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Amendments of Antioxidant Enzyme Status in Different Skeletal Muscle Fibers under Age induced Oxidative Stress Conditions with Reference to Exercise Training

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Abstract : The purpose of the present investigation was to ascertain the influence of exercise training and aging on antioxidant enzyme status in functionally different skeletal muscle fiber types. Wistar strain albino rats of two age groups (3months young and 18months moderately aged/old) were divided into two experimental groups from each age and treated as sedentary control (SC) and exercise trained (ExT; 23m/min, 30min/day, 5days/week for 12 weeks). After completion of the last training session, the antioxidant capacity was evaluated by the assay of total superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione (GSH) content in the rat hind limb muscle fibers of soleus (SOL), red gastrocnemius (RG) and white gastrocnemius (WG). The results revealed that the activities of all antioxidant enzymes and GSH content were decreased in SOL, RG and WG muscle fibers of old rats compared to young rats. Whereas, the same parameters were up-regulated with exercise training in all muscle fibers. However, the exercise induced elevation in SOD and CAT activities was greater in older rats than the young rats. This elevation even in old muscle fibers with exercised may help to cope from age induced oxidative stress. The results obtained in the current investigation are discussed in the light of functional physiology of the muscle fiber types. From this study it was concluded that 12 weeks period of treadmill exercise training has beneficial in preventing the age-associated amendments in antioxidant machinery of different locomotor muscle fiber types.

Key words : Aging, exercise, antioxidant enzymes, skeletal muscle fibers.

Introduction

In humans and animals, aging is associated with pronounced morphological and functional changes in skeletal musculature including loss of muscle mass, muscle strength and decreased speed of contraction (Fielding and Meydani, 1997; Song *et al.*, 2006). The loss of muscle mass is due to reduction in both the number and the size of muscle fibers. The rat hind limb muscle fibers, soleus (SOL), red gastrocnemius (RG) and white gastrocnemius (WG) are also considered as type-I (slow oxidative-SO), type-IIa (fast oxidative glycolytic-FOG) and type-IIb (fast glycolytic-FG) muscle fibers respectively (Delp and Duan, 1996). There

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are greater losses of type II fibers compared with type-I fibers with age and type-II fibers show more mitochondrial deletions and mitochondrial damage (Payne *et al*., 2003). The decline in mitochondrial content and function impairs muscle oxidative and endurance capacity and is therefore, likely to contribute to the increase in muscle fatigue ability that occurs with aging (Rooyackers *et al*., 1996).

Strenuous physical exercise and sports are associated with a dramatic increase in oxygen uptake both at the whole-body level and in skeletal muscle. Evidences indicate the increase of oxygen flux in locomotor muscle during strenuous exercise, results in the production of reactive oxygen species (ROS). Specifically muscular contraction has been shown to generate several reactive radicals, such as superoxide anion (O_2^{o-}), hydrogen peroxide (H_2O_2), hydroxyl radical (^oOH) and nitric oxide (NO) (Jackson, 1998). Mitochondria are in fact a major intracellular source for ROS production during oxidative phosphorylation not only in exercise but also in aging; and the increased production of ROS is implicated in the aging process (Bakala *et al*., 2003; Lee and Wei, 2007). The resulting large increase in ROS may stimulate antioxidant enzyme synthesis; influence their degradation or both and can alter the structure and function of lipids, proteins and nucleic acids, leading to cellular injury and even cell death (Powers *et al*., 2005).

To cope with oxidative stress, like other organs of the body, skeletal muscle also well equipped with highly sophisticated and complex defense mechanism known as "antioxidant defense system". This defense system includes antioxidant enzymes such as, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and non-enzymatic antioxidants like glutathione (GSH), vitamin C, α-tocopherol etc., (Mallikarjuna, 2005; Somani and Husain, 1996). Because of the relation between free radical production and exercise, some investigators have reported that endurance exercise results in elevated antioxidant defense system in skeletal muscle (Hammeren *et al*., 1992; Mallikarjuna *et al*., 2004). Although information exists on exercise training induced alteration in skeletal muscle antioxidant enzyme capacity, virtually no data is available for the antioxidant capacity with type-I, type-IIa and IIb muscle fibers in male rats of comparable age groups. Because of the importance of the specific muscle fibers involved in neuromuscular disease and in exercise science, the present study is aimed to investigate and to test the hypothesis that 'senescent animals would have greater alterations in skeletal muscle antioxidant capacity than young animals due to exercise training'.

Materials and Methods

Animal care and treatment

Pathogen free, Wistar strain male albino rats (*n = 24*) of two different age groups i.e., young (3 months old) weighing 170±10 gm and moderately aged/old (18 months old) weighing 240±10 gm were used in the current investigation. [Approved by the Institutional Animal Ethics Committee (Regd. No. 438/ 01/a/CP CSEA/dt.17.07.2001) in its resolution number 9/IAEC / SVU/2001/ dt.04.03.2002]. We assumed such a division of the age groups according to the studies of Cao and Catler (1995) and Koprowska *et al*. (2004). The skeletal muscle growth/

maturity occur in between 3-6 months and other physiological changes occur in the aging rats from 6 months onwards. The rats were housed in clean polypropylene cages, 6 rats per cage and maintained under temperature controlled room (27 ± 2°C) with a photoperiod of 12 hrs light and 12 hrs dark cycle. The rats were fed with a standard rat pellet diet and water *ad libitum*.

The animals (total *n* = 24) of both the age groups i.e., young (n = 12) and moderately aged/old (n = 12) were divided in to two groups of six each.

Group - I: Sedentary Control (SC): 12 rats from both age, young (*n* = 6) and moderately aged/old (*n* = 6) treated as sedentary control and put on a six channel motor driven treadmill for 5 days a week over 12 weeks and given 2 m/min exercise for 5 min for equivalent handling. These rats were maintained similar to the exercise trained rats.

Group - II: Exercise Trained (ExT): 12 rats from each age young (*n* = 6) and moderately aged/old (*n* = 6) treated as exercised rats and given exercise training on a six channel motor driven treadmill for 5 days/ week for a period of 12 weeks and given 23m/min exercise for 30 min. The running program was scheduled between 6.00-8.00 AM. Treadmill was custom built at University Scientific Instrumentation Centre (USIC), Sri Venkateswara University campus.

Muscle Sampling Procedure

The animals were sacrificed after 24 hrs of the last training session along with sedentary control rats by cervical dislocation and selected muscle fibers of soleus (SOL), red gastrocnemius (RG) and white gastrocnemius (WG) were quickly removed from the hind limb of rats by using dissecting microscope. The excised muscle fibers (SOL, RG and WG) were rapidly dissected free of fat, tendon and surface fascia in ice-cold rat ringer solution, blotted and immediately frozen them in liquid nitrogen. Tissues were stored at -80°C until biochemical analysis. Total time for muscle excision, dissection and freezing was < 5 min.

Chemicals and Solutions

Roboflavine, Nitroblue tetrazolium, Triton X-100, GSSG, NADPH, Dithio-Nitrobenzoic acid, GR were obtained from Sigma Chemicals (St. Louis, MO). All organic solvents were of spectral grade and general chemicals were of reagent grade.

Biochemical Assays

In this investigation the antioxidant enzyme status was evaluated in SOL, RG and WG muscle fibers of control and exercise trained rats of both young and moderately/ old rats. The total superoxide dismutase (SOD; EC-1.15.1.1) activity was measured as the inhibition of photoreduction of nitroblue tetrazolium (NBT) by the enzyme as per the method of Beauchamp and Fridovich, 1971. The catalase (CAT; EC-1.11.1.6) activity was assayed by the method of Chance and Maehly (Chance and Maehly, 1955). The glutathione peroxidase (GSH-Px; EC-1.11.1.9) activity was assayed following the NADPH oxidation by GR, using cumen hydrogen peroxide as described by Flohe and Gunzler, 1984. The glutathione reductase (GR; EC-1.6.4.2) activity was estimated by the method of Carlberg and Mannervik (1985) and the content of glutathione (GSH) was monitored by using the method of Theodorus *et al*. (1981).

Statistical Analysis

The ANOVA was carried out by using SPSS package and the data has been

analyzed for the significance of the main effects i.e., age and exercise along with their interaction. Results were expressed as the mean ± SD of six observations and the values of significance were evaluated with p values. The difference were considered significant at $p < 0.01$.

Results

In the present study, the SOD activity in SOL, RG and WG was decreased in moderately aged/old age rats compared to young rats. Endurance exercise training significantly ($p<0.01$) elevates the total SOD activity in all muscle fiber types of both young and moderately aged/old rats. However, the per cent increase in the activity of total SOD with exercise was more in old than that of young muscle fiber types. In both the age groups slow twitch oxidative muscle fibers showed greater elevation than fast oxidative and glycolytic muscle fibers with exercise training.

Table 2 depicts that as age advances CAT activity was decreased in all muscle fibers. But with exercise training a significant elevation of CAT activity was noticed in both age groups of SOL, RG and WG muscle fibers. The elevation of CAT with exercise training was more in SOL followed by RG and WG muscle fibers in both age groups.

GSH-Px activity was greater in young animals compared to old animals in all the muscle fiber types. We report that significant elevation ($p<0.01$) in GSH-Px activity due to exercise. The percent elevation of GSH-Px was more in RG (53%) followed by WG (49%) and SOL (40%) in young rats. The same trend was also observed in moderately aged/old exercised rats i.e., more in RG (37%) followed by WG (35%) and SOL (30%) (Table-3).

As result of age the activity of GR was decreased in SOL, RG and WG muscle fibers compared to their young rats. In the present study a significant ($p<0.01$) augmentation of GR activity in SOL (72%) followed by RG (61%) and WG (59%) in young rats, and SOL (39%) followed by WG (33%) and RG (31%) in moderately aged/old rats was observed with exercise training when compared to their respective sedentary controls (Table-4).

In this investigation the concentrations of GSH observed as a foot marker of oxidative stress, was dropped with advancement of age and elevated with exercise training. Slow twitch oxidative muscle fibers (SOL) have high amount of GSH content than the fast twitch oxidative glycolytic (RG) and fast twitch glycolytic (WG) muscle fibers in both age groups. Exercise induced elevation of GSH content even in old animals promotes the antioxidant status in muscle fibers (Table-5).

Discussion

To examine the effect of age and endurance exercise training on antioxidant defense system in slow and fast twitch muscle fibers, we studied the rat soleus (type-I) and gastrocnemius (red and white/ type-IIa and IIb respectively) muscles fibers in this investigation. These muscles were chosen for the study because they are actively recruited during treadmill exercise and represent a composite of the three major locomotor muscle fiber types of the rat. The major findings of this study were as follows.

Among various antioxidative mechanisms in the body, SOD is thought to be one of the major enzymes which protects against tissues damage caused by the potentially cytotoxic reactivities of radicals.

Table 1. : Changes in total Superoxide Dismutase (SOD) activity in the selected skeletal muscle fibers of sedentary control and exercise trained (30 min/ day/5 days/week of 12 weeks) male albino rats of two age groups.

Type of the Skeletal muscle fibers	Young rats (3 months)		Moderately aged/Old rats (18 months)	
	Sedentary control	Exercise trained	Sedentary control	Exercise trained
Soleus (SOL)	2.581 ± 0.005	3.194 ± 0.004** (+23.70)	2.169 ± 0.006	3.034 ± 0.004** (+39.88)
Red Gastrocnemius (RG)	2.243 ± 0.004	2.641 ± 0.003** (+17.84)	2.111 ± 0.004	2.624 ± 0.004** (+24.24)
White Gastrocnemius (WG)	2.326 ± 0.004	2.657 ± 0.006** (+14.23)	1.945 ± 0.004	2.343 ± 0.008** (+20.0)

Values are expressed in units of superoxide anion reduced /mg protein/min.
All values are mean ± SD of six individual observations.
Values in parentheses denote percent change over sedentary control
*** All the values are significant at p < 0.01 compare to sedentary control.*

Table 2. : Changes in Catalase (CAT) activity in the selected skeletal muscle fibers of sedentary control and exercise trained (30 min/day/5days/ week of 12 weeks) male albino rats of two age groups.

Type of the Skeletal muscle fibers	Young rats (3 months)		Moderately aged/Old rats (18	
	Sedentary control	Exercise trained	Sedentary control	Exercise trained
Soleus (SOL)	3.18 ± 0.255	4.72 ± 0.337** (+48.42)	2.84 ± 0.277	4.56 ± 0.217** (+ 60.56
Red Gastrocnemius (RG)	2.76 ± 0.183	3.99 ± 0.264** (+ 44.56)	2.20 ± 0.159	3.36 ± 0.264** (+52.72)
White Gastrocnemius (WG)	1.58 ± 0.161	2.19 ± 0.166** (+ 38.60)	1.16 ± 0.123	1.64 ± 0.142** (+41.37)

Values are expressed in m moles of H2O2 metabolized / mg protein/min
All values are mean ± SD of six individual observations.
Values in parentheses denote percent change over sedentary control
***All the values are significant at p < 0.01 compare to sedentary control.*

Table 3. : Changes in Glutathione-Peroxidase (GSH-Px) activity in the selected skeletal muscle fibers of control and exercise trained (30 min/day/5 days/ week of 12 weeks) male albino rats of two age groups.

Type of the Skeletal muscle fibers	Young rats (3 months)		Moderately aged/Old rats (18	
	Sedentary control	Exercise trained	Sedentary control	Exercise trained
Soleus (SOL)	0.832 ± 0.005	1.172 ± (+40.92)	0.592 ± 0.005	0.773 ± (+ 30.63)
Red Gastrocnemius (RG)	0.418 ± 0.099	0.642 ± 0.005** (+53.64)	0.204 ± 0.006	0.279 ± 0.005** (+37.22)
White Gastrocnemius (WG)	0.325 ± 0.007	0.486 ± (+49.84)	0.169 ± 0.006	0.228 ± (+35.24)

Values are expressed in m moles of NADPH oxidized /mg protein/min.
All values are mean ± SD of six individual observations.
Values in parentheses denote percent change over normal
***All the values are significant at p < 0.01 compare to sedentary control.*

Table 4. : Changes in Glutathione reductase (GR) activity in the selected skeletal muscle fibers of sedentary control and exercise trained (30 min/day/5 days/ week of 12 weeks) male albino rats of two age groups.

Type of the Skeletal muscle fibers	Young rats (3 months)		Moderately aged/Old rats (18 months)	
	Sedentary control	Exercise trained	Sedentary control	Exercise trained
Soleus (SOL)	1.284 ± 0.005	2.221 ± 0.007** (+72.97)	0.986 ± 0.005	1.374 ± 0.005** (+ 39.35)
Red Gastrocnemius (RG)	0.739 ± 0.005	1.196 ± 0.004** (+61.84)	0.628 ± 0.007	0.826 ± 0.006** (+31.53)
White Gastrocnemius (WG)	0.682 ± 0.005	1.086 ± 0.005** (+59.24)	0.572 ± 0.005	0.764 ± 0.005** (+33.57)

Values are expressed in m moles of NADPH oxidized /mg protein/min.
All values are mean ± SD of six individual observations
Values in parentheses denote percent change over sedentary control
*** All the values are significant at p < 0.01 compare to sedentary control.*

Table 5. : Changes in concentration of Glutathione (GSH) content in the selected skeletal muscle fibers of sedentary control and exercise trained (30 min/ day/5 days/week of 12 weeks) male albino rats of two age groups.

Type of the Skeletal muscle fibers	Young rats (3 months)		Moderately aged/Old rats (18 months)	
	Sedentary control	Exercise trained	Sedentary control	Exercise trained
Soleus (SOL)	4.688 ± 0.004	4.930 ± 0.005** (+5.16)	4.134 ± 0.004	4.281 ± 0.004** (+3.55)
Red Gastrocnemius (RG)	3.896 ± 0.004	4.123 ± 0.005** (+5.83)	3.532 ± 0.006	3.682 ± 0.005** (+5.81)
White Gastrocnemius (WG)	3.318 ± 0.029	3.552 ± 0.004** (+7.05)	3.127 ± 0.006	3.267 ± 0.003** (+4.47)

Values are expressed in moles of glutathione/gram wet weight of the tissue
All values are mean ± SD of six individual observations.
Values in parentheses denote percent change over sedentary control
*** All the values are significant at p < 0.01 compare to sedentary control.*

In this study age related decrease in SOD activity was observed in SOL, RG and WG muscle fibers, indicates either reduced synthesis of enzyme or elevated degradation or inactivation of enzyme as age advances. It is therefore possible that the decreases in SOD activities with age may be closely related to the aging of the organism. The reported decrease in SOD activities with age may further accelerate the aging process (Alper *et al*., 1998). Sawada and Carlson (1987), report that superoxide radical formation increases with age, therefore a decreased protection against toxic radical may have serious consequences for aging tissues. During aging process, tissues are damaged to some extent due to the oxidative processes primarily caused by ROS, in particular by superoxide anion radicals (Carillo *et al*., 1992). Endurance exercise training significantly elevates the SOD activity in both age groups of rats. As oxygen consumption increase during exercise, a concomitant increases occur in free radical production. The increased generation of free radical, *i.e*., superoxide anion radical would have triggered the induction of SOD enzyme and hence SOD activity was elevated during exercise (Powers *et al*., 1999). This elevation of SOD even in old exercised rats might be aimed at the removal of excess amount of superoxide radicals which are generating during aging process (Mallikarjuna, 2005). In the present investigation increased SOD activity with exercise training considered to be an adaptational change to mitigate superoxide toxicity in the muscle fiber types. We

observed that the exercise induction of SOD is fiber type specific with highly oxidative muscles being most responsive than the others. Therefore, tissue with a high oxidative capacity shows more antioxidant activity.

Catalase and superoxide dismutase are considered to be indispensable for the survival of cell against deleterious effects of hydroperoxides. In the present investigation decrease in the activity of CAT was observed in the muscle fibers of SOL, RG and WG of moderately aged/old rats when compared with young rats. The lower activity of CAT may be due to lower levels of SOD or may be due to inactivation of catalase owing to excess production of ROS. The decreased CAT activity in the present study may be because of high reactive oxygen metabolites production especially O_2^{o-} and H_2O_2 during aging process and cause oxidative stress to the tissue. Evidences suggest that O_2^{o-} it self affect directly the CAT activity (Kono and Fridovich, 1982). It is also been reported that CAT is inactivated by hydroxyl radical (Piegeolet and Corbisier, 1990). Age related decrease in hepatic CAT also reported by Sriram and Lakshmi (2001) in Wistar albino rats. However, skeletal muscle CAT activity was increased with exercise training of both age groups of rats. Several exercise physiologists reported the increased CAT activity due to exercise training in various tissues (Ji, 1999; Navarro *et al*., 2004; Somani and Husain, 1996). Regular exercise may capture the age induced hydrogen peroxides before escaping it from the cell and breakdown them to water and oxygen. In this way exercise can maintain the abundant catalase activity in the tissues under age induced oxidative stress condition. This elevation of CAT activity in aged rats due to exercise training indicates its active involvement in decomposition of hydrogen peroxide. Due to the fact that CAT has an important role in free radical detoxification, the age related decrease in the expression of this enzyme might predispose the tissue to increased free radical damage (Alper *et al*., 1998).

The glutathione peroxidase (GSH-Px) system is a critically important enzymatic defense system against oxidative stress in skeletal muscle. In the current investigation we observed, a decrease in the specific activity of GSH-Px in SOL, RG and WG muscle fibers of moderately aged/old rats when compared to young rats. The production of free radicals and other ROS are believed to increase with age in most cells (Lee and Wei, 2007). The age induced increased free radicals especially hydrogen peroxide may be responsible for the low activity of GSH-Px in older rats. Vohra *et al*., 2001 reported the decreased SOD and GSH-Px activities in aged guinea pigs. The deficiency of SOD has been shown to be associated with decrease in the activity of GSH-Px and vice-versa (Michiels *et al*., 1994). The lowered SOD activity in old rats which was also reported in the present study may also be responsible for the lower GSH-Px activity, because of their interrelation in detoxifying the toxic radicals. This age related decrease in GSH-Px activity was augmented with exercise training. In general, the literature concerning the effect of endurance training on skeletal muscle GSH-Px activity is consistent, with most studies indicating that regular exercise training results in increased the GSH-Px activity in different skeletal muscle fiber types (Mallikarjuna *et al*., 2004; Sen *et al*., 1992). The increased activities of

GSH-Px and SOD might account in part for the decline in protein oxidation in exercise trained animals and efficient elimination of organoperoxides (Leeuwenburgh and Heinecke, 2001). Exercise induced changes in the GSH-Px activity in the present study reveal its active participation in the decomposition of hydroperoxides, which causes the cell damage due to aging. Thus exercise training renders beneficial in cope with oxidative stress due to aging process.

Glutathione reductase (GR) is an ancillary antioxidant enzyme in the mammalian cells. It play an important role in converting GSSG to GSH, there by maintaining GSH-Px catalytic function and reduced intracellular redox status (Somani and Husain, 1996). In this study GR activity was dropped in SOL, RG and WG muscle fibers of older rats. Recently from our lab we (Mallikarjuna *et al*., 2007) reported the decreased hepatic GR activity with advancement of age. The decrease in enzyme activity also suggests the possible free radical mediated oxidative stress and consequent damage to the skeletal muscle mass. Age induced oxygen derived free radicals, which cause the disturbance of pro-oxidants and antioxidant homeostasis in the tissues and leads to decrease all the antioxidant enzymes including GR. The low levels of GR in older subjects reveal that the young age group rats exhibit lesser oxidative damage and this is increased with the advancement of age. In the present investigation we observed that 12 weeks treadmill exercise training significantly elevates the GR activity in SOL, RG and WG muscle fibers of both age groups of rats, suggest that GR actively, participating in converting of toxic GSSG to GSH using NADPH as a co-factor. Previous studies were also elucidates that exercise training has been shown to increase the GR activity in the rat skeletal muscle tissues (Ji and Leichtweis, 1997; Mallikarjuna *et al*., 2004; Sen *et al*., 1992). The increase in GR activity indicates the subcellular adaptive response of skeletal muscles during exercise training to maintain GSH turnover under oxidative stress condition. In our studies slow twitch oxidative (SOL) muscle fivers exhibits greater elevation of GR than the fast twitch oxidative (RG) and glycolytic (WG) muscle fibers in young as well in old aged rats. However, training provides possible adaptation by increasing GR activity, which is involved in the replenishment of glutathione.

Glutathione is the most abundant intracellular thiol based antioxidant present in milli molar concentration, and plays an important role in maintaining the integrity of cells (Powers *et al*., 2004). In the present study, like other antioxidant enzymes, GSH content also decreased in SOL, RG and WG muscle fibers with aging. Aging tissues has inadequate levels of synthesizing enzymes in the ã-glutamyl cycle and it leads to low content of GSH at old age (Martenson *et al*., 1990). Decreased concentrations of GSH have been reported in several diseased states and are associated with an increase risk to oxidative stress. GSH decrease may be due to increased oxidation of GSH or decreased in the synthesis of GSH and/or decreased availability of precursors for GSH formation (Mallikarjuna *et al*., 2007). Exercise training represents significant augmentation of GSH in skeletal muscle fibers of both age groups of rats. Increased GSH content with exercise training may also due to the increase in the synthesis of precursors for GSH formation and increase

the ã-Glutamyl-Cystineglycine enzyme, which is very essential for the GSH. The synthesis and degradation of GSH is referred as the ã-Glutamyl cycle. This cycle may responsible for the enhanced GSH concentration in the tissues with exercise training (Mallikarjuna, 2005). The increased GR activity is an indicative of increased GSH concentration via GSSG reaction at cellular level. These results were also concurring with previous finding who reported the increased GR activity in the tissues (Mallikarjuna *et al*., 2004; Sen *et al*., 1992).

Summary and conclusion

In the current investigation, the exercise training induced elevation of antioxidant capacity in different muscle fiber types may considered as a physiological significant change The greater change in the activities of SOD and CAT was observed in SOL, RG and WG of moderately aged/old rats. Whereas, up regulation of GSH-Px and GR activities with exercise training was greater in young rats compare to old rats. Even though the SOL, RG and WG muscle fibers exhibits different elevated levels with exercise training, but this elevation was in similar trend in both ages. From these results it is clearly envisaged that 12 weeks period of treadmill exercise training bestow a beneficial in preventing the cellular oxidative damage in skeletal muscle fibers and protect the muscle fibers by improving the antioxidant capacity during aging.

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