



## Suppressive effects of Active Hexose Correlated Compound on the increased activity of hepatic and renal ornithine decarboxylase induced by oxidative stress

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Received 2 April 2003; accepted 4 June 2003

### Abstract

Active Hexose Correlated Compound (AHCC), an extract derived from fungi of Basidiomycetes family has been shown to act as a biological response modifier in various disorders. In our present study, ferric nitrilotriacetate (Fe-NTA), which generates hydroxyl radicals *in vivo*, was given intraperitoneally to rats and AHCC was tested for its ability to suppress oxidative stress and the activity of ornithine decarboxylase (ODC) in the liver and kidney. Substantial increments in glutathione-related enzymes including glutathione reductase, glutathione peroxidase activity as well as oxidized glutathione contents were shown in the liver at 12 h after treatment with Fe-NTA (7.5 mg Fe/kg body weight). Effects of oxidative stress induced by Fe-NTA were also demonstrated by the increase in serum lipid peroxidation, aminotransferases and urinary 8-hydroxy-2'-deoxyguanosine. However, the increases in these parameters were restored to normal in AHCC-pretreated rats. The ODC activity in the liver and kidney was significantly increased by Fe-NTA, while the increased ODC activity induced by Fe-NTA was normalized in AHCC-pretreated rats. These results suggest AHCC acts as a potent antioxidant and protects against disorders induced by oxidative stresses.

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**Keywords:** Active Hexose Correlated Compound; Ferric nitrilotriacetate; Ornithine decarboxylase; 8-hydroxy-2'-deoxyguanosine; Lipid peroxidation; Antioxidant enzymes

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## Introduction

Clinical and epidemiological findings, as well as investigations in experimental systems have provided evidence supporting a role of free radicals in the etiology of cancer (Guyton and Kensler, 1993). The free radical-scavenging vitamin C and E as well as other scavengers of free radicals have been shown to protect against cancer development or to delay tumorigenesis in animal models, and may be chemoprotective in humans (Guyton and Kensler, 1993). The reactive oxygen species (ROS) generated as a result of exposure to various endogenous and environmental stimuli such as pesticides, herbicides, drugs, tobacco and other pollutants can damage DNA, membrane lipids and proteins (Borek, 1991). The DNA damage may lead to mutation and protein damage may lead to the impairment of growth regulatory enzymes, which are considered to be the prerequisite at early stages of carcinogenesis (Ciolino and Levine, 1997).

Chemoprevention with naturally occurring substances and synthetic chemicals appears to be promising for preventing, arresting, and reversing cancer development (Greenwald, 2002). It has been reported that about 30 classes of chemicals with cancer-preventive effects that may have practical implications in reducing cancer incidence in human population (Wattenberg, 1997). The efficacy of these chemopreventive agents has been related to their antioxidative potential of reducing/or inhibiting free radical-mediated damage to DNA, lipids and proteins. In addition, anticarcinogenic effects of these agents are also reported to be related to their potential of decreasing oxidative stress and/or inducing phase II detoxifying enzymes such as glutathione S-transferase and quinone reductase (Iqbal and Athar, 1998).

Recently, Active Hexose Correlated Compound (AHCC™, Amino UP Chemical Co. Ltd., Sapporo), an extract derived from fungi of Basidiomycetes family, which are widely used in Chinese traditional medicine, is drawing widespread attention (Kidd, 2000). AHCC is a mixture of polysaccharides, amino acids, lipids and minerals derived from fungi. It is obtained by hot water extraction after culturing mycelia of several basidiomycetes in a liquid culture media and then treating them with some enzymes (Wakame, 1999). The chemical analysis has revealed that oligosaccharides are the major components of AHCC, consisting about 74%, among which nearly 20% of the oligosaccharide are  $\alpha$ -1, 4-glucan and their acetylated forms with an average molecular weight of approximately 5000, which may be responsible for its biological activities (Matsushita et al., 1998; Matsui et al., 2002). A wide range of biological, pharmacological and clinical potentials of AHCC has been investigated so far. As a therapeutic agent, AHCC is well tolerated and largely free of adverse effects (Kidd, 2000). AHCC has been reported to enhance the activity of natural killer cell of cancer patients (Matsushita et al., 1998), to increase detoxification enzymes in the liver and protect the liver from CCl<sub>4</sub>-induced injury (Sun et al., 1997), to prevent the onset of diabetes induced by streptozotocin in animal models (Wakame, 1999), to suppress thymic apoptosis induced by dexamethasone (Burikhanov et al., 2000), to decrease ferric nitrilotriacetate (Fe-NTA)-mediated excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in rat urine (Wang et al., 2001). These results suggest AHCC acts as a promising biological response modifier (BRM), antioxidant, antimutagenic and/or anticarcinogenic agent. It was also found that AHCC reduced the metastasis rate of rat mammary adenocarcinomas (Matsushita et al., 1998). More recently, AHCC treatment after surgical operation has been shown to improve the quality of life (QOL) and prognosis of patients with hepatocellular carcinoma

(Matsui et al., 2002). However, the precise mechanisms by which AHCC exerts its beneficial effects remain to be elucidated.

Most of the toxicants including Fe-NTA that behave as tumor promoters act through the generation of free radicals, induction of ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, and by enhancing the rate of DNA synthesis with a simultaneous decrease in antioxidant defenses. Therefore, oxidative stress and ODC are widely used as biomarkers of tumor promotion (Iqbal et al., 1995). In the current study, we have evaluated the mechanisms by which AHCC suppresses early events of tumorigenesis in a rat model induced by Fe-NTA.

## Materials and methods

### *Chemicals*

AHCC was obtained from Amino UP Chemical Co. Ltd. (Sapporo, Japan). Reduced and oxidized glutathione (GSH and GSSG) and NADPH were obtained from Wako Pure Chemical Co., Inc. (Osaka, Japan). GSH reductase (GSH Rd) and L-ornithine were obtained from Sigma Chemical Co. (St Louis, MO). DL-[1-<sup>14</sup>C] ornithine (1.96 Bq/mmol) was obtained from NEN (USA). Fe-NTA solution was prepared immediately before use as our previous method (Wang et al., 2001). All other chemicals and reagents were of the highest analytical grade.

### *Animals and treatment*

Eight-week-old male Wistar rats weighting 200–250 g were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were treated according to guidelines for the Care and Use of Laboratory Animal of the Committee, Dokkyo University School of Medicine. The rats were housed (n = 5) in a ventilated room at 23 ± 2 °C and under an alternating 12 h light /dark cycle. All the animals were allowed to acclimatize for one week before study and had free access to standard laboratory chow and water ad libitum.

Twenty rats were divided into four groups. Two groups received 3% AHCC in drinking water for one week until their sacrifice. On the base of the water consumed, the AHCC given daily to each rat was roughly estimated to be 1.8 g/kg body weight. The concentration of AHCC and the duration of the treatment were chosen according to our previous results (Burikhanov et al., 2000). Control groups received only tap water for one week. Fe-NTA (7.5 mg/kg body weight) was injected intraperitoneally to two groups; one of them was AHCC-pretreatment group, and the other was the non-treated group. These animals were killed by decapitation at 12 h after the treatment with saline or Fe-NTA. The selection of dose regime of Fe-NTA was based on previously published data (Iqbal et al., 1995).

### *Assay for urinary 8-hydroxy-2'-deoxyguanosine(8-OHdG)*

Urine samples taken from the urinary bladder of the rats were centrifuged at 10,000 rpm, and the supernatants were used for 8-OHdG assay by ELISA kit (Institute for the Control of Ageing, Shizuoka, Japan). The results were expressed as 8-OHdG/creatinine ratio.

### *Blood biochemical determination*

Serum samples were obtained after centrifuging at 4 °C for 15 min at 3000 rpm. Serum levels of creatinine and urea nitrogen were assayed by using Creatinine-HR Kit and Urea-Nitrogen test kit (Wako Pure Chemical industries, Ltd., Osaka, Japan). Serum levels of lipid peroxidation were assayed by [Yagi's methods \(1976\)](#). Serum SOD activity was assayed by [Oyanagni's method \(1984\)](#). Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by [Henry et al.'s method \(1960\)](#).

### *Assay for ornithine decarboxylase (ODC) activity*

The ODC activity was determined using 100,000 × g supernatants of the liver and kidney homogenates by measuring the release of <sup>14</sup>CO<sub>2</sub> from the DL-[1-<sup>14</sup>C] ornithine hydrochloride by the method of [Murakami et al. \(1988\)](#). The ODC activity was expressed as pmol <sup>14</sup>CO<sub>2</sub> released/per hour mg protein.

### *Determination of glutathione (GSH) content*

The content of reduced GSH and oxidized GSH in 100,000 × g supernatants was measured by fluorometric method as described previously ([Hissin and Hilf, 1976](#)) with minor modifications. The intensity of fluorescence due to the GSH-OPT adduct at pH8.0 and GSSG-OPT adduct at pH12.0 was measured at an excitation-emission of 350–420 nm.

### *Assay for glutathione reductase (GSH Rd) and glutathione peroxidase (GSH Px)*

The activity of GSH Rd and GSH Px was measured by monitoring changes of NADPH oxidized to NADP at 340 nm per minute as described by [Hsiao et al. \(2001\)](#) and [Paglia and Valentine \(1967\)](#), respectively.

### *Assay for superoxide dismutase (SOD) activity*

The SOD activity in liver and kidney was measured using 78,000 × g supernatants by SOD Assay Kit-WST (Dojindo Molecular Technologies, INC., Japan) according to the manufacturer's instructions. Data were expressed as SOD inhibition (%).

### *Protein assay*

Protein contents of tissue homogenates were determined by [Lowry et al.'s method \(1951\)](#) using bovine serum albumin as the standard.

### *Statistical analysis*

All the data were expressed as means ± SD. Statistical analysis was performed by ANOVA method and significant difference was judged by Newman-Keuls test. A P value less than 0.05 was considered as significant difference.

## Results

### *General conditions in rats*

During the course of the study, the animals exhibited no apparent sign of toxicity. Body weight and weights of the liver, kidney and adrenal glands were not significantly different among the four groups (data not shown).

### *Urinary 8-OHdG*

An increase in urinary 8-OHdG was observed following exposure of animals to Fe-NTA. The AHCC pre-treatment significantly suppressed the increase in urinary 8-OHdG induced by Fe-NTA (Table 1).

### *Serum lipid peroxidation (LPO), creatinine, urea nitrogen and aminotransferases*

AHCC normalized the Fe-NTA-mediated increase in the levels of LPO (Table 1). Moreover, the Fe-NTA treatment led to an enhancement of 2.5- and 2.1-fold in the values of serum creatinine and urea nitrogen, respectively, as compared with control group (Table 1). Pre-treatment with AHCC resulted in significant decreases in these values. AHCC also significantly decreased serum AST and ALT levels to about 56% and 70% as compared with the Fe-NTA alone group under the same conditions. The recovery of these two values by AHCC is still above the control values, but the difference in values between Fe-NTA group and AHCC plus Fe-NTA group were statistically significant ( $P < 0.05$ ) (Table 1).

### *ODC activity*

ODC activity is generally considered to be a biomarker of tumor promotion. It was shown that Fe-NTA increased the activity of both hepatic and renal ODC maximally at 12 h after its administration in the rat (Iqbal et al., 1995). In this study, treatment with Fe-NTA alone resulted in 1.7- and 1.4-fold increase in hepatic and renal ODC activity as compared with the control group.

Table 1  
Effects of AHCC and Fe-NTA on oxidative stress-related parameters in urine and serum

Treatment groups	8-OHdG (ng/ml/creatinine)	LPO (nmol/ml)	BUN (mg/dl)	Creatinine (mg/dl)	AST (IU/l)	ALT (IU/l)
Control	1.04 ± 0.23	2.2 ± 0.2	14.9 ± 0.9	0.25 ± 0.02	208.4 ± 45.0	65.6 ± 8.3
AHCC	1.06 ± 0.20	2.3 ± 0.1	15.0 ± 0.8	0.25 ± 0.02	160.2 ± 10.9	55.4 ± 7.3
Fe-NTA	1.91 ± 0.32*	4.2 ± 0.3*	30.1 ± 4.3*	0.62 ± 0.03*	677.2 ± 206.0*	348.0 ± 171.3*
AHCC + Fe-NTA	1.24 ± 0.24**	2.9 ± 0.5**	18.3 ± 3.6**	0.36 ± 0.08**	296.2 ± 50.6**	104.5 ± 21.5**

Each value represents mean ± SD of five animals.

\* $P < 0.05$  vs. control group.

\*\* $P < 0.05$  vs. Fe-NTA treatment group.

Table 2

Effects of AHCC and Fe-NTA on ornithine decarboxylase activity (ODC) in the liver and kidney

Treatment groups	ODC activity (pmol of $^{14}\text{CO}_2$ released/hr/mg protein)	
	Liver	Kidney
Control	133.9 $\pm$ 14.9	275.3 $\pm$ 29.1
AHCC	142.9 $\pm$ 27.3	243.2 $\pm$ 25.0
Fe-NTA	462.5 $\pm$ 94.1*	475.4 $\pm$ 74.6*
AHCC + Fe-NTA	211.0 $\pm$ 91.1**	278.3 $\pm$ 41.7**

Each value represents mean  $\pm$  SD of five animals.

\*P &lt; 0.05 vs. control group.

\*\*P &lt; 0.05 vs. Fe-NTA treatment group.

The pretreatment of animals with AHCC resulted in a significant suppression of the Fe-NTA-induced increase in hepatic and renal ODC activity (Table 2).

#### GSH content, GSH Rd and GSH Px activities

In Fe-NTA-treated rats, the hepatic GSH content was significantly less than that of control group (Table 3). On the contrary, the levels of GSSG in the liver were significantly higher in the Fe-NTA treated group

Table 3

Effects of AHCC and Fe-NTA on glutathione content and glutathione-related enzymes in the liver

Treatment groups	GSH ( $\mu\text{g}/\text{mg}$ protein)	GSSG ( $\mu\text{g}/\text{mg}$ protein)	GSH Rd (nmol NADPH oxidized/min/mg protein)	GSH Px (nmol NADPH oxidized/min/mg protein)
Control	17.23 $\pm$ 0.58	5.52 $\pm$ 0.51	0.303 $\pm$ 0.034	0.105 $\pm$ 0.005
AHCC	17.49 $\pm$ 0.68	5.80 $\pm$ 0.47	0.305 $\pm$ 0.042	0.106 $\pm$ 0.009
Fe-NTA	9.23 $\pm$ 0.35*	10.39 $\pm$ 1.29*	0.399 $\pm$ 0.020*	0.133 $\pm$ 0.007*
AHCC + Fe-NTA	14.77 $\pm$ 1.38**	6.37 $\pm$ 0.58**	0.354 $\pm$ 0.031**	0.112 $\pm$ 0.007**

Each value represents mean  $\pm$  SD of five animals.

\*P &lt; 0.05 vs. control group.

\*\*P &lt; 0.05 vs. Fe-NTA treatment group.

Table 4

Effects of AHCC and Fe-NTA on superoxide dismutase activity in surum, liver and kidney

Treatment groups	SOD activity		
	Serum (U/ml)	Liver (%)	Kidney (%)
Control	25.3 $\pm$ 3.1	79.9 $\pm$ 2.2	76.4 $\pm$ 2.4
AHCC	24.2 $\pm$ 6.3	81.5 $\pm$ 2.5	75.5 $\pm$ 4.9
Fe-NTA	19.9 $\pm$ 3.2*	58.7 $\pm$ 3.4*	47.3 $\pm$ 6.6*
AHCC + Fe-NTA	22.5 $\pm$ 1.4**	69.6 $\pm$ 2.6**	58.5 $\pm$ 3.8**

Each value represents mean  $\pm$  SD of five animals.

SOD activity in liver and kidney is expressed as inhibition (%).

\*P &lt; 0.05 vs. control group.

\*\*P &lt; 0.05 vs. Fe-NTA treatment group.

than in the control group (Table 3). However, AHCC treatment restored the hepatic contents of GSH and GSSG to nearly normal. GSH Rd and GSH Px activities increased about 1.26- and 1.34-fold, respectively after Fe-NTA administration as compared with control groups. The pretreatment of rats with AHCC reduced the activity of GSH Rd and GSH Px to normal levels (Table 3).

### *SOD activity*

The activities of SOD were reduced to 20%, 38%, 26% of the control values in serum, liver and kidney, respectively, following Fe-NTA treatment. However, pretreatment with AHCC resulted in significant recovery of SOD activity ( $P < 0.05$ ) (Table 4).

## **Discussion**

The treatment with Fe-NTA has been reported to induce a variety of changes in vivo, i.e. enhanced lipid peroxidation with a concomitant decrease in tissue glutathione levels, increased formation of 8-OHdG and renal and hepatic ODC induction by generating hydroxyl radical (Toyokuni et al., 1994; Iqbal et al., 1995). The results of the present study have demonstrated that AHCC suppresses the Fe-NTA-mediated cellular oxidative stress, hepatic and renal ODC induction, and alleviates nephro- and hepatotoxicity.

Oxidative DNA damage is thought to be involved in ROS-induced carcinogenesis (Umemura et al., 1990). The most ubiquitous oxidative DNA base modification is 8-OHdG, and the increase in urinary excretion of 8-OHdG reflects the oxidative DNA damage in vivo (Umemura et al., 1990; Kasai, 1997). It has been established that Fe-NTA administration results in enhanced formation of 8-OHdG and high incidence of renal and hepatic carcinoma in rats and mice (Iqbal et al., 1995; Iqbal and Athar, 1998). LPO has been shown to promote the formation of 8-OHdG by production of 4-hydroxy-2-nonenal (HNE) in vitro (Toyokuni et al., 1994). In accordance with previous reports (Toyokuni et al., 1994; Umemura et al., 1996), we have demonstrated the levels of 8-OHdG in renal DNA change nearly in parallel with urinary 8-OHdG at 12 h after Fe-NTA treatment (Wang et al., 2000), and AHCC suppresses such an increase in 8-OHdG formation in a preliminary study. In the present study, our results revealed that AHCC ameliorated oxidative DNA damage and decreased serum LPO level induced by Fe-NTA. These findings are consistent with our previous reports (Wang et al., 2000, 2001) and other earlier observations that several antioxidants such as 2-mercaptoethane and N-acetylcysteine prevent Fe-NTA-mediated DNA damage (Umemura et al., 1996). Considering the number of reports demonstrating a close relation between 8-OHdG formation and carcinogenicity, it seems likely that AHCC may suppress Fe-NTA-mediated carcinogenicity by acting as an antioxidant.

Several lines of evidence have shown that certain naturally occurring substances possess significant antitumor-promoting activity because of their antioxidant properties in tumor promotion animal model (Iqbal et al., 1995; Umemura et al., 1996; Ansar et al., 1999). Fe-NTA is a potent renal and hepatic tumor promoter and acts through the generation of oxidative stress. Induction of ODC activity is associated with cell proliferation and is extensively used as a biomarker of many tumor promoters including Fe-NTA (Iqbal et al., 1995; Ansar et al., 1999). Pretreatment with AHCC results in a decrease in Fe-NTA-mediated induction of ODC activity, suggesting the AHCC exhibits a potent antitumor promoting activity in these two organs. The suppression of Fe-NTA-induced ODC activity by AHCC is in accordance with previous

data, which show that certain natural compounds inhibit cutaneous ODC activity due to their antioxidant activity (Saleem et al., 2001).

Some endogenous protective factors such as GSH Px, GSH Rd and catalase are activated in the defense against oxidative cell injury by scavenging free-radicals (Talalay, 1989). Glutathione as the main component of endogenous non-protein sulfhydryl pool participates in scavenging free radicals, reducing peroxides or being conjugated with electrophilic compounds, thus provides the cell with multiple defenses not only against ROS but also against their toxic products (Hayes and McLellan, 1999). Under oxidative stress, glutathione is largely consumed by the GSH-related enzymes, thereby resulting in induction of some intoxication (Nordberg and Arner, 2001). In the present study, a single dose of Fe-NTA decreases the glutathione content, enhances the activities of GSH-related enzyme. These results support the notion that depletion of tissue glutathione is one of the major factors leading to lipid peroxidation and subsequent tissue damage after Fe-NTA treatment (Toyokuni, 1996). Administration of AHCC maintained the glutathione levels, thus protected tissue against oxidative stress. Our results also demonstrated that SOD is significantly elevated by pretreatment with AHCC, suggesting that AHCC can restore and/or activate SOD activity in Fe-NTA-treated rat. These results indicated that AHCC might be used as a promising chemopreventive agent because of its antioxidant properties.

AHCC is a mixture of various oligosaccharides and other minor compounds. Interestingly, in contrast to conventional active components such as  $\beta$ -1, 3-glucan found in PSK and lentinan, which have been reported as strong antioxidants and anti-tumor agents in many systems (Kidd, 2000), the oligosaccharide in AHCC have an N-acetylated- $\alpha$ -1, 4-linkage structure (Matsushita et al., 1998). The results of our present study have shown that AHCC alone little affects oxidative status and the activities of enzymes scavenging ROS, but clearly ameliorates Fe-NTA-induced oxidative stress. These results are in good accordance with previous report (Paterna et al., 1998), which shows that some poorly metabolized hexoses such as D-tagatose protect Fe-NTA-induced oxidative cell injuries in hepatocytes. These antioxidant effects are mediated by its ability of scavenging hydroxyl radical, stabilizing intracellular levels of reduced GSH and preventing protein carbonyl formation and membrane lipid peroxidation (Paterna et al., 1998). Only weak iron-chelating property of D-tagatose has been shown in a cell-free system (Charley et al., 1963).

It seems unlikely that the oligosaccharides found in AHCC are taken up into circulation in its intact forms and interact with hydroxyl radicals generated from Fe-NTA in peripheral tissues. Therefore, it is possible that other mechanisms by which AHCC acts as an antioxidant are suggested. In fact, the beneficial effects of AHCC are shown first on the third day following its treatment in our experimental mode (Burikhanov et al., 2000), which implies the protection of AHCC against Fe-NTA-mediated oxidative injury requires certain period of time. However, all the enzymes that scavenge ROS remained unchanged and the levels of GSH were not effect by treatment with AHCC alone. Recently, AHCC has been shown to prevent the onset of diabetes induced by streptozotocin (Wakame, 1999). This experimental diabetes is thought be induced by streptozotocin-mediated free radicals in pancreatic  $\beta$ -cells (Nukatsuka et al., 1988). In addition, AHCC has also reported to suppress thymic apoptosis induced by dexamethasone, which is assumed be mediated by ROS (Burikhanov et al., 2000). These results raise the possibility that AHCC does not scavenge ROS directly but it stimulates certain antioxidant mechanisms in vivo, though one cannot neglect the possibility that the AHCC exerts its effects by chelating ferric iron at least partially.

In summary, our data indicate that AHCC suppresses renal and hepatic ODC induction and alleviate tissue damages induced by Fe-NTA. It is likely that AHCC has the potential to be used as an antioxidant agent in free radical-mediated disorders.

## Acknowledgements

We would like to thank Professor S. Matsuzaki, Department of Biochemistry, Dokkyo University School of Medicine for his critical review of the manuscript. This study was supported in part by research grants from Tsukushi Foundation.

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