*Free Rad. Res.*, Vol. 33, pp. 287–293
Reprints available directly from the publisher
Photocopying permitted by license only





# Age and Sex Differences in Human Skeletal Muscle: Role of Reactive Oxygen Species*

O. PANSARASA[a], L. CASTAGNA[a], B. COLOMBI[a], J. VECCHIET[b], G. FELZANI[b] and F. MARZATICO[a,†]

[a]*Department of Physiological and Pharmacological Sciences, Section of Pharmacology, University of Pavia, P.zza Botta 11, I-27100, Pavia, Italy;* [b]*Department of Internal Medicine and Aging "G. D'Annunzio", University of Chieti, Chieti, Italy*

Accepted by Prof. H. Sies

*(Received 3 November 1999; In revised form 31 January 2000)*

Previous studies, conducted on experimental animals, have indicated that reactive oxygen species (ROS) are involved in the aging process. The objective of this work was to study the relationship between oxidative damage and human skeletal muscle aging, measuring the activity of the main antioxidant enzymes superoxide dismutase (total and MnSOD), glutathione peroxidase (GPx) and catalase in the skeletal muscle of men and women in the age groups: young (17–40 years), adult (41–65 years) and aged (66–91 years). We also measured glutathione and glutathione disulfide (GSH and GSSG) levels and the redox index; lipid peroxidation and protein carbonyl content. Total SOD activity was lower in the 66–91 year-old vs. the 17–40 year-old men; MnSOD activity was significantly greater in 66–91 year-old vs. 17–40 year-old women. GPx activity remained unchanged. The activity of catalase was lower in adults than in young men but higher in the aged. We observed no changes in GSH levels and significantly higher GSSG levels only in aged men vs. adult men, and a significant decrease in aged women vs. aged men. The protein carbonyl content increased significantly in the 41–65 and 66–91 year-old vs. the 17–40 year-old men. Finally, young women have lower lipid peroxidation levels than young men. Significantly higher lipid peroxidation levels were observed in aged men vs. both young and adult men, and the same trend was noticed for women. We conclude that oxidative damage may play a crucial role in the decline of functional activity in human skeletal muscle with normal aging in both sexes; and that men appear to be more subject to oxidative stress than women.

*Keywords:* Skeletal muscle, lipid peroxidation, sex, aging, antioxidant enzymes

## INTRODUCTION

There are various theories about the possible causes of aging; many of which agree and each of which is supported by scientific evidence,[1] one addressing the involvement of oxidative damage induced by reactive oxygen species (ROS).[2]

Two tissues that are particularly vulnerable to oxidative stress are the central nervous system and muscle, both of them containing postmitotic

* The present study was carried out in association with other research teams as part of the Sigma Tau Neurigen Project, "Recent Drugs for Peripheral Neuropathies".

† Corresponding author. Tel.: ++39-(0382)-506390. Fax: ++39-(0382)-506385. E-mail: fulviomk@unipv.it.

cells which are liable to accumulate oxidative damage over time, and both of which use a large amount of oxygen. Muscle mass and strength decrease with age:[3] type II fiber atrophy has been observed in the elderly;[4] a decrease in the mitochondrial volume, in the muscle respiratory rate and in the muscle content of mitochondrial enzymes.[5,6] Thus, mitochondria are believed to be involved in the aging process.[7]

As the main source of cell energy, mitochondria are considered to be among the most frequently affected ROS targets. ROSs are produced in mitochondria during respiratory chain electron transport and they should be rapidly transformed into more inactive species by the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. However, the increased production of ROSs described during aging[8] might not be counteracted by a concomitant increase in antioxidant enzyme activity.[9]

In addition to enzymatic antioxidant activity, glutathione also plays a protective role. The concentrations and the ratio of GSH vs. glutathione disulfide can undergo dynamic changes in different physiological and pathological situations, thus it provides a sensitive measure of oxidative stress[10] and an index of cells' ability to cope with stressful conditions. Furthermore, muscle has little ability to synthesize GSH *de novo*; thus, muscle take up GSH mainly from extracellular sources.[11]

The degree of membrane lipid peroxidation, detected by means of malondialdehyde and 4-hydroxy-2-nonenal production, and the protein carbonyl content are useful indexes of tissue damage.[12,13]

The effects of aging on skeletal muscle antioxidant systems may be quite different from those in liver, kidney, brain and heart:[14] recent evidence reported a marked enhancement in rat muscle antioxidant enzymes in old age;[15] however, few data are available on human skeletal muscle aging.

There is also a significant difference between sexes in aging. Rikans and coauthors[16] reported that cytosolic SOD (CuZnSOD) and GPx activities displayed sex-dependent variations in rat liver. In human aging there are also sex differences: in women estrogens protect cultured neurons against lipid peroxidation induced both by $FeSO_4$ and amyloid $\beta$-peptide.[17]

The purpose of this study was to investigate the effects of aging on the activities of SOD, catalase and GPx in human muscle samples from men and women in three different age groups: young (17–40), adult (41–65) and aged (66–91). Secondly, we tried to identify whether the cellular GSH and GSSG levels and the redox index, are compromised during aging and whether a sex difference exists. Finally, we quantified the influences of age and sex on both protein carbonyl content and lipid peroxidation.

## MATERIALS AND METHODS

### Chemicals

All chemicals used in this study were from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

### Subjects

Human muscle biopsy samples were obtained with informed consent, from 120 hospitalized patients who had undergone orthopedic surgery under general anesthesia. On the whole, men and women who participated to this study were in a healthy state.

Muscle samples were essentially of three types: vastus lateralis, rectus abdominis and gluteus maximus.

Of the 120 samples 57 were from men and 63 were from women. Samples were divided into three groups according to the age of the donor: young (17–40, $n = 19$, 11 men and 8 women, average age 28.3), adult (41–65, $n = 53$, 26 men and 27 women, average age 53) and aged (66–91, $n = 48$, 20 men and 28 women, average age 75.8). According to age, no statistical differences were reported in the young group.

The total weight of each muscle sample obtained during the biopsy was about 3 g, of which we used 150–200 mg.

### Preparation of Tissue Samples

To measure the activity of the three main antioxidant enzymes (SOD, GPx and catalase) and the lipid peroxidation levels, muscle samples were weighed and diluted 20% w/v in 20 mM Tris–HCl, pH 7.4. Samples were homogenized twice: once with a Polytron and the second with a Potter Braun homogenizer. Part of the homogenate was used for lipid peroxidation level determination, and the rest was centrifuged at 5000× *g* for 10 min. The supernatant fraction was used to evaluate the total SOD activity; MnSOD activity was determined using the mitochondrial fraction obtained after two homogenizations of the muscle tissue in 0.32 M sucrose and 1 mM ethylenediaminetetraacetate (EDTA) buffer (IM) and a 800× *g* centrifugation for 15 min. Supernatants were centrifuged again at 14000× *g* for 20 min, and the mitochondrial sediment was resuspended in sucrose–EDTA to obtain a dilution of 10 mg supernatant in 0.1 ml sucrose.

The muscle samples used for the glutathione level evaluation were weighed, homogenized in 5% sulfosalicylic acid (SSA) 10% w/v, and centrifuged at 9500× *g* for 5 min. After the centrifugation we used the supernatant fraction to measure the glutathione content.

Finally, to assay protein carbonyl content, human muscle samples were homogenized in 5 mM phosphate buffer (pH 7.5) with protease inhibitors (leupeptin 0.5 μg/ml, aprotenin 0.5 μg/ml, and pepstatin 0.7 μg/ml) and 0.1% Triton X-100. The homogenate was centrifuged at 700× *g* for 20 min and the biochemical analysis performed on the resulting supernatant.

### Procedure

The total (10 μM KCN) and the MnSOD (1 mM KCN) activities were measured by monitoring at 550 nm the rate of cytochrome c reduction by superoxide radicals ($O_2^{\bullet -}$) produced by a xanthine/xanthine oxidase system.[18]

Catalase activity was detected spectrophotometrically at 240 nm by measuring $H_2O_2$ decay.[19]

GPx activity was evaluated by spectrophotometrically measuring at 340 nm NADPH oxidation in the presence of GSH and hydrogen peroxide ($H_2O_2$).[20] Malondialdehyde and 4-hydroxy-2-nonenal were evaluated colorimetrically at 586 nm according to Esterbauer and Cheeseman method.[21]

Total glutathione levels (data not shown) were assayed in the 5% SSA supernatant in the presence of 5-5′-dithionitrobenzoic acid (DTNB). The rate of DTNB reduction was monitored at 412 nm.[22] Glutathione disulfide (GSSG) was measured according to the same method after derivatization of GSH with 2-vinylpyridine. Both the reactions started after the addition of glutathione reductase. GSH was calculated as difference between total glutathione and GSSG.

Protein carbonyl content was detected using the 2,4-dinitrophenyl hydrazine (DNPH) procedure.[23] The difference between the DNPH-treated and the HCl-treated (blank) samples was determined at 366 nm and the results were expressed as nmol carbonyl groups/mg of protein using an extinction coefficient of 22,000 $M^{-1} \cdot cm^{-1}$. Sample protein concentration was evaluated using bovine albumin as a standard.[24]

### Statistical Analysis

Statistical analysis was carried out with the Student's *t* test; and differences were considered statistically significant at $p < 0.05$.

## RESULTS

### Total SOD and MnSOD

As reported in Table I, total SOD activity decreased in aged men, but no differences were observed between men and women. MnSOD

TABLE I Age-related changes in total, MnSOD, GPx activities and in GSH, GSSG levels and the redox index, ([GSH] + 2[GSSG])/(2[GSSG] × 100), evaluated in 17–40, 41–65 and 66–91 year-old men and women. The value are expressed as means ± SEM

| Age group (years) | Total SOD (U/mg pr) | MnSOD (U/mg pr) | GPx (mU/mg pr) | GSH (nmol/g tissue) | GSSG (nmol/g tissue) | Redox index |
|---|---|---|---|---|---|---|
| 17–40 Men | 38.4 ± 12.9 | 44.8 ± 11.2 | 160 ± 41.1 | 643 ± 56.2 | 63.1 ± 12.6 | 0.07 ± .01 |
| Women | 185 ± 4.7 | 33.3 ± 9.5 | 114 ± 25.7 | 879 ± 115 | 36.1 ± 6.5 | 0.13 ± .01$^{\S}$ |
| 41–65 Men | 20.1 ± 4.5 | 73.1 ± 14.5 | 222 ± 43.5 | 649 ± 86 | 58.7 ± 13.8 | 0.13 ± .03 |
| Women | 19.8 ± 15.6 | 64.7 ± 11.9 | 155 ± 21.4 | 803 ± 105 | 69.8 ± 14.6 | 0.07 ± .02 |
| 66–91 Men | 10 ± 3.6° | 80.3 ± 19.4 | 171 ± 27.2 | 690 ± 128 | 285 ± 83.9* | 0.03 ± .01°,* |
| Women | 17.1 ± 2.3 | 94.8 ± 21$^{\$}$ | 185 ± 23.8 | 788 ± 137 | 89.8 ± 32$^{\#}$ | 0.08 ± .02$^{\#}$ |

°$p < 0.05$, 66–91 year-old vs. 17–40 year-old men; $^{\$}p < 0.05$, 66–91 year-old vs. 17–40 year-old women; *$p < 0.05$, 66–91 year-old vs. 41–65 year-old men; $^{\S}p < 0.05$, 17–40 year-old men vs. 17–40 year-old women; $^{\#}p < 0.05$, 66–91 year-old men vs. 66–91 year-old women.

activity was significantly higher in the 66–91 year-old women vs. the 17–40 year-old women.

### GPx and Catalase Activity

GPx activity showed no differences between the experimental groups (Table I). Age-dependent changes in catalase activity are reported in Figure 1A. We observed a significant decrease ($p < 0.05$) in adult vs. young men, and a significant increase in old vs. adult men. No changes were obtained in the female groups, or between men and women.

### GSH, GSSG Levels and Redox Index

The age-related changes in GSH and GSSG levels are reported in Table I. GSH levels remained unchanged both with aging and between the two sexes. As regards GSSG, we observed a significant increase in 66–91 vs. 41–65 year-old men; and a significant decrease in the aged women vs. the aged men. Table I also reports the age-dependent changes in cell redox state expressed by the redox index: ([GSH] + 2[GSSG])/(2[GSSG] × 100). The redox index is significantly higher in young and aged women vs. young and aged men respectively. With regard to age-dependent changes in the cell redox state, aged men showed significantly lower levels vs. both young and adult men.

### Protein Carbonyl Levels

The protein carbonyl content increased significantly in the 66–91 year-old men compared to both the 17–40 and the 41–65 year-old men, Figure 1B.

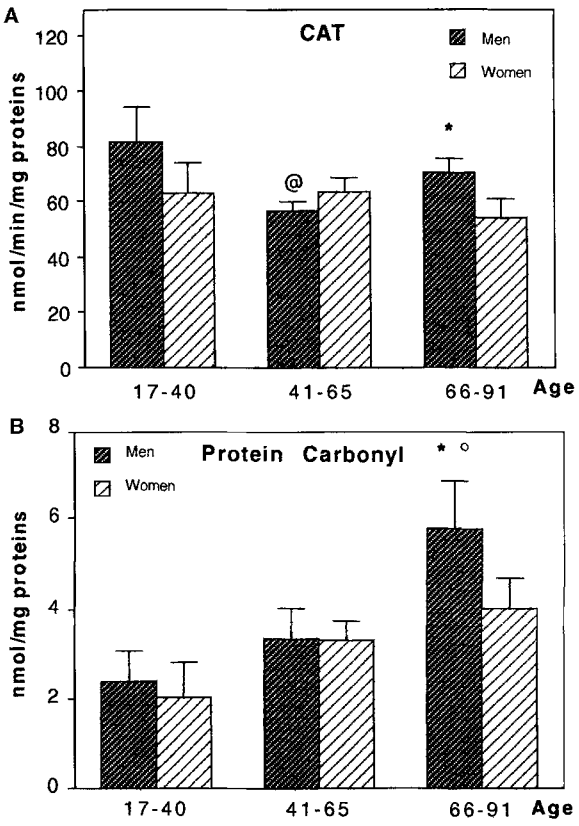


FIGURE 1A and B

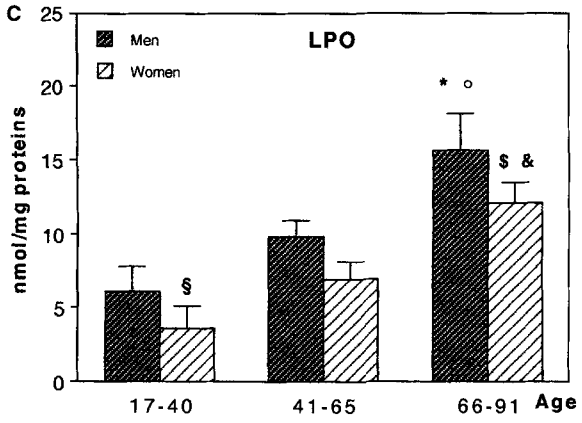


FIGURE 1C

FIGURE 1 Aging and sex differences on catalase activity (A), protein carbonyl content (B) and lipid peroxidation levels (C) in skeletal muscle of men and women in three experimental groups: 17–40 (young group), 41–65 (adult group) and 66–91 (old group). Values are expressed as means ± SEM. $*p < 0.05$, 66–91 year-old vs. 41–65 year-old men; $^{@}p < 0.05$, 41–65 year-old vs. 17–40 year-old men; $^{\circ}p < 0.05$, 66–91 year-old vs. 17–40 year-old men; $^{\$}p < 0.05$, 66–91 year-old vs. 17–40 year-old women; $^{\S}p < 0.05$, 17–40 year-old men vs. 17–40 year-old women; $^{\&}p < 0.05$, 66–91 year-old vs. 41–65 year-old women.

### Lipid Peroxidation Levels

A significantly lower level was observed in young women vs. their male counterpart, Figure 1C. In aged males we saw significantly higher LPO levels in aged men vs. both young and adult men. The same increasing trend was noted in the female groups.

## DISCUSSION

There is a controversy as to whether aging is associated with an increase or a decrease in cellular antioxidant defence mechanisms.[25] As regards age-dependent changes in SOD activity, Lammi-Keefe *et al.*[26] reported an increase both in total and MnSOD activity in several types of rat skeletal muscle; while Carillo *et al.*[9] described no changes in SOD activity. Few data are available on the relationship between sex differences and SOD activity: CuZnSOD activity was lower in male than female rat liver in a study by Ji *et al.*[27] As regards SOD activity and human skeletal muscle, Pansarasa *et al.*[28] have reported a decrease in total SOD and an increase in the MnSOD. In our study, we observed an age-dependent decrease in total SOD activity in 66–91 year-old men, while no changes were noted in women. This could be due to the fact that total SOD could be a limiting factor in muscle defences against oxidative damage in aged men. In 66–91 year-old women MnSOD activity was higher than in young women, thus the SOD dismutation mechanism seems to play an active role in women during aging.

Almost all of the studies previously carried out to examine GPx and catalase activity used rat skeletal muscles. Ji *et al.*[15] showed significantly higher GPx activity, but slightly lower mitochondrial isoform in aged rats (31 month-old). Our data show a non-significant tendency of GPx activity to increase in both adult men and women. Previous work on catalase activity showed no changes in 26 month-old rats; and an increase in 31 month-old rats.[15] In accordance with these data, we observed a two-phase trend: first a significant decrease in adult men and then a significant enhancement in aged men. No changes were noted in women, and no sex differences were observed in catalase activity. No significant changes were observed in GSH levels during aging and between the sexes. We noted that men have lower GSH levels than females. In aged men we obtained significantly higher GSSG levels with respect to adult men, and aging may cause significant alterations in glutathione status in male skeletal muscles. From our data it seems that GSSG levels increase only in aged men: 66–91 year-old women have lower GSSG levels than 66–91 year-old men. A sex-dependent alteration in the glutathione status is supported by the fact that the redox index is significantly higher in young women than in young men, and the same results were obtained in aged women vs. aged men. A decrease in the redox index was reported in aged men vs. young and adult men, suggesting that skeletal muscles, like other organs are under

oxidative stress during aging, but the pattern is quite complex.

Concerning alterations in LPO levels in human muscle during aging, our results indicate significantly higher LPO levels in aged men and women, in agreement with those obtained by Pansarasa *et al.*[28] and Mecocci *et al.*[29] Lower LPO levels were observed in young women vs. young men, consistent with the protective effect of female sexual hormones against lipid peroxidation. Our data show high LPO levels both in aged men and aged women: the two sexes show the same susceptibility to peroxidation because of the failure of the protective role of estrogens after menopause during female aging, which makes aged women more susceptible to LDL oxidation and to cardiovascular disease than aged men.[17]

Significantly higher protein carbonyl contents were observed in 66–91 year-old men compared to 17–40 year-old men. This agrees with results reported for the kidney, brain, and heart;[30] and for skeletal muscle.[28,29]

In summary, our data are consistent with previous evidence of the involvement of oxidative damage in the aging process. As regards sex differences, it seems that men are more susceptible than women to age-dependent changes in enzymatic and non-enzymatic antioxidant mechanisms in skeletal muscle.

## *References*


[1] N. Fabris, D. Harman, D.L. Knook, E. Steinhagen-Thiessen and I. Zs.-Nagy (1992) Physiopathological processes of aging towards a multicausal interpretation. In *Annals of the New York Academy of Sciences* (Eds. B. Boland, J. Cullinan and R. Stiefel), The New York Academy of Sciences, New York.

[2] D. Harman (1956) Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, **11**, 298–300.

[3] G. Grimbly and B. Saltin (1983) The ageing muscle (Mini review). *Clinical Physiology*, **13**, 209–218.

[4] A.R. Coggan, R.J. Spina, D.S. King, M.A. Rogers, M. Brown, P.M. Nemeth and J.O. Holloszy (1992) Skeletal muscle adaptation to endurance training in 60–70 year old men and women. *Journal of Applied Physiology*, **72**(5), 1780–1786.

[5] J. Orlander, K.H. Kiessling, L. Larsson, J. Karlsson and A. Aniansson (1978) Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men. *Acta Physiologica Scandinavica*, **104**, 249–261.

[6] D. Boffoli, S.C. Scacco, R. Vergari, G. Solarino, G. Santacroce and S. Papa (1994) Decline with age of respiratory chain activity in human skeletal muscle. *Biochimica Biophysica Acta*, **1226**, 73–82.

[7] M.K. Shigenaga, T.M. Hagen and B.N. Ames (1994) Oxidative damage and mitochondria decay in aging. *Proceeding of the National Academy of Sciences of USA*, **91**, 10771–10778.

[8] R.S. Sohal (1991) Hydrogen peroxide production by mitochondria may be a biomarker of aging. *Mechanisms of Ageing and Development*, **60**, 189–198.

[9] M.C. Carrillo, S. Kanai, Y. Sator and K. Kitani (1992) Age-related changes in antioxidant enzyme activities are region and organ, as well as sex, selective in the rat. *Mechanisms of Ageing and Development*, **65**, 187–198.

[10] S.M. Deneke and B.L. Fanburg (1989) Regulation of cellular glutathione. *American Journal of Physiology*, **257** (*Lung Cell. Mol. Physiol. 1*), L163–L173.

[11] A. Meister and M.E. Anderson (1983) Glutathione. *Annual Review of Biochemistry*, **52**, 711–760.

[12] H. Esterbauer (1982) Aldehydic products of lipid peroxidation. In *Free Radicals, Lipid Peroxidation and Cancer* (Eds. D.C.H. McBrein and T.F. Slater), Academic Press, London, pp. 101–128.

[13] E.R. Stadtman, P.E. Starke-Reed, C.N. Oliver, J.M. Carney and R.A. Floyd (1992) Protein modifications in aging. In *Free Radicals and Aging* (Eds. I. Emerit and B. Chance), Birkhauser, Basel, pp. 64–72.

[14] M. Matsuo (1993) Age-related alterations in antioxidant defence. In *Free Radical in Aging* (Ed. B.P. Yu), CRC Press, Boca Raton, Florida, pp. 143–181.

[15] L.L. Ji, D. Dillon and E. Wu (1990) Alteration of antioxidant enzymes with aging in rat skeletal muscle and liver. *American Journal of Physiology*, **267**, R918–R923.

[16] L.E. Rikans, D.R. Moore and C.D. Snowden (1991) Sex-dependent differences in the effects of aging on antioxidant and defence mechanisms of rat liver. *Biochimica Biophysica Acta*, **1074**, 195–200.

[17] Y. Goodman, A.J. Bruce, B. Cheng and M.P. Matison (1996) Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury and amyloid $\beta$-peptide toxicity in hippocampal neurons. *Journal of Neurochemistry*, **66**, 1836–1844.

[18] L. Flohé and F. Ötting (1984) Superoxide dismutase assay. *Methods in Enzymology*, **105**, 93–104.

[19] H. Aebi (1984) Catalase. *Methods in Enzymology*, **105**, 121–126.

[20] L. Flohé and W.A. Günzler (1984) Glutathione peroxidase. *Methods in Enzymology*, **105**, 114–121.

[21] H. Esterbauer and K.H. Cheeseman (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods in Enzymology*, **186**, 407–421.

[22] M. Anderson (1985) Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*, **113**, 548–552.

[23] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltier and E.R. Stadtman (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*, **186**, 464–478.

[24] O. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.

[25] D. Harman (1986) Free radical theory of aging: role of free radicals in the origination and evolution of life,

aging and disease process. In *Biology of Aging* (Eds. J. Johnson, R. Walford, D. Harman and J. Miquel), Liss, New York, pp. 3–50.

[26] C.J. Lammi-Keefe, P.B. Swan and P.V. Hegarty (1984) Copper-zinc and manganese superoxide dismutase activities in cardiac and skeletal muscle during aging in male rats. *Gerontology*, **30**, 153–158.

[27] L.L. Ji, R. Fu and E.W. Mitchell (1992) Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *Journal of Applied Physiology*, **73**, 1854–1859.

[28] O. Pansarasa, L. Bertorelli, J. Vecchiet, G. Felzani and F. Marzatico (1999) Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. *Free Radical Biology and Medicine*, **27**, 617–622.

[29] P. Mecocci, G. Fano, S. Fulle, U. MacGarvey, L. Shinobu, M.C. Polidori, A. Cherubini, J. Vecchiet, U. Senin and M.F. Beal (1999) Age-dependent increases in oxidative damage to DNA, lipids and proteins in human skeletal muscle. *Free Radical Biology and Medicine*, **26**, 303–308.

[30] L.D. Youngman, J. Park and B.N. Ames (1992) Protein oxidation associated with aging is reduced by dietary restriction of proteins or calories. *Proceedings of the National Academy of Sciences of USA*, **89**, 9112–9116.