L-malate Reverses Oxidative Stress and Antioxidative Defenses in Liver and Heart of Aged Rats.

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Short title:

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Summary

The intracellular levels of antioxidant and free radical scavenging enzymes are gradually altered during aging process. An age-dependent increase of oxidative stress occurring throughout the lifetime is hypothesized to be the major cause of aging. The current study examined the effects of L-malate on oxidative stress and antioxidative defenses in the liver and heart of aged rats. Sprague-Dawley male rats were randomly divided into 4 groups, each group consisting of 6 animals. Group Ia and Group IIa were young and aged control rats. Group Ib and Group IIb were young and aged rats treated with L-malate (0.210 g/kg body weight per day). L-malate was orally administrated via intragastric canula for 30 days, then the rats were sacrificed and the liver and heart were removed to determine the oxidant production, lipid peroxidation and antioxidative defenses of young and aged rats. Dietary L-malate reduced the accumulation of reactive oxygen species (ROS) and significantly decreased the level of lipid peroxidation in the liver and heart of the aged rats. Accordingly, L-malate was found to strengthen the antioxidative defense system with an increased activity of the antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and increased glutathione (GSH) level in the liver of the aged rats, phenomenon not observed in heart of aged rats. Our data indicate that oxidative stress

was reversed and the antioxidative defense system was strengthened by dietary supplementation with L-malate.

Key words

antioxidative defense system; glutathione peroxidase; L-malate; mitochondria; reactive oxygen species

Introduction

Reactive oxygen species (ROS) generated by electron transport chain (ETC) play a pivotal role in accelerating oxidative stress in biological systems (Kohl *et al*. 1995, Acharya *et al*. 2004). Physiological amounts of ROS are a cellular requirement (Hensley *et al*. 2000). However, ROS are not completely detoxified and therefore, have the potential to damage lipids, protein and DNA (Harman 1993). It is generally agreed that these intracellular damages are the major cause of aging. Normally, the cell is protected by virtue of an intricate antioxidative system, consisting of enzymatic and non-enzymatic systems, to maintain redox status homeostasis. Enzymic superoxide dismutase (SOD) and glutathione peroxidase (GPx) and non-enzymic reduced glutathione (GSH) play important roles during the process by scavenging reactive oxygen species (ROS) or preventing their formation (Kabuto *et al*. 2003, Veerappan *et al*. 2004). However, the intracellular levels of antioxidant and free radical scavenging enzymes are gradually altered during aging process (Machado *et al*. 1991, Semsei *et al.* 1991). These compounding factors lead to an age-dependent increase in the fraction of oxidants that may escape quenching by defense mechanism and cause oxidative damage to various biomolecules in the tissue. Thus, it is

conceivable that dietary interventions with antioxidants, which could augment endogenous antioxidant compounds to prevent the formation or quench the higher levels of oxidants, could provide an effective means to improve antioxidative defense system with age.

L-malate plays central role in fostering the transport of cytosolic reduced nicotinamide adenine dinucleotide (NADH) into mitochondria. Also, it is important as a spark for oxidation of acetyl-CoA and β-oxidation of fatty acid by virtue of its ability to form ATP(Witter *et al.* 1953, Masoro and Felts 1958).Previous studies in our laboratory have shown that supplementation of L-malate could improve physical stamina and enhance the activity of malate-aspartate shuttle and energy metabolism (Wu *et al.* 2007). Maybe, the increase in metabolic activity could bring about at the expense of increased generation of free radicals that increase oxidative stress. The relationship between oxygen consumption and ROS generation is complex and fundamental questions regarding what governs the relationship between overall metabolic rate and the production of ROS remain unclear (Balaban *et al*. 2005). Moreover, studies on the antioxidant effect of L-malate on aging are relatively scarce and are yet to be elucidated (Romanovich and Basieva, 1975). Therefore, the aim of the present study was designed to evaluate the effect of L-malate supplementation on the status of oxidant production, lipid peroxidation levels and enzymatic and non-enzymatic antioxidants in liver and heart of aged rats.

Materials and methods

Preparation of chemicals

L-malate, enzymes, bovine serum albumin and all substrates were purchased from Sigma

Chemical Company (St. Louis, MO, USA). Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was purchased from Molecular Probes (Guangdong, China). All other chemicals used were of analytical grade and obtained from Guangdong HuanKai Microbial Sci. & Tech. Co., Ltd., China.

Animal preparation

Male rats of Sprague-Dawley strain (SPF grade) were used in this study. They were healthy animals maintained and housed in large spacious cages and given food and water ad libitum. The animal room was well ventilated with a 12h light: 12h dark cycle and maintained at 21-23℃, throughout the experimental period.

Grouping of animals

The animals were divided into two major groups, Group I: Young rats (3-4 months old weighing about 180-220g), Group II: Aged rats (above 24 months old weighing approximately 480-560g). These groups were further sub-divided into two groups: one control group (Group Ia, IIa) and one experimental group (Group Ib, IIb). Each group consisted of six animals.

Group Ia: young control rats, Group Ib: young treated rats (L-malate administration), Group IIa: aged control rats, Group IIb: aged treated rats (L-malate administration). L-malate (0.210 g/kg body weight per day) was administrated via intragastric canula for 30 days. Control animals were administered physiological saline alone.

Cell Isolation and tissue homogenate preparation

On completion of experimental period, animals were killed by cervical decapitation. The liver

and heart samples were quickly dissected, rinsed with ice cold physiological saline and dried by blotting between two pieces of filter paper. An accurately weighed piece of liver and heart was dispersed into single cells by collagenase perfusion (Moldeus *et al*. 1978, Clark *et al.* 1978).

Another accurately weighed piece of liver and heart was minced and homogenized in nine vol (w/v) of MSE solution (220 mM mannitol, 70 mM sucrose, 5 mM potassium Hepes and 2 mM EDTA buffer, pH 7.4), using a Teflon pestle connected to a Braun homogenizer motor, to yield a 10% (w/v) tissue homogenate. The homogenate was centrifuged at 1 200 g at 4℃ for 10 min (Eppendorf centrifuge 5804R, Germany) and the pellet of nuclei and cell debris was discarded. This was repeated twice and the supernatant was stored at -80℃ and used for various biochemical assays. The protein concentration was determined by the method of Bradford (Bradford 1976) with bovine serum albumin as the standard.

Determination of oxidant production

Generation of ROS was evaluated by using DCFH fluorescent probe based on the methods of Schuessel K *et al*. (Schuessel *et al*. 2006) with some modifications. H_2DCF-DA is hydrolyzed and oxidized by various ROS to the fluorescent DCF (Hempel *et al.* 1999). H_2DCF-DA does not stain mitochondria (Diaz *et al.* 2003), but is oxidized mainly by peroxides in the cytosol (Walrand *et al*. 2003). The oxidized forms of H_2DCF-DA, DCF, could be excited with the 485 nm laser and emission at 530 nm laser of the Becton–Dickinson (BD Biosciences, San Jose, USA) FACSCalibur flow cytometer.

The H_2DCF-DA was used at a final concentration of 20 μM by adding 20μl of H_2DCF-DA solution to 1.0 ml cells suspension. The cells were incubated in dark at 37°C for 30 min. Then, the

cells were washed twice with PBS (150 g, 5 min) and resuspended in PBS solution at 1×10^6 cells/ml for flow cytometric analysis with Becton–Dickinson FACSCalibur utilizing CellQuest Pro software. A minimum of 8000 events were recorded per single measurement. Background fluorescence was corrected by the inclusion of parallel blanks. The level of ROS was expressed in arbitrary units (fluorescent intensity, FI). Cells were analyzed immediately after staining and always kept on ice in dark until measurement.

Determination of lipid peroxidation (LPO)

The concentrations of homogenate LPO were determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test (Ohkawa *et al.* 1979). To tissue homogenate (100 μl), 50 μl 8.1% SDS and 20 μl butylated hydroxytoluene (0.02% in 95% ethanol, w/v) were added. The samples were vortexed and incubated at room temperature for 10 min. Subsequently, 375 μl 20% acetic acid (pH 3.5) and 375 μl thiobarbituric acid (0.8% in 0.05 M NaOH) were added to the mixture, vortexed and heated at 95℃ for 60 min. The samples were then allowed to cool immediately under running tap water and centrifuged at 3 500 g for 10 min. A blank was also run simultaneously and tetramethoxy propane was used as an external standard. The absorbance of the clear supernatant was determined at 532 nm. The extent of lipid peroxidation was expressed as nmol of MDA formed per mg of protein.

Determination of GPx activity

The activity of glutathione peroxidase (GPx; EC 1.6.4.2) was estimated by the method of Sazuke *et al*. (Sazuke *et al.* 1989) with slight modification. Briefly, homogenates mixed with GSH

and H_2O_2 were incubated at 37℃ for 5min, followed by the addition 10% trichloracetric acid (TCA). After the samples centrifuged at 3 500 g for 10min, the supernatant were collected and mixed with disodium hydrogen phosphate and 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB). The absorbance of the samples was recorded against the blank at 412 nm. The unit of activity was expressed as μmol GSH oxidation per min per mg protein.

Determination of SOD activity

Total (Cu–Zn and Mn) superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to the method of Sun *et al.* (Sun *et al.* 1988) with a slight modification by Durak *et al.* (Durak *et al.* 1993). The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after a 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of a sample and centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. Activity was expressed as units per mg protein.

Determination of GSH

The GSH level of homogenate was measured at 412 nm run by the method of Moron *et al.* (Moron *et al.* 1979) with slight modification. Briefly, the protein in samples was precipitate with 50% TCA and then centrifuged at 1 000 g for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of 0.2 M Tris-EDTA buffer (pH 8.9) and 0.1 ml of 0.01 M DTNB. The solution was kept at room temperature for 5 min, and then read against the blank at 412 nm on the

spectrophotometer. The GSH concentration was calculated from a standard curve.

Statistical analysis

The original data were tested with SPSS software. All results were presented as Mean± standard deviation (SD). One –way analysis variance (ANOVA) was used for statistical analysis, and all tests were considered to be statistically significant at $P<0.05$ or $P<0.01$.

Results

Effect of L-malate on oxidant production

Fig. 1 represents the level of ROS in liver and heart of young and aged rats. For this experiment we used redox sensitive dye H_2DCF-DA, which fluoresces upon oxidation. The level of ROS was found to be enhanced 43.5% ($P<0.05$) in liver of aged control rats (Group IIa) than that of young control rats. Similarly, the level of ROS was found to be increased 84.3% ($P<0.01$) in heart of aged control rats. L-malate significantly reduced the level of ROS generation in liver and heart of aged treated rats ($P<0.01$). There was no significant effect on young rats.

Effect of L-malate on lipid peroxidation (LPO)

To assess whether L-malate modulated age-related oxidative stress, we measured steady-state levels of MDA, a marker of lipid peroxidation. Fig. 2 shows the lipid peroxidation status in the liver and heart tissue homogenate of young and aged rats supplemented with and without L-malate. Lipid peroxidation was found to increase 18.7% and 67.4% in liver and heart, respectively, of aged control rats compared with young controls. Administration of L-malate significantly

decreased the LPO in the liver and heart of aged treated rats. There was no significant difference between young treated rats and young control rats.

Effect of L-malate on antioxidant defenses

The effects of L-malate supplementation on antioxidant antioxidative defense in liver and heart of young and aged rats were examined. Fig. 3 and Fig. 4 show the antioxidant enzyme activities of GPx and SOD in liver and heart of control and L-malate treated rats. The activities of these antioxidant enzymes were found to be inhibited significantly in liver of aged control rats compared with young controls. The activity of GPx and SOD in liver decreased 40.5% and 29.5%, respectively, in aged controls. L-malate administration was found to enhance ($P<0.05$) the activities of these enzymes, an increase of 39.9% and 34.4% in GPx activity and SOD activity, respectively, in liver during aging. However, L-malate had no effect on activities of both the enzymes in heart.

The levels of GSH in liver and heart were measured to determine whether these low molecular weight antioxidants declined with age. As shown in Fig. 5, the level of GSH in liver was lower ($P<0.05$) in aged control rats compared with young controls. L-malate administration was found to enhance ($P<0.01$) the level of GSH, an increase of 68.1%, in liver of aged rats. The level of GSH in heart was higher ($P<0.05$) in aged control rats. L-malate had no effects on the level of GSH in heart of young and aged rats.

Discussion

The crucial role of L-malate in cellular metabolism is to control the influx of NADH into mitochondria for oxidative phosphorylation and subsequent energy production, its role as an

antioxidant in the aging process is not yet elucidated. The present investigation thus focused on the effects of L-malate on oxidative stress and antioxidant status in liver and heart of aged rats. The liver and heart was chosen as model systems for evaluation of L-malate effects on oxidative stress and antioxidant defense system since these tissues were particularly prone to oxidative damage and the malate-aspartate shuttle was the dominant shuttle in these two organs (Scholz *et al.* 2000).

Age-associated reductions in the activities of antioxidant enzyme related with increases in oxidant production and LPO that were found in the current study support previous findings (Hagen *et al.* 2002, Kalaiselvi and Panneerselvam 1998). The H_2DCF-DA assay has been adapted to measure oxidant production in various tissue cells (Schuessel *et al.* 2006, Bejma *et al.* 2000, Sethumadhavan and Chinnakannu 2006). A progressive increase in the production of ROS with aging has been correlated with decrease in the number of functional mitochondria per cell, as well as a decline in the production of ATP, protein synthesis and increase in peroxide leakage (Vorbeck *et al.* 1982, Herbener 1976, Sandhu and Kaur 2003). The main findings of our study were that supplementation of L-malate decreased H_2DCF-DA oxidation in liver and heart of aged rats. The decrease of ROS level may be attributed either to its reduced generation or to the enhancement in antioxidant level. Previous reports showed that electron transport efficiency decreased in aged rats (Kumaran *et al.* 2004). The loss of electron transport efficiency has the added detrimental effect of increasing the production of intracellular ROS and enhancing oxidative damage (Buttgereit and Brand 1995). Moreover, the recent studies of Speakman et al. (Speakman et al. 2004) have suggested that proton leak and augmented basal metabolism may protect from ROS production and aging. Early studies in our laboratory have shown that supplementation of L-malate enhance

the activity of malate-aspartate shuttle and energy metabolism (Wu *et al.* 2007). It can be assumed that L-malate could reduce the generation of ROS by increasing the efficiency of electron transport and enhancing the activity of energy metabolism.

Lipid peroxidation is used as an index for measuring the damage that occurs in membranes of tissues as a result of free radical infliction (Husain *et al.* 2001). The degree of LPO has been assessed according to the MDA formation, which has been routinely used as an index of LPO. Similar to other studies (Hagen *et al*. 2002, Kalaiselvi and Panneerselvam 1998, Hagen *et al.* 1999), age-associated increase in LPO was observed in our study. Lipids act as vital substrates for LPO, and the enhancement of lipid profile during aging (Leoper *et al*. 1983) may be the cause for increased LPO. The data of current study showed that L-malate administration decreased LPO in liver and heart of aged rats. It is possible that L-malate decreases the level of lipids in aged treated rats by enhancing metabolic rates and β-oxidation, thereby lowering the availability of lipids for peroxidation.

Age-related increase in LPO might be a reflection of decrease in enzymatic and non-enzymatic antioxidant protection (Yu 1994). GPx and SOD are the main enzymes of the antioxidant defense system. A significant decline in the levels of these enzymes in liver of aged rats observed in our study was supported by extensive studies (Kalaiselvi and Panneerselvam 1998, Bejma *et al.* 2000, Kumaran *et al.* 2004, Savitha *et al.* 2005). We found no changes in the activities of these enzymes in heart during age, although findings of previous reports (Kumaran et al. 2004, Savitha *et al.* 2005) showed a decrease. Such a difference between results may be caused by different experimental conditions. After treatment with L-malate, the aged rats were found to regain SOD activity in liver to a significant level. The increased SOD activity could be because of the

providing redox status of L-malate. GPx is a selenium-containing antioxidant enzyme, which effectively reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively，at the expense of reduced glutathione. The decreased GPx activity in liver of aged rats may be due to the decline of glutathione concentration. L-malate supplementation enhanced the level of glutathione in aged rat in our present study and the increased level of the substrate glutathione may be responsible for the increase in the activity of GPx in liver of aged treated rats. Also, the other possible reason could be the increased synthesis of GPx and SOD. As enzymes are proteins, the reduced protein synthesis during aging attributed to decreased ATP production (Nair 2005) may be the cause for the reduction in the activities of these enzymes. L-malate supplementation by virtue of its ability to enhance ATP production (Bendahan *et al.* 2002) might have improved the overall protein synthesis in cells. This may also explain why we observed a beneficial effect of L-malate only in old and not in young rats.

As a water-soluble tripeptide, GSH is the most abundant intracellular small thiol molecule and predominant defence against ROS in tissues. GSH reacts directly with ROS and electrophilic metabolites, protects essential thiol groups from oxidation, promotes the regeneration of α -tocopherol, and serves as a substrate for GSH-related enzymes, e.g., GPx and glutathione S-transferases (Townsend *et al.* 2003). GSH plays important roles in the maintenance of the intracellular redox state and in the cellular defence of oxidative damage. In our study, our findings that the level of GSH was decreased in liver of aged rats consist with previous reports (Kalaiselvi and Panneerselvam 1998, Mosoni *et al.* 2004). The observed decrease in glutathione concentration in liver of aged rats may be due to the enhanced oxidative damage due to free radicals. The decline in the levels of GSH seen in liver of aged rats was completely reversed with supplementation of

L-malate. The recycling of GSH from GSSG (oxidized glutathione) is catalyzed by the enzyme glutathione reductase using NADPH as a cofactor. L-malate increases the TCA metabolism which in turn increases NADPH generation (Bobyleva-Guarriero 1986). Therefore, the observed increase in GSH on treatment could be ascribed to the ability of L-malate to increase the TCA metabolism. Our results showing that levels of GSH increased in heart of aged rats were found by other authors (Fiebig *et al.* 1996) and the levels of GSH in heart were unaffected by L-malate treatment. The possible mechanism may be antioxidants in heart of aged rats have demonstrated considerable adaptability in response to prooxidant exposure (Ji *et al*. 1998).

In conclusion, our study strongly suggests that the supplementation of L-malate may considerably reduce oxidative stress in liver and heart of aged rats by alleviating LPO through scavenging of ROS and increasing the antioxidative defenses in liver of aged rats which then detoxify free radicals. Though our present findings suggest that L-malate supplementation may be a safe and increase protection against oxidative damage, long-term feeding studies with L-malate are needed to determine whether benefits of L-malate seen in aged animals can be sustained over time.

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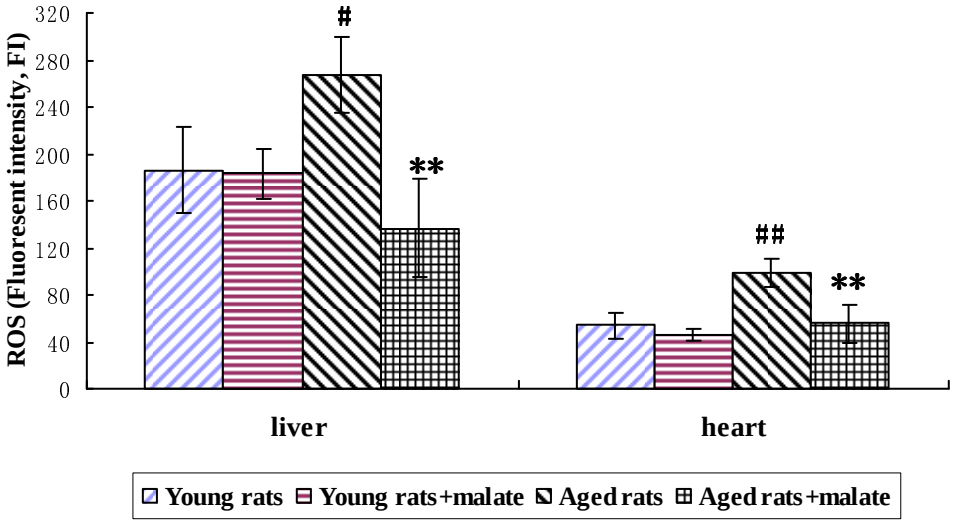

Fig. 1 Effect of L-malate on oxidant production in liver and heart of young and aged rats. Values are the means ± SD (n=4, respectively).ROS level was expressed in arbitrary units (fluorescent intensity, FI). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia); *P<0.05 and **P<0.01 indicates significant compared with aged rats (Group IIa).

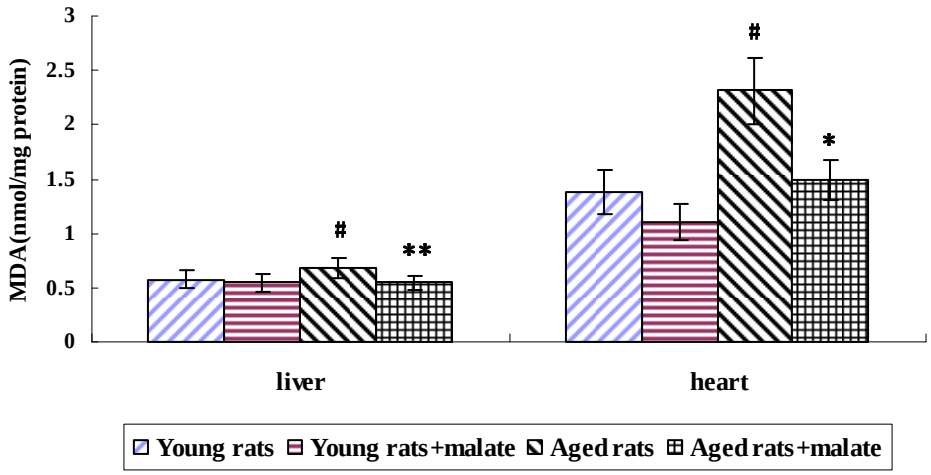

Fig. 2 Effect of L-malate on level of lipid peroxidation in liver and heart of young and aged rats. Values are the means ± SD (n=6, respectively). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia); $^{*}P<0.05$ and $^{**}P<0.01$ indicates significant compared with aged rats (Group IIa).

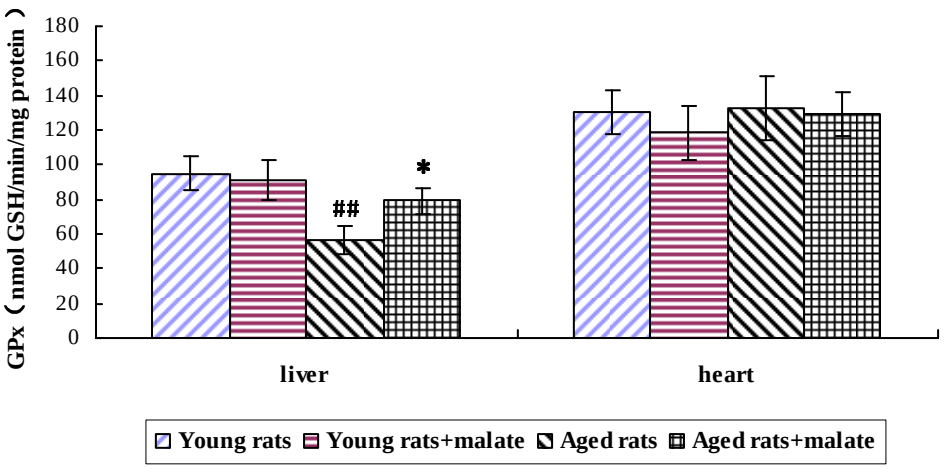

Fig. 3 Effect of L-malate on GPx activity in liver and heart of young and aged rats. Values are the means ± SD (n=6, respectively). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia); *P<0.05 and **P<0.01 indicates significant compared with aged rats (Group IIa).

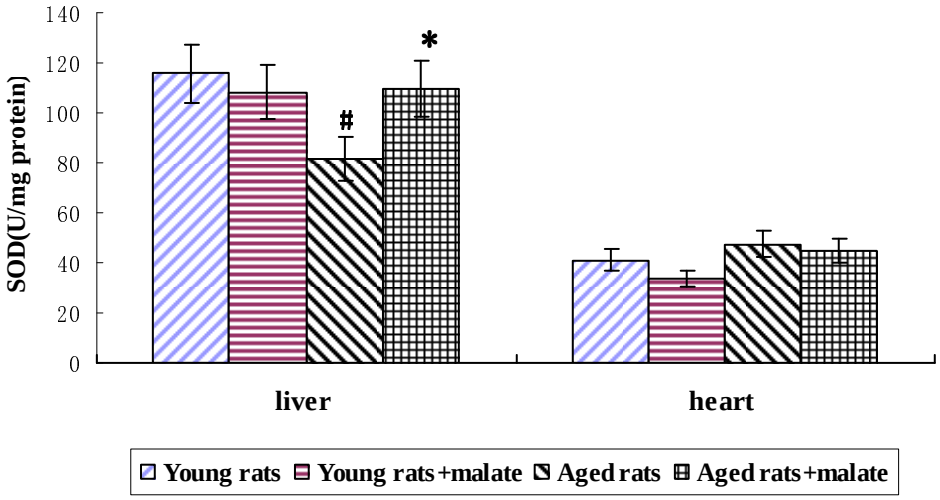

Fig. 4 Effect of L-malate on SOD activity in liver and heart of young and aged rats. Values are the means ± SD (n=6, respectively). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia); *P<0.05 and **P<0.01 indicates significant compared with aged rats (Group IIa).

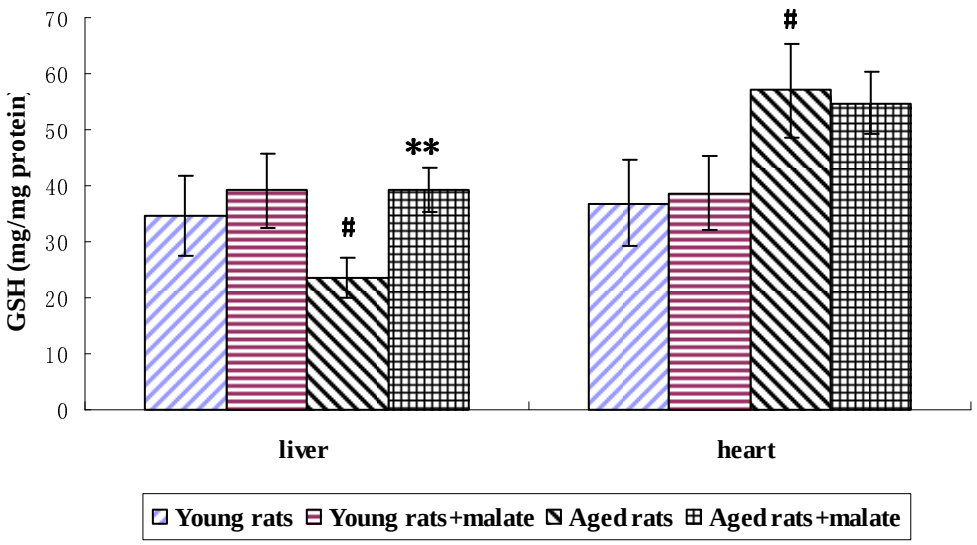

Fig. 5 Effect of L-malate on GSH in liver and heart of young and aged rats. Values are the means ± SD (n=6, respectively). $^{\#}P<0.05$ and $^{\#\#}P<0.01$ indicates significant compared with young rats (Group Ia); *P<0.05 and **P<0.01 indicates significant compared with aged rats (Group IIa).