Effect of Kokuto, a Non-Centrifugal Cane Sugar, on the Development of Experimental Atherosclerosis in Japanese Quail and Apolipoprotein E Deficient Mice

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Kokuto, a traditional cane sugar of Okinawa, has been reported to have antioxidative and lipid-lowering properties. In this experiment, we investigated the effect of three different kinds of Kokuto (KA, KB, and KC) on atherosclerosis in two different animal models: Japanese quail and apolipoprotein E deficient (apoE-/-) mice. Ingestion of Kokuto had no significant effect on the serum and liver lipid levels of Japanese quail. Dietary intake of atherogenic diet (AD) with KA and KB decreased the liver triglyceride level and body weight in apoE-/- mice. Quail fed on AD with KA developed less extent of lipid-containing aortic intimal thickening lesions than those fed on AD with sucrose. Dietary intake of AD with Kokuto or sucrose induced aortic atheromatous lesions in mice, but the extent of atheromatous lesions was roughly comparable between these dietary groups of apoE-/- mice. The present study suggests that Kokuto prevents lipid-containing aortic intimal thickening lesions in Japanese quail.

Keywords: Kokuto, atherosclerosis, Japanese quail, apolipoprotein E deficient mice

Introduction

Recently, atherosclerosis has been considered as the chief cause of morbidity and mortality in developed countries (Braunwald, 1997). More than 250 factors have been reported as risk factors associated with the development of coronary artery disease (Hopkins et al., 1981; Hackam et al., 2003). Austin et al. (1998) revealed the correlation between the serum triglyceride level as a risk factor and atherosclerosis by meta-analyses of epidemiological studies. An elevated serum cholesterol level, and particularly low-density lipoprotein cholesterol, has been also been reported to be one of the risk factors for atherosclerosis (Boullier et al., 2001). A number of studies have showed that oxidized LDL plays a critical role in an early event of atherosclerosis (Choy et al., 2004) and also oxidative stress is associated with several risk factors for atherosclerosis (Runge et al., 1999). Therefore, the importance of lowered serum lipid levels and suppressed oxidative stress has been established to be beneficial in preventing atherosclerosis (Wierzbicki et al., 2003; Kaliara et al., 2005).

Kokuto, a non-centrifugal cane sugar of Okinawa, Japan, has been manufactured by a traditional process of boiling sugarcane juice. Sugarcane cultivated in tropical and subtropical environments grows under various kinds of climatic conditions, and it is expected that Kokuto, as well as sugarcane juice, contain many antioxidants such as phenolic compounds. Several investigators have reported that Kokuto contains a lot of new, in addition to previously known, antioxidants (Takara et al., 2002). Non-sugar fraction derived from crude black sugar has been reported to inhibit the increase of serum TG level in rats (Kimura et al., 1982). It has been already reported that Okinawan sugar cane rinds and wax decrease the serum cholesterol level in rats fed on the diets containing 1% cholesterol (Sho et al., 1981 and 1984; Fukuda et al., 1986). As mentioned above, Kokuto is expected to possess antiatherosclerotic functions by exerting lipid-lowering effects and antioxidative activities. However, the effect of Kokuto on the development of atherosclerotic lesions has never been studied. Thus, the objective of this study was to investigate the effects of Kokuto on lipid metabolisms, epididymal fat accumulation, and the development of atherosclerotic lesions in two different animal models: Japanese quail and apolipoprotein E deficient mice.

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Materials and Methods

Animal treatments All animals ethically approved under the rules and regulations of the Animal Welfare Center, University of the Ryukyus, Okinawa, Japan, were kept in specific pathogen-free conditions with laminar airflow and humidity. All animals were individually kept in cages at room temperature (25°C) and ambient lightening was automatically controlled to provide 12-h light and dark cycles.

Phenolic contents and antioxidative activities in Kokuto In this study, three different samples of Kokuto prepared in different factories (Kokuto A, B, and C) were used. The total phenolic content of Kokuto was measured by the Folin-Denis assay method according to the method described by Tateyama et al. (1997). Briefly, the reaction mixture, containing 200 µL of Kokuto solution (2 g/100 mL water) with 200 µL of Folin-Denis reagent, 400 µL of saturated sodium carbonate solution and 3.2 mL water, was allowed to stand for 30 min at room temperature, and then its absorbance was measured at 700 nm by a spectrophotometer (UV160, Shimadzu Corp., Kyoto, Japan). The unit of total phenolic content was expressed as mg of (+)-catechin/100 g of Kokuto. The DPPH scavenging activity of the Kokuto was examined according to the method described by Oki et al. (2001). Briefly, 300 µL of Kokuto solution (1 g/100 mL-4 g/100 mL), 300 µL of 20% ETOH, and 300 µL of 0.2 M-morpholinoethanesulfonic acid (MES) buffer at pH 6.0 were placed in a test tube. The reaction was initiated by adding 300 µL of 400 mM DPPH in EtOH. Following that, the reaction mixture was left to stand for 20 min at room temperature and its absorbance at 525 nm was measured by a spectrophotometer. The unit of DPPH scavenging activity was expressed as μmols Trolox equivalent/g of Kokuto using the standard Trolox curve.

Grouping and feeding of quails/quail A total of 40 Japanese male quail (Coturnix japonica, 2-month old) were purchased from Tokaiyuki Co., Ltd. (Aichi, Japan). The birds were randomly divided into a control (CO) diet group and three different Kokuto diet groups consisting of Kokuto A (KA), Kokuto B (KB), and Kokuto C (KC) diet group. The Kokuto diets consisted of 63% basa commercial diet (Kyoei Co., Ltd, Okinawa, Japan), 30% Kokuto, 5% corn oil, and 2% cholesterol. For the CO diet, 30% Kokuto was substituted with 30% sucrose. During the course of experimentation, all birds were strictly pair-fed on the same amount of the diets and given water ad libitum. At the 12th week of the experimental period, blood serum, liver and the entire aorta with its branches along with the heart specimens, were collected from each mouse. The same procedure was carried out with respect to the quail also. Epididymal adipose tissues were excised carefully and then weighed.

Lipid analysis Serum total cholesterol (TC) and triglyceride (TG) levels were measured using the commercially available enzymatic kits (Wako Pure Chemical, Osaka, Japan).

Total lipids were extracted from the liver tissues using the chloroform-methanol (2:1, v/v) method as reported previously (Folch et al., 1957). The level of the liver TC and TG was determined by Shoeneheimer-Sperry (Sperry et al., 1950) and Fletcher’s method (Fletcher et al., 1968), respectively.

Histological and Immunohistochemical examinations The excised heart and 1 cm long proximal portion of ascending aorta and its large branches from quail and mice were fixed in 10% buffered formalin. The paraffin-embedded blocks were prepared from formalin-fixed tissues and underwent sectioning into 4-micrometer thickness for histological and immunohistochemical examinations. For histopathological study purposes, the tissue sections were stained by hematoxylin eosin (H.E.), Mallory azan (M.A.), and elastica van Gieson (E.V.). Immunohistochemical study was also carried out on the tissue sections using the Envision system (Dako, Kyoto, Japan), for which a panel of primary antibodies such as α-smooth muscle actin (αSMA) (Dako) and CD44 (Novocastra Lab., Newcastle, UK) were used in this study. To reduce the non-specific background staining, the blocking of endogenous peroxidase activity was performed with 3% hydrogen peroxidase. The tissue sections were incubated with the primary antibodies, and then allowed to react with labeled dextran polymer. The sections were stained with activated 3, 30-deaminobenzidine-tetrahydrochloride (DAB) solution, followed by counter-staining with Mayer-hematoxylin. Washing of the sections with Tris buffer saline three times was done after each step. The sections were then studied by light-
microscopy. To evaluate the degree of atherosclerosis, the total intimal thickness (I)/medial thickness (M) ratio was measured with three aortic segments from each bird (Fig. 2 and 3).

**Statistical analysis** Statistical analysis was performed using one-way ANOVA, followed by inspection of the differences between pairs of mean values by Tukey-Kramer’s test. All statistical analyses were performed using the SAS statistical software program (SAS Institute, Tokyo, Japan).

**Results**

The phenolic contents and antioxidative activities in Kokuto (Table 2) Three types of Kokuto used in this study showed high phenolic content and antioxidative activity. The phenolic content and antioxidative activity were the highest with KA, and the lowest with KB. Sucrose contained no phenolic substance and showed no antioxidative activity.

**Body weight, and serum and liver lipid profiles in each experimental group** In both the quail and mice experiments, no significant difference in energy intake was noted between each diet group (Table 3).

Table 3. Energy density and intake in each diet group.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Group</th>
<th>Energy density of diet (kcal/g)</th>
<th>Food intake (g/day)</th>
<th>Energy intake (kcal/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese quail</td>
<td>CO</td>
<td>3.921</td>
<td>10.3 ± 0.37</td>
<td>40.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>KA</td>
<td>3.826</td>
<td>10.7 ± 0.32</td>
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<td>10.8 ± 0.33</td>
<td>41.2 ± 1.3</td>
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<tr>
<td>apoE−/− mice</td>
<td>CO</td>
<td>4.344</td>
<td>2.76 ± 0.08</td>
<td>12.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>KA</td>
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<td></td>
<td>KB</td>
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<td>2.84 ± 0.07</td>
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**Results**

Body weight, and serum and liver lipid profiles in each experimental group

In both the quail and mice experiments, no significant difference in energy intake was noted between each diet group (Table 3).

Table 4 lists the growth and lipid level parameters for the quail experiment. There was no significant difference in body weight between each diet group. Liver TG levels of all Kokuto diet groups tended to be lower in comparison to that of the CO diet group.

Table 5 summarizes the results of the mice experiment. The body weight of the KC diet group was significantly lower in comparison to that of CO diet group.

The different letters in Table 2 indicate significant differences (P < 0.05).

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**Table 4. Body weight, and serum and liver lipid levels of Japanese quail.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Body weight (g)</th>
<th>Serum lipid (mg/dL)</th>
<th>Liver weight (g/100g BW)</th>
<th>Liver (mg/g liver)</th>
<th>Liver TG (g/mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>n = 10</td>
<td>103.6 ± 2.2</td>
<td>1997 ± 361</td>
<td>246.0 ± 32.5</td>
<td>3.04 ± 0.38</td>
<td>30.5 ± 1.9</td>
</tr>
<tr>
<td>KA</td>
<td>n = 10</td>
<td>103.1 ± 2.2</td>
<td>1633 ± 340</td>
<td>156.6 ± 28.6</td>
<td>2.42 ± 0.16</td>
<td>24.4 ± 3.1</td>
</tr>
<tr>
<td>KB</td>
<td>n = 10</td>
<td>105.3 ± 2.4</td>
<td>1872 ± 254</td>
<td>199.6 ± 28.6</td>
<td>3.31 ± 0.27</td>
<td>28.3 ± 5.2</td>
</tr>
<tr>
<td>KC</td>
<td>n = 10</td>
<td>105.9 ± 2.5</td>
<td>1673 ± 300</td>
<td>186.6 ± 22.8</td>
<td>2.35 ± 0.14</td>
<td>23.0 ± 2.7</td>
</tr>
</tbody>
</table>

**Table 5. Body, liver and epididymal adipose tissue weights, and serum and liver lipid levels of apoE−/− mice.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Body weight (g)</th>
<th>Serum lipid (mg/dL)</th>
<th>Liver weight (g/100g BW)</th>
<th>Liver (mg/g liver)</th>
<th>Liver TG (g/mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>n = 6</td>
<td>31.8 ± 0.6</td>
<td>723 ± 122</td>
<td>138.9 ± 8.2</td>
<td>3.90 ± 0.23</td>
<td>10.7 ± 0.9</td>
</tr>
<tr>
<td>KA</td>
<td>n = 7</td>
<td>27.6 ± 1.0</td>
<td>745 ± 160</td>
<td>116.6 ± 12.0</td>
<td>3.93 ± 0.28</td>
<td>9.4 ± 1.8</td>
</tr>
<tr>
<td>KB</td>
<td>n = 7</td>
<td>29.3 ± 1.0</td>
<td>816 ± 117</td>
<td>123.0 ± 14.1</td>
<td>4.21 ± 0.22</td>
<td>13.6 ± 0.5</td>
</tr>
<tr>
<td>KC</td>
<td>n = 7</td>
<td>25.8 ± 1.2</td>
<td>751 ± 126</td>
<td>93.8 ± 2.7</td>
<td>4.02 ± 0.32</td>
<td>12.8 ± 1.7</td>
</tr>
</tbody>
</table>

Data shown as mean ± S.E.

1CO: Control diet, KA: Kokuto A diet, KB: Kokuto B diet, KC: Kokuto C diet.

Different letters shows significant difference (P < 0.05).
ence in liver TC levels was statistically insignificant between all experimental groups, whereas the liver TG levels were significantly lower in the KA diet group compared with that of CO diet group.

**Histological and immunohistochemical findings**

Histological and immunohistochemical findings in Japanese quail are shown in figure 1. No significant intimal thickening lesions were observed in some birds (Fig. 1A), while significant atherosclerotic lesions were remarkable in most birds (Fig. 1B, C, and D). The most frequent type of atherosclerotic lesions were lipid-containing intimal thickening lesions (LI) which were consistent with the early stage of atherosclerosis (Fig. 1B). Lipid-containing cells in LI were positively stained with anti-CD44 antibodies (Fig. 1C) but not with anti-SMA antibodies (Fig. 1D), suggesting the macrophages origin. The degree of aortic intimal thickening lesions (I/M ratio) of quail in the KA diet groups was significantly lower in comparison to the CO diet group (Fig. 3A).

Histological and immunohistochemical findings of apoE-/- mice are shown in Figure 2. A few mice had shown almost normal architecture of aortas (Fig. 2A), and lipid-containing intimal thickening lesions (Fig. 2B). Many mice developed atheromatous lesions with numerous cholesterin crystals that were compatible with the advanced stage of atherosclerosis (Fig. 2C). All dietary groups developed the atheromatous lesions to the same extent with an almost comparable degree of I/M ratio (Fig. 3B).

**Discussion**

Our present study reports the effect of dietary Kokuto intake on lipid metabolisms, epididymal fat accumulation, and the development of atherosclerotic lesions for the first time. Kimura et al. (1982) have previously reported that non-sugar fraction, derived from crude black sugar (Kokuto), inhibited the increase of serum TG levels in high sugar diet-fed rats. They further suggested that non-sugar fractions in black sugar inhibited the elevation of serum TG levels in rats fed on a high sugar diet by the reduction of both glucose and fructose absorption from the small intestine. Thus, the present result that the dietary intake of several types of Okinawan crude Kokuto decreased the liver TG levels in apoE-/- mice may support
this previous observation. The accumulated reports of numerous researchers (Olefsky et al., 1974; Parekh et al., 1998; Leiber et al., 2004) have suggested that the excess dietary intake of culinary fat and oil is associated with hypertriglyceridemia, obesity, and nonalcoholic steatosis. Serum and liver cholesterol levels have been reported to be decreased by dietary feeding of Okinawan sugar cane rinds and wax in rats (Sho et al., 1981 and 1984). Fukuda et al. (1986) have examined the effects of wax, one component of sugar cane rinds, on the serum cholesterol level in lard fed rats and found insignificant effects of wax administration on the amount of fecal excretion of steroids in lard fed rats. They speculated that the cholesterol-lowering effect in sugar cane wax is due to the modification of cholesterol metabolisms instead of the fecal excretion of steroids. In the present experiment, Kokuto did not have any effects on the serum and liver cholesterol levels in both Japanese quail and in apoE-/- mice. Reliable evidence from animal model studies and the correlative data from human research have indicated that oxidative stress is the unifying mechanism for many atherosclerotic risk factors (Soccio et al., 2005; Singh et al., 2006). Steinberg et al. (1997) has suggested that the oxidation of lipoproteins, particularly low-density lipoprotein (LDL), is one of the important initial events in the pathogenesis of atherosclerosis. Therefore, antioxidants have been expected to be potentially useful therapeutic agents against atherosclerosis since they may inhibit lipoprotein oxidation involved in LDL, and reduce detrimental biological consequences caused by oxidative stress (Cynshi et al., 2005). Moreover, many investigators have reported the beneficial effects of the anti-atherosclerotic functions of numerous types of antioxidants such as phenolic antioxidant AGI-1067, the mono-succinate ester of probucol, (Sundell et al., 2003), BO-653, phenolic antioxidant (Cynshi et al., 1998), and Vitamin E and/or C (Kaliora et al., 2006). Kokuto has been recognized as a unique material in that it possesses various phenolic compounds and antioxidative activity (Takara et al., 2003). Takara et al. (2003) illustrated that antioxidants isolated from Kokuto had phenolic hydroxyl groups in their structure, and suggested that radical-scapenging activity of these antioxidants is mostly related to the phenolic hydroxyl group. The different types of Kokuto used in this study contained various amount of phenolic compound. Japanese quail fed on a phenolic compound-rich type of Kokuto developed less severe atherosclerotic lesions than the control one, whereas apoE-/- mice fed on all types of Kokuto developed advanced atherosclerotic lesions. Recently, it has been reported by Kaliora et al. (2006) that the antioxidant therapy is supposed to be effective in the early stages of atherosclerosis by preventing LDL oxidation and oxidative lesion of endothelium. Most atherosclerotic lesions induced in our Japanese quail experiment were lipid-containing intimal aortic lesions consistent with the findings of the early stage of atherosclerotic lesions. On the other hand, apoE-/- mice have been known to be a very susceptible animal model to atherosclerosis and develop the atherosclerotic lesions at 9 weeks-old when they are fed on a low fat and sucrose diet without cholesterol (Jawien et al., 2004). In our apoE-/- mice experiment, the most frequent type of atherosclerotic lesion was an atheromatous lesion that exhibits attributes similar to that of the advanced stage of atherosclerotic lesions. Furthermore, the species-dependant difference in susceptibility to atherosclerosis and the different predilection site of atherosclerotic lesions may exist in the anti-atherogenic effect of Kokuto as reported in probucol experiments (Daugherty et al., 1991; Tardif et al., 1993; Bird et al., 1998). In conclusion, we demonstrate that Kokuto may be effective to prevent atherosclerosis in comparison with sucrose in Japanese quail. However, further study is required to clarify how Kokuto prevents the development of atherosclerotic lesions and improves the deteriorated lipid metabolism in different species.

References
Boullier, A., Bird, D.A., Chang, M.K., Dennis, E.A., Eriedman, P., Gilleotre-Taylor, K., Horkko, S., Palinski, W., Quehenberger, O.,

Fig. 3. The degree of atherosclerotic lesions in Japanese quail and apoE-/- mice.
CO: Control diet, KA: Kokuto A diet, KB: Kokuto B diet, KC: Kokuto C diet.
Different letters shows significant difference (P<0.05).


