Milk whey protein decreases oxygen free radical production in a murine model of chronic iron-overload cardiomyopathy

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BACKGROUND: Chronic iron overload is a major cause of organ failure worldwide, but its pathogenesis remains to be elucidated.
OBJECTIVES: To examine in an experimental murine model of iron-overload cardiomyopathy the relation between milk whey protein and, first, the production of reactive oxygen free radical species and, second, antioxidant reserve status.
METHODS: B6D2F1 mice were randomly assigned to four treatment groups (n=8 per treatment group): placebo control; iron only; whey only; and iron with whey. Reactive oxygen free radical species in the heart were quantified by the cytotoxic aldehydes malondialdehyde (MDA), 4-hydroxy-nonenal (HNE) and hexanal, while antioxidant reserve status was quantified by glutathione (GSH) and glutathione peroxidase (GPx) activity in the heart tissue.
RESULTS: Significantly decreased concentrations (pmol/100 mg wet weight tissue) of MDA (2468±261), HNE (912±38) and hexanal (5385±927) were observed in the heart tissue of the group receiving iron with whey, in comparison with the iron-only treatment group (MDA 9307±387, HNE 1416±157, hexanal 14,874±2955; P<0.001). Significantly increased GPx (141±38 IU/L) and GSH (521±136 IU/L) activity were observed in mice receiving iron with whey, in comparison with mice receiving iron only (GPx 100±10 IU/L, GSH 446±33 IU/L; P<0.001).
CONCLUSION: Mice receiving iron treatments with whey supplementation had significantly lower concentrations of cytotoxic aldehydes and significantly higher cardiac levels of GPx and GSH activity than did iron-only treated mice. Additional basic research is warranted to examine the exact mechanisms by which milk whey protein protects the heart.

Key Words: *Antioxidants; Cardiomyopathy; Free radicals; Iron overload; Whey protein*

La protéine de lactosérum réduit la production de radicaux libres dans un modèle murin de cardiomyopathie par surcharge en fer chronique

HISTORIQUE : La surcharge en fer chronique est une cause importante d'insuffisance cardiaque de par le monde, mais sa pathogenèse demeure inconnue.
OBJECTIFS : Examiner, dans un modèle murin de cardiomyopathie par surcharge en fer, le lien entre la protéine de lactosérum et, d'abord la production des espèces de radicaux libres réactifs, puis le statut des réserves d'antioxydants.
MÉTHODOLOGIE : Des souris B6D2F1 ont été réparties au hasard entre quatre groupes de traitement (n=8 par groupe) : placebo, fer seulement, lactosérum seulement et fer et lactosérum. Les espèces de radicaux libres réactifs dans le cœur ont été quantifiées par la malone dialdéhyde des aldéhydes cytotoxiques (MDA), le 4-hydroxy-nonénal (HNE) et l'hexanal, tandis que le statut des réserves d'antioxydants l'a été selon l'activité de la glutathione (GSH) et de la glutathione péroxydase (GPx) dans le tissu cardiaque.
RÉSULTATS : Des concentrations considérablement plus faibles (pmol/100 mg de tissus de poids frais) de MDA (2 468±261), de HNE (912±38) et d'hexanal (5 385±927) ont été observées dans le tissu cardiaque du groupe recevant du fer et du lactosérum par rapport à celles du groupe ne recevant que du fer (MDA de 9 307±387, HNE de 1 416±157, hexanal de 14 874±2 955; P<0,001). Une augmentation marquée de l'activité de la GPx (141±38 UI/L) et de la GSH (521±136 UI/L) a été observée chez les souris recevant du fer et du lactosérum par rapport à celles ne recevant que du fer (GPx de 100±10 UI/L, GSH de 446±33 UI/L; P<0,001).
CONCLUSION : Les souris qui recevaient des traitements de fer accompagnés de suppléments de lactosérum présentaient des concentrations considérablement plus faibles d'aldéhydes cytotoxiques et des taux cardiaques d'activité GPx et GSH considérablement plus élevés que les souris traitées seulement au fer. Des recherches fondamentales supplémentaires s'imposent pour examiner les mécanismes exacts selon lesquels le lactosérum protège le cœur.

Iron is an essential element required by the human body for metabolic functions such as oxygen transport and is used by the cell as a catalyst for redox reactions required for energy production. Conversely, in excess quantities and unbound from proteins, 'free-iron' is highly cytotoxic (1-4). Iron toxicity in the heart tissue can lead to iron-overload cardiomyopathy, a prevalent cause worldwide of death due to heart failure in the second and third decades of life (5-7).

Hereditary (primary) hemochromatosis is the most common autosomal recessive disorder worldwide, with one in 200 white persons homozygous for the disease (8-10). Approximately 30% of patients with chronic iron overload die of iron-induced cardiac complications including heart failure and arrhythmias (6,9,11,12). The risk of developing iron-induced heart failure is also present in patients with secondary forms of hemochromatosis (for example, beta-thalassemia major

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and sickle cell disease) due to the chronic blood transfusion (5,6,13,14). African iron overload (Bantu siderosis) is a distinct form of iron overload that results from excessive ingestion of large amounts of traditional beer (kaffir) containing iron (8,9,15) and is estimated to affect up to 10% of the rural populations in sub-Saharan Africa.

Despite the prevalence of iron-overload disorders worldwide, the exact mechanism of iron-induced heart failure remains to be elucidated. The toxicity of nontransferrin-bound free-iron is thought to be a result of its ability to catalyze the conversion of hydrogen peroxide (H_2O_2), through Fenton-type or iron-catalyzed Haber-Weiss biochemical reactions, to reactive oxygen free radical species (ROS), such as the hydroxyl ($OH\bullet$) and superoxide radicals ($O_2^{-}\bullet$), as shown below (13,16-18):

$$O_2^{-}\bullet + \text{ferric iron } (Fe^{3+}) \rightarrow O_2 + Fe^{2+}$$
$$\text{Ferrous iron } (Fe^{2+}) + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH\bullet$$
$$\overline{O2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^{-} + OH\bullet}$$

It is conjectured that chronic states of iron overload result in increased ROS generation and decreased protective antioxidant reserves (such as vitamin E and glutathione peroxidase [GPx]), resulting in cellular injury and dysfunction (4,19-21). Indeed, free radicals are highly reactive biochemical species that contain unpaired electrons and have the ability to damage numerous macromolecules including cellular membranes, proteins and DNA (4,21).

Whey is a nontoxic protein concentrate from milk that includes alpha-lactalbumin, beta-lactalbumin and lactoferrin and is believed to have the ability to chelate trace metals, including iron (22,23). Preliminary evidence suggests that milk whey protein concentrate may possess beneficial antioxidant properties that can limit the production of harmful ROS by acting as a precursor source for glutathione (GSH) production in relevant tissues, thus also having immunoenhancing, anticarcinogenesis and anticancer properties (22,24-29).

However, no previous studies, to our knowledge, have examined the cardioprotective effects of milk whey protein on ROS production and antioxidant reserve status in an experimental model of iron-overload cardiomyopathy (30). Therefore, we first hypothesized that iron-overloaded mice receiving dietary supplementation with whey protein would have significantly decreased ROS production in the heart, as quantified by malondialdehyde (MDA), 4-hydroxy-nonenal (HNE) and hexanal, in comparison with nonsupplemented iron-overloaded mice. Our second hypothesis was that the iron-overloaded mice receiving whey supplementation would have significantly increased antioxidant reserve status in cardiac tissue, as quantified by GPx and GSH activity, in comparison with nonsupplemented iron-overloaded mice.

ANIMALS AND METHODS

The authors' laboratory developed a murine model of iron-overload cardiomyopathy that mimics the iron-loading pattern in the heart of patients with disorders of iron metabolism. This model was used for the experiment described below. The specific hemodynamic, biochemical and histopathological characteristics of this model have been described elsewhere (20,31-33). Male B6D2F1 mice (Charles River, Canada) five to eight weeks of age and weighing 20 g to 25 g were housed in stainless steel cages (five per cage) in a temperature- and humidity-controlled room with 12 h light 12 h dark cycles. The mice had access to water and food pellets (Laboratory Rodent Diet, PMI Nutrition International Inc, United States) ad libitum. The study received institutional approval (Queen's University Animal Care Committee) and conformed to the standards of the Animals for Research Act (Province of Ontario 1968-69, revised 1980) and the Canadian Council on Animal Care (34). All chemicals used were reagent grade and were obtained from the Sigma-Aldrich Chemical Company (St Louis, United States), unless otherwise stated.

Thirty-two mice were randomly assigned to one of four treatment groups: placebo control (0.5 mL normal saline intraperitoneally per mouse per day, n=8); iron only (10 mg iron dextran intraperitoneally per mouse per day, n=8); whey (Immunocal, IMMUNOTEC Research Corporation, Canada) only (100 mg dissolved in 250 mL distilled water orally, n=8); and iron with whey (n=8). Statistical power analysis previously showed that five mice per treatment group is sufficient to detect differences (effect size 0.35) between groups with a power of 0.80 and an alpha level of 0.05 (20,35). All mice received treatments for a total of four weeks (total cumulative dose of iron dextran: 200 mg). The dose of whey protein administered was based on a pilot investigation using this murine model (30). The dose of iron dextran administered was based on previous investigations with this murine model of iron-overload cardiomyopathy (20,31). Moreover, it was previously shown that the administration of iron dextran intraperitoneally for a period of three to four weeks results in significantly increased heart tissue concentrations of iron with accompanying histopathological changes to cellular morphology and altered cardiac function (20,31,32,35). Following their treatment period, surviving mice were killed by cervical dislocation and the hearts were harvested by rapid midsternal thoracotomy, cleared of excess tissue and dipped into a bath of liquid nitrogen. The hearts were subsequently stored at −75°C until analysis for ROS and antioxidant activity.

Oxygen free radical production

Oxygen free radical production in the heart was quantified by analysis of the cytotoxic aldehydes MDA, HNE and hexanal by capillary column gas chromatography-negative ionization mass spectrometry (31). Samples were compared with commercially available standards for control of accuracy and precision (32,35). This method has a detection limit between 50 fmol/mL and 100 fmol/mL of injected aldehyde (36,37).

Heart GPx and GSH activity

GPx activity was quantified by scanning fluorescence spectrophotometry, according to previously described methods (20,38). GSH was assessed according to the method of Leeuwenburgh et al (39). Samples were compared with commercially available standards (Randox Laboratories, United Kingdom) for control of accuracy and precision.

Descriptive statistics for the key end points (MDA, HNE, hexanal, GPx and GSH) are presented as mean ± SD. A two-step procedure was used for the data analysis. One-way analysis of variance was performed to compare overall treatment effect, and $P<0.05$ was deemed significant a priori. Second, when a statistically significant difference was observed, post hoc multiple pairwise comparisons were performed to determine the location and nature of the difference after analysis of variance (40).

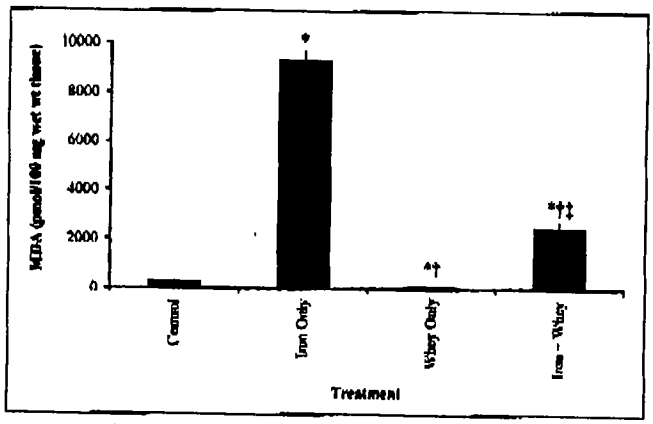

Figure 1) *Heart malondialdehyde (MDA) concentrations. Significantly lower MDA concentrations were observed in mice receiving iron with whey supplementation than in mice receiving iron only. All values shown are mean ± SD. *P<0.001 compared with control; †P<0.001 compared with iron only; ‡P<0.001 compared with whey only*

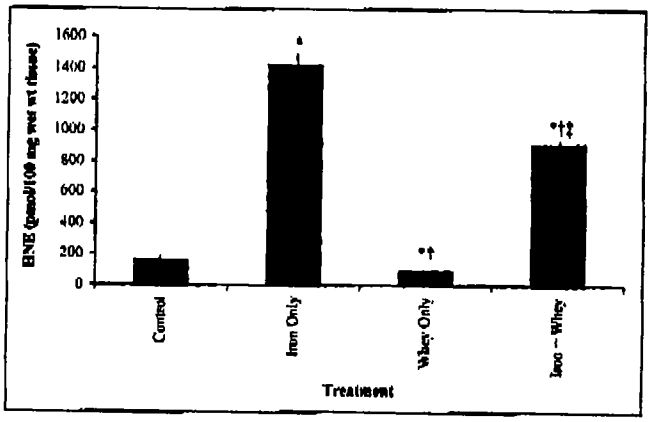

Figure 2) *Heart 4-hydroxy-nonenal (HNE) concentrations. Significantly lower HNE concentrations were observed in mice receiving iron with whey supplementation than in mice receiving iron only. All values shown are mean ± SD. *P<0.001 compared with control; †P<0.001 compared with iron only; ‡P<0.001 compared with whey only*

RESULTS

The mice receiving the iron-only treatment had slower growth rates. Their fur lost shine and colour, and was coarser than in other treatment groups. The hearts, livers and spleens of mice in the iron-only treatment group appeared significantly enlarged and had a pronounced bronze discolouration compared with all other treatment groups, which is consistent with previous investigations in this model (20,31,32,35). Moreover, the mice seemed to become progressively lethargic with increasing total iron burden (32,35).

The murine group receiving the iron-only treatment had a mortality rate of 40%. Conversely, no deaths were observed in placebo controls, mice supplemented with whey only or iron-loaded mice receiving supplementation with whey. No significant differences for either caloric intake from food pellets or oral intake of drinking fluids were observed between the various treatment groups.

MDA concentrations

MDA concentrations in the heart tissue of the various treatment groups are shown in Figure 1. Compared with placebo controls, iron-only mice had a 36.5-fold increase in MDA concentrations, whereas the combined iron and whey treated mice had only a 9.7-fold increase (P<0.001) in MDA concentrations. Furthermore, the iron and whey supplemented group had a 3.8-fold decrease (P<0.001) in heart tissue concentrations of MDA compared with the iron-only group. Significantly lower (P<0.001) concentrations of MDA were also found in the heart tissue of the whey-only group than in the iron-only group. The whey-only group had a significantly lower (P<0.001) concentration of MDA than did the saline control group.

HNE concentrations

Figure 2 shows the concentration of HNE by assigned treatment. In comparison with the control group, iron-only treated mice had a ninefold increase (P<0.001) in heart tissue concentrations of HNE, whereas the iron and whey supplemented mice had only a 5.8-fold increase (P<0.001) in heart tissue concentrations of HNE. In comparison with the iron-only treated mice, mice that received the whey supplementation had a 1.6-fold decrease (P<0.001) in heart tissue concentration of HNE. The whey-only group had a significantly lower (P<0.001) concentration of HNE in the heart tissue than did the saline control group and a significantly lower concentration (P<0.001) of HNE than did the iron-only treated mice.

Hexanal concentrations

Figure 3 shows concentrations of the cytotoxic aldehyde hexanal in heart by assigned treatment. In comparison with the control group, the iron-only treated mice had a 120-fold increase (P<0.001) in the concentration of hexanal in the cardiac tissue, whereas only a 43.4-fold increase in hexanal (P<0.001) concentration in cardiac tissue was observed in the iron and whey supplemented mice. In comparison with the iron-only treated mice, the iron-loaded mice receiving the whey supplementation had a significant 2.8-fold decrease (P<0.001) in hexanal concentration in the heart tissue. The whey-only treated mice had a significantly lower concentration (P<0.001) of cytotoxic hexanal in the heart tissue than did both the saline and the iron-only treated groups.

GPx and GSH activity

Heart GPx activity by assigned treatment is shown in Figure 4. In comparison with saline controls, iron-only treated mice had a 1.7-fold decrease (P<0.001) in GPx activity, whereas the combined iron and whey treated mice had a 1.2-fold decrease (P=0.05) in GPx activity. Furthermore, the iron-treated mice receiving whey supplementation had a significant 1.4-fold increase (P<0.005) in GPx activity in the heart tissue compared with the iron-only treated mice.

Figure 5 shows heart GSH concentrations in the control, iron only, whey only, and iron with whey treatment groups. In comparison with the placebo group, the iron-only group had a 1.5-fold decrease (P<0.001) in GSH concentration, while there was a 1.3-fold decrease in GSH concentration in the heart tissue of the combine iron and whey treated mice (P=0.007). A 1.2-fold increase (P=0.05) in GSH activity was observed in the iron and whey supplemented mice in comparison with the iron-only group.

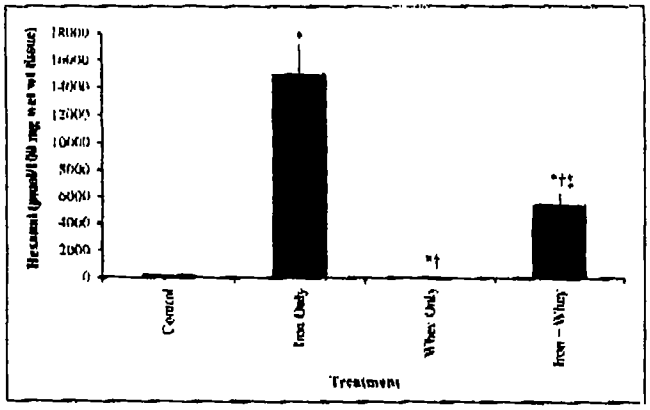

Figure 3) *Heart hexanal concentrations. Significantly lower hexanal concentrations were observed in mice receiving iron with whey supplementation than in mice receiving iron only. All values shown are mean ± SD. *P<0.001 compared with control; †P<0.001 compared with iron only; ‡P<0.001 compared with whey only*

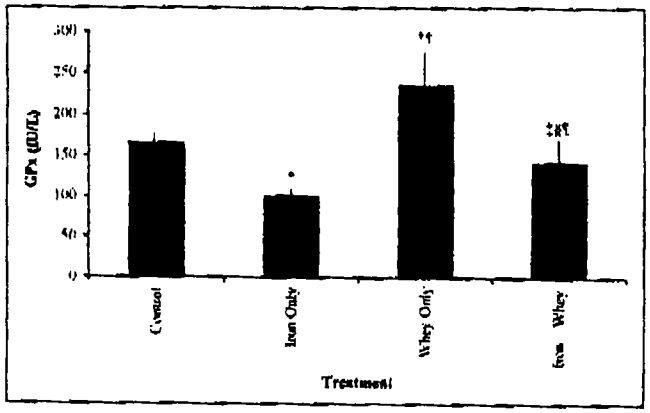

Figure 4) *Heart glutathione peroxidase (GPx) activity. Significantly decreased GPx activity was observed in mice receiving iron with whey supplementation compared to mice receiving iron only. All values shown are mean ± SD. *P<0.001 compared with control; †P<0.001 compared with iron only; ‡P<0.001 compared with whey only; §P<0.005 compared with iron only; ¶P<0.05 compared with control*

DISCUSSION

Although the exact mechanism of iron-induced heart failure remains to be explained, nonprotein-bound free iron may have a role as a result of its ability to catalyze the production of excessive ROS when antioxidant defence systems are overtaxed (21,41,42). The resulting cellular damage that free radicals inflict on the affected organs is thought to be responsible for the dysfunction of the organ. Excess ROS production has been implicated in the pathogenesis of numerous disorders, including cardiovascular disease (1,43-45). An improved understanding of the pathogenesis of iron-induced heart failure is essential for the development of therapeutic interventions that would help to stabilize and ultimately prevent clinical complications in patients who are affected by disorders of iron metabolism. Furthermore, a fundamental understanding of the underlying mechanisms of iron toxicosis is necessary to provide safe and effective patient care.

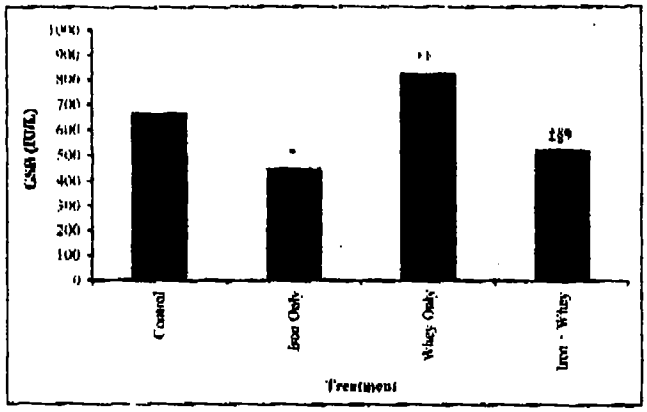

Figure 5) *Heart glutathione (GSH) concentrations. Significantly decreased GSH activity was observed in mice receiving iron with whey supplementation compared to mice receiving iron only. All values shown are mean ± SD. *P<0.001 compared with control; †P<0.001 compared with iron only; ‡P<0.001 compared with whey only; §P=0.05 compared with iron only; ¶P=0.007 compared with control*

Aldehydes such as MDA, HNE and hexanal are cytotoxic products that are formed by the reaction of ROS with proteins and lipid membranes (46,47). They are therefore used as biological markers for ROS-mediated damage and activity in vivo. Hence, an increase in cytotoxic heart aldehyde concentrations in our murine model implies that ROS-mediated activity and damage to the heart have recently increased. In the present investigation, we observed significant increases in all heart cytotoxic aldehydes measured in the iron-only treated group. In support of the first hypothesis, the findings show that the iron-treated mice receiving oral dietary supplement with milk whey protein have significantly lower ROS-mediated-damage to the heart. To our knowledge, this is the first study to examine the effects of milk whey protein supplementation on the production of ROS in an in vivo model of iron-overload cardiomyopathy.

The mechanism by which whey proteins may have cardioprotective effects against ROS includes its ability to chelate transition metals by lactoferrin (48). Among the whey proteins lactoferrin is regarded as the most significant iron-binding protein (49). It has also been reported that beta-lactoglobulin can inactivate pro-oxidative heme proteins, forming dityrosine the oxidation product of two tyrosines, thus suggesting that whey proteins can scavenge ROS (50).

GSH is a cysteine-containing tripeptide that is part of an important cellular defence mechanism against ROS (51,52). GSH helps to maintain the cellular redox state of protein thiol and additional low molecular mass protective antioxidants (such as vitamin E and ascorbic acid) (53,54). GPx is a selenoprotein that is critical in preventing the generation of ROS through Fenton-type reactions by scavenging H_2O_2 and other organic peroxides in the soluble component of cells (55,56).

In support of the second hypothesis, significantly higher levels of GPx activity were observed in the iron with whey supplemented group than in the iron-only group. Heart GSH activity was lower in iron-only treated mice than in iron-loaded mice receiving supplementation with milk whey protein, but this difference did not reach the level of statistical significance. Nonetheless, these findings suggest that milk whey protein may be important in preserving protection

antioxidant reserves in the iron-loaded heart. To our knowledge, this is the first study to examine the effects of milk whey protein on the preservation of GSH and GPx in the iron-loaded heart.

Taken together, the findings provide preliminary evidence that milk whey proteins have cardioprotective properties against iron-mediated ROS damage in a murine model of iron overload. The data suggest that dietary supplementation with milk whey protein may be of benefit for the clinical management of iron-overload disorders. Nonetheless, these findings cannot be extrapolated to patients with iron-induced heart failure because additional basic research is needed to clarify the exact mechanism by which milk whey protein may be cardioprotective in iron-overload disorders. Future research to investigate the dose-dependent effects of milk whey supplementation and the iron-binding capacity of specific milk whey proteins (for example, lactoferrin and albumin) is warranted. It would be premature to recommend dietary supplementation with milk whey proteins for patients with disorders of iron metabolism because additional basic and clinical investigations are necessary before guidelines for clinical practice can be developed.

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REFERENCES

1. Chau LY. Iron and atherosclerosis. Proc Natl Sci Counc China B 2000;24:151-5.
2. Hershko C, Link G, Cabantchik I. Pathophysiology of iron overload. Ann NY Acad Sci 1998;850:191-201.
3. Kehrer JP. The Haber-Weiss reaction and the mechanisms of toxicity. Toxicology 2000;149:43-50.
4. McCord JM. Effects of iron status at a cellular level. Nutr Rev 1996;54:85-8.
5. Aldouri MA, Hoffbrand AV, Flynn DM, Ward SE, Agnew JE, Hilson AJW. High incidence of cardiomyopathy in beta-thalassemia patients receiving regular transfusions and iron chelation: Reversal by intensified chelation. Acta Haematol 1994;84:113-23.
6. Liu P, Olivieri N. Iron overload cardiomyopathies: New insights into an old disease. Cardiovasc Drugs Ther 1994;8:101-10.
7. Olivieri NF, Nathan DG, MacMillan JH, et al. Survival in medically treated patients with homozygous beta-thalassemia. N Engl J Med 1994;304:319-24.
8. Andrews NC. Disorders of iron metabolism. N Engl J Med 1999;341:1986-95.
9. Bothwell TH, MacPhail AP. Hereditary hemochromatosis: Etiologic, pathologic, and clinical aspects. Semin Hematol 1998;35:55-71.
10. Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nature Genet 1996;13:399-408.
11. Adams PC, Valberg LS. Evolving expression of hereditary hemochromatosis. Semin Liver Dis 1996;16:47-54.
12. Madani TA, Bormanis J. Reversible severe hereditary hemochromatotic cardiomyopathy. Can J Cardiol 1997;13:391-4.
13. Lynch SR. Iron-overload: Prevalence and impact on health. Nutr Rev 1995;53:255-60.
14. Olivieri NF. The β-thalassemias. N Engl J Med 1999;341:99-109.
15. Gordeuk VR. Hereditary and nutritional iron overload. Baillieres Clin Haematol 1992;5:164-86.
16. Burkitt MJ, Mason RP. Direct evidence for in-vivo hydroxyl generation in experimental iron overload: An ESP spin-trapping investigation. Proc Natl Acad Sci USA 1991;88:8440-4.
17. Byler RM, Sherman NA, Wallner JS, Horwitz LD. Hydrogen peroxide cytotoxicity is iron dependent. Am J Physiol 1994;35:H121-7.
18. Demougeot C, Marie C, Beley A. Importance of iron location in iron-induced hydroxyl radical production by brain slices. Life Sci 2000;67:399-410.
19. Bartfay WJ, Bartfay E. Systemic oxygen-free radical production in iron overloaded mice. West J Nurs Res 2000;22:927-35.
20. Bartfay WJ, Butany J, Sole MJ, Hou D, Bartfay E, Liu PP. A biochemical, histochemical, and electron microscopic study on the effects of iron-overloading in heart of mice. Cardiovasc Pathol 1999;8:305-14.
21. Lesnefsky EJ. Tissue iron overload and mechanisms of iron catalyzed oxidative injury. Adv Exp Med Biol 1994;366:129-46.
22. Tong LM, Sasaki S, McClements DJ, Decker EA. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. J Agric Food Chem 2000;48:1473-8.
23. Vegarud GE, Langsrud T, Svenning C. Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. Br J Nutr 2000;84(Suppl 1):S91-8.
24. Bounos G. Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. Anticancer Res 2000;20:4785-92.
25. McIntosh GH, Regester GO, Le Leu RKK, Royle PJ, Smithers GW. Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. J Nutr 1995;125:809-16.
26. Zommara M, Toubo H, Sakono M, Imaizumi K. Prevention of peroxidative stress in rats fed on a low vitamin E-containing diet by supplementation with a fermented bovine milk whey preparation: Effect of lactic acid and β-lactoglobulin on the antiperoxidative action. Biosci Biotechnol Biochem 1998;62:710-7.
27. Micke P, Beeh KM, Schlaak JF, Buhl R. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. Eur J Clin Invest 2001;31:171-8.
28. Hakkak R, Korourian S, Ronis MJ, Johnston JM, Badger TM. Diets containing whey proteins or soy protein isolate protect against 7,12-dimethylbenz(a)anthracene-induced mammary tumors in female rats. Cancer Epidemiol Biomarkers Prev 2001;9:113-7.
29. Papenburg R, Bounos G, Fleiszer D, Gold P. Dietary milk proteins inhibit the development of dimethylhydrazine-induced malignancy. Tumor Biol 1990;11:129-36.
30. Davis MT, Bartfay WJ, Medves JM. Milk whey protein decreases oxygen free radical production in a murine model of chronic iron-overload cardiomyopathy. The 32nd Annual Heart & Stroke Clinical Update, Heart & Stroke Foundation. Toronto, December 6-8, 2001. (Abst)
31. Bartfay WJ, Bartfay E. Iron-overload cardiomyopathy: Evidence for a free radical mediated mechanism of injury and dysfunction in a murine model. Biol Res Nur 2000;2:1-11.
32. Bartfay WJ, Hou D, Brittenham GM, et al. The synergistic effects of vitamin E and selenium in iron-overloaded mouse hearts. Can J Cardiol 2000;14:937-41.
33. Bartfay WJ, Lehotay D, Hou D, Backx P, Liu PP. Cytotoxic aldehyde generation in heart following acute iron-loading. J Trace Elem Biol Med 2000;4:14-20.
34. Olfert ED, Cross BM, McWilliams AA. Canadian Council on Animal Care: Guide to Care and Use of Experimental Animals, 2nd edn. Ottawa: Bradda Printing Services, 1993.
35. Bartfay WJ, Dagwood FD, Wen WH, et al. Cardiac function and cytotoxic aldehyde production in a murine model of chronic iron-overload. Cardiovasc Res 1999;43:892-900.
36. Luo XP, Yazdanpanah M, Bhooi N, Lehotay DC. Determination of aldehydes and other lipid peroxidation products in biological samples by gas chromatography-mass spectrometry. Anal Biochem 1995;228:294-8.
37. Yazdanpanah M, Luo XP, Lau R, Greenberg M, Fisher LJ, Lehotay DC. Cytotoxic aldehydes as possible markers for childhood cancer. Free Radic Biol Med 1997;23:870-8.
38. McMaster D, Bell N, Anderson P, Love AH. Automated measurements of two indicators of human selenium status and applicability to population study. Clin Chem 1990;36:211-6.
39. Leeuwenburgh C, Fiebig R, Chandwaney R, Ji LL. Aging and exercise training in skeletal muscles. Response of glutathione and antioxidant enzymes systems. Am J Physiol 1994;36:R439-45.
40. Montgomery DC. Design and Analysis of Experiments, 3rd edn. Toronto: John Wiley & Sons, 1990.
41. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic

metal ions in human disease: An overview. Methods Enzymol 1990;186:1-85.
42. Halliwell B, Gutteridge JMC. Free radicals and antioxidant protection: Mechanisms and significance in toxicity and disease. Hum Toxicol 1988;7:7-13.
43. Ball A, Sole MJ. Oxidative stress and pathogenesis of heart failure. Cardiol Clin 1998;16:665-75.
44. Halliwell B. Antioxidants and human disease: A general introduction. Nutr Rev 1997;55:S44-55.
45. Singal PK, Khaper N, Palace V, Khumer D. The role of oxidative stress in the genesis of heart disease. Cardiovasc Res 1998;40:426-32.
46. Cheeseman KH. Mechanisms and effects of lipid peroxidation. Mol Aspects Med 1993;14:191-7.
47. Esterbauer H. Cytotoxicity and genotoxicity of lipid-peroxidation products. Am J Clin Nutr 1993;57:779S-86S.
48. Gutteridge JMC, Paterson SK, Segal AW, Halliwell B. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem J 1981;199:259-61.
49. Nagasko Y, Saito H, Tamura Y, Shimamura S, Tomita M. Iron-binding properties of bovine lactoferrin in iron rich solution. J Dairy Sci 1993;76:1876-81.
50. Osdal H, Daneshvar B, Skibsted LH. Reduction of ferrylmyoglobin by β-lactoglobulin. Free Radic Res 1996;24:429-38.
51. Halliwell B. Free radicals and antioxidants: A personal view. Nutr Rev 1994;52:253-65.
52. Sagara Y, Dargusch R, Chambers D, Davis J, Schuben D, Maher P. Cellular mechanisms of resistance to chronic oxidative stress. Free Radic Biol Med 1998;24:1375-89.
53. Bast A, Haenen GR. Regulation of lipid peroxidation by glutathione and lipoic acid: Involvement of liver microsomal vitamin E free radical reductase. Adv Exp Med Biol 1990;264:111-6.
54. Meister A. Glutathione deficiency produced by inhibition of its synthesis and its reversal: Application in research and therapy. Pharmacol Ther 1991;51:155-94.
55. Bartfay WJ, Hou D, Lehotay DC, Bartfay E, Luo XP, Liu PP. Cardioprotective effects of selenium and morin hydrate in a murine model of chronic iron overload. J Trace Elem Exp Med 2000;13:285-97.
56. Michiel C, Raes M, Toussaint O, Remacle J. Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. Free Radic Biol Med 1994;17:235-48.