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ORIGINAL ARTICLE

# Age- and time-dependent mitochondrial genotoxic and myopathic effects of beta-guanidinopropionic acid, a creatine analog, on rodent skeletal muscles

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**Abstract** Beta-guanidinopropionic acid (GPA) is a creatine analog suggested as a treatment for hypertension, diabetes, and obesity, which manifest primarily in older adults. A notable side effect of GPA is the induction of mitochondrial DNA deletion mutations. We hypothesized that mtDNA deletions contribute to muscle aging and used the mutation promoting effect of GPA to examine the impact of mtDNA deletions on muscles with differential vulnerability to aging. Rats were treated with GPA for up to 4 months starting at 14 or 30 months of age. We examined quadriceps and adductor longus muscles as the quadriceps exhibits profound age-induced deterioration, while adductor longus is maintained. GPA decreased body and muscle mass and mtDNA copy number while increasing mtDNA deletion frequency. The interactions between age and GPA treatment observed in the quadriceps were not observed in the adductor longus. GPA had negative mitochondrial effects in as little as 4 weeks. GPA treatment exacerbated mtDNA deletions and muscle aging phenotypes in the quadriceps, an age-sensitive muscle, while the adductor longus was spared. GPA has been proposed for use in age-associated diseases, yet the pharmacodynamics of GPA differ with age and include the detrimental induction of mtDNA deletions, a mitochondrial genotoxic stress that is pronounced in muscles

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that are most vulnerable to aging. Further research is needed to determine if the proposed benefits of GPA on hypertension, diabetes, and obesity outweigh the detrimental mitochondrial and myopathic side effects.

**Keywords** Guanidinopropionic acid · Aging · Skeletal muscle · Mitochondria · Genetics · Creatine

## Introduction

The prevalence of cardiometabolic disease increases dramatically with age and is greater than 49% in adults over age 65 in the USA [1]. This public health crisis is driving the search for interventions to improve metabolic health in older adults. Beta-guanidinopropionic acid (GPA) is a creatine analog that has been proposed as a therapeutic intervention for cardiometabolic diseases including type 2 diabetes and hypertension [2, 3]. GPA is also an additive in some commercially available, over-the-counter dietary supplements where it is touted to have beneficial metabolic and exercise-mimetic effects [4]. The use of GPA as an "exercise mimetic," and proposals to use GPA as a treatment for cardiometabolic disease in older adults necessitate a clear understanding of GPA effects in aged mammals.

Despite rodent studies of GPA and its effects on metabolism and skeletal muscle, and the proposed use of GPA for diseases of aging, there have been few studies of GPA in appropriately aged rodent models. In young rodents, GPA treatment has positive and negative effects on skeletal muscle structure, function, and metabolism [5, 6, 7, 8, 9] as well as myopathic alterations [10, 11]. The GPA-induced myopathy in young rodents includes abnormal mitochondria with paracrystalline inclusions similar to those observed in some human mitochondrial myopathies. In 28-month-old rats, markers of mitochondrial biogenesis in response to GPA were reduced compared to 3-month-old rats [12]. In 18-month-old mice, GPA delayed age-related declines in lean mass, improved gait performance, and increased fat metabolism [5]. A number of gaps remain in our understanding of GPA's effects on mammalian skeletal muscle. Little is understood about the effects of GPA on aged muscle. No comparisons have been made between muscles with different susceptibility to age-induced muscle mass loss, and a time course of these effects is lacking.

A spectrum of mtDNA deletion mutations increase with age. These deletions clonally accumulate to high abundance within individual cells. In muscle fibers, deletion accumulation results in a focal loss of electron transport and oxidative phosphorylation. This metabolic insufficiency activates apoptosis and leads to necrosis, fiber rupture, and fiber loss. In aged rat quadriceps, approximately 15% of muscle fibers harbor an accumulation of mtDNA deletion mutations [13], and genetic induction of these segmental biochemical defects in homozygous polymerase gamma mutator mice accelerates sarcopenia [14]. MtDNA deletion mutations are also observed in genetic diseases that arise from mutations in numerous nuclear genes required for normal mitochondrial function. The findings of a GPA effect in muscle that resembles mitochondrial genetic myopathies [15, 16, 17, 18] led us to compare the mitochondrial effects of GPA treatment initiated at different ages and in muscles that exhibit different aging phenotypes. Muscles such as the adductor longus are resistant to age-induced degeneration as compared to muscles of the quadriceps [19]. We hypothesized that GPA treatment would exhibit different effects depending on the age, length of treatment, and type of muscle examined. Because of the concerning muscle- and mitochondrial-specific side effects of GPA, we focused our methods on body, muscle, and muscle fiber morphometric properties, as well as mitochondrial respiratory function, mtDNA integrity, and gene expression profiles.

We found pleiotropic muscle and mitochondrial effects of GPA treatment when deployed at different ages, for different durations, and in different muscles. These outcomes interacted with age and GPA treatment in the quadriceps muscle with respect to mass, fiber number, and mtDNA deletion frequency, but were not observed in the adductor longus [19]. GPA increases mtDNA mutation frequency and increases fiber loss at old age more than at young age in rats, which may complicate the therapeutic use of GPA.

## Materials and methods

### Animals and GPA treatments

This study was carried out in accordance with the recommendations in the NIH Guide for Care and Use of

Laboratory Animals and the guidelines of the Canadian Council on Animal Care. The protocols used were approved by the Institutional Animal Care and Use Committees at the University of Alberta and UCLA. Male Fischer 344 × Brown Norway F1 hybrid rats of various ages were obtained from the National Institute on Aging (NIA) colony. Beta-guanidinopropionic acid (GPA, reagent grade, > 99% pure) was purchased from a commercial vendor (Green Stone Swiss Co., Ltd., Xiamen, China), and identity was verified by mass spectroscopy (data not shown). GPA was formulated to 1% by weight in NIH-31 rodent chow (Envigo, Madison, WI, USA) and fed for 1–4 months ad libitum. Oral delivery of GPA at this dose results in similar skeletal muscle GPA concentrations in young as compared to aged rodents [5]. Rats were housed on a 12-h light/dark cycle. No difference was observed in the survival of rats treated with GPA vs controls. Rat ages were selected based on our previously published molecular and histological data in aging rat quadriceps across the rat lifespan including measurements at three month intervals from 12 to 38 months of age [20].

### Tissue collection and preparation

We chose to study the quadriceps and adductor longus muscles. The quadriceps, one of the largest muscle groups in mammals, was chosen because this muscle group is susceptible to sarcopenia, has a mixed fiber type, can be cleanly dissected in the rat from origin and insertion landmarks, and is a component of human clinical assessments such as chair stands that predict disability, falls, and other health related outcomes such as hospitalization and death [21, 22, 23, 24, 25]. Adductor longus was chosen given the resistance of this muscle to sarcopenia as discussed above. Animals were euthanized, the tissues dissected, weighed, and embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen, and stored at − 80 °C. A minimum of one hundred 10-micron-thick consecutive transverse cross sections were cut with a cryostat at − 20 °C and placed on Probe-On-Plus slides (Thermo Fisher). Slides were stored at − 80 °C until needed. For the mouse studies, frozen quadriceps tissues from control or GPA-fed male mice were obtained from the NIA Intervention Testing Program (ITP) Collaborative Interactions Program. Treated mice had been fed a diet containing GPA at 3300 ppm (0.33%) starting at 6 months [26]. We examined quadriceps tissue from 12 and 22 months control and 22 months GPA-treated male UM-HET3 mice. Tissues for each age and treatment were pooled from across all three ITP testing sites.

### Histochemical and immunohistochemical staining

At 70-µm intervals, sections were stained for COX (brown) or SDH (blue) enzymatic activities as previously described [13]. A third slide was dual stained, first for COX and secondly for SDH. Electron transport chain (ETC) abnormal fibers appear blue on a brown background following dual staining. Two slides within the series were used for hematoxylin and eosin (H&E) staining. Fibrotic tissue was stained using Masson's trichrome, as previously described [27]. After histochemistry or immunohistochemistry, slides were imaged by a scanning microscope (Nanozoomer, Hamamatsu).

### Image analysis, counts, and quantitation

Cross-sectional area measurements and fiber counts of the rectus femoris were obtained from digital images at the mid-belly. Rectus femoris muscle is used for CSA measurements and fiber counts because it is entirely encapsulated by the vasti and has a low pennation angle to the muscle fibers, facilitating accurate and precise quantification. Individual fiber morphometric data was obtained by overlaying each rectus femoris image with ten regions of interest. Each ROI was a square with a length of 0.552 mm. Muscle fibers contained entirely within the ROI were measured using Image J software. Measurements obtained included the length of the minor and major axes, minimum ferret diameter, area and perimeter. From these values, the circularity ($4\pi$*CSA/perimeter$^2$), roundness (minor axis/major Axis) and solidity (CSA/area of fitted hull) were calculated. The fiber density is the area of fibers within a ROI divided by the contiguous area of fibers and interconnected interstitial tissue. The absolute number of ETC abnormal fibers (COX-/SDH + +) were identified and annotated across a 10-µm tissue section in both GPA treated and control rats.

### DNA isolation and quality control

Rat quadriceps muscle was ground to a powder using a mortar, pestle, and liquid nitrogen. Total DNA was

extracted using proteinase K digestion with SDS and EDTA, phenol/chloroform extraction, and ethanol precipitation as previously described [13]. Total DNA quantity and quality was measured using spectrophotometry at A230, A260, and A280 (Thermo Scientific Nanodrop 2000 Spectrophotometer), fluorometry (Thermo Fisher Qubit 2.0 Fluorometer), and integrity examined by gel electrophoresis or TapeStation 4200 (Agilent).

### MtDNA copy number and mtDNA deletion frequency by digital PCR

A 5-prime nuclease cleavage assay and chip-based digital PCR (dPCR) were used to quantitate copy numbers for nuclear DNA (nDNA), total mtDNA, and mtDNA deletions with specific primer/probe sets for each as previously described [20]. Samples were diluted to the manufacturer's recommended target range (200 to 2000 copies per microliter) in 17.5 µl reactions using QuantStudio 3D Digital PCR Master Mix v2 (Thermo Fisher; Waltham, MA) and loaded onto QuantStudio 3D Digital PCR 20 K Chip (Version 2, Thermo Fisher; Waltham, MA). Final primer and probe concentrations were 900 nM and 250 nM, respectively. Digital PCR cycling conditions were Taq-polymerase activation at 95 °C for 10 min, 40 cycles of denaturation at 94 °C for 30 s, and annealing/extension at 60 °C for 2 min. MtDNA copy number per microliter and the threshold were determined using QuantStudio 3D Analysis Suite Cloud Software (Version 3, Thermo Fisher; Waltham, MA). Direct quantitation of the major arc deletions by dPCR used the same cycling conditions but for 60 cycles. DPCR quantitation of all samples and for all targets was performed on blinded samples.

### RNAseq

Frozen muscle samples were cooled in liquid nitrogen and crushed to powder by a pestle in a liquid nitrogen–cooled mortar and 50 mg of powdered muscle transferred into 1 mL QIAzol Lysis Reagent (Qiagen) and homogenized using TissueRuptor (Qiagen) at full speed for 15 s, twice. Total RNA was isolated from the homogenate using RNeasy Mini Kit (Qiagen). RNA integrity and concentration were determined using Agilent Tapestation 4200 (Agilent Technologies Inc, Santa Clara, CA), and all samples had a RIN > 8. Using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA), 200 ng of total RNA was converted to cDNA and sequencing performed using the Illumina HiSeq 2000 system (Illumina, San Diego, CA).

Alignment of cDNA sequenced reads was carried out using Tophat v2.0.8. Reads counted by gene feature were performed by featureCounts in Rsubread 1.14.2. All RNAseq data were submitted to NCBI BioProject ID PRJNA793055.

### Respirometry assay on frozen tissue samples

Respirometry was performed on frozen skeletal muscle and cardiac tissue according to a validated protocol [28]. Briefly, frozen tissues were thawed in ice-cold PBS, minced, and homogenized. Protein concentration was determined by BCA Protein Assay (Thermo Fisher). Mitochondrial respiratory chain oxygen consumption rate (OCR) via complexes I, II, and IV were measured using a Seahorse XF96. Substrates used for the respirometry assays were NADH for Complex I, succinate for Complex II with rotenone to inhibit Complex I, TMPD for Complex IV, and ascorbic acid to keep TMPD in the reduced state. Seahorse Wave software (Version 2.6.1, Agilent) was used to calculate oxygen consumption rates normalized by protein concentration.

### Statistical analysis

All data are presented as means ± SEM. As some data were not normally distributed, we used rank transformation and Mann–Whitney *U* tests to make statistical comparisons. Comparisons were made between young and old controls as well as between young or old controls and age-matched GPA-treated rats. Unadjusted *p* values are reported, and no corrections are made for multiple comparisons. Because some data were not normally distributed, interactions between treatment and age were analyzed by linear regression and extra sum of squares *F*-tests. We tested the null hypothesis that the slopes were the same for control and GPA regressions with the alternative hypothesis that the slope were different for control and GPA.

## Results

Cohorts of 14-month-old and 30-month-old rats were randomized to control diet or diet compounded with GPA at 1% w/w in chow. Within age groups, body weights at baseline before treatment were not different between GPA-treated and control rats ($p=0.29$ and 0.44 for 14 months and 30 months, respectively; Fig. S1). After 4 months, tissues were collected from four experimental groups: 18-month-old rats on the control diet (18C), 18-month-old rats on the GPA diet (18G), 34-month-old rats on the control diet (34C), and 34 month-old rats on the GPA diet (34G). Following 4 months of GPA treatment, average rat body mass decreased by 14.4% at 18 month and by 19.1% at 34 months (Fig. 1A). Food consumption decreased upon GPA treatment in both the young and old rats (Fig. S2). With time, however, food consumption returned to a level similar to controls. In the quadriceps, GPA treatment decreased muscle mass by 19.8% at 18 months and 18.2% at 34 months (Fig. 1B). Quadriceps muscle mass also declined with age by 37.4%. By contrast, the adductor longus mass did not change with age (average loss of 2.6%, $p=0.6151$; Fig. 1C). GPA treatment induced a loss of adductor longus (AL) mass at 18 months and 34 months (12.1% and 14.2% average loss, respectively).

Following 4 months of GPA treatment, rectus femoris fiber number did not decrease at 18 months and decreased by 10.6% at 34 months (Fig. 2A). Between 18 and 34 months, fiber number declined by 7.5%. Rectus femoris muscle cross-sectional area (CSA) was decreased by both age and GPA treatment. With age, CSA decreased by 35.6% (Fig. 2B). GPA treatment reduced rectus femoris CSA by 20.6% and 23.7% at 18 and 34 months of age, respectively. Fiber density decreased 10.6% with age and 2.4% and 6.7% with GPA treatment at 18 and 34 months of age (Fig. 2C). Out of approximately 50,000 muscle fibers, there was one ETC-deficient fiber observed in one of five 18-month-old control rats (Fig. 2D). In the GPA-treated 18-month-old rats, 4 of 5 rats had ETC-deficient fibers with a mean of 3.8 fibers per rat rectus femoris tissue section. At 34 months of age, control rats had average of 36.5 ETC-deficient fibers per rectus femoris tissue section and this was further increased by GPA treatment to 148.4. The percentage of type I muscle fibers increased by 49.3% with GPA treatment at 18 months. There was no change in type I fiber percentage by age or with GPA treatment at 34 months of age (Fig. 2E). Muscle fiber morphology was affected by age, with decreases in average fiber cross-sectional area (49.4%), fiber circularity (10.5%), and minimum Feret diameter (33.1%) (Fig. 3). GPA treatment at 18 months also decreased each of these parameters; however, no effects of GPA treatment were observed at 34 months.

MtDNA copy number declined with GPA treatment in both quadriceps (21.7% at 18 months and 36.3% at 34 months (Fig. 4A) and AL (14.5% at 18 months and 36.3% at 34 months (Fig. 4B). The age-induced decline in mtDNA copy number in the quadriceps (Fig. 4A) was not observed in the AL (Fig. 4B). MtDNA deletion mutation frequency increased by 57.3 times in the quadriceps and 17.6 times in the adductor longus between

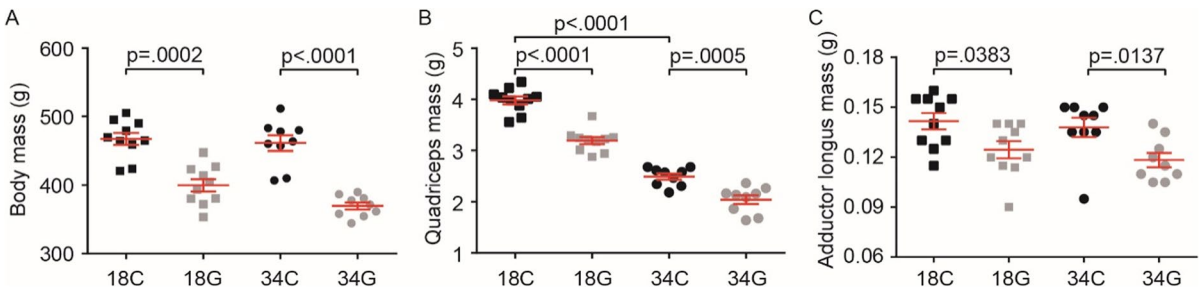


**Fig. 1** Effects of GPA treatment and age on (**A**) body mass, **B** quadriceps mass, and (**C**) adductor longus mass in 18- and 34-month-old rats. 18C, 18 months control; 18G, 18 months GPA-treated; 34C, 34 months control; 34G, 34 months GPA-treated. Black symbols denote control rats and gray symbols denote GPA-treated rats. Boxes represent data from 18-month-old rats and circles denote 34-month-old rats. Whisker plots denote mean and SEM. *P* values were calculated using Mann–Whitney tests. $N=9$–10 per experimental group

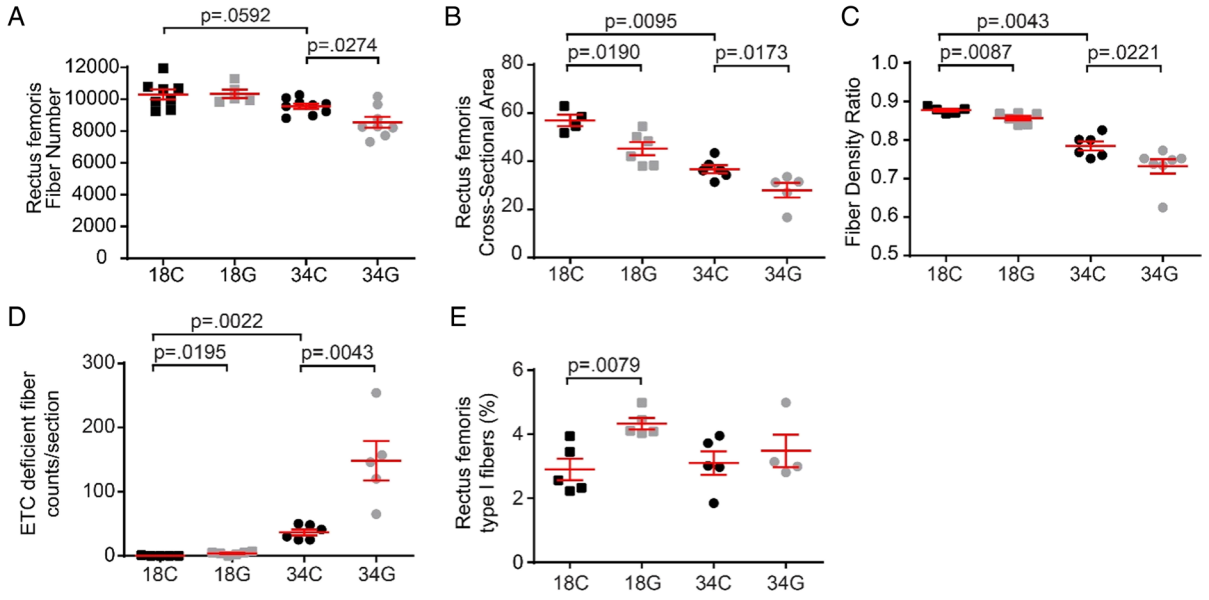


**Fig. 2** Effects of GPA treatment and age on rectus femoris muscle in 18- and 34-month-old rats. **A** Fiber number, **B** cross-sectional area, **C** fiber density, **D** type I fiber number, and (**E**) ETC-deficient fiber counts. 18C, 18 months control; 18G, 18 months GPA-treated; 34C, 34 months control; 34G, 34 months GPA-treated. Whisker plots denote mean and SEM. *P* values were calculated using Mann–Whitney tests. $N = 6–9$ per experimental group

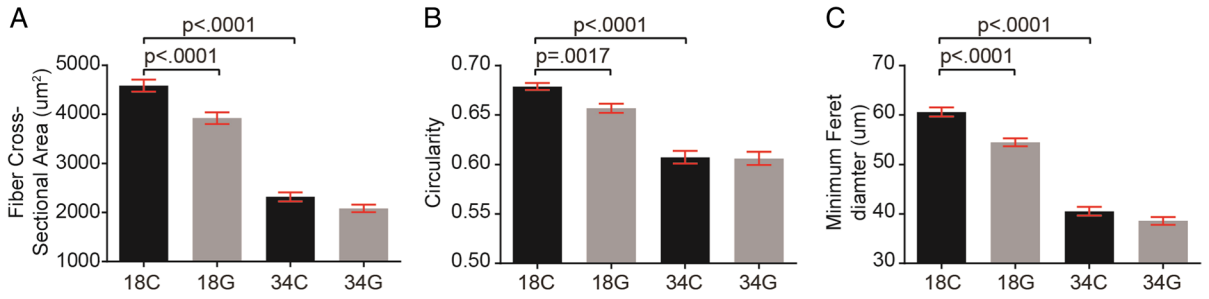


**Fig. 3** Effects of GPA treatment and age on rectus femoris muscle fiber morphology. **A** Fiber cross-sectional area, **B** fiber circularity, **C** fiber minimum Feret diameter. 18C, 18 months control; 18G, 18 months GPA-treated; 34C, 34 months control; 34G, 34 months GPA-treated. Whisker plots denote mean and SEM. *P* values were calculated using Mann–Whitney tests. $N = 6–9$ per experimental group

18 and 34 months of age. Similarly, GPA treatment increased deletion frequency in both muscles. GPA treatment increased mtDNA deletion frequency by 76% at 18 months and 185% at 34 months in the quadriceps (Fig. 4C). In the AL, GPA treatment increased mtDNA deletion frequency by 53% at 18 months and 125% at 34 months (Fig. 4D).

We observed no effect of age or GPA treatment on Complex II– or Complex IV–dependent respiration in the quadriceps muscle (Fig. 5 and S3). Complex I–dependent respiration decreased by 61.2% with age and 27.1% with GPA treatment but only at 18 months.

Some effects of GPA treatment interacted with age (Fig. 6), while other effects did not (Fig. S2). GPA has a greater impact on fiber cross-sectional area, minimum Feret diameter, oxygen consumption rate (OCR), and quadriceps mass loss at 18 months than at 34 months. At 34 months, GPA effects were greater in adductor longus mtDNA copy number, mtDNA deletion frequency in the quadriceps, ETC-deficient fiber number, and fiber loss. In the adductor longus,

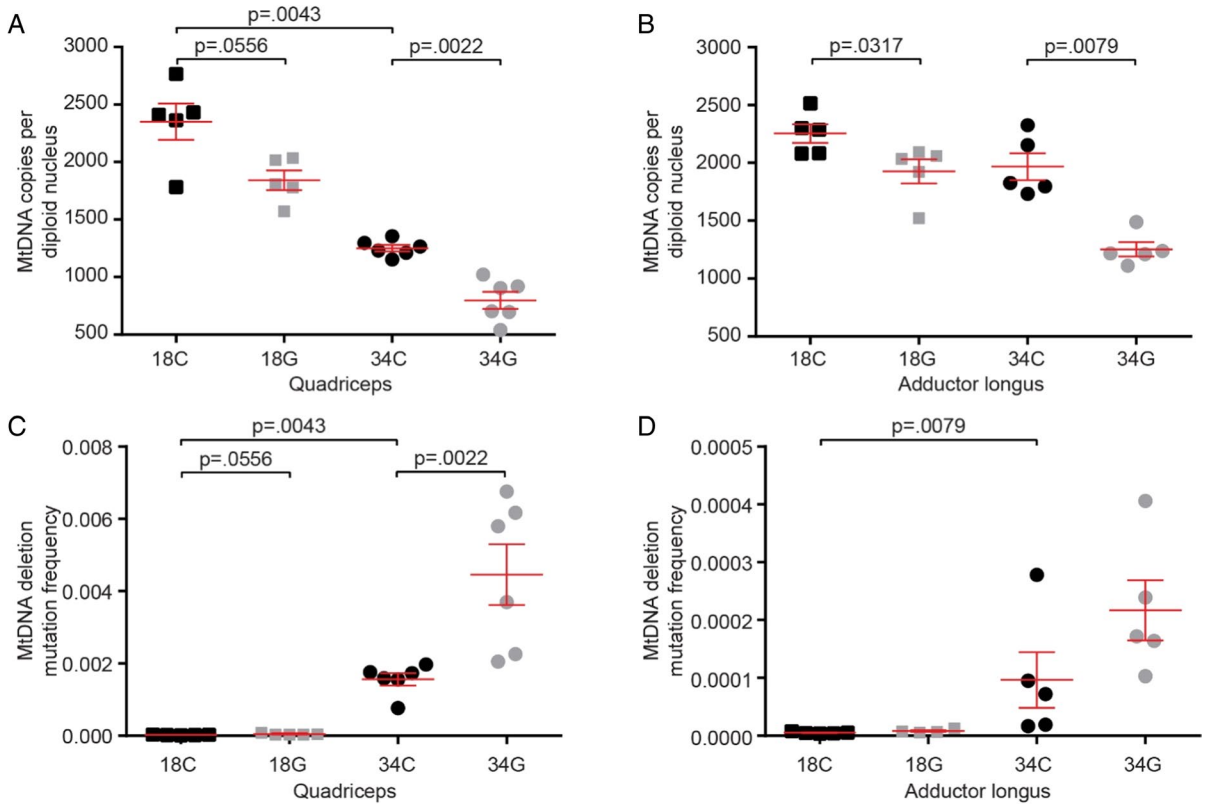


**Fig. 4** Effect of GPA treatment and age on mtDNA copy number (**A**, **B**) and mtDNA deletion frequency (**C**, **D**) in the quadriceps (**A**, **C**) and the adductor longus (**B**, **D**) muscles. 18C, 18 months control; 18G, 18 months GPA-treated; 34C, 34 months control; 34G, 34 months GPA-treated. Whisker plots denote mean and SEM. *P* values were calculated using Mann–Whitney tests. $N = 5–6$ per experimental group

the effects of GPA on mtDNA deletion frequency and mass did not change with age (Fig. S4).

To understand the kinetics of GPA treatment, we treated 30-month-old rats with GPA for 1, 2, 3 or 4 months (Fig. 7). The effect of GPA treatment was apparent after 1 month and worsened with additional time in parallel with the controls. The most rapid effect of GPA treatment on mass was observed in the

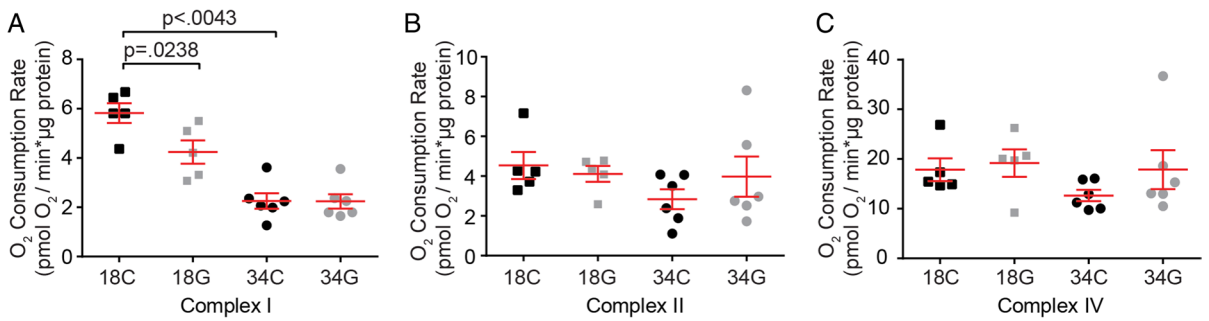


**Fig. 5** Effect of GPA treatment and age on quadriceps mitochondrial respiration. **A** Complex I–dependent, **B** Complex II–dependent, and (**C**) Complex IV–dependent oxygen consumption. 18C, 18 months control; 18G, 18 months GPA-treated; 34C, 34 months control; 34G, 34 months GPA-treated. Whisker plots denote mean and SEM. *P* values were calculated using Mann–Whitney tests. $N = 5–6$ per experimental group

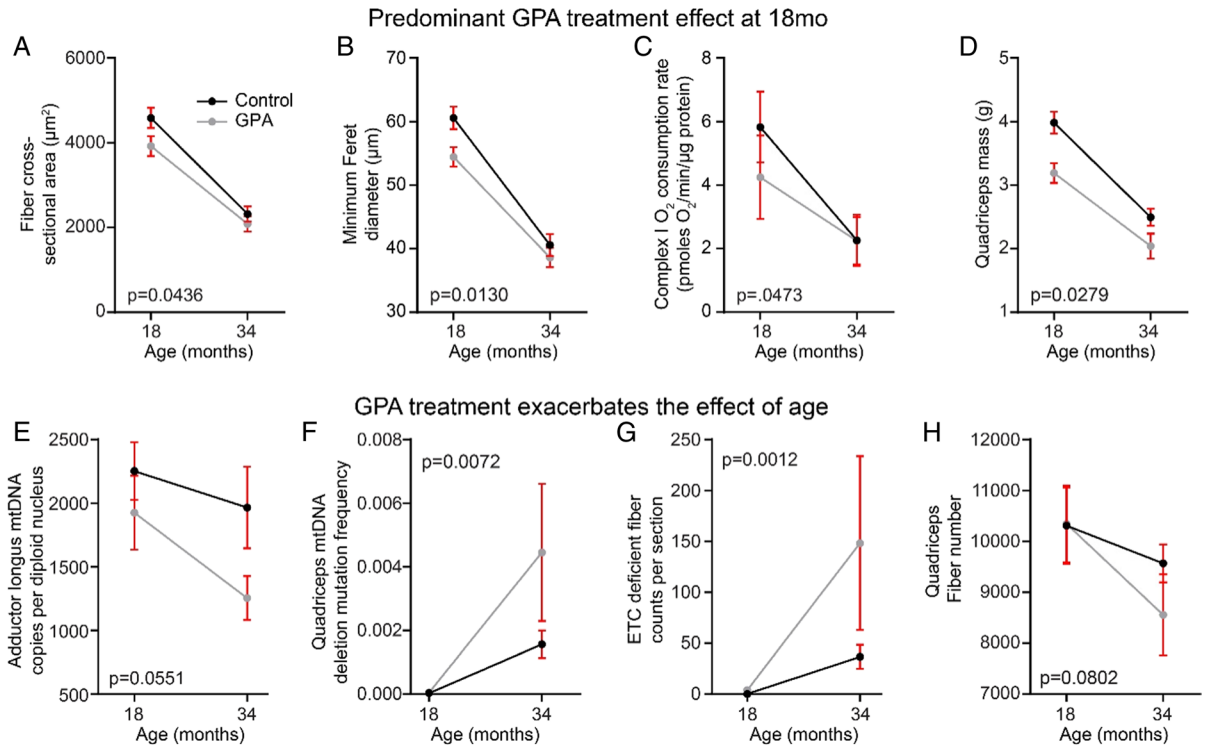


**Fig. 6** Divergent effects of GPA treatment in muscle on 18- and 34-month-old rats. Interactions plots of (**A**) fiber cross-sectional area, **B** minimum Feret diameter, **C** Complex I–related oxygen consumption rate, **D** quadriceps mass, **E** adductor longus mtDNA copy number, **F** quadriceps mtDNA deletion mutation frequency, **G** ETC-deficient fiber number, **H** quadriceps fiber number. *P* values were generated with extra sum of squares *F*-tests, Error bars are 95% confidence intervals. Sample sizes are as listed in the previous figures for each experimental group and outcome

quadriceps with delayed effects in the adductor longus. Similarly, mtDNA copy number and deletion frequency responded to GPA treatment within 1 month.

Differential gene expression analyses from the quadriceps showed four distinct clusters based on age and treatment (Fig. 8A). The intersections of these four DEG lists are visualized in an UpSet plot [29] in Fig. 8B and DEG lists are in Table S1. As the 34G rats have the highest abundance of ETC abnormal fibers and mtDNA deletion mutation frequency and based on the interaction plots in Fig. 6, we considered the DEG list intersection of 34C versus 34G the most informative for identifying genes that correlate with deletion frequency, ETC fiber accumulation, and fiber loss. Gene ontology analysis of the 34C/34G DEG intersection showed pathway changes in proteostasis, mitochondrial fission, autophagy, mitochondrial solute carriers, and muscle fiber development (Table S2).

The striking pathology induced by GPA in aging rats prompted us to examine skeletal muscle tissue available from a study of chronic GPA treatment in mice [26]. In the mouse study, GPA was given as 3300 ppm in the diet of UM-HET3 male mice starting at 6 months of age. After 16 months of treatment in the 22-month-old mice, chronic GPA had no effect on quadriceps mtDNA copy number (Fig. 9A), and mtDNA deletion frequency was lower ($p=0.0561$, Fig. 9B). By contrast GPA treatment in aged rats increased quadriceps deletion mutation frequency (Fig. 4C). Also, unlike GPA treatment in rats, long-term GPA treatment at the 3300 ppm dose in male UM-HET3 mice had no effect on body weight (Fig. S5).

## Discussion

We found that GPA has genotoxic and cytotoxic effects on skeletal muscle with age in male rats. The detrimental effects of GPA were pronounced in the

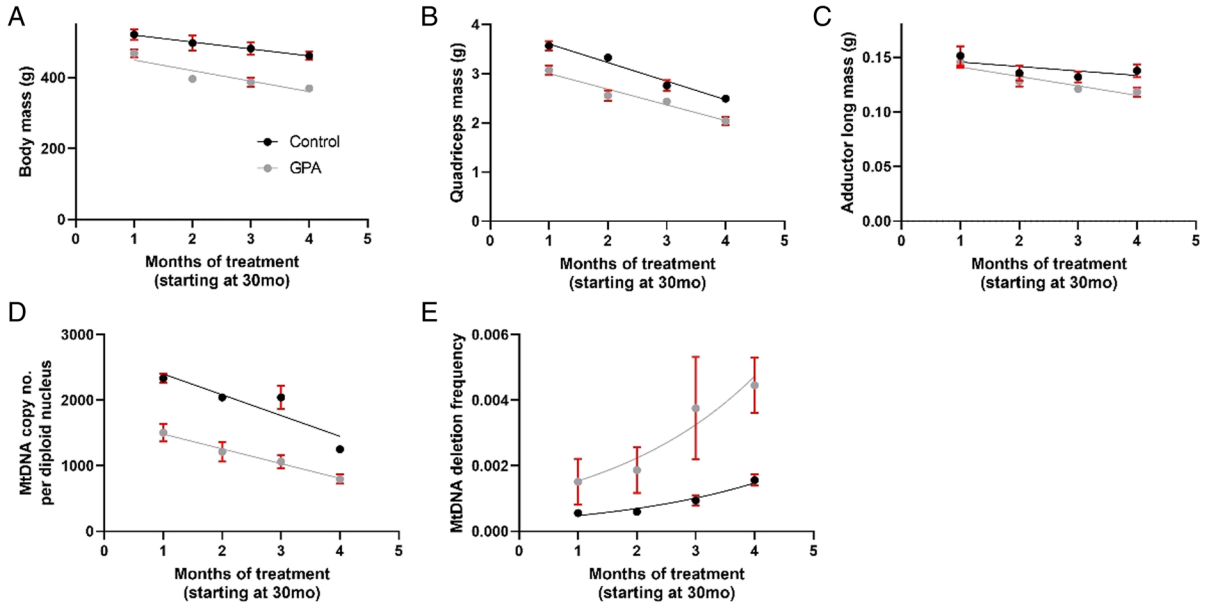


**Fig. 7** Effects of increasing length of GPA treatment starting at 30 months. **A** Body mass, **B** quadriceps mass, **C** adductor longus mass, **D** mtDNA copy number, **E** deletion frequency. Lines denote the best fit curves (linear except for E where they are exponential growth). Symbols denote mean ± SD. $N = 9–10$ per experimental group

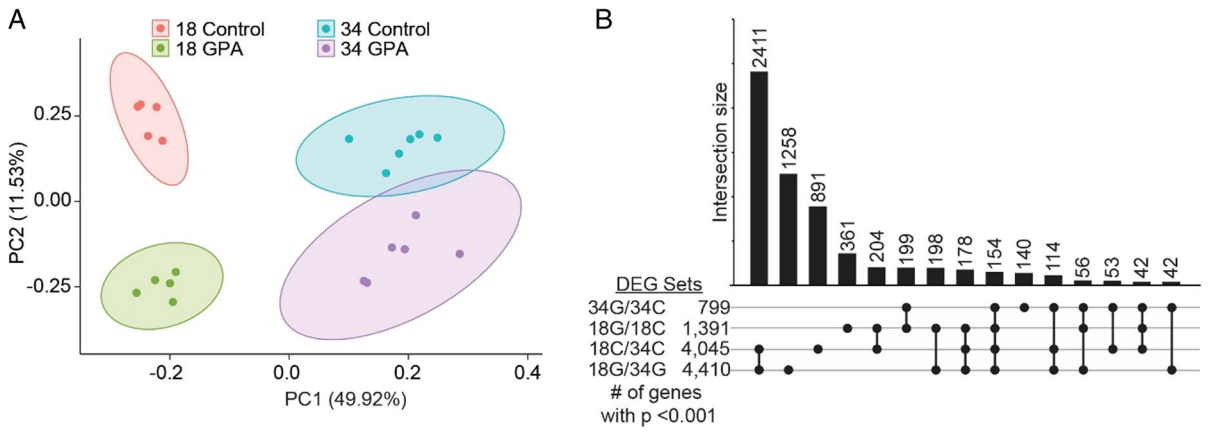


**Fig. 8** RNAseq analysis comparing age and GPA treatment in rat quadriceps. **A** Principal component analysis showing four distinct experimental groups. **B** Visualization of set intersections for differentially expressed genes (DEG) sets are shown in the UpSet plot. Each row corresponds to a DEG set. Each column corresponds to a possible intersection: filled-in cells show which set is part of an intersection. 18C, 18 months control; 18G, 18 months GPA-treated; 34C, 34 months control; 34G, 34 months GPA-treated

quadriceps, which undergoes substantial age-induced degeneration. In contrast, the detrimental effects of GPA were muted in the adductor longus, which exhibits resistance to aging. Reductions in mtDNA copy number and dramatic increases in mtDNA deletion mutations demonstrate the pathogenic effects of GPA on the mitochondrial genome. GPA is not a known chemical mutagen; however, it does inhibit creatine uptake and metabolism [30]. GPA alters skeletal muscle fibers to promote the proliferation of

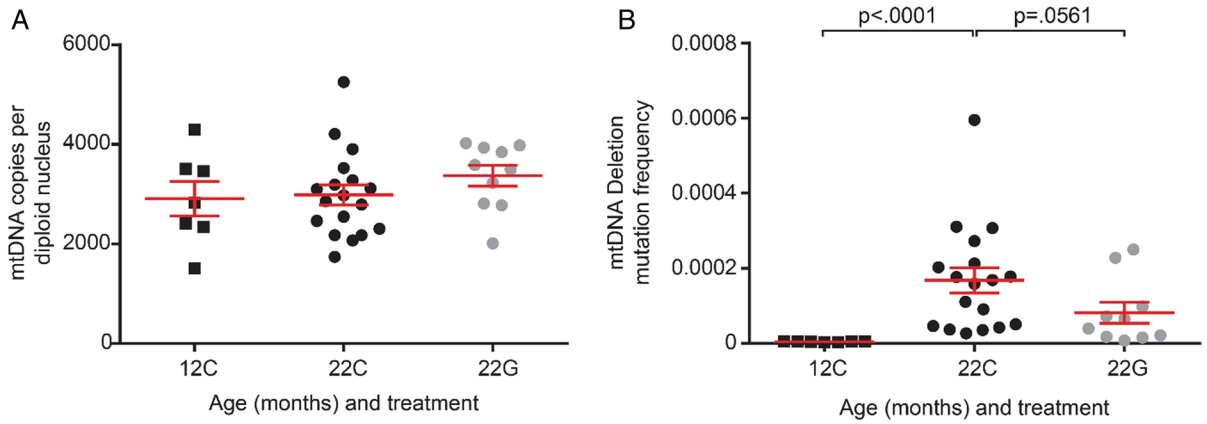


**Fig. 9** Effects of age and long-term GPA (3300 ppm in the diet) treatment on mtDNA copy number and deletion frequency in male mouse quadriceps. All mouse quadriceps tissues were obtained from the NIA ITP Collaborative Interactions Program. 12C, 12 months control. 22C, 22 months control. 22G, 22 months GPA-treated. Samples were pooled across ITP testing sites. **A** MtDNA copy and (**B**) MtDNA mutation frequency. Whisker plot denotes mean ± SEM. $N = 7–18$ per experimental group

genotoxic mtDNA deletion mutations [18] Although the mechanism remains unclear, the gene expression data suggest the involvement of mitochondrial homeostasis and muscle fiber development.

We found pleiotropic effects of GPA on skeletal muscles. While greatest in the 34-month-old rats, there were similar, but less severe perturbations in the 18-month-old rats. At the muscle fiber level, we observed myopathic changes with greater numbers of fibers containing high levels of mtDNA deletions following GPA treatment. These deletion-containing fibers lack the capacity for respiration and oxidative phosphorylation, resulting in fiber death [31] GPA treatment at old ages resulted in fiber loss. Together, these outcomes suggests that GPA treatment promotes mtDNA deletion mutation accumulation and accelerates muscle aging at old ages, with lesser effects at young ages.

Many of the main study outcomes have interaction effects that must be considered to interpret the effects of GPA treatment. For example, mtDNA copy number in the adductor longus is lower with age and with GPA treatment, but the interaction plot clarifies that GPA treatment exacerbates the age effects (Fig. 6E). Examination of the interaction plots revealed two categories, those outcomes the parameters where GPA treatment exhibits greater effects on young rats and those outcomes the parameters where GPA has greater effects on aged rats. For fiber cross-sectional area, minimum Feret diameter, Complex I oxygen consumption rate, and quadriceps mass, GPA has greater effects at 18 months, but little apparent effect at 34 months. A blunted effect on Complex I at 30 months, when Complex I activity in muscle is reduced, suggests that GPA-induced decreases may be biologically limited at certain points. For adductor longus mtDNA copy number, quadriceps mtDNA deletion frequency, ETC-deficient fiber number, and quadriceps fiber number, GPA has far greater effects at 34 months, as compared to 18 months. The more profound effects in the adductor longus further demonstrates the ability of GPA treatment to accelerate some domains of skeletal muscle aging.

The current study builds on earlier investigations of GPA by examining rodents of distinct ages and different lengths of GPA treatment. The current data show negative effects at 18 months, including a decline in complex I–dependent respiration, an effect not previously reported for GPA. The appearance of genotoxic and myopathic effects within a month of treatment in aged rats argues against the safety of even short-term treatments with GPA, particularly in aged mammals. Gene expression studies show changes expected from a treatment that impairs skeletal muscle energy flux, but do not reveal specific mediators for the cellular action of GPA at young or old age.

GPA treatment was noted to have some beneficial metabolic effects, but these do not translate to

increased lifespan. In skeletal muscle, GPA depletes the phosphocreatine pool, causing an energy deficit and a corresponding activation of AMP-activated protein kinase (AMPK). The resulting beneficial physiological effects of GPA include lower blood glucose, lower blood insulin, and lower blood pressure [8]. The energy deficit and physiological effects were thought to mimic calorie restriction (CR), an intervention known to extend rodent lifespan robustly. Due to these similarities to CR, the National Institutes on Aging Intervention Testing Program (ITP) examined the lifespan effects of GPA at a dose of 3300 ppm in the diet initiated at 6 months of age in the HET3 mouse strain (Strong, Miller et al.). There was no effect of this GPA regimen on mouse lifespan. The response to lifelong GPA treatment in mice differed greatly from our shorter treatments in aged rats (Fig. 9) with limited effects on quadriceps mtDNA copy number and lowered mtDNA deletion frequency. This was surprising because shorter GPA treatments in rats reduces copy number and increases deletion mutation frequency in the quadriceps [32].

There are many possible explanations for the differences between life-long and subacute GPA treatment including the differing doses, ages of initiation, and metabolic state of the muscles. The dose used in the ITP was 3300 ppm in the food, which is 1/3 of the dose we used. At this dose, the mice had no appreciable weight loss (Fig. S3) demonstrating that GPA treatment at 3300 ppm was not having similar physiological effects as the 10,000 ppm dose in aged rats used in this study. Young mice have greater resilience to stressors and may better tolerate the energy deficit caused by short-term GPA treatment. This youthful compensation may negate the age-induced responses to GPA. Many of the processes thought to be activated by GPA including mitochondrial function, mitochondrial biogenesis, mitophagy, fission/fusion decline with age [33], such that older rodents may be ill-equipped to handle the energy deficit. GPA in young rodents is a potent activator of AMPK, but this is not observed in aged rodent muscle [5, 12]. Future studies will examine short-term GPA treatment in aged mice.

Limitations of our study include our focus on male rates and glycolytic respiration. Data from this rat strain are predominantly in males and the female F344 x Brown Norway rats have a shorter lifespan than the males [34], which limits the generalizability to other rodents where females generally live longer. The lack of hybrid vigor in the female rats of the F344 x Brown Norway F1 is not widely appreciated as a possible confounder to the use of females from this cross. A genetically heterogeneous rat strain analogous to the UM HET3 mice may provide an improved model of rat aging that addresses sex-specific hybrid vigor in the F344 x Brown Norway F1 strain. We used substrates associated with glycolytic respiration (e.g., NADH and succinate), while GPA has been noted to modulate fatty acid oxidation [5]. Our data showing minimal GPA effects on glycolytic respiration may not reflect the full scope of mitochondrial effects.

In summary, our data demonstrate mitochondrial and skeletal muscle damage by GPA in both young and old rats. Together with the earlier reports of GPA's myopathic effects, the data suggest that GPA use by older adults may result in irreversible damage, which may warrant future testing. The proposed benefits of GPA in older adults would not seem to outweigh the potential risks. Future work should examine the pleiotropic mitochondrial DNA effects of other metabolic drugs preferentially used in older adults.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Declarations**

**Ethics approval** This study was carried out in accordance with the recommendations in the NIH Guide for Care and Use of Laboratory Animals and the guidelines of the Canadian Council on Animal Care. The protocols used were approved by

the Institutional Animal Care and Use Committees at the University of Alberta and UCLA.

**Conflict of interest** The authors declare no competing interests.

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