Whole-life bone percentage trajectory and longevity in rats: Role of insulin

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Abstract

The present study assessed body composition trajectory of rats (N = 96) ranked to 5 groups according to lifespan, using dual-energy x-ray absorptiometry every 6 months until end-life. A striking linearity between lifespan and bone mass percentage (not absolute bone mass or density) was observed. Long-lived rats show higher bone mass percentage with a delayed insulin rise to a similar peak level as short-lived counterparts followed by insulin declines and bone mass loss. Decreasing insulin by the streptozotocin (STZ)-treatment caused a rapid bone mass loss (-10.5%) with a decreased 5-day survival rate to 35% in old rats (20 months). Insulin replacement to STZ-treated rats completely blocked bone loss and increased the survival rate to 71%. Normal old rats showed faster lean mass loss despite greater myofiber regeneration (centronucleation) compared with the young rats (4 months). Increased $CD68^+$ and $CD163^+$ cell infiltration into insulin-depleted muscle suggests bone marrow cell exhaustion by aged muscle tissues. Bone produces stem cells and immune cells to rejuvenate aging peripheral tissues. Our data suggests that unsustainable life is associated with development of bone disproportionality to the growing body, partly due to insulin reversal from hyperinsulinemia during late life.

Key words: sarcopenia, osteopenia, longevity, frailty, cachexia, tumor

Introduction

Multicellular organisms may be viewed as society-like system made by living cells, where increased survival fitness comes from large-scale cooperation among highly differentiated cells. However, cells within a multicellular mammal are mostly short-lived [1]. Therefore, positive balance between cell death and cell proliferation is crucial for sustaining weight growth in a way similar to population curve. The weight reversal during late life represents a shift from positive to negative balance between cell proliferation and cell death, since animal weight is, in large part, determined by cell number [2]. Body composition continues to change during a protracted weight gain period followed by a short weight loss period before death, representing an imbalanced development among differentiated cells in an expanding and shrinking multicellular system. The relative importance of bone, muscle, and fat to sustain the longest survival duration of multicellular systems has not been strictly defined by a whole-life approach observation covering the weight loss periods of end-life.

Complying with physical law, size expansion during growth will eventually reach a critical point where structural stability can no longer persist. In humans, weight gain during early adulthood is generally regarded as an adverse metabolic condition [3] and associated with increased baseline inflammation as a sign of increased entropy [4]. However, unintentional weight loss, that occurs during late life, is also a strong predictor of precipitous health deterioration and increased mortality [5], associated with sarcopenia, osteopenia, and lipopenia. Bone and muscle mass loss may be inter-related [6]. Bone marrow produces stem cells and immune cells which are required for tissue renewal and muscle mass gain against weight load [7]. Macrophages are bone marrow derived immune cells residing in nearly all tissues [8]. Phagocytic macrophages (M1, $CD68^{+}$) function to recognize and clear unhealthy cells followed by cell regeneration in presence of (M2, $CD163^{+}$) macrophages in tissues [9-

11]. This program is essential to maintain a relatively younger cell population for bone and its surrounding tissues within a multicellular system.

Insulin produced from the pancreas is a pleiotropic anabolic hormone, which increases in circulation during the weight gain period of early life (termed hyperinsulinemia) [12]. This anabolic signal stimulates daily cell reconstruction to suppress entropy by lowering baseline inflammation [13]. The role of insulin in the regulation of muscle inflammation during the weight loss period of late life has rarely been studied. Insulin is essential for bone marrow cell production and bone formation [14]. In this study, we firstly examined relationship between body composition and insulin levels every 6 months until 24 months of age among laboratory rats. We secondly determined the role of insulin on bone mass in aged rats during weight loss period of end-life.

Results

Upper panel of Figure 1 shows the weight trajectory pattern of 96 rats ranked into 5 groups according to lifespan (LS). All groups of rats show a common trajectory pattern of two discrete life phases: weight gain (long) and weight loss (short) periods. A linear (inverse) relationship between mean survival time and peak weight of the groups ranked from shortest (LS1) to longest (LS5) lived groups was observed (Lower panel of Figure 1). Long-lived rats are characterized by the modest weight gain for the first three quarters of life followed by delayed weight loss until the end of life compared to the rest groups. Female-to-male ratio of each group increases as lifespan increases. Therefore, both male and female rats were further dichotomized into fast growth group and slow growth group. Growth rates (1^{st} year) are male (fast growth), 2.13 g/d; male (slow growth), 1.83 g/d; female (fast growth), 1.12 g/d; female

(slow growth), 0.92 g/d. Median lifespans are male (fast growth), 654 d; male (slow growth), 714 d; female (fast growth), 762 d; and female (slow growth), 780 d.

Evolutions of percent bone mass, percent muscle mass, and percent fat mass for the 5 groups ranked by lifespan are shown in Figure 2. At 6 months of age, the long-lived group is characterized by high percent bone mass (A), high percent muscle mass (B), and low percent fat (C) relative to their short-lived counterparts. However, survivors after 12 months of age show progressive decreases in percent muscle mass and percent bone mass concurrent with increase in percent fat mass. The most striking feature for the long-lived rats is the persistently highest percent bone mass across the entire observation period with modest decline at the end (Figure 2B). For these long-lived survivors (LS4 and LS5 groups), bone mass loss occurred after 18 months of age. The residual survival time after 18 months of age significantly correlated with bone mass loss including one outlier (Figure 3). The topmost 8 rats with the fastest bone mass loss exhibit apparent tumorigenesis and cachexia, indicated by X-ray image, compared with bottommost 8 counterparts showing slowest bone mass loss during the same observation period (Lower panel).

Fasting insulin levels from 6 to 24 months of age are shown in Figure 4. Short-lived rats (LS1) exhibit greatest hyperinsulinemia at 6 months of age compared with the rest groups (Figure 4A). Long-lived survivors (LS4 and LS5) show delayed hyperinsulinemia and reached similar insulin levels at 18 months of age, followed by ~35% decline at 24 months of age. Glucose was not significantly different among the 5 groups and was not changed overtime (Figure 4B). To determine the role of insulin decline in lean mass loss (sarcopenia and osteopenia) during late life, STZ was injected to young (4 months) and old (20 months) rats, with or without insulin co-injection. The survival rates in 5 days were 100%, 35%, and 71%

for the C, S, and SI groups, respectively (Figure 4C). Survival rate of young rats (4 months of age) was not affected by STZ injection (100% survival rate). STZ eliminated plasma insulin level to an undetectable level and decreased glycogen concentration of soleus muscle by ~30%. Insulin injection to STZ-treated rats replenished muscle glycogen in young rats but had minimal effect in old rats (Figure 4D).

To determine whether insulin loss during late life is causally related with muscle and bone mass loss, the whole-body DEXA scan was conducted for old rats at 20 months of age in contrast to young rats at 4 months of age. Supplemented Table 1 details the absolute mass changes for muscle, fat, and bone tissues. Body weight changes measured 10 days before STZ experiments were at a rate of 1.5 ± 0.7 g per day for young rats and -7.8 ± 0.7 g per day for old rats. Insulin loss by STZ caused greater weight loss for old rats, while the dose of insulin injection (0.25 IU/kg twice a day) was not sufficient to restore body weight to control level. This body weight decline occurred in parallel with muscle, fat, and bone mass loss. However, bone mineral density (BMD) was not affected by the short-term insulin loss.

In normal untreated rats, muscle fiber regeneration (centronucleation) of old rats was approximately 8-fold (Figure 5A) greater than that of young rats. Insulin depletion significantly lowered muscle fiber regeneration concurrent with ~50% greater cell infiltration for both young and old rats (main effect, $p < 0.01$) (Figure 5B). Insulin injection partially reversed these changes. Phagocytic M1 macrophage ($CD68^+$ positive cells) in soleus muscle of both young and old rats was significantly increased in the STZ injected group above their control groups ($p < 0.05$) (Figure 5C). Regenerative M2 macrophage ($CD163^+$ positive cells) in soleus muscle of old rats was substantially greater than those of young rats (Figure 5D). Insulin injection showed a small effect in reversing this change.

Discussion

The most striking finding of the study is the linearity between bone mass percentage and the longevity of rats. Unlike other body composition variables (percent fat and muscle), this relationship is consistent encompassing the weight gain and weight loss periods of life during aging. Furthermore, we observed ~ 35% decreases in plasma insulin during 18-24 months of age from hyperinsulinemic state after rats reached the peak weight of their life. This transition contributes to age-dependent bone loss and cachexia-like condition of multicellular mammals. Insulin replacement to old rats completely blocked bone mass loss and reversed mortality. In a contrary, bone mass and survival fitness of young rats were not severely decreased after insulin loss by STZ injection. The results of the study implicate the importance of insulin production for bone maintenance in sustaining increasingly greater weight during late life.

Insulin is required for bone marrow cell proliferation [15] which directly stimulates bone formation [16]. Bone marrow releases immune cells (i.e. macrophages) for clearing unhealthy cells and produces stem cells for tissue regeneration in bone and surrounding tissues [17]. Therefore, this cell-producing organ is essential for the balance between phagocytosis and cell regeneration to maintain youth state and size of all tissues. Despite concurrent increases in absolute bone mass and body weight during the first 3 quarters of life, the age-dependent decreases in bone mass percentage of during the same period reflects a relative deficit of the bone marrow cell producing organ in support of the increasingly greater cell population during growth. This deficit is expected to influence the degree of tissue senescence due to limited senescence cell clearance and cell renewal. Thus, we speculate that

development of the disproportionality between bone and the body is a likely origin of tissue aging during late life.

Hyperinsulinemia associated with weight gain during early period of life has been recognized as a common predictor of age-related metabolic conditions and premature death [18, 19]. In this life-long study, we have further found that insulin level reversed from its peak, yet glucose level was unchanged during weight loss period of end-life. It remains unclear why glucose is not elevated as insulin decline during this period. One possibility is increased basal glucose uptake due to higher rate of cell regeneration revealed by increased centrally nucleated fibers to compensate increased cell death in aged muscle. Newly generated muscle cells have substantially greater basal glucose uptake than differentiated muscle cells [20]. Similar to a previous study [21], our data also observed a substantially greater regenerative macrophage infiltration ($CD163^+$ cells) and cell regeneration (centronucleation) in muscle tissues of old rats, albeit a daily muscle loss occurred in contrast to those young rats. The increased muscle regeneration during lean mass loss period (20 month of age) may be an intrinsic compensatory mechanism to preserve muscle mass in the cost of bone marrow source.

Insulin loss to a certain level will eventually deplete the bone marrow cells and undermines this compensatory mechanism, resulting in sarcopenia. Increased catabolic stress after insulin declines further increases demand of bone marrow cells for tissue repair by pervasive inflammation [13], evidenced by a substantially increased immune cell infiltration ($CD68^+$ or $CD163^+$ macrophages) from bone marrow in the skeletal muscle.

Increasing cell population in an expanding multicellular system will eventually reach a point where cell death in the body dramatically rises [22]. The result of increased insulin level during weight gain period of early life appear to be physiologically essential to suppress entropy in expanding cell population of the multicellular system. Insulin is a pleiotropic anabolic hormone, which stimulates biosynthesis (e.g. DNA, glycogen, triglyceride, and protein) and cell survival against daily catabolic challenge. High insulin concentration in circulation ensures survival and regeneration of peripheral cells residing distal to capillary beds where oxygen and nutrients are delivered. At young age, other anabolic hormones (e.g. growth hormone and sex hormones) may remain at sufficient amount to suppress the thermodynamic pressure during weight gain period of early life. This may explain much better survival rate of young rats (100%) after STZ-induced insulin loss.

Another major finding of the study is the higher incidence of tumorigenesis among rats with fast bone mass loss (upmost 50%) during late life. Tumorigenesis is the result of random genetic mutation from DNA replication, normally emerged at high rate of cell turnover during persistent inflammation [23]. Inflammation is the inherent mechanism to maintain cell population of a tissue [24]. In normal tissues, cell senescence and death trigger inflammation by increasing phagocytosis and cell regeneration to ensure a relatively younger cell population of the tissues [25], which immediately demands bone marrow source [7]. We speculate that disproportionality between bone and the body size during late life results in senescent cell accumulation and inadequate tissue healing. Therefore, tumorigenesis may be a sign to reflect tissue imbalance in demanding relatively limited bone marrow source as a result of global competition.

Growth is an essential feature of life. Size expansion inevitably builds up thermodynamic pressure leading to structure imbalance and eventual collapse of the system until entropy of the growing body can no longer to be efficiently suppressed. In this study, we observed a linear association between the rise-and-fall pattern of weight and lifespan among 5 groups ranked by survival time, in which the long-lived rats are characterized by the modest weight gain-and-loss trajectory pattern during the entire life compared with the short-lived groups. This data suggests that the law of thermodynamics sets the upper ceiling of weight growth and thus determines the length of survival (time of expansion) in an evolved multicellular system, which explains the fact that females had longer lifespan than males due to modest body size expansion, supported by reduced gap in median lifespan with closer body weight between fast growing female rats and slow growing male rats.

The major limitation of the study is inability of DEXA to distinguish muscle mass, fibrosis, and other solid tissues in aged rats. It has been reported that DEXA may include other types of fat-free mass, such as fluid retention or fibrosis [26]. In this study, fat and bone were reversed by insulin injection in STZ treated rats, but not muscle mass. However, we could not definitely conclude outcomes of muscle mass change under insulin manipulation among old rats with DEXA assessment.

Conclusion

Bone produces stem cells and immune cells functioned to maintain youth of cells for bone itself and peripheral tissues. Longevity is closely associated with the development of disproportionality between bone mass and body mass. Decreases in bone mass and survival fitness are causally associated with insulin return from hyperinsulinemia during end-life.

Skeletal muscle exhausts bone marrow cells when insulin is declined, which may contribute rapid bone mass loss and death of aged rats.

Materials and Methods

Ethical Statement

All experimental procedure followed Animal Protection Act of Taiwan and approved by the Institutional Animal Care and Use Committee of the University of Taipei, Taipei, Taiwan.

Animal Care

Sprague–Dawley rats were obtained from BioLASCO Co., Ltd, Taipei City, Taiwan. They were housed in animal facility at University of Taipei. All animals were maintained in a temperature-controlled room (21 ± 2°C) under a 12-h light–dark cycle (6:00 a.m.–6:00 p.m.) and a relative humidity of 45-55%. Every 2 animals were housed for each cage. They were fed with standard rat chow (Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and normal tap water in feeding bottle, recorded the amount of food intake and body weight at the same time twice a day. Food and water intake were recorded.

Longevity Study

This study aimed to characterize body composition trajectories of long-lived and short-lived rats. One hundred Sprague-Dawley rats at one month of age with known date of birth were transferred to animal facility at University of Taipei in 2012 (N =50) and 2016 (N = 50) for entire life until natural death. Linearity between bone percentage (bone mass divided by body mass in percentage) and longevity (survival time) among 5 ranked group was first established in the first experiment in 2012. The second study in 2016 used the same number of rats to determine reproducibility of the first observation, which showed similar pattern. Taken

together, a total of 96 rats (M = 47; F = 49) were included to examine lifetime body weight changes among 5 ranked group according to their lifespan. The second half of animals was used to further determine insulin trajectories. To study aging associated death, rats survived < 12 months (N = 4) were excluded. For the repeated experiment, a total of 50 rats (M = 23; F = 27) was used for insulin analysis. Blood sample were collected at 6, 12, 18, and 24 months of age for serum insulin measurements to obtain trajectory. Body composition was measured following blood collection. There were 26 rats survived until the last measurement at 24 months of age.

Intervention Study

An intervention study was designed to examine the causal effect of insulin change on body composition (% muscle, fat, and bone mass) and muscle inflammation during weight loss period of late life. Weight loss rate (- 3.5 g per day) was measured 7 days before treatments. Since weight loss is highly associated with increased mortality among aged animals, 30 rats were classified to 10 weight levels and matched into 3 groups for each weight level. Additional 6 rats were allocated to the S group as STZ is expected to produce high mortality rate to aged rats. Control group (C, N = 8) received intraperitoneal injections of 0.1 M citrate buffer solution. Two rats in the C group died before intervention after grouping were precluded from mortality analysis. STZ group (S, N = 14) received intraperitoneal injections of STZ (50 mg/kg/ml in 0.1 M citrate buffer solution); STZ with insulin group (SI, N = 7) received intraperitoneal insulin injection (0.25 IU/kg in 0.1 M citrate buffer solution) every 12 h right after STZ injection. Due to high mortality among the aged rats, only the animals with complete data after tissue collection were included (C: N = 6; S, N = 5; SI: N = 6) for tissue analysis and pre-post comparison of body composition measures. Same number of young rats (N = 30) at 4 months of age was used as age control and was evenly assigned to

each group (N = 10). All rats were survived after treatments. Both insulin and vehicle solution were delivered daily at the same time of the day for treated rats. Soleus muscle was used for glycogen analysis and immunohistochemical analysis on myeloid cell infiltration.

Streptozotocin and insulin

Streptozotocin (Sigma-Aldrich, Darmstadt, Germany) was dissolved in 0.1 M citrate buffer at the dose of 50 mg/kg body weight to eliminate insulin to undetectable level. Insulin (Humulin®, Lilly, USA) was diluted with saline and injected 0.25 IU/ml every 12 h for 5 days. Animals were fasted overnight for 12 h before STZ injection. Food and water were made available immediately after injection. Loss of insulin was confirmed by measurements of serum insulin using enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) 5 days after STZ injection. Responsiveness of rats (SI group) to human insulin was confirmed by muscle glycogen concentration 3 h after glucose intubation with insulin injection. Survived rats were anesthetized with sodium pentobarbital (80 mg/kg) 5 d after intervention. Soleus muscle was surgically collected for immunohistochemically analysis and glycogen assay.

Body composition

This study used dual-energy x-ray absorptiometry (DXA) (Lunar iDXA, GE Medical Systems, Madison, WI) to measure percent muscle mass, percent fat mass, percent bone mass and bone density every 6 months for both the longevity study and the intervention study. Before taken dual-energy x-ray absorptiometry, each rat was given an intraperitoneal injection of sodium pentobarbital (80 mg/kg). Food and water were removed 12 hours before measurement.

Glycogen assay

To determine insulin responsiveness to human insulin injection, muscle glycogen was measured in soleus muscle. Soleus muscle was frozen in liquid nitrogen until glycogen analysis. Muscle glycogen content was broken down into glucose units using amyloglucosidase (Teco diagnostics, Anaheim, USA) to determine the amount of glycogen as glucose unit (55 mg of averaged muscle weight). The experimental protocol is according to our previous study (Kuo, Browning, & Ivy, 1999). Glucose concentration was measured by reading the optical density at 505 nm by spectrophotometer (Thermo, Genesis 10S UV-Vis, USA) after 18 minutes.

Immunohistochemical (IHC) analysis

Histology for leukocyte infiltration and IHC staining for macrophage infiltration were conducted by a professional pathologist at Taipei Institute of Pathology (Taipei, Taiwan). Middle section of soleus muscle was placed in 10% solution of formaldehyde for no more than 3 h before analysis. Soleus muscle was fixed in 4% formalin and then embedded in paraffin. All soleus sample in paraffin were sliced in serial cross-sections (3 μm each) for hematoxylin-eosin staining and immunohistochemistry analyses. For each sample, more than 500 myofibers were included for analysis. Tissue sections were transferred onto coated slides (Super Frost Plus, Braunschweig, Germany). Antigen retrieval occurred in boiled water for 15 min in 0.1 M sodium citrate (pH 7.2). These pretreated slides were blocked for 15 min at room temperature with 5% BSA and then incubated at 4°C overnight with primary antibodies. Antibodies against rat CD68 (dilution 1:50) (abcam, Cambridge, UK) and rat $CD163^{+}$ (dilution 1:100) (Bio-Rad, Hercules, USA). Briefly, specific antibody was purchased to perform IHC staining by using horseradish peroxidase-conjugated avidin biotin complex (ABC) from the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and DAB

chromogen (Vector Laboratories). The sections were counterstained with hematoxylin and mounted. Macrophage infiltration are expressed as positive cell number per myofiber.

Statistical Analysis

Analysis of variance (ANOVA) was used to compare difference among groups for all variables. A one-way analysis of variance (ANOVA) with a repeated measure was used to compare the mean differences in body composition variables between pre- and post-treatments. Fisher's protected least significant test, which holds the value of type I error to 5% for each test, was used to distinguish the differences between pairs of groups. All values are expressed as means ± standard errors. All statistical analyses were performed using SPSS.

Data availability statement

This data will be made available from authors upon reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contribution

Funder has no role in study design and manuscript preparation. YHL, CYH, and CHK designed the experiments. SCT, SJC, KM, PNC, CTL, and CCH performed the experiments. SJC, YHL, CCH, and TK conducted the statistical analyses. YHL drafts the manuscript.

MBH, CYH, and CHK help with the discussing, writing and editing of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Longevity is associated with weight trajectory pattern. Abbreviation: LS, lifespan; LS1 (short-lived, survival time < 579 days); LS2 (survival time 582-672 days); LS3 (survival time 672-738 days); LS4 (survival time 738-870 days); LS5 (long-lived, survival time 870-1032 days).

Figure 2. Development of disparity in muscle, bone, and fat percentage of short-lived and long-lived rats during aging. Long-lived rats (LS4 and LS5) are characterized by relatively higher % bone mass from 6 to 24 months of age (A). Short-lived rats had lowest % muscle mass (B) and highest % fat mass (C) at 6 months of age, whereas long-lived survivors showed progressive declines in % muscle mass and increases in % fat mass with age. Abbreviation: LS, lifespan; LS1 (short-lived, survival time < 579 days); LS2 (survival time 582-672 days); LS3 (survival time 672-738 days); LS4 (survival time 738-870 days); LS5 (long-lived, survival time 870-1032 days). ¶ Significant difference against long-lived rats (LS5), $P < 0.05$; * Significant difference against 6^{th} month, $P < 0.05$.

Figure 3. Bone mass loss is associated with tumor occurrence and residual survival time after 18 months of age (A). Lower panel shows x-ray images of the top 8 and bottom 8 rats (N = 17) on magnitudes of bone mass loss among rats survived after 18 months (B), demonstrating a severe increased tumorigenesis together with cachexia.

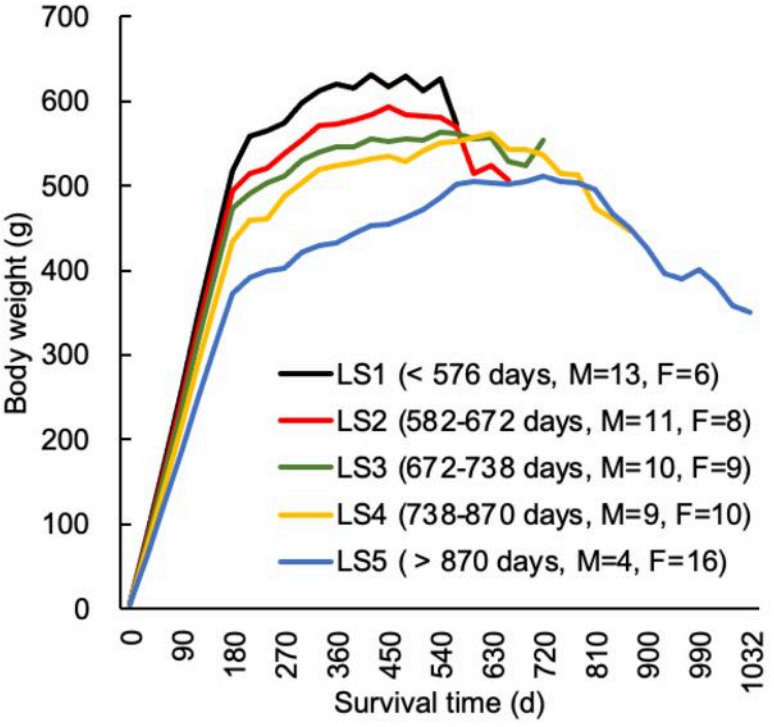
Figure 4. Role of insulin on survival fitness during end-life. Plasma insulin declines (A) from hyperinsulinemic state while glucose (B) remains unchanged from 18-24 months of age. Decreases in survival rate (C) and glycogen storage (D) after insulin loss by streptozotocin injection (S group) is reversed by insulin replacement (SI group, 0.25 IU/kg twice a day) for old rats (20 months of age). Abbreviation: LS, lifespan; LS1 (short-lived, survival time < 579 days); LS2 (survival time 582-672 days); LS3 (survival time 672-738 days); LS4 (survival time 738-870 days); LS5 (long-lived, survival time 870-1032 days). ¶ Significant difference

against long-lived rats (LS5), $P < 0.05$; * Significant difference against 6^{th} month, $P < 0.05$; † Significant difference against 18^{th} month, $P < 0.01$. ** Significant difference against control group, $P < 0.05$. Abbreviation: C, vehicle-injected control group; S, Streptozotocin-injected group; SI, Streptozotocin and insulin co-injected group.

Figure 5. Bone marrow derived cell infiltration ($CD68^+$ and $CD163^+$ cells) in skeletal muscle of old rats altered by insulin. Centrally nucleated fibers (reflecting cell regeneration)(A), cell infiltration (B), $CD68^+$ cells (C), and $CD163^+$ cells (D) in soleus muscle from young and old rats were measured 5 days after streptozotocin injection (50 mg/kg/ml) and/or insulin injection (0.25 IU/kg twice a day). * Significant difference against C group, $P < 0.05$; † Significant against Young group, $P < 0.05$. Abbreviation: C, vehicle-injected control group; S, Streptozotocin-injected group; SI, Streptozotocin and insulin co-injected group.

Figure 1

A.



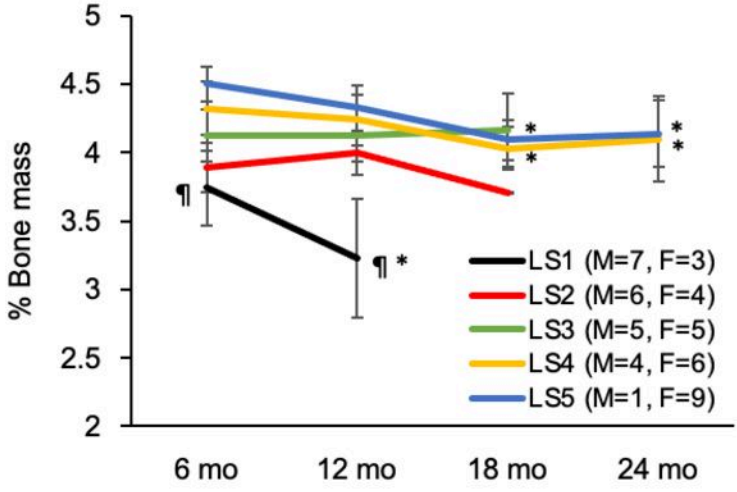
B.

Groups (Survival time)	Peak weight (g)		Time from birth (d)	
	Mean ± SE	P value	Mean ± SE	P value
LS 1 (< 576 d)	661 ± 41 g	0.03	400 ± 18 d	< 0.01
LS 2 (582-672 d)	646 ± 36 g	0.04	525 ± 22 d	< 0.01
LS 3 (672-738 d)	629 ± 35 g	0.08	537 ± 39 d	< 0.01
LS 4 (738-870 d)	627 ± 31 g	0.07	605 ± 39 d	0.01
LS 5 (870-1032 d)	562 ± 28 g	-	726 ± 29 d	-

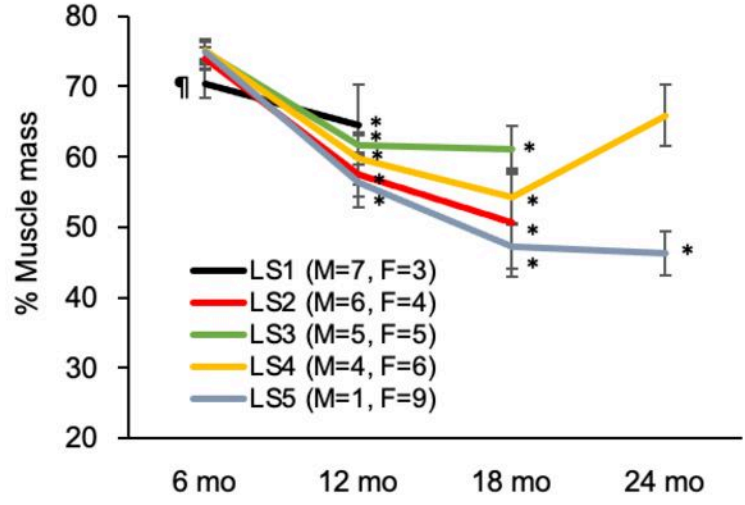
P value compared against LS5 group.

Figure 2

A.


% Bone mass
5
4.5
4
3.5
3
2.5
2
6 mo
12 mo
18 mo
24 mo
LS1 (M=7, F=3)
LS2 (M=6, F=4)
LS3 (M=5, F=5)
LS4 (M=4, F=6)
LS5 (M=1, F=9)

B.


% Muscle mass
80
70
60
50
40
30
20
6 mo
12 mo
18 mo
24 mo
LS1 (M=7, F=3)
LS2 (M=6, F=4)
LS3 (M=5, F=5)
LS4 (M=4, F=6)
LS5 (M=1, F=9)

C.

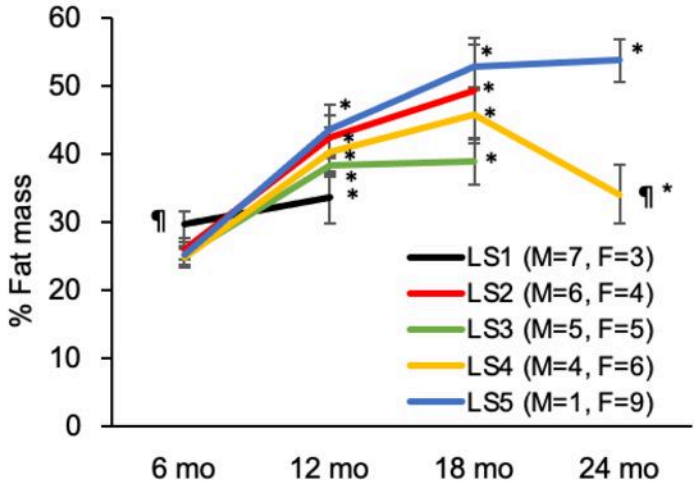
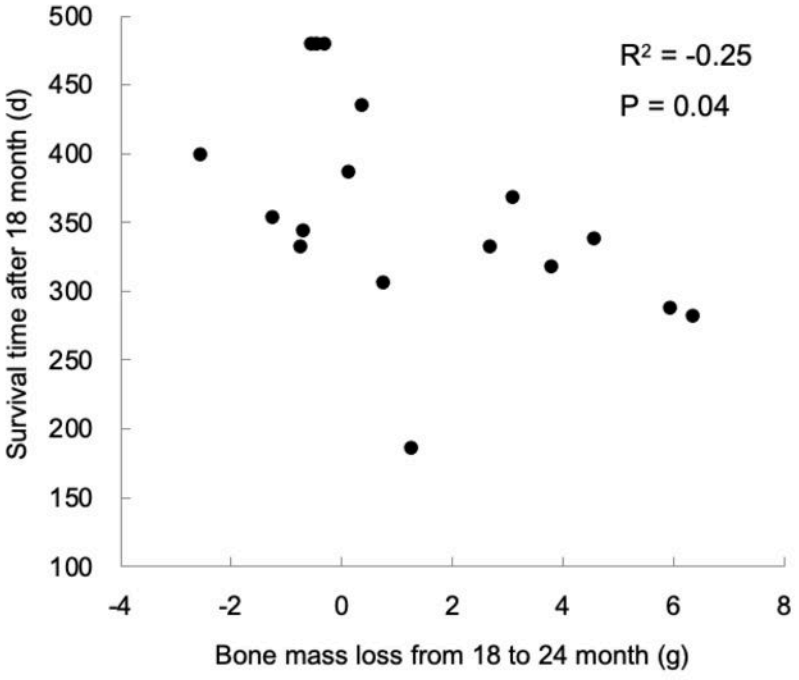

% Fat mass
60
50
40
30
20
10
0
6 mo
12 mo
18 mo
24 mo
LS1 (M=7, F=3)
LS2 (M=6, F=4)
LS3 (M=5, F=5)
LS4 (M=4, F=6)
LS5 (M=1, F=9)

Figure 3

A.


Survival time after 18 month (d)
500
450
400
350
300
250
200
150
100
-4
-2
0
2
4
6
8
Bone mass loss from 18 to 24 month (g)
R² = -0.25
P = 0.04

B.

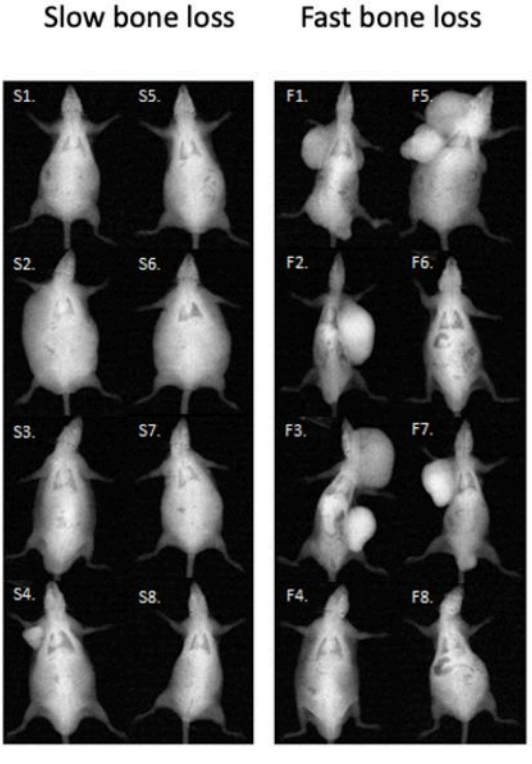

Slow bone loss
Fast bone loss
S1.
S5.
S2.
S6.
S3.
S7.
S4.
S8.
F1.
F5.
F2.
F6.
F3.
F7.
F4.
F8.

Figure 4

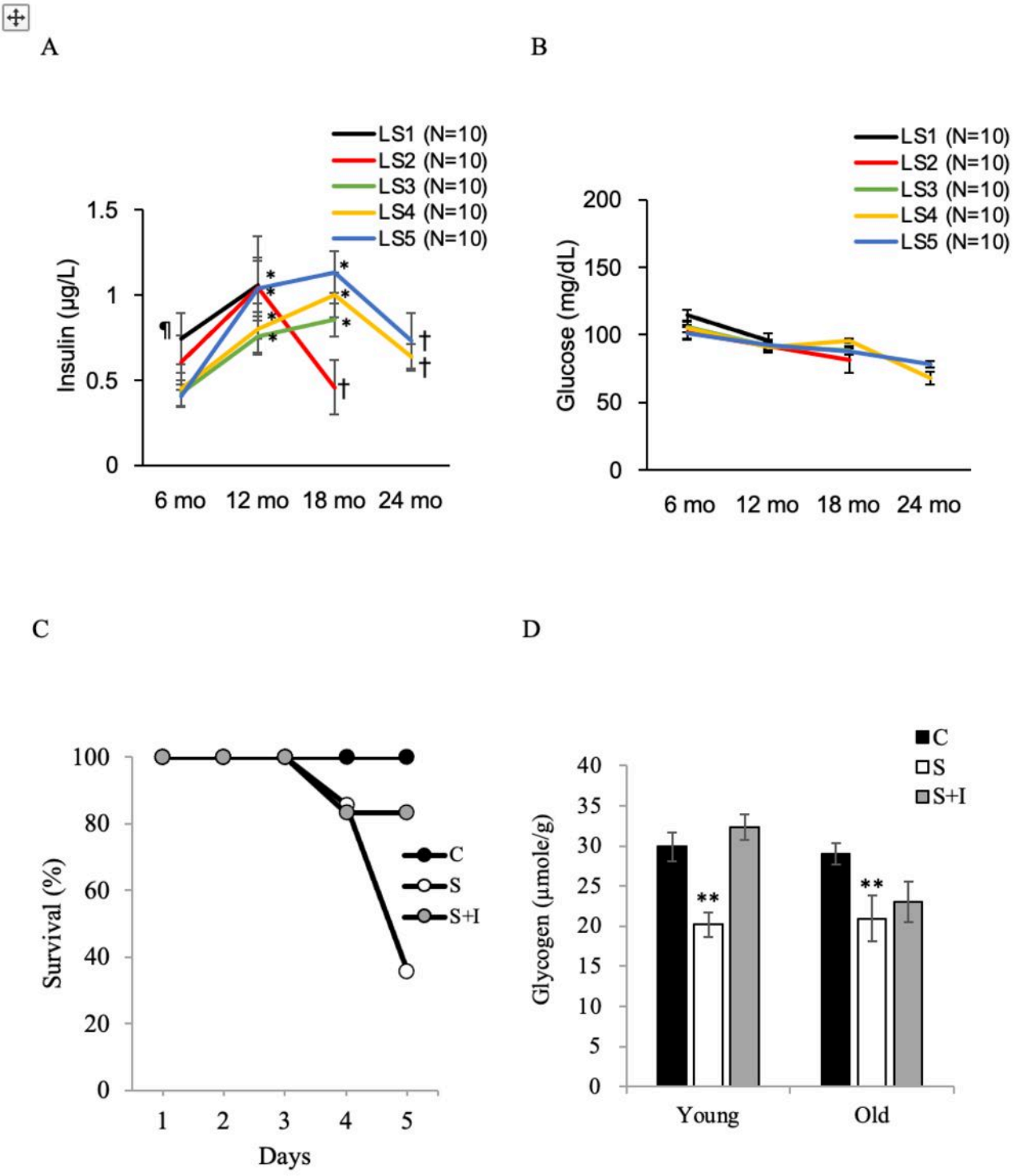
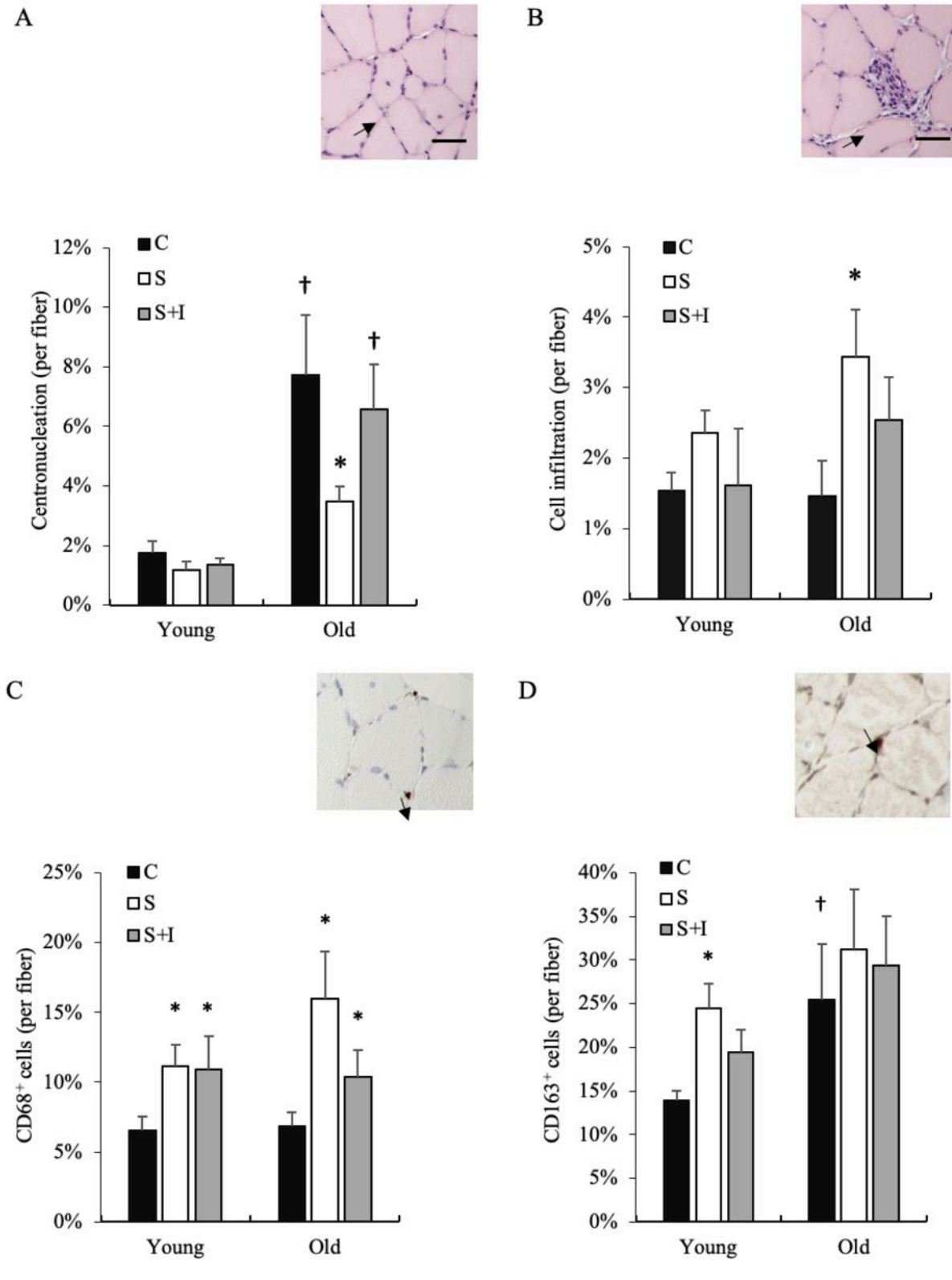

A
LS1 (N=10)
LS2 (N=10)
LS3 (N=10)
LS4 (N=10)
LS5 (N=10)
Insulin (µg/L)
1.5
1
0.5
0
¶
*
†
6 mo
12 mo
18 mo
24 mo
B
LS1 (N=10)
LS2 (N=10)
LS3 (N=10)
LS4 (N=10)
LS5 (N=10)
Glucose (mg/dL)
200
150
100
50
0
6 mo
12 mo
18 mo
24 mo
C
Survival (%)
100
80
60
40
20
0
C
S
S+I
1
2
3
4
5
Days
D
C
S
S+I
Glycogen (µmole/g)
40
35
30
25
20
15
10
5
0
**
Young
Old

Figure 5


A
B
C
D
Centronucleation (per fiber)
Cell infiltration (per fiber)
CD68+ cells (per fiber)
CD163+ cells (per fiber)
C
S
S+I
Young
Old
12%
10%
8%
6%
4%
2%
0%
5%
3%
1%
25%
20%
15%
40%
35%
30%