ROLE OF L-ARGININE SUPPLEMENTATION IN PREVENTING OBESITY "ULTRAHISTOCHEMCIAL STUDIES"

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Abstract

Obesity is an important complex and chronic disease. Dietary arginine (2-amino 5-guanidinovaleric acid) supplementation represents a potentially novel and useful strategy for the management of obesity and diabetes. Oral supplementation with L-arginine reduced inflammation and oxidative stress associated with high carbohydrate, high fat feeding in rats. The main goal of the present study was to investigate beneficial effects of L-arginine in the protection against intoxication on altered peroxisomal enzymes: D-amino acid oxidase (D-AAO) and catalase after high fat diet (H.F.D). 20 male Swiss albino rats (8 w old) were divided into two groups. Group 1: 5 rats (control), Group II a: 15 rats fed on H.F.D for 24 w. Group II b: 10 rats orally administered L-argenine in a dose of 622.14 mg/kg body weight /day for 12 weeks. 5 rats from each group were sacrificed and processed for ultrahistochemical studies of D-AAO and catalase enzymes. Our results revealed that in addition to lipid accumulation, the formation of myelin figures are related to impaired lipid degradation. After L-arginine supplementation, we observed that EM sections of liver have shown the formation of a peroxisomal reticulum at early times and the subsequent return of single peroxisome (PO). Also, we showed that following H.F.D, PO underwent significant changes in size, shape, and their contents of D-AAO and catalase. After L-arginine treatment of animals receiving H.F.D, D-AAO and catalase activities returned to nearly control levels. This recovery could be attributed to capability of L-arginine to alter the antioxidant defense system induced by H.F.D.

Keywords: Obesity, L.arg, PO. D-AAO, Catalase, EM

Introduction

Obesity is an important, complex and chronic disease. It is a severe metabolic disorder, characterized with increases in energy intake and a

decrease in energy output concerning body weight and glucose metabolism (Robert et al., 2006).

Fatty diet is a significant factor causing obesity. It is known that obesity is associated with many chronic disorders such as a non-alcoholic fatty liver disease (NAFLD), and many important complications such as diabetes and coronary heart disease (Abu-Abid et al., 2002). It is also associated with insulin resistance and hyperlipidemia (Bailhache et al., 2003). Liver steatosis is a well-known pathology in severely obese patients and is especially associated with visceral adiposity and diabetes (Adams et al., 2005). NAFLD is the most common liver disorder in the world, and in obesity, type 2 diabetes and related metabolic diseases, its incidence reaches 70-90%. The disease is characterized by the accumulation of triacylglycerols inside liver cells, and the condition can progress into more serious liver disease, such as liver fibrosis, cirrhosis, and more rarely, liver carcinoma (Adams and Angulo, 2005).[(5)] Oxidative stress is now believed to be an important factor in the development of non alcoholic fatty liver disease (NAFLD) (Videla et al., 2006).

Previous works have shown that feeding rats a high fat diet (57% of energy from fat) induces hepatic steatosis and liver damage, which are characteristic of NAFLD and thus provides a suitable model for the early stages of the disease (Safwat et al., 2009). In an electron microscopic examination; enlargement of microvilli, arising in number of lysosome, and cytoplasmic swelling were reported (Altunkaynak and Zbek, 2005). A liver damage was observed in rats fed with high-fat diet via increase of liver lipidperoxidation and decreased hepatic antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase).

Among type 2 diabetic patients, 50%-70% of individuals were diagnosed with NAFLD; in obese patients, that value increases to 95% (Bloomgarden, 2005). Prolonged exposure to free fatty acids damages pancreatic β-cells and hepatocytes (Lupi et al., 2002). Furthermore, there was excessive fat accumulation in the liver damages mitochondria, which are the primary cellular sites for fatty acid utilization (Bak et al., 2011).

Arginine (2-amino 5-guanidinovaleric acid) plays a role in the body as a free amino acid and as a component of most proteins, as well as the substrate for several non-proteins; nitrogen-containing compounds. As a free amino acid, arginine functions as an intermediate in the urea cycle. As one of the 20 common α-amino acids, arginine is an integral component of mammalian proteins. As a substrate for several non-proteins, nitrogen-containing compounds, arginine indirectly participates in the rapid regeneration of adenosine triphosphate, cell proliferation, vasodilatation, neurotransmission, calcium release, and ultimately immunity (Nieves and Langkamp-Henken, 2002).

Dietary arginine supplementation represents a potentially novel and useful strategy for the management of obesity and diabetes (Miguez et al. 2004). L-Arginine is an important dietary amino acid in both health and disease, especially of the cardiovascular system. However, supplementation may be needed in special conditions such as malnutrition, excessive ammonia production, burns, infections, peritoneal dialysis, rapid growth, urea synthesis disorders, and/or sepsis (Appleton, 2002). Tousoulis et al, 2007 suggested that L-arginine supplementation may help treat individuals with atherosclerosis risk factors, such as hypercholesterolemia, hypertension, diabetes mellitus, kidney failure, hyperhomocysteinemia, smoking, and aging, all of which are conditions that are associated with reduced NO biosynthesis. It also has several metabolic functions as a substrate in the synthesis of creatine and NO (McConell and Kingwell, 2006).

Peroxisomes (PO) are ubiquitous organelles contributing to various catabolic as well as anabolic pathways, for example, oxidation of long and very long-chain fatty acids, detoxification of reactive oxygen species, synthesis of plasmalogens (Schluter et al. 2007). Among the different tissues investigated so far, liver and kidney house the highest concentrations of these organelles (Islinger et al., 2007).

D-Amino acid oxidase (D-AAO), a FAD-dependent peroxisomal flavoenzyme, catalyzes oxidation of D-amino acids to hydrogen peroxide and expressed in a wide range of species from yeasts to human (Pollegioni et al., 2007; Huang et al., 2012). There is no significant amino acid homology within the substrate binding region of D-AAO which may reflect the broad substrate specificity of this enzyme. High levels of D-AAO expression and enzyme activity are found in the mammalian liver, kidney, and brain (Tishkov and Khoronenkova, 2005). Angermüller et al (2009) showed that D-AAO is significantly more active in preiportal than pericentral rat hepatocytes. Also, it concentrated in the central region of PO matrix compartment.

Catalase is one of the most efficient enzymes known. CAT reacts with H_2O_2 to form water and molecular oxygen; and with H donors using peroxide in a kind of peroxidase activity. H_2O_2 is enzymically catabolized in aerobic organism by catalase and several peroxidases (SOD and GPx) (Pandya et al., 2010).

The main goal of the present study is to investigate beneficial effects of L-arginine on two important enzymes D-AAO and Catalase after H.F.D and to show the role of L-argenine, a nitric oxide precursor, in inducing protection against toxicity of HFD.

Materials & Methods

20 male Swiss albino rats (8 w old) were obtained from Medical Research Institute Animal house unit, Alexandria University. Rats were housed in a temperature and humidity controlled facility on a 12-h light: dark cycle. At 9 w of age rats were divided into two groups.

Group 1: 5 rats considered as control

Group IIa: 15 rats fed on High fat diet (H.F.D) in gradient g/100g diet :(Basic diet 82.8, Lard 10, Cholesterol 0.5, Bile acid 0.5, Propylthiouracil 0.2, AIN 76 Vitamin mix and AIN 76 mineral mix) for 24 w . 5 rats were sacrificed at the end of this period.

Group IIb: 10 rats orally administered L-arg. In a dose of 622.14 mg/kg b.w /day for 12 wk. 5 rats from control and experimental groups were sacrificed and processed for ultrahistochemical studies of two peroxisomal markers:

1- D-amino acid oxidase enzyme (D-AAO) according to the method of Angermüller & Fahimi 1988. This method was summarized in brief: Liver was perfused with 0.9 ml NaCl saline via portal vein for 1 min, then slices from median lobe were fixed for 5 min. in 4F/G plus 2% sucrose were incubated for 60 min. at $37C^0$ in 3mM $CeCl_3$, 100mM Na azide and 10mM D-proline in 0.1M Tris maleate buffer pH 7.8 . Sections were then post fixed in 2% OsO_4 and processed for EM study. Control sections were incubated in the absence of D-proline.

2- Catalase enzyme according to the method of Fahimi & Baumgart 1993. After perfusion and fixation for liver tissue slices were incubated in DAB solution (without H_2O_2) for 1 min., after washing the slices were incubated in DAB solution (With H_2O_2) for 15 min. Slices were washed and intensificated in 0.1M phosphate buffer, pH 7.2 for 5 min, sections were then fixed in 2% OsO_4 and processed for EM study.

Results

Peroxisomes (PO) were numerous in the liver cells of control animals and usually appeared spherical and closely associated with smooth ER and mitochondria. They were filled with enzymes, especially catalase and D-AAO.

1. D-AAO

Rat liver sections were stained for D-AAO using D-proline as a substrate. H_2O_2 released, oxidized Ce^{3+} to cerium perhydroxide (an insoluble electron dense precipitate). Thus the staining intensity for cerium perhydroxide can be used as a marker to quantify the D-AAO activity in individual PO. PO with differential cerium staining appeared to be distributed throughout the whole cytoplasm. A direct association with other

organelles, such as SER or mitochondria was observed (Fig.1, 2). On prolonged administration of experimental diet, numerous lipid droplets appeared. In addition to lipid accumulation, rat liver after 24wk H.F.D. showing decreased activity of D-AAO (Fig.3). There are myelin figures that are related to impaired lipid degradation in the lysosomes in these conditions (Figs 3&4a). These figures originate from cisternal elements of the ER in the hepatocytes of intoxicated rats. After L-arginine supplementation (12wk) we observed that electron microscopic sections of regenerating rat liver showed the formation of peroxisomal reticulum at early times, the subsequent return of single PO and increased D-AAO activity around peroxisomal membranes (Fig.4b).

2. Catalase

In respect to catalase: The specificity of DAB staining of catalase was confirmed by the control reactions in which the specific substrate of the enzyme was omitted. The staining reaction of catalase was moderate in control liver cells, extensively localized in the matrix of PO and on their membranes (Fig 5). Following H.F.D. PO underwent significant changes in size, shape, and their contents of catalase. These alterations were mostly prominent after chronic dose of H.F.D. (24wk) and accompanied with strong catalase staining reaction in the matrix of PO and other organelles which were closely associated with them (SER and mitochondria).(Fig.6) We noticed that the ER and Golgi apparatus were well preserved (Fig.7). L-arginine treatment of animals receiving H.F.D. returned catalase activity to control levels (Figs 8&9b).

Discussion

The peroxisomal B -oxidation system may play an important role in the control of lipid metabolism. It has been calculated that the relative contribution of peroxisomal fatty acid oxidation is no more than 10% to overall hepatic oxidation of the physiologically important fatty acids palmitate and oleate, under normal conditions (Debeer and Mannaerts. 1983). The electron microscopic observations suggested that lipid metabolism may be an important factor in the control of peroxisome number and function in liver cells (Miyazawa et al., 1980).

The changes in the peroxisomal enzyme activities are due to changes in the content in peroxisomal proteins. The 'normal' turnover rate of peroxisomal enzymes is rather low (Miyazawa et al., 1980). In this respect, it was enlightening to observe that antilipolytic agents induce the formation of autophagic vacuoles in liver cells, often containing peroxisomes. Also, Yamamoto & Fahimi (1987) showed that the peroxisomes in regenerating rat liver showed several distinctive features: a) marked variation in shape and

size, e.g., peroxisomes with tail-like extensions and tortuously elongated rod-shaped ones, b) formation of peroxisomal clusters and, c) interconnections between adjacent peroxisomes suggesting cleavage or budding.

Limiting the generation of reactive oxygen species, especially superoxide, hydrogen peroxide, lipid peroxides, hydroxyl radical, and lipid peroxyl radicals, caused limitation in the oxidative inactivation of NO to nitrite, nitrate, peroxynitrite, and lipid peroxynitrites (Gocke et al., 1999).

The mechanisms responsible for the beneficial effects of L-arginine are likely complex, but ultimately involve altering the balance of energy intake and expenditure in favor of fat loss or reduced growth of white adipose tissue (Knight et al., 2010).

Previous studies conducted in different animal models demonstrated that L-arginine reduced generation of superoxide anion ($O_2^{\cdot -}$) and prevented the reduced expression of eNOS (Schneider et al., 2003). The balance between NO and $O_2^{\cdot -}$ plays an important role in the process of many cardiovascular and pulmonary diseases (Sud et al., 2007). Oral administration of L-arginine can also lower PH and prevent right ventricular hypertrophy (Sasaki et al., 2004). Hung et al (2008) have showed that supplementation with L-arginine significantly attenuated pulmonary and cardiac oxidative stress during exhaustive exercise in both young and aging rats.

Other experiments show that L-arginine has direct antioxidant effects and that this is due to the alpha-amino group, a chemical moiety different from that necessary for NO biosynthesis (Lass et al., 2002; Alvares et al., 2011). By simply acting as an antioxidant, L-arginine may scavenge superoxide or restore the crucial cofactor tetrahydrobiopterin (BH_4) and thereby prevent eNOS-mediated superoxide production in an "uncoupled" status. In this area, L-arginine seems to be a good antioxidant couple that has been shown to inhibit the Fenton reaction and thus preventing the formation of hydroxyl radical (Rubbo et al., 2009). Lu et al (2010) showed that D-AAO inhibitors produce analgesia by blocking spinal H_2O_2 production rather than interacting with spinal D-serin. Tripath et al (2009) observed an increase in the activity of SOD and decreased the activity of XO in myocardial ischemic patients on oral administration of L-arginine 3g/day for 7 days

Pharmacological treatment with L-arginine-induced protection against acute stress (Kurhalyuk, 2003), physical loading (Kurhalyuk and Tkachenko, 2006) and low resistance to hypoxia partially by reducing lipid hydroperoxides level (Tkachenko and Kurhalyuk, 2011).

Paddon et al (2004) reported that enhancing arginine availability stimulated muscle protein synthesis without affecting muscle proteolysis in rabbits, thereby decreasing the availability of long-chain fatty acyl-CoA for triglyceride synthesis and of acetyl-CoA for fatty acid synthesis result in an

overall increase in the oxidation of both glucose and fatty acids while possibly decreasing fat deposition.Several studies have also demonstrated that oral L-arginine has a protective function against attacks by reactive oxygen species (ROS), thereby attenuated or normalized obesity-related changes in heart, liver and pancrease (Lanteri et al., 2006; Alvares et al., 2012). In addition, oral l-arginine administration reduced blood pressure in both normo- (Siani et al., 2000) and hyperglycaemic (Huynh & Tayek, 2002) conditions. Mendez & Balderas (2001) noted beneficial l-arginine effects on hyperglycaemia and dyslipidaemia in alloxan-induced diabetes mellitus.

The lower lipolysis may have been due to greater NO production with L-arginine infusion since NO inhibits catecholamine-induced stimulation of lipolysis (Klatt et al., 2000). NO reduces glycerol release from isolated human adipocytes in vitro (Andersson et al., 1999), and also reduce fat mass in obese subjects with type-II diabetes mellitus (Fu et al., 2005). 21 days of oral L-arginine treatment augmented the beneficial effects of a hypocaloric diet and exercise training program on glucose metabolism, insulin sensitivity and markers of oxidative stress in obese type 2 diabetics (Lucotti et al., 2006). Arginine supplementation reduced the weight of abdominal (retroperitoneal) and epididymal adipose tissues (45 and 25% respectively) as well as serum concentrations of glucose (25%), triglycerides (23%), FFA (27%), homocysteine (26%,) dimethylarginines (18–21%), and leptin (32%).

D-AAO is a peroxisomal flavoprotein that catalyses the oxidative deamination of D-amino acids to a-keto acids and the byproduct H_2O_2 without producing ROS (Lu et al., 2012). Also the peroxisomal activity of D-amino acid oxidase may be directly associated with specific metabolic functions of individual hepatocytes. Moreover, Po with significant differences in the D-amino acid oxidase activity can be also frequently observed within the same cell. D-amino acid oxidase shows a heterogeneous distribution in rat liver tissue (Islinger et al., 2010).

Our present findings were in accordance with these previous studies where normal hepatocytes showed different D-AAO activity although there was heterogeneity in its distribution.

Our present results revealed that in addition to lipid accumulation, the formation of myelin figures are related to impaired lipid degradation in the lysosomes in these conditions. These figures originate from cisternal elements of the ER in the hepatocytes of intoxicated rats. After L-arginine supplementation we observed that electron microscopic sections of regenerating rat liver have shown the formation of a peroxisomal reticulum at early times and the subsequent return of single PO. Moreover, the findings revealed that rat liver after 24wk H.F.D. showed a decreased activity of D-

AAO while an increased D-AAO activity around peroxisomal membranes after L-arginine treatment was noted.

Respecting catalase, these alterations were mostly prominent after chronic dose of H.F.D. (24wk) and accompanied with strong catalase staining reaction in the matrix of PO and other organellae which were closely associated with them (SER and mitochondria). Also our results showed that following H. F. D. PO underwent significant changes in size, shape, and their contents of catalase. After L-arginine treatment of animals receiving H.F.D, catalase activity returned to control levels. This could reflect an altering in the antioxidant defense system induced by H.F.D. In accordance with our findings, the activities of SOD and CAT in the liver were significantly ($p < 0.05$) lowered in rats fed with high-fat diet than control group animals. Loss of CAT activity results in oxygen intolerance and triggers a number of deleterious reactions such as protein and DNA oxidation, and cell death (Nakbi et al., 2010).

Also, DeGaener et al (1995) showed that in human fatty liver, PO were found to increase in both number and density, whereas their size and catalase activity were decreased.

Conclusion, it is plausible to assume that L-arginine inhibit the lipid peroxidation process that is known to be triggered by ROS. In another word, L-arginine may have a beneficial prophylactic effect that altered lipid composition of membranes and may results in altered membrane integrity, permeability, and function.

Conflict of interest: There is no conflict of interest

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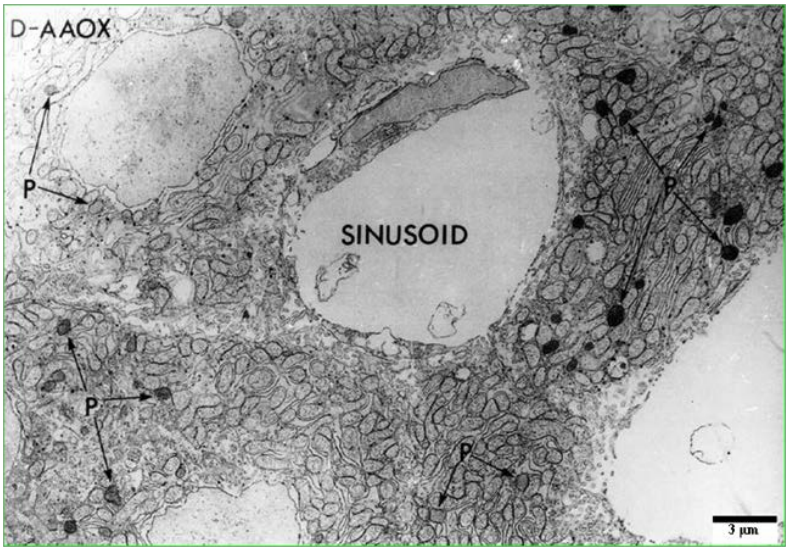

Fig.1: Visualization of D-AAO in ultrathin section of rat liver using the cerium-technique. Note PO (arrows) with different D-AAO activities, those in the left show generally weaker activities, whereas those in the right exhibit a dark stain. Individual PO are marked by arrows bar = 3 □m.

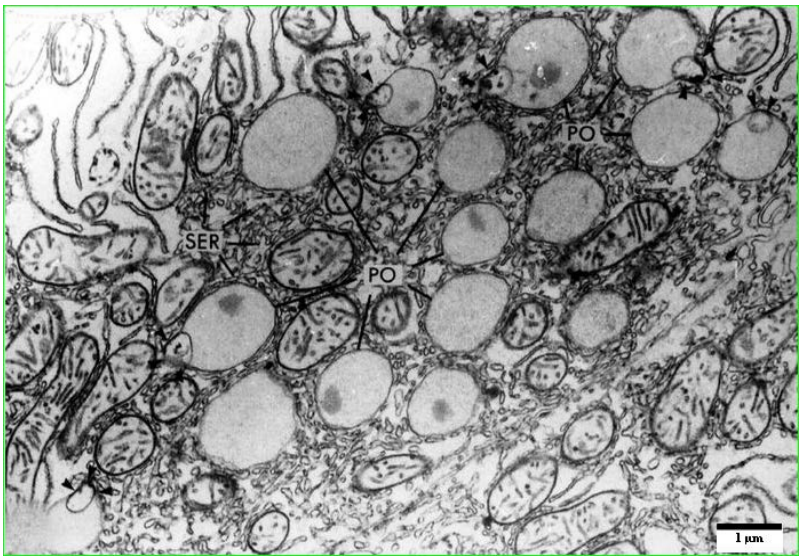

Fig 2: Higher magnification showing intracellular heterogeneity of peroxisomal D-AAO activity (arrow heads) and in smooth endoplasmic reticulum (SER). Note nascent PO budding from a proliferating peroxisomal reticulum (arrow head) bar = 1□□m.

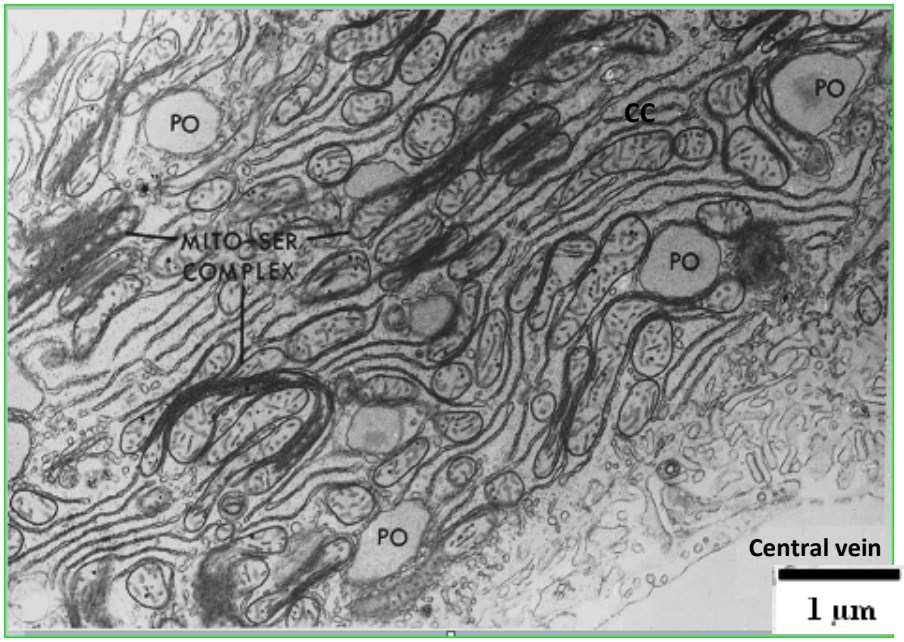

Fig.3: Ultrathin sections of rat liver after 24wk H.F.D. showing decreased activity of D-AAO. Lipid fragments and crystal clefts (cc) were also observed. Bar = 1 □m

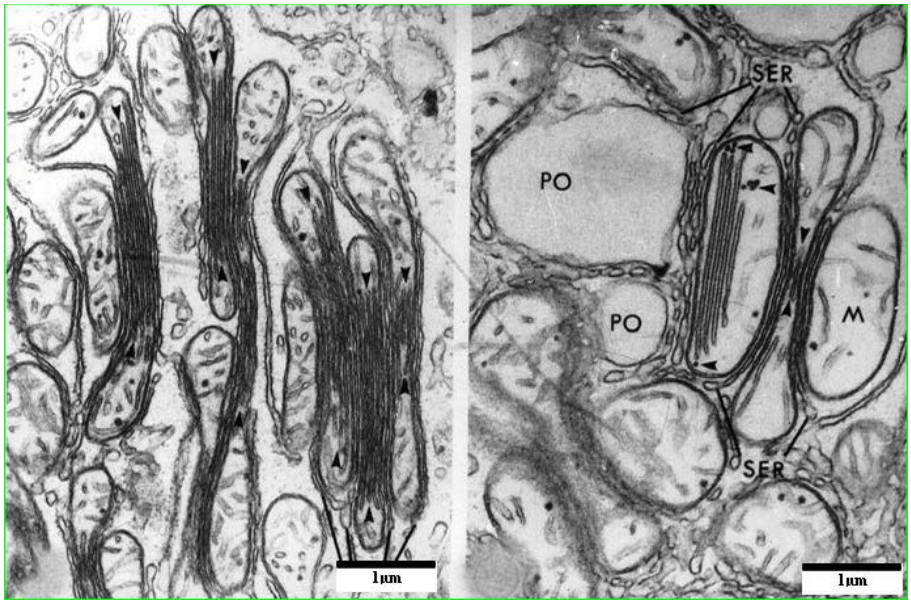

Fig.4a: EM of rat liver 24wk H.F.D showing details of hepatocytes contains myelin figures (arrow heads). Their outer layers are continuous with the dilated ER. Bar = 1 □m

Fig.4b: After L-arg. treatment we observed that regenerating rat liver showing the formation of a peroxisomal reticulum and the subsequent return of single PO. Note traces of myelin figures (arrow head) and increased D-AAO activity around peroxisomal membranes. Bar = 1 □m.

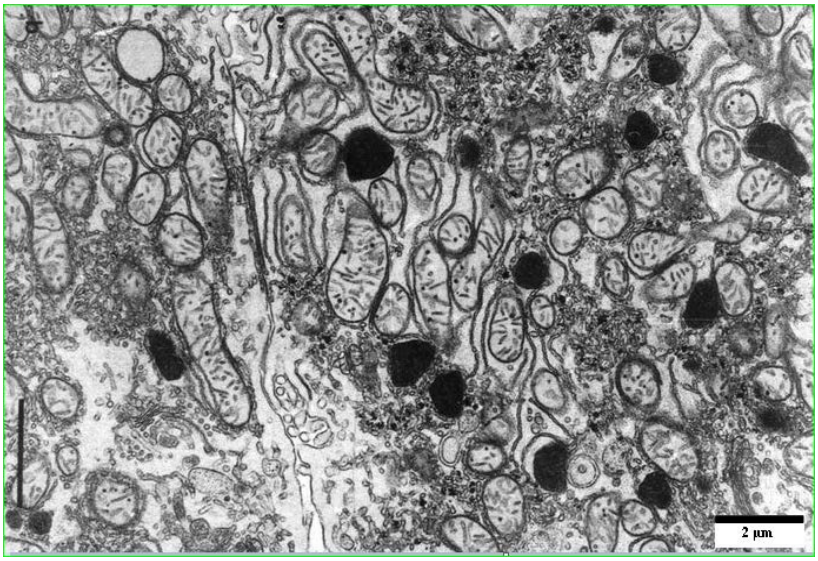

Fig.5: EM of control rat liver showing normal small PO with moderate catalase activity in the matrixand on the membrane of PO. Note mitochondria (M) RER,SER and microtubules(MT) Bar = 2□m.

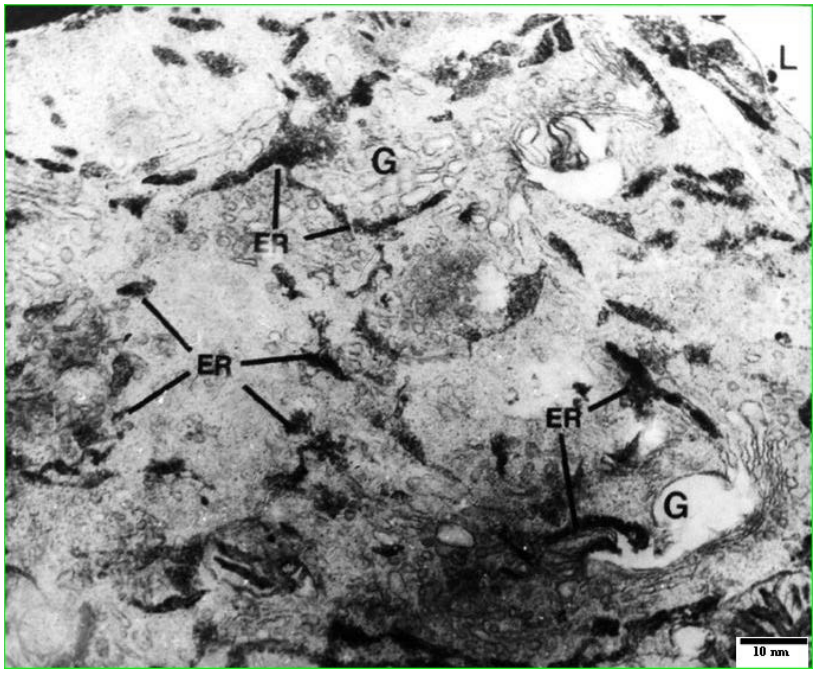

Fig.6: EM of rat liver 24wk H.F.D showing strong catalase activity especially in organelle closely associated with PO. Note also a few lipid droplets (L) in pericanalicular region. Bar = 10 nm.

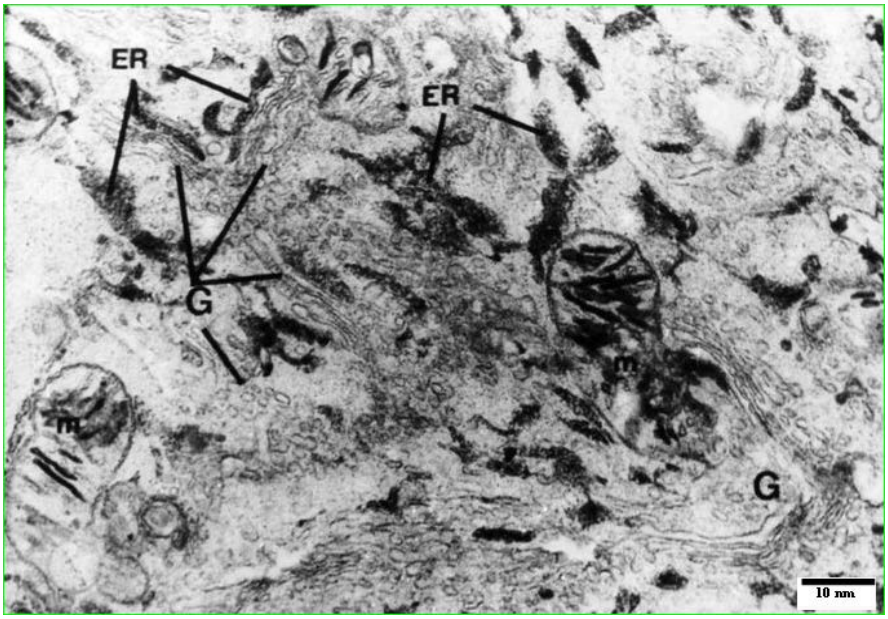

Fig.7: EM of rat liver 24wk H.F.D. Note ER well preserved and Golgi apparatus. Bar = 10 nm.

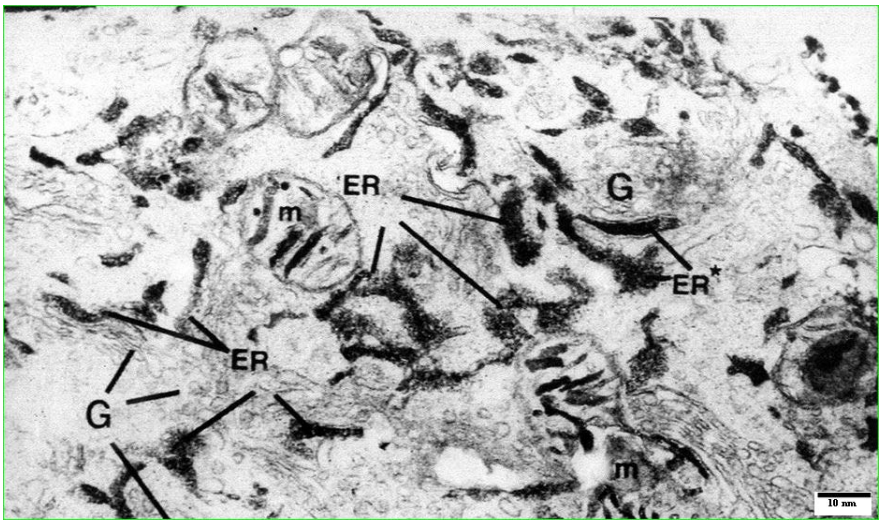

Fig.8: EM of rat liver after L-arg. treatment showing returned catalase activity to control levels. Bar = 10 nm.

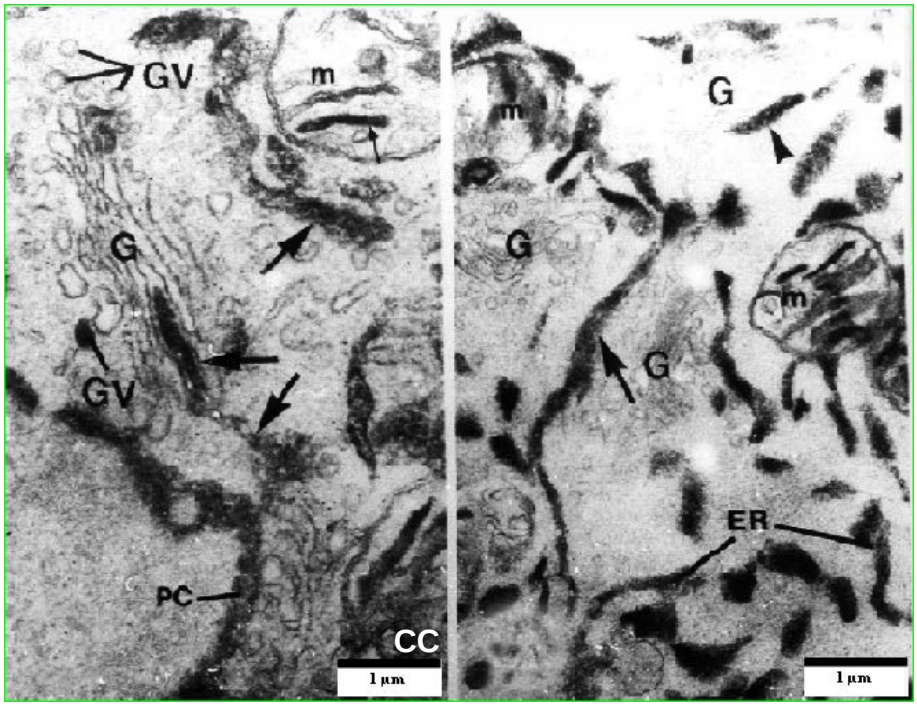

Fig.9a: EM of rat liver (H.F.D.) showing the appearance of crystal clefts (CC) in the cytoplasm. Bar = 1 □m.

Fig.9b: EM of rat liver after L-argenine treatment showing that catalase enzyme returned more or less to control activity. Bar = 1 □m.