1-1-2007

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Recommended Citation

Walker, Ernest M., et al. "Acetaminophen protects against iron-induced cardiac damage in gerbils." Annals of Clinical & Laboratory Science, 37.1 (2007): 22-33.

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Available online at www.annclinlabsci.org

Acetaminophen Protects Against Iron-Induced Cardiac Damage in Gerbils

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Abstract. There are few effective agents that safely remove excess iron from iron-overloaded individuals. Our goal was to evaluate the iron-removing effectiveness of acetaminophen given ip or orally in the gerbil iron-overload model. Male gerbils were divided into 5 groups: saline controls, iron-overloaded controls, iron-overloaded treated with ip acetaminophen, iron-overloaded treated with oral acetaminophen, and iron-overloaded treated with ip deferoxamine. Iron dextran was injected ip twice/wk for 8 wk. Acetaminophen and deferoxamine treatments were given on Mondays, Wednesdays, and Fridays during the same 8 wk and continued for 4 wk after completion of iron-overloading. Echocardiograms were performed after completion of the iron-overloading and drug treatments. Liver and cardiac iron contents were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). Iron-overloaded controls had 232-fold and 16-fold increases in liver and cardiac iron content, respectively, compared to saline controls. In iron-overloaded controls, echocardiography showed cardiac hypertrophy, right and left ventricular distension, significant reduction in left ventricular ejection fraction (-22%), and fractional shortening (-31%) during systole. Treatments with acetaminophen (ip or oral) or deferoxamine (ip) were equally effective in reducing cardiac iron content and in preventing cardiac structural and functional changes. Both agents also significantly reduced excess hepatic iron content, although acetaminophen was less effective than deferoxamine. The results suggest that acetaminophen may be useful for treatment of iron-induced pathology.

Keywords: acetaminophen, deferoxamine, echocardiogram, iron-overload, gerbils, hemochromatosis

Introduction

It is estimated that >2 million persons in the United States and up to 100 million persons worldwide acquire some degree of iron-overload [1]. There are 2 major causes of iron-overload in humans. The first is transfusional iron-overload, resulting from frequent blood transfusions in patients with thalassemia, sickle cell disease, or myelodysplastic syndrome. The second is hereditary hemochromatosis, an autosomal recessive disorder with a frequency of about 10% in persons of European descent [2-4]. While only 0.5% of subjects are homozygous for this trait, nearly 40 million people worldwide have iron-overload due to hereditary hemochromatosis and various types of hemosiderosis. In these cases, tissue iron levels progressively increase with age and may cause life-threatening complications. These include advanced liver disease and cirrhosis, diabetes mellitus likely due to accumulation of iron in pancreatic islets with direct damage to beta cells, and cardiotoxicity due to accumulation of iron in myocytes and interstitial cells of the myocardium, and within the fibers of the conduction system.

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Cardiomyopathy from excess cardiac iron is the major cause of death in thalassemia patients [5,6]. Excess iron accumulation in the heart may cause cardiac hypertrophy, dilatation, myocardial fiber degeneration, and fibrosis [7]. The survival of patients with beta thalassemia and sickle cell anemia is determined by the magnitude of cardiac iron-loading [8]. Removal of excess iron is necessary to improve morbidity and reduce mortality of patients with thalassemia and sickle cell disease [7]. Cardiotoxic effects may be severe, with cellular degeneration and fibrosis of the myocardium, disturbances of cardiac rhythm, and eventual death. However, even individuals with only mild to moderate elevations of tissue iron appear to have increased risk for diabetes mellitus, heart disease, G-I cancers, and hematological malignancies [1].

Treatment with phlebotomy results in some clinical improvement of subjects with elevations in tissue iron burden and, if used as preventive therapy, may ameliorate organ damage and result in normal life expectancy [9]. However, this treatment requires repeated removal of up to a liter of blood for periods of a year or longer, followed by lifetime maintenance phlebotomy. Chelation therapy with currently approved chelating agents is a poor substitute for phlebotomy and is used mainly in treating patients with secondary hemochromatosis or hemosiderosis. There are currently 3 iron chelators in clinical use or in late-stage development; these are Desferal (deferoxamine), deferiprone (L1), and Exjade (deferasirox, ICL670) [6].

Desferal (deferoxamine). The major chelator agent approved for iron removal in humans is deferoxamine, which may be beneficial as a cardio-protective agent through its ability to remove excess iron and possibly to act as an antioxidant. Deferoxamine has demonstrated efficacy in removing excess iron, preventing early death, and improving survival rates in thalassemia patients [6,10,11]. Deferoxamine must be administered by iv, sc, or im routes and is ineffective when given orally. It can be associated with side effects such as visual and auditory disturbances, urticaria, hypo-tension, poor patient tolerance, and low compliance [6,7,8,12,13]. Deferoxamine is a hexadentate chelator that combines with iron at a 1:1 ratio to form a stable inert complex [14].

Deferiprone (L1). Deferiprone is approved as a second-line therapy for patients unable to receive deferoxamine or in whom deferoxamine is less effective [6]. Deferiprone is effective orally, but has a short half-life so that multiple daily oral doses are required. It is associated with serious toxic side effects in some recipients, including increase of serum transaminase activity, joint problems, neutropenia, and agranulocytosis [6,8,14]. There is a risk of paradoxical aggravation of iron toxicity by deferiprone [14]. Liver iron levels and body burdens of iron may not be as well controlled for long periods (4-6 yr) with deferiprone vs deferoxamine and may remain at levels above the threshold associated with heart disease in 18% to 65% of patients [8,15-20]. Beneficial effects of deferiprone on heart disease are unclear and appear to be improved when the agent is given in combination with deferoxamine [8,21]. Three molecules of deferiprone are required to bind one molecule of iron [6,8]. The use of deferiprone may produce zinc deficiency in some patients [6].

Exjade (deferasirox, ICL670, Novartis). Deferasirox has recently been evaluated in phase IIb/III studies and approved for clinical use. It has a long half-life so that it can be used for once-daily dosing [6,22,23]. Early clinical results suggest that reasonable doses of deferasirox effectively control excess iron levels and that deferasirox has equivalent efficacy to deferoxamine [6]. Deferasirox also shows high efficacy and therapeutic safety in preclinical animal studies [8,24,25]. Two molecules of deferasirox bind one molecule of ferric iron [6].

There is a great clinical need for an orally active iron-chelating drug for treating cases of iron-overload. Based on earlier work by our laboratory [26], we tested the effectiveness of acetaminophen, given ip or orally, for the reduction of tissue iron levels and prevention of cardiopathology in the Mongolian golden gerbil iron-overload model.

Materials and Methods

Animals. Twelve-wk-old male pathogen-free Mongolian golden gerbils (Hilltop Laboratories, Scottsdale, PA) were housed on wood chip bedding in plastic cages in rooms kept at 23 ± 2°C with a 12 hr light/dark cycle. Purina gerbil mix and tap water were available ad libitum. All animal procedures were approved by the Marshall University Institutional Animal Care and Use Committee.

Table 1. Disposition of gerbils in the 5 experimental groups.

Experimental group	Mortality	ECHO studies	Used for histology	Analyzed for iron
Saline controls (SC) (n = 16)	0	16	6	10
Iron-overload controls (IO) (n = 16)	3	15	6	10*
Iron-overload acetaminophen ip (IOAi) (n = 16)	1	9	6	9
Iron-overload acetaminophen po (IOAo) (n = 16)	0	10	6	10
Iron-overload deferoxamine ip (IODF) (n = 16)	3	7	6	7

*Three iron-overloaded animals died during or after ECHO procedures and their hearts and livers were salvaged for determination of iron content. There were no statistically significant differences among the groups regarding mortality.

Treatments. Animals (average wt 60 g) were divided into 5 groups (16 gerbils/group). (SC)saline control gerbils received ip injection of 1% saline (0.15 ml/kg) twice/wk for 8 wk. (IO) iron-overloaded control animals were subjected to a similar schedule of twice/wk ip injections of iron dextran (120 mg/kg, ferric hydroxide dextran complex, Sigma Chemical Co., St. Louis, MO) dissolved in 1% saline. (IOAi) acetaminophen-injected animals received ip injections of iron dextran twice/wk and acetaminophen (150 mg/kg or 1.0 mM/kg, 4-acetaminophen, Sigma) 3 times/wk (Monday, Wednesday, Friday). Acetaminophen was injected 4 hr after the iron injection when both were given on the same days. The acetaminophen treatment was continued 3 times/wk (M,W,F) for 4 wk after the completion of iron-overloading. (IOAo) these animals, which received oral acetaminophen, were given ip iron dextran twice/weekly and acetaminophen (150 mg/kg in saline) by gavage 3 times/wk (M,W,F) for the first 9 treatments. Animal distress due to repeated gavages forced additional oral acetaminophen to be given in drinking water at an initial acetaminophen concentration of about 1.2 mg/ml. Water intake was monitored and averaged 5 ml/gerbil/day. Animals were periodically weighed and the acetaminophen concentration adjusted as necessary to regulate the oral dosage (approximately 100 mg/kg/day). Oral acetaminophen treatment was continued for 4 wk after completion of iron-overloading. (IODF) this group of gerbils received ip iron dextran twice/wk and deferoxamine (IODF) 3 times/wk. Deferoxamine in the form of the methanesulfonate salt (82 mg/kg or 0.125 mM/kg, Sigma) was administered about 4 hr after the iron injection when both were given on the same days. Deferoxamine treatment was continued 3 times/wk for 4 wk after the completion of iron-overloading, so that the total period of treatment and observation was 12 wk. ECHO studies were conducted on all gerbils 8 wk after completion of treatment and the gerbils were killed at 20-21 wk into the experiment for histopathologic studies and measurements of cardiac and hepatic iron contents. The disposition of gerbils in the 5 experimental groups is listed in Table 1.

ECHO studies. ECHO cardiac structural and functional values have not previously been reported for gerbils. Groups of control gerbils (n = 16) and iron-overloaded gerbils (n = 16 less one death, or n = 15) were studied to ensure the validity and statistical power of the results. Nine of 16 iron-overloaded, acetaminophen-injected (IOAi), 10 of 16 iron-overloaded, acetaminophen orally administered, and 7 of 16 iron-overloaded, deferoxamine injected animals were randomly selected for ECHO studies. One IOAi gerbil died during ECHO studies and 6 IOAi gerbils were used for non-ECHO studies. Six IOAo gerbils were used for non-ECHO studies. Three IODF gerbils died prior to, during, or just after ECHO studies and 6 were used for non-ECHO studies.

Echocardiography is a noninvasive ultrasound procedure in which harmless, high-frequency sound waves (frequency >20,000 cycles/sec) are emitted from a piezoelectric crystal or transducer, beamed in particular directions, and reflected back (echo) by small structures in the mm and sub-mm range [27]. These waves are beamed toward and penetrate the heart and are reflected back to the transducer as a series of echoes, which are amplified and displayed on a cathode ray tube [28]. Echocardiography is used to evaluate the position, size, and movement of cardiac valves, heart wall structure and function, and directional flow of blood within cardiac chambers [29].

Echocardiographic measurements were done with a Phillips Sonos 5500 echocardiogram using a S12 transducer (frequency range 8-12 MHz). Animals were anesthetized with ip injections of a 2:1 mixture of ketamine HCl (100 mg/ml) and xylazine (20 mg/ml) (approximately 0.3 to 0.4 ml/100 g gerbil wt). Gerbils were shaved in the chest area for adequate sonic transference, an ultrasonic transmission gel was applied to the chest area, and the animals were positioned on their left sides or backs. Echocardiographic images were obtained including two-dimensional, pulse wave Doppler and M-mode images. Two-dimensional echocardiography was used to image the cardiac structures in both the substernal long axis and short axis views. These echocardiographic views were then used to position the M-mode echocardiographic line. In the long axis, the probe was oriented toward the base of the heart projecting toward the apex (x-axis) with depth along the y-axis, thus allowing pulse wave Doppler evaluation of valvular blood flow velocities. In short axis procedures, the probe was oriented toward the left ventricle and across the heart for evaluation of wall structure, which was used in the calculation of ejection fraction and fractional shortening during systole. M-mode displays were analyzed by a digital echocardiographic analysis system. Six measurements were selected for each assessment of cardiac structure and function. Structural parameters included: diastolic (IVSd) and systolic (IVSs) left ventricular septal thickness, diastolic (LVIDd) and systolic (LVIDs) left ventricular internal dimension, diastolic (LVPWd) and systolic (LVPWs) left ventricular posterior wall thickness, and right ventricular diastolic internal dimension

Table 2. Echocardiographic evaluation of cardiac structural parameters in the experimental groups of gerbils.

Group	N	IVSd (cm)	IVSs (cm)	LVIDd (cm)	LVIDs (cm)	LVPWd (cm)	LVPWs (cm)	RV (cm)
SC	16	0.09 ± 0.01	0.15 ± 0.01	0.43 ± 0.01	0.27 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.14 ±0.01
IO	15	0.13 ± 0.01*	0.19 ± 0.01*	0.53 ± 0.02*	0.35 ± 0.01*	0.17 ± 0.01*	0.19 ± 0.01*	0.20 ± 0.01*
IOAi	9	0.09 ± 0.01+	0.14 ± 0.01+	0.44 ± 0.01+	0.30 ± 0.01*+	0.11 ± 0.01+	0.15 ± 0.01+	0.12 ± 0.01+
IOAo	10	0.09 ± 0.02+	0.15 ± 0.02+	0.48 ± 0.02+	0.31 ± 0.02*+	0.10 ± 0.01+	0.16 ± 0.02+	0.12 ± 0.02+
IODF	7	0.10 ± 0.01+	0.15 ± 0.01+	0.43 ± 0.02+	0.27 ± 0.01+	0.12 ± 0.01+	0.15 ± 0.01+	0.14 ± 0.01+

An asterisk (*) or cross (+) indicates significant difference ($p < 0.05$) from saline control (SC) and iron-overload (IO) groups, respectively. See Materials and Methods for abbreviations.

Table 3. Echocardiographic evaluation of cardiac functional parameters in the experimental groups of gerbils.

Group	EF (%)	FS %)	AV Max (cm/sec)	PV Max (cm/sec)	MV Max (cm/sec)	TV Max (cm/sec)
SC	77.5 ± 1.5	40.4 ± 1.5	47.2 ± 2.5	48.9 ± 3.2	41.0 ± 3.9	40.3 ± 2.6
IO	60.6 ± 1.8*	27.9 ± 1.3*	49.8 ±2.4	52.9 ± 4.2	38.0 ± 3.7	38.1 ± 3.1
IOAi	72.5 ± 1.7*+	35.0 ± 2.0*+	70.4 ± 4.0*+	66.2 ± 4.4*+	50.7 ± 2.9*+	51.0 ± 4.0*+
IOAo	76.4 ± 1.7+	39.3 ± 1.3+	58.6 ± 3.3*,+	58.1 ± 4.0*	44.3 ± 3.4	37.0 ± 1.8
IODF	67.1 ± 1.1*,+	34.1 ± 2.4*,+	53.4 ± 3.9	47.6 ± 3.2	39.8 ± 3.2	37.6 ± 2.3

An asterisk (*) or cross (+) indicates significant difference ($p < 0.05$) from saline control (SC) and iron-overload (IO) groups, respectively. See Materials and Methods for abbreviations.

(RV). Functional measurements included: ejection fraction (EF), left ventricular fractional shortening during systole (FS), maximal aortic (AVmax), pulmonary (PVmax), mitral (MVmax), and tricuspid (TVmax) valvular blood flow velocities. In addition to direct measurements of cardiac mass, echocardiographic measurements were utilized to estimate left ventricular mass (LVM) as previously described [30]: $LVM = 1.04 (LVIDd + IVSd + PWd)^3 - (LVId)^3$.

The wavelength of an ultrasonic pulse is calculated using the formula $\lambda = V/F$ = velocity of pulse in tissue/frequency of pulse. Pulse velocity in tissue is approximately 1,500 m/sec [28-30], so $\lambda = 0.125$ mm with a 12 MHz transducer. ECHO resolution is the smallest distance between 2 points at which the points can be distinguished as separate [28]. Resolution = wavelength times pulse length, and the pulse length was 1 sec. Therefore, we were able to accurately measure cardiac wall thicknesses of approximately 0.125 mm or greater.

Sensitivity is the ability of a system to image small targets located at specific depths in an attenuative medium [28]. It is determined by the transducer transmitting efficiency times the transducer receiving efficiency of the reflected pulse (echo). System efficiency is influenced by transducer beam geometry, frequency spectrum, and energy conversion efficiency. The typical transducer used in ECHOs of adult patients is about 2.25-MHz and has an efficiency of about 4.4% [28]. 3.5-MHz transducers are typically used in younger children and 5-MHz transducers in infants and neonates. In the gerbils, a 12-MHz transducer was used and the beam traveled a much shorter distance to detect gerbil hearts, compared to human hearts, so that the system efficiency should be considerably greater.

Liver and cardiac iron content. After completion of the echocardiographs (12-13 wk), the gerbils were euthanized by exsanguination via cardiac puncture or by carbon dioxide inhalation. Hearts and livers were removed by dissection, weighed to the nearest mg, frozen in liquid nitrogen, and stored at -70°C until analysis. Whole hearts and liver samples were digested in concentrated nitric and perchloric acids (70:30, v/v). Iron concentration was determined in tissue digestates by inductively coupled plasma-atomic emission spectrometry (ICP-AES) [31].

Histological techniques. Animals from each group were killed after completion of treatment and ECHO procedures. Six animals were histologically evaluated from each group. Whole hearts (breadloaf sectioned) and representative sections of liver, spleen, pancreas, and testes were fixed in 10% buffered formalin solution and processed into paraffin blocks by routine procedures. Representative 3-5 µm slices were cut with a cryostat and sections were mounted on glass slides for staining with hematoxylin and eosin (H & E), Perls iron stain, or Mallory trichrome stain. Bone marrow samples were obtained from cross-section blocks of gerbil spinal column and from femurs and were fixed in B-5 solution after light decalcification, processed, sections cut and mounted on glass slides, and stained with H & E or Perls iron stain. Slides were evaluated by light microscopy.

Studies in vitro. Five ml of acetaminophen (10^{-3} M, clear solution) was added to 5 ml of $Fe(II)SO_4 \bullet 7H_2O$ (10^{-3} M, clear solution) or $Fe(III)Cl_3 \bullet 6H_2O$ (10^{-3} M, light yellow solution) in glass scintillation vials that were capped, shaken periodically, and observed at 23°C for up to 48 hr.

Statistics. Data were expressed as means ± SD. Statistical analyses were performed using Sigma Stat statistical software (Jandel Corporation, San Rafael, CA). Differences among groups were assessed by 2-way ANOVA, followed by Tukey test. Values of $p \leq 0.05$ were deemed statistically significant.

Results

Cardiac structure and function. Iron overloading resulted in significant increases in left ventricular septal (IVSd, IVSs) and posterior wall (LVPWd, LVPWs) thicknesses as well as left (LVIDd, LVIDs) and right ventricular (RV) internal dimensions (Table 2). Treatments with either acetaminophen or deferoxamine by ip injection were equally effective in preventing iron overloading-induced changes in cardiac structure. Oral administration of acetaminophen also prevented iron-induced changes; the decreases in left ventricular dimensions (LVIDd, LVIDs) were statistically different from the values obtained in IO animals.

Iron-overloading resulted in significant decreases in the ejection fraction (-22%) and left ventricular fractional shortening during systole (-31%), with little or no change in valvular blood flow velocities (Table 3). Both oral administration and ip injection of acetaminophen prevented these losses in function. Deferoxamine ip significantly prevented or reversed iron-induced decreases in ejection fraction and left ventricular fractional shortening (FS) during systole. The ip injections of acetaminophen caused a significant increase in maximal aortic (AVmax), pulmonary (PVmax), and tricuspid (TVmax) valvular blood flow velocity, suggesting a positive inotropic effect of the drug.

Tissue iron evaluation. Iron overloading resulted in 232-fold increase in liver and 16-fold increase in cardiac iron content (Fig. 1). Treatments with either acetaminophen (ip, oral) or deferoxamine (ip) were roughly equal in partially reducing the cardiac iron burden in iron-overloaded animals. Average post-treatment cardiac iron concentrations were: SC, 15.11 ± 11.77 ug; IO, 243.36 ± 51.08 ug; IOAi, 130.38 ± 29.58 ug; IOAo, 133.76 ± 27.95 ug; IODF, 110.50 ± 7.47 ug (p values IOAi vs IOAo or DF >0.05; IOAo vs DF >0.05). As shown in Fig. 1, acetaminophen (ip, oral) appeared less effective than deferoxamine (ip) in reducing hepatic

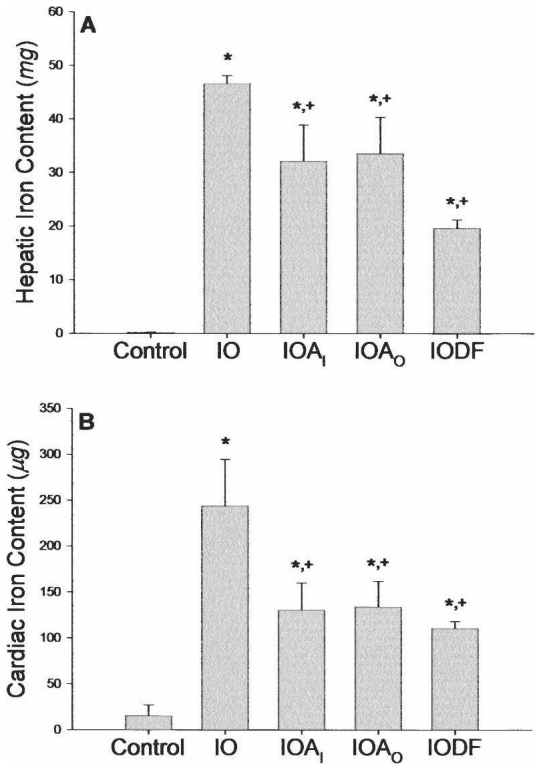

Fig. 1. Hepatic and cardiac iron content of gerbils in the experimental groups. The iron content of liver (A) and heart (B) of saline controls (SC, n = 10), iron-overloaded (IO, n = 10), acetaminophen-injected (IOAi, n = 9), acetaminophen administered by oral gavage (IOAo, n = 10), and deferoxamine-injected gerbils (IODF, n = 7).
An * or + indicates a significant difference ($p < 0.05$) from the controls or iron-overloaded gerbils, respectively.

concentrations of excess iron. Average post-treatment hepatic iron concentrations were: SC, 0.201 ± 0.016 mg; IO, 46.623 ± 1.457 mg; IOAi, 33.139 ± 6.780 mg; IOAo, 33.516 ± 6.812 mg; IODF, 19.567 ± 1.636 mg (p values IOAi vs IOAo >0.05; IOAi or IOAo vs DF <0.05).

Iron accumulation was noted in most gerbil tissues with the least in the central nervous system. In iron-overloaded gerbils, hemosiderin iron deposition was seen in gerbil liver (Fig. 3A), pancreas (Fig. 3B), spleen (Fig. 3D), myocardial cells and interstitial spaces (Fig. 2A,C), aorta (Fig. 2D,E), kidney (Fig. 3C), pituitary, thyroid, bone marrow, and testes. The cardiovascular system demonstrated iron accumulation in myocardial cells, and interstitial spaces (Fig. 2A,C), while the aortas accumulated iron in all vascular layers, most marked in tunica adventitias and tunica intimas

(Fig. 2D,E). Sections of left ventricle from iron-overloaded gerbils showed areas of iron-induced destruction and necrosis of cardiac muscle (Fig. 2B). Liver sections stained with iron stain (Prussian blue) demonstrated accumulation of hemosiderin iron granules in cytoplasm of hepatocytes, most marked in periportal areas (Fig. 3A) and often perinuclear in location (arrow). Pancreases were intensely pigmented with hemosiderin accumulation in acinar and islet cells (Fig. 3B). Renal sections revealed tubular and glomerular accumulations of hemosiderin granules (Fig. 3C). Spleens showed marked hemosiderin accumulation in both red and white pulp (Fig. 3D). Hemosiderin granules accumulated in sections of bone marrow (not shown). Testes accumulated iron chiefly in interstitial spaces among seminiferous tubules, but little within tubules (not shown). Hemosiderin iron accumulated in the adrenal and thyroid glands (not shown). Both acetaminophen (ip or oral) and deferoxamine (ip) produced substantial iron reductions in the tissues (liver, heart, aorta, spleen, pancreas, bone marrow, testis, adrenal, and thyroid), with the least reduction in liver and spleen, as judged from histological evaluations. The body weights of iron-overloaded gerbils were similar to saline controls, indicating that the injections of iron dextran did not result in debilitation and weight loss (Table 4). By comparison, liver and cardiac weights were increased 40% and 43%, respectively, in the IO group. Consistent with echocardiographic evidence of hypertrophy and increase in heart weight, calculation of left ventricular mass indicated an approximate increase of 130% in the left ventricular mass of iron overloaded animals compared to controls (Table 5). Acetaminophen (ip, oral) and deferoxamine (ip) appeared equally effective in reducing the increases in liver and cardiac weights (Tables 4 and 5).

Table 5. Left ventricular mass calculated from echocardiographic analysis of gerbils in the control (SC), iron overloaded (IO), acetaminophen treatment by ip injection (IOAi), acetaminophen treatment by gavage (IOAo), and deferoxamine treatment by ip injection (IODF) groups.

Group	N	LVM (g)
SC	16	0.177 ± 0.005
IO	15	0.408 ± 0.13*
IOAi	9	0.189 ± .007+
IOAo	10	0.205 ± .006*+
IODF	7	0.222 ± .017*+

An asterisk (*) or cross (+) indicates a significant difference ($p < 0.05$) from the groups of control (SC) or iron-overloaded (IO) gerbils, respectively.

Mortality and arrhythmias. During the total period of observation, of 16 saline control (SC) gerbils, 0 developed cardiac arrhythmias (ECHO studies) and there were 0 deaths. Of 16 iron-overloaded (IO) gerbils, 9 developed significant cardiac arrhythmias, chiefly premature ventricular contractions (PVCs), and 3 of the 9 gerbils died after completion of ECHOs and prior to the end of the experiments. The 16 iron-overloaded gerbils treated by acetaminophen ip (IOAi) showed 0 cardiac arrhythmias and 1 death. The 16 iron-overloaded gerbils treated orally with acetaminophen (IOAo) demonstrated 1 animal with cardiac arrhythmia (PVCs) and 0 deaths. The 16 iron-overloaded gerbils treated with deferoxamine ip showed 3 animals with cardiac arrhythmias (PVCs), which died prior to the end of the experiment.

Table 4. Body, liver, and heart weights of gerbils in the experimental groups.

Group	Body wt (g)	Liver per body wt (%)	Heart per body wt (%)
SC	83.7 ± 2.2	4.10 ± 0.06	0.31 ± 0.01
IO	80.5 ± 2.6	5.78 ± 0.32*	0.46 ± 0.01*
IOAi	85.3 ± 2.4	4.93 ± 0.13*+	0.40 ± 0.01*+
IOAo	84.2 ± 2.3	5.14 ± 0.20*+	0.40 ± 0.01*+
IODF	92.6 ± 5.6	4.94 ± 0.11*+	0.37 ± 0.01*+

An asterisk (*) or cross (+) indicates a significant difference ($p < 0.05$) from saline control (SC) and iron overload (IO) groups, respectively. See Materials and Methods for abbreviations.

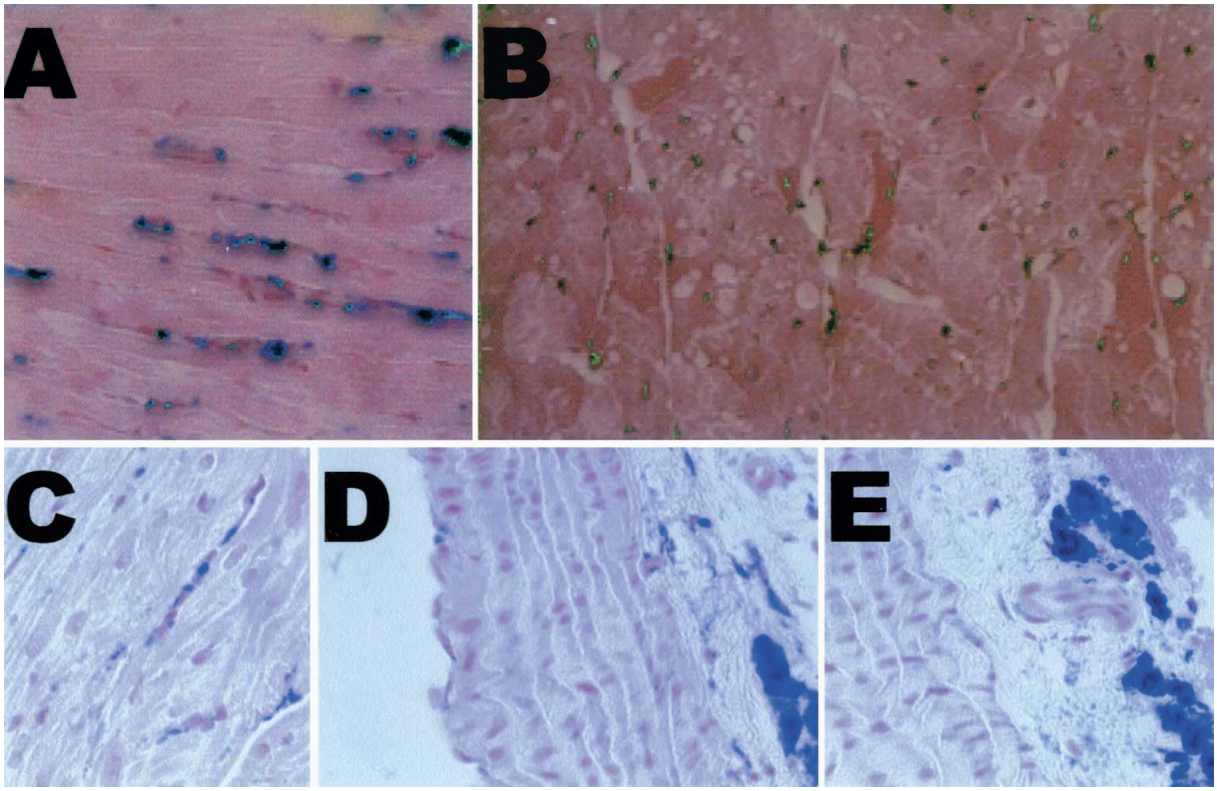

Fig. 2. Cardiac (A-C) and aortic (D and E) iron deposits in iron-overloaded gerbils (group IO): 2A depicts iron accumulations (blue granules) in cardiac interstitial cells and cardiomyocytes (Perls iron stain, 100X); 2B depicts iron-induced damage and necrosis of cardiac muscle (H&E, 100X); 2C shows iron accumulations (blue granules) in cardiac interstitial cells and cardiomyocytes (Perls iron stain, 100X); 2D demonstrates iron deposits in all layers of iron-overloaded aortic wall, but especially concentrated in the adventitial area (Perls iron stain, 40X); 2E is a higher power view of 2D (Perls iron stain, 400X).

In vitro study. Acetaminophen reacted in vitro with $Fe(II)SO_4 \bullet 7H_2O$ (ferrous) (10^{-3} M, clear solution) to produce a brown precipitate and with $Fe(III)Cl_3 \bullet 6H_2O$ (ferric) (10^{-3} M, light yellow solution) to yield a rusty-appearing solution, which gradually turned dark brown and formed a brown-black precipitate. These observations suggest that acetaminophen can bind to iron in vitro.

Discussion

The results of this study suggest that acetaminophen is equally effective as deferoxamine in cardiac iron removal and the prevention or reversal of cardiac functional and structural abnormalities in the iron-overloaded gerbil model. ECHO and radionuclide ventriculography are the most useful noninvasive diagnostic techniques to detect iron-overload-induced cardiomyopathy [32]. Our ECHO studies revealed easily discernable differences in cardiac structure and function between the saline control and iron-overloaded groups and between the iron-overloaded group and the iron-overloaded treated groups.

Iron localization in human hearts. Normally, there is no stainable iron within the myocardium [33]. In cases of iron-overload, iron-saturated transferrin attaches to transferrin receptors on cardiomyocytes and iron is released into the cell to be stored as hemosiderin-iron [32]. In iron-overloaded human patients, excess iron accumulates in cardiomyocytes, cardiac macrophages, in cells of the bundle of His and Purkinje system, and in interstitial spaces of the heart [32]. In cases of hemochromatosis or hemosiderosis, iron deposits tend to be more extensive in the epicardial third of the ventricle, followed by the subendocardium and papillary muscle, and least in the middle third of the ventricular wall. Hematoxylin and eosin as well as

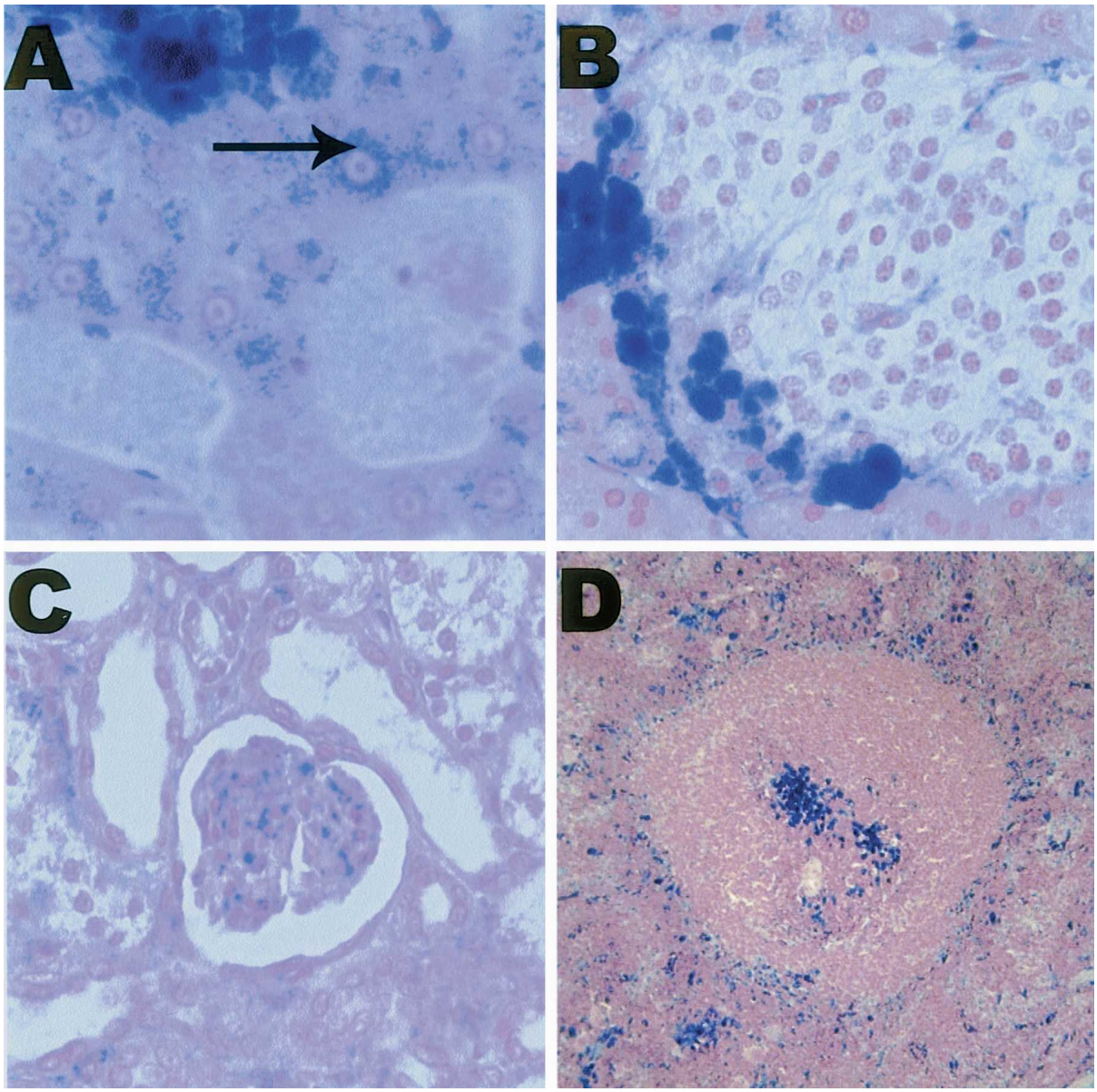

Fig. 3. Liver (A), pancreatic (B), kidney (C), and splenic (D) iron deposits in iron-overloaded gerbils (group IO): 3A reveals iron deposits in hepatocytes and Kupffer cells. Perinuclear iron distribution occurs in hepatocytes (arrow) (Perls iron stain, 40X); 3B shows iron distribution in pancreas, emphasizing iron deposits in pancreatic cells, including islet and beta cells (Perls iron stain, 400X); 3C illustrates iron in kidney (blue granules) including glomerular and tubular iron deposits and tubular damage (Perls iron stain, 100X); 3D shows iron distribution (blue granules) in splenic red and white pulp (Perls iron stain, 100X).

Prussian blue staining show hypertrophic myocytes with hemosiderin iron in perinuclear locations in cells, but with increased iron concentrations eventually occupying most of the cells and occasionally totally replacing myocytes [33]. Iron accumulations in the cardiac conduction system, coronary arteries, and valves are usually limited, but involvement of the conduction system is associated with cardiac arrhythmias. Iron accumulation in the bundle of His and Purkinje system may impair signal conduction from the atrial pacemaker to the ventricles and this may result in arrhythmias and sudden death [32]. Cardiac fibrosis may be present and result in restrictive cardiac hemodynamics [33]. There is significant correlation between serum ferritin concentrations and the endomyocardial biopsy grade. Patients with high ferritin levels and poor compliance to treatment

with chelating agents are at high risk of cardiac hemochromatosis and its complications [33].

Iron localization in gerbil hearts and aortas. Iron distribution in the gerbil hearts was almost identical to that reported in human hearts. Marked iron accumulations were seen in the ventricles, less in atria, and little in fibers of the conduction system, valves, or coronary arteries. Appreciable quantities of iron accumulated in gerbil aortas, especially in the adventitial region. Iron accumulations were evident in cardiomyocytes, cardiac macrophages, and interstitial spaces. Infiltrating fibrosis in gerbil heart ventricles was similar to that which sometimes occurs in hearts of iron-overloaded patients.

Hemosiderin iron is innocuous, but it is in equilibrium with a small pool of loosely bound ("free") iron in cardiomyocytes. Loosely bound iron is capable of catalyzing the formation of ROS (reactive oxygen species) through the Fenton reaction. The ROS can cause oxidant-mediated injury to cells. Cardiac cells are particularly sensitive to oxidant-induced damage since they contain a high concentration of mitochondria and maintain a high degree of metabolic function, which includes performing complex operations such as contraction and transmission of electrical impulses [32]. By binding iron, deferoxamine and acetaminophen may reduce or prevent the generation of ROS via the Fenton reaction, thus protecting against cellular damage and restoring normal cellular activity.

Pathogen-free Mongolian gerbils were used in this study to avoid the increased morbidity and mortality and advanced hepatocellular necrosis observed in gerbil strains (eg, *Psammomys obesus*) with coincidental laboratory infections [14]. Acetaminophen was as effective as deferoxamine in reducing cardiac excess iron content and preventing echocardiographic evidence of cardiotoxicity in iron-overloaded gerbils. Acetaminophen and deferoxamine treatments both reduced hepatic excess iron content, although acetaminophen was less effective than deferoxamine. This may be due to tight binding of iron to the high concentrations of ferritin and similar molecules in the liver.

The gerbil iron-overload model was first described by Carthew et al [34] and provides an excellent experimental model for the study of iron-overloading of the heart and liver. Iron overloading in the gerbil model closely simulates the features observed in transfusion iron-overloaded patients [34]. Weekly injections of iron dextran in gerbils result in accumulation of ferritin in perisinusoidal cells, Kupffer cells, and hepatocytes with subsequent scarring hepatic fibrosis [35] and suppression of mitochondrial respiratory enzyme activities [39]. Others have shown the development of cardiomyopathy in the iron-overloaded gerbil [14,37-41]. Subepicardial and subendothelial areas and the intraventricular septum in gerbil hearts were primarily affected, as occurs with severe iron overload in humans. Similarly, intracellular iron in individual myocytes showed a characteristic perinuclear pattern in gerbils, as seen in human hemochromatosis. Within 12 wk from the start of iron administration, iron-overload in the gerbil resulted in significant increase of cardiac iron content with progressive development of hypertrophy, electrocardiographic abnormalities, and reduced function. The similarities of iron accumulation, distribution, and associated pathology in gerbil and human hearts and their similar responses to iron-removing therapy emphasize the value of the Mongolian gerbil iron-overload model in studying iron-induced cardiovascular pathology and therapeutic interventions.

Our study showed iron-induced changes in cardiac structure and function in the gerbil model based on echocardiographic findings. Consistent with previous reports, the present results indicate cardiac hypertrophy with possible ventricular distension (Table 2) and significant reduction in function (Table 3). Similar to previous studies [14,36,39,41], we found that chelation therapy with deferoxamine prevents functional abnormalities in the gerbil model. Furthermore, we demonstrated that oral or ip administration of acetaminophen partially or totally prevents the changes in structure and cardiac function. Acetaminophen and deferoxamine were equally effective in reducing excess cardiac iron. Acetaminophen and deferoxamine both reduced the accumulation of iron in livers and hearts of iron-overloaded gerbils (Fig. 1). Acetaminophen provided significant cardio-protection when given either by ip injection or oral gavage.

Possible mechanisms of action. The mechanism by which acetaminophen provides cardioprotection against iron-overload is uncertain. At high doses and prolonged use, acetaminophen is a hepatotoxin due to its ability to form free radicals and deplete glutathione stores [42,43]. Conversely, acetaminophen can also be considered an antioxidant due to its ability to protect membranes from lipid oxidation by scavenging peroxyl radicals and peroxynitrite [44]. In addition to its actions against hydroxyl radicals and hydrogen peroxide, acetaminophen has also been shown to be efficacious against peroxynitrate [45], myeloperoxidase [46], cyclooxygenase [47], and other peroxides [48]. Acetaminophen provides cardioprotection to the postischemic, reperfused guinea pig myocardium [49,50]. Given before or during ischemia-reperfusion periods, acetaminophen treatment produced positive inotrophic effects, lower coronary perfusion pressure, lower coronary vascular resistance, retention or recovery of a greater percentage of left ventricular function, preservation of myofibrillar ultrastructure, attenuation of bursts of hydroxyl radicals during reperfusion, reduced release of creatine kinase during ischemia-reperfusion, reduced production of peroxynitrate, attenuation of damaging effects of peroxynitrate and hydrogen peroxide via protein oxidation, and reduced perturbations of myocardial electrical stability.

In conclusion, increases in ROS (reactive oxygen species) may represent the underlying mechanism of iron-induced cardiac and tissue damage. Therefore, the reduction of excess cardiac and tissue iron accumulations should combat the iron-associated organ and tissue damage. Previous observations suggest that acetaminophen provides significant functional and structural cardiac protection, probably by its antioxidant properties [26]. Therefore, the antioxidant characteristics of acetaminophen might provide cardioprotection and prevent tissue or organ damage in a number of conditions such as increased iron concentrations, which induce the release of free radicals and other oxidants [1]. In the present study, it seems likely that acetaminophen may have provided cardioprotection by dual mechanisms: (a) its antioxidant effects and (b) the removal of excess cardiac iron.

Speculative mechanisms of iron-binding by acetaminophen. Possible mechanisms of iron removal include (a) direct chelation or complexing of acetaminophen with iron (ferrous and ferric?) and (b) reaction of ferric iron with [NAPQI•] (N-acetyl-p-benzoquinoneimine), the hepatic toxic metabolite of acetaminophen, to form iron-NAPQI complexes. NAPQI normally reacts with sulfhydryl groups of glutathione to form a nontoxic complex. However, at very high levels, excess NAPQI, not detoxified by glutathione, may react with sulfhydryl groups of hepatic proteins to form covalent bonds, which lead to cellular necrosis and death [51]. If iron is capable of reacting with NAPQI to form a nontoxic complex, protection may be provided.

Future areas for study. That acetaminophen is able to mobilize excess iron from the heart and other organs is a novel observation. Acetaminophen has been used as an analgesic-antipyretic agent for many decades, so that much is known about its therapeutic and toxic characteristics. The demonstration that acetaminophen is orally effective as an iron removing agent is potentially important, since there is a shortage of approved oral agents that are therapeutically effective in iron removal. However, knowledge in key areas regarding acetaminophen removal of iron is lacking. These areas must be investigated to ensure the clinical safety and therapeutic efficacy of acetaminophen in its iron-removing role before human clinical trials can be recommended. The areas include: (a) mechanisms (chelation or complexing) of acetaminophen binding to iron and mobilization of excess tissue iron, (b) routes of elimination (urinary or biliary) of acetaminophen-iron complexes, and (c) studies to determine why acetaminophen is less effective in mobilizing iron from some organs (liver, spleen) than others (heart, kidney) and whether acetaminophen binding to hepatic iron is associated with redistribution of soluble iron chelates.

Acknowledgements

The authors thank Romaine R. Perdue, Lisa Hunt, and Lucy Dornon for assistance in the echocardiographic procedures on gerbils and interpretation of ECHO data. The authors thank Drs. Rameez

Sayyed, Imran Arif, Shewit Weldetensae, and A. Betts Carpenter for advice and assistance. The study was supported in part by the Cardiovascular Research Fund, Joan C. Edwards Medical School, Marshall University. Phillips Sonos Corp. kindly loaned the S12 transducer used in this study. Ernest M. Walker, Jr., holds US Patent #6,509,380 (2002): Method of treating iron overload with acetaminophen [26].

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