原　　著

Anti Type 2 Diabetic Effect and Anti-oxidation Effect in Active Hydrogen Water Administration KK-Ay Mice

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————Abstract————

This study aimed to examine the inhibitory effect of active hydrogen water (AH) on the increased blood glucose levels. Regarding the anti-oxidation effect, the inhibition rate of the SOD activity was measured in ICR mice using an SOD (superoxide-dismutase) test (Wako), by collecting blood at 37 days, 52 days and 107 days after the commencement of AH intake. Regarding the inhibitory effect on the blood glucose level increase, the changes in the blood glucose level at the time of glucose tolerance testing 30 days after the commencement of feeding was measured in KK-Ay mice and BALB/cA mice, which were divided into a control group and an AH intake group, using a glucose test (Wako). In addition, variations in the long-term blood glucose levels were measured every two weeks for 107 days after the commencement of feeding. Throughout the entire feeding

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period, the control group was given a free-intake of distilled water（DW）, and the AH group was given a free-intake of AH. Regarding the anti-oxidation effect, the AH intake group after the long-term administration tended to demonstrated a greater effect than the DW intake group. Regarding the blood glucose level controlling effect, a significant difference in the KK-Ay mice under long-term administration was observed after 86 days, and the controlling effect became clear three months after the commencement of intake. These findings suggest that AH is therefore capable of anti-oxidation and it also has a controlling effect on the blood glucose level in type 2 diabetes.

Introduction

Diabetes is becoming a lifestyle-related disease nationwide, this statement is supported by the fact that currently 6.9 million people are highly suspected of having diabetes and the number rises to 13.7 million when including those at significant risk for developing diabetes. Most cases of diabetes are type 2 diabetes with chronic hyperglycemia due to insulin resistance and a relative decrease in their insulin secretion ability from the beta cells of the pancreas, and long time suffering from this disease may induce complications typical of diabetes such as retinopathy, kidney disease and neuropathy.[1] For type 2 diabetes, diet therapy and kinesitherapy are the most common treatments, and the daily control of the blood glucose level is important for the management of type 2 diabetes.[1] Therefore, it is necessary to develop a convenient and safe treatment to be taken on a daily basis for type 2 diabetes. Since the reduction in the damage caused by free radicals to the beta cells of the pancreas *in vitro*[2] has been clarified, we conducted this study in order to examine the inhibitory effect of active hydrogen water on the blood glucose level *in vivo*.

Material and Methods

Animal

Five week old JcHCR mice (average body weight of 27–29 g), BALB/cA Jcl mice (average body weight of 20–23 g) and KK-Ay/Te Jcl mice (average body weight of 24–26 g) were purchased from CLEA Japan, Inc., and then were raised with normal mouse feed (CE-2 of CLEA Japan, Inc.) under conventional conditions (room temperature of 22 ± 3℃, humidity 60%). For the entire breeding period, the control group was left with a free intake of distilled water (DW), while the AH (Friendear, Inc.) intake group has free intake of AH.

KK-Ay/Ta Jcl is the II-type diabetes mellitus model mouse which introduced an Ay gene into KK mouse. Therefore, a KK mouse develops severe corpulence and hyperglycemia early.

The measurement principle of the anti-oxidation effect

Regarding the measurement of the SOD-like activity, the level of SOD-like activity in the blood was measured by the NBT reduction method using the Wako SOD test (Wako Pure Chemical Industries, Ltd.). The NBT reduction method using NO_2–TB (nitro blue tetrazolium) as a detection agent for O_2^- radicals is a method for measuring the SOD-like activity levels based on the inhabitation rate obtained by comparing the coloration reaction, thus indicating O_2^- radical reduction to the coloration reaction, and also indicating the O_2^- radical generation (Xanthine and Xanthine oxidase) and the disproportionation by SOD. This method is capable of quantitatively measuring the anti-oxidation activity.[3]

The superoxide anion radical (O_2^-) is generated by a chemical reaction between Xanthine and Xanthine oxidase (1). The resulting O_2^- effects the reduction of the co-existing nitro blue tetrazolium (NO_2–TB) and produces diformazan (2), but if SOD exists in the reaction solution, part of the O_2^- is disproportioned with hydrogen peroxide (H_2O_2) and oxygen (O_2) (3), and the production of diformazan is thereby reduced. Thus, the level of SOD activity in the test solution was considered to be the inhibition rate and it was measured by the degree of decrease in the formation of diformazan in the O_2^- and NO_2–TB reaction.

Measurement principles for the blood glucose level controlling effect

When a color developing solution is applied to a sample, the glucose in the sample immediately changes from α type to β type as a result of the action of mutarotase contained in the color developing solution. D-glucose in a balanced state consisting of 36.5% α-D-glucose, and 63.5% β-D-glucose.[4)5)] β-D-glucose is oxidized by the action of glucose oxidase (GOD), and thus producing hydrogen peroxide. GOD acts specifically on β-D-glucose, and not on the α type. Therefore, β-D-glucose is consumed first, then α-D-glucose commences conversion to β type. Accordingly, it is reported that the measurement is the most accurate when the method uses mutarotase, which effects the conversion of α-D-glucose into β type, in order to speed up the reaction.[6)] The resulting hydrogen peroxide thus helps to generate red pigment as a result of quantitative oxidation and the condensation of phenol and 4-aminoantipyrine in the color developing solution together with the action of co-existing peroxidase (POD). The concentration of glucose in the sample was determined by measuring the red light absorbance at 505 nm.

Measurement method for the antioxidation effect

Two free distilled water intake groups (hereinafter called "X-DW group") and a free active hydrogen water intake group (hereinafter called "X-AH group") of the ICR male mice were arranged.

For the measurements, blood was taken from the ocular fundus of each mouse through a capillary tube 37 days, 52 days and 107 days after administration, the collected blood was then placed in a centrifuge (at 1000rpm, for 10 minutes) for serum separation, and the serum used as samples of specimen (S), blind (BL), specimen-blind (S-BL), reagent blind (BL-BL), with each sample being dispensed into a 96 well micro-plate at 8 μl/well. The serum was used in S and S-BL samples, and distilled water in BL and BL-BL samples. In the above-mentioned drawing blood time, we started since 9 : 00 a.m.

After dispensing each sample, 80 μl/well of a luminescent reagent was added and stirred for one minute, then 80 μl/well of an enzyme solution was added to the S and BL samples, and 80 μl/well of a blank solution was added to the S-BL and BL-BL samples, and after further stirring for one minute, a micro-plate was heated in a heater to a temperature of 37℃ for 20 minutes to induce the reaction. After the reaction, 160 μl/well of a reaction stop solution was added to each sample and then light absorbance was measured using a Micro Plate Reader (Toyosada, MBR A4) at a wavelength of 560 nm. SOD-like activity was calculated by formula (1) using the value obtained from the measurement of light absorbance. The statistical analysis was conducted using the Donnett test.

Measurement method for the blood glucose level controlling effect

A total of four groups of type 2 diabetes test mice were created : a group of six KK-Ay mice (CLEA Japan, Inc.) given free intake of distilled water (DW-KK group), a group of seven BALB/cA mice (CLEA Japan, Inc.) given free intake of distilled water (DW-BA group), a group of eight KK-Ay mice given intake of active hydrogen water (AH-KK group) and a group of six mice given an intake of active hydrogen water (AH-BA group). All of the mice used were males.

In the glucose tolerance test at 16 days after administration, cane sugar (2 kg/kg) was orally administered to the mice in the KK-Ay and BALB/cA

groups.　The blood from the ocular fundus of the mice collected through capillary tubes was placed in a centrifuge for serum separation, then the glucose concentration was measured using the Wako Glucose CII-Test (Wako Pure Chemical Industries, Ltd.).　The measurement schedule was set to be immediately before and after glucose administration, and 2 hours, 4 hours and 7 hours after glucose administration.　In glucose administration time, we followed the above-mentioned methods since 9：00 a.m.　In fast time, we did it by 9：00 a.m. of the examination day since 9：00 p.m. on the examination day before.

For the study on long-term AH administration, the glucose concentration was measured without the glucose administration using the same measurement method as that used in the measurement of short-term AH administration.　The measurement period was set to begin 44 days after the administration and end 114 days after the administration, wherein the measurements were conducted every two weeks.　The statistical analysis was conducted using the Dunnett Test.

Principles and methods for insulin level measurement

The Glazyme Insulin-EIA Test was used to measure the insulin level.　This method is based on a one-step sandwich enzyme immunoassay with a solid phase of glass beads.　When the antibody beads ― the glass beads bound with the anti-insulin antibody and the enzyme (peroxidase)-labeled anti-insulin antibody are caused to react with the insulin in a sample, a sandwich-like "anti-insulin antibody (beads) - insulin - enzyme-labeled insulin antibody" compound is formed.　Since the quantity of enzymes bound with the antibody beads is in proportion to that of the insulin in the sample, the quantity of insulin in

the specimen can thus be determined by measuring the enzyme activity using a color developing reagent (o-phenylenediamine and H_2O_2) and then comparing the result to the standard curve prepared using the standard solution with a known quantity of insulin.

Regarding the method for measuring the insulin levels, after 3 weeks of repeated administration, 0.5 ml of blood from the heart of mice in the KK-Ay group was collected in a Fuji heparin tube, centrifuged (at 10,000 rpm for 10 minutes) using a CHIBITAN-II by Millipore Corporation, and then 500 μl of enzyme-labeled antibody solution and the antibody beads were added to 100 μl of the resulting serum.　After standstill heating at 37℃ for one hour, washing (removing the reaction liquid with an aspirator, adding 3 ml of buffer solution to wash the tube walls at the same time, and removing the buffer solution with the aspirator) was then conducted three times.　The antibody beads were transferred to a new test tube, to which 500 μl of substrate color developing solution was added.　The BL sample was created at the time.　After standstill heating at 37℃ for 30 minutes, 1.5 ml of enzyme reaction stopping solution was added and mixed well.　This was prepared as the subject for BL, after which the light absorbance was measured using an absorption meter (Shimadzu/UV-1200) at a wavelength of 492nm and the insulin concentration was determined by measuring the absorbance compared to the standard curve prepared using the standard solution.

Results

Antioxidation effect

The mean value and the standard error of the values measured for SOD activity (inhibition rate) are shown in table1.　Although no statistically significant difference was observed in table1, the

group of mice with an active hydrogen water intake shows a higher value, thus indicating a radical scavenging action on the O_2^-.

Blood glucose level controlling effect

Glucose tolerance test

The mean value and the standard error for the glucose concentrations obtained from measurements taken immediately before and after, and 2 hours, 4 hours and 7 hours after glucose administration, are shown in Figure 1 and Table 1, from which statistically significant differences were observed in

Table1. SOD activity（%）

Groups	Time	37dp	52dp	107dp
DW	average	46.015	56.384	43.272
	S. E.	4.204	6.464	4.306
AH	average	50.530	60.531	47.821
	S. E.	3.285	3.200	6.098

（dp；days post, S. E.；standard error）

the BALB/cA group mice with active hydrogen water intake 2 hours and 4 hours after glucose administration.

Regarding the long-term administration, the mean value and the standard error for glucose concentrations obtained from measurements every 2 weeks during the period from 44 days to 114 days after administration are shown in Figure 2, from which statistically significant differences were observed in comparison to the control group in the KK-Ay mice with active hydrogen water intake 86 days after commencing intake, and from which the controlling effect on the blood glucose level began to be observed 3 months after commencing intake.

Results of insulin level measurement

The effect on the insulin concentration after repeated administration to the KK-Ay group mice are shown in Figure3.

The AH group with active hydrogen water intake, compared to the control group mice, showed a
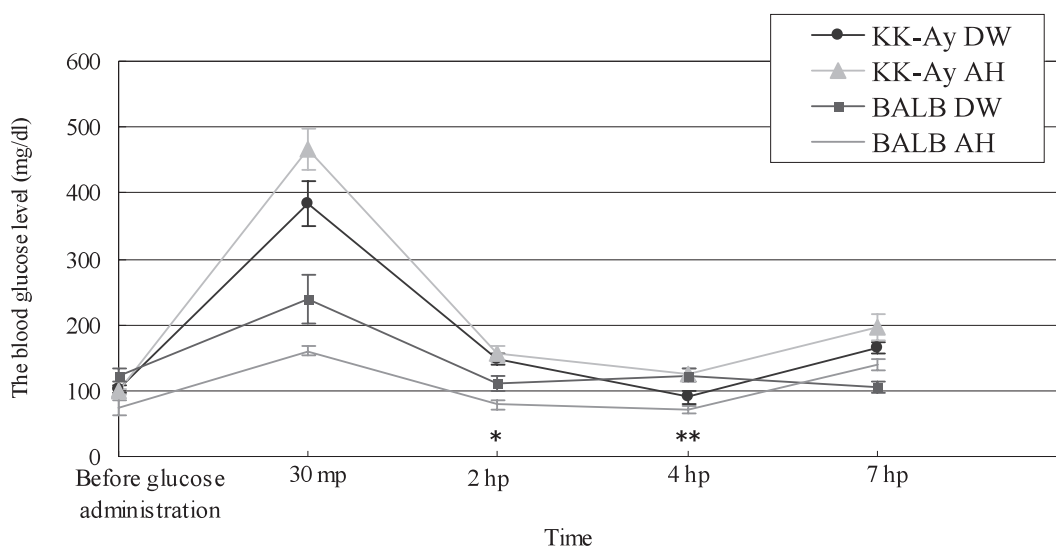

Fig. 1. These lineagrams representative the blood glucose level and elapsed time from carbohydrate tolerance test. The experiment started after AH and DW administration later on 16th. Lineagrams are mean value ± S. E. Analysed by Dunnett test vs DW. $^*p < 0.05$, $^{**}p < 0.01$ （mp；minutes post, hp；hours post）

Table1. Blood glucose level and elapsed time from carbohydrate tolerance test.（mg/dl）

Groups	Time	Before glucose administration	30mp	2hp	4hp	7hp
DW-KK	average	102.330	384.363	148.484	90.681	165.516
	S. E.	6.722	33.682	7.911	10.033	8.008
AH-KK	average	98.325	466.402	154.991	124.260	195.376
	S. E.	4.177	32.533	13.533	10.585	20.391
DW-BA	average	122.549	239.308	111.829	123.026	105.516
	S. E.	9.919	35.995	11.816	10.677	8.838
AH-BA	average	74.067	160.077	78.394*	72.330**	139.967
	S. E.	10.858	7.411	7.508	5.849	8.200

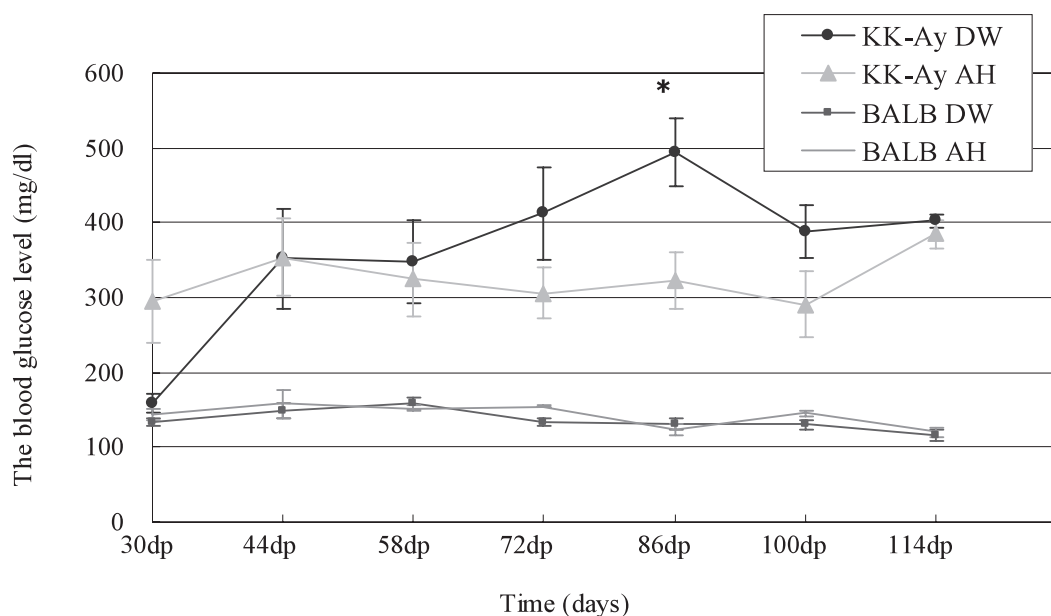Significantly different from *: $p < 0.05$, **: $p < 0.01$, Dunnett test.（mp ; minutes post, hp ; hours post）

Fig. 2. These lineagrams representative the blood glucose level and elapsed time from the first-time administarting. Lineagrams are mean value ± S. E. Analysed by Dunnett test vs DW. Difference of statistical are detected between the KK-Ay AH. group and the KK-Ay DW group on 86 days after the test. *$p < 0.05$, **$p < 0.01$（dp ; days post）

significant increase in the insulin concentration（$P < 0.05$）.

Discussion

Regarding the inhibitory effect on the blood glucose level, as the active hydrogen water is confirmed to be capable of anti-oxidation action both *in vivo* and *in vitro*, there is a possibility that active hydrogen water may reduce damage to the beta cells of the pancreas by means of active oxygen, as well as reducing insulin resistance. In long-term administration, a significant difference in the inhibitory effect was observed 86 days after commencing intake in the KK-Ay group mice, and the
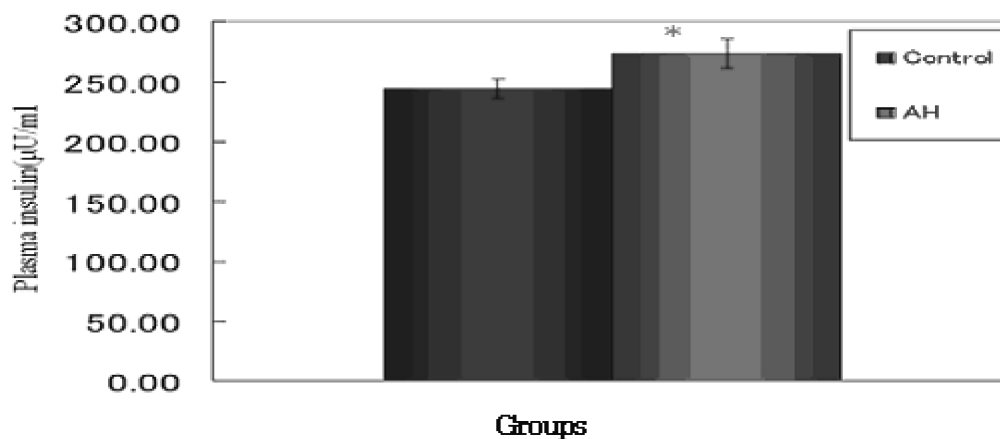
Fig. 3. Plasma insulin in KK-Ay mice. Each point indicates the mean ± S. E. from 6 mice. Significantly different from control, [*]: $P < 0.05$, [**]: $P < 0.01$, AH; Active Hydrogen Water

actual effects began to be observed 3 months after commencing intake, thus indicating an anti-diabetic effect. However, because an inhibitory effect on the blood glucose level was observed in the glucose tolerance tests in the BALB/ca group mice, which were healthy mice, it is considered that the active hydrogen water did not reduce insulin resistance, but instead inhibited the modification process involved in the increase of the blood glucose level.[7] The reason for this is considered to be that magnesium metal of 99.9% purity was used as an inorganic catalyst for the generation of the active hydrogen water source. Regarding the inhibitory effect of the magnesium metal on the blood glucose level, since it has been reported[8] that magnesium-rich bittern has a controlling effect on the blood glucose level, it is considered that the anti-oxidation action and the SOD-like activity of the active hydrogen water administered to the mice contributed to the controlling effect on the blood glucose level.[9] As for the controlling effect on the blood glucose level, a significant difference was observed in the BALB/cA group mice in the glucose tolerance test, and the same effect was also observed in the KK-Ay group mice after the long-term administration, and hence,

the intake of active hydrogen water can be expected to control the blood glucose level; however, such effects would not be seen immediately but only after repeated administration over a certain period of time. Therefore, further examination of the inhibitory effect over a longer period of time is necessary.

With respect to the effect on glucose concentration in the BALB/cA group mice after administration, hypoglycemic effects were observed in the AH group at 2 hours and 4 hours after glucose administration, in comparison to that of the control group. In the single administration, regarding the effect on the glucose concentration in the BALB/cA group mice, no change was observed in the blood glucose level observed in any of the groups. Therefore, within a short period of time, no blood glucose level reduction effect was observed.

Regarding the effect on the glucose concentration and the insulin concentration of the KK-Ay group mice after repeated administrations, significant hypoglycemic effects were observed in the AH intake KK-Ay group mice during the period from 58 days to 114 days after the administration, in comparison to that of the control group.

Regarding the effect on the glucose concentration

in the BALB/cA group mice after repeated administrations, there was no change observed in the blood glucose level in either of the groups. These results after both cases of repeated administration and after the single administration suggest that active hydrogen water lowers only the abnormal blood glucose levels, and therefore, the active hydrogen water is expected to be a safe anti-diabetic agent with no adverse effects. It is considered that the active hydrogen water will be absorbed by the human body, thus generating a large amount of anti-oxidant-like substances and activating the internal organs and tissues. Accordingly, the anti-diabetic effect is presumed to be attributable to homeostasis in the body after the long-term intake of active hydrogen water.

This insulin-like action demonstrated by the active hydrogen water was likely the result of the active hydrogen water inhibiting the wortmannin, the specific PI-3 kinase (phosphatidyl inositol 3-kinase) inhibitor that is the key enzyme in insulin signal transmittance. In addition, this activity could also be considered to be the result of the reduced active hydrogen water stimulating the translocation of GLUT-4 (glucose transporter-4) to the cell membrane. Furthermore, the insulin-like action could be considered the result of the reduced active hydrogen water inhibiting the protein tyrosine phosphatase, consequently activating the phosphorylation of the insulin receptor. Regarding the SOD-like activity, anti-oxidant ability, and radical scavenging action, the active hydrogen water administration group indicated a greater level (of SOD-like activity, anti-oxidant ability, and radical scavenging action) in comparison to that of the control group. The active hydrogen water is thus considered to activate an SOD-like enzyme serving as a radical scavenger to remove various free radicals,

such as O_2^--radicals, peroxy radicals, LOO (peroxy radical lipids) in the body.[9)10)] It is considered that the active hydrogen water acted as a radical scavenger to normalize the secretion of insulin by the anti-oxidation-induced activation of internal organs, especially in the Langerhans cells of the pancreas.[11)] Hypoglycemic effects were observed in the KK/Ay group mice after single and repeated administrations. However, no hypoglycemic effect was observed in the healthy mice after repeated administrations. In the KK-Ay group mice after repeated administrations, significant hypoglycemic effects were observed one week after the administration and later. Then, after repeated administrations, an increase in the insulin level was observed. The hypoglycemic effect of active hydrogen water is thus considered to be attributable to the SOD-like activity (induction of antioxidant enzymes) following intestinal absorption of the active hydrogen water.[12)13)] Furthermore, because no decrease in the blood glucose level was observed in healthy mice, the radical-blocking action of the active hydrogen water is also considered to contribute to an improvement in anti-oxidation, thereby elevating the insulin level and reducing insulin resistance. Since the active hydrogen water lowers the blood glucose level only when the level is abnormal, and it does not induce a reduction of a normal blood glucose level, it is therefore expected to be a safe medication with no adverse effects.

The active hydrogen water administration for KK-Ay mice group was effective in only repetition administration of a specific period. However, active hydrogen water administration group did stability than control group, but the strong effect of a blood glucose level fall was absent. Therefore, active hydrogen water does not treat serious diabetes mellitus such as KK-Ay mice and can expect protective efficacy. Therefore, we do not treat

serious diabetes mellitus such as KK-Ay mice, and active hydrogen water can expect protective efficacy.

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