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2 **Exercise training increases anabolic and attenuate catabolic and apoptotic processes in**  
3 **aged skeletal muscle of male rats**

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5 Mohammad Mosaferi Ziaaldini<sup>1</sup>, Erika Koltai<sup>1</sup>, Zsolt Csende<sup>2</sup>, Sataro Goto<sup>3</sup>, Istvan Boldogh<sup>4</sup>, Albert W.  
6 Taylor<sup>5</sup>, Zsolt Radak<sup>1</sup>  
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10 <sup>1</sup>Research Institute of Sport Science, Semmelweis University, Budapest, Hungary Department of  
11 Biomechanics, Semmelweis University, Budapest, <sup>3</sup>Department of Exercise Physiology, School of  
12 Health and Sport Science, Juntendo University, Chiba, Japan, <sup>4</sup>Department of Microbiology and  
13 Immunology, Sealy Center for Molecular Medicine, University of Texas Medical Branch at Galveston,  
14 Galveston, Texas 77555, USA, 5 Faculty of Health Sciences, The University of Western Ontario,  
15 London, Ontario, Canada  
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23 **Correspondence:** Zsolt Radak, Ph.D., DSc

24 Institute of Sport Science

25 Faculty of Physical Education and Sport Science

26 Semmelweis University

27 Alkotás u. 44. TF Budapest, Hungary

28 Tel: +36 1 3565764, Fax: +36 1 356 6337

29 Email: radak@tf.hu  
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1  
2 **Abstract**  
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5 Aging results in significant loss of mass and function of skeletal muscle, which negatively  
6 impacts the quality of life. In this study we investigated whether aerobic exercise training has  
7 the potential to alter anabolic and catabolic pathways in skeletal muscle. Five and twenty  
8 eight month old rats were used in the study. Aging resulted in decreased levels of  
9 follistatin/mTOR/Akt/Erk activation and increased myostatin/Murf1/2, proteasome subunits,  
10 and protein ubiquitination levels. In addition, TNF- $\alpha$ , reactive oxygen species (ROS), p53,  
11 and Bax levels were increased while Bcl-2 levels were decreased in skeletal muscle of aged  
12 rats. Six weeks of exercise training at 60% of VO<sub>2</sub>max reversed the age-associated activation  
13 of catabolic and apoptotic pathways and increased anabolic signaling. The results suggest that  
14 the age-associated loss of muscle mass and cachexia could be due to orchestrated down-  
15 regulation of anabolic and up-regulation of catabolic and pro-apoptotic processes. These  
16 metabolic changes can be attenuated by exercise training.  
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## Introduction

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4 Skeletal muscle is crucial for movement and also plays an important role in sugar and fat  
5 metabolism, and immune response. Age-associated loss in function and mass of skeletal  
6 muscle is well documented (Bijlsma and others 2012; Reid and Fielding 2012). However, the  
7 causative mechanism(s) controlling this complex process is not well understood. Enhanced  
8 generation of inflammation (Degens 2010), aging-related increases in the level of reactive  
9 oxygen species (ROS) (Hiona and Leeuwenburgh 2008), altered metabolism (Lawler and  
10 Hindle 2011) , and increased rates of protein degradation (Witt and others 2008) are also on  
11 the list of potential causative factors of sarcopenia. Indeed, it has been reported that  
12 administration of exogenous tumor necrosis factor alpha (TNF- $\alpha$ ) leads to a significant  
13 decrease in the mass of skeletal muscle (Llovera and others 1993). This cytokine can interfere  
14 with the contractile properties of skeletal muscle causing decreased force generating capacity  
15 (Reid and others 2002). Inflammation can readily increase the concentration of ROS, which  
16 above certain levels jeopardizes cellular function (Ji 2007; Langen and others 2003; Radak  
17 and others 2005). Recently it has been reported that myostatin, which is a negative regulator  
18 of muscle growth and is induced in aged skeletal muscle (Bowser and others 2013; Brioché  
19 and others 2013), can also add to higher levels of ROS (Sriram and others 2011). Increased  
20 levels of myostatin can readily reduce protein synthesis (Hitachi and others 2014) and it  
21 appears that the rate of protein degradation is enhanced in aged skeletal muscle (Goto and  
22 others 2007). It has also been shown that the ubiquitin-dependent proteasome system can be  
23 activated with aging (Radak and others 2002), and recent information indicates that muscle  
24 RING finger 1/2 (Murf1/2), which is an ubiquitin ligase, could have an important role in  
25 aging skeletal muscle (Sacheck and others 2007). Thus, it is obvious that the mechanism(s)  
26 affecting muscular atrophy is very complex and extremely complicated.

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46 Physical exercise has been shown to retard age-associated loss of muscle mass (Dickinson and  
47 others 2013), and supplementation of growth hormone (Brioché and others 2013; Nass 2013).

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51 Therefore the aim of the present study was to obtain a picture of the signaling anabolic,  
52 catabolic and apoptotic pathways of aged skeletal muscle. The role of aerobic exercise  
53 training on these pathways was investigated.  
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## Methods

### Animals and training protocol

Twelve young (three months old) and twelve eight month old male Wistar rats were used in the study and grouped into young control (YC), young exercised (YE), old control (OC), old exercised (OE).

The investigation was carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by the local ethics committee. Exercised rats were introduced to treadmill running for three days; then for the next two weeks the running speed was set at 10 m/min, with a 5% incline for 30 min/day. The running speed and duration of the exercise were gradually increased to 60% of VO<sub>2</sub>max of the animals. As a result, by the final week of the six weeks training program, young animals ran at 22 m/min, on a 10% incline, for 60 min, whereas old animals ran at 13 m/min, and a 10% incline for 60 min.

At the end of the study, the rats were anaesthetized with intraperitoneal injections of ketemine (50 mg/kg) and perfused by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). This procedure was carried out two days after the last exercise session to avoid the metabolic effects of the final run.

Quadriceps muscle was carefully excised and homogenized in buffer containing 137 mMNaCl, 20mM Tris-HCl pH 8.0, 2% NP 40, 10% glycerol and protease inhibitors. The protein content was measured by the Bradford method using BSA as a standard, and the samples were stored at -80 C.

### Estimation of Oxidant levels and Redox Active Iron

Intracellular oxidant and redox-active iron levels (Kalyanaraman and others 2011)) were estimated using modifications of the dichlorodihydrofluoresceindiacetate (H<sub>2</sub>DCFDA) staining method (Radak and others 2004). In brief, the H<sub>2</sub>DCFDA (Invitrogen-Molecular Probes #D399) was dissolved to a concentration of 12.5 mM in ethanol and kept at -80 °C in the dark. The solution was freshly diluted with potassium phosphate buffer to 125 μM before use. For fluorescence reactions, 96-well, black microplates were loaded with potassium phosphate buffer (pH 7.4) to a final concentration of 152 μM/well. Then 8 μl diluted tissue homogenate and 40 μl 125 μM dye were added to achieve a final dye concentration of 25 μM. The change in fluorescence intensity was monitored every five

1 minutes for 30 minutes with excitation and emission wavelengths set at 485 nm and 538 nm  
2 (Fluoroskan Ascent FL). The fluorescence intensity unit was normalized with the protein  
3 content and expressed in relative unit production per minute.  
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## 7 **Western blots**

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10 Ten to 50 micrograms of protein were electrophoresed on 8-12% v/v polyacrylamide SDS-  
11 PAGE gels. Proteins were electrotransferred onto PVDF membranes. The membranes were  
12 subsequently blocked and after blocking, PVDF membranes were incubated at room  
13 temperature with antibodies (1:500 #sc-6884 Santa Cruz GDF-8/11(C-20); 1:500 #sc-30194  
14 Santa Cruz Follistatin (H-114); 1:500 #sc-32920 Santa Cruz MuRF1(H-145); 1:500 #sc-  
15 49457 Santa Cruz MuRF2(N-15); 1:1000 #9272s cell signaling Akt; 1:1000 #9271s cell  
16 signaling Phospho-Akt (Ser473); 1:500 #sc-8319 Santa Cruz mTOR (H-266); 1:1000 #5536  
17 cell signaling Phospho-mTOR (Ser2448); 1:1000 #9102 cell signaling p44/42 MAPK  
18 (Erk1/2); 1:1000 #9106 cell signaling Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204);  
19 1:500 #sc-1350 Santa Cruz TNF $\alpha$ (N-19); 1:500 #sc-526 Santa Cruz Bax (P-19); 1:500 #sc-  
20 492 Santa Cruz Bcl-2 (N-19); 1:500 #sc-1311 Santa Cruz p53 (C-19); 1:1000 #2459 cell  
21 signaling PSMA6; 1:1000 #3936 cell signaling Ubiquitin (P4D1); 1:500 #sc-15404 Santa  
22 Cruz SIRT1 (H-300); 1:500 #sc-69359 Santa Cruz COX4 (D-20); 1:500 #sc-7159 Santa Cruz  
23 cytochrome c (H-104); 1:2000 #sc-81178 Santa Cruz  $\beta$ -Actin (ACTBD11B7). After  
24 incubation with primary antibodies, membranes were washed in TBS-Tween-20 and  
25 incubated with HRP-conjugated secondary antibodies. After incubation with the secondary  
26 antibody, membranes were repeatedly washed. Membranes were incubated with  
27 chemiluminescent substrate (Thermo Scientific, SuperSignal West Pico Chemiluminescent  
28 Substrate #34080) and protein bands were visualized on X-ray films. The bands were  
29 quantified by ImageJ software, and normalized to  $\beta$ -actin, which served as an internal control.  
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## 49 **Statistical analyses**

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51 Statistical significance was assessed by Kruskal-Wallis ANOVA followed by Mann-Whitney  
52 U test in case of those variables where post-hoc analysis was adequate. The significance level  
53 was set at  $p < 0.05$ .  
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## Results

### *The effects of aging*

Aging resulted in significant decrease in the protein content of cytochrome C (**Fig. 1A**) and COX4 (**Fig. 1B**), indicating decreased mitochondrial content. The ROS levels were appraised using the H<sub>2</sub>DCFDA staining method, and age-associated increase was detected (**Fig. 2**). Myostatin, which is a negative regulator of muscle growth significantly increased with aging ( $p < 0.01$ ) (**Fig. 3A**). An age-associated decrease in the follistatin levels, which is the antagonist of myostatin, was observed in OC group compared to YC (**Fig. 3B**). The ratio of pmTOR/mTOR, pAkt/Akt, did not change significantly as a result of aging (**Fig. 3C,D**). However the ratio of pERK/ERK increased in aged control group compared to young controls (**Fig. 3E**).

The assessment of protein degradation was made by measuring Murf1, Murf2, proteasome subunit alpha (PSMA6), and protein ubiquitination. Generally, all of these markers increased with aging **Fig. 4A-D**). Degradation of proteins is associated with apoptosis and an increase in p53 levels was detected as a result of aging (**Fig. 5A**). Bax is a pro-apoptotic protein and an age-associated increase in this protein was found in the skeletal muscle ( $p < 0.01$ ) (**Fig. 5B**). TNF- $\alpha$  is an adipokine which can relate to apoptosis and it has been found unaltered with aging (**Fig. 5C**). Bax induces apoptosis by binding the Bcl-2 family, which was found to be significantly lower in aged muscle than in young muscle (**Fig. 5D**). SIRT1 is anti-apoptotic protein, which levels was not altered by aging (**Fig. 5E**).

### *The effects of exercise training*

Six weeks running training at the intensity of 60% of VO<sub>2</sub>max resulted in an adaptive response in mitochondrial enzymes with significant elevation of cytochrome C levels in both young and aged groups. The training program eliminated the age-associated loss of cytochrome C (**Fig. 1A**) and COX4 (**Fig. 1B**). Exercise training did not significantly change the levels of ROS. Aerobic exercise training did not change the myostatin levels (**Fig. 3A**), however eliminated the age-associated increase. In accordance with this change, the follistatin levels increased by training in aged animals (**Fig. 3B**).

1 Exercise increased the pmTOR/mTOR levels in aged groups, while no statistical alteration  
2 was present in young groups, and this was true for pAkt/Akt ratio (**Fig. 3C,D**). However,  
3 exercise prevented the age related increase in the ratio pERK/ERK (**Fig. 3E**). Exercise  
4 training decreased the protein levels of Murf1 aged groups compared to aged control rats  
5 (**Fig. 4A**), while exercise decreased the levels of Murf2 in both age groups (**Fig. 4B**).  
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7 Interestingly statistical increase in PSMA6 and ubiquitination levels were found between  
8 young control and young exercise rats (**Fig. 4. C,D**), while in aged groups exercise does not  
9 significantly altered the levels of PSMA6 and protein ubiquitination. Exercise training did  
10 not result in significant alteration of p53, Bax, TNF- $\alpha$  and SIRT1 levels (**Fig. 5. A,B,D,E**),  
11 the only statistical difference in these apoptotic markers was that exercise decreased the  
12 Bcl2 levels in young group compared to young control rats (**Fig. 5C**).  
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## 23 **Discussion**

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27 Age-associated loss in function and size of skeletal muscle leads to a decreased quality of life.  
28 The findings of the present study suggest that the loss of muscle mass is due to decreased  
29 activity of anabolic pathways and increased activity of catabolic pathways in skeletal muscle.  
30 The follistatin mediated anabolic pathway was found to be down-regulated in aged skeletal  
31 muscle. The IGF pathway is known to promote myogenesis (Rosen and others 1993), and  
32 follistatin mediated inhibition of myostatin causes enhanced expression of IGF-1 (Gilson and  
33 others 2009) and activation of anabolic pathways, probably through an IGF-receptor (IGF-  
34 IR). Data from the present study demonstrate that aging results in down-regulation of  
35 follistatin mediated pathways. This is finding is in accordance with the observation, that  
36 administration of follistatin results in increased muscle protein synthesis (Suryawan and  
37 others 2006). Aerobic exercise has been shown to elevate serum levels of follistatin (Gorgens  
38 and others 2013), while exercise can activate Akt and Erk pathways (Boonsong and others  
39 2007; Fuentes and others 2011; Pasiakos and others 2010; Williamson and others 2006),  
40 leading to enhanced production of follistatin (Chen and Ruiz-Echevarria 2013). In the present  
41 study we have observed that exercise could counter act with the effects of aging on follistatin  
42 levels, and this could be an important means by which regular exercise could attenuate  
43 sarcopenia.  
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1 The significant decrease in mass of skeletal muscle could be also due to the enhanced level of  
2 catabolic processes. Myostatin is a powerful negative regulator of muscle growth. Myostatin  
3 signaling results in activation of Smad2 and Smad3 and consequently the regulation of  
4 MyoDas well as the ubiquitin-associated degradation (Attisano and others 2001). This  
5 pathway is activated in aged skeletal muscle, suggesting the involvement of myostatin in age-  
6 associated muscle loss. Indeed, blockage of myostatin also curbs the activity of catabolic  
7 pathways (Thomas and Mitch 2013). On the other hand, cancer-associated cachexia has been  
8 shown to increase myostatin and Murf2 levels in skeletal muscle (Bonetto and others 2009).  
9 These data suggest a functional link between myostatin and Murf(s) mediated catabolism.  
10 Murf1 and Murf2 are ubiquitin ligases but results from work using Murf1 transgenic mice  
11 suggest that Murf1 can interfere with the ROS production of mitochondria in the cardiac  
12 muscle (Mattox and others 2014). Similar interaction could be present in the skeletal muscle.  
13 Murf1/Murf2 has been implicated in the remodeling of type-II fibers in skeletal muscle  
14 (Moriscot and others 2010) as these fibers lose more total area and function than type-I  
15 fibers during the aging process (Deschenes 2004; Pak and Aiken 2004). The increased level of  
16 Murf1/Murf2, hence, can be a compensatory mechanism to try to remodel these fibers, which  
17 includes degradation of damaged fibers. Aging resulted in increased levels of ROS, which are  
18 initiators/consequences of muscle wasting (Eley and others 2008) and closely related to the  
19 activation of apoptosis (Favier and others 2008). It has been reported that age-associated  
20 increases in p53 in skeletal muscle leads to mitochondrial release of cytochrome c  
21 and apoptosis (Tamilselvan and others 2007). In the present study aging resulted in increased  
22 levels of pro-apoptotic proteins p53 and Bax and down-regulation of anti-apoptotic Bcl-2  
23 protein.  
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43 Exercise associated decrease in the levels of p53 and Bax in proteins could counteract the  
44 age-mediated pro-apoptotic pathways. SIRT1 is considered to be an anti-apoptotic protein  
45 (Radak and others 2013). However, an age-associated alteration of this protein was not  
46 observed, although exercise training increased the content of this protein in the older group.  
47 We have previously reported, using the same animals, that exercise increased the activity of  
48 SIRT1 (Koltai and others 2010). However, it is not clear if that finding affects the anti-  
49 apoptotic role of SIRT1. **In addition, it has to be mentioned that the role of sirtuins in aging  
50 is very complex, sirtuins belong to the vitagen family together with heat shock proteins and  
51 thioredoxin (Calabrese and others 2011; Calabrese and others 2010; Calabrese and others  
52 2012; Cornelius and others 2013). The U-shape dose response curve, which is often called**  
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hormesis, is very representative to oxidants, oxidative damage and vitagens, and without question vitagens could play an important role in aging process (Calabrese and others 2007; Radak and others 2011). Nevertheless, the role of SIRT1 in age-associated loss of muscle mass needs further verification.

In conclusion, we report that aging results in significant decreases in anabolic processes of skeletal muscle by activation of the follistatin pathway. This finding, together with the data that show enhanced activation of myostatin, Murf1/2, PMSA6, protein ubiquitinating pathway, and apoptosis in skeletal muscle of aged animals, suggests that the age-associated loss in muscle mass is a result of altered protein synthesis and degradation. Exercise training, can reverse the decline in anabolic processes and increases in catabolic and apoptotic processes, and serve as an important tool to fight sarcopenia and cachexia.

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2 **Figure legends**  
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5 **Fig. 1. The levels of cytochrome C and COX 4**  
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7 Mitochondrial content was evaluated by cytochrome c and COX 4. Groups: YC,  
8 young control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old  
9 control; OE, old exercised, OEI, old exercised IGF-1 treated. Values are means  $\pm$  SE  
10 for six animals per group. \* $p < 0.05$ , \*\* $p < 0.01$ .  
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16 **Fig. 2. The evaluation of ROS content**  
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18 The measurement of ROS levels was done by fluorescent detection of  
19 H2DCFDA. Groups: YC, young control; YE, young exercised; YEI, young exercised  
20 IGF-1 treated, OC, old control; OE, old exercised, OEI, old exercised IGF-1 treated.  
21 Values are means  $\pm$  SE for six animals per group. \* $p < 0.05$ , \*\* $p < 0.01$ .  
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27 **Fig. 3. Anabolic factors of skeletal muscle**  
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29 Myostatin (A) and follistatin (B) levels were evaluated by Western blot. The activities  
30 of mTOR (C), Akt (D) ERK (E), were measured by the ratio of phosphorylated and  
31 total levels of mTOR, Akt and ERK. Groups: YC, young control; YE, young  
32 exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised,  
33 OEI, old exercised IGF-1 treated. Values are means  $\pm$  SE for six animals per group.  
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66 **Fig. 4. Catabolic factors of skeletal muscle**  
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68 MuRF1 (A) and MuRF2 (B) PSMA6 (C) and protein ubiquitination (D) levels were  
69 evaluated as markers of protein degradation. Groups: YC, young control; YE, young  
70 exercised; YEI, young exercised IGF-1 treated, OC, old control; OE, old exercised,  
71 OEI, old exercised IGF-1 treated. Values are means  $\pm$  SE for six animals per group.  
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999 **Fig. 5. Proapoptotic and anti apoptotic markers in skeletal muscle**  
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1001 Pro-apoptotic factors p53 (A), BAX (B), and TNF-a (C) and anti-apoptotic factors  
1002 Bcl-2 (D) and SIRT1 (E) were measured by immunoblot. Groups: YC, young  
1003 control; YE, young exercised; YEI, young exercised IGF-1 treated, OC, old control;  
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OE, old exercised, OEI, old exercised IGF-1 treated. Values are means  $\pm$  SE for six animals per group. \*p<0.05, \*\*p<0.01.

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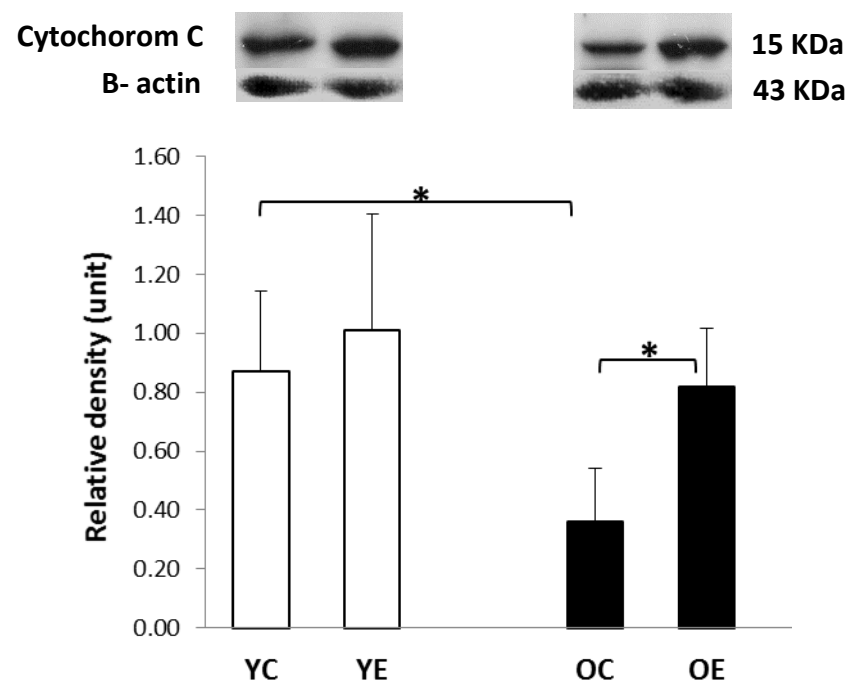
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There is no conflict of interest regarding the manuscript

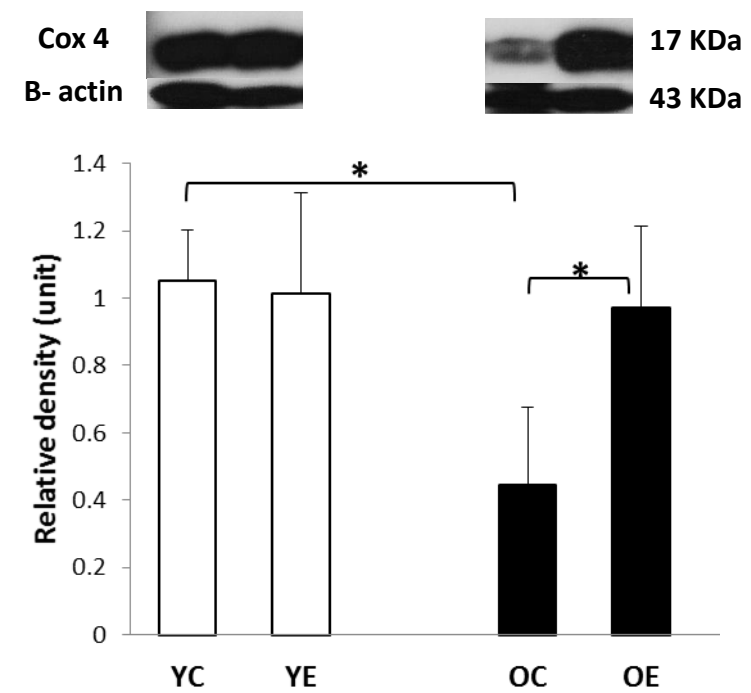


Fig 1.

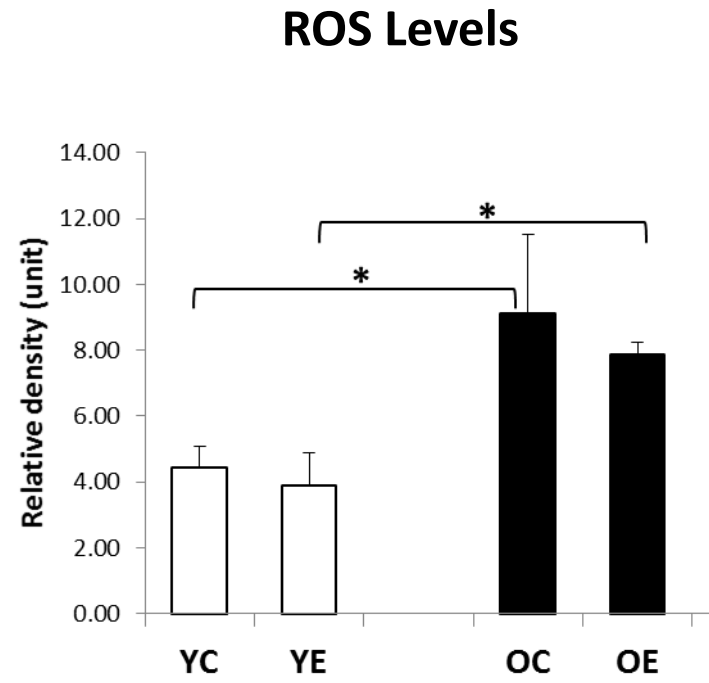
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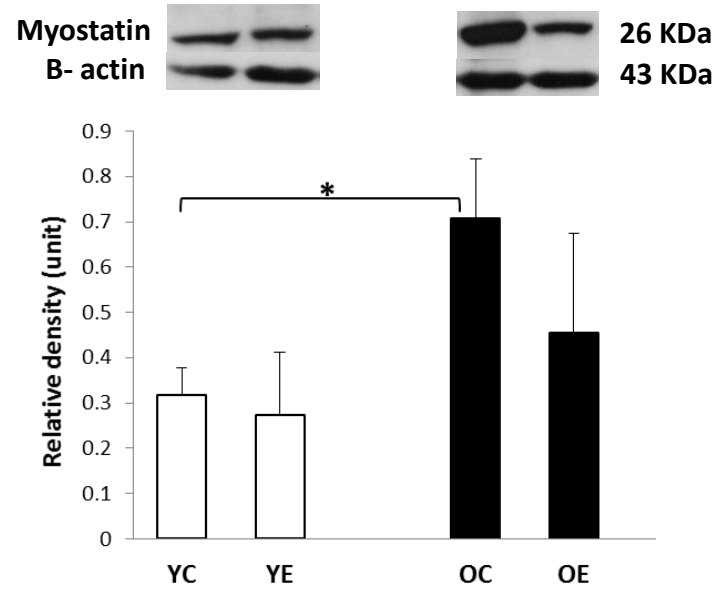
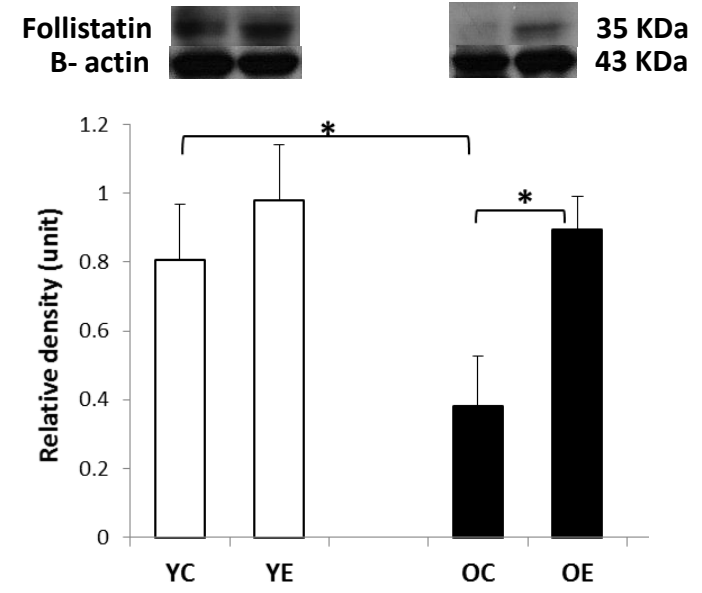
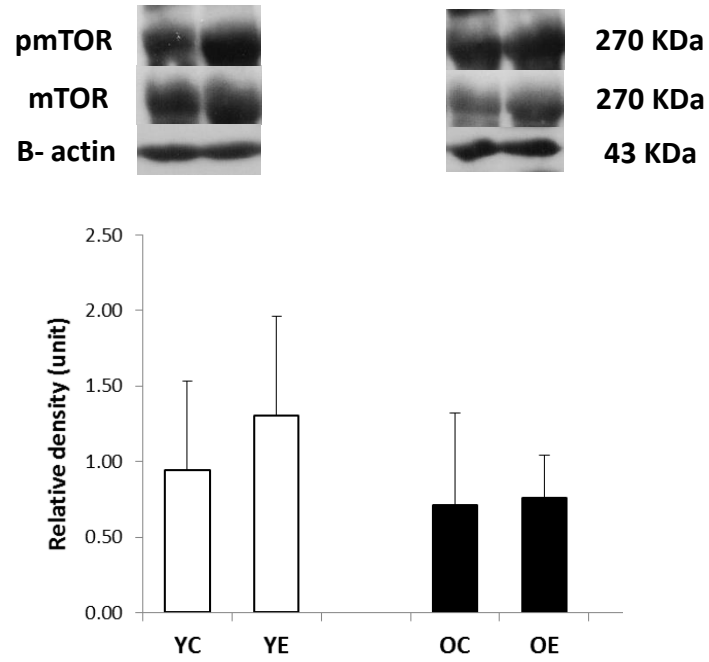
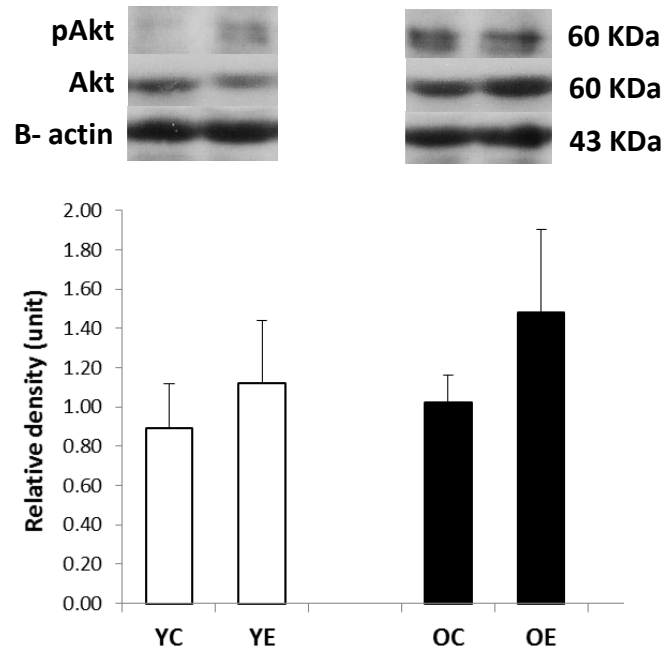
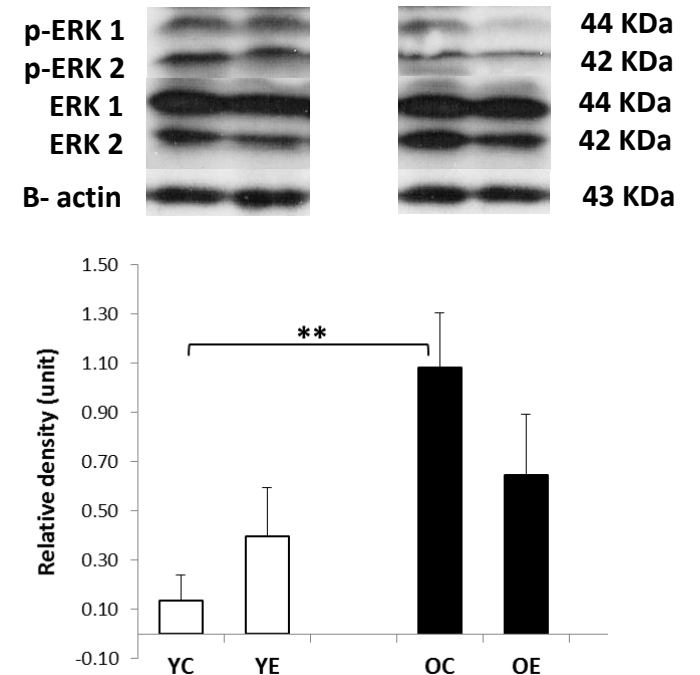


B



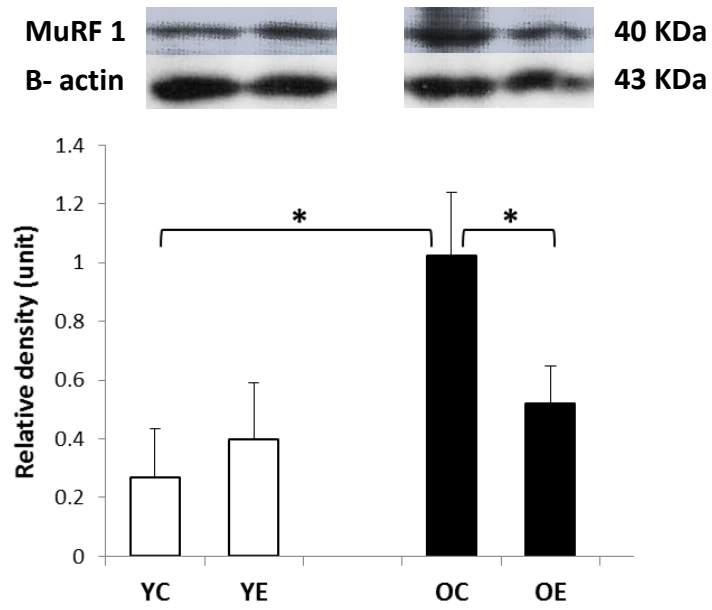
**Fig 2.**



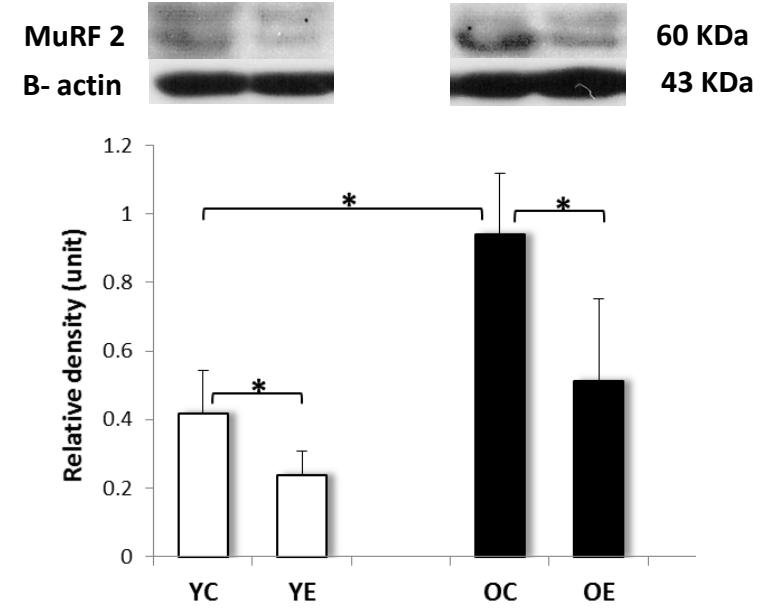
**Fig 3.****A****B****C****D****E**

**Fig 4.**

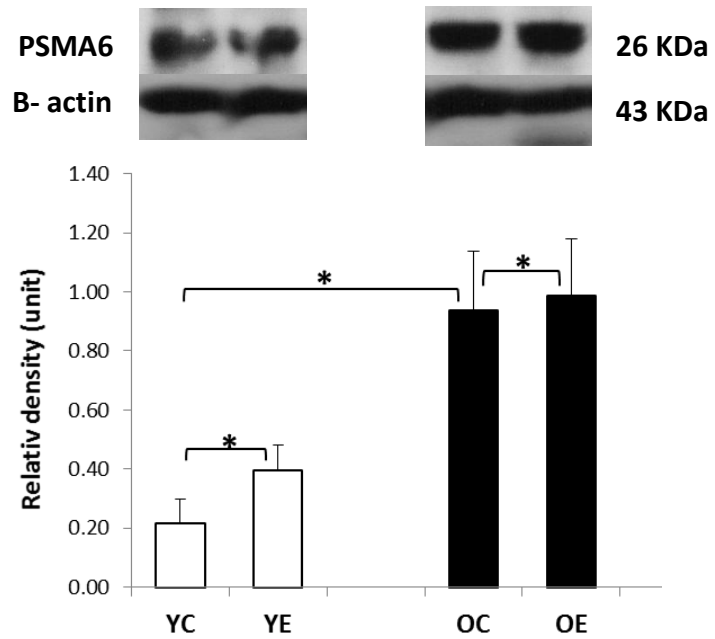
**A**



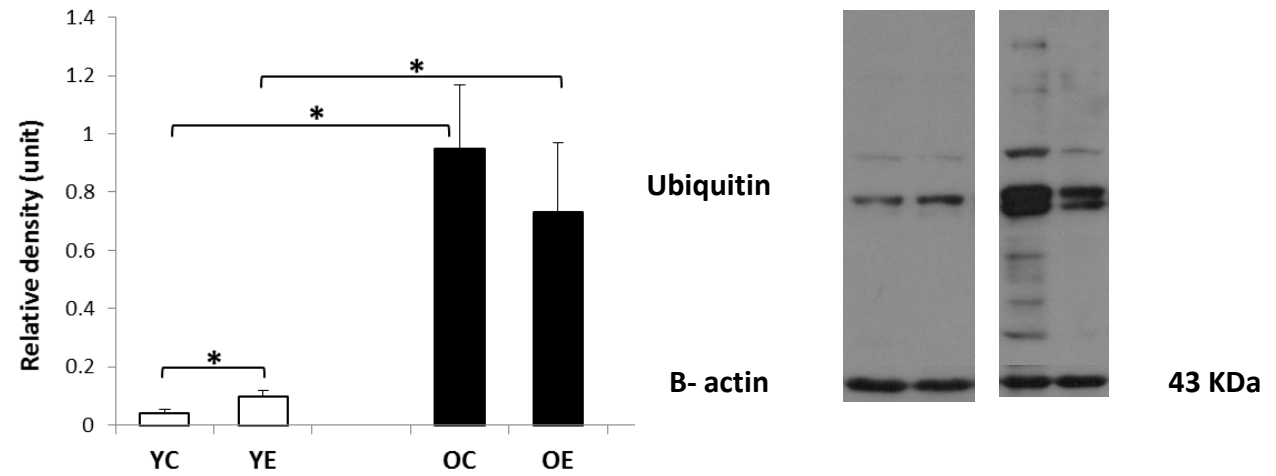
**B**



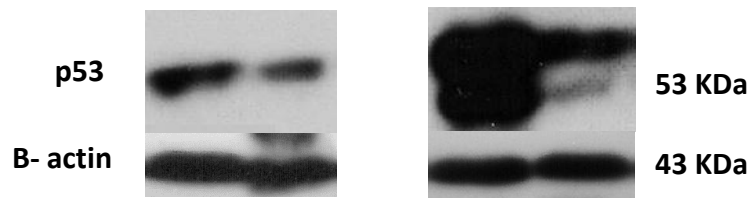
**C**



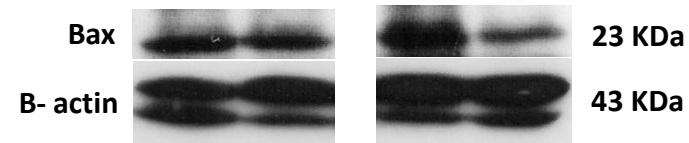
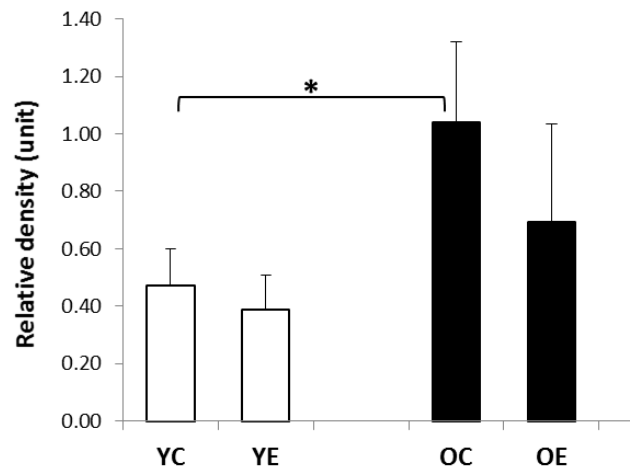
**D**



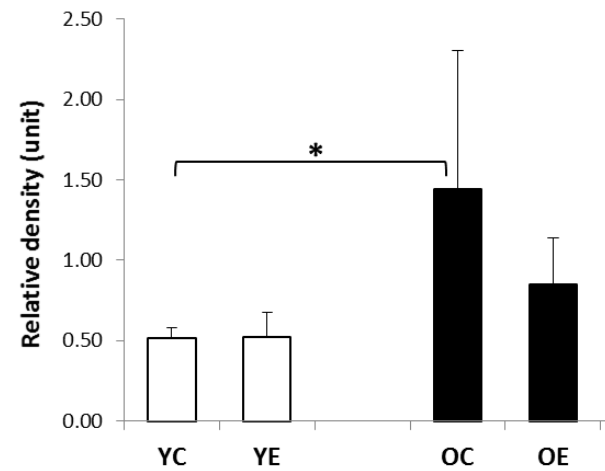
**Fig 5.**



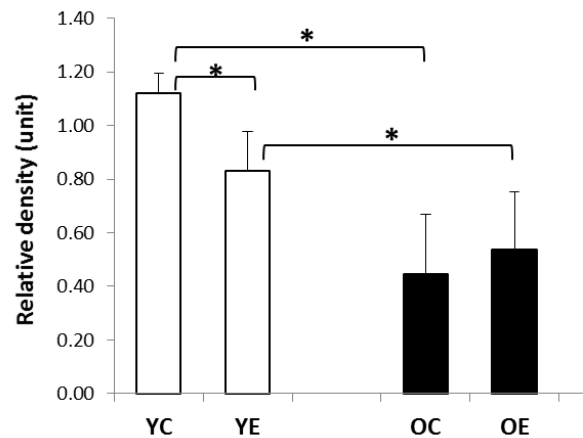
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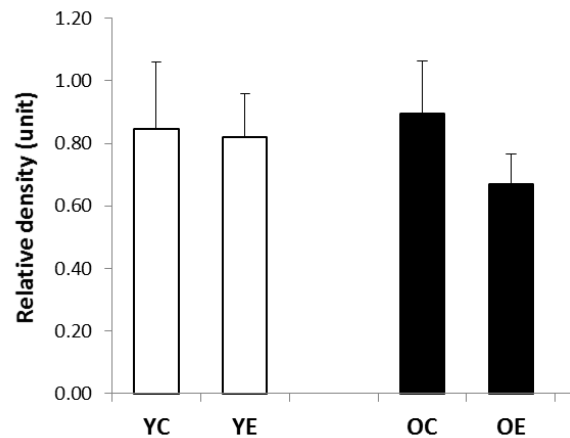
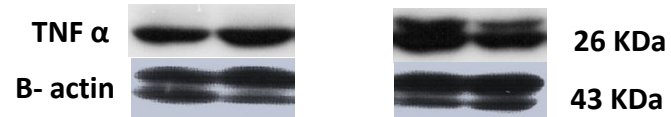
**B**



**C**



**D**



**E**

