Iron Chelation by Dibasic Amino Acid Prevents Glycoprotein Insolubilities: A Strategy to Inhibit Age-Related Macular Degeneration?

William H. Waugh, MD

Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, North Carolina

KEY WORDS: macular degeneration, endothelium, iron homeostasis, arginine chelation, endosomes

ABSTRACT

Nephelometric assays of human Cohn plasma protein fraction IV-1 (rich in glycoproteins and lipids) were performed at protein concentrations of 40 mg/dL of saline solutions after incubations with 40 µmol/L concentrations of H_2O_2. Induced turbidities were related to complete turbidities or insolubilities caused by 1.5 g/dL sulfosalicylic acid. Turbidity was induced when H_2O_2 was incubated with 100 µmol/L ferrous ion levels. Mean protein insolubilities of 7.28 ± 0.33% (n=12) (P<0.001) were induced when the ferrous sulfate reacted with 40 µmol/L H_2O_2. H_2O_2 alone at 40 µmol/L did not induce turbidities. Of 6 amino acids tested at acidity close to that in active endosomes and lysosomes, only arginine and lysine, by binding to iron inhibited markedly protein insolubilities, induced by micromolar Fenton-type oxidative reactions. Histidine inhibited lessly. The inhibition was mediated by oxidation-reduction chelation of bivalent iron by dibasic amino acid. The findings give credence to the hypothesis that Fenton-type oxidative reactions in the choroid vasculature and retinal pigment epithelium may be important early in the pathogenesis of age-related macular degeneration. Macronutritional trial with arginine or its precursor, citrulline, may be warranted in this disease.

INTRODUCTION

An estimated ten million Americans have age-related macular degeneration (AMD). About 30% of individuals over 75 years of age have early AMD in predominately white populations.[1,2] Causes of AMD are ill-defined. AMD may be an exaggerated stage of the normal process of senescence, which affects the eyes of all individuals with time.[2] Most cases may be due to vascular sclerosis and obliteration of capillaries in the capillary layer of the choroid (choriocapillaris). AMD may be due to dysfunctional capillary endothelium with a changing equilibrium between the rate of production of metabolic waste and its rate of removal.[3,4,5] There is an hypothesis that oxidative stress leads to AMD, but it is unclear what the precise linkage may be between oxidation-induced events and the onset and progression of AMD.[4,6] AMD may result primarily from oxidative injury to the retinal pig-

ment epithelium (RPE) or primarily from oxidative injury to the choriocapillary endothelium.[6]

Whatever the primary cause of injury in this maculopathy or of the hemodynamic change in the choroidal vasculature, there is strong evidence that a major lesion in AMD resides in the RPE with its high rate of molecular degradation and its gradual and progressive accumulation of molecular debris.[2] The debris results from *abnormal* molecules within the RPE cells, partly from phagocytized rods and cones membranes.[2] RPE cells are in continual flux and these cells destroy an enormous quantity of cytoskeletal membrane material shed from the tips of adjacent rods and cones.[2]

Absorptive pinocytosis, endocytosis, and secondary phagosomes are involved in this process and molecular degradation does not always go to completion.[2] Abnormalities in the choriocapillaris or Bruch's membrane may contribute to the progressive deterioration of the RPE.[2] Importantly, the digestive problems of the RPE cells are believed to be due to the harmful effects of oxygen metabolism, which causes oxidative stress and progressively engorged indigestible residues in residual bodies and lysosomes in the RPE and may result in choriocapillaris atrophy.[2,6]

In AMD, extracellular insoluble deposits termed drusen accumulate in Bruch's membrane between the basal membrane of the RPE and the basement membrane of the choriocapillaris.[2,6] Many insoluble glycoprotein and lipid complexes are present in the drusen. These complexes include apoprotein E, amyloid P component, Alzheimer's amyloid beta peptide component, and other plasma and cytomembrane proteins.[7,8]

Very important to the hypothesis of oxidative stress and free radical damage in AMD,[2,6] increased iron concentrations were recently reported both in RPE and in Bruch's membrane in maculas from patients who had drusen only, geographic macular atrophy, and exudative AMD in pathologic areas.[9] Some of the excess iron was chelatable with deferoxamine. Hahn et al made the novel suggestion that iron overload may be involved in the early pathogenesis of AMD rather than solely a result of late events in the disease process.[9] Hahn et al concluded that iron chelation warrants investigation as potential therapy for AMD.[9]

In the presence of free or loosely bound bivalent iron intracellularly and extracellularly, hydrogen peroxide (H_2O_2) can react very devastatingly by forming hydroxyl free radicals ($OH^{\bullet}$) by Fenton-type chemistry: H_2O_2 + Fe II → $OH^{\bullet}$ + OH^- + Fe III.[10] Hydroxyl free radical reactions are intrinsically site-specific at sites where iron is bound in Fenton-generated oxidations.[10,11] It is very likely that $OH^{\bullet}$ radicals are the most damaging free radicals in AMD.

Therefore, in order to study the possible interaction of bivalent iron and H_2O_2 to cause, at low micromolar concentrations, oxidative denaturation of glycoprotein components synergistically by Fenton-type reactions, the following model experiments were performed at moderate acidity. The acidity present in activated endosomes and lysosomes is between a pH of 5 and 6 and said to be near a pH of 5.6 in brain endosomes during iron homeostasis.[12] The results lead credence to the conclusion of Hahn et al that bivalent iron, in causing oxidative stress, may be important in the pathogenesis of AMD.[9]

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from Sigma: Human plasma Cohn protein fraction IV-1 (predominately alpha glycoglobulins and rich in lipids),[13] L-arginine free base, L-histidine free base,

Table 1. Percentages of Potential Insolubilities Induced by Reactions of Micromolar Levels of Ferrous Salt and Hydrogen Peroxide in Cohn Fraction IV-1 Protein Solutions In Vitro at Acidic pH in Absence and Presence of Amino Acid or EDTA*

Solute added before reaction of 100 µmol/L $FeSO_4$ + 40 µmo/L H_2O_2	% Turbidity values
None (control)	7.28 ± 0.33 (n=12)
L-Arginine, 400 µmol/L	0.00 ± 0.00 (n=4)†
L-Lysine, 400 µmol/L	0.00 ± 0.00 (n=4)†
Ethylenediaminetetraacetic acid disodium salt, 400 µmol/L	0.00 ± 0.00 (n=4)†
L-Arginine, 300 µmol/L	0.56 ± 0.29 (n=4)†
L-Lysine, 300 µmol/L	0.86 ± 0.42 (n=4)†
L-Histidine, 400 µmol/L	3.35 ± 0.17 (n=4)†
L-Glutamine, 400 µmol/L	7.46 ± 0.54 (n=4) NS
L-Citrulline, 400 µmol/L	7.39 ± 0.48 (n=4) NS
Glycine, 400 µmol/L	8.34 ± 0.32 (n=4) NS

*Mixtures were incubated at 37˚C for 30 minutes. All solutions contained 40 mg/dL of fraction IV-1 proteins in 140 mmol/L NaCl solutions, pH near 5.8, unbuffered except for the contained proteins. The low mass solutes were mixed with brief vortexing within 1 to 3 minutes before addition of the $FeSO_4$ and H_2O_2 aliquots at room temperature. Maximal possible induced turbidities were measured and placed at 100% after addition of sulfosalicylic acid to 1.5 g/dL to similar protein solutions kept at room temperature for 30 minutes. Values are mean ± SEM. Significance of mean difference from control: †$P < 0.001$; NS indicates not significant.

L-lysine free base, L-citrulline, L-glutamine, ethylenediaminetetraacetic acid disodium salt, and 5-sulfosalicylic acid. Glycine was purchased from Aldrich. 30 weight % H_2O_2 was purchased from Sigma-Aldrich. Ferrous sulfate • 7 H_2O, sodium chloride, and sodium bicarbonate of analytical grade were used. Glass distilled H_2O was also employed.

Methods

Test solutions of the protein fraction were prepared by dissolving the Cohn fraction IV-1 to 40 mg/dL in saline solutions of 140 mmol/L NaCl alone. The resulting pHs of the solutions were 5.7 to 5.8. As an index of denaturation change to insolubility, induced turbidities were measured in a Coleman Model 9 Nepho-Colorimeter, which measured the intensity of reflected white Tydall light. Reaction volumes of 10 mL were used in Coleman cuvets. Liquid turbidity was assayed (after brief vortexing) before and then after addition of H_2O_2 up to 40 µmol/L alone or with antecedent addition of $FeSO_4$ to 100 µmol/L concentration followed by 30-minute incubations of the cuvets at 37˚C. In select experiments, 300 µmol/L or 400 µmol/L of amino acid or 400 µmol/L of $EDTANa_2$ was included before the additions of ferrous salt and H_2O_2 to the saline-protein solutions. Maximal possible denaturations by nephelometry were standardized, using the unit valves as 100% that were found in control saline-protein fraction solutions without inclusion of ferrous salt or H_2O_2 and kept at room temperature for 30 minutes after

addition of sulfosallcylic acid to 1.5 g/dL.

Statistical Analysis

Data are reported as means ± SEM and accompanying n values refer to the number of determinations. Unpaired Student's two-tailed t-tests were used, with significance considered at *P* values of 0.05 or less.

RESULTS

Table 1 shows the turbidimetric results that developed in Cohn fraction IV-1 protein solutions in reaction to 100 µmol/L ferrous ion and 40 µmol/L H_2O_2. Induced protein turbidities averaged 7.28 ± 0.33% of the total protein potentially denaturable and were made insoluble by sulfosalicylic acid. These turbidity changes were *completely prevented* by the antecedent presence of 400 µmol/L of L-arginine or of 400 µmol/L L-lysine, or of 400 µmol/L of the chelating agent, $EDTANa_2$. These levels were fourfold greater than the 100 µmol/L concentrations used of free ferrous ion. Similar greater concentrations of L-histidine (400 µmol/L) inhibited the denaturations only about half as much. Induced turbidity in the presence of histidine averaged 3.35 ± 0.17% compared to the mean control turbidity of 7.28%. L-glutamine, L-citrulline, and glycine at levels of 400 µmol/L did not inhibit the protein insolubilities induced by 100 µmol/L $FeSO_4$ and 40 µmol/L H_2O_2.

At threefold greater concentrations than the employed 100 µmol/L concentrations of ferrous salt, both L-arginine and L-lysine exhibited marked but incomplete inhibitory effects. Inhibitions averaged 92% ([7.28-0.56]/ 7.28%) and 88% ([7.28-0.86]/ 7.28%) when 300 µmol/L levels of these 2 amino acids were used in the presence of 100 µmol/L ferrous ion (Table 1). Incubations of control protein solutions with 40 µmol/L H_2O_2 in the absence of $FeSO_4$ (n=12) failed to induce protein turbidities.

DISCUSSION

Insoluble proteinaceous deposits associated with excess iron deposits are present intracellularly and extracellularly in maculopathic areas in AMD.[2,9] Hahn et al suggested recently that excess iron accumulation within the RPE and Bruch's membrane causes RPE damage and death with secondary photoreceptor degeneration, related perhaps to oxidative damage from Fenton-type reactions which produce $OH^{\bullet}$ free radicals.[9] Most of the excessive non-heme iron found by Hahn et al[9] was likely ferritin iron.

Ferritin is normally a cytoprotective stratagem for cells including endothelium to protect against oxidant damage through iron sequestration in the trivalent state. However, oxidative stress in formation of superoxide free radicals and H_2O_2 may cause release of iron in the reduced ferrous form from protective ferritin molecules. Potential devastating damage from $OH^{\bullet}$ radicals may result.[10,11]

The interiors of endosomes after absorptive pinocytosis and of active lysosomes are acidified relative to the rest of cells and to the extracellular environment.[12] Increased by acidification, Fenton-type oxidations are pH dependent upon available free bivalent iron ions.[14] The 40 mg/dL concentrations of Cohn fraction IV-1 protein solutions used in this Fenton chemistry study in saline solutions had pH's of 5.7 to 5.8, without adjustment of pH by confounding buffer like Hepes which scavenges $OH^{\bullet}$.[15] Cohn fraction IV-1 protein lots are rich in alpha glycoproteins and lipoproteins and the plasma proteins are separated at pH of 5.2.[13,16]

Part of the oxidative stress in AMD may originate from circulating blood and from endothelium cells in the lamina choriocapillaris. H_2O_2 is present in circulating human plasma at levels of about 2.5 to over 55 µmol/L.[17-19] Select plasma apolipoproteins with cholesterol

and body iron overloads are risk factors in atherogenesis.[20] AMD has a positive association with cardiovascular disease.[21] Transport of cell iron for metabolic uses or else for storage as trivalent iron inside ferritin molecules takes place presumably by binding of vesicular bivalent iron cations to a permeant labile chelator to transit vesicular walls without ensuing oxidative stress normally.[12,22] The chemical nature of this metabolically active iron pool is ill-defined. The iron might be complexed with some amino acids, citrate, sugars, or nucleotides.[23] This labile pool of transit or chelatable iron is said to have appearances of which are "somewhat like the Loch Ness monster, only to disappear from view before its presence or indeed its nature, can be confirmed."[24]

This investigator suggests that dysfunction in endocytosis of macromolecules and proper transport of iron and/or from cell digestive lysosomes may result in undue Fenton-type oxidative stress and oxidative injury as seminal events in the causation of AMD. Notably, threefold or greater micromolar concentrations of L-arginine or of L-lysine inhibited markedly or prevented glycoprotein insolubilities from micromolar levels of bivalent iron conjointly with low levels of H_2O_2 in vitro (Table 1).

The available low molecular weight or labile pool of free bivalent iron inside cells is estimated to be no greater than about 1 µmol/L.[25] The usual cytosolic concentrations of dibasic amino acid may be rate-limiting for proper iron homeostasis in the retina and choroid normally and they may be defective in AMD.

The endothelial cell concentrations of L-arginine are reported to be normally about 2 to 4 mmol/L.[26] However, this intracellular pool of arginine can be depleted during periods of prolonged release of nitric oxide as endothelial-derived relaxing factor.[26] This depleted state might pertain in the endothelium of the lamina choriocapillaris particularly in aging in some individuals. Notably, plasma arginine and lysine levels tend to decline with human aging.[27,28] Endothelial–derived nitric oxide from L-arginine as substrate is inactivated by free radicals.[29] Furthermore, low-density lipoprotein increases endothelial release of $O_2^{\bullet}$.[30]

It is remarkable that micromolar concentrations of arginine and of lysine inhibited markedly or *prevented* glycoprotein insolubilities, which were induced by micromolar levels of ferrous ions and H_2O_2 in this study. Histidine exhibited lesser inhibititory effect while glycine, glutamine, and citrulline were not effective (Table 1). Visible yellow hues readily developed in the reaction solutions soon after the short arginine and lysine mixing periods, even before the addition of H_2O_2 aliquots. The visible change to yellow (xanthochromia) indicated a change of hydrated ferrous ions to ferric ions caused by charge transfer to ligand.[31] The changes occurred by coordinate complexing in oxidation-reduction reaction with arginine or lysine as polydentate ligand, with iron change to its typical yellow ferric color.[31] Subsequently, these glycoprotein solutions containing iron at moderate acidity were resistant to insolubilities in the presence of 40 µmol/L H_2O_2 (Table 1). In such acidity, arginine and lysine are protonated and react with trivalent iron with 3 functional groups for ready coordination or chelation. The pK_3 values of arginine and lysine of 12.5 and 10.8, respectively, are much greater than the pK_3 of histidine of 6.0 as another dibasic amino acid.[32]

The results give credence to the hypothesis of Hahn and co-workers that iron-induced oxidative damage by ferrous ions reacting with H_2O_2 is involved importantly in the pathogenesis of AMD.[9] The oxidant damage may occur

chiefly at mild to moderate acidic conditions with dysfunction of iron in endosomes and/or lysosomes under dibasic amino acid insufficiency. Macronutrient supplementation with L-arginine or with its much more efficient precursor given orally, L-citrulline.[33,34] may warrant clinical investigation in AMD because of 2 justifications: 1) to increase available plasma levels in the choroid vasculature in order to support vasodilator tone via the endothelial L-arginine-nitric oxide pathway during aging and 2) to make more cytoplasmic L-arginine available inside choriocapillary cells and RPE cells, in order to support endosomal and lysosomal functions as chelatable ligand to bivalent iron cations. Moderate supplementation with lysine might also be warranted for investigation (Table 1). However, histidine supplementation as support should probably not be tested in patients with AMD since histidine as chelated carrier of ferrous ions through cell membranes is prooxidant.[35]

Although Hahn et al suggested that investigation with synthetic iron chelators might be investigated as therapy in AMD,[9] serious concerns or caveats exist with use of synthetic chelators in iron overload states. Such chelators may have prooxidant adverse effects.[36] At the present time, AMD is the chief cause of severe and irreversible vision loss in developed countries.[37]

ADDENDUM

Since the preparation of this report, it is well to point out that citrulline taken orally to support higher available plasma arginine levels may decrease rates of choroidal neovascularization in patients with nonneovascular or "dry" AMD. Citrulline supplementation has been given to humans without adverse side-effects[33,34] and L-citrulline largely bypasses liver uptake and liver removal from circulating blood in contrast to dietary or parenteral uptake of L-arginine.[38]

ACKNOWLEDGEMENTS

This research was not supported by intramural or extramural grants. The investigator was in emeritus status and the experiments were done in a home laboratory.

REFERENCES

1. O'Connell SR, Bressler NM. Age-related macular degeneration. In: Regillo CD, Brown GC, Flynn HW Jr, eds. *Vitreoretinal Disease The Essentials.* New York, NY: Thieme;1999:213-240.
2. Young RW. Pathophysiology of age-related macular degeneration. *Survey Ophthamol.* 1987;31:291-306.
3. Sarks SH. Ageing and degeneration in the macular region: a clinico-pathological study. *Brit J Ophthal.* 1996;60:324-340.
4. Cho E, Hung S, Seddon JM. Nutrition. In: Berger JW, Fine SL, Maqure MG, eds. *Age-Related Macular Degeneration.* St Louis, Mo: Mosby; 1999:57-67.
5. Friedman E. Pathogenesis: a hemodynamic model. In: Berger, JW, Fine SL, Maquire MG, eds. *Age-Related Macular Degeneration.* St. Louis, Mo: Mosby; 1999:173-178.
6. Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. *Molecular Vis*. 1999;5:32-41.
7. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *FASEB J.* 2000;14:835-843.
8. Johnson LV, Leitner WP, Rivest AJ, Staples MK, Radeke MJ, Anderson DH. The Alzheimer's A□-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2002;99:11830-11835.
9. Hahn P, Milam AH, Dunaief JL. Maculas affected by age-related macular degeneration contain increased chelatable iron in the retinal pigment epithelium and Bruch's membrane. *Arch Ophthalmol.* 2003;121:1099-1105.
10. Fridovich I. The biology of oxygen radicals: general concepts. In: Halliwell B, ed. *Oxygen Radicals and Tissue Injury.* Bethesda, Md: FASEB; 1988:1-5.
11. Borg DC, Schaich KM. Iron and iron-derived radicals. In: Halliwell B, ed. *Oxygen Radicals*

and Tissue Injury. Bethesda, Md: FASEB; 1988:20-26.

12. Aisen P. Entry of iron into cells: a new role for the transferrin receptor in modulating iron release from transferrin. *Ann Neurol.* 1992;32:S62-S68.
13. Seibert FB, Pfaff ML, Seibert MV. A serum polysaccharide in tuberculosis and carcinoma. *Arch Biochem.* 1948;18:279-295.
14. Hardwick TJ. The rate constant of the reaction between ferrous ions and hydrogen peroxide in acid solution. *Canad J Chem.* 1957;35:428-436.
15. Puppo A, Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. *Biochem J.* 1988;249:185-190.
16. Green AA, Hughes WL. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. In: Colowick SP, Kaplan, NO, eds. *Methods in Enzymology.* Vol. 1. New York, NY: Academic Press; 1955:67-90.
17. Varma SD, Devamanoharan PS. Hydrogen peroxide in human blood. *Free Rad Res Comms.* 1991;14:125-131.
18. Smielecki J, Wykretowicz A, Minczykowski A, Kazmierczak M, Wysocki H. The influence of electrical cardioversion on superoxide anions ($O_2^{\bullet}$) production by polymorphonuclear neutrophils, hydrogen peroxide (H_2O_2) plasma level and malondialdehyde serum concentration. *Intl J Cardiol.* 1996;55:137-143.
19. Lacy F, O'conner DT, Schmit-Schonbein GW. Plasma hydrogen peroxide production in hypertensive and normotensive subjects at genetic risk of hypertension. *J Hypertens.* 1998;16:291-303.
20. De Valk B, Marx JJM. Iron, atherosclerosis and ischemic heart disease. *Arch Intern Med.* 1999;159:1542-1548.
21. Hyman LG, Lilienfeld AM, Ferris FL III, Fine SL. Senile macular degeneration: a case-control study. *Am J Epidemiol.* 1983;118:213-237.
22. Roberts R, Sandra A, Siek GC, Lucas JJ, Fine RE. Studies of the mechanism of iron transport across the blood-brain barrier. *Ann Neurol*. 1992;32:S43-S50.
23. Jacobs A. Low molecular weight intracellular iron transport compounds. *Blood*. 1977;50:433-439.
24. Crichton RR, Charloteaux-Wauters M. Iron transport and storage. *Euro J Biochem.* 1987;164:485-506.
25. Williams RJP. Free manganese (II) and iron (II) cations can act as intracellular cell controls. *FSBS Letters.* 1982;140:3-10.
26. Hecker M, Sessa WC, Harris HJ, Anggard, EE, Vane JR. The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: Cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci U S A.* 1990;87:8612-8616.
27. Ackermann PG, Kheim T. Plasma amino acids in young and older adult human subjects. *Clin Chem.* 1964;10:32-40.
28. Moriguti JC, Padovan GJ, Vannucchi H, Marchini JS. Fasting plasma free amino acid of elderly men. *Amino Acids.* 1995;9:46-47.
29. Mugge A, Elwell JH, Peterson TE, Harrison DG. Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. *Am J Physiol.* 1991;260:C219-C225.
30. Prichard KA, Groszek L, Smalley DM, et al. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res.* 1995;77:510-518.
31. Miessler Gl, Tarr DA. *Inorganic Chemistry.* 3rd ed. Upper Saddle River, NJ: Pearson Prentice Hall; 2004:197-198,440-446.
32. Rodwell VW. Amino acids. In: Murray RK, Granner DK, Mayes PA, Rodwell VW, eds. *Harper's Biochemistry.* 24th ed. Stanford, Conn: Appleton & Lange; 1996:23-31.
33. Waugh WH, Daeschner CW III, Files BA, McConnell ME, Strandjord SE. Oral citrulline as arginine precursor may be beneficial in sickle cell disease: early phase two results. *J Natl Med Assoc*. 2001;93:363-371.
34. Waugh WH. Simplified method to assay total plasma peroxidase activity and ferriheme products in sickle cell anemia, with initial results in assessing clinical severity in a trial with citrulline therapy. *J Pediatr Hematol Oncol.* 2003;25:831-834.
35. Winkler P, Schaur RJ, Schawenstein E. Selective promotion of ferrous ion-dependent lipid peroxidation in Ehrlich ascites tumor cells by histidine as compared with other amino acids. *Biochim Biophys Acta.* 1984;796:226-231.
36. Cole CM. Ironic fate: can a banned drug control metal heavies in neurodegenerative diseases? *Neuron.* 2003;37:889-893.
37. Fine SL, Berger JW, Maguire MG, Ho AC. Age-related macular degeneration. *N Engl J Med.* 2000;342:483-491.
38. Waugh WH, Daeschner CW III, Files BA, Gordon DW. Evidence that L-arginine is a key amino acid in sickle cell anemia-a preliminary report. *Nutr. Res.* 1999;19:501-518.