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Pyrroloquinoline quinone and imidazopyrroloquinoline intake diminish mortality risk during midlife and improve muscular dysfunctions with age in mice

Keiko Odera, *[a] Yuka Tanaka,[a] Kazuto Ikemoto, [b] Masanori Tamakoshi[c] and Ryoya Takahashi *[a,d]

Pyrroloquinoline quinone (PQQ) and its derivative imidazopyrroloquinoline (IPQ) are nutritionally important vitamin-like compounds that exert various physiological effects, including cell-growth promotion, neuroprotection, and mitochondriogenesis stimulation. This study investigated the potential of PQQ and IPQ as geroprotectors that promote healthy longevity, addressing the general lack of lifespan aging intervention experiments in mammals. We conducted lifelong and midlife experiments with 0.02% (w/w) PQQ and 0.02% (w/w) IPQ supplementation in the senescence-accelerated mouse P8 strain that is characterized by a short lifespan. In lifelong experiments, the survival days at the 75th percentile was prolonged by 73% and 36% in the PQQ and IPQ groups, respectively, compared with that in the control. In addition, significant delays in the appearance of aging and age-related muscular dysfunction were observed. Intake of PQQ and IPQ diets from midlife improved muscular function that had declined with age. IPQ intake reduced lipid accumulation in adipose tissue and the liver. To the best of our knowledge, this is the first study to demonstrate that PQQ and IPQ supplementation, whether initiated in the early or middle age, is effective in ameliorating age-related alterations, such as muscular function, and diminishes mortality risk during midlife in mice.

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1. Introduction

Maintaining and improving quality of life (QOL) and extending a healthy lifespan are among the most important objectives for an aging society. Sarcopenia, the age-related decline in muscle mass and strength, substantially reduces QOL in older adults.[1,2] This decline in muscle function restricts daily activities such as standing, walking, and posture control, while contributing to cognitive aging and shortened lifespan.[1–4] Notably, obesity in older adults (sarcopenic obesity) is closely associated with frailty, cardiometabolic dysfunction, physical disability, and increased mortality.[5–7]

Senescence-accelerated mouse (SAM) strains, developed by Dr T. Takeda's research team at Kyoto University, Japan, are a line of inbred mice characterized by a shorter lifespan of ~1 year and specific accelerated aging phenomena.[8–10] For example, the senescence-prone mouse P8 (SAMP8) strain has a shorter lifespan of ~1 year and exhibits age-related learning/memory deficits and sarcopenia similar to those observed in older adults.[8–12] Therefore, SAMP8 mice are frequently used to evaluate the effects of natural geroprotectors on lifespan, learning and memory, and sarcopenia over a short period of time.

In recent years, pyrroloquinoline quinone (PQQ), a novel coenzyme for various bacterial dehydrogenases, has attracted attention as a potential geroprotector.[13–15] Reduced PQQ has a stronger free radical scavenging ability than vitamin C[16] and has been reported to promote mitochondrial biogenesis[17] and improve brain function.[18,19] Moreover, PQQ-deficient diets cause growth retardation and impaired immune responses in mice.[20–22] Notably, imidazopyrroloquinoline (IPQ), converted from PQQ in the presence of glycine, lacks redox activity but shows almost the same biological activities as PQQ.[23] However, despite the potential anti-aging effects of both PQQ and IPQ, no long-term studies have investigated their impact on the aging process in mice.

In this study, we examined the effects of lifelong intake of PQQ and IPQ starting from weaning on lifespan, apparent

[a]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan.
E-mail: takahasi@phar.toho-u.ac.jp, k-odera@phar.toho-u.ac.jp
[b]Niigata Research Laboratory, Mitsubishi Gas Chemical Company, Inc., 182 Tayuhama, Kita-ku, Niigata-city, Niigata 950-3112, Japan
[c]Life Science Division, Green Energy & Chemicals Business Sector, Mitsubishi Gas Chemical Company, Inc., Mitsubishi building, 2-5-2 Chiyoda-ku, Tokyo 100-8324, Japan
[d]TaKaRyo Life Science Research and Education Lab (TaKaRyo Lab), 1-12-18 Asahicho, Funabashi, Chiba 273-0041, Japan

aging, and age-related changes in muscle function. We also investigated the effects of PQQ and IPQ administration starting in middle age on muscle function and age-related fat accumulation.

2. Materials and methods

2.1 Chemicals

PQQ disodium salt (4,5-dioxo-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid disodium) was obtained from Mitsubishi Gas Chemical (Tokyo, Japan). IPQ trisodium salt (7-oxo-7,10-dihydroimidazo[4,5,1-*ij*]pyrrolo[2,3-*f*]quinoline-1,3,9-tricarboxylic acid trisodium) was synthesized from BioPQQ and glycine (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), as previously described.[24]

2.2 Ethics statement

All animal experimental procedures were approved by the Toho University Animal Care and Use Committee (approval no. 21-41-486, 22-42-486, 21-486, 25-592) and conducted according to the guidelines of the Laboratory Animal Center of the Faculty of Pharmaceutical Sciences, Toho University (Chiba, Japan).

2.3 Animals and experimental design

SAMP8 strain mice were obtained from Dr T. Takeda (Chest Disease Research Institute, Kyoto University, Kyoto, Japan).[8] The mice were bred through brother–sister mating and maintained under controlled SPF conditions at 23 ± 1 °C, 50–60% humidity, a 12 h light/dark cycle (lights on from 08:00–20:00) at the Laboratory Animal Center of the Department of Pharmaceutical Sciences, Toho University. Five mice were placed in one cage [280 (*L*) × 170 (*W*) × 140 (*H*) mm] and fed a long-term maintenance diet (CE-7, CREA, Tokyo, Japan) and provided water *ad libitum*. Under these conditions, the mean lifespan (MLS) of male SAMP8/Toho (SAMP8) mice was ~13 months.

A schematic of the experimental timeline is shown in Fig. 1. The number and age of animals used in the following experiments are shown in Table S1. Experiment 1 investigated the effects of lifelong PQQ and IPQ intake on lifespan and age-related alterations. To minimize bias caused by placing the first mice selected by the researcher within the same group, or by placing pups from the same mother in the same experimental group, we randomly selected male pups born from the same dams and distributed them individually into three groups after weaning (3 weeks old). By repeating this process, the average weight (± standard error) of the mice became nearly identical between groups: control group, 18.9 ± 0.6 g (n = 15); PQQ group, 19.0 ± 0.5 g (n = 16); IPQ group, 19.0 ± 0.6 g (n = 15). After confirming the lack of statistically significant group differences in the average body weight, five mice were housed per cage. After 1 week acclimation, mice were provided purified diet pellets (AIN-93M, Oriental Yeast, Tokyo, Japan) containing 0.02% (w/w) PQQ or 0.02% (w/w) IPQ *ad libitum*. The average daily PQQ intake by young adult (3 months old) mice was ~25 mg per kg body weight. This dose was effective in our previous experiments on obese mice[25] and is within the safe dose range (100–200 mg kg^{-1}) for humans.[26] Control mice were provided with AIN-93M diet pellets *ad libitum*. Weight measurement, survival analysis, appearance evaluation, muscle function evaluation, and blood tests were performed periodically during the intake period (Fig. 1c).

Experiment 2 investigated the effects of midlife PQQ and IPQ intake on age-related changes. Male 31 week-old (7.1 month-old) SAMP8 mice maintained on a long-term maintenance diet (CE-7, CLEA, Tokyo, Japan) were randomly selected from a housing cage (5 mice per cage) and equally distributed individually into the control, PQQ, and IPQ groups; five mice were housed per cage. The average body weight (± standard error) of the control, PQQ, and IPQ groups was 33.2 ± 0.8 g (n = 12), 31.9 ± 0.5 g (n = 11), and 32.7 ± 0.8 g (n = 13), respectively, confirming no statistically significant group differences. The mice were allowed to acclimate for 2 weeks and then fed an AIM-93M diet containing 0.02% (w/w) PQQ or 0.02% (w/w) IPQ for 16 weeks. The control group was fed an AIM-93M diet. Weight measurements, survival analysis, blood analysis, and muscle function evaluations were conducted periodically during the intake period (Fig. 1d).

2.4 Evaluation of the degree of appearance aging

To evaluate the degree of appearance aging in the mice, we adopted the grading score system developed by Hosokawa *et al.*[27] In this experiment, three trained evaluators independently evaluated gloss, coarseness, hair loss, and skin ulcers on a five-point scale (grade 0, no particular change, to grade 4, most severe change). The sum of these scores was used as the integument ageing score. The evaluation was performed every 3–4 months.

2.5 Muscle function evaluation

Muscle function was evaluated using a four-limb hanging,[28] two-limb hanging,[29] and four-limb grip strength test[30] every 3 months (at 3, 6, 9, and 12 months of age) in Experiment 1, and before the start of intake, midway (week 6), and at the final week (week 12) in Experiment 2 (Fig. 1c and d).

The detailed muscle function evaluation methods were as follows:

(a) Four-limb hanging test: mice were placed on a stainless-steel mesh (size: 385 mm × 270 mm, mesh size: 10 mm × 10 mm, wire diameter: φ1.0 mm). When the mice settled in the center, the mesh was inverted, and the time (s) spent hanging upside down using all four limbs was measured. The test was terminated after 180 s. Each mouse was measured four times, with breaks in between, and the longest time was recorded as the test score. For mice that exceeded the test time limit, the score was recorded as 180 s. The score was calculated as hanging time (s) × body weight (g) to account for the effect of body weight.

(b) Two-limb hanging test: a single stainless steel wire [500 mm long (300 mm range of motion), 2.0 mm diameter]

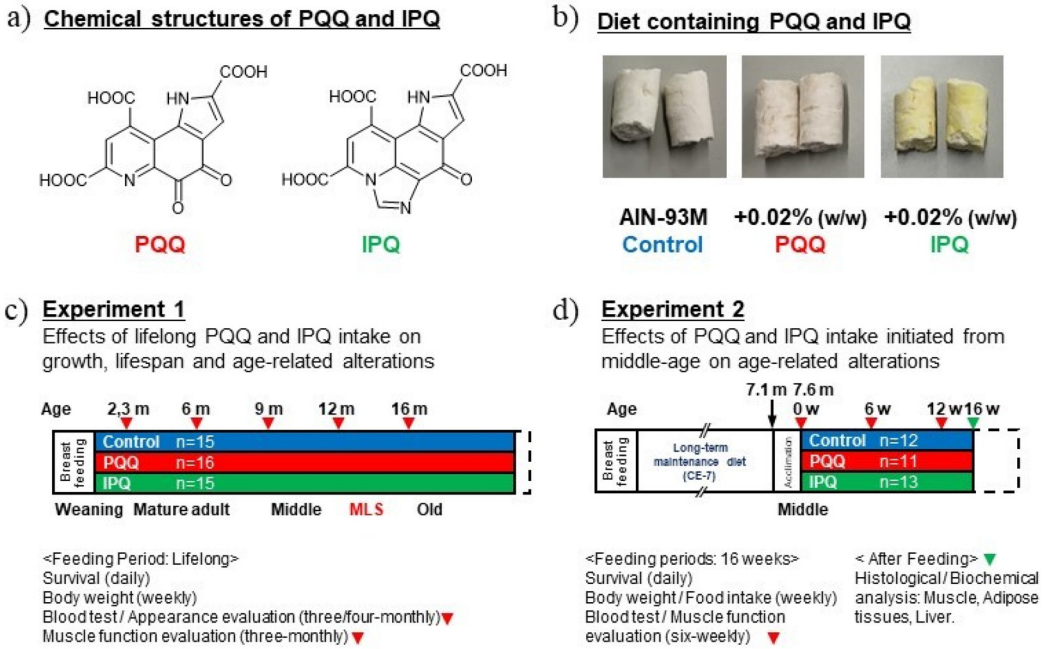

Fig. 1 Materials used and experimental designs for the male mouse study. (a) Chemical structures of pyrroloquinoline quinone (PQQ) and imidazopyrroloquinoline (IPQ). (b) Control (AIN-93M), 0.02% (w/w) PQQ-, and 0.02% (w/w) IPQ-containing diets (AIN-93M). (c) Experiment 1 investigated the effects of lifelong PQQ and IPQ intake on growth, lifespan, and aging-related alterations in 4 week-old male SAMP8 mice. Periodically, weight, food-intake, and survival were checked, blood tests were performed, and appearance and muscle function were evaluated. (d) Experiment 2 examined the effects of PQQ and IPQ intake on muscle function and lipid accumulation in middle-aged (7.6 months old) male mice. During the PQQ and IPQ intake period, survival and muscle function were monitored, and blood tests were performed, as in Experiment 1. After the intake period, muscle, adipose tissue, and liver were removed and analyzed histologically and biochemically. The number and age of animals used for Experiments 1 and 2 are shown in Table S1.

was suspended at a height of 50 cm, and the time (s) that a mouse could hang from it using its forelimbs was measured. The test was terminated at 180 s, which was the longest time allowed. Each mouse was measured four times, with a break in between, and the longest time was used as the test score. For mice that exceeded the longest time, the score was set to 180 s. To account for the effect of body weight, the score was calculated as hanging time (s) × body weight (g).

(c) Four-limb grip strength test: mice were allowed to grasp a stainless steel mesh (size: 100 mm × 100 mm, mesh size: 10 mm × 10 mm, wire diameter: φ1.0 mm) attached to a digital force gauge (DS2-50N, IMADA, Aichi, Japan) with all four limbs, and the force (N) exerted when the mice were pulled away from the mesh was measured. Each mouse was measured thrice, with a break of at least 1 min between two measurements, and the maximum value was recorded. The score was calculated as "force (N)/body weight (g).

2.6 Blood analysis

A small amount of blood (<50 μL) was collected from the tail vein of each mouse at 11:00–14:00 every 3 months in Experiment 1 and every 6 weeks in Experiment 2, and a microblood test was immediately performed. The levels of blood glucose, triglyceride, and high-density lipoprotein (HDL)-cholesterol were measured using a Cholestech LDX cassette on a compact rapid biochemistry testing device (Cholestech LDX Scan-Moni, Abbott Diagnostics Medical, Tokyo, Japan). The β-ketone level was measured using a Freestyle Precision Neo (Abbott) device with a β-ketone Measurement Electrode III. Plasma total cholesterol concentration was measured using a Total Cholesterol Assay Kit (CBL Cell Biolabs, San Diego, CA, USA).

2.7 Histochemical analysis

The mice were deeply anesthetized with isoflurane, the abdominal cavity was opened, and whole blood was collected from the inferior vena cava using a syringe. The livers and adipose tissues were immediately removed, submerged in ice-cold PBS, and weighed. The middle sections of the left lobe of the liver and adipose tissues were fixed in 10% neutral-buffered formalin for histological analysis of lipid droplets. The number and size of adipocytes and lipid droplets observed in hematoxylin–eosin (H&E)-stained paraffin sections of adipose tissues and the left lobe of the liver were analyzed using an all-in-one fluorescence microscope (BZ-X800) equipped with hybrid cell counting and analysis software (Keyence, Osaka, Japan).

2.8 Western blot analysis

The relative levels of target proteins in the muscle and liver were determined using western blot (WB) analysis, as previously described.[31] Briefly, equal amounts of homogenized

protein were subjected to SDS-PAGE on a 5–20% acrylamide gel (c-PAGEL, ATTO, Tokyo, Japan), and the protein-containing gel was transferred onto a PVDF membrane. Membranes were blocked with 3% BSA and incubated with the primary antibodies in Table S2. After washing, the membranes were incubated with an HRP-conjugated secondary antibody and visualized using Pierce ECL Plus Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Western blot signals were detected and analyzed using an Amersham Typhoon scanner (Cytiva, Marlborough, MA, USA).

2.9 Fatty acid oxidation assay

Liver fatty acid oxidation activity was determined using a fatty acid oxidation kit (Assay Genie, Dublin, Ireland) with octanoyl-CoA as a substrate, using the supernatant from frozen–thawed liver homogenate centrifuged at 10 000*g*, according to the manufacturer's protocol.

2.10 Statistical analysis

All statistical analyses were performed using the JMP statistical software package (SAS Institute, Cary, NC, USA). For the lifespan analysis, survival curves were estimated for each group separately using the Kaplan–Meier method, and compared statistically using the log-rank and Gehan tests. A one-way ANOVA was performed, followed by Dunnett's test, to compare the mean measurements between the treatment and control groups. A paired-sample *t*-test was used to compare the means between two related groups of samples. An ANCOVA was used to compare the regression lines. The Kruskal–Wallis test was used to determine statistically significant differences between the medians. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Effects of lifelong PQQ and IPQ intake on growth, lifespan, and age-related alterations (Experiment 1)

Experiment 1 investigated the effects of lifelong PQQ and IPQ intakes on age-related changes in growth, lifespan, blood biochemical properties, appearance, and muscle function.

3.1.1 Growth and lifespan. No significant differences were observed in body weight gain among the control, PQQ, and IPQ groups during the growth period from weaning (Fig. 2a and S1). After 4 months of age, weight gain in the control group nearly stopped, whereas that in the PQQ and IPQ groups continued to increase gradually. At 5 months of age, the mean body weights of the PQQ and IPQ groups were significantly higher (~10%) than that of the control group and remained so thereafter. Weight loss in older animals began at ~10 months of age, and the body weight of the IPQ group decreased to a value similar to that of the control group. However, the PQQ group maintained a value ~10% higher than that of the control group until an older age (Fig. 2a).

Food intake was ~3.8 g per mouse per d in all groups during the growth period (up to ~3 months of age, Fig. S1b) and then gradually decreased with age in all groups, reaching ~65% of the 3 month-old level (~2.5 g per mouse per d) at 20 months of age (Fig. S2). However, no apparent difference was observed in the overall changes in food intake over the lifespan among the control, PQQ, and IPQ groups, indicating that the differences in body weight after 5 months of age were not because of differences in food intake owing to food color or other factors (Fig. 1b).

Fig. 2b shows the Kaplan–Meier survival curves (uncensored) for the control, PQQ, and IPQ groups. Survival began to decline at 6.1 months of age in the control group, whereas the

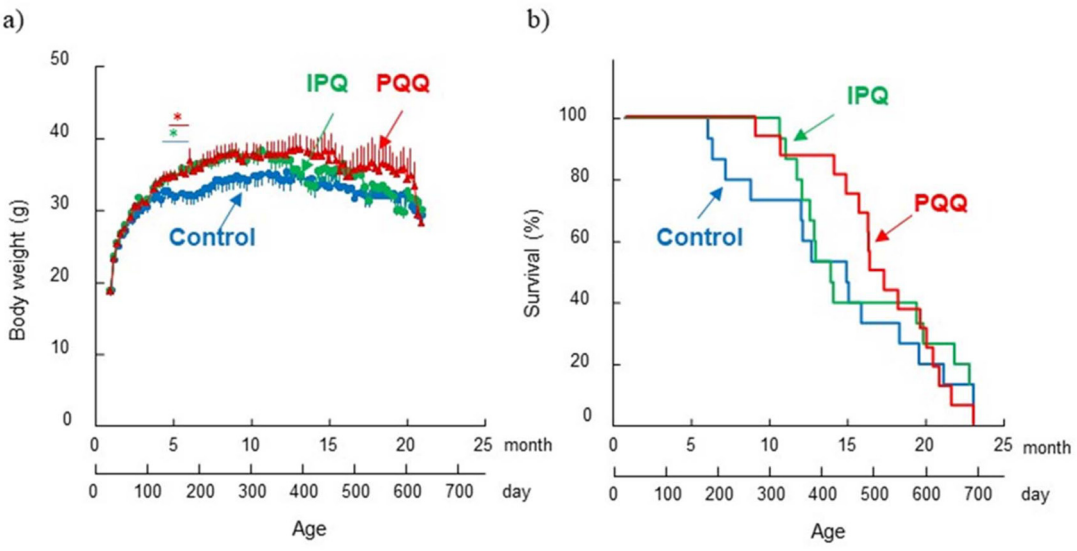

Fig. 2 Effects of PQQ and IPQ intake on body weight and lifespan of male SAMP8 mice. (a) Age-related change in body weights of control (blue), PQQ (red), and IPQ (green) groups. Compared with the control group, * $p < 0.05$ (Dunnett's *post-hoc* test). Data are presented as the mean ± SEM. (b) Kaplan–Meier survival curves of control (blue), PQQ (red), and IPQ (green) groups. The survival curves were analyzed by log-lank and Gehan tests (see section 3.1.1. Growth and lifespan).

onset of death was significantly prolonged to 9.1 and 10.6 months in the PQQ and IPQ groups, respectively, indicating a delay in the onset of death. The survival curves of the PQQ and IPQ groups were further analyzed using the log-rank test and Gehan (Gehan–Breslow–Wilcoxon) test, which emphasizes the change in early survival rate (Table 1). In the comparison of overall survival between the control and PQQ groups (log-rank test: p = 0.6570, Gehan test: p = 0.1559), and that between the control and IPQ groups (log-rank test: p = 0.7957, Gehan test p = 0.4247), the p-values for both PQQ and IPQ were smaller in the Gehan test than in the log-rank test, suggesting that the mortality risk from young to middle age may be diminished by PQQ and IPQ intake. Therefore, we examined survival days at the 75th, 50th, and 25th percentiles in the PQQ and IPQ groups compared with the control group (Table 2). The survival days at the 75th percentile were extended by 73.2% and 36.1% in the PQQ and IPQ groups, respectively. The PQQ group showed significant differences in both the log-rank and Gehan tests in the intervals up to the 75th and 50th percentiles compared with the control group (Table 3). In the IPQ group, significant differences were observed using both tests in the interval up to the 75th percentile compared with the control group. These results suggest that PQQ and IPQ intake, particularly PQQ intake, reduced mortality in middle-aged mice.

3.1.2 Blood biochemical properties. The effects of lifelong PQQ and IPQ intake on age-related changes in blood glucose, triglyceride, β-ketone, HDL-cholesterol, and plasma total cholesterol levels are shown in Fig. S3. The blood glucose (Fig. S3a) and β-ketone levels (Fig. S3c) remained relatively stable throughout life in all three groups. Triglyceride levels gradually decreased with age, reaching 50–60% of their young levels in old age, but no significant difference was observed among the three groups (Fig. S3b). Conversely, HDL-cholesterol levels increased with age in all groups, reaching ~1.5 times the levels at 12 months of age compared to those at 3 months of age (Fig. S3d). Plasma total cholesterol levels tended to be higher in the control group at young ages, but remained stable at ~150 mg dL^{-1} in the middle- and old-age groups. In the PQQ and IPQ groups, the levels remained ~150 mg dL^{-1} throughout life (Fig. S3e).

3.1.3 Appearance aging. Photographs of age-related changes in integument conditions are shown in Fig. 3a and S4. In all three groups, the coat condition gradually deteriorated with age. In particular, glossiness and coarseness showed significant age-related changes (Fig. S5b–S5e). Notably, in the PQQ and IPQ groups, the increase in the total grading score was significantly slower than in the control group (Fig. 3b). However, at older ages (after 12 months), no differences were observed among the three groups (Fig. S5). This is because mice with poor coat conditions tended to have relatively short lifespans, whereas mice with good coat conditions tended to have longer lifespans.

3.1.4 Muscle function. The four-limb hanging strength of the PQQ group was higher than that of the control group at 3 months of age. Limb hanging strength decreased with age at approximately the same rate as that in the control group. The performance of the IPQ group was similar to that of the control group at 3 months of age, but the age-related decrease in the rate of muscle strength was slower (Fig. 4a).

The two-limb hanging test showed a performance trend similar to that of the four-limb hanging test. Performance in the PQQ group was ~1.4 times higher than that in the control group at 3 months of age; however, both groups declined at approximately the same rate with age (Fig. 4b). The rate of decline in muscle function with age in the IPQ group was approximately half that in the control group.

Performance in the four-limb grip strength test declined with age in all three groups, with no significant differences between groups (Fig. 4c).

Table 1 Statistical results of log-rank and Gehan tests for overall Kaplan–Meier survival curves in male SAMP8 mice during PQQ and IPQ intake

Groups	Number	Median, d (95% CI)	Log-rank p-Value	Gehan p-Value
Control	15	455 (291–588)	—	—
PQQ	16	513 (453–610)	0.6570	0.1559
IPQ	15	422 (356–602)	0.7957	0.4247

Data are presented as median age in days, with 95% confidence interval (CI) in parentheses.

Table 2 Survival days in the 75th, 50th (median), and 25th percentiles for the control, PQQ, and IPQ groups

	75th percentile		50th percentile (median)		25th percentile	
Groups	d	% extension	d	% extension	d	% extension
Control	269	0	455	0	596	0
PQQ	466	73.2	513	12.7	617	3.5
IPQ	366	36.1	422	−7.3	633	6.2

Table 3 Log-rank and Gehan tests for Kaplan–Meier survival rates in the 75th, 50th, and 25th percentiles

		75th percentile		50th percentile (median)		25th percentile	
Control *vs.*	Number	Log-rank (p)	Gehan (p)	Log-rank (p)	Gehan (p)	Log-rank (p)	Gehan (p)
PQQ	16	0.0067**	0.0114*	0.0109*	0.0165*	0.0915	0.0533
IPQ	15	0.0067**	0.0114*	0.6197	0.2463	0.4010	0.4542

Log-rank and Gehan tests, * $p < 0.05$, ** $p < 0.01$.

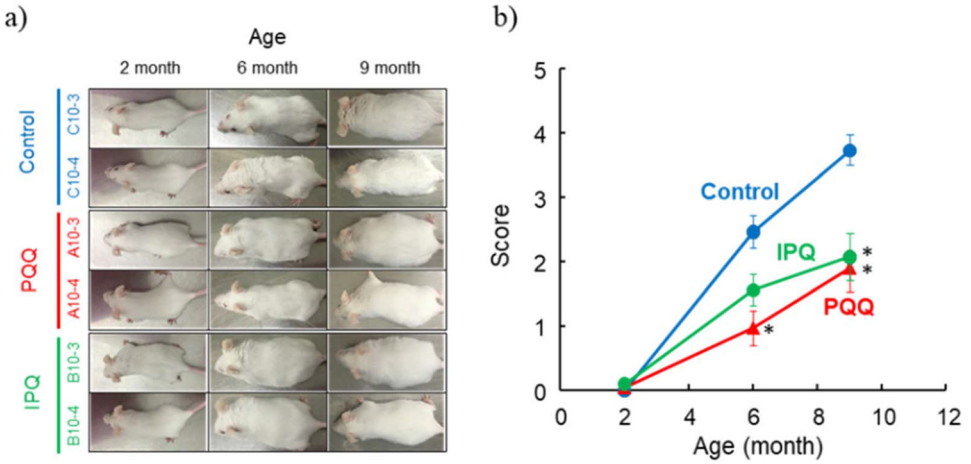

Fig. 3 Effects of PQQ and IPQ on the appearance aging of male SAMP8 mice. (a) Representative images of the backs of mice of different ages in the control, PQQ, and IPQ groups. (b) Age-related changes in integument grading scores with age. Compared with the control, * $p < 0.05$ determined using one-way ANOVA followed by Dunnett's *post-hoc* test. Data are presented as the mean ± SEM. Control, n = 11–15; PQQ, n = 14–15; IPQ, n = 13. Details of the number of mice used at each age in the experiment are shown in Table S1.

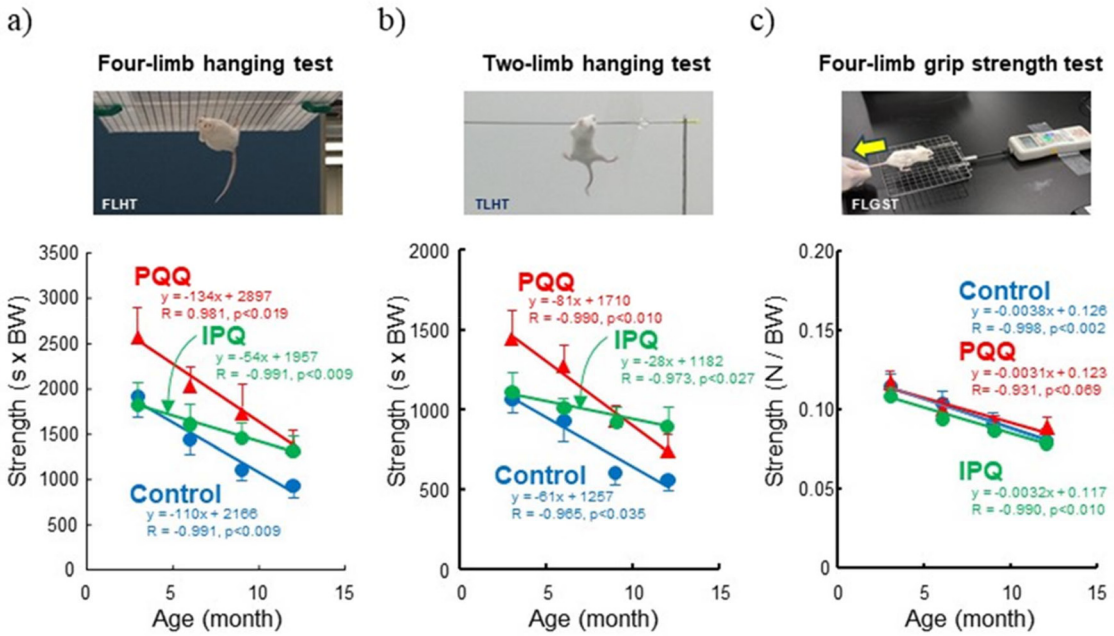

Fig. 4 Effects of PQQ and IPQ on age-related alterations in muscle function. (a) Four-limb hanging, (b) two-limb hanging, and (c) four-limb grip strength tests. The rate of change in muscle function with age was measured using a regression line calculated using the least-squares method. The linear regression equation ($y = ax + b$), Pearson's correlation coefficient (R), and significance level (p) for each test are shown. Control, n = 9–14; PQQ, n = 14–16; IPQ, n = 11–15. Details of the number of mice used at each age are shown in Table S1. Data are presented as the mean ± SEM. The differences in the slope and intercept between the three groups were tested using ANCOVA. In all three tests, no significant difference was observed in the slope between the control and PQQ groups and between the control and IPQ groups; a significant difference was observed in the intercept of the four- and two-limb hanging tests between the control and PQQ groups ($p < 0.05$).

3.2 Effects of PQQ and IPQ intake initiated from middle-age on age-related alterations (Experiment 2)

Experiment 2 investigated the effects of PQQ and IPQ intake on age-related changes from middle age onwards in mice fed a long-term maintenance diet after weaning and reaching 7.6 months (33 weeks) of age.

3.2.1 Changes in body weight and survival from middle-age. Fig. S6 shows the changes in body weight and amount of intake for diets containing PQQ and IPQ in 33 week-old mice after 2 weeks of acclimation. The daily food intake per mouse gradually decreased with age in all groups, falling to ~55% of that at the start of the experiment. However, the body weight remained relatively constant throughout the study in all three

groups. No significant differences in body weight or food intake were observed among the three groups.

Regarding survival rate, 3–4 deaths occurred in each group during the study period (Fig. S7). The first deaths occurred on days 41, 47, and 67 in the control, IPQ, and PQQ groups, respectively. Although no significant difference was observed between the survival curves of each group, the changes in the survival rate were similar to those observed in the long-term intake experiment (Experiment 1, Fig. 2b).

3.2.2 Blood biochemical properties. Blood glucose levels in the control, PQQ, and IPQ groups, which were 80–85 mg dL^{-1} before intake, increased to ~110 mg dL^{-1} in all groups after 6 weeks of treatment and remained elevated by week 12 (Fig. S8a). This level was similar to the mean blood glucose levels (100–115 mg dL^{-1}) of the age-matched mice in Experiment 1, suggesting that switching from the CE-7 long-term maintenance diet (CLEA, Japan) to the AIN-93M purified diet (Oriental Yeast) may have affected the increase in blood glucose levels.

In the control, PQQ, and IPQ groups, triglyceride levels decreased to 70–80% of the pre-treatment levels by week 12, but no significant differences were observed among the groups (Fig. S8b). Similar to blood glucose levels, HDL-cholesterol levels increased to 120–130% of the pre-treatment levels in all groups by week 12 (Fig. S8d). The changes in triglyceride and HDL-cholesterol levels may have been associated with age-related changes rather than the effects of dietary changes (see section 3.1.2. Blood biochemical properties).

The β-ketone and plasma total cholesterol levels remained relatively constant throughout the experimental period in the control, PQQ, and IPQ groups (Fig. S8c and S8e). No differences were observed in the hematocrit levels among the three groups at the end of the experiment (Fig. S8f).

These results indicate that no apparent change in blood biochemical properties occurred with PQQ or IPQ intake from middle-age onwards.

3.2.3 Muscle function. The muscle function of the 33 week (7.6 month)-old mice was approximately 30% lower than that of 3 month-old mice (Fig. 4).

The effects of PQQ and IPQ intake at middle-age on muscle function are shown in Fig. 5. The relative score in the four-limb hanging test was ~1.7 times higher after 6 weeks of PQQ intake than before the start of supplementation, but no significant difference was observed (Fig. 5a). In the IPQ group, the relative performance increased significantly by ~1.4-fold after 6 and 12 weeks of intake compared to that before the start of intake (Fig. 5a). Meanwhile, relative performance in the two-limb hanging test increased significantly by ~2.4-fold and 1.4-fold after 6 weeks of PQQ and IPQ intake, respectively, compared to that at the start of intake and remained high even after 12 weeks (Fig. 5b). No effect of PQQ or IPQ was observed on the four-limb grip strength score (Fig. 5c).

We investigated the relationship between muscle function, quantity and quality, and mitochondrial content in middle-aged mice fed PQQ and IPQ diets.

Fig. 5d shows representative photographs and weights of five hindlimb muscles [tibialis anterior (TA), quadriceps femoris (QF), soleus (SOL), plantaris (PL), and gastrocnemius (GC)] in mice fed PQQ and IPQ diets for 12 weeks from middle age. The average weights of the TA, QF, and GC muscles in the PQQ group were 5–15% higher than those in the control group, but this difference was not significant (Fig. 5d). No significant differences were observed in any of the muscle weights examined between the IPQ and control groups (Fig. 5d). To clarify the relationship between muscle weight and function, we conducted correlation analyses between muscle weight and four-limb hanging, two-limb hanging, and four-limb grip strength scores for each individual but no significant correlation was observed (Fig. S9).

Therefore, we investigated the differences in muscle quality by quantifying the fast-twitch myosin heavy chain isoform MYH4 and slow-twitch myosin heavy chain isoform MYH7 expressed in each muscle using western blot analysis. The expression level of MYH4 was high in the QF and GC, which are fast-twitch muscles, and low in the SOL, which is a slow-twitch muscle. Conversely, MYH7 was mainly expressed in the SOL (Fig. 5e and S10). However, no significant differences in MYH4 and MYH7 levels were observed between the control and the PQQ or IPQ groups in any muscle (Fig. 5e).

We investigated mitochondrial content, which affects muscle endurance, based on the electron transport chain proteins SDHA and ATP5A1, using western blot analysis. The levels of both mitochondrial proteins were ~2–3 times higher in the QF and SOL than in the GC (Fig. 5f and S11). However, no significant changes were observed between the PQQ and IPQ groups (Fig. 5f).

3.2.4 Adipose tissue weight and adipocyte size. We investigated the effects of PQQ and IPQ intake initiated from middle-age on the number and size of adipocytes in the subcutaneous and visceral fat. The weights of the subcutaneous fat (SUB), epididymal fat (EPI), retroperitoneal fat (RET), mesenteric fat (MES), and brown adipose tissue (BAT) are shown in Fig. 6a, b and S12.

The average weights of the five types of adipose tissues were slightly higher in the PQQ group than in the control group and, conversely, tended to be lower in the IPQ group. Although differences in adipose tissue weight were observed between the PQQ and IPQ groups, no significant differences were observed between the control and either treatment group (Fig. 6a, b and S12).

We analyzed the adipocyte size in the subcutaneous and epididymal fat of the PQQ and IPQ groups. Although no significant difference was noted in the adipocyte size of either tissue between the PQQ and control groups, a higher proportion of small cells was observed in the IPQ group (Fig. 6e–j and S13).

3.2.5 Accumulation of lipid droplets in liver. Since IPQ intake reduced the amount of adipose tissue, we examined the effects of PQQ and IPQ on the accumulation of lipid droplets in the liver during middle-age.

Fig. 7a–c and S14a show the livers in the control, PQQ, and IPQ groups. The livers in the control and PQQ groups were

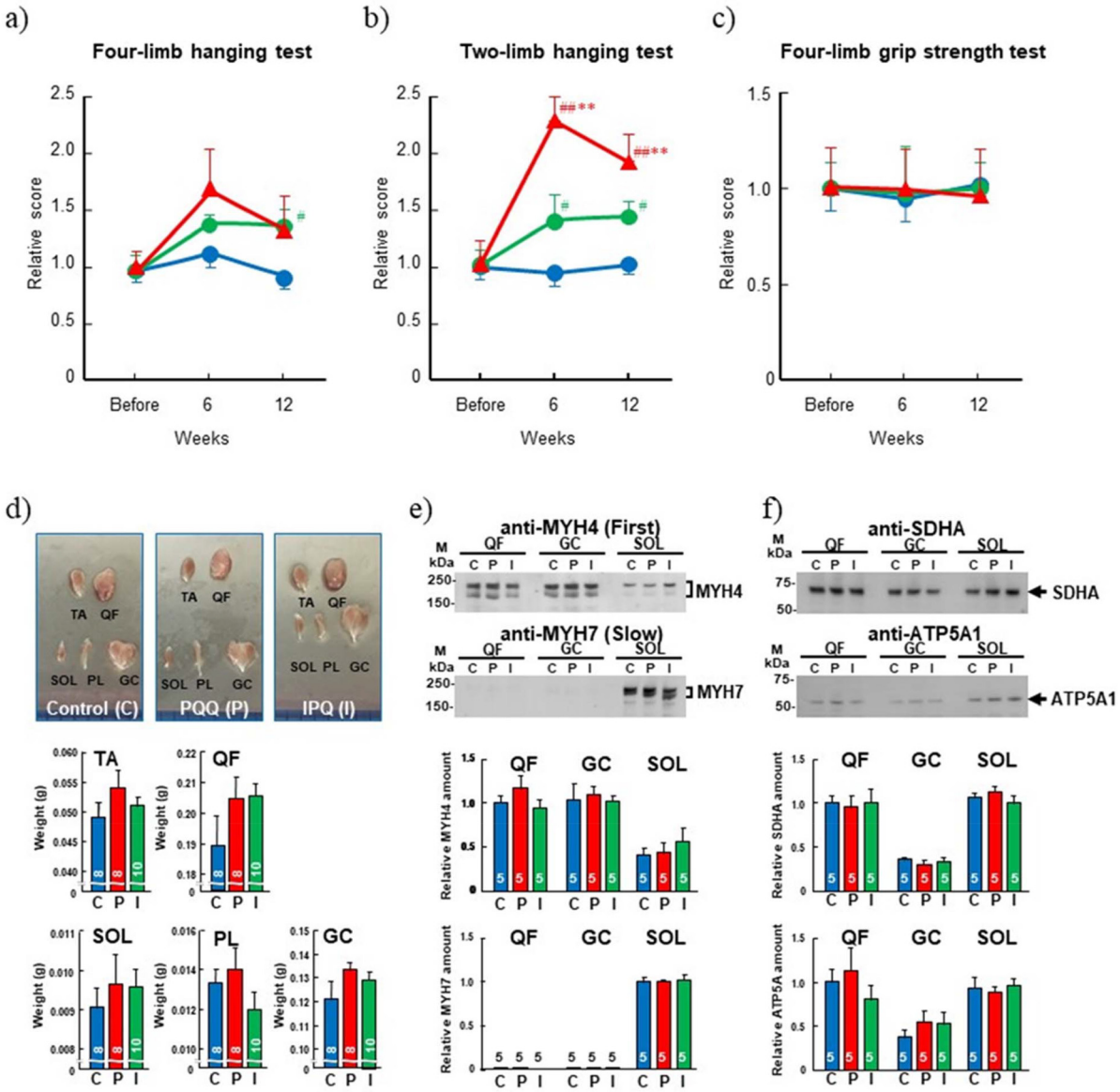

Fig. 5 Effects of PQQ and IPQ intake initiated from middle-age on muscle function in male SAMP8 mice. (a) Four-limb hanging, (b) two-limb hanging, and (c) four-limb grip strength tests. The scores for each test are expressed relative to the mean scores of the control (blue, n = 8–11), PQQ (red, n = 8–11), and IPQ (green, n = 10–13) groups before intake. (d) Muscle weight of hindlimb: tibialis anterior (TA), quadriceps femoris (QF), soleus (SOL), plantaris (PL), gastrocnemius (GC) muscles. (e) Western blot analysis of myosin heavy chain 4 (MYH4) and 7 (MYH7) in equal-amount mixtures of muscle homogenate from five mice in each group (raw data are shown in Fig. S10b). The relative levels in each group (Fig. S10c) were normalized to the relative level of pooled homogenate of each muscle. (f) Western blot analysis of succinate dehydrogenase complex subunit A (SDHA) and ATP synthase F1 subunit alpha (ATP5A1) (raw data and individual analysis are shown in Fig. S11b and c, respectively). C, control; P, PQQ; I, IPQ group. Compared with the group before initiation of diet, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ (paired t-test). Compared with the control group at the same age, * $p < 0.05$, ** $p < 0.01$ (Dunnett's *post-hoc* test). Data are presented as the mean ± SEM.

slightly whitish-ochre (Fig. S14), whereas the livers of many IPQ animals showed a dark ochre color, similar to that observed in young animals, and liver weight was slightly reduced (~10%, $p < 0.1$; Fig. 7d).

H&E-stained liver sections revealed the accumulation of lipid droplets of various sizes in all three groups. The average percentage of lipid droplet area in the liver was similar between the control (13.1 ± 1.8%) and PQQ (13.3 ± 3.7%) groups, but was reduced in the IPQ group (5.5 ± 4.2%, $p <$ 0.05) compared with the control group (Fig. 7a–c). In terms of the proportion of lipid droplet sizes, the IPQ group had a higher number of small lipid droplets relative to the control and PQQ groups (Fig. 7e–g).

3.2.6 Fatty acid oxidation activity in liver. Decreased mitochondrial quantity and function may lead to hepatic fat accumulation. The decrease in hepatic lipid droplets associated with the IPQ diet may be due to an increase in the mitochondrial number and/or enhancement of fatty acid metab-

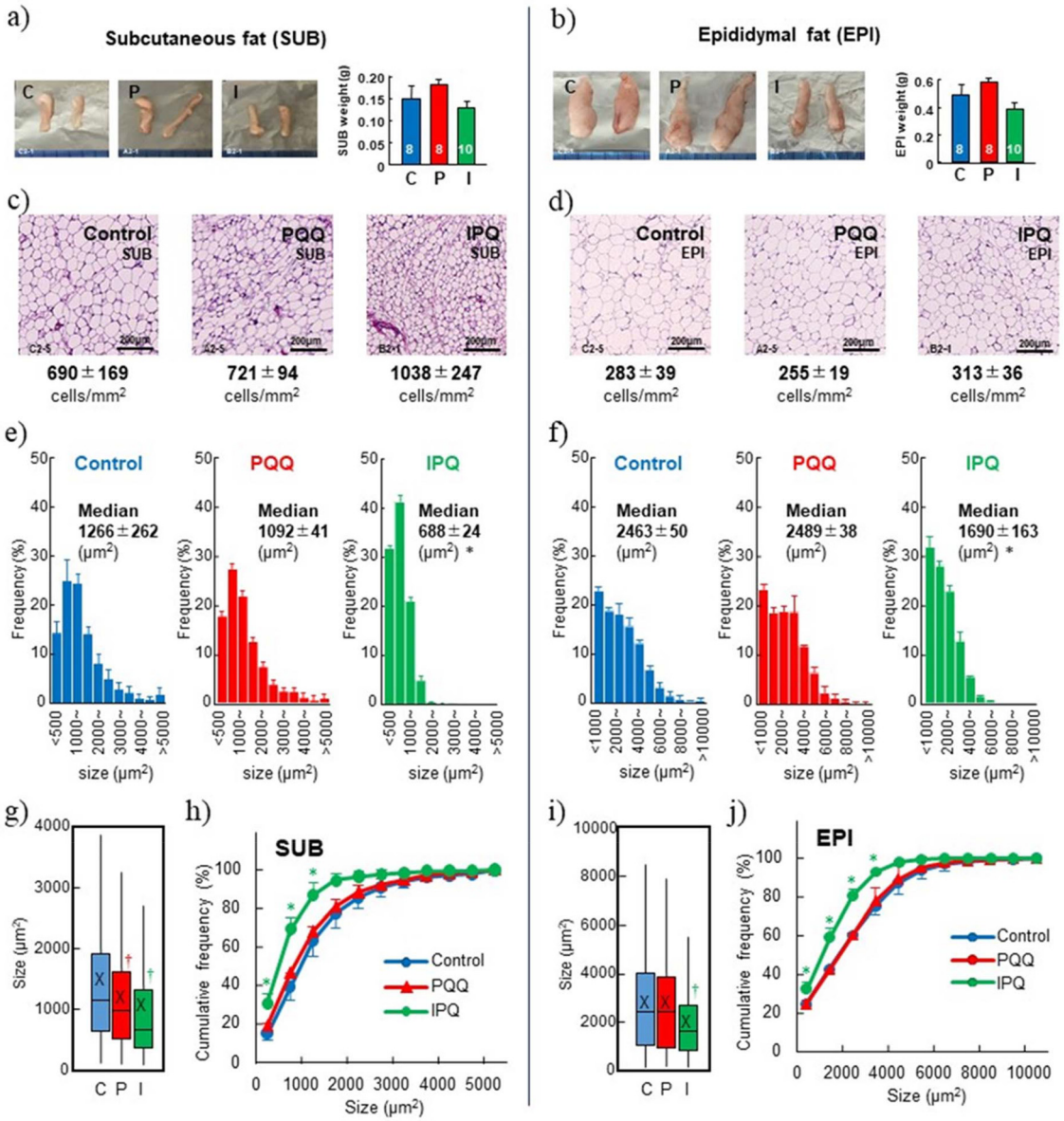

Fig. 6 Effects of PQQ and IPQ intake initiated from middle-age on adipose tissues in male SAMP8 mice. Weight of adipose tissue and adipocyte size distribution in subcutaneous fat (SUB) (left) and epididymal fat (EPI) (right). (a and b) Representative appearance and weight of adipose tissue. (c and d) Hematoxylin–eosin (H&E)-stained sections of adipose tissue and average number of adipose cells per mm^2. No significant difference was observed compared with control group (Dunnett's *post-hoc* test). (e and f) Relative frequency histograms and average of median. Compared with the control group in the same size range, * $p < 0.05$ (Dunnett's *post-hoc* test). (g and i) Box plots represent the median (bold line), mean (X), inter-quartile range (rectangle), and lower and upper limits (whiskers). Compared with the control group, † $p < 0.05$ (Kruskal–Wallis rank-sum test). (h and j) Cumulative distribution of adipocyte sizes. Compared with the control group in the same size range, * $p < 0.05$ (Dunnett's *post-hoc* test).

olism. Accordingly, we quantified mitochondrial levels *via* western blot analysis of the mitochondrial proteins SDHA and ATP5A1, and measured hepatic β-oxidation activity *in vitro* (Fig. S15a and S15b).

No significant differences were observed in the levels of the mitochondrial proteins among the control, PQQ, and IPQ groups. In contrast, mitochondrial β-oxidation was ~20% higher in the PQQ group than in the control group, whereas the activity in the IPQ group was similar to that in the control group (Fig. S15d).

These results suggest that IPQ intake reduced the amount of lipid droplets in the livers of middle-aged mice without affecting mitochondrial levels or fatty acid oxidation activity.

4. Discussion

4.1 Effects of lifelong PQQ or IPQ intake

Research into longevity-promoting compounds, such as geroprotectors, which may promote healthy aging and extend the

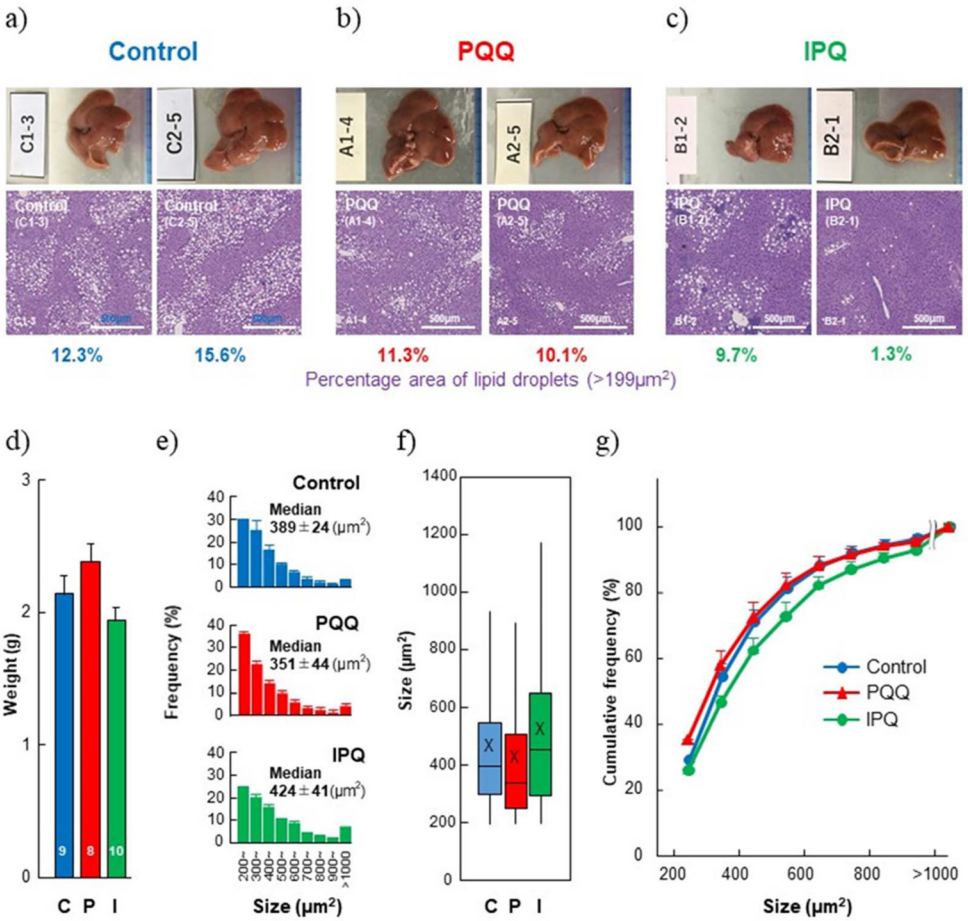

Fig. 7 Effects of PQQ and IPQ intake initiated from middle-age on accumulation of lipid droplets in the liver of male SAMP8 mice. Representative liver appearance and H&E-stained tissues in the control (a), PQQ (b), and IPQ (c) groups. The numbers below each H&E-staining image indicate the percentage area of the lipid droplets. (d) Liver weight. The number of animals used is shown on each bar. (e) Relative frequency, (f) box plots, and (g) cumulative distribution of lipid droplets (n = 3). Data are presented as the mean ± SEM. C, control group; P, PQQ group; I, IPQ group.

human lifespan, has actively focused on plant compounds and microbial metabolites.[32–34] To evaluate the lifespan-extending effects of candidate geroprotectors, yeast and nematodes (*Caenorhabditis elegans*) are often used because of their short lifespans and relatively easy maintenance.[35,36] Various compounds have been reported to extend the lifespan of nematodes, including plant compounds (*e.g.*, resveratrol, curcumin, quercetin, astragalus polysaccharide, and ginsenosides), microbial products (*e.g.*, rapamycin), and metabolic intermediates and drugs (*e.g.*, nicotinamide mononucleotide, alpha-ketoglutarate, and metformin).[36] In contrast, compounds that extend the lifespan of mice maintained under normal conditions are limited to metformin[37] and rapamycin,[38,39] while resveratrol[40,41] and curcumin[41,42] have not shown promising effects. Even if some candidate geroprotectors are effective in invertebrates such as insects, they may not be effective in mammals such as mice. Indeed, recent data on the lifespan-extending effects of 20 compounds revealed that studies on *C. elegans* are unlikely to identify compounds that extend mouse lifespan.[43]

In nematodes, PQQ exhibited significant lifespan-extending effects, whereas IPQ showed little activity.[24] The longevity-promoting effect of PQQ is attributed to its redox properties,[24] whereas IPQ lacks redox activity.[18,23]

In our mouse experiments, although there were differences in the degree of lifespan-extending effects between PQQ and IPQ, both effectively suppressed the decline in early survival rates, suggesting that the mechanisms underlying lifespan regulation differ between nematodes and mammals, and the existence of a lifespan-promoting mechanism that does not solely depend on redox reactions. Elucidating this novel mechanism is an important subject for future research.

In addition to reducing early mortality, PQQ and IPQ delayed the appearance of aging. Both PQQ and IPQ intake maintained a healthy and glossy integument even in middle-aged and older individuals, suppressing the progression of appearance aging. Studies on twins have shown that people who appear to be younger than their chronological age live longer.[44,45] Indeed, in this study, mice that lived longer tended to have less severe fur aging (Fig. S4). This may be because the

biological activities of PQQ and IPQ, such as cell proliferation-promoting and radical scavenging activities, have a beneficial effect on cells that affect fur growth and luster.[13–15]

A notable effect of PQQ and IPQ was the attenuation of age-related decline in muscle function, particularly in the four- and two-limb hanging tests but not in the four-limb grip strength test (Fig. 4). The hanging test evaluates muscle strength, coordination, and endurance by measuring the time spent hanging from a wire, and is particularly sensitive to neuromuscular disorders, making it useful for assessing neuronal function in rodents.[46] These results suggest that PQQ and IPQ may be effective in improving neuromuscular function with aging. However, in the two hanging tests, the PQQ and IPQ groups showed different effects on age-related alterations. Specifically, PQQ intake improved muscle performance without altering the rate of age-related decline in muscle function. In contrast, IPQ intake did not affect muscle function in young animals but reduced the rate of decline with age (Fig. 3). The mechanisms by which PQQ and IPQ affect age-related alterations in muscle function may vary, requiring further exploration in future studies.

4.2 Effects of PQQ and IPQ intake initiated from middle-age

Aging intervention experiments targeting middle-aged individuals are important for addressing the realistic health concerns experienced during this life phase. Accordingly, we focused on the effects of PQQ and IPQ on muscle function and fat accumulation in middle-aged SAMP8 mice. This experiment was initiated at 7.6 months of age, which corresponds to ~50 years of age in humans.[47]

The muscle function of middle-aged (7.6 months old) mice was ~30–50% lower than that of young (3 month-old) mice (Experiment 1, Fig. 4). Notably, after 6 weeks of PQQ and IPQ administration in middle-aged mice, the performance in the four- and two-limb hanging tests recovered in both groups to almost the same level as that of young mice. However, PQQ and IPQ administration had no significant effect on muscle weight and fast- and slow-twitch myosin heavy chain isoform levels, suggesting that the improvement in muscle function was not due to changes in muscle quantity or quality. Furthermore, PQQ and IPQ had no significant effects on the mitochondrial levels (in terms of SDHA and ATP5A1 expression). An increase in mitochondriogenesis has been observed in the liver of middle-aged rats administered PQQ for 8 months.[48] The difference in our mouse study may be due to the shorter administration period (12 weeks) compared to the rat experiment (8 months) and/or species differences.

Our analyses indicated that both lifelong and midlife administrations of PQQ and IPQ improved neuromuscular function in mice. Neuromuscular function refers to the coordinated activity of the nervous system and muscles.[49–51] Signals from the brain travel through nerves to the muscles, enabling overall physical performance. PQQ and IPQ have been reported to protect neurons from various stressors and improve brain function.[18,19] The improvement in neuromuscular function by PQQ and IPQ may be due to the prevention and improvement of age-related dysfunction of the nervous system and neuromuscular junctions. Elucidating the mechanisms by which PQQ and IPQ prevent and improve age-related decline in neuromuscular function is an important future challenge.

Regarding body fat and health, the accumulation of excess fat in the body increases the risk of developing various age-related diseases and, consequently, the risk of death.[4–7] However, maintaining a moderate amount of body fat is important for older adults to reduce their risk of death from disease and maintain physical activity.[52,53] Previous experiments have shown that PQQ suppresses high-fat diet-induced obesity in young adult (8 week-old) mice[25] and also suppresses the age-related decline in fat mass in aged (83 week-old) mice.[54] These findings suggest that PQQ plays an important role in the inhibition of excessive lipid accumulation and loss, and regulation of fat storage in the body. In this study, the adipose tissue weight of middle-aged mice administered PQQ tended to be slightly higher than that of the controls, which may have contributed to their longevity (Fig. S12). In contrast, IPQ tended to reduce fat accumulation in the body, particularly by reducing hepatic lipid droplet accumulation. However, IPQ intake did not induce mitochondriogenesis or β-oxidation activity, suggesting that the reduction in hepatic lipid droplet accumulation may be due to the suppression of fat absorption from the intestine and fat synthesis in the body; this requires experimental verification in the future.

In this study, the effects of PQQ and IPQ in ameliorating age-related alterations differed greatly. Importantly, orally consumed PQQ may be converted to IPQ compounds during food digestion in the alimentary canal because PQQ is a highly reactive compound that readily reacts with various amino group-containing substances.[55,56] In mice, orally administered radiolabeled PQQ was readily absorbed in the lower small intestine and accumulated in the skin within 24 h.[57] Furthermore, a rapid increase in PQQ concentrations in serum was observed within a few hours after a single oral administration of PQQ in humans.[58,59] These results suggest that a large proportion of orally administered PQQ is absorbed without changing its form in the small intestine, delivered to various tissues, and taken up by cells,[60] potentially exerting its physiological effects. In addition, even if some of the PQQ were to change into IPQ, the biological activity of IPQ is almost the same as that of PQQ,[18,23] and is thus unlikely to have any adverse effects on physiological functions.

4.3 Possible molecular mechanisms by which PQQ and IPQ ameliorate age-related changes

Even without a clear cause, such as an infection, high serum levels of inflammatory markers may persist in older adults, and this chronic inflammatory state can contribute to age-related diseases.[61,62] This process, known as inflammaging, is thought to cause widespread systemic chronic inflammation across tissues, promoting the development of various age-related diseases such as arteriosclerosis, sarcopenia, frailty, and cognitive impairment.[63] The main causes of this uncontrolled inflammatory environment are cytokines and chemo-

kines. In aged cells and tissues, factors such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are actively synthesized and secreted, acting on surrounding normal cells to induce chronic inflammation—a phenomenon known as senescence-associated secretory phenotype. Therefore, eliminating inflammation is considered a promising means of promoting anti-aging.[61–63] In our previous experiment on aged C57BL/6J mice (83 weeks old) fed the same PQQ-containing diet (0.02% w/w AIN-93M) for 2 months, age-related muscle function declines were restored, and the levels of proinflammatory genes (regulating IL-6, IL-1β, and TNF-α) in the muscles were significantly reduced.[54] Inhibition of inflammatory responses by PQQ intake has also been observed in obese rats,[64] mice,[65] weaned piglets,[66] and humans.[58] The suppression of pro-inflammatory gene expression by PQQ administration has been associated with the inhibition of NF-κB activation in an IL-1β-treated SW982 cell culture system.[67] In the current study, PQQ and IPQ intake may have contributed to suppressing the decline in survival rates and improving muscle function in middle-aged individuals by reducing systemic inflammatory responses, including those in the muscles.

Alteration of mitochondrial function is closely related to the progression of aging, since it not only affects energy production and overall metabolic function but also increases the production of reactive oxygen species.[68] PQQ has been reported to exert intracellular antioxidant activity, reducing intracellular reactive oxygen species levels[54] and protecting mitochondrial function.[69] Although little is known of the effects of IPQ on aging compared to PQQ, it has been shown to exert protective effects against oxidative cytotoxicity induced by hydrogen peroxide and 6-hydroxydopamine in nerve cells and hepatocytes, and to induce the expression of PGC-1α, which promotes mitochondrial synthesis.[18] In addition, IPQ has been reported to enhance memory and learning ability in aged mice, similar to PQQ.[18] Although no lifespan-promoting effect was observed for IPQ in *C. elegans*,[24] the reduction in middle-age mortality in mice may be related to the indirect protective effects of PQQ and IPQ against cytotoxic stress. Further research is needed to elucidate the detailed mechanism.

4.4 Safety of PQQ and IPQ, and human subject research

The PQQ (BioPQQ) used in this study is a functional food ingredient developed by Mitsubishi Gas Chemical. It has been approved for use by the U.S. Food and Drug Administration (certified as a New Dietary Ingredient in 2008), Japanese Ministry of Health, Labour and Welfare (permitted as a health food ingredient in 2014), and European Commission (approved as a Novel Food in 2018).[70] Its safety has been confirmed in multiple studies.

IPQ, synthesized from PQQ and glycine, shows almost the same biological activities as PQQ.[23] It is important to distinguish IPQ from imidazoquinoline derivatives,[71,72] which are known for their potent immunostimulatory effects. Despite the similarity in their names, IPQ is structurally distinct. Due to the presence of three carboxyl groups, IPQ exhibits high water solubility, likely preventing excessive bioaccumulation and reducing toxicological risk. Previous toxicological assessments have also demonstrated that IPQ has a very low toxicity profile.[18] Consistent with this, the lifespan extension observed in the current study suggests that the risk of severe side effects—such as cytokine storms or organ dysfunction often associated with excessive immune activation—is minimal under our experimental conditions.

Although clinical research on the physiological functions of PQQ in humans is limited, preclinical studies on rodents are gradually increasing. For example, PQQ has been shown to reduce low-density lipoprotein cholesterol and triglyceride levels,[73] improve skin barrier damage and dry skin,[74] improve sleepiness,[75] improve task performance,[76] and improve composite and verbal memory.[19] Many of these reports focus on brain function. On the other hand, the antioxidant effects of PQQ help reduce oxidative stress, which is involved in many diseases. Clinical studies have shown the potential benefits of PQQ supplementation, including improved cardiovascular health, cognitive function, weight management, improved insulin sensitivity, and prevention of metabolic syndrome.[26] Future research should focus on determining the optimal dosage of PQQ for specific health benefits and evaluating its long-term efficacy and safety. We believe that the current research findings on long-term administration in mice will be useful for guiding future clinical research.

5. Conclusions

This study demonstrates that lifelong supplementation of PQQ and IPQ initiated after weaning in mice reduces mortality risk during midlife and delays the progression of aging and age-related decline in muscular function. Furthermore, middle-age supplementation improves age-related decline in muscular function. IPQ reduced hepatic lipid accumulation, indicating a new physiological function. PQQ and IPQ improve age-related changes regardless of whether intake begins in early or middle-age, although intake from early age is particularly effective in diminishing mortality risk during midlife. However, further research is needed to determine the optimal dosage, long-term safety, and detailed mechanism of action of PQQ and IPQ before they can be prescribed to maintain a healthy lifespan.

Author contributions

M. T., K. I., K. O., and R. T. developed the study concept and design. K. O. and M. T. were responsible for the execution of the study. K. O., M. T., and R. T. were involved in animal experiments, analysis, and data collection. K. O. and R. T. wrote the draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Authors K. I. and M. T. are employed by Mitsubishi Gas Chemical Company Inc. (MGC) (Tokyo Japan). Toho University's joint research is sponsored by MGC. This study was sponsored only by MGC.

Data availability

All data generated or analyzed during this study are included in this manuscript and are available from the corresponding author upon reasonable request.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d6fo00788k.

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