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Effects of Calorie Restriction on Microsomal Mixed Function Oxidase System and Free Radical in Kidney of SAMP8 Mice

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This study investigated the antioxidative effect in kidney of senescence-accelerated prone SAMP8 mice with calorie restriction. 4-weeks-old SAMP8 female mice were divided into 4 groups according to the experimental feeding period: for 4, 8, 12 month, and at natural death. Each group was subdivided into 2 groups, with thirteen mice each one, as *ad libitum* group and as dietary restriction group (60% of *ad libitum* feeding amount). After feeding for a given period, the mice were sacrificed to get the following results: among the experimental groups, there wereno significant differences in xanthine oxidase (XOD) activity in their kidney tissues. The contents of cytochrome P_{450} decreased in *ad libitum* group and dietary restriction group by age. The activity of NADPH-cytochrome P_{450} reductase showed a trend similar to cytochrome P_{450}. Superoxide radical content increased with age. At the 4th, 8th and 12 months of the experimental period, the activity in the dietary restriction group was less than that of *ad libitum* group by as much as 17%, 14% and 14%, respectively. For hydrogen peroxide, the contents were increased in the *ad libitum* group with age, while no correlation between content and age was observed in the dietary restriction group. In the 8th and 12th months of the experimental period, the were in the dietary restriction group less than that of *ad libitum* group counterpart as much as 17% and 20%, respectively. For the cellular membrane stability of the kidney, no significant correlation with age was observed in either the dietary restriction group or the *ad libitum* group. However at the 12th month of the experiment, however, the stability in the dietary restriction group was 11% higher than that in the *ad libitum* group.
In conclusion, with these results obtained from the SAMP8 mouse model, we demonstrate that dietary restriction has the effects of anti-oxidation and anti-senescence in the kidney.

Key words: SAMP8, Dietary restriction, XOD, Cytochrome P_{450}, Superoxide radical

INTRODUCTION

We live in an aging society and Korea's elderly population accounted for 7.2% in 2000. It was persuasively indicated that half of the countries in the world will be aged by 2020. Since in society, elderly people usually have various chronic degenerative diseases and incurable diseases,[1] so preparation of measures of prevention and treatment for aging and age-related diseases are necessary.

Aging is a phenomenon, which physiological functions *in vivo* deteriorate with time and the capability to manage the stress from the environment become hard to support.[2] Individuals have deteriorated physiological functions due to increased age, with weaker immune functions that may cause sclerosis of the arteries, hypertension and diabetes.[3]

The kidney is one of the representative organs whose function declines after time, causing degenerative changes.[4] After aging, the numbers of glomerulus decrease, which may deteriorate the functions of kidneys, even resulting in probable death. Hypertension,[5] diabetes,[6] medications,[7] and diet[8] are major factors that influence the physiological functions of kidneys, but is know that dietary factors are especially close related with kidney function.[9]

It has been reported that overeating causes obesity, which is the cause of metabolic disorders in these obese overeating patients, resulting in damage to the cells and increased free radicals.[10,11] On the contrary, dietary restriction is characterized by a decrease of free radicals.[12]

It seems that diet is very closely related with aging, considering that free radical theory is most supported inside various others hypotheses about aging.[13] As it has been proven that free radical production and aging progress depend on types of diet, researchers are very concerned about dietary restriction. Recently, studies of

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calorie-controlled diet and anti-aging have been conducted as part of a general study about aging prevention and degenerative diseases.[14-16] Concerning reproducibility, efficacy, and genetic simplicity, it is regarded as an effective method of studying aging.[17] It has also been reported that dietary restriction is effective to extend lifespan and slow aging. The mechanisms to slow aging are still unclear, but it has been reported that changes in cells by free radicals is one of the principal factors.[16-20]

Therefore, the current study observed the production of xanthine oxidase, a microsomal mixed function oxidase system (MFO system), which plays a key role in production of free radicals, and also observed free radicals in kidneys of SAM (senescence-accelerated mice) with diet restriction to examine the effects of dietary restriction on anti-aging.

MATERIALS AND METHODS

1. Experimental Animals and Diets

Female specific pathogen-free-senescence-accelerated mice (SAMP8) were obtained from Korean Research Institute of Chemical Technology, and were randomly divided into two groups (13 each for 4, 8, or 12 months, and until death). Mice were either fed ad libitum (AL), or fed with dietary restriction (DR), equivalent to 60% of mean intake of AL. The mice were under controlled temperature (22±2 ℃), with 12-hr light/dark cycle beginning at 6:00A.M.

Table 1. Composition of Experimental diet (g/100g)

Component	AL(ad libitum)
Carbohydrate	**60.0**
Corn starch[1]	45.0
Sucrose[2]	15.0
Protein	**20.0**
Casein[3]	20.0
Lipid	**12.0**
Lard[4]	8.0
Corn oil[5]	4.0
Cellulose[6]	3.0
Vitamin mix (AIN-76)[7]	1.0
Mineral mix (AIN-76)[8]	3.5
DL-methionine[9]	0.3
Choline chloride[10]	0.2
Energy level(kcal/g)	**4.28**

1), 2) Sam Yang Co., Seoul, Korea
3) Lactic Casein, 30 mesh, New Zealand Dairy Board, Welligton, N. Z.
4) Cheiljedang Co., Seoul, Korea
5) Dong Bang Oil., Seoul, Korea.
6) Harlan TEKLAD Co., Madison, Wisconsin, USA
7) Vitamin mixture (g/kg mixture) : p-Aminobenzoic Acid 11.0132, Ascorbic Acid, coated (97.5%) 101.6604, Biotin 0.0441, Vitamin B_{12} (0.1% trituration in mannitol) 2.9736, Calcium Pantothenate 6.6079, Choline Dihydrogen Citrate 349.6916, Folic Acid 0.1982, Inositol 11.0132, Menadione 4.9559, Niacin 9.9119, Pyridoxine HCl 2.2026, Riboflavin 2.2026, Thiamin HCl 2.2026, Dry Vitamin A Palmitate (500,000 U/g) 3.9648, Dry Vitamin D3 (500,000 U/g) 0.4405, Dry Vitamin E Acetate (500 U/g) 24.2291, Corn Starch, Harlan TEKLAD Co.
8) AIN-76 salt mixture (g/kg mixture): $CaHPO_4 \cdot 2H_2O$ 500, NaCl 74, $K_3C_6H_5O_7 \cdot H_2O$ 220, K_2SO_4 52, MgO 24, $MgCO_3$ (45-48% Mn) 3.5, Fe citrate (16-17% Fe)6, Zn carbonate (70% Zn) 1.6, Cu carbonate (53-55% Cu) 0.3, KIO_3 0.01, $Na_2SeO_3 \cdot 5H_2O$ 0.01, $CrK(SO_4)_2 \cdot 12H_2O$ 0.55; filled up to 1,000 with sucrose.
9) Sigma Chem. Co., St. Louis, Missouri, U.S.A
10) ACROS. New Jersey, U.S.A

2. Measurement of Free Radical Production System in Kidney Tissues

Xanthine oxidase (XOD) activity

XOD activities of the kidney cytosol was measured utilizing the method developed by Stripe and Dellacorte[21] that analyzes the absorbance of uric acid, which is produced by operating the cytosol at 30 ℃ for 10 minutes with xanthine as its substrate, at 292 nm-wave. The unit of activity is indicated in nmol concentraction, which is the amount of uric acid produced from a substrate, after operating 1㎎ protein of kidney tissue for one minute.

Content of cytochrome P_{450}

To measure the cytochrome P_{450} content in kidney tissues, approximately 1g of kidney tissue was homogenized with 0.25 M sucrose solution and centrifuged at 8,000×g for four minutes. The supernatant obtained was also centrifuged at 105,000×g for one minute, resulting in cytosol and microsome. 4 mL of 0.25 M sucrose solution was then added to the microsome pellet layer for microsome floating, using the method of Omura and Sato.[22] The deoxidative carbon monoxide combination was measured by a spectrophotometer with 450 nm and 490 nm, after diluting 1 mL of the microsome solution with 6 mL of 0.1 mM phosphate (pH 7.4), thus obtaining 1 mg/mL protein concentration, which was the base line of the solution diluted with CO gas, and measured at 3-5 minutes.

The absorbance was measured at 450 nm 3 minutes after the sodium dithionite addition. At that moment, the molar absorbance coefficient was set to 91 $mM^{-1}cm^{-1}$.

NADPH-cytochrome P_{450} reductase activity

Using the method developed by of Masters and Kain,[23] the activities were measured by observation of any decrease in the absorbance of dichlorophenolindophenol (DCIP) for one minute. Solution of 0.1 mL microsome was diluted with 0.05 phosphate buffer (pH 7.7, 10-4 M EDTA included) to prepare 1 mg/ mL protein concentration and 0.5 mL of this diluted solution DCIP 96×10-9 mole was added in 1 mL volume capacity semimicro cell with addition of 0.1 mL of 10-3 M NADPH solution to prepare a final volume of 1.1 mL. The enzymatic activity was measured from any decrease in the absorbance at 30 ℃ for one minute after NADPH solution addition, while the absorbance was set to 21

$mM^{-1}cm^{-1}$.

3. Measurement of free radical contents in the kidney tissue

Superoxide radical ($O2^-$) production

Superoxide radical content was measured according to the method of Azzi *et al.*,[24] a specified amount of 50 mM K.P. buffer (pH 7.5) was added with 90 mM succinate, 150 mM KCl, 30 mM KCN, 0.3 mM cytochrome c, and mitochondria-enzymatic source, to obtain 3.0 mL of the final reaction mixture. The superoxide radical content was calculated by measuring the changes of the absorbance at 550 nm while operating the mixture at 37 ℃ for 2 minutes. The content was indicated in nmole of the reduced cytochrome amount, which were produced by 1 mg protein for one minute.

Hydrogen peroxide (H_2O_2) content

In vivo H_2O_2 production was measured from the increase change of the absorbance at 560 nm by using xylenol orange to oxidize ferrous (Fe^{2+}) ion to ferric (Fe^{3+}) ion. That is, the absorbance was measured at 25 ℃ and 560 nm by mixing FOX solution (0.1 M xylenol orange, 0.25 mM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H_2SO_4) into cytoplasm and mitochondria compartment and operating it at ambient temperature for 30 minutes. An H_2O_2 standard curve was obtained by observing the absorbance of H_2O_2 ranging 0~5 μm standard solution.[25]

4. Measurement of Membrane Fluidity

Mitochondria, a compartment from the kidney tissue and membrane fluidity, were measured according to the method of Heron *et al.*[26] using 1,6-diphenyl-1,3,5-hexatrine (DPH) as fluorescent probe. Using fluorescence spectrometer, the fluidity was measured at 360 nm of excitation wave length and 430 nm of emission wave length by adding and mixing 50 mM phosphate buffered solution (pH 7.2, 2750 μl), distilled water (250 μl), and sample (100 μl), leaving the mixture in a constant temperature cistern for 5 minutes, adding and mixing a probe, 0.167 nM TMA-DPH [1-(4-tri-methylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, p-toluenesulfonate] solution up to 6.67 μl to the solution, and operating it in a constant temperature cistern for 30 min while shaking, to maintain at 37 ℃.

5. Statistical Analysis

Results were assessed by ANOVA and Tukey's Honestly Significant Difference test.[27] Differences were considered significant at $p<0.05$.

RESULTS

1. Changes of Free Radical Production System in Kidney Tissues

Xanthine oxidase (XOD) activation in kidney tissues

Changes of XOD activities, an enzyme that produces superoxide radicals in cytosol, are shown in Fig. 1, in a process to produce uric acid from Xanthine as substrate. There was no significant differences between dietary restriction and age, among AL group and DR group ($p<0.05$).

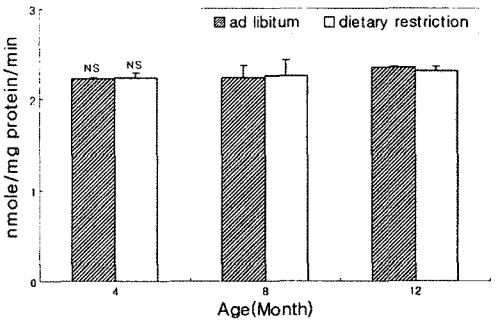

Fig. 1 Effects of age and dietary restriction on xanthine oxidase (XOD) activities of kidney in SAMP8

All values are mean±SE (n=13)
Bars with different letters are significantly different according to period by Tukey's test at $p<0.05$
NS : Not significant

Cytochrome P_{450} content in kidney tissues

Fig. 2 shows the results of cytochrome P_{450} content observation, which are the hemoprotein and the center of MFO system among Mixed Function Oxidase (MFO system) that exists as various types and engages in oxidization and deoxidization reactions with *in vivo* materials and external environmental materials as its substrate due to the wide specialty. In both the AL and DR groups, it was significantly reduced according to age, especially in the 12th month, and there was no significant difference between other groups ($p<0.05$).

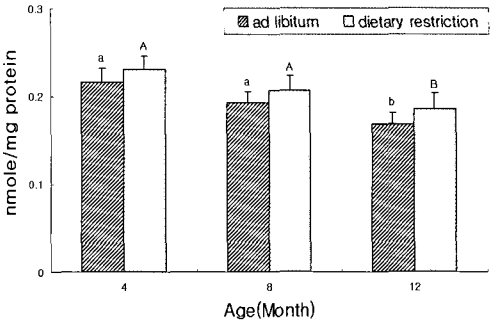

Fig. 2 Effects of age and dietary restriction on cytochrome P_{450} contents of kidney in SAMP8

All values are mean (n=13)
Bars with different letters are significantly different according to period by Tukey's test at $p<0.05$

Activities of NADPH-cytochrome P_{450} reductase in kidney tissues

The results of activities of NADPH-cytochrome P_{450} reductase observation, that consists of each FAD and FMN molecule and functions as a catalyst to transport electron from NADPH to cytochrome P_{450} in the kidney (Fig. 3), showed that the activities were similar to these of cytochrome P_{450} and they were significantly reduced by age increase in the AL group. However, there were no significant differences by age in the DR group.

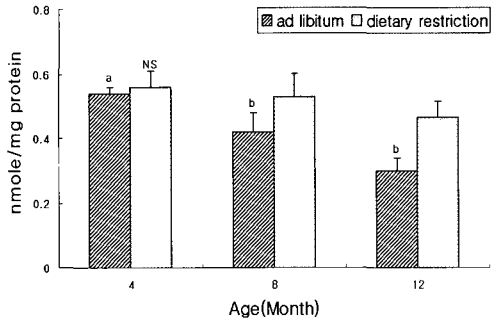

Fig. 3 Effects of age and dietary restriction on NADPH-cytochrome P_{450} reductase activities of kidney in SAMP8

All values are mean±SE (n=13)
*Compared with AL
Bars with different letters are significantly different according to period by Tukey's test at $p<0.05$

3. Changes of Free Radical Production in Kidney Tissues

Amount of superoxide radical (O2-.) production

Measurement of the content of superoxide radicals, which constitute one of factors causing aging and geriatric diseases, in the kidney (Fig. 4), indicated that the content was significantly increased in the 4th, 8th, and 12th month of both groups ($p<0.05$). Comparing the AL group with DR group, it was significantly reduced about 17%, 14%, and 14% in the 4th, 8th, and 12th month of DR group, respectively.

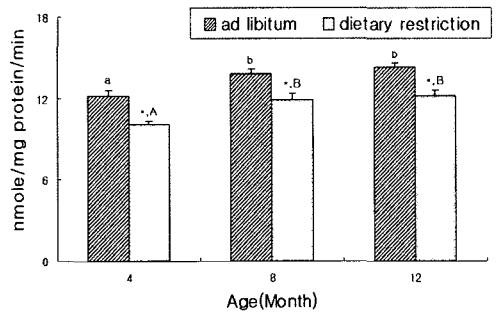

Fig. 4 Effects of age and dietary restriction on superoxide radical (O2-.) contents of kidney in SAMP8

All values are mean±SE (n=13)
*Compared with AL
Bars with different letters are significantly different according to period by Tukey's test at $p<0.05$

Hydrogen peroxide (H_2O_2) content

Fig. 5 shows the results of hydrogen peroxide content comparisons, present in fractions of kidney tissues due to the effect of age and dietary restriction. It was found increasing by age in the AL group, but there was no significant difference by age in the other group ($p<0.05$). However, in the 8th and 12th month of DR group, it significantly decreased about 17% and 20%, respectively, compared to the AL group ($p<0.05$).

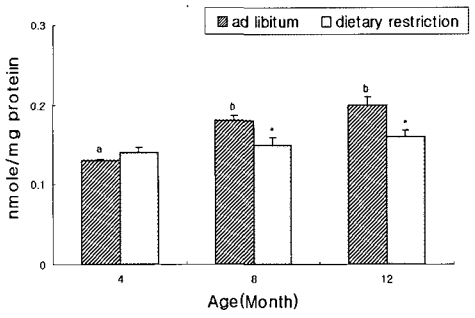

Fig. 5 Effects of age and dietary restriction on hydrogen peroxide (H_2O_2) contents of kidney in SAMP8

All values are mean±SE (n=13)
* Compared with AL
Bars with different letters are significantly different according to period by Tukey's test at $p<0.05$

4. Changes of Cell Membrane Fluidity

Homeostasis is very important in a healthy life, and maintenance of cell membrane fluidity is required for growth and reproduction of cells. Considering the effect of DR on the stability of cell membrane obtained from the kidney tissues fraction, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe (Fig. 6), there were no significant changes in all ages of both AL and DR groups ($p<0.05$), but it was indicated that it was higher in DR group than in AL group; especially, it increased about 11% in the 12 month, without significant differences.

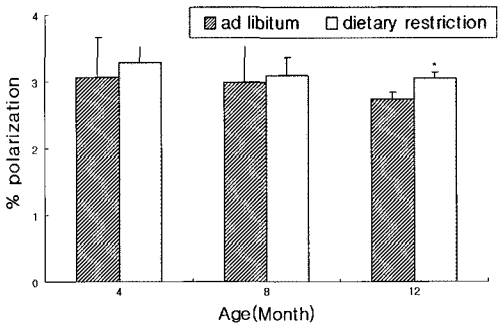

Fig. 6 Effects of age and dietary restriction on membrane fluidity of kidney in SAMP8

All values are mean±SE (n=13)
* Compared with AL
Bars with different letters are significantly different according to period by Tukey's test at $p<0.05$

DISCUSSION

This study investigated the activities of free radical production system and phases of free radical production to examine the effect of calorie-controlled diet on aging in kidney tissues. SAMP8, an animal widely used in aging studies, were divided into two groups as AL (ad libitum) and DR (dietary restriction [fed with 60% of AL]) and we examined the effect of calorie restriction at 4th, 8th, 12th month and at death.

According to free radical theory, one of the hypotheses about aging most supported is that oxygen free radicals are produced in the course of metabolic processes of cells using oxygen, forming peroxide from unsaturated fatty acid of membranes, and causing damage to protein or DNA, resulting in damage to cells oxidatively.[28,29] It is well known that the oxidative damage to cells is related with various chronic degenerative diseases[30] and aging.[31-33]

XOD, one of the systems to produce free radicals *in vivo*, is a non-specific enzyme involving metabolism of purine, pyrimidine, pteridine, aldehyd, and heterocyclic compounds. XOD function as a catalyst for the reaction that oxidizes hypoxanthine, a metabolite of purine, via xanthine produces uric acid.[34] In the current study, the results of measuring the activities of XOD, showed no significant difference between both groups. Therefore, it is concluded that XOD content is not influenced by dietary restriction.

MFO (mixed function oxidase) system is an enzyme system involved in oxidizing, deoxidizing, and hydrolyzing fat-soluble materials such as medications as well as drug metabolism and that metabolizes endogenous substances such as fat acid, steroids and materials coming from the outside such as medications and carcinogenic substances affecting the endoplasmic reticulum membrane of kidney cells. As an electron transport system enzyme, MFO creates O^{2-} and H_2O_2 in the course of counteracting poisonous effects and induces peroxide *in vivo*, possibly accelerating aging due to the activities of the MFO system. In this study, it was found that cytochrome P_{450} of kidney, one of the MFO systems, decreased in all ages of both AL and DR group, which clearly is confirmed in various clinical experiments as well as in experimental animals that the functions of MFO system decline as aging progresses.[35,36] As a result of observing the difference of cytochrome P_{450} content according to dietary restriction, there was no significant difference between AL group and DR group, but there was a tendency to increase in DR group, compared to the AL group in the content. This result was in accordance with a study performed by Yu *et al.*,[37] stating that about the resistance of cytochrome P_{450} against oxidative stress, white mice subjected to dietary restriction was higher than other mice. So, it may be hypothesized that white mice with restricted diet have more stable membrane against oxidative stress.

In addition, NADPH-cytochrome P_{450} reductase acted on the metabolism of endogenous and foreign substances, and functions as a catalyst to transport one of two electrons, which are required for oxidization reactions within a molecule and transfer, as flavoprotein components, an electron to the terminal oxidase, cytochrome P_{450}. It was shown that they were similar to these of cytochrome P_{450}. These results were in accordance with the results of Eden *et al.*[38] and Morgan *el al.*[39] stating that in DR white mice, the functions are maintained as MFO systems are protected from free radical damage.

Between various free radicals, which are known as a substance that cause geriatric disease, superoxide radical ($O_{2\cdot}$) content was significantly reduced in the DR group than in AL group at the 4th month. Since hydrogen peroxide (H_2O_2) features relatively high stability and neutral polarity, it may easily penetrate the mitochondria membrane up to the cytoplasm. H_2O_2 content increased by age in the AL group, but there was no significant difference by age in the DR group. In addition, it was found that the contents significantly decreased in DR group rather than AL group at 8th month. As well as Lee *et al.*[40] report, this result shows that dietary restriction produces more anti-oxidant enzymes than free radical remove, thus leading to restriction of accumulation of superoxide radicals and hydrogen peroxide. This is likely attributable to the activation of anti-oxidant systems in DR group, which were higher than in the AL group.[41]

In addition, declined membrane functions may reduce the fluidity, reducing substance transfer and penetration and also obstructing the maintenance of homeostasis. Therefore, it is important to stabilize it. Observation of effects of dietary restriction on cell membrane fluidity resulted that the fluidity of DR group was more stabilized than in AL group. This is opposite from the findings of Kim *et al.*[42] that showed no influence of dietary restriction on the fluidity. In the current study, it clearly increased the fluidity.

The current study demonstrated that dietary restriction increased NADPH-cytochrome P_{450} in kidney tissues of SAMP rather than AL group and it was effective as antioxidant to stabilize cell membrane fluidity and to produce free radicals. Such effects are probably attributably reinforced on anti-oxidative systems by dietary restriction as described above.

SUMMARY AND CONCLUSION

In the current study, changes of MFO system and free radical production in kidney tissues according to calorie

restriction were observed to examine the anti-aging effect of dietary restriction.

Additionally, concerning the activities of XOD in kidney tissues, there was no significant difference by age and dietary restriction between two groups. It was also found that Cytochrome P_{450} decreased across all ages in both groups, but there was no significant difference between the AL group and DR group. The activities of NADPH-cytochrome P_{450} reductase decreased by age in the AL group, but those of the DR group increased compared to the AL group, with no differences by age.

Superoxide radical content significantly increased by age in both groups and the content of the DR group significantly decreased compared to the AL group at 4th, 8th, and 12th month.

Comparing the hydrogen peroxide content, it was shown that it increased in AL group, without significant difference by ages ($p<0.05$). However, it significantly decreased about 17% and 20%, respectively, compared to the AL group at 8th and 12th months ($p<0.05$).

There were no significant changes in cell membrane fluidity by age in either the AL or DR groups ($p<0.05$), but it was also found that it was higher in DR group than in AL group.

In conclusion, it is proved that dietary restriction reduces free radical production in kidney tissues of SAMP and that stabilizes cell membrane fluidity.

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