LABORATORY STUDY

Antioxidant therapy improves non-thyroidal illness syndrome in uremic rats

Pingping Yang<sup>a,b,*</sup>, Yun Li<sup>c,*</sup> and Gaosi Xu<sup>a</sup>

<sup>a</sup>Department of Nephrology, Second Affiliated Hospital, Nanchang University, Nanchang, China; <sup>b</sup>Medical Center of the Graduate School, Nanchang University, Nanchang, China; <sup>c</sup>Department of Nephrology, Jiangxi Provincial People’s Hospital, Nanchang, China

ABSTRACT

Background The roles of antioxidant therapy on non-thyroidal illness syndrome (NTIS) in uremic rats is still unclear. Materials and methods Twenty-four Sprague-Dawley (SD) rats were randomly divided into blank, 5/6 nephrectomy (Nx), pyrrolidine dithiocarbamate (PDTC, 10 mg/100 g), sodium bicarbonate (SB, 0.1 g/100 g), N-acetylcysteine (NAC, 80 mg/100 g) and thyroid hormones (TH, levothyroxine 2 mg/100 g) groups. The serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), advanced oxidation protein products (AOPP), interleukin (IL)-1β, free triiodothyronine (FT3), and thyroid stimulating hormone (TSH) were detected in the sixth week. The expressions of IL-1β and deiodinase type 1 (DIO1) were assessed by western blotting. The nuclear factor kappa B (NF-κB) inflammatory signal pathway was confirmed by electrophoretic mobility shift assay (EMSA).

Results Compared with 5/6 Nx group, PDTC and NAC significantly reduced the levels (p < 0.01, respectively) of serum MDA, AOPP, TSH, and elevated levels of serum SOD (p < 0.01, respectively) and FT3 (p = 0.016 and p < 0.01). Neither had significant effects on serum IL-1β content (p = 0.612 and p = 0.582). PDTC and NAC markedly decreased the protein expression of IL-1β (p < 0.01) and increased the protein expression of DIO1 (p < 0.01), respectively. Both had been considerably blunted NF-κB activity (p < 0.01).

Conclusions In uremic rat model, PDTC and NAC can effectively improve oxidative stress level and NTIS. In terms of improving oxidative stress level, NAC is probably superior to PDTC.

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Introduction

Non-thyroidal illness syndrome (NTIS), known as low triiodothyronine (T3) syndrome, refers to low serum T3 and raised reverse T3 (rT3) level. Moreover, serum thyroxine (T4) level and thyroid stimulating hormone (TSH) level are normal or reduced in NTIS. Low serum free T3 (FT3) is primarily due to impaired extra-thyroidal conversion of T4 to active T3. Deiodinase type 1 (DIO1) is the crucial enzyme, which convert T4 to T3. NTIS is widely observed in end-stage renal disease (ESRD) patients. A recent study demonstrated that the accumulated uremic toxins inhibited the enzyme activity of DIO1.

The progression of chronic kidney disease (CKD) is closely linked to oxidative stress and chronic inflammation. By activating nuclear factor kappa B (NF-κB) signaling pathway, stimulating the release of pro-inflammatory cytokines and inducing expression of monocyte chemoattractant protein, oxidative stress promotes inflammation in ESRD. In addition, inflammatory cytokines could trigger the expression of the nicotinamide adenine dinucleotide phosphate oxidase, and intensify the oxidative stress subsequently, thus setting up a vicious cycle. Carrero et al. have found that a reduction in serum T3 was positively correlated with inflammation, malnutrition, and endothelial cell activation. Available data showed that T3 could reduce oxidative stress and weaken the damage mediated by reactive oxidative species (ROS).

Antioxidant treatment is regarded as a potential effective measure to attenuate the oxidative stress. Pyrrolidine dithiocarbamate (PDTC), a metal chelator and antioxidant, is a specific inhibitor of NF-κB. By inhibiting the activation of NF-κB signal pathways, PDTC thereby reduces the release of inflammatory cytokines. In non-immune proteinuria rats, PDTC can markedly decrease inflammation and tubulointerstitial injury. N-acetylcysteine (NAC) is a thiol-containing antioxidant, which increased intracellular glutathione (GSH) level. Added intracellular GSH level improves the ability of...
oxidant. Araki et al.\textsuperscript{12} have found that NAC inhibited NF-κB activation and decreased serum level of interleukin-6 (IL-6). In uremic rats, NAC attenuated the oxidative stress and the inflammatory reactions, finally reduced renal tissue damage.\textsuperscript{13}

Several studies showed that PDTC and NAC attenuated oxidative stress in 5/6 Nx rats.\textsuperscript{13,14} However, there have been no studies about the effects of antioxidant therapy on NTIS in ESRD rats. Therefore, to observe the effects of PDTC and NAC on rats with 5/6 Nx model in the present study, we detected the levels of serum malondialdehyde (MDA), superoxide dismutase (SOD), advanced oxidation protein products (AOPP), leukocytes interleukin-1β (IL-1β), FT3, and TSH. Furthermore, we investigated the effects of PDTC and NAC on the expression of IL-1β and DIO1, and their impacts on NF-κB signal pathways.

Materials and methods

Animals
Experiments were conducted on 7-week-old male and female Sprague-Dawley (SD) rats, weight ranging from 250 to 300 g. Animals were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were housed in a barrier facility at a temperature of 22–24 °C and a relative humidity of 45–65%. Artificial indoor lighting was provided by alternating 12 h light and 12 h dark with a luminance of 200 to 300 lx. Indoor ventilation frequency was 20 times/h, the ammonia concentration was below 14 mg/m\(^3\). The rats were fed with \(^{60}\)Co-irradiated pellet feeds and sterile drinking water. The study was approved by the ethics committee of the Second Affiliated Hospital to Nanchang University.

Study groups
These SD rats were equally randomly divided into six groups with equal number of males and females. Blood was collected from the tail vein for detecting preoperative serum creatinine level. 5/6 nephrectomy (Nx) were conducted on rats as previously described.\textsuperscript{15} Five groups were randomly selected for 5/6 Nx with surgical resections by performing a right Nx and two-thirds of the left kidney, and observed for four weeks under stable feeding. Blood was collected from the tail vein at the end of the fourth week after operation. The uremic rat models were successfully established if postoperative serum creatinine level was two times higher than the preoperative.\textsuperscript{16} The remaining group did not undergo 5/6 Nx was settled as Blank group. The 5/6 Nx rats were grouped and intervened respectively as follows: 5/6 Nx group, PDTC group [PDTC (Sigma-Aldrich, St. Louis, MO) were intraperitoneally injected at 10 mg/100 g, once daily], sodium bicarbonate (SB) group [SB was intragastric administrated at 0.1 g/100 g, once daily], NAC group [NAC effervescent tablet (Sigma-Aldrich, St. Louis, MO) was intragastrically administrated at 80 mg/100 g, once daily], thyroxin (TH) group (levothyroxine was intragastrically administrated at 2 μg/100 g, once daily). Animals were sacrificed after successful intervention, blood and liver were sampled. Serum samples were stored at −80 °C, liver samples were frozen in liquid nitrogen and transferred to dry ice for shipping. The level of MDA, SOD, AOPP, IL-1β, FT3, and TSH were detected. The protein expression of IL-1β and DIO1 was assessed by western blotting. NF-κB inflammatory signal pathways were confirmed by electrophoretic mobility shift assay (EMSA).

Blood sampling
Routine biochemical parameters were assayed in automated analyzer using commercial kits. MDA and SOD were determined according to the instructions on the kit (Sigma-Aldrich, St. Louis, MO). AOPP, IL-1β, FT3, and TSH production was measured in serum by using rat AOPP ELISA kit (CUSABIO, Barksdale, LA), rat IL-1β ELISA kit (R&D Systems, Wiesbaden, Germany), rat FT3 ELISA kit (CUSABIO, Barksdale, LA), and rat TSH ELISA kit (CUSABIO, Barksdale, LA), respectively. All samples were measured in triplicate.

Western blotting
In order to assess the effects of PDTC and NAC on uremic rats, the protein expressions of IL-1β and DIO1 were determined by western blot. Nuclear and cytosolic extracts were prepared using a Nuclear and Cytoplasmic Protein Extraction Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were determined by using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). Protein extractions of 50 μg were loaded onto 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membranes. In short, the membrane was blocked with 5% skimmed milk and incubated with primary rabbit antibody against IL-1β (Santa Cruz Biotechnology, Dallas, TX), DIO1 (Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. After washing with Tris-buffered saline with 0.1% Tween (TBS-T), the blots were incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The signals were visualized by using enhanced chemical luminescence. Western analysis of quantification
was performed with Image J (National Institutes of Health, Baltimore, MD).

**EMSA**

Nuclear protein was extracted and EMSA for the transcription factor NF-κB were carried out according to the manufacturer’s instructions. Protein concentrations were determined by the BioRad protein reagents (Hercules, CA). The NF-κB double-stranded consensus oligonucleotide sequence used was 5’-AGTTGAGGGACTTTCCCAGGC-3’. The NF-κB oligonucleotide probe was end-labeled with Cy5.5-lectin. Unincorporated nucleotides were removed by passing the reaction mixture through a Sephadex G-25 spin column (Amersham-Pharmacia, Uppsala, Sweden). Briefly, binding reactions were added to 1 μl of binding buffer, 2 μl of labeled probe, 1 μl of poly-dIdC, 1 μl of poly-L-Lysine, and 5 μg nuclear extracts for 15 min. Then, 5 μl of loading buffer was added to each sample. DNA protein complexes were separated by electrophoresis through a 6% native polyacrylamide gel in a running buffer containing 1.5 M Tris, pH 8.8, 1 M Glycine and 0.5 M EDTA for 50 min at 90 V. Acquire image using normal image scanning methods for colorimetric detection. Quantification was performed with Image J (National Institutes of Health, Baltimore, MD).

**Statistical analysis**

Data were presented as the mean ± standard deviation (SD) of triplicate experiments, unless otherwise specified. Statistically significant differences in mean values were analyzed by one-way ANOVA followed by the Least Significant Difference (LSD) test. The data were analyzed with SPSS 19.0 software (SPSS Inc., Chicago, IL). A p-value below 0.05 was considered statistically significant.

**Results**

**The serum levels of MDA, SOD, IL-1β, AOPP, T3, and TSH**

No rat died in each group. The levels of MDA, SOD, IL-1β, AOPP, T3, and TSH of each group are summarized in Table 1. PDTC showed benefit effects that reduced the levels (p < 0.01) of MDA, AOPP, and TSH (Figure 1A, C, and F), increased the levels of SOD (p < 0.01) and T3 (p = 0.016) (Figure 1B and E), but had no significant influence on the levels of IL-1β (p = 0.612) compared with the 5/6 Nx group (Figure 1D). NAC administration significantly reduced the contents (p < 0.01) of MDA, AOPP, and TSH, increased the contents (p < 0.01) of SOD and T3, but had no significant effect on the contents of IL-1β (p = 0.582) versus 5/6 Nx group. SB treatment notably decreased the levels of MDA (p = 0.018), AOPP (p < 0.01), and TSH (p = 0.038), ascended the contents of SOD (p = 0.042) and T3 (p = 0.012) compared with the 5/6 Nx group, but that of IL-1β level was no statistically different (p = 0.127). TH treatment notably reduced the levels of MDA (p < 0.01), AOPP (p = 0.021), and TSH (p < 0.01), added levels of SOD (p < 0.01) and T3 (p = 0.026) compared with the 5/6 Nx group. There was no statistical difference in IL-1β content between TH group and 5/6 Nx group (p = 0.168).

From the results, we learned that PDTC, SB, NAC, and TH significantly reduced the levels of serum MDA, AOPP, and TSH, elevated the levels of serum SOD and FT3 compared with 5/6 Nx group. However, the level of IL-1β had no significant difference between all drug intervention groups and 5/6 Nx group.

Although both PDTC group and SB group had the trend of increased level of SOD, there was statistically significant difference between the two groups (p = 0.033). This result showed that in terms of elevated serum SOD level, the effect of PDTC is superior to SB. At the level of AOPP, there was a significant statistical difference between NAC and PDTC (p < 0.01), NAC and SB (p < 0.01), NAC and TH (p < 0.01), PDTC and TH (p < 0.01), respectively. PDTC group and SB group had no statistical difference (p = 0.543). All of them could decrease the contents of AOPP effectively. This result illustrated that regarding to reduce the contents of AOPP, NAC was the best, followed by PDTC and SB, with TH the worst.

**Western blotting analysis of IL-1β and DIO1 expressions**

Western blot demonstrated that the IL-1β protein expression was significantly inhibited by approximately

| Parameters       | Blank (nmol/mL) | SOD (U/mL) | AOPP (μmol/L) | IL-1 (μg/mL) | FT3 (fmol/mL) | TSH (ng/mL) |
|------------------|----------------|------------|---------------|--------------|---------------|-------------|
| MDA              | 2.11 ± 0.78    | 156.06 ± 8.09 | 28.59 ± 1.70  | 88.02 ± 19.04 | 11.27 ± 2.55  | 0.063 ± 0.017 |
| SOD              | 6.13 ± 0.37    | 86.24 ± 5.76 | 41.39 ± 1.64  | 159.16 ± 42.01 | 6.12 ± 0.27   | 0.153 ± 0.040 |
| AOPP             | 3.66 ± 0.72    | 129.42 ± 15.72 | 35.62 ± 1.54  | 143.16 ± 58.37 | 9.34 ± 0.92   | 0.073 ± 0.017 |
| IL-1             | 4.70 ± 0.93    | 107.25 ± 17.12 | 36.28 ± 1.47  | 109.62 ± 28.43 | 9.51 ± 1.30   | 0.110 ± 0.037 |
| FT3              | 4.46 ± 0.94    | 114.40 ± 14.97 | 32.15 ± 0.79  | 141.77 ± 69.74 | 10.32 ± 2.59  | 0.088 ± 0.017 |
| TSH              | 3.84 ± 0.76    | 124.49 ± 15.67 | 38.69 ± 1.68  | 114.61 ± 17.93 | 9.05 ± 1.37   | 0.070 ± 0.022 |

Notes: Data are given as mean ± SD of four animals per group. Samples were measured in triplicate.
39% ($p<0.01$) under the NAC treatment, approximately 43% ($p<0.01$) under the SB treatment, approximately 10% ($p<0.01$) under PDTC treatment, and approximately 41% ($p<0.01$) under the TH treatment, compared with 5/6 Nx group (Figure 2A). However, the expression of IL-1β under NAC, SB, and TH treatment made a great statistical difference ($p<0.01$) versus PDTC treatment.

The DIO1 protein expression in 5/6 Nx group was reduced significantly ($p<0.01$) compared with Blank group. The western blot of DIO1 expressions suggested that the SB group had a dramatically difference between the NAC, PDTC, and TH group ($p<0.01$). As indicated in Figure 1(E), PDTC, SB, NAC, and TH had the prominent effects on ascending the serum FT3 levels. Owing to the elevated DIO1 protein expression induced by PDTC, SB, NAC, and TH treatments, it was demonstrated that the DIO1 function could be improved by antioxidant and TH therapy.

**NF-κB signal pathway by EMSA**

Accumulated uremic toxins in 5/6 Nx group activated the NF-κB signal pathway ($p<0.01$) in contrast with Blank group (Figure 3). The NF-κB signal was prominently reduced by approximate 75, 33, 50, and 42% after

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**Figure 1.** The levels of serum MDA (A), SOD (B), AOPP (C), IL-1β (D), FT3 (E), and TSH (F) in each group. Data are given as mean ± SD of four animals per group. # $p < 0.05$ compared with Blank group. * $p < 0.05$ compared with 5/6 Nx group.
PDTC, SB, NAC, and TH treatments, compared with 5/6 Nx group \((p < 0.01)\). There was an obvious inactivation of NF-κB while improving oxidative stress and/or serum T3 level was elevated.

**Discussion**

Several studies showed that PDTC and NAC attenuated oxidative stress in 5/6 Nx rats.\(^{13,14,17–19}\) At present, there is no study that explores the effects of PDTC and NAC on NTIS in uremic rats. Firstly, the present study showed the impact of PDTC and NAC on NTIS in 5/6 Nx rats.

Our study showed that in terms of increasing the serum SOD level, PDTC was superior to SB (Figure 1B). We speculated that via inhibit NF-κB activity, PDTC ameliorated inflammation and oxidative stress, scavenge oxygen-free radicals. SOD is the antioxidant enzyme and prime substance to remove free radicals.

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**Figure 2.** Effects of PDTC, SB, NAC, and TH treatments on uremic rats by western blot. Normalized densitometric data of IL-1β (A) and DIO1 (B) bands obtained from the protein extractions. RR stands for relative ratio. Values are expressed as fold changes relative to the appropriate controls. Values are given as mean ± SD of four animals per group. \(^{#}p < 0.05\) compared with Blank group. \(^{*}p < 0.05\) compared with 5/6 Nx group.

**Figure 3.** Effects of PDTC, SB, NAC, and TH treatments on uremic rats by EMSA. Normalized densitometric data of NF-κB band obtained from the extracts of proteins with unclear interaction. Data are given as mean ± SD of 4 animals per group. \(^{#}p < 0.05\) compared with Blank group. \(^{*}p < 0.05\) compared with 5/6 Nx group.
PDTC could directly reduce the consumption of SOD. SB probably through correcting metabolic acidosis, then indirectly decreased the oxidative stress level. The present study found in the field of lowering AOPP content, NAC was superior to PDTC and SB, PDTC and SB was superior to TH (Figure 1C). AOPP is the product of plasma albumin and chlorine oxide, and is regarded as a novel marker of protein oxidative damage. The antioxidant NAC properties, bases on its ability to prevent GSH depletion exposed to uremic serum and inhibit the pro-inflammatory transcription factor activators protein-1 (AP-1) and NF-κB. So, in terms of reducing the concentration of AOPP, the effect of NAC was the best among the four drugs. Several studies showed that TH could reduce the levels of ROS and attenuate the systemic oxidative stress. TH therapy appears to protect against lipid peroxidation and enhance mitochondrial aerobic capacity. Thus, indirectly improve oxidative stress level in uremic rats.

Inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α were higher with lower levels of glomerular filtration rate. The results showed IL-1β protein expression was markedly reduced under SB, TH, PDTC, and NAC administration, separately (Figure 2A). And the expression of IL-1β under NAC, SB, and TH treatment made a great difference compared with PDTC treatment. ROS may serve as signal transduction messengers for several important transcription factors, such as NF-κB and AP-1. PDTC is a specific NF-κB depressor, but that it has no influence on AP-1, cAMP response element-binding protein (CREB) or specificity protein (Sp-1). However, IL-1 was not only regulated by NF-κB but also by both activator protein AP-1 and CREB. Therefore, the specific NF-κB inhibitor, PDTC had little influence on IL-1β protein expression. Moreover, as indicated in Figure 1(D), each of the four interventions had very little effect on the serum level of IL-1β. The possible reason is that these treatments had a relatively prominent effect on the transcription level, but had little effect on the post-translational level of cytokine production.

SB, NAC, PDTC, and TH treatments were significantly up-regulated DIO1 expressions (Figure 2B). SB had been demonstrated to improve nutritional status and slow the rate of progression of renal failure to ESRD. A prospective randomized study found that SB could improve thyroid function by correcting metabolic acidosis. However, further study regarding the mechanism will be required. All of them exert direct or indirect anti-oxidative effects on promoting the expression of DIO1 protein in the ESRD.

NF-κB DNA binding activity was measured by EMSA (Figure 3). The results presented that PDTC and NAC significantly inhibited NF-κB signal pathways, nearly 75 and 50%, respectively. Combined with the effects of PDTC and NAC on oxidative stress parameters, which further verified NF-κB plays a crucial role in the process of oxidative stress.

According to the effects of the four drugs intervened in uremic rats, the present study showed that PDTC and NAC prominently improved oxidative stress level and NTIS. There are very limited studies about clinical application of PDTC. A high intravenous dose of PDTC was found to induce acute toxicity in mice and rats, which were mainly affected autonomous and central nervous system. NAC is a safe drug without any significant adverse effect. NAC was demonstrated safe and well-tolerated in hemodialysis patients. In the present study, PDTC and NAC could available improve oxidative stress level and NTIS. NAC is probably better than PDTC regarding the improved oxidative stress level and relatively safe. Based on the above reasons, prefer to recommend clinical application of NAC.

There are some limitations of the present study. Firstly, the number of animal models is relatively small. Secondly, there are three types of deiodinases. The protein expression of DIO2 and/or DIO3 could be investigated. Thirdly, pathological changes in rat kidney under the four drugs administration should be further investigated.

Conclusions

In CRF model, PDTC and NAC can effectively improve oxidative stress level and non-thyroidal illness syndrome (NTIS). In terms of improving oxidative stress level, NAC is probably superior to PDTC.

Disclosure statement

No conflict of interests is declared.

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