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Original article

Actions of various sweeteners on rat sperm.

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Abstract

***Purpose*:** Sweeteners other than sugar are often used in foods aimed at reducing calories. However, it has been reported that some of these sweeteners exhibit reproductive toxicity such as sperm count reduction and decreased sperm motility caused by the involvement of reactive oxygen species (ROS). In this study, we evaluated the actions of sucrose (Suc), sucralose (SCL), aspartame (APM), or neotame (NTM), which are food additive sweeteners frequently consumed on a daily basis, on rat sperm *in vitro*. In addition, rats were given APM and NTM to examine their actions on sperm.

***Methods*:** The animals used were Wistar male rats aged 12 to 15 weeks. Spermatozoa were removed from the epididymis and subjected to experiments. In the *in vitro* experiment, a stock solution of TYH medium containing sperm was prepared, and various concentrations of additives were added; after 5 min, the sperm motility was measured by a sperm motility analysis system (SMAS). Also, sperm stock solution containing sperm was diluted 5-fold, and L-012 (100 μM) was added as a fluorescent probe of ROS, followed by measuring the luminescence intensity by the chemiluminescence method. In *in vivo* studies, APM (250 mg/kg/day) or NTM (40 or 100 mg/kg/day) below the NOAEL (no observable adverse effect level) was administered as a mixed diet; after 2, 4, and 8 weeks, blood and epididymis were collected. As in the *in vitro* study, sperm motility and ROS production were measured, and the organs were analyzed for oxidative stress-related proteins.

***Results*:** In *In vitro* tests, ROS production had little effect on the addition of Suc (≦ 500 μM), SCL (≦ 1 μM) or APM (≦ 10 μM), while there was a tendency for NTM (1 μM) to decrease compared to the control group. Impacts were negligible on sperm motility by the addition of Suc (≦5 mM), SCL (≦10 μM), or APM (≦100 μM), however, sperm motility was significantly reduced by the addition of NTM (≧0.1 μM). In *in vivo* tests, APM administration did not change the sperm count, but ROS production was significantly increased at 2 weeks compared to the control group; at 8 weeks, the total sperm motility showed a declining trend. NTM significantly increased ROS production after 4 weeks of administration, and decreased sperm linear velocity, curve velocity, head amplitude, and head frequency at 8 weeks; the sperm count and total sperm motility were not affected; in parallel with the increase in ROS production, NTM increased the amount of lipid peroxidation-modified protein with positive 4-hydroxy-2-nonenal (4-HNE) in the cauda epididymis.

***Conclusion*:** Some sweeteners affected sperm where the administration of APM and NTM increased ROS production prior to sperm dysfunction. This mechanism could be caused by abnormalities in the electron transport system of mitochondria and oxidative stress due to decreased antioxidant capacity.

***KEY WORDS*:** sweeteners, oxidative stress, sperm motility, reactive oxygen production

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Introduction

With the expansion of the convenience store, fast food and restaurant industries, there are concerns about the influence of food additives. This reflects the Japanese social situation, such as aging society, working together, and single families. Concurrently, import / export and transportation of food from the production area are frequent, and an increase in food mileage is also a problem. For this reason, many efforts have been made to prevent food poisoning and the deterioration of foods. Typical examples include food additives, insecticides, preservatives, fungicides, and pesticides. Many chemicals are used in these and potential endocrine disrupting substances (environmental hormones) are considered to have *in vivo* adverse actions. It is reported that they act on reproductive toxicities such as genital abnormalities and reduced conception rate [1] and on the developmental brain, thus deteriorating learning ability and social recognition [2, 3]. A representative example of social properties is bisphenol A (BPA), which is used as a plastic plasticizer for food containers. BPA are shown to cause elevated reactive oxygen species (ROS) production due to reduced antioxidant enzyme activity and manifest reproductive toxicity such as reduced sperm motility and sperm count in rats [4, 5]. Furthermore, diethylstilbestrol (DES), which was once used as an abortion inhibitor, is reported to reduce sperm count and sperm motility in humans [6]. Also, some daily food additives have reproductive toxicity [7].

In recent years, the growth of the diabetic population is noted and an increasing number of people use artificial sweeteners for the purpose of calorie restriction.

Sucrose, a natural sweetener, is a major component of sugar and is one of the major sweeteners in developed countries. However, since 2002, the WHO (World Health Organization) has been monitoring daily sugar intake for the purpose of preventing lifestyle-related diseases and dental caries, and recommended sugar should comprise less than 10% of daily energy intake. Furthermore, a new guideline in March 2014 was issued to reduce the amount to less than 5% (about 25 g) from 2020. These guidelines imply that adults are restricted to about six teaspoons of sugar a day. This limit is limited to a very small intake given that a teaspoonful of tomato ketchup contains about 4 g of sugar and a carbonated drink contains about 40 g of sugar. Given that a teaspoon of tomato ketchup contains about 4 g of sugar and a carbonated drink contains about 40 g of sugar, it turns out that it is restricted to very small intake. Therefore, concerning the fact that the utilization rate of artificial sweeteners will increase, it is necessary to carefully evaluate the safety. In this study, we investigated the adverse actions of typical sweeteners, sucrose (Suc), sucralose (SCL), aspartame (APM) and neotame (NTM) on rat sperm. In addition, sperm contains a large amount of polyunsaturated fatty acids in the cell membrane, and thus is susceptible to oxidative damage and is said to be the most sensitive cell in a living body to external stimuli [8]. Therefore, when using this, the safety evaluation may be an excellent high-sensitivity safety test.

The artificial sweeteners, SCL, APM, and NTM are about 600 fold, 200 fold, and 10,000 fold more sweet than Suc, respectively [9-11], which are recently used frequently because the amount needed to feel the same sweetness is less than Suc and lower in calories. However, many artificial sweeteners have been questioned for their safety. For example, APM is absorbed from the intestinal lumen upon ingestion and is metabolized to phenylalanine, aspartic acid, and methanol; methanol produces ROS, causing liver damage; increased phenylalanine may affect brain dopamine and norepinephrine levels, thus modifying brain function [12-14]. NTM, on the other hand, is a dipeptide methyl ester derivative synthesized by reductive N-alkylation of APM and has a similar chemical structure to APM (***Fig.1***). Since NTM is not hydrolyzed to phenylalanine and aspartic acid, it can be used

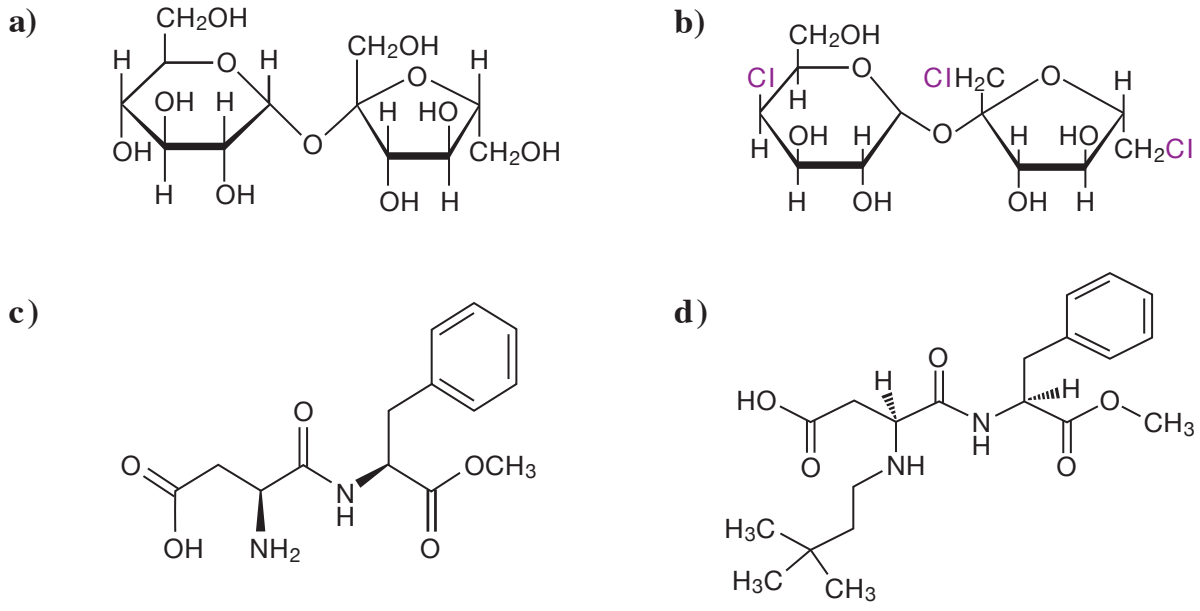

Fig.1. **Structural formula of sweeteners.**

a) Sucrose (Suc). **b)** Sucralose (SLC). **c)** Aspartame (APM). **d)** Neotame (NTM).

for patients with phenylketonuria, and its usage is expected to increase.

In 1992, Dr. Carlsen and his colleagues in Denmark reported that sperm counts and semen volume in men from 20 countries have declined by almost half in 50 years [15], suggesting that, as the cause, the influence of food additives and environmental hormones are present. Therefore, it is important to examine the presence or absence of sperm toxicity as a safety evaluation of food additives. In this study, we verified whether or not sperm toxicity is present and investigated the mechanism, using Suc, SCL, APM and NTM in the *in vitro* experiments and the rats administered APM and NTM.

Methods

Materials

• Reagents

APM and NTM were provided by AstaReal Co., Ltd. (Minato-ku, Tokyo, Japan). Other reagents were of biochemical grade.

• Animals

In this experiment, male Wistar rats (Clea Japan, Tokyo, Japan) of 12 to 15 weeks of age were used. CE-2 (Clea Japan) was used as a standard feed for the control group in the *in vivo* experiment. APM and NTM were mixed and fed freely at 250 mg/kg/day, 40 or 100 mg/kg/day, respectively. Water was freely available in all groups. After breeding for 2, 4 or 8 weeks, the rats were anesthetized by intraperitoneal injection of 5 g/kg of 25% ethyl carbamate (urethane) (Wako Pure Chemical, Osaka, Japan), and perfused with 0.9% saline at room temperature after blood collection from abdominal aorta. After sacrifice, blood, epididymis, and testes were collected for analysis. The animal experiments were performed with the approval of the Animal Experiment Committee of Doshisha University.

Radical scavenging ability in plasma

Each radical was measured using an X-band Microwave Unit ESR device (RE Series: JEOL, Tokyo, Japan) according to a previous report [16]. For analysis of each radical, WIN-RAD (Version 1.30: Radical Research Inc, Tokyo, Japan) was used.

Sperm function

A 2-mm incision was made on the surface of the cauda epididymis with scissors, and the sperm was extruded by pinching. The sperm obtained was collected with the tip of tweezers and placed in 1.5 mL of TYH medium (Mitsubishi Chemical Medience Co., Tokyo, Japan) preheated in a CO_2 incubator at 37°C for 3 hours or more, followed by slow tapping after 5-min incubation, thus preparing a stock solution of TYH medium containing sperm.

In the *in vitro* experiment, the sperm stock solution was diluted 5-fold with TYH at 37°C, to 200 µL of which 2 µL of a 100-fold concentration of each food additive was added, and 5 min later, put 4 µL of this solution into a dedicated chamber plate (SC20-01-04-B: Leja, Nieuw-Vennep, Netherlands). Using a sperm movement analysis system (SMAS; Detect, Tokyo, Japan), sperm motility (%), straight velocity (µm/sec), curve velocity (µm/sec), straightness (straight velocity / curve velocity), head amplitude (µm), and head frequency (Hz) were measured.

ROS production from sperm

The sperm stock solution prepared above was diluted 5 fold with TYH medium warmed in a CO_2 incubator at 37°C for 3 hours or more to prepare a 5-fold diluted sperm solution, 5 µL of which was placed in 945 µl of DMEM medium (D6046: Sigma, Osaka, Japan), and the luminescence intensity was measured by the chemiluminescence method (AccuFLEX Lumi400; Hitachi Healthcare Systems, Tokyo, Japan).

Thirty seconds after the start, 50 µL (final concentration 100 µM) of 2 mM L012 (Wako Pure Chemical) as a Luminal-based chemiluminescent probe, after 100 sec, 10 µL of each additive solution, and after 240 sec, 5 µL (final concentration 100 U/mL) of the prepared superoxide dismutase (SOD: Nippon Kayaku, Tokyo, Japan) solution readjusted to 10,000 U/mL was added. The amount of ROS production was evaluated based on the peak emission intensity.

The sperm count in the *in vivo* experiment was determined by cutting the left cauda epididymis and filtering the impurities using a 70 µm cell strainer (REF352350: BD Falcon, NJ, USA) and placing it in 50 mL Falcon. This solution was diluted 20-fold with phosphate buffer saline, PBS (–), and the sperm number was counted using a cell counter. Then, the total number of sperm contained in 1.5 mL of TYH medium was calculated.

Western blot analysis

The cytosol of the cauda epididymis was mixed with RIPA buffer so that the volume became 9 times that of the organ, and homogenized with a homogenizer (NS-360D: Microtech Nition, Chiba, Japan) at 20 krpm for 30 sec on ice. Thereafter, it was centrifuged at 4°C, 12,000 rpm for 10 min in a centrifuge (CT15RE: Hitachi Healthcare Systems, Tokyo, Japan). The supernatant was collected in 1.5 mL Eppendorf tubes and used as an organ cytosol stock solution. The appropriate amount of protein was subjected to SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis). As a molecular weight marker, a pre-stained protein marker (Wako Pure Chemical) was used.

After adding the primary antibody, anti-HNEJ-2 antibody (JaICA, Shizuoka, Japan), diluted 1: 5000 with Canget Signal Solution 1 (Toyobo, Osaka, Japan), the specimen was sealed to prevent air bubbles, stored at 4°C, and reacted overnight. After the overnight reaction, the plate was immersed in TBS-T (Tris buffered saline with Tween 20) and shaken at room temperature for 7 min with a shaker, followed by replacement of the solution. This procedure was repeated three times. Next, the membrane is placed in a hybridization bag, and a secondary antibody (Dako Japan, Kyoto, Japan) diluted 1: 20,000 with Canget Signal Solution 2 is added and sealed without air bubbles, followed by reaction at room temperature for 90 min. After the reaction,

the membrane was immersed in TBS-T and shaken with a shaker for 7 min. This was repeated three times, then, after washing, the membrane was immersed in the coloring solution for 2 min, followed by the detection of bands with Image Quant Las 4000 mini (GE Healthcare Lifescience, Buckinghamshire, UK).

Membrane potential of sperm mitochondria

The potential difference of mitochondria was measured using JC-1 Mitochondrial Membrane Potential Detection kit (Biotum, CA, USA). A stock solution was prepared in a TYH medium so as to have a cell solution (1×10^6 cells/mL). And then a NTM solution was added, and the mixture was incubated at 37°C in a CO_2 incubator for 30 min. Then, the mixture was centrifuged at 400 x g for 5 min at room temperature, removed the supernatant, added 500 µL of JC-1 reagent diluted 1:10 with assay buffer, and incubated at 37°C in a CO_2 incubator for 15 min. The mixture was centrifuged at 400 x g for 5 min at room temperature. After removing the supernatant, 2 mL assay buffer was added and centrifuged at 400 x g for 5 min at room temperature. Repeatedly 300 µL of assay buffer were added, then 100 µL was dispensed per well in a 96-well black plate (NUNC), followed by the measurement using a fluorescent plate-reader (live cells in red: excitation [Ex] 560 nm, emission [Em] 595 nm, apoptosis-inducing cells in green: Ex 485 nm, Em 535 nm).

Statistical analysis

A software, SPSS ver.22 (IBM Japan, Tokyo, Japan), was used for statistical analysis of data. Using the one-way analysis of variance's Turkey test, $p < 0.05$ was defined as the significance level.

Results

In vitro actions of food additives on sperm

Addition of Suc, SCL or APM had no effect on ROS production (***Fig.2***). The addition of NTM tended to rise ROS production below 10 nM, while, at higher concentrations, it tended to decrease. Addition of Suc, SCL or APM had almost no effect on sperm motility, which, in contrast, was significantly reduced by the addition of NTM with a concentration above 0.1 µM (***Fig.3***). Additives did not affect sperm linear velocity, curve velocity straightness, head amplitude, or head frequency. The decrease in sperm motility by NTM correlated with a drop in mitochondrial membrane potential (***Fig. 4***).

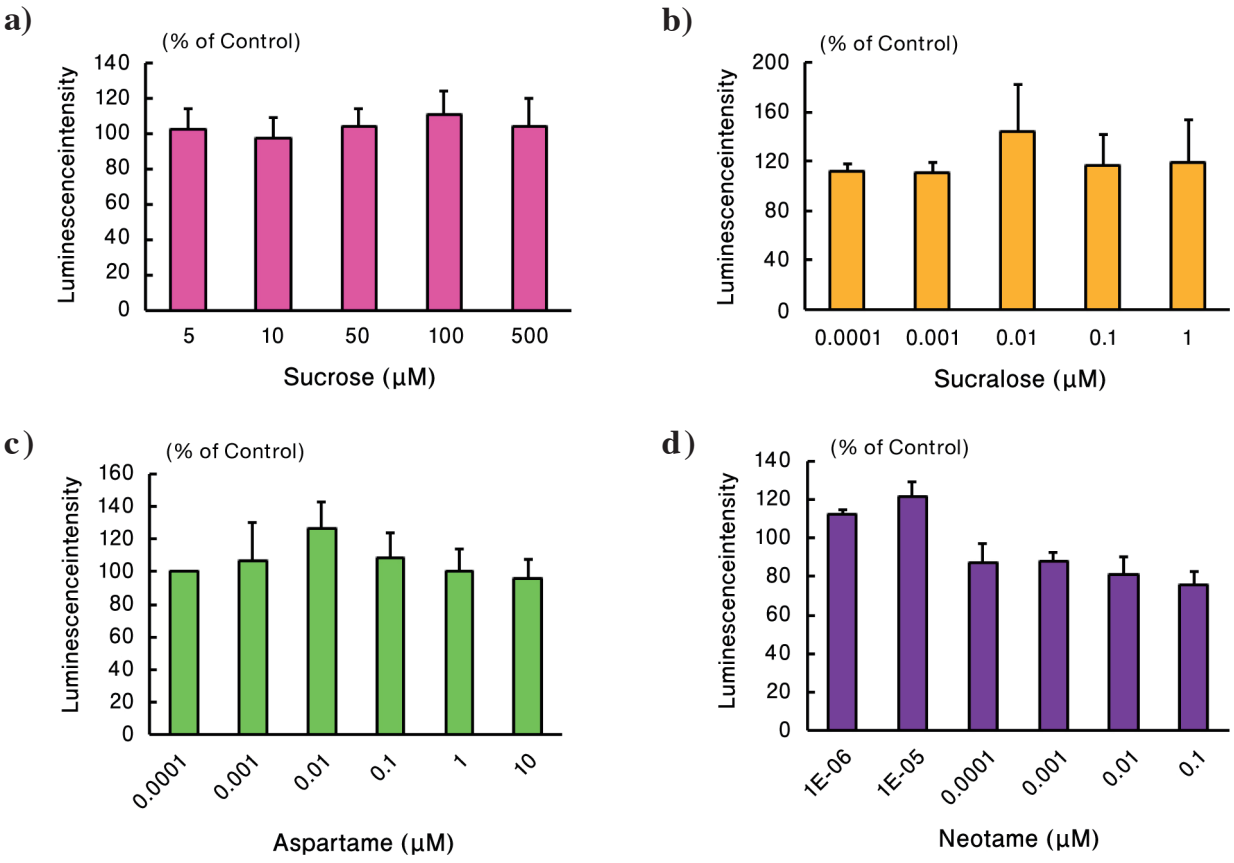

Fig.2. **ROS production from sperm by addition of sweeteners.**

a) Sucrose (Suc). **b)** Sucralose (SLC). **c)** Aspartame (APM). **d)** Neotame (NTM). Results are expressed as mean values ± SE, n = 4 ~ 8. ROS, reactive oxygen species; SE, standard error.

In vivo actions of APM and NTM

There was no significant difference in food intake and body weight between APM and NTM administration. In addition, the free radical scavenging activity of plasma was reduced by the administration of both additives for some radical species, and particularly after 2 weeks, the scavenging activities for ·OH and $O_2^{\cdot -}$ were reduced (***Fig. 5***).

ROS production from sperm and expression of lipid peroxide-modified protein by APM and NTM administration

At 2 weeks of APM administration, ROS production significantly rose compared to the control group (***Fig. 6-a***), while, at 8 weeks, it was not significantly different from that of the control. Two weeks after administration of NTM (40 mg/kg/day), ROS production was not changed compared to the control group, while it rose at 4 and 8 weeks (***Fig. 6-a***).

In the testis and cauda epididymis, bands of 4-HNE-modified protein were detected at 53 kDa and 28 kDa. Since the same shading tendency was observed in the bands of all organs, ***Fig. 6-b*** shows a band of 53 kDa in the cauda epididymis. Expression of HNE-modified protein was significantly elevated 2 weeks after APM administration. At 4 and 8 weeks of NTM (40 mg/kg/day) administration, the expression of HNE-modified protein was elevated compared to the control group, becoming significant at 4 weeks.

Actions on sperm function

Administration of APM and NTM did not change sperm count or total sperm motility (***Fig. 7***). APM administration did not change sperm straightness, head amplitude, curve speed, or straight speed. ***Figure 8*** shows the sperm straightness, head amplitude, and head frequency of the APM or NTM administration group when the control group was defined as 100%. Eight weeks after NTM administration, the sperm straightness showed an upward trend, and the head amplitude and head frequency decreased significantly, indicating the possibility of reduced fertility.

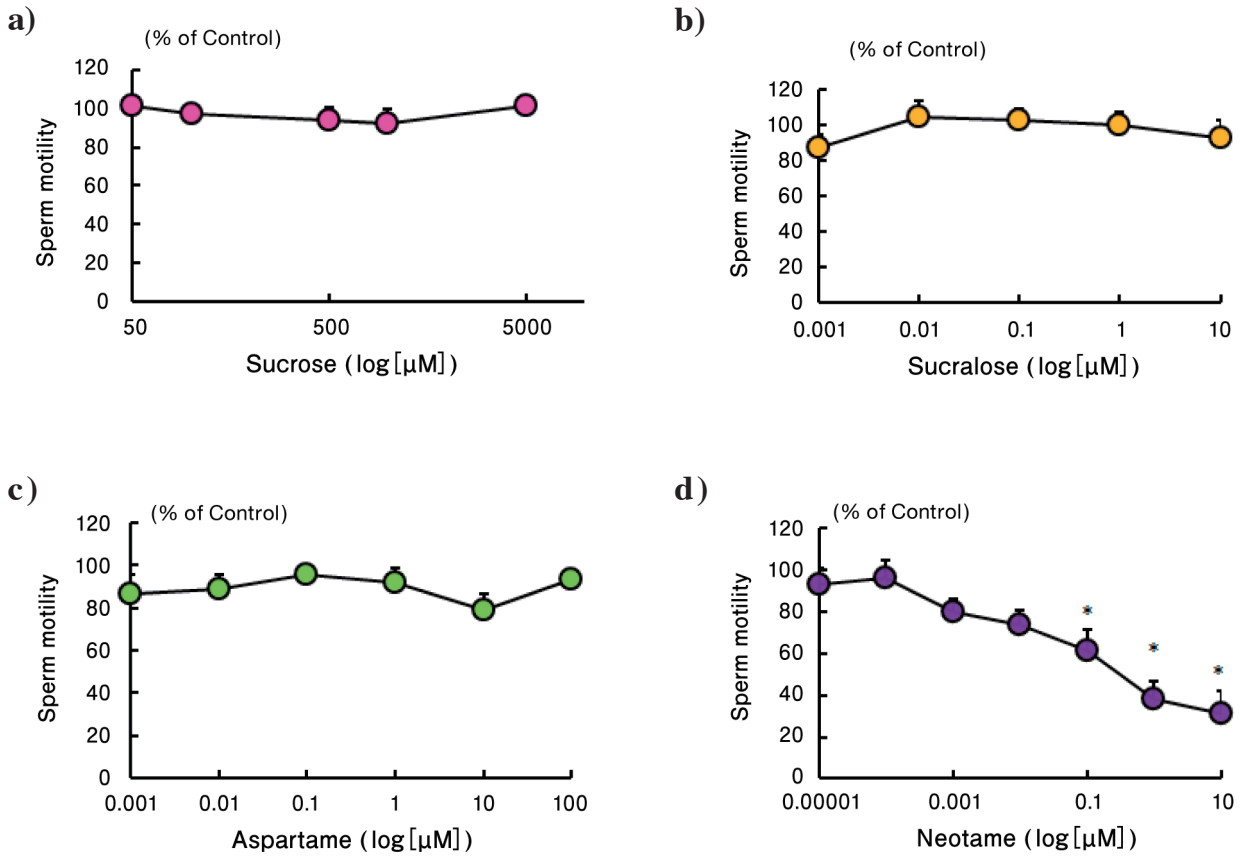

Fig. 3. **Actions of sweetener on sperm motility.**

a) Sucrose (Suc). **b)** Sucralose (SLC). **c)** Aspartame (APM). **d)** Neotame (NTM). Results are expressed as mean values ± SE, n = 4 ~ 8, * $p < 0.05$ vs control without sweeteners by Turkey test.

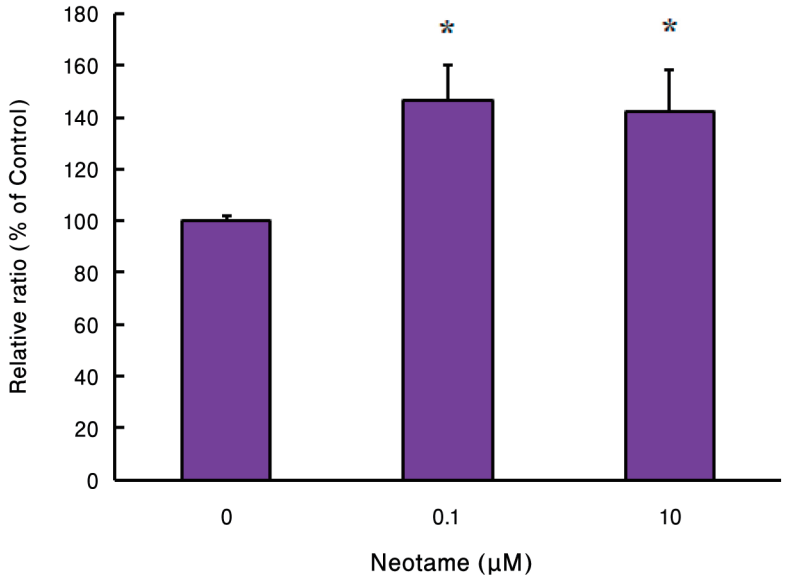

Fig. 4. **Decrease actions of NTM on the membrane potential of sperm mitochondria.**

NTM was added to the sperm suspension and incubated for 30 min. Results are expressed as mean values ± SE, n = 6, * $p < 0.05$ vs control without sweeteners by Turkey test. NTM, neotame; SE, standard error.

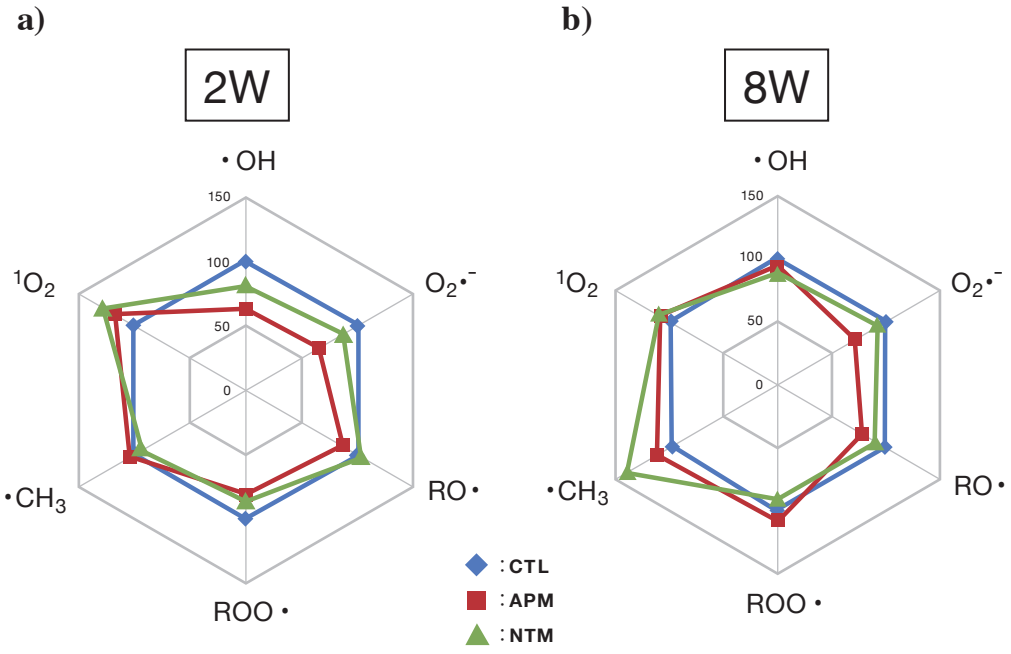

Fig. 5. **Radar chart of plasma free radical scavenging activity after administration of APM and NTM.**

a) At 2 weeks. **b)** At 8 weeks. Results are expressed as values when ROS elimination rate in control plasma defined as 100%. APM, aspartame; NTM, neotame; ROS, reactive oxygen species.

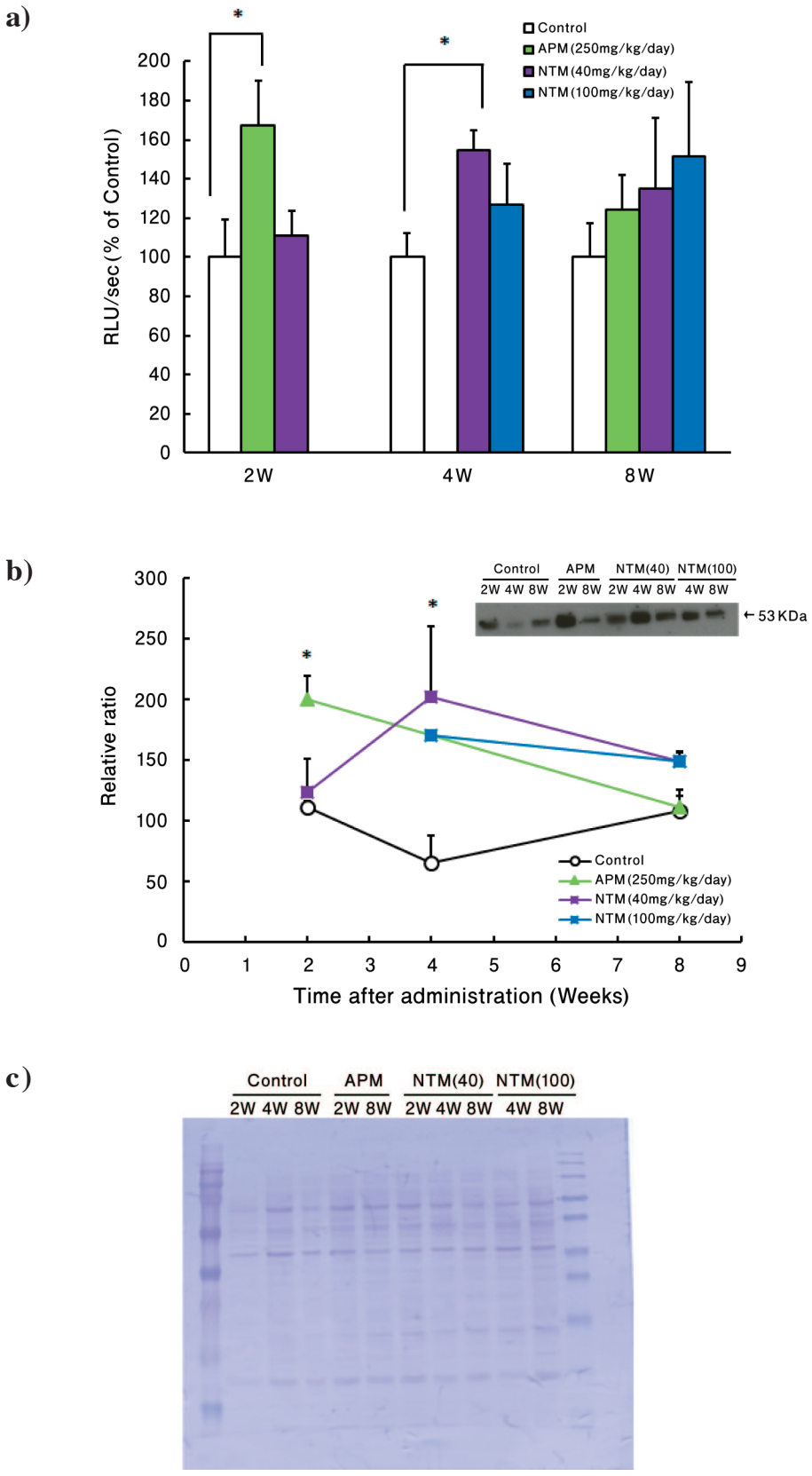

Fig. 6. **ROS production from sperm and 4-HNE-modified protein in the cauda epididymis induced by administration of APM and NTM.**

a) ROS production. **b)** 4-HNE-modified protein (53 kDa). **c)** Membrane image by Coomassie brilliant blue staining. Results are expressed as mean values ± SE, n = 3 ∼ 5, * $p < 0.05$ vs control by Turkey test. APM, aspartame; NTM, neotame; ROS, reactive oxygen species; 4-HNE, 4-hydroxy-2-nonenal; SE, standard error.

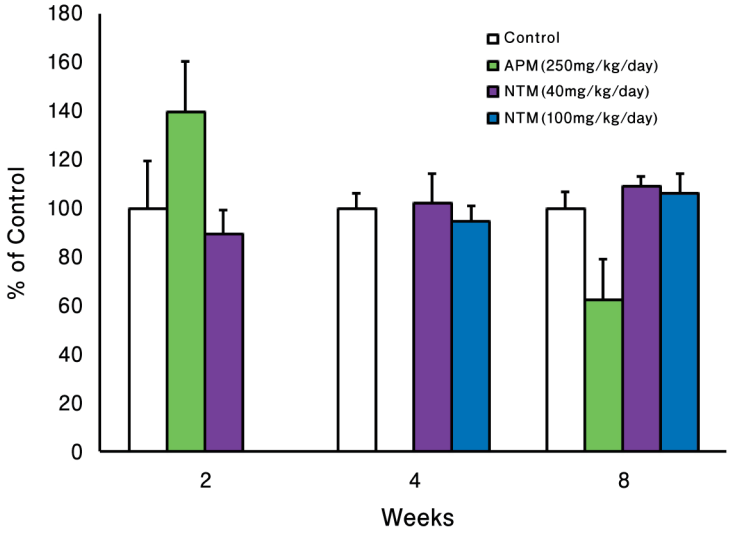

Fig. 7. **Actions of APM and NTM administration on total motile sperm count.**

The sperm motility was measured 2, 4 or 8 weeks after administration of APM (250 mg/kg/day) or NTM (40 or 100 mg/kg/day). Results are expressed as mean values ± SE, n = 3～5. APM, aspartame; NTM, neotame; SE, standard error.

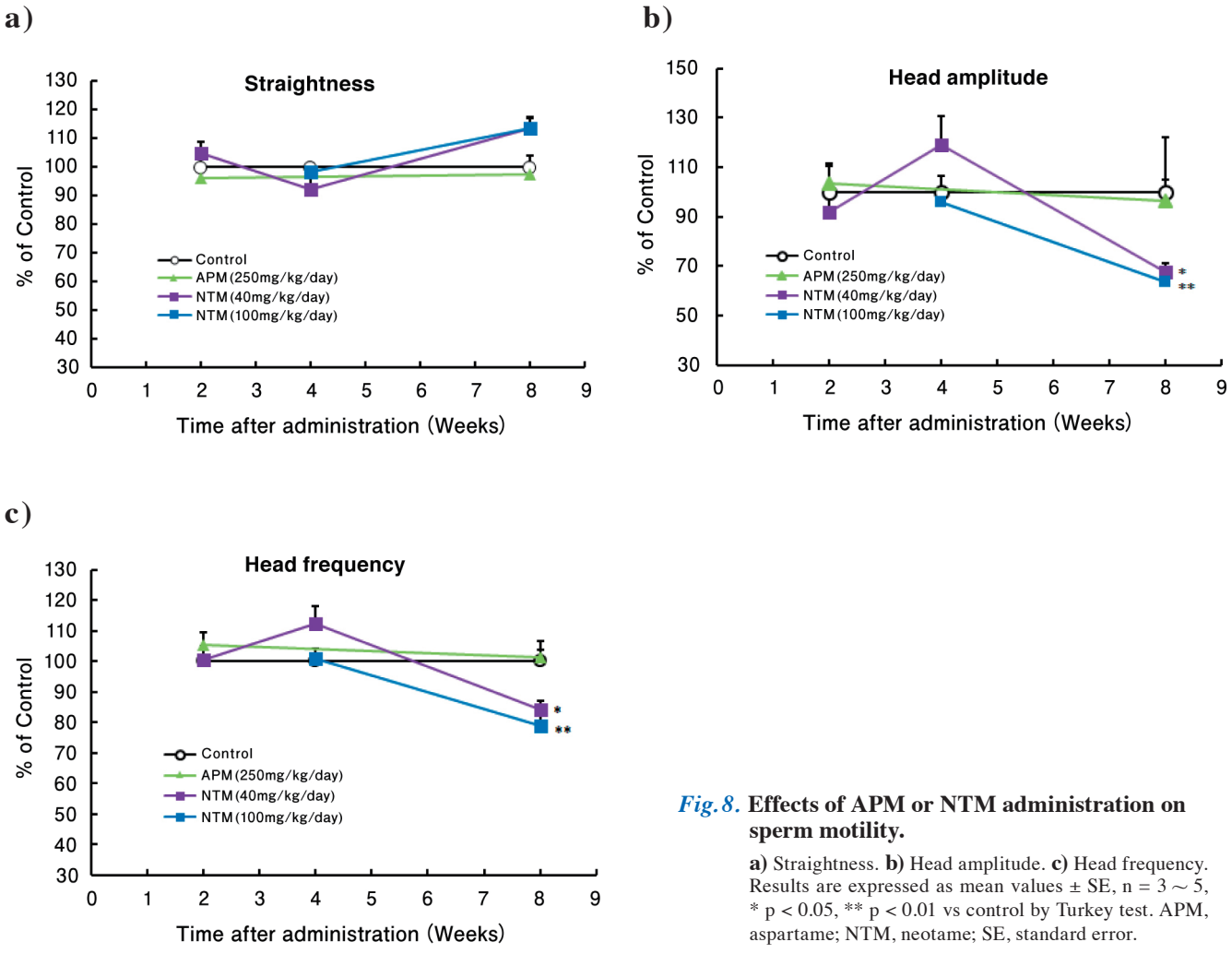

Fig. 8. **Effects of APM or NTM administration on sperm motility.**

a) Straightness. **b)** Head amplitude. **c)** Head frequency. Results are expressed as mean values ± SE, n = 3～5, * $p < 0.05$, ** $p < 0.01$ vs control by Turkey test. APM, aspartame; NTM, neotame; SE, standard error.

Discussion

In the present study, we investigated the actions of sweeteners on sperm in Wistar rats *in vitro* and *in vivo*. In the *in vitro* study, sperm motility was not affected by the addition of Suc or artificial sweeteners, SCL and APM, and no sperm toxicity was noted.

In contrast, a significant decrease in sperm motility and a rise in ROS production were observed with the addition of NTM. Furthermore, the sperm mitochondrial membrane potential dropped significantly at this time.

On the other hand, *in vivo* studies showed that sperm count was not changed by APM administration, while the total sperm motility showed a declining trend over 8 weeks. NTM administration did not change sperm count or total sperm motility, but increased sperm straightness, straight velocity, curve velocity, head amplitude, and head frequency, which are important for fertilization function. Furthermore, ROS production from sperm has been shown to increase ROS production prior to such a decline in sperm function. These findings are similar to the results of the administration test of endocrine disruptors such as bisphenol A in the previous report [17].

Sperm midpieces are abundant in mitochondria which produce ATP as an energy source for movement, concurrently, ROS is produced from the electron transport system. Therefore, when this ROS is overproduced, it causes leakage from mitochondria, resulting in impairment of sperm function and declined motility. This study has revealed that the administration of APM and NTM increased ROS production and HNE-modified protein, which is a secondary product of oxidation of ω6-polyunsaturated fatty acids. From the above, it was considered that the sperm membrane was oxidized due to the elevation in oxidative stress, which is one of the causes of the decline in sperm function.

SCL, which showed no sperm toxicity *in vitro*, has a structure where chlorine is attached to the molecule, and its safety has been questioned as a type of organochloride. Although some reports indicate that it is non-toxic and does not affect reproduction in gene expression [18, 19], considering the actions of metabolites, it may be necessary to study in terms of oxidative stress *in vivo*.

Currently, in Japan, the ADI (acceptable daily intake) of food additives is set as NOAEL(maximum non-toxic level in animal tests) x 1/100, with a safety factor of 100. The NOAELs for APM and NTM are 4,000 mg/kg/day and 96.5 mg/kg/day in rats, respectively. In contrast, spermatozoa toxicity was suggested in this intervention experiment, even though it was examined at low concentrations at 250 mg/kg/day in APM and 40 mg/kg/day in NTM, which were lower than NOAEL. According to a survey by the Ministry of Health, Labor and Welfare in 2011, the daily intake of APM was reported to be 0.019 mg/kg/day in humans. The toxicity of APM alone may be negligible, however, the actions of long-term combined intake need to be considered. Therefore, it is important to carefully evaluate the safety of food additives.

It has been reported that artificial sweeteners cause changes in the intestinal flora, dysbiosis, thus inducing impaired glucose tolerance [20]. In recent years, analyses of more than 4,000 people have identified that drinking soft drinks with artificial sweeteners is associated with an increased risk of stroke and dementia. The observational studies show that, in those who drink artificially sweetened beverages at least once a day, the risk of ischemic stroke almost triples and Alzheimer's risk is 2.9 times higher over 10 years, compared with those who do not [21]. They are correlated, however, it is not clear that the causes and effects may all correspond, and further tracking is needed. Furthermore, a meta-analysis of the ingestion sweeteners other than sugar has been reported to affect health, but no clear results have yet been obtained [22].

In this study, it was revealed that administration below the maximum non-toxic level caused a decline in the *in vivo* antioxidant capacity, an elevation in ROS production from sperm, and actions on sperm motility. It is also important to re-evaluate the previous toxicity tests. In addition, the concept of exposome has been proposed for all chemicals other than genetic factors that are exposed throughout life [23, 24]. Exposome widely includes extrinsic factors such as radiation as well as food additives and intrinsic factors such as oxidative stress and inflammation, where the association with inflammatory bowel disease [25] and various diseases has been recognized [26-28]. Conversely, transcriptome analysis has reported that dietary intervention can prevent exposome-related diseases [29]. Based on these concepts, further research on disease prevention and health maintenance is essential.

Conclusion

In the present study, some sweeteners have been revealed to have sperm toxicity due to oxidative stress.

Conflict of Interest Statement

The authors claim no conflict of interest in this study.

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