

Prevention of Alloxan-induced Diabetes by Se-Methylselenocysteine Pretreatment in Rats: The Effect on Antioxidant System in Pancreas

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Abstract

In this study, we assessed the effects of Se-methylselenocysteine (MSC) pretreatment on the antioxidant system in the pancreas and the development of alloxan-induced diabetes in rats. The rats were treated with MSC at a dose of 0.75 mg/rat/day for 2 weeks. The MSC-treated rats evidenced significantly increased glutathione content, GSH/GSSG ratio, and glutathione peroxidase (GPx) and glutathione reductase (GRd) activities in the pancreas. Diabetes was induced via alloxan injection. The alloxan-diabetic rats evidenced significantly reduced glutathione content and glucose 6-phosphate dehydrogenase (G6PD) activity and increased catalase activity in the pancreas, when measured 3 days after the alloxan injection. 2-week MSC pretreatment was shown to prevent the alloxan-induced hyperglycemia as well as changes in glutathione content, G6PD activity, and catalase activity. The results of this study indicate that the prevention of alloxan-diabetes by MSC pretreatment is associated with its effects on antioxidants in the pancreas, namely, the increase in cellular content and the reduction of glutathione by the facilitation of glutathione recycling induced via increased GPx, GRd, and G6PD activities.

Key words: Se-methylselenocysteine (MSC), diabetes, glutathione, antioxidants, alloxan

INTRODUCTION

Selenium (Se) is a naturally occurring trace mineral, small amounts of which are required for the maintenance of good health. Because Se is a constituent of many selenoproteins, including glutathione peroxidase, thioredoxin reductase, iodothyronine deiodinase, and selenoprotein P, Se deficiency has been implicated in a variety of diseases involving abnormalities in protection against oxidative stress, immune function, and thyroid function (1,2). The beneficial effects of supranutritional Se supplementation, on the other hand, are somewhat less clear. The first reports on the beneficial effects of Se supplementation were epidemiological studies that demonstrated the efficacy of Se in reducing the incidence of cancer (3).

The role of Se in diabetes has become the focus of a great deal of attention, because insulin resistance, impaired glucose tolerance, and type 2 diabetes are all linked with oxidative stress (4). Oxidative stress has been shown to reduce insulin secretion and increase insulin resistance in some experimental models, and thus performs a causal function in the pathogenesis of diabetes (5-7). It has been noted that selenate, an inorganic form of Se, mimics insulin activity in experimental models (8,9). The beneficial effects of other forms of Se, including selenite and selenomethionine, on glucose ho-

meostasis in diabetic animal models have also been reported in previous studies (10). Few population studies, however, are currently available for an in-depth evaluation of the association between Se and diabetes. Additionally, these reports are controversial. Some observational epidemiologic studies have reported a protective association of dietary or plasma Se against diabetic complications (11). Other researchers, on the other hand, have shown that high serum levels of Se are positively associated with the prevalence of diabetes (12). Therefore, the beneficial effects of taking Se supplements on top of an adequate dietary intake have come into question.

In the diet, Se exists principally in organic forms, whereas inorganic Se compounds such as selenite or selenate occur far less frequently and at extremely low concentrations. Organic Se is present principally in the form of selenoamino acids such as selenomethionine, selenocysteine, and Se-methylselenocysteine (MSC). Both organic and inorganic forms of Se appear to be utilized with similar efficacy in the body to produce selenoproteins, but they enter the metabolic pathway at different points depending on their chemical forms. In studies regarding the chemopreventive effects of Se, it has been demonstrated that MSC is more effective than either selenomethionine or selenite, and also does not exert some

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of the toxic effects associated with inorganic Se (13). Inorganic forms of Se and most of the organic Se compounds are metabolized into the key intermediate, hydrogen selenide (H_2Se), which either functions as a precursor for the synthesis of selenoproteins or undergoes stepwise methylation to generate the mono-, di-, and tri-methylated forms of Se (14). MSC is a common organic selenocompound and accumulates in plants such as broccoli, garlic, and onion. Unlike selenomethionine, MSC is not incorporated directly into proteins but is readily hydrolyzed by β -lyase to generate methylselenol (CH_3SeH), which has been tentatively identified as an active Se metabolite responsible for its anticarcinogenic effects (15).

Despite the proposed antidiabetogenic role of Se, Se supplementation has never been clearly demonstrated to prevent diabetes. In this study, we attempted to ascertain whether supranutritional MSC supplementation prevents alloxan-induced diabetes, and whether this prevention is associated with the effects of MSC on the pancreatic antioxidant system. Alloxan is the β -cell selective toxin that generates reactive oxygen species (ROS) and is used extensively to induce insulin-dependent diabetes mellitus in animal models. The antioxidant system is composed of low-molecular mass agents and antioxidant proteins. Glutathione (GSH) is the primary small molecular thiol antioxidant and is oxidized to glutathione disulfide (GSSG) upon reaction with ROS. The ratio of GSH/GSSG is lowered under oxidative stress, unless the GSSG is reduced by the glutathione reductase system or secreted from cells. Therefore, the cellular content and redox status of glutathione can show not only the antioxidant capacity of cells, but also the degree of oxidative stress being experienced by the cells. Superoxide dismutase (SOD) and catalase are major ROS-scavenging antioxidant enzymes which remove the superoxide anion and hydrogen peroxide by converting them to hydrogen peroxide and water, respectively. We determined that the pretreatment of rats with MSC at a dose of 0.75 mg/rat/day for 2 weeks prevented alloxan-induced hyperglycemia. Our results indicate that the glutathione in the pancreas may perform a pivotal role in this preventive effect.

MATERIALS AND METHODS

Animals and treatments

Male Sprague-Dawley rats (180~250 g) were used in this study. They were fed on a standard laboratory diet and water *ad libitum* and were maintained under a 12-hr light/dark cycle. A group of 10 rats received

MSC hydrochloride (Sigma, St. Louis, MO, USA) dissolved in water by oral gavage at a dose of 0.75 mg/rat/day for two weeks. A second group of 10 rats was given only water. After 2 weeks of MSC pretreatment, the rats were fasted overnight, and those in the control and MSC-treated groups were randomly divided into two subgroups, containing 5 rats each. The first subgroup of rats received i.p. injections with alloxan (Sigma, St. Louis, MO, USA) freshly dissolved in 100 mM citrate buffer, pH 4.5, at a dose of 100 mg/kg body weight. The second subgroup of rats was injected with citrate buffer alone. After the alloxan treatment, the rats were not administered any additional MSC. Blood samples were then obtained from the tail veins of overnight-fasted rats 1, 2, and 3 days after the alloxan treatment, and plasma glucose was measured via the glucose oxidase method using glucose assay kits from Sigma (St. Louis, MO, USA). The rats were sacrificed by CO_2 on the final day of blood collection. The pancreata were removed, washed, and immediately frozen by clamping with dry ice-cooled tongs. The tissues were then stored at $-80^\circ C$ until analysis.

Measurement of lipid peroxidation

The extent of lipid peroxidation in the pancreas was measured by the method of Ohkawa et al. that measures thiobarbituric acid-reactive substances (TBARS) in samples (16). The thiobarbituric acid-malondialdehyde (TBA-MDA) adduct was measured by fluorescence at an excitation wavelength of 515 nm and an emission wavelength of 553 nm and was calibrated with 1,1,3,3-tetraethoxypropane.

Measurement of glutathione

Glutathione in the pancreas was analyzed via a modified version of the HPLC method of Reed et al. (17). In brief, 5% perchloric acid (PCA) extract of the tissue was derivatized with 2,4-dinitrofluorobenzene, and reduced (GSH) and oxidized (GSSG) glutathione were HPLC-separated on a SupelcosilTM LC-NH₂ column (particle size 5 μm , 25 cm \times 4.6 mm; Supelco, Bellefonte, PA, USA). To avoid the effects of artifactual oxidation of glutathione during the sample treatment process, duplicate extracts with 5% perchloric acid containing 50 mM N-ethylmaleimide (NEM-PCA) were used to determine the GSSG. GSH was determined by the difference between the amount of total glutathione measured from the PCA extract and the GSSG measured from the NEM-PCA extract. Total glutathione was expressed as GSH equivalent to the sum of GSH and GSSG, that is, GSH+2 GSSG, per mg protein. The redox status of glutathione was presented as a GSH/GSSG ratio.

Enzyme assays

Supernatants from the pancreas homogenates with enzyme-specific buffers were utilized for enzyme assays. Homogenates for superoxide dismutase (SOD) and catalase (CAT) were prepared with buffers containing 1% Triton X-100.

The activity of glutamylcysteine ligase (GCL) in the pancreas was assessed by monitoring the oxidation of NADH at 340 nm in reaction mixtures containing 140 mM Tris-HCl (pH 8.2), 10 units/mL lactate dehydrogenase, 10 units/mL pyruvate kinase, 75 mM KCl, 25 mM MgCl₂, 10 mM ATP, 5 mM L-glutamate, 10 mM α -amino-L-butyrate, 0.2 mM NADH, 0.2 mM EDTA, and 1 mM phosphoenolpyruvate (18). Glutathione peroxidase (GPx) activity was measured by monitoring NADPH oxidation at 340 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1 unit/mL glutathione reductase (GRd), 1 mM GSH, and 0.25 mM H₂O₂. The reaction mixture except for H₂O₂ was incubated for 5 min, and the reaction was initiated via the addition of H₂O₂ (19). GRd activity was measured by monitoring NADPH oxidation in reaction mixtures containing 100 mM potassium phosphate (pH 7.4), 2 mM GSSG, 0.6 mM EDTA, and 0.5 mM NADPH (20). Glucose 6-phosphate dehydrogenase (G6PD) activity was measured by monitoring the reduction of NADP⁺ at 340 nm in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 50 mM MgCl₂, 2 mM NADP⁺, and 4 mM glucose 6-phosphate (21). The activity of total SOD was measured by monitoring the autooxidation of epinephrine at 480 nm in reaction mixtures containing 50 mM sodium carbonate (pH 10.2), 0.1 mM EDTA, and 0.4 mM epinephrine (22). One unit of SOD indicated the quantity of enzyme required to induce a 50% inhibition of oxidation. CAT activity was measured by monitoring the removal of H₂O₂ at 240 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.0) and 10 mM H₂O₂ (23).

Statistical analysis

Data are expressed as the means \pm standard deviations (SD). The significance of the differences between the experimental and control groups was determined using the Student's t-test. *p* values of <0.05 were considered significant.

RESULTS

Prevention of alloxan-induced diabetes by MSC pretreatment

A single treatment with alloxan induced a significant

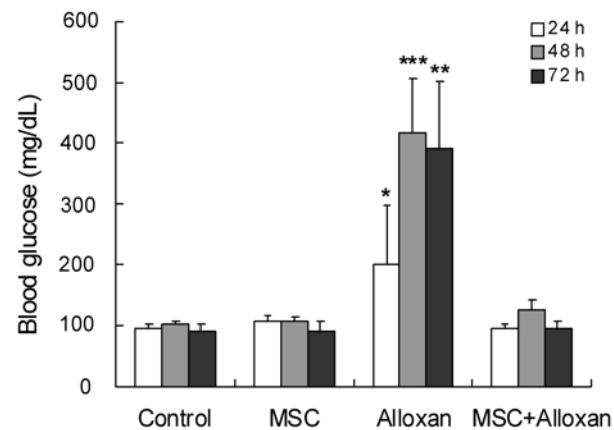


Fig. 1. Effect of Se-methylselenocysteine (MSC) pretreatment on blood glucose level of the control and alloxan-injected rats. The rats in the pretreatment group received MSC for two weeks at a dose of 0.75 mg/rat/day prior to alloxan injection. Blood glucose levels of the rats in the control, MSC-only, alloxan-diabetic, and MSC-alloxan groups were measured at the indicated time after alloxan injection. The results are expressed as means \pm SD (n=5). **p*<0.05, ***p*<0.01, ****p*<0.001.

increase in the blood glucose level in 80% of rats that were not pretreated with MSC (alloxan-diabetic group) at 24 hr after injection. On the other hand, none of the rats that received daily administrations of MSC at a dose of 0.75 mg/rat for 2 weeks prior to the alloxan injection (MSC-alloxan group) developed diabetes (Fig. 1). The MSC-alloxan rats did not exhibit the body weight losses and urine output increases that were observed in the alloxan-diabetic rats (data not shown). The rats receiving only MSC (MSC-only group) evidenced no apparent signs of increased stress or blood glucose levels as compared with the control rats.

Effect of MSC on glutathione in pancreas

To probe for the mechanism underlying the diabetes-preventive effects of MSC pretreatment, we analyzed markers for oxidative stress and antioxidant system in the pancreas. The lipid peroxidation level evidenced no statistically significant difference among the four rat groups (Fig. 2), which is probably attributable to the relatively short duration after alloxan injection. On the other hand, glutathione in the pancreas was significantly different among the groups (Fig. 3). Glutathione is not only a crucial antioxidant molecule but is also a sensitive indicator of oxidative stress, because glutathione is oxidized by both enzymatic and nonenzymatic reactions with ROS and is subsequently depleted from cells under oxidative stress. Fig. 3A shows the free glutathione content in the pancreas. The MSC-only group rats evidenced significantly increased glutathione content. On the other hand, the alloxan-diabetic rats evidenced significant reductions in glutathione content, thereby indicating in-

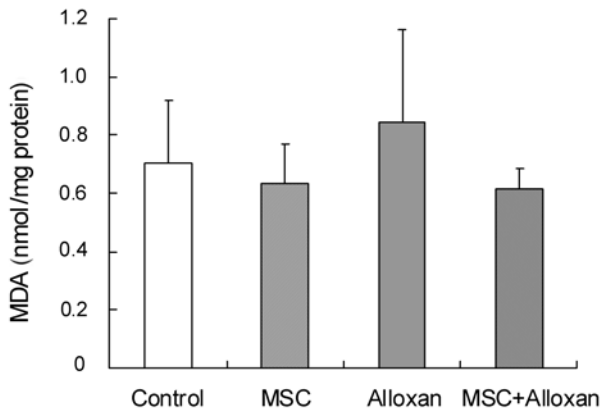


Fig. 2. Effect of Se-methylselenocysteine (MSC) pretreatment on lipid peroxidation levels in the control and alloxan-injected rat pancreas tissues. The rats in the pretreatment group received MSC for two weeks at a dose of 0.75 mg/rat/day prior to alloxan injection. Lipid peroxidation levels in the pancreata of the rats in the control, MSC-only, alloxan-diabetic, and MSC-alloxan groups were analyzed by measuring malondialdehyde (MDA) in terms of thiobarbituric acid-reactive substances (TBARS). The results are expressed as means \pm SD (n=5).

creased oxidative stress in the pancreas. The MSC-alloxan group rats did not manifest similar glutathione losses. Fig. 3B provides the GSH/GSSG ratio. In the MSC-only rat group, the ratio was significantly increased, whereas the alloxan-treated rats either with or without MSC pretreatment evidenced ratios similar to those of the control rats.

Effect of MSC on the activities of enzymes for glutathione synthesis and recycling

We also measured the activities of the enzymes relevant to glutathione metabolism. GCL activity (Fig. 4A), the rate-limiting enzyme in the glutathione synthesis pathway, did not differ significantly among groups, thereby indicating no differences in the glutathione synthesis rates. However, a clear difference was noted in the activities of the enzymes associated with glutathione recycling. GPx activity (Fig. 4B) was significantly increased in the MSC-pretreated rats, regardless of alloxan injection status. Conversely, the alloxan-diabetic rats evidenced GPx activity similar to that of the untreated control rats. GRd activity (Fig. 4C) was increased only in the MSC-only group rats. The activity of G6PD (Fig. 4D), the key enzyme that supplies NADPH, was reduced significantly in the alloxan-diabetic rats. No reduction in G6PD was observed in the MSC-alloxan rats. The MSC-only group rats exhibited G6PD activity similar to that of the control rats.

Effect of MSC on the activities of superoxide dismutase (SOD) and catalase (CAT)

SOD activity did not differ significantly among the

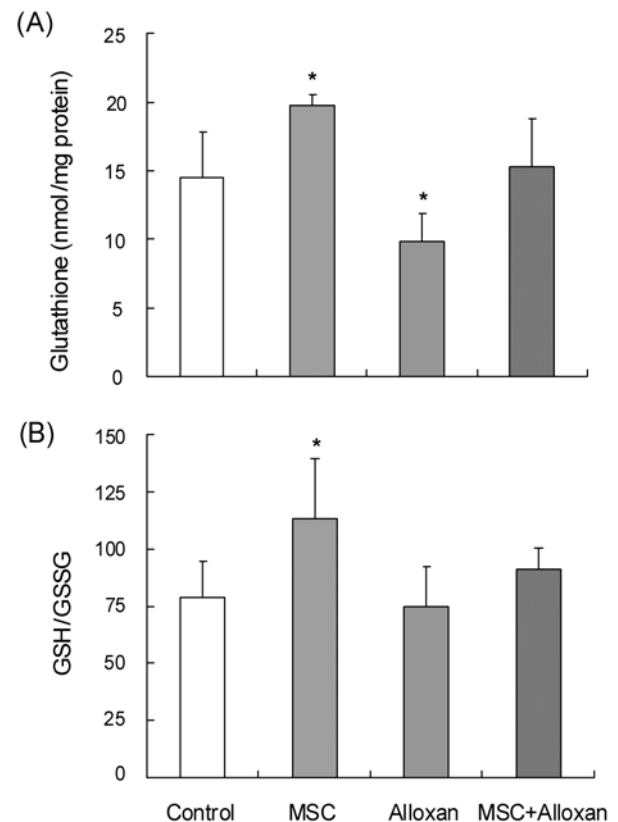


Fig. 3. Effect of Se-methylselenocysteine (MSC) pretreatment on glutathione in the control and alloxan-injected rat pancreas tissues. The rats in the pretreatment group received MSC for two weeks at a dose of 0.75 mg/rat/day prior to alloxan injection. Total glutathione content (A) and GSH/GSSG ratio (B) in the pancreata of the rats in the control, MSC-only, alloxan-diabetic, and MSC-alloxan groups were measured via the HPLC method. The results are expressed as means \pm SD (n=5). *p<0.05.

groups (Fig. 5A). The activity of CAT was, however, significantly increased in the alloxan-diabetic rats. This increase in CAT activity was not observed in the MSC-alloxan rats (Fig. 5B).

DISCUSSION

The death of pancreatic β -cells is the fundamental cause of type 1 diabetes and may also be a factor contributing to the reduced β -cell mass observed in type 2 diabetes. Although β -cell death can be induced by a variety of agents and substances, a growing body of evidence suggests that oxidative stress probably performs a pivotal role in β -cell death (24,25). Alloxan, which is used to induce diabetes in experimental animals, is relatively specific to β -cells and generates ROS by redox cycling with dialuric acid, thereby causing oxidative stress (26). Pancreatic β -cells are also known to harbor low levels of antioxidant enzymes, GPx and catalase in particular; this may explain their sensitivity to the toxic

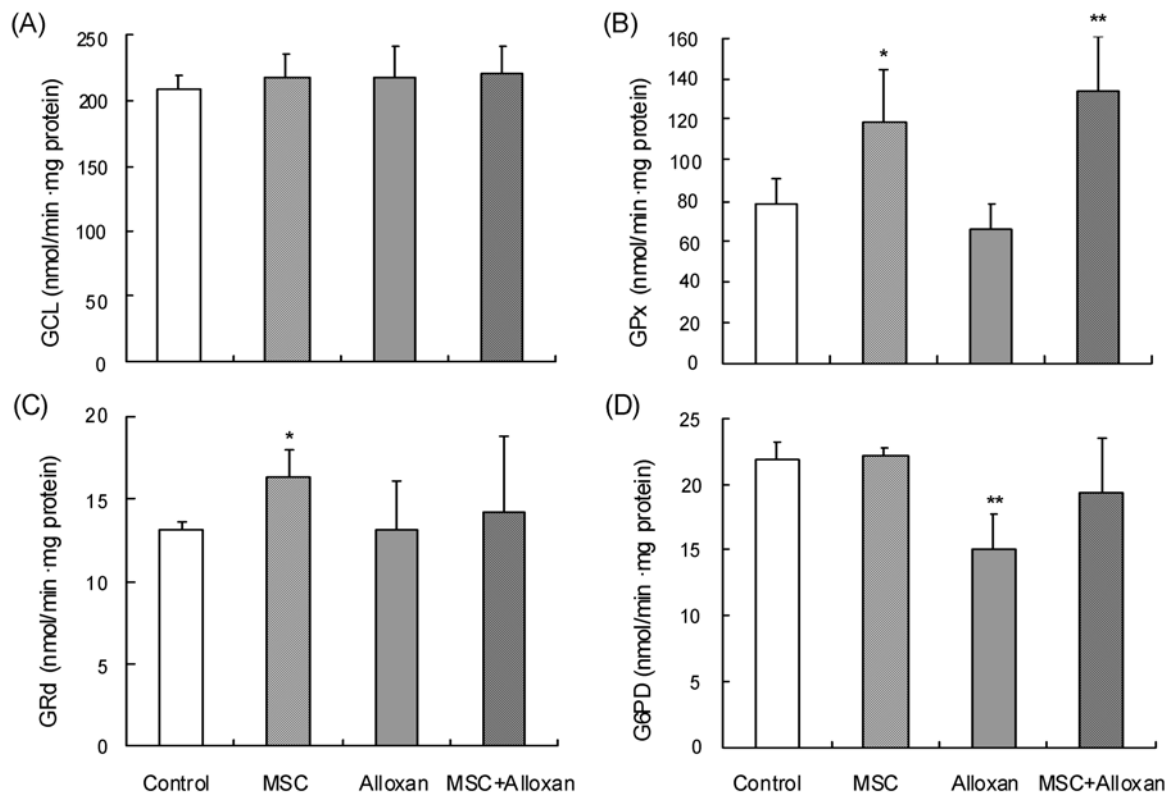


Fig. 4. Effect of Se-methylselenocysteine (MSC) pretreatment on the activities of enzymes for glutathione metabolism in the pancreas tissues of control and alloxan-injected rats. The rats in the pretreatment group received MSC for two weeks at a dose of 0.75 mg/rat/day prior to alloxan injection. Activities of glutamylcysteine ligase (GCL; A), glutathione peroxidase (GPx; B), glutathione reductase (GRd; C) and glucose 6-phosphate dehydrogenase (G6PD; D) in the pancreata of the rats in the control, MSC-only, alloxan-diabetic, and MSC-alloxan groups were measured. The results are expressed as means \pm SD (n=5). * $p < 0.05$, ** $p < 0.01$.

action of ROS (27).

The results of the current study clearly demonstrate that MSC pretreatment, for 2 weeks at a dosage of 0.75 mg/rat/day, can prevent alloxan-induced hyperglycemia in rats, and also that the preventive effect of MSC is associated with its effect on glutathione metabolism. The dose of MSC employed in this study was selected on the basis of previous reports. It was reported that the LD₅₀ of sodium selenate in mice was 3.6 mg/kg body weight, which is equivalent to approximately 20 μ mol Se/kg body weight (28). Azrak et al. reported that MSC dosage of up to 0.2 mg/mouse/day could be used for 35 days without causing any apparent adverse effects in mice (29). In our experiments, rats evidenced no apparent signs of stress as the result of MSC administration.

We determined that MSC supplementation induced significant increases in free glutathione content and GSH/GSSG ratio in the pancreas, which are indicative of increased antioxidant capacity and decreased oxidative stress. The increase in the glutathione content and GSH/GSSG ratio in the MSC-treated rats appears to be a consequence of enhanced glutathione recycling rather

than an increase in the synthesis, which is supported by the observed increase in the activities of GPx and GRd, but not GCL. Glutathione is involved in the removal of ROS by GPx in reaction with hydrogen peroxide, and also via direct reactions with a variety of oxygen free radicals without the aid of enzymes. The GSSG generated in the reactions with ROS can be removed from cells by export, unless it is disposed of via reduction by GRd and NADPH, which is generated by G6PD. Therefore, glutathione redox cycling is an important player in antioxidative defense. GSH is also consumed in the reaction with protein thiols to form mixed-disulfide under oxidative stress conditions. Therefore, the content of cellular glutathione is determined not only by the synthesis rate but also by redox cycling and removal under oxidative stress conditions. Efficient glutathione recycling can maintain cellular glutathione in its reduced state, thus preventing it from depletion as a form of GSSG, and eventually inducing increases in cellular glutathione content.

MSC pretreatment administered before alloxan injection prevented the decrease in pancreatic glutathione

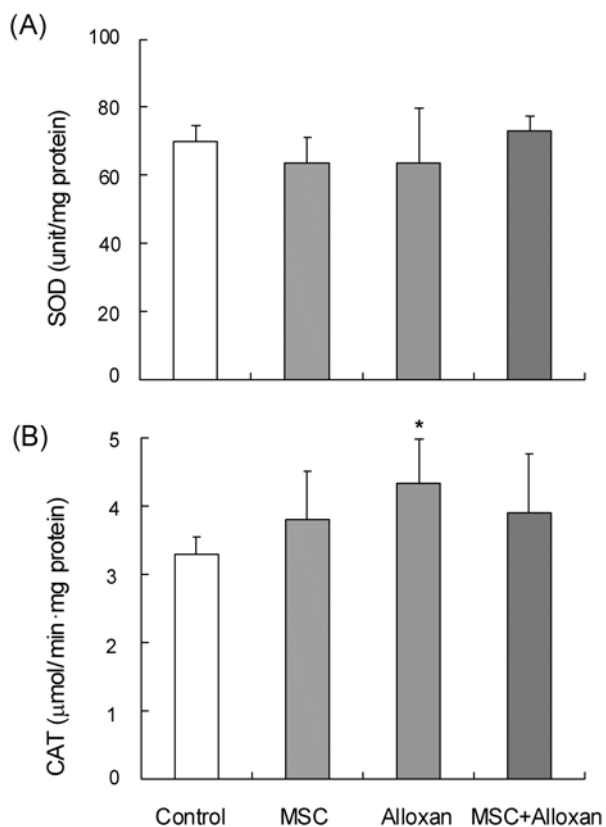


Fig. 5. Effect of Se-methylselenocysteine (MSC) pretreatment on the activities of superoxide dismutase (SOD) and catalase (CAT) in the pancreas tissues of control and alloxan-injected rats. The rats in the pretreatment group received MSC for two weeks at a dose of 0.75 mg/rat/day prior to alloxan injection. Activities of total SOD (A) and CAT (B) in the pancreata of the rats in the control, MSC-only, alloxan-diabetic, and MSC-alloxan groups were measured. The results are expressed as means \pm SD (n=5). *p<0.05.

observed in the alloxan-diabetic rats. The alloxan-diabetic rats maintained their GSH/GSSG ratios, however, probably as the result of the export of the formed GSSG. The alloxan-diabetic rats evidenced significantly reduced G6PD activity and increased catalase activity. This decrease in G6PD activity in animal diabetic models has also been reported in other studies, and the activation of protein kinase A and subsequent phosphorylation and inhibition of G6PD has been proposed as the mechanism underlying this phenomenon (30). G6PD is the principal source of NADPH, on which the antioxidant system relies heavily. Thus, a deficiency in G6PD activity will naturally result in augmented susceptibility to oxidative stress. MSC pretreatment administered before alloxan injection prevented the reducing in G6PD activity. The observed increase in the catalase activity may be attributed to stimulated peroxisomal function, as it has been previously shown that diabetes induces enhanced peroxisomal activities, including catalase and acyl-CoA oxi-

dase activity, in experimental diabetes models (31). No increase in catalase activity was noted in the MSC-pretreated rats.

The mechanism underlying the effects of MSC on pancreatic antioxidants appears to be separate from its role as a component of selenoproteins such as GPx, because MSC pretreatment also exerts a positive effect on the activities of GRd and G6PD. Reports concerning the effects of Se on the cellular redox state have been, thus far, rather controversial, and the results appear to depend heavily on the chemical forms and doses of Se compounds used, which can evoke either prooxidative or antioxidant effects (32,33). Thus, a more plausible explanation for the role of MSC might be that MSC functions as a moderate prooxidant, causing the antioxidant system to mount an adaptive response. Both inorganic and organic Se compounds have been previously noted to generate ROS when metabolized in cells (34). Methylselenol, the key metabolite of MSC, has been proposed as a source for the production of ROS either directly via reaction with oxygen or indirectly via a serial oxidation of cellular components (34,35).

In conclusion, this study showed that MSC pretreatment prevented alloxan-induced hyperglycemia in rats. Our results indicate that the role of MSC in the prevention of diabetes is associated with its effects on pancreatic antioxidants, that is, the increase in glutathione content and reduction by the facilitation of glutathione recycling as the result of increased GPx, GRd, and G6PD activities.

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