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Original Research Article

Roles of dietary supplementation with arginine or N-carbamylglutamate in modulating the inflammation, antioxidant property, and mRNA expression of antioxidant-relative signaling molecules in the spleen of rats under oxidative stress

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ABSTRACT

This study evaluated the effects of arginine (Arg) or N-carbamylglutamate (NCG) on inflammation, antioxidant property, and antioxidant-related gene expression in rat spleen under oxidative stress. A total of 52 rats were randomly distributed into 4 treatment groups with 13 replicates per group. Rats were fed a basal diet (BD) or BD supplemented with Arg or NCG for 30 days. On day 28, half of the BD-fed rats were intraperitoneally injected with sterile saline (control group), and the other half with 12 mg/kg body weight of diquat (DT; DT group). The other 2 diet groups were intraperitoneally injected with 12 mg/kg body weight of DT with either Arg (1%) (DT + Arg) or NCG (0.1%) (DT + NCG). Rat spleen samples were collected for analysis at 48 h after DT injection. Results showed that DT damaged the antioxidant defense in rats compared with the control group ($P < 0.05$). Compared with the DT group, the DT + Arg and DT + NCG groups manifested improved anti-hydroxyl radical, catalase, and total superoxide dismutase (T-SOD) activities, increased glutathione content ($P < 0.05$), and decreased malondialdehyde content ($P < 0.05$). Moreover, compared with the DT group, the DT + Arg and DT + NCG groups enhanced mRNA expression of superoxide dismutase (*SOD*), glutathione peroxidase 1 (*GPx1*), glutathione reductase (*GR*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), Kelch-like ECH-associated protein 1(*Keap-1*), and mammalian target of rapamycin (*mTOR*) ($P < 0.05$). Both NCG and Arg significantly increased anti-inflammatory cytokine mRNA level but suppressed the pro-inflammatory cytokine mRNA expression under oxidative stress ($P < 0.05$). In summary, NCG and Arg effectively alleviated oxidative stress, improved the antioxidant capacity and regulated the antioxidant-related signaling molecular expression in rat spleen. N-carbamylglutamate and Arg reduced the inflammation in the spleen by mediating the gene expression of anti-inflammatory and pro-inflammatory cytokines and transforming growth factor-β (*TGF-β*).

1. Introduction

Oxidative stress generally occurs when intra- and extracellular antioxidants cannot eliminate excessive reactive oxygen species (ROS) (Cao et al., 2016). Excessive ROS severely impairs cell function, accelerates cell death, and strengthens inflammatory responses (Circu and Aw, 2010; Bulua et al., 2011). Thus, animal health can be promoted by mitigating oxidative damage. Previous works have found that nutritional interventions (e.g., spermine and

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putrescine) can be utilized to overcome problems caused by oxidative stress (Wang et al., 2015b; Wu et al., 2017).

Although L-arginine (Arg) induces affirmative effects on animal growth and health, Arg is considered insufficient for most animals under oxidative stress (Wu et al., 2009). Recent studies found that Arg facilitates protein synthesis, accelerates intestinal cell migration, and promotes reproductive performance (Rhoads et al., 2008; Gao et al., 2012; Kong et al., 2012). In addition, Arg can elevate the antioxidant status of the serum and skeletal muscles in pigs (Ma et al., 2010), the antioxidative capacities of the muscles in fish (Wang et al., 2015a), and the antioxidant properties of the jejunum and liver in rats under stress conditions (Cao et al., 2016; Xiao et al., 2016). Therefore, Arg is beneficial for alleviating oxidative damage. In addition, spermine (downstream metabolite of Arg) can improve the antioxidant status in the spleen of rats and piglets (Wu et al., 2017; Cao et al., 2017). These findings suggest that Arg may affect the antioxidant defenses in the spleen. Nonetheless, no study has elucidated the effects of Arg intake on the antioxidant statuses in animal spleen. Antioxidant statuses mainly depend on enzymatic antioxidant defense systems. Enzymatic antioxidant activities partly depend on the gene expression of antioxidant enzymes (Wang et al., 2015a). Nevertheless, no study has clarified the correlation between Arg and the gene levels of antioxidant enzymes in animal spleen. The transcription of antioxidant enzyme genes is typically modulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which is suppressed by the Kelch-like ECH-associated protein1 (Keap1) (Li and Kong, 2009). Nonetheless, no study has examined the effects of Arg supplementation on the Nrf2 signaling pathway in animal spleen. Although Nrf2 accumulation can be regulated by mammalian target of rapamycin (mTOR), the effects of Arg on the mTOR in mammalian spleen remain unclear (Shay et al., 2012; Gorrini et al., 2014). A possible explanation is that Arg intake enhances the antioxidant properties in the spleen by influencing signaling pathways (e.g., Nrf2–Keap1 and mTOR), and this hypothesis requires further investigation.

Antioxidant capacity is related to inflammatory reactions (Bub et al., 2003), which are regulated by cytokines. In pigs, Arg reduced the transcription of the interleukin (*IL*)-*6* and tumor necrosis factor α (*TNF*-α) genes in the jejunum caused by lipopolysaccharide challenge (Liu et al., 2008). Moreover, previous works revealed that Arg intake enhanced transforming growth factor-β (*TGF*-β) and *IL-10* gene expression in carps (Chen et al., 2015). These studies suggested that Arg influenced the pro-inflammatory and anti-inflammatory cytokines in animals, but they failed to clarify the actual effects of Arg intake on the cytokines in animal spleen. N-carbamylglutamate is considered an effective precursor for Arg synthesis (Schwahn et al., 2010). N-carbamylglutamate has the clinical capacity to alleviate certain diseases (Kalkan Ucar et al., 2009). Moreover, NCG facilitates muscle protein synthesis (Frank et al., 2007), protects the morphological structure of the small intestine (Xiao et al., 2016), promotes reproductive performance (Jiang et al., 2011), and stimulates endogenous Arg synthesis (Frank et al., 2007). In addition, recent evidence indicate that NCG can improve the antioxidant statuses in the liver, plasma, and jejunum of rats (Cao et al., 2016; Xiao et al., 2016). However, no study has specified the effects of NCG intake on the antioxidant status in animal spleen, and the relationship between NCG intake and antioxidant enzyme gene expression remains unclear. Moreover, the mechanisms by which NCG induces an antioxidant effect through the Nrf2–Keap1 signaling pathway and mTOR in the spleen is generally unknown.

The current study is part of a series of studies evaluating the effects of nutritional support (e.g., NCG and Arg) on the antioxidant defenses in the jejunum and liver, and the metabolomic changes of rats in combination with diquat (Liu et al., 2016a; Cao et al., 2016; Xiao et al., 2016). This study aimed to investigate the effects of Arg and NCG on the antioxidant property in rat spleen. We also determined the effects of NCG and Arg on the inflammatory cytokines, TOR, and Nrf2 signaling molecules in rat spleen at the gene expression to gain useful theoretical evidence for the correlation between nutritional support (e.g., NCG and Arg) and animal health.

2. Materials and methods

2.1. Animal experiment and sample collection

The feeding management utilized in this study was in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care Advisory Committee of Sichuan Agricultural University. After 3 days of acclimatization, 52 healthy female Sprague–Dawley rats (Dossy Experimental Animals Co., Ltd., Chengdu, China) weighing 238 to 270 g were randomly divided into 4 experimental groups (i.e., diquat [DT], DT + Arg, DT + NCG, and control groups) of 13 replicates each. The experiment lasted for 30 days. The DT + Arg group was given a basal diet supplemented with 1% Arg, the DT + NCG group received a basal diet supplemented with 0.1% NCG, and the rest of the groups (i.e., diquat and control groups) were fed the basal diet. On day 28, the rats of the 3 experimental groups (i.e., DT, DT + Arg and DT + NCG groups) were intraperitoneally injected with 12 mg/kg body weight of DT according to a previous report (Cao et al., 2016). The rats of the control group were intraperitoneally injected with the same amount of saline. The rats were housed in an individual stainless-steel wire-bottomed cage in a controllable room, as was conducted by Cao et al. (2016). The room temperature ranged from 24 to 25 °C, and the relative humidity was maintained at 50% to 70% according to a previously reported method (Liu et al., 2016b). A 12 h light/dark cycle was maintained throughout the experimental period. In addition, the rats were given *ad libitum* access to food and drinking water during the duration of the experiment according to a previous method (Cao et al., 2015). The 52 rats were induced with ether anesthesia and then slaughtered at the end of the 30-day experiment. The spleen samples obtained from rats were temporarily stored in liquid nitrogen for analysis of inflammation and antioxidant-relative parameters. The doses of diquat, Arg, and NCG used in the current study followed the described doses in previous studies (Cao et al., 2016; Xiao et al., 2016; Wu et al., 2017).

2.2. Analysis of antioxidant-relative parameters in the spleen

The antioxidant enzyme and non-enzyme antioxidant activities were determined by using the same methods as those in our previous study (Xiao et al., 2016; Wu et al., 2017). The anti-superoxide anion (ASA) and anti-hydroxyl radical (AHR) capacities were evaluated by using a previously reported method (Jiang et al., 2009). Malondialdehyde (MDA) content was determined according to the method proposed by Cao et al. (2017). The protein and glutathione (GSH) content of the spleen samples were analyzed by using previously described methods by Bradford (1976) and Akerboom and Sies (1981), respectively. Glutathione reacts with 5, 5′-dithiobis-p-nitrobenzoic acid and produces the yellow-colored 5-thio-2-nitrobenzoic acid, which can be measured spectrophotometrically at 412 nm. Catalase (CAT) activity was evaluated through a method applied in a previous experiment (Xiao et al., 2016). The total superoxide dismutase (T-SOD) activity was determined by using the method described by Cao et al. (2016). The T-AOC capacity was measured as described by Özmen et al. (2002).

2.3. Real-time PCR analysis

The methods for isolating the total RNA of the spleen and evaluating quantitative real-time polymerase chain reaction (RT-PCR) were the same as those in a previous experiment (Cao et al., 2017). Briefly, the total RNA of frozen spleen (0.1 g) was extracted with 1 mL of RNAiso Plus (Takara, Dalian, China). The quality of the total RNA was evaluated through agarose gel electrophoresis (1%). The total RNA purity of the spleen samples was assessed through spectrophotometric analysis at 260 and 280 nm according to a previous method (Fang et al., 2017). The absorption ratios (260/280 nm) were between 1.8 and 2.0. The concentration of the total RNA was analyzed by using a nucleic acid/protein analyzer (Beckman DU-800, Brea, CA, USA). Subsequently, 1 μg of the total RNA of each spleen sample was reverse transcribed into cDNA by using the PrimeScript TM RT reagent kit with gDNA Eraser (Takara, Dalian, China). The primers for the antioxidant-related and housekeeping genes (β-actin) were designed on the Primer Express Software (version 3.0; Applied Biosystems, Foster City, CA, USA) and synthesized by TaKaRa Biotechnology Company (Takara, Dalian, China). Table 1 lists the forward and reverse primers of the corresponding genes. Quantitative RT-PCR was performed with a real-time PCR system (ABI 7900HT, Applied Biosystems). Amplification was completed with a total volume of 8 μL, which included cDNA (0.8 μL), primer pairs (1.6 μL), SYBR Premix Ex Taq_II (4 μL) (Takara, Dalian, China), and ddH_2O (1.6 μL). The PCR procedure was in accordance with a previous study (Fang et al., 2017). The expression results were assayed by using the method described by Livak and Schmittgen (2001).

2.4. Statistical analysis

All of the obtained results were subjected to one-way ANOVA, followed by Duncan's multiple range test on SPSS 21.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as means ± standard error. Significant differences among the treatment groups were identified at $P < 0.05$.

3. Results

3.1. Antioxidant parameters

Table 2 shows the differences in the spleen antioxidant parameters among the 4 experimental groups. The DT group manifested a significantly decreased GSH content (by 29.98%) ($P < 0.05$), reduced CAT (by 35.70%) ($P < 0.05$), ASA (by 26.65%) ($P < 0.05$), and T-SOD (by 41.46%) activities ($P < 0.05$), and increased MDA content (by 37.07%) ($P < 0.05$) in comparison with the control group. The DT + NCG and DT + Arg groups displayed an enhanced AHR (by 26.57% and 16.80%), CAT (by 62.98% and 49.07%), T-SOD (by 78.39% and 47.13%), and GSH (by 34.30% and 23.77%) activities, and decreased MDA content (by 37.46% and 28.31%) compared with the DT group ($P < 0.05$). The DT + NCG group showed an enhanced ASA activity (by 53.83%) compared with the DT group ($P < 0.05$). In additional, the DT + NCG group exhibited significantly enhanced ASA (by 44.79%), AHR (by 8.36%), and T-SOD (by 21.24%) activities relative to the DT + Arg group ($P < 0.05$).

3.2. Antioxidant-related gene expression

Fig. 1 presents the mRNA expression of antioxidant-related genes in rat spleen under oxidative stress. The DT group presented decreased expression levels of *SOD1* ($P < 0.05$), *CAT* ($P < 0.05$), glutathione reductase (*GR*) ($P < 0.05$), *Nrf2* ($P < 0.05$), *Keap-1* ($P < 0.05$), and *mTOR* ($P < 0.05$) in the spleen relative to the control group. Compared with the DT group, the DT + NCG and DT + Arg groups exhibited higher *SOD1*, glutathione peroxidase 1 (*GPx1*), *GR*, *Nrf2*, *Keap-1*, and *mTOR* mRNA levels in the spleen ($P < 0.05$). The expression of *SOD1*, *CAT*, *GPx1*, *GR*, *Nrf2*, *Keap-1* and *mTOR* mRNA did

Table 1
Primers sequences of target and reference genes selected for analysis by real-time PCR.

Genes	Primers	Sequences (5′ to 3′)	Size, bp	Temperature, °C	Accession No.
β-actin	Forward	CGCAGTTGGTTGGAGCAAA	61	58	V01217.1
	Reverse	ACAATCAAAGTCCTCAGCCACAT			
SOD1	Forward	CATTCCATCATTGGCCGTACT	62	58	BC082800.1
	Reverse	CCACCTTTGCCCAAGTCATC			
CAT	Forward	GTACAGGCCGGCTCTCACA	57	58	NM_012520.2
	Reverse	ACCCGTGCTTTACAGGTTAGCT			
GPx1	Forward	GCGCTGGTCTCGTCCATT	56	58	NM_030826.3
	Reverse	TGGTGAAACCGCCTTTCTTT			
GR	Forward	TTGGCTGCGATGAGATGCT	56	58	NM_053906.2
	Reverse	GGTGGCCCCCATTTTCA			
Nrf2	Forward	CCCATTGAGGGCTGTGATCT	60	58	NM_031789.2
	Reverse	GCCTTCAGTGTGCTTCTGGTT			
Keap-1	Forward	GGCTGGGATGCCTTGTAAAG	57	58	NM_057152.2
	Reverse	GGGCCCATGGATTTCAGTT			
mTOR	Forward	CCCTGTCTTCACTTGTGTTTCAAC	103	58	NM_019906.1
	Reverse	TCGTAGCGCTGGTGATTGATC			
IL-1β	Forward	TGACAGACCCCAAAAGATTAAGG	61	58	NM_031512.2
	Reverse	CTCATCTGGACAGCCCAAGTC			
IL-6	Forward	CCACCAGGAACGAAAGTCAAC	64	58	NM_012589.2
	Reverse	TTGCGGAGAGAAACTTCATAGCT			
IL-10	Forward	GCCCAGAAATCAAGGAGCATT	65	58	L02926.1
	Reverse	CAGCTGTATCCAGAGGGTCTTCA			
TNF-α	Forward	CAGCCGATTTGCCATTTCA	61	58	L19123.1
	Reverse	AGGGCTCTTGATGGCAGAGA			
TGF-β1	Forward	CCAGCCGCGGGACTCT	56	58	NM_021578.2
	Reverse	TTCCGTTTCACCAGCTCCAT			
Caspase-3	Forward	TTTGCGCCATGCTGAAACT	59	58	NM_012922.2
	Reverse	ACGAGTGAGGATGTGCATGAATT			

SOD1 = superoxide dismutase 1; *CAT* = catalase; *GPx1* = glutathione peroxidase 1; *GR* = glutathione reductase; *Nrf2* = nuclear erythroid 2-related factor 2; *Keap1* = Kelch-like ECH-associated protein 1; *mTOR* = mammalian target of rapamycin; *IL-1β* = interleukin 1β; *IL-6* = interleukin 6; *IL-10* = interleukin 10; *TNF-α* = tumor necrosis factor α; *TGF-β1* = transforming growth factor β1.

Table 2
Effect of arginine (Arg), N-carbamylglutamate (NCG) on antioxidant status in the rat spleen under oxidative stress.

Item	Treatments[1]			
	CG	DT	DT + NCG	DT + Arg
AHR, U/mg protein	112.71 ± 7.71	97.12 ± 3.07^{c}	122.92 ± 2.95^{a}	113.44 ± 3.02^{b}
ASA, U/g protein	62.40 ± 2.43	45.77 ± 1.16^{b*}	70.41 ± 2.28^{a}	48.63 ± 1.70^{b}
CAT, U/mg protein	12.52 ± 0.57	8.05 ± 0.42^{b*}	13.12 ± 0.57^{a}	12.00 ± 0.61^{a}
GSH, mg/mg protein	6.37 ± 0.18	4.46 ± 0.08^{b*}	5.99 ± 0.21^{a}	5.52 ± 0.14^{a}
MDA, nmol/mg protein	5.18 ± 0.25	7.10 ± 0.34^{a*}	4.44 ± 0.31^{b}	5.09 ± 0.31^{b}
T-AOC, U/mg protein	1.80 ± 0.07	1.58 ± 0.08	1.83 ± 0.09	1.65 ± 0.06
T-SOD, U/mg protein	27.11 ± 0.47	15.87 ± 0.85^{c*}	28.31 ± 1.07^{a}	23.35 ± 0.70^{b}

AHR = anti-hydroxyl radical; ASA = anti-superoxide anion; CAT = catalase; GSH = glutathione; MDA = malondialdehyde; T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase.
$^{a-c}$ Within a row, mean values with different letters are significantly different ($P < 0.05$) for comparison between DT, DT + NCG, and DT + Arg. Data are stated as means ± SEM.
* Within a row, mean values with an asterisk are significantly different ($P < 0.05$) for comparison between DT and CG.
[1] CG: control group, DT: diquat treatment, DT + NCG: diquat plus 0.1% N-carbamylglutamate treatment, DT + Arg: diquat plus 1% arginine treatment.

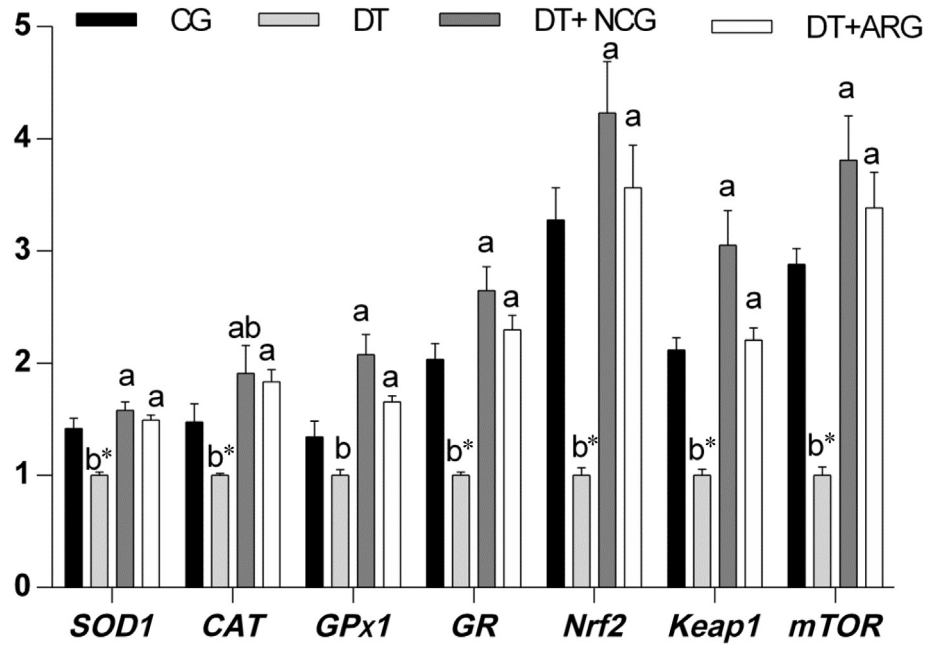

Fig. 1. Relative mRNA levels in the rat spleen under oxidative stress fed diets with arginine and *N*-carbamylglutamate. CG = control group, DT = diquat treatment, DT + NCG = diquat plus 0.1% N-carbamylglutamate treatment, DT + Arg = diquat plus 1% arginine treatment; *SOD1* = superoxide dismutase 1; *CAT* = catalase; *GPx1* = glutathione peroxidase 1; *GR* = glutathione reductase; *Nrf2* = nuclear erythroid 2-related factor 2; *Keap1* = Kelch-like ECH-associated protein 1; *mTOR* = mammalian target of rapamycin. The values are means ± SEM ($n = 6$). The asterisk (*) above bars denotes significant differences ($P < 0.05$) for comparison between DT and CG. [a,b,c] The different superscript letters above the bars show significant differences between DT, DT + NCG, and DT + Arg ($P < 0.05$).

not differ in the DT + Arg group compared with the data of DT + NCG group ($P > 0.05$).

3.3. Cytokine mRNA expression

Table 3 shows the cytokine mRNA expression of the spleen. DT significantly upregulated the mRNA level of *IL-1β* ($P < 0.05$), *IL-6* ($P < 0.05$), *TNF-α* ($P < 0.05$) and caspase-3 ($P < 0.05$), but it downregulated the gene transcription of *IL-10* ($P < 0.05$) and *TNF-α* ($P < 0.05$) relative to their corresponding levels in the control group. Compared with results from the DT group, the DT + NCG and DT + Arg groups exhibited higher *TGF-β1* mRNA levels and significantly lower *IL-1β*, caspase 3, and *TNF-α* mRNA levels ($P < 0.05$).

4. Discussions

4.1. Diquat decreased the antioxidant property

Diquat is a widely used inducer of oxidative stress in pig or rats. To evaluate whether the antioxidant statuses rat spleen was impaired after DT injection, the antioxidant-relative parameters were assayed in rat spleen. The results exhibited that the activity of antioxidant-relative parameters (i.e., ASA, SOD and CAT) and the GSH content were lower, whereas the MDA content was higher in the spleen after DT injection. The results suggested that DT can decrease the antioxidant defenses of the spleen, and that rat spleen suffered from oxidative stress after DT injection.

4.2. Arginine and N-carbamylglutamate improved the antioxidant status

Excessive ROS caused by oxidative stress can oxidize cyto-membrane lipids. Malondialdehyde content is used to reflect the degree of lipid oxidative damage in the body (Cynamon et al., 1985; Liu et al., 2016b). The current study demonstrated that the MDA content was decreased by nutrient intake (e.g., Arg or NCG) in the spleen under oxidative stress. This finding is consistent with the previous result obtained by Xiao et al. (2016) that the MDA content was decreased by Arg or NCG intake in rat jejunum. These findings demonstrated that the lipid oxidative damage of the spleen can be

Table 3
Effect of arginine (Arg), N-carbamylglutamate (NCG) on the cytokine mRNA expressions in the rat spleen under oxidative stress.

Item	Treatments[1]			
	CG	DT	DT + NCG	DT + Arg
IL-1β	0.33 ± 0.06	1.00 ± 0.02^{a*}	0.53 ± 0.04^{c}	0.69 ± 0.06^{b}
IL-6	0.36 ± 0.07	1.00 ± 0.08*	0.85 ± 0.11	0.91 ± 0.05
IL-10	1.77 ± 0.11	1.00 ± 0.04*	1.28 ± 0.12	1.17 ± 0.07
TNF-α	0.63 ± 0.05	1.00 ± 0.02^{a*}	0.62 ± 0.04^{c}	0.74 ± 0.06^{b}
TGF-β1	1.82 ± 0.26	1.00 ± 0.03^{b*}	3.27 ± 0.29^{a}	2.77 ± 0.29^{a}
Caspase-3	0.77 ± 0.04	1.00 ± 0.06^{a*}	0.74 ± 0.03^{b}	0.79 ± 0.02^{b}

IL-1β = interleukin 1β, *IL-6* = interleukin 6, *IL-10* = interleukin 10, *TNF-α* = tumor necrosis factor α, *TGF-β1* = transforming growth factor β1.
[a-c] Within a row, mean values with different letters are significantly different ($P < 0.05$) for comparison between DT, DT + NCG, and DT + Arg. Data are stated as means ± SEM.
[*] Within a row, mean values with an asterisk are significantly different ($P < 0.05$) for comparison between DT and CG.
[1] CG: control group, DT: diquat treatment, DT + NCG: diquat plus 0.1% N-carbamylglutamate treatment, DT + Arg: diquat plus 1% arginine treatment.

decreased due to supplementation with Arg or NCG. Lipid oxidative damage is induced by abundant quantities of superoxide anions and hydroxyl radicals (Abdollahi et al., 2004). The ASA and AHR activities are used to reflect the scavenging activity of free radicals (i.e., superoxide anions and hydroxyl radicals) (Kohen and Nyska, 2002). In the present study, Arg or NCG significantly improved the AHR activity in the spleen. This result contradicts our previous finding that Arg intake had no effect on the AHR activities of the rat plasma (Cao et al., 2016). The possible reasons for this discrepancy are the tissue and body fluid differences. In addition, NCG intake effectively improved the ASA activity of the rat spleen. Overall, these data revealed that Arg or NCG can contribute to scavenge free radicals.

Enzymatic antioxidants (e.g., SOD and CAT) participate in the scavenging of free radicals. Catalase is a key enzymatic antioxidant that can mitigate hydroxyl radical toxicity (Bagnyukova et al., 2005). In this study, Arg or NCG intake was effective in improving the CAT activity of spleen. This result was in accord with the finding of our previous study that the DT + Arg and DT + NCG groups manifested increased CAT activity in rat liver (Cao et al., 2016). Superoxide dismutase is another useful enzymatic antioxidant that cooperates with CAT to convert superoxide anions into H_2O (Winston and Giulio, 1991). In the present study, dietary supplementation with 1% Arg or 0.1% NCG increased the T-SOD activity in the spleen. This result disagrees with that of our previous study that supplementation with Arg and NCG had no effect on the T-SOD activity of rat liver (Cao et al., 2016). This discrepancy may be partially attributed to the larger ROS scavenging capacity in the spleen than that in the liver of rat. The enhancement T-SOD activity in the spleen is beneficial for protecting the spleen from oxidative damage and maintaining animal immunity. These results indicated that supplementation with Arg or NCG can alleviate oxidative stress by enhancing the activities of antioxidant enzymes.

Aside from enzymatic antioxidants, non-enzymatic antioxidants also play a crucial role in preventing oxidative damage. Glutathione, which is the most important and abundant non-enzymatic antioxidant, can directly scavenge hydroxyl radicals and singlet oxygen molecules (Das et al., 2005). In this study, dietary supplementation with Arg or NCG markedly increased the GSH content in the spleen. This result agrees with the finding of our previous study that supplementation with Arg and NCG improved the GSH content in rat jejunum (Xiao et al., 2016). Overall, according to our results, dietary supplementation with Arg and NCG can improve the non-enzymatic antioxidant defense of spleen.

The current study demonstrated that dietary supplementation with Arg or NCG effectively promoted the antioxidant ability in rat spleen. The increased enzymatic antioxidant activity in the tissues may be related to the increased mRNA expression. Thus, we evaluated the effects of supplementation with Arg or NCG on the gene expression of antioxidant enzymes.

4.3. Arginine and N-carbamylglutamate regulated the gene expression of antioxidant enzymes

To examine whether Arg and NCG regulated the activities of antioxidant enzymes at the gene level in rats, we investigated the mRNA levels of various antioxidant enzymes (i.e., *SOD*, *CAT*, *GPx1*, and *GR*). The results showed that the dietary supplementation with Arg upregulated the expression of *SOD*, *GR*, *GPx1*, and *CAT* genes in the spleen. The results are in accord with a previous report that dietary supplementation with Arg enhanced the *SOD1*, *GPx1* and *CAT* gene expression in grass carp muscle (Wang et al., 2015a). The enhanced *SOD1*, *GR*, *GPx1*, and *CAT* gene expression of rats after Arg intake may be proof of the increased activity of antioxidant enzymes in rat spleen. Supplementation with NCG significantly enhanced the expression of *SOD*, *GPx1*, and *GR*, leading to reduced oxidative damage, enhanced T-SOD and CAT activities, and increased GSH content. In conclusion, supplementation with Arg and NCG enhanced the enzymatic antioxidant capacity partially because of the upregulation of their mRNA expression in the spleen. Previous studies have demonstrated that the transcriptions of antioxidant enzyme genes are regulated by intracellular *Nrf2* (Wang et al., 2015a). Our study explored the effects of supplementations with Arg and NCG on transcription of related signaling molecules (i.e., *Nrf2*, *Keap1* and *mTOR*) in rat spleen.

Nuclear erythroid 2-related factor 2 can regulate the transcription of endogenous antioxidant enzyme genes (Nguyen et al., 2009). The present results showed that supplementation with Arg upregulated the mRNA level of *Nrf2*, which displayed similar patterns to the gene expression of *SOD*, *GR*, *GPx1*, and *CAT* in the spleen. The obtained results suggested that Arg possibly enhanced the *SOD* and *CAT* gene expression by increasing the *Nrf2* mRNA level. This hypothesis is supported by the result of a previous study that Arg intake promoted the *SOD* and *CAT* gene expression by enhancing the *Nrf2* mRNA level in fish (Wang et al., 2015a). Kelch-like ECH-associated protein 1 is defined as an Nrf2-binding protein that prohibits Nrf2 nuclear translocation and controls Nrf2 stability (Reisman et al., 2009). In the current study, the *Keap1* mRNA level was increased and showed a similar pattern to the gene expression of *Nrf2* in the spleen supplemented with Arg. This finding contradicts the result of a previous study that Arg may down regulate the *Keap1* expression to promote the *Nrf2* expression in fish (Wang et al., 2015a). The possible reason for this discrepancy is unknown. The *Nrf2* expression can be modulated by *mTOR*, which serves as upstream signaling molecules of *Nrf2* (Shay et al., 2012; Gorrini et al., 2014). The results revealed that the *mTOR* expression was enhanced by the supplementation with Arg or NCG. This finding was in accord with the result of a previous literature that an optimum Arg upregulated the mRNA levels of *TOR* in grass carp muscle (Wang et al., 2015a). The upregulation of *mTOR* expression by the supplementation with Arg or NCG possibly contributed to the enhanced *Nrf2* expression in the spleen. However, this hypothesis requires further investigation.

4.4. Arginine and N-carbamylglutamate regulated the gene expression of inflammatory cytokines

In addition to improving the antioxidant capacity, dietary supplementation with NCG or Arg may affect the immune capacity.

Immune status refers to the innate immune response correlated with an inflammatory response (Guedes and Gebhart, 2003; Wang et al., 2015a). In this study, supplementation with NCG and Arg decreased the *IL-1β* and *TNF-α* mRNA levels in rat spleen under oxidative stress. This result was consistent with a previous finding that dietary Arg ameliorated the enhancement of the jejunal and ileum *TNF-α* mRNA abundance induced by lipopolysaccaride challenge in weaned pigs (Liu et al., 2008). Therefore, these results suggested that NCG and Arg alleviated inflammatory response by downregulating the *IL-1β* and *TNF-α* mRNA expression.

Transforming growth factor-β, which is a multi-purpose set of peptide signal molecules, plays an idiographic and crucial role in the immune system of body. Transforming growth factor-β is involved in regulating the antigen presentation, proliferation, and differentiation of T cells (Letterio and Roberts, 1998). In the present study, NCG- or Arg-supplemented rats exhibited a significantly increased transcription of *TGF-β1* in the spleen. This result suggested that NCG or Arg may be conducive for strengthening the immune function. A previous study showed that mTOR inhibition with rapamycin reduced anti-inflammatory cytokines but increased pro-inflammatory cytokines (Weichhart et al., 2011). These findings supported the results of the present study that Arg or NCG downregulated the *IL-1β* and *TNF-α* mRNA expression but upregulated the *TGF-β* mRNA expression partly because of the upregulated *mTOR* gene expression. Furthermore, our previous study on piglets observed that spermine supplementation downregulated the mRNA expression of pro-inflammatory cytokines and enhanced the gene expression of anti-inflammatory cytokines by decreasing the gene expression of nitric oxide synthase (*iNOS*) (Cao et al., 2017). In rats, iNOS catalyzed the production of NO and modulated the secretion of inflammatory cytokines by activating the guanylate cyclase (Boczkowski et al., 1995). These findings implied that Arg or NCG possibly downregulated the *IL-1β* and *TNF-α* mRNA expression and upregulated the *TGF-β* mRNA expression by decreasing the *iNOS* mRNA level of the spleen. This hypothesis requires further investigation. Furthermore, our data revealed that supplementation with NCG or Arg significantly decreased the caspase-3 mRNA level under oxidative stress. Synthesized as a dormant proenzyme, caspase-3 plays a major role in regulating programmed cell death (Xu et al., 2016). The decreased expression of caspase-3 as a result of the supplementation with NCG or Arg may reduce cell apoptosis in the spleen. The mechanism may be influenced by the transcription factor nuclear factor *NF-κB*, which is involved in resisting to programmed cell death by activating anti-apoptotic gene expression (Manu and Kuttan, 2008).

5. Conclusions

This study demonstrated that DT can induce oxidative stress by decreasing the antioxidant status in rat spleen. Our results also suggested that supplementation with 1% Arg and 0.1% NCG effectively alleviated oxidative stress and enhanced the antioxidant capacity, as evidenced by the increased scavenging activities of free radicals and the enhanced enzymatic and non-enzymatic antioxidant functions. The increased antioxidant enzyme activities by the supplementation with Arg and NCG were related to enhanced antioxidant enzyme gene expression and signaling molecules, including *Nrf2*, *Keap1*, and *TOR*. Treatment with NCG or Arg alleviated the inflammation by regulating the transcription of anti-inflammatory and pro-inflammatory cytokine mRNA. To the best of our knowledge, this study is the first to evaluate the antioxidant status and underlying mechanism of Arg and NCG supplementation in animal spleen. In addition, this work is the first to demonstrate the anti-inflammatory ability and partial molecular mechanism by NCG supplementation in animal spleen. Nonetheless, further investigations must be conducted to elucidate the detailed mechanism by which NCG supplementation mitigates the inflammation.

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