

Oxidative damage lipid peroxidation in the kidney of choline-deficient rats

Georgina Ossani¹, Marianela Dalghi² and Marisa Repetto²

¹Centro de Patología Experimental, Departamento de Patología, Facultad de Medicina and ²Laboratorio de Estrés Oxidativo y Canales Iónicos, Cátedra de Química General e Inorgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Spain

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1. ABSTRACT

Phosphatidylcholine is the most abundant phospholipid constituent of cell membranes and choline is a quaternary amine required for phosphatidylcholine synthesis. The impairment of membrane functions is considered as an indication of oxidative damage. In order to kinetically analyze the time course of the pathogenesis of renal necrosis following to choline deficiency in weanling rats, we determined markers of membrane lipid peroxidation (thiobarbituric acid reactive substances; TBARS and hydroperoxide-induced chemiluminescence (BOOH-CL)) and studied the histopathological damage. Plasma TBARS ($t_{1/2}$ = 2.5 days) was an early indicator of systemic oxidative stress, likely involving liver and kidney. The levels of TBARS and BOOH-CL increased by 80 % and by 183 %, respectively, in kidney homogenates with $t_{1/2}$ = 1.5 days and 4 days, respectively. The levels of BOOH-CL were statistically higher in rats fed a choline-deficient diet at day 6, in a mixture of membranes (from plasmatic, smooth and rough endoplasmic reticulum and Golgi), in mitochondrial membranes and in lysosomal membranes. The results indicate that choline deficiency produces oxidative damage in kidney subcellular membranes. Necrosis involved mainly convoluted tubules and appeared with a $t_{1/2}$ = 5.5 days. An increase in the production of reactive oxygen species, triggered by NADH overproduction in the mitochondrial dysfunction associated with choline deficiency appears as one of the pathogenic mechanism of mitochondrial and cellular oxidative damage in choline-deficiency.

2. INTRODUCTION

Cell membranes are formed by a phospholipid bilayer with proteins and carbohydrates in varying proportions. Approximately, and in terms of dry weight, 38-42% of membranes is accounted by phospholipids and 52-60% by constitutive proteins that are linked to phospholipids by non-covalent interactions. Carbohydrates, accounting for 1-10 % of dry weight, are covalently linked to phospholipids and proteins by covalent bonding (1).

Membrane phospholipids are referred as amphipathic, since one part of them is hydrophilic and the other part is hydrophobic. Both parts are linked through a bridging moiety that can be a glycerol, a sphingoid, or a sterol molecule. Amphipathic lipids form the lipid bilayer, with the polar groups exposed at the surface and the hydrophobic chains occupying the interior of the bilayer (2).

The most abundant glycerophospholipid in eukaryotic membrane cell is phosphatidylcholine (1,2-diacyl-sn-glycero-3-phosphocholine) and the main phosphosphingolipid is sphingomyelin (ceramide1-phosphocholine) (3). The lipid composition of eukaryotic cell membranes varies both with their tissue source and subcellular location. Cell membranes include plasma, nuclear, lysosomal, peroxisomal and Golgi membranes as well as the inner and outer mitochondrial membranes and the membranes of rough and smooth endoplasmic reticulum. For example, in rat liver cells, phospholipids represent 60%

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of total lipid composition in plasma and Golgi membranes, 80% in nuclear and rough endoplasmic reticulum membranes and 85% and 90% in outer and inner mitochondrial membranes, respectively; phosphatidylcholine constitutes 45% in plasma and inner mitochondrial membranes, 50% in Golgi membranes and 60% in rough endoplasmic reticulum, nuclear and outer mitochondrial membranes (3).

Each membrane has one or more particular physiological function: the plasma membrane is the site of transport of nutrients into the cell, the mitochondrial inner membrane is the site of oxidative phosphorylation and Golgi membrane participates in glycoprotein biosynthesis and in protein secretion (3).

Choline is a quaternary amine required for the synthesis of phospholipids, phosphatidylcholine and sphingomyelin, that are the main constituents of cell membranes, though betain, it is a source of labile methyl groups and is needed for the synthesis of acetylcholine (4).

It is known that weanling rats fed a choline-deficient diet show morphological alterations in kidneys, liver, brain, heart, and eyes. Renal pathology is associated with acute renal failure and varies from focal tubular necrosis to massive cortical necrosis (5-7), liver alterations are characterized by fatty changes, cirrhosis and eventually cancer (8, 9) and heart damage by necrosis (10, 11).

The process of lipid peroxidation plays an important role in the development of the renal damage. Renal lipid peroxidation was found in the necrotic as well in the pre-necrotic stages of kidneys of choline-deficient weanling rats (12). In addition, antioxidants such as N, N'-diphenyl-p-phenylenediamine (DPPD) (12) or butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (13) have a protective effect.

Reactive oxygen species (ROS), such as superoxide radical anion (O_2^-), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO) are produced as by-products of oxidative metabolism. These chemical species have been implicated in various cell dysfunctions with impairment of the physiological functions of plasma and subcellular membranes. The mechanism of ROS-induced oxidative modifications in cell membranes involves: a) lipid peroxidation of membrane phospholipids with rupture of carbon double bonds and formation of oxidation products, b) inhibition of the activity of membrane-bound enzymes, c) inhibition of mitochondrial respiratory enzymes with impairment of oxidative phosphorylation and decrease of ATP levels, and d) oxidation of sulphhydryl groups located in membrane-bound proteins (14-16).

Mitochondria are the subcellular organelles of eukaryotic cells that produce the energy required to drive the endergonic biochemical processes of cell life, the most important cellular source of free radicals, and the main target for free radical cytotoxic actions. The inner mitochondrial membrane is composed, in terms of dry weight, by 68% of

protein and 32% of lipids, this latter with a 94% of phospholipids. In such way, the biochemical basis of inner mitochondrial membrane structure and function lies in the structural relationship, afforded by non-covalent and hydrophobic bonding, of the phospholipid and protein components of the membrane (17).

In order to study the pathogenesis of the renal damage of weanling rats fed a choline-deficient diet we determined markers of oxidative damage in cellular homogenates and subcellular components of membranes in kidneys of choline-deficient rats as well as in choline-supplemented controls (18-21).

Since chemiluminescence and TBARS determinations in kidney homogenates allow the evaluation of oxy-radical and hydroperoxide generation these measurements were performed in parallel with morphological and biochemical studies in rats fed a choline-deficient diet to establish the kinetic sequence of lipid peroxidation, cytological damage and necrosis.

3. MATERIALS AND METHODS

3.1. Animals

Weanling Wistar male rats from the Center of Experimental Pathology, Department of Pathology, School of Medicine, University of Buenos Aires were divided in two groups. One of them was fed a choline-deficient diet *ad libitum* (Diet 1, Table 1) (n = 16; 4 rats killed on the third day of diet and 12 rats on the sixth day). The other group was fed a choline supplemented diet *ad libitum* as control (Diet 2, table 1) (n = 8; 4 rats sacrificed on the third day of diet and 4 on the sixth day). Body weight was recorded daily. Animals were sacrificed after 3, 5 or 6 days of having choline-deficient or supplemented diets, considering that the renal lesions due to choline deficiency appear after 5 days (12). Animals were anesthetized with sodium pentobarbital (40 mg/kg).

3.2. Histopathology

Both kidneys were removed and weighted, the right kidney and the posterior half of the left kidney were frozen in liquid nitrogen for oxidative stress determinations. The anterior half of the left kidney was fixed in buffered-formalin and embedded in paraffin. Thin sections were cut and stained with hematoxylin and eosin in order to analyze histopathological alterations. Histological classification of renal necrosis includes four categories: A: Kidney without necrosis (grade 0); B: Acute tubular necrosis, with 4 grades, being grade 1, isolated foci of cellular necrosis in some tubules, grade 2, small groups of tubules with necrosis, grade 3, zones of tubular necrosis, and grade 4, confluent zones of tubular necrosis; C: Cortical necrosis, with 4 groups, being grade 5, grade 4 plus isolated foci of cortical necrosis, grade 6, grade 4 plus multiple foci of cortical necrosis, grade 7, grade 4 plus confluent foci of cortical necrosis and grade 8: massive cortical necrosis; and D: repair, that is characterized by different degrees of interstitial fibrosis, tubular atrophy, tubular regeneration, glomerular fibrosis, etc. According to its extension it is also divided into four grades (22).

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Table 1. Composition of diets (g/100g)

Components	Diet 1	Diet 2
Soybean protein ¹	20.0	20.0
Hydrogenated vegetable oil ²	14.3	14.3
Corn oil ³	5.7	5.7
Sucrose	49.5	49.15
Cellulose ⁴	4.0	4.0
Vitamin mixture (without choline) ⁵	4.0	4.0
Salts ⁶	2.0	2.0
L-cystine ⁷	0.5	0.5
Choline chloride	0.0	0.35

¹ Grade II, US Biochem. Corp.; ² Flora Dánica, Buenos Aires; ³ Mazola, Refinerías de Maíz, Buenos Aires; ⁴ Celufi, non-nutritive bulk, U.S. Biochem. Corp.; ⁵ Test diet 234312 57MV; ⁶ Salt Mixture, Wesson modification, U.S. Biochem. Corp.; ⁷ U.S. Biochem. Corp.

3.3. Tissue homogenate preparation

It is necessary to disrupt the outer plasma membrane and to fragment the endoplasmic reticulum in order to release the enclosed mitochondria, lysosomes and nuclei. Cell disruption was controlled to prevent destruction of the subcellular organelles, however, microsomal, Golgi and plasma membranes are broken into fragments or vesicles during homogenization and are recovered by centrifugation of the homogenate.

Kidneys were excised, weighed and tissue homogenates were prepared in a medium consisting of 30 mM phosphate buffer, 120 mM potassium chloride, pH 7.40. The tissue-buffer ratio was 1 g/9 mL of buffer. The homogenates were centrifuged at 600 g for 10 minutes at 4°C to discard nuclei and cell debris. The supernatant, a suspension of mixed and preserved organelles and plasma membrane, was used as homogenate for tert-butyl hydroperoxide-initiated chemiluminescence and malondialdehyde determinations (23).

3.4. Mitochondria, plasma membrane and lysosomes.

Mitochondrial fraction was obtained from the renal cortex, separated from medulla and papilla, homogenized in 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.40. The homogenate (1g organ/9 mL buffer) was centrifuged at 700 g for 10 minutes. The supernatant was separated, the pellet was suspended in the same buffer and the suspension was centrifuged at 8000 rpm for 10 minutes to precipitate the mitochondrial fraction that was washed with the same buffer and centrifugation conditions (24).

Separation of the mitochondrial, lysosomal and plasma membranes with a minimum of contamination from smooth and rough endoplasmic reticulum and Golgi membranes was performed in kidney cortex homogenates by differential centrifugation. Renal cortex was distinguished by its light brown color and separated, in the excised kidney cut in the middle with a razor blade, from the outer stripe of the outer zone of the renal medulla, which is yellow-brown. The cortical tissue was immediately weighed, minced with scissors and diluted 1:9 (w/v) with 0.30 M sucrose. It was homogenized in a Potter-Elvehjem glass homogenizer by 10 complete stokes with a loosely fitting teflon pestle rotation at about 1000 rpm. All tissue fractions were maintained at 0-4°C through the isolation procedure. The homogenate was

first centrifuged at 800 g for 10 minutes to sediment nuclei and unbroken tissue. The supernatant was decanted and centrifuged once again in the same form. The supernatant was divided in 12 mL portions, placed in cellulose nitrate tubes and centrifuged at 10,000 rpm for 30 minutes. The pellet formed during this centrifugation was macroscopically layered and consist of three differently colored layers. The bottom layer was dark brown and was clearly distinguishable from the middle layer, which was yellow-brown. The bottom layer was the semipurified lysosomal fraction and the middle layer the mitochondrial fraction. The top layer which was almost white contained a mixture of plasma membrane with other membranes. The separation of these fractions was a critical step in the procedure and was carried out with a Pasteur pipette and guided by the color difference between the layers. First, the supernatant was carefully removed and the plasma membranes, mainly brush border membranes, was resuspended using a few milliliters of 0.30 M sucrose. Then, when this fraction was removed, a second aliquot of a few milliliters of 0.30 M sucrose was added for resuspending and obtaining the mitochondrial fraction. The last fraction, the brown pellet and the walls of the tubes were rinsed once with 0.30 M sucrose and the residual semipurified lysosomal fraction was finally resuspended in 2.5 mL of 0.30 M sucrose (25). These preparations were used for the assay of the tert-butyl hydroperoxide-initiated chemiluminescence.

3.5. Tert-butyl hydroperoxide-initiated chemiluminescence

Tert-butyl hydroperoxide-initiated chemiluminescence (CL) was measured with a Packard Tri carb model 3355 liquid scintillation counter in the out of coincidence mode. The sample was suspended in 4 mL of 30 mM phosphate buffer, 120 mM potassium chloride, pH 7.40. Vials of 25 mm diameter, 50 mm height low-potassium glass filled with the suspension were checked for background emission. The background in the absence of vials was 2400 ± 60 counts per minutes (cpm) and the emission from the empty vials was 3000 ± 60 counts per minutes. Chemiluminescence measurements were started by the addition of 3 mM tert-butyl hydroperoxide and the counting continued until maximal level of emission was reached. Determinations were carried out at 30°C. The results are expressed as cpm /mg protein (26).

3.6. Malondialdehyde determination

This was estimated by a spectrophotometric assay by determining TBARS (27). This compound binds to malondialdehyde, a secondary product of lipid peroxidation, showing maximal absorbance at 535 nm. The reaction medium consists on 1mL of homogenate (1g organ/9 mL) or plasma, 30 mM phosphate buffer, 120 mM potassium chloride, pH 7.40, buthilhydroxytoluene 4 % w/v in ethanol, trichloroacetic acid 20 % w/v and thiobarbituric acid 0.7 % w/v). The mixture was heated at 100°C for 15 minutes. Absorbance at 535 nm was expressed as nmol/mg protein with $E = 156 \text{ mM}^{-1}\text{cm}^{-1}$.

3.7. Protein measurements

Protein concentration was determined by the method of Lowry *et al.* (28) using bovine serum albumin as standard.

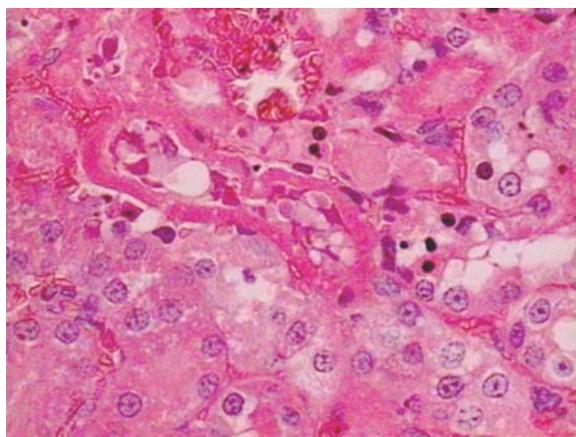


Figure 1. Acute tubular necrosis grade 2 (40X). See text, the picture is representative of kidney histological damage at day 6 after a choline-deficient diet in weanling rats.

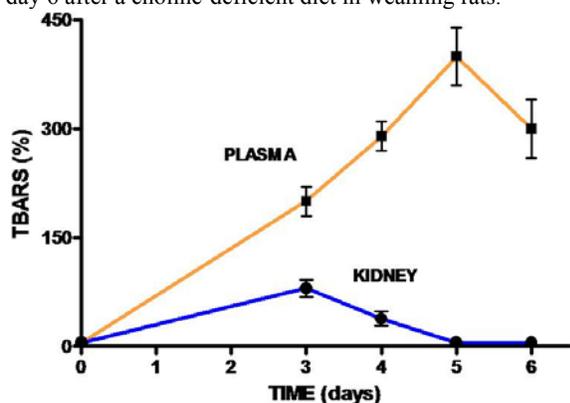


Figure 2. Thiobarbituric acid reactive substance (TBARS) levels in plasma and kidney of weanling rats fed a choline-deficient diet. The orange line with squares (■) represents the percentage of increase in plasma TBARS levels, and the blue line with circles (●) indicate the percentage of increase in kidney homogenates. The abscissa indicates time after weaning. Symbols and bars indicate mean values \pm S.E.M. The normal rat plasma level (1.5 μ M) was increased by 200% at day 3, ($p < 0.02$), 400% at day 5 ($p < 0.04$) and 300% at day 6 ($p < 0.05$). TBARS in kidney homogenates were increased 80% at day 3.

3.8. Chemicals

Thiobarbituric acid, buthylhydroxytoluene and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA), tert-butyl hydroperoxyde was from Aldrich Chem. Co. (Milwaukee, MI, USA). Other reagents were of analytical grade.

3.9. Statistical analysis

Data were expressed as mean \pm standard error of mean (SEM). The Student's t-test for unpaired samples was used for comparison between control and experimental group. Differences were considered statistically significant at $p < 0.05$. The statistical program used was Graphpad Instat, version 4, Windows version of Instat (San Diego, California, USA).

4. RESULTS

The acute choline deficiency in weanling rats produces morphological damage in kidney, which is the most sensitive organ, and in other organs such as liver, heart and brain (5-11). Renal damage was characterized by increased size and weight and by purplish red discoloration. Necrosis involved mainly convoluted tubules and was characterized by pyknosis, kariolysis and increased eosinophilia. Figure 1 shows renal cortex with necrosis grade 2. At the sixth day, from a total of 12 rats: 3 had no necrosis (grade 0), 1 showed isolated foci of cellular necrosis in some tubules (grade 1), 2 had small groups of tubules with necrosis (grade 2), 4 showed zones of tubular necrosis (grade 3), and 2 had confluent zones of tubular necrosis (grade 4). None of the choline-deficient rats showed cortical necrosis at day 6. It is noteworthy that choline-deficient rats did not show histological damage until day 6.

It has been recognized that renal necrosis following to choline deficiency is associated with augmented rates of lipid peroxidation and of oxidative damage (12, 13). The phenomenon reaches systemic significance since choline deficiency was followed by 4-times increased plasma levels of TBARS with a $t_{1/2}$ (the time for half maximal effect) of 2.5 days. The normal rat plasma level (1.5 μ M) was increased by 200% at day 3, 400% at day 5 and 300% at day 6 (Figure 2). The phenomenon is understood as a diffusion of malonaldehyde, the product of lipid peroxidation, from the well perfused kidney to the blood.

The content of TBARS in the kidney homogenates of choline-deficient rats reached the highest values at day 3 with an increase of 80% ($p = 0.0489$) (Table 2 and Figure 3), a condition that returned to normal values at days 5 and 6. It is worth noting that the increases in plasma TBARS ($t_{1/2} = 2.5$ days) and in kidney TBARS ($t_{1/2} = 1.5$ days) are along with the fatty liver some of the earlier signals reported for acute choline deficiency in weanling rats.

The other indicator of kidney oxidative damage was hydroperoxide-induced chemiluminescence (26). This indicator reached the highest value at day 5 with an increase of 183% and a $t_{1/2}$ of 4 days. (Table 2 and Figure 3).

The kinetics of the changes of two indicators of kidney oxidative damage, TBARS levels and hydroperoxide-initiated chemiluminescence, are compared with the kinetics of the appearance of kidney morphological damage. It is clear that increased TBARS ($t_{1/2} = 1.5$ days), interpreted as an exhaustion of the endogenous pools of phosphatidylcholine that are susceptible to lipid peroxidation, is the earlier sign of the pathogenic effect of choline deficiency, followed by the increase in hydroperoxide-induced chemiluminescence ($t_{1/2} = 4$ days), interpreted as decreased tissue levels of lipid soluble antioxidants, *i.e.* endogenous alpha-tocopherol, and by the histological damage ($t_{1/2} = 5.5$ days). The precedence of increased lipid peroxidation over histological damage was also reported in the liver damage following to vitamin E and selenium double deficiency in weanling rats (18).

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Table 2. Thiobarbituric acid-reactive substances (TBARS) and tert-butyl hydroperoxide-initiated chemiluminescence (BOOH-CL) in kidney homogenates from choline-deficient and choline-supplemented rats

Group	Day 3	Day 6
	TBARS (nmol/mg protein)	
Control (choline-supplemented)	14.7 ± 1.1	13.8 ± 1.3
Choline-deficient	26.6 ± 4.0	11.2 ± 1.3
P	0.0489	0.1044
	BOOH-CL (cpm/mg protein) × 10 ³	
Control	61 ± 8	52 ± 4
Choline-deficient	52 ± 6	95 ± 6
P	0.4166	0.0015

Results are expressed as X ± SEM

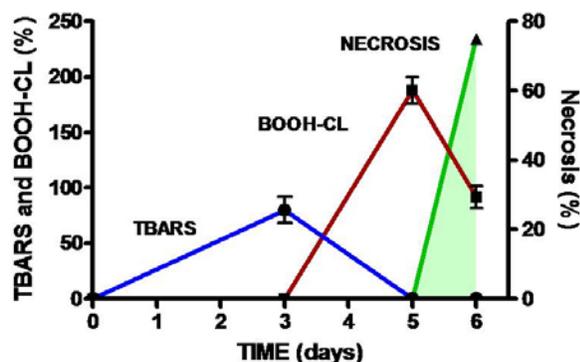


Figure 3. Thiobarbituric acid reactive species (TBARS) and hydroperoxide-induced chemiluminescence (BOOH-CL) in kidney homogenates of weanling rats fed a choline-deficient diet. The blue line with circles (●) indicate the percentage of increase in TBARS, the red line with the squares (■) indicate the percentage of increase in BOOH-CL, the green colored area with the triangles (▲) indicate the cumulative percentage of rats that developed necrosis. The abscissa indicates time after weaning. Symbols and bars indicate mean values ± S.E.M.

The assay of hydroperoxide-initiated chemiluminescence was applied to subcellular membranes isolated from choline-deficient rats at days 3 and 6. The assay showed statistically significant differences in the kidney subcellular membranes of rats fed a choline-deficient diet and sacrificed on day 6, similarly to what was observed in kidney homogenates (see Table 2). Plasma membranes (containing also smooth and rough endoplasmic reticulum and Golgi membranes) showed a 140% increased chemiluminescence, whereas mitochondria exhibited a 42% higher emission, and lysosomes a 143% increased chemiluminescence (Table 3). The interpretation is that all kidney subcellular membranes are affected by the oxidative stress and damage and have decreased levels of alpha-tocopherol following to choline-deficiency.

5. DISCUSSION

Early studies on the mechanism of renal lesions and cell necrosis in weanling rats fed a choline-deficient diet found evidence of oxidative damage and lipid peroxidation with subsequent degeneration of cell membranes. So, lipid peroxidation was early implicated in the mechanism of this cellular necrosis, because renal lipid peroxidation was found

in the necrotic as well as in the pre-necrotic stages of kidneys of choline-deficient weanling rats (12). An increased generation of ROS associated with choline deficiency induced cellular death of rat hepatocytes in culture (29).

The central point of the mechanism of choline deficiency is the vulnerability of cell membranes, due to its high content of polyunsaturated fatty acids, to free radical-mediated reactions that lead to lipid peroxidation and loss of membrane structure and function. Polyunsaturated fatty acids are essential components of mammalian kidney and confer fluidity, flexibility and selective permeability to kidney subcellular membranes (30-32). They exhibit the highest sensitivity to oxidative damage as a function of the proportion of double bonds per fatty acid molecule. Mammalian membranes contain appreciable amounts of phosphatidylcholine with polyunsaturated fatty acids, 18% in plasmatic, 23% in lysosomal, 44% in nuclei, 48% in endoplasmic reticulum, 38% in mitochondrial and 25% in Golgi membranes. The consequence of the peroxidation of unsaturated fatty acids is the impairment of membrane functions, such as ion transport and enzymatic activity, by changes in the biophysical properties of membrane-bound proteins (33). Lipid peroxidation proceeds by a free-radical mediated chain reaction that includes initiation, propagation and termination reactions. The chain reaction is initiated by the abstraction of a hydrogen atom from a methylene group of an unsaturated fatty acid. Propagation is cycled through rounds of lipid peroxy radical abstraction of the bis-methylene hydrogen atoms of a polyunsaturated fatty acyl chain to generate new radicals, after O₂ addition, resulting in the conversion of alkyl radical in hydroperoxy radical. Termination involves the reaction of two hydroperoxy radicals to form non-radical products. This reaction is particularly interesting since it is accompanied, although at low yield, by emission of light or chemiluminescence. Some lipid peroxidation products are light-emitting species and their luminescence is used as an internal marker of oxidative stress (19-21,34). The measurement of light emission derived from ¹O₂ and excited triplet carbonyl compounds, which are the most important chemiluminescent species in the lipid peroxidation of biological systems, is directly related to the rate of lipid peroxidation and allows an indirect assay of the content of lipophilic antioxidants in the sample (19-21, 34). Lipophilic antioxidants react with lipid peroxy radicals and a lower antioxidant content is associated with higher chemiluminescence (26, 34, 35).

Lipid hydroperoxides, in the presence or absence of catalytic metal ions, produce a large variety of products including short and long chain aldehydes and phospholipids and cholesterol ester aldehydes. They can be used to assess the degree of lipid peroxidation in a system (36).

In kidney homogenates, malondialdehyde (as TBARS) were higher in rats fed a choline-deficient diet already at day 3, (t_{1/2} = 1.5 days) well before other indicators of oxidative damage or histopathological lesion. The initial signs were followed by a significant increase in hydroperoxide-induced chemiluminescence (t_{1/2} = 4 days) and the evidence for histological damage (t_{1/2} = 5.5 days).

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Table 3. Tert-butyl hydroperoxide-initiated chemiluminescence in plasma membranes, mitochondria and lysosomes (cpm/mg protein x 10³)

Group	Day 3			Day 6		
	Control	Experimental	P	Control	Experimental	p
Plasma membranes	26 ± 2	32 ± 7	0.43	25 ± 1	60 ± 1	0.002
Mitochondria	15 ± 2	17 ± 1	0.58	24 ± 1	34 ± 2	0.03
Lysosomes	24 ± 5	20 ± 2	0.55	14 ± 1	34 ± 3	0.05

Results are expressed as X ± SEM.

The increased hydroperoxide induced chemiluminescence was also observed in subcellular membranes, indicating that all types of subcellular kidney membranes were subjected to oxidative damage. However, it could be recognized that lysosomal membranes and plasma membranes were more affected than the mitochondrial membranes.

In conclusion, the observations indicate that choline deficiency produces an oxidative damage in all kidney membranes. The membranes with lower phosphatidylcholine concentration are those in which it was observed a higher oxidative damage (plasma membranes containing a mixture of other membrane structures). Mitochondrial membranes have the higher levels of phosphatidylcholine among the membrane systems studied and the lower oxidative damage (lower chemiluminescence).

Perhaps the clearest demonstration of the independence from ionic bonding between membrane protein and phospholipids is provided by the experiments of Fleischer *et al* (37) who reported that phosphatidylcholine (amphipathic), cardiolipin (acidic) and phosphatidylethanolamine (basic) all have equivalent capacities to reactivate electron transfer in mitochondrial membranes (38).

Choline is an essential nutrient and plays an important role in biological functions, including the regulation of proliferation, transformation and cell death (39, 40). Most choline in the body is found in phospholipids such as phosphatidylcholine and sphingomyelin. Humans and animals fed a choline-deficient diet deplete choline stores and develop liver dysfunction and fatty liver that occurs because choline is required to make the phosphatidylcholine portion of the very-low-density lipoprotein (VLDL) particle, needed to export triacylglycerol from the liver. Animals fed a choline deficient diet also develop growth retardation, renal dysfunction and hemorrhage or bone abnormalities or development of memory (41). The nutritional requirements of choline have been established for human beings (42).

The exact amount of choline that the human diet must contain to sustain life is modulated by the pathway (most active in liver) for the *de novo* biosynthesis of the choline moiety via the sequential methylation of phosphatidylethanolamine using *s*-adenosylmethionine as the methyl donor. Choline deficiency depletes cells of methyl-pholate and methionine, and increases intracellular *s*-adenosylhomocysteine and homocysteine concentrations (41).

Choline-deficient diet effects in hepatocytes and liver mitochondria have been well studied. Mechanistic studies have reported that in isolated mitochondria from the

livers of rats fed a choline-deficient diet much more H₂O₂ is produced per NADH oxidized and that this induces hepatocarcinogenesis (43). The enhanced H₂O₂ generation by altered mitochondrial electron transfer chain activity may be responsible for the role of ROS in this choline-deficient diet model (44).

Mitochondrial membrane lipid peroxidation in liver shows maximal levels after five days whereas nuclear membrane lipid peroxidation peaked at the first day and the DNA lesions began at the second day. The choline-deficient diet caused a large drop (60 %) in vitamin E levels (45).

Hensley *et al* (46) reported that a choline-deficient diet resulted in impaired respiratory function, complex I (NADH-ubiquinone reductase) dysfunction and increased H₂O₂ in liver mitochondria and that oxidative damage plays a central role in the choline-deficient diet model of hepatocellular carcinoma (43). Choline-deficient diets result in the elevation of the production of ROS in a time and dose-dependent manner (43) in liver, so ROS may be involved in signaling choline-deficient-induced damage and apoptosis in hepatocellular carcinoma; this increase in ROS production appears related to mitochondrial dysfunction and causing apoptosis.

Reactive oxygen species includes O₂⁻, H₂O₂ and HO[·], which are physiologically generated as by-products of mitochondrial electron transfer. The formation of O₂⁻ is originated from the auto-oxidation of the ubisemiquinone of complexes I and III and the production of H₂O₂ occurs by Mn-SOD catalysis (24). When the electron transfer process is blocked at complexes I and III, electrons pass directly to O₂ producing O₂⁻. The reactive oxygen and nitrogen species, although kept in low steady-state concentrations by antioxidant systems, are able to react and damage biomolecules. Mitochondria are to be considered the main intracellular source of oxidizing reactive oxygen species (47).

Reduced glutathione (GSH) participates in the detoxification of naturally occurring free radicals and peroxides and is formed by two consecutive ATP-requiring reactions, synthesis from glutamate and cysteine and a second reaction, the incorporation of glycine. The rate limiting step is cysteine incorporation. The maintenance of a high intracellular levels of GSH requires two main mechanisms to provide cysteine: a) the transformation of methionine into homocysteine and then into cysteine through the transmethylation and transulfuration pathway and b) uptake of cysteine by hepatocytes. In the transulfuration pathway, methionine is converted into *S*-adenosyl-methionine by a reaction catalyzed by *S*-adenosyl methionine synthetase, it donates its methyl group via a

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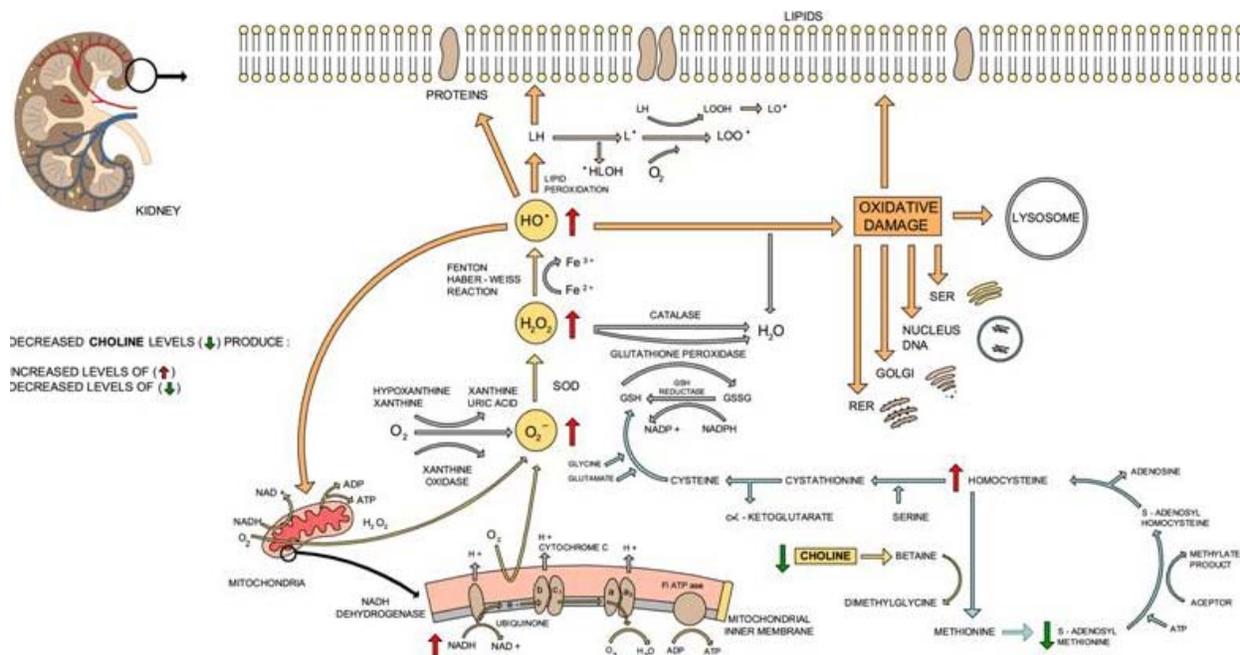


Figure 4. Oxidative damage pathways in the renal cells of choline-deficient rats. LHOH: hydroxy-lipid radical; L[•]: alkyl radical; LH: lipid (unsaturated fatty acid); LO[•]: lipid alkoxy radical; LOO[•]: lipid peroxy radical; LOOH: lipid hydroperoxide; NAD⁺: nicotinamide adenine dinucleotide (oxidized); NADH: nicotinamide adenine dinucleotide (reduced); O₂⁻: superoxide radical; HO[•]: hydroxyl radical; SOD: superoxide dismutase; H₂O₂: hydrogen peroxide.

number of methylation reactions catalyzed by methyltransferases. One of the methylation reactions is the transformation of phosphatidylethanolamine into phosphatidylcholine, essential for the regulation of cell membrane function and structure. The final product of S-adenosyl-methionine demethylation, homocysteine, is an intermediate in the transulfuration pathway. Homocysteine can follow two routes: re-methylation into methionine, which lead to its preservation or transformation to cysteine for GSH synthesis (48).

Chronic administration of diets marginally low in methionine or choline to rats has been shown to reduce by 50-60 % both hepatic s-adenosyl methionine and glutathione (GSH) levels, producing severe liver injury, fibrosis, cirrhosis and hepatocellular carcinoma. In experimental animals with alcoholic cirrhosis phosphatidylcholine administration present protective effect on methionine levels (48). In kidney, indeed, carcinogenesis was not observed, but hemorrhagic necrosis usually appears in weanling rats fed choline-deficient diets (Figure 1).

Mitochondrial membrane alterations are apparently the main consequence of subcellular lipid peroxidation and thiol residue damage. In the inner mitochondrial membrane, at complexes I and III, O₂⁻ is released into the mitochondrial matrix and subsequently dismutated into H₂O₂ that is able to diffuse across the membranes, leading to HO[•] generation in the cytosol. This highly reactive radical is able to react with any biomolecule. In kidney, the increase of these oxygen reactive species, triggered by NADH overproduction, might afford the main

cause of mitochondrial and cellular oxidative damage in rats fed a choline-deficient diet (Figure 4).

Choline deficiency in weanling rats leads sequentially to increased lipid peroxidation and kidney necrosis, as indicated by the kinetics of the markers of lipid peroxidation and of histological damage. Lipid peroxidation leads to membrane disruption and cell death, which initiate the necrotic process. The determination of chemiluminescence in kidney homogenates allowed the assessment of kidney as a target organ for increased lipid peroxidation. At day 5 after weaning, homogenate emission in rats fed a choline-deficient diet was 183% higher than in control animals. At this time, TBARS levels were diminished to control values although they were increased at day 3. At day 5, it was possible to define a pre-necrotic stage with a rate of excited-species generation, without observable morphological changes. Necrosis appeared 12-24 hours after this pre-necrotic stage, and the close relationship observed between kidney homogenates luminescence and necrosis (Figure 3) indicates that lipid peroxidation is a promoter of tissue damage.

Increased malondialdehyde (TBARS) levels preceding kidney necrosis indicates an increased rate of free-radical reactions in cellular membranes, which lead to depletion of membrane antioxidants, as indicated by the increased values of hydroperoxide-induced chemiluminescence, that promote membrane disruption and histological damage. Guo *et al.*, (43) reported that a choline-deficient diet induces an increased ROS generation in hepatocytes cultured *in vitro* in a time-dependent

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manner, resulting in a 1-fold increase at day 1, 3-fold at day 2, and 5-fold at day 3.

Plasma TBARS levels indicated an early situation of systemic oxidative stress, a condition that may be related to simultaneous liver damage, with lipid peroxidation and membrane rupture. The liver process could be reflected on TBARS plasma levels and these aldehydes may be involved in the renal necrosis associated to choline-deficiency. The higher plasma TBARS levels observed in rats fed choline-deficient diet would indicate a shift to a systemic pro-oxidative balance in the plasma of these animals previous to the increased lipid peroxidation and the morphological damage of kidney necrosis.

6. PERSPECTIVES

In summary, the observations reported here as well as the previous reports in the literature, clearly indicate that choline-deficiency causes increased lipid peroxidation with oxidative stress and damage. Those biochemical changes are the basis of the observed histological damage and necrosis that are characteristic of kidney and liver. It is worth noting that the quantity as well as the quality of lipids are important in the development of renal necrosis, according to the observations with this experimental model (5). It is interesting to bring into focus the influence of dietary lipids for the development of oxidative stress. It is likely that the point may bear a clinical implication since about 40% of the acute renal failure are intrahospitalary (49) and this brings the possibility of modulating kidney failure by an adequate diet in order to reduce mortality.

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Send correspondence to: Marisa Repetto PhD, Laboratorio de Estrés Oxidativo y Canales Iónicos. Cátedra de Química General e Inorgánica. Facultad de Farmacia y Bioquímica. Universidad de Buenos Aires. Junín 956, 1113 Buenos Aires, Argentina, Tel: 54-11-4964-8249, Fax: 54-11-4508-3653, E-mail: mrepetto@ffybu.uba.ar

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