

Arginine metabolism and its protective effects on intestinal health and functions in weaned piglets under oxidative stress induced by diquat

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Abstract

The intestine plays key roles in maintaining body arginine (Arg) homeostasis. Meanwhile, the intestine is very susceptible to reactive oxygen species. In light of this, the study aimed to explore the effects of Arg supplementation on intestinal morphology, Arg transporters and metabolism, and the potential protective mechanism of Arg supplementation in piglets under oxidative stress. A total of thirty-six weaned piglets were randomly allocated to six groups with six replicates and fed a base diet (0.95% Arg) or base diet supplemented with 0.8% and 1.6% L-Arg for 1 week, respectively. Subsequently, a challenge test was conducted by intraperitoneal injection of diquat, an initiator of radical production, or sterile saline. The whole trial lasted 11 d. The diquat challenge significantly decreased plasma Arg concentration at 6 h after injection ($P < 0.05$), lowered villus height in the jejunum and ileum ($P < 0.05$) as well as villus width and crypt depth in the duodenum, jejunum and ileum ($P < 0.05$). Oxidative stress significantly increased *cationic amino acid transporter (CAT)-1*, *CAT-2* and *CAT-3*, mRNA levels ($P < 0.05$), decreased *arginase II (ARGII)* and *inducible nitric oxide synthase* mRNA levels, and increased *TNF- α* mRNA level in the jejunum ($P < 0.05$). Supplementation with Arg significantly decreased crypt depth ($P < 0.05$), suppressed *CAT-1* mRNA expression induced by diquat ($P < 0.05$), increased *ARGII* and *endothelial nitric oxide synthase* mRNA levels ($P < 0.05$), and effectively relieved the *TNF- α* mRNA expression induced by diquat in the jejunum ($P < 0.05$). It is concluded that oxidative stress decreased Arg bioavailability and increased expression of inflammatory cytokines in the jejunum, and that Arg supplementation has beneficial effects in the jejunum through regulation of the metabolism of Arg and suppression of inflammatory cytokine expression in piglets.

Key words: Arginine: Intestines: Metabolism: Oxidative stress: Weaned piglets

It is well known that oxidative stress can be very harmful to mammalian tissues, particularly the intestine^(1,2). Health status is significantly affected and performance is decreased because of oxidative stress in pigs^(3–7). Weaning piglets are subjected to various challenges, such as change in the nutritional source, feed contamination with mycotoxins, pathogenic micro-organisms and some chemical agents as well as the use of drugs and vaccine factors, which might produce excessive amounts of reactive oxygen species (ROS) and induce oxidative stress^(8,9). Oxidative stress induces cell apoptosis, inhibits cell proliferation, suppresses intestinal development and disturbs intestinal function^(10–13). Further, the intestinal cells are rich in mitochondria⁽¹⁴⁾, which are the main sites for ROS generation⁽¹⁵⁾. At the same time, the intestine is highly susceptible to oxidative damage because of its frequent interaction with O₂ metabolism or luminal

oxidants from the intake of nutrients, micro-organisms or through infections^(16,17).

L-Arginine (L-Arg) is not only an essential amino acid in young piglets but also a functional amino acid. Numerous research studies show that L-Arg can function as a potential substance against oxidative stress^(18–21). Our previous study showed that supplementation with Arg can ameliorate the negative effect induced by diquat, an initiator of ROS production, through the enhancement of antioxidant capacity and attenuation of negative effects of feed intake in piglets⁽⁵⁾. The intestine plays a key role in Arg absorption, endogenous synthesis and metabolism in addition to functioning as the site for maintaining Arg homeostasis⁽²²⁾. Studies show that Arg is predominantly transported across the intestinal membrane via Na⁺-independent cationic amino acid transporters (CAT) including CAT-1, CAT-2 and

Abbreviations: Arg, arginine; ARGII, arginase II; ArgH, basal diet and supplementation with 1.6% synthetic L-Arg; ArgL, basal diet; ArgM, basal diet and supplementation with 0.8% synthetic L-Arg; CAT, cationic amino acid transporter; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

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CAT-3 isoforms^(23,24). Changes in Arg transport activity in the intestine reflect the status of Arg metabolism in intestines as well as in the whole body. Research studies also found that catabolic disease increased intestinal Arg transport in rats and in Caco-2 cell cultures⁽²⁴⁾. Besides, the intestine is the most important site for Arg and citrulline synthesis by pyrroline-5-carboxylate (P5C) synthase⁽²⁵⁾, because other major tissues including the liver, kidney and skeletal muscle lack P5C synthase for synthesising citrulline from glutamine or glutamate⁽²⁶⁾. As arginase and nitric oxide (NO) synthase use Arg as the common substrate, arginase may play a role in regulating NO synthesis by modulating Arg availability^(27,28). Therefore, Arg availability is regulated by many factors, and dietary Arg supplementation may be a necessary strategy to maintain Arg homeostasis for good health and body functions under many physiological and pathological conditions⁽²⁹⁾.

Our previous studies already found that oxidative stress decreased the level of Arg in circulation in piglets⁽³⁰⁾, and supplementation with Arg could attenuate oxidative stress by enhancing the total antioxidative capacity in piglets⁽⁵⁾. However, little is known about the effect of dietary Arg supplementation on Arg metabolism in the intestine under oxidative stress conditions in piglets. The aim of this study was to investigate the effects of supplementation with Arg on Arg transport and metabolism, and potential mechanisms underlying the Arg-induced protective effects in the intestine under the oxidative stress conditions in piglets.

Methods

Experimental animals and diets

The samples for the current study were obtained from the animal experiment conducted in our previous study. The experimental piglets and diets are described in detail in our previous study⁽⁵⁾. In brief, a total of thirty-six crossbred weaned male piglets (8.67 (SEM 0.43) kg, 28 (SEM 1) d) (Pig Improvement Company, PIC) were housed individually and randomly allocated into six groups with six replicates per group (*n* 6). Piglets were subjected to three dietary treatments (two groups per treatment) in the 1st week and fed a basal diet supplemented with varying concentrations of Arg. The diets were the same as in our previous report⁽⁵⁾. Dietary treatments were as follows: basal diet (ArgL), basal diet and supplementation with 0.8% synthetic L-Arg (ArgM) and basal diet and supplementation with 1.6% synthetic L-Arg (ArgH).

Experimental procedure

The experimental procedure was the same as in our previous study⁽⁵⁾. In brief, at 08.00 hours on day 8, piglets in each dietary treatment were intraperitoneally injected with diquat at a concentration of 10 mg/kg body weight or with a sterile 0.9% NaCl solution of the same amount, respectively. Diquat (Diquat dibromide monohydrate, PS365; Sigma, Co.) was dissolved in isotonic saline and filter-sterilised. The concentration of the diquat solution was 10 mg/ml. The whole trial lasted for 11 d. This study was approved by the Animal Care Advisory committee of Sichuan Agricultural University.

Blood sampling and analyses

Before injection (0 h) and at 6, 24, 48 and 96 h post injection, a blood sample (10 ml/pig) was collected from the portal vein precava into heparinised polyethylene tubes (Axygen biotechnology Co., Ltd). Plasma was prepared by centrifuging the blood (3000 **g** at 4°C for 15 min) and immediately stored at -20°C.

Plasma Arg concentration was assayed using the amino acid automatic analyser L8800 (Hitachi). Frozen plasma samples were thawed at 4°C and 3 ml of a 10% (w/v) solution of sulfosalicylic acid was added to 1 ml of plasma sample and centrifuged (12 000 **g** for 1 h) at 4°C. The Arg concentration of deproteinised plasma was determined by ion-exchange chromatography⁽³¹⁾.

Tissue sample collection

After the blood was collected at 96 h post injection, piglets were euthanised with an intravenous injection of pentobarbital sodium (50 mg/kg body weight) and then slaughtered by exsanguination protocols approved by the Sichuan Agricultural University Animal Care Advisory Committee.

A midline laparotomy was performed. The abdomen was incised, and the entire small intestine starting from the pylorus to the ileocaecal valve was removed and divided into three segments: duodenum, jejunum and ileum. The segment from the pylorus to the suspensory muscle of duodenum was defined as duodenum, the last three-fifths segment was defined as ileum, and the middle segment was defined as jejunum. Segments of 2 cm length were cut from the middle of the duodenum, jejunum and ileum, respectively. The segments were flushed gently with ice-cold PBS (pH 7.4) and then fixed in 10% fresh, chilled formalin solution. Following this, 0.5 cm jejunum samples were removed and snap-frozen in N₂ and then stored at -80°C until RNA isolation.

Intestinal morphology analysis

The samples were fixed in neutral-buffered formaldehyde, embedded and stained according to the method of Luna⁽³²⁾. The sections were stained with haematoxylin-eosin. In each section, villus height, villus width and crypt depth were examined in ten well-orientated villi and crypts using an Olympus CK 40 microscope (Olympus Optical Company).

Intestinal gene expression

Total RNA was extracted from samples of the jejunum using the TRIzol reagent (TaKaRa), according to the manufacturer's instructions. The concentration of RNA in the final preparations was calculated from the OD₂₆₀. The integrity of RNA was verified using denaturing agarose gel electrophoresis. Reverse transcription was performed using the Prime ScriptTM RT reagent Kit (TaKaRa) with a 2- μ g RNA sample, according to the manufacturer's instructions. Complementary DNA was used as the template for PCR.

Real-time quantitative PCR was performed in an Option Monitor 3 Real-Time PCR Detection System (Bio-Rad) using the SYBR Green Supermix (TaKaRa). Expression levels of



CAT-1, *CAT-2*, *CAT-3*, arginase II (*ARGII*), inducible nitric oxide synthase (*iNOS*), endothelial nitric oxide synthase (*eNOS*), *IL-6*, *TNF-α* and *PPAR-γ* in the jejunum were analysed using real-time quantitative PCR with SYBR Green PCR reagents (TaKaRa) carried out in the Option DNA Engine (Bio-Rad) using the following cycle parameters: 95°C for 10 s, followed by forty cycles at 95°C for 5 s, annealing temperature (Table 1) for 20 s, and a final extension at 72°C for 15 s. The gene-specific primers used are listed in Table 1. All primers were purchased from TaKaRa. Fluorescence detection was carried out immediately at the end of each annealing step, and the purity of the amplification was confirmed by analysing the melting curves. Gene expression relative to the housekeeping gene *β-actin* was measured in order to correct for the variance in amounts of RNA input in the reactions. *β-Actin* was used as the reference gene according to the stability test of Hillig *et al.*⁽³³⁾.

Each primer pair used yielded a single peak in the melting curve and a single band of the expected size in the agarose gel. The relative gene expression levels compared with the housekeeping gene *β-actin* were calculated using the Pfaffl (2001) method⁽³⁴⁾.

Statistical analysis

Data were analysed by two-way ANOVA using the general linear model procedure. The main effects of the model included Arg supplementation levels (0, 0.8 and 1.6%) and oxidative stress processing (injection of diquat or saline). $P < 0.05$ was considered to indicate a significant difference and values between 0.05 and 0.10 to indicate a trend. Variable means for treatments showing significant differences in ANOVA were separated by Duncan's test ($P < 0.05$). Values were expressed as means with their standard errors. All statistical analysis was performed using SPSS17.0.

Results

Concentrations of plasma arginine

The data on plasma Arg are shown in Table 2. Compared with the basal diet, supplementation with Arg had no significant influence on plasma Arg concentration ($P > 0.05$). Injection of diquat resulted in an acute reduction in plasma Arg concentration at 6 h after diquat injection ($P < 0.05$). There was no significant effect of interaction between Arg supplementation and injection of diquat on Arg concentration.

Intestinal morphology

Data on morphology of the small intestine are shown in Table 3. Injection of diquat resulted in a reduction of villus height in the jejunum and ileum ($P < 0.05$) as well as in villus width and crypt depth in the duodenum, jejunum and ileum ($P < 0.05$). Supplementation with Arg (ArgM and ArgH) significantly decreased crypt depth in the jejunum with or without injection of diquat ($P < 0.05$). There was no significant interaction effect of Arg supplementation and injection of diquat on intestinal morphology.

Cationic amino acid transporter gene expressions in the jejunum

As shown in Fig. 1, supplementation with Arg had no effect on *CAT-1* mRNA level in the jejunum ($P > 0.05$) under non-oxidative stresses conditions. Injection of diquat significantly increased *CAT-1* mRNA level ($P < 0.05$), whereas supplementation with Arg along with injection of diquat in the jejunum significantly decreased *CAT-1* mRNA level ($P < 0.05$).

ArgM significantly increased *CAT-2* mRNA level in the jejunum compared with ArgL without injection of diquat ($P < 0.05$), and injection of diquat significantly increased *CAT-2* mRNA level compared with injection of saline ($P < 0.05$).

Table 1. Primers used for real-time analyses

Gene names	Primer sequence 5'–3'	Product size (bp)	Annealing temperature (°C)	Accession no.
<i>CAT-1</i>	F: gagcaagaccaaactctcctc R: agcctatcagcatccacactg	137	57.2	NM_001012613
<i>CAT-2</i>	F: agtgagtaagaggcagtcaccag R: accagagagtaggccagaagg	135	61	EU155140
<i>CAT-3</i>	F: gaggaacgaggaaggtgaagt R: aggacactcagcagtagagcaag	165	60.6	NM_001130973
<i>eNOS</i>	F: acaggctctcaccttctcct R: aaccactccactcctcatagc	150	61	NM_214295
<i>iNOS</i>	F: acaccccaatacagagtgttc R: cccatgtaccagccattgaag	129	61	U59390
<i>ARGII</i>	F: gtcagtagagcgggtcaggt R: gatgaagtggtaggggtgtat	161	60	XM_001928679
<i>TNF-α</i>	F: gctctctgcctactgcacttc R: ggcttatctgaggttgagacg	123	61	X57321
<i>IL-6</i>	F: ggagacctgcttgatgagaatc R: gtactaatctgcacagcctcgac	117	61	M80258
<i>PPAR-γ</i>	F: ccagcatttccactccacacta R: gacacaggctccactttgatg	124	58	AJ006757
<i>β-Actin</i>	F: ccacgaaactcctcaactcc R: gtgatctcctctgcatcctgt	132	60	DQ845171

CAT, cationic amino acid transporter; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; ARGII, arginase II.

Table 2. Effects of arginine (Arg) supplementation and diquat injection on the concentration of Arg in the plasma of piglets (nmol/ml)*

Responses	SS			OS			SEM	Analysis by effect (P)		
	ArgL	ArgM	ArgH	ArgL	ArgM	ArgH		Arg	OS	Arg × OS
0 h	46.4	55.7	38.3	–	–	–	4.03	0.335	–	–
6 h	65.6	80.5	68.2	39.6	58.6	58.7	4.42	0.267	0.031	0.711
24 h	50.0	34.3	35.5	46.5	40.8	42.2	2.22	0.108	0.461	0.559
48 h	32.0	33.4	30.2	33.6	30.1	33.7	1.19	0.932	0.806	0.522
96 h	22.0	24.8c	22.9	25.6	25.4	22.7	1.06	0.711	0.560	0.733

SS, injection with sterile saline; OS, injection with diquat; ArgL, basal diet; ArgM, basal diet and supplementation with 0.8% synthetic L-Arg; ArgH, basal diet and supplementation with 1.6% synthetic L-Arg; Arg × OS, Arg × OS interaction effect.

* 0h, n 12, others, n 6.

Table 3. Effects of arginine (Arg) supplementation on villus height, villus width and crypt depth of weaned piglets after 96 h oxidative stress induced by diquat, n 6

Responses	SS			OS			SEM	Analysis by effect (P)		
	ArgL	ArgM	ArgH	ArgL	ArgM	ArgH		Arg	OS	Arg × OS
Villus height (µm)										
Duodenum	367	373	411	293	342	353	17	0.495	0.115	0.797
Jejunum	302 ^b	387 ^a	406 ^a	295 ^b	295 ^b	292 ^b	14	0.221	0.004	0.109
Ileum	388 ^a	378 ^{a,b}	315 ^{b,c}	288 ^c	283 ^c	320 ^{b,c}	11	0.741	0.002	0.067
Villus width (µm)										
Duodenum	127 ^a	130 ^a	132 ^a	120 ^{a,b}	99 ^b	113 ^{a,b}	3	0.387	0.002	0.266
Jejunum	139 ^a	139 ^a	123 ^{a,b}	94 ^b	105 ^b	105 ^b	5	0.659	0.018	0.926
Ileum	131	127	128	111	107	112	3	0.611	0.008	0.948
Crypt depth (µm)										
Duodenum	381	359	349	343	299	306	10	0.282	0.022	0.899
Jejunum	268 ^a	148 ^{b,c}	163 ^{b,c}	184 ^b	118 ^c	106 ^c	12	<0.001	<0.001	0.200
Ileum	258 ^a	260 ^a	245 ^a	206 ^{a,b}	230 ^{a,b}	192 ^b	9	0.873	0.004	0.640

SS, injection with sterile saline; OS, injection with diquat; ArgL, basal diet; ArgM, basal diet and supplementation with 0.8% synthetic L-Arg; ArgH, basal diet and supplementation with 1.6% synthetic L-Arg; Arg × OS, Arg × OS interaction effect.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

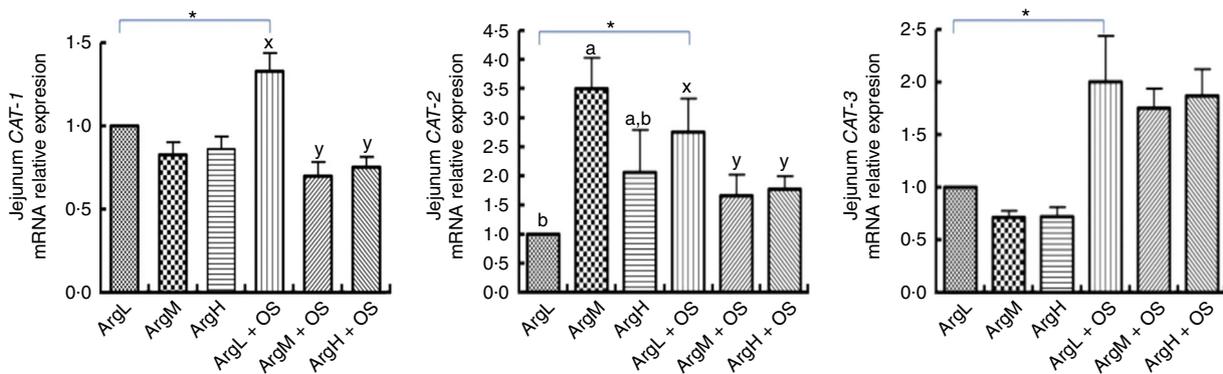


Fig. 1. Effects of arginine (Arg) supplementation and diquat injection on relative expressions of *CAT-1*, *CAT-2* and *CAT-3* mRNA in the jejunum of piglets. CAT, cationic amino acid transporter; ArgL, basal diet; ArgM, basal diet and supplementation with 0.8% synthetic L-Arg; ArgH, basal diet and supplementation with 1.6% synthetic L-Arg; OS, injection with diquat. Values are means (n 6), with their standard errors represented by vertical bars. ^{x,y} or ^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$). * Mean values were significantly different between two groups ($P < 0.05$).

Supplementation with Arg had no effect on *CAT-3* mRNA level in the jejunum ($P > 0.05$) but injection of diquat significantly increased *CAT-3* mRNA level ($P < 0.05$).

Arginine catabolism-related gene expressions

As shown in Fig. 2, supplementation with Arg did not affect *ARGII* mRNA level in the jejunum compared with supplementation with

ArgL under non-oxidative stress ($P > 0.05$). Injection of diquat significantly decreased *ARGII* mRNA level in the jejunum ($P < 0.05$). Supplementation with Arg significantly reversed *ARGII* mRNA level induced by diquat to normal level in the jejunum ($P < 0.05$).

However, ArgH significantly decreased *iNOS* mRNA level in the jejunum compared with ArgL under non-oxidative stress ($P < 0.05$). Injection of diquat significantly decreased *iNOS*

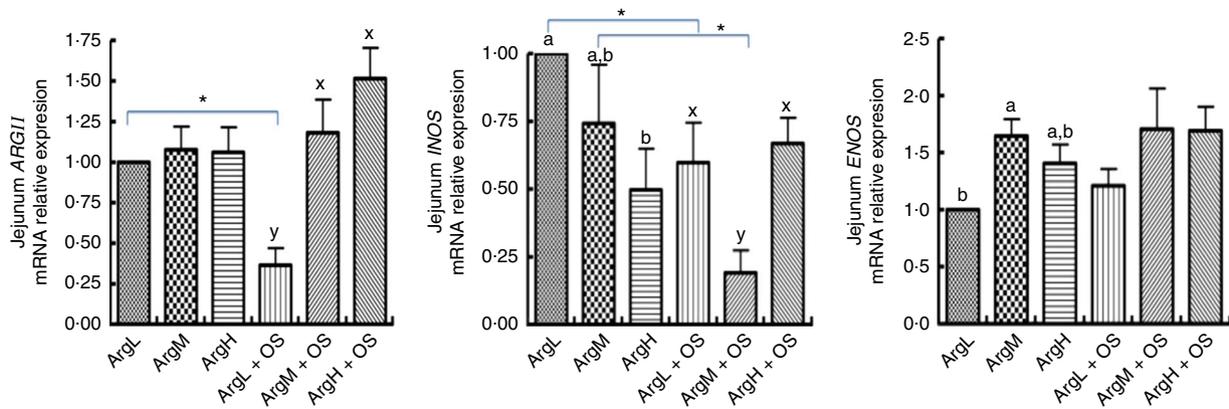


Fig. 2. Effects of arginine (Arg) supplementation and diquat injection on relative expressions of arginase II (*ARG1I*), endothelial nitric oxide synthase (*eNOS*) and inducible nitric oxide synthase (*iNOS*) mRNA in the jejunum of piglets. ArgL, basal diet; ArgM, basal diet and supplementation with 0.8% synthetic L-Arg; ArgH, basal diet and supplementation with 1.6% synthetic L-Arg; OS, injection with diquat. Values are means (n 6), with their standard errors represented by vertical bars. ^{x,y} or ^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$). * Mean values were significantly different between two groups ($P < 0.05$).

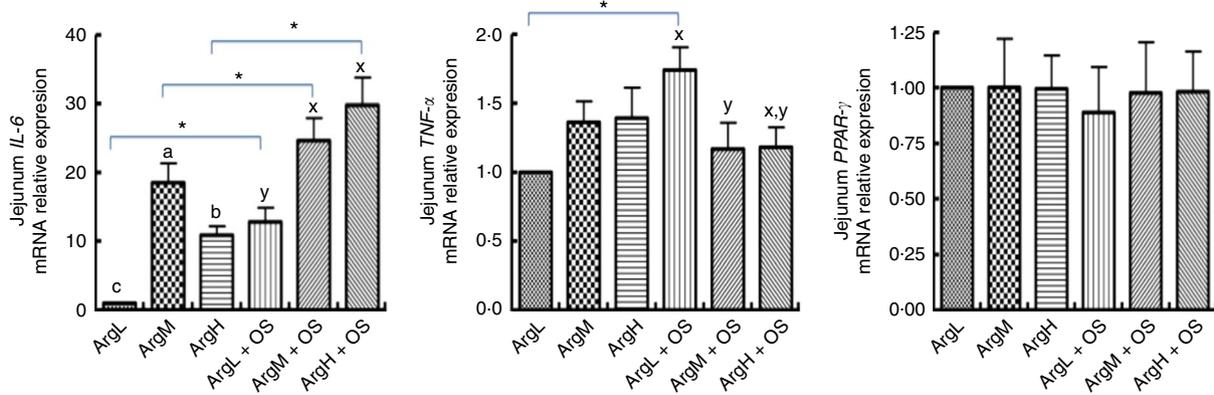


Fig. 3. Effects of arginine (Arg) supplementation and diquat injection on relative expressions of *IL-6*, *TNF-α* and *PPAR-γ* mRNA in the jejunum of weaned piglets. ArgL, basal diet; ArgM, basal diet and supplementation with 0.8% synthetic L-Arg; ArgH, basal diet and supplementation with 1.6% synthetic L-Arg; OS, injection with diquat. Values are means (n 6), with their standard errors represented by vertical bars. ^{x,y} or ^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$). * Mean values were significantly different between two groups ($P < 0.05$).

mRNA level in the jejunum ($P < 0.05$) as well. ArgM significantly decreased *iNOS* mRNA level in the jejunum with injection of diquat ($P < 0.05$) but ArgH did not have the same effect.

ArgM significantly increased *eNOS* mRNA level compared with ArgL under non-oxidative stress. Injection of diquat did not significantly affect *eNOS* mRNA level in the jejunum ($P > 0.05$).

Inflammation-related gene expressions

To investigate the possible protective effects of Arg on jejunum injury caused by oxidative stress induced by diquat, we quantified mRNA levels of *IL-6*, *TNF-α* and *PPAR-γ* in the jejunum.

As shown in Fig. 3, ArgM and ArgH significantly increased *IL-6* mRNA level in the jejunum of piglets, compared with ArgL with or without injection of diquat ($P < 0.05$). Injection of diquat significantly increased *IL-6* mRNA level in the jejunum of piglets ($P < 0.05$).

Supplementation with Arg did not affect *TNF-α* mRNA level in the jejunum under non-oxidative stress ($P > 0.05$). Injection of diquat significantly increased *TNF-α* mRNA level in the jejunum ($P < 0.05$). Supplementation with Arg could effectively relieve the increment of *TNF-α* mRNA level induced by diquat ($P < 0.05$).

Arg and injection of diquat had no effect on *PPAR-γ* mRNA level in the jejunum of pigs ($P > 0.05$).

Discussion

L-Arg is a multifunctional substance, which is not only a precursor for the synthesis of protein, NO, urea, polyamines and creatine but also regulates gene expression and antioxidation^(22,35–37). Arg plays an important role in improving the growth performance and morphological development of the small intestine in suckling piglets⁽³⁸⁾, weaning piglets⁽⁹⁾ and growing pigs fed a mould-contaminated diet⁽³⁹⁾. Previous research studies showed that supplementation with Arg could increase the weight of the small intestine, enhance intestinal development⁽⁴⁰⁾, improve microvascular development of the intestine in weaning piglets⁽⁴¹⁾ and alleviate the impairment induced by deoxynivalenol in growing pigs⁽⁴²⁾. Intestinal function in piglets was greatly disturbed by oxidative stress, which resulted in reductions in nutrient absorption⁽⁷⁾. Our previous study proved that piglets suffered oxidative stress upon diquat injection⁽⁵⁾. Injection with diquat significantly increased the concentration of cortisol and MDA and decreased

the activities of glutathione peroxidase and superoxide dismutase in the plasma of piglets⁽⁵⁾. At present, we have found that oxidative stress induced by diquat significantly decreased villus height, villus width and crypt depth in the jejunum and ileum. Supplementation with Arg significantly mitigated jejunum morphology impairment (e.g. lower villus width). Our previous study found that supplementation with Arg had a trend to increase ADFI of piglets under oxidative stress⁽⁵⁾. This result further proved that the increase in nutrient intake was beneficial to the health of piglets under oxidative stress. This result is also consistent with those from previous research studies that showed that supplementation with Arg can relieve dysfunction of the intestine caused by LPS-induced stress⁽⁴³⁾. Besides, Viana *et al.*⁽⁴⁴⁾ found that Arg was able to preserve barrier integrity and reduce bacterial translocation in the intestine of mice. We also detected the mRNA expression of inflammation-related genes and found that oxidative stress significantly increased *IL-6* and *TNF- α* mRNA levels in the jejunum of piglets; supplementation with Arg could effectively relieve the increment of *TNF- α* mRNA level induced by oxidative stress. *TNF- α* is a proinflammatory cytokine involved in systemic inflammation⁽⁴⁵⁾. Further, research studies found *TNF- α* caused an increase in barrier permeability of the intestinal epithelium through activation of the ERK1/2 signalling pathway⁽⁴⁶⁾. Therefore, it is reasonable to speculate that supplementing Arg in piglets can partially reduce oxidative injury by suppressing proinflammatory cytokine *TNF- α* expression in the jejunum.

Arg within the body is derived from the diet, endogenous synthesis and from turnover of proteins. The intestine plays a central role in maintaining Arg homeostasis by providing exogenous Arg into the system. Animal studies showed that Arg was predominantly transported across the intestinal membrane via an Na⁺-independent system: that is, via γ^+ CAT isoforms^(47,48). In this study, we measured *CAT* expression and found that oxidative stress induced by diquat significantly increased gene expression of *CAT-1*, *CAT-2* and *CAT-3* in the jejunum of piglets. Duan *et al.*⁽⁶⁾ found that oxidative stress induced by injection of H₂O₂ tended to increase mRNA levels of anionic amino acid transporters and that dietary Arg supplementation enhances intestinal anionic amino acid transporter expression⁽³⁹⁾. Supplementation with Arg increased gene expression of *CAT-2* but not *CAT-1* and *CAT-3* in the jejunum under the non-oxidative stress condition. However, under oxidative stress situations, supplementation with Arg significantly suppressed the increase in gene expression of *CAT-1* and *CAT-2* induced by oxidative stress but not of *CAT-3*. These results suggest that Arg transport is very complicated and influenced by many factors. The regulatory mechanisms of Arg absorption could be mainly in the following two ways. First, Arg absorption was mediated by the level of substrate⁽³⁶⁾. In this study, increasing the concentration of Arg in diet significantly increased the gene expression of *CAT-2* under non-oxidative stress conditions and *CAT-3* in oxidative stress conditions in the jejunum, respectively. On the other hand, the gene expressions of *CAT-1*, *CAT-2* and *CAT-3* in the jejunum were increased when the concentration of Arg in plasma was reduced by oxidative stress. Second, Arg absorption was mediated by the cytokine *TNF- α* pathway⁽⁴⁹⁾. In this study,

oxidative stress significantly increased the expression of the *TNF- α* gene as well as of *CAT-1*, *CAT-2* and *CAT-3* genes, whereas supplementation with Arg significantly inhibited the expression of *TNF- α* and *CAT-1*. Research studies indicated that *TNF- α* increased Arg transport in a time- and dose-dependent manner in human vascular endothelium by activation of protein kinase C⁽⁴⁹⁾. PKC regulated Arg transport activity via a mechanism of activating transporter mRNA and translational processes in Caco-2 intestinal cells⁽⁵⁰⁾. The data from this study suggested that *TNF- α* was involved in the regulation of Arg transport mainly by affecting the activity of *CAT-1* but not *CAT-2* and *CAT-3* in the jejunum under oxidative stress.

The small intestine is the most important site not only for Arg absorption but also for metabolism of Arg. Arg homeostasis is achieved principally through regulation of Arg catabolism. Both NOS and arginase use Arg as a common substrate, and arginase may down-regulate NO production by competing with NOS for Arg. In this study, oxidative stress significantly decreased *iNOS* and *ARGII* mRNA levels in the jejunum, which could be a result of the low availability of Arg. This conclusion is supported by the observation that Arg can regulate gene expression as a substrate for enzymes⁽³⁶⁾ and that Arg deprivation results in decreased expression of *iNOS*⁽⁵¹⁾. Supplementation with Arg could significantly increase *eNOS* and *ARGII* mRNA levels but not *iNOS* levels, probably because elevated *ARGII* expression inhibited the expression of *iNOS*⁽⁵²⁾. A previous study reported that the large amount of NO synthesised by *iNOS* lead to arthritis in humans⁽⁵³⁾, which indicated that the role of NO *in vivo* depended on its origin. Hence, in this study, supplementation with Arg could significantly increase the expression of *eNOS* but not *iNOS*, which could be one of the reasons for relieving the influences of oxidative stress with Arg supplementation. In conditions such as oxidative stress, which increase the metabolic demand for Arg beyond the maximum Arg synthetic capacity, dietary Arg supplementation is necessary. Hu *et al.*⁽⁵⁴⁾ also studied the safe dose of Arg supplementation in weaned pigs and indicated that supplementing up to 2% L-Arg (as either L-Arg-HCl or L-Arg-base) in the diet for 91 d did not have any adverse effect on postweaning pigs. However, Arg is expensive; therefore, it is important to research the proper dose of Arg to be supplemented in different situations. In this study, ArgM showed the same effects as ArgH in relieving oxidative stress in the intestine of piglets; thus, ArgM could be the best choice in this model, and the related research studies deserve more attention in the future.

In conclusion, oxidative stress induced by diquat can influence absorption and metabolism of Arg in the jejunum of weaned piglets, and dietary supplementation with Arg has beneficial effects for intestinal health and functions through improvement in the morphology of the intestine, regulation of Arg availability and reduction of inflammatory cytokine levels. This study illustrates that dietary Arg supplementation in piglets is necessary for increasing the metabolic demand of Arg in piglets under oxidative stress. Adequate L-Arg provision may be a novel and effective means to ameliorate oxidative injury in the small intestine.

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The author contributions are as follows: P. Z., Y. L. G. T. and Q. Z. conducted the experiments. B. Y. and D. C. participated in the design of the study. J. L. and Z. H. assisted in analyses. J. H. and J. Y. assisted in manuscript preparation. L. C and X. M. assisted with all data analyses.

The authors declare that there are no conflicts of interest.

References

1. Circu ML & Aw TY (2012) Intestinal redox biology and oxidative stress. *Semin Cell Dev Biol* **23**, 729–737.
2. Yin J, Ren W, Liu G, *et al.* (2013) Birth oxidative stress and the development of an antioxidant system in newborn piglets. *Free Radic Res* **47**, 1027–1035.
3. Lykkesfeldt J & Svendsen O (2007) Oxidants and antioxidants in disease: oxidative stress in farm animals. *Vet J* **173**, 502–511.
4. Jiang SZ, Yang ZB, Yang WR, *et al.* (2011) Effects of purified zeaxenone on growth performance, organ size, serum metabolites, and oxidative stress in postweaning gilts. *J Anim Sci* **89**, 3008–3015.
5. Zheng P, Yu B, He J, *et al.* (2013) Protective effects of dietary arginine supplementation against oxidative stress in weaned piglets. *Br J Nutr* **109**, 2253–2260.
6. Duan J, Yin J, Ren W, *et al.* (2016) Dietary supplementation with L-glutamate and L-aspartate alleviates oxidative stress in weaned piglets challenged with hydrogen peroxide. *Amino Acids* **48**, 53–64.
7. Yin J, Liu M, Ren W, *et al.* (2015) Effects of dietary supplementation with glutamate and aspartate on diquat-induced oxidative stress in piglets. *PLOS ONE* **10**, e0122893.
8. Askew EW (1995) Environmental and physical stress and nutrient requirements. *Am J Clin Nutr* **61**, 631S–637S.
9. He Q, Tang H, Ren P, *et al.* (2011) Dietary supplementation with L-arginine partially counteracts serum metabolome induced by weaning stress in piglets. *J Proteome Res* **10**, 5214–5221.
10. Yang H, Xiong X, Wang X, *et al.* (2016) Effect of weaning on intestinal crypt epithelial cells in piglets. *Sci Rep* **6**, 36939.
11. Yin J, Duan J, Cui Z, *et al.* (2015) Hydrogen peroxide-induced oxidative stress activates NF-κB and Nrf2/Keap1 signals and triggers autophagy in piglets. *RSC Adv* **5**, 15479.
12. Tang Y, Li J, Li F, *et al.* (2015) Autophagy protects intestinal epithelial cells against deoxynivalenol toxicity by alleviating oxidative stress via IKK signaling pathway. *Free Radic Biol Med* **89**, 944–951.
13. Yin J, Wu M, Li Y, *et al.* (2017) Toxicity assessment of hydrogen peroxide on Toll-like receptor system, apoptosis, and mitochondrial respiration in piglets and IPEC-J2 cells. *Oncotarget* **8**, 3124–3131.
14. Jaynes BJ & Altmann G (1975) Region of mitochondrial division in the epithelium of the small intestine of the rat. *Anat Rec* **182**, 289–296.
15. Figueira TR, Barros MH, Camargo AA, *et al.* (2013) Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. *Antioxid Redox Signal* **18**, 2029–2074.
16. Duan J, Yin J, Wu M, *et al.* (2014) Dietary glutamate supplementation ameliorates mycotoxin-induced abnormalities in the intestinal structure and expression of amino acid transporters in young pigs. *PLOS ONE* **9**, e112357.
17. Hussain T, Tan B, Yin Y, *et al.* (2016) Oxidative stress and inflammation: what polyphenols can do for us? *Oxid Med Cell Longev* **4**, e7432797.
18. Suschek CV, Schnorr O, Hemmrich K, *et al.* (2003) Critical role of L-arginine in endothelial cell survival during oxidative stress. *Circulation* **107**, 2607–2614.
19. Lin CC, Tsai WC, Chen JY, *et al.* (2008) Supplements of L-arginine attenuate the effects of high-fat meal on endothelial function and oxidative stress. *Int J Cardiol* **127**, 337–341.
20. Maggio M, Guralnik JM, Longo DL, *et al.* (2006) Interleukin-6 in aging and chronic disease: a magnificent pathway. *J Gerontol A Biol Sci Med Sci* **61**, 575–584.
21. Southern LL & Baker DH (1983) Arginine requirement of the young pig. *J Anim Sci* **57**, 402–412.
22. Wu G, Bazer FW, Davis TA, *et al.* (2007) Important roles for the arginine family of amino acids in swine nutrition and production. *Livest Sci* **112**, 8–22.
23. Closs EI, Simon A, Kony NV, *et al.* (2004) Plasma membrane transporters for arginine. *J Nutr* **134**, 2752S–2759S.
24. Pan M, Choudry HA, Epler MJ, *et al.* (2004) Arginine transport in catabolic disease states. *J Nutr* **134**, 2826S–2829S.
25. Wu G, Borbolla AG & Knabe DA (1994) The uptake of glutamine and release of arginine, citrulline and proline by the small intestine of developing pigs. *J Nutr* **124**, 2437–2444.
26. Wu G, Davis PK, Flynn NE, *et al.* (1997) Endogenous synthesis of arginine plays an important role in maintaining arginine homeostasis in postweaning growing pigs. *J Nutr* **127**, 2342–2349.
27. Morris SM (2000) Regulation of arginine availability and its impact on NO synthesis. In *Nitric Oxide: Biology and Pathobiology*, pp. 187–197 [LJ Ignarro, editor]. San Diego, CA: Academic Press.
28. Mori M & Gotoh T (2000) Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem Biophys Res Commun* **275**, 715–719.
29. Wu G, Bazer FW, Davis TA, *et al.* (2009) Arginine metabolism and nutrition in growth, health and disease. *Amino Acids* **37**, 153–168.
30. Zheng P, Yu B, Lv M, *et al.* (2010) Effects of oxidative stress induced by diquat on arginine metabolism of postweaning pigs. *Asian-Australas J Anim Sci* **23**, 98–106.
31. Ertingshausen G, Adler H, Reichler A, *et al.* (1969) Fully automated high-speed ion-exchange chromatography of amino acids. *J Chromatogr A* **42**, 355–366.
32. Luna LG (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. New York: McGraw-Hill Book Company.
33. Hillig A, Jørgensen C, Cirera S, *et al.* (2007) Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol* **8**, 67–73.
34. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, 2002–2007.
35. Montanez R, Rodriguez-Caso C, Sanchez-Jimenez F, *et al.* (2008) *In silico* analysis of arginine catabolism as a source of nitric oxide or polyamines in endothelial cells. *Amino Acids* **34**, 223–229.
36. Morris SM (2006) Arginine: beyond protein. *Am J Clin Nutr* **83**, 508S–512S.
37. Ma X, Zheng C, Hu Y, *et al.* (2015) Dietary L-arginine supplementation affects the skeletal longissimus muscle proteome in finishing pigs. *PLOS ONE* **10**, e0117294.



38. Kim SW, McPherson RL & Wu G (2004) Dietary arginine supplementation enhances the growth of milk-fed young pigs. *J Nutr* **134**, 625–630.
39. Yin J, Ren W, Duan J, *et al.* (2014) Dietary arginine supplementation enhances intestinal expression of SLC7A7 and SLC7A1 and ameliorates growth depression in mycotoxin-challenged pigs. *Amino Acids* **46**, 883–892.
40. Yao K, Guan S, Li T, *et al.* (2011) Dietary L-arginine supplementation enhances intestinal development and expression of vascular endothelial growth factor in weaning piglets. *Br J Nutr* **105**, 703–709.
41. Zhan Z, Ou D, Piao X, *et al.* (2008) Dietary arginine supplementation affects microvascular development in the small intestine of early-weaned pigs. *J Nutr* **138**, 1304–1309.
42. Wu L, Wang W, Yao K, *et al.* (2013) Effects of dietary arginine and glutamine on alleviating the impairment induced by deoxynivalenol stress and immune relevant cytokines in growing pigs. *PLOS ONE* **8**, e69502.
43. Liu Y, Huang J, Hou Y, *et al.* (2008) Dietary arginine supplementation alleviates intestinal mucosal disruption induced by *Escherichia coli* lipopolysaccharide in weaned pigs. *Br J Nutr* **100**, 552–560.
44. Viana ML, Santos RGC, Generoso SV, *et al.* (2010) Pretreatment with arginine preserves intestinal barrier integrity and reduces bacterial translocation in mice. *Nutrition* **26**, 218–223.
45. Ma T, Boivin M, Ye D, *et al.* (2005) Mechanism of TNF- α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *Am J Physiol Gastrointest Liver Physiol* **288**, G422–G430.
46. Al-Sadi R, Guo S, Ye D, *et al.* (2013) TNF- α modulation of intestinal epithelial tight junction barrier is regulated by ERK1/2 activation of Elk-1. *Am J Pathol* **183**, 1871–1884.
47. White MF (1985) The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochem Biophys Acta* **822**, 355–374.
48. Stevens BR (1992) Amino acids transport in intestine. In *Mammalian Amino Acid Transport*, 149–163 [MS Kilberg and D Haussinger, editors]. New York, NY: Plenum Press.
49. Pan M, Wasa M, Lind DS, *et al.* (1995) TNF- α stimulated arginine transport by human vascular endothelium requires activation of protein kinase C. *Ann Surg* **221**, 590–601.
50. Pan M & Stevens BR (1995) Protein kinase C-dependent regulation of L-arginine transport activity in Caco-2 intestinal cells. *Biochim Biophys Acta* **1239**, 27–32.
51. El-Gayar S, Thuring-Nahler H, Pfeilschifter J, *et al.* (2003) Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. *J Immunol* **171**, 4561–4568.
52. Lee J, Ryu H, Ferrante RJ, *et al.* (2003) Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. *Proc Natl Acad Sci U S A* **100**, 4843–4848.
53. Meininger CJ, Kelly KA, Li H, *et al.* (2000) Glucosamine inhibits inducible nitric oxide synthesis. *Biochem Biophys Res Commun* **279**, 234–239.
54. Hu S, Li X, Rezaei R, *et al.* (2015) Safety of long-term dietary supplementation with L-arginine in pigs. *Amino Acids* **47**, 925–936.