AMELIORATION BY ACTIVE HEXOSE CORRELATED COMPOUND OF ENDOCRINE DISTURBANCES INDUCED BY OXIDATIVE STRESS IN THE RAT

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Objective. Active hexose correlated compound (AHCC), an extract derived from fungi of *Basidiomycetes* family, has been found to be a potent antioxidant. Since the secretion of some hormones can be affected by reactive oxygen species, the objective of this study was to examine how ferric nitrilotriacetate (FeNTA), which generates hydroxyl radicals *in vivo*, modulates the hormone secretion and the effects of AHCC.

Methods. AHCC at 3 % in drinking water was given to male rats for one week, and the animals were decapitated at different time intervals after the treatment with FeNTA intraperitoneally. Serum levels of hormones (corticosterone, testosterone, thyroxine and triiodothyronine), adrenal ascorbic acid as well as changes in hepatic oxidative status were evaluated by immunoassay and spectrometry.

Results. Serum corticosterone levels increased significantly following FeNTA treatment, while AHCC reduced the increased levels to normal. Adrenal ascorbic acid levels that reflect ACTH secretion, were decreased by FeNTA and restored to normal by AHCC. Serum levels of testosterone and thyroxine (T_4) decreased rapidly after FeNTA treatment, while AHCC pretreatment prevented this fall. Serum triiodothyroxine (T_3) levels remained unchanged either by FeNTA or AHCC treatment. The hepatic oxidized glutathione, glutathione-related enzymes and also serum lipid peroxide were greatly enhanced after FeNTA treatment. All of these changes were restored to normal by AHCC pretreatment.

Conclusion. FeNTA induces various endocrine disorders and AHCC ameliorates these effects by acting as an antioxidant.

Key words: Oxidative stress – Corticosterone – Testosterone – Thyroid hormone – Fungi extract – Endocrine disorders

There is considerable evidence suggesting that reactive oxygen species (ROS) are involved in the pathogenesis of a variety of chronic diseases including carcinogenesis, atherosclerosis, senescence and dysfunctions of endocrine systems (Mates et al. 1999). Enzymatic and non-enzymatic systems preserve the oxidant/antioxidant status, but they are overcome during oxidative stress (Ciolino and Levine 1997). ROS injures the integrity of various biomolecules including proteins, lipids and DNA, which leads to their structural and functional impairments (Ciolino and Levine 1997; Greenwald 2002). Epidemiological studies have suggested that dietary supplementation with the antioxidants are capable of preventing oxidative stress-mediated disorders (Guyton 1993; Mates et al. 1999; Greenwald 2002). Therefore, the naturally occurring sources of antioxidants appear to be promising agents for preventing, attenuating, and reversing dysfunctions in many systems caused by oxidative challenges.

Active Hexose Correlated Compound (AHCC) is a mixture of oligosaccharides, amino acids, lipids and minerals derived from fungi (Wakame 1999). The

chemical analysis has revealed that oligosaccharides are the major components of AHCC with an average molecular weight of approximately 5000, which may be responsible for its biological activities (Matsushita et al. 1998; Matsui et al. 2002). AHCC has been reported to enhance natural killer (NK) cell activity of cancer patients (Matsushita et al. 1998), to increase detoxification enzymes in the liver and protect the liver from CCl_4 –induced injury (Sun et al. 1997), to prevent the onset of diabetes induced by streptozotocin in the rat (Wakame 1999), to suppress thymic apoptosis induced by dexamethasone (Burikhanov et al. 2000), and to decrease ferric nitrilotriacetate (FeNTA)-mediated increase of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in rat urine (Wang et al. 2001a). These findings suggest that AHCC act as a promising biological response modifier (BRM), antioxidant, antimutagenic and/or anticarcinogenic agent by preventing ROS-mediated damages.

FeNTA is a chemical that induces severe oxidative damages by generating hydroxyl radical *in vivo* (Ansar et al. 1999). It behaves as a potent nephrotoxin and hepatoxin as well as renal and hepatic tumor promoter through oxidative stress-mediated mechanisms (Iqbal et al. 1995). The damages done to these organs are ameliorated by antioxidants (Iqbal et al. 1995; Ansar et al. 1999). In our present study, we have examined if FeNTA treatment would induce any dysfunction in endocrine systems and how AHCC modulates these disturbances induced by FeNTA.

Materials and Methods

Chemicals. AHCC was obtained from Amino UP Chemical Co. Ltd. (Sapporo, Japan). Reduced and oxidized glutathione (GSH and GSSG), NADPH and L(+)–ascorbic acid were obtained from Wako Pure Chemical (Osaka, Japan); GSH reductase (GSH Rd) was obtained from Sigma (St Louis, MO, USA). FeNTA solution was prepared immediately before use as described previously (Wang et al. 2001a, 2001b). 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 (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 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 kept free access to standard laboratory chow and water *ad libitum*.

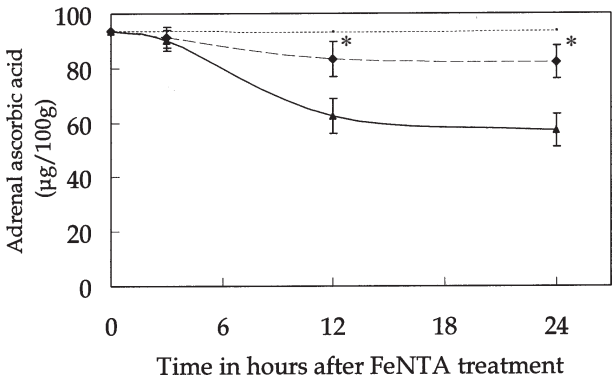

Fig.1 Effects of AHCC and FeNTA on ascorbic acid content in the adrenal. Each value represents mean ± SD of five rats. ···, control group; ◆, AHCC+FeNTA group; ▲, FeNTA group. *, p<0.05 vs. FeNTA group.

To assay serum levels of hormones and adrenal levels of ascorbic acid, twenty-four rats were divided into three groups: control group, FeNTA-treated group and AHCC plus FeNTA-treated group. One group received 3 % AHCC in drinking water for one week until their sacrifice. Three rats from each group were injected FeNTA intraperitoneally (7.5 mg/kg body weight), and then decapitated at 3 h, 12 h and 24 h after its injection. Our preliminary study revealed that AHCC alone did not affect the serum levels of any hormones tested.

To study the effects of AHCC and FeNTA on oxidative-stress status, twenty rats were divided into four groups. Two groups received treatment with AHCC for one week. Control groups received only tap water for one week. FeNTA was injected intraperitoneally to two groups; one of them was AHCC-pretreated group, and the other was the non–treated group. These animals were decapitated at 12 h after treatment with saline or FeNTA.

Estimation of ascorbic acid (AA). The content of AA in adrenal homogenate was assayed using Folin phenol reagent as described by Jagota and Dani (1982). The AA concentrations were expressed as μg/100g of the adrenal gland.

Serum biochemical determination. The serum levels of corticosterone, thyroxine (T_4), triiodothyronine (T_3), testosterone, lipid peroxidation (LPO), urea nitrogen (BUN), creatinine and aspartate aminotrans-

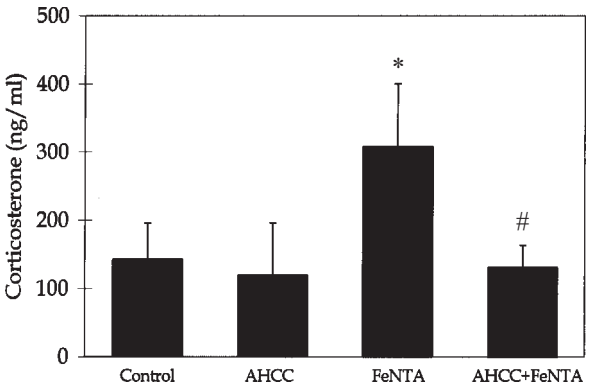

Fig. 2 Effects of AHCC and FeNTA on serum corticosterone 12 h after FeNTA treament. Each value represents mean ± SD of five rats. *, p<0.05 *vs.* control group; #, p<0.05 *vs.* FeNTA group.

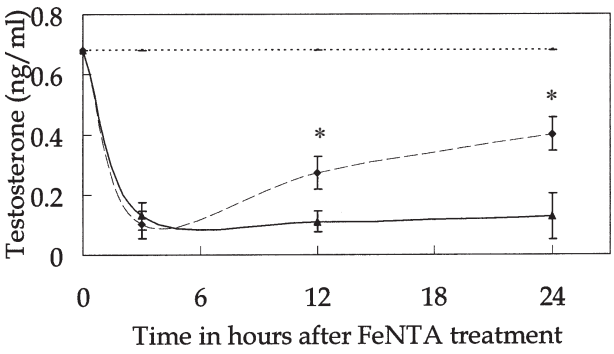

Fig. 3 Effects of AHCC and FeNTA on serum testosterone. Each value represents mean ± SD of five rats. ···, control group; ◆, AHCC+FeNTA group; ▲, FeNTA group. *, p<0.05 vs. FeNTA.

ferase (AST) and alanine aminotransferase (ALT) were determined by commercial kits.

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.

Determination of glutathione (GSH). The content of reduced GSH and oxidized GSH in 100,000 x 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 pH 8.0 and GSSG-OPT adduct at pH12.0 was measured at an excitation-emission of 350-420 nm.

Protein assay. Protein contents of tissue homogenates were determined by Lowry'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 between the four groups (data not shown).

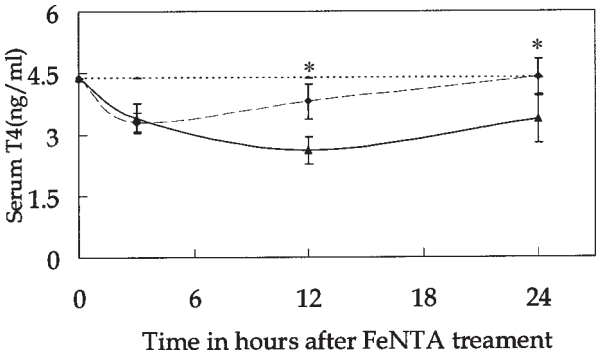

Fig. 4 Effects of AHCC and FeNTA on serum thyroxine (T4). Each value represents mean ± SD of five rats. ···, control group; ◆, AHCC+FeNTA group; ▲, FeNTA group. *, p<0.05 vs. FeNTA group.

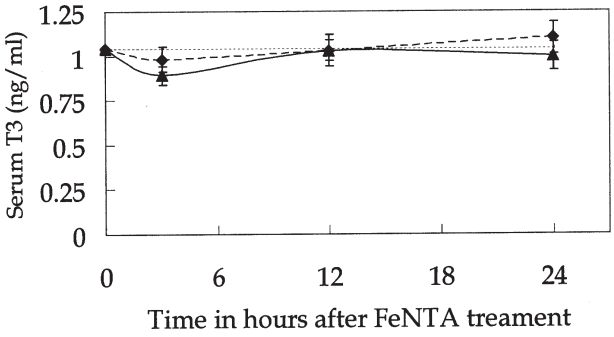

Fig. 5 Effects of AHCC and FeNTA on serum triiodothyronine (T3). Each value represents mean ± SD of five rats. ···, control group; ◆, AHCC+FeNTA group; ▲, FeNTA group. No significant difference was noted between AHCC+FeNTA group and FeNTA group at any time point within 24 h.

Table 1

Treatment groups	LPO (nmol/ml)	BUN (mg/dl)	Creatinine (mg/dl)	AST (IU/1)	ALT (IU/1)
Control	2.2 ± 0.2	14.9 ± 0.9	0.25 ± 0.02	208.4 ± 45.0	65.6 ± 8.3
AHCC	2.3 ± 0.1	15.0 ± 0.8	0.25 ± 0.02	160.2 ± 10.9	55.4 ± 7.3
FeNTA	4.2 ± 0.3*	30.1 ± 4.3*	0.62 ± 0.03*	677.2 ± 206.0*	348.0 ± 171.3*
AHCC+FeNTA	2.9 ± 0.5$^{\#}$	18.3 ± 3.6$^{\#}$	0.36 ± 0.08$^{\#}$	296.2 ± 50.6$^{\#}$	104.5 ± 21.5$^{\#}$

Effects of AHCC and FeNTA on oxidative stress-related parameters in serum at 12 h after FeNTA treatment. Each value represents mean ± SD of five rats. *, $p<0.05$ *vs.* control group; $^{\#}$, $p<0.05$ *vs.*FeNTA group.

Table 2

Treatment	GSH (µg/mg protein)	GSSG (µg/mg protein)	GSH Rd (nmol NADPH oxidized / min per mg protein)	GSH Px (nmol NADPH oxidized / min per mg protein)
Groups				
Control	17.23 ± 0.58	5.52 ± 0.51	0.303 ± 0.034	0.105 ± 0.005
AHCC	17.49 ± 0.68	5.80 ± 0.47	0.305 ± 0.042	0.106 ± 0.009
FeNTA	9.23 ± 0.35*	10.39 ± 1.29*	0.399 ± 0.020*	0.133 ± 0.007*
AHCC+FeNTA	14.77 ± 1.38**	6.37 ± 0.58**	0.354 ± 0.031**	0.112 ± 0.007**

Effects of AHCC and Fe-NTA on hepatic oxidative status at 12h after FeNTA treatment. Each value represents mean ± SD (n=5). *, $p<0.05$ *vs* control group; **, $p<0.05$ *vs*. FeNTA group.

Ascorbic acid (AA) content and corticosterone level. The decrease in the adrenal content of AA accompanied by increase in serum corticosterone level is an important response to many types of stress. In this study, the adrenal AA was decreasing gradually over 24 h after FeNTA treatment, while AHCC pretreatment significantly inhibited the depletion of adrenal AA (Fig 1). The serum corticosterone levels were dramatically increased at 12 h after FeNTA treatment, and the administration of AHCC resulted in a significant suppression on serum corticosterone levels (Fig 2).

Serum testosterone level. The serum testosterone level was reduced markedly at 3 h following a single injection of FeNTA. However, AHCC pretreatment restored the decreased secretion of testosterone gradually to nearly normal (Fig 3).

Serum T3 and T4 level. Serum levels of T_4 were greatly suppressed by FeNTA at 3 h after its injection, and the pretreatment with AHCC restored the serum T_4 levels nearly normal at 24 h (Fig 4). In contrast, the serum T_3 levels remained nearly constant all through the experiment (Fig 5).

Serum lipid peroxides (LPO), creatinine, urea nitrogen and transaminases. AHCC normalized the FeNTA mediated increase in the levels of LPO (Table 1). The FeNTA treatment led to an increase of 2.5- and 2.1-fold in the values of serum creatinine and urea nitrogen, respectively (Table 1). Pretreatment 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 FeNTA alone group under the identical conditions. The recovery of these two values by AHCC is still above the control values, but the differences in the activity of these enzymes between FeNTA group and AHCC plus FeNTA group were statistically significant (Table 1).

GSH content, GSH Px and GSH Rd activities. In FeNTA-treated rats, the hepatic GSH content was significantly less than that of control group ($p<0.05$, Table 2). On the contrary, the levels of GSSG in the liver were significantly higher in the FeNTA treated group than in the control group ($p<0.05$, Table 2). 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 FeNTA treatment as compared with control groups. The pretreatment of rats with AHCC reduced the activity of GSH Rd and GSH Px to normal levels ($p<0.05$, Table 2).

Discussion

The results of our present study have first provided evidence that various disturbances of endocrine sys-

tems can be induced by FeNTA, as demonstrated by increased serum corticosterone, decreased serum T_4 and testosterone. These disorders may be closely associated with oxidative stress, because FeNTA produces hydroxyl radicals *in vivo* (Ansar et al. 1999). However, pretreatment with AHCC ameliorated these disorders induced by FeNTA. In support of earlier data (Wang et al. 2001a), our present results have also demonstrated that AHCC ameliorates FeNTA-mediated nephro- and hepatotoxicity.

Our present data have shown that FeNTA treatment enhances the secretion of serum corticosterone. Oxidative stress induced by FeNTA apparently stimulated the hypothalamic-pituitary-adrenal (HPA) axis, resulting in an increased secretion of ACTH and corticosterone. However, the precise mechanism by which hydroxyl radicals stimulate HPA axis remains to be elucidated. In human and animals, the adrenal cortex contains a higher concentration of ascorbic acid than other tissues (Kipp and Rivers 1987), and the acute administration of ACTH decreases ascorbic acid levels in the adrenal (Overbeek 1985). This response is closely associated with corticosterone secretion in response to most stresses (Kipp and Rivers 1987), and has long been used as bioassay of ACTH (Overbeek 1985). Our data demonstrated that FeNTA treatment caused a significant depletion of adrenal ascorbic acid, suggesting that the secretion of ACTH was greatly stimulated. AHCC pretreatment significantly restored this decrease in adrenal ascorbic acid levels induced by FeNTA. It is likely that AHCC suppresses the secretion of not only corticosterone but also ACTH in response to oxidative stress. These results seem to suggest that AHCC restores oxidative stress-mediated hyperactivity of HPA axis to normal.

Oxidative stress plays an important role in senescence related diseases, which are commonly accompanied by a decreased ability to produce hormones including testosterone and thyroid hormone. Some antioxidants have been shown to retard the aging process by scavenging free radicals (Wang and Xie 1992; Bonnefoy et al. 2002). Our results have shown that oxidative stress induced by FeNTA decreases the serum levels of testosterone and T_4 under identical conditions. In contrast, the serum T_3 levels remained nearly constant all through the experiment. Because T_3 is the main active form among thyroid hormones, it is possible that FeNTA little affects the thyroid states. The mechanisms by which FeNTA induced the abnormal secretion of testosterone and T_4 are poorly understood. One can assume that these changes are closely related to ROS induced by FeNTA, because ROS has been reported to damage the cell membranes of the thyroid gland and testis, resulting in reduction of circulating thyroid hormones (Sadani et al. 1997) and sex hormones (Sikka 2001). The results of our present study, however, suggest that all the endocrine organs are not injured by hydroxyl radicals to the same extent. The response to oxidative stress may vary from gland to gland. AHCC restored the decreased secretion of these hormones to nearly normal, suggesting that protective effects of AHCC may be due to its ability of scavenging free radicals.

Our previous study revealed that FeNTA induced high excretion of urinary 8-OHdG at 3 h after its treatment (Wang et al. 2001a). The FeNTA-induced changes in serum levels of testosterone and corticosterone at 3 hr were not affected by AHCC. These results are consistent with our previous report (Wang et al. 2001a), which showed that AHCC was ineffective in depressing serum corticosterone levels at 1 hr after immobilization in the rat. In contrast, the increased urinary 8-OHdG was restored to normal at 3 hr after AHCC treatment (Wang et al. 2001a). Interestingly, the facts that FeNTA decreases testosterone and increases corticosterone in the serum simultaneously, suggest that FeNTA affects the secretion of the two kinds of steroids in different ways. Moreover, AHCC shows no apparent preventive effects on adrenal ascorbic acid levels at 3 hr after FeNTA treatment, while AHCC suppressed the FeNTA-mediated increase in urinary 8-OHdG under the same experimental conditions (Wang et al. 2001b).

AHCC is a mixture of oligosaccharides, amino acids, lipids and minerals derived from fungi (Wakame 1999). Our results are in good accordance with the previous report (Paterna et al. 1998), which shows that some poorly metabolized hexoses such as D-tagatose protect FeNTA-induced oxidative cellular injuries. However, only weak iron-chelating property of D-tagatose has been shown in a cell-free system (Charley at al. 1963). Some endogenous protective factors such as glutathione-related enzymes, catalase, and SOD are active in defense against oxidative cell injury by scavenging free radicals. The most important hepatic enzymes for detoxication of ROS are glutathione-related enzymes (Hsiao et al. 2001). Under oxidative stress, glutathione is largely consumed by glutathione-related enzymes, thereby resulting in induction of intoxication. In the present study, a single dose of FeNTA decreased the hepatic glutathione con-

tent, increased the activities of glutathione peroxidase and glutathione reductase, whereas AHCC markedly reverses these parameters. The effects of AHCC may be due initially to a reduction in hepatic oxidative stress followed by inhibition of the activities of glutahione-related enzymes, thereby leading to restoration of GSH content. Moreover, the beneficial effects of AHCC are shown first on the third day after its treatment (Burikhanov et al. 2000), and AHCC alone does not affect the serum levels of any hormones tested in our preliminary study. These results imply the protection of AHCC against FeNTA-mediated various disorders requires certain period of time. 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 FeNTA induces various endocrine disorders, while AHCC ameliorates these effects. AHCC may act as a potential antioxidant in many free radical-mediated disorders.

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