Modulation of Monocrotaline-Induced *Cor Pulmonale* by Grape Juice

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Abstract: The study was designed to test whether the ingestion grape juice (GJ) could modulate monocrotaline (MCT)-induced *Cor pulmonale* resulting from antioxidant properties. Three-week-old male Wistar rats received GJ (10 mL/kg/day) by gavage for 6 weeks. A single injection of MCT (60 mg/kg body weight intraperitoneally) was administered at the end of the third week. Animals were divided in four groups: control, MCT, GJ, and GJ + MCT. MCT promoted a significant increase in right ventricle (36%) and lung (70%) weight to body weight ratio. There was an increase in the right systolic (38%) as well as in the end diastolic (70%) ventricular pressures. MCT caused a significant decrease in lung endothelial nitric oxide synthase (20%) but increase in lipid peroxidation (13%) and catalase (43%). MCT-induced decrease in the endothelial nitric oxide synthase and increase in the right ventricular end diastolic pressure were prevented by GJ, whereas right systolic ventricular pressure and lung weight to body weight ratio were corrected only partially. MCT-induced increase in heart and right ventricle to body weight ratios was not changed by GJ. GJ blunted MCT-induced increase in lipid peroxidation but had no effect on the changes in catalase and superoxide dismutase activities. GJ appears to offer some protection against MCT-induced *Cor pulmonale* and right ventricle function changes.

Key Words: *Cor pulmonale*, grape juice, monocrotaline, nitric oxide, oxidative stress

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INTRODUCTION

Monocrotaline (MCT) is a pyrrolizidine alkaloid present in a variety of plant sources, including seeds of *Crotalaria spectabilis*. When ingested, it is metabolized in the liver to dehydromonocrotaline.[1] This metabolic product is the major cause of MCT-induced toxicity in rats, leading to a sustained elevation of pulmonary pressure and right ventricular hypertrophy[2] with clinical features that resemble human idiopathic pulmonary hypertension.[3] Pulmonary vascular remodeling after the administration of MCT appears to be an important manifestation characterized by endothelial degeneration, hypertrophy of medial smooth muscle cells accompanied by the thickening of the medial layer in the pulmonary arteries.[4] The pulmonary arterial hypertension (PAH) thus caused results in right ventricular hypertrophy and/or dilatation and may lead to right heart failure.[3–5] Involvement of reactive oxygen species in PAH, right ventricular hypertrophy, and failure induced by MCT have been demonstrated.[2]

MCT-induced *Cor Pulmonale*[6] exhibits various alterations in the expression of endothelial nitric oxide synthase (eNOS), leading to the impairment of nitric oxide (NO)-mediated signaling.[7] NO is recognized for its involvement in diverse biologic processes, including vasodilation, bronchodilation, neurotransmission, antimicrobial defense, and regulation of inflammatory-immune processes.[8] As a result of the exposure to high O_2 levels in the lungs, there is high flux of reactive oxygen species. NO plays an active role in the quenching of superoxide radical, thus modulating reactive oxygen species levels. Pulmonary oxidative stress is suggested to be involved in many pulmonary disease conditions, including acute lung injury, adult respiratory distress syndrome, hyperoxia, ischemia-reperfusion, sepsis, radiation injury, lung transplantation, chronic obstructive pulmonary disease, and inflammation.[9]

Consumption of foods rich in flavonoids is associated with a reduced risk of various chronic diseases.[10–12] Protective benefits of dietary flavonoids may in part be the result of their antioxidant properties and thus, their ability to reduce oxidative stress.[13] Flavonoids also increase the expression of eNOS and NO production.[14] Grape juice (GJ) is a rich source of the antioxidant flavonoids catechin, epicatechin, quercetin, and anthocyanins.[15] In vitro and in vivo studies have shown that GJ has antioxidant potential.[16]

This study was designed to examine whether increased consumption of GJ could modulate the MCT-induced *Cor Pulmonale* in terms of NO bioavailability, oxidative stress in the lungs, and right heart function.

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MATERIALS AND METHODS

Concentrated Purple Grape Juice

Concentrated purple GJ, commercially available, ready to drink, was purchased from Adega Casa de Madeira Ltda (Bento Gonçalves, Rio Grande do Sul, Brazil) in glass bottles and kept refrigerated until use. To eliminate any source of variability, the same lot of the juice was used throughout the study. One hundred milliliters of the concentrate contained 26 g of carbohydrate, 1 g of protein, and 1 g of total fat. Amount of total phenols (0.43 g/100 mL) was confirmed using the Folin-Ciocalteau method,[17] in which gallic acid was used as a standard.

Experimental Design

Male Wistar rats, weighing 40 ± 5 g, were housed to a maximum of four animals per cage under standard laboratory conditions (controlled temperature and humidity, 12-hour light/dark cycle). Animals were divided into four groups: 1) control-saline (1 mL/kg, intraperitoneally); 2) monocrotaline (60 mg/kg, intraperitoneally); 3) GJ (10 mL/kg, by gavage); and 4) GJ + monocrotaline. Total study duration was 6 weeks. Drug was administered in a single injection of MCT (60 mg/kg in saline intraperitoneally) or an equal volume of saline at the end of the third week of the experimental protocol. GJ (10 mL/kg/day) or an equal volume of tap water was administered for 6 weeks starting on Day 1.[18,19] Standard rat chow and tap water were given ad libitum during the experimental protocol. All studies were done 21 days after MCT injection. All animal procedures used in this study were in accordance with the Principles of Laboratory Animal Care (COBEA-Brazilian College of Animal Experimentation) and the experimental protocol was approved by the University of Rio Grande do Sul Animal Care Committee. Administration of grape juice before MCT injection was chosen as a preventive strategy once there is evidence favoring the consumption of grape extracts rich in polyphenols in the prevention of cardiovascular disease.[20]

Morphometric Analysis and Tissue Preparation

At the end of 6 weeks, animals were catheterized for hemodynamic assessment and were euthanized by cervical dislocation for tissue collection. After euthanasia, the heart, right and left lung, and liver were rapidly collected in the saline solution to wash out excess blood and placed on filter paper to remove excess saline. For recording heart weight, both atria were removed. After this, the right ventricle free wall was dissected out and weighed. Left ventricle along with the septum was also weighed. The liver and right lung were weighed to estimate the congestion in these organs in terms of tissue weight to body weight ratio. The left lung was homogenized (1.15% w/v KCl and phenyl methyl sulphonyl fluoride PMSF 20 mmol/L) in Ultra-Turrax homogenizer (Marconi Equipamentos de Laboratório Ltda., Piracicaba, SP, Brazil). The homogenate was centrifuged at 600 g for 10 minutes at 0°C to 4°C to remove the nuclei and cell debris[21] and supernatant was used for further analysis.

Hemodynamic Measurements

For hemodynamic measurements, rats were anesthetized (ketamine 90 mg/kg; xylazine 10 mg/kg, intraperitoneally) and the right jugular vein was cannulated with a PE 50 catheter connected to a strain gauge transducer (Narco Biosystem Pulse Transducer RP-155, Houston, TX) linked to a pressure amplifier (HP 8805C; Hewlett Packard, Andover, MA). Pressure readings were taken in a microcomputer equipped with an analog-to-digital conversion board (Windaq 1 KHz sampling frequency; Dataq Instruments Inc., Akron, OH). A catheter was advanced into the right ventricle for recording right ventricular systolic and end diastolic pressures. Mean arterial pressure was assessed in the right carotid artery using a similar cannulation procedure.

NADPH Diaphorase Histochemistry

Because the three known nitric oxide synthase (NOS) isoforms (nNOS, eNOS, and iNOS) possess NADPH-diaphorase (NADPH-d) activity, NADPH-d histochemistry is used as a commonly accepted procedure for NOS identification.[22] Animals (n = 4/group) were prepared for the NADPH-d histochemical studies by perfusion fixation as follows.

Anesthetized animals (90 mg/kg ketamine; 10 mg/kg xylazine; intraperitoneally) were cannulated through the apex of the right heart and a drain was put in the left atria. Perfusion with saline solution was followed by a fixative solution containing 4% paraformaldehyde in 0.1m/L phosphate buffer (pH 7.4). Lungs were quickly dissected out, postfixed in the same fixative solution for 4 hours at room temperature, and cryoprotected by immersion in 15% and 30% sucrose solutions in phosphate buffer at 4°C. Transverse serial sections (50 μm) were obtained on a cryostat (Leitz Digital 1702) at –20°C and collected on the gelatinized slides. These slides were incubated in 0.1 mol/L phosphate buffer with 12 μL Triton X-100 for 10 minutes and transferred to a freshly prepared medium containing 0.5 mg/mL β-NADPH (Sigma) 0.2 mg/mL nitro blue tetrazolium (Sigma) and 0.2 mol/L phosphate buffer containing 12 μL Triton X-100. After preincubation at room temperature for 5 minutes, the slides were incubated at 37°C for 4 hours. The sections were washed in 0.1 mol/L phosphate buffer and dehydrated, cleared with xylene, and covered with Entellan and coverslips. Control reaction to NADPH histochemistry was performed by incubating the sections without NADPH. No histochemical reaction was seen in these sections. Sections were examined and photographed with Nikon Optiphot-2 microscope equipped with a Nikon FX-35DX camera.

Western Blot Analysis

Tissue homogenization, electrophoresis, and protein transference were performed as described elsewhere.[23,24] Nitrocellulose membranes were processed for immunodetection using rabbit anti-eNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound primary antibody was detected using donkey antirabbit horseradish peroxidase–conjugate secondary antibody. Membranes were revealed for chemiluminescence and quantitatively analyzed with an image densitometer (Imagemaster VDS CI; Amersham Biosciences Europe, IT). Molecular weights of bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, Hercules, CA). Results from each

membrane were normalized through the Ponceau red method.[25]

Tert-Butyl Hydroperoxide-Initiated Chemiluminescence

To determine lipid peroxidation (LPO), chemiluminescence was measured in a liquid scintillation counter in out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB–Produkter AB, Sweden). Measurements were started by the addition of tert-butyl hydroperoxide to lung homogenate and data expressed as counts per second per milligram of protein (cps/mg protein).[26]

Determination of Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of superoxide radical reaction with pyrogallol.[27] Catalase activity was determined by following the decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm. It was expressed as nanomole of H_2O_2 reduced per minute per milligram of protein.[28]

Proteins and Statistical Analysis

Protein was measured by the method of Lowry[29] using bovine serum albumin as the standard. Data were expressed as mean ± standard deviation. To compare multiple groups, we used the one-way analysis of variance with post hoc Student-Newmann-Keuls. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Morphometric Characteristics

Data on the animal and tissue weights are summarized in Table 1. Animals in the MCT group showed a significant lower final body weight (BW) as compared with all other groups ($P < 0.05$). GJ prevented this loss in BW resulting from MCT. This improvement in BW in the GJ + MCT group must not be the result of the additional caloric intake, because animals in the GJ group receiving only GJ did not show any increase in BW over the control group receiving water. There was a significant increase in heart as well as right ventricle to BW ratios in the MCT group. GJ had no effect on these changes. Left ventricle + septum to BW ratio was not different among the four groups. Significant increase in lung weight to BW ratio was observed in MCT group (70%). Grape juice in the GJ + MCT group caused a significant reduction (29%) in this gain in the lung weight. However, this ratio in the GJ + MCT group was still higher than the control as well as the GJ group. With respect to the liver weight to BW ratio, there was no significant difference among the groups ($P > 0.05$).

Hemodynamic Assessment of the Right Ventricle Function

MCT caused a significant rise in the right ventricular end diastolic pressure as well as in the right ventricular systolic pressure, 70% and 38%, respectively, as compared with the control. GJ prevented this change as a result of MCT in right ventricular end diastolic pressure, whereas right ventricular systolic pressure was corrected only partially. Mean arterial pressure was not influenced by the treatments applied (Table 1), as) as has also been reported by others that systemic blood pressure at this stage of posttreatment does not change.[30]

NADPH Diaphorase Activity and Endothelial Nitric Oxide Synthase

A NADPH-d-positive reaction was detected in the lung of all experimental groups. Intense staining for NADPH-d occurred in the endothelium of capillaries and arterioles (Fig. 1). A faint staining was also seen in the smooth muscle layer of these animals. MCT caused a significant increase in the thickness of tunica media resulting from hypertrophy of the smooth muscle cells (Fig. 1B). GJ prevented this thickening as a result of the MCT (Fig. 1D). Lung airway epithelial cells from bronchioles exhibited a strong NADPH-d reaction in all experimental groups (Figs. 2A–D). The smooth muscle layer of bronchi as well as in cells of the alveolar region of all animal groups also showed faint staining (data not shown).

TABLE 1. Effects of Grape Juice (GJ) on the Monocrotaline (MCT)-Induced Changes in the Morphometric and Hemodynamic Parameters

	Groups			
Parameters	**Control (N = 8)**	**MCT (N = 6)**	**GJ (N = 6)**	**GJ + MCT (N = 8)**
BW (g)	248 ± 6	221 ± 10*	249 ± 8	236 ± 9†
Heart/BW (mg/g)	2.5 ± 0.1	2.7 ± 0.1*	2.5 ± 0.1	2.8 ± 0.1‡
RV/BW (mg/g)	0.4 ± 0.1	0.6 ± 0.1*	0.4 ± 0.1	0.6 ± 0.1‡
LV + septum/BW (mg/g)	2.1 ± 0.1	2.1 ± 0.2	2.2 ± 0.2	2.2 ± 0.2
Liver/BW (mg/g)	41.7 ± 2.1	43.4 ± 2.9	41.9 ± 4.1	44.9 ± 3.4
Lung/BW (mg/g)	2.7 ± 0.1	4.6 ± 0.3*	2.8 ± 0.3	3.9 ± 0.6†‡
RVEDP (mm Hg)	1.2 ± 0.3	2.1 ± 0.5*	0.9 ± 0.3	1.2 ± 0.3†
RVSP (mm Hg)	29.8 ± 1.9	41.3 ± 2.4*	28.9 ± 2.5	36.9 ± 3.3†‡
MAP (mm Hg)	119.7 ± 7.7	120.5 ± 13.8	105.1 ± 13.9	119.5 ± 8.2

BW, body weight; RV, right ventricle; LV, left ventricle; RVEDP, right ventricular end diastolic pressure; RVSP, right ventricular systolic pressure, MAP, mean arterial pressure; N, number of animals. Values are means ± standard deviation. Significantly different ($P < 0.05$) as compared with *control; †MCT; and ‡GJ. One-way analysis of variance with post hoc Student-Newmann-Keuls.

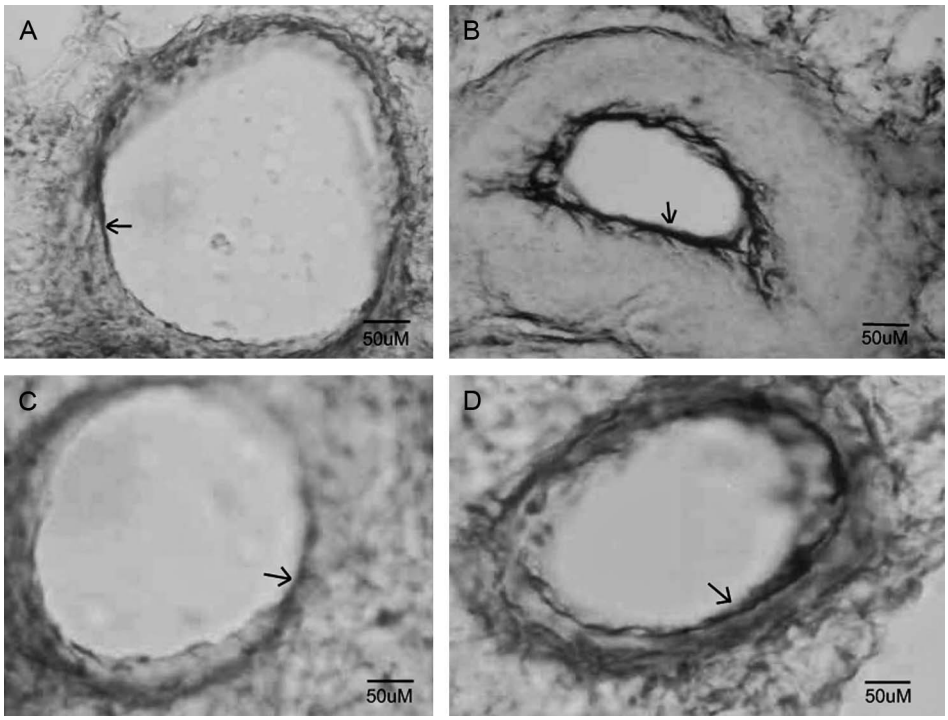

FIGURE 1. Histochemical localization of NADPH diaphorase activity in the lung arteriole of rats in different groups. (A) Saline; (B) monocrotaline; (C) grape juice; and (D) grape juice + monocrotaline. Intense staining in the endothelial layer (arrows) and faint staining is seen in the smooth muscle layer. Note a thickening of the smooth muscle layer in the MCT group (B) and a significant improvement in the GJ + MCT group (D).

A Western blot analysis of eNOS was approximately 20% lower in the MCT group compared with the control group. Grape juice in the GJ + MCT group prevented this change resulting from MCT (Fig. 3).

Lipid Peroxidation and Antioxidant Enzyme Activities

Changes in peroxidation of lipids and antioxidant enzyme activities in the lung tissue are shown in Table 2.

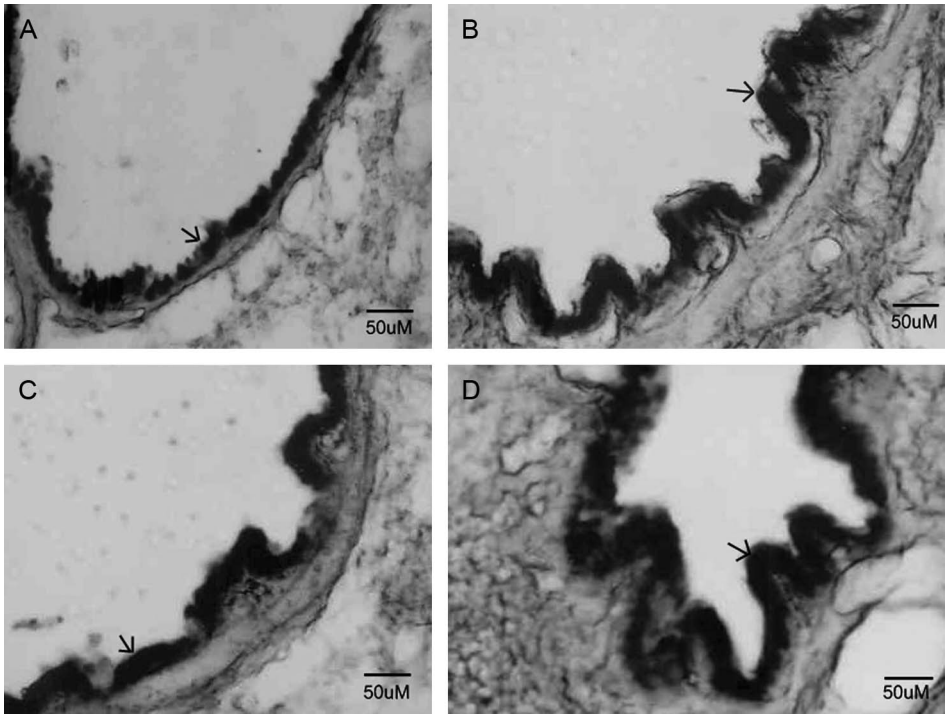

FIGURE 2. Histochemical localization of NADPH diaphorase activity in the bronchioles. The reaction is very intense on the lumen side in the epithelial cells (arrows). There is not much difference among different groups in the thickness of the airways smooth muscle cell layer. Symbols are the same as in Figure 1.

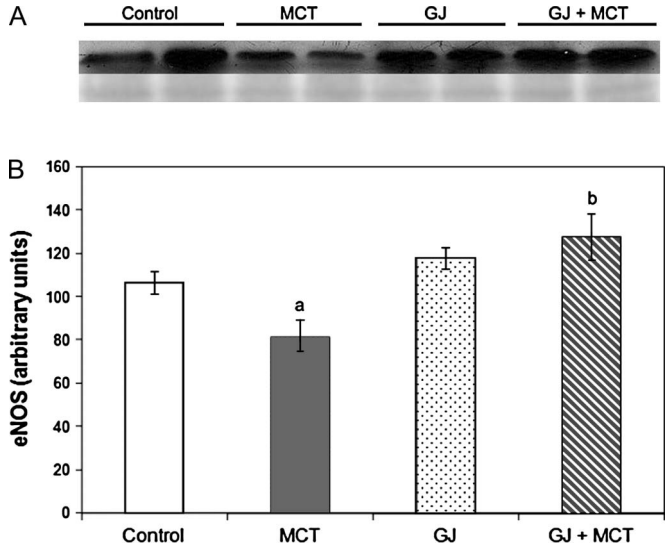

FIGURE 3. A, Western Blot analysis of lung homogenates. Duplicate gels from each group, endothelial nitric oxide synthase antibody for 135 kD and Ponceau S staining for 60 kD. B, Differences in the optical density. Data are mean ± SD from 4 animals in each group. Significantly different ($P < 0.05$) as compared with (a) control and (b) MCT. Symbols are the same as in Figure 1.

Lipid peroxidation was significantly ($P < 0.05$) increased (13%) in the MCT group as compared with the control group. Grape juice administration resulted in a reduction in LPO by approximately 8.5% as compared with the control group. Grape juice did reduce MCT-induced increase in LPO, but the levels were not back to normal.

In terms of antioxidant enzyme activities, a significant increase in catalase activity (43%) was observed in the MCT group. GJ by itself also caused a significant increase (48%) in the GJ group and had no effect on the MCT-induced increase in the catalase activity. On the other hand, SOD activity did not change significantly in the MCT group, whereas this activity was significantly decreased in the GJ and GJ + MCT, 22% and 31%, respectively, as compared with the controls.

DISCUSSION

Monocrotaline-induced remodeling in the lungs has been used to evaluate the effects of PAH on the neointimal proliferation, ventricular hypertrophy, regulation of gene expression, neuroendocrine modulation, and hemodynamic changes.[2,30,31] In the lungs, most common changes resulting from MCT include the pulmonary endothelial degeneration, hypertrophy of medial smooth muscle layer in the pulmonary arteries and arterioles, adventitial edema, which augments pulmonary vascular resistance, and increase the right ventricle afterload resulting from PAH.[32] Morphometric and hemodynamic changes seen in the MCT group, in this study, are in agreement with these findings, demonstrating that the experimental model was reproduced. The increase in muscular layer of arterioles resulting from MCT seen in the histologic studies supports smooth muscle cell hypertrophy. It is significant to note that in the GJ + MCT group, use of GJ blunted this hypertrophy of the tunica media.

One of the mechanisms involved in the pathogenesis of *Cor pulmonale* in the MCT model is the reduction in the NO bioavailability.[7] Clinical studies confirm that eNOS isoform is present in the endothelium of pulmonary vessels of healthy subjects. Expression of eNOS is downregulated in patients with primary PAH, exerting a pulmonary vasoconstriction effect.[33] In the present study, localization of the NADPH-d confirms the presence of eNOS in the endothelial cells of the arterioles and epithelial cells of the bronchioles. To further analyze both endothelial cells of the arterioles and the epithelial cells of the bronchioles, the whole lung homogenates were subjected to Western blot analysis. In the whole lung homogenates, a significant reduction in eNOS resulting from MCT was shown. Our data also showed that GJ mitigated this change. It has been shown that overproduction of NO can inhibit not only the increase in right ventricular systolic pressure, but also the remodeling of pulmonary vasculature and right ventricular hypertrophy.[34] Although in the present study, there is no evidence of overproduction of NO resulting from GJ, there was some attenuation of right ventricular pressures and lung congestion demonstrating relief of *Cor pulmonale* symptoms. The lack of any correction of the right ventricular hypertrophy may be a reflection of weak effect of the GJ in the correction of pulmonary arterial function. In this regard, a stronger dose of resveratrol (30 mg/kg/day for 3 weeks) was reported to inhibit right ventricular hypertrophy.[35]

Oxidative stress is involved in the pathogenesis of heart failure in the MCT model.[2] Many studies have demonstrated an increase in oxidative stress in the right ventricle subsequent to MCT exposure.[2,30] However, there is not much data on oxidative stress changes in the lungs in this model. To examine the role of oxidative stress in *Cor pulmonale* and how GJ could modulate this phenomenon, we analyzed lungs for some of the

TABLE 2. Oxidative Stress and Antioxidants in the Lung Tissue From Different Animal Groups

	Groups			
Parameters	**Control**	**MCT**	**GJ**	**GJ + MCT**
CL (cps/mg prot)	14,689 ± 651	16,780 ± 962*	13,442 ± 1289*	15,219 ± 620†‡
CAT (nmol/min/mg prot)	53.5 ± 8.6	76.3 ± 9.5*	79.4 ± 4.9*	78.4 ± 3.3*
SOD (U/mg prot)	6.4 ± 1.1	7.2 ± 1.1	4.9 ± 0.8*	4.4 ± 0.7†

CL, chemiluminescence for lipid peroxidation; CAT, catalase enzyme activity; SOD, superoxide dismutase enzyme activity. Values are means ± standard deviation from six to eight animals/group. Significantly different ($P < 0.05$) as compared with: *control; †MCT; ‡GJ. One-way analysis of variance with post hoc Student-Newmann-Keuls.

most important primary enzymatic antioxidant defenses and a parameter of lipid oxidative damage. We did find a small but significant increase in LPO resulting from MCT. Interestingly, GJ caused a small but significant decrease not only in the MCT-induced increase in LPO, but also in the control hearts. These data suggest an antioxidant effect of the GJ. The decrease in LPO resulting from GJ in the control as well as the MCT-treated animals may have been supported by an increase in lung catalase activity seen in these groups in the present study. Antioxidant effect of the GJ has also been reported by others.[36,37]

There was a significant increase in the lung catalase activity in the MCT-exposed animals with and without GJ. Grape juice by itself also caused a significant increase in lung catalase. An increase resulting from MCT most likely is an adaptive response.[38] On the other hand, there was a reduction in the lung SOD resulting from GJ both in the control as well as MCT animals. Exogenous antioxidants present in GJ could act as singlet oxygen and superoxide radical quenchers.[39] Such a reduction in the substrate may result in the reduced activity of SOD. In this regard, GJ has been reported to decrease superoxide levels in platelets of healthy subjects who consumed 7 mL/kg/day of purple GJ for 14 days.[40]

This study suggests that consumption of GJ may be important in the attenuation of *Cor pulmonale* by reducing pulmonary congestion and thus improving cardiac function. These effects may also involve an improvement in the antioxidant status in lungs by inhibiting MCT-induced reduction in eNOS. GJ is not only is a pleasant beverage, but it could also be a potentially new therapeutic agent for the prevention and treatment of pulmonary hypertension with its secondary beneficial effects to the heart function. However, for such thinking, great caution should be exercised until more experimental as well as clinical evidence is in hand.

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