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Food hardness influences the progression of age-related hearing loss in mice

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ABSTRACT

C57BL/6J and DBA/2J mice are often used for hearing research because of their early onset and progression of age-related hearing loss (AHL). Here, we report that the hardness of the diet affects the progression of AHL in these mice. When C57BL/6J mice and DBA/2J mice were fed a pellet-type or powder-type standard AIN93M diet, the pellet diet significantly promoted AHL. AHL promotion was eliminated by crushing the pellet diet to a powder. Subsequently, when C57BL/6J mice were fed the pellet-type AIN93M diet obtained from three different manufacturers, two of them significantly promoted AHL. The hardness of the diets was measured, and it was found that the two diets that promoted AHL were significantly harder than the other diet. Next, we attempted to reduce diet hardness by replacing some nutritional ingredients with dried eggs or phosphatidylcholine (PC), and we succeeded in obtaining brittle diets with lower hardness values. Then, C57BL/6J mice were bred with brittle diets for 6 months and the promotion of AHL was suppressed to the equivalent level as the powder diet. Furthermore, when senescence-accelerated mice, SAMP8, were fed a brittle diet for one year, the progression of AHL was also suppressed; however, it did not affect other aging indexes, such as mental and physical performance. We also confirmed that a high-fat pellet diet, which is soft even in pellet form, did not promote AHL. Time-restricted feeding (tRF), which is a chrono-nutritional method to delay aging, ameliorated the promotion of AHL by the hard AIN93M pellets in C57BL/6J mice. These results indicate that the physical form and hardness of diets affect the progression of AHL in mouse models.

1. Introduction

Age-related hearing loss (AHL) is an aging phenomenon common to many animals, including humans. The progression of AHL is due to loss of sensory hair cells, spiral ganglion neurons, and/or stria vascularis cells in the cochlea of the inner ears (Yamasoba et al., 2013). Sensory hair cells and auditory neurons that project onto hair cells do not recover once they die, so the number of these cells decreases with age, and as their function, hearing also declines. It is also known that loud acoustic stimulus induces the loss of sensorineural cells in the cochlea, causing noise-induced hearing loss (NIHL) (Kurabi et al., 2017). Recent studies indicate that oxidative stress and associated mitochondrial dysfunction contribute to the development of sensorineural hearing loss (SNHL), such as AHL and NIHL (Honkura et al., 2016; Oishi et al., 2020; Someya and Kim, 2021).

In experimental mice, the onset and progression of AHL and sensitivity to NIHL significantly differ depending on the strain (Myint et al., 2016; Zheng et al., 1999). Some of these strains have mutations in the *Cdh23* gene, whose protein is expressed in the tip link of hair cells, which significantly accelerates the onset of AHL and the subsequent progression of hearing loss (Johnson et al., 2008; Noben-Trauth et al., 2003). For example, AHL onset in CBA/CaJ mice carrying the wild-type *Cdh23* gene is over one year of age, whereas C57BL/6J mice carrying the mutant *Cdh23* gene typically develop AHL from 2 to 3-mo-old (Vlajkovic et al., 2011; Zheng et al., 1999). DBA/2J mice show faster onset of AHL, which develops shortly after weaning and progresses on a weekly timescale (Zheng et al., 1999).

One-third of persons over 65 years of age are estimated to have disabling hearing loss (WHO, 2018). There is concern that the number of people throughout the world with disabling hearing loss will grow over the years up to 630 million by 2030 and over 900 million in 2050. Research on the prevention of hearing loss is underway, and the nutritional approach is one of the successful methods in animal models. For example, calorie restriction or feeding with antioxidants such as coenzyme Q10, α-lipoic acid, and *N*-acetylcysteine ameliorate the development of SNHL (Marie et al., 2018; Someya et al., 2009; Someya et al.,

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2010; Tian et al., 2014).

We have fed mice various diets from the perspective of advancing research on hearing loss prevention using a nutrition/food-tech approach. In this process, we noticed that the physical form of the diet could affect the progression of AHL. Therefore, in this study, we examined the progression of AHL in C57BL/6J mice and DBA/2J mice by feeding pellets and powder diet, and found that the pellet diet significantly promoted AHL. Pellet diets are popular because of reduced stress by gnawing; however, the hardness of the pellets promotes AHL in the mice. We also reported that a pellet diet with reduced hardness via additives or pellets of a high-fat diet (HFD) did not promote AHL. The chrono-nutritional approach, time-restricted feeding (tRF), was also effective in improving the promotion of AHL owing to the hard pellets.

2. Materials and methods

2.1. Animals

Animals were handled according to the guidelines of the Japanese Ministry of Agriculture, Forestry and Fisheries for laboratory animal studies, and the studies were reviewed and approved by the Animal Care and Use Committee of the Food Research Institute, NARO (approval number: H27–046, H28–010, H28–011, H29–008, H29–050, and 20C091FRI). Female SAMP8 mice (3-wk-old) and male DBA/2JJcl mice (3-wk-old) were obtained from an institute for animal reproduction (Japan SLC, Hamamatsu, Japan). Male C57BL/6J mice (5-wk-old) were obtained from another institution (Charles River Japan, Yokohama, Japan). All mice were housed at 25 ± 1 °C, 50 ± 5% humidity, and a 12 h light-dark photocycle and had *ad libitum* access to food and water. Body weight and water and diet consumption (per cage) were measured once a week during the experimental period.

2.2. Diets and hardness measurement

All diets were obtained from the manufacturer: AIN93M powder (Oriental Yeast, Tokyo, Japan), AIN93M pellets (Oriental Yeast, CLEA Japan, Tokyo, Japan, and Research Diet, New Brunswick, NJ, USA), high-fat diet (HFD-60, Oriental Yeast), and brittle diets containing 8% dried egg powder (Kewpie, Tokyo, Japan) or 1% egg yolk-derived phosphatidylcholine (PC; Kewpie) instead of the equivalent nutritional amount of casein and cornstarch (custom order, Oriental Yeast). Yolk oil was also obtained from Kewpie.

All pellets were cylindrical with a diameter of approximately 12–14 mm and a length of approximately 2–3 cm. The water content was about 7–9% according to catalog information by manufactures. The hardness of the pellet diets was measured using a Kiya-type hardness tester (Fujiwara Scientific, Tokyo, Japan) at normal room temperature and humidity. The cross section of the pellet was placed sideways, and the tip of the stick-type pressure attachment (5 mm diameter) was in contact with the center of the pellet, and then pressure was manually applied for measurement. The hardness was defined as the force required for breakage of the pellet.

2.3. Hearing measurement

Auditory brainstem response (ABR) hearing tests were performed using TDT System 3 equipped with BioSigRP (Tucker-Davis Technologies, Alachua, FL, USA), as previously described (Oike et al., 2016). In

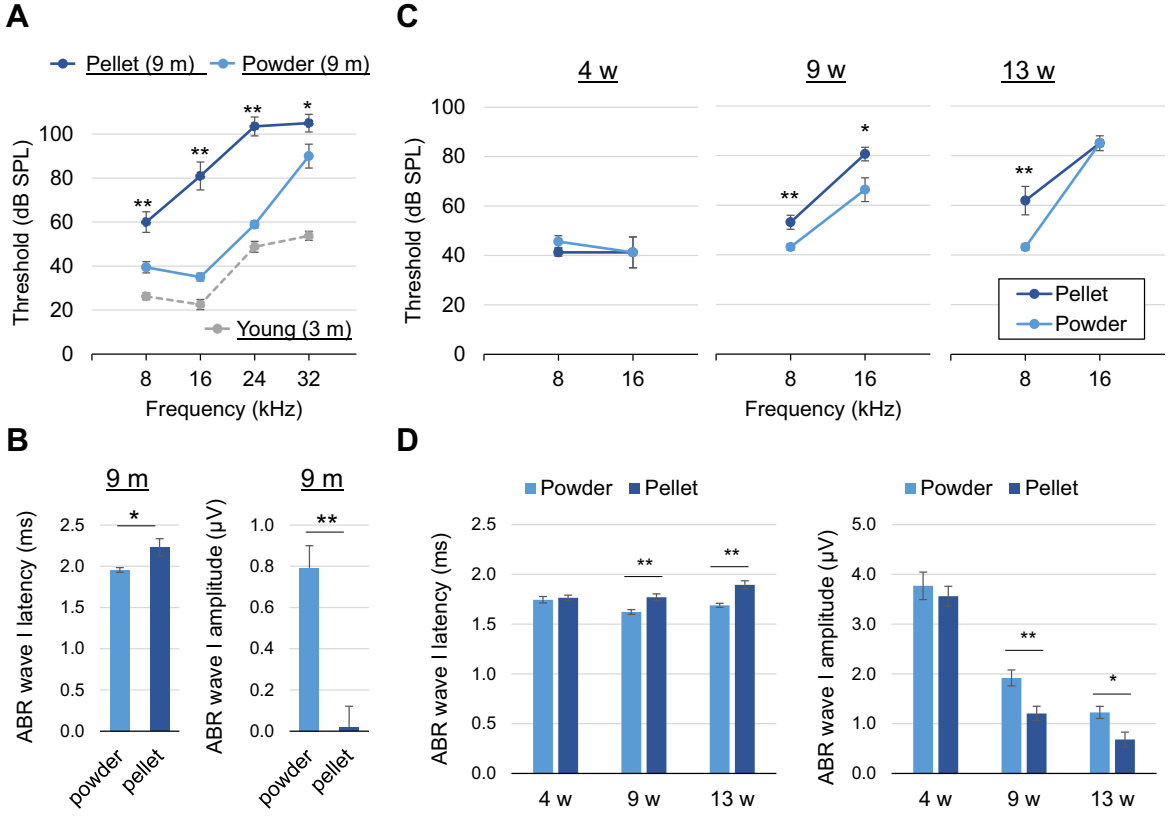
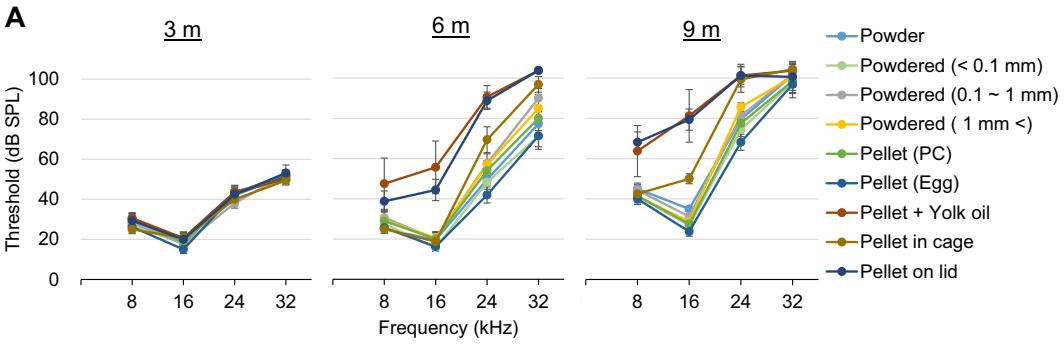
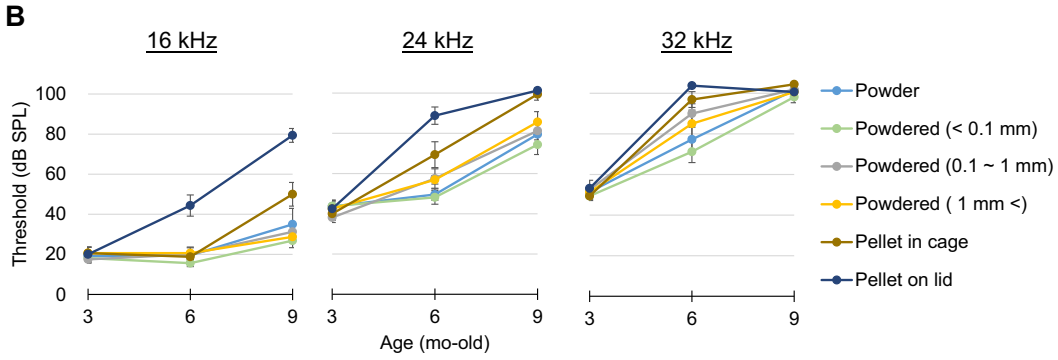
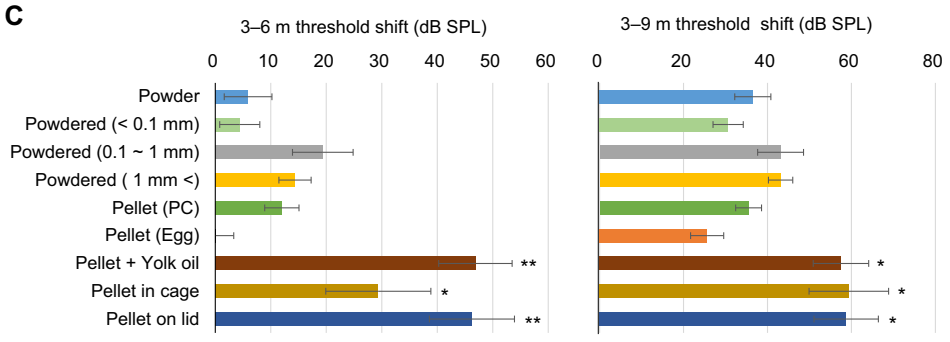
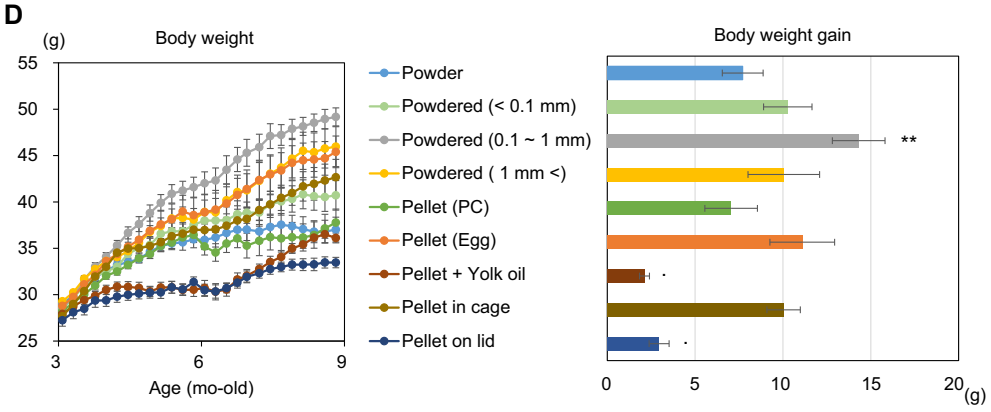


Fig. 1. Pellet diets promotes AHL in C57BL/6J and DBA/2J mice.
A: Auditory brainstem response (ABR) was measured at 8, 16, 24, and 32 kHz at the age of 3-mo-old (young control group, dotted line, $N = 8$) or 9-mo-old (pellet or powder group, $N = 8$ each) in male C57BL/6J mice. The latter groups were fed pellets or powder diets from 3 to 9-mo-old for 6 months (*t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).
B: The latencies and amplitudes for ABR wave I were analyzed from the data of 8 kHz - 80 dB stimulation at 9-mo-old (*t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).
C: ABR was measured at the ages of 4, 9, and 13-wk-old in male DBA/2J mice fed pellets or powder diet ($N = 8$ each, *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).
D: The latencies and amplitudes for ABR wave I were analyzed from the data of 8 kHz - 100 dB stimulation at 4, 9, and 13-wk-old (*t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).









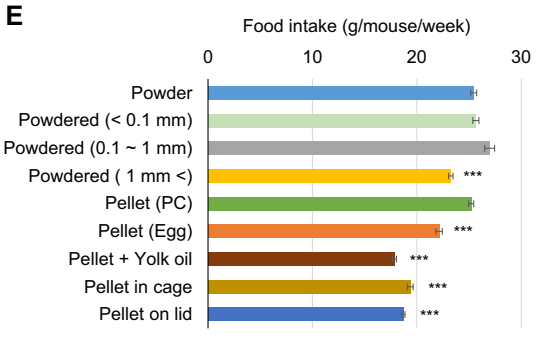


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Fig. 2. Pellet form and hardness affects AHL promotion in C57BL/6J mice.
A: ABR was measured at the age of 3, 6, and 9 m in male C57BL/6J mice fed physically different diets ($N = 8$ each). The experimental conditions are summarized in Table 1. Powder: purchased AIN93M powder diet, Powdered: purchased AIN93M pellets grained into three different sizes, Pellet (PC or Egg): brittle pellets containing PC or Egg, Pellet: purchased AIN93M pellets, Pellet + Yolk oil: purchased AIN93M pellet on the lid with 3.4% yolk oil containing drinking water.
B: ABR data shown in A is rearranged by aging axis.
C: Threshold shift at 24 kHz from 3 to 6-mo-old (left panel) and 3 to 9-mo-old (right panel) in each group (one-way ANOVA with Dunnett's *post hoc* test, $*p < 0.05$, and $**p < 0.01$ vs. powder group).
D: Body weight change during experimental period (left panel) and body weight gain from 3 to 6-mo-old (right panel) with statistics (one-way ANOVA with Dunnett's *post hoc* test, $\cdot p < 0.1$ and $**p < 0.01$ vs. powder group).
E: Average of weekly food intake from 3 to 6-mo-old with statistics (one-way ANOVA with Dunnett's *post hoc* test, $***p < 0.001$ vs. powder group).

brief, mice were intraperitoneally anesthetized with a mixture of medetomidine (0.3 mg/kg), midazolam (4.0 mg/kg), and butorphanol (5.0 mg/kg), and the subdermal needle electrodes were placed at the vertex (reference), beneath the pinna of both ears (active), and lower back (ground). After the ABR measurement, atipamezole (0.3 mg/kg) was injected intraperitoneally to awaken the mice from anesthesia. The sound stimulus consisted of a 5 ms tone burst with a rise-fall time of 1.5 ms at frequencies of 8, 16, 24, and 32 kHz (10–100 dB SPL). The responses to 500 sweeps were averaged at each intensity level (5 dB SPL steps) to assess the threshold. The hearing threshold was defined as the lowest stimulus intensity that produced reliable peaks in the ABR waveforms. If no peak was observed even at 100 dB, the threshold was recorded as 105 dB SPL. A better score was adopted as the threshold among the right and left ears.

2.4. Behavioral tests in SAMP8 mice

All behavioral tests were performed in SAMP8 mice at 14 months of age. NCT is index as exploration and activeness, TST as depression, and NORT as short-term memory.

2.4.1. New cage test (NCT)

Mice were individually placed in a new cage (W18 × D25 × H13 cm), and the activity transition was measured for 30 min with an infrared sensor system (AS10D and CIFIII; Melquest, Toyama, Japan).

2.4.2. Tail suspension test (TST)

TST was accomplished as described by Steru et al. (Steru et al., 1985). Briefly, each mouse was suspended for 8 min by the tail using a plastic clip. Cushioning materials were placed between the tail and clip to reduce the stress of pinching. After the first 2 min of the test, the total duration of immobility was measured (6 min). An animal was judged to be immobile when it ceased moving limbs and body, making only movements to facilitate breathing. The test session was recorded and analyzed using SMART 3.0 software.

2.4.3. Novel object recognition test (NORT)

The NORT method was based on a previously described method (Oike et al., 2020). In brief, the test was performed in a dark cabinet equipped with an infrared camera. The mice were allowed to explore freely for 5 min in a cage with two fixed 50 mL plastic conical tubes (training session). After 10 min, one of the plastic tubes was randomly replaced with a conical flask, after which point the mouse was put back in the box and allowed to explore for 5 min (test session). We confirmed beforehand that there was no preference difference between both objects. The total time spent exploring was measured during the training and test sessions. Exploration was defined as pointing the nose at the object at a distance of 2 cm or less or touching the object with the nose.

Table 1
Summary of the experimental conditions in Fig. 2.

Group name	Diet and description
Powder	AIN93M powder
Powdered (< 0.1 mm)	AIN93M pellets grained to a size smaller than 0.1 mm
Powdered (0.1–0.1 mm)	AIN93M pellets grained to a size in the range of 0.1 mm to 1 mm
Powdered (1 mm <)	AIN93M pellets grained to a size greater than 1 mm
Pellet (PC)	AIN93M pellets with 1% replaced by phosphatidylcholine
Pellet (Egg)	AIN93M pellets with 8% replaced by dried egg
Pellet + Yolk oil	AIN93M pellets and 3.4% yolk oil by drinking water
Pellet in cage	AIN93M pellets in the cage with the feeding bottle
Pellet on lid	AIN93M pellets on the wire lid

2.5. Blood biochemistry

Plasma glucose and cholesterol were measured using a LabAssay glucose kit and LabAssay cholesterol kit, respectively (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions.

2.6. Statistical analysis

All statistical analyses were carried out using EZR 1.41, based on R (Kanda, 2013). The test methods applied and the significant differences are described in each figure. Data are expressed as mean values and standard error (SE).

3. Results

3.1. Feeding on pellet diet impaired hearing in C57BL/6J and DBA/2J mice

When mice are bred for a long period of time, pellet diet is often preferred over powder diet because of the ease of handling, deterioration of hygiene due to manure contamination, and reduction of stress due to gnawing. On the other hand, when examining the effects of functional food ingredients, a powder diet is often used because of the ease of preparation. We noticed that mice bred on a pellet diet had worse hearing than mice bred on a powder diet. Surprisingly, 9-mo-old C57BL/6J mice fed on a AIN93M pellet diet from 3-mo-old displayed significantly higher ABR thresholds than those of age-matched mice fed on a same nutritional powder diet (Fig. 1A). Analysis of the amplitude and latency of the cochlear-derived ABR wave I revealed that the pellet diet was significantly delayed in latency and significantly reduced in amplitude compared to the powder diet (Fig. 1B). These results suggest that the pellet diet may promote sensorineural hearing loss due to cochlear dysfunction compared to the powder diet.

The effects of pellet and powder diets on AHL progression were also examined in DBA/2J mice, which show early progression of hearing loss even in juveniles. When DBA/2J mice were fed these diets for 2 months from 4-wk-old, the pellet diet promoted the progression of hearing loss as well as in C57BL/6J mice (Fig. 1C and D).

Table 2
Summary of the experimental conditions in Fig. 3.

Group name	Diet and description
X	AIN93M pellets manufactured and sold by company X
Y	AIN93M pellets manufactured and sold by company Y
Z	AIN93M pellets manufactured and sold by company Z

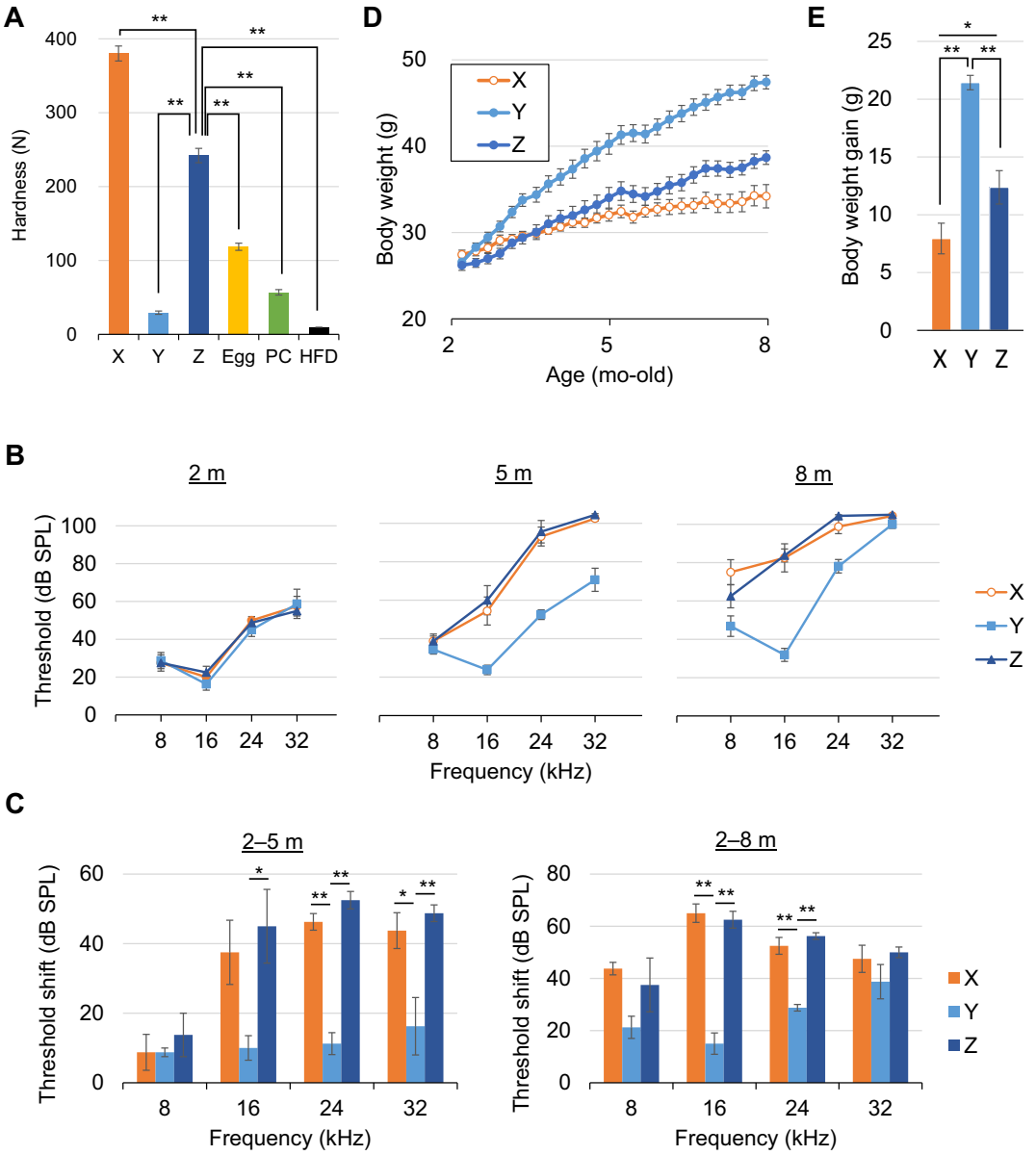


Fig. 3. Pellet hardness affects AHL promotion in C57BL/6J mice.
A: Hardness of pellets was measured using a Kiya-type hardness tester (average of 11–12 pellets each, one-way ANOVA with Dunnett's *post hoc* test, $^{**}p < 0.01$ vs. pellet-Z). Egg and PC means the brittle pellets containing 8% dried-egg powder and 1% PC, respectively.
B: ABR was measured at the age of 2, 5, and 8 m in male C57BL/6J mice fed commercially available AIN93M pellets manufactured by three different companies (X, Y, and Z; $N = 8$ each).
C: Threshold shift from 2 to 5-mo-old or 8-mo-old in each group (one-way ANOVA with Tukey's *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$).
D: Body weight change during experimental period.
E: Body weight gain from 2 to 8-mo-old is shown with statistics (one-way ANOVA with Tukey's *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$).

3.2. Gnawing pellets promoted AHL

First, in order to confirm whether the step of gnawing the pellet affected the progression of hearing loss or whether the ototoxic component was produced when the powder diet was hardened into pellets, we prepared grained pellet diets of three different sizes: coarse grains with a particle size greater than 1 mm, fine grains with a size in the range of 0.1 mm to 1 mm, and powder with a size smaller than 0.1 mm. C57BL/6J mice were fed from 3-mo-old with one of these grained diets, intact pellets, or intact powder diet. In addition, two methods were applied with intact pellet diets: one was placed on the wire mesh at the top of the cage and the other was placed in the cage with a feeding bottle as well as the powder diet. As a result of 6 months of feeding, hearing was significantly worse in the pellet feeding groups compared to the groups fed on the powder or grained diets. (Fig. 2A–C). Although there was not as much difference between grained diets, the hearing threshold tended to be higher when the pellet size exceeded 0.1 mm. These results indicated that gnawing the diet affected the progression of hearing loss, not the ototoxicity of the diet. Furthermore, it should be noted that the pellet diet placed on the wire mesh above the cage had a significantly stronger hearing loss promoting effect than the pellets placed in the cage. Since the pellets placed in the cage were partially softened by soaking in saliva, urine, and drinking water, it was suggested that the hardness of the diets affected the progression of AHL. It is also noted that the pellet diet groups (on the lid) gained less body weight than the other groups (Fig. 2D and E).

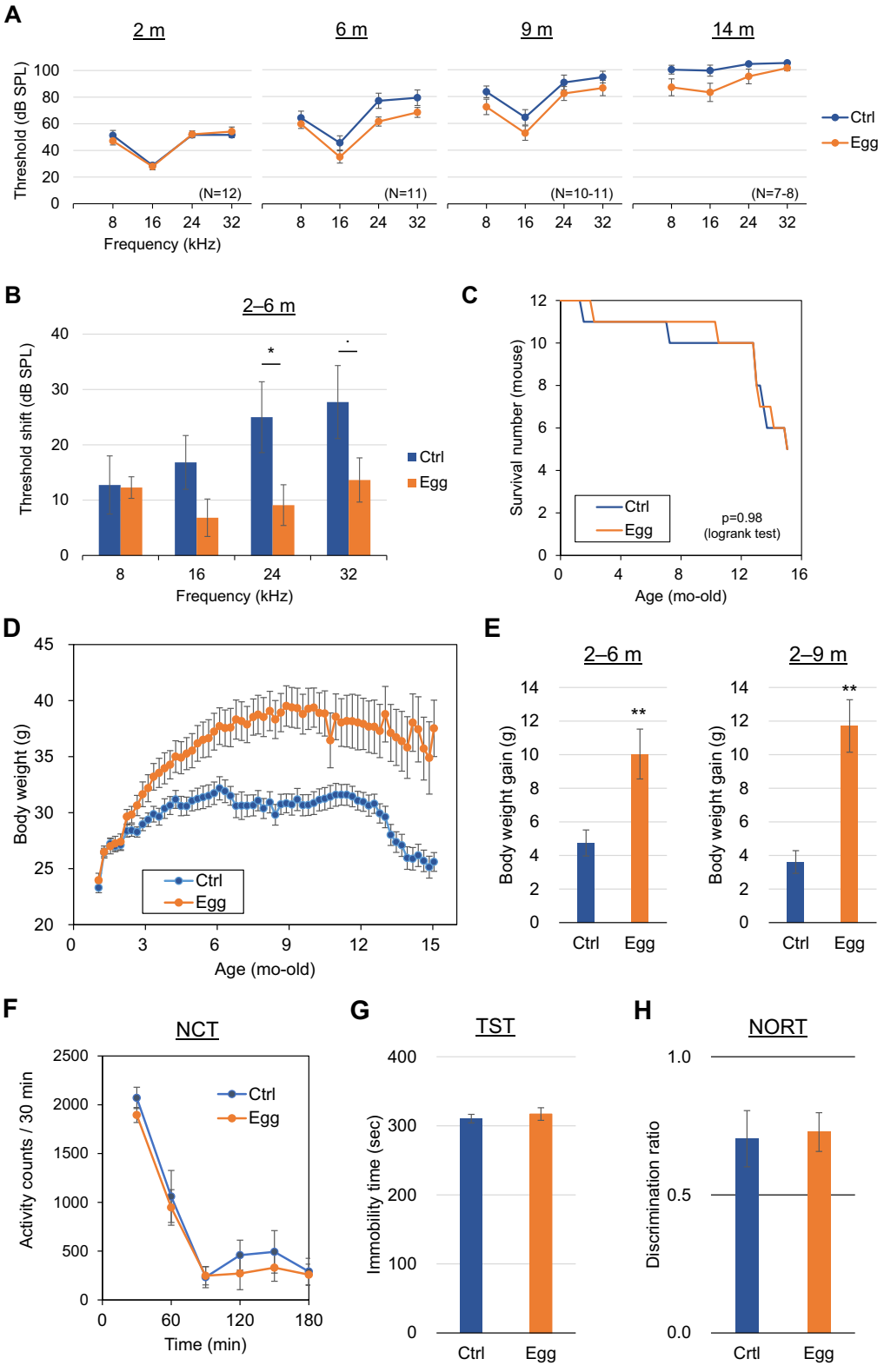


Fig. 4. Brittle pellet diet suppressed AHL in SAMP8 mice.
A: ABR was measured at the age of 2, 6, 9, and 14 m in female SAMP8 mice fed a control pellet diet or a brittle egg diet ($N = 7$–12 each).
B: Threshold shift from 2-mo-old to 6-mo-old ($N = 11$ each, t-test, $p < 0.1$, $^{*}p < 0.05$).
C: Changes in survival with statistics.
D: Body weight change during experimental period.
E: Body weight gain from 2 to 6-mo-old and 2 to 9-mo-old (t-test, ** $p < 0.01$).
F–H: Results of performance tests; curiosity and activity by new cage test (NCT), depression by tail suspension test (TST), and cognitive function measured by novel object recognition tests (NORT). There are no statistical differences (One-way repeated measures ANOVA for NCT, and t-test for TST and NORT).

3.3. Brittle pellets did not promote AHL

AIN93M, which is a standard purified diet for nutritional studies in rodents, was obtained from three manufacturers (Table 2; X, Y, and Z). The hardness of the pellet was measured, and the pellet of one manufacturer Y was more likely to collapse, and the hardness was lower than the others (Fig. 3A). When C57BL/6J mice were fed using these pellet diets with the same composition for 6 months, AHL was significantly promoted by the two hard pellet diets (Fig. 3B and C). It is also noted that these hard pellet diet groups (X and Z) gained less body weight than Y group (Fig. 3D and E).

Next, we controlled the hardness of the pellets. We succeeded in reducing the hardness of the pellets by replacing part of the diet with dried egg or egg yolk-derived phosphatidylcholine (PC) without changing the nutritional PFC balance, that is, protein, fat, and carbohydrate (Fig. 3A). When these brittle diets were administered to C57BL/6J mice for 6 months, the low hardness diets containing egg or PC did not promote the progression of AHL (Fig. 2A and C). On the other hand, when PC-containing yolk oil was administered by drinking water with the hard pellet diet on the lid, the AHL promotion was not improved. This indicated that prevention of AHL promotion by the egg- or PC-containing diet was not due to functional components such as antioxidants in the egg, but rather to the hardness of the pellets.

Moreover, when SAMP8 (senescence-accelerated mouse prone 8) was bred on the normal pellet diet or the egg-containing brittle pellet diet, the egg-containing pellets delayed the progression of AHL (Fig. 4A and B). The egg-containing pellets increased body weight than control pellets (Fig. 4D and E). On the other hand, survival and the results of behavioral tests, new cage test (NCT), tail suspension test (TST), and novel object recognition test (NORT), which are often used to evaluate aging, illustrated no difference between the two diets (Fig. 4C and F–H). It was suggested that the hardness of the diet promotes AHL not only in C57BL/6J mice but also in SAMP8 mice.

3.4. Effects of HFD and body weight on promotion of AHL

High-fat pellets were soft enough to be broken with fingers, and in fact, as a result of the hardness measurement, they showed the lowest hardness among all the diets (Fig. 3A). Therefore, we hypothesized that pellets of the high-fat diet (HFD) also did not promote AHL and examined it in C57BL/6J mice. In the above experiments, AHL progression and body weight suppression were coincident; thus, we also examined the effect of time-restricted feeding (tRF), which is a chrono-nutritional method that prevents body weight gain without caloric restriction (Hatori et al., 2012). Specifically, a total of four groups were set for each of the HFD pellets and the AIN93M normal diet (ND) pellets, with tRF or *ad libitum* feeding (ALF); HFD-tRF, HFD-ALF, ND-tRF, and ND-ALF (Table 3). As expected, ALF of the HFD resulted in a significant increase in body weight and blood biomarkers, glucose and cholesterol, and tRF prevented them (Fig. 5D–F). The HFD-tRF group showed equivalent body weight transition to the ND-ALF group. In hearing, the HFD pellets significantly suppressed the progression of AHL compared to ND pellets, regardless of dietary restrictions (Fig. 5A–C). This is probably due to the lower hardness of the HFD than the ND. However, body weight gain and deterioration of blood metabolic indexes were found not to be associated with the progression of AHL. Notably, the ND-tRF group demonstrated better hearing than the ND-ALF group, indicating that tRF is effective in preventing AHL due to the hard ND pellets.

Table 3
Summary of the experimental conditions in Fig. 5.

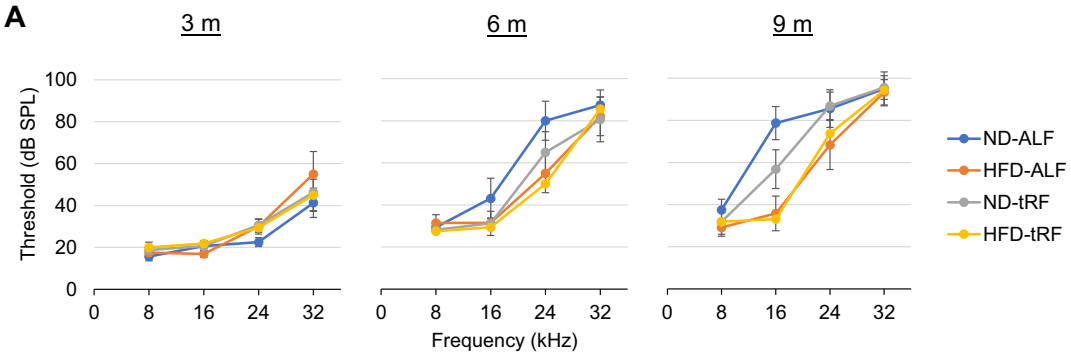
Group name	Diet	Feeding condition
ND-ALF	AIN93M pellets	*Ad libitum*
HFD-ALF	HFD-60 pellets	*Ad libitum*
ND-tRF	AIN93M pellets	12 h time-restricted during dark phase
HFD-tRF	HFD-60 pellets	12 h time-restricted during dark phase

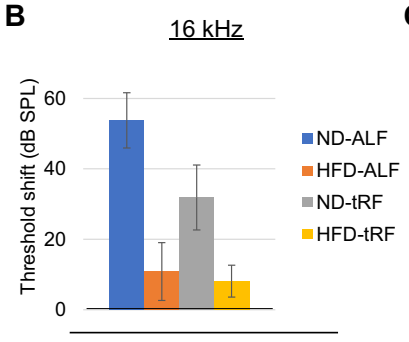
4. Discussion

This study revealed the relationship between dietary hardness and AHL progression, which has rarely been considered in hearing research; however, the physical form of the diets is known to affect growth, sleep, cognitive function, and metabolism (Anegawa et al., 2015; Oka et al., 2003; Yan et al., 2011). The AIN93 diet used in this study is a standardized diet used in nutritional studies around the world, but with no restrictions on physical form and hardness. Indeed, the hardness of the pellets varies from manufacturer to manufacturer, and we have found that harder diets promote AHL. It is unknown exactly why pellets of the same composition vary in hardness depending on the manufacturer, but compression pressure, moisture, temperature conditions, drying conditions, etc. might affect the hardness. We aware that simply increasing or decreasing the amount of water in the powder bonding process had a slight effect on hardness (data not shown). Similar problems occur more often with diets that have been replaced with some ingredients. Indeed, the hardness of HFDs is low enough to be broken with fingers. Therefore, we have succeeded in making brittle pellets with reduced hardness by replacing some of the ingredients in the AIN93M diet with eggs or PC. However, in most cases, the difference in hardness is not large enough to be perceived by the fingers. In fact, the difference in hardness between AIN93M diets from different manufacturers was not noticeable by touch. Nonetheless, when administered to mice, the progression of AHL and body weight gain were clearly different. This is likely to be misunderstood as a function due to different diet ingredients, and indicates that it is necessary to pay attention to hardness in experiments examining the effects of food ingredients and drugs on AHL prevention as well as energy metabolism. To avoid this risk, it is helpful to consider administration of the sample by injection, sonde, dissolution in drinking water, or mixing with a powder diet.

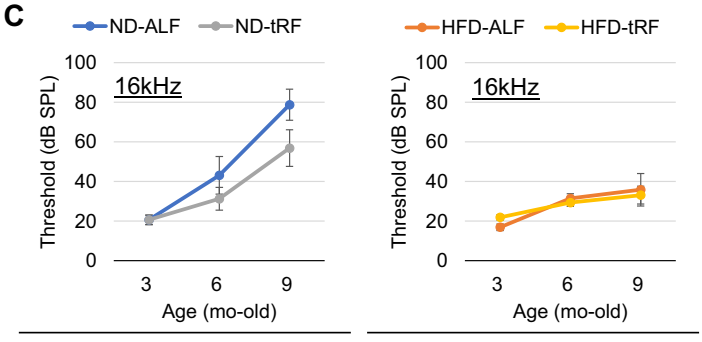
Powder diets and soft diets are known to increase body weight compared to pellet diets (Oka et al., 2003; Yan et al., 2011). In fact, breeder's weight reports include data for both powder and pellet diets. In this study, it was confirmed that even pellet diets with the same nutritional composition had different effects on body weight owing to their different hardness, which is consistent with previous reports. Interestingly, hard pellets that promoted AHL resulted in lower body weight compared to brittle pellets or powder diets that did not promote AHL. Food intake also tended to be lower with hard pellets, as it was associated with lower body weight. This might have been due to the fact that the hard pellets are physically difficult to gnaw or to continue gnawing. Therefore, we performed tRF to evaluate the effect of feeding and weight gain on AHL. tRF resulted in comparable or slight reductions in food intake and body weight compared to ALF in both ND and HFD groups. This is similar to the condition of the hard pellets, but tRF did not promote AHL. This means that dietary intake and weight suppression are not related to the promotion of AHL. In addition, tRF rather ameliorated the promotion of AHL in the ND. A previous study has shown that imposing calorie restrictions on C57BL/6 mice suppresses the progression of AHL (Someya et al., 2010); thus, tRF might induce similar effects. Calorie restriction increases protection against ROS and their damage by improving mitochondrial function, enhancing antioxidants, and increasing autophagy. Similarly, tRF has been shown to improve energy metabolism and anti-inflammatory effects (Chaix et al., 2019). Thus, tRF might activate these pathways and suppress the promotion of AHL.

Another interesting finding is that HFD exacerbated metabolic markers, but not AHL. This means that adipose tissue-derived systemic inflammation does not affect the progression of AHL, at least in the short term. This is consistent with our previous report in which AHL was suppressed rather than promoted when C57BL/6J, SAMP8, and DBA/2J mice were fed a powder HFD (Oike et al., 2020). Fat is the major energy source during rest phase under normal circadian rhythm and is



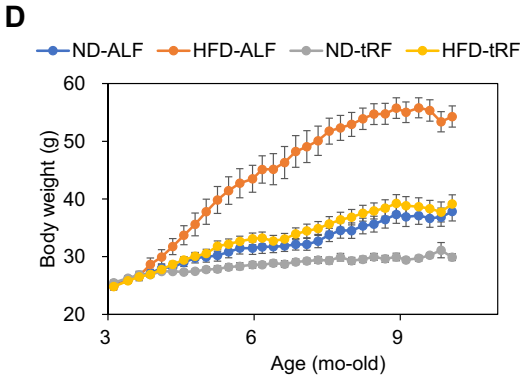


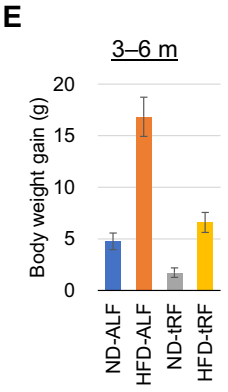
Two-way ANOVA	
Diet (ND/HFD) **	$p<0.01$
Feeding (ALF/tRF)	$p=0.12$



One-way repeated measures ANOVA	
Age (3/6/9) **	$p<0.01$
Feeding (ALF/tRF)	$p=0.08$

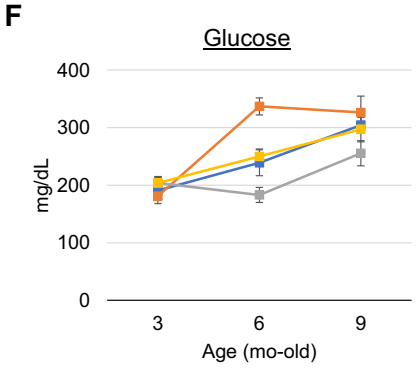
One-way repeated measures ANOVA	
Age (3/6/9) **	$p<0.01$
Feeding (ALF/tRF)	$p=0.84$



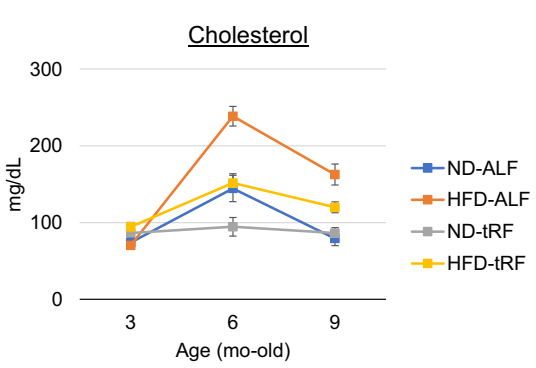


Two-way ANOVA at 6-mo-old	
Diet (ND/HFD) **	$p<0.01$
Feeding (ALF/tRF) **	$p<0.01$

Two-way ANOVA at 9-mo-old	
Diet (ND/HFD) **	$p<0.01$
Feeding (ALF/tRF) **	$p<0.01$



Two-way ANOVA at 6-mo-old	
Diet (ND/HFD) **	$p<0.01$
Feeding (ALF/tRF) **	$p<0.01$



Two-way ANOVA at 6-mo-old	
Diet (ND/HFD) **	$p<0.01$
Feeding (ALF/tRF) **	$p<0.01$

(caption on next page)

Fig. 5. High-fat pellet diet and time-restricted feeding suppresses AHL in C57BL/6J mice.
A: ABR was measured at the age of 3, 6, and 9 m in male C57BL/6J mice fed a normal pellet diet (ND) or a high-fat pellet diet (HFD) with *ad libitum* feeding (ALF) or 12-h time-restricted feeding (tRF) ($N = 8$ each).
B: Threshold shift of 16 kHz from 3 to 9-mo-old in each group with the statistic results.
C: ABR data at 16 kHz are compared in terms of feeding type (ALF/tRF) for each diet (ND/HFD) along the aging axis.
D: Body weight change during experimental period.
E: Body weight gain from 3 to 6-mo-old or 3 to 9-mo-old with statistics.
F: Change of plasma glucose and cholesterol levels and their statistic results at 6 months old.

metabolized in mitochondria, which are the major source of ROS production. It is assumed that energy metabolism is constantly and slowly progressing in the rest phase because it does not require a large amount of energy rapidly as in the active phase. Therefore, when energy is produced mainly from fat by consuming an HFD, ROS production might be slow and mild even in the active phase. In this regard, it is consistent that long-term feeding of HFD pellets in SAMP8 ameliorated not only AHL but also grip strength, cognitive function, and gene expression in the brain (Oike et al., 2020). In this study, in which SAMP8 mice were fed brittle pellets for a long duration, AHL was ameliorated, but there were no effects on other typical aging indicators. This supports the possibility that dietary hardness directly affects the progression of AHL. The auditory and masticatory systems are anatomically related, as the mammalian middle ear bone serves as the temporomandibular joint and its supporting tissues in its ancestors (Kitazawa et al., 2015). The impact and noise of the jaw when gnawing on hard pellets might cause direct damage to the inner ear. NIHL is known to cause an increase in ROS in the inner ear and subsequent inflammation owing to acoustic damage (Kurabi et al., 2017), and it is possible that the promotion of AHL by the hard pellets is a phenotype close to that of NIHL.

In this study, we found that a hard diet promoted the progression of hearing loss in three different mouse strains with early AHL onset. This suggests that hard pellet diets may promote hearing loss at least for mouse strains that are genetically vulnerable to hearing loss (model of early-onset hearing loss). Similarly, we might need to pay attention to hearing loss when feeding pets hard pellets. Although further research on the exact mechanism remains, we found that egg-derived ingredients can reduce the pellet hardness, and tRF can ameliorate the promotion of AHL owing to the hard pellets. We expect that these findings will be useful for future AHL studies in both humans and animals.

CRediT authorship contribution statement

Hideaki Oike: conceptualization, resources, investigation, formal analysis, validation, writing - original draft, supervision, project administration, funding acquisition. **Kaoru Kohyama**: Investigation, formal analysis, supervision, writing - review, and editing. **Hiroko Mochizuki-Kawai**: Investigation, formal analysis, and writing - review. **Kayo Azami**: Investigation and formal analysis.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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