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"Age-related effect of cell death on fiber morphology and number in tongue muscle"[1]

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Abstract

Introduction—Multiple pathways may exist for age-related tongue muscle degeneration. Cell death is one mechanism contributing to muscle atrophy and decreased function. We hypothesized with aging, apoptosis and apoptotic regulators would be increased, and muscle fiber size and number would be reduced in extrinsic tongue muscles.

Methods—Cell death indices, expression of caspase-3 and Bcl-2, and measures of muscle morphology and number were determined in extrinsic tongue muscles of young and old rats.

Results—Significant increases in cell death, caspase-3, and Bcl-2 were observed in all extrinsic tongue muscles along with reductions in muscle fiber number in old rats.

Discussion—We demonstrated that apoptosis indices increase with age in lingual muscles and that alterations in apoptotic regulators may be associated with age-related degeneration in muscle fiber size and number. These observed apoptotic processes may be detrimental to muscle function, and may contribute to degradation of cranial functions with age.

Keywords

age-related pathology; biology of aging; apoptosis; muscle; animal model; speech; swallowing

Introduction

In the next 30 years, the number of individuals 60 years and older will increase to more than 2 billion, representing 22% of the population.[1] Sarcopenia, the age-related degeneration of skeletal muscle that culminates in reductions in muscle mass, strength, and function,[2] may contribute to communication and swallowing deficits in elderly people, negatively impacting

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health, nutrition, respiratory function, and quality of life.[3-8] Processes primary to the age-related decline in the cranial muscles have been understudied. Knowledge of underlying cellular mechanisms contributing to age-related muscular degeneration in the cranial muscles is a clinical priority due to the high incidence of communication and swallowing disorders in the aging population.

The tongue has an important role in speech, respiration, and swallowing. During speech, tongue position and movement is crucial to the production of vowels, consonants, and constant-vowel combinations.[9-11] For respiratory actions, the tongue's primary function is to maintain patency of the upper airway through the dilation and/or narrowing of the pharynx during breathing.[9,12-14] Additionally, the tongue is active during the swallow, contributing to the formation, transport, and propulsion of the bolus.[9,15,16] Due to the cross-system role of the tongue musculature, age-associated reductions in tongue muscle structure and function may have deleterious effects on these critical actions.

Multiple causal pathways may exist for age-related degeneration of the tongue musculature that manifest as alterations in muscle fiber biochemistry and a transition toward more slowly contracting muscle fibers,[17-19] reductions in neuromuscular junction size and number,[20] decreased strength and force production,[17,21,22] and increased fatigue.[17,22] However, mechanisms underlying these morphological and physiological changes remain elusive. Possible causal pathways suggested by recent findings in limb muscles include cell apoptosis or programmed cell death.[23-26] Apoptosis of single myonuclei along the length of the muscle fiber has been implicated as a cause of age-related reductions in total muscle fiber number and in muscle fiber diameter.[23,27-33] It has been well established in limb musculature that apoptosis of myonuclei increases with age.[26,33-35] In addition, resulting imbalances and/or misregulation in the capability of aged muscle to replace nuclei eliminated via apoptosis may be primary processes leading to muscle atrophy and ultimately to reductions in muscle mass, strength and function.[23,25,34,36,37] Mechanisms that regulate apoptotic processes, such as mitochondrial dysfunction, mitochondrial damage, and increased caspase activity in the cell cytosol, may contribute to age-related muscle degeneration.[38-40] The protein family Bcl-2 has a major role in regulating mitochondrial mediated cell apoptosis, and activation of caspase-3 by mitochondrial and/or the death receptor mediated apoptotic pathways execute cell death.[39-43] However, processes identified in limb skeletal muscles may not be found in cranial skeletal muscles due to distinctions in muscle fiber types and geometric arrangements.[44-48] Age-induced apoptosis has never been characterized in muscles of tongue and may represent a converging mechanism through which age-related cranial muscle degeneration ensues.

The purpose of this study was to examine the effect of age on cell apoptosis, protein regulators of cell apoptosis, and muscle fiber size and number in extrinsic tongue muscles of young adult and old rats. We hypothesized that with aging, cell death would be increased, protein expression of apoptotic regulators involved in intrinsic and extrinsic pathways of cell death would be altered, and that there would be a reduction in muscle fiber size and number in the genioglossus (GG), styloglossus (SG), and hyoglossus (HG) muscles of old compared to young adult rats.

Materials and Methods

Animals

This study was performed in accordance with the NIH Guide for Care and Use of Laboratory Animals, 8th Edition, 2011. The animal care and use protocol was approved by University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee. A total of 8 young adult (9 months old) and 8 old (32 months old) male Fischer 344/Brown Norway rats were obtained from the National Institute on Aging colony (Harlan Laboratories, Indianapolis, IN). The Fischer 344/Brown Norway rat median life span is approximately 36 months of age.[49] Rats were housed in standard polycarbonate cages in pairs in a light controlled environment with a 12:12-hour light-dark reversed light cycle with food and water provided ad libitum. Animals were obtained 2 weeks before experimentation to allow for acclimation to the vivarium and light cycle reversal.

Tissue collection

Rats were anesthetized with isoflurane (4%), and euthanized by an overdose of Beuthanasia (0.3 mL, IP injection). The genioglossus (GG), styloglossus (SG), and hyoglossus (HG) muscles were extracted and rinsed in 0.9% saline solution. Muscles from the right or left side of the tongue were either individually embedded in optimum cutting temperature compound (OCT) and snap-frozen in 2-methylbutane cooled by liquid nitrogen or immediately frozen in liquid nitrogen, and stored at -80°C until future use.

TUNEL assay and laminin staining analysis

DNA fragmentation was determined using a standard TUNEL assay (TdT-mediated dUTP nick end labeling; *In Situ* Cell Death Detection Kit, Roche). OCT embedded tissue was cut into serial 10 μm cross-sections using a -16°C cryostat (Leica CM 1850, Leica Biosystems) and mounted on slides. Tissue was then fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS). Tissue sections were permeabilized for 2 minutes on ice in a solution consisting of 0.1% Triton X-100 and 0.1% Triton, and washed with PBS. The TUNEL reaction mixture was then prepared per manufacturer's recommendations and was applied to cross-sections. Tissue sections were then incubated for 60 minutes at 37°C. Positive-controls were incubated for 10 minutes with DNase I recombinant (1000 U/ml in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 1 mg/ml BSA) to induce DNA strand breaks prior to TUNEL labeling. Negative-controls were incubated for 60 minutes at 37°C with label solution.

Following TUNEL labeling, tissue sections were washed with PBS and blocked in a solution of 10% normal goat serum and 0.3% Triton X-100 suspended in PBS for 20 minutes. Sections were then incubated for 1 hour at room temperature in diluted primary antibody (polyclonal rabbit anti-laminin IgG, Sigma) for cross-sectional area analysis and washed in PBS. Slides were then incubated with the fluorescent secondary antibody (Cy3 conjugated goat anti-rabbit IgG, Roche) for 1 hour at room temperature. Following PBS wash, slides were mounted with ProLong Gold Antifade with DAPI (Life Technologies) to visualize nuclei and stored at 4 °C to preserve fluorescence until microscopy was performed. Negative

control slides were stained following the same procedure with omission of the primary antibody.

TUNEL- and DAPI-stained nuclei and laminin staining were examined under a fluorescent microscope (Nikon N-STORM) equipped with a digital camera (Andor iXon 897 EMCCD). The microscopist (HK) was masked to the animal age group. Photographs of four random non-overlapping images at an objective magnification of 20× were taken.[50] FIJI (LOCI, University of Wisconsin Madison) was used to analyze images for quantification of indices of apoptosis. The number of TUNEL- and DAPI-positive nuclei were counted for each image. The TUNEL index was calculated by counting the number of TUNEL-positive nuclei divided by the total number of nuclei (DAPI-positive nuclei) multiplied by 100 for each of the four images from each muscle (GG, SG, and HG).[50]

The MATLAB application SMASH (Semi-automatic Muscle Analysis using Segmentation of Histology) was used to calculate GG and SG muscle fiber cross-sectional area (CSA; μm^2), minimal Feret's diameter (minimum distance of parallel tangents at opposing borders of the muscle fiber; μm), and the coefficient of variance ((standard deviation/mean)ˆ10^3; %) in four non-overlapping images.[51-55] The HG was excluded from muscle fiber morphometric analyses due to the high number of elongated fibers in each tissue cross-section which would not permit and confound accurate calculation of muscle fiber cross sectional area. True cross-sections were difficult to obtain because of the geometry of the HG muscle. Data analysis was constrained to muscle fibers between 150 and 2,500 μm^2 to allow exclusion of non-muscle connective tissue bodies and vasculature.[52] Elongated and elliptically shaped regions were also excluded.

Western Blot Analysis

Western blot analyses were performed to determine the protein expression of caspase-3 and Bcl-2 in the GG, SG, and HG muscles.[56] Following tissue homogenization, the protein concentration was determined (DC Protein Assay; Bio-Rad), 50 μg of protein from each tissue sample was loaded into a 4-20% precast gradient gel (Criterion TGX, Bio-Rad), and gel electrophoresis was performed. Proteins were then transferred to a nitrocellulose membrane and washed in TBST (Tris buffered saline, 0.15% Tween 20). Membranes were blocked in 5% non-fat dry milk in TBST for one hour at room temperature, and then incubated with caspase-3 (Cell Signaling Technology) or Bcl-2 (BioLegend) antibodies overnight at 4°C. Following primary antibody incubation, membranes were washed in TBST and incubated for 1 hour in horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). After final TBST washes, membranes were developed (SuperSignal West Femto Chemiluminescent Substrate, Thermo Scientific) for 5 minutes using a ChemiDoc-It^2 Imaging System (UVP). Following immunodetection, membranes were washed with TBST and stained with SimplyBlue SafeStain (Invitrogen) to determine the total amount of protein transferred for data normalization.[57-59] Blots were analyzed with FIJI to quantify caspase-3 and Bcl-2 protein expression; data are represented as a ratio of protein band relative to the total protein in lieu of loading controls.

Statistical Analysis

T-tests were used to compare TUNEL indices, protein expression, the number of muscle fibers per unit area between each age group, and body weights between age groups. Pearson correlation coefficients were used to evaluate the relationship between body weight and all muscle fiber morphometric measures. Since weight is often associated with age, we used a one-way analysis of covariance (ANCOVA) with a body weight covariate to compare dependent variables between groups. The critical value of obtaining statistical significance was set at α=0.05. Data were presented as means ± SE. SPSS 23 was used for all analyses (IBM SPSS Statistics, IBM Corp., Armonk, NY).

Results

Body Weight

Body weights were significantly greater in the old rat group (t_{14}=-9.82, p<.001). Because muscle fiber morphometric measures can be highly correlated with body size,[60,61] Pearson correlation statistics were used to evaluate the relationship between body weight and all muscle fiber morphometric measures. We found moderate-to-high correlations and thus used a one-way analysis of covariance (ANCOVA) with a body weight covariate to compare dependent variables between groups (cross-sectional area, R = 0.576; minimal Feret's diameter, R = 0.617; coefficient of variance, R = -0.877).

TUNEL Index

In the old rat group, the number of nuclei staining positively for DNA fragmentation, an indication of cell apoptosis or programmed cell death, was significantly greater in the GG, SG, and HG muscles than in the young adult rat group (Fig. 1a-c: t_{14}=-3.018, p=0.009; t_{14}=-6.028, p=0.008; t_{14}=-8.389; p<0.001; respectively). That is, with increasing age there was a significant increase in the index of cell death for all muscles studied in the extrinsic tongue. Representative images of immunostained cross-sections from young adult and old HG muscles are found in Figure 2.

Protein Expression

The expression of full-length caspase-3 was significantly higher in all extrinsic tongue muscles of old compared to young adult rats (Fig 3a-c: GG, t_{14}=-3.93, p<0.001; SG, t_{13}=-6.38, p<0.001; and HG t_{14}=-9.037, p<0.001; respectively). There was no evidence of caspase-3 cleavage in the extrinsic tongue muscles. With aging, there was also significantly increased expression of Bcl-2 in the GG (t_{14}=-6.819, p<0.001; Fig. 4a), SG (t_{13}=-6.494, p<0.001; Fig. 4b), and HG muscles (t_{14}=-8.07, p<0.001; Fig. 4c). As demonstrated in Figure 5, protein expression of both caspase-3 and Bcl-2 increased in the extrinsic tongue musculature of old rats compared to young adult rats.

Muscle Morphology

Muscle fiber CSA and the minimum Feret's diameter were measured in a total of 1678 muscle fibers in the GG and 1604 muscle fibers in the SG. For each rat on average, 104 muscle fibers from each GG image and 100 muscle fibers from each SG image were

measured. A coefficient of variance was determined for both GG and SG muscle fiber CSA and minimum Feret's diameter.

Genioglossus

With increasing age, muscle fiber diameter and CSA were not significantly reduced (respectively, $F_{1, 15} = 4.58$, $p = 0.052$; $F_{1, 15} = 3.857$, $p = 0.072$; Fig. 6a-b). Variability in muscle fiber diameter and size did not significantly change with increased age ($F_{1, 15} = 0.028$, $p = 0.87$; $F_{1, 15} = 0.016$, $p = 0.90$, respectively; Fig. 6c-d). However, there was a significant reduction in muscle fiber number in the old group versus the young adult group ($t_{61} = 2.12$, $p=0.038$; Figure 7a).

Styloglossus

Muscle fiber diameter was not significantly decreased with age ($F_{1, 15} = 3.66$, $p = 0.078$; Fig. 6e). There was no difference in muscle fiber CSA with increasing age ($F_{1, 15} = 2.913$, $p = 0.11$; Fig. 6f). The old group had more variability in muscle fiber CSA compared to the young adult group ($F_{1, 15} = 4.59$, $p = 0.052$; Fig. 6h). However, variations in muscle fiber diameter were not observed with increased age ($F_{1, 15} = 2.22$, $p = 0.16$; Fig. 6g]. The old group had a significant reduction in muscle fiber number compared with the young adult group ($t_{62} = 2.05$, $p=0.044$; Fig. 7b).

Discussion

Our results supported our hypotheses in part. We observed increased cell death and expression of apoptotic regulator proteins in all muscles of the extrinsic tongue with age, a reduction in muscle fiber number in the genioglossus and styloglossus, and higher variability of myofiber size in the styloglossus muscle. The results are indicative of an underlying cellular mechanism that may contribute to age-related lingual muscle degeneration, and, if also found in humans, may be associated with the development of oromotor decline evidenced in elderly people.[62-64]

Cell apoptosis, or programmed cell death, is characterized by a series of molecular, biochemical, and morphological changes that result in cellular self-destruction.[34,65-67] Activated by two main pathways, intrinsic (mitochondrial mediated) or extrinsic (death receptor mediated), apoptosis is a conserved molecular process that occurs normally during development and aging, and is an important homeostatic mechanism that maintains cellular populations in a variety of tissues.[34,67,68] The mitochondrial mediated pathway to cell death is highly regulated by the Bcl-2 protein family.[38,40,42,43] Cytotoxic stress results in the imbalance of apoptotic regulator proteins, such as Bcl-2, and initiates a cascade of events that lead to cell death through the release of apoptogenic factors from the mitochondria into the cell cytosol. Mitochondrial and death receptor mediated pathways of cell death intersect at the activation of caspase-3 in the cell cytosol.[40] The main executioner of cell death, caspase-3 coordinates the destruction of key cellular proteins and initiates apoptotic DNA fragmentation.[39,41,42] Our findings in muscles of the tongue were consistent with cell death pathways proposed in the limb literature.[24,69] With aging, we also observed upregulated expression of apoptotic regulator proteins, Bcl-2 and caspase-3, suggesting that both

extrinsic and intrinsic mediated pathways of cell death may contribute to the increased DNA fragmentation we detected in extrinsic tongue muscles of old rats.

Age-related increases in muscle cell apoptosis have been found in the soleus, extensor digitorum, and gastrocnemius muscles in aging Fischer 344/Brown Norway rats,[23-25] and in the vastus lateralis muscle of elderly humans.[27,28] In our study, significant increases in nuclear DNA fragmentation, indicative of cell apoptosis, were observed in the genioglossus, styloglossus, and hyoglosuss muscles of old rats compared to young adult rats. We observed elevated Bcl-2 and caspase-3 protein expression with increasing age, that is comparable to previously reported data in aged rat limb muscle.[24,69] The increase in Bcl-2, an anti-apoptotic regulator, may be a compensatory measure to combat an increase in pro-apoptotic events occurring during mitochondrial mediated cell death, in order to prevent the age-related loss of muscle mass and size.[70-72] However, an alternative explanation for elevated Bcl-2 may be related to the protein's loss of function as an anti-apoptotic regulator.[69,71] Previous studies have reported that phosphorylation of Bcl-2 inactivates the anti-apoptotic function of the protein, and that Bcl-2 phosphorylation increases with age in skeletal muscles in conjunction with increased Bcl-2 expression.[69] This suggests that Bcl-2 expression may be upregulated to counteract the increase in the biologically inactive phosphorylated form of the protein. To further elucidate the mechanism of increased Bcl-2 elevation, it would be helpful in the future to examine the ratio of pro- and anti-apoptotic regulators of the Bcl-2 family of proteins, and determine the level of Bcl-2 phosphorylation. With aging, we also observed increased expression of full length caspase-3, without a concomitant increase of the activated and cleaved form, in the GG, SG, and HG muscles. This is consistent with previous reports from the limb literature.[24] An age-related increase in the full length caspase-3 protein may suggest that extrinsic tongue muscles are more susceptible to apoptosis.[70,71,73,74] The absence of the activated, cleaved form of caspase-3, may mean that few muscles fibers are actively undergoing apoptosis at the 32 month time-point[24] or may, in fact, be indicative of a caspase-independent pathway of age-related myonuclear loss.[38,70,71,73,74] Misregulation of these apoptotic processes may have deleterious consequences in the affected tissue.[36,66]

In aging tongue muscle, alterations in the apoptotic regulator proteins, Bcl-2 and caspase-3, resulting in increased activation of apoptotic processes, may have led to the removal of individual myonuclei, the associated sarcoplasm, [65,66] and contributed to the eventual atrophy and loss of muscle fibers we observed in the genioglossus and styloglossus muscles of old rats. When an imbalance exists between mechanisms of muscle regeneration and degeneration, an increase in apoptotic signaling may lead to protein degradation, fiber atrophy, myofiber loss, and decreased function in that muscle.[32,34-37,68,75-77] As such, the morphological changes in muscle fiber size and reductions in myofiber number, consistent with age-related sarcopenia, that we observed in GG and SG muscles of our study may be associated with increased muscle cell death.[24,29] Consistent with limb literature, we observed reductions in muscle fiber cross-sectional area (CSA), minimal Feret's diameter, a robust measure of muscle fiber size due to its insensitivity to deviations from an optimal cross-section, and a significant reduction in muscle fiber number with increasing age in the GG and SG muscles. Because of the high variability of muscle fiber size in the cranial musculature, we also determined the variance coefficient for muscle fiber CSA and minimal

Feret's diameter.[53,54,78] We identified significantly greater variability of muscle fiber CSA in the SG of old rats, which could be indicative of atrophic muscle fibers and a disruption in the ability of the muscle to replace and/or repair damaged or degenerating fibers.[52-54] Our results suggest that age-related muscle atrophy may be associated with increased activation or the misregulation of apoptotic processes in the extrinsic tongue muscles. The ramifications of age-related myonuclear loss and reductions in myofiber size and number may be detrimental to muscle function, leading to losses in muscle mass and contractile function, and must be investigated further.

Apoptosis may be an underlying cellular mechanism contributing to age-related cranial muscle degeneration. Understanding how age-related processes contribute to other manifestations of aging, the destruction of myonuclei and myofibers in tongue muscles, and functional deficits is crucial to the development of potential pharmacological and exercise-based therapies for communication and swallowing disorders.

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Abbreviations

ANCOVA	analysis of covariance
CSA	cross-sectional area
GG	genioglossus
HG	hyoglossus
MHC	myosin heavy chain
OCT	optimum cutting temperature compound
PBS	phosphate-buffered saline
SG	styloglossus
SMASH	Semi-automatic Muscle Analysis using Segmentation of Histology
TUNEL	TdT-mediated dUTP nick end labeling
TBST	Tris buffered saline

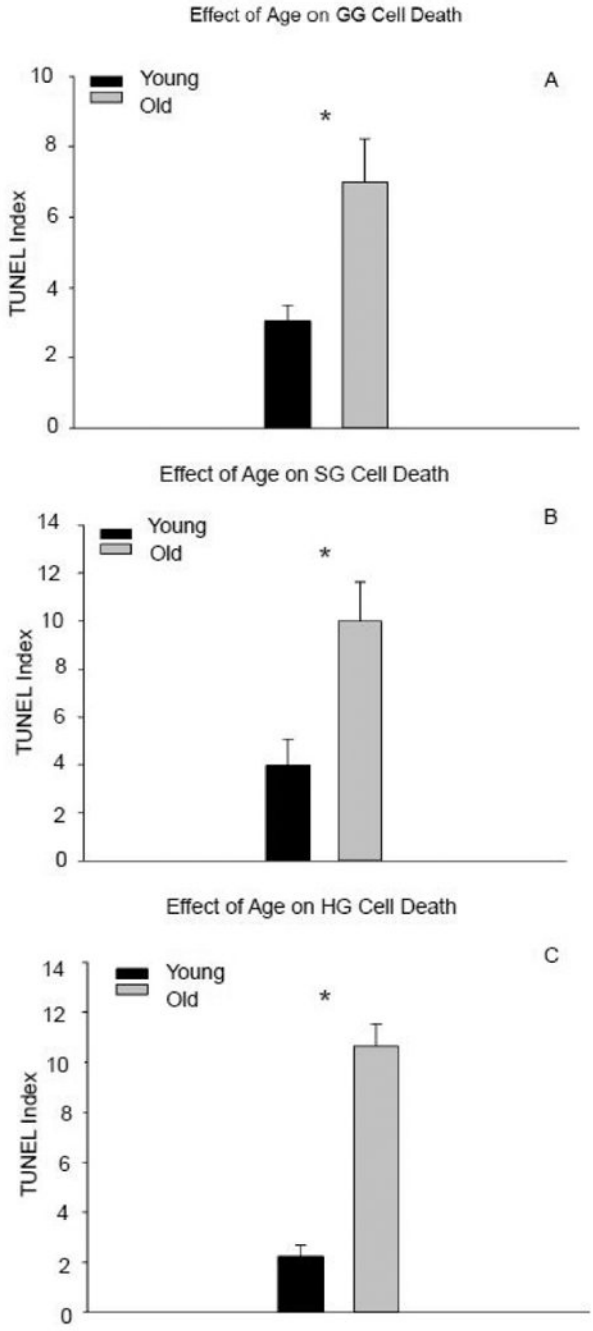

Figure 1.

Cell death indices were significantly increased in the genioglossus (A), styloglossus (B), and hyoglossus (C) muscles of old versus young adult rats. (* signifies $p<0.05$)

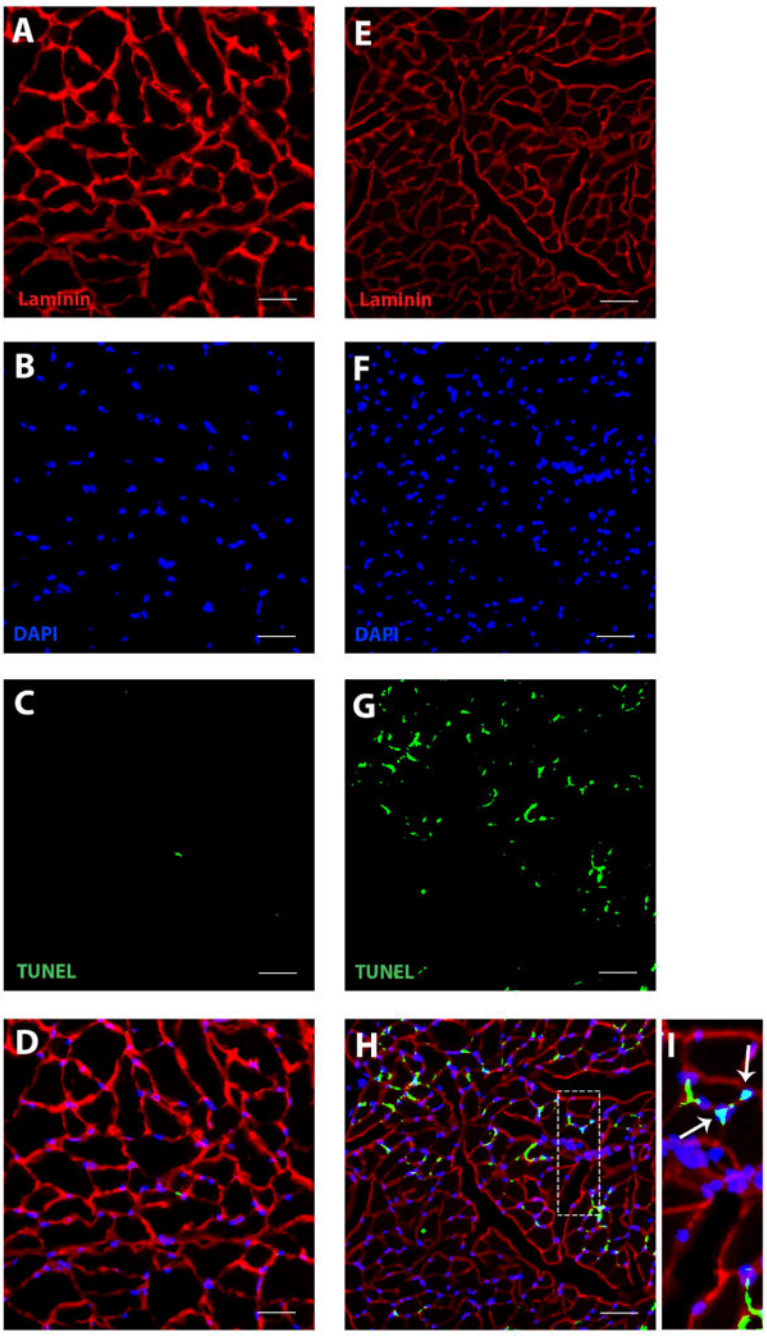

Figure 2.

Representative images for TUNEL labeling for young adult (A, B, C, D) and old rat (E, F, G, H) hyoglossus muscles. Immunostaining of laminin (A, E) and myonuclei (B, F) was performed following TUNEL staining (C, G) to identify co-localization of TUNEL-positive myonuclei with regard to the basal lamina. Images were super imposed to allow for the identification of apoptotic muscle nuclei. The enlarged box demonstrates TUNEL-positive nuclei (arrow) with myonuclei in relation to the sarcolemma (I). (scale bar = 100 μm)

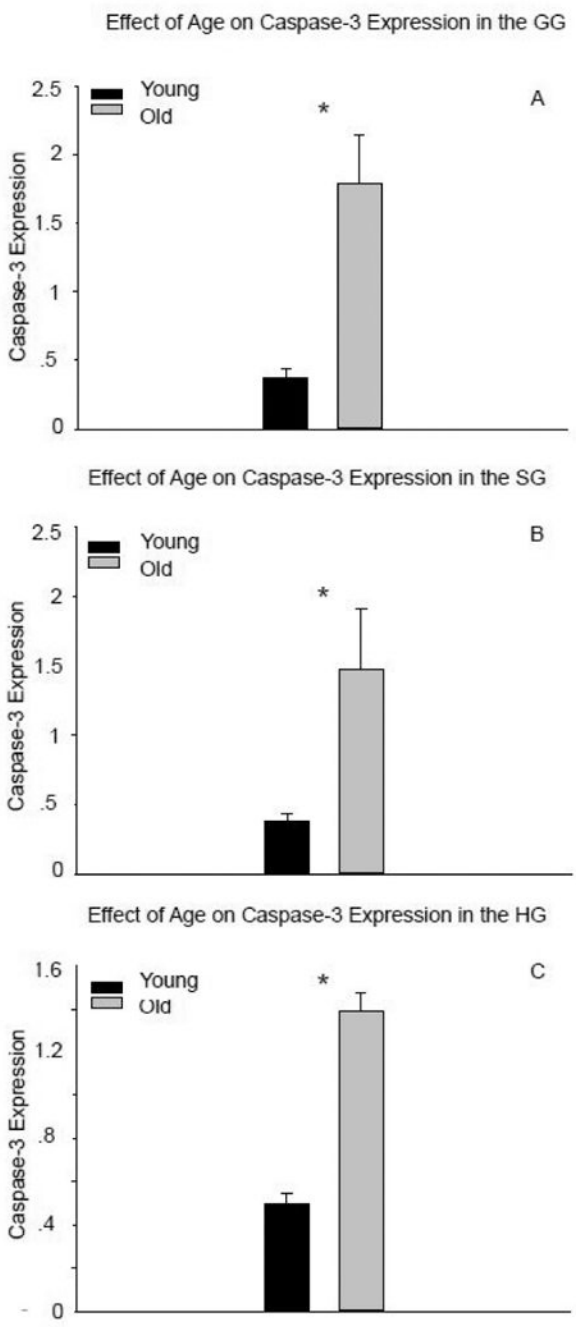

Figure 3.

Caspase-3 protein expression was significantly elevated in all extrinsic muscles of old rats compared to young adult rats (genioglossus, A; styloglossus, B; hyoglossus, C). (* signifies $p<0.05$)

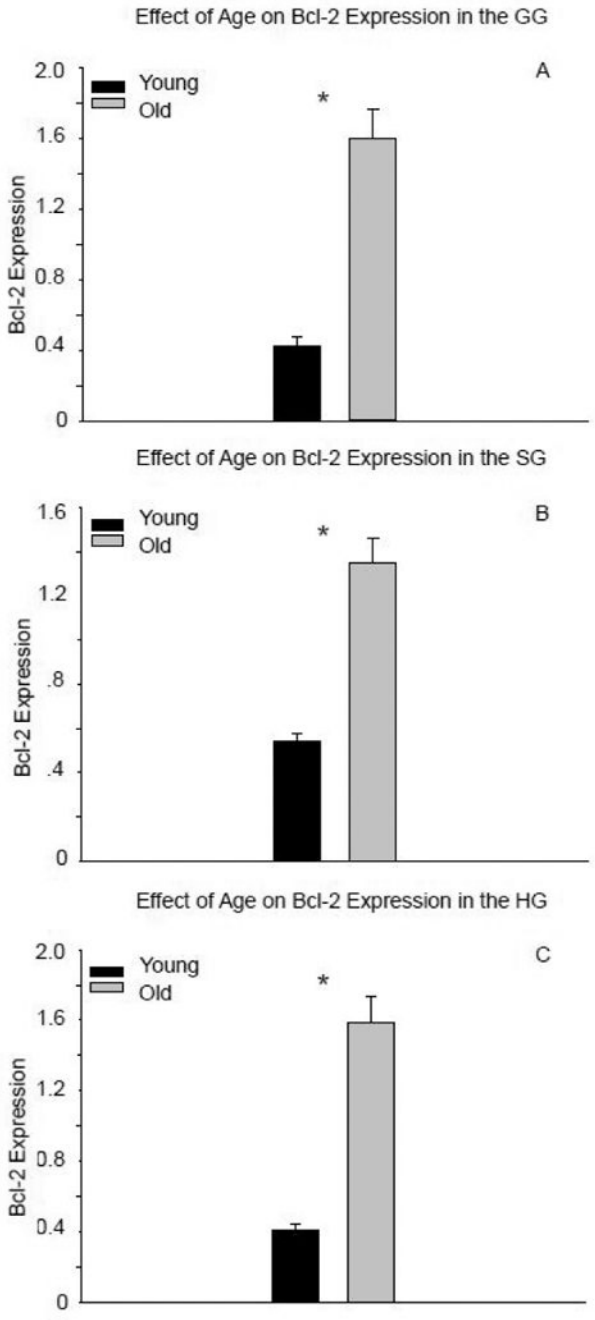

Figure 4.

Expression of Bcl-2 significantly increased in the genioglossus (A), styloglossus (B), and hyoglossus (C) with age. (* signifies $p<0.05$)

Figure 5.
Representative immunoblots from the genioglossus muscle of 4 young adult and 4 old rats.

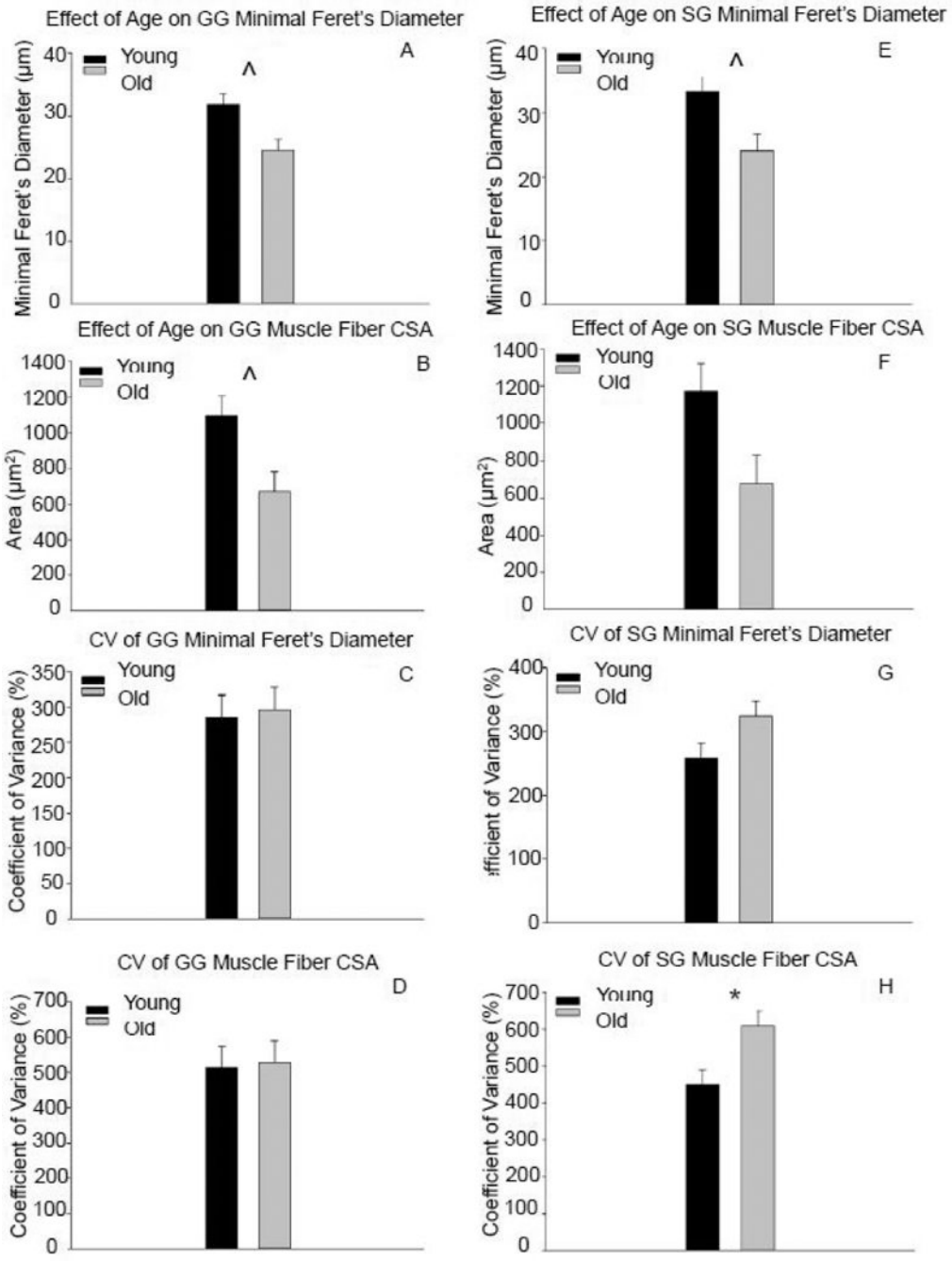

Figure 6.

With increasing age, reductions in genioglossus muscle diameter and cross-sectional area were observed, but were not significantly different (A, B). In the styloglossus muscle, muscle fiber diameter was not significantly reduced with increasing age (E), and significantly more variability in muscle fiber cross-sectional area (H) was observed in the old rat group. (* signifies $p<0.05$; ^ signifies $p<0.08$)

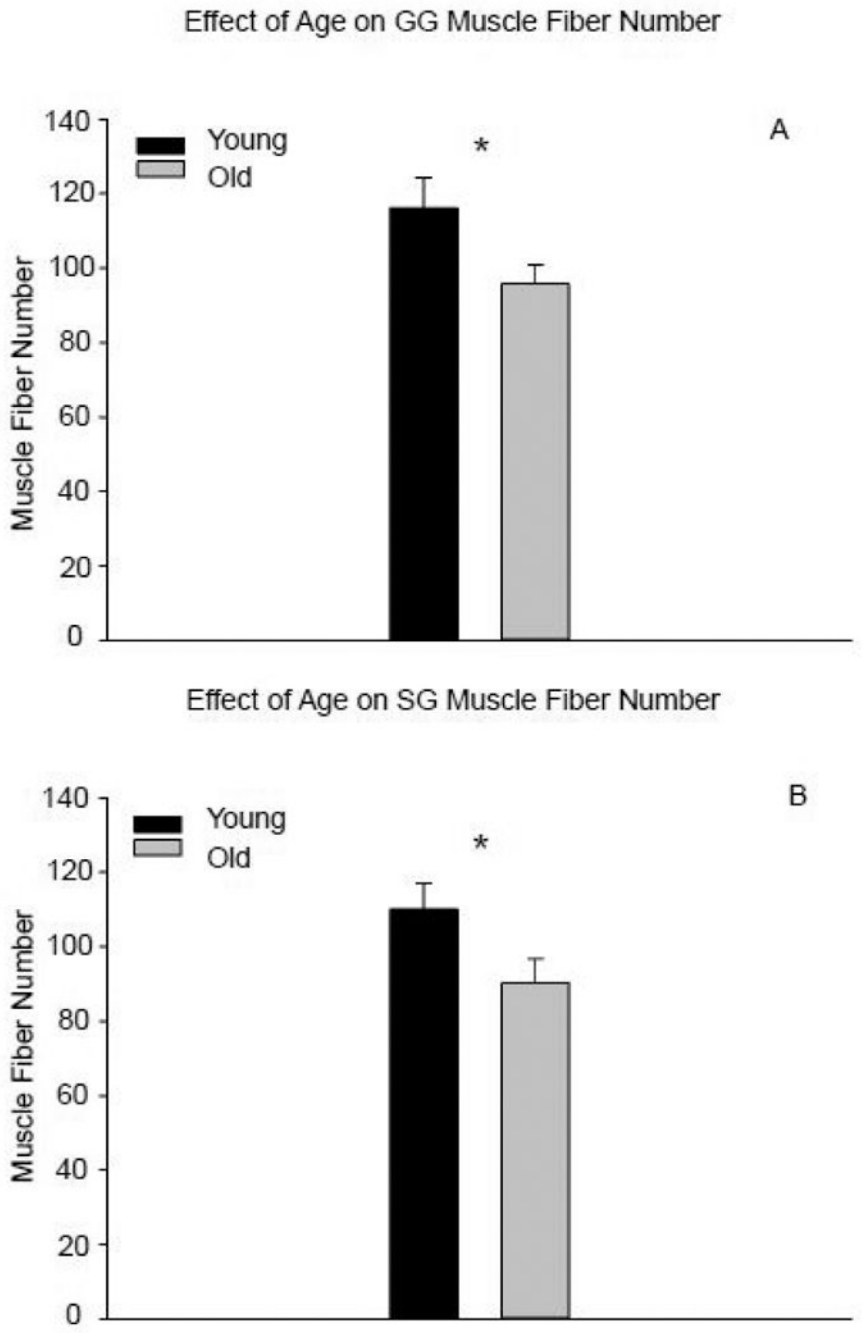

Figure 7.

There was a significant reduction in the number of muscle fibers in the old versus young adult rats in the genioglossus (A) and styloglossus muscles (B). (* signifies $p<0.05$)