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Reversal of Age-associated Oxidative Stress in Rats by MRN-100, a Hydro-ferrate Fluid*

NARIMAN K. BADR EL-DIN[1], EMAN NOAMAN[2], SALMA M. ABDEL FATTAH[3] and MAMDOOH GHONEUM[4]

[1]Department of Zoology, Faculty of Science, University of Mansoura, Mansoura 35516 Egypt;
Departments of [2]Radiation Biology, and [3]Drug and Radiation Research,
National Center for Radiation and Technology, Cairo, Egypt;
[4]Department of Otolaryngology, Charles Drew University of Medicine and Science,
Los Angeles, California, U.S.A.

Abstract. *Aging is associated with an increase in the level of reactive oxygen species (ROS), which cause cellular damage. This study examined the protective effects of MRN-100, an iron-based hydro-ferrate fluid, against age-associated oxidative stress using young (4 months old) and aged (22 months old) rats . The effect of MRN-100 intake on lipid peroxidation, protein oxidation, and antioxidant status was studied in the blood, liver, and brain. Aged rats were treated with tap water or MRN-100 (100%) orally daily for 40 days. Results revealed that in aged rats treated with MRN-100 (100%) there was: (i) an inhibition of the levels of oxidative stress biomarkers: malondialdehyde, nitric oxide, protein carbonyl groups and total free radicals; (ii) an augmentation in glutathione and total thiol contents; (iii) an enhancement in the activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase; and (iv) an enhancement in plasma iron and ferritin concentrations, and in transferrin saturation. MRN-100 reversed age-related oxidative changes, bringing levels to within the limits of the young control rats in the blood, liver and brain. MRN-100 showed effectiveness in modulating age-associated oxidative stress in rats, and it may therefore be useful to reduce age-associated disorders where free radicals and oxidative stress are the major cause.*

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Correspondence to: Nariman K. Badr El-Din, Ph.D., Department of Zoology, Faculty of Science, University of Mansoura, Mansoura 35516, Egypt. Tel: -20 101016511, e-mail: na_ri_eg@yahoo.com

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Oxidative stress has been implicated in the aging process, which is postulated to be closely related to the formation of reactive oxygen species (ROS) (1-3). These ROS are highly reactive and capable of oxidatively damaging many biological macromolecules such as nucleic acids, protein, and lipids (4-6), which may lead to genetic mutation and cellular senescence (7). Higher levels of free radicals have been reported in aged rats (2, 8), which was attributed to the reduction in antioxidant levels. An age-dependent decline in antioxidant function has been well documented in rats (2, 3, 7, 9), and in humans (10). The mRNA level for the enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were quantified in the liver of aged rats and found to be diminished (11).

The cellular redox state, which is changed during aging, may be modified by diet. Iron and other nutritional deficiencies, which currently affect over 2 billion people worldwide, have been shown to induce oxidative stress (12, 13). In addition, iron deficiency is common in the elderly, who have reduced quantities of stomach acid and therefore a reduced ability to absorb nutrients such as iron (14). Thus far, the ability of iron to protect against oxidative stress has only been studied to a limited extent. Iron has the capacity to accept and donate electrons readily, and this characteristic makes it a useful component of cytochromes and oxygen-binding molecules. Previous studies have suggested ferritin can act as a protectant against oxidative damage in endothelial cells as well as murine and human leukemia cells (15, 16).

The present study was performed in order to examine the antioxidant effects of an iron-based hydro-ferrate fluid (MRN-100) derived from bivalent and trivalent ferrates. Recently, it was shown that MRN-100 exhibits protective effects against oxidative stress-induced apoptosis in murine splenic cells *in vitro* (17). The present study therefore aimed to examine the *in vivo* effect of MRN-100 on oxidative stress

by measuring lipid peroxidation and oxidative protein damage in aged rats. Plasma iron, ferritin and transferrin levels were also evaluated.

Materials and Methods

MRN-100. MRN-100 was prepared in distilled water (DW) with the concentration of Fe^{2+} and Fe^{3+} ions at about 2×10^{-12} mol/l. In tap water, the iron concentration, in the form of ferric, is typically found to be between 0 and 0.03 mg/l ($0\text{-}5.4\times10^{-7}$ mol/l). MRN-100 is obtained from phytosin, a plant extract that contains iron and neutral lipid compounds and is found in rice, wheat or radish seeds (18). When phytosin is dispersed in distilled water, ferric chloride is added, the lipid compounds are removed and the iron compound thus obtained is subjected to fractional determination with respect to bivalent ferrate and trivalent ferrate in order to generate MRN-100. MRN-100 was provided by ACM Co., Ltd., Japan.

Animals. Male Wistar rats aged 4 months (~120 g body weight) and 22 months (~370 g body weight) were used in this study. The animals were obtained from the Research Institute of Ophthalmology (Giza, Egypt) and acclimatized for one week prior to experimentation. Rats were caged individually in a room with light and temperature control (20±2˚C) and were fed standard laboratory cube pellets. The experimental protocol was approved by the Animal Care and Use Committee, National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA) Cairo, Egypt, in accordance with the guidelines set by the World Health Organization, Geneva, Switzerland.

Experimental design. Twenty-four animals were divided into two equal groups, each consisting of twelve rats: Group I, normal young; and Group II, normal aged rats. Each group was further subdivided equally into two groups, each consisting of six rats: control untreated group and MRN-100-treated group. Control groups and treated groups had free access to tap water or MRN-100, respectively, for 40 days. Water consumption and body weights of both the young and aged animals were monitored throughout the duration of the experiment. Water consumption and body weights of both young and aged animals treated with MRN-100 were comparable with their corresponding control groups throughout the duration of the experiment.

Sample collection. On day 41, the animals were sacrificed after being fasted for 16 hours and blood was collected in heparinized tubes. Liver and brain were excised, washed in ice-cold normal saline and homogenized in ice-cold phosphate buffer (0.1 mol/l, pH 7.4) using a Potter-Elvehjem homogenizer to give a 10% w/v homogenate. Whole blood was collected by heart puncture after light anesthesia using heparinized syringes, and one volume was used for measurement of total free radicals. Hemolysates were prepared and used to obtain reduced glutathione (GSH), SOD, CAT and GPx estimations. The separated plasma from heparinized blood was used for the determination of the end product of lipid peroxidation (malondialdehyde; MDA) and nitric oxide (NO) levels. In addition, total thiols (TSH), total protein, protein carbonyls (PCO) and iron profile evaluation were assessed.

Analytical procedures. Lipid peroxidation (LPx) in plasma, liver and brain homogenate was ascertained by the formation of MDA and measured as described in (19). Reduced GSH content in erythrocytes, liver and brain homogenates was determined according to Beutler *et al*. (20). SOD activity in erythrocytes, liver and brain homogenates was estimated according to Minami and Yoshikawa (21). CAT activity in erythrocytes, liver and brain homogenates was measured by the method of Luck (22). GPx activity was assayed in erythrocytes, liver and brain homogenates as described by Lawrence *et al*. (23). NO, TSH, PCO and total protein levels were assessed in plasma, liver and brain homogenates according to the methods of Miranda *et al*. (24), Ellman (25), Levine *et al*. (26) and Lowry *et al*. (27), respectively. Plasma iron and total iron binding capacity (TIBC) were estimated colorimetrically according to the method of Kunesh and Small (28). Ferritin was measured in plasma with a rat ferritin test kit (Ramco Labs., Houston, TX, USA), a sandwich solid-phase enzyme immunoassay. Transferrin saturation index was calculated according to the following formula: saturation (%)=(Plasma iron/TIBC)×100.

Detection of blood total free radicals by electron spin resonance (ESR). Using lyophilization, water was removed by sublimation from the frozen tissue. Freeze drying of samples was carried out using Edwards Pirani 501 Freeze dryer Super Modulyo (Edwards Ltd., Crawley, UK). ESR signals were recorded at room temperature using a Bruker EMX spectrometer (X-band; Bruker, Rheinstetten, Germany). The operating conditions were: microwave power=1.008 mW, modulation amplitude=4 G, modulation frequency=100 kHz, sweep width=200 G, microwave frequency=9.717 GHz, time constant=327.68 ms and sweep time=41.943 s. The detection limits of the ESR technique depend on the sample material, sample size, detector sensitivity, frequency of the incident radiation and the electronic circuits of the instrument. Samples were inserted into ESR quartz tubes and measured using suitable instrument parameters. The peak height of the radiation-induced ESR signals was determined for each sample. The reading intensities were divided by sample weight of each sample to calculate the normalization values (29).

Analysis of ESR Data. For monitoring variations in the peak height of ESR signals as a function of the magnetic field, intensities were measured as the distance between top and bottom points of the first derivative recorded according to Pascual *et al*. (30). Quantitative assessments of free radical concentrations were, however, made according to the following equation:

$$Nd=K[Ho(\Delta H^2)\ A/2]/[Hm\times Ge\ \sqrt{}\ PH\ ]$$

Where: Nd: number of radicals, K: factor depending on the experimental condition of spectrophotometer=10^3/cm, Ho: magnetic field at peak in gauss, ΔH: width peak to peak, Hm: modulation field, PH: power in mW=1.008, Ge: gain of the detector=3.17×10^5. Concentration=unpaired electrons/lyophilized blood (g) or spin/lyophilized blood (g), A=peak height of signals/weight.

Statistical analysis. Values were reported as mean±SEM (standard error of the mean) for six rats in each group, and significance of the differences between mean values was determined by one-way analysis of variance (ANOVA) coupled with the Newman-Keuls multiple comparison test. Values of $p<0.05$ were considered to be significant. Statistical significance of differences between the young control and young MRN-100 treated groups or between the aged control and aged treated with MRN-100 groups for body weight change and water consumption were determined by Student's *t*-test. Levels of significance were evaluated with *p*-values.

Table I. *Effect of MRN-100 administration on water consumption and body weight of young and aged rats.*

	Young				Aged			
	Control		MRN-100		Control		MRN-100	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Water consumption (ml/day)	17.6	0.60	18.3	0.68	26.8	1.07	25.3	0.95
Initial body weight (g)	118	1.0	117	1.6	395	16.5	397	23.0
Last body weight (g)	208	3.0	213.6	3.9	418	17.3	419	18.4
Body weight gain (g)	90.4		95.6		23.2		22.7	
% Change in body weight			5.75%*				–2.20%*	

Each value represents the mean±SEM for 6 animals per group. Body weight gain=(last body weight) – (initial body weight). % Change is the change in final body weight gain as compared to the corresponding control group. *$p>0.05$ Compared to the corresponding control group. %Change calculated from unrounded values to four decimal places.

Table II. *Effect of MRN-100 administration on blood total free radicals levels of young and aged rats.*

	Young				Aged			
	Control		MRN-100		Control		MRN-100	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total free radicals (radical/g) $\times 10^{15}$	1.5^{C}	0.02	1.5^{C}	0.05	$1.9^{A,B,D}$	0.08	1.4^{C}	0.04
% Change			–0.4%		28.2%		–5.7%	

Each value represents the mean±SEM for 6 animals per group. A, Significantly different from young control at $p<0.01$; B, significantly different from young treated at $p<0.01$; C, significantly different from aged control at $p<0.01$; D, significantly different from aged treated at $p<0.01$. Change is compared to that of the young control group, calculated from unrounded values at four decimal places.

Results

Water consumption and body weight. No significant change was observed in water consumption per day or body weight between control animals and their corresponding treated rats, either young or aged animals, for the entire duration of the study (Table I). Both young and aged animals treated with MRN-100 showed a healthy appearance throughout the period of the study, without any signs of toxicity or adverse side-effects.

Total free radical levels. The total levels of free radicals in the blood were measured by ESR and are presented in Table II and Figure 1. Aged rats demonstrated a significant increase in total free radicals (28.2 %, $p<0.01$) as compared to control young rats. However, aged rats treated with MRN-100 showed a significantly decreased level of total free radicals (–5.7%, $p<0.01$) as compared to control aged animals, bringing the level to within the range of young rats.

MDA levels. Lipid peroxidation levels measured as MDA in the plasma, liver and brain of control and MRN-100 treated young and aged rats are shown in Table III. The mean MDA values were markedly elevated in untreated aged rats in the plasma (27.2%, $p<0.05$), liver (54.8%, $p<0.01$), and brain (31.8%, $p<0.01$), as compared to control young rats. Treatment with MRN-100 significantly reduced the level of MDA in the aged animals to be comparable with that the young control rats, and this effect of MRN-100 was noticed in all tissues.

NO levels. As shown in Table III, NO levels showed marked elevation in aged untreated rats. Mean NO values in the plasma, liver and brain were significantly higher than those of young controls by 17.2%, ($p<0.05$), 23.0%, ($p<0.01$) and 25.6%, ($p<0.01$), respectively. Treatment with MRN-100 significantly lowered the levels of NO in the plasma, liver and brain of the aged animal group to be within the values of young untreated rats.

PCO levels. PCO content is an indicator of oxidative protein damage. As shown in Table III, comparisons of PCO levels followed tissue-dependent variations. Aged untreated rats demonstrated a significant increase in PCO content in the plasma (48.1%, $p<0.05$), liver (32.8%, $p<0.01$) and brain

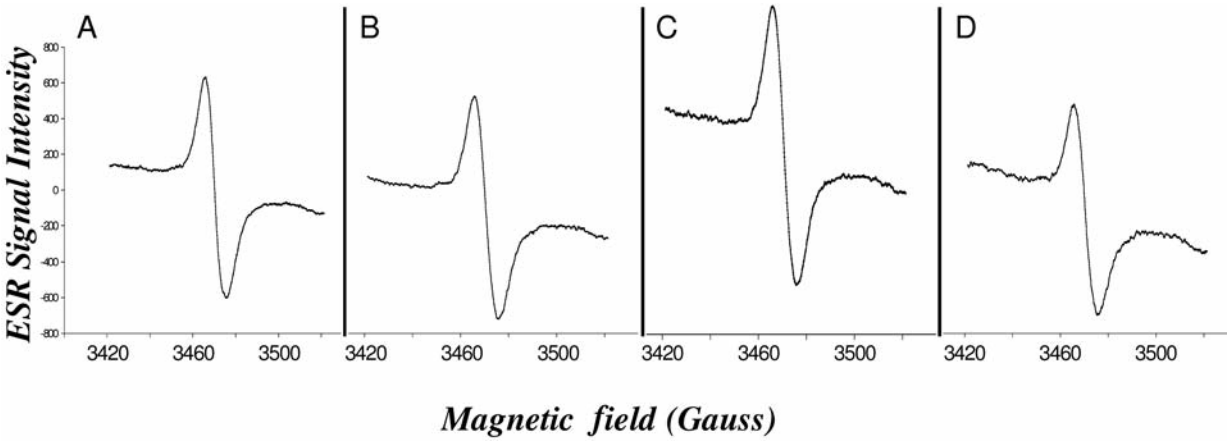

Figure 1. *ESR spectra of lyophilized blood samples. Young and aged animals were treated with MRN-100 or given tap water only (control) for 40 days. Whole blood was collected by heart puncture after light anesthesia using heparinized syringes and was analyzed for total free radical levels by ESR. A: Young control (untreated) group; B: young MRN-100 treated group; C: aged control (untreated)group; D: aged MRN-100-treated group.*

Table III. *Effect of MRN-100 administration on MDA, NO, and PCO, in different tissues of young and aged rats.*

Tissue	Parameter	Young				Aged			
		Control		MRN-100		Control		MRN-100	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Plasma	MDA	6.2^{c}	0.5	5.9^{c}	0.6	7.9^{a,b,d}	0.4	6.3^{c}	0.5
	% Change			(–4.0%)		(27.2%)		(2.2%)	
	NO	9.3^{c}	0.4	8.7^{C}	0.5	11.0^{a,B}	0.6	9.6	0.5
	% Change			(–6.7%)		(17.2%)		(3.4%)	
	PCO	0.82^{c}	0.08	0.80^{C}	0.07	1.2^{a,B,d}	0.15	0.90^{c}	0.09
	% Change			(–2.9%)		(48.1%)		(9.2%)	
Liver	MDA	20.4^{C}	2.5	20.1^{c}	3.1	31.6^{A,B,d}	2.7	23.4^{c}	1.4
	% Change			(–1.8%)		(54.8%)		(14.8%)	
	NO	0.94^{C}	0.05	0.90^{C}	0.05	1.2^{A,B,d}	0.03	0.96^{c}	0.06
	% Change			(–3.7%)		(23.0%)		(2.9%)	
	PCO	1.98^{C}	0.12	1.91^{C}	0.10	2.63^{A,B,d}	0.12	2.06^{c}	0.24
	% Change			(3.4%)		(32.8%)		(4.0%)	
Brain	MDA	73.4^{C}	5.6	67.2^{C}	3.6	96.7^{A,B,D}	6.1	70.0^{C}	5.8
	% Change			(–8.4%)		(31.7%)		(–4.6%)	
	NO	1.1^{C}	× 0.03	1.0^{C,d}	± 0.03	1.4^{A,B,D}	± 0.04	1.1^{b,c}	± 0.04
	% Change			(–6.0%)		(25.6%)		(4.5%)	
	PCO	0.88^{C}	0.07	0.84^{C}	0.05	1.22^{A,B,D}	0.13	0.86^{C}	0.05
	% Change			(–4.1%)		(38.7%)		(–2.4%)	

Each value represents the mean±SEM for 6 animals per group. $^{a, A}$Significantly different from young control at $p<0.05$ and $p<0.01$, respectively; $^{b, B}$, significantly different from young treated at $p<0.05$ and $p<0.01$, respectively; $^{c, C}$, significantly different from aged control at $p<0.05$ and $p<0.01$, respectively; $^{d, D}$, significantly different from aged treated at $p<0.05$ and $p<0.01$, respectively. MDA expressed as μM/mg protein; NO expressed as μmol/l for plasma and μmol/mg for liver and brain; PCO expressed as nmol/mg protein. % Change is compared to young control group, calculated from unrounded values at four decimal places.

(38.7%, $p<0.01$) compared with the young control group. MRN-100 administration significantly suppressed the levels of carbonyl groups in the aged animals to reach young control values.

TSH levels. Table IV details the levels of TSH in the plasma, liver and brain of the various animal groups. In the aged untreated group a significant reduction in TSH level was noticed in comparison with the young control rats. There was

Table IV. *Effect of MRN-100 administration on TSH and GSH in different tissues of young and aged rats.*

Tissue	Parameter*	Young				Aged			
		Control		MRN-100		Control		MRN-100	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Plasma	TSH	2.1^{C}	0.11	2.2^{C}	0.11	$1.6^{A,B,D}$	0.08	2.0^{C}	0.08
	% Change			(3.5%)		(–26.3%)		(–4.3%)	
RBC	GSH	$79.8^{b,C}$	3.8	$89.8^{a,C,D}$	2.2	$55.6^{A,B,D}$	3.6	$71.1^{B,C}$	3.3
	% Change			(12.6%)		(–30.2%)		(–10.9%)	
Liver	TSH	4.2^{C}	0.6	4.3^{C}	0.04	$2.2^{A,B,d}$	0.14	3.7^{c}	0.49
	% Change			(1.0%)		(–47.6%)		(–12.0%)	
	GSH	1.5^{C}	0.13	1.7^{C}	0.07	$0.95^{A,B,D}$	0.03	1.5^{C}	0.03
	% Change			(7.7%)		(–38.1%)		(–5.0%)	
Brain	TSH	2.2^{c}	0.36	2.3^{C}	0.16	$1.4^{a,B}$	0.15	1.9	0.15
	% Change			(5.6%)		(–38.0%)		(–13.7%)	
	GSH	$1.1^{C,d}$	0.05	1.2^{C}	0.03	$0.87^{A,B,D}$	0.05	$1.2^{a,C}$	0.03
	% Change			(7.2%)		(–19.7%)		(14.0%)	

Each value represents the mean±SEM for 6 animals per group. ^{a,A} Significantly different from young control at $p<0.05$ and $p< 0.01$, respectively; ^{b,B} significantly different from young treated at $p<0.05$ and $p<0.01$, respectively; ^{c,C} significantly different from aged control at $p<0.05$ and $p< 0.01$, respectively; ^{d,D} significantly different from aged treated at $p<0.05$ and $p<0.01$, respectively. TSH is expressed as µM/mg protein; GSH is expressed as µM/Hb for RBC and µmol/mg protein for brain and liver. % Change is compared to young control group, calculated from unrounded values at four decimal places.

Table V. *Effect of MRN-100 on antioxidant scavenger enzymes SOD, CAT and GPx at different tissues of young and aged rats.*

	Parameter	Young				Aged			
		Control		MRN-100		Control		MRN-100	
Tissue		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
RBC	SOD	5.3^{C}	0.16	$5.5^{C,D}$	0.27	$4.1^{A,B}$	0.20	4.7^{B}	0.16
	% Change			(4.6%)		(–22%)		(–11%)	
	CAT	5.3^{C}	0.09	5.6^{C}	0.3	$4.1^{A,B,D}$	0.03	5.4^{C}	0.4
	% Change			(5.7%)		(–22%)		(1.8%)	
	GPx	43.6^{C}	0.64	44.3^{C}	0.6	$38.4^{A,B,D}$	1.6	43.1^{C}	0.7
	% Change			(1.6%)		(–12.1%)		(-1.3%)	
Liver	SOD	24.0^{C}	3.3	25.8^{C}	1.5	$15.0^{A,B,d}$	1.6	23.3^{c}	2.0
	% Change			(7.5%)		(–38%)		(–2.9%)	
	CAT	3.2C	0.27	$3.4^{C,d}$	0.07	$2.2^{A,B,D}$	0.08	$2.8^{b,C}$	0.08
	% Change			(4.9%)		(–32%)		(–13%)	
	GPx	6.5^{C}	0.01	6.6^{C}	0.11	$5.6^{A,B,D}$	0.36	6.4^{C}	0.04
	% Change			(1.9%)		(–14%)		(–1.6%)	
Brain	SOD	24.7^{C}	1.4	26.2^{C}	2.5	$17.2^{A,B,d}$	1.6	22.5^{c}	1.3
	% Change			(6.2%)		(–30%)		(–8.6%)	
	CAT	4.4^{C}	0.04	4.4^{C}	0.2	$3.0^{A,B,D}$	0.0	4.0^{C}	0.16
	% Change			(0.18%)		(–31.0%)		(–9.5%)	
	GPx	8.7c	0.3	9.3^{C}	0.31	$7.6^{a,B,D}$	0.39	9.0^{C}	0.23
	% Change			(7.0%)		(–13%)		(3.4%)	

Each value represents the mean ± SEM for 6 animals per group. ^{a,A} Significantly different from young control at $p<0.05$ and $p<0.01$, respectively; ^{b,B} significantly different from young treated at $p<0.05$ and $p<0.01$, respectively; ^{c,C} significantly different from aged control at $p<0.05$ and $p<0.01$, respectively; ^{d,D} significantly different from aged treated at $p<0.05$ and $p<0.01$, respectively. SOD is expressed as units enzyme required for 50% inhibition of NBT reduction/min, oxidized per mg Hb for RBC and per mg protein for liver and brain; CAT is expressed as µmoles of H_2O_2 utilized/min per mg Hb for RBC and per mg protein for liver and brain; GPx is expressed as µg of GPx consumed for 1 µmole of GSHoxidized/min per mg Hb for RBC and per mg protein for liver and brain. % Change is compared to young control group, calculated from unrounded values at four decimel places.

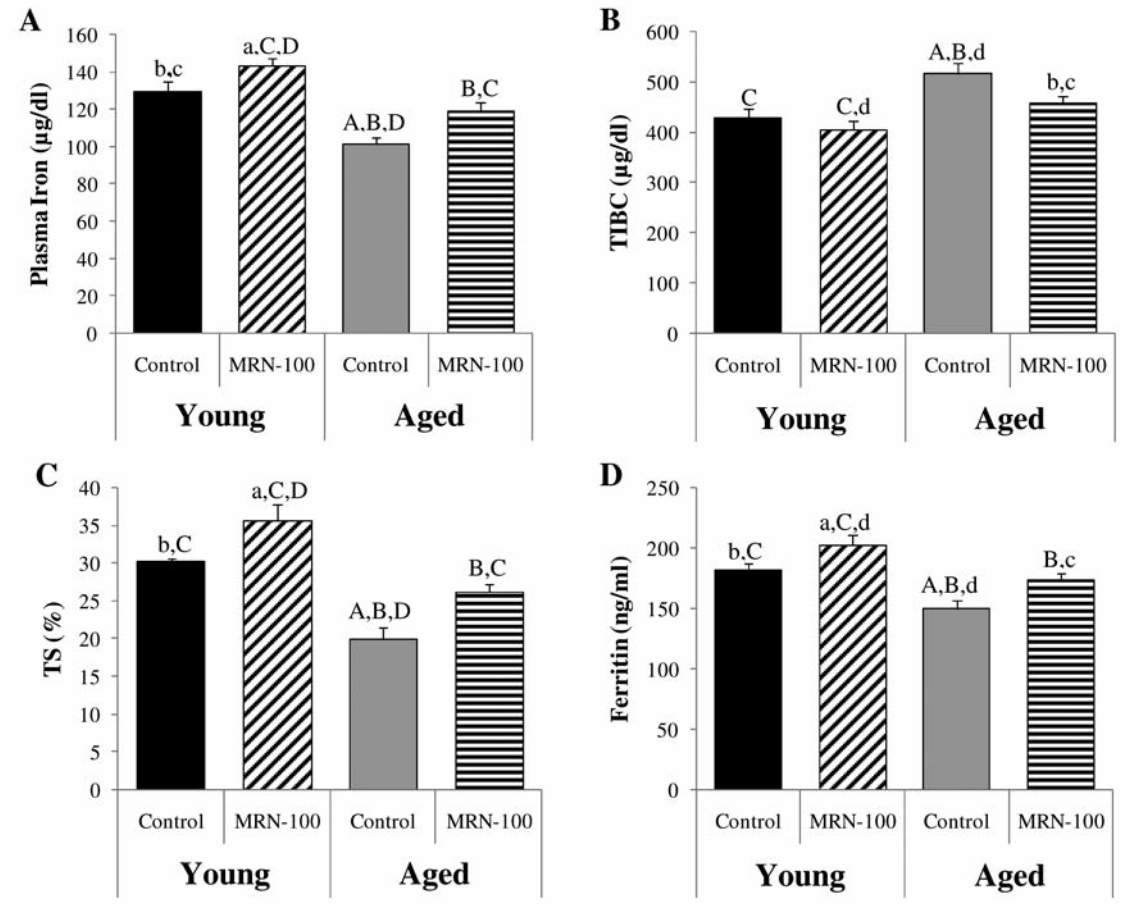

Figure 2. *Effect of MRN-100 administration on plasma iron status of young and aged rats. Young and aged animals were treated with MRN-100 or given tap water only (control) for 40 days. Whole blood was then collected by heart puncture after light anesthesia using heparinized syringes. Hemolysates were used to assess A: total plasma iron levels, B: total iron-binding capacity (TIBC), C: transferrin saturation (TS) and D: ferritin levels. Data are means of 6 rats/group±SEM and are shown as a percentage of the young control rat group.* [a, A] *Significantly different from young control at p<0.05 and p<0.01 respectively;* [b, B] *significantly different from young treated at p<0.05 and p<0.01 respectively;* [c, C] *significantly different from aged control at p<0.05 and p<0.01 respectively;* [d, D] *significantly different from aged treated at p<0.05 and p<0.01 respectively.*

a differential response among the three biological samples with respect to the level of TSH in aged rats. The TSH levels were reduced by 26.3% ($p<0.01$) in the plasma, 47.6% ($p<0.01$) in the liver, 38.0% ($p<0.05$) in the brain, as compared to untreated young rats. MRN-100 administration revealed a marked elevation in the TSH levels of aged animals to be comparable with the young ones.

GSH levels in red blood cells (RBCs) and tissues. Aged rats demonstrated a highly significant depletion in GSH level ($p<0.01$) in RBCs (–30.2%), liver (–38.1%) and brain (–19.7%), as compared to young rats (Table IV). MRN-100 supplementation significantly increased the GSH level in aged rats. There was also a noticeable increase in GSH content in the young rats treated with MRN-100.

SOD, CAT and GPx activity in RBCs and tissues. The effect of MRN-100 intake and aging on the activities of the antioxidant enzymes SOD, CAT and GPx in plasma, liver and brain of young and aged rats are shown in Table V. Generally, MRN-100 treatment in aged rats resulted in a remarkable enhancement in the activity of these antioxidant enzymes. SOD activity in untreated aged rats was significantly ($p<0.01$) decreased in RBCs (–22.2%), liver (–37.5%), and brain (–30.1%), as compared to control young rats. MRN-100 treatment in aged rats resulted in a marked increase in SOD activity when compared to aged untreated rats and was comparable with the young groups. RBC, liver and brain tissues of aged animals also showed a highly significant ($p<0.01$) decline in CAT activity (–22%, –32% and –31.0%, respectively). Similarly, GPx activity showed a slight but

significant decrease in RBCs, liver and brain tissues of aged animals (–12.0%, –14.4%, –13.0%, respectively). Daily intake of MRN-100 significantly enhanced CAT and GPx activity to reach the control young rat values.

Iron status. As shown in Figure 2, plasma iron TS, and ferritin concentrations were significantly reduced, whereas plasma TIBC was significantly elevated ($p<0.01$) in the untreated aged rats when compared with the young control group. MRN-100 supplementation significantly enhanced plasma iron and ferritin values and elevated TS to be comparable with the young control group and significantly lowered plasma TIBC. In addition, MRN-100 treatment caused a slight but significant increase ($p<0.05$) in the levels of plasma iron, ferritin and TS in young treated rats as compared to the young control group.

Discussion

Several agents have been used to reverse age-related changes associated with ROS. These include synthetic catalytic scavengers of ROS (EUK-189 and EUK207) (31), vitamins A, C and E, and selenium (32, 33). It has been reported recently that MRN-100 has a protective effect against oxidative stress-induced apoptosis in lymphocytes *in vitro* (17). In the current study, it was found that daily supplementation of aged rats with MRN-100 for 40 days resulted in a reversal in the levels of lipid peroxidation, protein oxidation biomarkers, total free radicals, GSH, and the antioxidant enzymes. Several studies showed that the levels of NO, PCO and MDA are increased in the plasma, liver and brain of aged animals (2, 3, 34, 35). This increase of carbonyl group content was correlated with diminished antioxidant enzymes, GSH content and TSH of aged animals. Interestingly, MRN-100 was able to counteract the age-related increase in the levels of PCO, free radicals, MDA and NO.

Age-related increases in lipid peroxidation and protein oxidation may be a reflection of decreases in enzymatic and non-enzymatic antioxidant protection (1, 36, 37). The mechanisms by which MRN-100 exerts its effect may involve enhancement of the enzymatic and non-enzymatic antioxidant defense systems, GSH and iron, to counteract and regulate overall ROS levels. The antioxidant scavenger enzymes include CAT, SOD and GPx. Data in the current study revealed that aged rats exhibit significant depletion in antioxidant enzymes SOD, CAT and GPx, as compared with young rats. The decline was observed in the blood, liver and brain of untreated aged rats and is supported by earlier studies (9-11, 37). However, MRN-100 treatment in aged rats resulted in a marked increase in activity of these enzymes, which were within the limits of the enzyme activity of the young rats. MRN-100-induced activation of these enzymes may increase the reaction rates of SOD-mediated conversion of superoxide to hydrogen peroxide and CAT- and GPx-mediated conversion of hydrogen peroxide to water (38, 39).

MRN-100 may exert its effect *via* a non-enzymatic antioxidant defense system, which may include GSH. GSH is an important protective endogenous antioxidant that combats free radicals and other oxidants, and has been implicated in immune modulation and inflammatory responses (40). It is known that restoring the intracellular GSH levels *via* exogenous administration of GSH or its precursors presents one possibility for protecting cells from damage caused by ROS and has a potential therapeutic role in protecting against lung oxidant stress, inflammation and injury (41-43). The data of the present study showed depletion in GSH content in aged animals, which is in accordance with other studies (2, 44). However, treatment with MRN-100 restored the levels of GSH in aged rats to the levels of young control rats.

The mechanism by which MRN-100 neutralizes the action of reactive oxygen species may involve the iron contained in this product. Thus far, the ability of iron to protect against oxidative stress has only been studied to a limited extent. Iron has the capacity to accept and donate electrons readily, and this characteristic makes it a useful component of cytochromes and oxygen-binding molecules. MRN-100 may exert its effect by improving the levels of ferritin, the key cellular iron storage protein. Iron captured within ferritin or transferrin does not generate the reactive radicals that it might otherwise form. Iron is known to interact with free radicals in the Haber-Weiss and Fenton reactions, producing highly reactive hydroxyl radicals and carbon-centered radicals. The iron-catalyzed Haber-Weiss reaction generates •OH from H_2O_2 and superoxide ($\bullet O_2^-$) (45). This reaction can occur in cells and is therefore a possible source of oxidative stress (45). Previous studies have suggested that ferritin can act as a protectant against oxidative damage in endothelial cells as well as murine and human leukemia cells by sequestering reactive free iron (15, 16). The results of the present study showed that MRN-100 improved the ferritin levels in the aged rats, suggesting that the increased ferritin is cytoprotective, serving to prevent the accumulation of protein carbonyl groups (a principal product of metal-catalysed oxidation of proteins). Ferritin may act as an antioxidant protein, since its iron-storing function prevents excess iron from taking part in the Fenton reaction, which would lead to production of ROS (46). Consistent with this idea, ferritin overexpression or knockdown in mammalian cells has demonstrated the cytoprotective role of ferritin under prooxidative conditions (47, 48). MRN-100, may increase the levels of cellular iron, and it has been demonstrated that an increase in cellular iron can cause an upregulation of ferritin protein translation (49). Transferrin, another important iron binding compound, is responsible for sequestering and transporting iron through the bloodstream (50). The results of

the present study showed that aged rats demonstrated lower levels of TS which was corrected post-treatment with MRN-100 (Figure 2). It is possible that the MRN-100-induced enhancement in ferritin and transferrin may have reduced the levels of free iron and therefore prevented the accumulation of reactive radicals, such as protein carbonyl groups (a principal product of metal-catalysed oxidation of proteins). This may suggest another mechanism by which MRN-100 exerts its effect. Altogether, the results of the present study strongly suggest, for the first time, that MRN-100 has a free radical-scavenging effect *in vivo*.

In conclusion, the present study demonstrated that MRN-100 augments endogenous antioxidant enzymes and reduces oxidative stress biomarkers. Thus, it appears that daily supplementation of the iron-based beverage MRN-100 may protect against age-associated oxidative stress.

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