1-1-2009

Effects of aging on regulators of muscle apoptosis in the female F344BN rat

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TITLE

Effects of aging on regulators of muscle apoptosis in the female F344BN rat

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A thesis submitted to the

Graduate faculty of the Department of Biology

At

Marshall University

In partial fulfillment of the requirements for the degree

Of

Master of Science

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Date: 07-27-2009

Summer 2009

Marshall University

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ABSTRACT

Age-related muscle atrophy is a consequence of normal aging characterized by decreases in muscle mass and strength. The mechanism(s) underlying the loss of muscle mass with increasing age is not fully understood, however recent data has suggested that muscle cell apoptosis may be involved. Here we investigate how aging affects the regulation of muscle apoptosis in the extensor digitorum longus (EDL) and soleus muscles of young (6-month), aged (26-month), and very aged (30-month) female Fischer 344/NNiaHSD X Brown Norway / BiNia (F344BN) rats. EDL and soleus muscle mass/body weight ratios were lower in aged animals but not different between 26- and 30-months of age. Decrease in muscle mass was associated with increased TdT-mediated dUTP nick-end labeling (TUNEL) positive immunoreactivity in both EDL and soleus. With advancing age the time course and magnitude of changes in Bax, Bcl-2, caspase-3, caspase-9, caspase-12 and cleavage of α-fodrin protein were regulated differently between muscles. These data demonstrated that decreases in muscle mass, and increases in muscle cell apoptosis appear to be caspase independent and differ between fiber types in the female F344BN rats with aging.

Keywords: Aging; Apoptosis; F344BN; Female; Bax; Bcl-2; Muscle;

ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Eric Blough, for his guidance during my research and study at Marshall University. Without his support, my ambition to study abroad could hardly have been realized. Eric was always there to listen and to give advice. He taught me how to ask questions and express my ideas. He showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. I was delighted to interact with Dr. David Mallory by attending his classes and having him as my co-advisor and thesis committee member. Dr. Bin Wang and Dr. Kevin M. Rice deserve special thanks as my thesis committee members for their valuable comments and suggestions on the thesis. All my lab buddies at the Laboratory of molecular physiology made it a convivial place to work. In particular, I would like to thank Sunil Kakarla, Anjaiah Katta, Anil Gutta, Satyanarayana Paturi, Madhukar Kolli, Ravi Arvapalli, Siva Nalabotu, Jacqueline Fannin and Miaozong Wu for their friendship and help in the past two years. Working side by side with each of you made the experiments an enjoyable experience. I would like to acknowledge National Institute on Aging as my research was supported by their grant to Eric Blough. Last, but not least, my deepest gratitude goes to my family: Bhushaiah, Sambrajyam, Ramchand and Nageswara Rao Kodali and friends: Paramesh Kurapati, Vijay Udataneni, Vinay Akkina, Dinesh Jasti, Anil Kommineni, Kranthi uppaturi, Tulluri sreenivas, Hari Addagarla and Chanrasekhar Bathina for their unflagging love and support throughout my life.

ABBREVIATIONS

ANOVA	One-way analysis of variance on ranks
AAALAC	Association for assessment and accreditation of laboratory animal care
BSA	Bovine serum albumin
CSA	Cross sectional area
ECL	Enhanced chemiluminiscence
EDL	Extensor digitorum longus
F344BN	Fischer 344/NNiaHSD X Brown Norway / BiNia
KRB	Krebs-Ringers Buffer solution
NIA	National Institute of Aging
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.5% tween
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SEM	Standard Error of Mean
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TUNEL	TdT-mediated dUTP nick-end labeling
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.5% tween

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CHAPTER 1

Introduction

Sarcopenia is the loss of muscle mass and strength due to the aging process [1-3]. Sarcopenia contributes to physical disability, a loss of independence and increases the risk of injury in the elderly [4,5]. Age-associated muscle atrophy is emerging as a major health concern: during the 20th century, the number of persons in the United States under age 65 has tripled. According to the U.S. census bureau (1995) it is anticipated that the number of elderly will more than double between now and the year 2050. It has been estimated that the direct healthcare cost of sarcopenia in United States was $18.5 billion ($ 10.8 billion in men, $ 7.7 billion in women) in 2000, and this number is expected to increase [6].

Sarcopenia occurs at an earlier age in women [7]. Why sex may affect the incidence of sarcopenia is not well understood. Recent data has demonstrated that apoptosis may play a considerable role in mediating the progression of muscle loss in both rats and humans [8,9]. Apoptosis is programmed cell death that is characterized by DNA fragmentation, nuclear condensation, proteolysis, membrane blebbing and cell fragmentation [10-12]. Age associated adaptations like disuse, denervation, deficient satellite cell recruitment, mitochondrial dysfunction, oxidative stress, and decline in anabolic hormones synthesis may be responsible for activation of muscle apoptosis [13-17]. It has been postulated that mitochondria are centrally involved in activating apoptosis via caspase-dependent and independent mechanisms [18-20]. The

mitochondrial pathway of apoptosis can be initiated by reactive oxygen species [21], which can cause the mitochondria to release cytochrome c into the cytosol and formation of the apoptosome. Once the apoptosome is formed, procaspase-9 can undergo auto cleavage into an activate caspase-9 [22,23]. Caspase-9 then cleaves and activates procaspase-3. Active caspase-3 subsequently cleaves a wide range of protein substrates resulting in characteristic morphological changes in the nucleus, DNA fragmentation and cytoskeletal reorganization [24]. This caspase cascade activation results in disintegration of cell into apoptotic bodies. Recent data suggests that Bcl-2 family members can either promote (e.g., Bax, Bak, Bid, Bad and Bim) or prevent (e.g., Bcl-2 and Bcl-XL) the release of cytochrome c from mitochondria [25]. In particular, levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 and their ratio determines the fate of cytochrome c release [26]. In a caspase-independent manner, mitochondria can also release proapoptotic proteins, such as apoptosis inducing factor (AIF), Omi, and endonuclease G (endo G) [23]. Once in cytosol, these proteins translocate to the nucleus and induce apoptosis by DNA fragmentation. Alternatively, increased endoplasmic reticulum stress with aging can contribute to calcium dyshomeostasis [27,28] which can lead to the activation of procaspase-12 [29]. Recent data has demonstrated that caspase-independent apoptosis is responsible for sarcopenia in male F344BN rats [30]. In addition it has been shown that fast twitch and slow twitch muscles in male animals respond differently during aging [31]. Whether similar mechanisms and differences between muscle types are observed in the aging female F344BN rat has not been investigated.

Purpose

Our long term goal is to elucidate intracellular signaling mechanisms responsible for age-associated muscle atrophy. The purpose of the present study was to determine how aging affects apoptotic associated signaling pathways in female F344BN rats and whether differences in apoptotic signaling exist between muscle types. These goals will be accomplished by following specific aims.

Specific Aim # 1

To determine if age-related muscle atrophy is associated with increased myocyte apoptosis in the extensor digitorum longus (EDL) and soleus muscles of female F344BN rats.

Hypothesis:

Age-associated muscle atrophy will be associated with apoptosis in the EDL and soleus muscles of female F344BN rats.

Specific Aim # 2

To determine if the expression of muscle apoptosis regulators Bax and Bcl-2 is altered with aging in the EDL and soleus muscles of female F344BN rats.

Hypothesis:

Age-associated muscle atrophy will be associated with alterations in the expression of Bax and Bcl-2 in the EDL and soleus muscles of female F344BN rats.

Specific Aim # 3

To determine if the expression of caspase-3, caspase-9 and caspase-12 is altered with aging in the EDL and soleus muscles of female F344BN rats.

Hypothesis:

Aging will be associated with alterations in the amount of caspase-3, caspase-9, and caspase-12 in the EDL and soleus muscles of female F344BN rats.

Significance of study:

Sarcopenia is associated with increased health care costs and negatively impacts quality of life for many of the United States aging population. According to 1995 US census, the number of elderly women outnumbered elderly men by a ratio of 3 to 2. Sarcopenia is also a greater public health problem for women since they live longer and have higher rates of disability. This study will determine how aging alters the regulation of proteins thought to govern muscle apoptosis in females. This study will increase our understanding of the basic cellular mechanism underlying sarcopenia and be of potential use for the development of novel pharmacological and nutritional interventions to reverse or prevent sarcopenia.

CHAPTER 2

Review of Literature

Introduction:

A review of the pertinent literature concerning the present study will be presented in the following chapter. The following areas will be addressed: 1. Effect of aging and gender on skeletal muscle mass and strength and 2. Molecular mechanisms of age-related muscle atrophy.

Effect of aging and gender on skeletal muscle mass and strength

Older people experience difficulty in performing daily living activities due to a decline in the force-generating capacity of their skeletal muscles [32]. To evaluate this age-associated muscle loss several researchers have examined the cross sections of various limb muscles by measuring the whole muscle cross sections from cadaveric specimens or by using imaging techniques such as ultrasound, computer tomography or magnetic resonance imaging. Young et al.,1985 using ultrasonic imaging, reported reductions in the cross-sectional areas (CSA) of the quadriceps muscles in older men (25% less) and women (33% less) compared to young controls [33,34]. Computerized tomographic scanning showed similar reductions in the CSA of thigh (12.5%), all thigh muscles (14.7%), quadriceps femoris muscle (16.1%) , and flexor muscles (14.9%) in men [35]. Rice et al., 1989 reported decrease in elderly limb muscles size is associated with increase in non-muscle tissue (fat and connective tissue) [36]. It has been reported that men (14.8%) exhibited larger age-related decreases in total appendicular skeletal muscle mass than women (10.8%) [37]. Janssen et al. observed similar gender

differences by measuring skeletal muscle mass in a sample of 268 men and 200 women between 18 and 88 years of age using whole body magnetic resonance imaging [38]. This age-related muscle atrophy is due to reduction of fiber size, and reductions in fiber number with a preferential loss of type II fibers [39]. Thomas et al., 1985 studied the age-associated changes in fiber number and fiber type composition in EDL and soleus and diaphragm muscles of male F344BN rats using histochemical myosin ATPase fiber typing [40]. His data suggested that age-related losses in the number of muscle fibers and the preferential loss of type II (a and b) fibers may not be universally applicable. Jan Lexell et al., (1987), evaluated cross-sections of vastus lateralis muscles from 43 physically healthy men between 15 and 83 years of age, and suggested that sarcopenia begins around 25 years of age before accelerating thereafter [41]. This work also suggested that muscle atrophy is caused by a loss of fibers, with no predominant effect on any fiber type, and that it is accompanied by a reduction in fiber size, mostly of type II fibers. Although many studies have reported age-related muscle loss the mechanisms responsible for these changes have not been completely investigated.

Aging studies using humans are complicated by ethical issues and by the fact that human aging occurs over many decades. Because of this a great deal of research on aging has been performed on the aged rats because of its small size, limited life span and cost. The Fisher 344/NNiaHSd X Brown Norway/BiNia (F344BN) rat model has been recommended by the National Institue of Aging (NIA) for aging studies given that it exhibits fewer age-related pathologies (e.g., glomerulonephritis, retinal atrophy, and leukemia) than other inbred strains [42]. Rice et al., 2005 reported that the F344BN provides a better model of the alterations seen in aging human muscle than the

F344/NNiaHSd rat model [43]. Pistilli et al., 2006 used fast plantaris of male F344BN rats to study molecular regulation of apoptosis in aging and muscle unloading models [44]. Lushj et al., 2008 suggested that this rat model show age dependent decline in muscle mass and fiber and an increase in fiber atrophy and nonmuscle tissue after analyzing the three of quadriceps muscle (vastus lateralis, rectus femoris, and vastus medialis) at different ages [45].

Summary:

The age associated deterioration in size, mass, and strength of skeletal muscle that has been observed in human skeletal muscle can affect the quality of life. These age-associated changes in skeletal muscle may differ with gender and fiber type. The molecular mechanisms underlying the sarcopenia are not well understood.

Molecular mechanisms responsible for age-related muscle atrophy

The molecular mechanism(s) underlying sarcopenia are only poorly understood. It has been suggested that changes in proteolytic activity, neurologic deficits (loss of α-motor neurons), hormonal alterations (decline in growth hormone, Insulin like growth factor-1, testosterone or estrogen) and degree of physical inactivity likely contribute to loss of skeletal mass and contractile function with aging [46,47]. Recent data has suggested that an increased incidence of apoptosis might represent a key mechanism driving the onset and progression of muscle loss [8,23]. It has been demonstrated that reductions in myonuclear number per fiber and increased incidence of DNA fragmentation (as assessed by TUNEL staining) are associated with muscle atrophy

caused by immobilization and denervation [48,49]. Although apoptosis may occur via several mechanisms, the mitochondria are thought to play a major regulatory role [18-20]. Yasuhara et al., 2000 reported the role of mitochondria and caspase mediated mechanisms in muscle weight loss using burn injury model. This study suggests that increases in mitochondrial outer membrane permeabiliability leads to release of cytochrome c into the cytosol which activates caspase-3 [50]. After release of cytochrome c from the mitochondria, an apoptosis-initiating complex, the “apoptosome”, is assembled which consist of apoptotic protease activating factor-1 (Apaf-1), procaspase-9 and dATP. The apoptosome, when activated, results in cleavage and activation of procaspase-9 [22,23]. Following the activation of caspase-9, caspase-3 becomes activated which in turn leads to DNA fragmentation [24,47]. Reorganization of the cytoskeleton occurs concomitantly and results in disintegration of the cell into apoptotic bodies, eventually destroying the cell.

Tews et al., 1997 reported that denervated muscle contained a greater number of apoptotic myonuclei and an increased BAX to Bcl-2 ratio compared to innervated muscle [51]. This shift of BAX/Bcl-2 ratio towards apoptosis may be one determining factor in influencing the cytochrome c release [24]. In addition, caspase-independent apoptosis has been shown to occur via release of apoptosis inducing factor (AIF), endonuclease G (endo G) from the mitochondria [8,52]. Once released in to cytosol they translocate to the nucleus and cleaves the chromatin DNA in to nucleosomal fragments independently of caspases. Endoplasmic reticulum stress could also partly contribute to apoptosis by releasing the calcium into the cytosol [29] This leads to activation of caspase cascade by activated caspase-12. Nonetheless, the specific

molecular mechanisms underlying the progression of muscle atrophy with aging remain to be determined. Strasser et al., 2000 observed that the age-related loss of skeletal muscle cells in human rhabdosphincter muscle was associated with increased apoptosis [53]. Similarly, Dirks et al., 2001 reported an increase in the number of apoptotic cells in the skeletal muscle of aging male Fischer 344 rats [8]. Rice et al., 2006 suggested that mitochondrial-dependent apoptosis pathways may not play a primary role in the loss of muscle nuclei in the aging male F344BN rat model. In addition they also provide evidence suggesting that both proteolytic and apoptotic regulatory events are different between fiber types with aging [31]. Lushaj et al., 2008 reported age-related changes in fiber number, muscle mass, CSA and nonmuscle tissue of vastus lateralis, rectus femoris and vastus medialis (three of quadriceps muscles) at 3-month intervals in male F344BN rats [45]. Marzetti et al., 2008 reported that mitochondrial caspase-independent apoptotic pathway may play a more prominent role in skeletal muscle loss than caspase-dependent apoptotic pathway in skeletal muscles of male F344BN rats [30]. To date, no reports have examined if the mechanisms responsible for age-related sarcopenia differ across gender.

Summary:

The molecular mechanisms responsible for age-related alterations in skeletal muscle remain elusive. Caspase-independent apoptosis appear to play a role in mediating sarcopenia in male rats. Additional evidence also suggests that apoptotic mechanisms may differ across muscle fiber type. How aging affects the regulation of apoptotic signaling in female muscle has not been investigated.

CHAPTER 3

Effects of aging on regulators of muscle apoptosis in the female F344BN rat

(Note: chapter 3 has been formatted for publication purpose)

Abstract

Age-related muscle atrophy is a consequence of normal aging characterized by decreases in muscle mass and strength. The mechanism(s) underlying the loss of muscle mass with increasing age is not fully understood, however recent data has suggested that muscle cell apoptosis may be involved. Here we investigate how aging affects the regulation of muscle apoptosis in the extensor digitorum longus (EDL) and soleus muscles of young (6-month), aged (26-month), and very aged (30-month) female Fischer 344/NNiaHSD X Brown Norway / BiNia (F344BN) rats. EDL and soleus muscle mass/body weight ratios were lower in aged animals but not different between 26- and 30-months of age. Decrease in muscle mass was associated with increased TdT-mediated dUTP nick-end labeling (TUNEL) positive immunoreactivity in both EDL and soleus. With advancing age the time course and magnitude of changes in Bax, Bcl-2, caspase-3, caspase-9, caspase-12 and cleavage of α-fodrin protein were regulated differently between muscles. These data demonstrated that decreases in muscle mass, and increases in muscle cell apoptosis appear to be caspase independent and differ between fiber types in the female F344BN rats with aging.

Keywords: Aging; Apoptosis; Bax; Bcl-2; F344BN; Female; Muscle;

Introduction

Aging in humans is characterized by losses in muscle mass and strength that can impair the ability of the aged to perform every day activities. This age-related muscle atrophy is characterized by muscle fiber loss with the elderly exhibiting nearly a 30-40% decrease in total fiber number between the second and eighth decade of life [54]. The degree of muscle atrophy with aging appears to be greater in men than in women [38,55,56]. Although not completely elucidated, recent data has demonstrated that apoptosis may play a considerable role in mediating the progression of muscle loss in both rats and humans [8,9]. The mechanism(s) regulating this process are not well understood. Similarly, whether differences in the regulation of signaling processes involved in controlling apoptosis may differ across gender or between muscle types has not been clarified.

Muscle apoptosis has been shown to occur in both a caspase-dependent and independent manner [18]. Recent data using male rats has demonstrated that the caspase-independent apoptotic pathway may play a prominent role in skeletal muscle loss [30,31]. In addition, other data has demonstrated that age-related apoptotic signaling may differ between different muscle types [31]. Whether similar mechanisms operate in aging female muscle is not known.

On the basis of findings from our laboratory [57,58] and others [59] indicating that the male Fischer 344/NNiaHSD X Brown Norway / BiNia (F344BN) rats exhibits a similar level of sarcopenia to that seen in aging humans we examined the time course and regulation of apoptotic signaling in the fast-twitch muscle extensor digitorum longus (EDL) and the slow-twitch soleus muscles of adult, aged, and very aged female F344BN

rats. Consistent with previous reports that have employed male animals [31], our findings demonstrate that the regulation of age-related apoptotic signaling may differ across muscle fiber type.

Material and methods

Animals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society as well as the Animal Use Review Board of Marshall University. Procedures were conducted in strict accordance with Public Health Service animal welfare policy. Fully mature adult (6-months; n=4), post-menopausal aged (26-months; n=4) and very aged (30-months; n=4) female F344BN rats were obtained from the National Institute of Aging. Rats were housed two per cage in an AAALAC approved vivarium. Housing conditions consisted of a 12:12 hour dark-light cycle and temperature was maintained at 22 ± 2 °C. Animals were provided food and water *ad libitum*. Rats were allowed to recover from shipment for at least two weeks before experimentation and during this time the animals were carefully observed and weighed weekly. None of the older animals exhibited signs of failure to thrive such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations.

Materials

Primary antibodies against Bax [#2772], Bcl-2 [#2876], caspase-3 [#9662], caspase-9 [#9506], caspase-12 [#2202], α-fodrin [#2122], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [# 2118], HRP-linked anti-rabbit IgG [#7074] and NIH-3T3 control cell extracts [#9203] were obtained from Cell Signaling Technology (Beverely, MA). The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end

labeling) assay kit was purchased from Roche Diagnostics Corporations (Indianapolis, IN). Antibody against dystrophin (C-terminus) was from Novocastra Laboratories Ltd. (Newcastle, UK). Texas Red anti-mouse secondary antibody [#TI-2000] and mounting medium with DAPI [# H-1500] was acquired from Vector Laboratories, Inc. (Burlingame, CA). Precast 10% and 15% SDS-PAGE gels were procured from Lonza (Rockland, ME) while the Enhanced Chemiluminescence (ECL) Western Blot Detection Reagents, Hyperfilm and Hybond nitrocellulose membranes were attained from Amersham Biosciences (Piscataway, NG).Tissue protein extraction reagent (T-PER) was obtained from Pierce (Rockford, IL). Dual Color Molecular Weight Markers were from Bio-Rad (Hercules, CA). All other chemicals were purchased form Sigma (St. Louis, MO).

Tissue Isolation

Rats were anesthetized with ketamine-xylazine (4:1) cocktail (50 mg/kg intraperitoneal injection) and supplemented as necessary for reflexive response. Soleus and extensor digitorum longus (EDL) were rapidly removed, blotted dry, weighed, and immediately frozen in liquid nitrogen. Tissues were stored at -80° C until use.

***In situ* TUNEL Staining**

Cross sections of 8µm thickness were obtained from mid-belly of the soleus and EDL muscles using an IEC Minotome Cryostat. After fixing with 4% paraformaldehyde, sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4^0C. The TUNEL reaction mixture (50µl) containing terminal deoxynucleotidyl

transferase (TdT) and fluorescein-dUTP was added to the sections and incubated for 60 min at 37^0 C in a dark humidified chamber. After washing with phosphate buffered saline (PBS), pH 7.4, tissues were incubated with an anti-dystrophin antibody (1:500) for 30 min at room temperature, washed, and then incubated with secondary antibody for 30 min at room temperature. After rinsing with PBS, sections were mounted and counterstained DAPI (4, 6-diamidino-2-phenylindole) to visualize nuclei. Three randomly selected regions from each cross-section were visualized with Olympus fluorescence microscope (Melville, NY) using a 20X objective. Control experiments performed in parallel using DNase 1 or without TdT were used to verify specificity of labeling. Images were digitally recorded using a CCD (Olympus, Melville, NY) camera.

Immunoblotting

EDL and soleus muscles were homogenized on ice, twice for 30 seconds in T-PER (1 mL/100mg tissue weight) supplemented with 1mM PMSF, 1mM Na_3VO_4, and 1mM NaF. After centrifugation (10,000 X g for 15 min at 4ºC), the supernatant was separated from the pellet and stored in aliquots at -80ºC until use. Protein concentrations were determined in triplicate using the Pierce 660 nm protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Samples were diluted to a concentration of 2.0 mg/ml in SDS-loading buffer, boiled for 5 minutes, and 40 µg of total protein was separated using SDS-PAGE. Transfer of protein onto nitrocellulose membranes was performed using standard conditions [60]. After transfer, membranes were blocked in 5% milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 1h at room temperature and then incubated with the appropriate primary antibody

overnight at 4^0C. After washing with 1X TBS-T, membranes were exposed to horseradish peroxidase-labeled IgG secondary antibody for 1h at room temperature. Protein bands were visualized with ECL (Amersham Biosciences) and the exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and non-saturated range. Band signal intensity was quantified by densitometry using Imaging software (Alpha Ease FC) and normalized to GAPDH to verify equal protein transfer to membranes. Molecular weight markers (Bio-rad) were used as molecular mass standards and NIH-3T3 cell lysates were included as positive controls.

Data analysis

Results are presented as mean ± SEM. Differences among age groups were evaluated by one way analysis of variance (ANOVA) followed the student-Newman-Keuls test using Sigma Stat 3.5 statistical program. The level of significance accepted for differences was set at $P<0.05$.

Results

Aging related muscle atrophy is associated with an increase in the number of TUNEL positive myonuclei

Compared to 6-month rats, soleus muscle to body weight ratio was 25% lower in both 26-months and 30-months ($P<0.05$). Similarly, EDL muscle to body weight ratio was 24% and 28% less in the 26- and 30-month animals ($P < 0.05$). EDL and soleus muscle to body weight ratio was unchanged between 26- and 30-months (Table 1, Figure 1)

TUNEL staining was used to detect and quantify apoptosis in the soleus and EDL muscles of aging F344BN female rats. Compared to 6-month old animals, the percentage of TUNEL positive nuclei was significantly increased by 28.5 and 62.7-fold ($P < 0.05$) in the 26- and 30-month soleus (Figure 2). Similarly in the EDL, the number of TUNEL positive nuclei was increased by 2.2 fold $(P < 0.05)$ in 30-month animals (Figure 2).

The regulation of Bax and Bcl-2 protein expression with aging differs across muscle type

Compared to 6-month animals, the expression of pro-apoptotic Bax was 37% and 53% higher ($P < 0.05$) in the soleus muscles from 26- and 30-month old animals. Conversely, the amount of the anti-apoptotic Bcl-2 was 61% and 48% lower $(P < 0.05)$ in 26- and 30-month solei compared to that observed in 6-month animals. Aging increased the ratio of Bax to Bcl-2 in the soleus by 263% and 206% at 26- and 30-months, respectively $(P < 0.05)$.

In the EDL, Bax expression was 31% higher ($P < 0.05$) in 30-month old animals compared to that found in muscles from 6- and 26-month animals. Unlike the soleus, Bcl-2 expression in the EDL was not changed with aging. The ratio of Bax to Bcl-2 in the aging EDL was 31% higher at 30-months compared to 6-months (Figure 3).

Age-related changes in caspase and α-fodrin expression are regulated differently across muscle type

Compared to 6-month animals, caspase-3 protein levels were 29% and 61% higher ($P < 0.05$) in 26- and 30-month solei muscles. Similarly, aging increased the amount of caspase-3 in the EDL by 60% ($P < 0.05$) at 30-months. Contrary to what was found in 6-month animals, caspase-9 protein expression was 28% and 36% higher ($P < 0.05$) in the 30-month soleus and EDL muscles (Figure 4). Total Caspase-12 expression was not changed with aging in either the soleus or EDL muscles (Figure 5). Compared to soleus muscles from 6-months animals, the amount of cleaved α-fodrin was 83% higher ($P < 0.05$) at 30-months. Conversely, the amount of alpha-fodrin cleavage in the EDL muscle was unchanged with aging (Figure 6).

Muscle type has an influence on the correlation between muscle apoptosis and the expression of apoptosis regulatory factors

The relationships among Bax, Bcl-2, Bax to Bcl-2 ratio, caspase-3, caspase-9, α-fodrin and percentage of TUNEL positive nuclei were analyzed using the Pearson's correlation (R^2). In the soleus and EDL muscles, the expression of Bax (soleus: $R^2 = 0.75$, ($P < 0.05$); EDL: $R^2 = 0.21$, ($P < 0.05$)), Bax to Bcl-2 ratio (soleus: $R^2 = 0.41$, ($P <$

0.05)*;* EDL: R^2 = 0.55, (*P < 0.05*)), caspase-3 (soleus: R^2 = 0.69, *(P < 0.05);* EDL: R^2 = 0.05, (*P < 0.05*)), caspase-9 (soleus: R^2 = 0.37, *P < 0.05*)*;* EDL: R^2 = 0.15, (*P < 0.05*)) and the amount of cleaved α-fodrin (soleus: R^2 = 0.49, *P< 0.05*)*;* EDL: R^2 = 0.22, (*P < 0.05*)) were positively correlated with apoptosis (Figures 7, 8). Conversely, Bcl-2 expression (soleus: R^2 = 0.476, (*P< 0.05*); EDL: R^2 = 0.5, *P< 0.05*) was negatively correlated with the percentage of TUNEL positive nuclei.

Discussion

To our knowledge, this is the first report to examine the regulation of muscle apoptosis between muscle types in an aging female animal. Similar to previous work we employed the F344BN rat model because of its increased longevity and decreased cumulative lesion incidence compared to other strains [42]. Furthermore, F344BN rats display an age-related atrophy that is similar to that observed in humans [43]. Consistent to that observed in the aging male F344BN animals, the data of the present study demonstrate that the regulation of apoptotic signaling mechanisms may be different in fast and slow muscle types.

Age-related apoptotic mechanisms appear to differ in fast- and slow-twitch muscles of the female rat

Previous reports in humans and rats have suggested that the degree and rate of muscle atrophy may differ between muscle types and across gender [38,55]. We observe a similar finding here. Using animal survivability curves developed by the National Institutes on Aging that were based on large, long term studies examining F344BN mortality rates we selected animals that corresponded roughly to humans in the 3rd, 7th and 8th decade of life [42,43]. This latter time point was chosen because the World Health Organization defines this age group as ‘‘elderly’’, a time where muscle atrophy and dysfunction are present and accelerating in humans [61]. Although different in absolute age, it should also be noted that the 6-, 26-, and 30-month female animals used in the present study are likely to be of similar “physiological age” to 6-, 30- and 36-month male animals given that these age groups occupy similar positions on

their respective probability of survival curves [42]. Using these crude measures as a means to compare across male and female animals, the findings of the present study are consistent with the notion that muscle loss with aging is less in the female than male F344BN animal. For example, previous data has suggested that the degree of muscle atrophy continues to increase with age in the male F344BN rat [43]. Conversely, here we demonstrate that age-related muscle loss in female animals plateaus at 26-months and remains constant thereafter (Figure 1). Although similar differences between the rates of muscle atrophy with aging between genders have been demonstrated in humans [38,55] it is clear that muscle atrophy in human females is a progressive process that appears to continue even at advanced age. Why the loss of muscle mass appears to remain constant after a certain age in the female F344BN is not clear, however it is possible that the examination of animals older than 30-months of age could have yielded different results. Future studies perhaps employing female animals older than the ones used in this study will no doubt be useful in clarifying this possibility.

Previous data examining the regulation of muscle apoptosis with aging has suggested that the degree of apoptosis may vary by muscle type [30,31]. Our findings support this contention. For example, in the aging soleus muscle the amount of TUNEL positive nuclei increases sharply at 26-months and then again at 30-months of age (Figure 2). Conversely, in the aging female EDL the number of TUNEL positive nuclei does not appear to significantly increase until the animals are 30-months of age. In addition the incidence of apoptotic nuclei are less in the aging EDL than soleus. This latter finding is similar to our previous data when examining the incidence of apoptosis in the aging F344BN male. Why the amount of apoptosis might differ between muscle

types is not entirely clear. Given that different muscle types exhibit differences in their resistance to muscle atrophy, metabolic profile, and degree of usage it would not be surprising that that fast- and slow-twitch muscles may also exhibit different proclivities to nuclei loss during aging. Additional investigation using other muscles or muscles that contain a mixture of fiber types will be useful in expanding our understanding of this finding.

It has been suggested that the mechanisms of age-related apoptosis may differ in fast- and slow-twitch muscle types [29,62,63]. The data of the present study are consistent with this notion. Indeed, one of the main findings of the present study is that the regulation of apoptotic regulators appears to be at variance between muscle types with aging. For example, Bcl-2 content in the soleus is decreased with aging, whereas in the EDL, Bcl-2 content remained constant (Figure 3). Similarly, in the soleus, Bax content was significantly increased at 26- and 30-months, while in the EDL; Bax levels did not change until 30-months (Figure 3). Support for this notion is given by our analysis of the Bax to Bcl-2 ratio; in the aging EDL the ratio of Bax to Bcl-2 remains constant until 30-months of age while in the soleus this ratio is elevated significantly at 26-months (Figure 3). Finally, we observed what appears to be a higher degree of correlation between Bax and the number of TUNEL positive cells in the soleus than in the EDL (Figure 7). Why the content of apoptotic regulators or the relationship between regulators and the extent of apoptosis may differ between muscle types is not entirely clear. Recent data has suggested that enhanced production of reactive oxygen (ROS) and nitrogen species (RNS) may induce a pro-apoptotic shift of the pattern of expression of Bcl-2 proteins (e.g., increased Bax to Bcl-2 ratio) [64]. Given the fact that

the soleus muscle contains a higher concentration of mitochondria and a greater reliance on oxidative activity to produce energy than the EDL it is possible that it also experiences a higher elevation of age-related ROS. This finding, if present, may help to explain the differences we see between the soleus and EDL in the regulation of Bax and Bcl-2. Further experimentation to directly evaluate these factors is likely to increase our understanding of this possibility.

Increases in mitochondrial dysfunction with aging is considered a powerful stimulus for apoptosis [23]. Impairment of mitochondrial function has been shown to trigger the release of cytochrome-c [65]. It is thought that this process is controlled, at least in part, by the ratio of Bax to Bcl-2 with the release of cytochrome-c and cell death favored as the balance shifts toward Bax [66]. In the aging F344BN female EDL and soleus muscles the alterations we observe in the ratio of Bax to Bcl-2 suggest that mitochondrial-mediated processes may play a role in regulation of age-related muscle apoptosis. It is postulated that cytosolic cyotochrome-c couples with apoptotic protease activating factor-1 (Apaf-1) which results in formation of apoptosome and the cleavage of caspase-9. Caspase-9 activation in turn activates caspase- 3 resulting in proteolytic disassembly of the cell [67]. Similar to our findings for Bax and Bcl-2, it appears that the the amount of caspase-3 and its upstream activator caspase-9 with aging (Figure 4) and the degree of correlation between caspase-3, -9 levels and the percentage of TUNEL positive nuclei may differ between the EDL and soleus (Figure 7). Nonetheless, a common theme in both muscles was no significant alteration in the levels of cleaved caspase-3 and caspase-9 in both the aging EDL and soleus muscles (data not shown). Taken together, these data suggest that there is no activation of caspase with aging in

these muscles. This latter finding is similar to what we [31] and others have previously found regarding the regulation of apoptotic signaling in male F344BN rats [8,29]. Nonetheless, these data suggest that the mechanisms of age-related muscle apoptosis may differ with gender. To our knowledge, this finding has not been reported before.

Calpain cleavage of α-fodrin is highly correlated with age-related apoptosis in the slow-twitch soleus muscle

Age-related changes in the ability of skeletal muscle to regulate intracellular calcium (calcium dyshomeostasis) have been demonstrated and it is thought that this phenomenon is capable of causing cellular apoptosis [23]. Increases in cellular calcium, if excessive, can result in the activation of calpains. Calpains are a family of calcium-dependent, non-lysosomal cysteine proteases that can participate in the breakdown of numerous proteins and have been postulated to play a role in muscle atrophy [68,69]. The α-fodrin protein is a 240 kDa protein that can be cleaved by activated calpains to yield a N-terminal fragment of 150 kDa [70,71]. The cleavage of α-fodrin has been observed in many types of cell death and the use of immunoblotting to detect calpain-dependent proteolysis of α-fodrin fragment has proven to be a reliable method to demonstrate calpain activation in cell lysates [72,73]. Similar to our findings for Bax and Bcl-2, the aged EDL and soleus appear to regulate calpain activation differently with aging. For example, we show increased calpain-dependent cleavage of α-fodrin in the soleus with aging but not the EDL (Figure 6). No evidence of capase-dependent cleavage of α-fodrin was present in either muscle (data not shown). These data are different from what has previously been shown in the aging male F344BN where

evidence of caspase-dependent cleavage was evident in both the soleus and EDL [31]. The possibility that disparity exists between the two muscle types in the degree of calpain activation with aging is also represented by differences in the strength of the correlation between the cleavage of α-fodrin and the number of TUNEL positive nuclei (Figure 8). Taken together, these data suggest that proteolytic activities are regulated differently between muscle types with aging. Further, these data also support our hypothesis that the mechanism(s) underlying age-related muscle loss may differ between fiber types and with gender. Whether other signaling pathways involved in regulating muscle atrophy exhibit similar age-related muscle type differences in regulation is unknown. Additional studies examining other possible regulators of apoptosis such as apoptosis inducing factor (AIF) or endonuclease G will certainly be helpful in furthering our understanding if or how gender may influence the mechanism(s) involved in muscle apoptosis.

Conclusion

These data suggest caspase-independent apoptosis may play a role in the age-related loss of muscle nuclei in the skeletal muscles of the female F344BN rat. In addition, we confirm previous observations demonstrating that proteolytic and apoptotic regulatory events are regulated differently in fast- and slow-twitch muscles. Further research directed against determining the role of other apoptotic signaling pathways as a mechanism of age-related muscle nuclei loss is warranted.

Acknowledgements

This study was supported by National Institute on Aging to Eric Blough.

TABLE AND FIGURE LEGENDS

Table 1: Muscle weight and muscle to body weight ratio in young adult (6-month), aged (26-month) and very aged (30-month) female F344BN rats. Values are expressed as mean ± SEM, n = 4 per each group. * indicates significant difference from young adult (6 month) age group ($P < 0.05$).

Figure 1: Changes in the muscle to body weight ratio with age in A) Soleus B) EDL of female F344BN rats. Values are mean ± SEM, n = 4 per each group. * indicates significant difference from young adult (6-month) age group ($P < 0.05$).

Figure 2*:* Quantification of apoptosis with age is shown in A) Soleus B) EDL of female F344BN rats. Apoptotic myonuclei were visualized with TUNEL staining. Muscle borders were visualized using mouse monoclonal antibody dystrophin (C-terminus) and all nuclei were stained with 4', 6-diamidino-2-phenyllindole (DAPI). Arrows highlight TUNEL-positive nuclei in the image. Data are presented as mean ± SEM. * indicates significant difference from young adult (6-month) age group ($P < 0.05$). † indicates significant difference from aged (26-month) group ($P < 0.05$). n = 4 per each group.

***Figure 3**:* Mitochondrial content of pro-apoptotic Bax shown in A) Soleus B) EDL and anti-apoptotic Bcl-2 shown in C) Soleus D) EDL of female F344BN rats with age as determined by western blot analysis. Expression of Bax and Bcl-2 were normalized for GAPDH. Representative Immunoblot images of Bax, Bcl-2 and GAPDH were showed for each group in insets. C) Bax to Bcl-2 ratio changes with age in both soleus and EDL muscles. Data are presented as mean ± SEM. * indicates significant difference from young adult (6-month) age group ($P < 0.05$). † indicates significant difference from aged (26-month) group ($P < 0.05$). n = 4 per each group.

***Figure 4**:* Expression of tissue content of caspase-3 shown in A) Soleus B) EDL and caspase-9 shown in C) soleus D) EDL of female F344BN rats with age as determined by western blot analysis. Expression of caspase-3 and caspase-9 were normalized for GAPDH. Representative Immunoblot images of caspase-3, caspase-9 and GAPDH were showed for each group. Data are presented as mean ± SEM. * indicates significant difference from young adult (6-month) age group ($P < 0.05$). † indicates significant difference from aged (26 month) group ($P < 0.05$). n = 4 per each group.

***Figure 5**:* Expression of tissue content of cleaved (42 kDa) compared to total (55 kDa) caspase-12 with aging in A) soleus and B) EDL muscles of female F344BN rats as determined by western blot analysis. GAPDH blots were shown below the figure to show equal loading in all lanes along with cleaved (42 kDa) and total (55 kDa) caspase-12 blots. Data are presented as mean ± SEM. * indicates significant difference from young adult (6-month) age group ($P < 0.05$). † indicates significant difference from aged (26-month) group ($P < 0.05$). n = 4 per each group.

***Figure 6**:* Expression of cleaved α-fodrin (150 kDa) compared to total α-fodrin (240 kDa) with aging in A) soleus and B) EDL muscles of female F344BN rats as determined by western blot analysis. GAPDH blots were shown below the figure to show equal loading in all lanes along with cleaved and total α-fodrin blots. Data are presented as mean ± SEM. * indicates significant difference from young adult (6-month) age group ($P < 0.05$). † indicates significant difference from aged (26 month) group ($P < 0.05$). n = 4 per each group.

***Figure 7**:* Relationships between TUNEL staining (% of TUNEL positive nuclei) and protein expression of Bax, Bcl-2, and Bax to Bcl-2 ratio were investigated in the soleus (A, C, E) and EDL (B, D, E) by examining the Pearson product-moment correlation coefficient (R^2).

***Figure 8**:* Relationships between TUNEL staining (% of TUNEL positive nuclei) and protein expression of caspase-3, caspase-9, and cleaved to total α-fodrin were investigated in the soleus (A, C, E) and EDL (B, D, E) by examining the Pearson product-moment correlation coefficient (R^2).

Table 1

Age groups	Body wt (g)	Soleus (mg)	Soleus to body wt ratio	EDL (mg)	EDL to body wt ratio
6m	228.00±8.7	109.13±7.5	0.48±0.02	115.88±6.6	0.51±0.02
26m	322.25±25.9	116.38±9.2	0.36±0.04 *	124.63±1.1	0.39±0.03 *
30m	310.00±11.5	111.75±2.2	0.36±0.01 *	114.25±11.1	0.37±0.02 *

Figure 1

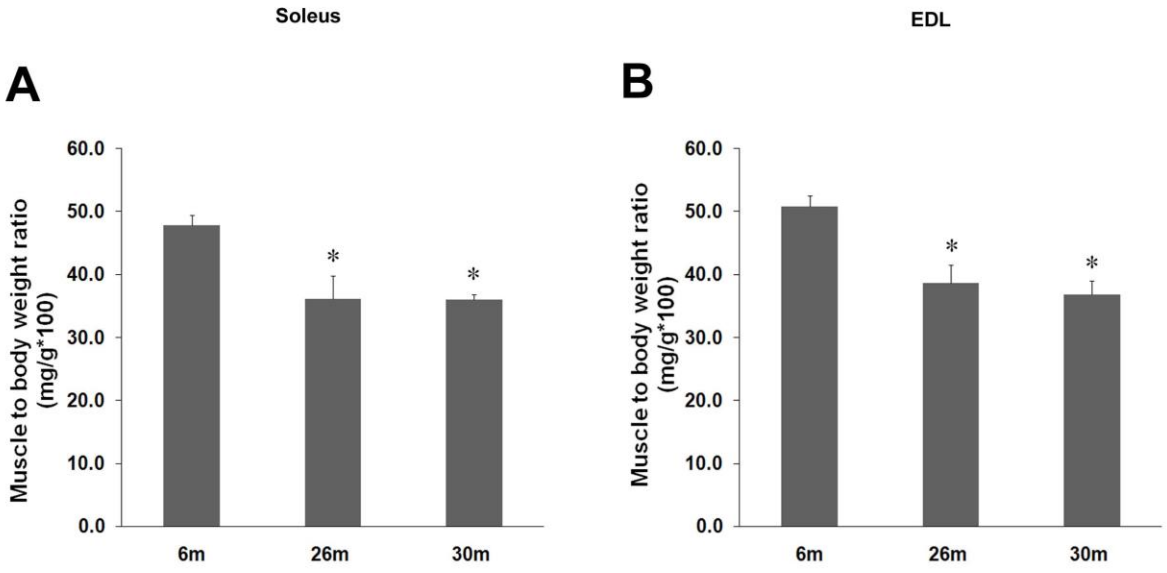

Soleus
A
Muscle to body weight ratio (mg/g*100)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
6m
26m
30m
*
*
EDL
B
Muscle to body weight ratio (mg/g*100)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
6m
26m
30m
*
*

Figure 2

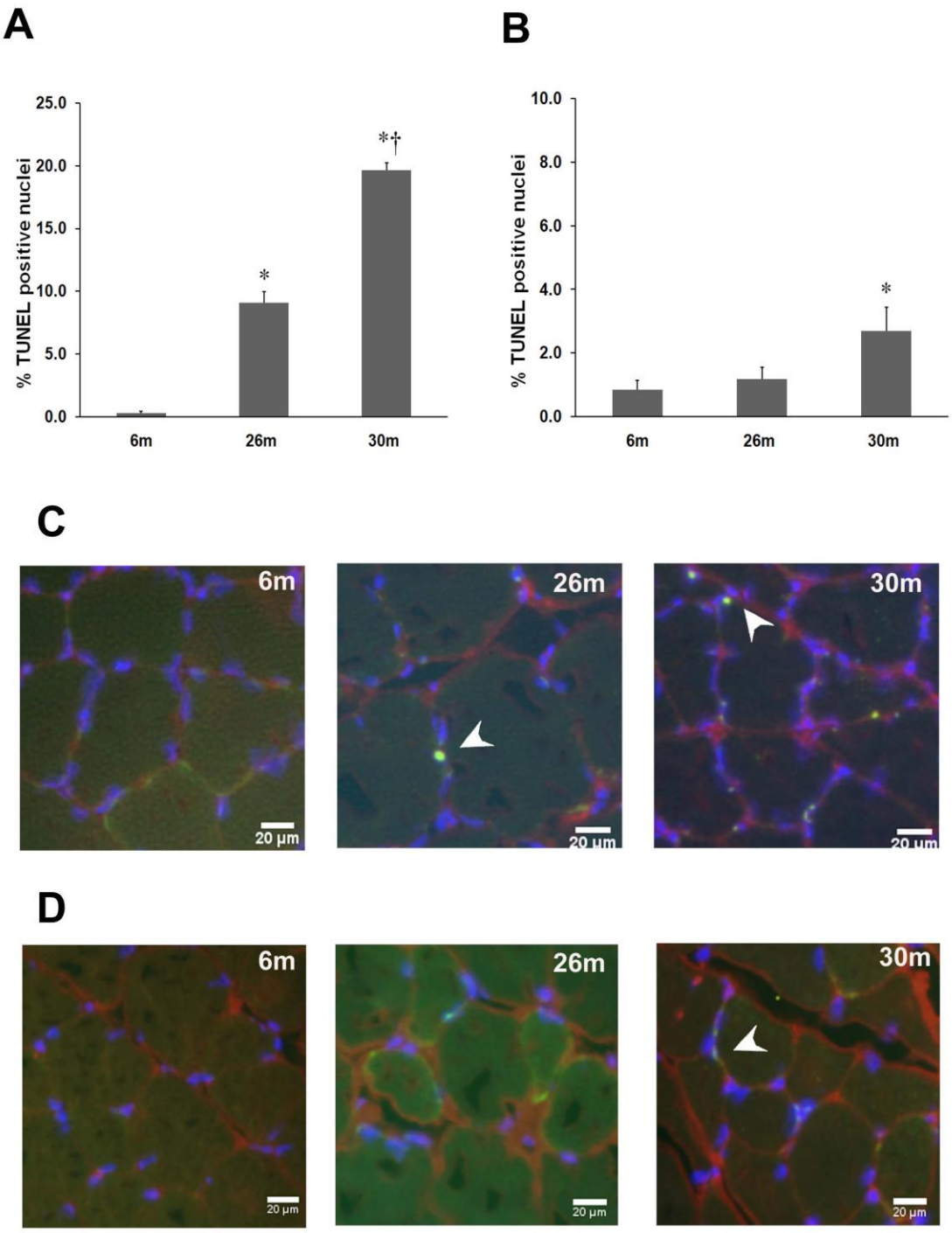

A
% TUNEL positive nuclei
25.0
20.0
15.0
10.0
5.0
0.0
*
*†
6m
26m
30m
B
% TUNEL positive nuclei
10.0
8.0
6.0
4.0
2.0
0.0
*
6m
26m
30m
C
6m
26m
30m
20 µm
D
6m
26m
30m
20 µm

Figure 3

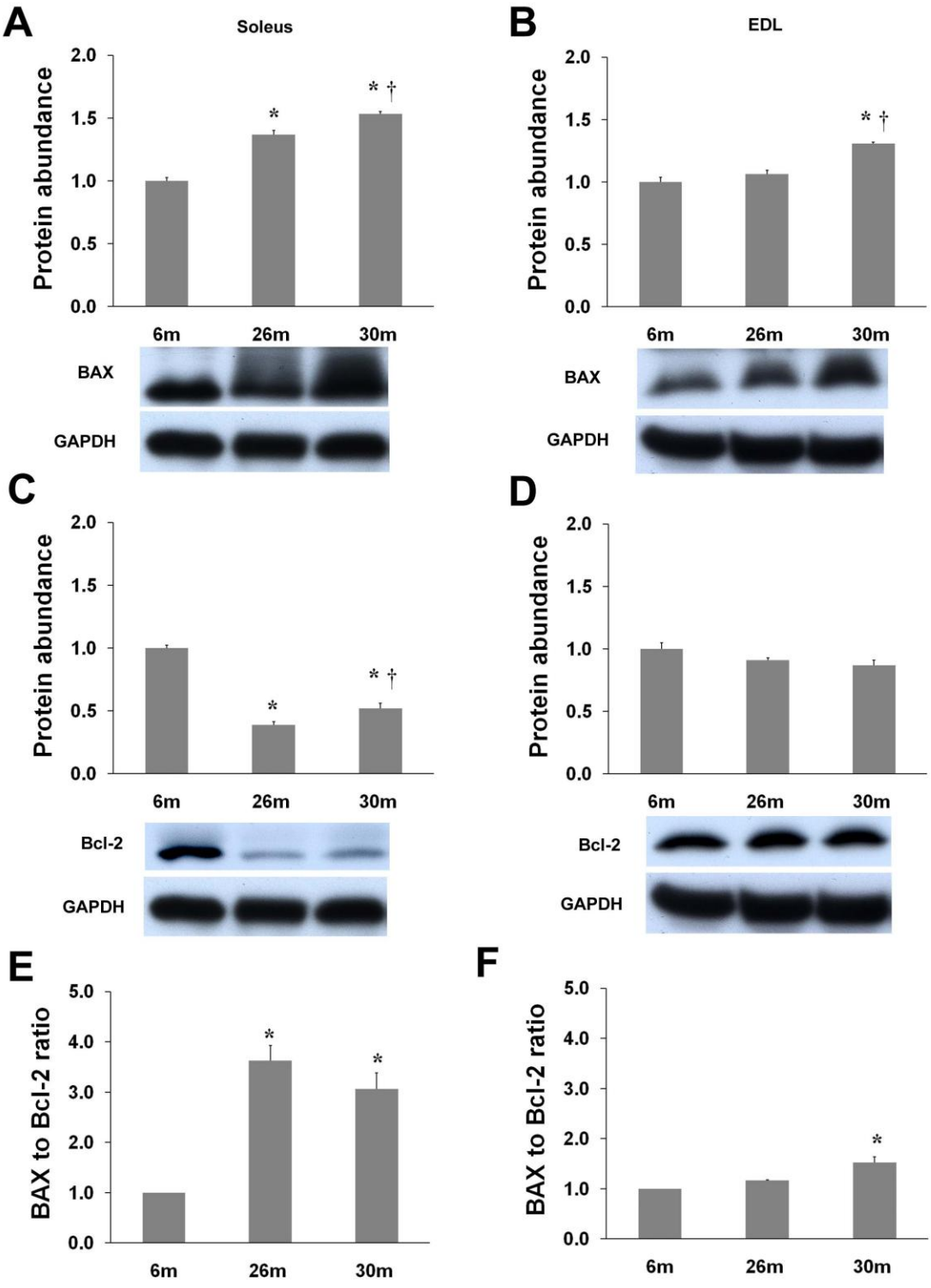

A
Soleus
Protein abundance
2.0
1.5
1.0
0.5
0.0
* †
*
6m
26m
30m
BAX
GAPDH
B
EDL
Protein abundance
2.0
1.5
1.0
0.5
0.0
* †
6m
26m
30m
BAX
GAPDH
C
Protein abundance
2.0
1.5
1.0
0.5
0.0
* †
*
6m
26m
30m
Bcl-2
GAPDH
D
Protein abundance
2.0
1.5
1.0
0.5
0.0
6m
26m
30m
Bcl-2
GAPDH
E
BAX to Bcl-2 ratio
5.0
4.0
3.0
2.0
1.0
0.0
*
*
6m
26m
30m
F
BAX to Bcl-2 ratio
5.0
4.0
3.0
2.0
1.0
0.0
*
6m
26m
30m

Figure 4

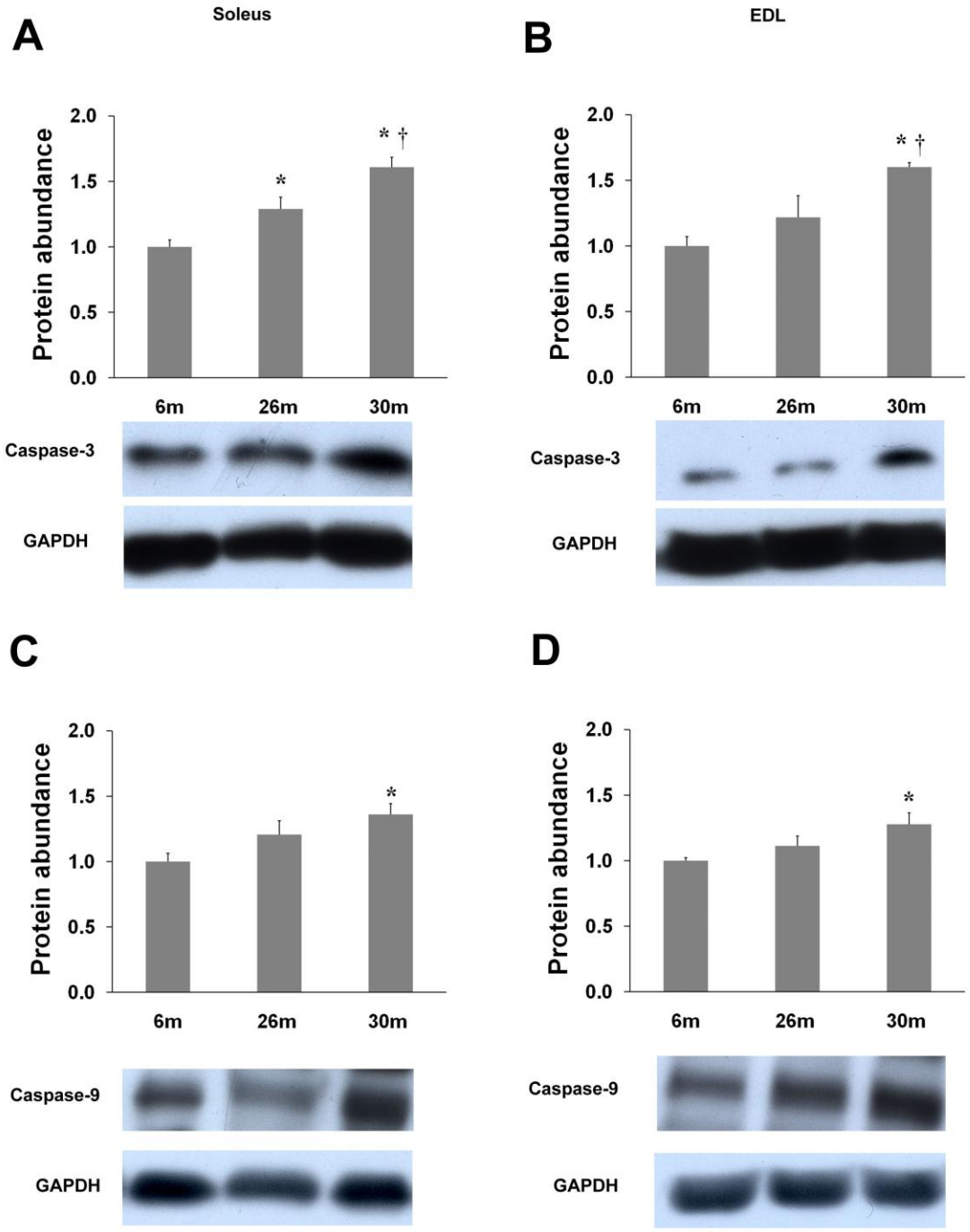

A
Soleus
Protein abundance
2.0
1.5
1.0
0.5
0.0
* †
*
6m
26m
30m
Caspase-3
GAPDH
B
EDL
Protein abundance
2.0
1.5
1.0
0.5
0.0
* †
6m
26m
30m
Caspase-3
GAPDH
C
Protein abundance
2.0
1.5
1.0
0.5
0.0
*
6m
26m
30m
Caspase-9
GAPDH
D
Protein abundance
2.0
1.5
1.0
0.5
0.0
*
6m
26m
30m
Caspase-9
GAPDH

Figure 5

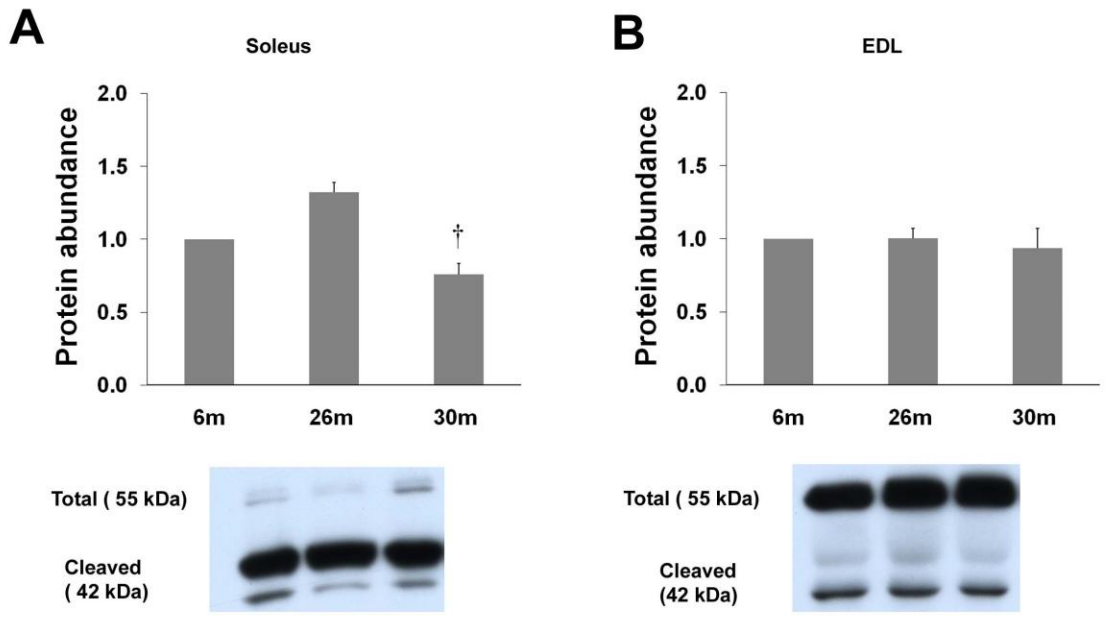

A
Soleus
Protein abundance
2.0
1.5
1.0
0.5
0.0
†
6m
26m
30m
Total (55 kDa)
Cleaved
(42 kDa)
B
EDL
Protein abundance
2.0
1.5
1.0
0.5
0.0
6m
26m
30m
Total (55 kDa)
Cleaved
(42 kDa)

Figure 6

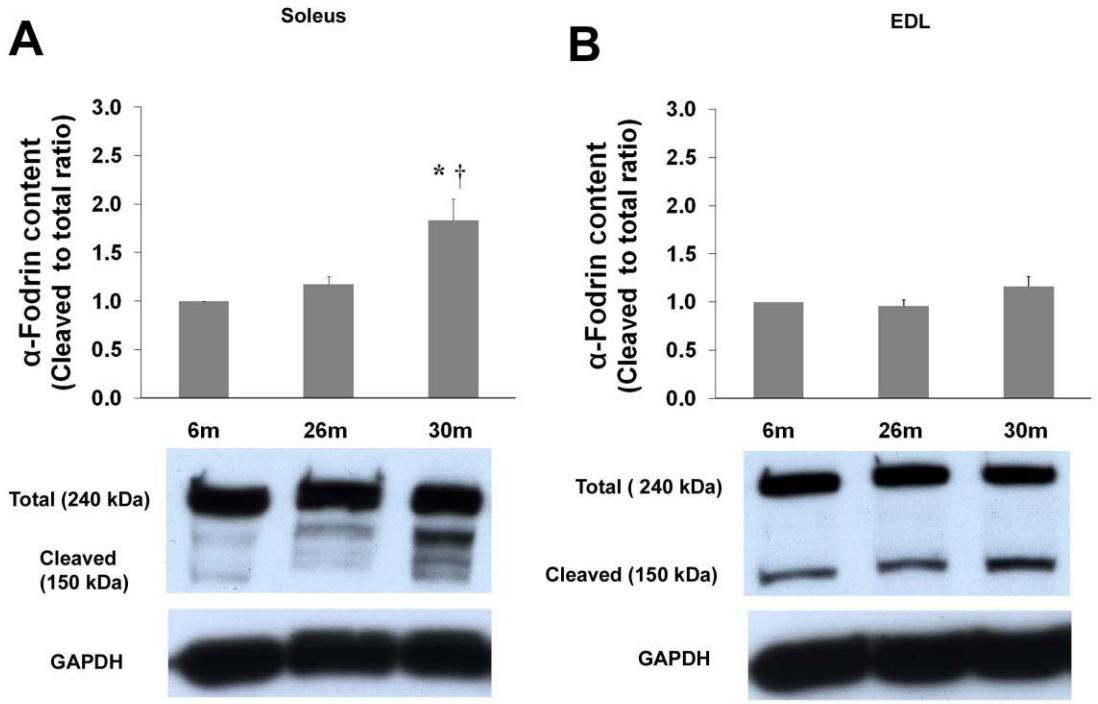

A
Soleus
α-Fodrin content
(Cleaved to total ratio)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
* †
6m
26m
30m
Total (240 kDa)
Cleaved
(150 kDa)
GAPDH
B
EDL
α-Fodrin content
(Cleaved to total ratio)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6m
26m
30m
Total (240 kDa)
Cleaved (150 kDa)
GAPDH

Figure 7

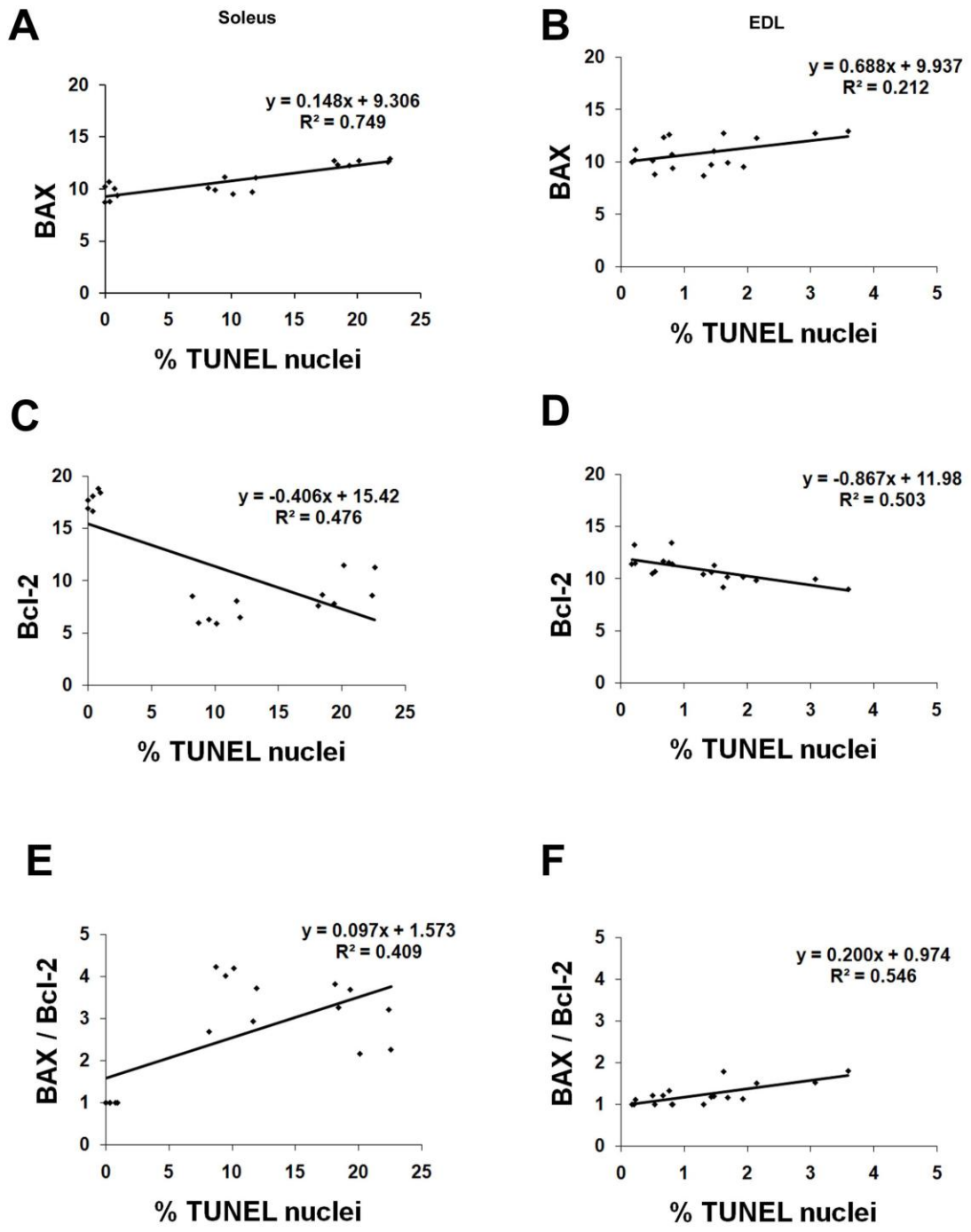
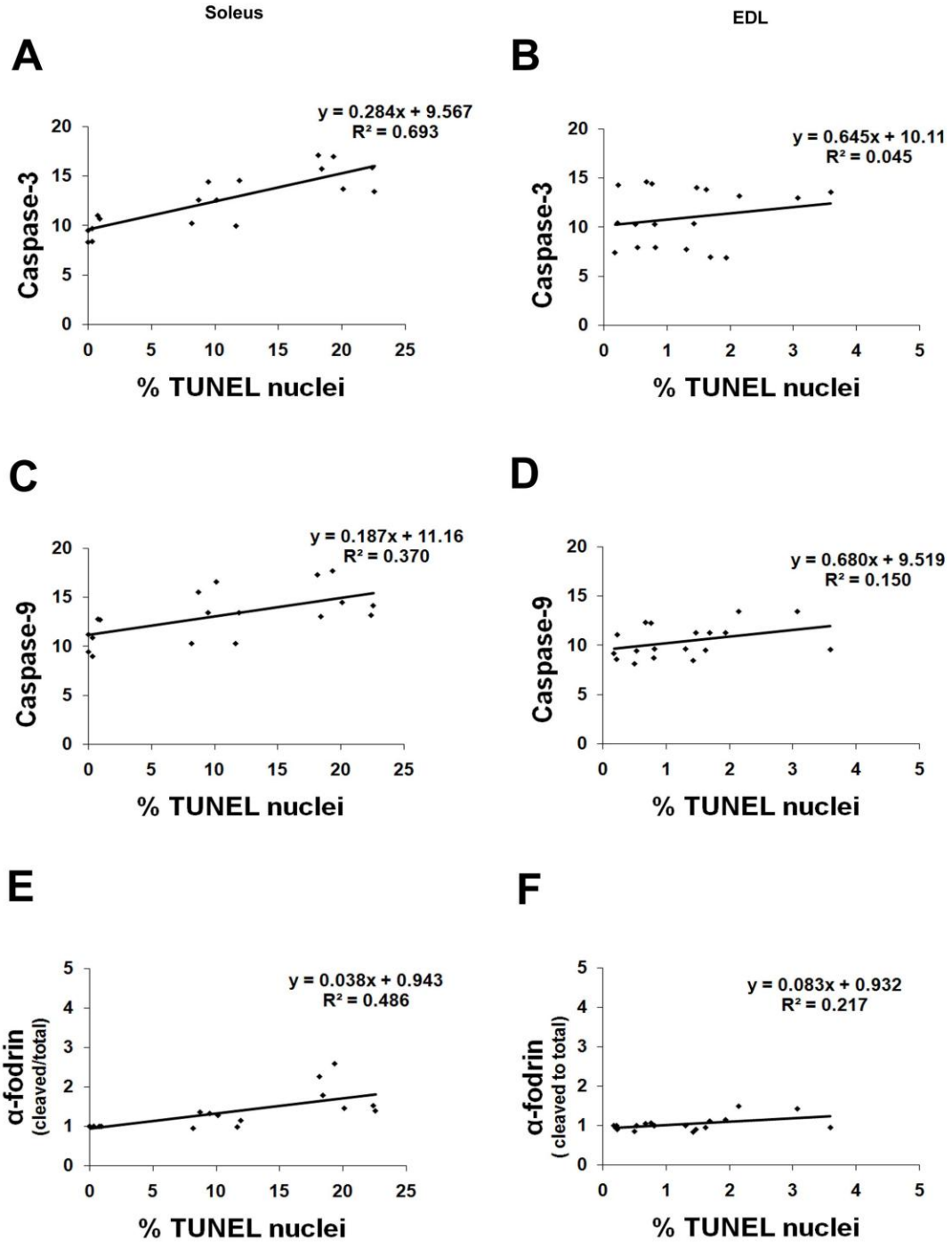

A
Soleus
y = 0.148x + 9.306
R² = 0.749
BAX
% TUNEL nuclei
B
EDL
y = 0.688x + 9.937
R² = 0.212
BAX
% TUNEL nuclei
C
y = -0.406x + 15.42
R² = 0.476
Bcl-2
% TUNEL nuclei
D
y = -0.867x + 11.98
R² = 0.503
Bcl-2
% TUNEL nuclei
E
y = 0.097x + 1.573
R² = 0.409
BAX / Bcl-2
% TUNEL nuclei
F
y = 0.200x + 0.974
R² = 0.546
BAX / Bcl-2
% TUNEL nuclei

Figure 8


Soleus
EDL
A
B
C
D
E
F
y = 0.284x + 9.567
R² = 0.693
y = 0.645x + 10.11
R² = 0.045
y = 0.187x + 11.16
R² = 0.370
y = 0.680x + 9.519
R² = 0.150
y = 0.038x + 0.943
R² = 0.486
y = 0.083x + 0.932
R² = 0.217
Caspase-3
Caspase-9
α-fodrin (cleaved/total)
α-fodrin (cleaved to total)
% TUNEL nuclei

CHAPTER 4

Conclusions

1. Although we demonstrate an increase in the expression levels of caspase-9 and caspase-3 with aging there is no change in the level of active caspases (caspase-3, caspase-9 and caspase-12). These data suggests that age-associated muscle apoptosis in the female F344XBN may occur in a caspase-independent manner.

2. We confirm previous observations in male rats [31] demonstrating that proteolytic and apoptotic regulatory events are regulated differently in fast- and slow-twitch muscles. We observe the alterations in the number of apoptotic cells, Bcl-2, and the ratio of Bax to Bcl-2 across muscle type. In addition, we also found that the calpain-dependent cleavage of α-fodrin is highly correlated with age-related apoptosis in the slow-twitch soleus.

Future Directions

Future directions for research based on this study should focus on the further understanding of molecular mechanisms underlying the sarcopenia in females.

1. We observed that caspase-independent apoptosis may be responsible for age-related muscle nuclei loss in skeletal muscles of female F344BN rats. To further investigate this possibility, it may be useful to examine other upstream molecules that may be involved in this pathway like AIF and EndoG. Similarly, the examination of other caspase-dependent apoptogenic factors like cytochrome c and apaf-1 may lead to a greater understanding of apoptosis may be occurring in the aged female F344BN rats.

2. It is well known that Bcl-2 family proteins and mPTP are involved in the regulation of mitochondrial membrane stability. Investigating the changes in expression of mPTP components and Bcl-2 family proteins other than Bax and Bcl-2 over the course of aging process may also reveal new and interesting information regarding our understanding of how aging affects skeletal muscle in female F344BN rats.

3. The free radical theory of aging suggests that age-related increases in the concentration of reactive oxygen species (ROS) may play a role in aging [74]. It has been shown that enhanced production of reactive oxygen species (ROS) and nitrogen species (RNS) may induce a pro-apoptotic shift in the expression of Bcl-

2 proteins. To address this possibility, the effects of these ROS with aging in the skeletal muscle could be analyzed by oxyblot analysis / or by some immunohistochemical methods such as 8-OHdG (8-Hydroxy-2'-deoxyguanosine) staining. Similarly, the effects of RNS with aging in the skeletal muscle could be analyzed by dot blot analysis for nitrotyrosine. Additional data regarding how aging affects ROS and RNS levels in the skeletal muscles of aging male and female F344BN rats will no doubt be useful in increasing our understanding of gender differences because of direct estrogen effects of skeletal muscle in women.

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APPENDIX

This section includes western blot film properties reports, raw data tables, and statistics of various molecules in soleus and EDL used for this study.

Soleus:

Laboratory of Molecular Physiology
Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde				
Date	2009/02/17	Project	Aging female		
Report Number	1A	Tissue/ cell line/ etc.	Soleus		
Gel type	15%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hours	Duration	1 hour

Primary Antibody

Name (Do Not Abbreviate) Bcl-2

Dilution 1: 1000 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

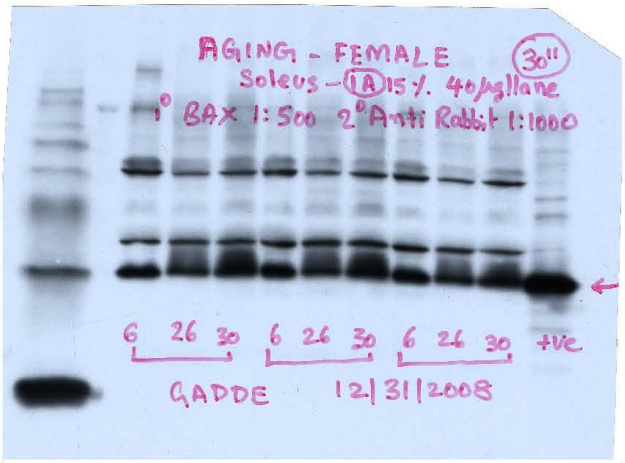
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Exposure time

1 min.

Molecular Wieght

26 KDa

Place Scan of Film Here

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\Soleus\Bcl-2

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\Soleus\Bcl-2

Notes

Murali Krishna Gadde Digitally signed by Murali Krishna Gadde DN: cn=Murali Krishna Gadde, o=Marshall University, ou=Biology, email=gadde@marshall.edu, c=US Date: 2009.07.05 17:00:06 -04'00'

Signature

email Report

Laboratory of Molecular Physiology, Marshall University, Science Bldg. Suite 311, 1 John Marshall Drive, Huntington, WV, 25755 * Phone: 304-696-3267 Fax: 304-696-7136
http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films.

%C	6 m	26 m	30 m
1a	8.20	11.14	13.04
1b	9.10	11.96	13.62
1c	9.00	12.38	13.78
2a	7.90	11.24	12.94
2b	9.00	12.06	13.52
2c	9.20	12.84	13.46
N	6.00	6.00	6.00
Mean	8.73	11.94	13.39
SD	0.54	0.66	0.33
SEM	0.24	0.29	0.15
%RE	100.00	136.68	153.34
SEM	2.78	3.36	1.70

Statistics:

One way analysis of variance (ANOVA)

Normality Test: Passed (P = 0.112)

Equal Variance Test: Passed (P = 0.537)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	6	0	8.733	0.543	0.222
26 months	6	0	11.936	0.655	0.267
30 months	6	0	13.391	0.332	0.135

Source of Variation	DF	SS	MS	F	P
Between Groups	2	68.150	34.075	122.582	<0.001
Residual	15	4.170	0.278		
Total	17	72.319			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
30 months vs. 6 months	4.658	3	21.641	<0.001	Yes
30 months vs. 26 months	1.455	2	6.760	<0.001	Yes
26 months vs. 6 months	3.203	2	14.881	<0.001	Yes

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Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2009/02/17	Project	Aging female
Report Number	1A	Tissue/ cell line/ etc.	Soleus

Gel type	15%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hours	Duration	1 hour

Primary Antibody

Name (Do Not Abbreviate) Bcl-2

Dilution 1: 1000 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

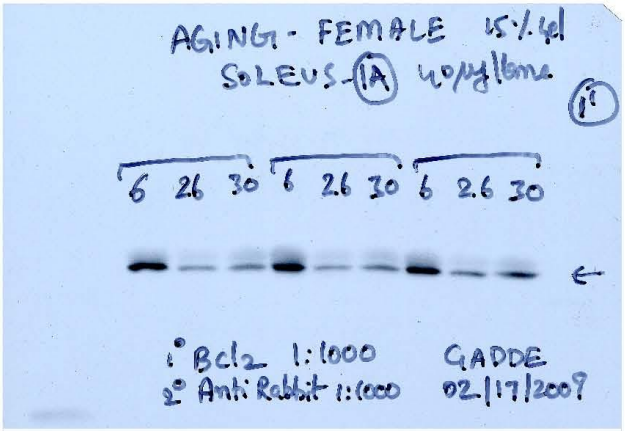
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Place Scan of Film Here

Exposure time

1 min.

Molecular Wieght

26 KDa

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\Soleus\Bcl-2

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\Soleus\Bcl-2

Notes

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

%C	6 m	26 m	30 m
1a	18.10	5.86	7.80
1b	18.40	6.49	8.57
1c	16.60	8.52	11.24
2a	17.70	5.96	7.61
2b	18.80	6.29	8.67
2c	16.90	8.06	11.45
N	6.00	6.00	6.00
Mean	17.75	6.86	9.22
SD	0.86	1.14	1.69
SEM	0.38	0.51	0.76
%RE	100.00	38.67	51.97
SEM	2.17	2.86	4.27

Stastics:

One way ANOVA

Normality Test: Passed (P = 0.183)

Equal Variance Test: Passed (P = 0.567)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	6	0	17.750	0.860	0.351
26 months	6	0	6.865	1.136	0.464
30 months	6	0	9.224	1.693	0.691

Source of Variation	DF	SS	MS	F	P
Between Groups	2	393.504	196.752	120.568	<0.001
Residual	15	24.478	1.632		
Total	17	417.982			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
6 months vs. 26 months	10.885	3	20.873	<0.001	Yes
6 months vs. 30 months	8.526	2	16.348	<0.001	Yes
30 months vs. 26 months	2.360	2	4.524	0.006	Yes

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Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2008/12/05	Project	Aging female
Report Number	3	Tissue/ cell line/ etc.	Soleus

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hours	Duration	1 hour

Primary Antibody

Name (Do Not Abbreviate) caspase-3

Dilution 1: 500 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

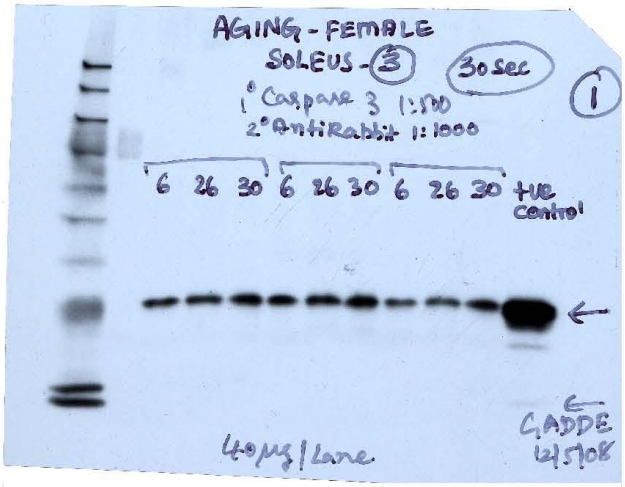
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Exposure time

30 sec.

Molecular Wieght

35 KDa

Place Scan of Film Here

☒ Used For Analysis

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Notes

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

%C	6 m	26 m	30 m
1a	9.70	12.58	16.95
1b	10.70	14.51	15.83
1c	8.40	10.19	13.45
2a	9.50	12.58	17.09
2b	11.00	14.39	15.72
2c	8.30	9.97	13.66
N	6.00	6.00	6.00
Mean	9.60	12.37	15.45
SD	1.12	1.96	1.57
SEM	0.50	0.88	0.70
%RE	100.00	128.87	160.94
SEM	5.24	9.15	7.34

Stastics:

One way ANOVA

Normality Test: Passed (P = 0.415)

Equal Variance Test: Passed (P = 0.568)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	6	0	9.600	1.124	0.459
26 months	6	0	12.372	1.963	0.801
30 months	6	0	15.450	1.575	0.643

Source of Variation	DF	SS	MS	F	P
Between Groups	2	102.777	51.389	20.289	<0.001
Residual	15	37.993	2.533		
Total	17	140.770			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
30 months vs. 6 months	5.850	3	9.004	<0.001	Yes
30 months vs. 26 months	3.079	2	4.739	0.005	Yes
26 months vs. 6 months	2.772	2	4.266	0.009	Yes

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2009/02/18	Project	Aging female
Report Number	2A	Tissue/ cell line/ etc.	Soleus

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) caspase-9

Dilution 1: 500 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

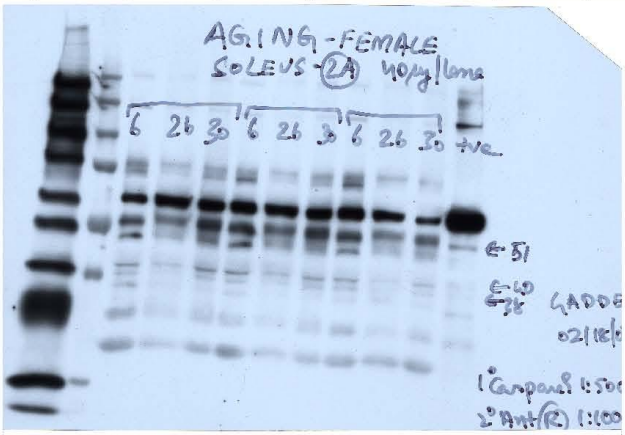
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



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Exposure time

30 sec.

Molecular Wieght

51 KDa

☒ Used For Analysis

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Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\Soleus\caspase-9

Notes

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films.

%C	6 m	26 m	30 m
1a	9.00	16.58	17.67
1b	12.70	13.42	13.16
1c	10.90	10.31	14.16
2a	9.40	15.54	17.29
2b	12.80	13.42	13.06
2c	11.20	10.31	14.48
N	6.00	6.00	6.00
Mean	11.00	13.27	14.97
SD	1.60	2.60	2.02
SEM	0.71	1.16	0.91
%RE	100.00	120.59	136.07
SEM	6.49	10.55	8.23

Statistics:

One way ANOVA

Normality Test: Passed (P = 0.543)

Equal Variance Test: Passed (P = 0.658)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	6	0	11.000	1.596	0.652
26 months	6	0	13.265	2.595	1.059
30 months	6	0	14.968	2.025	0.827

Source of Variation	DF	SS	MS	F	P
Between Groups	2	47.543	23.772	5.329	0.018
Residual	15	66.915	4.461		
Total	17	114.458			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.018).

Power of performed test with alpha = 0.050: 0.659

All Pair wise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
30 months vs. 6 months	3.968	3	4.601	0.014	Yes
30 months vs. 26 months	1.702	2	1.974	0.183	No
26 months vs. 6 months	2.265	2	2.627	0.083	No

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2009/02/18	Project	Aging female
Report Number	3A	Tissue/ cell line/ etc.	Soleus

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) caspase-12

Dilution 1: 500 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

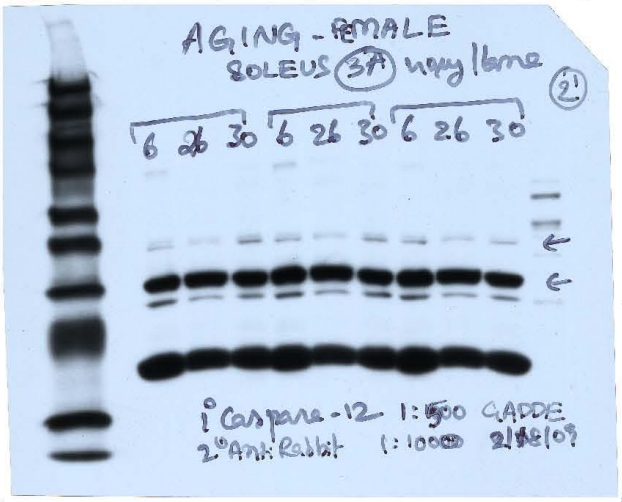
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Exposure time

2 min.

Molecular Wieght

55, 42 KDa

Place Scan of Film Here

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\Soleus\caspase-12

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\Soleus\caspase-12

Notes

Murali Krishna Gadde Digitally signed by Murali Krishna Gadde DN: cn=Murali Krishna Gadde, o=Marshall University, ou=Biology, email=gadde@marshall.edu, c=US Date: 2009.07.05 17:00:06 -04'00'

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email Report

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

Total caspase-12:

%C	6 m	26 m	30 m
1a	1.00	0.67	1.40
1b	1.00	0.71	1.06
1c	1.00	0.76	0.82
2a	1.00	0.73	1.41
2b	1.00	0.71	1.58
2c	1.00	0.75	0.81

Cleaved caspase-12

%C	6 m	26 m	30 m
1a	1.00	1.04	1.03
1b	1.00	0.92	0.85
1c	1.00	0.89	0.73
2a	1.00	1.06	1.10
2b	1.00	0.89	0.69
2c	1.00	0.89	0.72

Cleaved to total caspase-12 ratio

%C	6 m	26 m	30 m
1a	1.00	1.55	0.73
1b	1.00	1.30	0.81
1c	1.00	1.18	0.89
2a	1.00	1.45	0.78
2b	1.00	1.26	0.43
2c	1.00	1.18	0.90
N	6.00	6.00	6.00
Mean	1.00	1.32	0.76
SD	0.00	0.15	0.17
SEM	0.00	0.07	0.08
%RE	100.00	132.24	75.83

Statistics:

One way ANOVA

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Tuesday, July 07, 2009, 11:34:44 PM

Data source: Data 1 in Notebook 7

Group	**N**	**Missing**	**Median**	**25%**	**75%**
6months	6	0	1.000	1.000	1.000
26months	6	0	1.283	1.185	1.452
30months	6	0	0.795	0.734	0.895

H = 15.726 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	**Diff of Ranks**	**q**	**P<0.05**
26months vs 30months	72.000	5.506	Yes
26months vs 6months	36.000	2.753	No
6months vs 30months	36.000	2.753	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2008/12/05	Project	Aging female
Report Number	1	Tissue/ cell line/ etc.	Soleus

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) alpha-fodrin

Dilution 1: 1000 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

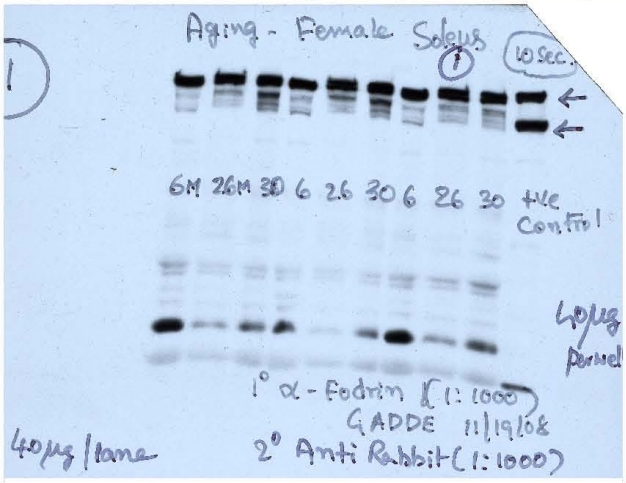
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Place Scan of Film Here

Exposure time

10 sec.

Molecular Wieght

240, 150

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\Soleus\alpha-fodrin

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\Soleus\alpha-fodrin

Notes

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Signature

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

Total α-fodrin:

%C	6 m	26 m	30 m
1a	1.00	1.10	0.95
1b	1.00	1.18	1.10
1c	1.00	1.40	0.99
2a	1.00	1.03	1.16
2b	1.00	1.12	1.03
2c	1.00	1.45	1.00

Cleaved α-fodrin:

%C	6 m	26 m	30 m
1a	1.00	1.41	2.45
1b	1.00	1.35	1.67
1c	1.00	1.34	1.37
2a	1.00	1.40	2.63
2b	1.00	1.48	1.85
2c	1.00	1.42	1.46

Cleaved to total α-fodrin ratio:

%C	6 m	26 m	30 m
1a	1.00	1.28	2.58
1b	1.00	1.14	1.52
1c	1.00	0.95	1.39
2a	1.00	1.36	2.27
2b	1.00	1.32	1.79
2c	1.00	0.98	1.46
N	6.00	6.00	6.00
Mean	1.00	1.17	1.83
SD	0.00	0.17	0.49
SEM	0.00	0.08	0.22
%RE	100.00	117.24	183.44

Statistics:

One way ANOVA

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Tuesday, July 07, 2009, 11:52:21 PM

Data source: Data 1 in Notebook 3

Group	**N**	**Missing**	**Median**	**25%**	**75%**
6months	6	0	1.000	1.000	1.000
26months	6	0	1.210	0.983	1.319
30months	6	0	1.656	1.461	2.266

H = 12.231 with 2 degrees of freedom. (P = 0.002)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method):

Comparison	**Diff of Ranks**	**q**	**P<0.05**
30months vs 6months	60.000	4.588	Yes
30months vs 26months	48.000	5.435	Yes
26months vs 6months	12.000	1.359	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

EDL:

Laboratory of Molecular Physiology
Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde				
Date	2008/12/31	Project	Aging female		
Report Number	1A	Tissue/ cell line/ etc.	EDL		
Gel type	15%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

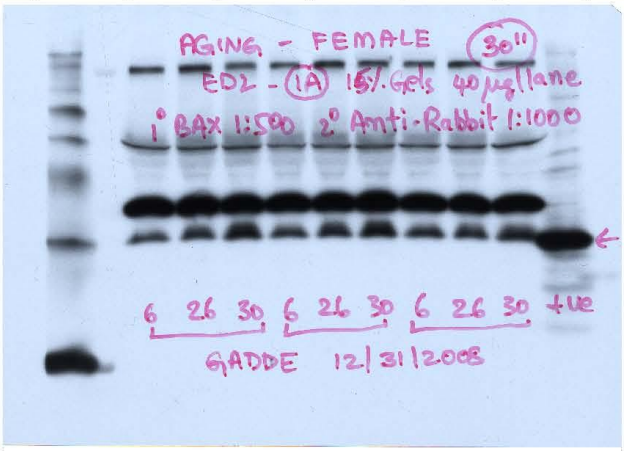
Primary Antibody

Name (Do Not Abbreviate)	BAX		
Dilution	1: 500	Medium	5% BSA in TBST
Incubation time	12 : 00 hr.		

Secondary Antibody

Name	Anti-Rabbit		
Dilution	1:1000	Medium	5% Milk in TBST
Incubation time	1 : 00 hr.		

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Exposure time

30 sec.

Molecular Wieght

20 KDa

Place Scan of Film Here

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\EDL\BAX

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\EDL\BAX

Notes

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

%C	6 m	26 m	30 m
1a	8.80	9.51	12.25
1b	9.40	11.05	12.60
1c	10.70	10.10	12.91
2a	8.70	9.89	12.71
2b	10.00	11.15	12.31
2c	10.20	9.70	12.72
N	6.00	6.00	6.00
Mean	9.63	10.23	12.58
SD	0.80	0.70	0.26
SEM	0.36	0.31	0.11
%RE	100.00	106.23	130.63
SEM	3.72	3.25	1.19

Statistics:

One way ANOVA

Normality Test: Passed (P = 0.849)

Equal Variance Test: Passed (P = 0.083)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	6	0	9.633	0.802	0.327
26 months	6	0	10.233	0.700	0.286
30 months	6	0	12.584	0.257	0.105

Source of Variation	DF	SS	MS	F	P
Between Groups	2	29.193	14.596	36.524	<0.001
Residual	15	5.995	0.400		
Total	17	35.187			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
30 months vs. 6 months	2.951	3	11.435	<0.001	Yes
30 months vs. 26 months	2.351	2	9.110	<0.001	Yes
26 months vs. 6 months	0.600	2	2.325	0.121	No

Laboratory of Molecular Physiology
Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2008/12/31	Project	Aging female
Report Number	1A	Tissue/ cell line/ etc.	EDL

Gel type	15%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) Bcl-2

Dilution 1: 1000 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

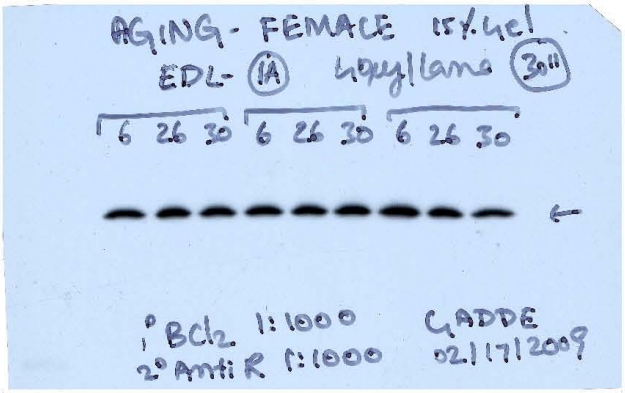
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Exposure time

30 sec.

Molecular Wieght

26 KDa

Place Scan of Film Here

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\EDL\Bcl-2

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\EDL\Bcl-2

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

%C	6 m	26 m	30 m
1a	10.70	10.17	9.86
1b	11.40	11.25	11.53
1c	13.40	10.50	8.99
2a	10.40	10.17	9.95
2b	11.40	11.45	11.63
2c	13.20	10.60	9.18
N	6.00	6.00	6.00
Mean	11.75	10.69	10.19
SD	1.26	0.54	1.14
SEM	0.57	0.24	0.51
%RE	100.00	90.98	86.73
SEM	4.81	2.07	4.34

Statistics:

One way ANOVA

Normality Test: Passed (P = 0.051)

Equal Variance Test: Passed (P = 0.401)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
6 months	6	0	11.750	1.265	0.516
26 months	6	0	10.691	0.544	0.222
30 months	6	0	10.190	1.141	0.466

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	2	7.609	3.805	3.571	0.054
Residual	15	15.983	1.066		
Total	17	23.592			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.054).

Power of performed test with alpha = 0.050: 0.426

The power of the performed test (0.426) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2008/12/05	Project	Aging female
Report Number	2	Tissue/ cell line/ etc.	EDL

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) caspase-3

Dilution 1: 1000 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

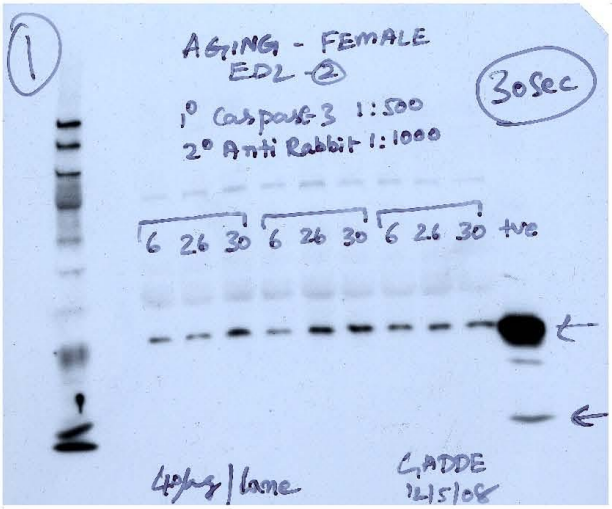
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Place Scan of Film Here

Exposure time

30 sec.

Molecular Wieght

35 KDa

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\EDL\caspase-3

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\EDL\caspase-3

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Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

%C	6 m	26 m	30 m
1a	7.90	6.88	13.19
1b	7.90	14.00	14.41
1c	10.30	10.27	13.54
2a	7.70	6.97	12.98
2b	7.40	14.29	14.61
2c	10.40	10.37	13.84
N	6.00	6.00	6.00
Mean	8.60	10.46	13.76
SD	1.37	3.23	0.65
SEM	0.61	1.44	0.29
%RE	100.00	121.67	160.00
SEM	7.11	16.79	3.39

Statistics:

One way ANOVA

Normality Test: Passed (P = 0.187)

Equal Variance Test: Passed (P = 0.052)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
6 months	6	0	8.600	1.368	0.559
26 months	6	0	10.464	3.229	1.318
30 months	6	0	13.760	0.652	0.266

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	2	81.933	40.966	9.658	0.002
Residual	15	63.627	4.242		
Total	17	145.560			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Power of performed test with alpha = 0.050: 0.937

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method):

Comparisons for factor:

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
30 months vs. 6 months	5.160	3	6.137	0.002	Yes
30 months vs. 26 months	3.297	2	3.921	0.014	Yes
26 months vs. 6 months	1.864	2	2.216	0.138	No

Laboratory of Molecular Physiology
Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2008/12/05	Project	Aging female
Report Number	2A	Tissue/ cell line/ etc.	EDL

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) caspase-9

Dilution 1: 500 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

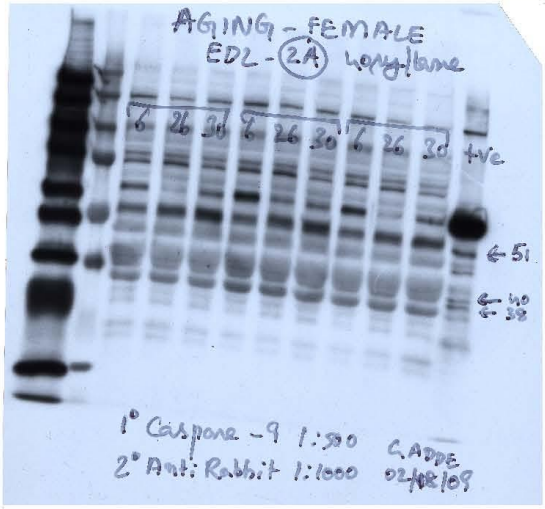
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Place Scan of Film Here

Exposure time

2 min.

Molecular Wieght

51 KDa

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\EDL\caspase-9

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\EDL\caspase-9

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

%C	6 m	26 m	30 m
1a	9.40	11.25	13.43
1b	9.60	11.29	12.22
1c	8.70	8.11	9.59
2a	9.60	11.25	13.43
2b	9.20	11.09	12.31
2c	8.60	8.43	9.51
N	6.00	6.00	6.00
Mean	9.18	10.23	11.75
SD	0.44	1.53	1.78
SEM	0.20	0.68	0.80
%RE	100.00	111.45	127.91
SEM	2.14	7.44	8.68

Statistics:

One way ANOVA

Normality Test: Passed (P = 0.155)

Equal Variance Test: Passed (P = 0.326)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	6	0	9.183	0.440	0.180
26 months	6	0	10.235	1.528	0.624
30 months	6	0	11.747	1.783	0.728

Source of Variation	DF	SS	MS	F	P
Between Groups	2	19.927	9.964	5.238	0.019
Residual	15	28.534	1.902		
Total	17	48.461			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.019).

Power of performed test with alpha = 0.050: 0.649

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
30 months vs. 6 months	2.564	3	4.553	0.015	Yes
30 months vs. 26 months	1.512	2	2.685	0.077	No
26 months vs. 6 months	1.051	2	1.867	0.207	No

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2009/02/18	Project	Aging female
Report Number	3A	Tissue/ cell line/ etc.	EDL

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) caspase-12

Dilution 1: 500 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

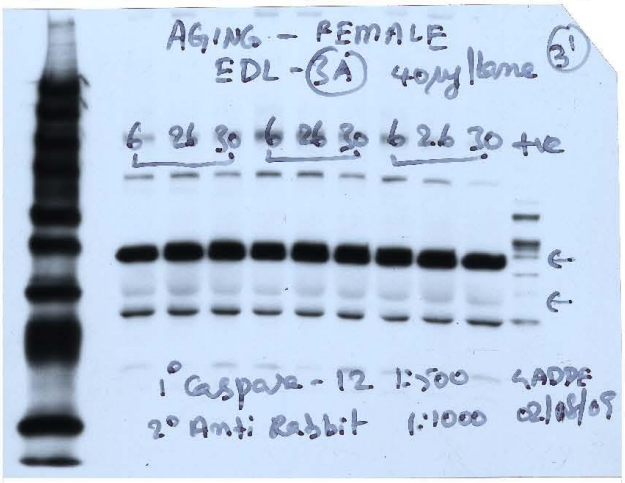
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Place Scan of Film Here

Exposure time

2 min.

Molecular Wieght

55, 42 KDa

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\EDL\caspase-12

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\EDL\caspase-12

Notes

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Signature

email Report

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http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

Total caspase-12

%C	6 m	26 m	30 m
1a	1.00	1.21	1.19
1b	1.00	1.08	1.05
1c	1.00	1.00	0.90
2a	1.00	1.23	1.21
2b	1.00	1.09	1.06
2c	1.00	1.00	0.90

Cleaved caspase-12:

%C	6 m	26 m	30 m
1a	1.00	1.06	0.89
1b	1.00	1.03	0.81
1c	1.00	1.19	1.19
2a	1.00	1.02	0.87
2b	1.00	1.06	0.77
2c	1.00	1.20	1.20

Cleaved to total caspase-12:

%C	6 m	26 m	30 m

1a	1.00	0.87	0.75
1b	1.00	0.95	0.77
1c	1.00	1.19	1.32
2a	1.00	0.83	0.72
2b	1.00	0.97	0.73
2c	1.00	1.20	1.33
N	6.00	6.00	6.00
Mean	1.00	1.00	0.94
SD	0.00	0.16	0.30
SEM	0.00	0.07	0.14
%RE	100.00	100.30	93.57

Statistics:

One way ANOVA

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6months	6	0	1.000	1.000	1.000
26months	6	0	0.961	0.874	1.193
30months	6	0	0.758	0.728	1.324

H = 1.747 with 2 degrees of freedom. (P = 0.417)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.417)

Laboratory of Molecular Physiology

Western Blot Film Record

Print Form

Experimenter	Murali K. Gadde		
Date	2008/11/19	Project	Aging female
Report Number	1	Tissue/ cell line/ etc.	EDL

Gel type	10%	Electrophoresis Voltage	124	Transfer Voltage	25
Protien Load per Well	40 ug	Duration	2 : 30 hr.	Duration	1 hr.

Primary Antibody

Name (Do Not Abbreviate) alpha-fodrin

Dilution 1: 1000 Medium 5% BSA in TBST

Incubation time 12 : 00 hr.

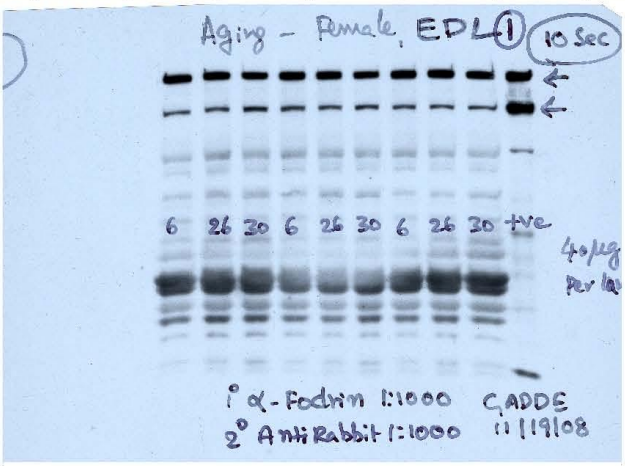
Secondary Antibody

Name Anti-Rabbit

Dilution 1:1000 Medium 5% Milk in TBST

Incubation time 1 : 00 hr.

Lane # 1	Lane # 2	Lane # 3	Lane # 4	Lane # 5	Lane # 6	Lane # 7	Lane # 8	Lane # 9	Lane # 10	Lane # 11	Lane # 12
Standards	Protein Standards	6m	26m	30m	6m	26m	30m	6m	26m	30m	Positive Control



Exposure time

2 min.

Molecular Wieght

240, 150 KDa

Place Scan of Film Here

☒ Used For Analysis

Location of Scan \\Blough-raid\exp-data\Project folder\Aging Female\FILMS\EDL\alpha-fodrin

Location of Excel Data \\Blough-raid\exp-data\Project folder\Aging Female\Excel\EDL\alpha-fodrin

Notes

Murali Krishna Gadde Digitally signed by Murali Krishna Gadde DN: cn=Murali Krishna Gadde, o=Marshall University, ou=Biology, email=gadde@marshall.edu, c=US Date: 2009.07.05 17:00:06 -04'00'

Signature

email Report

Laboratory of Molecular Physiology, Marshall University, Science Bldg. Suite 311, 1 John Marshall Drive, Huntington, WV, 25755 * Phone: 304-696-3267 Fax: 304-696-7136
http://www.science.marshall.edu/blough

Raw data:

This section represents the raw data tables produced from spot densitometry of the immunoblot films

Total α-fodrin:

%C	6 m	26 m	30 m
1a	1.00	0.93	0.84
1b	1.00	0.93	0.83
1c	1.00	1.07	1.02
2a	1.00	0.93	0.83
2b	1.00	0.92	0.82
2c	1.00	1.05	1.02

Cleaved α-fodrin:

%C	6 m	26 m	30 m
1a	1.00	1.08	1.25
1b	1.00	0.84	0.89
1c	1.00	0.92	0.97
2a	1.00	1.03	1.19
2b	1.00	0.83	0.86
2c	1.00	0.87	0.97

Cleaved to total α-fodrin ratio:

%C	6 m	26 m	30 m
1a	1.00	1.15	1.49
1b	1.00	0.90	1.07
1c	1.00	0.85	0.95
2a	1.00	1.11	1.43
2b	1.00	0.90	1.05
2c	1.00	0.84	0.96
N	6.00	6.00	6.00
Mean	1.00	0.96	1.16
SD	0.00	0.14	0.24
SEM	0.00	0.06	0.11
%RE	100.00	95.84	115.81

Statistics:

One way ANOVA

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source:

Group	N	Missing	Median	25%	75%
6 months	6	0	1.000	1.000	1.000
26 months	6	0	0.900	0.855	1.109
30 months	6	0	1.061	0.956	1.429

H = 3.106 with 2 degrees of freedom. (P = 0.212)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.212)