007). Chitosan has been reported to reduce diarrhea during weaning (Xu et al. 2017) and have beneficial effects on relieving the oxidative stress (Anandan et al. 2012). In addition, it is used in nursery diets for growth promotion. However, the causal link between CS and the improvement in piglets' health status remains largely unknown. Diquat dibromide is a commercially available herbicide that is used extensively to induce animal oxidative stress. Thus, the protective roles of dietary CS in diquat-induced oxidative stress in piglets were investigated in the present study.

Materials and methods

Animal and experimental design

The experiment was arranged as a randomized design. A total of 36 healthy piglets (Duroc × Yorkshire × Landrace, 18 males and 18 females) weaned at the age of 28 ± 2 days with an average initial body weight (BW) of 8.80 ± 0.53 kg were selected from a commercial herd. The animals were housed in 36 pens (1 piglet/pen) taking litter and initial BW into account and randomly divided into six treatments with six replicates in each group. Each pen contained a nipple drinker and a self-feeder to provide ad libitum access to water and feed. The temperature was controlled between 25 and 29 °C. The treatments were as follows: control (C, basal diet), negative control (NC, basal diet fed to piglets injected with 10 mg diquat/kg BW), and CS group (basal diet treatments containing either 250, 500, 1000, or 2000 mg/kg of CS administered to piglets injected with 10 mg diquat/kg BW). Basal diet was shown in Table 1 and formulated to meet or exceed the nutrient requirements recommended by the National Research Council (2012). The experiment lasted for 21 days which consisted of a pre-starter period (14 days) and a starter period (7 days). At 08.00 h on day 15, the piglets were injected intraperitoneally (i.p.) with diquat at 10 mg/kg BW (NC and CS groups) or sterile saline (control group). Diquat (diquat dibromide monohydrate, PS365; Sigma Co.) was dissolved in isotonic saline and filter-sterilized.

Measurement of growth performance and diarrhea

Piglets were weighed before the morning feeding on days 1, 15, and 22. Feed intake was recorded, and orts were collected and weighed daily. All feed was mash. Average daily feed

Table 1 Composition and nutrient levels of experimental diets (air-dry basis, g/kg)

Ingredients	Content	Nutrient levels	
Maize	600.9	Digestive energy (MJ/kg)	13.94
Soybean meal	265.7	Crude protein (N × 6.25)	203.7
Wheat bran	50.0	Lysine	14.5
Fish meal	30.0	Methionine	4.7
Soybean oil	10.0	Methionine + cysteine	8.7
Limestone	13.9	Threonine	9.6
CaHPO ₄	8.9	Tryptophan	2.4
NaCl	3.0	Calcium	9.0
L-lysine HCL	4.5	Phosphorus	6.1
DL-methionine	1.6		
L- threonine	1.5		
Premix ¹	10.0		

¹ The premix provided the following per kilogram of the diet: vitamin A, 8000 IU; vitamin D₃, 2000 IU; vitamin E, 34 mg; vitamin K₃, 2.8 mg; vitamin B₁, 2.6 mg; vitamin B₂, 6.0 mg; vitamin B₆, 7.0 mg; vitamin B₁₂, 0.02 mg; pantothenic acid, 12 mg; nicotinic acid, 50 mg; biotin, 0.47 mg; folic acid, 0.85 mg; Cu (as copper sulfate), 11 mg; Zn (as zinc sulfate), 80 mg; Mn (as manganese sulfate), 40 mg; Fe (as ferrous sulfate), 94 mg; I (as potassium iodide), 0.35 mg; Se (as sodium selenite), 0.35 mg; Co (as cobalt sulfate), 0.3 mg; choline chloride, 750 mg; phytase, 500 FTU

intake (ADFI), average daily gain (ADG), and the ratio of gain to feed intake (G:F) were calculated.

The fecal consistency was classified using a score ranking from 1 to 5 (Pierce et al 2005): with (1) hard firm feces; (2) slightly soft feces; (3) soft, partially formed feces; (4) loose, semi-liquid feces (diarrhea); and (5) watery, mucous-like feces (serious diarrhea). Feces were scored daily for individual piglets by a trained individual with no prior knowledge of the dietary treatment.

Biochemical analysis of sample

Blood samples were collected from venepuncture into non-heparinized tubes at 5 h after injection of diquat and on the morning (08:00 h) of 7 days after injection and were allowed to coagulate for 30 min before centrifugation (3000×g, 15 min, and 4 °C). Serum samples were frozen and stored at −20 °C until analysis. The serum was used to determine MDA content and the enzymatic activity of CAT, SOD, and GSH-Px using commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China). The parameters of oxidative stress in serum including cortisol and adrenocorticotrophic hormone (ATCH) levels, immune parameters including IL-1β, IL-2, IL-4 IL-6, TNF-α, and γ-INF, and immunoglobulin (Ig) including IgM, IgA, and IgG were determined using an ELISA kit (Nanjing Boerdi Institute, Jiangsu, China). All kits used were validated for pig serum, following manufacturer's standard procedures. All samples were analyzed in duplicate.

Statistical analysis

Fecal consistency data were analyzed using the mixed procedure of statistical analysis system. The mixed model included the fixed effects of the diet treatment, experimental day, and the interaction between the dietary treatment and sampling day. Other data were tested for normality of distribution (PROC UNIVARIATE). Differences between treatment groups for normally distributed data were determined by using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer honest significant difference test using SAS (SAS Institute, Cary, NC, USA). A value of $P < 0.05$ was considered to be statistically significant. Values were expressed as mean $\pm$ standard error.

Results

Growth performance and diarrhea

As shown in Table 2, during the pre-starter period, the ADG of piglets fed with the 250–1000 mg CS/kg diets was greater than piglets fed with the non-CS diet ($P = 0.001$), and the G:F of piglets was enhanced in 500 and 1000 mg CS/kg diet groups ($P = 0.012$) compared with piglets fed with the non-CS diet. During the starter period, compared with the piglets in control group, diquat exposure reduced ADG. But in diquat-treated piglets, dietary CS increased the ADG from days 14 to 21 ($P < 0.05$).

CS treatment at 1000 mg/kg decreased fecal score at day 2 compared with NC group ($P < 0.05$) (Fig. 1). Fecal score of piglets in the 250 mg CS/kg diet group was lower than that of

control group at day 10 ($P < 0.05$). Feeding 500 mg CS/kg significantly decreased the fecal score of piglets compared with control group at day 12 ($P < 0.05$). For the first week after injection, fecal score decreased in the CS group compared with that in the NC group ($P < 0.001$). The dietary treatment and sampling day, respectively, had significant effects on fecal scores during the overall experimental period ($P < 0.05$), whereas there was no interaction effect between the dietary treatment and sampling day ($P > 0.05$).

Blood stress indicators

As shown in Fig. 2, at 5 h post-injection, exposure to diquat increased the concentrations of serum ACTH in piglets of NC and CS diet treatments compared with those in the control group ($P < 0.05$). But the supplementation of CS decreased serum concentration of ACTH in piglets fed with CS diet with diquat treatment compared with NC group (Fig. 2a). At 7 days post-injection, no differences ($P > 0.05$) were observed in the serum concentrations of ACTH and cortisol among piglets fed with the basal diet and 500–2000 mg/kg CS diets with diquat treatment.

The parameters of oxidative stress in serum

At 5 h post-injection, the serum SOD activity of piglets in NC group was lower than piglets in the control and CS diets with diquat treatment groups ($P < 0.05$, Fig. 3a). Exposure to diquat without CS decreased ($P < 0.05$) the CAT activity in piglets, but the supplementation of CS increased ($P < 0.05$) the CAT activity compared with NC group (Fig. 3b). The serum GSH-Px activity was lower in piglets challenged with

Table 2 Growth performance of weaned piglets

Items		Diquat (10 mg/kg BW)					SEM	<i>P</i> value
C	NC	Chitosan mg/kg diet						
		250	500	1000	2000			
Pre-starter (1–14 days)								
ADG (g/day)	277 ^{bc}	266 ^c	345 ^a	375 ^a	345 ^a	329 ^{ab}	23.4	0.001
ADFI (g/day)	569	574	590	614	566	569	28.5	0.859
G:F (g/g)	0.50 ^b	0.48 ^b	0.59 ^{ab}	0.61 ^a	0.61 ^a	0.58 ^{ab}	0.04	0.012
Starter (15–21 days)								
ADG (g/day)	423 ^a	249 ^c	314 ^b	346 ^b	340 ^b	337 ^b	27.6	<0.001
ADFI (g/day)	744 ^a	602 ^b	658 ^{ab}	685 ^{ab}	654 ^{ab}	656 ^{ab}	33.6	0.076
G:F (g/g)	0.57 ^a	0.41 ^c	0.48 ^b	0.51 ^{ab}	0.52 ^{ab}	0.52 ^{ab}	0.03	<0.001

The treatments were as follows: C, control (basal diet without CS and diquat); NC, negative control (basal diet with diquat), and CS (basal diet supplemented with 250, 500, 1000, and 2000 mg/kg diet CS and 10 mg/kg body weight diquat). Data are means of six piglets

Different letters within the same line denote significant differences between diets

Fig. 1 Fecal consistency in piglets receiving a control diet (C) or the same diet supplemented with 0, 250, 500, 1000, and 2000 chitosan mg/kg diet during the initial 21 days post-weaning and 10 mg diquat/kg body weight during the last 7 days (1 = hard firm feces to 5 = watery, mucous-like

feces). Values represent means $\pm$ standard error. Different letters in the same sampling day denote significant differences between diets. *P* values for treatment, day, and treatment $\times$ day are from the ANOVA for the days 1–14 and days 15–21 periods

diquat compared with piglets fed with control diet ($P < 0.05$, Fig. 3c).

At 7 days post-injection, the serum SOD activity of piglets in NC group was lower than that in the control group ($P < 0.05$), and piglets fed with the CS diets with diquat treatment were similar to piglets fed with the control diet (Fig. 3a).

The serum CAT activity was greater in piglets fed with 500 mg/kg CS diet with diquat treatment compared with piglets fed with control diet ($P < 0.05$, Fig. 3b). The serum GSH-Px activity was lower in piglets in NC group than those in the control group ($P < 0.05$), but no differences ($P > 0.05$) were observed in serum GSH-Px activity among piglets fed with the basal diet and CS diets with diquat treatment (Fig. 3c). Serum MDA concentration in piglets fed with CS diets and challenged with diquat was significantly higher than that in the control group, but lower than that in the NC group ($P < 0.05$) both at 5 h and 7 days post-injection (Fig. 3d).

Fig. 2 a, b Stress hormones in piglets receiving a control diet (C) or the same diet supplemented with 0, 250, 500, 1000, and 2000 chitosan mg/kg diet during the initial 21 days post-weaning and 10 mg diquat/kg body weight during the last 7 days. Values represent means $\pm$ standard error. a,b,c,d Mean values with unlike letters were significantly different ($P < 0.05$)

Determination of serum immune parameters

At 5 h post-injection, exposure to diquat increased ($P < 0.05$) the serum IL-1 β concentration in piglets, but serum IL-1 β content was decreased ($P < 0.05$) when piglets were fed with the CS diet compared with those in NC group (Fig. 4a). The concentrations of serum IL-6 (Fig. 4d) and TNF- α (Fig. 4e) were greater in piglets after diquat challenged than those in the control group ($P < 0.05$, $P < 0.05$).

At 7 days post-injection, the serum IL-1 β concentration was lower in piglets fed with CS diets with diquat injection than that in the NC group ($P < 0.05$), but was similar to that in the control group (Fig. 4a). Exposure to diquat increased the serum TNF- α concentration in piglets ($P < 0.05$), but TNF- α content was decreased ($P < 0.05$) when piglets were fed with the 500–2000 mg/kg CS diets with diquat injection compared with piglets in NC group (Fig. 4e). The levels of IL-2 (Fig. 4b), IL-6 (Fig. 4d), γ -INF (Fig. 4f), IgM, IgG, and IgA (Fig. 5) in serum of diquat injection were measured, but no significant difference was observed among treatments both at 5 h and/or 7 days post-injection ($P > 0.05$).

Fig. 3 a–d Serum antioxidant enzyme and MDA in piglets receiving a control diet (C) or the same diet supplemented with 0, 250, 500, 1000, and 2000 chitosan mg/kg diet during the initial 21 days post-weaning and 10 mg diquat/kg body weight during the last 7 days. Values represent means $\pm$ standard error. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$)

Discussion

Oxidative stress induced by diquat has been reported to affect growth performance (Lu et al. 2010; Lv et al. 2012; Qiang et al. 2014). In study of Yuan et al. (2007), at the beginning of post-injection, vomiting and anorexia occurred for all the diquat-treated piglets. Similar to that, in this study, injecting diquat significantly decreased ADG and G:F, whereas it increased the diarrhea. Meanwhile, supplementation with CS relieved the inhibitory effect of diquat treatment on growth performance in piglets, suggesting a potentially important role for CS inhibiting adverse effects of oxidative stress in weaning piglets.

Under stress, the hypothalamus secretes corticotrophin-releasing hormone, and this provokes the release of ACTH from the pituitary. ACTH triggers the secretion of glucocorticoids from the adrenal cortex (Kudielka et al. 2005). Cortisol is the main glucocorticoid of the hypothalamus-pituitary-adrenal (HPA) axis in piglets. The elevation of cortisol activates the HPA axis providing a convenient index for monitoring stress status of farm animals (Mormède et al. 2007). Cortisol regulates or impacts other important physiological systems, like the immune system and the sympathetic-adrenal-medullary axis. Chitosan can alleviate animal stress and improve the antioxidant function of animals. And it can reduce the level of ACTH and cortisol in weaned piglets (Li et al. 2013), indicating that it has the function of reducing stress. This experiment indicated that CS significantly

decreased the elevation of ACTH in piglets caused by diquat challenge, suggesting that CS could alleviate stress. Although the mechanism of CS to relieve stress of weaning piglets is still unclear, we hypothesize that the piglets fed with CS can save a portion of their energy to resist stress, and this saved energy may be invested into the better growth performance and health status. In addition, we noticed a decrease of cortisol content in serum of piglet, which can alleviate the inhibition of immunity as well as improve the immune function.

The largest amounts of reactive oxygen species are produced in mitochondria by the transfer of unpaired electrons to O_2^- , then converted to H_2O_2 by manganese-dependent SOD that exists in various tissues and organisms, which protect cells from damage caused by O_2^- (Kadenbach et al. 2009). Catalase and GSH-Px can act as catalysts to transform H_2O_2 into non-toxic H_2O (Rajat and Panchali 2014). In the present study, the activities of SOD and CAT in serum of diquat-treated piglets were lower than those in the control group at 5 h post-injection, indicating that antioxidative capabilities of piglets were damaged. At 7 days post-injection, dietary CS treatment significantly decreased the decline of the activities of serum SOD and GSH-Px of piglets caused by diquat challenge; the serum CAT, however, was greater in piglets fed with 500 mg/kg CS diet compared with piglets fed with the control diet. This is analogous to the results of Tao et al. (2013) who indicated that the CS could enhance the SOD activity in serum. In addition, investigations using rats as models have suggested that CS can prevent lipid peroxidation

Fig. 4 a–f Cytokines in piglets receiving a control diet (C) or the same diet supplemented with 0, 250, 500, 1000, and 2000 chitosan mg/kg diet during the initial 21 days post-weaning and 10 mg diquat/kg body weight during the last 7 days. Values represent means $\pm$ standard error. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$)

induced by lipopolysaccharide and retain GSH content and CAT activity (Qiao et al., 2011). Piglets receiving diquat had significantly higher concentration of MDA than those receiving isotonic saline in the serum. The serum MDA was lower in piglets fed with CS diets compared with piglets in the NC group. Malondialdehyde is the most common indicator of lipid peroxidation, and its level can be regarded as an evaluation index of lipid oxidative injury in tissues (Pirinciglu et al. 2010). The overall decrease of MDA values with the supplementation of CS showed that CS might provide a protective impact against the damaging effects of oxidation reactions in piglets. Hydroxy and amino group in CS can be served as hydrogen donors to the proxy radicals, hence protecting cells from damage (Feng et al. 2008). Indeed, in our study, CS supplementation in diets had pre-protective effects against oxidative stress induced by diquat in weaned piglets, suggesting that CS might contribute to the enhancement of antioxidative functions.

Inflammation is induced by the innate immune system in response to invading pathogens and tissue trauma. Systemic

inflammation arises secondary to local inflammation if pro-inflammatory substances such as pro-inflammatory cytokines are not successfully functioned in local inflammatory processes (Seeley et al. 2012). A systemic response might be due to release of locally produced pro-inflammatory cytokines to circulation (Gabay and Kushner 1999), so serum cytokine profiles could be used as markers of inflammation (Burton et al. 2009; Pusterla et al. 2006). Serum levels of cytokines and immunoglobulins can show the immune system status of animals; therefore, our study measured the concentrations of cytokines and immunoglobulins in serum. Previous studies demonstrated that dietary CS supplementation, under normal conditions, enhanced immune response in early weaned piglets (Li et al. 2013) and beef cattle (Li et al. 2015) as well as the human (Baek et al. 2007) by modulating the production of cytokines and antibodies, such as IL-1, IL-2, and TNF- α . In this study, diquat injection significantly increased the contents of serum IL-1 β , IL-6, and TNF- α in piglets, whereas decreased the production of serum IL-4. Meanwhile, diet supplemented with CS significantly reduced the concentrations of serum IL-1 β and TNF- α . Bits of cytokines

Fig. 5 a–c Serum immunoglobulins in piglets receiving a control diet (C) or the same diet supplemented with 0, 250, 500, 1000, and 2000 chitosan mg/kg diet during the initial 21 days post-weaning and 10 mg diquat/kg body weight during the last 7 days. Values represent means $\pm$ standard error

may serve as cell-signaling molecules, which trigger a disease-fighting response from nuclear factor of kappa B signal pathway, hence promoting the formation of immunoglobulins (Epinat and Gilmore 1999) and cytokines, such as IL-1, IL-2, IL-6, and TNF- α , as well as growth factor (Huang and Miyamoto 2001). Inflammation itself cannot be viewed as a disease but should be rather viewed as a biological process. Our results indicated that CS supplementation exerted a positive influence on immune function in the weaned piglets. These cytokines promoted the proliferation and differentiation of lymphocytes and the secretion of antibodies (Malek et al. 2001).

It is known those amino groups, which are found in CS, can be recognized by B cell receptors and promote the secretion of corresponding antibody (Tokura et al. 1999). Immunoglobulins,

secreted by B cells, are the main antibody isotypes of the serum and extracellular fluid immune system, therefore allowing them to control the infection of body tissues. However, in the present study, there were no significant changes in the serum IgG, IgA, and IgM after the consumption of CS diets in piglets under diquat-induced oxidative stress, in contrast to the reports showing that the serum levels of IgG (David et al. 2007), IgA, and IgM (Yin et al. 2008) were markedly increased in animals fed with CS. The different humoral immune response to CS addition may depend on the dose and exposure duration of CS, and animal species (piglets, rats, chicken), or other experimental conditions. Moreover, immune response may also vary according to animal health status and management conditions.

In conclusion, CS showed antioxidative activities and anti-inflammatory effects on piglets in the present study. The results indicated that dietary supplementation with CS relieved the growth performance impairment, and alleviated oxidative stress and inflammatory response induced by diquat in piglets. It demonstrated that CS was an accessible source of natural antioxidants and could be used in feed and as a therapeutic agent.

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Compliance with ethical standards

The experimentation was carried out at the experimental farms of the Inner Mongolia Agricultural University in China and was approved by the Animal Care and Use Committee of Inner Mongolia Agricultural University.

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