Protective effect of chitosan against growth inhibition and pancreatic oxidative stress in weaned piglets

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ABSTRACT
This study was undertaken to determine the protective effect of chitosan against oxidative injury in pancreas of weaned piglets. A 2 × 2 factorial experiment was conducted. Twenty-four weaned piglets were randomly divided into one of two dietary treatments (0 or 0.5 g/kg chitosan). After 2 weeks of feeding, half of the piglets on each dietary treatment were given an intraperitoneal injection of diquat or saline. Piglets were killed and samples were collected to assess enzyme activity, redox parameters and nutrient apparent digestibility. The results showed that diquat decreased growth performance and induced pancreatic injury, markedly increased pancreas weight, the pancreatic MDA content, activities of pancreatic lipase, serum amylase and lipase, but decreased antioxidant enzyme activity of pancreas. However, chitosan improved growth performance, decreased the relative pancreas weight, activities of serum amylase and lipase, pancreatic lipase activity and crude fat apparent digestibility, but increased the pancreatic T-AOC and activities of antioxidant enzyme, pancreatic trypsin activity, and crude protein apparent digestibility. Furthermore, in the weaned piglets challenged with diquat, chitosan inclusion alleviated the activities of serum amylase and lipase, and pancreatic MDA concentration. The results of the present study indicated that chitosan could be a safe and potent new additive for alleviating pancreatic oxidative injury.

Introduction
Oxidative stress, caused by excessive reactive oxygen species (ROS), can lead to damage of all biological molecules, including DNA, RNA, bio-membrane lipids, carbohydrates, proteins and other macromolecules (Cadenas and Davies 2000), and then potentially damage the whole organism’s function. Many natural antioxidants can prevent or delay the oxidation of oxidizable substrates. Among these, several biological polysaccharides, such as chitosan, were reported to have strong scavenging effect on free radical in vitro (Xie et al. 2001; Je and Kim 2006). Chitosan, an abundant marine origin polysaccharide isolated from the exoskeleton of crab, prawns, oysters, fungi and crustaceans (Cárdenas et al. 2001), which is an alkaline glucosamine biopolymer and chemically similar to that of cellulose. Previous reports have shown that chitosan has antitoxic (Santhosh et al. 2006), antibacterial (Benhabiles et al. 2012) and antioxidant (Xie et al. 2001) potentials. Though the beneficial effects of chitosan have been extensively studied, the protective effect of chitosan against oxidative damage in vivo has not yet been explored. In addition, diquat, a commonly used bipyridylium herbicide, could convert molecular oxygen into superoxide anion that subsequently forms hydrogen peroxide by redox metabolism, which has been widely used as an effective model chemical agent for studies of oxidative stress in animal (Yuan et al. 2007). Therefore, in the present study, diquat was used as an agent to establish oxidative stress model by injection, and chitosan was used as the dietary source to mitigate the pancreatic injury of diquat-induced oxidative stress. It was hypothesized that supplementation of the diet with chitosan before and after diquat injection would relieve pancreatic oxidative injury by improving antioxidant function.

Materials and methods
This experiment was approved by the Animal Care and Use Committee of Inner Mongolia Agricultural University.

Chitosan
Chitosan powder used in this experiment was a natural product extracted from shrimp shells and contains active amino and hydroxyl group, which contribute to its many biological activities. The deacetylation degree of chitosan was approximately 85% and average molecular weight was 232 kDa.

Experimental design and dietary treatments
The experiment was designed as a 2 × 2 factorial arrangement. A total of 24 healthy crossbred weaned piglets (Duroc × Yorkshire × Landrace, 12 boars and 12 sows) initially weighing 10.20 ± 0.76 kg were allotted randomly into one of two dietary treatments (0 or 0.5 g/kg chitosan; n = 12 pigs/dietary treatment). Ingredients composition and chemical analysis...
composition of the basal diet are given in Table 1. The diets were formulated to meet or exceed the requirements of 10–20 kg of pigs according to NRC (2012). On d 15, half of the piglets (three male and three female) on each dietary treatment were given an intraperitoneal injection of 10 mg diquat/kg body weight in 0.5 mL saline, the other half were injected with the same volume of saline. The experiment lasted for 21 d.

### Sampling and measurements

At the end of the experimental period, body weight of all piglets was recorded and blood samples were collected via venipuncture for harvesting serum. After that, all piglets were anesthetized with intravenous injection of sodium phenobarbital and slaughtered, pancreas samples were weighted, collected, then immediately placed in liquid nitrogen and stored at −20°C for enzyme activity analysis. The feed and rectal content samples were collected to determine nutrient apparent digestibility.

The activities of amylase and lipase in serum, the total antioxidant capacity (T-AOC), activities of superoxide dismutase (SOD: including T-SOD, CuZn-SOD and Mn-SOD), glutathione peroxidase (GPx), catalase (CAT), trypsin and lipase, and the content of malonaldehyde (MDA) in pancreas were measured spectrophotometrically using commercial assay kits (Nanjing Jiangcheng Bioengineering Institute, China), according to the manufacturer’s instructions.

Acid insoluble ash (AIA) was used as an endogenous marker to determine the apparent digestibility. The AIA in feed and fecal samples were determined using a method described by the Standards Press of China (2009). All feed and fecal samples were analyzed for crude protein and crude fat as described by the AOAC (2000).

### Statistical analysis

The experimental data were analyzed using a 2 × 2 factorial with the general linear model procedures of the SAS (Version 8.1; SAS Institute, Inc.). The statistical model consisted of the main effects of dietary treatment (chitosan vs. no chitosan), diquat challenge (saline vs. diquat) and their associated interactions. The individual piglet served as the experimental unit for all variables measured. P < 0.05 was considered statistical significance, whereas P < 0.10 was considered to represent a tendency. Data were presented as mean and the standard error of the mean.

### Results

As shown in Table 2, before diquat injection, piglets offered diets containing chitosan had a higher ADG than piglets offered basal diets (P = 0.09). After diquat injection, there was no interaction between chitosan and diquat on the growth performance of piglets (P > 0.05). Diquat injection decreased the final body weight (P = 0.05), ADG (P < 0.01), ADFI (P < 0.01) and G:F (P = 0.01) of piglets. However, dietary chitosan supplementation increased the ADG of piglets (P = 0.09).

As shown in Table 3, diquat injection increased the relative pancreas weight (P = 0.06), activities of serum amylase (P < 0.01) and lipase (P < 0.01), pancreatic lipase activity (P < 0.01) and MDA concentration (P = 0.05), and decreased the activities of antioxidant enzymes in pancreas including T-SOD (P = 0.09), GPx (P < 0.01) and CAT (P = 0.04), and pancreatic trypsin activity (P = 0.09). However, dietary chitosan supplementation decreased the relative pancreas weight (P = 0.09), activities of serum amylase (P = 0.03) and lipase (P < 0.01), pancreatic lipase activity (P < 0.01), crude fat apparent digestibility (P < 0.01), but increased pancreatic T-AOC (P = 0.05) and activities of antioxidant enzyme including T-SOD (P = 0.07), CuZn-SOD (P = 0.03) and GPx (P = 0.01), pancreatic trypsin activity (P < 0.01) and crude protein apparent digestibility (P = 0.05). Furthermore, in the weaned piglets challenged with diquat, dietary chitosan supplementation could alleviate activities of serum amylase (P = 0.02) and lipase (P = 0.02), and pancreatic MDA concentration (P = 0.09).

### Discussion

Pancreas is one of the important secretory organs and the normal operation of its function is essential for the digestion and absorption of nutrients. However, pancreas is a main target of free radicals based on its high synthesis and secretion activity, resulting in oxidative damage and negative effect on endocrine and exocrine function. This study was carried out to investigate the therapeutic effects of chitosan on the pancreatic oxidative damage induced by diquat in weaned piglets. The elevated pancreas weight, serum amylase and lipase were observed in pigs injected with diquat which is evidenced for pancreatic damage (Walgren et al. 2007). The inclusion of chitosan declined serum amylase and lipase as well as pancreatic enlargement induced by diquat injection, which implicated the therapeutic effect of chitosan for pancreatic injury. Furthermore, in the present study, oxidative damage was generated in pancreas of piglets after exposure to diquat, as evidenced by the decreased activities of T-SOD, CAT as well as GPx, and the increased MDA production. However, chitosan inclusion reduced the level of MDA in pancreas of piglets injected with diquat, and increased pancreatic T-AOC and activities of antioxidant enzymes including T-SOD, CuZn-SOD.
Endogenous antioxidant defense systems regulate the level of ROS to maintain normal physiological homeostasis. As free radical scavengers, SOD removes superoxide radical by converting it into hydrogen peroxide that is rapidly converted to nontoxic water by CAT and GPx, and provides protection in pancreas. Similarly, chitosan treatment decreased the MDA level and showed a protective role against anti-tubercular drugs-induced hepatotoxicity (Santhosh et al. 2006). Again, chitosan oligosaccharide (low molecular chitosan) exerts greater SOD activity and decreases the MDA level in glycerol-induced acute renal failure rats (Yoon et al. 2008). Taken together, chitosan plays an antioxidant role in oxidative stress induced by diquat, which may be attributed to the scavenging ability of its amino and hydroxyl groups (Xie et al. 2001; Je and Kim 2006). Moreover, oxidative stress induced by ROS is thought to be critically involved in pancreatic dysfunction (Wang and Roper 2014). In this study, oxidative damage induced by diquat decreased pancreatic trypsin activity but increased lipase activity, which represents exocrine function of pancreas. However, the inclusion of chitosan increased pancreatic trypsin activity and crude protein apparent digestibility, but decreased pancreatic lipase activity and crude fat apparent digestibility. This concurs with the findings of our previous study (Xu et al. 2014), which indicated that chitosan improved crude protein apparent digestibility and decreased crude fat apparent digestibility of weaned piglets, which was mainly due to change in the digestive enzyme activities under normal physiological conditions. On the other hand, a higher growth performance observed in this study inversely supported the improved digestive enzyme activities. Indeed, the underlying mechanism of this inconsistent result in regulating digestive enzymes is unknown.

### Conclusions

In summary, the present study provided some evidence that chitosan is capable of reducing the levels of pancreas MDA and serum markers in diquat-induced pancreatic damage, and increasing the endogenous antioxidant defense mechanisms. These results also show that pancreatic physiological and GPx. Endogenous antioxidant defense systems regulate the level of ROS to maintain normal physiological homeostasis. As free radical scavengers, SOD removes superoxide radical by converting it into hydrogen peroxide that is rapidly converted to nontoxic water by CAT and GPx, and provides protection in pancreas. Similarly, chitosan treatment decreased the MDA level and showed a protective role against anti-tubercular drugs-induced hepatotoxicity (Santhosh et al. 2006). Again, chitosan oligosaccharide (low molecular chitosan) exerts greater SOD activity and decreases the MDA level in glycerol-induced acute renal failure rats (Yoon et al. 2008). Taken together, chitosan plays an antioxidant role in oxidative stress induced by diquat, which may be attributed to the scavenging ability of its amino and hydroxyl groups (Xie et al. 2001; Je and Kim 2006). Moreover, oxidative stress induced by ROS is thought to be critically involved in pancreatic dysfunction (Wang and Roper 2014). In this study, oxidative damage induced by diquat decreased pancreatic trypsin activity but increased lipase activity, which represents exocrine function of pancreas. However, the inclusion of chitosan increased pancreatic trypsin activity and crude protein apparent digestibility, but decreased pancreatic lipase activity and crude fat apparent digestibility. This concurs with the findings of our previous study (Xu et al. 2014), which indicated that chitosan improved crude protein apparent digestibility and decreased crude fat apparent digestibility of weaned piglets, which was mainly due to change in the digestive enzyme activities under normal physiological conditions. On the other hand, a higher growth performance observed in this study inversely supported the improved digestive enzyme activities. Indeed, the underlying mechanism of this inconsistent result in regulating digestive enzymes is unknown.

### Table 2. Effect of chitosan on growth performance of piglets challenged with diquat.

| Item            | Saline | Diquat | SEM | P-value |
|-----------------|--------|--------|-----|---------|
|                 | No chitosan 0.5 g/kg chitosan | No chitosan 0.5 g/kg chitosan |     |         |
| Before diquat-challenged BW, kg |
| d 1             | 10.22 | 10.28 | 0.25 | 0.87    |
| d 14            | 14.46 | 14.94 | 0.37 | 0.38    |
| ADG, g/d        | 326   | 359   | 13.8 | 0.09    |
| ADFL, g/d       | 747   | 724   | 71.5 | 0.66    |
| G:F             | 0.44  | 0.51  | 0.03 | 0.14    |
| After diquat-challenged BW, kg |
| d 15            | 14.38 | 15.12 | 14.54 | 14.76 | 0.53 | 0.86 | 0.40 | 0.65 |
| d 21            | 17.86 | 18.74 | 16.68 | 17.30 | 0.46 | 0.05 | 0.24 | 0.84 |
| ADG, g/d        | 497   | 517   | 306  | 363    | 45.2 | <0.01| 0.09 | 0.39 |
| ADFL, g/d       | 1070  | 1045  | 816  | 896    | 71.5 | <0.01| 0.64 | 0.37 |
| G:F             | 0.46  | 0.50  | 0.38 | 0.42   | 0.03 | 0.01 | 0.21 | 0.89 |

Notes: BW = body weight; ADG = average daily weight gain; ADFI = average daily feed intake; G:F = gain to feed ratio.

### Table 3. Effect of chitosan on pancreatic antioxidant capacity and endocrine function of piglets challenged with diquat.

| Item                                    | Saline | Diquat | SEM | P-value |
|------------------------------------------|--------|--------|-----|---------|
|                                          | No chitosan 0.5 g/kg chitosan | No chitosan 0.5 g/kg chitosan |     |         |
| Pancreas weight*, g/kg                   | 2.78   | 2.75   | 3.26 | 2.80   | 0.16 | 0.06 | 0.09 | 0.13 |
| Serum amylase and lipase                 | 188.7  | 191.7  | 303.2 | 229.0 | 25.2 | <0.01| 0.03 | 0.02 |
| Lipase, U/L                              | 29.4   | 24.6   | 42.8  | 25.7   | 4.0  | <0.01| <0.01| 0.02 |
| Antioxidative function                    |        |        |      |        |      |      |      |      |
| T-AOC, U/mg prot.                        | 0.60   | 0.64   | 0.48  | 0.74   | 0.08 | 0.89 | 0.05 | 0.12 |
| T-SOD, U/mg prot.                        | 121.1  | 137.5  | 113.2 | 122.4  | 7.2  | 0.09 | 0.07 | 0.59 |
| CuZn-SOD, U/mg prot.                     | 88.1   | 100.7  | 81.2  | 93.7   | 5.8  | 0.20 | 0.03 | 0.99 |
| Mn-SOD, U/mg prot.                       | 33.0   | 36.8   | 32.0  | 28.8   | 3.7  | 0.25 | 0.94 | 0.37 |
| GPx, U/mg prot.                          | 19.5   | 22.0   | 16.2  | 18.9   | 1.3  | <0.01| 0.01 | 0.94 |
| CAT, U/mg prot.                          | 0.76   | 0.74   | 0.62  | 0.72   | 0.04 | 0.04 | 0.28 | 0.12 |
| MDA, nmol/mg prot.                       | 0.29   | 0.32   | 0.43  | 0.33   | 0.04 | 0.05 | 0.33 | 0.09 |
| Digestive enzymes                        |        |        |      |        |      |      |      |      |
| Tryptsin, U/mg prot.                     | 161.7  | 194.3  | 133.9 | 176.6  | 14.1 | 0.09 | <0.01| 0.70 |
| Lipase, U/g prot.                        | 771.7  | 537.3  | 960.1 | 705.1  | 80.6 | <0.01| <0.01| 0.86 |
| Apparent digestibility                   |        |        |      |        |      |      |      |      |
| Crude protein, %                         | 77.2   | 79.7   | 78.9  | 80.8   | 1.1  | 0.21 | 0.05 | 0.75 |
| Crude fat, %                             | 47.3   | 37.4   | 45.5  | 36.6   | 3.3  | 0.64 | <0.01| 0.86 |

Notes: BW = body weight; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; GPx = glutathione peroxidase; CAT = catalase; MDA = malonaldehyde.

*OS = oxidative stress, indicating main effect of oxidative stress induced by diquat.

*CS = chitosan, indicating main effect of chitosan.

*Relative weight to body weight.
characters including weight and secretory function are modulated by chitosan. Thus, these findings suggest that chitosan could be developed as an effective feed additive for preservation of pancreas in oxidative stress.

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